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Protein Serine/Threonine Phosphatase Ptc2p Negatively Regulates the Unfolded-Protein Response by Dephosphorylating Ire1p Kinase

AJITH A. WELIHINDA,[1] WITOON TIRASOPHON,[1] SARAH R. GREEN,[1] AND RANDAL J. KAUFMAN[1,2]*

Department of Biological Chemistry[1] and Howard Hughes Medical Institute,[2] University of Michigan Medical Center, Ann Arbor, Michigan 48109-0650

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Cells respond to the accumulation of unfolded proteins in the endoplasmic reticulum (ER) by increasing the transcription of the genes encoding ER-resident chaperone proteins. Ire1p is a transmembrane protein kinase that transmits the signal from unfolded proteins in the lumen of the ER by a mechanism that requires oligomerization and *trans*-autophosphorylation of its cytoplasmic-nucleoplasmic kinase domain. Activation of Ire1p induces a novel spliced form of *HAC1* mRNA that produces Hac1p, a transcription factor that is required for activation of the transcription of genes under the control of the unfolded-protein response (UPR) element. Searching for proteins that interact with Ire1p in *Saccharomyces cerevisiae*, we isolated *PTC2*, which encodes a serine/threonine phosphatase of type 2C. The Ptc2p interaction with Ire1p is specific, direct, dependent on Ire1p phosphorylation, and mediated through a kinase interaction domain within Ptc2p. Ptc2p dephosphorylates Ire1p efficiently in an Mg^{2+}-dependent manner in vitro. *PTC2* is nonessential for growth and negatively regulates the UPR pathway. Strains carrying null alleles of *PTC2* have a three- to fourfold-increased UPR and increased levels of spliced *HAC1* mRNA. Overexpression of wild-type Ptc2p but not catalytically inactive Ptc2p reduces levels of spliced *HAC1* mRNA and attenuates the UPR, demonstrating that the phosphatase activity of Ptc2p is required for regulation of the UPR. These results demonstrate that Ptc2p downregulates the UPR by dephosphorylating Ire1p and reveal a novel mechanism of regulation in the UPR pathway upstream of the *HAC1* mRNA splicing event.

In eukaryotic cells, the endoplasmic reticulum (ER) is the site where folding of the newly synthesized proteins that are destined for cell surface occurs. A number of cellular proteins, such as immunoglobulin binding protein, protein disulfide isomerase, glucose-regulated protein 94, peptidyl-prolyl-*cis-trans*-isomerase, and Erp72, are localized to the lumen of the ER and are proposed to act as chaperones to promote proper folding and/or to prevent aggregation of the folding intermediates (12). Consistent with the proposed chaperone functions, the accumulation of unfolded or misfolded proteins in the ER activates the transcription of the genes encoding the ER chaperones and thereby upregulates their synthesis. A conserved promoter element, the unfolded-protein response (UPR) element (UPRE), was identified in yeast (23) and mammalian (5) cells as being necessary and sufficient to mediate transcriptional induction in response to unfolded proteins in the ER. In the yeast *Saccharomyces cerevisiae*, a basic leucine zipper protein, Hac1p (8, 25), that binds to the UPRE and a transcriptional coactivator complex, Gcn5-Ada (42), are required for the transcriptional induction of *KAR2* in response to unfolded proteins in the ER.

In yeast, transcriptional induction of the ER chaperone genes also requires a transmembrane serine/threonine kinase, Ire1p (7, 24). Ire1p is structurally similar to class I growth factor receptors and has three distinct domains, an N-terminal lumen domain, a transmembrane domain that spans the ER membrane, and a C-terminal domain that is either in the cytoplasm or in the nucleoplasm. The cytoplasmic-nucleoplasmic domain has intrinsic serine/threonine kinase activity (41), undergoes oligomerization and *trans*-autophosphorylation in response to unfolded proteins in the ER (30), and contains a region in the extreme carboxy terminus that has homology to RNase L (35). Thus, Ire1p appears to be the proximal sensor of unfolded proteins in the ER that initiates the UPR.

Recently, it was shown that the UPR is regulated by Hac1p levels in the cell (8, 16). When the UPR is inactive, Hac1p is not produced, as the *HAC1* precursor mRNA is not translated (16). Upon activation of the UPR, *HAC1* mRNA is spliced in an Ire1p-dependent manner to generate Hac1p, which binds the UPRE and activates transcription of the chaperone genes. As *HAC1* mRNA splicing is not affected by mutations that affect the splicesome function, a novel splicing pathway appears to be involved in the splicing of *HAC1* mRNA. Recently, it was shown that Ire1p has a site-specific endonuclease activity that cleaves *HAC1* mRNA (35) and that the cleaved intermediates are subsequently ligated by the tRNA ligase Rlg1p (36).

Reversible protein phosphorylation is a major mechanism that modulates protein function in a variety of signal transduction pathways by the opposing actions of protein kinases and phosphatases. In eukaryotes, dephosphorylation at serine and threonine residues is catalyzed by gene products of two distinct families, PPP and PP2C (serine/threonine phosphatases of type 2C) (2). Members of the PP2C family show significant homology to mitochondrial pyruvate dehydrogenase phosphatase and show no apparent homology to the PPP family, comprised of PP1, PP2A, and PP2B. PP2C enzymes are unique in that they exist as monomers (15, 39), require Mg^{2+} or Mn^{2+} for catalytic activity (15, 39), and have no known inhibitors. The recently described crystal structure of human PP2Cα demonstrated that the catalytic domain is composed of conserved acidic residues that are proposed to coordinate Mg^{2+} or Mn^{2+} (9). Although the absence of specific inhibitors and genetic approaches has delayed the analysis of these enzymes, recent

* Corresponding author. Mailing address: Howard Hughes Medical Institute, University of Michigan Medical Center, MSRB II Room 4570, 1150 W. Medical Center Dr., Ann Arbor, MI 48109-0650. Phone: (313) 763-9037. Fax: (313) 763-9323. E-mail: kaufmanr@umich.edu.

TABLE 1. Yeast strains

Strain	Genotype	Source or reference
EGY48	*MATα his3 leu2::3LexAop-LEU2 ura3 trp1 LYS2*	13
W3031b	*MATα ade 2-1 can1-100 leu2-3,112 his3-11,15 trp1-1 ura3-52*	P. Walter, University of California
AWY500	Same as W3031b except *his3-11,15::HIS$^+$ UPRE-lacZ*	This study
AWY446	Same as W3031b except *his3-11,15::HIS$^+$ UPRE-lacZ ptc2-1::kan^r*	This study
AWY506	Same as W3031b except *his3-11,15::HIS$^+$ UPRE-lacZ ptc2-Δ1::LEU2*	This study
AWY516	Same as W3031b except *his3-11,15::HIS$^+$ UPRE-lacZ ire1-Δ1*	This study

studies have revealed that they are key components of a variety of cellular signal transduction pathways. In eukaryotes, PP2C has been implicated in the reversal of protein kinase cascades that are activated upon environmental stress. In both fission and budding yeasts, a PP2C-like phosphatase negatively regulates the PBS2/HOG1-MAP kinase pathway that is activated in response to osmotic and heat shock (19, 20, 33, 34). In plants, PP2C positively regulates signal transduction by the plant hormone abscisic acid, leading to stomatal closure (18), seed dormancy (18, 22), and growth inhibition (18). Although these studies have identified pathways that are regulated by the PP2C family of phosphatases, no known specific substrates have been identified for these phosphatases to date.

To gain insight into the regulation of the UPR pathway, we searched for proteins that interact with the cytoplasmic-nucleoplasmic domain of Ire1p kinase. In this paper, we describe the identification of a novel PP2C-like protein serine/threonine phosphatase, Ptc2p, by virtue of its interaction with Ire1p. Ptc2p specifically interacts with phosphorylated Ire1p and dephosphorylates Ire1p in vitro. Cells devoid of Ptc2p have a hyperactive Ire1p receptor that leads to deregulation of the UPR pathway. We propose that Ptc2p downregulates the UPR by dephosphorylating Ire1p, thus functioning as an off switch in the UPR pathway.

MATERIALS AND METHODS

Yeast strains, general methods, and plasmid constructions. *Escherichia coli* DH5α was used for the propagation of plasmids. The genotypes of the *S. cerevisiae* strains used in this study are shown in Table 1. The genetic methods and standard media used were previously described (31).

The construction of fusions containing different subdomains of Ire1p with either the LexA DNA binding domain or glutathione *S*-transferase (GST) were previously described (41). PCR primers are shown in Table 2. To construct pYES2PTC2, full-length *PTC2* was amplified by PCR with 5′ primer PTC2N and 3′ primer PTC2C and subcloned into the *Bam*HI and *Eco*RI sites of the yeast expression vector pYES2 (Invitrogen, Carlsbad, Calif.). The authenticity of the clones were confirmed by DNA sequence analysis. Overlap-extension PCR-mediated mutagenesis was performed to construct the E37A/D38A double mutant. *PTC2* was amplified as two fragments with a combination of mutagenic (ptc2$^{E37A/D38A}$N and ptc2$^{E37A/D38A}$C) and wild-type (PTC2N and PTC2C) primers, and the products were mixed and reamplified with the wild-type primers. Similarly, the D234A mutant was constructed with mutagenic primers ptc2^{D234A}N and ptc2^{D234A}C. The constructs described above contained T7 epitope-tagged versions of the phosphatase, and their expression was confirmed by Western blotting with anti-T7 epitope antibodies.

