Ligand-independent Dimerization Activates the Stress Response Kinases IRE1 and PERK in the Lumen of the Endoplasmic Reticulum*

IRE1 and PERK are type I transmembrane serine/threonine protein kinases that are activated by unfolded proteins in the endoplasmic reticulum (ER) to signal adaptive responses. IRE1 is present in all eukaryotic cells and signals the unfolded protein response through its kinase and endoribonuclease activities. PERK signals phosphorylation of a translation initiation factor to inhibit protein synthesis in higher eukaryotic cells but is absent in the Saccharomyces cerevisiae genome. The amino acid sequences of the amino-terminal ER luminal domains (NLDs) from IRE1 and PERK display limited homology and have diverged among species. In this study, we have demonstrated that the NLD of yeast Ire1p is required for signaling. However, the NLDs from human IRE1α and murine IRE1β and the Caenorhabditis elegans IRE1 and PERK function as replacements for the S. cerevisiae Ire1p-NLD to signal the unfolded protein response. Replacement of the Ire1p-NLD with a functional leucine zipper dimerization motif yielded a constitutively active kinase that surprisingly was further activated by ER stress. These results demonstrate that ER stress-induced dimerization of the NLD is sufficient for IRE1 and PERK activation and is conserved through evolution. We propose that ligand-independent activation of IRE1 and PERK permits homodimerization upon accumulation of unfolded proteins in the lumen of the ER.

In response to accumulation of unfolded proteins in the endoplasmic reticulum (ER),1 cells activate an intracellular signaling transduction pathway called the unfolded protein response (UPR) that culminates in the induction of nuclear genes encoding ER resident folding catalysts and chaperones to correct protein folding defects. In Saccharomyces cerevisiae, Ire1p has been implicated as the receptor that transmits the stress signal from the ER to the nucleus. Ire1p is a transmembrane Ser/Thr protein kinase/RNase with its amino-terminal domain residing in the ER lumen (NLD) and the kinase/RNase domain in the cytoplasm/nucleoplasm (1, 2). The accumulation of unfolded proteins initiates Ire1p oligomerization to induce autophosphorylation in trans with subsequent activation of the kinase/RNase activities (3, 4). Activated Ire1p functions as a sitespecific RNase that cleaves at the 5’- and 3’-splice site junctions of HAC1 mRNA to initiate its splicing (5). Spliced HAC1 mRNA encodes Hac1p, a basic leucine zipper (bZIP) transcription factor that binds to the unfolded protein response element (UPRE) to activate the transcription of target genes. In S. cerevisiae, the UPRE is conserved in the promoters of all known UPR target genes including KAR2 (encoding yeast BIP/GRP78), PDI1, and FKBP2 (6–8).

Considerably less is understood concerning the mechanism of UPR signaling in higher eukaryotes. Signaling is more complex in that both transcriptional and translational processes are regulated and survival and death responses are activated (9). Two homologs, Ire1α and Ire1β, were recently identified in both murine and human genomes that both exhibit kinase/RNase activities and have a subcellular localization similar to that of yeast Ire1p (10–12). In mammalian cells, an ER stress response element is necessary and sufficient for transcriptional induction of genes encoding protein chaperones such as BIP and GRP94 (13, 14). However, a mammalian homolog of yeast HAC1 has not been identified. Recently, a gene encoding the ER-localized protein PEK/PERK was identified that has limited homology to Ire1p in the luminal domain. PERK is a protein kinase that is activated by ER stress to phosphorylate the eukaryotic translation initiation factor 2 on its α subunit (eIF2α) (15–17). Phosphorylated eIF2α inhibits protein synthesis at the level of initiation, thereby limiting the amount of protein that requires folding. IRE1 and PERK are structurally similar to type I growth factor receptors that are activated by ligand-induced dimerization (18, 19). Therefore, it was proposed that ER stress generates a signal that is transduced through the NLD within the lumen to induce IRE1 and PERK kinase dimerization that will initiate downstream signaling (3, 15, 20). In support of this hypothesis, the Ire1p NLD was shown to oligomerize in S. cerevisiae (20). In the present study, we have examined the functional role of the NLD in IRE1 and PERK for intracellular signaling from the ER to the nucleus by studying its function in yeast. Our results show that NLDs of IRE1 and PERK provide an evolutionarily conserved dimerization function that is necessary and sufficient to initiate signaling. Thus, we propose a ligand-independent negative regulatory model for the mechanism of IRE1 and PERK dimerization.

