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The HOG MAPK Pathway and Yeast Stress Responses: Roles in Oxidative Stress and Heat Shock

by

Qiang Zhao

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE

Doctor of Philosophy

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HOUSTON, TEXAS

JULY, 2001
ABSTRACT

The HOG MAPK Pathway and Yeast Stress Responses:

Roles in Oxidative Stress and Heat Shock

by

Qiang Zhao

The HOG MAP kinase pathway in the budding yeast *Saccharomyces cerevisiae* senses and responds to high osmolarity. Here we demonstrated that HOG pathway mutants are hypersensitive to K1 killer toxin, which implies certain defects in their cell wall. Overexpression of the *PBS2* gene leads to enhanced resistance to K1 killer toxin. Treating yeast cells with a pore-forming antifungal agent, amphotericin B, lowers the cellular turgor pressure. More importantly, amphotericin B treatment leads to activation of the HOG pathway, supporting the hypothesis that loss of turgor pressure activates the HOG pathway. Deficiencies in the HOG pathway also cause hypersensitivity to hydrogen peroxide and the superoxide-generating drug plumbagin. Hydrogen peroxide, menadione and plumbagin all activate the HOG pathway. The HOG pathway acts parallel to Skn7p and Yap1p in oxidative stress response, evidenced by the additive effect of *hgl1Δ, skn7Δ* and *yap1Δ* on hydrogen peroxide sensitivity. Both *ssn6Δ* and *sko1Δ* suppress *hgl1Δ* mutant sensitivity to oxidants. Oxidative stress induces transcription of *HSP12* and *HSP26*. The HOG pathway regulates *HSP12* transcription in this response. Msn2p and Msn4p are important for the oxidative stress-induced transcription of *HSP12* and *HSP26*. The HOG pathway is also involved in heat shock response. Cells lacking the *HOG1* gene
are hypersensitive to heat stress. A temperature shift from 25°C to 37°C activates the HOG pathway. Such an increase in temperature also induces transcription of \textit{HSP12} and \textit{HSP26}. The HOG pathway regulates \textit{HSP12} transcription in the heat shock response. Msn2p and Msn4p are important for the heat shock-induced transcription of \textit{HSP12} and \textit{HSP26}. 
ACKNOWLEDGEMENTS

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Last, but certainly not least, I want to thank my committee. Their advice has helped me stay on the right track and eventually finish this work.
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<tbody>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>b-ZIP</td>
<td>basic leucine zipper</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethyldimethyltetraacetic acid</td>
</tr>
<tr>
<td>FRE</td>
<td>filamentation and invasion response element</td>
</tr>
<tr>
<td>HOG</td>
<td>high osmolarity glycerol</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HSE</td>
<td>heat shock element</td>
</tr>
<tr>
<td>HSF</td>
<td>heat shock factor</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
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<td>MEK</td>
<td>MAPK and ERK kinase</td>
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<td>MEK kinase</td>
</tr>
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<td>optical density</td>
</tr>
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<td>messager RNA</td>
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<td>polyacrylamide gel electrophoresis</td>
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<td>phosphate buffered saline</td>
</tr>
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<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>STRE</td>
<td>stress response element</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBS/T</td>
<td>TBS + Tween 20</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet light</td>
</tr>
<tr>
<td>YRE</td>
<td>Yap1p response element</td>
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CHAPTER 1
INTRODUCTION

The survival of all living cells is dependent on their ability to sense alterations in the environment and appropriately respond to the new condition. Often the process of adaptation involves various signal transduction pathways. Mitogen-Activated Protein Kinase (MAPK) pathways are among the most thoroughly studied of signal transduction pathways and have been shown to participate in a diverse array of cellular activities in response to the constantly changing environment. The work described here details studies of the High Osmolarity Glycerol (HOG) MAPK pathway and its roles in yeast stress responses. This chapter provides relevant background information regarding MAPK pathways in the budding yeast *Saccharomyces cerevisiae* and especially the HOG pathway. The stress response pathway in the fission yeast *Schizosaccharomyces pombe* and the p38 pathway in mammalian cells are also described because they are homologues of the S. cerevisiae HOG pathway. Last, various stress conditions and how yeast respond to them are briefly summarized.

1.1 MAPK PATHWAYS IN THE BUDDING YEAST *SACCHAROMYCES CEREVISIAE*
MAPK pathways can be found in all eukaryotes (Widmann et al., 1999). Although different MAPK pathways carry out distinct functions, they all contain the same kinase cascade, which consists of three kinases, the MAP kinase kinase kinase (MAPKKK or MEKK), the MAP kinase kinase (MAPKK or MEK), and the MAP kinase (MAPK). The biochemical mechanisms mediating signal transduction among these three kinases have been determined (Robinson and Cobb, 1997). MEKK has a regulatory domain at the NH$_2$ terminus and a protein kinase domain at the COOH terminus. When activated, MEKK phosphorylates two conserved residues in the NH$_2$ terminus of MEK, a serine and a threonine. The phosphorylated and now activated MEK then phosphorylates MAPK on a threonine and a tyrosine residue, separated by a single amino acid, within the activation loop of the conserved kinase domain, thereby activating MAPK.

Our understanding of the MAPK pathways in the budding yeast is more complete than that in other organisms (Gustin et al., 1998). There are five different MAPK pathways in _S. cerevisiae_ (Figure 1-1). They are the mating pathway, the filamentation-invasion pathway, the cell integrity pathway, the HOG pathway, and the spore wall assembly pathway. Four of these pathways, the mating pathway, the filamentation-invasion pathway, the cell integrity pathway, and the HOG pathway, are present in growing cells. The Smk1p MAPK, part of the spore wall assembly pathway, is not present in growing cells but functions during the sporulation process.
Figure 1-1. MAPK pathways in the budding yeast *S. cerevisiae*. 
<table>
<thead>
<tr>
<th>Signal</th>
<th>Mating Pheromone</th>
<th>Filamentation-Invasion Pathway</th>
<th>Cell Integrity Pathway</th>
<th>HOG Pathway</th>
<th>Spore Wall Assembly Pathway</th>
</tr>
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<tr>
<td>MEKK</td>
<td>Ste11p</td>
<td>Ste11p</td>
<td>Bck1p</td>
<td>Ssk2p/Ssk22p/Ste11p</td>
<td>Unknown</td>
</tr>
<tr>
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<td>Ste7p</td>
<td>Ste7p</td>
<td>Mkk1p/Mkk2p</td>
<td>Pbs2p</td>
<td>Unknown</td>
</tr>
<tr>
<td>MAPK</td>
<td>Fus3p</td>
<td>Kss1p</td>
<td>Slt2p</td>
<td>Hog1p</td>
<td>Smk1p</td>
</tr>
</tbody>
</table>
The Mating Pathway

Yeast cells generally exist as either diploids or haploids. The haploid cells can be one of two mating types, a or α. The haploid cells of one mating type secrete a short peptide known as mating pheromone (a-mating factor or α-mating factor), which binds receptors on cells of the opposite mating type. Upon binding of the mating pheromone, the haploid cells stop growing and differentiate into mating competent cells by inducing transcription of mating genes (Elion, 2000; Leberer et al., 1997). Genes whose disruption inhibits mating and causes sterility are designated as sterile genes (STE). The membrane receptors for the a- and α-mating factors are Ste2p (Jenness et al., 1983) and Ste3p (Sprague et al., 1983), respectively, and they are coupled to a heterotrimeric G protein. Pheromone activation of the G-protein induces the dissociation of the subunits, Gpa1p (α-subunit), Ste4p (β-subunit), and Ste18p (γ-subunit) (Nomoto et al., 1990; Sommers and Dumont, 1997). The released βγ-subunit complex activates Ste20p (Leeuw et al., 1998; Wu et al., 1995) and interacts with the scaffold protein Ste5p (Choi et al., 1994; Pryciak and Huntress, 1998), resulting in the stimulation of the MAPK module Ste11p-Ste7p-Fus3p (Errede et al., 1993; Inouye et al., 1997b; Wu et al., 1995)

Ste5p acts as a scaffold for the mating pathway. Analyses of point mutations and deletions have shown Ste5p has separate sites for binding Ste11p, Ste7p, and Fus3p (Choi et al., 1994). Such a scaffold protein is considered to increase the specificity of the kinase cascade by blocking inappropriate interactions with other, related cascades (Pawson and Scott, 1997). Although the MEKK Ste11p also serves in the HOG pathway (Posas and
Saito, 1997), pheromone-activated Ste11p is unlikely to activate the HOG pathway because it forms a stable complex with Ste5p. The existence of Ste5p oligomers was first suggested by observations of interallelic complementation of different ste5 mutants that did not complement ste5Δ on their own (Yablonski et al., 1996), and confirmed by two-hybrid analysis and co-precipitation experiments (Feng et al., 1998; Inouye et al., 1997b). Oligomerization seems to be important for signal relay from Ste11p to Ste7p, for co-expression of two different nonfunctional ste5 mutants, one that can not bind Ste11p and one that can not bind Ste7p, fully complements the sterile phenotype of ste5Δ mutant (Inouye et al., 1997a).

It was initially thought that Fus3p and Kss1p were redundant kinases in the mating pathway (Elion et al., 1991; Ma et al., 1995), but Madhani et al. have proposed that only in null mutant lacking Fus3p can Kss1p substitute Fus3p in the mating pathway (Madhani et al., 1997a). The expression of a kinase inactive fus3p mutant in the fus3Δ background hampers the ability of Kss1p to fulfill mating function, indicating that Kss1p does not activate the mating pathway in wild-type cells because it is excluded from this pathway by Fus3p (Madhani et al., 1997a).

Stimulation of the mating pathway leads to activation or repression of different proteins. For example, Far1p, phosphorylated by Fus3p, is a negative regulator of the Cdc28p/cyclins kinase complex that controls cell cycle progression (Gartner et al., 1998; Tyers and Futcher, 1993). This is important because cells that are ready to undergo mating have to stop growing. Ste12p, a transcription factor mediating transcription of
pheromone-response genes including *FAR1*, is phosphorylated and activated by Fus3p (Elion *et al.*, 1993).

**The Filamentation-Invasion Pathway**

When starved for nitrogen, diploid cells of certain yeast strains undergo profound morphologic changes, forming a linear chain of elongated cells called pseudohyphae (Gimeno *et al.*, 1992). Pseudohyphae grow as filaments of rough-edged colonies and invade solid medium. Haploid cells can also invade the agar and grow beneath the surface, but this invasive growth does not require nutritional starvation (Roberts and Fink, 1994). The signaling pathway responsible for filamentation and invasive growth happens to be the same, and is therefore called the filamentation-invasion pathway. The kinase cascade of the filamentation-invasion pathway includes Ste11p, Ste7p, and Kss1p (Liu *et al.*, 1993; Roberts and Fink, 1994). In contrast to the mating pathway, no scaffold protein has been identified for the filamentation-invasion pathway. Upstream of the kinase cascade, there are two small GTP-binding proteins, Ras2p (Gimeno *et al.*, 1992) and Cdc42p (Mosch *et al.*, 1996). Signaling from Ras2p requires the 14-3-3 proteins Bmh1p and Bmh2p (Gelperin *et al.*, 1995; Roberts *et al.*, 1997). Cdc42p is directly downstream of Ras2p, and acts together with Ste20p to transmit signal to the kinase cascade.
Two promoters have been identified as targets of the filamentation-invasion pathway, an upstream activating sequence in the Ty1 transposon (Baur et al., 1997) and the promoter of the TEC1 gene (Madhani and Fink, 1997). Both sequences contain the pheromone-response element (PRE) (Hagen et al., 1991; Kronstad et al., 1987), the binding site for the transcription factor Ste12p (Dolan et al., 1989). They also have a binding site for another transcription factor Tec1p (Baur et al., 1997; Madhani and Fink, 1997). The regulatory DNA sequence containing both Ste12p and Tec1p binding sites is termed as the filamentation and invasion response element (FRE). An FRE is both necessary and sufficient for transcriptional regulation by the filamentation-invasion pathway (Madhani and Fink, 1997).

The MAPK Kss1p seems to play dual roles in signal transduction. By binding the transcription factor Ste12p, the unphosphorylated Kss1p inhibits diploid pseudohyphal development on low-nitrogen medium, haploid invasive growth, and expression of FRE-lacZ reporter (Cook et al., 1997). The MEK Ste7p-catalyzed phosphorylation of Kss1p not only removes a repressor but also creates an activator, the phosphorylated Kss1p. Deletion of KSS1 does not block filamentation, invasive growth or FRE-lacZ expression, but an inactivated Kss1p (with STE7 deleted or expressing a non-phosphorylatable mutant kss1 in the kss1Δ background) inhibits all these responses (Cook et al., 1997; Madhani et al., 1997a). Expression of hyperactive forms of either Ste11p or Ste7p induces a strong pseudohyphal response and increased FRE-lacZ expression, while kss1Δ cells show no response to the expression of these hyperactive mutants (Madhani et al.,
1997b). The Kss1p-dependent mechanism well represents the complexity of cellular signaling.

The Cell Integrity Pathway

The cell integrity pathway mediates cell wall synthesis and responds to various signals including cell cycle regulation, growth temperature, changes in external osmolarity, and mating pheromone. Signaling proteins in the cell integrity pathway include the GTP-binding protein Rho1p (Kamada et al., 1996), the protein kinase C homologue Pkc1p (Levin et al., 1990), the MEKK Bck1p (Lee and Levin, 1992) (Costigan et al., 1992), the redundant pair of MEKs Mkk1p and Mkk2p (Irie et al., 1993), and the MAPK Slt2p (Lee et al., 1993; Torres et al., 1991). Membrane proteins that potentially provide input signals to the cell integrity pathway include the Wsc and Mid families of proteins (Rajavel et al., 1999; Verna et al., 1997).

Downstream targets of the cell integrity pathway include Rlm1p, Swi4p and Swi6p. Rlm1p, a member of the MADS box family of transcription factors, is a substrate of Slt2p in vitro (Watanabe et al., 1997). It also shows heat stress-induced, Slt2p-dependent phosphorylation in vivo. Functional analysis indicates Rlm1p has three domains, the NH2-terminal MADS box DNA binding domain, the central domain that is the target for phosphorylation and regulation by Slt2p, and the COOH-terminal domain that is required for transcriptional activation. Mutants lacking a functional cell integrity pathway are
usually sensitive to high temperature and caffeine, and Rlm1p appears to mainly affect the caffeine sensitivity. Cells with rlm1 null mutation are sensitive to caffeine but not high temperature, while over-expression of the RLM1 gene suppresses the caffeine sensitivity of the bck1Δ mutant but not its temperature sensitivity. Swi4p and Swi6p together form the transcription factor SBF (Andrews and Herskowitz, 1989; Primig et al., 1992). Swi4p is the DNA binding subunit and transcriptional activator, required for expression of cyclin genes CLN1, CLN2, PCL1, and PCL2 at the G1/S transition (Koch et al., 1996; Nasmyth and Dirick, 1991). Swi6p is more of a regulatory subunit, because swi6Δ leads to constitutive intermediate level of CLN1 and CLN2 expression (Dirick et al., 1992). The cell integrity pathway regulates SBF through Slt2p-catalyzed phosphorylation of Swi4p and Swi6p (Madden et al., 1997).