To construct fusions between the transcriptional activator B42 tagged with the hemagglutinin epitope (B42-HA) and Ptc2p, different regions of *PTC2* were amplified by PCR with primers PTC2^{174}N, PTC2^{312}C, and PTC2^{355}C. The PCR fragments were subcloned into either the *Eco*RI or the *Eco*RI and *Xho*I sites of pJG4-5. To create GST-Ptc2p fusion constructs, PCR-amplified fragments of the wild type and the mutants were subcloned into the *Bam*HI and *Eco*RI sites of the bacterial expression vector pGEX1λT (Pharmacia Biotech Inc., Piscataway, N.J.). The expression of B42-HA–Ptc2p and GST-Ptc2p fusion constructs was confirmed by Western blotting with anti-influenza hemagglutinin (HA) epitope and anti-GST antibodies, respectively.

To construct pGEM-4ZACT1, a 210-bp fragment of *ACT1* was amplified by PCR with primers ACT1N and ACT1C and subcloned into the *Xba*I and *Hin*dIII sites of pGEM4-Z. Similarly, a 456-bp fragment of *HAC1* spanning the 5′ splice junction was amplified by PCR with primers HAC1N and HAC1C and subcloned into the same sites of pGEM-4Z to construct pGEM-4ZHAC1.

To create pPTC2KO1, the *Not*I-*Not*I fragment of pFAMX2 (40) containing the *kan*^{r} gene was inserted into the unique *Tth*III1 site in pYES2PTC2 after overhangs were filled in with T4 DNA polymerase. pPTC2KO2 was created by replacing the 935-bp *Xba*I-*Nru*I fragment of pGEX1λTPTC2 with the *Nhe*I-*Sma*I fragment of YDp-L (3) containing the *LEU2* gene. pBSIRE1 was constructed by subcloning PCR-generated *IRE1* (with primers IRE1N and IRE1C) into the *Sal*I and *Xba*I sites of pBluescript II KS+/− (Stratagene, Menasha, Wis.). To construct pIRE1KO, the 2,052-bp *Eco*RI-*Bgl*II internal fragment of pBSIRE1 was replaced by the *hisG-URA3-hisG* cassette (1) with the same restriction sites. This construct replaced 684 amino acids of Ire1p.

All yeast strains carried a single integrated copy of the UPRE-LacZ reporter, constructed by homologous recombination of *Nae*I-linearized pJC002 (7) at the *HIS3* locus. Strains carrying null alleles of *PTC2* and *IRE1* were created by one-step gene disruption (28). AWY500 cells were transformed with *Eco*RI-*Bam*HI fragments of pPTC2KO1 and pPTC2KO2 containing disrupted *ptc2* and selected for kanamycin resistance and leucine prototrophy, respectively. To create the *ire1-Δ1* disruption, AWY500 cells were transformed with the *Sal*I-*Xba*I fragment containing disrupted *ire1* and selected against uracil prototrophy. The stable Ura$^+$ integrants were grown in rich media and selected for uracil prototrophy (4). The gene disruptions were confirmed by PCR and Southern-Northern blot analysis.

Western blot analysis. Yeast cell lysates were made according to Williams et al. (43). Western blotting was performed by standard procedures (14) with anti-influenza HA epitope (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), anti-GST (Santa Cruz Biotechnology, Santa Cruz, Calif.), and anti-T7 epitope (Novagen, Madison, Wis.) primary antibodies and horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (Gibco BRL, Gaithersburg, Md.). Bands were detected with an enhanced chemiluminescence kit (Amersham Corp., Arlington Heights, Ill.) and quantified with National Institutes of Health Image 1.55b program.

Protein phosphatase assays. Ptc2p, Ptc2p^{D234A}, Ptc2p$^{E37A/D38A}$, and Ptc2p$^{E37A/D38A/D234A}$ were expressed as GST fusion proteins in *Escherichia coli*, purified, and cleaved with thrombin to release the phosphatase fragments. Purified recombinant GST-Ire1p (GST-WC) fusion protein was phosphorylated with [γ-^{32}P]ATP as described before (41). Phosphatase assays were performed as described by McGowan and Cohen (21) with buffer containing 50 mM Tris-HCl (pH 7.0), 0.1 mM EGTA, 0.1% (vol/vol) 2-mercaptoethanol, 60 mM magnesium acetate, and 1 mg of bovine serum albumin per ml and with ^{32}P-labeled GST-WC (1 μg) as the substrate. The phosphorylated proteins were analyzed by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE) and autoradiography.

TABLE 2. PCR primers

Primer	Sequence
PTC2N	5′ACCGGATCCAGAAAAATGGGACAAATTCTATC3′
PTC2C	5′AACGGCCGGCACCGAATTCTTAACCCATTTGCTGTCCACCAGTCATGCTAGCCATTCCTTGCAAGTGGGAACGGCCGGC3′
PTC2^{174}N	5′GTGGAATTCGATGGCTTCGTAGAAATGGAT3′
PTC2^{312}C	5′AGAACGGAATTCTCAGCGGTGGGCCTTGGACTT3′
PTC2^{355}C	5′GGAAGGATCCTCAGTGGTCGCGAGTGAATTTGTC3′
ptc2$^{E37A/D38A}$N	5′GCAATGCAAGGGTGGCGGATGTCAATGGCGGCGTCACACATTCTA3′
ptc2$^{E37A/D38A}$C	5′CTTTGTCAAAACATTAGGCTCTAGAATGTGTGACGCCGCCATTGACA3′
ptc2^{D234A}N	5′GATTACGATAGGGACGAGTTTGTAATCTTAGCCTGTGCAGGTATCTG3′
ptc2^{D234A}C	5′AATCTTGGGAAGTCAAACAATCCCAGATACCTGCACAGGCTAA3′
IRE1N	5′ACTTGTCGACATGCGTCTACTTCGAA3′
IRE1C	5′GCTCTAGATTATGAATACAAAAATTCACG3′
ACT1N	5′GGTTCTAGATTCTCCACCACTGCTGAAAGA3′
ACT1C	5′GTCAATACCGGCAGATTCC3′
HAC1N	5′AAATCTAGAAGAGTCGACGACGCTACCTG3′
HAC1C	5′AAATTCGAATACCCTCTTGCGATTGTCTTC3′

In vitro pull-down assays. Plasmids carrying *PTC2*, *ptc2*^{D234A}, *ptc2*$^{E37A/D38A}$, or *ptc2*$^{E37A/D38A/D234A}$ under the control of the T7 promoter were used in a coupled in vitro transcription-translation assay to create [^{35}S]methionine-labeled products. Translated products were separated by SDS-PAGE, treated with En^{3}Enhance, and quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). Equal amounts of recombinant proteins were incubated with glutathione-Sepharose beads containing equal amounts of phosphorylated and nonphosphorylated GST-Ire1p cytoplasmic domain fusion proteins at 4°C for 2 h in binding buffer (phosphate-buffered saline [PBS], 10% glycerol, 2 mM EDTA). As the control, beads containing comparable amounts of GST were used. Beads were recovered, washed twice with PBS containing 10% glycerol and 0.05% Triton X-100 and once with PBS, and boiled for 3 min in 1× Laemmli buffer (17). Extracts were electrophoresed on reducing SDS–10% polyacrylamide gels, treated with En^{3}Enhance, and analyzed by autoradiography and PhosphorImager scanning.

Two-hybrid assays. Yeast two-hybrid assays were performed as described previously (13). The yeast reporter strain EGY48 was sequentially transformed with derivatives of pEG202 and pJG4-5 containing different regions of the Ire1p cytoplasmic domain. Interactions were monitored by the ability of the reporter strain to grow on media lacking leucine.

β-Galactosidase activity and protein assays. β-Galactosidase activity was quantified with a β-galactosidase assay kit (Promega Corp., Madison, Wis.) according to the instructions provided by the manufacturer. Proteins were quantified with a protein assay kit (Bio-Rad Laboratories, Hercules, Calif.).

RNase protection analysis. Total RNA was isolated (29) from cells treated or not treated with tunicamycin for 90 min and analyzed with an RNase protection kit (Boehringer). pGEM-4ZHAC1 and pGEM-4ZACT1 were linearized with *Xba*I, and antisense RNA probes were synthesized with T7 RNA polymerase (Boehringer) and [α-^{32}P]CTP (Amersham). For each sample, 10 μg of RNA was hybridized for 14 h at 45°C with 2×10^5 cpm of *HAC1* and *ACT1* RNA probes, digested with RNase A and RNase T_1, processed as suggested by the manufacturer, and analyzed on a 6% polyacrylamide gel containing 8 M urea. The band intensities were quantitated by PhosphorImager scanning and normalized to that of actin (*ACT1*) mRNA. A DNA sequencing ladder of a known template was used as a size marker.