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The abbreviations used are: ER, endoplasmic reticulum; NLD, amino-terminal luminal domain; bZIP, basic leucine zipper; UPR, unfolded protein response; UPRE, UP element; PERK/Pch, gene that encodes a eukaryotic translation initiation factor 2 kinase (α subunit) that is selectively activated on ER stress; WT, wild type; Tm, tunicamycin; BIP or GRP78, immunoglobulin heavy chain-binding protein; GRP, glucose-regulated protein; NLD193 and NLD35, truncated forms of the NLD with 193 and 35 amino acids, respectively; AD and BC, mutant IRE1 expression products; hIRE1α, human IRE1α protein; mIRE1β, mouse IRE1β protein; ceIRE1, C. elegans IRE1 protein; cePERK, C. elegans PERK protein.

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MATERIALS AND METHODS

Strains and Materials—The yeast strain AWY19 (W303-1A, UPRE CYC1 Leu2, UPRE CYC1 lacZ, \(\Delta ire1\)) was used.\(^2\) Rat-Maf and rat-Jun cDNAs were obtained from Dr. Tom K. Kerppola (University of Michigan Medical School). Vent DNA polymerase (New England BioLabs) was used in all polymerase chain reactions, and all mutations were confirmed by automated DNA sequencing. Tunicamycin (Tm) was obtained from Calbiochem.

Plasmid Constructions—To construct pRS316-IRE1, a 4380-base pair fragment including 755 base pairs of the promoter sequence derived from pRNIGL (2) was ligated into the HindIII site of pRS316 (21) by standard techniques. In pRS316-IRE1-NLD193, NLD was deleted to the \(\Delta Nhel\) site leaving a region that codes for 193 amino acids of the NLD. In NLD35, the NLD was deleted to the \(\Delta Ecori\) site leaving a region that codes for 35 amino acids of the NLD. In pRS316-IRE1-BC, CGCACC (encoding Arg1-Thr27) was mutated to CTTAAG (BsiWI site, encoding Arg1-Thr27), and CTAAAG (encoding Leu196-Lys203) was mutated to CTTAAG (BsiWI site, encoding Ile196-Lys203). In pRS316-IRE1-AD, TCCATC (encoding Ser20-Ile21) was mutated to TCCGGA (BsiWI site, encoding Ser202 (double mutation), Asp221, and Lys282/Thr283 (double mutation) was determined as described previously (27). Protein concentration was determined as described previously (27).

RESULTS

The NLD of Ire1p Is Required for the UPR in Yeast—The same stimuli, the accumulation of unfolded proteins in the ER lumen, activate both IRE1 and PERK. We reasoned therefore that the NLDs of both molecules may function in a common manner to sense ER stress and regulate kinase activity in vivo. Because the genome of \(S.\) cerevisiae encodes only one ER stress-signaling molecule (Ire1p), we chose this model system to study the functional role of the NLD. Alignment of the NLD sequences of IRE1 and PERK proteins from divergent species identified only weak homology (6% identity/similarity with less than 12% homology overall) although 4 conserved motifs of about 12 identical amino acids could be aligned (Fig. 1). We examined the requirement of these limited numbers of identical residues by functional analysis in yeast that harbor a single copy of the lacZ gene under the control of the UPR from \(\Delta ire1\). Expression of either WT Ire1p or the mutants containing alanine at residues Glu129, Trp144, Asp176, Pro192, Leu198/ Ser202 (double mutation), Asp231, and Lys282/Thr283 (double mutation) was able to rescue Tm-dependent \(\beta\)-galactosidase induction in \(\Delta ire1\) cells (data not shown). In addition, mutation of the highly conserved and single N-glycosylation site (N298Q) that is utilized (2) did not affect Tm-dependent \(\beta\)-galactosidase induction when compared with WT Ire1p (data not shown).