The HOG Pathway

The HOG pathway is required for appropriate response to an increase in osmolarity (Brewster et al., 1993). The internal osmolarity of growing yeast cells is normally maintained higher than the external osmolarity. The resulting osmotic gradient across the plasma membrane brings in water for cell expansion. In the natural environment, however, yeast cells frequently encounter increasing external osmolarity. High extracellular osmolarity causes a disruption of the osmotic gradient and leads to a decrease in cell volume roughly proportional to the magnitude of the stress (Meikle et al., 1988). The HOG pathway senses the stress condition and activates gene expression (Albertyn et al.,
1994b; Rep et al., 1999), thereby helping resist the toxic effects and promoting survival and eventual cell growth.

The basic structure of the HOG pathway is fairly well described (Figure 1-2). The MAP kinase cascade of the HOG pathway consists of three MEKKs, Ssk2p, Ssk22p, (Maeda et al., 1995), Ste11p (Posas and Saito, 1997), one MEK Pbs2p (Boguslawski and Polazzi, 1987) and one MAPK Hog1p (Brewster et al., 1993). There are two incoming and partially redundant branches upstream in the HOG pathway. One branch contains a three-component signaling protein complex composed of Sln1p, Ypd1p, and Ssk1p (Maeda et al., 1995; Maeda et al., 1994). These three proteins are structurally and functionally similar to the regulatory proteins of the two-component phosphorelay system in bacteria (Alex et al., 1996; Appleby et al., 1996). The Sln1p has two trans-membrane domains at the NH2 terminus, a histidine kinase domain in the middle, and a receiver domain at the COOH terminus. The overall structure of Sln1p is similar to the E. coli osmosensor EnvZ, except that EnvZ lacks the COOH-terminal receiver domain (Pratt and Silhavy, 1995). Sln1p catalyzes the transfer of phosphate from ATP to a conserved histidine residue in its histidine kinase domain, and this phosphate is transferred to a conserved aspartate residue in the receiver domain of a second Sln1p (Posas et al., 1996). The phosphate attached to the aspartate residue is then transferred to a histidine residue of Ypd1p and from there transferred to an aspartate residue of Ssk1p. Sln1p and Ypd1p act as negative regulators of the HOG pathway. Deletion of SLN1 or YPD1 is lethal, and this lethality can be blocked by deletion of downstream genes (Posas et al., 1996). The Sln1p-Ypd1p-Ssk1p protein complex regulates the function of two closely related and
functionally redundant MEKKs Ssk2p and Ssk22p (Posas and Saito, 1998). A second upstream branch of the HOG pathway involves Sho1p, Ste11p, (Maeda et al., 1995), Ste50p (O'Rourke and Herskowitz, 1998), Cdc42p, Ste20p (Raitt et al., 2000b). Sho1p has four transmembrane domains at the NH2 terminus and an SH3 domain at the COOH terminus. The SH3 domain binds to the proline-rich domain of Pbs2p, and through this interaction Sho1p anchors the kinase module to the membrane (Maeda et al., 1995; Reiser et al., 2000). In response to osmotic stress, Cdc42p-bound, membrane-localized Ste20p phosphorylates Ste11p, leading to the activation of Pbs2p (Raitt et al., 2000b). However, how osmotic stress activates Ste20p is still not clear.

The HOG pathway is negatively regulated by protein phosphatases. When activated, Hog1p is phosphorylated at two residues in the activation loop, a threonine and a tyrosine. Ptp2p and Ptp3p mediate the tyrosine dephosphorylation of Hog1p (Jacoby et al., 1997; Wurgler-Murphy et al., 1997). The threonine-specific phosphatase for Hog1p has not yet been identified. The late decrease in Hog1p phosphorylation after osmotic stress is partially blocked by deletion of PTP2 and fully blocked by deletion of both PTP2 and PTP3. Ptp3p seems more important in regulating Fus3p activity (Zhan et al., 1997). Another protein phosphatase, Ptc1p, also negatively regulates the HOG pathway (Warmka et al., 2001).

Exposure of yeast cells to high osmolarity results in a global transcriptional response. DNA microarray analysis has revealed that about 7% of the genes encoded in the yeast genome are induced more than five fold after treatment with 0.4 M NaCl for 10 minutes
(Posas et al., 2000; Rep et al., 1999; Yale and Bohnert, 2001). Well represented are genes encoding proteins involved in glycerol synthesis, protein synthesis, trehalose metabolism, glycogen metabolism, ion homeostasis, and signal transduction. The variety of genes induced by osmotic stress suggests it affects many aspects of cellular activity.

**The Spore Wall Assembly Pathway**

When diploid cells are exposed to a nitrogen-deficient medium that lacks a fermentable carbon source, they form spores that are resistant to various stresses. Upon completion of meiosis, the spore wall assembles around each of the four haploid nuclei. The MAPK Smk1p is important for spore cell assembly (Krisak et al., 1994). Cells deficient in SMK1 fail to properly assemble the spore wall and show reduced expression of late sporulation genes. Although the MEKK or MEK of the spore wall assembly pathway has not yet been identified, two other protein kinases, SpS1p (Friesen et al., 1994) and Cak1p (Wagner et al., 1997), may be part of this pathway. SpS1p is a homologue of the PAK protein kinase family. Similar to smk1Δ mutants, sps1Δ cells proceed normally through meiosis but then fail to assemble the spore wall (Friesen et al., 1994). Cak1p is an essential protein kinase required for cell cycle progression during vegetative growth. CAK1 was isolated as a gene which, when overexpressed, suppresses the sporulation defect of a partial-loss-of-function smk1 mutation (Wagner et al., 1997). CAK1 is expressed to a high level during the same time that SMK1 is expressed, and cak1 mutants have a spore wall assembly defect similar to that of smk1 cells.
Figure 1-2. The HOG pathway.
1.2 THE STRESS RESPONSE PATHWAY IN THE FISSION YEAST
SCHIZOSACCHAROMYCES POMBE

The fission yeast has a general stress response pathway similar to the HOG pathway. It is activated not only by high osmolarity (Millar et al., 1995) but also by UV, heat shock, oxidative stress, and nutrient limitation (Degols et al., 1996; Shiozaki and Russell, 1995). The fission yeast pathway contains an upstream response regulator protein Mcs4p (Cottarel, 1997), which is structurally and functionally homologous to Ssk1p of the HOG pathway. Mcs4p activates the kinase cascade composed of two MEKKs Wak1p (Shieh et al., 1997) and Win1p (Shieh et al., 1998), the MEK Wis1p (Warbrick and Fantes, 1991), and the MAPK Sty1p (Millar et al., 1995). Protein phosphatases negatively regulate the MAPK pathway. The tyrosine phosphatases Pyp1p and Pyp2p dephosphorylate Sty1p (Degols et al., 1996), while Ptc1p and Ptc3p act down stream of the kinase cascade (Gaits et al., 1997).

The MAPK cascade stimulates the expression of many stress response genes, mediated by transcription factors Atf1p and Pap1p. Atf1p regulates induction of osmotic stress genes such as GPD1 (Shiozaki and Russell, 1996). It is directly phosphorylated by Sty1p both in vivo and in vitro (Wilkinson et al., 1996). Atf1p is most homologous to ATF2 (Gupta et al., 1995; Raingeaud et al., 1995), a key substrate of the mammalian stress activated protein kinases (SAPKs), p38 MAPK, and c-Jun N-terminal kinases (JNKs). Pap1p has significant homology and similar DNA-binding specificity to mammalian c-Jun. Pap1p mediates transcription of CTTL and other genes in response to
oxidative stress (Toone et al., 1998). It accumulates in the nucleus in a stress-induced, Stylp-dependent manner.

1.3 THE MAMMALIAN P38 PATHWAY

Four types of MAPK pathways have been defined to date in mammalian cells, the ERK pathway, the JNK pathway, the p38 pathway, and the MEK5/ERK pathway (Kyriakis and Avruch, 2001). They are involved in a diverse set of responses affecting cell fate, including cell proliferation and differentiation, adaptation to environmental stress, and apoptosis. In mammalian cells, different MEKKs, MEKs, and MAPKs within a given pathway are usually interchangeable. For example, there are 3 isoforms and 10 different splicing variants of MAPKs in the JNK pathway (Gupta et al., 1996). The relevance of this complexity is poorly understood and difficult to assess.

The mammalian p38 MAPK family consists of at least four different homologous proteins, p38α, p38β, p38γ, and p38δ (Jiang et al., 1996; Jiang et al., 1997; Stein et al., 1997). These p38 MAPKs have very high homology to the yeast MAPK Hog1p. A variety of cellular stresses activate the p38 pathway, including UV, lipopolysaccharide, protein synthesis inhibitors, heat shock, osmotic stress, and certain cytokines. Upon activation, p38 MAPK phosphorylates and stimulates the MAPK-activated protein kinases 2 and 3 (Rouse et al., 1994). Other substrates of p38 MAPK have been identified as transcription factors. ATF2 is phosphorylated by p38 MAPK at two threonine residues.
within its NH₂-terminal activation domain, resulting in increased transcriptional activity (Casillas et al., 1993). Elk1 (Raingeaud et al., 1996), an effective ERK substrate, and Chop (Wang and Ron, 1996), a member of the C/EBP family of transcription factors, are also phosphorylated and activated by p38 MAPK. Interestingly, one substrate of the p38 MAPK, the transcription factor Max, forms a heterodimer with c-Myc, an ERK substrate, raising the possibility that ERK MAPK pathway and p38 MAPK pathway integrate at the transcription level (Zervos et al., 1995).

The variety of substrates indicates that the p38 pathway affects many different biological functions (Obata et al., 2000). For instance, p38 MAPK is activated by thrombin in platelets, suggesting a role in platelet activation (Kramer et al., 1995). The p38 MAPK pathway may also be involved in the cardiac hypertrophic growth program, since expression of an active form of MEK, MEK<sup>met6</sup>, leads to augmentation of cell size, induction of genes encoding A- and B-natriuretic peptides, and increase of α-skeletal actin expression (Zechner et al., 1997).

1.4 STRESS CONDITIONS AND YEAST RESPONSES

Being a unicellular, nonmotile organism, yeast is often challenged by various stress conditions, including osmotic stress, oxidative stress, heat shock, high or low pH, nutrient depletion, etc. The nature of oxidative stress and heat shock will be described in more detail in Chapters 3 and 4. Of course, these classifications are simplified and the natural
situations are more complex. For example, nutrient depletion is a very broad phenomenon dealing with depletion of carbon, nitrogen, sulfur or phosphorus sources. The detrimental effects caused by them overlap but are not exactly the same. In the case of osmotic stress, cation or anion toxicity also contributes to the adversity. Oxidative stress triggers a number of responses, some by superoxide and other by hydrogen peroxide or indirectly by heavy metal ions.

The budding yeast *S. cerevisiae* is a simple eukaryotic model system. It has an extremely well-characterized genetic system. Classical yeast genetics and molecular biology have made it possible for a tremendous amount of information to accumulate over the years, covering almost every aspect of biological research. Many basic and fundamental biological processes can be found in *S. cerevisiae*, and most of them are conserved among yeast, plants and animals. Studies of yeast stress responses will undoubtedly increase our understanding of how eukaryotic cells sense, respond and resist stress conditions. Historically, *S. cerevisiae* has been widely used in beer brewing, wine making, food production and synthesis of many valuable compounds. A complete knowledge of yeast stress responses will help engineer strains and improve growth conditions, leading to higher yield and better quality.

An intrinsic aspect of yeast stress response is the phenomenon called acquired resistance. Cells can withstand a severe stress condition more easily when they are previously exposed to a mild form of the same stress. For instance, a short pre-treatment of cells with 0.7 M NaCl leads to an increase in the number of surviving cells when they
are subsequently exposed to 1.4 M NaCl (Blomberg and Adler, 1992). The same has been described for cells exposed to a severe heat shock when first treated with a mild, non-lethal rise in temperature (Coote et al., 1991), or in the case of hydrogen peroxide treatment prior to an oxidative stress challenge (Davies et al., 1995). Mild stress conditions most likely trigger the pertinent cellular responses in order to get cells prepared to cope with a severe stress.

Cross protection is considered the first evidence for the occurrence of a general stress response. For example, pre-treatment of cells with a mild osmotic shock conferred resistance to heat shock (Varela et al., 1992). Exposure of yeast to high ethanol concentrations or weak acids conferred thermostolerance (Coote et al., 1991). Cross protection is not always reciprocal. Treatment of yeast cells with the superoxide generating drug menadione evokes resistance to hydrogen peroxide, but the opposite treatment does not lead to cross protection (Jamieson, 1992). Similarly, pre-exposure to heat shock results in acquisition of ethanol resistance but the converse does not hold true (Coote et al., 1991). Therefore, not all the stress responses appear to be shared or to lead to a certain level of cross protection.
CHAPTER 2

LOSS OF TURGOR PRESSURE ACTIVATES THE HOG PATHWAY

2.1 INTRODUCTION

The yeast cell wall has a highly dynamic nature. Cell wall weakening is sensed by the Wsc and Mid families of membrane proteins and results in activation of the cell integrity pathway (Rajavel et al., 1999; Verna et al., 1997). In this chapter we present experimental evidence suggesting the HOG pathway is also implicated in cell wall organization.

Yeast Cell Wall

The yeast cell wall is a complex structure essential for cell growth and viability (Klis, 1994). It makes up to 15 to 30% of the dry weight of the cell and 25 to 50% of the volume (Orlean, 1997). The main components of the cell wall are mannoproteins and β-linked glucans. Some chitin is also present.

Mannoproteins are secreted glycoproteins commonly modified by the attachment of three kinds of carbohydrate side chains: N-linked oligosaccharide chains, O-linked oligosaccharide chains, or β-1,6 glucan chains (Herscovics and Orlean, 1993;
Mannoproteins are heavily glycosylated, often containing 50 to 95% carbohydrate by weight (van der Vaart et al., 1995). As the exterior layer of the yeast cell wall, mannoproteins function as a barrier to retain periplasmic proteins and protect against damaging enzymes from outside.

β-linked glucans account for approximately half of the dry weight of yeast cell wall (Lipke and Ovalle, 1998). They provide the mechanical strength to maintain cell shape. Cells treated with pronase rapidly lose the mannoprotein layer of the cell wall, but they still maintain their shape (Zlotnik et al., 1984). Based on the chemical linkage, β-glucans can be divided into β-1,3 glucans and β-1,6 glucans. The β-1,3 glucans consist of predominantly 1,3-linked glucose residues, with only 3% 1,6-linked glucose residues at branch points (Manners et al., 1973a). The average degree of polymerization is 1,500, corresponding to a molecular mass of 240,000 and a maximum fiber length of 600 nm (the length of a yeast cell is about 3 μm). The β-1,3 glucans are synthesized by plasma membrane bound complexes, which release the polymers through the plasma membrane as they are formed (Cabib et al., 1982). The β-1,6 glucans are highly branched molecules comprised largely of 1,6-linked glucose residues with a small portion of 1,3-linked glucose residues. The average size of β-1,6 glucans is 140-200 residues per molecule, much shorter than that of β-1,3 glucans (Boone et al., 1990; Manners et al., 1973b). β-1,6 glucans serve as the primary targets of yeast K1 killer toxin in vivo (Bussey, 1991; Hutchins and Bussey, 1983).
Chitin is a linear homopolymer of β-1,4-linked N-acetylglucosamine. It is glycosidically linked to non-reducing branches of the β-1,3 glucans and β-1,6 glucans (Kollar et al., 1997). The linkage between chitin and β-glucans is important for the stability of the cell wall, since cells treated with the chitin inhibiting drug calcofluor white are very sensitive to perturbation in the rest of the cell wall structure (Ram et al., 1994). Like β-1,3 glucans, chitin is synthesized by plasma membrane bound complexes and released to the periplasmic space as it is formed (Cabib et al., 1983). Interestingly, chitin synthesis appears to be cell cycle controlled, peaking during cytokinesis when septa forms between mother and daughter cells (Pammer et al., 1992; Shaw et al.,1991).