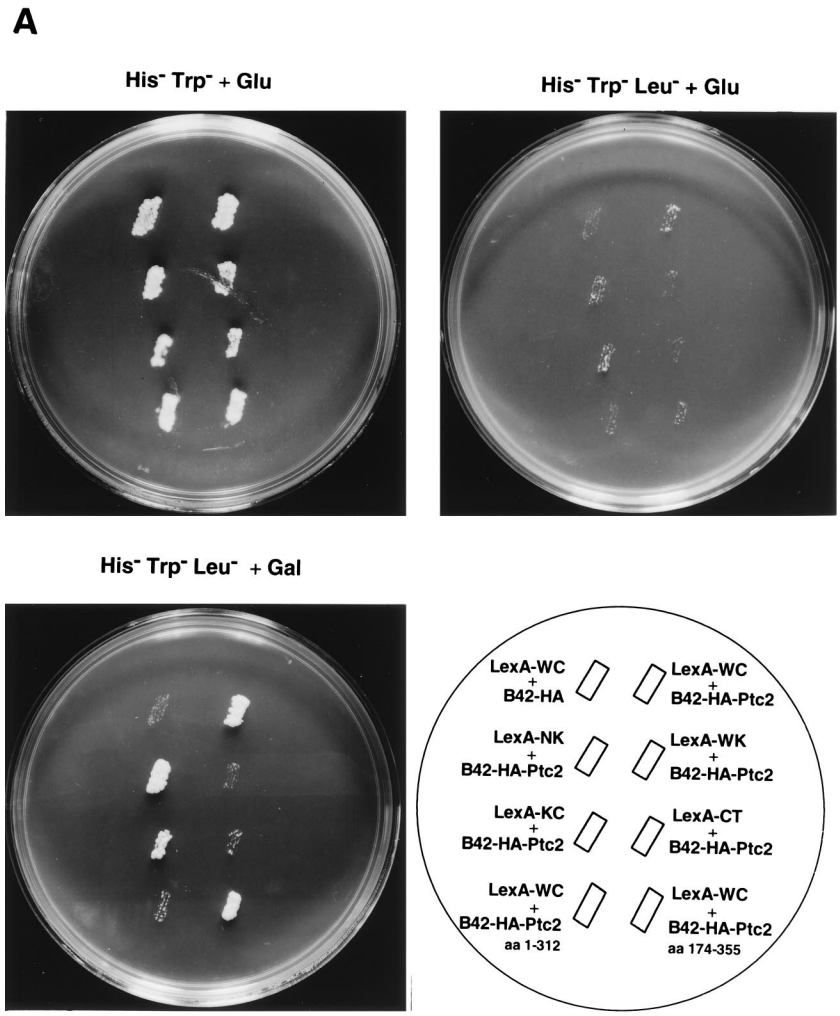
RESULTS

Ptc2p interacts with Ire1p in vivo. To elucidate mechanisms that regulate the UPR, we searched for molecules that interact with Ire1p. As conventional biochemical methods have often failed to identify such serine/threonine kinase receptor molecules, we used a modified version of the yeast two-hybrid system (13). The cytoplasmic-nucleoplasmic domain of Ire1p containing the intact kinase domain was used as the bait (LexA-WC) to screen a yeast genomic library. The kinase that showed the highest homology to Ire1p, Cdc28p (27% identity and 46% similarity within the kinase domains), was used as a negative control bait. *PTC2* was isolated three times independently in interactions with Ire1p in this screen. When tested in the yeast two-hybrid system, B42-HA–Ptc2p specifically interacted with LexA-WC (Fig. 1A) but not with LexA-Cdc28p (data not shown). To examine the molecular details of the Ire1p-Ptc2p interaction, a series of N-terminal and/or C-terminal truncations were constructed in both IRE1p and Ptc2. The summary of the interaction data is presented in Fig. 1B. Both LexA-NK (N linker plus the kinase domain) and LexA-KC (kinase domain plus the C tail) interacted with B42-HA–Ptc2p, and the binding properties were quantitatively similar to those observed with LexA-WC, indicating that deletion of either the N-linker region or the C-tail region does not destroy the interaction. In contrast, neither LexA-WK (kinase domain alone) nor LexA-CT (C-tail alone) interacted with B42-HA–Ptc2p. Although we cannot rule out the possibility that these subdomains had altered secondary structures that did not permit detectable interactions, these results suggest that there are multiple Ptc2p interaction sites disseminated within the cytoplasmic-nucleoplasmic domain of Ire1p and that the loss of one or more such sites can significantly perturb the detectable interaction. The N-linker region alone could not be used in the two-hybrid assays because it was transcriptionally active.

Ptc2p is a 464-amino-acid (aa) protein that contains a region (aa 1 to 300) showing high homology to PP2C. Since Ptc2 clones isolated in the two-hybrid screens did not contain aa 1 to 173, we questioned whether the N terminus of Ptc2p also contributes to the interaction. As detected by the two-hybrid assays, neither B42-HA–Ptc2p$^{1–312}$ (aa 1 to 312; Fig. 1A) nor B42-HA–Ptc2p$^{1–217}$ (aa 1 to 217; data not shown) interacted with LexA-WC, suggesting that the N-terminal 312 aa of Ptc2p do not mediate the interaction with Ire1p. The carboxy terminus of Ptc2p (aa 355 to 464) is also not required for the interaction, since B42-HA–Ptc2p$^{174–355}$ (aa 174 to 355) interacted with LexA-WC as well as the original clone, B42-HA–Ptc2$^{174–464}$ (aa 174 to 464). However, B42-HA–Ptc2p$^{355–464}$ did not interact with LexA-WC (data not shown). These results indicated that the Ire1p interaction domain resides within aa 174 to 355 of Ptc2p.

Ptc2p physically associates with phosphorylated Ire1p. To substantiate the genetic evidence for a protein-protein interaction between Ire1p and Ptc2p, affinity adsorption experiments were performed with products from coupled in vitro transcription-translation of *PTC2* in the presence of [^{35}S]methionine. The Ire1p cytoplasmic-nucleoplasmic domain was produced as a soluble GST fusion protein (GST-WC) in *E. coli*, adsorbed to glutathione-Sepharose beads, and used for adsorption of the in vitro-translated Ptc2p from reticulocyte lysates. Glutathione-Sepharose beads impregnated with comparable amounts of GST were used as a control. In these assays, the amount of Ptc2p brought down by GST-WC was not different from the amount obtained with the control beads containing GST alone (Fig. 2, lanes 4 and 2, respectively). However, when GST-WC was autophosphorylated by incubation in kinase buffer with ATP prior to the adsorption, the binding of GST-WC to Ptc2p was increased by 4.5-fold (Fig. 2, lane 5) over that of the GST control. When EDTA was removed from the binding buffer, GST-WC did not bring down Ptc2p (data not shown). It is possible that in the presence of Mg^{2+} the phosphatase activity of Ptc2p dephosphorylates Ire1p and consequently results in the dissociation of Ptc2p from Ire1p (see below). These results suggest that Ire1p physically associates with Ptc2p as a result of a direct interaction between the two proteins. More importantly, these results indicate that Ptc2p discriminates between the phosphorylated and the nonphosphorylated forms of Ire1p and specifically interacts with the phosphorylated receptor kinase.

Ptc2p is a genuine protein serine/threonine phosphatase that dephosphorylates Ire1p in vitro. Ire1p kinase is autophosphorylated at serine and threonine residues both in vitro (41) and in vivo (30). The specific interaction between the phosphorylated form of Ire1p and Ptc2p, a putative protein serine/threonine phosphatase, implied two possible functional consequences. First, to investigate whether Ire1p is a substrate for Ptc2p, in vitro dephosphorylation assays were performed. Ptc2p was produced by expressing a GST-Ptc2p fusion protein in *E. coli*; the fusion protein was purified and subsequently cleaved with thrombin to remove GST. Ire1p was also expressed as a GST fusion protein (GST-WC) in *E. coli*, purified, and autophosphorylated in the presence of [γ^{32}-P] ATP. A fixed amount of labeled GST-WC was incubated with increasing amounts of Ptc2p. The degree of phosphorylation or dephosphorylation was analyzed by SDS-PAGE followed by autoradiography. In these assays, Ptc2p efficiently removed covalently attached phosphate groups from GST-WC (Fig. 3). The dephosphorylation was evident, with a GST-WC/Ptc2p ratio as low as 1:0.2 (data not shown), and increased steadily with increasing amounts of Ptc2p (Fig. 3, lanes 2 to 5). Since EDTA completely inactivated the dephosphorylation of GST-WC (see Fig. 5A, lane 3), the phosphatase activity of Ptc2p is Mg^{2+} dependent. These results demonstrate that Ptc2p is in-


A
His- Trp- + Glu
His- Trp- Leu- + Glu
His- Trp- Leu- + Gal
LexA-WC + B42-HA
LexA-WC + B42-HA-Ptc2
LexA-NK + B42-HA-Ptc2
LexA-WK + B42-HA-Ptc2
LexA-KC + B42-HA-Ptc2
LexA-CT + B42-HA-Ptc2
LexA-WC + B42-HA-Ptc2 aa 1-312
LexA-WC + B42-HA-Ptc2 aa 174-355