These data support our observations that neither the highly conserved residues nor the N-linked glycosylation site within the NLD is required for Ire1p structure and/or function.

To determine whether the NLD is required for UPR signaling, we analyzed two amino-terminal deletion mutants retaining the signal peptide and either 193 (with NLD193 in which most of the conserved residues were deleted) or 35 (NLD35) amino acids at the carboxyl terminus of the NLD (Fig. 2A). Whereas \(\Delta ire1\) WT rescued the \(\Delta ire1\) phenotype monitored by \(\beta\)-galactosidase induction in response to Tm treatment, a mutant (\(\Delta ire1\)-K1058A) that is defective in RNase activity did not induce \(\beta\)-galactosidase activity in response to Tm treatment (Fig. 2A). Indeed, expression of NLD193 (Fig. 2B) and NLD35 (Fig. 2B) did not induce \(\beta\)-galactosidase activity in response to Tm treatment (Fig. 2B). Although we were unable to localize and quantify the mutant protein because of the low level expression of Ire1p (1, 2), the results suggest that the NLD is required for Ire1p function.

NLD Function of IRE1 and PERK Is Conserved through Evolution—To elucidate whether the NLDs of IRE1 and PERK

\(^{2}\) A. A. Welihinda and R. J. Kaufman, unpublished data.

\(^{3}\) C. Y. Liu and R. J. Kaufman, unpublished data.
use a similar mechanism(s) for sensing ER stress, chimeric expression vectors were constructed so that the yeast Ire1p NLD was replaced by the NLD of hIRE1, mIRE1β, ceIRE1, or cePERK while retaining the Ire1p signal peptide sequence (Fig. 2A). All four of the resultant chimeric NLDs were able to restore the UPR in ΔIRE1 cells, and similar levels of β-galactosidase activity were observed within 3–4 h after Tm treatment (Fig. 2, C and D). The Tm dose responses for ceIRE1-NLD and cePERK-NLD activation in yeast were strikingly similar although ceIRE1-NLD displayed a higher basal activity (Fig. 2F). These results support the observation that both ceIRE1 and cePERK utilize a similar sensing mechanism for activation, which responds to the same level of unfolded proteins in the ER.

A Leucine Zipper Dimerization Motif Can Replace NLD Function within Ire1p—To determine whether dimerization is sufficient for Ire1p activation, we generated IRE1-MafL and JunL, in which the NLD was replaced by a leucine zipper dimerization motif from the bZIP-containing transcription factors Maf and Jun (Fig. 2A) (28). As expected, expression of these chimeras was able to activate β-galactosidase expression in the absence of Tm (Fig. 2E). However, β-galactosidase expression was induced by Tm to similar levels in yeast that express WT Ire1p (Fig. 2, B and E). Substitution of one or two leucine residues within the leucine zipper with prolines (IRE1-MafL(P)) abolished UPR induction (Fig. 2E). These results support the finding that Ire1p dimerization is necessary and sufficient to initiate Ire1p activation.

To extend the analysis from the reporter gene experiments, Northern blot analysis of KAR2 induction and HAC1 splicing were performed. Upon Tm treatment, induction of KAR2 transcription and HAC1 mRNA splicing was defective in Δire1 cells compared with cells expressing WT Ire1p (Fig. 3A). Δire cells expressing NLD193 and NLD35 showed significantly reduced KAR2 induction and HAC1 mRNA splicing. Cells that express the chimeric proteins containing the NLD from hIRE1, mIRE1β, ceIRE1, or cePERK, or MafL induced KAR2 transcription and HAC1 mRNA splicing with Tm treatment. Consistent with the β-galactosidase assay, the basal levels of KAR2 expression were elevated for hIRE1α, ceIRE1, and MafL when compared with WT Ire1p, K1058A, mIRE1β, and cePERK.

