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The unfolded protein response in yeast

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The Unfolded Protein Response in Yeast

by

Caroline E. Shamu

DISSERTATION

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DOCTOR OF PHILOSOPHY

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Cell Biology

in the

GRADUATE DIVISION

of the

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San Francisco

I dedicate this thesis to my parents, Robert and Judith Shamu.

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Abstract

The accumulation of unfolded proteins in the endoplasmic reticulum (ER) triggers the increased production of several ER-resident proteins. This signalling pathway exists in organisms as divergent as mammals and yeast, and is the only known example of an intracellular signalling system that links the ER and the nucleus. This thesis describes a genetic screen initiated to look for *S. cerevisiae* mutants that cannot induce the unfolded protein response. As a result of the screen, the gene *IRE1* was identified as a key component of the pathway. It encodes a transmembrane serine/threonine kinase similar in structure to growth-factor receptor kinases: Ire1p has a single transmembrane domain and its kinase domain, located in the C-terminal half of the protein, is cytoplasmic. *IRE1* is essential for cell viability under stress conditions that cause unfolded proteins to accumulate in the ER. The gene is also required for inositol protophy, suggesting that the induction of ER resident proteins is coupled the biogenesis of new ER membrane. Molecular genetic and biochemical studies of Ire1p are described which suggest that, as in the case of growth-factor receptors, Ire1p oligomerizes in response to the accumulation of unfolded proteins in the ER, and that it is phosphorylated *in trans* by other Ire1p molecules as a result of oligomerization. The C-terminal protein tail of Ire1p is required for induction of the unfolded protein response and the role of the tail is probably to bind other proteins that transmit the unfolded protein signal toward the nucleus. Finally, experiments designed to examine the nature of the unfolded protein signal that originates in the ER are described and, in particular, the role of the ER-resident proteins BiP (encoded by the *KAR2* gene) and protein disulfide isomerase (PDI, encoded by the *PDI1* gene) is explored. The data presented are consistent with the

possibility that BiP and PDI (and possily other ER chaperone proteins) monitor the level of unfolded proteins in the ER and, when unfolded proteins accumulate, that BiP and PDI initiate the unfolded protein response by modulating their binding to Ire1p.

Peter Walter 6/27/95

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Chapter I:

Introduction

When unfolded proteins accumulate in the endoplasmic reticulum (ER), the transcription of genes encoding ER-resident proteins is induced in the nucleus. This "unfolded protein response" was first studied in animal cells, where the expression of BiP/GRP78, GRP94, PDI/ERp59, and ERp72 is induced by a variety of different treatments whose common property is that they cause the accumulation of unfolded proteins in the ER (Dorner et al., 1990; Kozutsumi et al., 1988).

In mammalian cells, unfolded proteins are produced in the ER and the unfolded protein response is induced 1) by preventing protein glycosylation with drugs such as tunicamycin or 2-deoxyglucose, or by starving cells for glucose, 2) by preventing disulfide bond formation with reducing agents such as 2-mercaptoethanol, 3) by overexpressing mutant secretory proteins that are incapable of folding properly and thus accumulate in the ER, or 4) by causing a rapid flux of calcium out of the ER with the calcium ionophore A23187 (Drummond et al., 1987; Lee, 1987).

The unfolded protein response is regulated at the level of transcription (Lee, 1987), and enhancer-like elements required for the response have been identified in promoters of BiP/GRP78 and GRP94 (Chang et al., 1989; Resendez et al., 1988) All of the ER-resident proteins induced by the unfolded protein response appear to help secreted proteins fold as they pass through the ER. BiP/GRP78 is a homologue of the heat shock protein hsp70, and GRP94 is a homologue of hsp90. Heat shock proteins promote protein folding apparently by using the energy released by ATP hydrolysis to prevent inappropriate inter- and intra-molecular protein aggregation (for a review of hsp's and further discussion on their role in protein folding, see Gething and

Sambrook, 1992). PDI/ERp59 (protein disulfide isomerase) is thought to help proteins fold, at least in part, by promoting correct disulfide bond formation (Freedman, 1989; LaMantia and Lennarz, 1993). ERp72 is a PDI-like protein of as yet unknown function. It makes sense that the expression of these ER-resident proteins is augmented when unfolded proteins accumulate in the ER: by inducing the unfolded protein response, the cell increases its capacity to fold proteins and to assemble multi-protein complexes in the ER.

The unfolded protein response also exists in the yeast *Saccharomyces cerevisiae*, where the expression of a similar set of ER resident proteins is induced, including BiP (encoded by *KAR2* ; Normington et al., 1989; Rose et al., 1989), PDI (encoded by *PDI1*; Cox et al., 1993; LaMantia et al., 1991), Eug1p (a PDI-like protein encoded by *EUG1*; Tachibana and Stevens, 1992), and peptidyl-prolyl cis-trans isomerase (PPIase, encoded by *FKB2*; Partaledis and Berlin, 1993). No yeast homologue of GRP94 has yet been identified.

In yeast cells the unfolded protein response is induced by tunicamycin, 2-deoxyglucose, 2-mercaptoethanol, and overexpression of mutant secretory proteins. In addition to these treatments, temperature sensitive mutations in the genes *SEC53* and *SEC11* cause the accumulation of unfolded proteins in the ER. At the non-permissive temperature, *sec53* mutants lack phosphomannomutase and are defective for protein glycosylation (Kepes and Schekman, 1988); *sec11* mutants lack signal peptidase activity (Bohni et al., 1988). Mutations in *SEC18* also result in the induction of the unfolded protein response. *SEC18* encodes the yeast homologue of NSF, a protein required for vesicle fusion and thus for ER to Golgi transport (Wilson et al., 1989) . Although it is not known whether the proteins that accumulate in the

ER in *sec18* mutants are unfolded, the ER does expand in the mutants at the non-permissive temperature (Novick et al., 1980).

The promoter element required for the unfolded protein response in yeast was first identified by a deletion analysis of the *KAR2* promoter (Kohno et al., 1993; Mori et al., 1992). A single 22 bp element, the unfolded protein response element (UPRE), is necessary and sufficient to activate transcription in response to agents that cause the accumulation of unfolded proteins in the ER. Other genes regulated by the unfolded protein response also have promoter elements similar in sequence to the *KAR2* UPRE (Figure I-1; Partaledis and Berlin, 1993; Tachibana and Stevens, 1992 and J. Cox unpublished observations). Of these others, only the *FKB2* sequence has been demonstrated experimentally to function as a UPRE (Partaledis and Berlin, 1993).

At the time we began work on the unfolded protein response, in addition to the UPRE the identity of only one other component of the pathway had been proposed: BiP itself might monitor the levels of unfolded protein in the ER. Specifically, Ng et al. have suggested that the cell monitors the concentration of "free" BiP (BiP not bound to unfolded proteins) in the cell as a measure of the amount of unfolded protein there (Ng et al., 1992). Mutations were made in the glycosylation sites of a viral protein that passes through the ER of animal cells on its way to the cell surface. As a result, the mutant proteins do not fold properly in the ER (as monitored by conformation-specific monoclonal antibodies) and accumulate there. All but two were shown to bind BiP; expression of these proteins induced the unfolded protein response. Interestingly, the two mutants that do not bind to

BiP do not induce the unfolded protein response. Thus, it was proposed that the cell monitors the levels of free BiP in the ER to determine when to induce the unfolded protein response: when there are few unfolded proteins in the ER, the level of free BiP is high; as unfolded proteins accumulate, the level of free BiP falls and the cell induces the unfolded protein response. This hypothesis has been supported by work on yeast. If BiP levels in the ER are artificially lowered by expressing a BiP mutant that is not retained in the ER and is instead secreted, cells induce transcription of *KAR2*, presumably by inducing the unfolded protein response (Hardwick et al., 1990). Also, overexpression of BiP in yeast cells that accumulate unfolded proteins in their ER diminishes their unfolded protein reponse (Kohno et al., 1993).

Because few components of the response were known, the path followed by the "unfolded protein" signal from the ER to the nucleus was unknown. Two plausible routes are shown in Figure I-2. The signal might pass directly from the ER to the nucleus by crossing the inner nuclear membrane (route a). A signal following this path has never before been described. Alternatively, the signal might pass across the ER membrane, through the cytoplasm and into the nucleus through the nuclear pore (route b). Because the ER lumen is topologically identical to the outside of the cell, a signal travelling along this second path would be following essentially the same route through the cytosol as the signals that eminate from receptors in the plasma membrane (for example, pathways that mediate the response of animal cells to growth factors or of *S. cerevisiae* cells to mating pheromones).

To begin to elucidate the path of the unfolded protein signal between the ER and the nucleus and to learn more about the mechanism by which

cells monitor the accumulation of unfolded proteins in the ER, we sought to identify new components of the *S. cerevisiae* unfolded protein response pathway. This thesis records the progress that we have made. Chapter II describes a simple color assay for the unfolded protein response that was developed and then used to carry out a genetic screen for mutants unable to induce the unfolded protein response. As a result of the screen, we discovered that the transmembrane kinase Ire1p is an important component of the pathway and that its role is likely to transmit the unfolded protein signal out of the ER. In Chapter III, models for the mechanism of Ire1p activation are tested and we show that, consistent with the models, Ire1p oligomerizes and is phosphorylated. The role of BiP and other ER resident chaperone proteins in monitoring the unfolded protein response is explored in Chapter IV and models discussed in previous chapters are revised to accommodate new data. Finally, in Chapter V, future experimental directions for the study of the unfolded protein response pathway in yeast are proposed.

Figure I-1:

A comparison of unfolded protein response elements (UPRE's) from the *S. cerevisiae* genes *KAR2*, *FKB2*, *EUG1*, and *PDI1* (Kohno et al., 1993; Mori et al., 1992; Partaledis and Berlin, 1993; Tachibana and Stevens, 1992 and J. Cox unpublished observations). Each sequence is numbered relative to the translational start site. The *KAR2* and *FKB2* promoter elements are the only sequences that have been demonstrated experimentally to act as UPRE's.

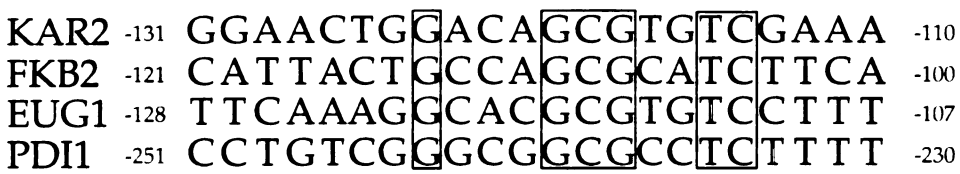

Figure I-1

Figure I-2:

Two possible routes (***a*** and ***b***) along which the signal transmitted by the unfolded protein response pathway might travel. Several components of the pathway, unfolded proteins (UP), a transcription factor (Txf), and the unfolded protein response element (UPRE), are labelled, as are the ER, the nucleus (N), and the cytoplasm (C).

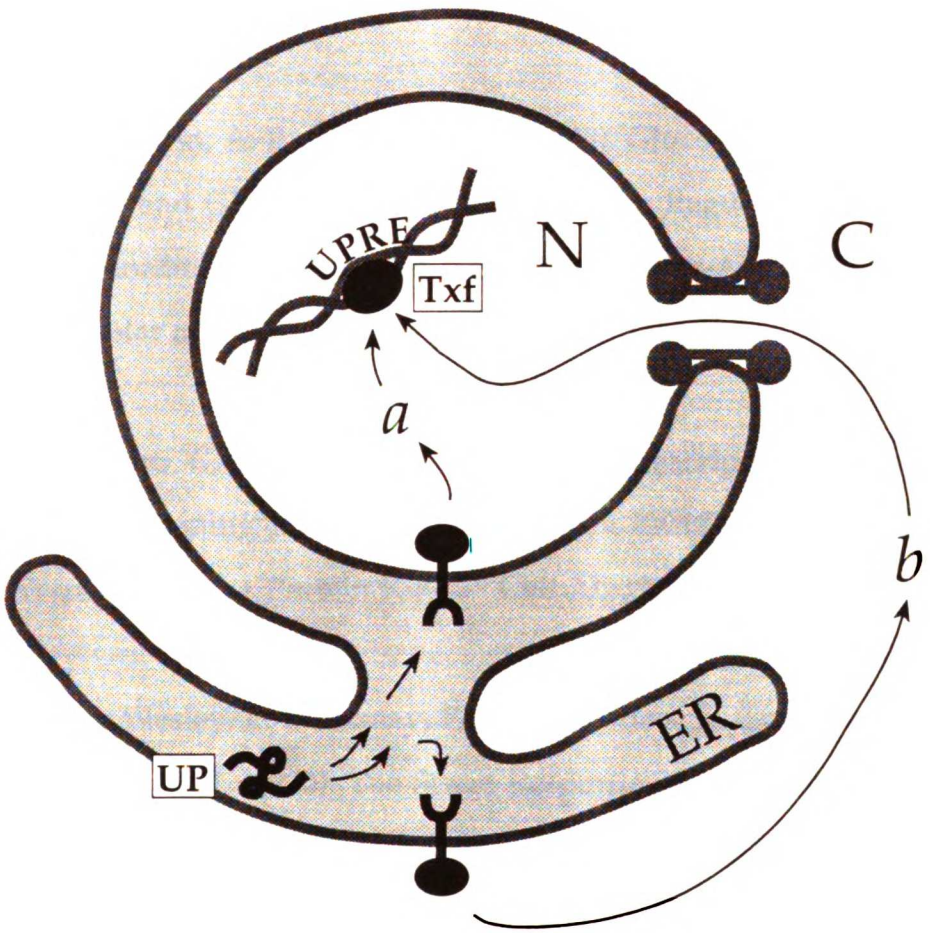

Figure I-2

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Chapter II:

Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase

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Transcriptional Induction of Genes Encoding Endoplasmic Reticulum Resident Proteins Requires a Transmembrane Protein Kinase

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Summary

The transcription of genes encoding soluble proteins that reside in the endoplasmic reticulum (ER) is induced when unfolded proteins accumulate in the ER. Thus, an intracellular signal transduction pathway must exist that mediates communication between the ER lumen and the nucleus. We have identified a gene in S. cerevisiae, *IRE1*, that is required for this pathway: *Ire1*$^-$ mutants cannot activate transcription of *KAR2* and *PDI1*, which encode the ER resident proteins BiP and protein disulfide isomerase. Moreover, *IRE1* is essential for cell viability under stress conditions that cause unfolded proteins to accumulate in the ER. *IRE1* encodes a transmembrane serine/threonine kinase that we propose transmits the unfolded protein signal across the ER or inner nuclear membrane. *IRE1* is also required for inositol prototrophy, suggesting that the induction of ER resident proteins is coupled to the biogenesis of new ER membrane.

Introduction

In all eukaryotic cells, secreted and transmembrane proteins must be folded and assembled correctly in the endoplasmic reticulum (ER) before they can exit the ER. Folding in the ER is assisted by a set of enzymes including BiP, a member of the HSP70 family of molecular chaperones (reviewed in Gething and Sambrook, 1992). BiP is thought to bind transiently to nascent proteins as they are translocated into the ER lumen to assist proper folding and to prevent aggregation of folding intermediates (Pelham, 1986; Haas and Wabl, 1983; Ng et al., 1989; Machamer et al., 1990). BiP also binds to partially assembled and misfolded proteins and thereby may help target dead-end products to degradative pathways in the ER (Ng et al., 1990; Gething et al., 1986; Knittler and Haas, 1992). Another well-characterized ER lumenal protein that is required for proper protein folding is protein disulfide isomerase (PDI), which catalyzes the formation of disulfide bonds (Freedman, 1989).

Interestingly, the synthesis of these ER resident proteins is regulated according to demand for them inside the organelle. In mammalian cells, for example, synthesis of BiP, PDI, and glucose-related protein GRP94, a member of the HSP90 family, is induced when unfolded proteins accumulate in the ER (Lee, 1987; Kozutsumi et al., 1988; Dorner et al., 1992). Experimentally, the accumulation of unfolded proteins in the ER is induced by various treatments: the inhibition of glycosylation either by glucose starvation or by the addition of drugs such as tunicamycin or 2-deoxyglucose; the addition of reducing agents, such as β-mercaptoethanol, which are thought to affect protein folding by preventing proper disulfide bond formation; the expression of folding-defective mutant secretory proteins; or the addition of calcium ionophores that deplete calcium stores in the ER. Induction of the ER resident proteins occurs at the transcriptional level (Lee, 1987). Thus, an unfolded protein response pathway must exist that allows the transduction of a signal from the ER lumen, where unfolded proteins accumulate, to the cell nucleus, where transcription is activated.

Lee and colleagues have identified a promoter element in the BiP gene of mammalian cells that is required for BiP induction in response to unfolded proteins. The sequence of this element is conserved among different mammalian species (Resendez et al., 1988). Genes encoding other ER lumenal proteins are coordinately regulated with the BiP gene. For example, GRP94 contains similar upstream promoter elements, and it has been proposed that the two genes are regulated by a common transcription factor(s) (Chang et al., 1989; Li and Lee, 1991; Li et al., 1992).

In the yeast Saccharomyces cerevisiae, BiP is encoded by the *KAR2* gene, and, as in higher cells, its transcription is induced when unfolded proteins accumulate in the ER (Normington et al., 1989; Rose et al., 1989). However, unlike the mammalian BiP gene, *KAR2* transcription is also induced by heat shock. An analysis of the *KAR2* promoter has revealed that the unfolded protein response is regulated by a 22 bp unfolded protein response element (UPRE) that is distinct from the heat shock element in the *KAR2* promoter (Kohno et al., 1993; Mori et al., 1992). The UPRE is sufficient to activate transcription from a heterologous promoter in response to the accumulation of unfolded proteins in the ER lumen. Here, we describe the results of a genetic screen in which we used a UPRE-controlled reporter gene to isolate yeast mutants defective in the unfolded protein response pathway.

Results

Construction of a *lacZ* Reporter Gene That Is Activated by the Accumulation of Unfolded Proteins in the ER Lumen

To isolate S. cerevisiae mutants defective in *KAR2* induction, we first made a *lacZ* reporter gene that is induced when unfolded proteins accumulate in the ER. Because transcription from the intact *KAR2* promoter occurs at a relatively high basal level even in the absence of *KAR2* induction, we chose to construct a hybrid promoter with a lower basal level. To this end, we took advantage of the observation that the UPRE in the *KAR2* promoter can function as an upstream activating sequence when fused to a heterologous promoter (Mori et al., 1992). Thus, we synthesized a 38 bp DNA fragment bearing the UPRE from *KAR2* and inserted it upstream of a crippled *CYC1* promoter that, in the absence of an upstream activating

Table 1. Yeast Strains

Strain	Genotype	Source/Reference
W303-1A	*leu2-3,-112; his3-11,-15, trp1-1; ura3-1; ade2-1; can1-100; MATa*	R. Rothstein, Columbia University
W303-1B	*leu2-3,-112; his3-11,-15, trp1-1; ura3-1, ade2-1; can1-100; MATα*	R. Rothstein, Columbia University
JC103[a]	same as W303-1A, except *his3-11,-15::HIS⁺UPRE-lacZ; leu2-3,-112::LEU⁺UPRE-lacZ; met⁻*	This study
JC104	same as W303-1B, except *his3-11,-15::HIS⁺UPRE-lacZ*, and *leu2-3,-112::LEU⁺UPRE-lacZ*	This study
CS165	same as JC103, except *ire1::URA3*	This study
CS171[b]	same as JC103, except *ire1-1*	This study
CS172	same as CS171, except *MATα*	This study
CS181	diploid product of CS165 × CS172	This study
BRS1015	*ino1-13; trp1; leu2; ura3; MATα*	Culbertson and Henry, 1975
JRY318	*tun1-1; his4-260,-39; leu2-1; ura3; thr4; met⁻; MATα*	Barnes et al., 1984

[a]Spontaneous *met⁻* derivative of W303.

[b]CS171 is the product of the third backcross of our original *ire1-1* isolate, but for simplicity we designate all of the *MATa* products of *ire1-1* backcrosses by this name. In practice, the data in Figure 2 were obtained using the original *ire1-1* isolate and products of the first backcross, the *ire1-1* strain rescued by pJC012 (from the yeast genomic library) was a product of the second backcross, and the data in Figures 3B, 3C, and 4 were obtained using the product of the third backcross. CS172 is a product of the third backcross.

sequence, is transcriptionally silent (Guarente and Mason, 1983). Single copies of this reporter construct were integrated at two different locations in the genome of yeast strain W303 to create the reporter strain JC103 (see Experimental Procedures; Table 1).

We can detect the induction of *lacZ* expression from the reporter construct using 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) plates that contain tunicamycin, a drug that blocks protein glycosylation and causes the accumulation of unfolded proteins in the ER. JC103 colonies turn blue when transferred to X-Gal–tunicamycin indicator plates, but are white when no drug is present (data not shown). This induction is due to the presence of the UPRE, because an isogenic strain bearing a *lacZ* construct that lacks this element fails to turn blue on X-Gal–tunicamycin plates (data not shown). This result indicates that the integrated *lacZ* reporter constructs respond properly to the presence of unfolded proteins in the ER.

To confirm this observation, we analyzed the accumulation of *KAR2* and *lacZ* messenger RNAs (mRNAs) in JC103 cells after treatment with tunicamycin. Figures 1B and 1C show the results from a quantitative S1 nuclease protection assay. When cells were grown in the absence of tunicamycin, the amount of *KAR2* mRNA was constant at all time points analyzed (Figure 1B, lanes 1, 3, and 5; Figure 1C, Tm⁻), and, as expected from the color assay described above, *lacZ* mRNA was barely detectable. In contrast, when cells were grown in the presence of tunicamycin, a 3.5- to 5-fold increase in the amount of *KAR2* mRNA was seen (Figure 1B, compare lanes 2 and 4; Figure 1C, Tm⁺). *lacZ* mRNA levels were induced as well, increasing more than 150-fold after the addition of tunicamycin. This greater induction is due to the lower basal transcription of the UPRE–*lacZ* reporter gene. Both *KAR2* mRNA and *lacZ* mRNA amounts remained elevated at a constant level for at least 6 hr after the addition of tunicamycin (Figure 1B, lane 6). Thus, there is a strict correlation between the induction of the *lacZ* reporter gene and the induction of the endogenous *KAR2* gene upon tunicamycin treatment.

Isolation of Mutants Defective in the Unfolded Protein Response

We used JC103 cells in a genetic screen to isolate mutants that fail to induce *KAR2* transcription in response to the accumulation of unfolded proteins in the ER. We reasoned that the unfolded protein response pathway might not be essential for cell viability under normal, nonstress conditions and that loss-of-function mutations in components that participate in the signaling pathway could be obtained. In the primary screen, JC103 cells were mutagenized with ethyl methanesulfonate (to 30% survival) and plated on rich medium. Colonies were grown at room temperature and replica-plated from the master plates onto X-Gal–tunicamycin indicator plates. Mutant cells unable to induce transcription from the UPRE–*lacZ* reporter gene formed white colonies on the indicator plates. The corresponding colonies were picked from the master plates for further analysis. Approximately 45,000 mutagenized JC103 colonies were screened in this way.

To eliminate mutants that are not defective for *KAR2* induction, we applied a series of secondary criteria to the isolated mutants. First, we eliminated mutants that were unable to take up tunicamycin or were resistant to its activity. Such mutants have been described previously (Barnes et al., 1984). We required that cell growth was still sensitive to tunicamycin and that 2-deoxyglucose, another drug that leads to impaired protein glycosylation in wild-type cells, would fail to induce the reporter gene. Therefore, we discarded mutants that grew well on plates containing tunicamycin and those that turned blue on X-Gal plates containing 2-deoxyglucose.

Next, we eliminated mutants that were unable to induce transcription of *lacZ* from a second regulated promoter. Such mutants might be unable to carry out the β-galactosidase enzymatic color reaction or might fail, in general, to induce transcription. To this end, we took advantage of the rat glucocorticoid receptor (GR), which can function as a hormone-inducible transcriptional activator in yeast (Schena and Yamamoto, 1988). Each prospective mutant

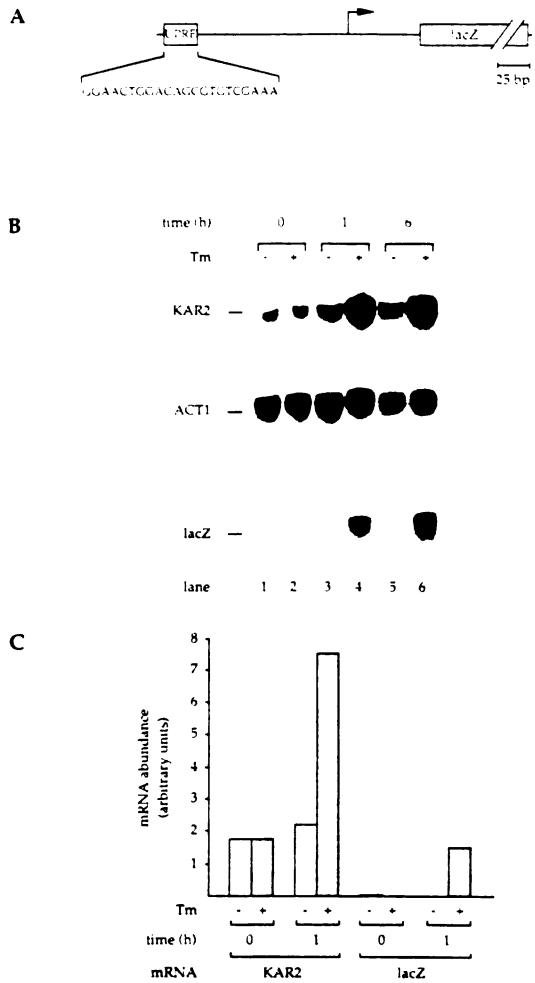

Figure 1. Characterization of the Reporter Strain JC103

(A) The UPRE–*lacZ* reporter construct. An oligonucleotide bearing the 22 bp UPRE defined by Mori et al. (1992) was inserted upstream of a disabled *CYC1* promoter and *lacZ* gene fusion. Two copies of this construct were integrated into the genome to produce reporter strain JC103 (see Experimental Procedures). The approximate position of the transcription start site is indicated by an arrow.

(B) Induction of *lacZ* transcription parallels that of *KAR2*. Midlog phase cultures of JC103 were incubated at room temperature in the absence (minus Tm) or presence (plus Tm) of 1 μg/ml tunicamycin for 0, 1, or 6 hr. Total RNA was harvested, and the abundance of *KAR2*, *lacZ*, and *ACT1* mRNAs was analyzed by S1 nuclease protection assays (see Experimental Procedures).

(C) The data from the 0 and 1 hr time points in (B) were quantitated using the PhosphorImager (Molecular Dynamics, Sunnyvale, California). *KAR2* and *lacZ* mRNA levels were normalized to *ACT1* mRNA levels. The relative induction of *KAR2* and *lacZ* mRNA did not change significantly between the 1 and 6 hr time points.

was transformed with two plasmids, one encoding GR under the control of a constitutively active promoter and a second bearing the *lacZ* gene controlled by glucocorticoid response element upstream activating sequences. GR-dependent transcription from the glucocorticoid response

mones, such as deoxycorticosterone, and wild-type yeast cells bearing these constructs are blue on X-Gal plates containing deoxycorticosterone. Mutants that carried these plasmids and produced white colonies on X-Gal–deoxycorticosterone plates were eliminated from further analysis.

Finally, each mutant was tested directly for its ability to induce transcription of the *KAR2* gene. RNA was harvested from cells grown in the presence of tunicamycin, and the level of *KAR2* transcript present was determined by S1 nuclease protection. This test provided the most direct confirmation that the induction pathway of endogenous *KAR2*, and not just the *lacZ* reporter, was compromised in the mutant cells. We obtained two mutant strains that passed all three secondary tests. The mutations in the strains are recessive with regard to their white phenotype on X-Gal–tunicamycin indicator plates, and the mutants fall into different complementation groups (data not

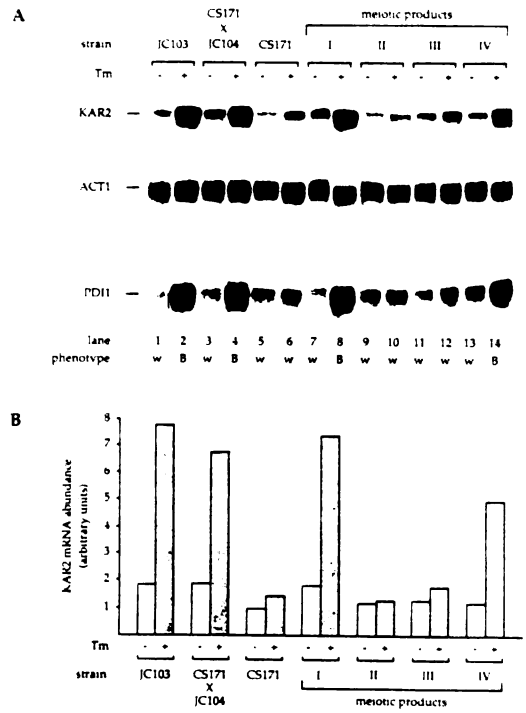

Figure 2. Strain CS171 Carries a Recessive Mutation That Abolishes UPRE-Dependent Induction of the *KAR2* and *PDI1* Genes

(A) S1 protection analysis was performed on RNA isolated from the parent JC103, haploid mutant CS171, the heterozygous diploid product of CS171 crossed with reporter strain JC104, and the four meiotic products of sporulation of the diploid. RNA from two tetrads that were products of different backcrosses of CS171 was analyzed by S1 nuclease protection; the data in lanes 7–14 are a representative result for one tetrad. Each strain was grown for 1 hr in the absence or presence of 1 μg/ml tunicamycin (Tm), and the amount of *KAR2*, *ACT1*, and *PDI1* mRNA was analyzed. The phenotype of each strain on X-Gal indicator plates made without or with 1 μg/ml tunicamycin is indicated under each lane as either blue (B) or white (w).

(B) The data from (A) were quantitated and normalized to actin mRNA

shown). In this study, strain CS171 was chosen for further analysis. The second mutant will be described elsewhere.

Characterization of CS171 Cells

When analyzed by S1 nuclease protection, CS171 cells show a severely impaired level of *KAR2* mRNA induction upon tunicamycin treatment as compared with the parent JC103 cells (Figure 2A, compare bands labeled KAR2 in lanes 5 and 6 with lanes 1 and 2). The mutation in CS171 cells is recessive with regard to *KAR2* induction: the diploid formed by mating of CS171 cells with the wild-type reporter strain JC104 showed the same amount of tunicamycin-dependent *KAR2* mRNA induction as did JC103 cells (Figure 2A, compare bands labeled KAR2 in lanes 3 and 4 with lanes 1 and 2). Furthermore, analysis of the four colonies derived from spores of a single tetrad after sporulation of the heterozygous diploid strain (CS171 × JC104) showed a 2:2 segregation of the *KAR2* induction phenotype (Figure 2, lanes 7–14), consistent with the idea that the mutant phenotype results from a mutation in a single gene. As expected, the inability of the mutant to induce *KAR2* transcription correlated with its white colony color on X-Gal indicator plates containing either tunicamycin or 2-deoxyglucose (Figure 2A).

In mammalian cells, the expression of soluble ER resident proteins other than BiP (e.g., PDI) is also induced upon accumulation of unfolded proteins in the ER. We therefore wished to determine whether corresponding yeast ER resident proteins are similarly coregulated and, if this is the case, whether CS171 cells are impaired in their induction. To address this question, we first established with the S1 nuclease protection experiment shown in Figure 2 that yeast PDI, encoded by the *PDI1* gene (LaMantia et al., 1991), is induced in wild-type cells upon tunicamycin treatment (Figure 2A, compare lanes 1 and 2, bands labeled PDI1). Analysis of CS171 cells showed that the tunicamycin-dependent induction of *PDI1* is impaired (Figure 2). These results suggest that the unfolded protein response pathway in yeast regulates a set of genes whose products are involved in protein folding and assembly in the ER and that the product of the gene mutated in CS171 cells is required for the induction of both *KAR2* and *PDI1* transcription.

The *IRE1* Gene Restores Induction of *KAR2* Transcription in CS171 Cells

To identify the gene that is defective in CS171 cells, we screened a yeast genomic library for complementation of the mutant phenotype. CS171 cells were transformed with a high copy plasmid library and replica-plated onto X-Gal–tunicamycin indicator plates. One plasmid, pJC012, containing a 9 kb insert complemented the white phenotype of CS171 cells, yielding blue colonies on indicator plates. The 9 kb DNA insert was moved to a low copy vector that also complemented the CS171 phenotype on X-Gal–tunicamycin plates. This result ruled out the possibility that the observed complementation was the result of high copy suppression.

To identify the region of the 9 kb DNA insert required for complementation, a pool of pJC012 was generated that

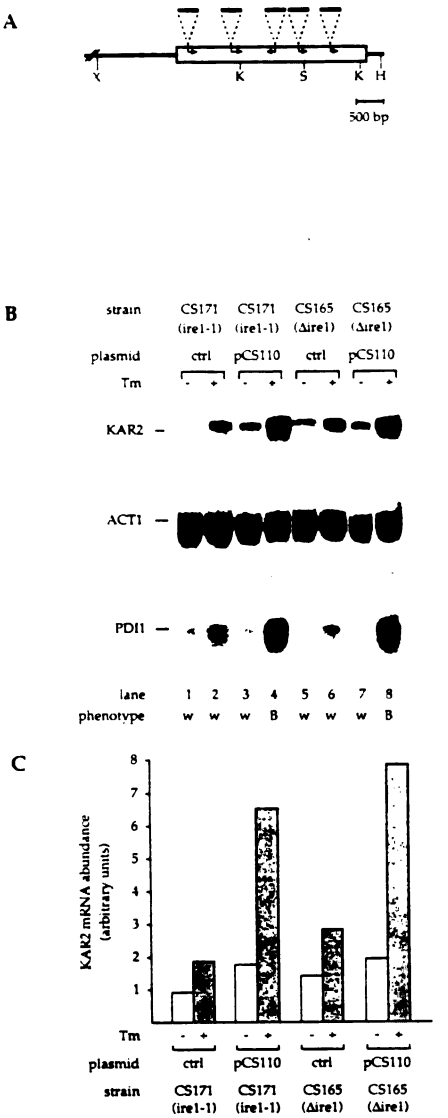

Figure 3. Cloning of *IRE1*

(A) Restriction map of the 9 kb insert of plasmid pJC012, the plasmid from the genomic library that complements the mutant phenotype of CS171. The open reading frame encoding Ire1p is represented by an open box. The positions of Tn10LUK insertions (solid lines above box) that abolish complementation are indicated. Arrows denote direction of sequencing and approximate length of DNA sequence obtained using primers from the ends of the inserted transposons. Restriction sites: HindIII (H), KpnI (K), SacI (S), and XhoI (X). The region between the XhoI and the HindIII site is drawn to scale except for the lines representing Tn10LUK.

(B) *IRE1* restores tunicamycin (Tm)-dependent *KAR2* and *PDI1* mRNA induction to CS171 (*ire1-1*) and CS165 (*Δire1*) cells. *KAR2* and *PDI1* mRNA levels were analyzed in CS165, and CS171 cells that carried either the CEN/ARS control plasmid pRS314 (ctrl) or pRS314 bearing the XhoI–HindIII fragment shown in (A) (pCS110). Tunicamycin induction followed by S1 nuclease protection analysis was performed as in Figure 2A. The phenotype of each strain on X-Gal indicator plates is indicated as in Figure 2B.

(C) The data from (A) were quantitated and normalized to *ACT1* mRNA levels as in Figure 1C.

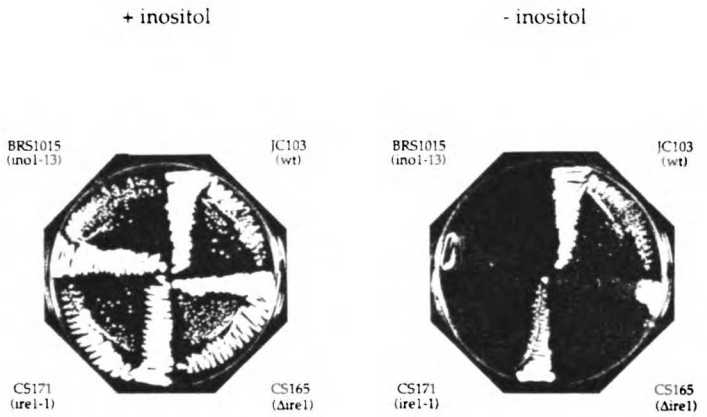

Figure 4. Inositol Auxotrophy of *ire1-1* and *Δire1* Cells

The yeast strains indicated were streaked for single colonies on media containing either 100 μg/ml inositol or no inositol. Plates were incubated at room temperature for 3 days and then photographed.

had a bacterial transposon, Tn10LUK, inserted at random positions (see Experimental Procedures). CS171 cells were transformed with this plasmid pool. Colonies containing plasmid were selected and then screened for complementation of the CS171 phenotype by replica-plating onto X-Gal–tunicamycin indicator plates. Five independent pJC012::Tn10LUK plasmids were isolated that were unable to complement the CS171 defect, presumably because the transposon had inserted within the region of the plasmid insert required for complementation. We mapped the position of these transposon insertions by restriction enzyme digestion and found that all five insertions were clustered in a 2.5 kb segment of the 9 kb insert (Figure 3A). Moreover, when a 5 kb XhoI–HindIII fragment of pJC012 (Figure 3A, X→H) that includes the essential region identified by the transposon insertion was subcloned into a low copy plasmid (generating plasmid pCS110), it was found to be sufficient to restore the tunicamycin-dependent induction of *KAR2* mRNA in CS171 cells (Figure 3B, compare lanes 3 and 4). As expected, the control plasmid lacking the insert had no effect (Figure 3B, lanes 1 and 2).

These results strongly suggested that the transposon insertions disrupted a gene sufficient to complement the defect in CS171 cells. Using DNA primers complementary to regions close to the ends of the transposon, we determined the nucleotide sequences of regions flanking the transposon insertion sites (Figure 3A, arrows). A comparison of these sequences with GenBank entries revealed that all five transposition events disrupted the coding region of a previously sequenced gene, *IRE1* (Nikawa and Yamashita, 1992; GenBank accession number Z11701). The restriction map of the 5 kb insert in pCS110 is also in complete agreement with the published sequence of *IRE1*.