**Yeast K1 Killer Toxin**

Some yeast strains secrete killer toxins that kill other sensitive yeast strains. Among them, the K1 killer toxin system of *S. cerevisiae* is well characterized (Bussey, 1991). K1 killer strains secrete a small pore-forming toxin that kills other sensitive strains, while they themselves are immune to the toxin. K1 killer strains maintain a double-stranded RNA (dsRNA) viral genome of two distinct species: M₁, a medium-size dsRNA (1.8 kb) encoding the killer toxin and immunity precursors, and L-A, a larger dsRNA (4.6 kb) required for the replication and maintenance of M₁. These two dsRNAs are packaged separately with a common capsid protein (encoded by L-A) to form virus-like particles, which are transmitted cytoplasmically during vegetative growth and conjugation (Somers and Bevan, 1969).
A two-step model for killer toxin action came from the following observations (Hutchins and Bussey, 1983). K1 toxin can kill sphaeroplasts derived from some resistant mutant strains, and mutant forms of the toxin deficient in receptor binding retain the ability to kill sphaeroplasts. These findings suggest that the K1 toxin initially binds to a receptor at the cell surface, and in a subsequent and lethal step, inserts into the plasma membrane. The receptor molecule has been identified as β-1,6 glucan by several experiments. K1 toxin can bind to the β-1,6 glucanase sensitive fraction of yeast cell wall, which is either altered in structure or reduced in amount in the resistant mutant strains. In addition, β-1,6 glucan is a strong competitive inhibitor of toxin action on sensitive cells. Affinity chromatography provides direct evidence of toxin interaction with β-1,6 glucan, as judged by a pH-dependent binding of K1 toxin to the β-1,6 glucan, pustulan.

Toxin-treated cells are found to leak intracellular ATP and K⁺ (Bussey and Sherman, 1973). Studies also show that H⁺/amino acid symport is rapidly inhibited, which causes a reduction in the electrochemical gradient across the plasma membrane, ultimately resulting in acidification of the cytoplasm and K⁺ efflux. Patch clamp analysis has directly demonstrated that highly purified toxin preparations are capable of forming ion conductance channels in both sphaeroplasts and artificial liposomes (Martinac et al., 1990). A potassium channel on the plasma membrane, Tok1p, has been identified as one of the molecular targets for K1 toxin (Ahmed et al., 1999; Sesti et al., 2001).
The HOG Pathway Activation

The HOG pathway is activated by an increase in extracellular osmolarity. Since both sorbitol and NaCl can trigger HOG pathway activation, the chemical nature of the osmolyte appears to be irrelevant (Brewster et al., 1993). High extracellular osmolarity causes many effects on yeast cells such as water loss (Brown, 1976) and cell shrinkage (Albertyn et al., 1994a). Water loss affects hydration of molecules, and cell shrinkage decreases intracellular space, both potentially altering protein conformation and activity. Unfolded proteins are known to stimulate stress responses, as in the case of heat shock (Becker and Craig, 1994; Piper, 1995).

High extracellular osmolarity also causes loss of turgor pressure. Under normal conditions, the internal osmolarity of growing cells is maintained higher than the external osmolarity. The resulting osmotic gradient across the plasma membrane brings in water for cell expansion and creates turgor pressure. However, if the external osmolarity suddenly increases, turgor pressure will drop accordingly. To test the hypothesis that loss of turgor pressure activates the HOG pathway, we use the pore-forming reagent amphotericin B as a tool.

Amphotericin B

From early 1950s until the discovery of azoles, the polyene antifungal agent amphotericin B (Figure 2-1) was the only drug to control serious fungal infection. It has
been proposed that the interaction of amphotericin B with membrane sterols results in the production of aqueous pores consisting of an annulus of eight amphotericin B molecules linked hydrophobically to the membrane sterols (de Kruijff and Demel, 1974; Holz, 1974). This configuration gives rise to a pore in which the polyene hydroxyl groups face inwards, leading to altered permeability, leakage of vital cytoplasmic components, and death of fungal cells. For example, the antibacterial RNA polymerase inhibitor rifampin, which demonstrates no intrinsic activity against fungi, appears quite active against several fungal species when used in combination with amphotericin B (Beggs et al., 1976). This synergistic activity has been attributed to increased uptake of the rifampin into fungal cells resulting from the action of amphotericin B on the plasma membrane. Similar synergism has also been observed between amphotericin B and 5-fluorocytosine in a murine model of candidiasis (Polak et al., 1982). The fatty acyl composition of membrane phospholipids might also contribute to the polyene susceptibility of fungi (HsuChen and Feingold, 1973).
Figure 2-1. Amphotericin B.
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### Table 2-1. Strains and Plasmids

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<th>Strains</th>
<th>Genotype</th>
<th>Source</th>
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<tr>
<td>T158C/S144a</td>
<td>MATα/MATα HIS4/his4c-864 ADE2/ade2-5</td>
<td>H. Bussey</td>
</tr>
<tr>
<td>W303</td>
<td>MATα ura3-1 ade2-1 trp1-1 his3-11 leu2-3,112 can1-100</td>
<td>lab stock</td>
</tr>
<tr>
<td>hog1Δ</td>
<td>MATα hog1Δ::TRP1</td>
<td>lab stock</td>
</tr>
<tr>
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<td>MATα pbs2Δ::LEU2</td>
<td>lab stock</td>
</tr>
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<td>P. Hieter</td>
</tr>
<tr>
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<td>URA3, CEN, PBS2</td>
<td>J. Brewster</td>
</tr>
<tr>
<td>pJB42</td>
<td>URA3, 2μ, PBS2</td>
<td>J. Brewster</td>
</tr>
</tbody>
</table>
Yeast Transformation

Yeast transformation was accomplished using the lithium acetate – polyethylene glycerol (PEG) procedure (Chen et al., 1992). Yeast culture was grown overnight in YEPD or appropriate dropout medium. 0.5 ml culture was spun down in the 1.5-ml Eppendorf tube at top speed for 10 seconds, and the supernatant was removed completely by aspiration. Cells were re-suspended in 100 µl one-step buffer (0.2 M LiAc, 0.1 M dithiothreitol (DTT), 40% PEG). 50 ng to 1 µg foreign DNA was added along with 5 µg sheared/boiled/chilled salmon sperm DNA as the carrier. The Eppendorf tube was vortexed and then incubated at 45°C for 30 minutes. Cells were spun down again, re-suspended in 0.2 ml YEPD, and plated on selective medium.

Competent Cell Preparation

CaCl₂ competent cells: A single bacterial colony was picked from a fresh plate and inoculated in 3 ml LB medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl) over night. 500 µl of the overnight culture was transferred into 50 ml fresh LB medium and grown until OD₆₀₀ reached 0.4. The culture was poured into a 50 ml pre-chilled, sterile conical centrifuge tubes, and kept on ice for 5 to 10 minutes. Cells were spun down at the speed of 1,600 g in a 4°C centrifuge for 7 minutes. The supernatant was discarded and cells were re-suspended in 4 ml ice-cold, filter-sterilized CaCl₂ solution (60 mM CaCl₂, 15% glycerol, 10 mM PIPES buffer, pH7.0). Cells were centrifuged again at 4°C for 5 minutes, re-suspended in 4 ml ice-cold CaCl₂ solution, and kept on ice for 30 minutes.
Cells were spun down again, re-suspended in 2.5 ml CaCl₂ solution, and put into sterile, pre-chilled Eppendorf tubes as 50 μl aliquots. All the tubes were flash frozen in liquid nitrogen and stored at −80°C up to one year.

Electroporation competent cells: A single colony was picked and grown in liquid YENB medium (7.5 g/L yeast extract, 8g/L nutrient broth). 10 ml overnight culture was added to 1 L fresh YENB medium and grown at 37°C until OD₆₀₀ 0.5 to 0.9. The culture was chilled on ice for 5 minutes. Cells were harvested at 4000 g for 10 minutes at 4°C, washed twice in 100 ml ice-cold sterile water, and re-suspended in 20 ml ice-cold 10% glycerol. After centrifugation at 4000 g for 10 minutes, cells were re-suspended in 2-3 ml ice-cold glycerol. 40 μl/tube aliquots were made and flash frozen in liquid nitrogen. These competent cells were stored at −80°C and good for one year.

**Bacterial Transformation**

CaCl₂-competent cells: 1-3 μl DNA was added to the 50-μl aliquot of competent cells and mixed gently. The tube was left on ice for 30 minutes. Cells were heat shocked for 1 minute at 42°C and immediately transferred to ice. 1 ml LB medium was added and cells were grown at 37°C for 1 hour with shaking. A proper amount of cells were plated on LB medium supplemented with antibiotic and incubated overnight at 37°C.

Electroporation-competent cells: 1-3 μl DNA was added to the 40-μl aliquot of competent cells and mixed well. The tube was left on ice for approximately 1 minute.
Cells were transferred to a cold 0.1 cm electroporation cuvette (Bio-Rad, Hercules, CA) and electroporated in a Bio-Rad Gene Pulser according to the manufacturer’s instruction. The cuvette was removed from the chamber immediately and 1 ml SOC medium (20 g/L tryptone, 5 g/L yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) was added. Cells were transferred to a sterile 1.5-ml Eppendorf tube and grown for 1 hour at 37°C with shaking. A proper amount of cells were plated on LB medium supplemented with antibiotic and incubated overnight at 37°C.

Large Scale Toxin Preparation

A single colony of the killer strain T158C/S14a was inoculated in 10 ml Halvorson minimal medium and grown overnight at 30°C until it reached the stationary phase. 1 ml overnight culture was added to each of 3 flasks containing 100 ml Halvorson minimal medium, and grown at 30°C until the cultures reached the stationary phase. 12 big flasks were prepared, each containing 1.6 L Halvorson minimal medium with 5% glycerol. 20 ml overnight culture was added to each of the 12 big flasks, and grown for 36 hours at 18°C until late log phase was reached. The cultures were kept on ice from this point. Cells were spun down at 1000 g for 10 minutes and discarded. The 20 L supernatant was concentrated using an Amicon ultra-filtration unit with a PM10 membrane and 20 p.s.i. pressure. The filtration process took about 36 hours to complete. After the ultra-filtration unit was disassembled, the membrane was washed repeatedly using a total of 20 ml ice-cold toxin storage buffer (15% glycerol, 50 mM sodium sodium acetate, pH 4.7). The 20
ml toxin was spun in a refrigerated centrifuge at 2500 g for 10 minutes to remove any remaining cells or debris, and sterilized using a 0.45 μm filter.

**Seeded Plate Test**

The seeded plate test was described previously (Brown et al., 1993a). Yeast cultures were grown to mid-log phase and cells were counted using a hemacytometer. 1 x 10⁶ cells were spun down and re-suspended in 100 μl sterile water. The cells were added to 10 ml of Halvorson buffered seeded plate medium (5 g/L yeast extract, 5 g/L peptone, 20 g/L glucose, 200 ml/L Halvorson 5X salt solution, 0.001% methylene blue, 10 g/L agar), which was melted and held at 45°C. The cells and medium were mixed well and quickly poured into a Petri dish and allowed to cool to room temperature. 20 μl toxin was spotted on the surface of the solidified medium and the plate was left at room temperature overnight. The plate was moved to the 30°C incubator on the following morning and grown for 24 hours. The size of the killing zone was then measured.

**Cellular Turgor Pressure Measurement**

Cellular turgor pressure (Π) was calculated using the formula below.

\[
Π = R \times T \times \text{Osmolarity}
\]

R: the universal molar gas constant, 0.082L.atm.K⁻¹.mol⁻¹.

T: temperature of the solution in degree Kelvin (°C + 273).
Osmolarity: intracellular osmolarity (Osm).

The intracellular osmolarity was determined using an automatic osmometer (MICRO-OSMETTE™, Model No. 5004, Precision Systems Inc., Natick, MA). Yeast cultures were grown to mid-log phase. Cells were spun down and the osmolarity of the medium (O₁) was measured. The same amount of sample was boiled for 1.5 minutes and cell debris was spun down. The osmolarity of the supernatant (O₂) was measured and the increase in osmolarity (ΔO) after cell lysis was determined (ΔO = O₂ – O₁). The intracellular osmolarity was higher than the osmolarity of the medium, and the difference between them was calculated by multiplying ΔO with the concentration factor (the total volume of medium divided by the total volume of cells). The volume of a single yeast cell was estimated to be ~ 0.3 pL (Gancedo and Gancedo, 1973), which was multiplied by the cell number to determine the total volume of cells.

**Western Blot Analysis**

Yeast cells were grown and treated as described in the text and figure legends. Cells were spun down by centrifugation and the supernatant was aspirated. Cell pellets were re-suspended in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 50 mM sodium fluoride, 0.1 mM sodium vanadate) plus protease inhibitors (1 mM phenylmethylsulfonylfluoride, 50 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin). Glass beads (525-600 microns, Sigma, St. Louis, MO) were added, and cells were lysed by three rounds of agitation, 50 seconds each, using a
bead-beater (BioSpec, Bartlesville, OK). Extracted proteins were separated from cell
debris by 10-minute spin at top speed in a refrigerated microcentrifuge. The supernatant
was added to the same volume of 2X SDS sample buffer (125 mM Tris-HCl, pH 6.8,
6.7% SDS, 10% glycerol, 10% β-mercaptoethanol, 0.01% bromophenol blue) and boiled
for 5 minutes. The Bradford reagent (BioRad) was used to determine the protein
concentration.

Equal amount of proteins were loaded into each lane of a 10% SDS-PAGE gel with a
5% stacking gel. The electrophoresis was run at 125 V for about 1 hour. The gel was
briefly washed in the transfer buffer (3.03 g/L Tris, 14.4 g/L glycine, 0.1% SDS), and
proteins were transferred to a nitrocellulose membrane (Schleicher & Schull, Keene, NH)
using a semi-dry transfer apparatus (BioRad). The transfer took place at 20 V for 1 hour.

Following the transfer, the membrane was washed with 25 ml TBS solution (2.42 g/L
Tris-HCl, pH 7.6, 8 g/L NaCl) for 5 minutes and incubated in 25 ml blocking buffer
(TBS plus 0.1% Tween-20 and 5% nonfat dry milk) for 1 hour at room temperature. Then
the membrane was washed three times for 5 minutes each with 15 ml TBS/T (TBS plus
0.1% Tween-20). 10 μl phospho-p38 MAPK antibody (New England Biolabs, Beverly,
MA) was added to 10 ml primary antibody dilution buffer (TBS plus 0.1% Tween-20 and
5% BSA) and incubated with the membrane over night at 4°C. On the next day, the
membrane was washed 3 times for 5 minutes each with 15 ml TBS/T. 5 μl horseradish
peroxidase-conjugated secondary antibody (New England Biolabs) was added to 10 ml
blocking buffer and incubated with the membrane for 1 hour at room temperature. The
membrane was again washed 3 times for 5 minutes each with 15 ml TBS/T. For
detection, equal volumes of ECL reagents 1 and 2 (Amersham Pharmacia Biotech,
Piscataway, NJ) were mixed and poured on the membrane. Membrane was drained of
excessive developing solution after 1 minute at room temperature, wrapped in Saran
Wrap, and exposed to X-ray film.