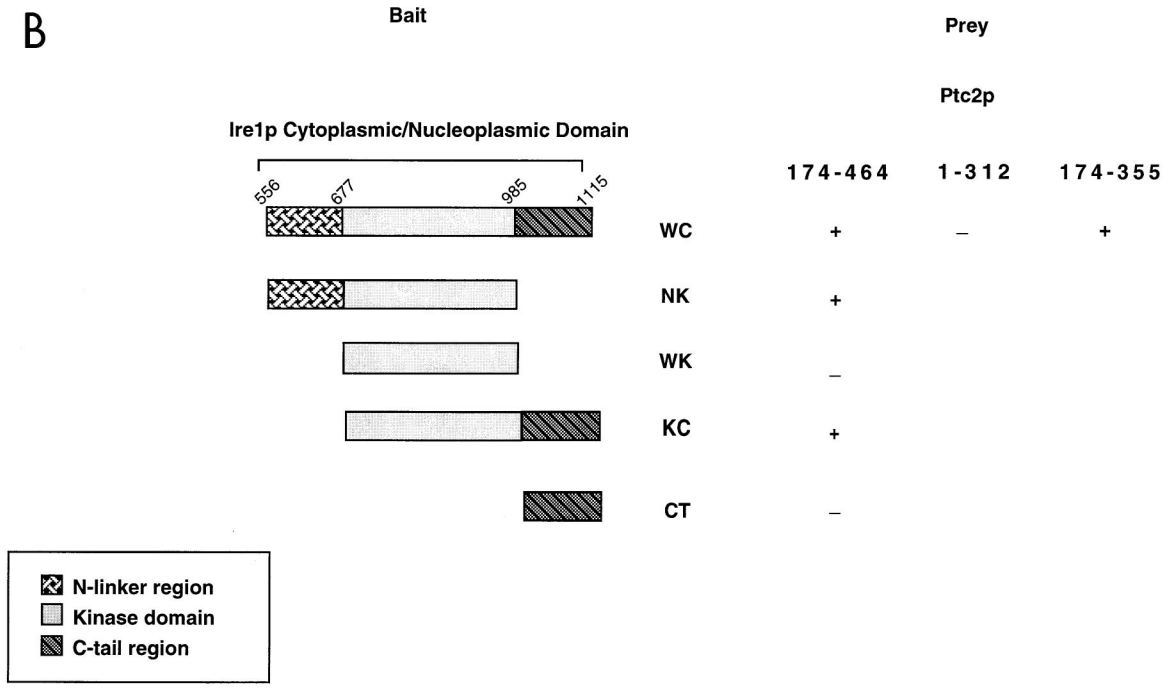

B
Bait
Prey
Ptc2p
Ire1p Cytoplasmic/Nucleoplasmic Domain
556
677
985
1115
174-464
1-312
174-355
WC + – +
NK +
WK –
KC +
CT –
N-linker region
Kinase domain
C-tail region

FIG. 1. Analysis of Ire1p interaction with Ptc2p and mapping of interaction domains. (A) Two-hybrid analysis. LexA fusion proteins with intact and truncated forms of the cytoplasmic domain of Ire1p (bait) were tested for interaction with the original clone B42-HA–Ptc2p (aa 174 to 464), B42-HA–Ptc2p containing aa 1 to 312, or B42-HA–Ptc2p containing aa 174 to 355. Transformants harboring *IRE1* and *PTC2* fusions were patched onto His^- Trp^- plates and replica plated onto His^- Trp^- Leu^- plates containing either glucose or galactose. The growth of strain EGY48 harboring different regions of the Ire1p cytoplasmic domain as LexA fusions (in pEG202) and as B42-HA fusions (in pJG4-5) on His^- Trp^- medium (control) and His^- Trp^- Leu^- medium containing either galactose plus raffinose (Gal) or glucose (Glu) was monitored. (B) Schematic representation of LexA-Ire1p fusion proteins (bait) and their interactions with B42-HA–Ptc2p (prey) as detected by two-hybrid analysis. WC, wild-type cytoplasmic-nucleoplasmic domain; NK, N linker plus kinase domain; WK, wild-type kinase domain; KC, kinase domain plus C-terminal tail; CT, C-terminal tail.

deed a PP2C-like protein serine/threonine phosphatase that can efficiently dephosphorylate Ire1p kinase in vitro.

To investigate whether Ptc2p acts as a substrate for Ire1p, we performed in vitro phosphorylation assays by coincubation of GST-WC and Ptc2p in the presence of [γ-^{32}P]ATP. Although GST-WC was autophosphorylated in these assays, Ptc2p was not phosphorylated by GST-WC (data not shown). To rule out the possibility that the phosphatase activity of Ptc2p diminished the kinase activity of Ire1p and thus the substrate phosphorylation, catalytically inactive Ptc2p mutants were used as substrates. Ire1p did not *trans*-phosphorylate Ptc2p under these conditions. These results suggest that Ptc2p is not a substrate for Ire1p in vitro.

Mutations in the metal binding sites affect the substrate binding and catalytic activity of Ptc2p. The crystal structure of human PP2Cα reveals that its catalytic domain is composed of a binuclear metal center that is coordinated by four invariant aspartate residues and a glutamate residue that are conserved among some members of the PP2C family (9). Since metal ions (Mn^{2+} and Mg^{2+}) are required for the catalytic activity of the PP2C-like enzymes, substitution of these residues would predict catalytically inactive enzymes. To establish whether the phosphatase activity of Ptc2p is required for the in vivo regulation of Ire1p, we substituted the Glu^{37}, Asp^{38}, and Asp^{234} residues corresponding to the conserved residues that coordinate metal ions in the human counterpart (Fig. 4) with alanine. GST-Ptc2p fusion proteins carrying single ($Ptc2p^{D234A}$), double ($Ptc2p^{E37A/D38A}$), or triple ($Ptc2p^{E37A/D38A/D234A}$) mutations were expressed as soluble proteins in *E. coli*, indicating that the structures of these proteins were not severely misfolded. Dephosphorylation assays revealed that the single ($Ptc2p^{D234A}$; Fig. 5A, lane 5), double ($Ptc2p^{E37A/D38A}$; Fig. 5A, lane 4), and triple ($Ptc2p^{E37A/D38A/D234A}$; data not shown) phosphatase mutants could not catalyze the removal of covalently attached phosphate residues from GST-WC, similar to the results obtained in the absence of Mg^{2+} (Fig. 5A, lane 3). These results demonstrate that the above-listed mutations render the phosphatase catalytically inactive and are consistent with its predicted tertiary structure and the deduced catalytic mechanism.

To test the ability of the catalytically inactive Ptc2p mutants to interact with Ire1p, we performed in vitro adsorption experiments. Reticulocyte lysates containing the ^{35}S-labeled in vitro-translated mutant proteins were incubated with either phosphorylated or nonphosphorylated GST-WC, and the proteins adsorbed to GST-WC were analyzed by SDS-PAGE and fluorography. Like wild-type Ptc2p, the $Ptc2p^{E37A/D38A}$ double mutant was specifically adsorbed by phosphorylated GST-WC (Fig. 5B, lanes 7 and 9) but not by nonphosphorylated GST-WC (Fig. 5B, lanes 4 and 6), indicating that the substitution of both residues Glu^{37} and Asp^{38} with alanine did not destroy the interaction. This result is consistent with the observation that amino acid residues 1 to 173 of Ptc2p were not required for the interaction with Ire1p detected by the two-hybrid analysis. These results are also in agreement with the observation that Mg^{2+} was not required for the interaction, suggesting that the Ptc2p-Ire1p interaction differs from a canonical enzyme-substrate interaction. In contrast, substitution of the invariant aspartic acid residue at 234 with alanine completely abrogated the Ptc2p interaction with phosphorylated GST-WC (Fig. 5B, lanes 5 and 8), demonstrating the specificity and the selectivity of the interaction. The interaction properties of the Ptc2p triple mutant were almost indistinguishable from those of the single mutant, and the triple mutant failed to interact with Ire1p (data not shown). These results are consistent with the two-hybrid data and reconfirm that the Ptc2p-Ire1p interaction is mediated through amino acid residues 174 to 355 of Ptc2p.

***PTC2* is not essential for cell viability.** If the interaction between Ire1p and Ptc2p and the dephosphorylation of Ire1p by Ptc2p were physiologically significant for the regulation of the UPR pathway, cells deficient in Ptc2p should have an altered UPR. To investigate this hypothesis, two yeast strains carrying null alleles of *PTC2* were constructed. In AWY446, a kan^r gene was inserted into the unique *Tth111*I site in the 5′ end of the *PTC2* coding region. In the other null mutant, AWY506, the open reading frame was disrupted by replacing the 935-bp *Xba*I-*Nru*I fragment with a *LEU2* gene. This construction removed amino acid residues 42 to 353 of *PTC2*. PCR and Northern blot analyses confirmed that these mutants contained the disrupted *PTC2* gene (data not shown). Both null mutants were viable, indicating that *PTC2* is not an essential gene for cell viability. In rich liquid medium, Δ*ptc2* cells exhibited growth rates identical to those of wild-type and Δ*ire1* cells (Fig. 6A). Under the same growth conditions, a low concentration of tunicamycin, a drug that inhibits N-linked glycosylation and causes the accumulation of unfolded proteins in the ER, severely impaired the growth of the Δ*ire1* cells (Fig. 6A). In contrast, tunicamycin did not significantly affect the growth of either wild-type or Δ*ptc2* cells, and the growth curves for these two strains were indistinguishable. These observations suggest that Δ*ptc2* cells have no apparent growth defects and are capable of effectively protecting themselves from the lethal

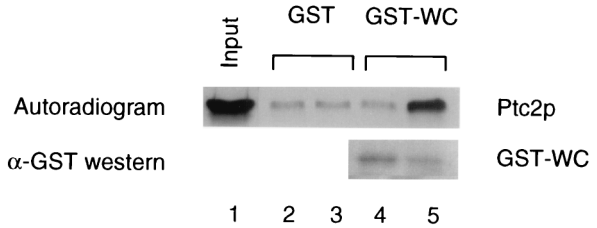


FIG. 2. Direct physical association of Ire1p and Ptc2p in vitro. Aliquots of an in vitro translation reaction mixture containing ^{35}S-labeled Ptc2p were incubated with glutathione-Sepharose beads impregnated with either phosphorylated (lane 5) or nonphosphorylated (lane 4) GST-WC. As a control, beads coated with GST alone and either with (lane 3) or without (lane 2) prior incubation in in vitro kinase buffer (41) were used to adsorb Ptc2p. Beads were collected, washed, and boiled to release the bound proteins. Equal proportions of the samples were analyzed by SDS-PAGE, Western blotting, and fluorography. A portion (8.3%) of the Ptc2p input is shown in lane 1.

