Identification of Mitogen-Activated Protein Kinase Signaling Pathways That Confer Resistance to Endoplasmic Reticulum Stress in Saccharomyces cerevisiae

Yijun Chen, Douglas E. Feldman, Changchun Deng, James A. Brown, Anthony F. De Giacomo, Allison F. Gaw, Gongyi Shi, Quynh T. Le, J. Martin Brown, and Albert C. Koong

Department of Radiation Oncology, Center for Clinical Sciences Research, Stanford University Medical Center, Stanford, California

Abstract

Hypoxia activates all components of the unfolded protein response (UPR), a stress response initiated by the accumulation of unfolded proteins within the endoplasmic reticulum (ER). Our group and others have shown previously that the UPR, a hypoxia-inducible factor–independent signaling pathway, mediates cell survival during hypoxia and is required for tumor growth. Identifying new genes and pathways that are important for survival during ER stress may lead to the discovery of new targets in cancer therapy. Using the set of 4,728 homozygous diploid deletion mutants in budding yeast, Saccharomyces cerevisiae, we did a functional screen for genes that conferred resistance to ER stress–inducing agents. Deletion mutants in 56 genes showed increased sensitivity under ER stress conditions. Besides the classic UPR pathway and genes related to calcium homeostasis, we report that two additional pathways, including the SLT2 mitogen-activated protein kinase (MAPK) pathway and the osmosensing MAPK pathway, were also required for survival during ER stress. We further show that the SLT2 MAPK pathway was activated during ER stress, was responsible for increased resistance to ER stress, and functioned independently of the classic IRE1/HAC1 pathway. We propose that the SLT2 MAPK pathway is an important cell survival signaling pathway during ER stress. This study shows the feasibility of using yeast deletion pools to identify relevant mammalian orthologues of the UPR. (Mol Cancer Res 2005;3(12):669–77)

Introduction

The endoplasmic reticulum (ER) maintains an oxidative environment that is optimized to promote the efficient folding of proteins destined for the secretary pathway. This environment is enriched in chaperones, glycosylation enzymes, and oxidoreductases (1). Occasionally, the protein load in the ER exceeds the folding capacity and misfolded proteins accumulate. At least two evolutionarily conserved processes have been described to help eukaryotic cells cope with this stress: unfolded protein response (UPR; refs. 2-4) and ER-associated degradation (5-9). Although considerably more complex in mammalian cells, the classic UPR pathway in yeast consists of three genes: IRE1, HAC1, and RLG1. Gene profiling studies revealed that the scope of UPR-related genes was more extensive than initially believed. The induced genes are involved in translocation, protein glycosylation, vesicular transport, cell wall biosynthesis, vacuolar protein targeting, and ER-associated degradation (10).

Accumulating evidence suggests that activation of the UPR is important in tumor development and growth. In a variety of human tumors, investigators have reported elevated expression of UPR targets, such as Grp78 (also called binding protein or BiP) and Grp94 (11). The expression of these genes and other components of the UPR is also correlated with increased malignancy (12, 13).

Previously, we showed that hypoxia activates the UPR and mediates survival under these conditions. Inhibition of BiP using an antisense strategy sensitized cells to hypoxic stress (14). More recently, we showed that cells deficient in XBP-1 were also more sensitive to hypoxic stress and unable to grow as tumor xenografts, suggesting that XBP-1 plays an essential role in tumorigenesis (15). Recent studies also support an important role of another major branch of the UPR, PKR-like ER kinase, in regulating protein translation and survival under hypoxic stress as well as tumor growth (16, 17). As a potential therapeutic strategy, Park et al. showed that blocking activation of UPR with a small-molecule inhibitor (versipelostatin) could potentiate the effects of cisplatin and inhibit tumor xenograft growth (18). Taken together, ER stress–activated pathways are essential for the development and growth of solid tumors and inhibition of these pathways may lead to new anticancer therapies.

In this study, we used the Saccharomyces deletion pool, constructed by an international consortium, in which all known
open reading frames (ORF) were deleted by a PCR-based deletion strategy (19) to perform a functional screen for genes involved in promoting survival during ER stress. This pool has been used by previous investigators to identify novel genes required for UV or ionizing radiation resistance (20, 21). We exposed this pool of 4,728 homozygous deletion strains to a variety of chemical agents known to induce ER stress. We hypothesized that screening the deletion pool with this method would result in the identification of novel UPR-related genes and pathways responsible for survival during ER stress that could represent potential new targets for cancer therapy.