2.3 RESULTS

HOG Pathway Mutants Are Hypersensitive to K1 Killer Toxin

We used the seeded plate assay to examine the sensitivity of HOG pathway mutants
to K1 killer toxin. K1 toxin is thought to first bind the β-1,6 glucans of yeast cell wall and
then insert itself into the plasma membrane (Hutchins and Bussey, 1983). Changes in
killer toxin sensitivity often reflect alterations in the cell wall structure.

To quantify the assay, we first determined the relationship between the concentration
of K1 toxin and the size of the killing zone. 1 x 10^6 W303 cells were added into the
buffered agar medium, and 20 µl K1 toxin of various concentrations was spotted on the
plate. The vital stain, methylene blue, was included in the media to highlight the “killing
zone”, appearing as a blue halo of dead cells (Figure 2-2A). A reduced zone size, or
confluent lawn of cells, indicates strong resistance to killer toxin, while an enlarged zone
size indicates hypersensitivity. It was determined that the diameter of the killing zone is in linear relationship with the logarithm of K1 toxin concentration (Figure 2-2B).

We then used the same assay to test HOG pathway mutants for their sensitivity to K1 toxin. The diameter of the killing zone was used as the measurement of sensitivity, with the sensitivity of the isogenic wild-type strain arbitrarily set to be 100% (Figure 2-3). Our data suggests that HOG pathway mutants are hypersensitive to K1 toxin.

**PBS2 Overexpression Leads to Enhanced Resistance to K1 Killer Toxin**

Since deficiency in various HOG pathway genes causes hypersensitivity to K1 toxin, we next tested whether overexpression of HOG pathway genes could lead to enhanced resistance. For comparison, the *PBS2* gene was cloned in a single-copy yeast vector and a high-copy yeast vector respectively, and these two constructs (pJB41 and pJB42) were both transformed into the *pbs2Δ* mutant strain. An empty high-copy yeast vector (pRS426) was also transformed into the *pbs2Δ* mutant strain and used as a control. As shown in Figure 2-4, a single copy of the *PBS2* gene restored the resistance to normal level, and multiple copies of the *PBS2* gene led to enhanced resistance to K1 toxin. This dosage-dependent resistance to K1 toxin suggests HOG pathway genes such as *PBS2* are important for K1 toxin resistance.
Figure 2-2. Seeded plate test. (A) Killing zone formation. (B) Linear relationship between killing zone diameter (cm) and the logarithm of K1 killer toxin concentration. C₀ represents the concentration of the most diluted K1 killer toxin sample (designed as 1X).
Figure 2-3. HOG pathway mutants are hypersensitive to K1 toxin. 20 μl concentrated K1 toxin was spotted on plates containing 1 x 10^6 yeast cells. The diameters of killing zones were determined and used as the measurement of sensitivity, with the sensitivity of the wild-type strain W303 arbitrarily set to be 100%. The error bars represented standard deviation.
Figure 2-4. *PBS2* overexpression leads to enhanced resistance to K1 toxin.

An empty vector (A), a single-copy vector expressing *PBS2* (B), and a high-copy vector expressing *PBS2* (C) were transformed into the *pbs2Δ* mutant strain and tested for resistance to K1 toxin.
Amphotericin B Treatment Lowers the Cellular Turgor Pressure

As a widely used polyene antifungal agent, amphotericin B interacts with sterols to form aqueous pores on the plasma membrane (Ghannoum and Rice, 1999). Therefore, it is likely that amphotericin B treatment would lead to a decrease in cellular turgor pressure. This was tested through direct measurement.

Cellular turgor pressure under various growth conditions was determined using an automatic osmometer. The presence of glucose increases the osmolarity of growth medium, and as expected, the turgor pressure of yeast cells grown in glucose-containing medium (YEPD) is higher than that of those cells grown in medium without any glucose (YEP). Treatment with 1 µg/ml amphotericin B lowers the cellular turgor pressure dramatically (Figure 2-5). This decrease could be due to leakage of cellular components.

Amphotericin B Treatment Activates the HOG Pathway

The effect of amphotericin B on turgor pressure makes it a useful tool to test our hypothesis that loss of turgor pressure activates the HOG pathway. Activation of the HOG pathway is best represented by an increase in Hog1p phosphorylation. As shown in Figure 2-6, the phosphorylation level of Hog1p increases after amphotericin B treatment, suggesting activation of the HOG pathway. There are two in-coming branches upstream
of the HOG pathway, one involving Sln1p, Ypd1p, Sskn1p, Ssk2p, Ssk22p, and the other involving Sho1p, Cdc42p, Ste20p, Ste 50p, Ste11p (Figure 1-2). Deficiency in either one of these two branches does not block the activation of the HOG pathway by osmotic stress. In contrast, the Sln1p branch appears to transmit most of the signal generated by amphotericin B treatment. Blocking the Sln1p branch by deletion of SSK1 or SSK2/22 greatly reduces the phosphorylation level of Hog1p, while blocking the Sho1p branch has little effect (Figure 2-6).

2.4 DISCUSSION

The HOG Pathway and Yeast Cell Wall

In this chapter we described a new phenotype of HOG pathway mutants, hypersensitivity to K1 killer toxin. HOG pathway mutants are more sensitive to K1 toxin than the isogenic wild-type strain, evidenced by enlarged sizes of killing zones (Figure 2-3). Although the molecular basis for killer toxin hypersensitivity is still not clear, it is known that β-1,6 glucans in the yeast cell wall are the receptors for K1 killer toxin. Thus, one can imagine that changes in the yeast cell wall that increase functional toxin receptors might lead to hypersensitivity. It is known that reduced levels and/or altered structures of β-1,6 glucans can lead to killer toxin resistance (Bussey, 1991). Even a modest reduction in the β-1,6 glucan level can result in a very strong resistance phenotype (Brown and Bussey, 1993; Roemer and Bussey, 1991).
Figure 2-5. Amphotericin B treatment lowers cellular turgor pressure.

Wild-type W303 yeast cells were grown in YEP medium, YEPD medium, or YEPD medium containing 1 μg/ml amphotericin B. Samples were taken at different time points and cellular turgor pressure was measured as described in Materials and Methods. The error bars represented standard deviation.
<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>ssk2,22Δ</th>
<th>sho1Δ</th>
</tr>
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<tr>
<td>Control</td>
<td>Amp B</td>
<td>NaCl</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>NaCl</td>
<td>NaCl</td>
</tr>
</tbody>
</table>

Figure 2-6. Amphotericin B treatment activates the HOG pathway. Different yeast strains were treated with 1 μg/ml amphotericin B for 10 minutes (Amp B), 400 mM NaCl for 10 minutes (NaCl), or left untreated (Control). Hog1p phosphorylation was detected using the phospho-p38 MAPK antibody from New England Biolabs.
The cell integrity pathway regulates cell wall synthesis. Signals of cell wall weakening are sensed by the Wsc and Mid families of membrane proteins and relayed via Rho1p to the cell integrity pathway, resulting in enhanced expression of many cell wall-related genes (Rajavel et al., 1999; Verna et al., 1997). However, evidence is also accumulating that the HOG pathway modulates cell wall organization. The yeast Golgi enzyme, α-1,3-mannosyltransferase (Mnn1p), catalyzes the addition of terminal α-1,3-mannose residues to N- and O-linked oligosaccharides. A mutation in HOG1 perturbs Mnn1p localization, resulting in its loss from early Golgi compartments and a concomitant increase of Mnn1p in late Golgi compartments (Reynolds et al., 1998). Cullen et al. have proposed that a pathway involving Sho1p and other components from the mating pathway and filamentation-invasion pathway contributes to maintenance of cell wall integrity in vegetative cells (Cullen et al., 2000). One of their key observations is that defects in protein glycosylation lead to Sho1p-dependent activation of the pheromone-responsive FUS1 gene, whereas neither high osmolarity nor nutrient limitation has the same effect. Recently Kapteyn et al. have shown that low extracellular pH affects the molecular organization of the cell wall, and these low pH-induced structural changes rely on a functional Hog1p (Kapteyn et al., 2001). In our experiments, we observed a dosage-dependent effect of the PBS2 gene on K1 toxin resistance. Together, these data indicate that the HOG pathway participates in the regulation of cell wall structure.
Activation of the HOG Pathway

Although the structure of the HOG pathway has been well determined, the exact mechanism by which the HOG pathway senses a change in extracellular osmolarity remains a mystery. Here we have presented evidence suggesting that the loss of turgor pressure is the physical parameter sensed by the HOG pathway. Turgor pressure is a stress that creates tension in the cell surface, and therefore alterations of turgor pressure may cause conformational changes in proteins associated with cell surface, including the cell wall, the plasma membrane, and even the plasma membrane-associated actin cytoskeleton. Two putative sensors of the HOG pathway, Sln1p and Sho1p, are both membrane proteins. It is possible that loss of turgor pressure alters the conformation of Sln1p and/or Sho1p and starts signal transduction through the HOG pathway.

Studies of another yeast MAP kinase pathway, the cell integrity pathway, might also shed some light on the mechanism of pathway activation. The cell integrity pathway can be activated by different stresses such as heat shock, hypotonic stress and mating pheromone. Kamada et al. provided evidence that membrane stretch leads to activation of the cell integrity pathway. Chlorpromazine, an amphipathic drug that induces membrane stretch by inserting into the cytosolic face of the lipid bilayer, strongly activates the cell integrity pathway (Kamada et al., 1995). The putative sensors for the cell integrity pathway are the Wsc and Mid families of membrane proteins. As predicted from their amino acid sequences, these proteins all have long extracellular domains rich in cysteine, serine and threonine. The serine and threonine residues are highly O-mannosylated,
usually consisting of four or five mannosyl groups in α-1,2 and 1,3 linkage. The glycosylation prompts the peptide to adopt a stiff and extended conformation, and thus makes it long enough to span the periplasmic space between the plasma membrane and the cell wall (Rajavel et al., 1999; Verna et al., 1997). Therefore, despite their plasma membrane nature, changes in the cell wall could be sensed by these proteins and transformed into a signal to activate the pathway.
CHAPTER 3

HOG PATHWAY PLAYS AN IMPORTANT ROLE IN OXIDATIVE STRESS RESPONSE

3.1 INTRODUCTION

Homologues of the HOG pathway have been found in both fission yeast and mammalian cells (Widmann et al., 1999). They are both activated by multiple stresses, such as high osmolarity, oxidative stress, heat shock, and UV radiation. In a high-copy suppressor screen, TRX1 and TRX2 were identified as high-copy suppressors of the HOG pathway mutant pbs2-3 (Davenport et al., 1999). When expressed from a high-copy yeast vector, TRX1 and TRX2 both suppress pbs2-3 sensitivity to high osmolarity. TRX1 and TRX2 encode thioredoxins, small proteins important for yeast antioxidant defense. The identification of the thioredoxin genes as suppressors of a HOG pathway mutant raises the possibility that the S. cerevisiae HOG pathway might be involved in oxidative stress response. Experimental evidence presented in this chapter confirms this prediction.

Oxidative Stress and Yeast Defenses

The exposure to oxygen is essential but also dangerous for organisms that live in an aerobic environment. Oxidative stress has been linked to the process of aging and a
number of diseases (Ames et al., 1993; Halliwell, 1994). The detrimental effects of oxidative stress are mostly caused by a group of highly active molecules called reactive oxygen species (ROS), which include superoxide anion (O$_2^-$), hydroxyl radical (HO•), and hydrogen peroxide (H$_2$O$_2$). ROS are generated during normal cellular metabolism by the mitochondrial respiratory chain and H$_2$O$_2$-generating reactions catalyzed by oxidases (Gralla and Kosman, 1992). ROS can also originate from the presence of superoxidedenerating drugs such as plumbagin and menadione as well as pro-oxidants such as heavy metals (Halliwell and Gutteridge, 1984). ROS are capable of damaging DNA, producing sugar damage, single-strand breaks, abasic sites, and DNA-protein crosslinking (Demple and Harrison, 1994). ROS cause damage to lipids and proteins too. Lipid peroxidation impairs the structural integrity of membrane and increases membrane fluidity (Coyle and Puttfarcken, 1993). Oxidative damage to proteins involves oxidation of amino acids and protein crosslinking, which lead to increased proteolytic susceptibility and loss of biological activity (Stadtman, 1992).

To keep ROS at a physiological, unharmed level, yeast cells possess both enzymatic and non-enzymatic antioxidant defenses (Estruch, 2000), as listed in Table 3-1. The enzymatic antioxidant defenses include enzymes that are capable of removing ROS and/or repairing the damages caused by ROS. For example, catalase catalyzes the breakdown of H$_2$O$_2$ to H$_2$O and O$_2$. Two catalases exist in yeast, catalase A and catalase T, encoded by the CTA1 and CTT1 genes, respectively. Catalase A is in the peroxisome, where it removes H$_2$O$_2$ produced by fatty acid β-oxidation (Jamieson, 1998). The physiological role of the cytosolic catalase T is less clear, though CTT1 expression is
induced by oxidative stress, osmotic stress, and starvation (Davidson et al., 1996; Schuller et al., 1994). Superoxide dismutase (SOD) disproportionates two molecules of superoxide anion to \( \text{H}_2\text{O} \) and \( \text{O}_2 \). *S. cerevisiae* contains two forms of SOD. Sod1p is a cytoplasmic, \( \text{Cu}^{2+} \)- and \( \text{Zn}^{2+} \)-bound SOD, while Sod2p is a mitochondrial, \( \text{Mg}^{2+} \)-bound SOD. Sod2p is the primary defense against the toxicity of superoxide generated in the mitochondria under physiological conditions. Accordingly, sod2 mutants show hypersensitivity towards oxygen, and this hypersensitivity can be suppressed by mutations that block respiration (Guidot et al., 1993). The roles of Sod1p are more complicated. Mutants lacking *SOD1* are sensitive to oxygen (van Loon et al., 1986) and superoxide-generating drugs, such as plumbagin (Jamieson et al., 1994) and paraquat (Gralla and Kosman, 1992). In addition, they are also auxotrophic for some amino acids including cysteine and methionine (Strain et al., 1998). Another role of Sod1p appears to be buffering intracellular \( \text{Cu}^{2+} \), since sod1 mutants show increased sensitivity to copper (Culotta et al., 1995).