CS171 Cells Are Mutant in the *IRE1* Gene

To confirm that CS171 indeed has a defective *IRE1* gene, we first disrupted the chromosomal copy of *IRE1* in JC103 cells. In the resulting strain, CS165, approximately 70% of the *IRE1* coding sequence was removed and replaced by the *URA3* gene. The integration of the selectable marker at the expected locus and the concomitant disruption of *IRE1* were confirmed by Southern blot. CS165 cells are viable, consistent with the previous finding that *IRE1* is a nonessential gene under normal growth conditions (Nikawa and Yamashita, 1992).

When we analyzed the phenotype of CS165 cells on X-Gal–tunicamycin indicator plates, we found that the colonies remained white, indicating that the UPRE-driven *lacZ* reporter gene was not induced upon tunicamycin treatment. Moreover, analysis by S1 nuclease protection showed that neither *KAR2* nor *PDI1* transcription was induced in CS165 cells after tunicamycin treatment (Figures 3B and 3C). Thus, cells bearing the null allele of *IRE1* are defective in the unfolded protein response pathway. We next crossed CS165 cells with a strain carrying our original mutation, CS172 (isogenic to CS171, but *MATα*), generating diploid strain CS181. CS181 cells are also white on indicator plates and do not induce *KAR2* transcription upon tunicamycin treatment, as determined by S1 nuclease protection (data not shown). This indicates that the *ire1* disruption and the gene defective in our original isolate affect the same complementation group. Finally, CS181 cells were sporulated, the resulting tetrads were dissected, and the dissection products were analyzed on X-Gal–tunicamycin and X-Gal–2-deoxyglucose indicator plates. Of 48 tetrads analyzed, each of which gave rise to four viable spores, the white phenotype on indicator plates always segregated 4:0. This demonstrates that *IRE1* is allelic to the gene defective in CS171 cells, and we henceforth refer to the mutant allele in CS171 cells as *ire1-1*.

The *IRE1* gene was originally cloned by complementation of a yeast mutant auxotrophic for inositol (Nikawa and Yamashita, 1992). Although at present we can only speculate on how the two phenotypes (the requirement for inositol and the inability to induce the unfolded protein response) are related (see Discussion), the reported inositol auxotrophy of *ire1*$^-$ cells provided us with an additional phenotype to confirm that CS171 cells are defective in *IRE1*. As shown in Figure 4, both CS171 and CS165 cells, but not the parent JC103 cells, require inositol for growth. The inositol auxotrophy of CS165 cells is as severe as

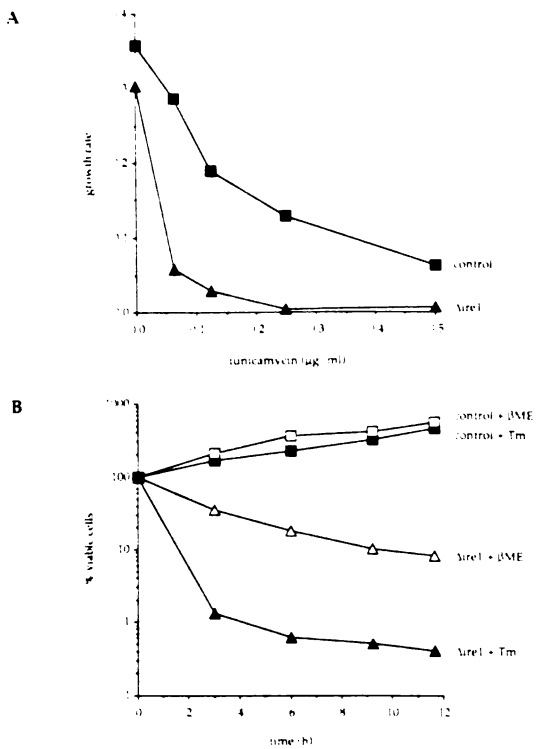

Figure 5. *Δire1* Cells Are Supersensitive to Tunicamycin and β-Mercaptoethanol

Tunicamycin and β-mercaptoethanol were added to JC103 and CS165 cultures growing in midlog phase.

(A) Growth rate of JC103 (closed squares) and CS165 (*Δire1*) (closed triangles) cells versus tunicamycin concentration. The growth rate is defined as the inverse of the doubling time for each strain at a given concentration of tunicamycin. The doubling time for cells in each culture was determined by the change in optical density at 600 nm that occurred between 7.5 hr and 10.75 hr of growth in the presence of tunicamycin.

(B) Cell viability was determined for strains JC103 and CS165 at various times after the addition of tunicamycin (to 0.125 μg/ml) or β-mercaptoethanol (to 15 mM). Aliquots from cultures were removed at different time points, and cells were plated on rich media. The fraction of viable cells was determined by counting colonies after 3 days of growth at 25°C. The viability of untreated cells at time 0 was set at 100%. The data were collected from cultures treated in parallel. Each data point represents the average of at least two determinations.

that of *ino1*⁻ cells, which are defective in an enzyme essential for inositol biosynthesis (Donahue and Henry, 1981). CS171 cells grow slightly better than CS165 and *ino1*⁻ cells on plates lacking inositol, indicating that, with regard to the inositol requirement, *ire1-1* is a slightly weaker allele than the *IRE1* disruption. Importantly, the addition of extra inositol to X-Gal–tunicamycin indicator plates does not cause CS165 cells to turn blue, indicating that the failure of these cells to induce transcription of the *lacZ* reporter gene is not due to the low concentration of inositol in X-Gal–tunicamycin plates.

IRE1 Is Required to Protect Cells from the Accumulation of Unfolded Proteins in the ER

With the exception of the requirement for inositol, *IRE1* seems to be dispensable for normal cell growth: cells deleted for *IRE1* grow at approximately the same rate in rich medium as wild-type cells (Figure 5A, point at Tm = 0) and are not sensitive to either low or elevated temperatures (data not shown). We wished to determine whether cells impaired in *IRE1* function are more sensitive to the specific stresses caused by the accumulation of unfolded proteins in the ER lumen. To this end, we tested the sensitivity of wild-type and CS165 cells to tunicamycin and β-mercaptoethanol, a compound that induces BiP in mammalian cells (Whelan and Hightower, 1985). β-Mercaptoethanol also induces UPRE-dependent transcription in yeast: JC103 cells are blue on X-Gal–β-mercaptoethanol plates. This response requires *IRE1* because CS171 and CS165 cells are white on X-Gal–β-mercaptoethanol plates.

The data in Figure 5 demonstrate that growth of CS165 cells was inhibited by low tunicamycin concentrations (Figure 5A, triangles) that only slightly impair the growth of JC103 control cells (Figure 5A, squares), indicating that *ire1*⁻ cells are hypersensitive to tunicamycin. A qualitatively similar effect was observed when cells were grown in medium containing β-mercaptoethanol.

To distinguish whether these treatments lead to growth arrest or cell death, CS165 cells and wild-type JC103 cells were incubated in medium containing low concentrations of either tunicamycin or β-mercaptoethanol. After various lengths of time in the presence of the drugs, cells from each culture were plated on rich medium, and the number of viable cells was determined by colony-forming ability. The addition of either compound led to rapid cell death of CS165 cells (Figure 5B, triangles) but did not significantly impair the viability of the control JC103 cells (Figure 5B, squares). We conclude from this experiment that *IRE1* function is required to protect cells from the stress resulting from the accumulation of unfolded proteins in the ER and that this stress, in the absence of the protective unfolded protein response, is lethal to the cells. Hence, under conditions where unfolded proteins accumulate in the ER, *IRE1* is essential for cell viability.

Ire1p Is a Transmembrane Protein with a Cytosolic Kinase Domain

The data presented here suggest that the *IRE1* gene product, Ire1p, is an essential component in the unfolded protein response pathway. *IRE1* encodes a protein with a predicted molecular mass of 127 kd that bears a classic N-terminal signal sequence and has an internal stretch of 29 hydrophobic amino acids that is likely to be a single membrane-spanning region (Figure 6A). These features suggest that the N-terminal half of Ire1p is translocated into the ER lumen during its biogenesis and that the C-terminal half of the protein is exposed in the cytosol. Interestingly, the C-terminal portion of Ire1p contains a 300 amino acid domain that has the sequence characteris-

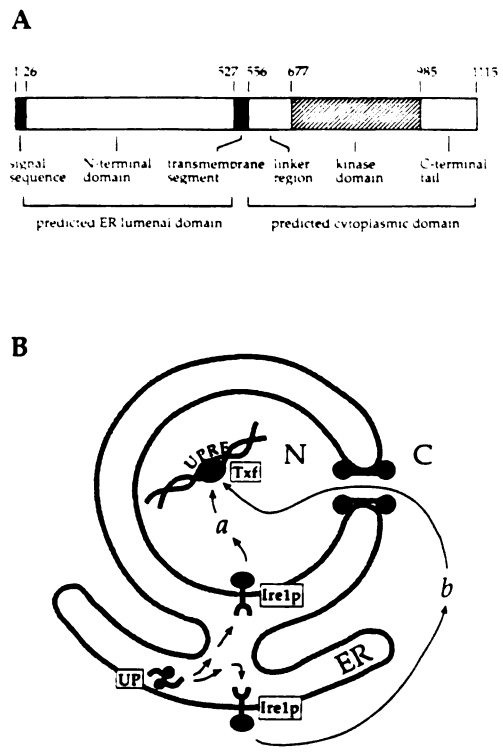

Figure 6. Predicted Domain Structure of Ire1p and Models for the Role of Ire1p in the Unfolded Protein Response Pathway

(A) A linear representation of Ire1p as deduced from the nucleotide sequence of the *IRE1* gene.

(B) Two possible routes (a and b) are depicted, along which the signal transmitted by the unfolded protein response pathway might travel. The routes are distinguished by the subcellular location of Ire1p. In route a, the ER membrane and the nuclear envelope are a continuous membrane system, and the kinase domain of Ire1p may face into the nucleus (N), where it transduces the signal generated by unfolded proteins (UP) across the inner nuclear membrane; activation of the kinase would lead to UPRE-dependent transcription, presumably by modulating the activity of a transcription factor (Txf). In route b, alternatively, the kinase domain of Ire1p may be in the cytoplasm (C), where its activation leads to a signal that traverses the nuclear envelope, most likely by traveling through nuclear pores.

tics of a serine/threonine protein kinase (Hanks et al., 1988; Nikawa and Yamashita, 1992). Other regions of Ire1p are not similar in sequence to any known protein. Thus, Ire1p appears to have the topology of a membrane protein kinase similar to that of many growth factor receptors in higher eukaryotic cells.

Discussion

We have identified a gene required for the unfolded protein response pathway in yeast and have shown that it is the previously identified gene *IRE1*. The sequence of Ire1p suggests that it is a serine/threonine protein kinase similar in structure to receptor kinases involved in transmembrane signaling. Ire1p thus belongs to a class of transmembrane kinases that, to date, includes only five other proteins: the activin and transforming growth factor β type II receptors from vertebrates (Mathews and Vale, 1991; Lin et al., 1992), the *daf-1* gene product from Caenorhabditis elegans (Georgi et al., 1990), the TMK1 kinase from Arabidopsis (Chang et al., 1992), and the Zea mays protein kinase 1 from maize (Walker and Zhang, 1990). Ire1p is the only known transmembrane kinase in yeast.

Because the accumulation of unfolded proteins in the ER lumen sends a signal to activate transcription in the nucleus and because of the similarity of Ire1p to plasma membrane receptors involved in other signaling events, we propose that Ire1p transmits the unfolded protein signal across the membrane surrounding the ER lumen. Ire1p could transmit the signal directly to the nucleus (Figure 6B, route a) or to the cytosol to components that then enter the nucleus through nuclear pores (Figure 6B, route b). Thus, we predict that Ire1p is localized either in the inner nuclear membrane or in the ER membrane. These possibilities can be readily distinguished by localization studies once antibodies that recognize Ire1p become available.

If Ire1p functions as proposed in Figure 6B, it must detect unfolded proteins in the ER lumen. It is likely that BiP plays a key role in the detection process. A growing body of evidence suggests that in both yeast and mammalian cells, the concentration of free BiP in the ER is monitored: when the concentration of free BiP falls, transcription of the genes encoding ER resident proteins is induced via the unfolded protein response pathway (Dorner et al., 1992; Hardwick et al., 1990; Kohno et al., 1993; Ng et al., 1992). It is possible that Ire1p senses the level of free BiP in the ER lumen directly. The activation of transmembrane receptor kinases is thought to involve their dimerization (Ullrich and Schlessinger, 1990; Hemmati-Brivanlou and Melton, 1992), and, by analogy, the Ire1p kinase may also be activated in this manner. One hypothesis is that when the level of free BiP in the ER is high, BiP binds the ER lumenal domain of Ire1p, thereby preventing dimerization and keeping Ire1p in an inactive state. In contrast, under conditions that cause the accumulation of unfolded proteins in the ER, preferential binding of BiP to unfolded proteins rather than to Ire1p may permit the dimerization of Ire1p. Dimerization would activate the kinase and transmit a signal via phosphorylation of nuclear or cytosolic proteins. In turn, the Ire1p-initiated phosphorylation events would activate UPRE-dependent transcription of genes in the nucleus. This simple model is consistent with the available data and with current ideas about transmembrane kinase function; however, we recognize the possibility that the pathway may be more complex.

The identification of *IRE1* as an essential gene in the unfolded protein response pathway has allowed us to address a number of issues regarding the physiological role of the pathway. For example, we have shown that *IRE1* is not essential for normal growth in rich medium. However, when cells are challenged with compounds that cause unfolded proteins to accumulate in the ER, we observe

that *ire1*⁻ cells die quickly. By contrast, *IRE1*⁺ cells remain viable under the same conditions. These observations demonstrate unambiguously that the unfolded protein response pathway is required for cell survival under conditions of stress in the ER.

We have also shown that *PDI1* transcription is induced in response to unfolded proteins in the ER. This is in contrast with arguments made by others that *PDI1* and *KAR2* are not coregulated (Tachibana and Stevens, 1992). Moreover, Ire1p is required for the tunicamycin-dependent induction of both *KAR2* and *PDI1* transcription, suggesting that the two genes are regulated by the same pathway or by pathways that are at least partially overlapping. While this work was in progress, another ER resident protein, Eug1p, was identified and shown to be related to PDI. Interestingly, transcription of *EUG1* is also induced by the accumulation of unfolded proteins in the ER and there is a UPRE-like sequence in the *EUG1* promoter (Tachibana and Stevens, 1992). Thus, as has been postulated for mammalian cells, it seems likely that the unfolded protein response pathway in S. cerevisiae operates on a set of genes that encode ER resident proteins.

Finally, with the identification of *IRE1*, we have discovered a surprising link between the unfolded protein response pathway and inositol metabolism. In yeast, phosphatidylinositol is a major constituent of membranes, and free inositol levels play a central role in the regulation of phospholipid synthesis (White et al., 1991). This suggests the possibility that the regulation of synthesis of ER resident proteins and the regulation of phospholipid biogenesis may be coupled. Such a link seems reasonable because as a cell synthesizes more ER proteins, it might also need to produce more ER membrane, and Ire1p may coordinate the two processes.

Experimental Procedures

Media and Genetic Methods

YPD (complete) and synthetic minimal media are described by Sherman (1991). X-Gal indicator plates are described by Chien et al. (1991) and contained 40 mg/ml of X-Gal (Biosynth International, Skokie, Illinois) and either 1 μg/ml tunicamycin (Boehringer Mannheim, Indianapolis, Indiana), 10 mM 2-deoxyglucose, 15 mM β-mercaptoethanol (Sigma Chemical Company, St. Louis, Missouri), or 1 mM deoxycorticosterone (gift of K. Yamamoto, University of California, San Francisco). Minimal medium lacking inositol was made as described by Culbertson and Henry (1975). When added, inositol (myo-inositol; Sigma, St. Louis, Missouri) was at a final concentration of 100 μg/ml. Yeast transformations were performed by lithium acetate procedures (Ito et al., 1983; Elble, 1992). Plasmids were isolated from yeast as described by Strathern and Higgins (1991).

Plasmid Construction

To make the UPRE–*lacZ* reporter construct, the following double-stranded oligonucleotide bearing the UPRE (Mori et al., 1992) was synthesized. Its protruding termini are complementary to those generated by the restriction endonucleases BglII and XhoI:

5′-GATCTGTCGACAGGAACTGGACAGCGTGTCGAAAAAGC-3′
3′-ACAGCTGTCCTTGACCTGTCGCACAGCTTTTTCGAGCT-5′

One copy of this oligonucleotide was inserted into the BglII and XhoI sites of plasmid pGA1696 to create plasmid pJC005. pGA1696 is a plasmid identical to pGA1695, described by Sorger and Pelham (1987), but it has a 5′ BglII site and a 3′ XhoI site. It is a derivative of plasmid pLG-Δ178 (Guarente and Mason, 1983) and bears the Escherichia coli *lacZ* gene under the control of a disabled *CYC1* promoter.

To create reporter plasmids that could integrate into the yeast genome, a 4 kb BglII–Tth111I fragment of pJC005 containing the UPRE, disabled *CYC1* promoter, and *lacZ* gene sequences was subcloned into the yeast-integrating vectors pRS303 (*HIS3*) and pRS305 (*LEU2*; Sikorski and Hieter, 1989) to replace the BamHI–NaeI fragments in both plasmids. The resulting plasmids were named pJC002 (*HIS3* UPRE–*lacZ*) and pJC003 (*LEU2* UPRE–*lacZ*).

pCS110 was constructed by inserting the XhoI–HindIII fragment bearing *IRE1* (see Figure 3) into the low copy *TRP1*⁺ yeast vector pRS314 (Sikorski and Hieter, 1989) to replace the XhoI–EcoRI fragment in the polylinker.

Yeast Strains

Yeast strains used in this study are listed in Table 1. Reporter strains JC103 and JC104 were constructed by homologous recombination of both pJC002 (cut with NheI) and pJC003 (cut with BstEII) into the chromosomal copies of the *HIS3* and *LEU2* genes of strains W303-1A and W303-1B. Correct integration of the reporter genes was confirmed by Southern blot.

To disrupt the *IRE1* gene in strain CS165, a plasmid, pCS109, was constructed in which the 2.1 kb KpnI fragment of *IRE1* is replaced by the *URA3* gene. The haploid reporter strain JC103 was transformed with the XhoI–HindIII fragment from pCS109 that bears the disrupted *IRE1* gene, and cells were plated on *URA*⁻ plates that had been supplemented with inositol. Inositol was added to a final concentration of 20 μg/ml in the selection plates to allow growth of the inositol auxotrophs that were created by disrupting the chromosomal copy of *IRE1*. The disruption of the *IRE1* gene with *URA3* in strain CS165 was confirmed by Southern blot.

RNA Analysis

RNA Preparation

Cultures were grown in log phase in the absence or presence of tunicamycin. Yeast cells from a 25 or 50 ml sample from each culture were collected by centrifugation and washed with water, and the cell pellets were stored at −80°C until processed for RNA. Whole-cell RNA was made according to a scaled-down version of the hot phenol method described by Kohrer and Domdey (1991).

Probes

The probes used for S1 analysis protect the following sequences: bases 375–561 of *KAR2* (Rose et al., 1989), bases 878–1016 of *ACT1* (Ng and Abelson, 1980), bases 207–306 of *PDI1* (LaMantia et al., 1991), and bases 2231–2330 of *lacZ* (Kalnins et al., 1983). In a three-fragment ligation, DNA fragments bearing these sequences were cloned into the polylinker of the phagemid pUC119 (Vieira and Messing, 1987) along with a DNA fragment from Staphylococcus aureus nuclease that is not homologous to yeast or *lacZ* DNA sequences. To make end-labeled probes, oligonucleotides specific for the mRNA sequences were labeled with [γ-³²P]ATP, annealed to single-stranded plasmid bearing the sense strand of the sequences to be analyzed, and extended with the Klenow fragment of DNA polymerase. The double-stranded product was cut with a restriction enzyme and probes of discrete lengths bearing *KAR2*, *ACT1*, *PDI1*, or *lacZ* sequences fused to 150 bp of S. aureus nuclease sequences were isolated by preparative denaturing gel electrophoresis (Sambrook et al., 1989).

S1 Nuclease Protection Assay

S1 nuclease protection assays were carried out as described by Favaloro et al. (1980). In brief, 15 μg of RNA was hybridized at 37°C overnight to 0.01 pmol of each probe. S1 digestion was carried out at 18°C for 2 hr using 500 U/ml of S1 nuclease (Sigma, St. Louis, Missouri). Samples were then fractionated on polyacrylamide (8%) denaturing gels and analyzed by autoradiography and by quantitation using the PhosphorImager (Molecular Dynamics, Sunnyvale, California).

Primary Screen

Strain JC103 was mutagenized with ethyl methanesulfonate (Sigma, St. Louis, Missouri) to 30% survival according to the protocol of Lawrence (1991). Cells were plated onto YPD plates and allowed to grow at room temperature for 3 days. The colonies were then replica-plated onto X-Gal–tunicamycin indicator plates and incubated overnight at 30°C. The desired mutants failed to turn blue on the indicator plates

and were patched from the master YPD plates onto fresh YPD plates. They were rescreened on X-Gal–tunicamycin indicator plates.

Secondary Screens

2-Deoxyglucose

Yeast patches were replica-plated from YPD onto X-Gal–2-deoxyglucose plates. Mutants that turned blue on these indicator plates were discarded.

Tunicamycin Resistance

Roughly equivalent numbers of yeast cells from each mutant strain were transferred to YPD plates containing 1 μg/ml tunicamycin by spotting diluted suspensions of cells onto the plates. The plates were then incubated at room temperature for 3 days in the dark. Wild-type JC103 failed to grow at this concentration of tunicamycin, and any mutants that grew equally well as the tunicamycin-resistant strain JRY318 (Barnes et al., 1984) were discarded.

Glucocorticoid Response

To identify those mutants that were able to activate transcription of a reporter construct regulated by the glucocorticoid response element, mutant cells were transformed with two plasmids. pRS314/F260SGR bears a mutant version of the GR driven by the glyceraldehyde-3-phosphate dehydrogenase promoter in a low copy *TRP1*⁺ yeast vector. The F620S mutation in the GR seems to increase the affinity of the receptor for its ligand and is described by Garabedian and Yamamoto (1992). The reporter plasmid pΔS26X (Schena et al., 1989) has three copies of the glucocorticoid response element fused to a disabled *CYC1* promoter and the *lacZ* gene in a high copy *URA3*⁺ yeast vector. Both plasmids were provided by S. Bohen. When replica-plated onto X-Gal indicator plates containing the GR ligand deoxycorticosterone, yeast that can carry out the GR response turn blue. We discarded mutants that failed to turn blue in this assay.

Measuring Tunicamycin-Dependent KAR2 mRNA Induction

Cultures (25 ml) of mutant cells were grown to midlog phase at room temperature in YPD. Tunicamycin was added to a final concentration of 1 μg/ml, and cells were grown for 1 hr in the presence of the drug before being harvested. Whole-cell RNA was isolated, and S1 nuclease protection experiments were carried out as described above. Mutants that induced expression of *KAR2* mRNA to similar levels as (control) JC103 cells were discarded.

Rescuing the CS171 Mutant Phenotype

Strain CS171 was transformed with a high copy yeast genomic library (Carlson and Botstein, 1982), and colonies were replica-plated to X-Gal–tunicamycin plates. One colony (out of 10,000 screened) turned blue, and the complementing plasmid, pJC012, was isolated. The 9 kb yeast genomic insert from pJC012 was subcloned into the low copy vector pRS314 (Sikorski and Hieter, 1989), and this new plasmid also complemented the CS171 defect. Tn10LUK mutagenesis of pJC012 was performed essentially as described in Huisman et al. (1987) except that pools of transposon-containing pJC012 were transformed into the *recA*–E. coli strain DH5α and amplified before being transformed into yeast. Plasmids containing integrated transposons were screened for their ability to complement the white phenotype of CS171 cells on X-Gal–tunicamycin plates. Ten plasmids that were unable to complement the defect were isolated from the yeast cells, transformed into DH5α cells, and reamplified. Restriction digest mapping of the pJC012::Tn10LUK plasmids revealed five different integration events that clustered within a 2.5 kb segment in the genomic insert. DNA sequencing across the junctions of the Tn10 integrations was carried out using a primer oligonucleotide specific to the 5′ end of the Tn10LUK *lacZ* gene. The identity of *IRE1* was determined by comparison of these sequences with the GenBank data base. The accession number of the *IRE1* sequence is Z11701.

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Concluding Remarks

Shortly after this work was published, several more things were learned about Ire1p and the products of our genetic screen. They are described here.

First, we have shown, by immunoprecipitating Ire1p from ^{35}S-labelled *ire1-1* mutant cells (see Chapter III Materials and Methods), that the mutant Ire1p migrates faster than the wild-type protein during PAGE. This observation suggests that the *ire1-1* mutation produces a truncated Ire1p protein. Its mobility relative to wild-type suggests that the truncation occurs somewhere in the Ire1p kinase domain. We have not, however, confirmed this by sequencing *ire1-1*. Second, complementation group two from our screen is *SPT4*, which encodes a transcription factor thought to play a role in the transcription of many genes by affecting chromatin strucuture in the promoters of the genes (Malone et al., 1993; Swanson and Winston, 1992). Thus, although *SPT4* may play a role in UPRE-dependent transcription, its role is probably not specific to the unfolded protein response and we have not studied it any further. Third, completion of the nucleotide sequence of *S. cerevisiae* chromosome VIII revealed that *IRE1* is located on that chromosome (Johnston et al., 1994).

Contemporaneous with the work described in this chapter, Mori *et al.* (1993) set up a similar color assay for the unfolded protein response and carried out a similar genetic screen for *S. cerevisiae* mutants. Instead of using the *KAR2* UPRE linked to the *cyc1* promoter to drive expression of their reporter, they fused a near full-length *KAR2* promoter to *lacZ*,. This regulatory region was deleted for the *KAR2* heat shock element and GC-rich region but contained the UPRE. Rather than using tunicamycin to induce the unfolded protein response,

Mori *et al.* carried out their screen in cells bearing a temperature sensitive mutation in the *SEC53* gene (encoding phosphomannomutase) and looked for cells unable to turn blue at the semi-permissive temperature of 30°C. Despite these differences from our screening strategy, Mori *et al.* also identified Ire1p as a component of the unfolded protein response. They refer to *IRE1* as *ERN1* in their paper.

Further characterization of Ire1p

Mori *et al.* studied *ire1* mutant cells in some of the same ways in which we did, but they also carried out other experiments that further characterized the Ire1p kinase and its role in the unfolded protein response. In particular, they showed that *ire1* mutants are unable to induce transcription of *EUG1* in response to the accumulation of unfolded proteins in the ER (*FKB2* transcription has not yet been examined in *ire1* mutants) and that the *ire1* cells are capable of inducing transcription in response to other stimuli, such as heat shock, demonstrating that they are not generally defective for stress responses. Mori *et al.* found that the *IRE1* transcript itself is not very abundant and that its transcription is not induced either by the accumulation of unfolded proteins in the ER or by heat shock. Using strains that overexpress the Ire1p kinase and polyclonal antibodies directed against Ire1p, Mori *et al.* showed that Ire1p, as predicted, is an integral membrane protein oriented with its N-terminus inside a membrane-bound compartment. Finally, their analysis of mutations in Ire1p demonstrated that its kinase activity is required for the unfolded protein response. It has been shown in other kinases that single point mutations in the lysine residues that lie in the catalytic site diminish or inactivate phosphotransfer activity (Robbins et al., 1993; Ullrich and Schlessinger, 1990). Such mutations inactivate Ire1p as well. The

catalytic lysine in Ire1p lies at residue 702. Yeast strains bearing Ire1p mutants that have alanine or arginine substitutions at position 702 are defective for the unfolded protein response.

In the title and text of their paper, Mori *et al.* emphasize the "significant identity and similarity" between members of the cdk family of kinases (specifically *S. pombe* cdc2+ and *S. cerevisiae* Cdc28p) and Ire1p. It should be noted, however, that the Ire1p kinase domain is structurally unique. It is distantly related to the cdk group of kinases but has no close relative on the kinase tree (Steven Hanks, personal communication).

Refining the models for Ire1p activation

As stated previously, similarities between the structure of Ire1p and those of receptor transmembrane kinases suggest similarities in mechanism. Growth factor receptors are thought to be activated by oligomerization (Heldin, 1995). Ligand binding to the receptor induces oligomerization and activation of the kinase (Figure II-7a). By analogy, both Mori *et al.* and we proposed that the Ire1p kinase is also activated by oligomerization. A prediction of this proposal is that it should be possible to construct dominant negative mutations in Ire1p by making C-terminal truncations that lack a functional kinase domain but have intact ER-lumenal and transmembrane domains (Figure II-7b; Herskowitz, 1987). Indeed, when we made such a mutant (truncated just before the Ire1p kinase domain and expressed under control of the *GAL1* promoter; see Chapter III Materials and Methods for a more detailed description of this mutant), we found that it had a dominant negative effect on the unfolded protein response (Figure II-8). Cells grown in galactose, expressing the truncated Ire1p in addition to wild-type Ire1p,

do not carry out the unfolded protein response (lanes 3 and 4) while control cells, grown in galactose but not expressing dominant negative Ire1p, carry out a normal unfolded protein response (lanes 7 and 8). Mori *et al.* report a similar observation in their paper. Thus, it seems likely that the Ire1 kinase is activated by oligomerization.

The oligomerization of growth factor receptors causes autophosphorylation of the intracellular domain and association of the receptors with cytoplasmic proteins. This association leads to the assembly of an active signalling complex (Heldin, 1995; Ullrich and Schlessinger, 1990). If Ire1p and growth factor receptors do in fact operate by similar mechanisms, one would predict that activated Ire1p is phosphorylated and that it associates with other proteins involved in sending the "unfolded protein" signal.

There are at least two possible models to explain how Ire1p oligomerization might be regulated in response to the accumulation of unfolded proteins in the ER. In the first (Figure II-9a), when there are few unfolded proteins in the ER, Ire1p exists primarily as a monomer, with its kinase inactive. The accumulation of unfolded proteins in the ER produces a ligand that, when bound to Ire1p, causes oligomerization and activation of the kinase. The ligand in this case might be unfolded proteins themselves, unfolded proteins bound to BiP or to other ER-resident chaperones, or an as yet undetermined entity. A second possibility, mentioned briefly in the Discussion section of this chapter and diagrammed in Figure II-9b is that a chaperone such as BiP binds to Ire1p when there are few unfolded proteins in the ER and thereby prevents Ire1p from dimerizing. The chaperone dissociates from Ire1p when unfolded proteins accumulate in the ER, because the chaperone binds to unfolded proteins with

greater affinity or because the concentration of unfolded proteins is much greater than that of Ire1p.

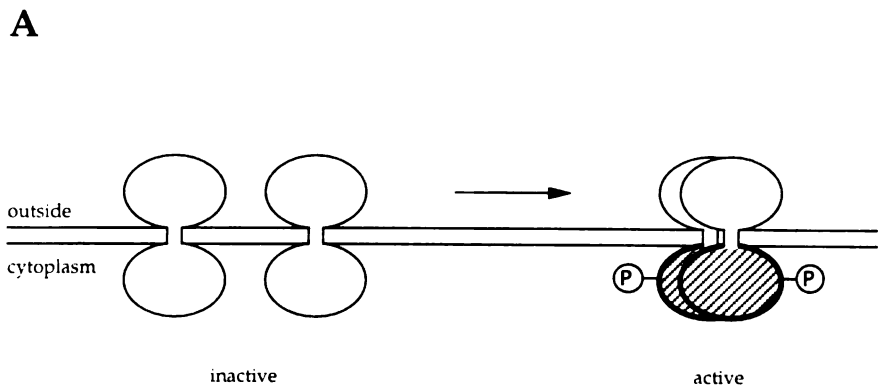
Although both models are consistent with evidence suggesting that BiP itself may play a role in signalling the accumulation of unfolded proteins in the ER, the second is more appealing because it is the simplest. This model is similar to a model that has been proposed to explain the activation of another stress response, the heat shock response. To activate transcription in response to heat shock, the heat shock factor (HSF) must trimerize (Perisic et al., 1989; Sorger and Nelson, 1989) It has been proposed that, at least in animal cells, one circumstance preventing this multimerization in the absence of heat shock might be the binding of cytoplasmic chaperone proteins, such as hsp70 and/or hsp90, to HSF monomers (see, for example Morimoto, 1993 and Sorger, 1991). According to this model, upon heat shock, the chaperones would bind the newly unfolded proteins in the cytoplasm, thereby freeing HSF and allowing it to trimerize.

There are other similarities between the two stress responses. Both are regulated at the level of transcription and both involve phosphorylation; phosphorylation is required for the activation of HSF (Larson et al., 1988; Sorger et al., 1987). Furthermore, at least in yeast, both UPRF-1 (the protein(s) that bind to the UPRE; Mori et al., 1992) and HSF bind to their respective promoter elements constitutively, that is, both in the absence and in the presence of the stress response (Mori et al., 1992; Sorger et al., 1987). Because of these parallels between the two stress reponses, it seems possible that by studying one, we may learn about the other. For example, by identifying the ligand for Ire1p, we may learn not only how the cell can detect the accumulation of unfolded proteins in

the ER but, by extension, how cells detect unfolded proteins in other compartments as well.

In conclusion, the discovery that Ire1p, a transmembrane kinase, is involved in the unfolded protein response has refined our thinking about the signal transduction pathway that links the ER and the nucleus. Ire1p is probably the component of the system that transduces the "unfolded protein" signal out of the ER. The requirement for Ire1p in both the unfolded protein response and inositol prototrophy suggests that Ire1p may be part of a general mechanism for ER maintenance. Interorganellar regulatory networks that mediate communication between the nucleus and either mitochondria or chloroplasts have been described (for example, see Liao and Butow, 1993 and Susek et al., 1993). The unfolded protein response may provide a paradigm for these and other as yet-undiscovered intracellular signalling pathways.

Figure II-7: (a) Receptor transmembrane kinases are activated by dimerization. Upon binding of ligand to a receptor on the cell surface, receptors dimerize, bringing the two cytoplasmic domains together. This leads to activation of the kinase (grey region) by autophosphorylation. **(b)** A dominant negative mutant prevents activation. Truncation mutants missing the kinase domain (black receptor) are able to bind ligand and to dimerize with full-length receptor, creating a complex that cannot be activated.


A
outside
cytoplasm
P
P
inactive
active

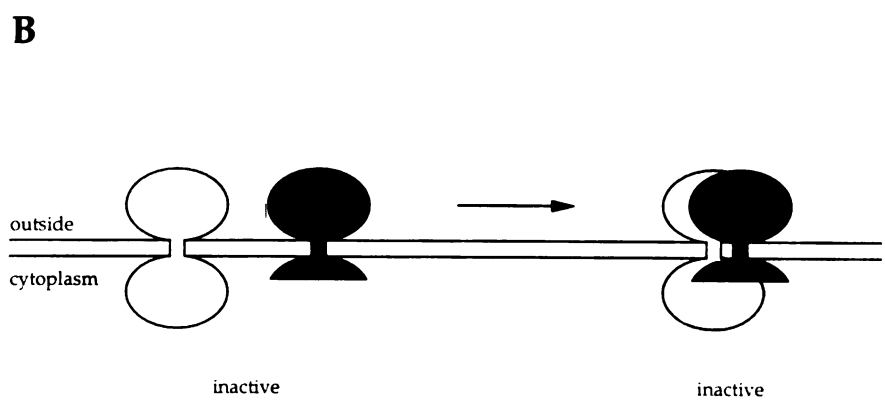

B
outside
cytoplasm
inactive
inactive

Figure II-7

Figure II-8: A truncated Ire1p lacking the kinase domain has a dominant negative phenotype with respect to the unfolded protein response. **(a)** S1 protection analysis was performed on RNA isolated from strains CS191 and CS192. In addition to its wild-type chromosomal copy of *IRE1*, strain CS191 carries a CEN/ARS plasmid bearing truncated, "dominant negative" (Dom. neg.) *ire1* under control of the *GAL1* promoter. The control strain, CS192, is identical except that its CEN/ARS plasmid lacks any *IRE1* sequences. Cells were grown to mid-log phase for 6 hours in either dextrose, to repress transcription of the *GAL1* promoter, or galactose, to induce transcription of the *GAL1* promoter. Tunicamycin was added or not, and the cells were grown for an additonal hour before RNA was harvested. **(b)** The data from (a) were quantitated and normalized to *ACT1* mRNA levels as in Figure II-1c.