Results
Deletion Pool Analysis Reveals That >50 Mutants Are Sensitive to Agents That Induce ER Stress

To identify genes that were essential for mediating resistance to ER stress and not just one specific drug, we treated the deletion pool with three chemical agents that induce unfolded proteins in the ER through different mechanisms: DTT and $\beta$-mercaptoethanol, both reducing agents, and tunicamycin, a N-linked glycosylation inhibitor. The yeast deletion pool is composed of ~4,700 diploid strains, each with homozygous deletion of a nonessential gene. Each mutant strain can be identified based on a “molecular barcode,” and following exposure of this pool to various chemical inducers of ER stress, we determined the relative frequency of each mutant in the pool before and after the treatment.

We ranked the 4,728 viable homozygous diploid deletion mutants according to their sensitivities to tunicamycin, DTT, and $\beta$-mercaptoethanol. The rankings were based on a ratio of mean signal intensity between treated and untreated cells. As expected, ire1A/ΔA and hac1A/ΔA were two of the most sensitive mutants to all three ER stress-inducing agents. These results showed the validity of this screen in identifying novel UPR-related genes and pathways using this approach. Although some mutants only displayed sensitivity to a single drug, many showed reproducible sensitivity to all three agents. Table 1 is a rank list of the sensitive mutants for all three UPR-inducing drugs.

To confirm the sensitivity of the deletion strains, we selected 150 of the most sensitive mutants and tested their sensitivity individually on YPD plates containing tunicamycin, DTT, and $\beta$-mercaptoethanol. We also tested an additional 50 mutants that may not have been among the most sensitive mutants but were related to some functional group relevant to ER stress. Overall, we confirmed that deletions in 56 genes caused increased sensitivity to agents that induce ER stress. To further verify that deletion of these genes conferred sensitivity to ER stress, we replaced the deleted ORF of shl2A/ΔA and showed that the sensitivity of this yeast strain to tunicamycin was similar to that of the wild-type. We selected several other mutants and replaced the deleted ORFs of the pbs2, rlm1, mkk2, and skl1 mutants. These cells were also found to have tunicamycin sensitivity similar to the wild-type cells (data not shown).

The major functional categories that we identified using this screen were the SLT2 mitogen-activated protein kinase (MAPK) pathway, the osmosensing MAPK pathway, genes related to calcium signaling and homeostasis, and the classic UPR pathway. Although ire1 and hac1 deletion mutants were the most sensitive mutants on this list, the majority of the genes that we identified in this study have not been shown previously to be involved in the response to ER stress (Table 2). Other major functional categories of genes that were required for survival during ER stress included protein glycosylation, transcription regulation, and transport genes. Several protein glycosylation gene mutants, including rhk1, oxi3, kar7, alg9, and alg8, were sensitive to both reducing agents and some were also sensitive to tunicamycin. Several genes related to transcriptional repression and regulation were also required for survival, but their exact function during ER stress has yet to be determined.

Deletions in Genes Involved in the SLT2 MAPK Pathway Cause Extreme Sensitivity to ER Stress

Figure 1 (top) shows the individual sensitivities of yeast strains with mutations in the SLT2 MAPK pathway genes. Deletions in two genes in this pathway (BCK1 and SLT2) revealed extreme sensitivity to tunicamycin, $\beta$-mercaptoethanol, and DTT. These mutants exhibited similar sensitivity to these agents compared with ire1A/ΔA. Signaling to Slt2p occurs through either Mkk1p or Mkk2p and deletion in either of these genes resulted in partial sensitivity to ER stress. Deletion of other components of this pathway, including cell surface sensor genes, such as HCS77 and MID2, also conferred partial sensitivity. Rlm1p and the SBF complex (a heterodimer composed of Sbi4p and Swi6p) are two known transcription factors downstream of Slt2p (22, 23). However, deletion of RLM1 resulted in moderate sensitivity to tunicamycin and had no effect on sensitivity to $\beta$-mercaptoethanol. The swi4 and swi6 mutants were not sensitive to ER stress (data not shown). These results suggest that Slt2p must be responsible for regulating another survival pathway during ER stress that is independent of Rlm1p and the SBF complex.