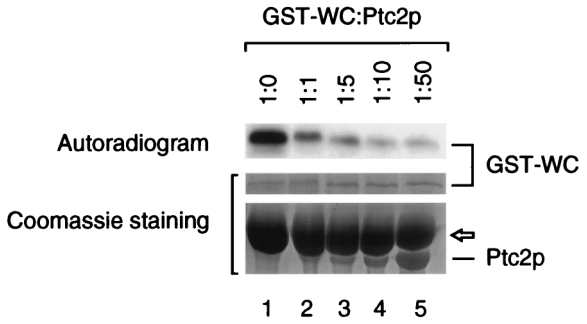


FIG. 3. Dephosphorylation of Ire1p by Ptc2p. A fixed amount of ^{32}P-labeled GST-WC (1 μg) was incubated with increasing amounts of Ptc2p in dephosphorylation buffer (21). Samples were boiled, and equal portions were analyzed by SDS-PAGE and autoradiography. The GST-WC/Ptc2p ratios used were determined by a protein assay and were confirmed by scanning of the Coomassie blue-stained gel with NIH Image. The migration of bovine serum albumin is indicated by an arrow.

consequences of the accumulation of unfolded proteins in the ER.

Overexpression of *PTC2* retards cell growth. To further elucidate the role of *PTC2* in yeast biology, T7 epitope-tagged Ptc2p was overexpressed with an inducible *GAL1* promoter. Overexpression of wild-type Ptc2p inhibited cell growth, whereas overexpression of both Ptc2p mutants did not affect the growth rate relative to that of wild-type cells (Fig. 6B). Since the levels of expression of the mutant and wild-type Ptc2p proteins were similar (Fig. 6C), this difference was not due to a difference in expression. These results support the conclusion that overproduction of the phosphatase is growth inhibitory due to excessive phosphatase activity. It is possible that Ptc2p is also involved in other signaling pathways that regulate cell growth, and its overproduction may therefore inhibit cell growth. The addition of tunicamycin significantly retarded the growth of the yeast cells overexpressing wild-type Ptc2p but did not significantly affect the growth of either wild-type cells or cells that overexpressed mutant Ptc2p, indicating that the UPR pathway is compromised upon Ptc2p overexpression, a phenotype common to Δ*ire1* cells.

The UPR pathway is sensitized in *ptc2* cells. In order to investigate whether null mutants of *PTC2* have an elevated UPR, yeast strains harboring a single integrated copy of a *lacZ* reporter gene that has an upstream 22-bp UPRE were constructed. The UPR was monitored by the levels of tunicamycin-inducible β-galactosidase expression in the cell extracts. In wild-type cells, the expression of β-galactosidase increased steadily with increasing concentrations of tunicamycin (Fig. 7A). Although both the deletion (*ptc2-Δ1::LEU2*) and the insertion (*ptc2-1::kan*^{r}) mutants showed similar dose-dependent levels of β-galactosidase expression, the levels of expression were approximately three- to fourfold higher than that of wild-type cells, indicating that cells devoid of Ptc2p were hypersensitive to unfolded proteins. These observations suggest that *PTC2* is a negative regulator of the UPR pathway.

Overexpression of wild-type Ptc2p but not the catalytically inactive mutants of Ptc2p downregulates the UPR. To study the functional effects of the catalytically inactive Ptc2p mutants on the UPR, T7 epitope-tagged versions of the catalytically inactive mutants were overexpressed in a wild-type strain that harbors a UPRE-LacZ reporter construct. In comparison with the vector control, yeast cells that overexpressed Ptc2p displayed a reduction in tunicamycin-dependent β-galactosidase

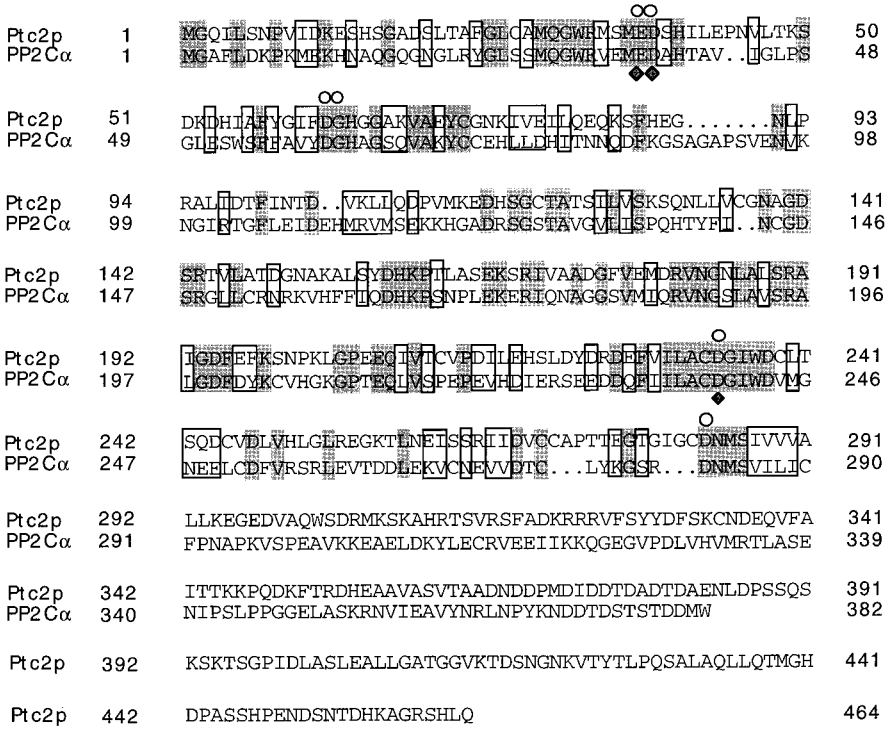


FIG. 4. Sequence alignment of the catalytic domains of human PP2Cα and Ptc2p. The GenBank accession numbers for the PP2Cα gene and *PTC2* nucleotide sequences are S87759 and U18839, respectively. Invariant residues are shaded in gray. Conserved residues are boxed. Residues that coordinate metal ions are indicated by circles. Residues that were mutated in this study are denoted by diamonds. The alignment was made with the Wisconsin sequence analysis package (Genetics Computer Group Inc., Madison, Wis.).

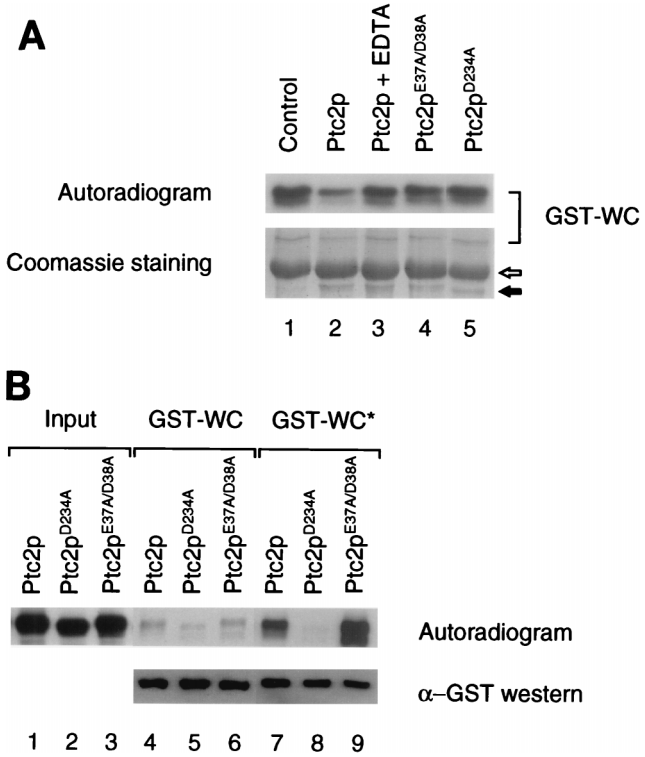


FIG. 5. Effect of mutations at the predicted Mg^{2+} binding sites on the Ire1p interaction and phosphatase activity. (A) In vitro dephosphorylation assay. Equal amounts (1 μg) of ^{32}P-labeled GST-WC were incubated with dephosphorylation buffer (control, lane 1), Ptc2p (1 μg, lane 2), Ptc2p (1 μg) with EDTA (lane 3), Ptc2p$^{E37A/D38A}$ (1 μg, lane 4), or Ptc2p^{D234A} (1 μg, lane 5). Samples were boiled, and equal portions were analyzed by SDS-PAGE Coomassie staining, and autoradiography. The migration of the wild-type and mutant phosphatases is indicated by a solid arrow. The open arrow indicates the migration of bovine serum albumin. (B) In vitro binding assay. Beads impregnated with equal amounts of either nonphosphorylated (lanes 4 to 6) or phosphorylated (lanes 7 to 9) GST-WC were tested for interaction with equal amounts of in vitro-translated Ptc2p (lanes 4 and 7), Ptc2p^{D234A} (lanes 5 and 8), or Ptc2p$^{E37A/D38A}$ (lanes 6 and 9). Bound proteins were analyzed by SDS-PAGE, Western blotting, and fluorography. Ten percent of the Ptc2p input is shown in lanes 1 to 3. The phosphorylated form of GST-WC is indicated by an asterisk.