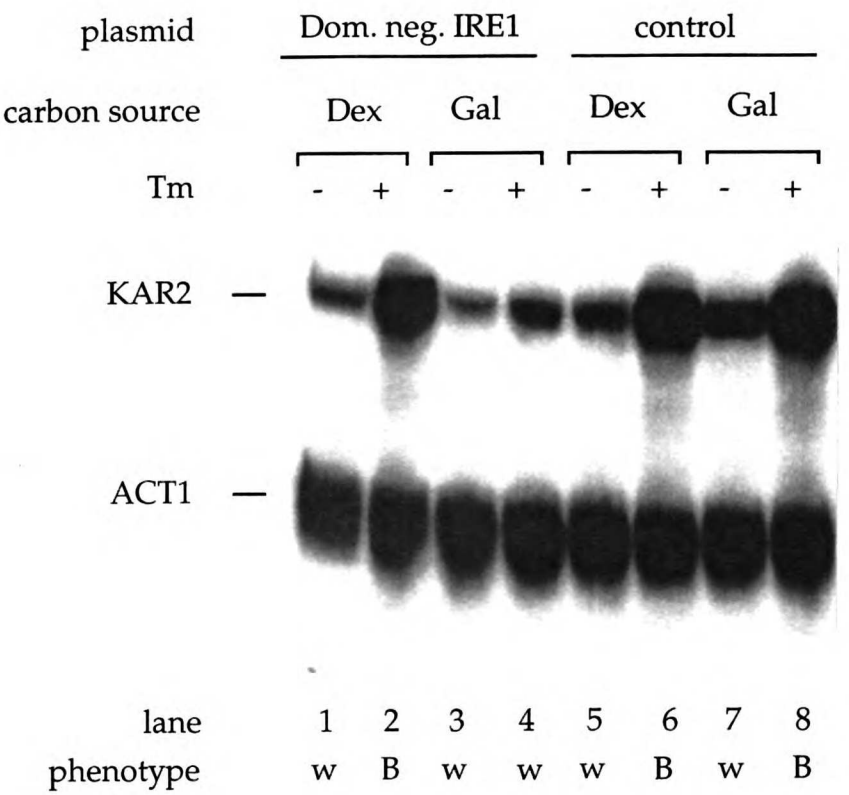

plasmid
Dom. neg. IRE1
control
carbon source
Dex
Gal
Dex
Gal
Tm
- + - + - + - +
KAR2
ACT1
lane
1 2 3 4 5 6 7 8
phenotype
w B w w w B w B

Figure II-8a

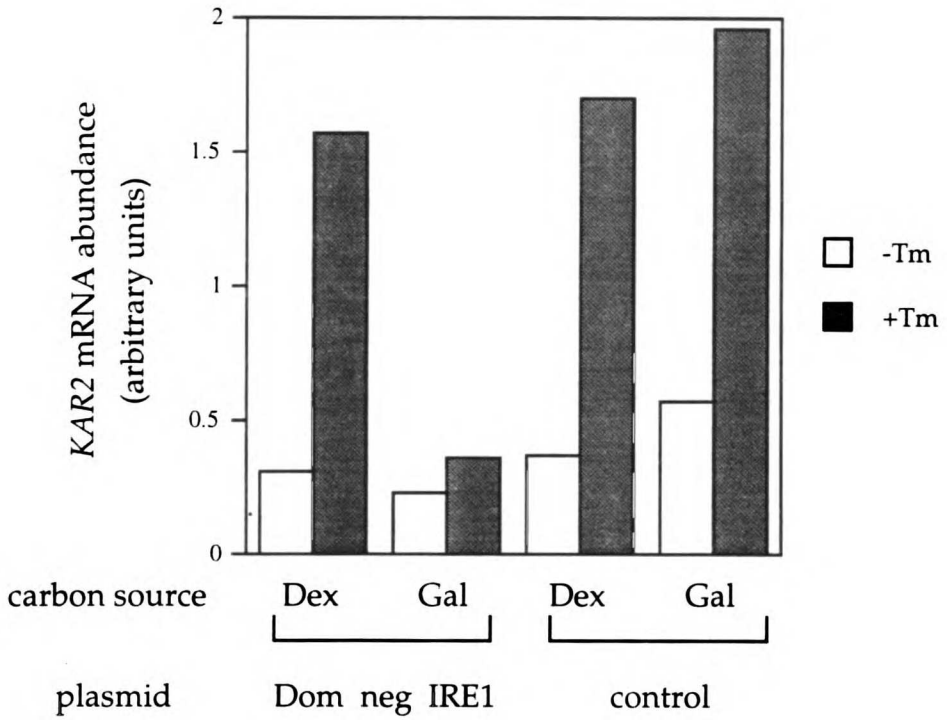


Figure II-8b

Figure II-9: Models for Ire1p activation. **(a)** When unfolded proteins accumulate in the ER, a ligand for Ire1p is generated that binds to Ire1p and induces it to oligomerize, thus activating the kinase. **(b)** When the level of unfolded proteins in the ER is low, Ire1p is occupied by a ligand that prevents its oligomerization. When unfolded proteins accumulate in the ER, the ligand is released from Ire1p, because it binds preferentially to unfolded proteins, allowing Ire1p to oligomerize.

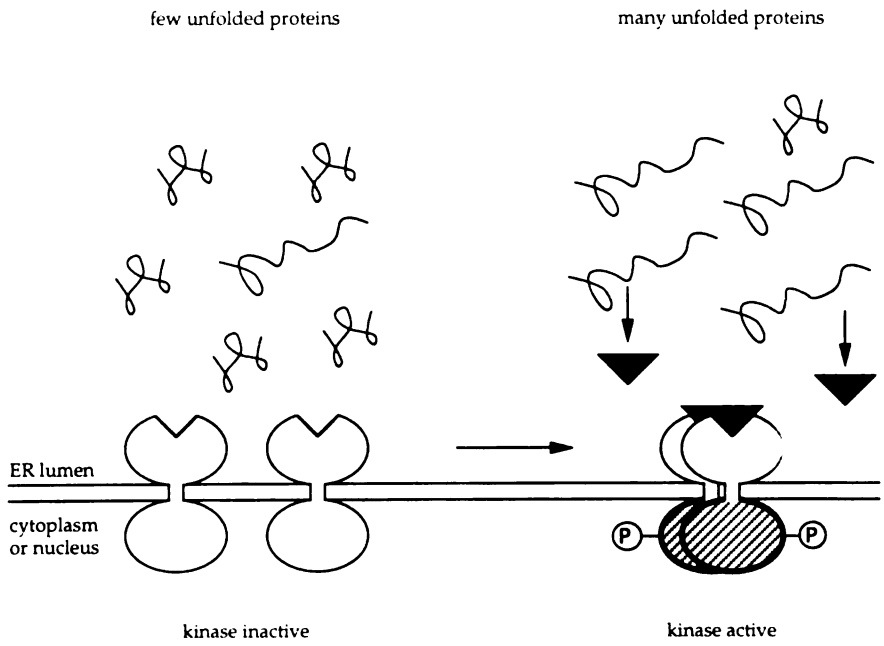

few unfolded proteins
many unfolded proteins
ER lumen
cytoplasm
or nucleus
P
P
kinase inactive
kinase active

Figure II-9a

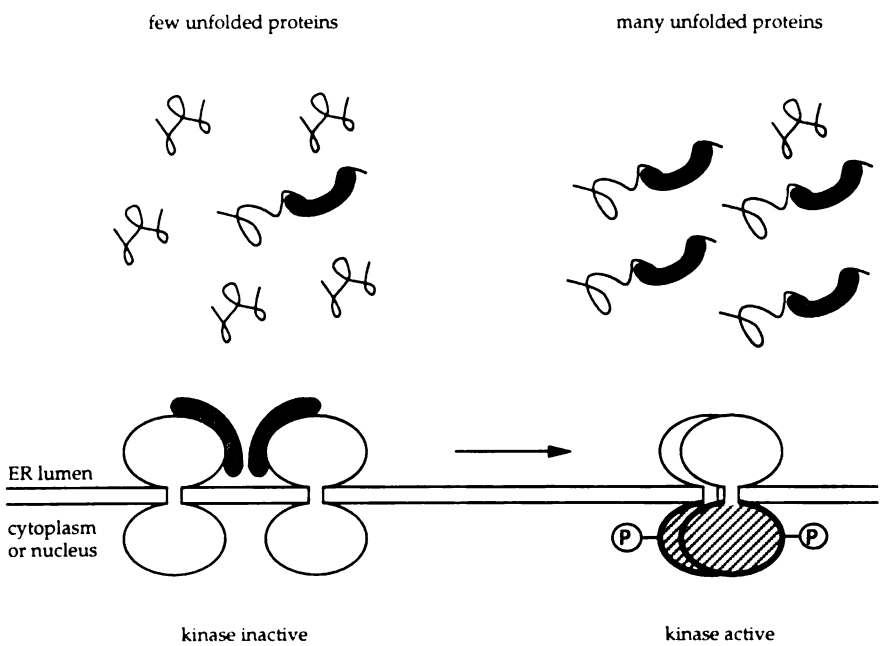

few unfolded proteins
many unfolded proteins
ER lumen
cytoplasm
or nucleus
P
P
kinase inactive
kinase active

Figure II-9b

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Chapter III:

Oligomerization and phosphorylation of Ire1p

Introduction

The similarity in the structure of Ire1p to the structures of receptor transmembrane kinases suggests that they may function by similar mechanisms. In particular, it is possible that Ire1p oligomerizes as unfolded proteins accumulate in the ER, that it is phosphorylated in *trans* by other Ire1p molecules as a result of oligmerization, and that both oligomerization and phosphorylation of Ire1p are required for activation of the unfolded protein response. In this chapter, we test these possibilities using a combination of molecular genetic and biochemical approaches.

Results

As a first step in characterizing the role of the Ire1p kinase in the unfolded protein response, we produced polyclonal antibodies directed against two non-overlapping domains of Ire1p: α-Ire1Nterm, directed against amino acids 20 to 521, and α-Ire1tail, directed against amino acids 976 to 1115 (Figure III-1). Although the antibodies do not detect Ire1p on Western blots of wild-type yeast, the antibodies do immunoprecipitate Ire1p from cells expressing wild-type levels of the protein (Figure III-2a), and they recognize Ire1p on Western blots of extracts made from cells overexpressing the protein. As has been proposed previously (Mori et al., 1993), these results suggest that Ire1p is not very abundant. However, it appears that the polyclonal antibodies do not have very high affinity for Ire1p (see Materials and Methods and Appendix A). Thus accurate determination of the abundance of Ire1p awaits quantitative studies.

There is a potential nuclear localization signal in the C-terminal half of Ire1p, located after the transmembrane domain. It has been proposed that this signal might localize the kinase domain to the nucleus following the accumulation of unfolded proteins in the ER (Mori et al., 1993). This could occur as a result of the movement of Ire1p in the plane of the membrane to the inner nuclear membrane. Alternatively, nuclear localization could occur if the C-terminal half of Ire1p was cleaved from the rest of the protein in the cytoplasm and was imported into the nucleus through a nuclear pore. One precedent for this possibility has been described in which the N-terminus of a membrane-bound transcription factor, SREBP-1, is released by a regulated proteolysis event and thus freed to translocate to the nucleus (Wang et al., 1994). To examine whether proteolysis of Ire1p occurs upon induction of the unfolded protein response, Ire1p was immunoprecipitated from extracts of wild-type cells that had been labelled with ^{35}S-methionine and grown in the presence or absence of tunicamycin. 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 (in fact, the mobility of Ire1p may decrease after treatment with tunicamycin; Figure III-2a, lanes 2 and 8). We also observed that the levels of Ire1p do not change with the induction of the response. Thus, the unfolded protein response is probably not mediated by proteolysis of Ire1p. However, other post-translational modifications of the kinase, such as phosphorylation (see below), may occur.

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

Like Ire1p, many receptor kinases have protein tails that lie C-terminal to their kinase domains. In most cases, these tails interact with downstream components of the signalling pathway. The function of the Ire1p tail is unknown; sequence comparisons with 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 domains (Derynck, 1994; Massague et al., 1994). At least in the case of the type II TGF-β receptor, deletion of the tail does not diminish signalling activity (Wrana et al., 1994).

To investigate the function of the Ire1p tail, we constructed a mutant kinase that is deleted for the C-terminal 133 amino acids (Ire1p-tailless; Figure III-1). Like members of the cdk kinase family, the coding sequence of this truncated Ire1p ends less than ten amino acids after the kinase domain. When *ire1*-tailless is introduced into yeast cells, replacing wild-type *IRE1* as the only copy of the gene, cells can no longer carry out the unfolded protein response as determined by color assays on X-Gal-tunicamycin plates and by S1 nuclease protection assays (Figure II-2b and 2c). Furthermore, cells bearing Ire1p-tailless 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-tailless is expressed as well as full-length Ire1p (Figure III-2a, lanes 3 and 4), there are two explanations for this result. One is that the C-terminal tail is required for Ire1p kinase activity and that the tailless mutant does not carry out the unfolded protein response simply because its kinase is non-functional. A more interesting possibility is that the kinase is active in the tailless mutant and that the tail is required for

propagation of the unfolded protein signal. Thus, tailless Ire1p might be unable to interact with downstream components of the signalling pathway.

Intragenic complementation of some *ire1* alleles

Because oligomerization of Ire1p may be required for activation of the unfolded protein response, we asked whether a tailless mutant, bearing a wild-type kinase domain, could be complemented by either of two full-length *ire1* alleles that have wild-type tails but mutant kinase domains with single amino acid substitutions in the catalytic lysine. The two kinase mutants, Ire1p-K702A and Ire1p-K702R, have been described previously (Mori et al., 1993; Chapter II), and each has a different effect on the unfolded protein response (Figure III-3a and 3b). Cells bearing Ire1p-K702A have the same phenotype as control cells that lack Ire1p completely: they are severely compromised in their ability to carry out the unfolded protein response and are inositol auxotrophs. Cells bearing Ire1p-K702R carry out a diminished response to the accumulation of unfolded proteins in the ER that is about 25% of the wild-type response. Consistent with this finding, the K702R mutants can grow in the absence of inositol but do so at a slightly slower rate than wild-type cells. Although it has not been demonstrated, we assume that the unfolded protein response phenotypes in these mutants reflect the activity of the Ire1p kinase in each. Ire1p-K702A most likely has an inactive kinase whereas that of Ire1p-K702R is partially acitve.

To determine whether the mutant alleles could compensate for each other when combined, cells bearing Ire1p-tailless were transformed with a full length version of either wild-type, K702A, or K702R Ire1p. The ability of

the transformants to carry out the unfolded protein response was assayed both on X-Gal-tunicamycin plates and by S1 nuclease protection. The results are presented in Figures III-3c and 3d. Interestingly, the combination of the K702R mutant and the tailless mutant in the same cell produce an unfolded protein response that is much greater than that in cells bearing only Ire1p-K702R and that is almost as strong as that produced when wild-type Ire1p and tailless are combined (Figure III-3c, lanes 2 and 6). However, the K702A mutant has no effect on the tailless phenotype: the level of induction of the response in Ire1p-K702A + Ire1p-tailless cells is essentially the same as that in cells bearing only the tailless construct (Figure III-3c, lanes 4 and 8). The complementation of the K702R mutant and Ire1p-tailless is also evident in the phenotypes of the cells on media lacking inositol: Ire1p-K702R + Ire1p-tailless cells grow while Ire1p-K702A+Ire1p-tailless cells do not (data not shown).

There are two straightforward explanations for this intra-allelic complementaion. The most interesting possibility is that the tailless Ire1p kinase is active but cannot signal because it lacks a tail and that oligomerization of the truncated and full-length proteins allows cross talk between the two and compensation for the defect in each (Figure III-4). In this scenario, one wild-type tail would be sufficient for transduction of the signal but two at least partially functional kinase domains would be required. The K702A mutant, whose kinase is probably less active than that of K702R, cannot "talk" to the tailless mutant and so complementation does not occ ". In fact, further experiments have shown that the Ire1p-tailless must have a wild-type kinase domain in order to complement: if either the K702A or the K702R mutation is introduced into Ire1p-tailless, the intragenic

complementation with full-length Ire1p-K702R is no longer observed (Figure III-5a, compare lanes 2, 4 , and 6).

A second, less appealing model to explain the intra-allelic complementation must also be considered. Although we believe that the phenotype of cells bearing full-length Ire1p with the K702R mutation is due to reduced kinase activity of the protein resulting from the point mutation, we cannot rule out the possibility that it is due to increased binding of a factor that negatively regulates the unfolded protein response. If this were the case, the intragenic complementation described above might be the result of titration of the negative factor by Ire1p-tailless, revealing almost full kinase activity of Ire1p-K702R. We feel this is unlikely because Ire1p-tailless is probably present in the cells at the same level as K702R: both are expressed from genes present in single (Ire1p-tailless) or low (Ire1p-K702R) copy number. Furthermore, it is unlikely that mutation of a single catalytic residue in the Ire1p-tailless kinase domain would alter the ability of truncated kinase to titrate negative factors away from K702R. However, as shown in Figure III-5a, such mutations do prevent Ire1p-tailless from complementing Ire1p-K702R. Finally, we have measured the unfolded protein response 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 might overcome the inhibition. We found that overexpressing Ire1p-K702R does not restore the unfolded protein response to wild-type levels: the response in such cells is less than 50% of that in cells overexpressing the wild-type kinase even though the proteins are expressed at similar levels in each strain (Figure III-5c and 5d, and data not shown).

Thus, our data argue against this second model and confirm the first, suggesting strongly that oligomerization and transphosphorylation of the Ire1p monomers is required for the unfolded protein response.

Truncated Ire1p associates with full-length Ire1p

Further evidence that the oligmerization of Ire1p is required for the unfolded protein response was obtained from biochemical experiments in which a truncated, dominant-negative version of Ire1p (Ire1p-DomNeg; see Figure III-1 and Chapter II) was shown to crosslink to full-length Ire1p. Strains bearing both Ire1p-DomNeg and wild-type Ire1p and a control strain bearing only wild-type Ire1p were labelled with ^{35}S methionine in the presence of tunicamycin and lysed under non-reducing conditions in the absence of detergent. The reversible crosslinker DSP was then added to the whole-cell extracts. After crosslinking, membranes were isolated, solubilized in SDS, and non-native immunoprecipitations were carried out with either α-Ire1tail, which binds only to full-length Ire1p and and not to Ire1p-DomNeg, or with α-Ire1Nterm, which binds to both versions of Ire1p. The products of the immunoprecipitations were run on reducing gels that reverse the crosslinker. The results, shown in Figure III-6, demonstrate that Ire1p-DomNeg can be crosslinked to wild-type Ire1p (lane 2). This experiment shows that the two proteins are closely associated in the whole-cell extract and demonstrates that oligomers of Ire1p are present in cells carrying out the unfolded protein response.

Ire1p is phosphorylated

Ire1p has many of the same properties as other transmembrane receptor kinases. Its tail is required for signal transduction and oligomerization may regulate its function. We therefore wished to examine whether it shares a third characteristic of transmembrane receptors, phosphorylation. We wished to determine if Ire1p is phosphorylated and, if so, whether phosphorylation increases when the kinase is activated by induction of the unfolded protein response. We made extracts from wild-type cells that had been labelled with ^{32}P orthophosphate either in the presence or absence of tuncamycin. Labelled Ire1p was immunoprecipitated with α-Ire1Nterm antibody, the products separated by PAGE, and then visualized by autoradiography. As shown in Figure III-7a, Ire1p is a phosphoprotein and its phosphorylation increases two to three fold with induction of the unfolded protein response.

We next looked at phosphorylation of the full-length Ire1p-K702A and Ire1p-K702R mutants (Figure III-7b, 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. However, on the basis of these data, we cannot rule out the more unlikely possibility that the Ire1p kinase domain is required for phosphorylation of second protein, itself a kinase or one that activates another kinase, which then phosphorylates Ire1p.

If Ire1p phosphorylates itself, is it an intra- or inter-molecular phosphorylation event? Given the previously described interaction between

the Ire1p-tailless and Ire1p-K702R alleles, we wished to see whether Ire1p-K702R is phosphorylated in cells in which Ire1p-tailless is also present. We labelled with ^{32}P-orthophosphate a yeast strain bearing both versions of the kinase (Ire1p-K702R + Ire1p-tailless) and strains bearing either version alone (Ire1p-K702R or Ire1p-tailless) and immunoprecipitated Ire1p as described above. First, we observed that phosphorylation of Ire1p-tailless can be detected in cells expressing only that construct (Figure III-7c, lane 3). Assuming that Ire1p phosphorylates itself, this result is further evidence that the tailless kinase is active even though it cannot propigate the unfolded protein response. It also demonstrates that not all, if any, of the sites of phosphorylation on Ire1p are in the tail. We also observed that, in the presence of Ire1p-tailless, Ire1p-K702R is phosphorylated (Figure III-7c, lane 1). These results suggest that phosphorylation of Ire1p occurs in *trans* and is probably carried out by other Ire1p molecules. Furthermore, they provide confirmation for our model (Figure III-4) to describe the basis of the intragenic complementation between the two Ire1p alleles.

Ire1p is phosphorylated primarily on serine

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 labelled with ^{32}P-orthophosphate (see Materials and Methods). Only phosphoserine could be detected in Ire1p isolated 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 III-8a,b).

However, 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 that were grown in the presence of tunicamycin. 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 III-8c). Thus, Ire1p is primarily phosphorylated on serine and, keeping in mind the caveat that cells overexpressing Ire1p do not behave exactly like wild-type cells, it is possible that phosphothreonine is also present.

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 residue(s) are phosphorylated. The cdk kinases, to which Irep is most closely related, and many transmembrane tyrosine kinases have activating phosphorylation sites on a protein loop that stretches between conserved kinase domains VII and VIII (Figure III-9; 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 phophorylation sites by mutating them to alanine and then examining the phenotypes of cells bearing the different Ire1p mutations.

We first tested Ire1p-T844A and the double mutant, Ire1p-S840A/S841A. Interestingly, as measured on tunicaycin-X-Gal plates and by S1 nuclease protection assay, the T844A mutation disrupted the unfolded protein response only slightly whereas the S840A/S841A mutation had a much more severe effect on the response (Figure III-10a, lanes 5 to 8). The Ire1p-S840A/S841A mutant carries out only a weak unfolded protein response. These phenotypes correlated with the ability of the mutants to grow on plates lacking inositol: Ire1p-T844A mutants grew essentially as well as wild-type while Ire1p-S840A/S841A mutants barely grew (data not shown). When we examined the phosphorylation state of the mutant Irep proteins, we found that Ire1p-T844A is phosphorylated while Ire1p-S840A/S841A is not (Figure III-7b, lanes 5 and 6). Thus, given the phosphoamino acid data presented above, we thought it likely that S840 and/or S841 might be the activating phosphorylation site(s) in Ire1p. Our hypothesis was supported by sequence comparisons of *S. cerevisiae* Ire1p and *C. elegans* Ire1p, the sequence of which was entered into the protein sequence database only recently (Figure III-11): a serine in the vicinity of amino acid 840 or 841 is conserved while there is no threonine nearby in the *C. elegans* gene.

To test this hypothesis, we made two additional Ire1p mutants, Ire1p-S840A and Ire1p-S841A, in which the two serines were mutated separately. When we examined the unfolded protein response 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 III-10a, lanes 5, 6, 9, 10, 13, and 14). Furthermore, both Ire1p-S840A and Ire1p-S841A are phosphorylated *in vivo* , although to reproducibly lower levels than the wild-type protein (Figure III-7b, lanes 7 and 8). This result has

two interpretations. First, the two serines may be 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. On the other hand, it is also possible that neither serine is the activating phosphorylation site on Ire1p and that the almost null phenotype of the S840A/S841A double mutant is a result of structural problems in the kinase that simply destroy its activity.

To attempt to distinguish between these two possibilities, we made another mutant, Ire1p-S840D/S841D, in which both of the serines are changed to aspartic acid. Aspartic acid has been shown, in some cases, to substitute functionally for phosphoserine (Casanova et al., 1990; Huang and Erikson, 1994) and we hoped that by making this mutant, we could create a version of Ire1p kinase that was not phosphorylated but that was active. To say conclusively that S840 and/or S841 are the activating phosphorylation sites on Ire1p, we needed either of two possible positive results: 1) the S840D/S841D mutant induced the unfolded protein response constitutively, both in the absence and presence of tunicamycin, or 2) the mutant was not phosphorylated at all but could still induce the unfolded protein response. We found that the S840D/S841D mutant can carry out the unfolded protein response, but the response is not constitutive (Figure III-10a lanes 11 and 12) and that the mutant protein is phosphorylated (Figure III-12), although to a lesser extent than wild-type. Thus, based on these data, we have not identified unambiguously any phosphorylation sites in Ire1p, but we have shown that S840 and S841 are important for the unfolded protein response.

Discussion

In this chapter, we have tested and confirmed the model that activation of Ire1p and transmission of the unfolded protein signal occurs by mechanisms similar to those found in many other receptor kinases. We have shown that the Ire1p tail domain is required for the unfolded protein response and that its role is likely to bind other components of the pathway that transmit the unfolded protein signal to the transcription machinery in the nucleus. We have presented molecular genetic and direct biochemical evidence that Ire1p oligomerizes, although we do not yet know whether the oligomerization state changes with the induction of the response. Finally, we have shown that Ire1p is a phosphoprotein that is phosphorylated primarily on serine and that Ire1p kinase activity is required for Ire1p phosphorylation.

The study of other receptors suggested that phosphorylation of Ire1p might correlate with the activity of the unfolded protein response and, given the partial activity of Ire1p-K702R, we had expected to detect some phosphorylation of the mutant. We found instead that Ire1p-K702R is not phosphorylated. One explanation for this result is that phosphorylation of Ire1p correlates more closely with the activity of the Ire1p kinase than with the unfolded protein response as measured by induction of *KAR2* transcription. Thus, K702R may have a very weak kinase activity, while Ire1p-K702A may have virtually none, and the magnitude of the unfolded protein response in cells bearing Ire1p-K702R could reflect an amplification of the weak kinase activity.

We had hoped to identify kinase-activating phosphorylation sites in Ire1p in the loop that connects conserved domains VII and VIII of the kinase. 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 homologous loop occupies the active site of each kinase, 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 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 are consistent with the possibility that S840 and S841 both are activating phosphorylation sites in Ire1p. The presence of more than one phosphorylation site in the VII to VII loop would not be unprecedented as several kinases, such as insulin receptor and Mek1, have multiple activating phosphorylation sites in the VII to VIII loop. In the insulin receptor, two of the phosphorylated residues lie side by side (Huang and Erikson, 1994; White et al., 1988). However, we also acknowledge that other serine and/or threonine residues in the Ire1p VII to VIII loop (e.g. S837, T844, S850, or T852) may be activating phosphorylation sites as well as or in addition to S840 and S841.

In the case of Mek1, changing the activating phosphorylation sites (both serines) to aspartic acids creates a mutant kinase that is constitutively active as determined by *in vitro* kinase assays (Huang and Erikson, 1994) Strains bearing Ire1p-S840D/S841D do not induce the unfolded protein response constitutively, but it is still possible that the mutant Ire1p kinase is constitutively active. Constitutive kinase activity might be measured with an *in vitro* assay, and, as described above, it is likely that Ire1p kinase activity is

not reflected in the measurement of a downstream event such as the unfolded protein response. This might be because, in the absence of an accumulation of unfolded proteins in the ER, Ire1p-S840D/S841D monomers cannot oligomerize and transduce the response even though their kinases are fully active. Further experiments, in particular, development of an *in vitro* kinase assay for Ire1p and phosphopeptide analysis of the protein should allow identification of any activating phosphorylation sites in Irep.

Many confirmatory experiments remain to be done but we have presented here data that support the model for activation of Ire1p diagrammed in Figure III-13. 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, both on activating phosphorylation sites in the kinase domain that increase kinase activity and on sites in the Ire1p tail domain or on proteins that bind to the Ire1p tail that allow transmission of the unfolded protein signal towards the nucleus.

Materials and Methods

Strains and plasmids

The *ire1* disruption in strain CS236 is the same as that in CS165. The *ire1* disruptions in strains CS243 and CS309 remove the Ire1p coding sequence only and were created by homologous recombination using PCR-amplified fragments of *IRE1* in plasmid pRS306 (CS243) and plasmid pRS304 (CS309;

Sikorski and Hieter, 1989). Sequences truncating Ire1p at amino acid 982 (Ire1p-tailless, 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 in place of the *IRE1* terminator.

Plasmid pCS114 expresses a mutant Ire1p that is truncated at amino acid 674 and the amino acids AMA are added just before the stop codon. The truncated *ire1* is cloned between the Bam HI and XbaI sites in CEN/ARS plasmid pTS210 (gift of Tim Stearns) which places the *ire1* gene under control of the *GAL1* promoter and *ACT1* transcriptional terminator. Site-directed mutagenesis of Ire1p was carried out by standard methods (Kunkel et al., 1987) and all mutated fragments of *IRE1* were sequenced to confirm that only the desired mutations had been introduced into the gene. All plasmids expressing full-length Ire1p bear the XhoI-HindIII fragment of the *IRE1* gene (see Chapter II). Plasmid pCS122 is described in Appendix A. pCS178 is identical to pCS122 except the K702R mutation has been introduced into *ire1*. pCS170, pCS175, and pCS188 all bear wild-type *IRE1* in the CEN/ARS vector YCplac33 (Gietz and Sugino, 1988). The three constructs are identical but were made at different times using different cloning strategies. Plasmids pCS145 (K702A), pCS146 (K702R), pCS171 (S840A/S841A), pCS172 (T844A), pCS176 (K702A), pCS177(K702R), pCS185 (S840A), pCS186 (S840D/S841D), and pCS187(S841A) are identical to pCS170 except the indicated mutations were introduced into *ire1*. Plasmid pCS179, expressing Ire1p-tailless (with a wild-type kinase domain) bears *IRE1* truncated exactly as described for yeast strain CS240. Expression of the ire1-tailless gene is driven by the *IRE1* promoter (starting at the upstream XhoI site, Chapter II) 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 (tailless-K702A) and pCS181 (tailless-K702R) 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 and, because Vent polymerase has relatively high fidelity, the rest of the each construct was not sequenced further. *E.coli* transformed with the GST-Ire1 plasmids produced GST fusions of the expected molecular weight (approximately 83kD for Ire1Nterm and 43 kD 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* labelling and immunoprecipitation**

Yeast cells, grown at room temperature in medium containing appropriate supplements and lacking methionine, were labelled with ^{35}S methionine and non-native immunoprecipitations (IP's; done after SDS denaturation) were carried out essentially as described by Hann and Walter (1991) . The cells were labelled for a total of 1.5 hours with 100mCi of ^{35}S methionine per OD_{600} unit of cells (Pro-mix ^{35}S cell labelling mix, Amersham) before harvesting. In the IP's shown in Figures III-2a, III-7, III-12 and done for phosphoamino acid analysis (Figure III-8), α-Ire1p antibodies were collected with heat inactivated Staph A (Pansorbin cells, Cal Biochem, San Diego CA). α-Ire1p antibodies were collected with Protein A sepharose in the IPs shown in Figure III-6. Staph A seems to be more efficient in collecting α-Ire1p antibodies than Protein A. Under the conditions shown in Figure III-2a, greater than 85% of Ire1p in each 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 labelled with ^{32}P orthophosphate (NEX-053, DuPont, Wilmington DE) at room temperature. Cells grown overnight in the appropriate high phophate medium to log phase were washed into low phosphate medium and grown for 4 hours. 5 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 100μCi/ml. Cells were incubated, shaking, at room temperature for 15 minutes before tunicamycin was added (or not) and the cultures grown for an additional 30 minutes before harvesting. Extracts and non-native immunoprecipitations were carried exactly as described for ^{35}S methionine labelled cells except phosphatase inhibitors (10mM NaF, 80mM

β-glycerol phosphate, 1 mM sodium vanadate, and 10μm 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 Staph A pellet. Under these immunoprecipitation conditions, greater than 65% of Ire1p in each lysate was precipitated. When necessary, the protein concentration of the yeast cell lysates was determined using the Micro BCA protein assay (Pierce, Rockford, IL).

Crosslinking

Chemical crosslinking was carried out on extracts of cells that had been labelled, as described above, with ^{35}S methionine. 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 PMSF) and then spinning the lysate at 1000 rpm (Beckman JS13 rotor) for 4 minutes to remove unbroken cells. The reversible crosslinker DSP (Pierce, Rockford, IL) was added to the extract equivalent of 10 OD_{600} of cells in each crosslinking reaction. Reactions proceeded for 20 minutes 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 minute 35000 rpm (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 were then carried out exactly as described above.

Phosphoamino acid analysis

Two dimensional phophoamino acid analysis (PAA) was carried out on Ire1p that had been immunoprecipited from extracts of cells labelled with ^{32}P orthophosphate and eluted from gel slices. The PAA protocol followed is

the same as that described by Boyle et al. (1991). To analyze Ire1p from JC102 cells, the appropriate bands from 3 separate lanes of an acrylamide gel (loaded with 30µl of resuspended immunoprecipitate/lane) were combined before eluting the protein and, after hydrolysis etc., 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 and one-half of the resulting hydrolyzed sample was loaded on a TLC plate.

Table III-1 Yeast strains

Strain	Genotype	Source/Reference
JC102	leu2-3,-112; his3-11,-15; trp1-1; ura3-1; ade2-1; can1-100; MAT<u>a</u>; leu2-3,-112::LEU$^+$UPRE-lacZ; MET$^+$	This study
CS165	same as JC102, except his3-11,-15::HIS$^+$UPRE-lacZ; ire1::URA3 (only 2/3 of Ire1p coding sequence deleted); met$^-$	Chapter II
CS236	same as JC102, except ire1::URA3 (only 2/3 of Ire1p coding sequence deleted)	This study
CS240	same as JC102, except ire1:: TRP$^+$ire1-tailless	This study
CS243	same as JC102, except ire1::URA$^+$pRS306 (entire Ire1p coding sequence deleted; Sikorsi and Hieter, 1989)	This study
CS309	same as JC102, except ire1::TRP$^+$pRS304 (entire Ire1p coding sequence deleted; Sikorsi and Hieter, 1989)	This study

Figure III-1 The epitopes used to produce the α-Ire1p antibodies α-Ire1Nterm and α-Ire1tail, and the Ire1p mutants tailless and Dominant negative are diagrammed.

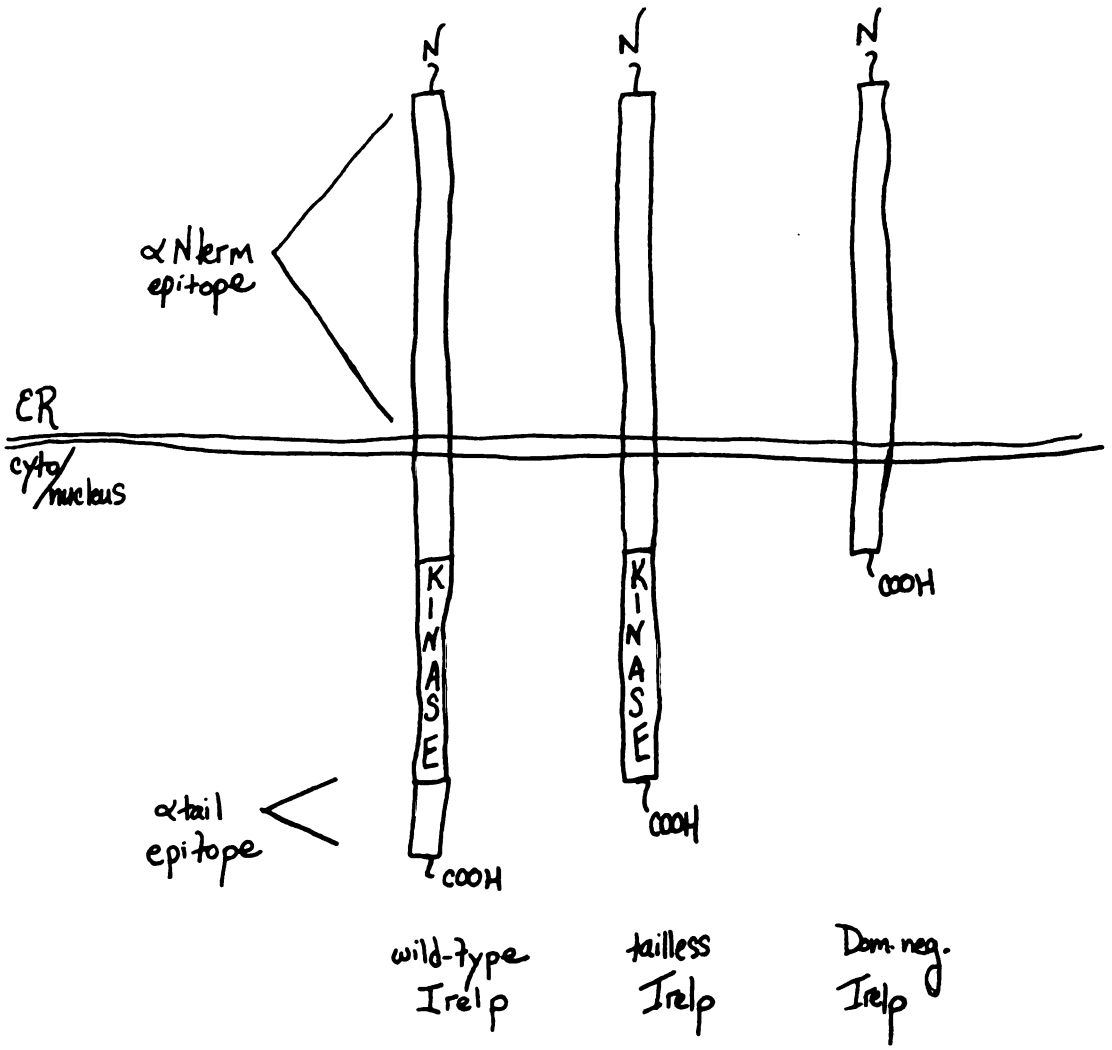

Figure III-1

Figure III-2 (a) Immunoprecipitations, using the indicated antibodies, from extracts made from ^{35}S methionine-labelled yeast cells. The strains were wild-type (JC102), *ire1*-tailless (CS240), and Δ*ire1* (CS309). The numbers on the right side of the gel indicate the mobilities of molecular weight standards (kD). **(b)** S1 nuclease protection analysis of RNA made from three strains: wild-type (JC102), Δ*ire1* (CS236), and *ire1*-tailless (CS240). Tunicamycin induction followed by S1 nuclease protection analysis was performed as in Figure II-2a. **(c)** *KAR2* mRNA levels from (b) were quantitated and normalized to *ACT1* mRNA levels as in Figure II-1c.