Table 1. Rank List of Deletion Mutant Sensitivities to UPR-Inducing Agents

<table>
<thead>
<tr>
<th>ORF</th>
<th>Gene</th>
<th>Sensitivity Rank Among 4,728 Strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tunicamycin</td>
<td>$\beta$-Mercaptoethanol</td>
<td>DTT</td>
</tr>
<tr>
<td>YFL031W</td>
<td>HAC1</td>
<td>1</td>
</tr>
<tr>
<td>YHR879C</td>
<td>IRE1</td>
<td>2</td>
</tr>
<tr>
<td>YFL032W</td>
<td>hac1A</td>
<td>3</td>
</tr>
<tr>
<td>YJL095W</td>
<td>BCK1</td>
<td>4</td>
</tr>
<tr>
<td>YOL031C</td>
<td>SLS1</td>
<td>5</td>
</tr>
<tr>
<td>YHR039C</td>
<td>SLT2</td>
<td>7</td>
</tr>
<tr>
<td>YNL219C</td>
<td>Alg9</td>
<td>10</td>
</tr>
<tr>
<td>YML035C-A</td>
<td>YML034C-A</td>
<td>15</td>
</tr>
<tr>
<td>YJR177W</td>
<td>STE24</td>
<td>6</td>
</tr>
<tr>
<td>YEL031W</td>
<td>SPY1</td>
<td>8</td>
</tr>
<tr>
<td>YKL190W</td>
<td>CNB1</td>
<td>11</td>
</tr>
<tr>
<td>YJL187C</td>
<td>SWE1</td>
<td>25</td>
</tr>
<tr>
<td>YPL140C</td>
<td>MKK2</td>
<td>14</td>
</tr>
<tr>
<td>YOR093C</td>
<td>YOR093C</td>
<td>19</td>
</tr>
<tr>
<td>YML085C</td>
<td>YRL085C</td>
<td>74</td>
</tr>
<tr>
<td>YML033W</td>
<td>src1</td>
<td>29</td>
</tr>
<tr>
<td>YKR003C</td>
<td>YKR003C</td>
<td>1,948</td>
</tr>
<tr>
<td>YMR006C</td>
<td>MIV1</td>
<td>391</td>
</tr>
<tr>
<td>YOR085W</td>
<td>OST3</td>
<td>1,447</td>
</tr>
<tr>
<td>YJL128C</td>
<td>PBS2</td>
<td>27</td>
</tr>
</tbody>
</table>

NOTE: List of the most sensitive yeast mutants to UPR-inducing agents as determined by yeast deletion pool analysis.
Our data indicate that the SLT2 MAPK pathway genes play a significant role in mediating survival to ER stress. This pathway is activated by Pkc1p, which phosphorylates specific serine and threonine residues on Bck1p, to activate downstream phosphorylation of Mkk1p, Mkk2p, and Slt2p. Homozygous deletion mutants of PKC1 in the BY4743 genetic background were not viable and the heterozygous mutant did not exhibit increased sensitivity to ER stress.

Table 2. Genes Whose Deletion Cause Sensitivity to UPR-Inducing Agents

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>ORF</th>
<th>Protein Function</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tunicamycin</td>
</tr>
</tbody>
</table>

**SLT2 MAPK pathway**

- **SLT2** YHR030C Cell wall organization and biogenesis, response to stress and nitrogen starvation SSS SSS SSS
- **BCK1** YJL095W Cell wall organization and biogenesis, response to stress and nitrogen starvation SSS SSS SSS
- **RL1** YPL089C Transcription factor for cell wall organization and biogenesis SS S WT
- **HCS77** YOR008C Plasma membrane protein for maintenance of cell wall integrity SS SS WT
- **MID2** YLR332W Plasma membrane protein for maintenance of cell wall integrity SS S S
- **MKK2** YPL140C MAPK kinase S WT S
- **MKK1** YOR231W MAPK kinase S WT S

**Omosensing MAPK pathway**

- **SSK2** YCR073C MAPK kinase WT SS S
- **SSK1** YLR006C Osmosensory signaling pathway via two-component system S SS S
- **PSB2** YJL128C Protein threonine/tyrosine kinase S S S
- **HOG1** YLR113W MAPK of the high-osmolarity signal transduction pathway SS S S
- **SSK2** YNR031C MAPK kinase S WT S

**Calcium channel**

- **PMR1** YGL167C Calcium-transporting ATPase, ER-to-Golgi transport SS SS S
- **SPF1** YEL031W Calcium-transporting ATPase SS SS WT
- **CNB1** YKL190W Calcineurin regulatory (B) subunit SSS SS S

**UPR**

- **IRE1** YHR079C Response to unfolded protein SSS SSS SSS
- **HAC1** YPL031W Transcription factor that activates UPR SSS SSS SSS

**Protein glycosylation**

- **BHI1** YBL082C α-1,3-Mannosyltransferase activity WT S S
- **ALG9** YNL219C Protein amino acid glycosylation S S SS
- **KTR7** YIL085C Glycoprotein biosynthesis SS S S
- **OST3** YOR085W N-linked glycosylation protein amino acid glycosylation WT S S SS
- **ALG8** YOR067C Glucosyltransferase of the ER WT S S SS

