Essential role of calcineurin in response to endoplasmic reticulum stress

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Depletion of calcium ions (Ca\textsuperscript{2+}) from the endoplasmic reticulum (ER) of yeast cells resulted in the activation of the unfolded protein response (UPR) signaling pathway involving Ire1p and Hac1p. The depleted ER also stimulated Ca\textsuperscript{2+} influx at the plasma membrane through the Cch1p–Mid1p Ca\textsuperscript{2+} channel and another system. Surprisingly, both Ca\textsuperscript{2+} influx systems were stimulated upon accumulation of misfolded proteins in the ER even in the presence of Ca\textsuperscript{2+}. The ability of misfolded ER proteins to stimulate Ca\textsuperscript{2+} influx at the plasma membrane did not require Ire1p or Hac1p, and Ca\textsuperscript{2+} influx and signaling factors were not required for initial UPR signaling. However, activation of the Ca\textsuperscript{2+} channel, calmodulin, calcineurin and other factors was necessary for long-term survival of cells undergoing ER stress. A similar calcium cell survival (CCS) pathway operates in the pathogenic fungi and promotes resistance toazole antifungal drugs. These findings reveal an unanticipated new regulatory mechanism that couples ER stress to Ca\textsuperscript{2+} influx and signaling pathways, which help to prevent cell death and promote resistance to an important class of fungistatic drugs.

**Keywords:** Candida albicans/capacitative calcium entry/cell death/ER stress/Saccharomyces cerevisiae

**Introduction**

The endoplasmic reticulum (ER) of mammalian cells accumulates high levels of Ca\textsuperscript{2+} from the cytoplasm through the action of high-affinity Ca\textsuperscript{2+} pumps of the sarco-endoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA) family (Moller et al., 1996; MacLennan et al., 1997). This pool of Ca\textsuperscript{2+} is important for the translocation, folding, glycosylation, disulfide bonding and sorting of secretory proteins in the ER through its direct interactions with a variety of ER-resident molecular chaperones and enzymes (Meldolesi and Pozzan, 1998). Agents that cause sustained depletion of Ca\textsuperscript{2+} from the ER, such as Ca\textsuperscript{2+} ionophores and chelators, inhibitors of SERCA pumps and activators of Ca\textsuperscript{2+} release channels, can all affect the efficiency of protein folding in the ER and thereby stimulate the unfolded protein response (UPR) signaling pathways (Lamarche et al., 1985; Drummond et al., 1987; Price et al., 1992; Gill et al., 1996). UPR signaling in mammalian cells involves the transmembrane protein kinases Ire1\textalpha{}, Ire1\textbeta{} and PERK, which dimerize and become activated in response to the accumulation of unfolded proteins in the ER (Shi et al., 1998; Tirasophon et al., 1998; Wang et al., 1998; Harding et al., 1999). The activated Ire1\textalpha{} and Ire1\textbeta{} kinases induce the transcription of the genes encoding ER-resident molecular chaperones and inhibit the translation of mRNAs encoding secretory proteins (Harding et al., 1999; Kaufman, 1999). Therefore, activation of UPR signaling pathways serves to enhance ER functioning on demand.

A second response to depletion of Ca\textsuperscript{2+} from the ER is the stimulation of Ca\textsuperscript{2+} influx through the plasma membrane, a process known as store-operated or capacitative Ca\textsuperscript{2+} entry (CCE) (Putney, 1986, 1990). This process serves to replenish the Ca\textsuperscript{2+}-depleted organelles and to activate calcium signaling pathways. The molecular mechanisms by which plasma membrane Ca\textsuperscript{2+} channels become activated upon depletion of Ca\textsuperscript{2+} from the ER are not yet clear.