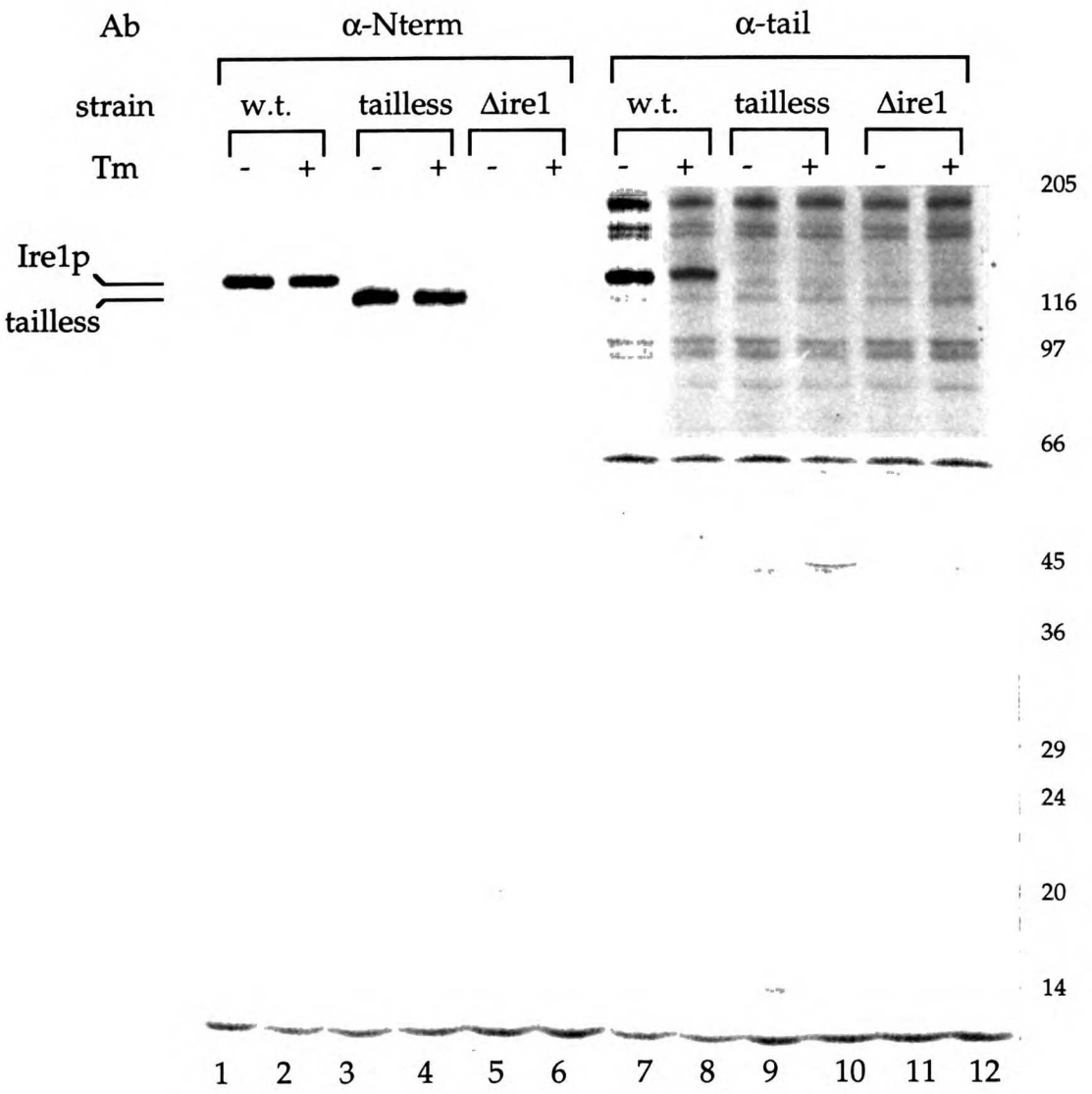

Figure III-2a

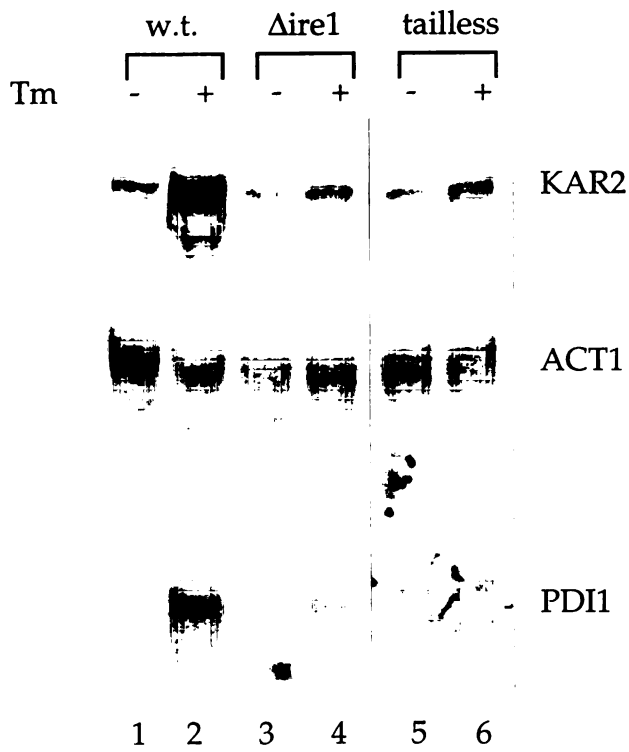

Figure III-2b

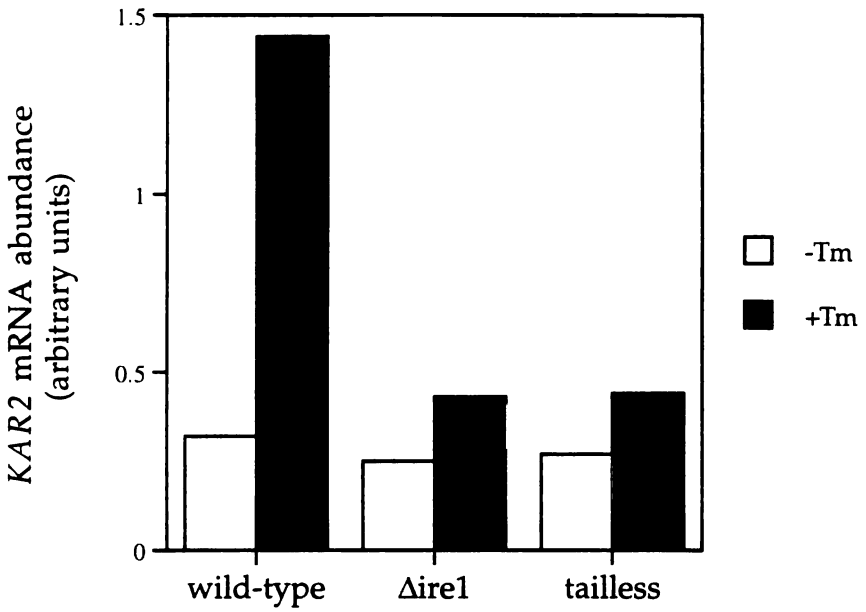

Figure III-2c

Figure III-3(a) S1 nuclease protection analysis of RNA made from strain CS309 (which is deleted for the Ire1p coding sequence) bearing a CEN/ARS plasmid with either*IRE1*-K702A (plasmid pCS145), *IRE1*-K702R (pCS146), wild-type *IRE1* (pCS170) or no *IRE1* (control; YCplac33; Gietz and Sugino, 1988). **(b)** *KAR2* mRNA levels from (a) were quantitated and normalized to *ACT1* mRNA levels as in Figure II-1c. **(c)** S1 nuclease protection analysis of RNA made from strain CS240 (which carries *ire1*-tailless) bearing a CEN/ARS plasmid with either wild-type *IRE1* (pCS175), *IRE1*-K702A (pCS176), *IRE1*-K702R (pCS177), or no *IRE1* (control; YCplac33) **(d)** *KAR2* mRNA levels from (c) were quantitated and normalized to *ACT1* mRNA levels as in Figure II-1c. Tunicamycin induction followed by S1 nuclease protection analysis was performed as in Figure II-2a.

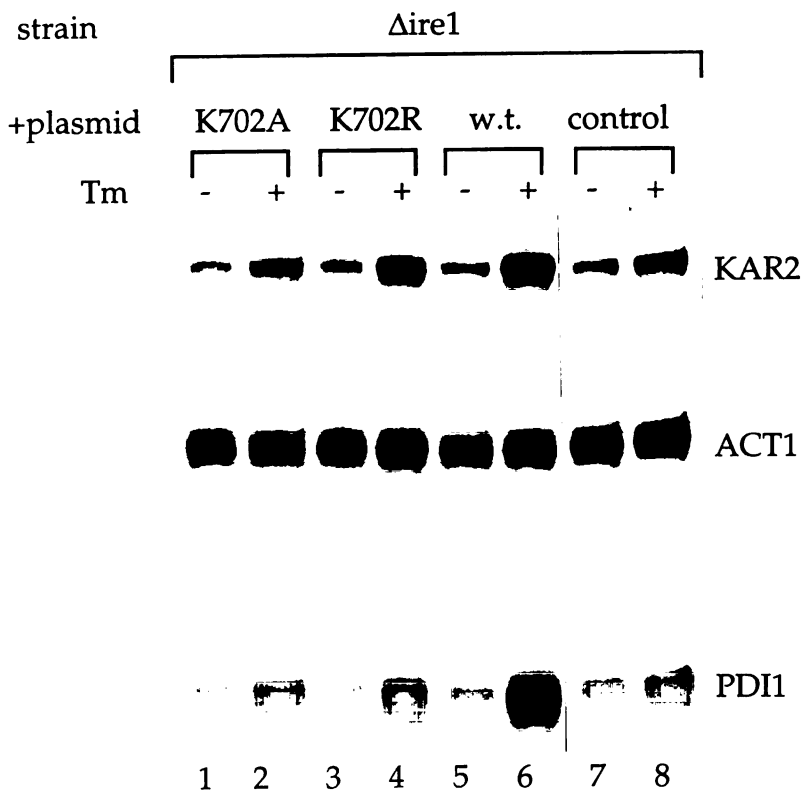

Figure III-3a

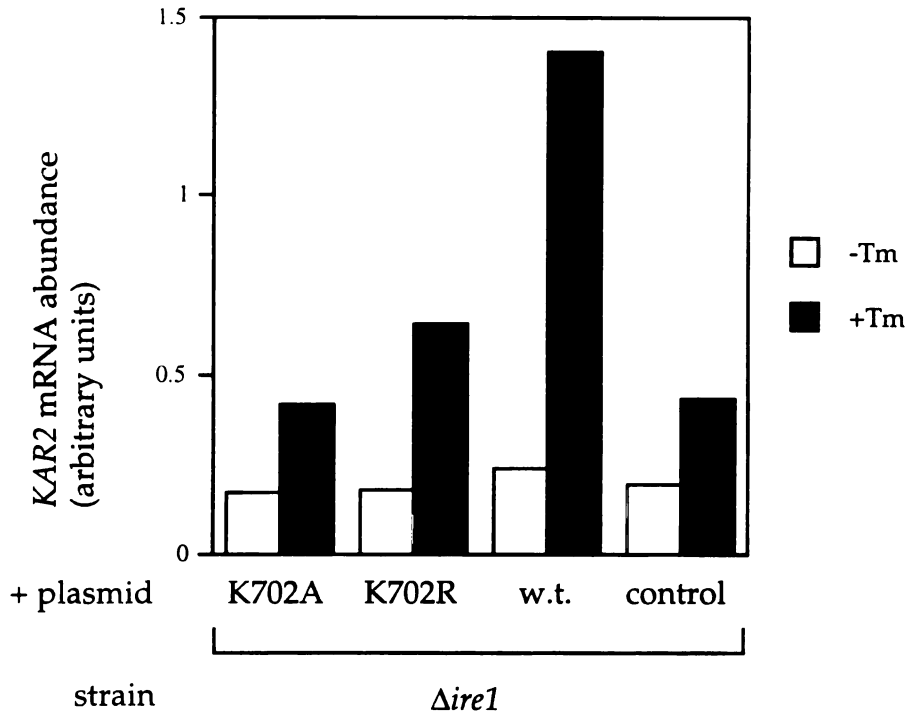

Figure III-3b

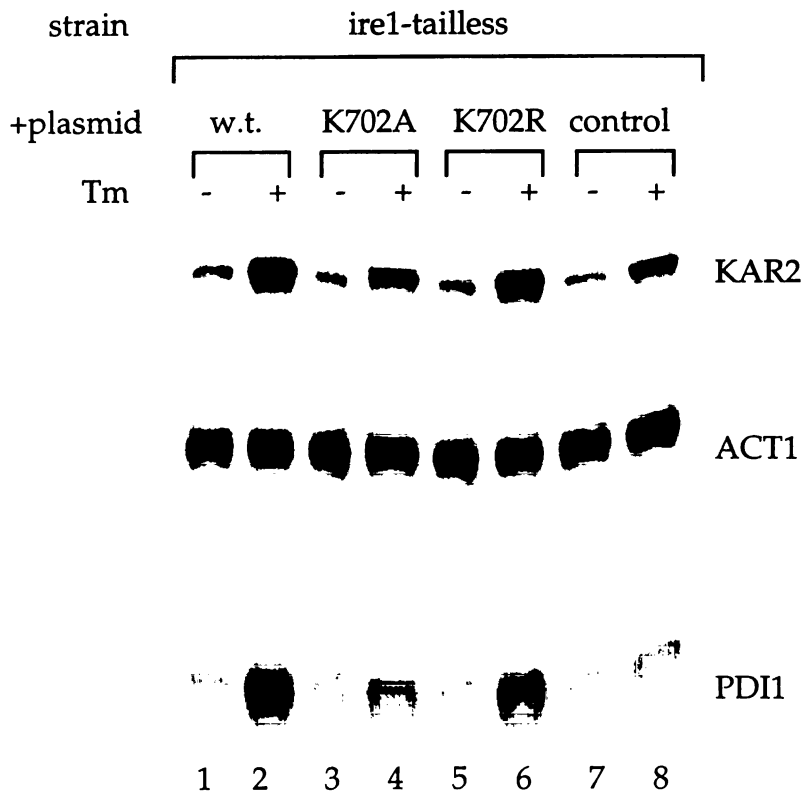

Figure III-3c

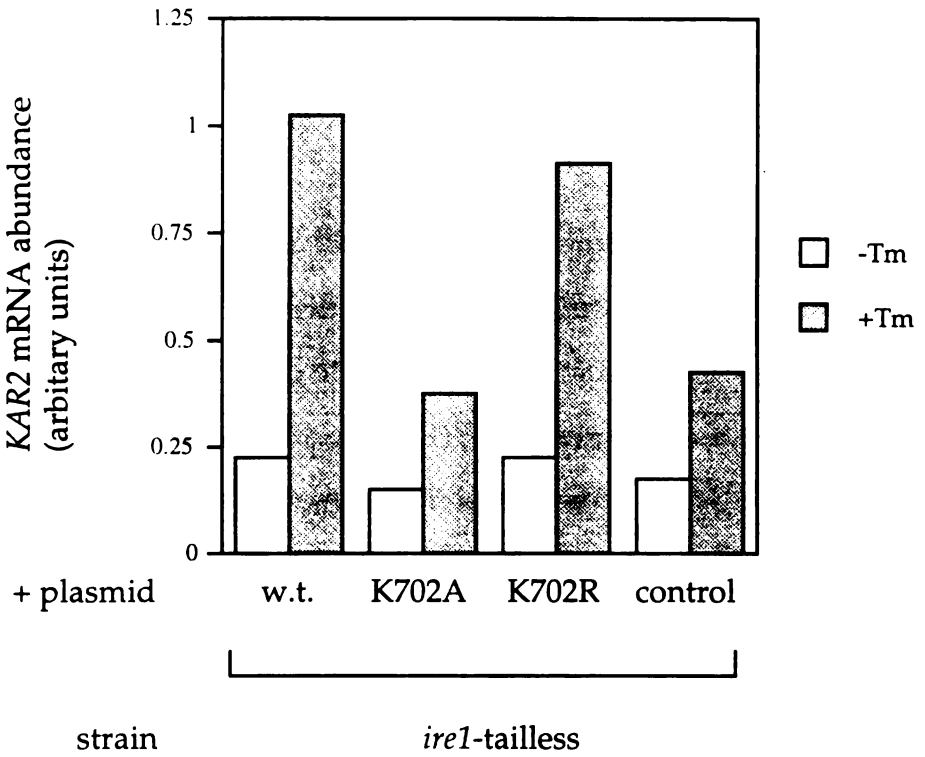

Figure III-3d

Figure III-4 A model for the intragenic complementation between *ire1* alleles. When the unfolded protein response is induced in cells bearing both Ire1p-tailless and Ire1p-K702R, oligomers of the two mutant proteins form and each kinase phosphorylates the other (1). Additionally, the truncated Ire1p (that has a wild-type kinase domain) phosphorylates either the tail of Ire1p-K702 R or a protein that binds to the tail (2). This second phosphorylation event is required for transmission of the unfolded protein signal.

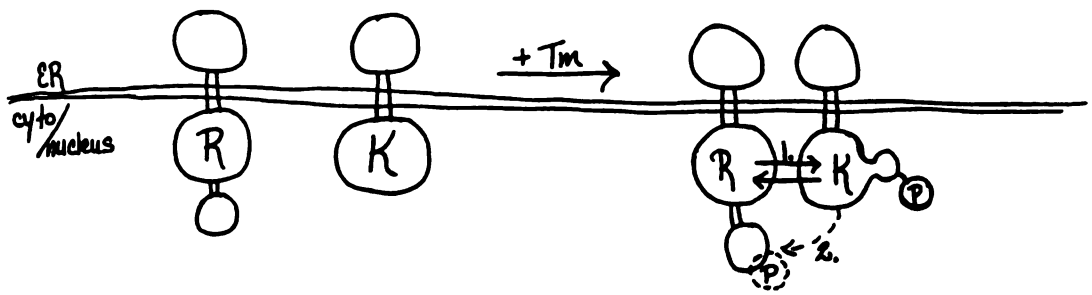

Figure III-4

Figure III-5 (a) S1 nuclease protection analysis of RNA made from strain CS309 (which is deleted for the Ire1p coding sequence) bearing CEN/ARS plasmids expressing the indicated tailless-Ire1p and full-length-Ire1p proteins. The plasmids bearing Ire1p tailless alleles are pCS179 (w.t.), pCS180 (K702A), and pCS181 (K702R). Plasmid pCS177 bears the full-length Ire1p-K702R allele. The control plasmids (-) are pRS313 (vector backbone of pCS179-181; Sikorski and Hieter, 1989) and YCplac33 (vector backbone of pCS177; Gietz and Sugino, 1988). **(b)** *KAR2* mRNA levels from(a) were quantitated and normalized to *ACT1* mRNA levels as in Figure II-1c. **(c)** S1 nuclease protection analysis of RNA made from strain CS309 bearing 2 μm plasmids with either wild-type *IRE1* (w.t., pCS122),or *IRE1*-K702R (pCS178) or made from a related Δire1 strain (CS165) bearing a control 2 μm plasmid (YEplac112; Gietz and Sugino, 1988) **(d)** *KAR2* mRNA levels from(c) were quantitated and normalized to *ACT1* mRNA levels as in Figure II-1c. Tunicamycin induction followed by S1 nuclease protection analysis was performed as in Figure II-2a.

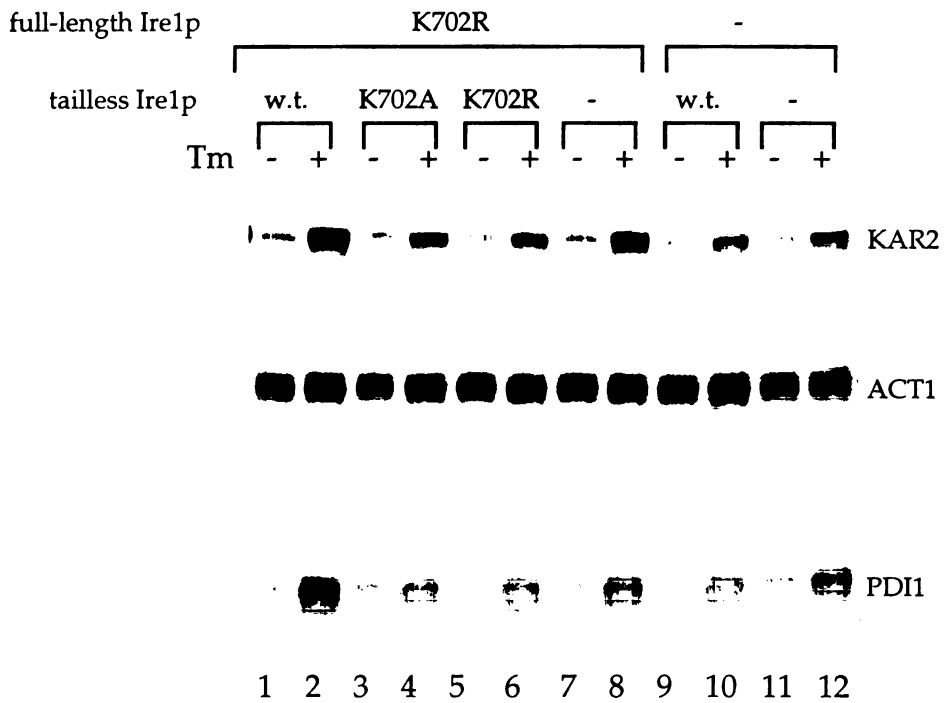

Figure III-5a

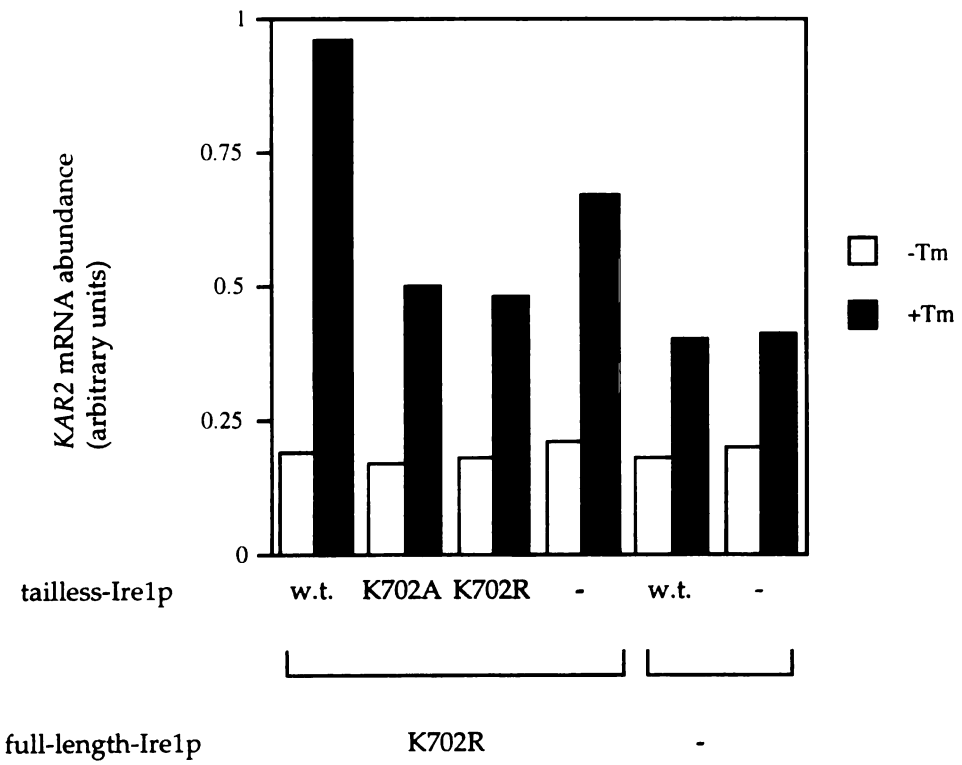

Figure III-5b

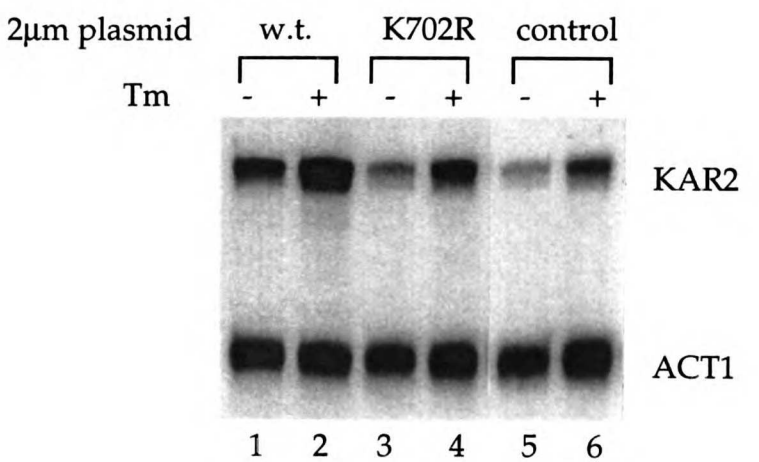

Figure III-5c

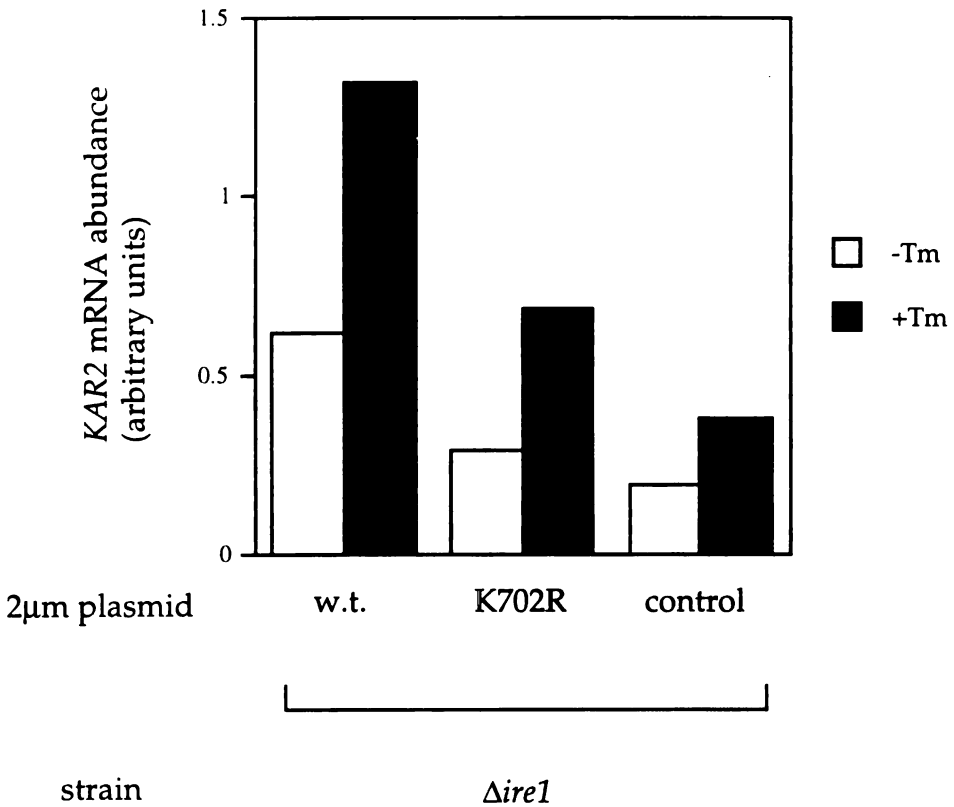

Figure III-5d

Figure III-6 Truncated Ire1p associates with full-length Ire1p. The indicated strains, JC102 (*IRE1*) and CS243 (Δ*ire1*)bearing a plasmid expressing Dominant negative Ire1p (pCS114) or a control plasmid (pTS210) were grown in galactose in the presence of ^{35}S methionine and tunicamycin and then extracts were made. Chemical crosslinking was carried out with the reversible crosslinker DSP (at a concentration of 200μg/ml in each reaction) and Ire1p was immunoprecipitated from the extracts with the indicated antibodies. The crosslinker was reversed before PAGE and the gel was autoradiographed to visualize the products of the immunoprecipitations. This figure is a composite of non-consecutive lanes of same exposure of one gel. The numbers on the left side of the gel indicate the mobilities of molecular weight standards (kD).

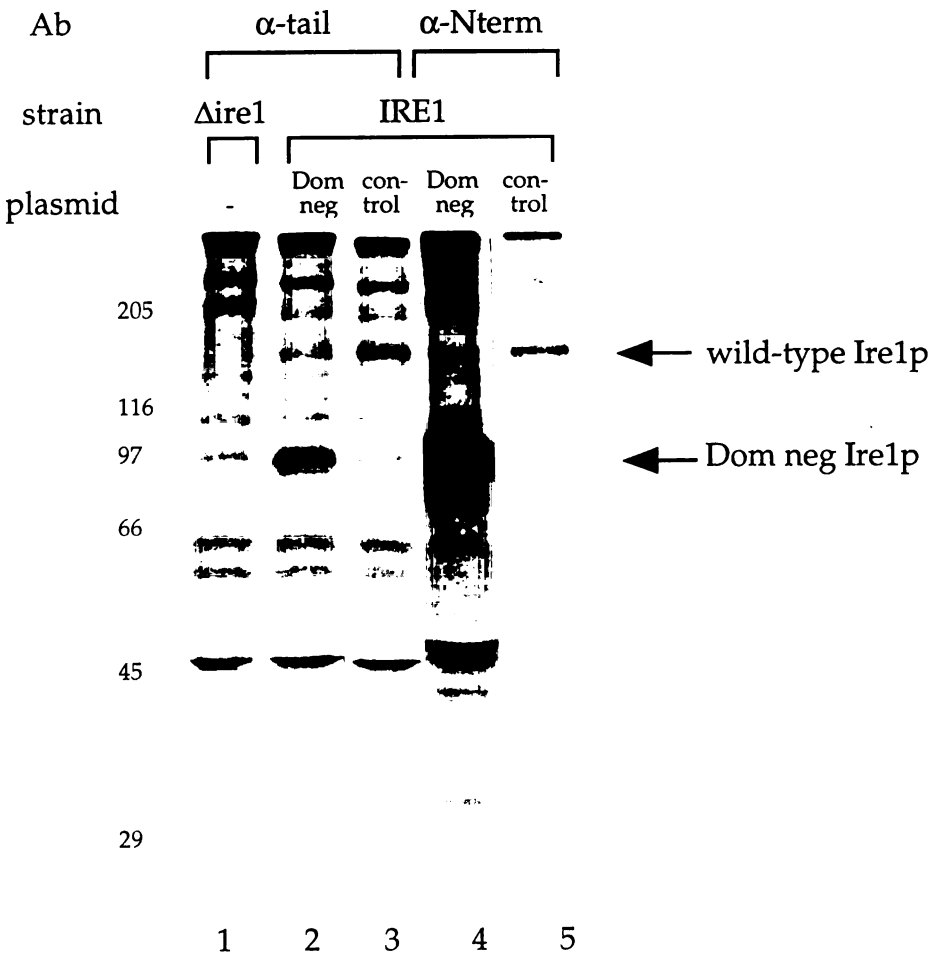
Ab
α-tail
α-Nterm
strain
Δire1
IRE1
plasmid
-
Dom neg
con-trol
Dom neg
con-trol
205
116
97
66
45
29
wild-type Ire1p
Dom neg Ire1p
1
2
3
4
5

Figure III-6

Figure III-7 (a) Phosphorylation of Ire1p increases with the unfolded protein reponse. Immunoprecipitaion of Ire1p with α-Ire1Nterm antibodies from strain CS243 (Δ*ire1*), deleted for the Ire1p coding sequence, and wild-type strain JC102 (*IRE1*). The cells were labelled with ^{32}P orthophosphate in the presence or absence of tunicamycin (Tm) before being harvested. A very dark background band is visible in all lanes that migrates faster than Ire1p. **(b)** Some Ire1p mutants are not phosphorylated. Immunoprecipitations on ^{32}P orthophosphate-labelled 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 (Δire1) 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 on ^{32}P orthophosphate-labelled cells were carried out as described in (a) except all strains were grown in the presence of tunicamycin. In lanes 1-3, the strains used are all based on strain CS309 (Δire1) and bear two CEN/ARS plasmids with the indicated *IRE1* alleles (w. t. tailless, pCS179 or full-length K702R, pCS177) or no *IRE1* allele (pRS313; Sikorski and Hieter, 1989; or YCplac33; Gietz and Sugino, 1988). The strain in lane 4 is JC102 bearing a control plasmid (YCplac22; Gietz and Sugino, 1988).

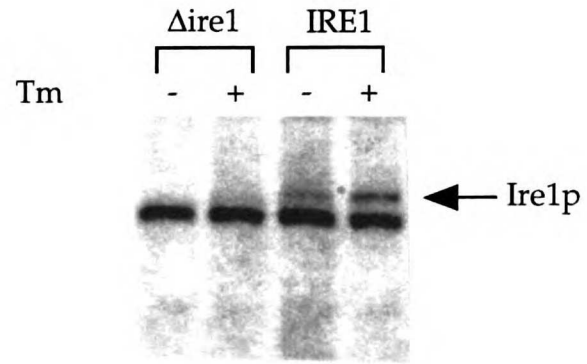

Figure III-7a

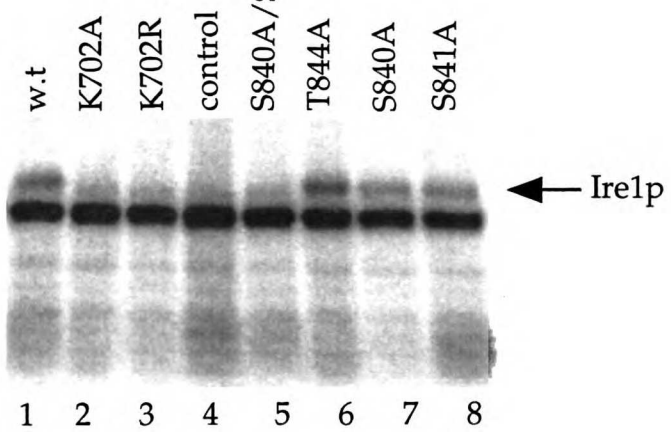

Figure III-7b

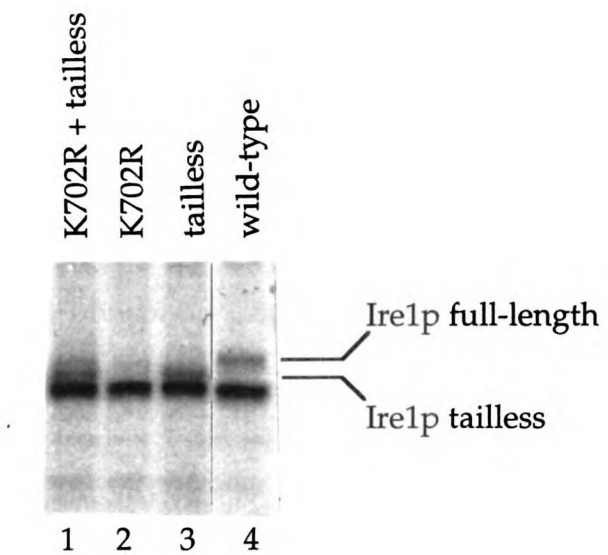

Figure III-7c

Figure III-8 Ire1p is phosphorylated primarity on serine. Phosphoamino acid analysis was carried out on phosphorylated Ire1p immunoprecipitated from strains either, **(a)** and **(b),** expressing wild-type levels of Ire1p (JC102) or **(c)** overexpressing the protein from a 2 μm plasmid (pCS122 in strain CS243)

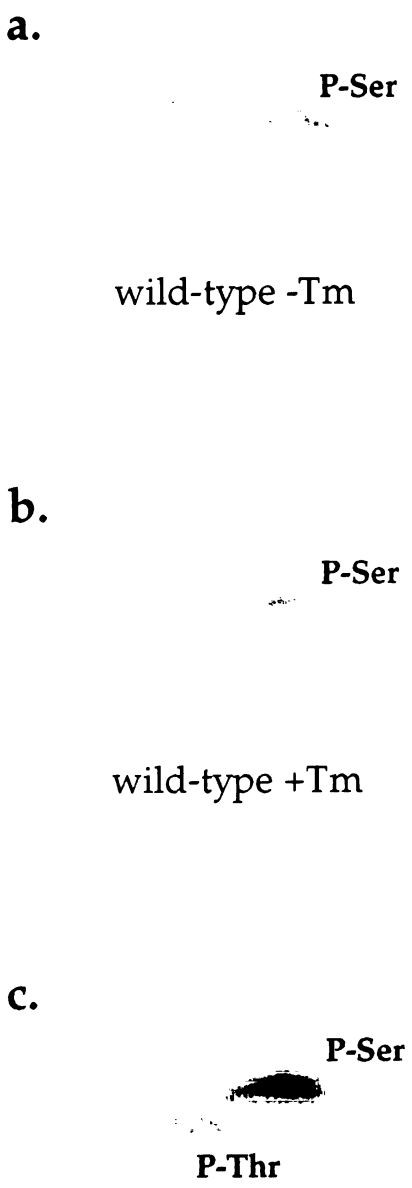

Figure III-8

Figure III-9 Possible activating phosphorylation sites in Ire1p. A sequence comparison of the kinase domain VII to VIII loops from Ire1p, *S. cerevisiae* Cdc28p, and human CDK2. Known activating phosphorylation sites are starred and candidate activating sites in Ire1p are indicated with arrows.

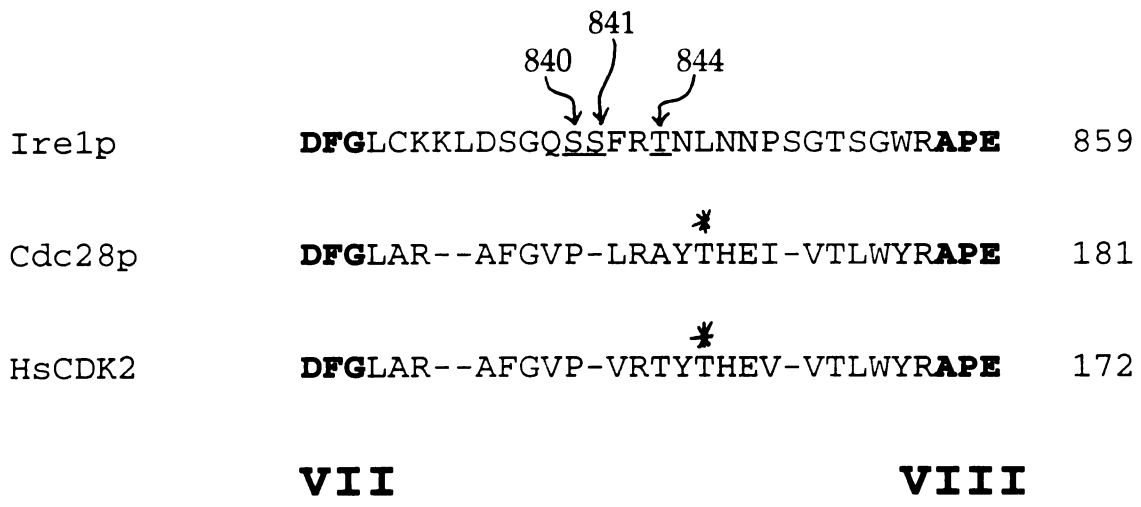

Figure III-9

Figure III-10 (a) S1 nuclease protection analysis of RNA made from strain CS309 (Δ*ire1*) bearing CEN/ARS plasmids expressing the indicated Ire1p alleles (w.t. , pCS175; control, YCplac33; Gietz and Sugino, 1988); S840A/S841A, pCS171;T844A, pCS172; S840A, pCS185; S840D/S841D, pCS186; S841A, pCS187). Tunicamycin induction followed by S1 nuclease protection analysis was performed as in Figure II-2a. **(b)** *KAR2* mRNA levels from (a) were quantitated and normalized to *ACT1* mRNA levels as in Figure II-1c. **(c)** *PDI1* mRNA levels from (a) were quantitated and normalized to *ACT1* mRNA levels as in Figure II-1c.