**Transcription**

- **ISW2** YOR304W Transcriptional repressor WT S S
- **RM301** YHL027W Specific transcriptional repressor S S S
- **MMS4** YKL062W Transcription factor that response to stress S WT WT
- **PHO80** YLO1001W Cyclin-dependent protein kinase SS WT WT
- **DAL81** YIR023W Specific RNA polymerase II transcription factor S WT SS
- **PPI2** YOR363C DNA binding, transcriptional activator activity WT S S
- **ARD1** YHR013C Regulation of transcription, mating-type specific SS SS SS

**Transport**

- **FPS3** YLL043W Glycerol transporter S SS S
- **DNM2** YKR035W-A Nonselective vesicle transport, protein-vacuolar targeting SS S SS
- **SCC28** YIL076W Vesicle-mediated transport, retrograde (Golgi-to-ER) transport WT S WT
- **SLT1** YOL031C ER protein of unknown function S S WT
- **TAT2** YOL020W Aromatic amino acid transporter activity WT S SS
- **ERV23** YML012W ER-to-Golgi transport S WT S
- **MED1** YBL047C Polar budding, endocytosis, protein transport WT WT S
- **UGO1** YDR470c Mitochondrial outer membrane; transporter WT WT S
- **VPS10** YPL120W Vesicle-mediated transport, protein-Golgi targeting S WT SS

**Ungrouped or unknown genes**

- **RGA1** YOR127W Cytokinesis, regulation of cell shape WT WT SS
- **RMD1** YDL001W Protein possibly involved in meiotic nuclear division WT SS SS
- **RGG1** YBR260C Rho GTPase activator, establishment and/or maintenance of cell polarity SS S WT
- **SPC2** YML055W Component of the signal peptidase complex S SS WT
- **SWE1** YJR187C Protein tyrosine kinase SS S S
- **FYY8** YGR196C Protein of unknown function SS SS S
- **SKL1** YML013W Protein involved in secretion SS WT S
- **YKR035C** YKR035C Protein of unknown function SS SS SS
- **YMR007W** YMR007W Glucosyltransferase of the ER SS S WT
- **YMR029C** YMR029C Protein of unknown function S WT WT
- **YMR032C** YMR029C Protein of unknown function S WT WT
- **YOL031C** YOR313C Protein of unknown function S WT S

**NOTE:** Individual sensitivities of deletion mutants to ER stress organized by functional categories. Each mutant is ranked by relative sensitivity to UPR-inducing agents. For example, IRE1 is classified as “SSS” where “S” indicates impaired colony-forming ability that corresponds to one dilution (~10-fold), “SS” corresponds to two dilutions and “SSS” to three or more dilutions. “R” refers to a resistant phenotype and “WT” indicates the same sensitivity as the wild-type. All references to the primary data and the known function of the proteins are given in the proteome database (https://www.yeastgenome.org).
However, the haploid pck1 mutant in the EG123 genetic background showed nearly identical sensitivity to ER stress as the bck1 homozygous mutant from the same genetic background (data not shown).

Previous investigators have reported that, because of defects in the cell wall, deletions in SLT2 and BCK1 resulted in increased sensitivity to elevated temperature, Congo red, and caffeine. This phenotype can be complemented by the addition of an osmotic stabilizer, such as sorbitol (24, 25). In Fig. 1 (bottom), we tested the sensitivity of bck1 and slt2 mutants to ER stress in the presence of sorbitol. The addition of 1 mol/L sorbitol completely reversed the sensitivity of slt2Δ/Δ and bck1Δ/Δ to caffeine and Congo red but minimally affected the tunicamycin and β-mercaptoethanol sensitivity. These data suggested that the sensitivity of bck1 and slt2 mutants to ER stress—inducing agents was not due to an intrinsic defect in the cell wall. We hypothesized that the BCK1/SLT2 signaling pathway mediated a protective response to ER stress that was largely independent of any defect in the cell wall.

**SLT2 MAPK Pathway Is Activated during ER Stress**

SLT2 mRNA is induced by ER stress through an IRE1/ HAC1-independent mechanism (10, 26). Because SLT2 is activated by phosphorylation, we investigated the possibility that phosphorylated Slt2p might be a direct target of ER stress signaling. We did Western blot analysis using an antibody that specifically recognized the phosphorylated (active) form of Slt2p (Thr202/Tyr204)-p44/42 MAPK; refs. 24, 27). We used this antibody to assay for the phosphorylation status of Slt2p in cell lysates from wild-type yeast treated with tunicamycin, DTT, and β-mercaptoethanol (β-Mer).