Recent evidence indicates that yeast may employ a CCE-like mechanism that helps to replenish secretory organelles with Ca\textsuperscript{2+} and to activate calcium signaling pathways (Locke et al., 2000). Yeast cells lack homologs of SERCA-type Ca\textsuperscript{2+} pumps, but express Pmr1p, a homolog of mammalian secretory pathway Ca\textsuperscript{2+} ATPases (SPCAs) (Rudolph et al., 1989; Antebi and Fink, 1992; Strayle et al., 1999). Pmr1p localizes to the Golgi complex and is important for supplying the Ca\textsuperscript{2+} and Mn\textsuperscript{2+} required for efficient glycosylation, processing and sorting reactions in this compartment (Duhr et al., 1998). Like mammalian cells treated with inhibitors of SERCA, pmr1 null mutants exhibit high rates of Ca\textsuperscript{2+} influx via a high-affinity Ca\textsuperscript{2+} channel composed of Cch1p and Mid1p (Locke et al., 2000). Cch1p is homologous to the catalytic subunit of voltage-gated Ca\textsuperscript{2+} channels in animals and, together with a putative regulatory subunit Mid1p, forms a Ca\textsuperscript{2+} influx pathway that can also be stimulated during the mating response (Iida et al., 1994; Fischer et al., 1997; Paidhungat and Garrett, 1997; Muller et al., 2001). The elevated Ca\textsuperscript{2+} influx in pmr1 mutants activates the calcineurin-dependent transcription factor Tcn1p (Matheos et al., 1997; Stathopoulos and Cyert, 1997; Mendizabal et al., 1998), which induces Pmc1p, a homolog of plasma membrane Ca\textsuperscript{2+} ATPases (PMCA) localized to the vacuole (Cunningham and Fink, 1994, 1996; Halachmi and Elam, 1996; Marchi et al., 1999). All these phenotypes of pmr1 mutants can be suppressed by expression of mammalian SERCA1a (Degand et al., 1999; Locke et al., 2000). Together, these findings indicate that Pmr1p is the functional equivalent of mammalian SERCA and that the Cch1p–Mid1p Ca\textsuperscript{2+} channel can be activated by a CCE-like mechanism.

Roles for Ca\textsuperscript{2+} in the yeast ER have been difficult to elucidate. The yeast calnexin homolog appears to function...
well without Ca\(^{2+}\) (Parlati et al., 1995). Additionally, free Ca\(^{2+}\) in the lumen of the yeast ER is 10–100 times lower than that of mammalian ER (Strayle et al., 1999). Mutants lacking Pmr1p accumulated half as much Ca\(^{2+}\) in the ER, but this decrease failed to stimulate UPR signaling (Dürr et al., 1998), indicating that high luminal Ca\(^{2+}\) may not be required for protein folding in the ER of yeast. Because SERCA homologs have been identified in animals, plants, protozoa and even other fungi (Burk et al., 1989), the loss of SERCA-like enzymes in the evolution of budding yeast may reflect decreased necessity for Ca\(^{2+}\) in ER folding reactions. Alternatively, other Ca\(^{2+}\) ATPases such as Pmr1p, Pmc1p or possibly the newly identified P-type ATPase Cod1p/Spf1p (Cronin et al., 2000) may have compensated for the ancient loss of SERCA in yeast.

Here, we re-examined the roles of Ca\(^{2+}\) in the yeast ER using more stringent conditions and confirm that Ca\(^{2+}\) accumulation in the ER can enhance protein folding there much as in mammalian cells. Surprisingly, inhibitors of either protein folding or ergosterol biosynthesis in the ER were sufficient to stimulate Ca\(^{2+}\) influx and/or activate calcium signaling pathways that were essential for cell survival. This calcium cell survival (CCS) pathway was also active in the pathogenic yeasts Candida albicans and Candida glabrata, where it provides resistance to common antifungal drugs. Thus, existing antifungal therapies might be improved by blocking the CCS pathway with drugs that inhibit either the mechanism linking ER stress to Ca\(^{2+}\) influx or the essential targets of calcineurin.