expression (Fig. 7B). Although this difference was observed throughout the entire range of concentrations, it was more pronounced at lower doses (Fig. 7C). In contrast, yeast cells that overexpress either Ptc2p^{D234A} (the single mutant that did not interact with Ire1p) or Ptc2p$^{E37A/D38A}$ (the double mutant that could interact with Ire1p) displayed increased and tunicamycin-inducible β-galactosidase activity (Fig. 7B). These results are consistent with the notion that *PTC2* is a negative regulator of the UPR pathway and that the phosphatase activity of Ptc2p is essential for the regulation of the UPR.

***PTC2* is required for regulated *HAC1* mRNA splicing.** Since activation of the UPR pathway is regulated by Ire1p-dependent *HAC1* mRNA splicing and subsequent generation of Hac1p (8, 16), we questioned whether *PTC2* plays a role in *HAC1* mRNA splicing. To investigate this possibility, splicing of *HAC1* mRNA was monitored by an RNase protection assay. In this assay, detection of an RNase-resistant 147-nucleotide fragment is diagnostic of cleavage of the 3′ splice site of *HAC1* mRNA. In agreement with Cox and Walter (8), cleaved *HAC1* mRNA was detected in a tunicamycin-dependent fashion in wild-type control cells (Fig. 8, lanes 1 and 2) but not in Δ*ire1* cells (lanes 3 and 4). Interestingly, Δ*ptc2* cells displayed a 64% increase in tunicamycin-dependent cleavage of *HAC1* mRNA (Fig. 8, lane 6), and tunicamycin-dependent cleavage of *HAC1* mRNA was reduced to 57% in cells overexpressing wild-type Ptc2p (Fig. 8, lane 8). In addition, cleaved *HAC1* mRNA was detected in Δ*ptc2* cells as well as in cells overexpressing either Ptc2p^{D234A} or Ptc2p$^{E37A/D38A}$ in the absence of tunicamycin (Fig. 8, lanes 5, 9, and 11), indicating that *HAC1* mRNA processing is deregulated in these cells. These results confirm the role of *PTC2* as a negative regulator of the UPR pathway and, more importantly, demonstrate that *PTC2* acts upstream of the *HAC1* mRNA splicing event.

DISCUSSION

Ptc2p is a novel phosphatase that binds to and dephosphorylates Ire1p. In the budding yeast *S. cerevisiae*, the Ire1p kinase is activated in response to unfolded protein in the ER to transmit a signal that induces the transcription of genes encoding ER-resident protein chaperones. We have identified a

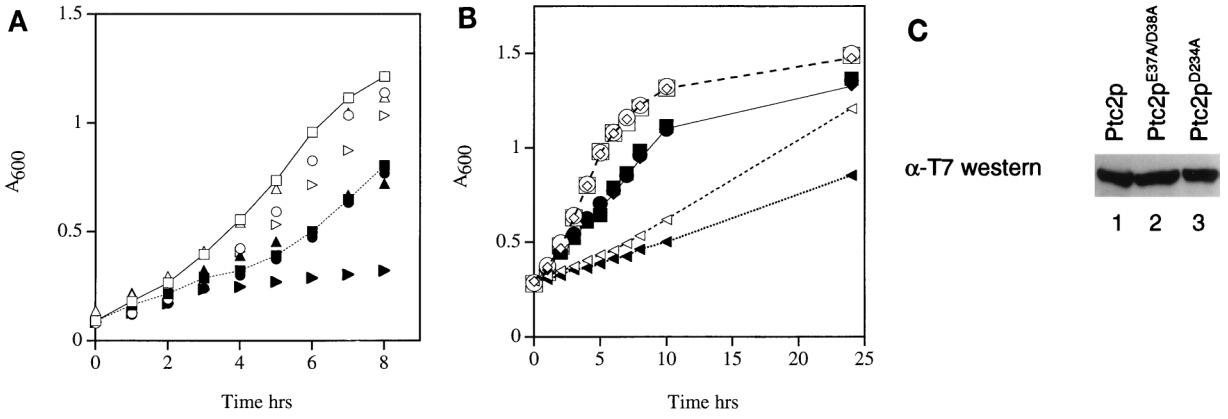


FIG. 6. Effect of *PTC2* on the growth of *S. cerevisiae* in the presence (closed symbols) or absence (open symbols) of tunicamycin. (A) *PTC2* deletion does not affect the growth rate. AWY446 (*ptc2-1::kan*^{r}) (triangles), AWY500 (*PTC2*) (squares), AWY506 (*ptc2-Δ1::LEU2*) (circles), and AWY 516 (*ire1Δ1*) (arrowheads) were grown in 1% yeast extract–2% peptone–2% dextrose (YPD) medium to an A_{600} of 0.1, and the cultures were divided into flasks. Tunicamycin (final concentration, 0.25 μg/ml) was added to one set of flasks, and incubation was continued at 30°C. Aliquots were removed at the indicated times, and the A_{600} was measured. (B) Ptc2p overproduction inhibits cell growth. AWY500 harboring *PTC2* (arrowheads), *ptc2*^{D234A} (circles), or *ptc2*$^{E37A/D38A}$ (squares) in a 2μm vector, pYES2 (diamonds), was grown in Ura$^-$ medium containing 2% galactose and 1% raffinose for 10 h. Cultures were divided and treated as in panel A. (C) Wild-type Ptc2p and single and double Ptc2p mutants are expressed equally in *S. cerevisiae*. AWY500 harboring *PTC2* (lane 1), *ptc2*$^{E37A/D38A}$ (lane 2), and *ptc2*^{D234A} (lane 3) in the pYES2 vector was grown in Ura$^-$ medium containing 2% galactose and 1% raffinose for 10 h. Cells were harvested and lysed, and the extracts were analyzed by Western blotting with anti-T7 antibody.

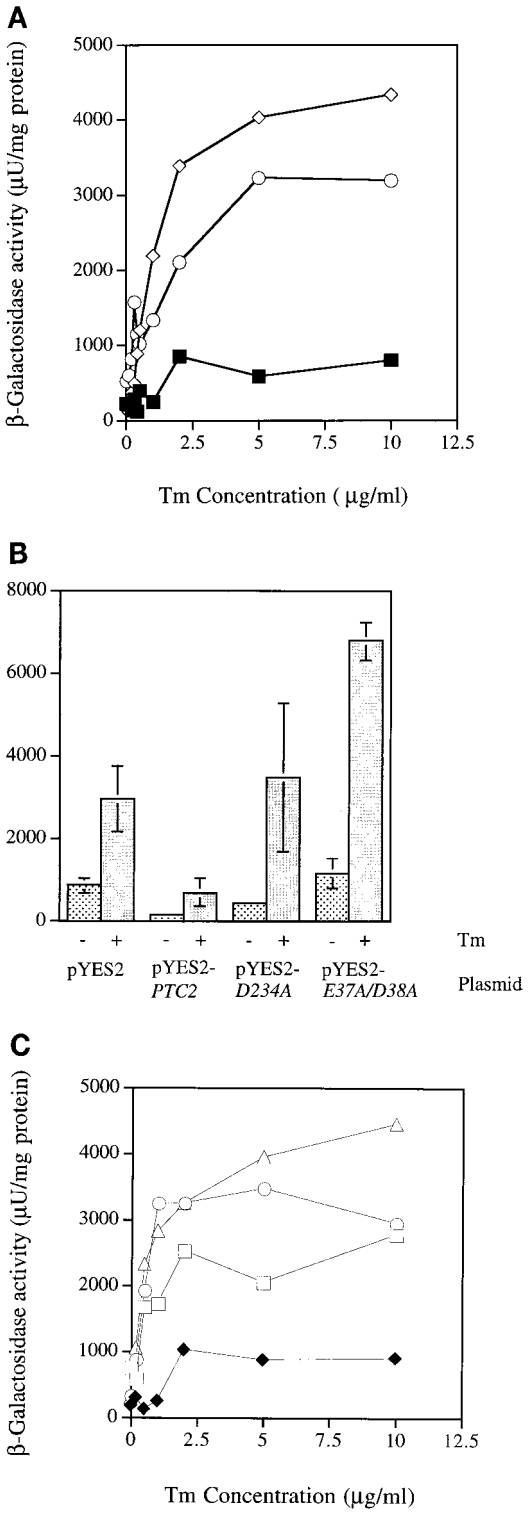


FIG. 7. *PTC2* downregulates the UPR. (A) Null mutants of *PTC2* exhibit an elevated UPR. Cultures of strains AWY446 (*ptc2-1::kan*^{r}) diamonds, AWY500 (*PTC2*) (squares), and AWY506 (*ptc2-Δ1::LEU2*) (circles) were grown in YPD to the early log phase and divided, and tunicamycin (Tm) was added to the final concentrations indicated. After 90 min of incubation at 30°C, cells were harvested and lysed and β-galactosidase activity was measured. (B) Overexpression of wild-type Ptc2p and catalytically inactive Ptc2p deregulates the UPR. Strains expressing either wild-type or mutant Ptc2p were grown in Ura$^-$ medium containing 2% galactose and 1% raffinose for 10 h to the early log phase. Cultures were divided, Tm was added to a final concentration of 2 μg/ml, and incubated was continued for 90 min. Cells were harvested and lysed, and β-galactosidase activity was measured. Specific β-galactosidase activity represents an average of three independent experiments. Bars indicate standard deviations. (C) Tm dose dependence of the UPR in wild-type cells overexpressing wild-type or mutant Ptc2p. The UPR was monitored as described in panel B, except that different concentrations of Tm were used for induction. Symbols: squares, pYES2 vector; diamonds, pYES2 carrying *PTC2*; circles, pYES2 carrying the D234A allele; triangles, pYES2 carrying the E37A/D38A allele.