If the NLD requirement for Ire1p function is physiologically significant, cells deficient in NLD function should be sensitive to ER stress. To investigate this hypothesis, yeast cells were grown on plates containing different concentrations of Tm (Fig. 3B). In the absence of Tm, all the tested strains displayed growth rates similar or identical to WT and ΔIRE1 cells. Under the same growth condition, a low concentration of Tm (0.25 μg/ml) severely impaired the growth of ΔIRE1. Similarly, a low concentration of Tm dramatically reduced the growth of NLD193 and NLD35, which is consistent with β-galactosidase assay and Northern blot analysis. In contrast, Tm did not significantly affect the growth of hIRE1α-NLD, mIRE1β-NLD, ceIRE1-NLD, and cePERK-NLD when compared with WT Ire1p even though the observed growth rates were slightly different among the yeast expressing the four NLD-Ire1p chimeras.

In the presence of Tm, the growth rates of Ire1p and MafL were comparable (Fig. 3B) suggesting that the leucine zipper
motif functions effectively to protect cells from ER stress. The growth of MafL(P) was significantly impaired at Tm concentrations of 0.25 and 0.5 \( \mu \text{g/ml} \) compared with Ire1p and MafL. However, MafL(P) survived Tm treatment better than NLD35. In addition, we also observed a residual UPR induction with MafL(P) (Fig. 2D). These results suggest that the mutations in MafL(P) did not completely abolish dimer formation.

**DISCUSSION**

All eukaryotic cells respond to the accumulation of unfolded proteins in the ER by activation of transmembrane protein kinases that transmit the signal across the ER membrane. IRE1 and PERK mediate different downstream effects although both are activated by similar ER stresses (9). Interestingly, although *S. cerevisiae* does not have a *Perk* gene, replacement of the yeast Ire1p-NLD from cePERK was able to accurately signal the UPR. Therefore, NLD-mediated activation of IRE1 and PERK occurs through a common sensing mechanism that is conserved through evolution. In addition, dimerization of the NLD does not require gene products that are not expressed in *S. cerevisiae* such as presenillin-1 (12) or GRP94 (30), which have been implicated in the UPR in higher eukaryotic cells.

IRE1 dimerization may be regulated through one of two mechanisms (2, 8, 9). In a positive regulatory model, dimerization of IRE1 is activated by ligand binding to the NLD. The ligand could be unfolded protein itself, a complex between unfolded protein and a chaperone(s), or a yet unidentified ligand generated on ER stress. Because the NLDs of IRE1 and PERK are interchangeable, they should share a common ligand under this model. Replacement of the NLD with a bZIP motif from Maf or Jun constitutively activated Ire1p, which supports the requirement for dimerization. Surprisingly, this leucine zipper also conferred ER stress-dependent activation onto Ire1p similar to the WT Ire1p. It is unlikely that the bZIP motif would have specificity to bind an ER stress-induced ligand. An alternative model proposes that a negative effector maintains IRE1 as a monomer in the absence of ER stress. When unfolded proteins accumulate, they bind the negative regulator and release Ire1p for dimerization (Fig. 4).

What might function as a negative regulator of IRE1 and PERK dimerization? It is possible that structures such as the chaperone matrix of the lumen of the ER (31) or the phospholipid bilayer itself may prohibit lateral diffusion to prevent dimerization. However, data indicate that BIP may prevent IRE1 and PERK dimerization. First, overexpression of BIP in yeast and in mammalian cells blocks activation of the UPR (32, 33) whereas reduction in BIP levels induces the UPR (5, 34–36). Second, BIP binds to a broad spectrum of short hydrophobic polypeptides and interacts transiently with exposed hydrophobic patches on protein-folding intermediates as well as interactive surfaces on unassembled protein subunits of multisubunit protein complexes (37–39). The leucine zipper motif may provide a hydrophobic surface, thereby explaining the ER stress induction of the bZIP-IRE1 chimeric molecules. Finally, the accumulation of properly or improperly folded mutant glycoproteins to which BIP does not bind does not induce the UPR.
(39–41). This suggests that only unfolded proteins that bind BIP are capable of competing with the NLD for BIP binding.

In summary, our studies support a novel ligand-independent mechanism to induce IRE1 and PERK kinase dimerization. This mechanism may provide a paradigm for the mechanism of activation of additional protein kinases.

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