Because SLT2 was activated by various UPR-inducing agents and deletion mutants of this pathway resulted in increased sensitivity to these agents, we next addressed whether constitutive activation of this pathway would confer resistance to ER stress. To test this hypothesis, we transformed wild-type cells with a plasmid (BCK1-20) overexpressing BCK1, an upstream activator of SLT2. Other investigators have shown that transformation with BCK1-20 resulted in constitutive activation of the SLT2 pathway (28). As expected, the transformed cells were more resistant to tunicamycin than the untransformed cells (Fig. 2B). In Fig. 2C, we showed that reintroduction of the deleted ORF in the slt2 mutant resulted in complementation of the mutant sensitivity back to that of the wild-type. Collectively, these data show that the SLT2 pathway is directly activated by ER stress and that activation of this pathway confers resistance to ER stress.

**Deletions in Genes That Result in Increased Sensitivity to ER Stress Do Not Correlate with Transcriptional Changes during ER Stress**

Genome-wide expression profiling of yeast in response to UPR-inducing agents revealed a complex transcriptional program that consists of at least 381 induced genes with a variety of functions (10). We characterized the relationship between genes that are increased by ER stress and genes that are necessary for survival during ER stress. By comparing our deletion pool data with the microarray data reported by Travers et al. (10), we found that of the 56 genes that we identified in this study to be required for resistance to ER stress, 25% showed a significant transcriptional increase during ER stress. These genes included SLT2, PBS2, HOG1, PMR1, SPFI, RHK1, SEC28, SLS1, ERV25, FTV8, SEL1, YMR295C, RGA1, and RGD1. Furthermore, only 11% of these genes, including HOG1, PBS2, SPFI, RHK1, ERV25, and SEL1, were induced in an IRE1/HAC1-dependent manner (10). These findings indicated that the genes required for cell survival during ER stress were not necessarily the same genes that undergo transcriptional activation during ER stress.

UPR element (UPRE) reporter analysis revealed that the mutants sensitive to ER stress had available UPRE-mediated...
inducible response. Mutants with increased sensitivity to tunicamycin, DTT, and β-mercaptoethanol were tested for transactivation of a UPRE-dependent reporter plasmid for basal activity before and after exposure to ER stress. The reporter construct consisted of the lacZ gene under transcriptional control of the UPRE, originally identified from the promoter of KAR2, an ER chaperone protein. Deletion of IRE1 resulted in impaired transactivation of this reporter construct during ER stress. Similarly, deletion of BCK1 and SLT2 also resulted in reduced ability to transactivate this UPRE reporter (Fig. 3A). Interestingly, we identified other deletion mutants that had higher basal UPRE activity, suggesting that these mutants had a higher level of ER stress at baseline. In addition, we found several mutants that had both a blunted UPR response and an exaggerated UPR response (data not shown).

Although deletions in the SLT2 MAPK pathway resulted in impaired UPRE-regulated transcription, Northern blot analysis showed that HAC1 splicing was intact in the slt2 mutant (Fig. 3B). Furthermore, KAR2 induction during ER stress was identical between the SLT2 mutant and the wild-type cells (data not shown). These data indicate that SLT2 is regulated in an IRE1/HAC1-independent manner during ER stress (10, 26) and that the classic UPR and SLT2 MAPK pathways contribute to survival during ER stress through separate mechanisms.

Mutations in UPR and SLT2 MAPK Pathway Have Synthetic Effects during ER Stress

To further support our observations that the UPR and SLT2 MAPK pathways were required for survival during ER stress through different mechanisms, we constructed haploid strains containing deletions in IRE1 and SLT2. Although the double mutants grew more slowly, they were still viable. When treated with tunicamycin, the double mutants showed increased sensitivity compared to the parental strains (Fig. 4). In the range of tunicamycin concentrations used, deletion of IRE1 and SLT2 resulted in additive sensitivity. Similar results were obtained for these strains after treatment with DTT (data not shown). Our results indicate that these genes are in separate epistasis groups, supporting our conclusion that the classic UPR and SLT2 MAPK signaling pathways are distinct from each other and may serve different functions to promote survival during ER stress.