Glutathione is the most abundant low-molecular-mass intracellular thiol compound. Depletion of cellular glutathione leads to increased sensitivity to hydrogen peroxide. Glutathione biosynthesis requires the *GSH1* and *GSH2* genes, encoding \( \gamma \)-glutamylcysteine synthetase and glutathione synthase respectively. Cells lacking *GSH1* are hypersensitive to hydrogen peroxide (Grant et al., 1996). In addition, gsh1 mutants cannot grow in non-fermentable carbon sources. Cells lacking *GSH2* are not sensitive to oxidative stress, suggesting that \( \gamma \)-glutamylcysteine can be a substitute anti-oxidant for glutathione (Moradas-Ferreira et al., 1996).
Table 3-1. Yeast Antioxidant Defenses

<table>
<thead>
<tr>
<th></th>
<th>Proteins</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glutathione</strong></td>
<td>Gsh1p</td>
<td>glutathione synthesis</td>
</tr>
<tr>
<td></td>
<td>Gsh2p</td>
<td>glutathione synthesis</td>
</tr>
<tr>
<td><strong>Thioredoxin</strong></td>
<td>Trx1p</td>
<td>thioredoxin (cytoplasm)</td>
</tr>
<tr>
<td></td>
<td>Trx2p</td>
<td>thioredoxin (cytoplasm)</td>
</tr>
<tr>
<td></td>
<td>Trr1p</td>
<td>thioredoxin reductase (cytoplasm)</td>
</tr>
<tr>
<td></td>
<td>Trx3p</td>
<td>thioredoxin (mitochondria)</td>
</tr>
<tr>
<td></td>
<td>Trr2p</td>
<td>thioredoxin reductase (mitochondria)</td>
</tr>
<tr>
<td><strong>Glutaredoxin</strong></td>
<td>Grx1p</td>
<td>glutaredoxin</td>
</tr>
<tr>
<td></td>
<td>Grx2p</td>
<td>glutaredoxin</td>
</tr>
<tr>
<td></td>
<td>Grx3p</td>
<td>glutaredoxin</td>
</tr>
<tr>
<td></td>
<td>Grx4p</td>
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</tr>
<tr>
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<td>Grx5p</td>
<td>glutaredoxin</td>
</tr>
<tr>
<td><strong>Superoxide dismutase</strong></td>
<td>Sod1p</td>
<td>superoxide dismutase (cytoplasm)</td>
</tr>
<tr>
<td></td>
<td>Sod2p</td>
<td>superoxide dismutase (mitochondria)</td>
</tr>
<tr>
<td><strong>Catalase</strong></td>
<td>Cta1p</td>
<td>catalase (peroxisome)</td>
</tr>
<tr>
<td></td>
<td>Ctt1p</td>
<td>catalase (cytoplasm)</td>
</tr>
</tbody>
</table>
Thioredoxins are small proteins with the conserved sequence (Trp-Cys-Gly-Pro-Cys) in the active site. The dithiol group is able to catalyze the reduction of disulfides in some proteins. The thioredoxin system is composed of thioredoxins and thioredoxin reductases. Two thioredoxins, Trx1p and Trx2p, and one thioredoxin reductase Trr1p are found in the cytoplasm, while thioredoxin Trx3p and thioredoxin reductase Trr2p are found in the mitochondria (Pedrajas et al., 1999). Besides sensitivity to hydrogen peroxide, deletion of TRX1 and TRX2 also causes defects in methionine/cysteine metabolism and in the cell cycle (Muller, 1991). Mutants lacking TRR1 or TRR2 are hypersensitive to hydrogen peroxide (Lee et al., 1999b; Machado et al., 1997).

Acting in a similar way to thioredoxins, glutaredoxins consist of two subfamilies that differ in the number of cysteine residues at the active site. The first subfamily has two members, Grx1p and Grx2p. Grx1p protects cells against superoxide anions and Grx2p protects cells against hydrogen peroxide (Luikenhuis et al., 1998). The second subfamily includes three additional members, Grx3p, Grx4p and Grx5p. Among them, Grx5p appears to be important in both normal growth conditions and after exposure to hydrogen peroxide and menadione (Rodriguez-Manzaneque et al., 1999).

**Transcriptional Regulation of Oxidative Stress Response**

Several transcription factors are involved in the regulation of oxidative stress response. The best characterized are Skn7p and Yap1p. Skn7p contains a receiver motif
similar to the response protein of the bacterial two-component system (Brown et al., 1994), indicating its activity could be regulated by an upstream histidine kinase. In this connection, it has been reported that the Sln1p-Ypd1p branch of the HOG pathway regulates Skn7p activity (Ketela et al., 1998; Li et al., 1998). Skn7p also contains a region homologous to the DNA-binding domain of the heat shock factor Hsf1p (Brown et al., 1993b; Morgan et al., 1995), suggesting a direct role in gene expression. A Gal4p-Skn7p hybrid construct, consisting of the Gal4p DNA-binding domain fused to Skn7p receiver and activation domains, is sufficient to induce the GAL1-lacZ reporter gene expression upon oxidative stress (Morgan et al., 1995).

Yap1p is a b-ZIP transcription factor. When overexpressed, YAP1 is able to confer resistance to several toxic agents (Grey and Brendel, 1994; Hussain and Lenard, 1991; Schnell et al., 1992); conversely, yap1 mutants are hypersensitive to oxidative stress (Schnell et al., 1992). Under normal growth conditions, Yap1p is localized in the cytoplasm. It translocates to the nucleus in response to oxidative stress (Kuge et al., 1997). Once inside the nucleus, Yap1p binds to the Yap1p response element (YRE), which has been found in the promoters of several genes encoding antioxidant defense (Toone and Jones, 1999).

Both ska7 and yap1 mutants show increased sensitivity to oxidative stress (Krems et al., 1995; Krems et al., 1996; Kuge and Jones, 1994), probably because they are required for the expression of many genes encoding antioxidant defense. Two-dimensional gel electrophoresis studies have revealed that the induction of genes such as TSA1, TRRI or
TRX2 requires both Skn7p and Yap1p (Godon et al., 1998). In contrast, induction of other genes such as GLRI or GSH1 depends on Yap1p but not Skn7p. Recent DNA microarray experiments have also shown that Yap1p is indispensable for the strong induction of many genes by both hydrogen peroxide and menadione (Gasch et al., 2000). It regulates both Skn7p-dependent and Skn7p-independent gene induction in response to oxidative stress. Yap1p is also important for cadmium resistance, whereas Skn7p is not only dispensable but also likely to be a negative regulator of this response (Godon et al., 1998).

Transcription driven by the stress response element STRE can be induced by a variety of stress conditions such as osmotic stress, oxidative stress, nitrogen starvation and heat shock (Moskvina et al., 1998; Treger et al., 1998). The STRE-mediated transcription appears to be under negative control of protein kinase A (Kobayashi and McEntee, 1993; Marchler et al., 1993). Two homologous zinc-finger transcription factors, Msn2p and Msn4p, bind specifically to STRE and are required for the stress-induced transcriptional activation (Martinez-Pastor et al., 1996; Schmitt and McEntee, 1996). Importantly, msn2Δmsn4Δ double mutants are hypersensitive to various stresses, suggesting Msn2p and Msn4p are key components of the general stress response system (Martinez-Pastor et al., 1996; Schmitt and McEntee, 1996).
3.2 MATERIALS AND METHODS

Strains, Media and General Methods

Yeast strains used in this chapter are described in Table 3-2. All strains are of the W303 background. To disrupt the SKN7 gene, the skin7::HIS3 fragment was PCR amplified from an SKN7 disruption plasmid (a gift from Dr. L. H. Johnson) (Kuge and Jones, 1994). The PCR product was used to transform the wild-type strain. To disrupt the YAPI gene, the plasmid pSM27 (a gift from Dr. W. Scott Moye-Rowley) (Wemmie et al., 1997) was cut with EcoRI and used to transform the wild-type strain. Gene deletion was confirmed by PCR. Yeast growth media (YEPD and dropout media) were prepared as described in section 2.2. Common procedures were followed for PCR, DNA cloning, etc. (Sambrook et al., 1989). Standard techniques for yeast crosses, sporulation, dissection and propagation were used (Kraiser et al., 1994). Yeast transformation was accomplished using the lithium acetate – polyethylene glycerol (PEG) procedure (Chen et al., 1992).

Oxidant Sensitivity Assay

Two different methods were used to assay oxidant sensitivity. In the first method, overnight cultures were diluted to OD$_{600}$ 0.1 and grown to OD$_{600}$ 0.4. Cultures were diluted back to exactly OD$_{600}$ 0.1 and serial dilutions (1:1, 1:5, 1:25) were made in fresh
YEPD medium. 5 µl of each dilution was spotted on YEPD plates with or without hydrogen peroxide. In the second method, overnight cultures were diluted to OD₆₀₀ 0.05 and grown to exactly OD₆₀₀ 0.15. Cells were spun down and resuspended in the same amount of YEPD medium containing either hydrogen peroxide or plumbagin. Grown at 30°C for 1 hour, cells were spun down and washed once with phosphate-buffered saline (90 g/L NaCl, 1.44 g/L KH₂PO₄, 7.95 g/L Na₂HPO₄, pH 7.4). Serial dilutions (1:1, 1:10, 1:100, 1:1,000) were made in phosphate-buffered saline and 5 µl of each dilution was spotted on YEPD plates. Plates were incubated at 30°C for 2 days.

**Western Blot Analysis**

Western blot analysis was performed as described previously (see section 2.2). Briefly, protein extracts were prepared from treated and untreated cells. 20 µg protein of each sample was separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was washed in TBS buffer and incubated in the blocking buffer for 1 hour at room temperature. After three washes in TBS/T buffer, the membrane was incubated with phospho-p38 antibody at 4°C overnight. The membrane was washed again in TBS/T buffer and incubated with the secondary antibody for 1 hour at room temperature. Following three more washes in TBS/T buffer, ECL reagents were added to the membrane. The membrane was wrapped up and exposed to X-ray film.
<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
<td>hog1Δ</td>
<td>MATα hog1Δ::TRP1</td>
<td>lab stock</td>
</tr>
<tr>
<td>pbs2Δ</td>
<td>MATα pbs2Δ::LEU2</td>
<td>lab stock</td>
</tr>
<tr>
<td>ssk1Δ</td>
<td>MATα ssk1Δ::LEU2</td>
<td>lab stock</td>
</tr>
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</tr>
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<th>Plasmids</th>
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<td>URA3, 2μ, HOG1</td>
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</table>
Northern Blot Analysis

Yeast cells were grown and treated as described in the text and figure legends. Cells were spun down by centrifugation and the supernatant was aspirated. Cell pellets were flash frozen in a dry ice-ethanol bath and kept at −80°C until use. Cell pellets were thawed on ice and resuspended in 1 ml of ice-cold water. The 1 ml suspension was spun for 10 seconds at top speed in a 4°C microcentrifuge and the supernatant was discarded. To the pellet was added 200 μl glass beads, 350 μl ice-cold lysis buffer (0.3 M NaCl, 1 mM EDTA, 0.2% SDS and 10 mM Tris-HCl, pH 7.5), and 350 μl of phenol-chloroform-isopropanol (PCI, 25:24:1, pH 7.8). The tubes were then placed in a bead beater (BioSpec Products) and agitated for 50 seconds. This process was repeated 3 times with cooling on ice at the end of each pulse. The phases were separated by 10-minute spin at top speed in the 4°C microcentrifuge. The upper aqueous phase was transferred to a new tube and extracted twice with the same volume of PCI. After the PCI extraction, 1 ml ice-cold ethanol was added to the sample. RNA was allowed to precipitate at −20°C overnight, and collected by 4°C centrifugation at top speed for 20 minutes. The pellet was washed with 70% ethanol and dried at room temperature for 10 minutes. Once dry, RNA was re-suspended in DEPC-treated water. The nucleic concentration was determined by measuring the absorbance at 280 nm.

RNA samples were run on formaldehyde agarose gel consisting of the following: 1 g agarose, 100 ml 1X NBC buffer (0.05 M boric acid, 5 mM NaOH, 1 mM sodium citrate),
4 ml formaldehyde, and 5 μl ethidium bromide. 5 μg RNA of each sample was put in the 1.5-ml Eppendorf tube before 10 μl formamide, 3 μl 10X NBC buffer (0.5 M boric acid, 50 mM NaOH, 10 mM sodium citrate), and 3 μl formaldehyde were added. The mixtures were heated at 70°C for 5 minutes. Prior to loading, 3 μl of 10X loading buffer (0.1 M EDTA, 0.25% bromophenol blue, 15% ficoll) was added to each tube. Samples were loaded on the gel and run at 80-100 V in 1X NBC buffer. Once the bromophenol blue reached 2/3 length of the gel, electrophoresis was stopped. A photograph was taken under UV illumination to determine the RNA quality and verify equal loading.

The formaldehyde agarose gel was washed in 20X SSC (175.3 g/L NaCl, 88.2 g/L sodium citrate, pH 7.0) for 5 minutes. The RNA was then transferred to a Hybond-N+ Nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ) in 20X SSC overnight. Once the transfer was complete, a Stratalinker (Stratagene, La Jolla, CA) was used to crosslink the RNA to the membrane.

The desired genes were PCR amplified from genomic DNA, gel purified, and then used as template for generating radioactively-labeled probes. A random decamer kit (Ambion, Austin, TX) was used along with $^{32}$P-α-dATP to produce radioactive DNA probes. The newly synthesized probe was denatured by boiling for 5 minutes and flash frozen in dry ice-ethanol bath. The probe was added to the ULTRAhyb hybridization buffer (Ambion) and hybridized at 42°C overnight. After the hybridization, the membrane was washed twice in the low stringency wash buffer and then twice in the high stringency buffer (Ambion). The membrane was left on filter paper and allowed air dry. It
was then wrapped in a piece of Saran Wrap and exposed to a Fuji phosphoimaging plate. After the initial exposure was obtained, the membrane was exposed to X-ray film.

3.3 RESULTS

HOG Pathway Mutants Are Hypersensitive to Hydrogen Peroxide and Plumbagin

To determine whether the HOG pathway is involved in oxidative stress response, we tested the sensitivity of HOG pathway mutants to different oxidants. We spotted yeast cells on hydrogen peroxide-containing medium and compared their growth with the wild-type strain. HOG pathway mutants did not grow well on YEPD medium containing 1.2 mM hydrogen peroxide (Figure 3-1). We also tested the superoxide-generating drug plumbagin, but the same assay did not produce consistent results. Therefore, we designed a second assay, in which yeast cells were treated with plumbagin in liquid culture and then spotted on YEPD medium to determine the survival rate. This assay worked well for both hydrogen peroxide and plumbagin (Figure 3-2). HOG pathway mutants have a lower survival rate to 4 mM hydrogen peroxide or 60 μM plumbagin than the isogenic wild-type strain.
Figure 3-1. HOG pathway mutants are hypersensitive to hydrogen peroxide. All yeast cultures were adjusted to OD₆₀₀ 0.1 before serial dilutions were made (1:1, 1:5, 1:25). 5 µl of each dilution was spotted on YEPD plate (Control) or hydrogen peroxide-containing plate (1.5mM H₂O₂). Plates were incubated at 30°C for two days before pictures were taken.
Figure 3-2. HOG pathway mutants are hypersensitive to the superoxide-generating drug plumbagin. All yeast cultures were adjusted to OD$_{600}$ 0.15 before 60μM plumbagin was added. Cells were grown at 30°C for an hour and serially diluted in PBS (1:1, 1:10, 1:100, 1:1000). 5 μl of each dilution was spotted on YEPD plates. Untreated cells were used as a control. Plates were incubated at 30°C for two days before pictures were taken.
Oxidative Stress Activates the HOG Pathway

The hypersensitivity of HOG pathway mutants to hydrogen peroxide and the superoxide-generating drug plumbagin indicates that the HOG pathway is involved in resistance to oxidative stress. To determine whether the HOG pathway plays an active role in oxidative stress response, we added various oxidants to yeast culture and measured Hog1p phosphorylation level using the phospho-p38 antibody. Treatment with hydrogen peroxide or the superoxide-generating drugs plumbagin and menadione all resulted in activation of the HOG pathway (Figure 3-3). Hog1p phosphorylation starts increasing 5 minutes after addition of oxidants, and usually reaches the maximal level around 30 minutes. Even at the 1-hour time point, Hog1p phosphorylation level is still higher than the basal level. The two incoming branches both contribute to the activation of the HOG pathway, evidenced by a decrease of Hog1p phosphorylation in sho1Δ and ssk1Δ mutants (Figure 3-4). However, the Sln1p branch that involves Ssk1p seems to be more important for signal transduction.

