

**TRANSCRIPTION FACTORS UNDER THE CONTROL OF THE**  
**YEAST Hog1 MAPK**

Laura Casadomé Burriel

Tesis Doctoral

Departament de Ciències Experimentals  
i de la Salut  
Programa de Doctorat en Ciències de la Salut  
i de la Vida  
Universitat Pompeu Fabra  
Barcelona, 2004

Laura Casadomé Burriel

Tesis Doctoral

Barcelona, 2004

**TRANSCRIPTION FACTORS UNDER THE CONTROL OF THE  
YEAST Hog1 MAPK**

Departament de Ciències Experimentals  
i de la Salut  
Programa de Doctorat en Ciències de la Salut  
i de la Vida  
Universitat Pompeu Fabra

Laura Casadomé Burriel

Tesis Doctoral

Barcelona, 2004

**TRANSCRIPTION FACTORS UNDER THE CONTROL OF THE  
YEAST Hog1 MAPK**

Memòria presentada per optar a l'obtenció al títol de Doctor per la

Universitat Pompeu Fabra (UPF)

Treball dirigit pels Drs Francesc Posas Garriga

i Eulàlia de Nadal Clanchet

Unitat de Senyalització Cel·lular

Departament de Ciències Experimentals

i de la Salut (CEXS)

Programa de Doctorat en Ciències de la Salut

i de la Vida de la UPF 2000-2002

Dr. Francesc Posas Garriga  
Director de la Tesi

Dr. Eulàlia de Nadal Clanchet  
Director de la Tesi

Dipòsit legal: B.40015-2005  
ISBN: 84-689-3829-7



---

## SUMMARY

Yeast cells are exposed to a wide variety of environment stresses, among them changes in the osmotic conditions. An osmolar upshift leads to fast loss of intracellular water, so living cells have developed mechanisms to counteract this loss. In *Saccharomyces cerevisiae* changes in the osmotic conditions are sensed by the HOG pathway. The HOG pathway is a MAPK signalling pathway and the functional homolog of the stress activated MAPK JNK MAPK and p38 present in mammals. Because there is a high degree of conservation of these cascades, the HOG pathway is a good model to study osmotic adaptation processes.

Recent reports have shown that the Hog1 MAPK can regulate several processes such as cell cycle control, metabolic adaptation or regulation of gene expression.

At the beginning of this work, the mechanisms by which the Hog1 MAPK was controlling gene expression were unclear because transcription factors under the control of the MAPK were not well characterized. Our goal was the identification of new transcription factors under the control of the MAPK.

Therefore, we designed a genetic screen and selected clones from a multicopy genomic library that were able to induce the expression of Hog1 dependent genes in non stress conditions. One of these clones was the *SMP1* gene. Smp1 encodes for a MEF2-like transcription factor. Its overexpression induced the expression of osmoresponsive genes such as *STL1*, whereas *smp1* cells were defective in their expression. *smp1* cells showed reduced viability upon osmotic shock. Smp1-Hog1 interaction was checked by coprecipitation. Moreover,

Smp1 was phosphorylated upon osmotic stress in a Hog1-dependent manner and *in vitro* phosphorylation experiments showed that Hog1 phosphorylated Smp1 at the C-terminal region. This phosphorylation was important for Smp1 osmoadaptation functions.

Moreover Hog1 was implicated in cell adaptability to stationary phase through Smp1.

On the other hand, microarrays studies showed that *HXT1* hexose transporter was upregulated upon an osmotic shock in a Hog1 dependent manner. Expression of the *HXT1* gene, which encodes a low affinity glucose transporter in *Saccharomyces cerevisiae*, is induced in response to glucose by the general glucose induction pathway, involving the Snf3/Rgt2 membrane glucose sensors, the SCF-Grr1 ubiquitination complex and the Rgt1 transcription factor. In addition to the glucose signalling pathway, we have found that, regulation of *HXT1* expression also requires the HOG pathway. Deletion of components on both pathways results in impaired *HXT1* expression. Genetic analyses identified Sko1 as the transcription factor under the control of Hog1 that was modulating *HXT1* expression.