Validation of Genes Identified from Yeast in Mammalian Tumor Cells

Although SLT2 shares sequence homology (up to 50%) with several of the MAPK family proteins, it does not have an identified orthologue in mammalian cells. Therefore, to show that this functional screen in yeast was relevant to mammalian cells, we used a small interfering RNA approach to inhibit the HAC1 functional orthologue (XBP-1) in human tumor cells. Deletion mutants of HAC1 were sensitive to all forms of ER stress (Table 1) and ranked at the top of our list in all of the screens. We have shown previously that hypoxia activates UPR signaling pathways in mammalian cells. In addition, we showed that XBP-1 in mouse embryonic fibroblasts mediated survival during hypoxia and was required for tumor growth (15). In the current study, we selected two different XBP-1 small interfering RNA clones (targeting different sequences) in HT1080 cells (human fibrosarcoma). Inhibition of XBP-1 resulted in
decreased clonogenic survival under hypoxia and impaired tumor growth (Fig. 5). These findings show the potential of identifying important targets for cancer therapy through a functional yeast screen of deletion mutants with increased sensitivity to ER stress.

Discussion

The classic UPR pathway in yeast consists of signaling through Ire1p and Hac1p. To date, the only identified substrate for Ire1p is Hac1p (29). Ogawa and Mori reported that Hac1p is autoregulated by itself through a UPRE-like sequence in its promoter (30). Cells expressing the HAC1 gene under the control of a mutant HAC1 promoter lacking the UPRE were unable to sustain high levels of HAC1 mRNA and were sensitive to prolonged ER stress. In addition to HAC1 itself, there are at least 381 downstream targets that are transcriptionally regulated in an Ire1p/Hac1p-dependent manner. These genes were identified by microarray studies and seem to involve different elements of the ER-associated degradation and secretory pathways (10). In a follow up study, Patil and Walter identified two other UPRE-like sequences (UPRE-2 and UPRE-3) through a computational analysis of the promoter sequences of all UPR target genes. Gen4p (through direct binding to promoter target sequences) and its activator Gen2p were required for the induction of most UPR target genes during ER stress (31). In addition to the classic UPR pathway, other pathways also exist to account for the transcriptional activation of another subset of ER stress–activated genes (10, 26).

Other investigators have observed no correlation between the transcriptional response of yeast to DNA-damaging agents and the function of these genes in regulating survival to the same DNA-damaging agents (32). In the present study, among the 56 genes that we have identified as important in mediating survival during ER stress, 25% were increased at the transcriptional level. Furthermore, only 11% of these 56 genes were induced in an Ire1p/Hac1p-dependent manner. This suggests that transcriptional response pathways, including the classic UPR pathway and the newly described Ire1p/Hac1p-independent pathway, were only partially responsible for mediating the overall response to ER stress.

In addition to the classic UPR pathway and calcium signaling pathway, this study has implicated at least two other pathways to be involved in the response to ER stress. These pathways include the SLT2 MAPK pathway and the osmosensing MAPK pathway. Surprisingly, deletion of most ER-associated degradation genes did not result in increased sensitivity to ER stress, suggesting that alternative pathways may function to remove misfolded proteins. For example, ER-to-vacuole transport may function in this capacity to reduce the stress of protein accumulation in the ER (33).

Because of their extreme sensitivity to ER stress, we chose to characterize in greater detail the deletion mutants of the SLT2 MAPK pathway. In this report, we show by Western blot analysis that SLT2 is activated directly by ER stress. The sensitivities of SLT2 pathway deletion mutants to ER stress cannot be explained purely as a defect in the cell wall, because their sensitivities were minimally affected by the addition of sorbitol, a cell wall–stabilizing agent. Moreover, deletions in the majority of genes important for the synthesis of cell wall proteins and cell wall maintenance (FKS1, FKS2, KRE6, MNN9, GAS1, KNR4, and
KRE1) did not show increased sensitivity to ER stress. This finding is consistent with other studies concluding that the SLT2 MAPK pathway regulates important biological functions other than cell wall biogenesis and maintenance. Some of these functions include promoting survival during nitrogen/carbon starvation, regulating progression through G2-phase cell cycle checkpoint, and controlling life span (34-36).

The SLT2 MAPK pathway is distinct and separate from the classic UPR pathway. The evidence for this conclusion comes from several different lines of evidence. First, in SLT2 MAPK pathway deletion mutants, HAC1 splicing and KAR2 induction was intact. Next, all of the genes in the SLT2 MAPK pathway were regulated in an IRE1/HAC1-independent manner. Finally, double mutants of IRE1 and SLT2 showed additive sensitivity to ER stress compared with the parental mutants, indicating that these genes reside in different epistasis groups.