The HOG Pathway Acts Parallel to Skn7p and Yap1p in Oxidative Stress Response

Skn7p and Yap1p are two transcription factors important for protecting yeast cells from oxidative stress (Lee et al., 1999a). Cells with mutation in either of these two genes are hypersensitive to oxidative stress (Krems et al., 1995; Krems et al., 1996; Kuge and Jones, 1994). Skn7p activity is modulated by the Sln1p-Ypd1p branch and contributes
Figure 3-3. Activation of the HOG pathway by various oxidants. *HOG1* was expressed from the high-copy yeast vector pRS426. Yeast cultures were grown to mid-log phase and treated with 1mM H$_2$O$_2$, 100mM menadione, or 3.5μM plumbagin. Samples were taken at the indicated time points. Protein samples were prepared and Western blots performed as described in MATERIALS AND METHODS.
<table>
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<tr>
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**Figure 3-4. Two incoming branches both contribute to HOG pathway activation.** All yeast strains were transformed with a high-copy yeast vector expressing *HOG1*. Yeast cultures were grown to mid-log phase when 1mM H₂O₂ was added. Samples were taken either before H₂O₂ addition or 20 minutes after H₂O₂ addition. Cells treated with 0.4M NaCl for 5 minutes were included as a positive control.
Figure 3-5. Additive effects of hog1Δ, skn7Δ and yap1Δ on H₂O₂ sensitivity. All yeast cultures were adjusted to OD₆₀₀ 0.1 before serial dilutions were made (1:1, 1:5, 1:25). 5 µl of each dilution was spotted on a YEPD plate (Control) or hydrogen peroxide-containing plate (1.2mM H₂O₂). Plates were incubated at 30°C for two days before pictures were taken.
to the regulation of the HOG pathway (Ketela *et al*., 1998; Li *et al*., 1998), but the exact role of Skn7p in the signal transduction pathway is still not clear. Since the HOG pathway, Skn7p and Yap1p are all involved in oxidative stress response, we want to determine whether the HOG pathway acts upstream or parallel to Skn7p and Yap1p. Double or triple mutants of *hog1Δ, skn7Δ* and *yap1Δ* were made and tested for hydrogen peroxide sensitivity. As shown in Figure 3-5, double mutants are more sensitive to hydrogen peroxide than any of the single mutants, indicating the effects of *hog1Δ, skn7Δ* and *yap1Δ* are additive. The data suggest the HOG pathway acts parallel to Skn7p and Yap1p in oxidative stress response. However, it does not rule out the possibility that the HOG pathway regulates the activity of Skn7p or Yap1p.

**Suppressors of *hog1Δ* Hypersensitivity to Oxidative Stress**

The Ssn6p-Tup1p co-repressor is important for negative regulation of stress gene expression (Smith and Johnson, 2000). Deletion of *SSN6* or *TUP1* suppresses *hog1Δ* sensitivity to high osmolarity (Marquez *et al*., 1998). We examined whether loss of the Ssn6p-Tup1p co-repressor also suppresses *hog1Δ* sensitivity to oxidative stress. The result shows that the introduction of the *ssn6Δ* mutation improves the growth of *hog1Δ* mutant on medium containing 2 mM hydrogen peroxide (Figure 3-6). Therefore, loss of the Ssn6p-Tup1p co-repressor suppresses *hog1Δ* sensitivity to oxidative stress.
**Figure 3-6.** *ssn6Δ* suppresses *hog1Δ* mutant sensitivity to *H₂O₂*. All yeast cultures were adjusted to OD₆₀₀ 0.1 before serial dilutions were made (1:1, 1:5, 1:25). 5 µl of each dilution was spotted on YEPD plate (Control) or hydrogen peroxide-containing plate (2mM *H₂O₂*). Plates were incubated at 30°C for two days before pictures were taken.
Sko1p, a transcription factor of the ATF/CREB family, is a downstream target of the HOG pathway. It is directly phosphorylated by the Hog1p MAPK at multiple sites within its NH2-terminal region (Proft et al., 2001). This phosphorylation disrupts the Sko1p-Ssn6p-Tup1p complex, thereby elevating the expression level of osmotic stress genes. We tested if loss of Sko1p suppresses hog1Δ sensitivity to oxidative stress. As shown in Figure 3-7, sko1Δ suppresses hog1Δ mutant sensitivity to both hydrogen peroxide and plumbagin. The hog1Δsko1Δ double mutant withstands 4 mM H2O2 and 60 μM plumbagin better than the hog1Δ single mutant.

Taken together, our data suggests the Ssn6p-Tup1p complex probably represses oxidative stress genes under normal growth conditions via the transcription factor Sko1p. Loss of the Ssn6p-Tup1p complex or Sko1p elevates the expression level of oxidative stress genes and leads to enhanced resistance towards oxidative stress.

The HOG Pathway Regulates the Transcriptional Activation of HSP12 in Oxidative Stress Response

Transcription of TRX2 and CTT1 are induced by osmotic stress, and the induction of these two genes is dependent on a functional HOG pathway (Schuller et al., 1994). TRX2 and CTT1 are also likely downstream targets of the HOG pathway in oxidative stress response, for their protein products are part of the yeast antioxidant defenses. We treated yeast cells with oxidants and examined the mRNA level of TRX2 and CTT1. Although
Figure 3-7. skolΔ suppresses hog1Δ mutant sensitivity to H₂O₂ and plumbagin.

All yeast cultures were adjusted to OD₆₀₀ 0.15 before 4mM H₂O₂ or 60μM plumbagin was added. Cells were grown at 30°C for an hour and serially diluted in PBS (1:1, 1:10, 1:100, 1:1000). 5 μl of each dilution was spotted on YEPD plates. Untreated cells were used as a control. Plates were incubated at 30°C for two days before pictures were taken.
TRX2 and CTT1 mRNA both increase after oxidant addition, this increase in their transcription is not affected by the deletion of HOGL (data not shown).

Raitt et al. have reported that a group of heat shock protein genes are strongly induced by oxidative stress (Raitt et al., 2000a). We observed that one of them, HSP12, requires a functional HOG pathway for its expression (Figure 3-8). HSP12 mRNA level rises after the addition of 0.2 mM H₂O₂ to the medium, and this H₂O₂-induced increase in HSP12 mRNA diminishes in the hog1Δ mutant. Interestingly, such a dependence on HOGL was not observed in the H₂O₂-induced transcription of HSP26, a closely related small heat shock protein gene. Our data suggests the HOG pathway only regulates a subset of the oxidative stress genes.

Msn2p and Msn4p Are Important for the Transcriptional Activation of HSP12 and HSP26 in Oxidative Stress Response

Msn2p and Msn4p are two zinc-finger transcription factors that mediate transcriptional activation through STRE. Since STRE is found in the promoters of heat shock protein genes, we expect that the STRE-binding transcription factors Msn2p and Msn4p contribute to the regulation of heat shock protein genes in oxidative stress response. Deletion of MSN2 and MSN4 dramatically reduces the hydrogen peroxide-induced transcription of HSP12 and HSP26 (Figure 3-9). Judging by the magnitude of decrease in mRNA levels, Msn2p and Msn4p-mediated transcriptional activation appears to be very important for expression of HSP12 and HSP26. This result shows that the
Figure 3-8. The HOG pathway regulates the transcriptional activation of *HSP12* in oxidative stress response. Wild-type strain (WT) and *hog1Δ* mutant were grown to mid-log phase when 0.2mM H₂O₂ was added. Samples were taken at the indicated time points. Total RNA was isolated and probed for *HSP12*, *HSP26*, or *ACT1* (loading control), as described in MATERIALS AND METHODS.
Figure 3-9. Msn2p and Msn4p are important for the transcriptional activation of *HSP12* and *HSP26* in oxidative stress response. Wild-type strain (WT), *hog1Δ*, and *msn2Δmsn4Δ* mutants were grown to mid-log phase when 0.2mM H₂O₂ was added. Samples were taken at the indicated time points. Total RNA was isolated and probed for *HSP12*, *HSP26*, or *ACT1* (loading control), as described in MATERIALS AND METHODS.
HOG pathway and Msn2/4p are both required for gene transcription in oxidative stress response, but it is not known whether the HOG pathway regulates Msn2p and Msn4p in this response.

3.4 DISCUSSION

MAP kinase pathways allow cells to quickly adapt to a constantly changing environment. This is especially important for yeast due to its non-motile existence. At least five MAP kinase pathways have been identified in *S. cerevisiae*, and they respond to different external stimuli (Gustin *et al.*, 1998). Among them, the HOG pathway is important for adaptation to high osmolarity (Brewster *et al.*, 1993). Upon exposure to high osmolarity, Hog1p is phosphorylated by Pbs2p and translocates from cytoplasm to nucleus. Once inside the nucleus, Hog1p activates transcription of osmotic stress genes such as *GPD1* (Albertyn *et al.*, 1994b; Schuller *et al.*, 1994).

In this chapter we have demonstrated that the HOG pathway plays an important role in oxidative stress response. Mutants lacking a functional HOG pathway are hypersensitive to hydrogen peroxide and the superoxide-generating drug plumbagin. We have also shown that addition of hydrogen peroxide and superoxide-generating drugs to the culture increases Hog1p phosphorylation. Although the two incoming branches of the HOG pathway both contribute to the activation of the HOG pathway, the Sln1p branch seems to be more important for signal transduction in oxidative stress response. The
HOG pathway regulates the oxidative stress-induced transcription of \textit{HSP12} but not another closely related HSP gene \textit{HSP26}. Msn2p and Msn4p are important for the transcription of both \textit{HSP12} and \textit{HSP26}.

\textbf{The HOG Pathway and Oxidative Stress}

Cells lacking a functional HOG pathway display growth defects when challenged with hydrogen peroxide or superoxide-generating drugs (Figure 3-1,2). More importantly, addition of oxidants to the cell culture leads to an increase in Hog1p phosphorylation, indicating activation of the HOG pathway (Figure 3-3). It would appear that it is oxidative stress that generates the signal, and not some other aspect of the oxidants, since different oxidants activate the HOG pathway in a similar fashion. Hog1p phosphorylation starts increasing around 5 minutes after addition of oxidants and this increase lasts as long as 1 hour. Therefore, the dephosphorylation of Hog1p in this process seems to be rather slow. When yeast cells are exposed to 400 mM NaCl, Hog1p phosphorylation increases very quickly and reaches the maximal level in 10 minutes, after which it drops back to the basal level within 30 minutes. The maximal level of Hog1p phosphorylation is also lower in the case of oxidative stress (Figure 3-4). These differences might reflect how cells perceive various forms of stress. High osmolarity presents a sudden and severe threat to the survival of yeast cells, requiring a prompt and magnified response. ROS are continuously produced during normal respiration, and therefore might be perceived as a mild form of stress. Notably, the HOG pathway is not very sensitive to oxidative stress,
for its activation requires H$_2$O$_2$ at a relatively high concentration (1 mM). Only 0.3 mM H$_2$O$_2$ is needed to activate Yap1p (Delaunay et al., 2000).

**Transcriptional Regulation by the HOG Pathway in Oxidative Stress Response**

Oxidative stress induces *HSP12* transcription, and normal activation requires an intact HOG pathway. The level of *HSP12* mRNA after the addition of 0.2 mM H$_2$O$_2$ is much lower in the *hog1Δ* mutant than in the isogenic wild-type strain (Figure 3-8). Transcription of another highly inducible heat shock protein gene, *HSP26*, is not affected by the deletion of *HOG1*. Analysis of the promoter sequence reveals that *HSP12* and *HSP26* have multiple copies of STRE in their promoters. Transcriptional activation through STRE is known to be mediated by two transcriptional factors Msn2p and Msn4p (Martinez-Pastor et al., 1996; Schmitt and McEntee, 1996). Our data confirms that Msn2p and Msn4p are indispensable for oxidative stress-induced transcription of *HSP12* and *HSP26* (Figure 3-9). Interestingly, *HSP12* promoter has an ATF binding site (ACGTCA) that is not found in *HSP26* promoter. We speculate this sequence element might be responsible for *HSP12* regulation by the HOG pathway. There are examples in both mammalian cells and yeast suggesting ATF proteins are downstream targets of MAPK pathways. For instance, mammalian ATF2 is directly phosphorylated by the p38 MAPK (Casillas et al., 1993; Raingeaud et al., 1995). ATF2-mediated transcription is also under the regulation of the p38 pathway. In the fission yeast, Atf1p is a substrate for the Sty1p MAPK (Shiozaki and Russell, 1996). Atf1p mediates transcription in osmotic
stress response. A transcriptional repressor in *S. cerevisiae*, Sko1p is also a member of the ATF protein family. It is bound and phosphorylated by Hog1p in vitro, qualifying itself as a substrate of Hog1p (Proft *et al.*, 2001). Our data shows that deficiency in *SKO1* suppresses *hog1Δ* mutant sensitivity to hydrogen peroxide and plumbagin (Figure 3-7), suggesting Sko1p is probably responsible for repression of oxidative stress genes under normal growth conditions. Whether Sko1p-mediated repression involves *HSP12* still needs to be examined.

Hsp12p has not yet been linked to any distinct function. Although it is strongly induced under many stress conditions, loss of *HSP12* produces no apparent phenotype. Perhaps Hsp12p works in parallel with other proteins to help cells resist stress conditions. Loss of *HSP12* could be compensated by the functions of other proteins. Mutation in an *HSP12* homologue of *S. pombe* suppresses *cdc4* mutant phenotype, suggesting it might participate in formation of the F-actin contractile ring in cytokinesis (Jang *et al.*, 1996). We won’t be able to appreciate the significance of *HSP12* regulation by the HOG pathway until we have a better understanding of its function.
CHAPTER 4

THE HOG PATHWAY IS INVOLVED IN HEAT SHOCK RESPONSE

4.1 INTRODUCTION

In the previous chapter, we have described that the HOG pathway plays an important role in oxidative stress response. Specifically, the HOG pathway is required for the oxidative stress-induced transcription of the heat shock protein gene \( HSP12 \). This raises the possibility that the HOG pathway might also be involved in heat shock response.

Heat Shock and Yeast Response

Heat shock is another form of stress yeast cells have to face in the natural environment, and it causes disruption of a large number of cellular assemblies and processes, an increase in protein unfolding and aggregation, and membrane structure alteration (Lindquist, 1986; Morano et al., 1998). Yeast heat shock response serves to counteract these events and thus help cells withstand heat stress. It involves accumulation of trehalose in the cytoplasm and strong induction of heat shock proteins (HSPs).

Trehalose, a disaccharide composed of two \( \alpha-\alpha \)-linked glucose units, helps preserve membrane structure and enzyme activity (Colaco et al., 1992; Hottiger et al., 1994). It
accumulates during desiccation, freezing, and heating, basically all conditions that lower growth rate. Consistent with this, there is good correlation between trehalose level and thermotolerance in stationary phase and non-fermentative yeast cultures (Van Dijck et al., 1995).

At least 52 different proteins are highly induced by a temperature shift from 25°C to 38°C, and many of them are HSPs (Miller et al., 1982). Both transcriptional and translational mechanisms are thought to operate to ensure that HSP synthesis bypasses the more general inhibition of synthesis of most other proteins under stress conditions, but the exact mechanisms remain to be established. Cells do not maintain high HSP synthesis for a very long time. In cells heat shocked and then maintained at a high temperature, HSP synthesis is gradually reduced to a new steady-state level, which is still considerably higher than the level of HSP synthesis before heat stress is applied.