**Results**

**Activation of the UPR upon depletion of secretory Ca\(^{2+}\) stores**

Mutants lacking the secretory pathway Ca\(^{2+}\) ATPase Pmr1p exhibit only mild defects in the sorting and processing of secretory proteins and no significant activation of UPR signaling during growth in standard media (Dürr et al., 1998). However, such conditions permit increased Ca\(^{2+}\) influx from the medium along with up-regulation and mislocalization of the vacuolar Ca\(^{2+}\) ATPase Pmc1p (Marchi et al., 1999; Locke et al., 2000), which might partially replenish secretory Ca\(^{2+}\) and thus compensate for the loss of Pmr1p. To address this possibility, we measured expression of UPRE-lacZ, a reporter of UPR signaling, in *pmr1 pmc1* double mutants and in *pmr1* mutants grown in medium deficient in Ca\(^{2+}\). Under these conditions, UPRE-lacZ expression was induced in *pmr1* mutants and in *pmr1 pmc1* double mutants but not in wild-type yeast cells (Figure 1A). Induction of UPRE-lacZ in the mutants was suppressed by supplementing the medium with high concentrations of CaCl\(_2\) or MnCl\(_2\) (Figure 1A), by expression of rabbit SERCA1a, or by disruption of the UPR signaling factor Ire1p (data not shown). Thus, genetic depletion of Ca\(^{2+}\) and Mn\(^{2+}\) from the secretory pathway led to activation of UPR signaling.

Chelators of Ca\(^{2+}\), Mn\(^{2+}\) and other metal ions were also able to induce UPRE-lacZ expression in wild-type cells. Cells treated with either the membrane-impermeant chelator bis-(o-aminophenoxy)-ethane-N,N',N\(^\prime\),N\(^\prime\)-tetra-acetic acid (BAPTA) (Figure 1B) or the membrane-permeant chelator N,N',N\(^\prime\),N\(^\prime\)-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN) (Figure 1C) exhibited high levels of expression of UPRE-lacZ (Figure 1B). In both cases, UPRE-lacZ induction was partially suppressed by over-expression of Pmr1p (data not shown) and abolished in *ire1* mutants (Figure 1B and C). Taken together, these results provide evidence for a role of Ca\(^{2+}\) and the intracellular Ca\(^{2+}\) pump Pmr1p in preventing activation of
the UPR, presumably by promoting the activities of Ca^{2+}-dependent chaperones in the ER.

**TPEN treatment stimulates Ca^{2+} influx and signaling**

In cultured mammalian cells, TPEN treatment rapidly diminishes ER free Ca^{2+} concentration ([Ca^{2+}]_{er}) and activates the CCE pathway, which leads to elevation of cytosolic free Ca^{2+} concentration ([Ca^{2+}]_{c}) within 1 min of application (Hofer et al., 1998). We have previously reported evidence of a CCE-like pathway in yeast (Locke et al., 2000), but the use of genetic methods prevented a kinetic analysis of the CCE-like response. Therefore, we tested whether TPEN addition could stimulate Ca^{2+} influx and [Ca^{2+}]_{c} elevation in yeast on a time scale similar to that observed in mammalian cells. TPEN treatment stimulated 45Ca^{2+} accumulation in wild-type cells ~10-fold in the presence of the calcineurin inhibitor FK506 and ~2.5-fold in the absence of FK506 (Figure 2A). FK506 was used because it prevents calcineurin-dependent feedback inhibition of the Cch1p–Mid1p Ca^{2+} channel (Locke et al., 2000; Muller et al., 2001). Mutants lacking Cch1p exhibited a markedly diminished response to TPEN in the presence of FK506 (Figure 2A), indicating that TPEN may activate this Ca^{2+} channel in much the same way as a pmr1 mutation. Mutants lacking Ire1p or Hac1p exhibited a wild-type response to TPEN (Figure 2A and data not shown). Thus, TPEN appears to phenocopy pmr1 mutants and to trigger the CCE-like pathway in yeast, which is independent of UPR signaling.

In time course experiments, the effect of TPEN treatment on 45Ca^{2+} accumulation became apparent after a lag of at least 30 min and reached a maximum after ~150 min (Figure 2B). Similar kinetics were observed using cytoplasmic aequorin as a probe for [Ca^{2+}]_{c} (data not shown). Therefore, the Ca^{2+} influx observed in response to TPEN appears to be much slower in yeast relative to mammalian cells.