In mammalian systems, the MAPK pathways are evolutionary conserved pathways that integrate extracellular signals, resulting in cellular responses that regulate proliferation, differentiation, development, inflammation, and apoptosis (37). As part of the Ras-Raf-MAPK/extracellular signal-regulated kinase kinase-extracellular signal-regulated kinase signaling cascade, MAPK family members are critical regulators of oncogenesis and represent novel targets for anticancer therapy (38-41). MAPK signaling cascades also play an important role in cell survival during ER stress (42-44). It was shown recently that the extracellular signal-regulated kinase pathway can be activated by ER stress and that inhibition of the MAPK/extracellular signal-regulated kinase kinase-extracellular signal-regulated kinase pathway could protect tumor cells from ER stress—induced cell death (43). Yeast SLT2 shares the greatest sequence homology with mammalian MAPK family members; however, no mammalian gene has >50% identity to SLT2, suggesting that significant differences exist in the regulation of this pathway across different species.

To show the feasibility of using the yeast deletion pool as a functional screen to identify novel mammalian signaling pathways involved in cell survival during ER stress, we first studied HAC1, which conferred extreme sensitivity to a variety of ER stress when deleted (Table 1). In mammalian cells, XBP-1 is the functional homologue of HAC1. Inhibition of XBP-1 expression by XBP-1 antisense oligonucleotides in human embryonic fibroblasts (15). Hypoxia is an important activator of the UPR in solid tumors and has been shown to regulate multiple branches of the mammalian UPR (15-17, 45). Further studies are needed to characterize the significance of other mammalian orthologues of yeast genes that mediate survival during ER stress. A similar approach may be used to identify and characterize other signaling pathways that are relevant in cancer.

Materials and Methods

Yeast Strains, General Procedures, and Reagents

Genotypes of the BY4743 parental yeast strain and construction of the homozygous diploid deletion strains have been described previously (19). Information is also available in the Saccharomyces Genome Deletion Project Web site (http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html). In the present study, we used a pool of 4,728 strains representing homozygous deletion of all nonessential genes. Some haploid mutants in the BY4741 background were also used. Strain CP193 (Mata ire1Δ::LEU2) was from Dr. Peter Walter’s laboratory (University of California at San Francisco, San Francisco, CA). DL100 (1783a::bck1Δ::URA3), DL1101 Matα 1783 [YEp3525(MPK1::H4)], and DL376 (1783a pck1Δ::LEU2) were from Dr. David E. Levin’s laboratory (Johns Hopkins University, Baltimore, MD). Yeast manipulations were carried out using standard methods, except that in some cases the pH of YPD was lowered to pH 5.4 to inhibit oxidation of DTT. All incubations were at 30°C unless otherwise stated. KAR2, ACT1, and HAC1 probes and pJC005 plasmid were generously provided by Dr. Peter Walter. The p636 plasmid was generously provided by Dr. David E. Levin.

Drug Treatment

For experiments involving the deletion pool, aliquots of the deletion pool representing 10⁴ cells of each of the individual strains were grown in YPD medium in an orbital shaker at 30°C and 300 rpm for 2 hours. Cells were divided equally and treated with tunicamycin (1 μg/mL), DTT (4 mmol/L), β-mercaptoethanol (30 mmol/L), and mock control for 2 hours, respectively. The cells were washed, pelleted, and inoculated into prewarmed YPD, and the remaining resistant strains were allowed to grow for an additional 18 hours. We did three identical experiments with tunicamycin and β-mercaptoethanol and two identical experiments with DTT.

PCR Amplification, Hybridization, and Data Analysis

PCR amplification, microarray hybridization, and data analysis were done as described previously (19). Briefly, the genomic DNA was isolated and amplified by two PCR reactions that amplify the two tags from each strain in the pool. The PCR products were combined with oligonucleotides complementary to nontag regions of the PCR product, heat denatured, and hybridized to purpose-built oligonucleotide microarrays (DNA TAG3, Affymetrix, Santa Clara, CA) for 16 hours at 42°C. After staining, arrays were scanned at an emission wavelength of 560 nm. To assess the degree of sensitivity to these folding agents, a ratio of treated to untreated substrate and detected as an increase in absorbance at 420 nm.

UPRE Assays

Strains assayed for β-galactosidase activity were transformed with pJC005, a 2μ plasmid carrying the lacZ gene under the control of the UPRE from the KAR2 promoter. Wild-type and mutant cells were harvested and lysed 1 hour after addition of 1 μg/mL tunicamycin. β-Galactosidase activity was measured from lysates using 2-nitrophenyl-β-D-galactopyranoside as a substrate and detected as an increase in absorbance at 420 nm.

Northern Blot Analysis

To generate RNA samples for Northern blot analysis, 50 mL cultures of BY4743 were grown in YPD. At early log phase, where indicated, cultures were exposed to 1-hour incubation in
1 µg/mL tunicamycin. Total RNA was isolated by a glass bead disruption method. Probes for KAR2, HAC1, and ACT1 were prepared by 32P radiolabeling with the Ready-to-Go kit (Pharmacia, New York, NY). Quantitation was done using a Bio-Rad (Hercules, CA) phosphorimaging system.