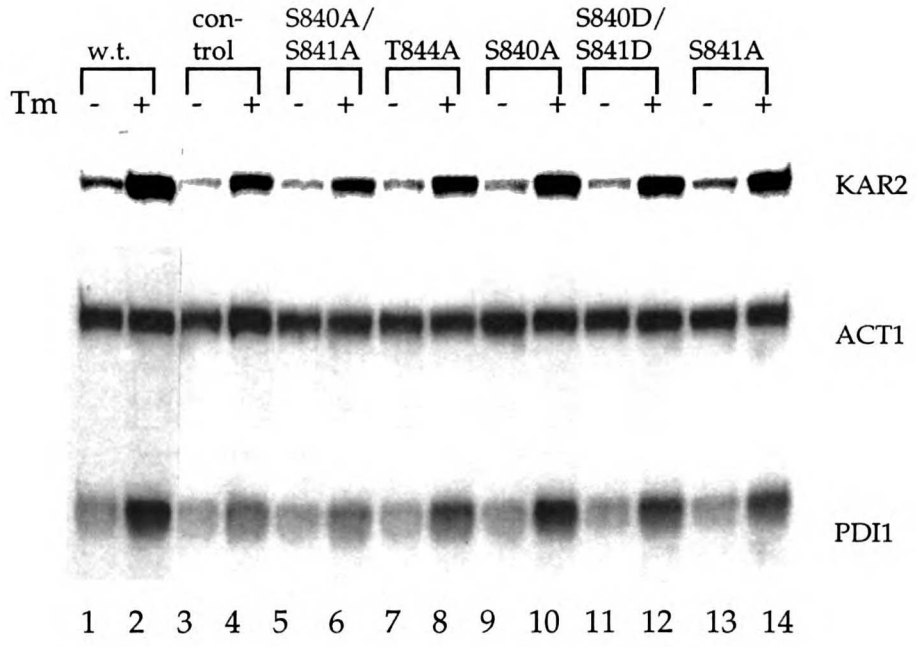

Figure III-10a

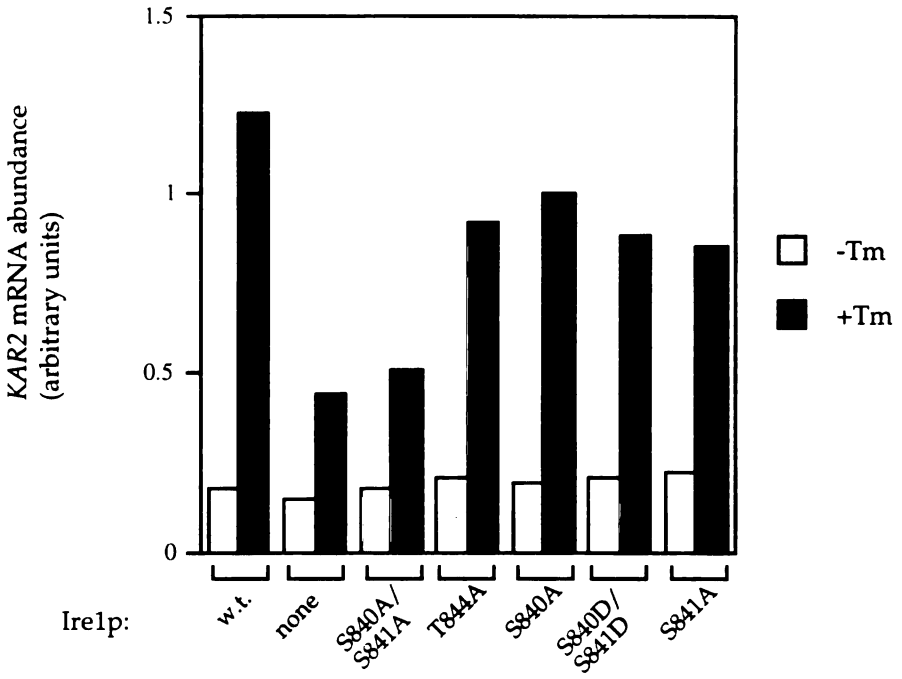

Figure III-10b

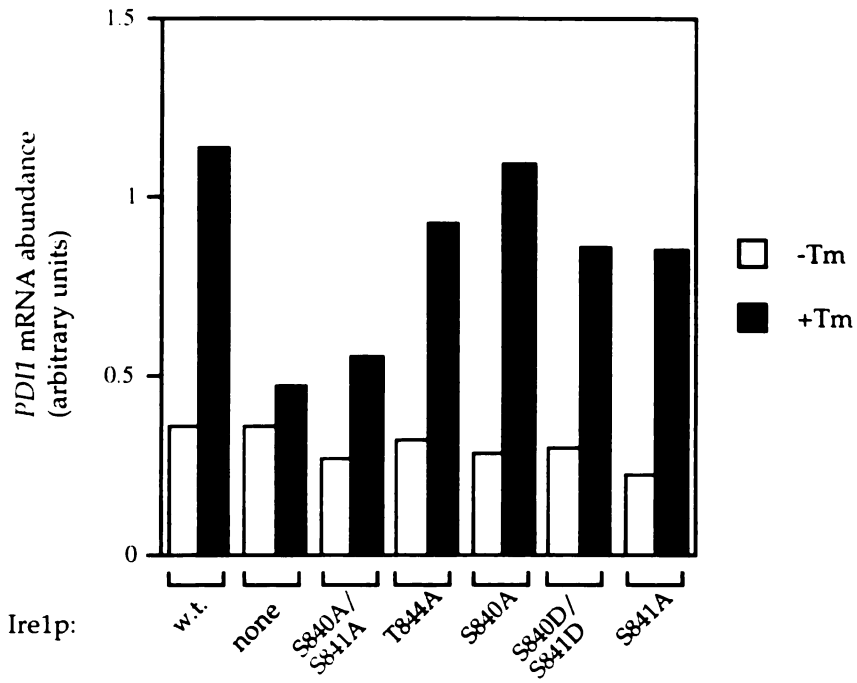

Figure III-10c

Figure III-11 A comparison of the kinase domain VII to VIII loops in Ire1p from *S. cerevisiae* and from *C. elegans*.

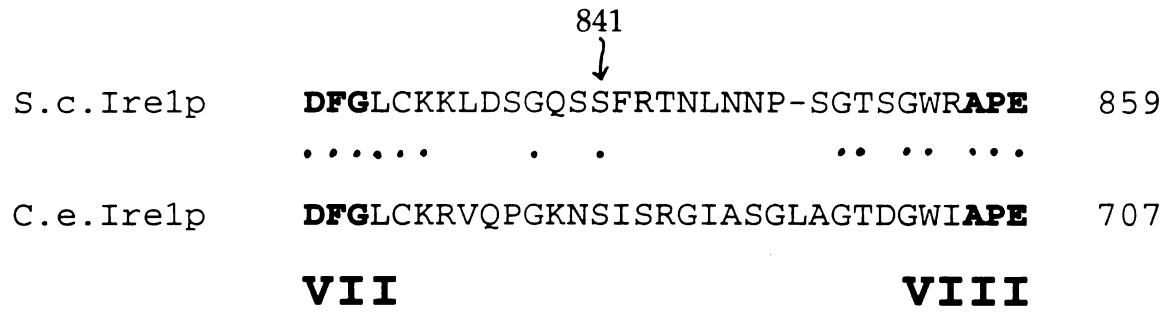

Figure III-11

Figure III-12 Ire1p-S840D/S841D is phosphorylated to a lesser extent than wild-type. Immunoprecipitations of Ire1p from ^{32}P orthophosphate-labelled cells bearing either Ire1p-S840D/S841D (p CS186), wild-type Ire1p (pCS188), or no Ire1p (control, YCplac33; Gietz and Sugino, 1988)) were carried out as described in Figure III-7a except all strains were grown in the presence of tunicamycin.

Figure III-12

Figure III-13 A model for the role of Ire1p in the unfolded protein response pathway. Induction of the unfolded protein response (+Tm) causes Ire1p monomers to oligomerize and phosphorylate each other. Phosphorylation of the Ire1p C-terminal tail or of other, as yet unidentified proteins that bind to the tail (shaded proteins), allows transmission of the unfolded protein signal toward the nucleus and induction of transcription (Tx) of the genes encoding ER resident proteins.

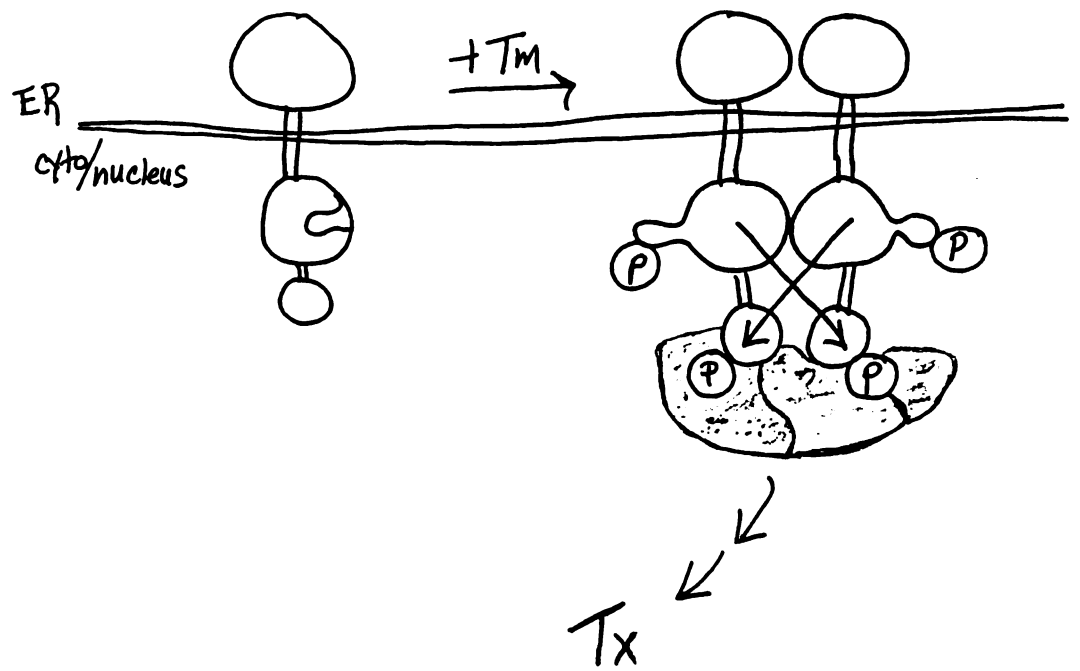

Figure III-13

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Chapter IV:

Testing the models proposed for the role of Ire1p in the unfolded protein response pathway

Introduction

This chapter consists of three sets of experiments that were designed to test the model, described in Chapter II, that the ER resident protein BiP plays a role in monitoring the accumulation of unfolded proteins in the ER. According to the model, BiP binding to Ire1p prevents oligomerization of the kinase in the absence of the unfolded protein response, but, as unfolded proteins accumulate in the ER, BiP binds preferentially to the unfolded proteins, releasing Ire1p and allowing it to oligomerize. First, we examined the association of Ire1p with BiP and other proteins. Next, we attempted to determine the subcellular localization of Ire1p and thus to chart the route taken by the unfolded protein signal as it travels to the nucleus. Finally, we addressed the function of the unfolded protein response by testing the requirement for the pathway in cells that have lowered amounts of the essential proteins BiP and PDI in their ER. The work described in this chapter is not yet at an endpoint--many experiments still remain to be done--but the results are consistent with the proposed model and suggest two important refinements.

Results

Ire1p association with BiP and other proteins

To identify additional components of the unfolded protein response, we looked for proteins that associate with Ire1p. In particular, we looked for BiP binding to Ire1p and determined whether its binding changed with the

induction of the unfolded protein response. We labelled cells with ^{35}S methionine, made whole-cell lysates in the absence of detergent, crosslinked the lysates using the reversible crosslinker DSP, isolated membranes, solubilized the crosslinked membranes with SDS, carried out immunoprecipitations with antibodies to Ire1p, and then ran the immunoprecipitation products on protein gels under conditions that reverse the crosslinker. In initial experiments with cells overexpressing Ire1p, several proteins were observed to bind to Ire1p in a crosslinker-dependent manner (Figure IV-1). Of these, the most prominent band has a molecular weight of approximately 80 kD, the same as BiP. That this band is BiP was confirmed by Western blotting using α-BiP polyclonal antibodies (data not shown). The identities of the other bands have not yet been determined.

That BiP, a very abundant ER resident protein, binds to Ire1p is interesting. However, because its role is to bind non-specifically to secretory proteins as they pass through the ER, the observed binding of BiP to Ire1p is not necessarily regulatory. We therefore asked whether this binding could be observed in cells expressing wild-type levels of Ire1p and whether the binding changed after induction of the unfolded protein response. The model we are testing predicts that BiP binding to Ire1p should be greatest when the response is not induced and should fall as unfolded proteins accumulate in the ER. We repeated the crosslinking experiment described above with wild-type cells grown in the presence and absence of tunicamycin and found that the amount of BiP binding to Ire1p is identical under both conditions (Figure IV-2).

Our observation that the amount of BiP crosslinked to Ire1p does not change does not necessarily mean that *in vivo*, BiP binding to Ire1p does not change after induction of the unfolded protein response. Our inability to detect a change in BiP bound to Ire1p may reflect a limitation in the *in vitro* binding experiment. Almost thirty minutes elapse from the time that cells are removed from the growth medium to the time that cross-linker is added to the lysates. During this time, the conditions in the ER might change such that they no longer resemble the conditions under which the unfolded protein response is (or is not) induced. On the other hand, our results suggest that we cannot exclude the possibility that BiP is always bound to Ire1p, whether or not unfolded proteins have accumulated in the ER.

Subcellular localization of Ire1p

As discussed previously, there are two possible routes that the "unfolded protein" signal can follow between the ER and the nucleus. It can pass directly to the nucleus through the inner nuclear membrane or it can travel through the cytoplasm. Because subcellular fractionation of yeast nuclei from non-nuclear-associated ER is difficult, it is not possible using biochemical methods to determine whether Ire1p is localized exclusively to the inner nuclear membrane, exclusively to ER membrane that contacts cytoplasm, or to both. Thus, we sought to determine the subcellular localization of Ire1p using immunofluorescence and immunoelectron microscopy (immuno-EM).

To confirm that Ire1p is indeed localized to the ER, we carried out immunofluorescence on yeast cells expressing a myc epitope-tagged version of Ire1p (see Appendix A). Because we were not sure at first whether our antibodies would detect the protein after formaldehyde fixation, we used a strain in which Ire1p-myc is carried on a 2μm high copy number plasmid but is expressed from its own promoter (strain CS200: CS165 cells transformed with plamid pCS118, see Table IV-1 and Materials and Methods). We found that Ire1p could be detected in CS200 cells with the α-myc monoclonal antibody and also with the α-Ire1Nterm and α-Ire1Cterm polyclonal antibodies (see Chapter III and Appendix B) and that all three antibodies show the same staining pattern (Figure IV-3a). The pattern is reticular and similar to, but much fainter than, the staining pattern observed when cells from the same strain are stained with α-BiP antibodies (Figure IV-3b). Thus, Ire1p, at least when overexpressed, seems to localize to the ER, both around the nucleus (as demonstrated by co-staining with the DNA-intercalating dye DAPI, data not shown) and at the periphery of the cell. We also attempted to examine the localization of Ire1p under a more physiologically relevant situation, in cells bearing only one copy of the *IRE1* gene. Unfortunately, in such cells, neither Ire1p-myc nor wild-type Ire1p was detectable by immunofluorescence with the α-myc monoclonal or α-Ire1p polyclonal antibodies.

To determine exactly where Ire1p resides, in the inner nuclear membrane or in the ER membrane contacting the cytoplasm, immuno-EM must be carried out because individual membranes cannot be resolved by immunofluorescence microscopy. To this end, we began a collaboration with Jon Mulholland and David Botstein (Stanford University). We provided

yeast strains, α-myc antibodies, and affinity purified α-Ire1Nterm and α-Ire1Cterm antibodies to Jon Mulholland and he carried out the immuno-EM.

Even though immuno-EM is a more sensitive technique than immunofluorescence, we were unable to detect Ire1p over background staining when Ire1p was expressed at wild-type levels in the cell. We obtained this negative result whether the three antibodies were used individually or as a mixture of α-Ire1Nterm and α-Ire1Cterm antibodies. However, Ire1p was detectable when it was overexpressed in CS200 cells. There, it was localized both to the inner nuclear membrane and to parts of the ER membrane that contact the cytoplasm (Figure IV-4). Thus, Ire1p is clearly present in the ER when it is overexpressed. This is consistent with our hypothesis that Ire1p detects the accumulation of unfolded protein in the ER and then transmits the signal out of the ER. Unfortunately, because the kinase is overexpressed and therefore may be localized aberrantly, these results do not allow us to draw any conclusions about the route of the unfolded protein signal.

In the electron micrographs, we noticed that cells overexpressing Ire1p-myc look less healthy than wild-type control cells: CS200 cells have enlarged vacuoles and seem to have extra ER membranes. We hypothesize that the additional ER may be produced to accommodate the extra Ire1p present in CS200 cells in the same way that cells overexpressing other membrane proteins produce additional membranous structures called karmellae (Wright et al., 1988). This observation emphasizes the importance of determining the localization of Ire1p in cells expressing only wild-type levels of the protein. To do this, antibodies with a higher affinity for Ire1p must be raised both

because the current α-Ire1p antibodies seem to have low affinity for Ire1p and because Ire1p is probably not very abundant (see Chapter III and Appendix A for a discussion of this point). An alternative way to distinguish between the two possible routes for the unfolded protein signal is to wait until other components of the pathway have been identified and then to determine their subcellular localization.

Secretion of BiP or PDI and the unfolded protein response

Lumenal ER proteins are retained in the ER by virtue of an amino acid signal present at their C-terminus. In *S. cerevisiae*, this signal is HDEL. It has been shown that removal of these amino acids from BiP or from PDI causes secretion of the proteins without significantly affecting cell viability (Hardwick et al., 1990; LaMantia and Lennarz, 1993; Pelham et al., 1988). Cells expressing BiP that lacks the C-terminal HDEL (hereafter referred to as "HDEL-less" BiP) as their only version of the protein have an increased rate of BiP protein synthesis (Hardwick et al., 1990). This increase in expression allows intracellular levels of BiP to remain essentially identical to wild-type even though large amounts of BiP are also secreted from the cell. The location of the intracellular BiP in these mutants has not been determined. It is likely that the levels of BiP in the ER are slightly lower than wild-type, while BiP levels in other compartments of the secretory pathway are higher than normal. As described in Chapters I and II, it has been suggested that the increase in BiP synthesis in HDEL-less BiP strains is mediated by the unfolded protein response because most of the BiP in the ER of the mutants is bound to

unfolded proteins and is not "free" to downregulate the response (Ng et al., 1992).

If the unfolded protein response causes the increase in BiP production in HDEL-less BiP mutants, then the level of transcription of the coordinately regulated *KAR2* and *PDI1* genes must be greater in the mutants than in wild-type cells. To examine this possibility, we obtained the HDEL-less BiP strain (6210.2 YFGR) and its wild-type parent (SEY6210) described by Hardwick et al. (1990) and carried out S1 nuclease protection assays to measure the relative amounts of *KAR2* and *PDI1* transcripts (Figure IV-5a and 5b). We observed that, in the absence of tunicamycin, the level of transcription of both loci is reproducibly higher in the HDEL-less BiP strain than in the control strain, a difference of almost two-fold. Interestingly, the response is not fully induced under these conditions--the HDEL-less BiP mutant increases *KAR2* and *PDI1* transcription further when tunicamycin is added.

Because *PDI1* is an essential gene (LaMantia et al., 1991), PDI may play as important a role as BiP in folding proteins in the ER and possibly in monitoring the levels of unfolded protein there. We wanted to test whether secretion of PDI might also induce the unfolded protein response. We constructed a strain that expresses HDEL-less PDI as its only version of the protein (see Materials and Methods), and examined transcription of *KAR2* by S1 nuclease protection assays (Figure IV-5c and 5d). Just as for HDEL-less BiP, we saw an increased basal level of *KAR2* transcription in HDEL-less PDI cells. This level increased further upon addition of tunicamycin.

These results suggest that the unfolded protein response mediates the increase in BiP and PDI production in cells bearing HDEL-less proteins. Further evidence supporting this possibility comes from experiments that suggest that the HDEL-less alleles of BiP or PDI are synthetically lethal with *ire1* mutations. When diploids heterozygous for a null mutation in *ire1* and either HDEL-less BiP or HDEL-less PDI are sporulated, no spores that bear both the *ire1* and the HDEL-less mutation grow (Duncan Wilson, personal communication and data not shown). Thus Ire1p, and probably the unfolded protein response, seems to be required for germination of spores that do not retain BiP or PDI in their ER. It is reasonable to speculate that the unfolded protein response is required for the viability of any cell that constitutively secretes BiP or PDI, but further experiments must be carried out to verify this. Synthetic lethality of *ire1* with HDEL-less alleles would make sense if increased production of BiP and PDI (and probably other ER resident proteins) is mediated by the unfolded protein response in cells secreting these important proteins: the *ire1* mutation prevents the cells from inducing the unfolded protein response, and the cells die as a result of the diminished levels of chaperone proteins in their ER.

Discussion

Although we were not able to determine the subcellular location of Ire1p, we have shown that BiP associates with Ire1p and that BiP might bind to Ire1p whether or not the unfolded protein response is induced. Our data also suggest that cells secreting BiP or PDI seem to induce a low level

unfolded protein response constitutively and that this response is required for their survival.

To accommodate this new information, two important alterations must be made in the model for the role of BiP in the unfolded protein response that was proposed in Chapter II. First, our results suggest that PDI may be as important as BiP in monitoring the levels of unfolded protein in the ER. The BiP molecules in the model should thus be generalized to be chaperones, including PDI and other ER resident proteins. Second, although the data are not definitive, it is possible that there is no quantitative change in the amount of BiP bound to Ire1p with the induction of the response. As diagrammed in Figure IV-6, this lack of change does not rule out a regulatory role for BiP in the revised model. When few unfolded proteins are present in the ER, binding of chaperone(s) to Ire1p would prevent the kinase from oligomerizing, as proposed before. However, when unfolded proteins accumulate in the ER, chaperone binding to an unfolded protein at the same time as it bound to Ire1p would allow a local conformational change in Ire1p that promoted oligomerization and activation of the kinase.

This revised model, while incorporating the current data on BiP, PDI, and the unfolded protein response, would also explain how small changes in the number of chaperone molecules binding to unfolded proteins could affect the oligomerization state of a protein of such relatively low abundance as Ire1p. Furthermore, it makes several predictions that can be tested experimentally: PDI and other ER resident chaperones bind to Ire1p; the chaperones have Ire1p binding sites that are distinct from their unfolded

protein binding sites; and unfolded proteins associate with Ire1p in complexes with the chaperones.

Finally, the observation that the unfolded protein response is induced when levels of ER resident proteins fall re-emphasizes the importance of Ire1p and the unfolded protein response in the life of a cell. The unfolded protein response seems to be conserved through evolution because it is required for maintaining the integrity of the ER when cells are subjected to stress.

Materials and Methods

Plasmids and strains

Plasmids pCS118 and pCS122 are describe in Appendix A. Sequences changing the C-terminal amino acids of PDI from AIHDEL to AIGR (strain CS297) were introduced into the *PDI1* gene by homologous recombination (Sikorski and Hieter, 1989) . The integrating vector used was YIplac204 (TRP^+; Gietz and Sugino, 1988) and the resulting *pdi1* gene also has the *ACT1* transcriptional terminator in place of the *PDI1* terminator. It has not been not confirmed that PDI is actually secreted from strain CS297.

Antibodies

The α-Ire1p antibodies are described in Chapter III and Appendix B. α-BiP antibodies were made exactly as described by Rose et al. (1989). α-Ire1Nterm and α-Ire1Cterm antibodies were affinity purified following the protocol described by Matlack (1994) The antisera were first passed over a GST

column to deplete α-GST antibodies. Partially purified GST-Ire1Nterm and GST-Ire1Cterm fusions (the antigens used to innoculate the rabbits) was coupled to Affigel-10 (Bio-Rad Laboratories, Hercules CA) in 50mM Hepes pH 7.6, 150 mM SDS. The flow through from the GST columns was then passed over either the GST-Ire1Nterm or GST-Ire1Cterm column and, after washing with CTBS/Tween (50 mMTris pH 7.5, 150 mM NaCl, 0.5% Tween 20), the affinity pure antibodies were eluted with 200mM Glycine pH2.5, 200 mM NaCl. The pH was neutralized immediately with high pH Tris, the antibodies were dialyzed into CTBS, glycerol was added to 30% and the antibodies were stored at -80°C in small aliquots.

Immunofluorescence

Immunofluorescence was performed essentially as described previously (Pringle et al., 1989). α-Ire1p and α-myc antibodies were used at dilutions of 1:250 to 1:1000. α-BiP/Kar2p antibodies were used at a dilution of 1:10,000. Bound primary antibodies were decorated with either FITC-conjugated donkey α-mouse IgG or FITC-conjugated donkey α-rabbit IgG (Jackson Immunoresearch, West Grove PA) diluted 1:100. Samples were stained briefly with a solution of 1mg/ml DAPI and then washed well before being mounted in 90% glycerol containing 1 mg/ml phenylenediamine, pH 9.0. Light micrographs were taken on a Nikon Microphot-FXA (Nikon Inc., Melville NY) using TMAX P3200 film (Eastman Kodak Co., Rochester NY).

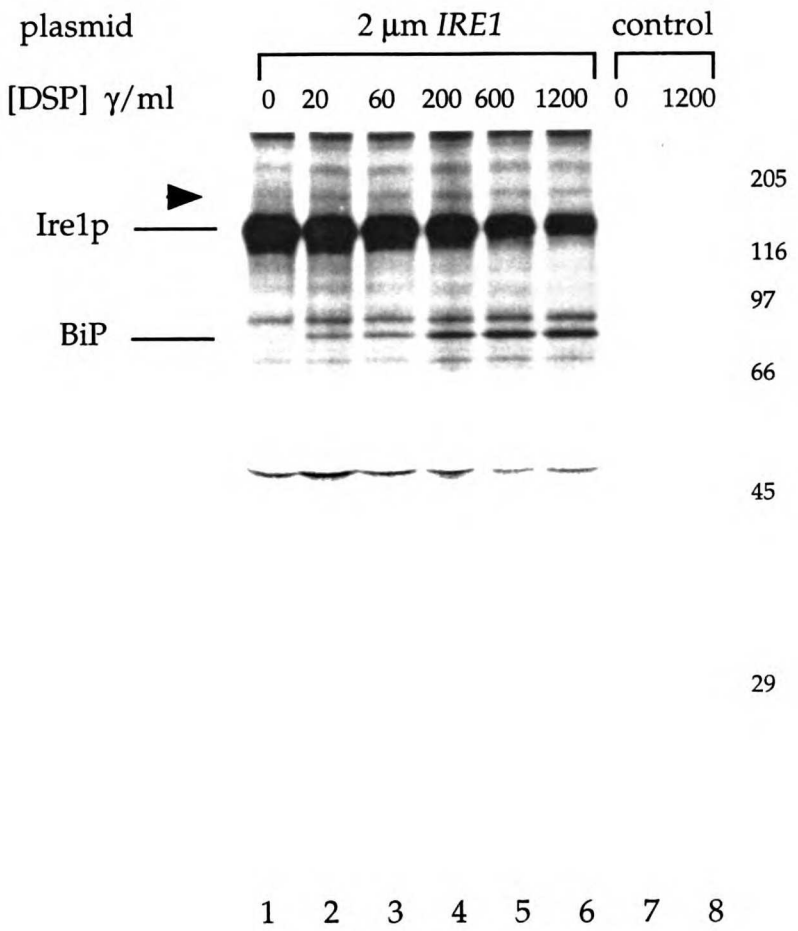
Immunoelectron microscopy

Immunoelectron microscopy was carried out according to Method IV described by (Mulholland et al., 1994). Supernatant from cell line 9E10 (Evan et al., 1985), expressing the α-myc antibody was used at a dilution of 1:1. A 1:1

Table IV-1 Yeast strains

Strain	Genotype	Source/Reference
JC102	leu2-3,-112; his3-11,-15; trp1-1; ura3-1; ade2-1; can1-100; MAT<u>a</u>; leu2-3,-112::LEU$^+$UPRE-lacZ; MET$^+$	Chapter III
JC103	same as JC102, except his3-11,-15::HIS$^+$UPRE-lacZ; met$^-$	Chapter II
CS228	same as JC102, except MATα	This study
CS243	same as JC102, except ire1::URA$^+$pRS306 (entire Ire1p coding sequence deleted; Sikorsi and Hieter, 1989)	Chapter III
CS297	same as CS228, except pdi1::TRP$^+$pdi1-HDELless	This study
SEY6210	leu2-3,112; his3-Δ200; trp1-Δ901; ura3-52; suc2Δ9; lys2-801; MATα	Scott Emr
6210.2YFGR	same as SEY6210, except kar2::BiP FGR (BiP HDELless)	Hardwick et al., 1990

Figure IV-1 BiP associates with Ire1p. Cells deleted for their chromosomal copy of *IRE1* (strain CS243) and either overexpressing *IRE1* on a 2 μm plasmid (pCS122) or bearing a control plasmid with no *IRE1* gene (YEplac112; Gietz and Sugino, 1988) were labelled with ^{35}S methionine and extracts were made. Crosslinking was carried out with the indicated amount of DSP, membranes were isolated and then solubilized, and non-native immunoprecipitations were carried out with with α–Ire1Nterm antibodies (the protocol is described in more detail in Chapter III). The crosslinker was reversed before PAGE, and the gel was autoradiographed to visualize the products of the immunoprecipitations. **(a)** is a shorter exposure of the same gel shown in **(b)**. Bands corresponding to Ire1p and BiP are indicated. Other proteins that associate with Ire1p in a crosslinker-dependent manner are indicated with arrowheads. The mobility of molecular weight standards is indicated at the right edge of each gel.


plasmid
2 µm IRE1
control
[DSP] γ/ml
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Ire1p
BiP
205
116
97
66
45
29
1 2 3 4 5 6 7 8

Figure IV-1a

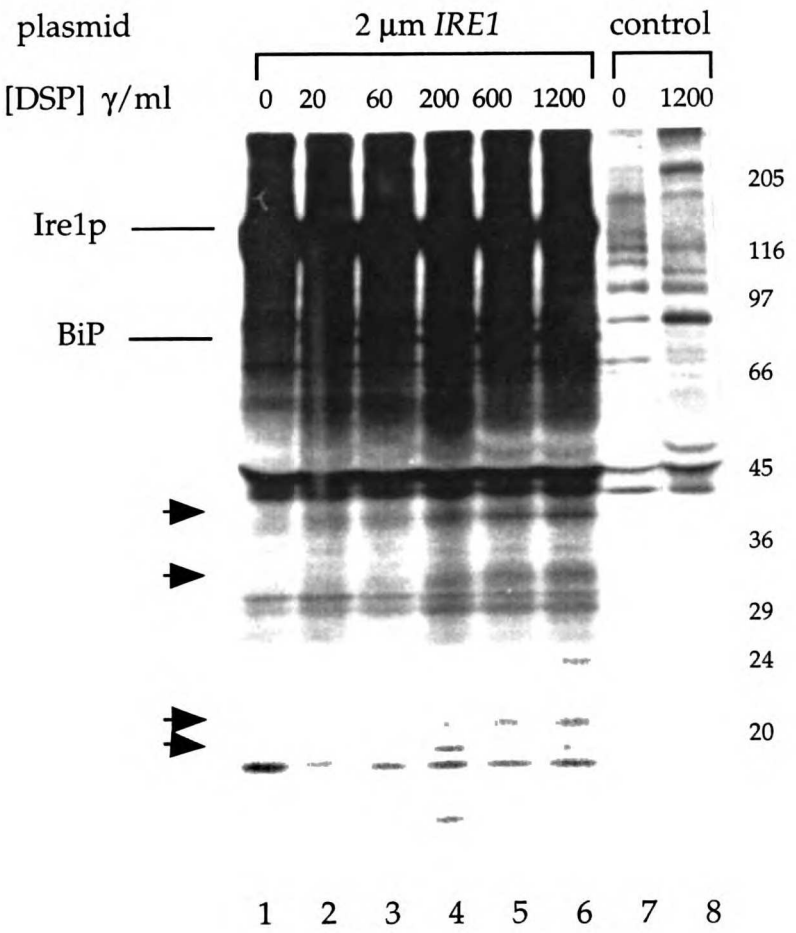

plasmid
2 µm IRE1
control
[DSP] γ/ml
0 20 60 200 600 1200 0 1200
Ire1p
BiP
205
116
97
66
45
36
29
24
20
1 2 3 4 5 6 7 8

Figure IV-1b

Figure IV-2 The association of BiP with Ire1p does not change with the unfolded protein response. Wild-type cells (strain JC102), cells deleted for the Ire1p coding sequence (Δ*ire1*; strain CS243), and cells overexpressing Ire1p (CS243 cells transformed with pCS122) were labelled with ^{35}S methionine for one hour. The cultures were divided into two equal portions, tunicamycin was added to one, and cells were grown for an additional 30 minutes before whole-cell extracts were made. Crosslinking and PAGE were carried out as described in Chapter III. A Western blot of the gel was done and the blot probed with α-BiP antibodies. Bands corresponding to BiP are indicated. Two-fold less extract from 2μm *IRE1* cells than from wild-type or Δ*ire1* cells was loaded in the indicated lane. The mobilities of molecular weight standards are indicated on the left side of the blot.

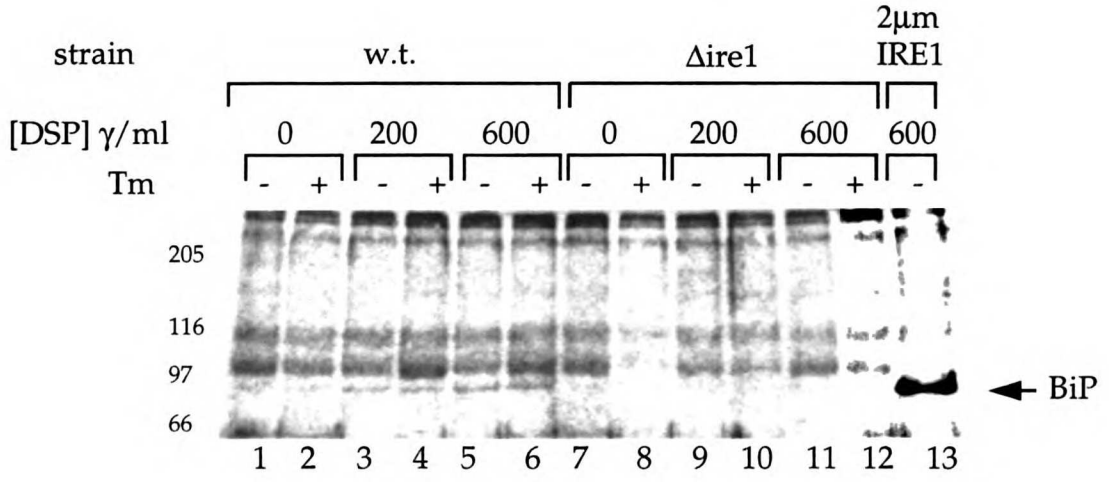
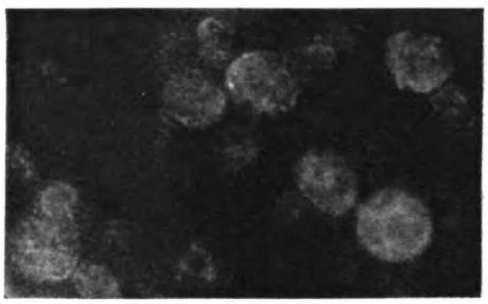

strain
w.t.
Δire1
2μm IRE1
[DSP] γ/ml
0
200
600
0
200
600
600
Tm
- + - + - + - + - + - + -
205
116
97
66
BiP
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure IV-2

Figure IV-3 Immunofluorescence microscopy shows Ire1p localized to the ER **(a)** CS200 cells (overexpressing Irep-myc) stained with α-Ire1Cterm antibodies. **(b)** CS200 cells stained with α-BiP antibodies.

a. α-Ire1Cterm



b. α-BiP

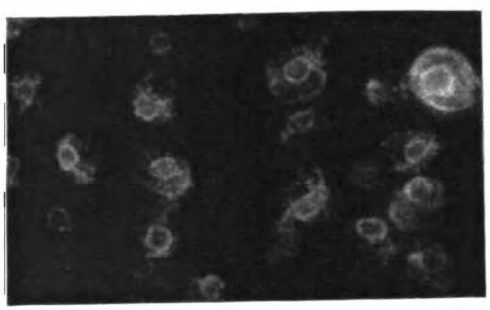


Figure IV-3

Figure IV-4 Immunoelectron microscopy shows Ire1p localized to the ER. Cell nuclei (N) and vacuoles (V) are indicated as are gold particles presumably bound to α-myc or α-Ire1p primary antibodies (arrows). A negative control for the α-Ire1p antibodies is not shown, but few gold particles were visible on sections of JC103 cells stained with α-Ire1p antibodies (J. Mulholland, personal communication). (a) JC103 cells (wild-type) stained with α-myc antibodies, magnification 60,000x. (b) CS200 (overexpressing Irep-myc) cells stained with α-myc antibodies, magnification 50,000 x. (c) CS200 cells stained with a mixture of α-Ire1Nterm and α-Ire1Cterm antibodies, magnification 35,000 x. (d) CS200 cells stained with a mixture of α-Ire1Nterm and α-Ire1Cterm antibodies, magnification 55,000 x.