**Misfolded secretory proteins stimulate Ca^{2+} influx and signaling**

The relatively slow response of yeast cells to TPEN indicates that these cells may utilize a distinct mechanism for coupling Ca^{2+} influx to depletion of [Ca^{2+}]_{er}. To test whether this coupling involves the accumulation of misfolded proteins, we measured 45Ca^{2+} accumulation in cells treated with tunicamycin (TM), an inhibitor of N-glycosylation in the ER (Tkacz and Lampen, 1975; Lehle and Tanner, 1976) that causes accumulation of misfolded proteins and stimulation of UPR signaling. TM treatment significantly stimulated 45Ca^{2+} accumulation in wild-type cells and in cch1 mutants, mid1 mutants and cch1 mid1 double mutants (Figure 3A), suggesting the existence of a Ca^{2+} influx pathway that is independent of Cch1p–Mid1p. In the presence of FK506, TM stimulated even greater levels of 45Ca^{2+} accumulation, which was abolished in cch1 and mid1 mutants (Figure 3A). These effects of TM were completely independent of Ire1p and Hac1p (Figure 3B) but were clearly diminished in cells overexpressing Alg7p, the enzyme in the ER that is the direct target of TM (Figure 3C). The time course of Ca^{2+} accumulation in response to TM was similar to that of TPEN (data not shown). These findings indicate that specific inhibition of Alg7p and N-glycosylation in the ER can activate two Ca^{2+} influx systems through a mechanism that is independent of Ire1p and Hac1p. Identical results were obtained using dithiothreitol (DTT) as an inhibitor of disulfide bond formation in the ER and its target Ero1p (Figure 3B and data not shown). Thus, multiple inhibitors of protein folding or processing in the ER (e.g., TPEN, TM and DTT) all stimulated Ca^{2+} accumulation independently of a functional UPR signaling pathway.

To test whether accumulation of a misfolded protein in the ER is sufficient to stimulate Ca^{2+} responses, the effects of overexpressing a folding-incompetent mutant of carboxypeptidase Y termed CPY* (Hiller et al., 1996) were measured. Overexpression of CPY* in wild-type or ire1 mutants stimulated 45Ca^{2+} accumulation (data not shown) and [Ca^{2+}]_{c} elevation as assessed by monitoring expression of a calcineurin-dependent reporter gene CDRE-lacZ (Figure 3C). Thus, accumulation of a single misfolded protein species in the ER appeared to be sufficient to stimulate Ca^{2+} influx and to activate calcineurin signaling. These findings indicate the existence of a novel UPR-independent regulatory pathway emanating from the ER that can sense the accumulation of
Fig. 3. Accumulation of misfolded proteins in the ER stimulates Ca^{2+} influx and signaling independently of Ire1p. (A) 44Ca^{2+} accumulation in wild type, cch1 and mid1 mutants was measured as in Figure 2A except that TM (2.5 μg/ml) was used instead of TPEN. (B) 44Ca^{2+} accumulation in wild type, ire1 and hac1 mutants (strains K1257, K1259 and RGO5650) was measured as in (A) except that DTT (2.5 mM) was included as indicated. (C) 44Ca^{2+} accumulation in wild-type (open symbols) and ire1 mutant cells (closed symbols) that were overexpressing Alg7p, Ero1p or no additional proteins in the ER (plasmids pMK13XRV, pKT001 or vector control) was measured after treatment with varying amounts of TM in the presence of 2 μg/ml FK506. Complementary results were obtained using DTT instead of TM (data not shown). (D) Expression of the CDRE-lacZ reporter gene (plasmid pAMS366) was measured in wild-type cells and ire1 mutants expressing a folding-incompetent mutant of carboxypeptidase Y (denoted CPY*) under control of the galactose promoter (strains DNY1047 and DNY1048) after growth for 4 h in SC medium lacking uracil but supplemented with either 2% glucose or 2% galactose as indicated. Induction of CPY* in galactose medium stimulated CDRE-lacZ expression through a process that was sensitive to both 4 mM BAPTA and FK506 (data not shown). Wild-type cells lacking CPY* (strain K501) were included as a control. Bars represent the mean of three independent transformants (± SD).

misfolded proteins and stimulate Ca^{2+} influx at the plasma membrane as well as calcineurin signaling.