novel serine/threonine phosphatase, Ptc2p, as a specific interactor of Ire1p by interaction-trap (two-hybrid) screening. This is the first demonstration of a protein serine/threonine phosphatase as a component of the signal transduction pathway from the ER to the nucleus. Several genetic and physical methods demonstrated that the association between Ptc2p and Ire1p was specific and selective. First, the yeast two-hybrid assay demonstrated that Ptc2p interacted with Ire1p but not with Cdc28p, a kinase that shows the highest homology to Ire1p. Second, in vitro physical assays demonstrated that Ptc2p interacted with phosphorylated Ire1p but not with nonphosphorylated Ire1p. Third, a single amino acid mutation in the Ire1p interaction domain of Ptc2p disrupted the interaction between the two proteins in vitro. On the basis of the in vitro interaction between the bacterially expressed Ire1p and the in vitro-translated Ptc2p, we suggest that the interaction is direct and specific and depends on the phosphorylation status of Ire1p.

A typical PP2C enzyme requires Mg^{2+} or Mn^{2+} for substrate binding (21). However, the Ptc2p-Ire1p interaction differs from a typical PP2C-substrate interaction because the interaction is Mg^{2+} and Mn^{2+} independent. This finding is not completely unprecedented, as the interaction between a PP2C enzyme in *Caenorhabditis elegans*, FEM-2, and its interaction partner, FEM-3, is also Mg^{2+} and Mn^{2+} independent (6). Our data support the idea that the Ptc2p-Ire1p interaction is mediated through a kinase interaction domain mapped within aa 174 to 355 of Ptc2p (Fig. 9A). A similar kinase interaction domain has been described for a PP2C-like enzyme, KAPP (kinase-associated protein phosphatase), in *Arabidopsis thaliana* (38). Like the Ptc2p-Ire1p interaction, the interaction between KAPP and the RLK5 kinase is phosphorylation dependent. However, there is no significant homology between these two kinase interaction domains. It is not clear how Ptc2p discriminates between the phosphorylated and nonphosphorylated forms of Ire1p for interaction. It is possible that a phosphorylation-induced change in the secondary structure of Ire1p creates a Ptc2p binding site that mediates the interaction. On the other hand, phosphoserine or threonine(s) in Ire1p may act as a docking site(s) for Ptc2p. In fact, Muslin et al. (26) recently demonstrated that the interaction between the 14-3-3 family of proteins and their interacting partners is mediated by the recognition of phosphoserine.

We have established that Ptc2p is a genuine serine/threonine phosphatase and that Ire1p is the target for the Ptc2p phosphatase. This is the first description of a physiological substrate for the PP2C class of enzymes. As revealed by the crystal structure, the catalytic domain of human PP2Cα contains a binuclear metal ion center that is coordinated by four invariant aspartate residues and a semiconserved glutamate residue. It is proposed that in PP2C-like enzymes, the metal ion-activated water molecules act as nucleophiles and acids to catalyze dephosphorylation. Our data demonstrated that the substitution of aspartate and glutamate residues in Ptc2p at positions equivalent to those of the metal ion-coordinating aspartate residues in the human counterpart destroyed catalytic activity, support-

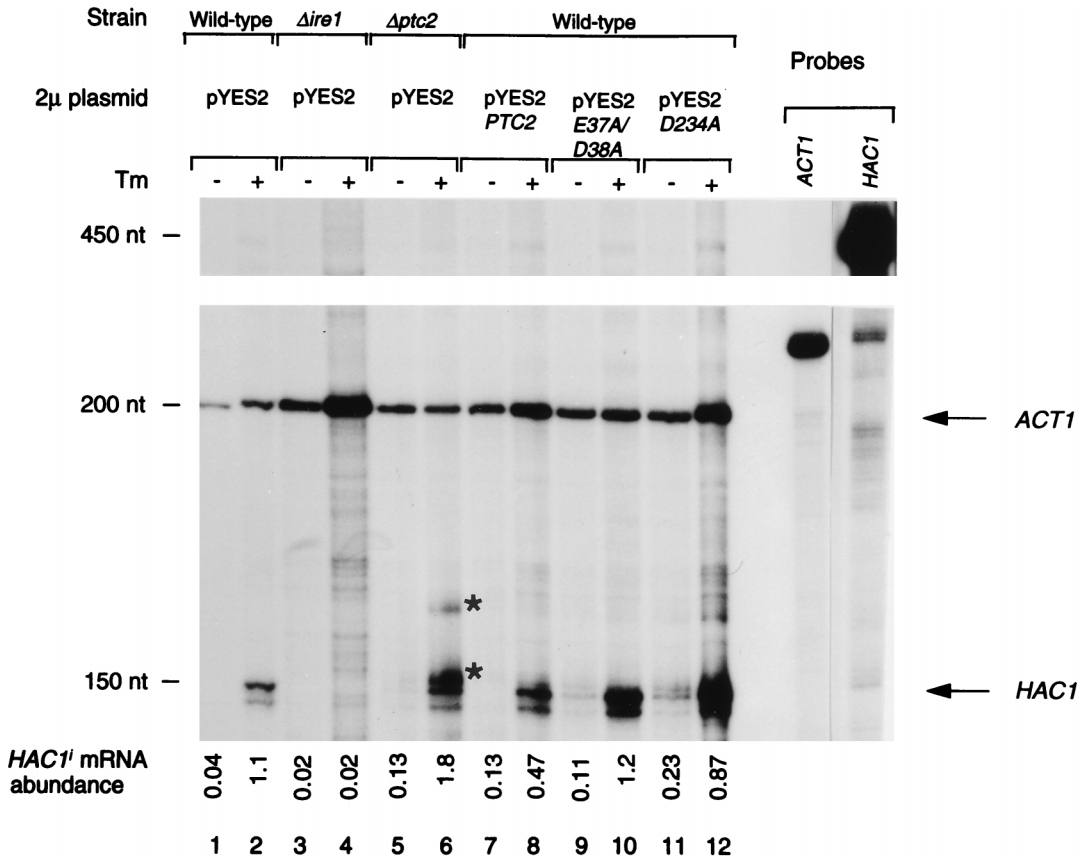


FIG. 8. *PTC2* regulates *HAC1* mRNA splicing. Cultures of strains AWY500, AWY506, and AWY500 overexpressing either wild-type Ptc2p or Ptc2p mutants were grown in synthetic complete medium with or without uracil to the early log phase, divided, and induced with tunicamycin (Tm) (2 μg/ml) for 90 min. Cells were harvested, RNA was isolated, and cleavage of *HAC1* mRNA was assayed by an RNase protection assay with a probe that is colinear with the *S. cerevisiae HAC1* gene. The 147-nucleotide (nt) fragment represents the 3′ portion of (*HAC1*) mRNA that extends to the 3′ cleavage site. The 74-nucleotide fragment derived from the 5′ side of the spliced *HAC1* mRNA is not shown in this analysis. The abundance of spliced *HAC1* mRNA *HAC1*^{i} relative to *ACT1* mRNA is indicated. Products of higher molecular weights may represent intermediates in the splicing reaction that are observed only in Δ*ptc2* cells (asterisks). 2μ, 2μm.

ing the requirement for a binuclear metal ion center in the proposed catalytic mechanism for the PP2C family of enzymes.

Ptc2p negatively regulates the UPR pathway upstream of *HAC1* mRNA splicing. An increasing body of evidence suggests that the PP2C-like enzymes play multiple roles in regulating a number of signal transduction pathways. In mammals, PP2C is implicated in Ca^{2+}-related signaling in the brain (11). In *C. elegans*, the PP2C enzyme FEM-2 is required to promote male development (6). The *ABI1* gene product of *A. thaliana*, which features an EF hand (two alpha helices) Ca^{2+}-binding site at the amino terminus and a PP2C domain at the carboxy terminus, is required for abscisic acid-mediated responses, such as stomatal closure (18), the maintenance of seed dormancy (18, 22), and the inhibition of plant growth (22). The *S. cerevisiae* genome encodes six PP2C-like enzymes (37) whose physiological functions are unknown, with the exception of Ptc1p and Ptc2p. Ptc1p downregulates the osmosensing signal transduction pathway (20) and is required for tRNA splicing (27). In *Schizosaccharomyces pombe*, the Ptc1p counterpart is encoded by *ptc1*$^{+}$ and is important for survival of heat shock as well as for osmoregulation (32). *S. cerevisiae* Ptc2p is most homologous to Ptc3p of *S. pombe*, and both proteins are implicated in osmosensing signal transduction pathways (19, 20, 33, 34). In this report, we have described the role of Ptc2p in the UPR, a hitherto-unknown function for this enzyme.