Protein Extraction and Western Blots

After exposure to the indicated stress, cells were rapidly pelleted and frozen in dry ice. The cell pellet from a 50 mL culture was lysed by vortexing with glass beads in 0.6 mL lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 10% glycercer, 1% Triton X-100, 0.1% SDS, 150 mmol/L NaCl, 50 mmol/L NaF, 5 mmol/L EDTA, 15 mmol/L Na2HPO4, 20 mmol/L p-nitrophenyl phosphate, 0.2 mmol/L sodium orthovanadate] with a protease inhibitor cocktail (Sigma, St. Louis, MO). The protein concentrations were determined by the Bio-Rad Bradford assay. For detection of diphospho-Slt2p, a three-antibody protocol was used to enhance sensitivity as reported before (35). The rabbit anti-phospho-p44/p42 MAPK antibody (Cell Signaling, Beverly, MA) was used at 1:1,000 dilution followed by mouse anti-rabbit immunoglobulin G (Jackson Immunoresearch Laboratories, West Grove, PA) at 1:5,000 dilution and finally horseradish peroxidase–conjugated goat anti-mouse immunoglobulin G at 1:5,000 dilution. Goat anti-Mpk1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used to detect the total Slt2p level at 1:200 dilution. Immunoblots were developed with horseradish peroxidase–conjugated secondary antibody and SuperSignal Chemiluminescent Substrate kit (Pierce, Rockford, IL).

RNA Interference Knockdown Vectors

RNA interference knockdown constructs targeting human XBP-1 were generated by ligating annealed RNA interference hairpin oligonucleotides into a modified pU6.pro vector (46) containing a puromycin resistance marker. The small interfering RNA hairpin constructs were designed to target distinct, nonoverlapping regions in human XBP-1 and were confirmed by sequencing. The sequences targeted are as follows: XBP-1 RNAi-1 (5'-CAGCCATCTTTCTGGCTAATTTCAAGA-GAAGTAGCCAGGAGATGCTTTTGTACGT-3') and XBP-1 RNAi-2 (5'-CGATGACCTCGTTCCGGAGCCTAA-GAGAGGTCCGGAACGGTACCTTTTGTACGT-3').

Tumor Cell Lines and Culture Conditions

The human fibrosarcoma cell line HT1080 was cultured at 37°C with 5% CO2 in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin antibiotics. The human XBP-1 RNA interference lines were generated by transfecting pU6.pro-hXBp-1 and pU6.pro-hXBp-2 into HT1080 cells using the calcium phosphate method. Total RNA isolated from individual puromycin-resistant clones was analyzed by Northern blot to confirm knockdown of XBP-1. For hypoxia survival assays, cells were grown in 60-mm dishes until reaching at 40% to 50% confluence and shifted to anoxia for 48 hours. Cells were trypsinized, counted using a hemocytometer, and replated in triplicate at 1,000 to 20,000 per plate in normal culture medium. After 14 days, colonies were stained with 0.2% crystal violet and counted. All experiments were repeated at least thrice.

For tumor xenograft experiments, 2 × 10^6 HT1080 fibrosarcoma cells were resuspended in 0.1 mL PBS and injected s.c. in the dorsal flanks of 6-week-old, female BALB/c nu/nu mice, with n = 4 for the control group and n = 5 for experimental groups. Tumors were measured with a caliper. Tumor volume was calculated using the formula: Volume (mm^3) = (Width) × (Height) × (Length) × 0.52.

Acknowledgments

We thank Davis Ng, Dr. David E. Levin, and Dr. Peter Walter for providing strains, plasmids, and antibodies; Franco Guisetti and Nicholas C. Denko for technical assistance with the yeast deletion pool experiments; Jo-Sook Hahn and Dennis J. Thiéle for helpful suggestions with the phospho-Slt2 Western blotting studies; and Amato J. Giaccia and Nicholas C. Denko for critical comments regarding this article.

References


Identification of Mitogen-Activated Protein Kinase Signaling Pathways That Confer Resistance to Endoplasmic Reticulum Stress in <i>Saccharomyces cerevisiae</i>


Updated version
Access the most recent version of this article at:
http://mcr.aacrjournals.org/content/3/12/669

Cited articles
This article cites 43 articles, 16 of which you can access for free at:
http://mcr.aacrjournals.org/content/3/12/669.full.html#ref-list-1

Citing articles
This article has been cited by 31 HighWire-hosted articles. Access the articles at:
/content/3/12/669.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.