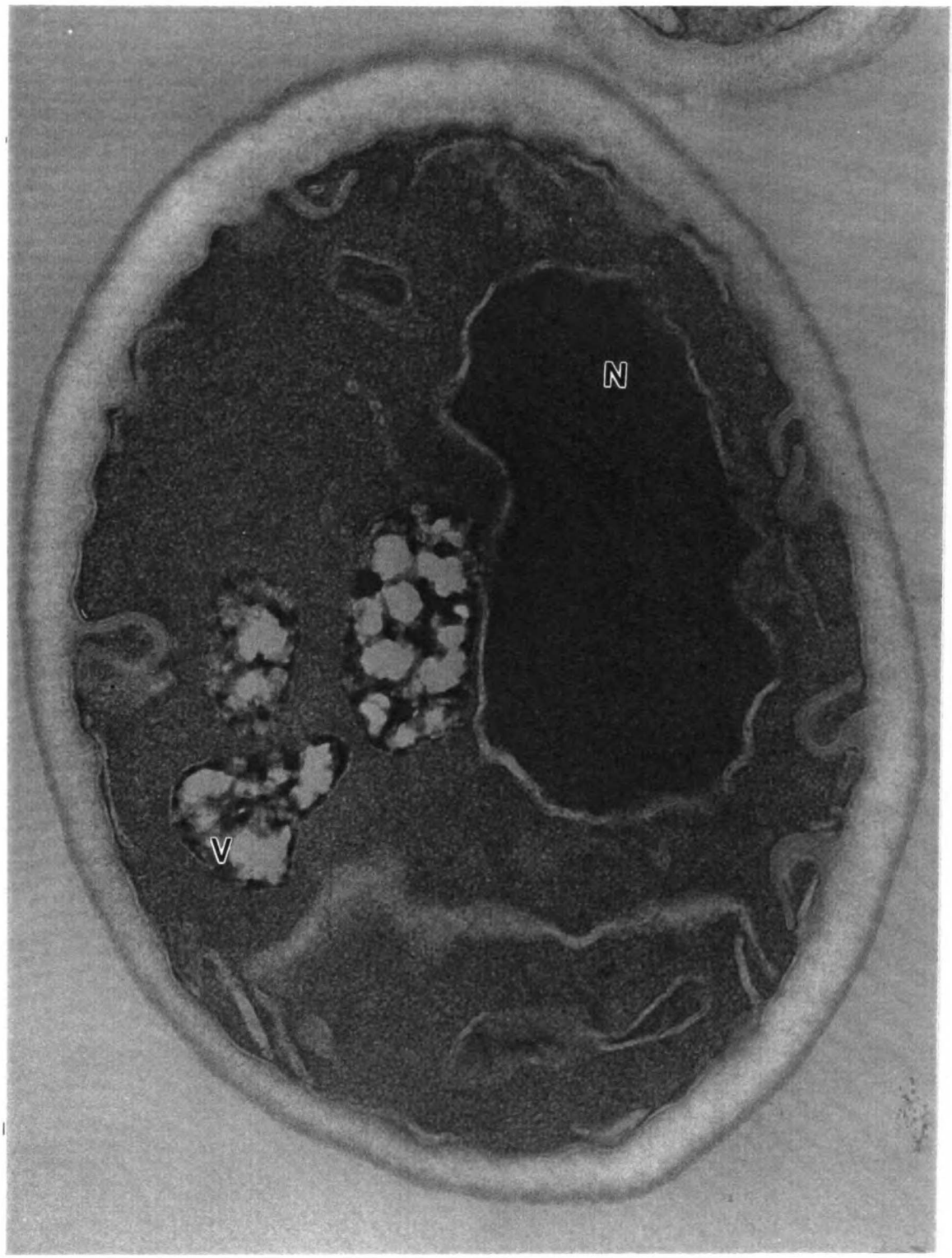


Figure IV-4a wild-type, α-myc

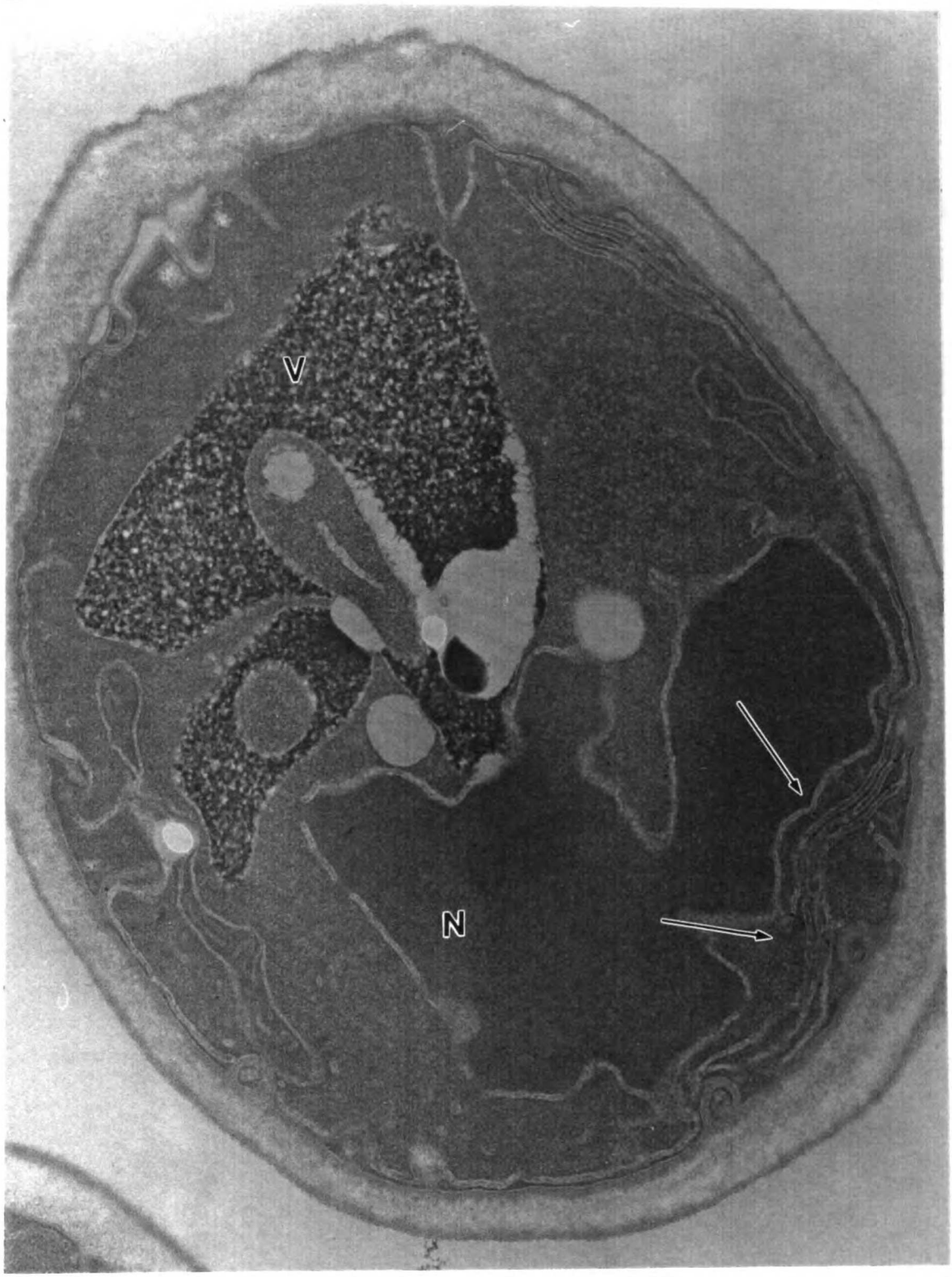


Figure IV-4b 2µm Ire1p-myc, α-myc

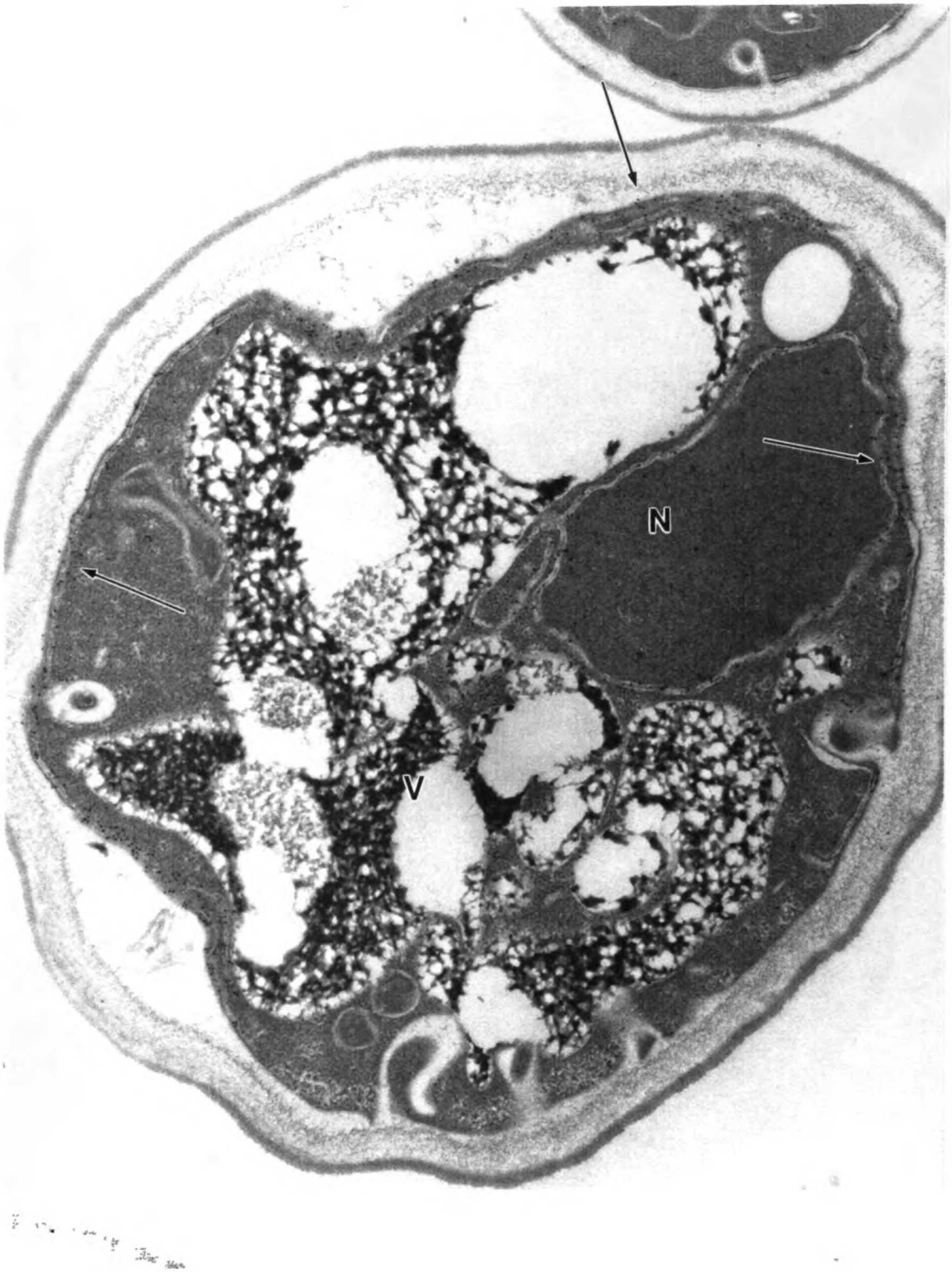


Figure IV-4c 2μm Ire1p-myc, α-Ire1p

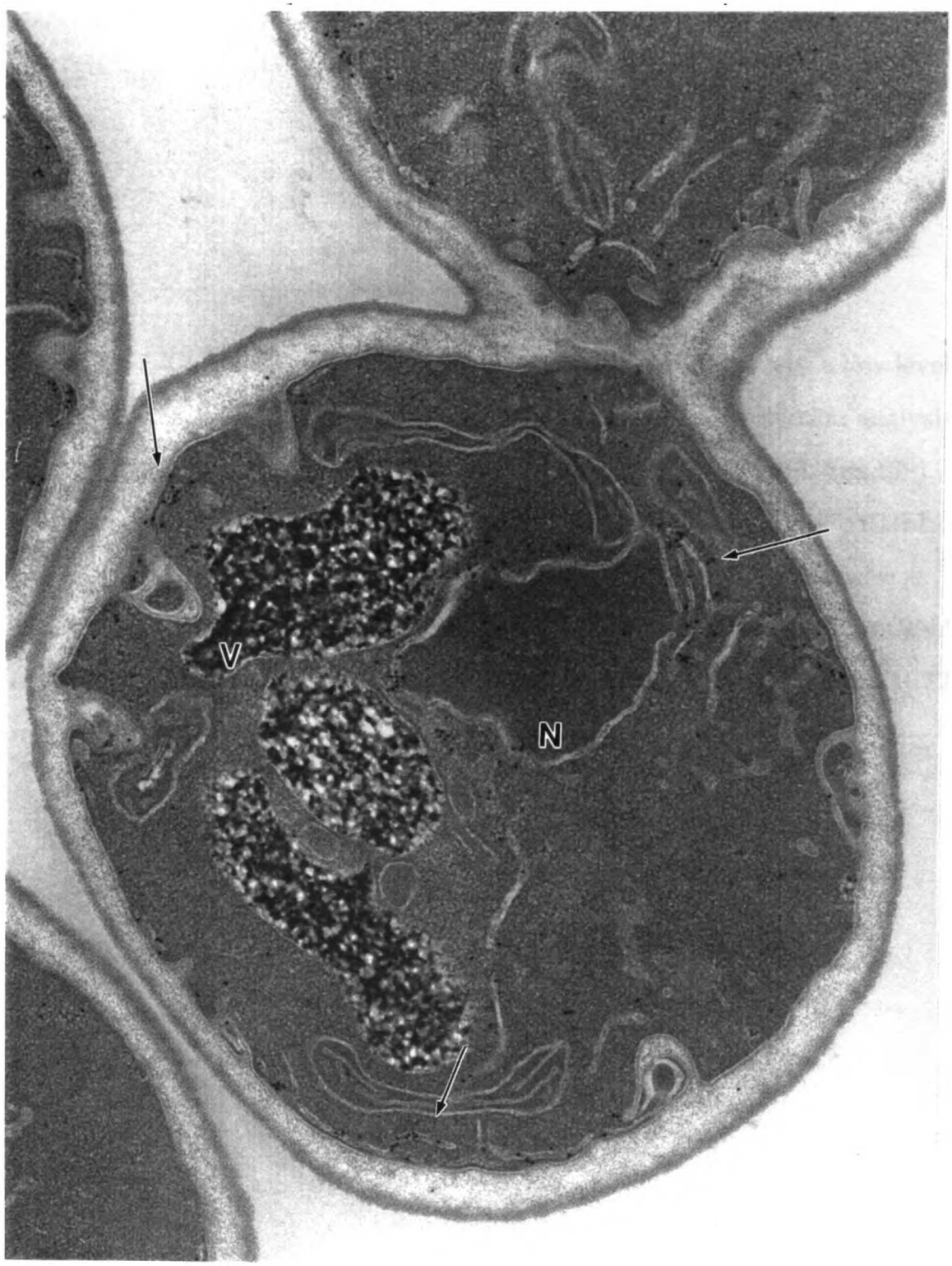


Figure IV-4d 2μm Ire1p-myc, α-Ire1p

Figure IV-5 Cells bearing HDEL-less alleles of BiP and PDI induce a low level unfolded protein response constitutively. **(a)** S1 nuclease protection analysis of RNA made from strains SEY6210 (w.t.) and 6210.2 YFGR (HDEL-less BiP). **(c)** S1 nuclease protection analysis of RNA made from strains CS297 (HDEL-less PDI) and CS228 (w.t.). Tunicamycin induction followed by S1 nuclease protection analysis was performed as in Figure II-2a. **(b)** and**(d)** *KAR2* mRNA levels from (a) and (c) respectively were quantitated and normalized to *ACT1* mRNA levels as in Figure II-1c.

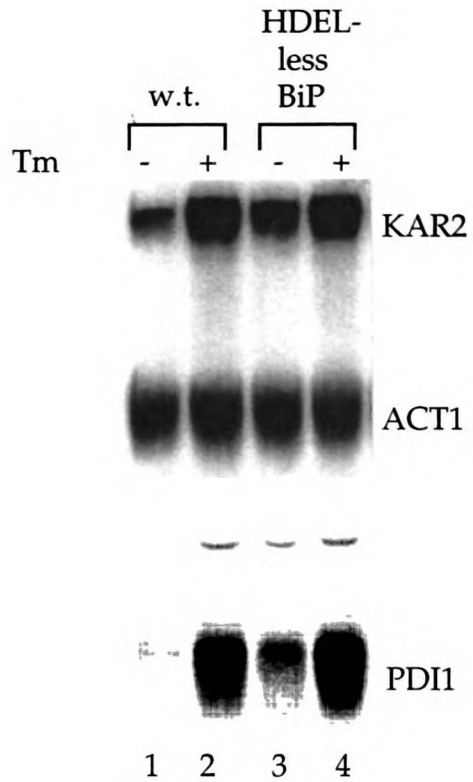


Figure IV-5a

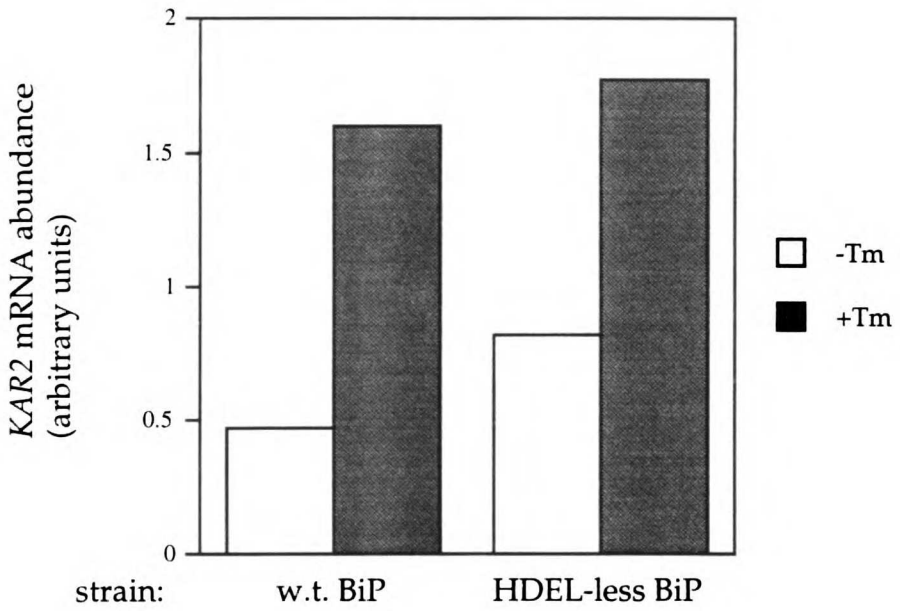

KAR2 mRNA abundance
(arbitrary units)
2
1.5
1
0.5
0
strain:
w.t. BiP
HDEL-less BiP
-Tm
+Tm

Figure IV-5b

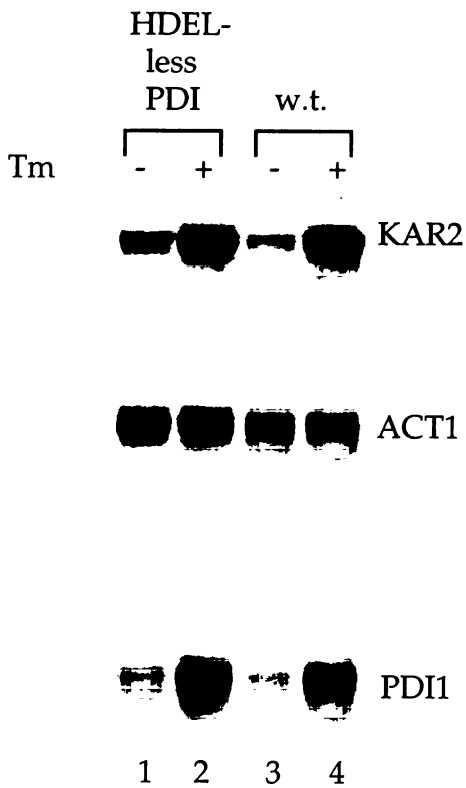


Figure IV-5c

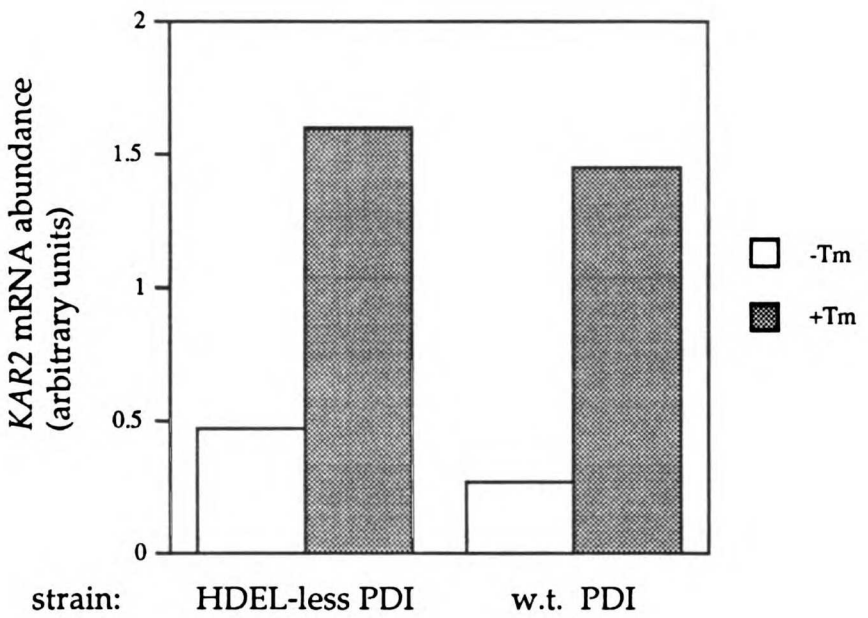

KAR2 mRNA abundance
(arbitrary units)
2
1.5
1
0.5
0
-Tm
+Tm
strain:
HDEL-less PDI
w.t. PDI

Figure IV-5d

Figure IV-6 A model to explain how accumulation of unfolded proteins in the ER might trigger Ire1p multimerization. The uncolored protein represents Ire1p and the cross-hatched protein represents ER resident chaperone proteins such as BiP and PDI. The squiggles are unfolded proteins and shaded proteins are depicted binding to the Ire1p C-terminal tail. When few unfolded proteins are present in the ER, binding of chaperone proteins to Ire1p prevent the kinase from oligomerizing. When unfolded proteins accumulate, chaperone binding to an unfolded protein at the same time as it binds to Ire1p allows a local conformational change in Ire1p that promotes oligomerization and activation of the kinase.

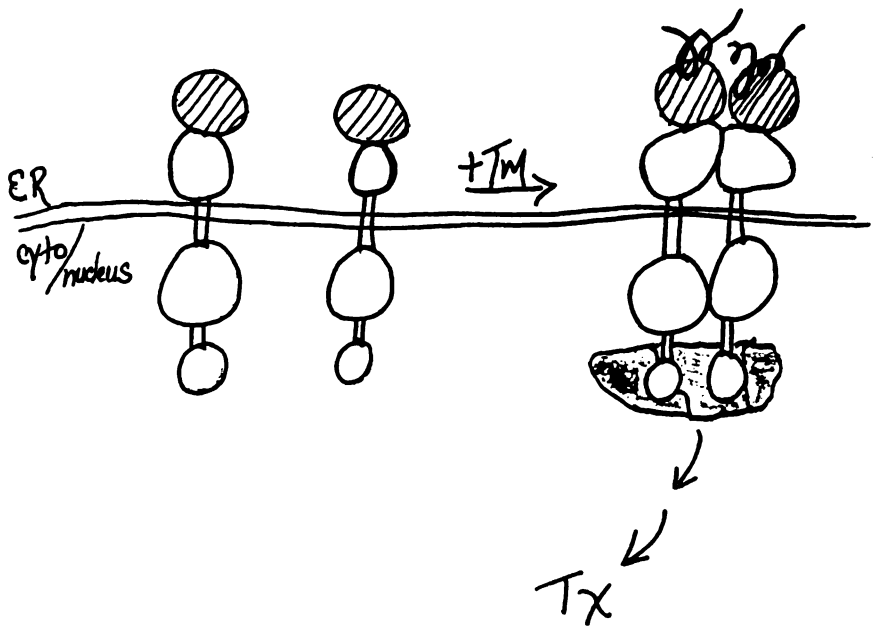


Figure IV-6

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Chapter V:

Summary and future prospects

This thesis describes the progress that we have made in the last four years in studying the unfolded protein response in yeast. The serendipitous discovery that the transmembrane kinase Ire1p is required for the response has given us clues as to how the unfolded protein signal is transmitted out of the ER. The structural similarity between Ire1p and receptor protein kinases suggests a testable model for the mechanism of action of Ire1p. This model has withstood experimental scrutiny. However, we have have just begun to understand the intricacies of the unfolded protein response pathway. Only two components of the pathway have been identified unambiguously: Ire1p and the UPRE upstream activating sequence. To determine how cells sense the accumulation of unfolded proteins in the ER and induce transcription in the nucleus, more components of the pathway must be identified.

Because Ire1p lies half in the ER and half in the cytoplasm or nucleus, identifying proteins that interact directly with Ire1p will help us to understand what happens on each side of the ER membrane. As described in Chapter III, it seems likely that cytoplasmic or nuclear proteins bind to the Ire1p C-terminal tail and are important for transmitting the unfolded protein signal toward the transcription machinery. As shown in Chapter IV (Figure IV-1) several proteins that can be crosslinked to Ire1p have already been observed. One of the crosslinked proteins is BiP, and although more experiments must be done to determine the physiological significance of the interaction, it will be interesting to see whether any of the other Ire1p-associated proteins are ER chaperones. Proteins that interact with Ire1p may be found by standard biochemical methods or by genetic methods (for example by using the two-hybrid approach (Fields and Song, 1989).

Development of an *in vitro* assay for Ire1p kinase activity will be useful for finding Ire1p substrates and regulators.

The identification of Ire1p as a component of the unfolded protein response pathway allows more sophisticated genetic screens to be designed to identifiy new components of the pathway, components that do not necessarily interact directly with Ire1p. To find downstream proteins that negatively regulate the unfolded protein response, one could look for mutants that, in the absence of Ire1p, constitutively activate the pathway. Also, primary screens for mutants unable to carry out the unfolded protein response might be repeated using UPRE reporter constructs but new secondary screens can now be applied to interesting candidate mutants before time-consuming S1 nuclease protection assays confirm the lack of transcriptional induction of *KAR2* and *PDI1*. For example, mutants unable to carry out the unfolded protein response might be inositol auxotrophs and supersensitive to ER stress conditions such as growth in tunicamycin or the presence of BiP-HDELless. As this thesis is being written, new genetic screens such as these are already underway.

The link between inositol biosynthesis and the unfolded protein response is being explored as well. We know that the unfolded protein response is important for cell survival after ER stress. It seems likely that the response protects the cell not only by increasing production of ER resident chaperones, but also by synthesizing more ER membrane to accommodate the new proteins. Recently, Ire1p homologues have been found in the nematode *C. elegans* and in the yeast *K. lactis* (J. Cox, unpublished observations). Thus, because the unfolded protein response is conserved through evolution, study

of the unfolded protein response and the metabolic pathways that it coordinates may give us insight into the mechanisms used by higher cells to maintain organellar homeostasis.

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Appendix A:

Epitope tags on Ire1p

In order to detect Ire1p using monoclonal antibodies directed against well-characterized protein epitopes, five different epitope tags were fused to the C-terminus of the protein: 1 copy of the influenza hemagglutinin (HA) epitope, three copies of the HA epitope, one copy of the *c-myc* (myc) epitope, three copies of the *c-myc* epitope, and two copies of the polyoma (Py) tag (see Table A-I; Bollag et al., 1993; Evan et al., 1985; Field et al., 1988; Grussenmeyer et al., 1985). All of the tags were fused to Ire1p after the last amino acid (serine 1115).

TABLE A-1

Tag	Protein Sequence*
HA	AMAYPYDVPDYASLGPGL
3 x HA	AMAYPYDVPDYAGYPYDVPDYAGSYPYDVPDYAAQC
myc	AMEQKLISEEDLN
3 x myc	AMEQKLISEEDLNGEQKLISEEDLNGSEQKLISEEDLN
2 x Py	AMEYMPMEMEYMPMESR

*the underlined sequences correspond to the epitope recognized by the monoclonal antibodies.

Tagged versions of Ire1p were introduced into yeast cells such that only tagged Ire1p was expressed in each cell. Using these strains, we found, unfortunately, that adding a C-terminal tag to Ire1p diminishes the cell's ability to carry out the the unfolded protein response (Figure A-1). In their paper, Mori et al. (1993) report a similar finding. Interestingly, at least in the case of Ire1p-myc, overexpression the tagged version of the kinase does not

entirely compensate for the defect (Figure A-2). We do not know whether the presence of the tag affects Ire1p kinase activity, affects the ability of Ire1p to interact with other components of the signal transduction machinery, or both. The unfolded protein response was diminished to different degrees with different tags, and the severity of the effect did not correspond directly to the length of the tag. The single copy of the HA epitope had the least negative effect on the unfolded protein response. Strains bearing Ire1p-HA are reproducibly able to carry out an unfolded protein response that is 80-90% that of wild-type as measured by induction of *KAR2* and *PDI1* transcription. Because the signal sequence of Ire1p is required for its proper localization, tags cannot be placed at its N-terminus. Thus, the only hope to epitope tag Ire1p without disrupting its function seems to be to insert the tag somewhere within the protein. The feasibility of this will have to be determined experimentally by trial and error.

The defect in the unfolded protein response in cells bearing tagged Ire1p does not seem result from poor expression of the proteins. As monitored by immunoprecipitation using polyclonal antibodies directed against Ire1p (see Chapter III), the levels of expression of the HA-, 3 x HA-, and polyoma-tagged Ire1p were approximately the same as that of wild-type Ire1p in control cells. Similarly, when myc-tagged Ire1p was introduced into cells on a multi-copy plasmid, its level of expression was the same as or greater than that of wild-type Ire1p expressed from a multi-copy plasmid. The levels of expression of myc- or 3 x myc- tagged Ire1p in cells bearing only one chromosomal copy of the tagged gene were not checked. However, because the copy of *ire1* in those cells differs from the HA- or polyoma-tagged *ire1* version only in the bases that encode the epitope tag and because myc-tagged

Ire1p can be overexpressed to at least the same levels as wild-type, it is likely that expression of myc- and 3 x myc-tagged Ire1p from genes present in single copy in the genome is also similar to wild-type.

By Western blot, probing with monoclonal antibodies directed against the HA epitope, HA- and 3 x HA-tagged Ire1p can be detected in extracts made from cells bearing only one chromosomal copy of the tagged gene. Neither myc-tagged Ire1p nor Py-tagged Ire1p can be detected with antibodies directed against the appropriate epitope on Western blots of extract from cells in which the tagged gene is present in single copy in the genome. These results suggest that the α-HA antibodies might have a relatively higher affinity for their epitope under our blotting conditions than do the α-myc and α-Py antibodies. Furthermore, they suggest that Ire1p might not be present in the cell in impossibly low amounts and that high affinity polyclonal antibodies directed against Ire1p could be produced and would be very useful.

Materials and Methods

Strains and plasmids

Sequences encoding epitope tags were introduced at the C-terminus of Ire1p in strains CS189 (IRE1-myc), CS225 (IRE1- 3 x HA), CS226 (IRE1-HA), CS239 (IRE1-3 x myc), and CS295 (IRE1-2 x Py) by homologous recombination (Sikorski and Hieter, 1989) The myc, 3 x HA, HA, and 3 x myc tags fused to IRE1 coding sequences were introduced on vector YIplac204 (TRP^+) and the 2 x PY tag was introduced on vector YIplac211 (URA^+; Gietz and Sugino, 1988) . In all cases, the DNA sequence of each tag is followed by the transcription

terminator from the *ACT1* gene. Plasmids pCS118 (2μm IRE1-myc) and pCS122 (2μm wild-type IRE1) are based on the high copy vector backbone YEplac112; Gietz and Sugino, 1988) . pCS122 carries the XhoI to HindIII fragment of *IRE1* (see Chapter II). pCS118 carries the same *IRE1* promoter region and coding sequences but the construct is different from pCS122 in that the myc tag is added at the C-terminus of the protein and the *ACT1* transcription terminator replaces the *IRE1* sequences 3′ to the translation stop site.

Table A-2 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$^+$	Chapter III
CS189	same as JC102, except his3-11,15:: HIS$^+$UPRE-lac Z; met$^-$; ire1:: TRP$^+$ire1-myc	This study
CS225	same as JC102, except ire1:: TRP$^+$ire1-3 x HA	This study
CS226	same as JC102, except ire1:: TRP$^+$ire1-HA	This study
CS228	same as JC102, except MAT α	Chapter IV
CS236	same as JC102, except ire1::URA3 (only 2/3 of Ire1p coding sequence deleted)	Chapter III
CS239	ame as JC102, except ire1:: TRP$^+$ire1-3 x myc	This study
CS243	same as JC102, except ire1::URA$^+$pRS306 (entire Ire1p coding sequence deleted; Sikorsi and Hieter, 1989)	Chapter III
CS289	same as CS228, except ire1:: URA$^+$ire1-2 x Py	This study

Figure A-1 C-terminal epitope tags on Ire1p diminish the unfolded protein response. **(a)** and **(c)** S1 nuclease protection analysis of RNA made from strains expressing the indicated Ire1p alleles (see Table A-2). The wild-type and Δire1 controls in (a) are strains JC102 and CS236 respectively. In (c), they are CS228 and CS243. Tunicamycin induction followed by S1 nuclease protection analysis was performed as in Figure II-2a. **(b)** and**(d)** *KAR2* mRNA levels from (a) and (c) respectively were quantitated and normalized to *ACT1* mRNA levels as in Figure II-1c.

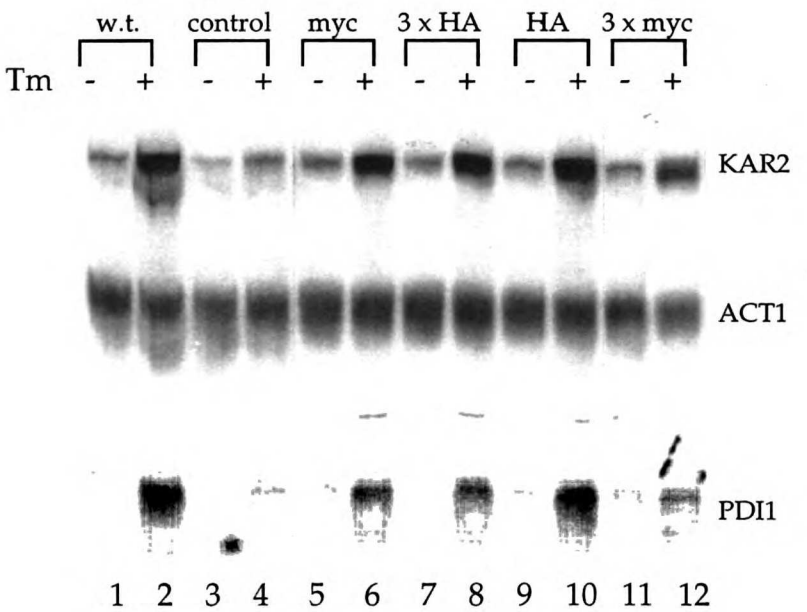
w.t.
control
myc
3 x HA
HA
3 x myc
Tm
- + - + - + - + - + - +
KAR2
ACT1
PDI1
1 2 3 4 5 6 7 8 9 10 11 12

Figure A-1a

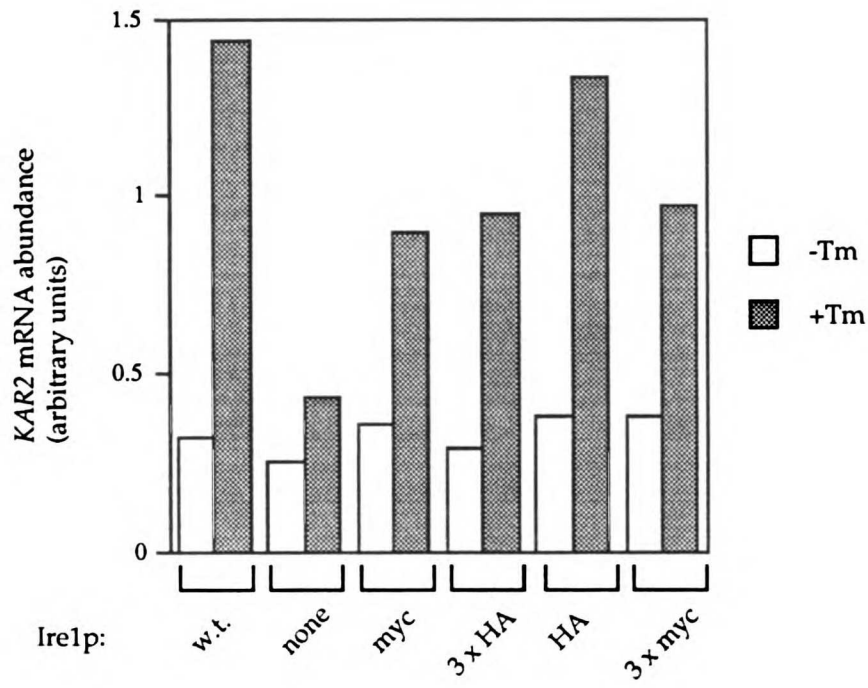
1.5
1
0.5
0
KAR2 mRNA abundance
(arbitrary units)
-Tm
+Tm
Ire1p:
w.t.
none
myc
3 x HA
HA
3 x myc

Figure A-1b

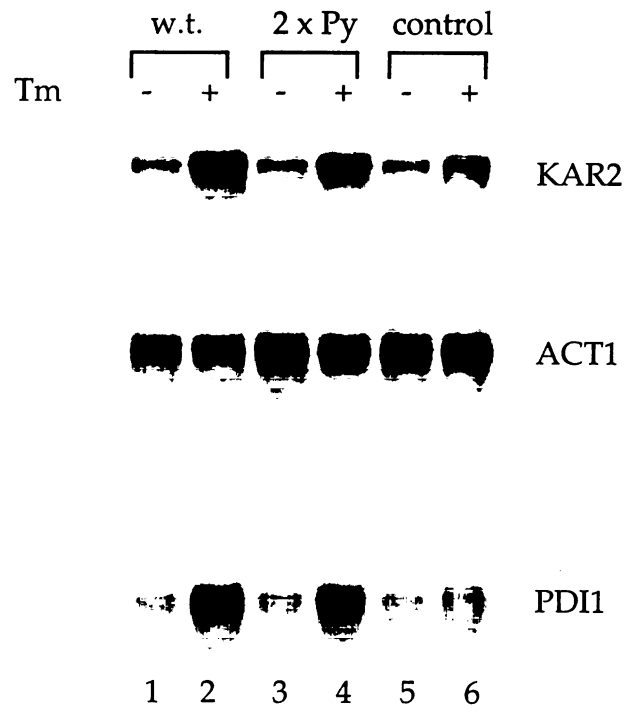

Figure A-1c

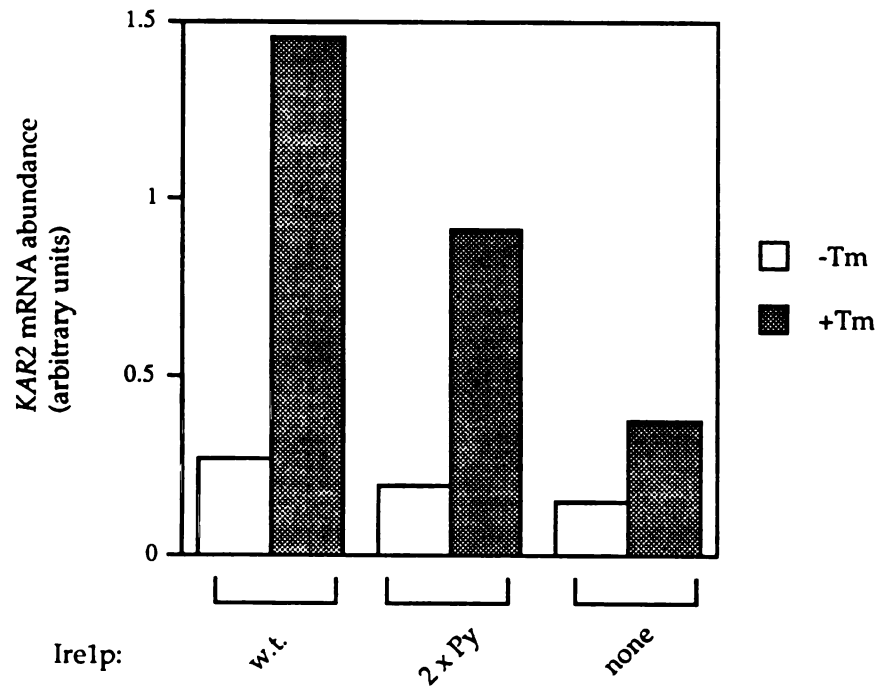
1.5
1
0.5
0
KAR2 mRNA abundance
(arbitrary units)
-Tm
+Tm
Ire1p:
w.t.
2 x Py
none

Figure A-1d

Figure A-2 **(a)** S1 nuclease protection analysis of RNA made from strains deleted for the Ire1p coding sequence and bearing 2 μm plasmids with either wild-type *IRE1* (w.t.; plasmid pCS122),*IRE1*-myc (pCS118), or no *IRE1* (control; YEplac112; Gietz and Sugino, 1988). Tunicamycin induction followed by S1 nuclease protection analysis was performed as in Figure II-2a. **(b)** The data from (a) were quantitated and normalized to *ACT1* mRNA levels as in Figure II-1c.