**Roles for Ca^{2+} influx and signaling during the response to unfolded protein accumulation**

What are the roles of Ca^{2+} during the response to ER stress? One possibility is that UPR signaling itself may depend on Ca^{2+} influx or signaling. Consistent with this possibility, UPRRE-lacZ induction was 5-fold lower in response to BAPTA relative to TPEN, the former of which prevents Ca^{2+} elevation (Figures 1 and 2). To determine whether UPR signaling might involve calcium signaling factors, several assays of UPR signaling were performed on a panel of yeast strains lacking either Ca^{2+}/calmodulin (cmd1-6 mutants), Ca^{2+}/calmodulin-dependent protein kinases (cnkl cnk2 double mutants), calcineurin (cnbl1 mutants), calcineurin-dependent transcription factor (ten1 mutants) or combinations thereof.

Early events in the UPR signaling pathway include the Ire1p-dependent splicing of the immature HAC1a pre-mRNA to the mature HAC1 mRNA, which is efficiently translated to promote accumulation of the Hac1p transcription factor (Sidrauski and Walter, 1997). Northern blot analysis of HAC1 transcripts in wild type, cnbl1 mutants, cnkl cnk2 and cnkl cnk2 cnbl1 triple mutants revealed no significant differences in either the kinetics or extent of TM-induced HAC1 mRNA splicing (Figure 4A and data not shown), although the basal levels of HAC1 transcription were slightly decreased. Western blot analysis of a functional epitope-tagged Hac1p–HA also revealed no significant differences between cmd1-6 mutants, cnkl cnk2 cnbl1 triple mutants and wild-type cells after treatment with TM (Figure 4B and data not shown). Finally, northern blot analysis of ERO1 transcripts, a target of Hac1p that is highly induced by UPR signaling (Frand and Kaiser, 1998), was performed in wild type, cmd1-6 mutants and cnkl cnk2 cnbl1 triple mutants at various times after treatment with TM. At early time-points, ERO1 expression was strongly induced in all these strains but ERO1 expression was not fully maintained in cmd1-6 mutants or cnkl cnk2 cnbl1 triple mutants at later time-points (Figure 4C and data not shown). The induction
of UPR-lucZ was markedly decreased in cmdl-6 mutants and cmk1 cmk2 cnbl triple mutants as measured after 4 h of TM treatment (Figure 4D). These results indicate that calcium signaling factors are not required for the initial stages of UPR signaling. However, calcium signaling factors may be important to sustain UPR signaling during long periods of TM treatment.

**Calcineurin signaling prevents cell death in TM**

Wild-type yeast cells treated with TM remain viable for extended periods of time but fail to proliferate, apparently due to reversible arrest in either the G1 or G2 phase of the cell division cycle (Klebl et al., 1984). Mutants deficient in Alg7p behaved similarly (Lennon et al., 1997). We therefore explored the possible role of calcium signaling factors in cell growth and cell death after TM treatment. In growth assays (measurement of culture turbidity by optical density at 600 nm), cmk1 cmk2 cnbl triple mutants were approximately four times more sensitive to TM than the wild-type strain (Figure 5A). However, in cell viability assays (exclusion of the vital dye methylene blue) wild-type cells remained almost completely viable after 6 h of treatment with TM whereas the cmk1 cmk2 cnbl triple mutant cells exhibited striking levels of cell death (Figure 5B). In time course experiments, ech1 and mid1 mutants died rapidly in response to TM (Figure 5C). Therefore, Ca\(^{2+}\) influx and signaling factors were required for survival of arrested cells during prolonged treatment with TM.

The relative contributions of individual calcium signaling factors were estimated by determining the time after TM treatment that resulted in death in 50% of various mutants. Wild-type cells and cmk1 cmk2 double mutants remained arrested but highly viable for at least 20 h of TM treatment but died rapidly if FK506 was also included (Figure 5D). In contrast, cmdl-6 mutants died rapidly with or without FK506. These findings indicate that cell survival requires activation of calcineurin by Ca\(^{2+}/\) calmodulin. Mutants lacking the calcineurin-dependent transcription factor Tcnlp behaved like wild-type cells in survival assays (Figure 5D), indicating that calcineurin promotes cell survival independently of Tcnlp. Interestingly, Irelp was not required for this role of calcineurin because ire1 mutants survived much longer without FK506 than with FK506 (Figure 5D). These findings demonstrate that calcium signaling factors such as Cchlp, Midlp, calmodulin and calcineurin promote long-term survival of cells treated with TM and that this role is not shared with UPR signaling factors. This CCS pathway has not been described previously.