Among all the HSPs, the Hsp70p family represents a diverse and multifunctional group of highly related proteins involved in protein translocation, folding, assembly and degradation. *S. cerevisiae* possesses at least 14 different Hsp70ps, grouped into five subcategories on the basis of sequence similarity and functional interchangeability (Boorstein et al., 1994; James et al., 1997). The overall structure of these proteins is highly conserved and consists of an NH₂-terminal ATPase domain, a central peptide-binding domain, and a so-called "variable" domain at the COOH terminus (Becker and Craig, 1994). The Ssa family of Hsp70p proteins is required for protein translocation into both the endoplasmic reticulum and mitochondria, while members of the Ssb family are
often found in association with actively translating polysome complexes (Horton et al., 2001; Liu et al., 2001).

Yeast Hsp90p is an essential component of receptor complexes in vivo (Picard et al., 1990). Two different genes, the heat-inducible HSP82, and the constitutively expressed HSC82, encode Hsp90p (Borkovich et al., 1989). Loss of either gene has little effect, whereas the double deletion strain is not viable. Studies of the Hsp90p receptor purified from mammalian cells have identified additional components of this large, multi-protein complex (Pratt and Toft, 1997). This complex is, to a large extent, conserved in yeast, and includes Hsp90p, Hsp70p, the DnaJ homologue Ydj1p, Sti1p, Cdc37p, and at least two different cyclophilin homologues, Cpr6p and Cpr7p (Chang and Lindquist, 1994).

One major class of HSPs, the low-molecular-mass Hsp12p and Hsp26p, has not yet been shown to have any function. Although both of them are induced under many stress conditions, loss of either HSP12 or HSP26 produces no apparent phenotype. Mutation in an HSP12 homologue of S. pombe suppresses cdc4 mutant phenotype, suggesting it might participate in formation of the F-actin contractile ring in cytokinesis (Jang et al., 1996). Hsp26 assembles into large 20S multimers of uniform size and molecular weight (Bentley et al., 1992). Its COOH terminus shares homology with small HSPs of other organisms.

Transport of bulk mRNA from the nucleus to the cytoplasm is blocked during heat shock, resulting in an accumulation of polyadenylated RNA in the nucleus (Liu et al.,
1996; Tani et al., 1995). However, SSA1 and SSA4 mRNAs, encoding members of the Hsp70p family, are exported to the cytoplasm under the same experimental conditions, suggesting the presence of signals and factors that may allow for selective nucleocytoplasmic transport during heat stress (Saavedra et al., 1996). At least some of the cytoplasmic export signals lie within the mRNA sequence, demonstrated by the observation that the hybrid GAL1-SSA4 mRNA is exported to the cytoplasm during heat shock.

Transcriptional Regulation in Heat Shock Response

Two distinct promoter elements are involved in gene induction by heat shock, the heat shock element (HSE) and the general stress response element (STRE). HSEs are the binding sites for heat shock factor (HSF) and composed of at least three copies of the 5-bp sequence NGAAAN, arranged in alternating orientation (Sorger, 1991). The number of the repeating sequence may vary but usually ranges from three to six. HSF is a transcription factor that binds HSEs and mediates gene induction in response to heat shock. The yeast HSF, Hsf1p, contains a helix-turn-helix DNA binding domain, and a coiled-coil hydrophobic repeat domain that mediates trimerization (Wu, 1995). In most eukaryotes, HSF exits as an inactive monomer under normal conditions. Only after heat shock is applied does it trimerize and acquire high DNA-binding affinity (Mager and De Kruijff, 1995). Yeast Hsf1p is fundamentally different from the HSFs of other eukaryotes. First, yeast Hsf1p is constitutively present as trimers bound to HSEs (Gross

As described previously, transcription driven by STRE can be induced by a variety of stress conditions including osmotic stress, oxidative stress, nutrient limitation and heat shock. This is in contrast with HSE-mediated transcription, which appears to only respond to high temperature.

4.2 MATERIALS AND METHODS

Strains, Plasmids, Media and General Methods

Yeast strains and plasmids used in this chapter are described in Table 4-1. All strains are of the W303 background. Yeast growth media (YEPD and dropout media) were prepared as described in section 2.2. Common procedures were followed for PCR, DNA cloning, etc (Sambrook et al., 1989). Standard techniques for yeast crosses, sporulation,
**Table 4-1. Strains and Plasmids**

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</tr>
<tr>
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<td>MATα hog1Δ::TRP1</td>
<td>lab stock</td>
</tr>
<tr>
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<td>URA3, 2μ, HOG1</td>
<td>J. Brewster</td>
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dissection and propagation were used (Kaiser et al., 1994). A lithium acetate-based
procedure was used for all the yeast transformation (Chen et al., 1992).

Heat Shock Sensitivity Assay

The heat shock sensitivity assay was similar to that described by Raitt et al. (Raitt et al., 2000a). Yeast cultures were grown to mid-log phase at 25°C, and then shifted to
51°C. Samples were taken at different time points, diluted into ice-cold YEPD and plated.
Plates were incubated at 30°C for 2-3 days and colonies were counted to determine the
survival rate.

Western Blot Analysis

Western blot analysis of Hog1p was performed as described previously (see section
2.2). Briefly, protein extracts were prepared from treated and untreated cells. 20 µg
protein of each sample was separated by SDS-PAGE and transferred to a nitrocellulose
membrane. The membrane was washed in TBS buffer and incubated in the blocking
buffer for 1 hour at room temperature. After three washes in TBS/T buffer, the membrane
was incubated with phospho-p38 antibody at 4°C overnight. The membrane was washed
again in TBS/T buffer and incubated with the secondary antibody for 1 hour at room
temperature. Following three more washes in TBS/T buffer, ECL reagents were added to the membrane. The membrane was wrapped up and exposed to X-ray film.

**Northern Blot Analysis**

Northern blot analysis was performed as described previously (see section 3.2). Briefly, RNA samples were prepared from treated and untreated cells. 5 µg RNA of each sample was separated on a formaldehyde agarose gel and transferred to a Nylon membrane. RNA was crosslinked to the membrane, and the membrane was pre-hybridized before radioactively-labeled probe was added. The desired gene fragment was amplified from genomic DNA and used as template to make the probe. The radioactive probe was added and hybridized to the membrane at 42°C overnight. The membrane was washed and dried before exposed to the phosphoimager or X-ray film.

**4.3 RESULTS**

**hog1Δ Cells are Hypersensitive to Heat Stress**

Challenging yeast cells with extreme high temperature is commonly used to screen for genes important for heat shock response. For example, the SKN7 gene is important for heat shock response, while skn7Δ mutants show no growth defect at all at 37°C. Only
after the temperature rises to 51°C do skn7Δ mutants become some 10 times more sensitive than the wild-type strain (Raitt et al., 2000a). Also, by swiftly rising to the lethal temperature, the process of adaptation can be avoided, thereby enhancing the effects of heat stress (Davidson et al., 1996).

Following this rationale, we compared the survival rate of the wild-type strain and hog1Δ mutants at 51°C. Cells deleted for HOG1 are more vulnerable to acute heat stress than the wild-type cells (Figure 4-1). The difference in survival rate between the wild type and hog1Δ cells is about 5 fold. We confirmed that cells lacking SKN7, to a similar degree, are also hypersensitive to heat stress. In addition, the skn7Δ hog1Δ double mutant has a survival rate lower than either of the two single mutant, suggesting effects of SKN7 and HOG1 are additive.

**Heat Shock Activates the HOG Pathway**

The fact that hog1Δ cells are hypersensitive to heat stress suggests the HOG pathway might be involved in heat shock response. Increase in Hog1p phosphorylation is a good indication of HOG pathway activation. As shown in Figure 4-2, when yeast cells were shifted from 25°C to 37°C, there was an increase in Hog1p phosphorylation. Hog1p phosphorylation level starts rising quickly after heat shock is applied, reaches the maximal level around 10 minutes, and then drops back to the basal level within 30 minutes. The overall kinetics of increase in Hog1p phosphorylation after heat shock is
Figure 4-1. *hog1Δ* cells are hypersensitive to heat stress. Yeast cells were grown to mid-log phase at 25°C, and then shifted to 51°C. Samples were taken at the indicated time points, diluted in ice-cold YEPD medium and plated to assess viability. The error bars represented standard deviation.
Figure 4-2. Heat shock activates the HOG pathway. *HOG1* was expressed from a high-copy yeast vector pRS426. Yeast culture was grown to mid-log phase at 25°C and shifted to 37°C. Samples were taken at the indicated time points and Western blot was performed as described previously.
very similar to that after osmotic stress, but the magnitude of increase in Hog1p phosphorylation after heat shock is smaller.

The HOG Pathway Regulates the Transcriptional Activation of HSP12 in Heat Shock Response

Heat shock induces expression of heat shock proteins. An increase in HSP12 mRNA level was observed when yeast cultures were shifted from 25°C to 37°C (Figure 4-3). HSP12 mRNA started increasing 15 minutes after the heat shock was applied, and at the 1-hour time point, HSP12 mRNA level was still much higher than the basal level. This heat shock-induced increase in HSP12 mRNA level was dramatically reduced in hog1Δ cells, suggesting its dependence on a functional HOG pathway. For comparison, we also measured HSP26 mRNA level in the same experiment. Increase of HSP26 mRNA after heat shock is indistinguishable from that of HSP12. However, deletion of HOGL has no effect on the heat shock-induced HSP26 transcription.

Msn2p and Msn4p Are Important for the Transcriptional Activation of HSP12 and HSP26 in Heat Shock Response
Figure 4.3. The HOG pathway regulates the transcriptional activation of 
HSP12 in heat shock response. Wild-type strain and hog1Δ mutant were 
grown to mid-log phase at 25°C and then shifted to 37°C. Samples were taken 
at the indicated time points. Total RNA was isolated and probed for HSP12, 
HSP26, or ACT1 (loading control), as described previously.
Msn2p and Msn4p are transcription factors important for general stress response. As demonstrated in the previous chapter, Msn2p and Msn4p are indispensable for the oxidative stress-induced transcription of *HSP12* and *HSP26*. We compared the *msn2Δ msn4Δ* double mutant with the isogenic wild-type strain for *HSP12* and *HSP26* induction under heat shock conditions. After a temperature shift from 25°C to 37°C, the mRNA levels of *HSP12* and *HSP26* in the *msn2Δ msn4Δ* double mutant are much lower than those in the wild-type strain (Figure 4-4). Therefore, Msn2p and Msn4p participate in the regulation of *HSP12* and *HSP26* transcription under heat shock conditions.
Figure 4-4. Msn2p and Msn4p are important for the transcriptional activation of HSP12 and HSP26 in heat shock response. Wild-type strain, hog1Δ and msn2Δ msn4Δ mutants were all grown to mid-log phase 25°C and then shifted to 37°C. Samples were taken at the indicated time points. Total RNA was isolated and probed for HSP12, HSP26, or ACT1 (loading control).
4.4 DISCUSSION

Involvement of the HOG pathway in oxidative stress response suggests its role is not only limited to osmotic stress response. Interestingly, the HOG pathway regulates the transcription of at least one of the HSP genes, *HSP12*, in response to oxidative stress. Therefore, it is of great interest to determine if the HOG pathway is also involved in heat shock response.

We first demonstrated that the HOG pathway is required for survival under acute heat stress conditions. Cells deleted for *HOGL* are found to be more vulnerable at 51°C than the isogenic wild-type strain (Figure 4-1). More importantly, a temperature shift from 25°C to 37°C rapidly activates the HOG pathway (Figure 4-2). This result suggests the role of the HOG pathway in heat shock response is related to signal transduction. Our data also shows the HOG pathway regulates the transcription of one HSP gene *HSP12*, but it has no effect on the transcription of another closely related HSP gene *HSP26*. The HOG pathway is not the only regulator of HSP gene expression. Msn2p and Msn4p mediate the transcription of both *HSP12* and *HSP26* under heat stress conditions.

HSFs are important for heat shock-induced gene expression (Wu, 1995). Studies of mammalian HSF1 have revealed that cellular kinases regulate its activity. A conserved central repression domain of mammalian HSF1 modulates repression of its transactivation domain under normal growth temperature. The repression domain consists of 24% serine residues, phosphorylation of which determines HSF1 activity. Stimulation
of Raf/Erk MAPK pathway increases phosphorylation of these serine residues (He et al., 1998). Mivechi and Giaccia have shown that overexpression of a dominant negative allele of Erk1 MAP kinase increased the activity of an HSP70 reporter (Mivechi and Giaccia, 1995). They have also found that the administration of sodium vanadate, an inhibitor of MAP kinase phosphatase, results in increased phosphorylation of HSF1, delayed activation of the HSP70 transcription, and a defect in induced thermotolerance. It is still not clear whether any MAPK pathway of S. cerevisiae modulates the activity of Hsf1p. Our data shows that although the heat shock-induced transcription of HSP12 requires the HOG pathway, the transcription of HSP26 is not affected by deletion of HOG1. Therefore, it is unlikely that the HOG pathway directly regulates the activity of Hsf1p.

Heat shock also activates another MAPK pathway in yeast, the cell integrity pathway (Zarzov et al., 1996). While the HOG pathway responds to hypertonic osmotic stress, the cell integrity pathway responds to hypotonic osmotic stress (Davenport et al., 1995). The fact that the same stress activates two antagonizing pathways is intriguing. Kamada et al. have found that activation of the cell integrity pathway by heat shock does not occur when cells are grown in high-osmolarity medium (Kamada et al., 1995). We think that cross regulation might exist between these two pathways. Specifically, an activated HOG pathway could suppress the activation of the cell integrity pathway and vice versa. Such cross regulation among different MAPK pathways has been observed before. For instance, addition of pheromone activates not only the mating pathway but also the cell integrity pathway (Buehrer and Errede, 1997; Zarzov et al., 1996). However, activation
of the cell integrity pathway by pheromone does not occur in cells lacking components of the mating pathway. Another example is that the MAPK of the mating pathway, Fus3p, becomes phosphorylated in response to high osmolarity in cells lacking HOG1 (O'Rourke and Herskowitz, 1998).
CHAPTER 5
DISCUSSION

Molecular Mechanism of the HOG Pathway Activation

The work described in Chapter 2 is consistent with the hypothesis that loss of turgor pressure contributes to the activation of the HOG pathway under osmotic stress condition. The evidence came from experiments involving the anti-fungal drug amphotericin B. Direct measurement of turgor pressure revealed that amphotericin B treatment lowered the cellular turgor pressure (Figure 2-5). More importantly, the phosphorylation level of Hog1p MAPK starts increasing after addition of amphotericin B (Figure 2-6), suggesting activation of the pathway. How membrane proteins such as Sln1p or Sho1p sense a change in turgor pressure is still not clear. Since turgor pressure is related to tension on the cell surface, alterations in turgor pressure might cause conformational changes in these proteins, leading to activation of the pathway.