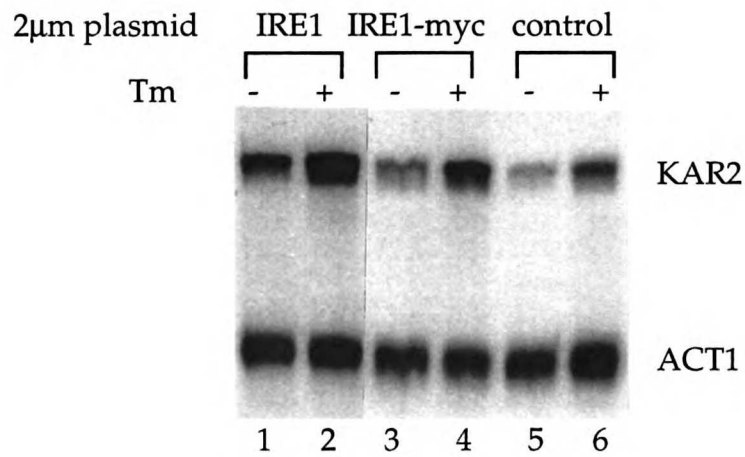

Figure A-2a

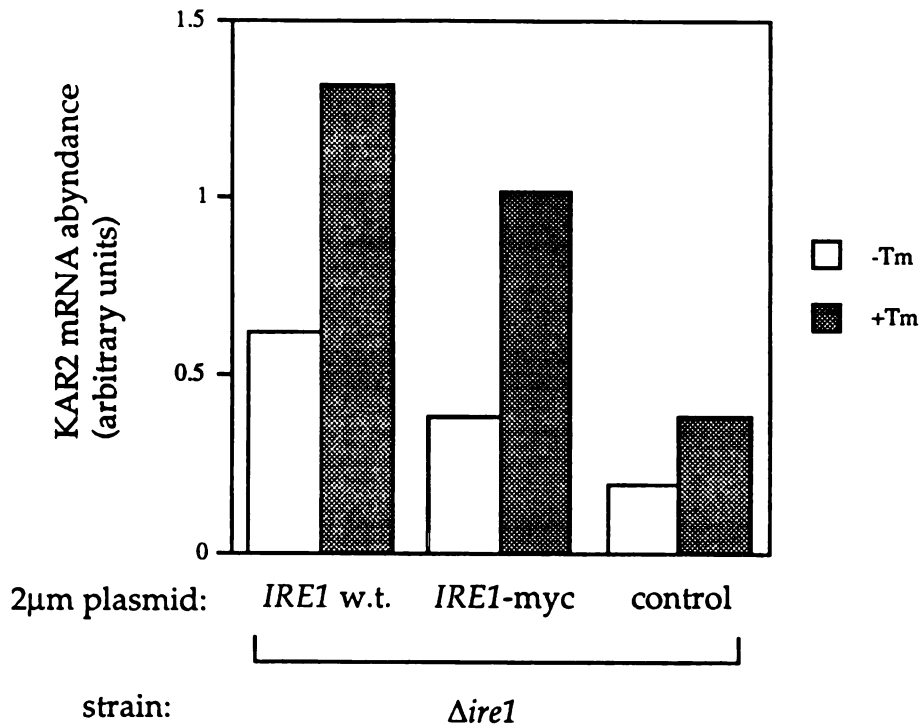
1.5
1
0.5
0
KAR2 mRNA abyndance
(arbitrary units)
-Tm
+Tm
2μm plasmid:
IRE1 w.t.
IRE1-myc
control
strain:
Δire1

Figure A-2b

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Appendix B:

Polyclonal antibodies to the C-terminal half of Ire1p

In addition to the two anti-Ire1p polyclonal antibodies described in Chapter III, α-Ire1Nterm and α-Ire1tail, a third polyclonal antibody was made that is directed against the entire C-terminus of Ire1p starting after the transmembrane region (amino acids 556-1115). This antibody, called α-Ire1Cterm, was produced by rabbits inoculated with a fusion of glutathione-S transferase (GST) to the indicated Ire1p amino acids (Smith et al., 1986; Smith and Johnson, 1988). As judged by Western blots and immunoprecipitation experiments, α-Ire1Cterm binds to Ire1p with approximately the same affinity as α-Ire1Nterm and α-Ire1tail (i.e. it does not detect Ire1p on Western blots of extracts from cells bearing only one copy of *IRE1*) but gives higher background in immunoprecipitations than the α-Ire1Nterm antibody.

Materials and Methods

A plasmid encoding the GST-Ire1Cterm fusion was made using Vent polymerase (New England Biolabs, Beverly MA) and PCR to amplify the appropriate fragment of the *IRE1* gene (bases 2021-3700; Nikawa and Yamashita, 1992) and the fragment was cloned into the pGEX2T GST expression vector (Pharmacia, Piscataway NJ). The DNA sequence of the GST-Ire1Cterm junction was determined to be correct in plasmid constructs derived from two independent PCR reactions and because Vent polymerase has relatively high fidelity, the rest of the each construct was not sequenced further. *E.coli* transformed with the GST-Ire1Cterm plasmids produced GST fusions of the expected molecular weight (approximately 89kD). The GST-Ire1Cterm fusion protein was almost completely insoluble in buffers containing 1% Triton X-100 and so was isolated from bacterial inclusion

bodies and partially purified by PAGE in the same way as the GST-Ire1Nterm and GST-Ire1tail fusions (Chapter III).

References

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Appendix C:

A screen for high copy suppressors of the Ire1p K702R mutation

The work described in this appendix was carried out in collaboration with Shelley Chu and Carmela Sidrauski while they were rotation students in the Walter lab.

Because the K702R mutation in Ire1p diminishes significantly, but does not completely prevent, the cell's ability to carry out the unfolded protein response (see Mori et al. (1993) and Chapter III), it seemed a good candidate to use in a screen for genes that, when expressed in high copy, would suppress defects in Ire1p. We hoped that such suppressors would identify new components of the unfolded protein response pathway, in particular ones that interact directly with Ire1p such as kinase substrates or regulatory subunits.

Yeast strain Y220 was constructed that 1) carries a single copy of the UPRE-lacZ reporter construct integrated into the genome, 2) is deleted for two thirds of the Ire1p coding sequence, and 3) bears the Ire1p K702R allele expressed from the wild-type *IRE1* promoter on a CEN/ARS plasmid (plasmid pSC104.17). This strain is light blue on tunicamycin/X-Gal plates and can be distinguished easily from strains bearing the wild-type version of Ire1p, which are blue.

A high copy number yeast genomic library carrying the 2 μm origin of replication (Nasmyth and Tatchell, 1980) was transformed into strain Y220, and colonies carrying suppressors of the K702R phenotype were identified because they turned blue (or at least bluer than the K702R mutant) on tunicamycin/X-Gal plates. Approximately 58,000 transformants, representing roughly 28 yeast genomes from the library, were screened, and 212 possible

supressors were identified. Initially, 38 of these were checked for the plasmid dependence of suppression. In 33 the plasmid was required for suppression of the mutant phenotype as monitored on Tm/X-Gal plates. The ability of the plasmids to restore the unfolded protein response was then confirmed by S1 nuclease protection assay of *KAR2* and *PDI1* transcription (as described in Chapter II). After 2.5 hours of tunicamycin treatment, in only one of the 33 candidate suppressors was transcription of these genes induced more than in the unsuppressed K702R control. This suppressing plasmid was isolated and shown by restriction digest and by Southern blot to bear the *IRE1* gene.

A further 80 suppressors were then examined. Unfortunately, only one of these 80 was both plasmid dependent and restored the unfolded protein response at the level of *KAR2* and *PDI1* transcription. It was also shown to be *IRE1* itself. With over half of the candidate suppressors examined and with *IRE1* having been found twice, it seemed unlikely that any novel components of the unfolded protein response pathway would be found by this method and so we stopped work on the products of the screen at this point.

Because we do not know the basis for the K702R mutant phenotype, we cannot explain with certainty why we did not find any high copy suppressors of it. However, it is likely that the Ire1p-K702R mutant allows only a diminished unfolded protein response because it has reduced kinase activity. A cell might be able to overcome this defect if a limiting substrate or regulatory subunit were overproduced, as was attempted in the screen described here. Thus, that we did not find any high copy suppressors of Ire1p-

K702R might indicate that Ire1p substrates or regulatory subunits are not limiting in the unfolded protein reponse.

It should be noted that the Ire1p-K702R plasmid present in strain Y220 (pSC104.17) had a point mutation in the *IRE1* gene in addition to the one that changed amino acid 702. This second mutation was also created during the process of site-directed mutagenesis and changed histidine 470, located in the ER-resident portion of the protein, to leucine. It was not detected until the K702R plasmid was fully sequenced, sometime after the primary screen was carried out. However, in assays monitoring β-galactosidase activity, Y220 has exactly the same phenotype as a second otherwise identical strain (Y218) that carries the "correct" version of Ire1p-K702R (on plasmid pSC104.5). It seems unlikely that the second point mutation affected our ability to identify suppressors of Ire1p K702R, but we cannot rule out this possibility and that, if the screen were repeated with the correct strain, we would find interesting new genes.

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Appendix D:

Sister chromatid separation in frog egg extract requires DNA topoisomerase II activity during anaphase

Preface

The work described in this appendix was carried out when I was a graduate student under the supervision of Dr. Andrew Murray. Although it does not pertain to the unfolded protein response in yeast, my committee has agreed that it should constitute part of this thesis.

Sister Chromatid Separation in Frog Egg Extracts Requires DNA Topoisomerase II Activity during Anaphase

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Abstract. We have produced metaphase spindles and induced them to enter anaphase in vitro. Sperm nuclei were added to frog egg extracts, allowed to replicate their DNA, and driven into metaphase by the addition of cytoplasm containing active maturation promoting factor (MPF) and cytostatic factor (CSF), an activity that stabilizes MPF. Addition of calcium induces the inactivation of MPF, sister chromatid separation and anaphase chromosome movement. DNA topoisomerase II inhibitors prevent chromosome segregation at anaphase, demonstrating that the chromatids are catenated at metaphase and that decatenation occurs at the start of anaphase. Topoisomerase II activity towards exogenous substrates does not increase at the metaphase to anaphase transition, showing that chromosome separation at anaphase is not triggered by a bulk activation of topoisomerase II.

ACCURATE chromosome segregation requires that the linkage between sister chromatids is regulated during the cell cycle. Sister chromatids must remain associated with each other from the time they are replicated until they have been correctly aligned on the metaphase plate, with the sister kinetochores attached to opposite poles of the mitotic spindle. At the onset of anaphase, the linkage between sisters must be promptly destroyed, allowing them to segregate to opposite poles of the spindle.

At metaphase, a polewards force is exerted on each kinetochore (McNeill and Berns, 1981; Rieder and Alexander, 1990). Because the sister chromatids are linked to each other, and attached to opposite poles of the spindle, these forces will tend to collapse the spindle and must be opposed by forces that tend to move the spindle poles apart (Cande and McDonald, 1985; Hiramoto and Nakano, 1988; Hyman and White, 1988). At anaphase, dissolution of the linkage between the sister chromatids destroys the opposition between the polewards force on the kinetochores and the forces tending to move the spindle poles apart. As a result, the forces in the spindle could move the chromosomes towards the poles (anaphase A) and the spindle poles apart from each other (anaphase B). In principle, breaking the linkage between sisters could initiate all the events of anaphase, without any change in the directions or magnitudes of the forces acting on components of the spindle. Indeed, measurements of the forces acting on kinetochores suggest that they do not change between metaphase and anaphase (Alexander and Rieder, 1991; Nicklas, 1988).

Recent advances have greatly increased our understanding of the regulation of the cell cycle. The entry into mitosis and the initiation of spindle assembly requires the activation of maturation promoting factor (MPF)[1] (for review see Murray and Kirschner, 1989*b*; Nurse, 1990). Active MPF is a complex between a catalytic subunit, $p34^{cdc2}$, and cyclin B. The onset of anaphase coincides with the destruction of cyclin B and with the consequent inactivation of MPF (Hunt and Ruderman, 1992; Lehner and O'Farrell, 1989; Murray et al., 1989). Unfertilized eggs of the frog *Xenopus laevis* are arrested in metaphase of meiosis II by cytostatic factor (CSF) (Masui and Markert, 1971) that is intimately related to the product of the c-*mos* proto-oncogene (Sagata et al., 1989), and stabilizes MPF activity by preventing cyclin degradation (Murray et al., 1989). Fertilization of the egg induces an increase in the intracellular concentration of calcium that leads to the destruction of cyclin, the inactivation of MPF and CSF (Lorca et al., 1991; Meyerhof and Masui, 1977; Watanabe et al., 1991), and the initiation of chromosome separation. CSF-arrested extracts are prepared from unfertilized frog eggs, and remain stably arrested with high levels of MPF. These extracts can be induced to inactivate MPF and progress to interphase by the addition of calcium (Lohka and Maller, 1985; Murray et al., 1989).

In contrast to our understanding of the activities that regulate progress through the cell cycle, we do not know what holds sister chromatids together, nor how this linkage is broken at anaphase. In mammalian cells, a number of proteins (inner centromere proteins [INCENP]) have been characterized that are located between sister chromatids during metaphase and that remain in the center of the spindle as chromosomes move towards the poles at anaphase (Cooke et

1. *Abbreviations used in this paper*: CSF, cytostatic factor; kDNA, kinetoplast DNA; MPF, maturation promoting factor.

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al., 1987). Sister chromatids may also be linked to each other by interlinking (catenation) of their DNA duplexes (Sundin and Varshavsky, 1980). Catenation arises from incomplete unwinding of the parental DNA duplex during DNA replication. Experiments in yeast and mammalian cells have established that the activity of type II DNA topoisomerases is required during mitosis to allow sister separation (DiNardo et al., 1984; Downes et al., 1991; Holm et al., 1985, 1989; Uemura et al., 1987), suggesting that sister chromatids are still catenated in mitosis. However, it is not known whether DNA catenation is sufficient or even necessary to maintain the linkage between sister chromatids, nor whether any of the identified INCENP proteins play a role in holding sisters together.

To investigate what holds sister chromatids together, we have developed a method for studying sister chromatid separation at anaphase in vitro. Sperm nuclei were allowed to undergo DNA replication in interphase frog egg extracts and induced to form metaphase spindles by the addition of CSF-arrested extract. Calcium was added to these extracts to inactivate MPF and initiate anaphase. We show here that chromosome separation follows the inactivation of MPF, and that the chromosomes separating from each other in these spindles are sister chromatids. The activity of type II topoisomerases is required at the metaphase-anaphase transition for successful sister chromatid separation, although topoisomerase II activity actually decreases during the progression from metaphase to anaphase.

Materials and Methods

Materials

Sperm nuclei were prepared as described (Murray, 1991) and stored at a concentration of $10^4/\mu l$ in small aliquots at −80°C. Before use, they were diluted in sperm dilution buffer (100 mM KCl, 1 mM $MgCl_2$, 150 mM sucrose) to a concentration of $\sim 10^3/\mu l$. Bovine brain tubulin was labeled, to one fluorochrome per tubulin dimer, with tetramethylrhodamine by the high pH labeling method (Hyman et al., 1990) and stored in small aliquots in injection buffer (50 mM K-glutamate, 0.5 mM glutamic acid, 0.5 mM $MgCl_2$). Aphidicolin (Sigma Chemical Company, St. Louis, MO) was dissolved at 20 mg/ml in DMSO. The topoisomerase II inhibitors VP-16 (demethylepipodophyllotoxin ethylidene-β-D-glucoside) and VM-26 (demethylepipodophyllotoxin thenylidene-β-D-glucoside) were obtained from Bristol-Myers Squibb (Wallingford, CT) and dissolved at 10 mM in DMSO; doxorubicin (Ben Venue Laboratories, Bedford, OH) was dissolved at 1 mg/ml in 0.45% NaCl; novobiocin (Sigma Chemical Company) was dissolved at 100 mg/ml in water. Inhibitors were stored in small aliquots at −20°C.

Preparation of Extracts

CSF-arrested extracts were made from freshly squeezed frog eggs as described previously (Murray, 1991; Murray et al., 1989) except that the crushing spin was carried out for 15 min at 10,000 *g* at 15°C and no clarifying spin was performed.

In Vitro Anaphase

CSF-arrested extracts were freshly prepared and rhodamine-labeled tubulin was added to 120 μg/ml. Extract was dispensed into microfuge tubes, diluted sperm nuclei were added to a concentration of 100/μl, and extract and sperm were incubated at room temperature (~20°C) for 10 min. To drive the extracts into interphase and to start DNA replication, 4 mM $CaCl_2$ in sperm dilution buffer was added to each reaction to a final concentration of 0.4 mM. The reactions were incubated at room temperature for 80 min before adding 0.5 vol of CSF-arrested extract (which contained rhodamine-labeled tubulin, but no sperm nuclei) to induce nuclear envelope breakdown and spindle assembly. We believe that the added CSF and MPF are stable because the calcium that was added at the start of the reaction has been sequestered. Metaphase spindles were allowed to assemble for 80 or 90 min. To induce anaphase, a fraction of the metaphase extract was placed in new microfuge tubes and calcium was added to 0.4 mM.

The morphology of the nuclei was determined by taking samples at various timepoints and fixing with formaldehyde in the presence of Hoechst 33342 (Murray and Kirschner, 1989*a*). Samples were examined by fluorescence and by phase contrast microscopy. Light micrographs were taken on a Nikon Microphot-FXA (Nikon Inc., Melville, NY) with 40× or 63× objectives, using TRI-X Pan or Technical Pan film (Eastman Kodak Co., Rochester, NY).

MPF Activity

To assay MPF activity, 1-μl aliquots of extract were frozen in liquid nitrogen at the desired time points and stored at −80°C until assayed. MPF activity was assayed as H1 kinase activity as described by Murray (1991). H1 kinase activity was determined by scintillation counting slices of dried gels, and is expressed as arbitrary units.

DNA Replication

To monitor DNA synthesis, 5-μl samples of extract were removed from the reaction at the desired time points, added to 1 μl of 100 mM KCl, 1 mM $MgCl_2$ that contained 0.25 μCi of α-^{32}P-dCTP, and incubated for 10 min at room temperature. Reactions were stopped by the addition of 300 mM sucrose, 10 mM EDTA, pH 8.0, 50 μg/ml RNase A and incubated at room temperature for 10 min. 5 μl of freshly prepared 3× TAE loading buffer (120 mM Tris-Acetate, pH 8.0, 33 mM EDTA, 30% wt/vol glycerol, 1% SDS) was added to each reaction, the reactions were heated to 65°C for 10 min, and then run on a 0.7% agarose-TAE gel until the dye front had run ~5 cm into the gel. Under these conditions, the sperm DNA remained in the wells of the gel. Gels were washed twice, for 1 h each, in 1× TAE, dried at 60°C onto paper, and autoradiographed. To inhibit DNA synthesis, aphidicolin (a specific inhibitor of DNA polymerase-α) (Ikegami et al., 1978) was added to a final concentration of 20 μg/ml, yielding a final DMSO concentration of 0.1%. Aphidicolin inhibited DNA synthesis by >95% (see Fig. 2 *B*).

Topoisomerase Inhibition

To test their effects on chromosome segregation, topoisomerase inhibitors were used in fresh extracts and were added after the extracts had reached metaphase. The final concentration of DMSO in the extract never exceeded 0.3%; this concentration has no effect on chromosome or spindle morphology (data not shown). The same lot of DMSO was used both to dissolve the topoisomerase inhibitors and for controls for the effect of added DMSO.

The ability of inhibitors to interfere with action of type II DNA topoisomerase was monitored by measuring the rate at which kinetoplast DNA was decatenated in the presence and absence of inhibitors. These assays were carried out in CSF-arrested extracts that had been frozen at −80°C after the addition of sucrose to 200 mM. Topoisomerase activity is identical in fresh and frozen extracts (data not shown). Extracts were diluted 50-fold into dilution buffer (100 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes, pH 7.7, 1 mM ATP, 8 mM creatine phosphate, 1 mM DTT, 200 mM sucrose) containing 5 ng/ml kinetoplast DNA (kDNA) from *Crithidia fasciculata*, (kindly provided by V. Klein and P. Englund, Johns Hopkins University School of Medicine, Baltimore, MD). Reactions were incubated at room temperature and at each time point, 25-μl samples were added to 0.25 ml of digestion buffer (50 mM Tris-HCl, pH 8.0, 20 mM EDTA, pH 8.0, 0.5% (wt/vol) SDS, 0.5 mg/ml proteinase K) and incubated at 37°C for 1 h to digest the proteinaceous component of any DNA-topoisomerase complexes. After digestion the samples were successively extracted with phenol and chloroform, ethanol precipitated, redissolved, and run on 0.7% agarose-TAE gels in the absence of ethidium bromide. Gels were stained with ethidium bromide and photographed. For some inhibitors a simpler assay, using undiluted extracts and in which the samples were not proteinase K digested before phenol extraction, was used.

To determine the level of topoisomerase II activity during metaphase and anaphase, we measured the rate of kDNA decatenation in fresh extracts that contained sperm nuclei and rhodamine-labeled tubulin. 80-μl samples of an extract were removed at metaphase or during anaphase, and added to 80 μl of ice-cold decatenation buffer (100 mM KCl, 1 mM $MgCl_2$, 10 mM K-Hepes, pH 7.7, 50 mM sucrose, 1 mM DTT, and 1.25 mM ATP) and 40 μl of ice-cold kDNA diluted to 24 μg/ml in decatenation buffer. Decatena-

ion reactions were performed on ice to prevent cell cycle progression during the course of the assay, and 25-μl samples were withdrawn at various times and processed for electrophoresis as described above, with the exception that they were not digested with proteinase K. H1 kinase activity was assayed at the beginning and end of the reaction to confirm that no cell cycle progression had occurred during the decatenation assay.

Electron Microscopy

100 μl of extract containing metaphase or anaphase spindles was diluted with 3.8 ml of BRB 80 (80 mM KPipes, pH 6.8, 1 mM $MgCl_2$, 6 mM EGTA) containing 0.3% Triton X-100 and 30% glycerol, and allowed to incubate at room temperature for 2.5 min. 1.3 ml of EM-grade 8% glutaraldehyde (Ted Pella Inc., Redding, CA) was then added, the reactions mixed gently, and then layered onto a 3-ml cushion of BRB 80, containing 40% glycerol, in Corex tubes that had been modified to hold a 12-mm-round acid-washed coverslip at the bottom (Evans et al., 1985). The tubes were spun at 13,000 *g* at 20°C for 1 h in a swinging bucket rotor. The spindles that had affixed to the coverslips were washed in 0.05 M Millonig's phosphate buffer (Hayat, 1970) in a humid chamber at 4°C overnight. The spindles and coverslips were then dehydrated and flat embedded in Epon/Araldite (Hayat, 1970). Sections 0.15–0.2 μm thick were cut on an ultramicrotome (Ultracut-E; Reichert Jung, Vienna) using a glass knife and placed on formvar-coated copper mesh grids. Sections were stained with 5% uranyl acetate at 60°C for 1 h and then destained with 0.2 M EDTA at room temperature for 0–15 min. Sections were then poststained for 10 min at room temperature with 0.6% lead citrate and examined at 100 kV on an electron microscope (model 100C, JEOL USA, Inc., Peabody, MA).

Results

We used frog egg extracts to prepare metaphase spindles that could be induced to enter anaphase (Fig. 1). Sperm nuclei were added to CSF-arrested extracts that were then induced, by the addition of calcium, to enter interphase and replicate their DNA. To induce entry into mitosis and the formation of metaphase spindles, CSF-arrested extract was added to the reaction 80 min after the initial calcium addition. To inactivate MPF and to induce the metaphase spindles to undergo anaphase, a second calcium addition was made 170 min after the initial calcium treatment. Samples were removed throughout the reaction. Nuclear morphology, and the distribution of DNA and microtubules were monitored by phase contrast and fluorescence microscopy. DNA replication was monitored by the incorporation of labeled nucleotides and MPF activity was assayed as histone H1 kinase activity.

The time course of DNA replication, H1 kinase activity and spindle morphology in a typical experiment are shown in Fig. 2. 35 min after the first calcium addition, H1 kinase activity was low, and the nuclei had decondensed and acquired nuclear envelopes. DNA synthesis typically began 30–40 min after calcium addition and continued for 20–40 min. The DNA synthesis inhibitor, aphidicolin (Ikegami, 1978), prevented DNA replication (Fig. 2 *B*), but did not affect progression of the cell cycle as monitored by the activity of H1 kinase (data not shown). No organized microtubule arrays were seen associated with interphase nuclei. This may simply reflect the instability of interphase microtubule arrays under our fixation conditions. In all the experiments shown in this paper, mitosis was induced by adding CSF-arrested extract 80 min after the initial calcium addition. After this addition, no further DNA replication was detected (data not shown). Spindles were allowed to assemble for 90 min after the addition of CSF arrested extract, and by the end of this period, the extract contained numerous well organized bipolar spindles with the chromosomes aligned on a metaphase plate, equidistant from the two spindle poles (Fig. 2 *C*). Depending on the experiment, between 40 and 75% of the nuclei gave rise to bipolar spindles. Once assembled, these spindles were stable for several hours and MPF activity remained high (Fig. 2 *A*, and data not shown).

Anaphase was induced by the addition of calcium to the spindle containing extracts, 170 min after the initial calcium addition. Within 10 min of the second calcium addition, MPF was inactivated and anaphase A occurred: the chromosomes separated and moved towards the spindle poles (Fig. 2 *C*). We have not reproducibly observed anaphase B in these extracts. During anaphase the density of astral microtubules increased, while that of microtubules in the center of the spindle decreased. Telophase typically started between 190 and 200 min and was marked by the disappearance of spindle microtubules and by the decondensation of individual chromosomes and nuclear envelope formation around them to form karyomeres. DNA replication was detected again starting between 200 and 210 min (data not shown), and the fusion of karyomeres to form interphase nuclei was completed between 210 and 230 min. In all experiments, the fraction of bipolar spindles that underwent anaphase and progressed to form daughter interphase nuclei after the second calcium addition exceeded 90%. Thus, we have shown that the formation of metaphase spindles and the induction of anaphase can be reproducibly obtained in vitro.

Chromosomes Separate Soon after Histone H1 Kinase Levels Fall

To determine when MPF levels fall relative to the onset of anaphase, we compared spindle morphology and H1 kinase activity every 2 min after the second addition of calcium. We could distinguish seven morphological stages during the progression to interphase: (*1*) metaphase; (*2*) metaphase-anaphase transition, in which part of the chromosomes had moved towards the poles but no separation of DNA staining could be seen in the center of the spindle; (*3*) early anaphase, in which a separation between the groups of segregating chromosomes could be seen at the former site of the metaphase plate; (*4*) mid-anaphase, in which the chromosomes were approximately equidistant between the center of the

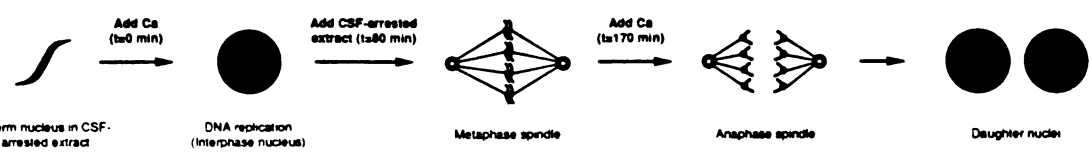


Figure 1. A schematic representation of the protocol used to produce anaphase in frog egg extracts. Times are relative to the time of first calcium addition.

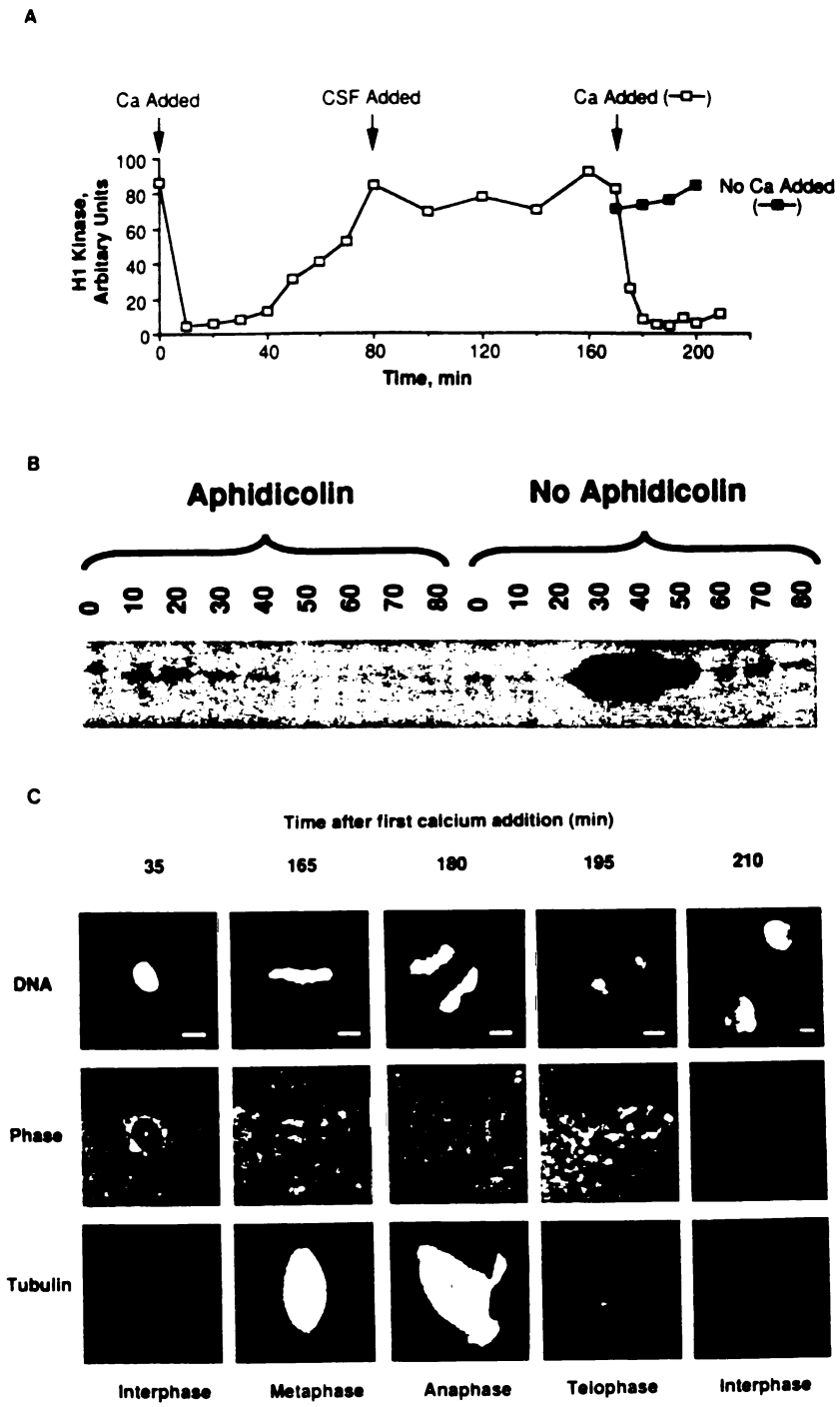
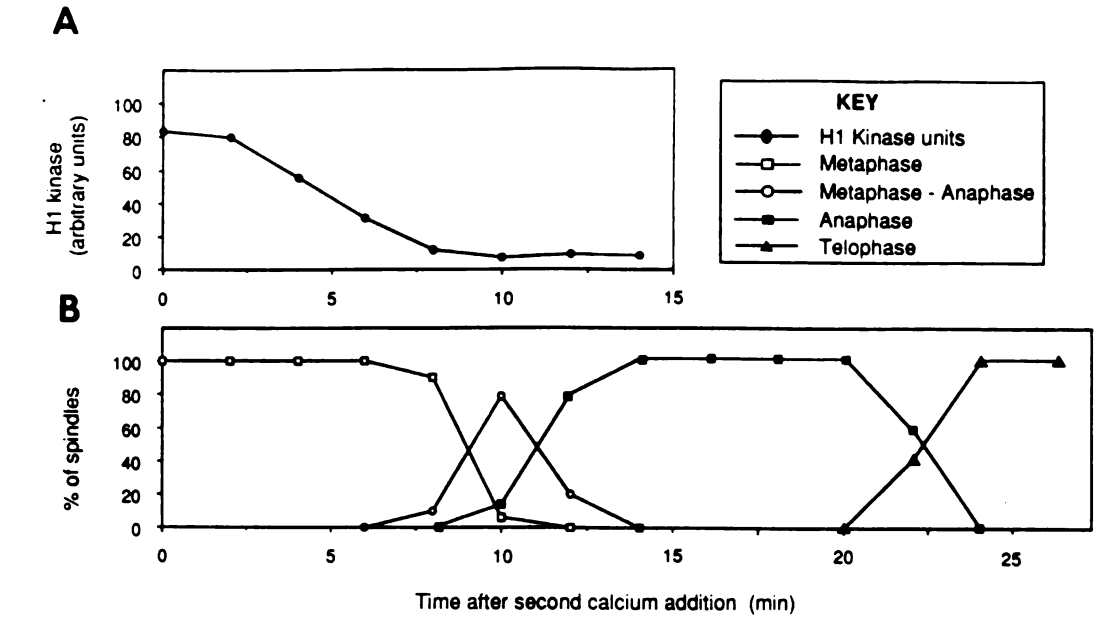


Figure 2. MPF activity and nuclear morphology during the course of a typical experiment. (*A*) MPF activity (measured as histone H1 kinase activity) graphed as a function of time (□). If calcium addition at 170 min is omitted, MPF activity remains high (■). (*B*) Timing of DNA replication during interphase and the inhibition of DNA replication by aphidicolin. Aphidicolin was added to a concentration of 20 μg/ml at the beginning of the experiment. Times are in minutes after the first addition of calcium. (*C*) Nuclear morphology at various time points; Note the change of magnification in 210 min time point. Data in *A* and *C* are from the same experiment, while that in *B* is from a different experiment. The time at which DNA replication begins and ends varies by ~15 min between experiments. Bars, 10 μm.