**The CCS pathway is required for resistance to azole antifungal drugs and operates in pathogenic fungi**

To appreciate the range of conditions that stimulate the CCS pathway, we tested several additional inhibitors and mutations that affect ER function. Like TM, addition of DTT caused much more rapid cell death in mutants lacking the calcium signaling factors in comparison with wild type (data not shown). To inhibit protein folding without the use of inhibitors such as TM or DTT, we examined the survival of temperature-sensitive kar2 mutants in which the ER-localized Hsp70-family chaperone homologous to mammalian BiP could be conditionally inactivated. After a shift from 23°C to the semi-permissive temperature of 30°C, two different kar2-ts mutants rapidly arrested growth relative to wild type, but remained viable for at least 24 h (Figure 6A). Viability of the mutants was rapidly lost at the semi-permissive temperature if FK506 was included (Figure 6A). Thus, genetic deficiencies in the ER protein folding machinery appear to require calcium signaling pathways for cell survival.

To test whether the CCS pathway might operate in other fungi, cell survival assays were performed on pathogenic strains of *C. albicans* and *C. glabrata* treated with TM and FK506. In both of these species, treatment with TM plus
FK506 resulted in rapid cell death whereas treatment with TM alone arrested growth without causing cell death (Figure 6B). Treatment with FK506 alone had no effect on growth or death of these cells. Thus, the CCS pathway appears to operate in diverse fungi.

The cytotoxic effects of FK506 during TM treatment resemble the cytotoxic effects of FK506 observed in pathogenic strains of *C. albicans* that had been treated with azole-class antifungal drugs that inhibit ergosterol biosynthesis in the fungal ER (Marchetti *et al.*, 2000). To determine whether the CCS pathway can provide tolerance to azole-class antifungal drugs in *Saccharomyces*, we measured the survival of wild-type and *cmd1*-6 mutant cells after a 6 h treatment with various concentrations of miconazole (Figure 6C). Wild-type yeast cells arrested but remained highly viable at concentrations of miconazole up to 20 μM whereas *cmd1*-6 mutant cells arrested and died at concentrations >0.2 μM. Miconazole treatment stimulated 45Ca2+ accumulation in wild-type cells, but did not stimulate expression of UPR-lacZ (data not shown). These results indicate that the CCS pathway becomes activated in response to miconazole and promotes cell survival. The CCS pathway may therefore be an important determinant of cellular resistance to azole-class antifungal drugs.

**Discussion**

This report provides evidence that Ca2+ accumulation in the ER is important for folding or processing of secretory proteins in yeast, much as it is in other eukaryotes. Accumulation of unfolded proteins or inhibition of ergosterol synthesis in the ER of yeast was sufficient to stimulate Ca2+ influx via the Cch1p–Mid1p channel and other pathways, which promoted cell survival through the activation of calcineurin. The existence of this CCS pathway in fungi may provide new opportunities for treatment of fungal infections as well as a model system for studies of similar pathways in human cells.

**Roles of Ca2+ in the ER**

Until now, little evidence was available to support the notion that the ER of yeast employs Ca2+ for the folding or processing of secretory proteins. Previously it was shown that mutants lacking Pmr1p failed to retain a heterologous secretory protein in the ER (Rudolph *et al.*, 1989) and failed to degrade misfolded proteins in the ER, although UPR signaling was not elevated (Dürr *et al.*, 1998). Our findings indicate that high extracellular Ca2+ and consequent up-regulation of Pmc1p suppresses the loss of Pmr1p and thus obscures a role for Ca2+ in the modifica-
**Communication between the ER and plasma membrane**