As to the sensor for oxidative stress, Delaunay et al. recently demonstrated that yeast cells sense hydrogen peroxide through oxidation of Yap1p (Delaunay et al., 2000). Upon hydrogen peroxide treatment, Yap1p is activated by oxidation of two cysteine residues. The current model is that oxidation of Yap1p leads to disulfide bond formation and results in conformation changes that mask recognition of the nuclear export signal, thereby promoting nuclear accumulation of the protein. Similar scenarios have also been
found in *E. coli* (Zheng and Storz, 2000). OxyR, the sensor for hydrogen peroxide in *E. coli*, is a transcription factor that can be directly oxidized by hydrogen peroxide. Oxidation by hydrogen peroxide leads to formation of a disulfide bond in OxyR, which triggers the activation of this transcription factor (Zheng *et al.*, 1998). SoxR, the sensor for superoxide and nitric oxide, is also a transcription factor. Its [2Fe-2S] clusters function as a redox switch (Ding *et al.*, 1996). During normal aerobic growth, the SoxR [2Fe-2S] clusters are predominantly in the reduced state. When *E. coli* cells are exposed to superoxide or nitric oxide, the [2Fe-2S] clusters become oxidized and SoxR is activated. The transcription factor NF-κB appears to be responsible for sensing oxidative stress in mammalian cells (Li and Karin, 1999), though there is no biochemical data available showing direct oxidation of NF-κB. Several lines of evidence suggest a role of ROS as common and critical intermediates for NF-κB activation. Exposure of mammalian cells to hydrogen peroxide induces NF-κB activation (Manna *et al.*, 1998; Schreck *et al.*, 1991), while activation of NF-κB by other stimuli can be inhibited by a variety of antioxidants as well as overexpression of antioxidant enzymes (Schreck *et al.*, 1992).

It has long been suspected that a sudden increase in the level of aberrant, misfolded or aggregated proteins in the cell triggers heat shock response (Parsell and Lindquist, 1993). However, the plasma membrane also plays an important role. Carratu *et al.* showed that the degree of fatty acid saturation in the plasma membrane strongly influences sensing of heat stress (Carratu *et al.*, 1996). Curran and Khalawan demonstrated alcohols lower the threshold temperature for heat shock response (Curran and Khalawan, 1994). Their data
supports a model in which heat shock disturbs the plasma membrane and produces a
temporary reorganization of the lipid bilayer, resulting in presentation of membrane
proteins to the cytoplasm and a cascade reaction that eventually activates HSF.

Although oxidative stress and heat shock activate the HOG pathway, this activation is
different from that by osmotic stress. First, the increase in Hog1p phosphorylation under
osmotic stress is much stronger than under oxidative stress or heat shock (Figure 3-3, 4-
2). Second, osmotic stress leads to translocation of Hog1p from the cytoplasm to the
nucleus, but such translocation could not be detected in oxidative stress and heat shock
(data not shown). It is possible that, due to the mild nature of HOG pathway activation
during oxidative stress and heat shock, the amount of Hog1p moving into the nucleus is
very small and therefore difficult to detect.

Transcriptional Regulation of Yeast Stress Responses

Genes whose expression is induced by osmotic stress have been identified, and many
of them show strong dependence on the HOG pathway (Posas et al., 2000). In this work,
we have demonstrated that one HSP gene, *HSP12*, is regulated by the HOG pathway in
both oxidative stress and heat shock (Figure 3-8, 4-3). Identification of additional genes
will clarify how the HOG pathway regulates specific stress responses. The DNA micro-
array technique will allow identification of all the genes induced by certain stress
condition in one simple experiment. By comparing the expression profiles of wild type
strain and *hog1A* mutant, future studies will reveal which genes are regulated by the
HOG pathway in response to oxidative stress or heat shock. Transcription factors downstream of the HOG pathway still remain to be identified. Hog1p is a strong transcriptional activator when fused to the Gal4p DNA binding domain (data not shown). While this activation has prevented us from screening for Hog1p-interacting proteins, it might also reflect part of its roles in vivo.

Hog1p regulates the b-ZIP transcriptional repressor Sko1p. Under osmotic stress conditions, activated Hog1p MAPK directly phosphorylates Sko1p and thus disrupts its association with the general repressor complex Ssn6p-Tup1p, leading to elevated expression of stress genes such as ENA1, HAL1, and GRE2 (Proft et al., 2001). Our data suggests the regulation of Sko1p by Hog1p is important for resistance to oxidative stress too (Figure 3-7). One would expect that activation of the HOG pathway relieves the repression of oxidative stress genes by Sko1p. Sko1p binds specifically to the ATF binding site in vitro (Nehlin et al., 1992), and thus analysis of promoter sequences will help identify those oxidative stress genes repressed by Sko1p.

Msn2p and Msn4p are very important for STRE-mediated gene expression under stress conditions. Although deletion of MSN2 and MSN4 causes reduction in the magnitude of osmotic stress-induced gene transcription, the fold induction is relatively unchanged (Martinez-Pastor et al., 1996). Msn2p moves into the nucleus upon osmotic stress, and this translocation is not affected by the deletion of HOG1 (Gorner et al., 1998). Our data suggests the HOG pathway and Msn2/4p are both involved in the
transcriptional activation of oxidative stress and heat shock genes, but it is still not clear whether the HOG pathway regulates Msn2p and Msn4p in these responses.

Skn7p and Yap1p are important transcription factors in oxidative stress response. While Yap1p regulates the transcription of many oxidative stress genes, only a subset of these genes are controlled by Skn7p (Lee et al., 1999a). The Skn7p-dependent subset includes most of the known reactive oxygen intermediate scavenging activities, while the Skn7p-independent subset comprises of genes involved in glutathione production and in the pentose phosphate pathway. Yap1p belongs to the AP-1 family of transcription factors, all of which recognize the same AP-1 response element. Members of the AP-1 family such as Jun and ATF proteins have been shown to be phosphorylated and regulated by p38 MAP kinase and SAPK, but whether Yap1p is a substrate of Hog1p is still not clear. Interestingly, Yap1p is also required for the stress-induced gene expression through the STRE element (Martinez-Pastor et al., 1996).

One Pathway and Multiple Stresses

The HOG pathway was initially identified as a signal transduction pathway responding to osmotic stress. Cells lacking a functional HOG pathway cannot grow on high-osmolarity medium. It was not noticed until very recently that the HOG pathway was also involved in responses to other forms of stress. For example, Kapteyn et al. have shown that low pH alters yeast cell wall architecture, and this alteration requires the HOG
pathway (Kapteyn et al., 2001). Illustrated in Chapters 3 and 4, the HOG pathway also plays important roles in oxidative stress and heat shock responses. Involvement of one pathway in multiple stress responses raises the question of specificity. Studies of S. pombe provide a very good example. There is only one general stress response MAPK pathway in the fission yeast, which can be activated by multiple signals. It turns out that S. pombe utilizes different transcription factors for induction of specific genes. Atf1p mediates the induction of osmotic stress genes, while Pap1p mediates the induction of oxidative stress genes (Toone et al., 1998). Identification of transcription factors downstream of the HOG pathway will help us solve the puzzle of its involvement in multiple stresses.

Various stresses might be linked to one another. For instance, it has been reported that the main cause of cell death under acute heat shock condition is the generation of toxic intermediates of oxygen metabolism (Davidson et al., 1996). Mutants deficient in the key antioxidant enzymes catalase, superoxide dismutase and cytochrome c peroxidase are sensitive to heat shock. Overexpression of catalase and superoxide dismutase leads to protection from heat shock. Anaerobically grown cells are more resistant to heat shock, and this protection was immediately abolished upon air exposure. Cross protection among different stresses is another good example. Pretreatment of cells with a mild osmotic shock conferred resistance to heat shock (Varela et al., 1992). Exposure of yeast to high ethanol concentrations or weak acids conferred thermo-tolerance (Coote et al., 1991).
Studies on mammalian cells have also implicated ROS in normal physiological signaling. A variety of ligands including platelet-derived growth factor, epidermal growth factor, angiotensin II and cytokines all trigger the rapid production of intracellular ROS (Bae et al., 1997; Speir et al., 1994; Sundaresan et al., 1995). Inhibition of ROS production following ligand addition abolishes downstream signal transduction. One piece of evidence linking ROS to specific signaling pathways came from the analysis of ASK1, an MEKK involved in the activation of p38 MAP kinase as well as the stress-activated protein kinase (Saitoh et al., 1998). Thioredoxin forms a complex with ASK1 and inhibits its activity. The rise in ROS level following tumor necrosis factor stimulation results in dissociation of ASK1 from thioredoxin and the subsequent activation of ASK1 (Gotoh and Cooper, 1998). Therefore, in addition to being ROS scavengers, antioxidant proteins also interact with specific signaling molecules and regulate their activity in a redox-dependent fashion. Consistent with this notion, the superoxide dismutase Sod1 can interact and regulate calcineurin activity (Wang et al., 1996). Another way that ROS can regulate signaling pathways is to alter the oxidation state of reactive cysteine residues in proteins. The bacterial transcription factor OxyR and yeast transcription factor Yap1p are well-characterized examples, whose activities are sensitive to the redox state. Cellular enzymes can be the targets for ROS too. All members of protein tyrosine phosphatase family have a reactive cysteine in their active site, and oxidation of this reactive cysteine leads to reversible inaction of enzymatic activity.
REFERENCES


Friesen, H., Lunz, R., Doyle, S., and Segall, J. (1994). Mutation of the SPS1-encoded protein kinase of Saccharomyces cerevisiae leads to defects in transcription and morphology during spore formation, Genes Dev 8, 2162-75.


beta(1two head right arrow6)-glucan interconnects mannoprotein, beta(1two head right arrow3)-glucan, and chitin, J Biol Chem 272, 17762-75.


Kuge, S., and Jones, N. (1994). YAP1 dependent activation of TRX2 is essential for the response of Saccharomyces cerevisiae to oxidative stress by hydroperoxides, EMBO J 13, 655-64.


APPENDIX

The structure of the HOG pathway is well understood. Many efforts, including the DNA microarray experiment, have defined downstream gene targets of the HOG pathway. However, a big gap still exists in our knowledge, i.e., how the activation of the HOG pathway leads to the expression of downstream genes. It's likely that transcription factors act between Hog1p and its downstream gene targets. By modulating the activity of transcription factors, the HOG pathway regulates gene expression in response to stress conditions. Needless to say, identification of such transcription factors will increase our understanding of the HOG pathway.

Sho1p is one of the two putative sensors of the HOG pathway. It anchors Pbs2p to the membrane through the interaction between its SH3 domain and the proline-rich domain of Pbs2p. The central region between its NH2-terminal transmembrane domains and its COOH-terminal SH3 domain appears to be important, since deletion in this region causes Sho1p to lose its function (Albertyn and Gustin, unpublished data). However, this 179-aa peptide is not linked to any distinct function.

We used two different methods to screen for proteins that interact with Hog1p or Sho1p, but neither of them produced satisfactory results. Our attempts are described below.
Two-hybrid Screen

The two-hybrid system is a genetic method that uses transcriptional activity as a measure of protein-protein interaction (Bartel and Fields, 1995). It is based on the observation that the DNA-binding domain and the transcriptional activation domain of many transcriptional activators don’t need to be covalently linked. They can be brought together by the interaction of any two proteins and still activate transcription. A variety of versions of the two-hybrid system exist, commonly involving DNA binding domains that derive from the yeast Gal4p protein or the E. coli LexA protein. Transcriptional activation domains are usually from the Gal4p protein or the herpes simplex virus VP16 protein. Reporter genes include the E. coli LacZ gene and selectable yeast genes such as HIS3, LEU2, and ADE3.

We made the HOG1 bait construct by cloning the HOG1 gene into a Gal4p-based DNA binding-domain vector (James et al., 1996). When the HOG1 bait construct was transformed into the two-hybrid host strain, it strongly activated the transcription of reporter genes in the absence of any interacting protein partner. McLaughlin et al. have reported that p38 MAPK also activates the transcription of reporter genes in the two-hybrid system, but this activation can be relieved by mutating a conserved aspartate residue in the kinase domain and thus inactivating the kinase (McLaughlin et al., 1996). Inspired by their strategy, we introduced an alanine residue to replace the aspartate residue using site-directed mutagenesis. Surprisingly, the mutated version of the HOG1 bait still strongly activated the reporter gene transcription. Hog1p shares very high
sequence homology in the kinase domain with p38 MAPK, but it also has an extra 80-aa sequence at the COOH terminus that is not found in p38 and other MAPKs. It was speculated that this COOH terminal "tail" was responsible for the strong transcriptional activation. We used restriction enzymes to remove the COOH terminus-coding sequence from HOG1. However, the shorter bait construct, with or without the aspartate-to-alanine point mutation in the kinase domain, remained a strong transcriptional activator. We also tried a LexA-based two-hybrid system (Vojtek et al., 1993), but changing the DNA-binding specificity did not solve the problem.

The DNA sequence encoding the Sho1p cytoplasmic domain was cloned into the DNA binding vector. Unfortunately, the SHO1 bait was also a strong transcriptional activator, preventing us from carrying out the conventional two-hybrid screen. Therefore, we came up with a three-hybrid screen strategy, in which PBS2 was cloned into the DNA binding vector and used as the bait, while the DNA sequence encoding Sho1p cytoplasmic domain was expressed from another yeast vector. Since the SH3 domain of Sho1p binds to the proline-rich domain of Pbs2p, any additional protein that interacts with Sho1p will be brought to the proximity of Pbs2p and thus activate reporter gene transcription. We made the PBS2 bait construct and determined that it didn't activate reporter gene transcription in the presence of over-expressed Sho1p cytoplasmic domain. Transformation of a Gal4p activation domain-based genomic DNA library produced over 100 positives clones. Plasmids were isolated from some of the positive clones. When we transformed these plasmids back the host strain, we were unable to confirm the interaction. At this point, we decided to give up the two-hybrid screen.
Expression Cloning

Expression cloning is based on the fact that proteins immobilized on a nitrocellulose membrane can be directly screened for kinase substrates or protein binding partners (Fukunaga and Hunter, 1997; Ma et al., 1999). The idea was to first obtain active Hog1p MAPK or radioactively labeled Sho1p and then use them to screen a phage library that expresses yeast cDNA.

Hog1p forms inclusion body and remains inactive when expressed in bacteria (Brewster and Gustin, unpublished data). Instead, we chose to use the baculovirus expression system. We cloned HOG1 into a baculovirus expression vector and used it to transfect Sf9 insect cells. Expression of Hog1p was confirmed by Western blot. The expression level of Hog1p was very low. Limited by the scale of cell culture, we were only able to obtain a small amount of purified Hog1p. Hog1p was expressed as a fusion protein to a cellulose-binding domain, which facilitated protein purification. However, it also caused loss of purified protein, since the spin-column and dialysis membrane used in sample handling were all made of cellulose. In the end, we did not have enough purified Hog1p to perform the screen.

The Sho1p cytoplasmic domain (Sho1p-c) was expressed as a GST fusion protein in bacteria. A Protein Kinase A (PKA) phosphorylation site was inserted between the GST tag and Sho1p-c for in vitro labeling. The GST-Sho1p-c was purified using glutathione agarose and radioactively labeled using PKA and γ-32P-ATP. The GST tag was cleaved
using biotinylated thrombin, and once the protease digestion was complete, streptavidin agarose was added to capture the thrombin.

The yeast cDNA library was constructed by inserting yeast cDNA into a phage expression vector (a gift from Dr. S. Elledge). We packaged this yeast cDNA library into phage particles and used them to transfect bacteria. Once plaques were formed, nitrocellulose membrane was put on top of the plate to replicate recombinant proteins. The membrane was hybridized with radioactively labeled Sh1p-c, washed, and exposed to X-ray film. Based on the positions of hybridization signal, putative positive plaques were picked and subject to a second-round screen. After several rounds of screen, all the positive plaques that we identified turned out to be false. They originated from nonspecific binding of the radioactively labeled Sh1p-c to the membrane. We tried increasing the hybridization stringency, but the problem still remained.

Summary

Due to technical difficulties, we did not obtain any results from the screens described above. However, we still believe that identification of interactions between an enzyme and its substrate or one protein and another protein will provide information that might not be readily gained by genetic analysis.
References