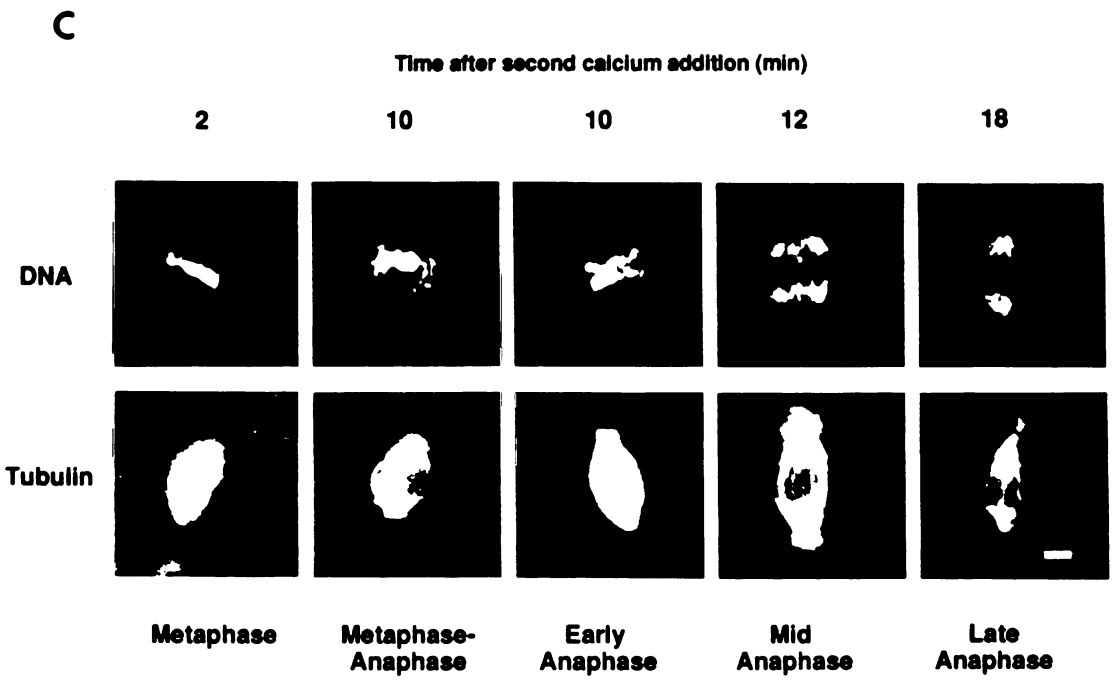


Figure 3. A detailed time course of MPF inactivation and nuclear morphology after the second calcium addition. Data from one of three experiments that demonstrated that MPF activity falls 2–4 min before anaphase chromosome movement begins. (*A*) MPF activity (measured as histone H1 kinase activity) graphed as a function of time after second calcium addition. (*B*) A quantitation of the number of spindles in each phase of the cell cycle at each time point. The categories of early, mid, and late anaphase, described in the text, have been expressed as a single category, anaphase. (*C*) Nuclear morphology in stages from metaphase to late anaphase. Bar, 10 μm.

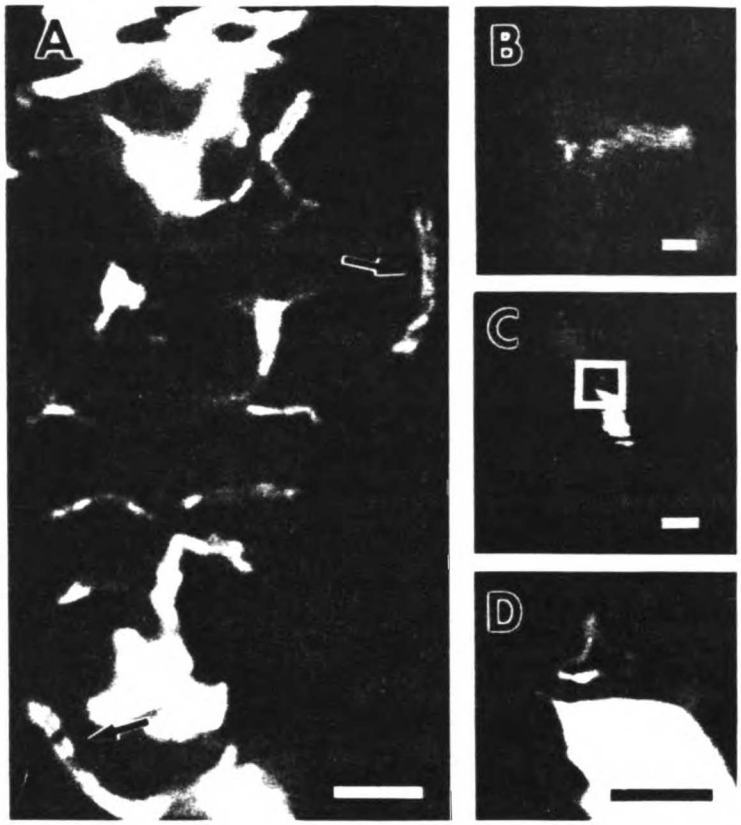


Figure 4. Metaphase and anaphase chromosomes in frog egg extracts. (*A*) Chromosomes at the metaphase plate of a large spindle; the spindle is not shown but is oriented horizontally. The arrows indicate chromosomes that are composed of paired chromatids of equal length. (*B*) A spindle (visualized with rhodamine tubulin), and (*C*) its chromosomes at the metaphase–anaphase transition. This spindle was from a sample taken 8 min after the second calcium addition in the experiment described in Fig. 3. (*D*) Enlargement of the boxed region of the spindle in *C*, showing a pair of chromatids separating from each other. Bars: (*A* and *D*) 5 μm; (*B* and *C*) 10 μm.

spindle and the poles; (*5*) late anaphase, in which the chromosomes were still condensed but located at or near the spindle poles; (*6*) telophase, in which the clustered chromosomes had begun to form karyomeres that fused with each other; and (*7*) interphase, in which fusion had been completed to yield spherical interphase nuclei bounded by membranes (Figs. 2 *C* and 3 *C*). In Fig. 3 *B* early, mid-, and late anaphase spindles are presented as a single class. In this experiment, H1 kinase activity begins to decline 4 min after the second calcium addition, and has reached interphase levels by 8 min. The metaphase–anaphase transition was first observed at 8 min, and clear chromosome separation (early anaphase) was first observed at 10 min, demonstrating that H1 kinase activity begins to fall before the morphological transition from metaphase to anaphase. Nuclei proceeded through the pathway from metaphase to interphase synchronously: at any time point, the majority of nuclei were in the same phase of mitosis (Fig. 3 *B*).

Sister Chromatids Separate during Anaphase in vitro

Having demonstrated that we could induce anaphase chromosome movement in vitro, we next asked whether the chromosomes that were segregating from each other were indeed sisters. The behavior of spindles as observed by light microscopy is entirely consistent with sister chromatid segregation. In metaphase spindles, we often observed chromosomes composed of two paired chromatids of equal length (Fig. 4 *A*) (see also Sawin and Mitchison, 1991). After the second addition of calcium, equal amounts of DNA moved to each pole, as judged by the intensity of Hoechst staining (e.g., Fig. 2 *C* and 3 *C*). The individual chromosome arms that could be seen at this point were about the width of one member of the paired chromatids that we had observed at metaphase. In extracts to which no calcium was added, the width of the chromosomes did not decrease, indicating that this decrease was not the result of progressive chromosome condensation with increasing length of time in mitosis. In addition, some chromosomes showed mirror symmetry about the metaphase plate as they were being pulled away (Fig. 4 *B–D* and Fig. 2 *C*, 180 min), suggesting that they were sister chromatids in the act of separating.

To extend these observations, we examined sections of metaphase and anaphase spindles by EM. We could see paired chromatids of equal length apparently attached to the metaphase spindle (Fig. 5 *A*). At the interface between the microtubules and chromosomes, we did not observe the trilaminar structure characteristic of many kinetochores. Sawin and Mitchison (1991) were also unable to find trilaminar kinetochores by EM in metaphase spindles assembled in *Xenopus* egg extracts. In anaphase spindles, we observed individual chromosomes fixed in the process of moving toward the poles and each of these chromosomes was one-half the

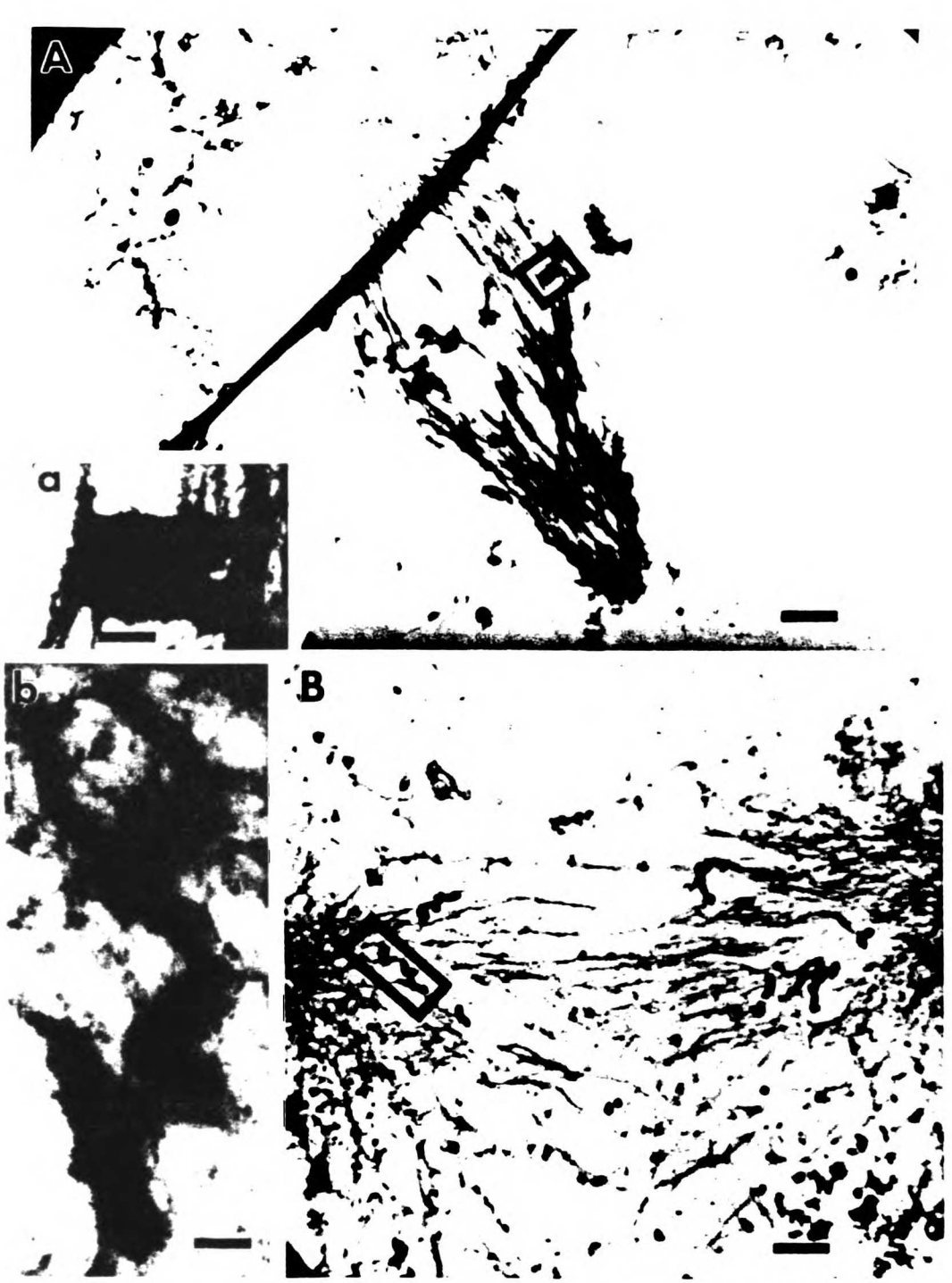


Figure 5. Electron micrographs of metaphase and anaphase spindles. (*A*) A metaphase spindle. The dark bar running across the spindle is a fold in the section. (*a*) An enlargement of the area inside the box in *A* showing a pair of chromatids at the metaphase plate. (*B*) A late anaphase spindle. (*b*) An enlargement of the area inside the box in *B* showing chromosomes segregating to the pole at anaphase. Note that the width of each chromosome in *b* is one-half that of the chromosome in *a*. Bars: (*A* and *B*) 2.9 μm; (*a* and *b*) 0.4 μm.

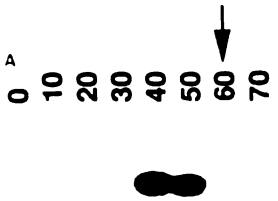


Figure 6. DNA replication and nuclear morphology in the presence and absence of aphidicolin. (*A*) DNA replication in a reaction without aphidicolin. The times listed above each well correspond to the number of minutes after the first calcium addition. The arrow indicates the time at which aphidicolin was added to a parallel reaction, whose spindles are shown in *C*. (*B*) Nuclear morphology in a control reaction and (*C*) in a reaction to which aphidicolin had been added to 20 μg/ml 60 min after the first calcium addition (as indicated in *A*). Metaphase spindles were fixed immediately before calcium addition. Anaphase spindles were fixed 22 min after the second calcium addition and interphase samples were taken 52 min after the second calcium addition. Bars, 10 μm.

width of a metaphase chromosome pair. In some sections, the chromosomes had a V-shaped morphology, as though they were being pulled to the spindle poles by their kinetochores (Fig. 5 *B*). The simplest interpretation of these observations is that the paired chromosomes seen at metaphase are indeed sister chromatids and that the sisters separate from each other at anaphase.

To confirm that the segregating entities were sister chromatids, we devised a treatment that would interfere with chromosome separation only if the chromosomes were sisters. We added aphidicolin, an inhibitor of DNA replication, late during DNA replication to create pairs of sister chromatids that would be mostly replicated, but held together by some unreplicated regions. In these regions sister chromatids are held together by base pairing between DNA strands, as well as by the normal linkage between sisters, and cannot be easily separated from each other. Therefore, the separation of sister chromatids will be prevented by inhibiting the completion of chromosome replication, while that of homologs, or randomly associated chromosomes will not.

We found that extracts to which aphidicolin had been added late in S phase (60 min after the initial calcium addition) (Fig. 6 *A*) formed morphologically normal metaphase spindles. However, when these spindles were induced to en-

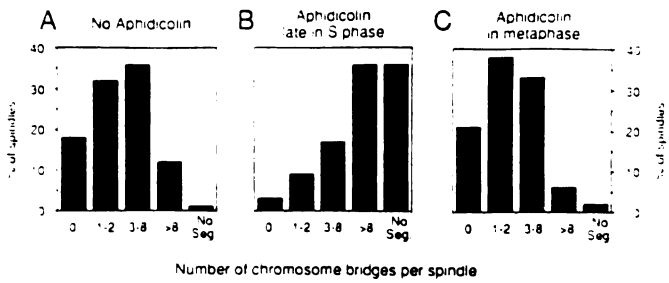


Figure 7. The distribution of chromosome bridges on spindles in the absence and presence of aphidicolin. The number of cross-bridges per spindle was scored for spindles fixed at mid-anaphase and the percent of the total number of spindles in each category was determined. The spindles in the "no segregation" (*No Seg.*) category appeared not to segregate their chromosomes. Chromosome bridges stretched all over these spindles and the bulk of chromatin was in the middle of the spindle, not near the poles. The data presented here are compiled from four separate experiments. (*A*) No aphidicolin added; 136 spindles scored. (*B*) Aphidicolin added late in S phase, as determined by DNA replication assays; 70 spindles scored. (*C*) Aphidicolin added at the time of second calcium addition, after DNA replication had been completed; 48 spindles scored.

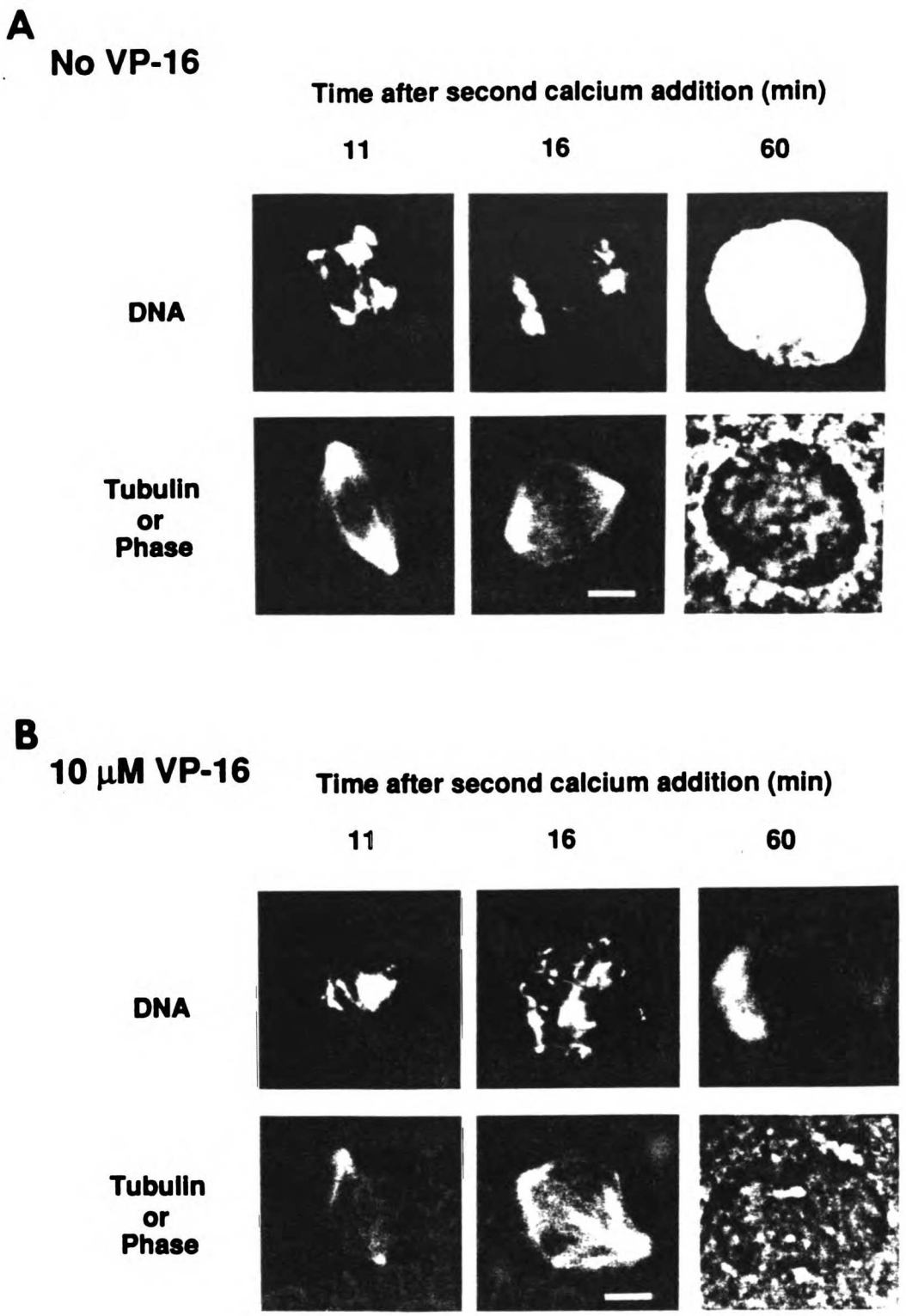
ter anaphase, many chromosome bridges, representing incompletely separated chromosomes, were seen (Fig. 6 *C*), compared to spindles in which DNA synthesis had not been inhibited (Fig. 6 *B*). To quantify this effect, we compared the number of bridges present in anaphase in aphidicolin-treated extracts with the number in untreated extracts. A substantial increase in both the number of bridges per spindle and in the number of affected spindles was seen after aphidicolin treatment (Fig. 7, *A* and *B*). However, the timing of MPF inactivation, chromosome decondensation, and nuclear envelope formation was identical in aphidicolin-treated and untreated extracts (Fig. 6 and data not shown).

As a control for the effects of the drug, we added aphidicolin at the time of second calcium addition, long after DNA replication had been completed. This did not interfere with anaphase, demonstrating that aphidicolin blocks chromosome separation by interfering with DNA replication rather than by inhibiting activities involved in destroying the linkage between fully replicated chromosomes (Fig. 7 *C*). These data strongly suggest that the chromosomes that segregate at anaphase in our extracts are indeed sisters.

Topoisomerase II Activity Is Required for Sister Chromatid Separation

To examine the role of DNA topoisomerase II at anaphase, we tested the ability of topoisomerase inhibitors to block chromosome segregation. Metaphase spindles were formed, and topoisomerase II inhibitors were added immediately before the calcium addition that induces anaphase. The addition of 10 μM VP-16, a potent inhibitor of topoisomerase II (Chen et al., 1984), had no effect on the morphology of metaphase spindles but grossly disrupted chromosome segregation when anaphase was induced (Fig. 8). In VP-16 treated extracts, although some chromosome movement occurred, no clear chromosome segregation had occurred by the time that control spindles had reached mid-anaphase (Fig. 8, *A* and *B*). The addition of DMSO from the same lot that had been used to dissolve the VP-16 had no effect on chromosome segregation (data not shown). The amount of chromosome movement was further reduced at higher concentrations of VP-16 (30 μM). By the time that control spindles had reached late anaphase, chromosomes had moved away from the metaphase plate in VP-16–treated extracts but were still associated by multiple chromosome bridges (Fig. 8 *B*), suggesting that the linkage between sister chromatids could not be completely dissolved in these extracts. Delays in chromosome movement and the subsequent formation of chromosome bridges were observed even when metaphase spindles were incubated for 45 min before VP-16 addition (data not shown). VP-16–treated extracts still carried out other aspects of the progression to interphase: MPF inactivation, chromosome decondensation, and interphase nuclei formation were initiated at the same time in untreated and in VP-16–treated extracts (Fig. 8). The addition of three other compounds that have been reported to inhibit topoisomerase II, VM-26 (10 μM or 30 μM) (Chen, 1984), doxorubicin (1 μg/ml) (Tewey et al., 1984), or novobiocin (250 μg/ml) (Hsieh and Brutlag, 1980; Osheroff et al., 1983) had essentially the same effect on chromosome movement as VP-16 (data not shown). The aberrations in chromosome morphology seen with topoisomerase inhibitors are more severe than those seen with late inhibition of DNA replication (Fig. 6). We believe this reflects the fact that most DNA was allowed to replicate in the experiments with inhibitors of DNA replication, while the topoisomerase inhibitors were added before the beginning of chromosome separation. Therefore, the number of points at which sister chromatids remain attached to each other may be smaller in the experiments where DNA replication was inhibited than it is in those where topoisomerase inhibitors were added.

To demonstrate that VP-16 was acting to inhibit type II topoisomerase in our experiments, we assayed topoisomerase activity in the presence and absence of the drug. To measure topoisomerase II activity, we examined the ability of our extracts to release small DNA circles from kinetoplast DNA (kDNA) (Marini et al., 1980), the highly catenated networks of DNA isolated from the kinetoplasts of hemoflagellate protozoans. Topoisomerase activity was inhibited by the following compounds: VP-16 (10 μM, Fig. 9), VM-26 (10 μM, data not shown), doxorubicin (1 μg/ml, data not shown), novobiocin (250 μg/ml) (data not shown). The observation that these compounds block topoisomerase II activity at the same concentration at which they prevent chromosome segregation, strongly suggests that the topoisomerase II–mediated decatenation of sister chromatids is required for their separation. In vivo metaphase lasts less than 10 min during the early embryonic cell cycle. The ability of topoisomerase II inhibitors to block chromosome segregation, even when added after spindles have spent as much as 45 min in metaphase, demonstrates that sister chromatid decatenation cannot be completed during metaphase, but requires the events that induce anaphase.


A
No VP-16
Time after second calcium addition (min)
11
16
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DNA
Tubulin
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B
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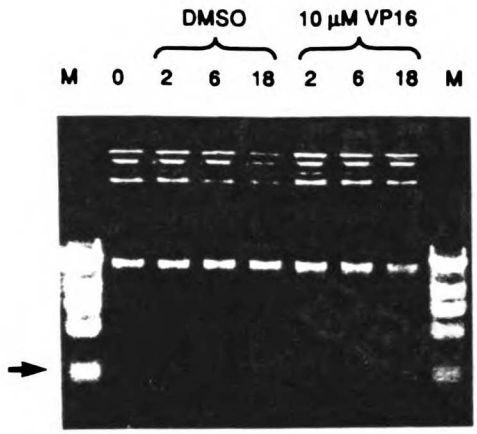

Figure 9. VP-16 inhibits topoisomerase II activity in frog egg extracts. Decatenation assays were carried out using frozen CSF-arrested extracts with kinetoplast DNA as a substrate, as described in Materials and Methods. Samples were taken at the indicated times (in min) and run on 0.7% agarose gels. The position at which the released mini-circles run is indicated by an arrow, while the catenated DNA networks are retained in the wells of the gel. Reactions contained either 1% (vol/vol) DMSO or 10 μM VP-16 (final DMSO concentration 1% (vol/vol). The markers (M) are a HindIII digest of lambda DNA.

Topoisomerase II Activity Does Not Increase at the Metaphase–Anaphase Transition

Does topoisomerase II activity increase at the metaphase to anaphase transition to cause sister chromatid separation? We assayed topoisomerase II activity by measuring the rate of kDNA decatenation. Topoisomerase II activity and spindle morphology were determined at metaphase and at three different times after the induction of anaphase: just before the earliest visible metaphase–anaphase transition; in mid-anaphase, soon after sister chromosomes had separated; and in late anaphase, well after sister chromosomes had separated. We found that the rate of kDNA decatenation was highest in samples taken from metaphase, slightly lower in the first two time points after calcium addition, and lowest in late anaphase (Fig. 10). Thus, topoisomerase II activity appears to decrease as extracts leave mitosis, although we cannot exclude the possibility that we have failed to detect a transient increase in topoisomerase activity during the metaphase–anaphase transition.

Discussion

We have assembled metaphase spindles in frog egg extracts and shown that these spindles can be induced to undergo anaphase. Sister chromatid separation is prevented by incomplete DNA replication, or the presence of topoisomerase II inhibitors during the induction of anaphase.

Anaphase In Vitro Resembles Anaphase In Vivo

To what extent does the anaphase we observe in extracts resemble anaphase in intact cells? We have used CSF, a natural inhibitor of the exit from mitosis, to arrest extracts with high levels of MPF and allow sufficient time for spindle assembly. Sawin and Mitchison (1991) have shown, using conditions similar to ours, that each bipolar metaphase spindle forms from a single nucleus. The length of the spindles assembled in the frog egg extracts (~30 μm) is similar to that seen in intact frog eggs (Karsenti et al., 1984). There are no direct measurements of the rate of anaphase chromosome movement in frog embryos, but the observation that spindle size and the duration of anaphase are not grossly different between embryos and extracts (A. W. Murray, unpublished observations) argues that the rate of anaphase chromosome movement is similar in these two systems.

Because CSF-arrested extracts are made from cells arrested in metaphase of meiosis II, the spindles that we have assembled by adding CSF-arrested extracts to our reactions might be regarded as meiotic rather than mitotic spindles. Whether these spindles are truly in mitosis or meiosis II is irrelevant to studies on the mechanism of sister chromatid segregation, because sister chromatids separate from each other in both mitotic and meiosis II anaphases. When induced to enter anaphase, spindles formed in extracts undergo morphological changes similar to those seen in cells: as chromosomes move to the poles, astral microtubules become more prominent and microtubule density at the center of the spindle decreases.

If frog egg extracts are to be used to study the nature of sister chromatid linkage and the mechanism of sister chromatid segregation, it is crucial to demonstrate that the segregating chromosomes are indeed sisters. A number of observations demonstrate that sister chromatid segregation occurs during the in vitro anaphase. When examined by light or by electron microscopy, spindles assembled in vitro contain chromatid pairs aligned at the metaphase plate; the two members of each pair have identical lengths and morphologies. In early anaphase these pairs are observed to split and their two members are pulled towards opposite poles of the spindle. When the DNA polymerase inhibitor aphidicolin is added to extracts late in DNA replication, pairs of chromatids held together by unreplicated regions are formed. When spindles formed under these conditions are induced to undergo anaphase, chromatin bridges are formed that stretch between the segregating chromosomes, demonstrating that the chromosomes attempting to segregate from each other are indeed sisters.

The following considerations confirm that the chromosomes whose segregation we observe must be sisters. One alternative possibility is that the paired chromosomes that separate are not sisters but nonhomologous chromosomes that are being paired and segregated by a distributive disjunction mechanism. Such a system operates in meiosis I to segregate chromosomes that have failed to undergo mitotic recombination (Grell, 1962; Dawson et al., 1986). Recent observations on

Figure 8. The effect of inhibiting topoisomerase II on nuclear morphology at anaphase. (*A*) Control anaphase. (*B*) Anaphase in the presence of the topoisomerase II inhibitor VP-16. VP-16 was added to the extract to 10 μM, just before the second calcium addition. Bars, 10 μm.

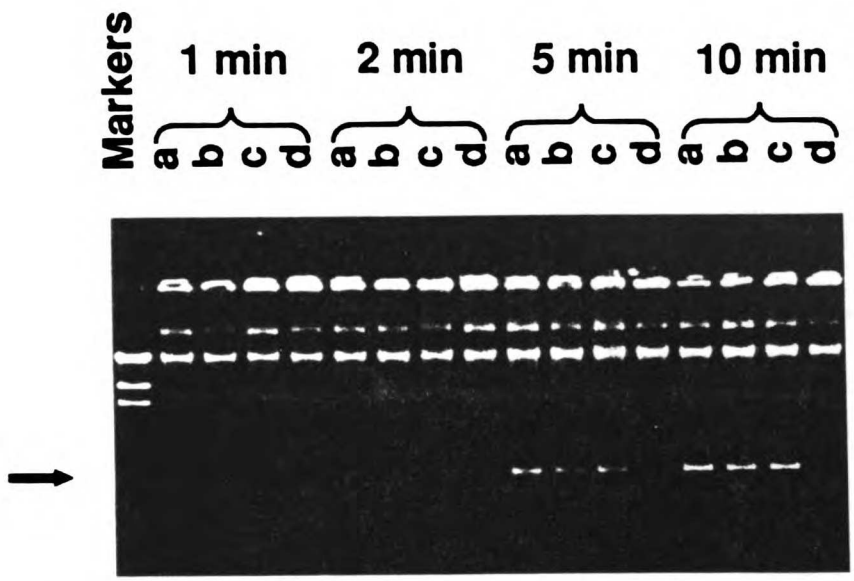

Figure 10. Topoisomerase II activity falls after the metaphase to anaphase transition. Topoisomerase II activity was assayed in fresh extract at four different time points: (*a*) metaphase; (*b*) after the induction of anaphase before chromosome movement had begun (8 min after second calcium addition); (*c*) in mid-anaphase (13 min after second calcium addition); and (*d*) in late anaphase (18 min after second calcium addition). A sample of extract from each time point was mixed with kDNA to begin decatenation reaction. Reactions were carried out on ice to halt progression through the cell cycle and to slow the rate of decatenation. Topoisomerase activity was monitored as the extent of decatenation that had occurred after 1, 2, 5, and 10 min. The markers are a HindIII digest of lambda DNA. The arrow indicates the position in the gel to which decatenated minicircles migrate.

meiotic spindles in *Drosophila* by Theurkauf and Hawley (1992) show that the chromosomes segregated by the distributive pairing mechanism, lie not on the metaphase plate but in between the plate and the spindle poles. We do not observe chromosomes in this position. In addition, inhibition of DNA replication, or type II topoisomerase activity should have no effect on the segregation of nonhomologous chromosomes. The possibility that the segregating chromosomes are homologous chromosomes that have become paired with and linked to each other by meiotic recombination can also be eliminated. Using conditions similar to ours, Sawin and Mitchison (1991) demonstrated that each spindle is formed from a single sperm nucleus. Since sperm nuclei are haploid no chromosome will have a homolog with which it could pair.

In summary, by a variety of criteria, the anaphase that we have observed in vitro resembles anaphase in vivo, strongly suggesting that anaphase in vitro proceeds by the same mechanism as anaphase in vivo. We believe that the extracts described here will be valuable for studying the mechanisms of sister chromatid separation and anaphase chromosome movement.

The Role of DNA Catenation in Sister Chromatid Linkage

Are sister chromatids at metaphase linked by DNA catenation? Incomplete unwinding of DNA duplexes during replication produces replicated DNA molecules that are linked by catenation (Sundin and Varshavsky, 1980, 1981), and that can only be separated from each other by the action of type II DNA topoisomerases. Experiments in budding and fission yeast and, more recently, in mammalian cells, show that type II DNA topoisomerase activity is required during mitosis for successful chromatid separation (DiNardo et al., 1984; Downes et al., 1991; Holm et al., 1989; Uemura et al., 1987). This suggests that, in vivo, sister chromatids are catenated and that this catenation must be resolved in mitosis to allow them to segregate from each other. However, none of these experiments address the question of when, in a normal spindle, topoisomerase activity is required to ensure complete sister chromatid segregation. In both mammalian cells and yeasts, the metaphase–anaphase transition cannot be experimentally regulated without depolymerizing spindle microtubules. In such cells, chromosomes do not experience normal metaphase forces. On the other hand, without experimentally controlling the metaphase–anaphase transition, it is not possible to determine whether topoisomerase activity is inhibited before or after the induction of anaphase. We have used CSF, a physiological regulator, to control the transition between metaphase and anaphase without affecting spindle function. We find that topoisomerase activity is required during anaphase, even in spindles that have been arrested in metaphase before the addition of topoisomerase inhibitors, unequivocally demonstrating that topoisomerase activity is required during anaphase for sister chromatid segregation.

The observation that topoisomerase II inhibitors block sister chromatid segregation strongly suggests that sister chromatids are catenated at metaphase. We cannot exclude the possibility that topoisomerase II has a role in chromatid segregation other than decatenating sister chromatids and that we have inhibited this activity. However, because the topoisomerase inhibitors used in this study have different mechanisms of action, we feel that this possibility is unlikely (Osheroff et al., 1983; for review see Liu, 1989).

It has been difficult to establish the role that DNA catenation plays in the linkage between sister chromatids. On one hand it has been proposed that the primary linkage between sister chromatids is DNA catenation (Murray and Szostak, 1985). On the other, experiments on the segregation of mini-chromosomes in yeast suggest that although catenation may be a prerequisite for bipolar attachment of chromosomes to the mitotic spindle, it need not be continuously maintained to ensure accurate chromosome segregation (Koshland and Hartwell, 1987). Because of the inability to visualize individual chromosomes in budding yeast, it is impossible to exclude the possibility that mini-chromosomes segregate as soon as they are decatenated, even though the natural chromosomes are still on the metaphase plate.

If catenation is the only linkage between sister chromatids, then sister chromatid segregation could be induced by the activation of topoisomerase II at the onset of anaphase. However, by monitoring the decatenation of an exogenous substrate (kDNA), we find that there is no increase in topoisomerase II activity at the metaphase-anaphase transition. Thus, an increase in bulk topoisomerase activity at anaphase cannot be the trigger for chromosome segregation. This observation can be explained in a number of ways. The first is that there are two populations of topoisomerase II: one which is soluble and a second, in the nuclear scaffold, which is closely associated with the chromosomes (Earnshaw et al., 1985). The soluble pool, whose activity we have assayed using an exogenously added substrate (kDNA), may be regulated differently from the chromosome-associated pool. The second possibility is that, during metaphase, the catenation between sister chromatids is protected from topoisomerase activity, and that the protecting factors are removed when anaphase is induced. Finally, catenation may not be the only entity holding sister chromatids together. Instead there might exist another linkage, involving unknown components, whose dissolution is the primary trigger for sister chromatid separation. This linkage would maintain a high local concentration of DNA, so that strand passages catalyzed by topoisomerase II would be as likely to increase catenation as to decrease it. At anaphase the destruction of the other linkage would allow sister chromatids to move apart, decreasing the local DNA concentration and favoring decatenation (Holm et al., 1989). In this view, decatenation would be triggered by anaphase and would be a consequence rather than a cause of chromosome segregation and would not require an increase in topoisomerase activity. Candidates for a proteinaceous linkage between the chromosomes must be present along the chromosome arms as well as the kinetochore, since acentric sister chromatids remain paired throughout metaphase (Bajer and Mole-Bajer, 1972). The INCENP proteins that are localized between sister chromatids along their length are candidates for such a linkage. However, the recent observation that the association of these proteins with chromosomes is abolished considerably before sister chromatid separation and the onset of anaphase (Earnshaw and Cooke, 1991), suggests that they cannot be the only components holding sister chromatids together.

Models for chromosome segregation in which chromosomes are held together only by catenation of the sister chromatids, and separation is triggered by an increase in the force acting at the kinetochores can be eliminated. Earlier studies on mitosis demonstrate that the initial separation of sister chromatids does not require kinetochore activity. In cells arrested in mitosis by colchicine, the sister chromatids eventually separate from each other in the absence of spindle microtubules, although there is no directed movement of the kinetochores towards the spindle poles (Mole-Bajer, 1958; Sluder, 1979). In these mitoses, the kinetochores often remain linked at the centromeres after the arms have separated, suggesting that there may be special components that hold sister kinetochores together in addition to the components that hold the chromosome arms together. In normal mitoses careful time-lapse cinematography has shown that the initial separation of the sisters occurs synchronously all along the length of a chromosome, rather than beginning at the kinetochore and then moving towards the poles, as would be expected if the sisters had to be pulled apart by their kinetochores (Bajer and Mole-Bajer, 1972). Acentric sister chromatids do separate from each other at anaphase, although after their initial separation they fail to show directed movements towards the spindle poles (Bajer and Mole-Bajer, 1972). Finally, experimental measurements suggest that force acting on kinetochores does not increase in anaphase (Alexander and Rieder, 1991; Nicklas, 1988).

The event that triggers sister chromatid separation is unknown. As a first step towards identifying this event we have examined the timing of sister chromatid separation relative to the inactivation of MPF. The first stages of sister separation are seen 4 min after H1 kinase activity begins to fall. It is tempting to conclude from this temporal correlation that the inactivation of H1 kinase is a prerequisite for sister chromatid separation, as it is for chromosome decondensation and nuclear envelope assembly (Murray et al., 1989). However, it is also possible that sister separation is triggered, not by the inactivation of MPF, but by the events that trigger cyclin B degradation and that the proteolytic system that degrades cyclin B also degrades proteins involved in holding sisters together. The existence of nondegradable forms of cyclin should make it possible to distinguish between these possibilities (Murray, 1989). Anaphase in meiosis II of frog oocytes, and in our in vitro extracts, is induced by an increase in the intracellular calcium concentration (Kline, 1988; Meyerhof and Masui, 1977). Although a role for calcium in inducing mitosis in other systems has been reported (Hepler, 1983; Izant, 1983; Poenie et al., 1986; Schollmeyer, 1988; Tombes and Borisy, 1989), it is not clear whether an increase in the intracellular calcium levels is a universal prerequisite for the induction of anaphase (Tombes and Borisy, 1989).

The ability to induce chromosome segregation in vitro will facilitate studies both on the initial separation of sister chromatids at the metaphase-anaphase transition, and on the anaphase movement of the chromosomes towards the poles. In vitro studies will help to clarify the role of DNA catenation, and the INCENP proteins in holding sister chromatids together. In addition, they will allow a biochemical dissection of the linkage between sister chromatids and of the mechanism by which this linkage is destroyed at anaphase.

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