Most mammalian cell types monitor Ca\(^{2+}\) levels in the ER and rapidly activate the CCE mechanism in response to even transient Ca\(^{2+}\) depletion (Putney, 1986). For example, TPEN treatment depletes [Ca\(^{2+}\)]\(_{er}\) very rapidly, and within seconds the depleted organelle triggers CCE at the plasma membrane and elevation of [Ca\(^{2+}\)]\(_{cyt}\) (Hofert et al., 1998). In yeast, TPEN stimulated Ca\(^{2+}\) entry and elevated [Ca\(^{2+}\)]\(_{cyt}\) (Figure 2A), but these effects required at least 1 h of treatment (Figure 2B). The different kinetics might reflect a lower permeability of yeast cells to TPEN, or possibly a distinct mechanism in yeast cells for regulating Ca\(^{2+}\) entry such as ER stress. Several other agents and conditions that stimulate UPR signaling in yeast, including TM, DTT, CPY*, kar-2-ts mutation and pmr1 mutation, also stimulated Ca\(^{2+}\) accumulation. Their effects on Ca\(^{2+}\) influx and signaling were completely independent of Ire1p and Hac1p, two crucial components of the UPR signaling pathway. Furthermore, an inhibitor of ergosterol biosynthesis in the ER also stimulated calcium signaling but did not activate UPR signaling (data not shown). These findings suggest that more general forms of ER stress can regulate Ca\(^{2+}\) entry in yeast. Though the signaling pathways employed to couple ER stress to Ca\(^{2+}\) influx remain to be identified, our results exclude the UPR signaling pathway.

All of the ER stress agents and conditions reported here appeared to stimulate two separate Ca\(^{2+}\) influx systems, one of which required Mid1p and Cch1p and the other remaining unidentified. Mid1p homologs are conserved in other fungi and are required for resistance to echinocandin-class drugs in *Schizosaccharomyces pombe* (Carnero et al., 2000; Tasaka et al., 2000). Cch1p is homologous to the catalytic subunit of voltage-gated Ca\(^{2+}\) channels (VGCCs) conserved in fungi and animals, but is most closely related to an uncharacterized family of four-repeat channels that is conserved in the genomes of nematodes, fruit flies and mammals (Lee et al., 1999). The possibility that ER stress activates these channels in mammalian cells has not been ruled out. In fact, ER stresses such as TM treatment or even Alzheimer’s disease mutations in presenilin-1 can affect CCE (Pahl and Baeuerle, 1996; Yoo et al., 2000) and can also affect the activity of Cch1p homologs that contribute to neuronal cell death and neurodegenerative disease (Paschen and Frandsen, 2001). Defining the signal transduction pathway that couples ER stress to Cch1p-Mid1p activation in yeast may therefore help to elucidate similar mechanisms in human cells.

**Roles for Ca\(^{2+}\) influx in response to ER stress**

Yeast mutants lacking Ca\(^{2+}\)/calmodulin (cmd1-6) or its effectors (cml1 cml2 cml1 triple mutants) appeared indistinguishable from wild-type cells in their ability to execute the early stages of UPR signaling (Figure 4). However, these mutants did not maintain long-term expression of target genes and largely failed to accumulate β-galactosidase activity expressed from the UPR-\(lacZ\) reporter gene. These defects might reflect a role for Ca\(^{2+}\) and calcium signaling factors at a late step in the UPR signaling pathway. Alternatively, the inability to maintain UPR signaling may be a consequence of the dramatically
accelerated cell death observed in the mutants during ER stress. Yeast strains lacking Cch1p, Mid1p, calmodulin or calcineurin functions died rapidly in response to ER stress agents whereas mutants lacking Tcn1p, the only known calcineurin-dependent transcription factor, remained fully viable for long periods of time, like wild-type cells (Figure 6). This Tcn1p-independent cell survival pathway appears to be distinct from the calcineurin-dependent growth of cells with cell wall defects (Mazur et al., 1995; Carnero et al., 2000), which requires Tcn1p (Matheos et al., 1997). Survival of yeast undergoing ER stress was also independent of Vcx1p and Hsl1p (M.Bonilla and K.W.Cunningham, unpublished results), two other suspected targets of calcineurin (Cunningham and Fink, 1996; Mizunuma et al., 2001). Because a similar pathway appears to operate in pathogenic fungi and promote resistance to the azole class of antifungal drugs (Figure 6; Del Poeta et al., 2000; Marchetti et al., 2000; Cruz et al., 2002), identification of the targets of calcineurin responsible for cell survival under these conditions may be useful in the development of new antifungal therapies.