The UPR pathway is required for cell survival under conditions of ER stress (7). Since null mutants of *PTC2* grow in the presence of tunicamycin, a condition that requires induction of the UPR, *PTC2* is not a positive regulator of the UPR. Instead, the evidence presented here suggests that *PTC2* is a negative regulator of the UPR. In comparison to wild-type cells, *ptc2* null mutant cells had a three- to fourfold-increased UPR, and this response correlates with increased levels of spliced *HAC1* mRNA, a phenotype qualitatively similar to that of Ire1p-overexpressing cells (8). In addition, overexpression of wild-type Ptc2p but not the catalytically inactive Ptc2p mutants reduced the UPR, with coincident reduced levels of *HAC1* mRNA, consistent with inactivation of signaling from Ire1p. Overexpression of the catalytically inactive Ptc2p mutants actually elevated the UPR and increased the levels of spliced *HAC1* mRNA, possibly by competition for interactions with critical regulatory components of the UPR pathway. Expression of the catalytically inactive mutant that was capable of binding to Ire1p, Ptc2p$^{E37A/D38A}$, caused the greatest increase in the UPR, suggesting that the interaction of inactive Ptc2p with Ire1p prevents turning off of the UPR. The results of these experiments are consistent with a requirement for Ptc2p in preventing signaling from Ire1p.

Since permanent activation of the UPR pathway is detrimental to cell growth, the UPR pathway must be negatively regulated (8). This fact may explain in part the slow growth of Ptc2p-overexpressing cells. Although *ptc2* mutant cells exhib-

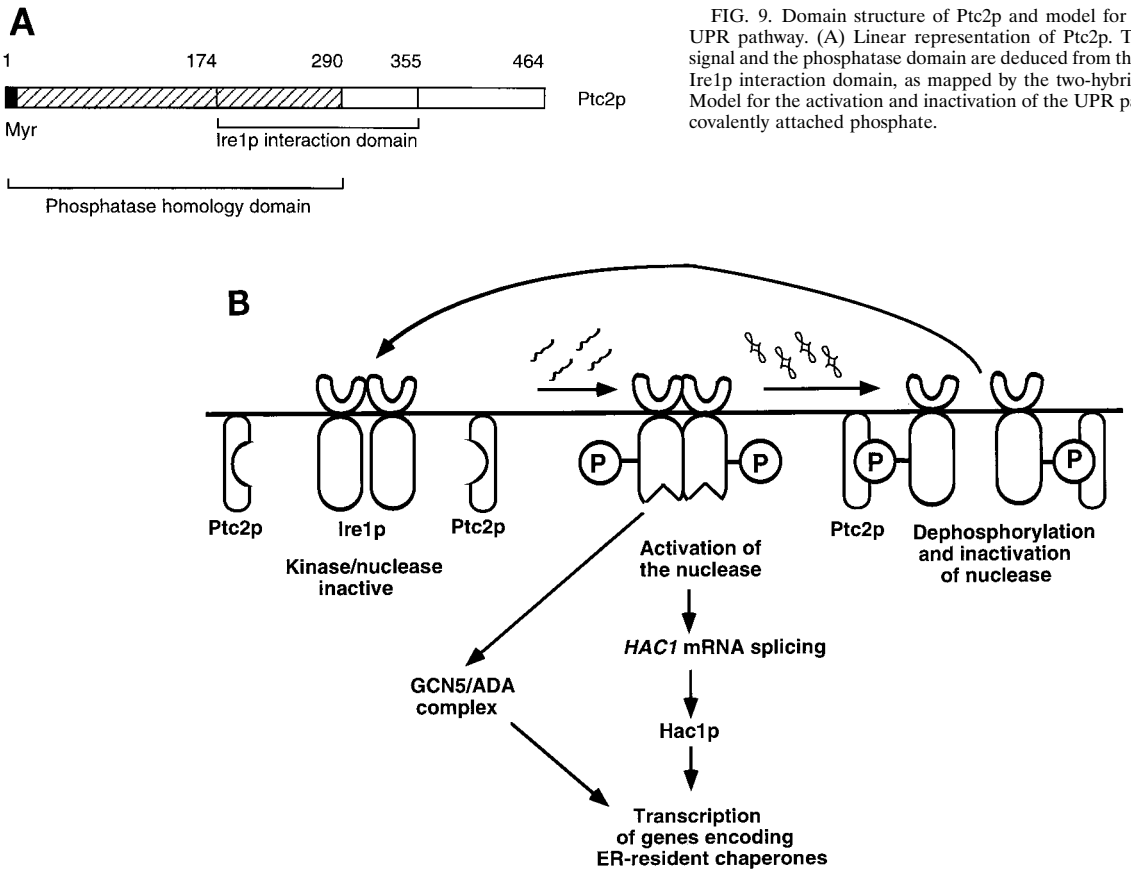


FIG. 9. Domain structure of Ptc2p and model for the role of Ptc2p in the UPR pathway. (A) Linear representation of Ptc2p. The myristoylation (Myr) signal and the phosphatase domain are deduced from the sequence of *PTC2*. The Ire1p interaction domain, as mapped by the two-hybrid analysis, is shown. (B) Model for the activation and inactivation of the UPR pathway in *S. cerevisiae*. P, covalently attached phosphate.

ited an elevated UPR, the pathway was not completely deregulated, as their growth rates in either the absence or the presence of tunicamycin were identical to that of wild-type cells. Perhaps, like the *S. pombe* PP2C enzymes (33, 34), the *S. cerevisiae* counterparts may have overlapping functions. It is also possible that a completely unrelated serine/threonine phosphatase(s) could substitute for Ptc2p. Such partial or complete redundancy is not uncommon in phosphorylation- or dephosphorylation-mediated signal transduction (19).

The results presented here reveal that Ptc2p plays a novel regulatory role upstream of *HAC1* mRNA splicing in the UPR pathway. Important issues that remain concern the mechanisms that regulate Ptc2p activity. Since *PTC2* transcription is not induced in response to stress in the ER (data not shown), the expression of Ptc2p is not regulated at the transcriptional level through Ire1p. It is not known whether Ptc2p is regulated at posttranscriptional levels that may include translation and/or a mechanism of activation or inactivation. A common regulatory mechanism controlling protein phosphorylation is the compartmentalization of the kinases and the counteracting phosphatases to allow maximum efficiency and specificity of the signaling events. Compartmentalization is achieved through a targeting moiety that directs an enzyme to a preferred site. In contrast to PP1, PP2A, and PP2B serine/threonine phosphatases, which utilize targeting subunits for localization (for a review, see reference 10), Ptc2p has a kinase interaction domain that directs it to Ire1p. In addition, Ptc2p contains a potential myristic acid lipid anchor at the amino terminus (Fig. 9A) that may facilitate the association of Ptc2p with membranes and that may further enhance targeting to Ire1p. The targeting of Ptc2p to phosphorylated Ire1p could provide a major regulatory event that controls Ire1p activity in the UPR pathway.

Reversible protein phosphorylation is the underlying theme in the regulation of protein function in a variety of signaling pathways leading to diverse biological responses. This process is accomplished by the opposing actions of protein kinases and phosphatases. In yeast, upon accumulation of unfolded proteins in the lumen of the ER, the transmembrane Ire1p kinase undergoes oligomerization and *trans*-autophosphorylation at serine and threonine residues to initiate the UPR. Since Ire1p kinase activity is required to induce *HAC1* mRNA splicing, we propose that activation of the UPR generates phosphorylated Ire1p, which directly induces the cleavage of *HAC1* mRNA (35). Subsequently, the tRNA ligase, Rlg1p, ligates the spliced products to complete the splicing reaction. Spliced *HAC1* mRNA serves as a template for the synthesis of Hac1p, which binds to the UPRE and induces transcription of the genes encoding ER-resident chaperones. Since permanent induction of the UPR is detrimental to cell growth (8), Ire1p must be inactivated so that the UPR pathway is downregulated upon removal of the stimulus. The data presented here support the idea that Ptc2p dephosphorylates Ire1p kinase to inactivate endonuclease activity and downregulate the UPR pathway. Our current model for this regulation is shown in Fig. 9B. When Ire1p is phosphorylated, it oligomerizes and its endonuclease activity is activated, and Ptc2p is recruited to the receptor kinase via its kinase interaction domain. The interaction results in the dephosphorylation and depolymerization of Ire1p and the subsequent inactivation of *HAC1* mRNA splicing. Dephosphorylation leads to the dissociation of Ptc2p from Ire1p and prevents further signaling from Ire1p. Since the

general features of the UPR pathway are conserved among eukaryotes, it will be interesting to determine the role of PP2C in the UPR of higher eukaryotes.

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