Materials and methods

Yeast strains, plasmids and growth conditions
All yeast strains used in this study (Table I) were obtained from original sources or derived from the parental strain W303-1A (Wallis et al., 1989) or BY4741 (Brachmann et al., 1996) using standard molecular and genetic methods (Sambrook et al., 1989). The CMK1 and CMK2 genes were disrupted in wild-type strain K601 by transformation with fragments of plasmids pBKScmk1A1 and pBKScmk2A2 (Pausch et al., 1991) to yield strain K481 and a MATα derivative K523. The CNBl gene was disrupted in strain K523 as described (Cyr et al. and Thorner, 1992) to yield strain K488. Strains K1257 and K1259 (ire1::G418) were recovered after sporulation of strain RG21907 (Research Genetics, Inc.).

Overexpression of Ero1p and Alg7p in yeast was achieved by transformation with high-dosage plasmids pKT001 (2μ URA3 ERO1) (Pollard et al., 1998) and pMK13×RV (2μ LEU2 ALG7) (gift of Maria Kukuruzinska), respectively. Activation of the UPR signaling pathway was monitored in yeast transformed with plasmid pCZY1 (2μ URA3 UPRE-lacZ) (Mori et al., 1996). Activation of the calcium signaling pathway was monitored in yeast transformed with pAMS366 (2μ URA3 4×CDRE-lacZ) (Stathopoulos and Cyr, 1997). Plasmid pMB004, which expresses epitope-tagged Hap1p-HA, was constructed by inserting two repeats of the 96 bp triple-tandem HAP1 sequence (Field et al., 1988) into the SpeI site at position +25 in the HAP1 open reading frame of plasmid YCP-HAC1WT (Mori et al., 1996).

Yeast strains were grown in standard yeast peptone dextrose (YPD) medium, synthetic complete (SC) medium containing 2% glucose, galactose or raffinose, as described previously (Sherman et al., 1986), using reagents from Difco (Detroit, MI, USA) and Sigma Chemical Co. (St Louis, MO, USA). Calcium-free SD medium was made by replacing YNB with divergent cation-free yeast nitrogen base (Q-Biogene Inc.) and supplementing with the chloride salts of Cu²⁺, Fe²⁺, Mg²⁺ and Zn²⁺ (but not Mn²⁺ or Ca²⁺) to their standard concentrations. The Ca²⁺ chelators BAPTA and TPEN were obtained from Sigma, dissolved in water and dimethylsulfoxide (DMSO), respectively, and diluted into SC culture medium pH 6.5, supplemented with 50 mM MES–KOH, to final concentrations of 3 mM and 130 μM, respectively. TM (Sigma) was dissolved in methanol and diluted into medium to a final concentration of 2.5 μg/ml DTT (GibcoLife Technologies, Inc.) was dissolved in water and diluted into YPD medium to a final concentration of 2.5 mM. These concentrations were chosen based on their maximal stimulation of UPR-lacZ expression in strain W303-1A.

β-galactosidase assays
Yeast strains transformed with plasmids carrying either the UPRE-lacZ or CDRE-lacZ reporter gene were grown to log phase in SC media (shaking overnight at 30°C), harvested by centrifugation, then cultured in SC medium containing the indicated concentrations of chelators or inhibitors. After incubation at 30°C for 4 h, cells were harvested by centrifugation, permeabilized with chloroform and assayed for β-galactosidase activity using the colorimetric substrate ONPG as described previously (Gurante, 1983). The effects of CPY* overexpression in wild-type (DNY1047) or ire1 mutant (DNY1048) cells were monitored after cells were grown to log phase in SC medium supplemented with 2% raffinose, washed twice, then shifted to SC medium supplemented with 2% galactose or 2% glucose for 4 h.

Cd²⁺ accumulation measurements
Total cellular accumulation of Cd²⁺ was measured as described previously (Cunningham and Fink, 1996). Briefly, yeast cells were grown to log phase in YPD medium, harvested and resuspended in fresh YPD medium supplemented with tracer quantities (~20 μCi/ml) of
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References


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