Our studies here have shown that both Smp1 and Sko1 are transcription factors under the control of the MAPK.





## Table of contents

<b>SUMMARY</b> .....	6
<b>INTRODUCTION</b> .....	11
<b>OSMOTIC STRESS</b> .....	12
Hyperosmotic Shock.....	13
Osmotic Signalling Pathways.....	14
<b>MAPK PATHWAYS</b> .....	15
Components of a MAPK Signalling Pathway.....	15
Other Signalling Components.....	17
<b>THE HOG PATHWAY</b> .....	18
<b>Components of the HOG Pathway</b> .....	20
Sln1 Branch.....	20
Sho1 Branch.....	22
Sln1 vs. Sho1.....	24
<b>Signalling Through the HOG Pathway</b> .....	25
<b>Signal Regulation</b> .....	28
PTP.....	28
PTC.....	30
<b>PHYSIOLOGICAL ROLES</b> .....	30
Transcription.....	33
<b>TRANSCRIPTION FACTORS</b> .....	36
Msn2/4.....	37
Msn1.....	38
Hot1.....	38

Sko1.....	39
<b>RESULTS.....</b>	<b>43</b>
<b><u>Paper 1:</u></b>	
Tareting The MEF2-Like Transcription Factor Smp1by The Stress-Activated Hog1 Mitogen-Activated Protein Kinase.....	44
<b><u>Paper 2:</u></b>	
Expression of the <i>HXT1</i> Low Affinity Glucose Transporter Requires the Coordinated Activities of the HOG and Glucose Signalling Pathways.....	53
<b>DISCUSSION.....</b>	<b>63</b>
<b>BIBLIOGRAPHY.....</b>	<b>82</b>

# **INTRODUCTION**

---

## **Osmotic Stress**

Yeast cells are exposed to a highly variable environment: changes in temperature, pH, radiation, availability and quality of nutrients, access to oxygen, and especially water activity (Hohmann, S.). These variations are called stresses.

In response to these stresses in the extracellular environment, cells coordinate intracellular activities to respond, adapt and maximize their probability of survival and proliferation.

Changes in water activity are one of the most common stresses. Water activity is defined as the chemical potential of free water in solution. In nature, the water activity can change widely and rapidly, due external influences and the activity of organisms.

For *Saccharomyces cerevisiae*, water activity of the cytosol and organelles has to be lower than that of the surrounding medium, for maintaining an appropriate cell volume, biochemical reactions and driving water inside the cell ((Blomberg and Adler 145-212); (Wood 230-62)). For that reason yeast cells have developed different mechanisms to survive a sudden change in water activity. These survival mechanisms need to operate within the first seconds after a sudden osmotic shift because passive water loss or uptake occurs very fast ((Blomberg and Adler 145-212); (Brown 803-46); (Brown A. D. 1990 Microbial water stress physiology: principles and perspectives. J Wiley and Sons Ltd., Chichester England 66). In this context, two different situations can occur: an osmotic upshift or an osmotic downshift. Osmotic shift or hyperosmotic shock on yeast cells is accompanied by rapid water outflow and cell shrinking. On the

---

other hand, the osmotic downshift or hypo-osmotic shock increases the water concentration gradient and leads to rapid influx of water, cell swelling, and hence increased turgor pressure. Within wide limits, the yeast cell wall prevents cell bursting (Smits et al. 348-52).

Research in our laboratory is mainly focused in hyperosmotic shock.

### ***Hyperosmotic Shock***

In an hyperosmotic situation (osmotic stress), cellular water follows its concentration gradient by passive diffusion, so that the cells lose water and the concentration of biomolecules and ions in the cell increases, resulting sometimes in an arrest of cellular activity. Yeast cells have developed mechanisms to adjust to high external osmolarity and maintain or re-establish an inside-directed driving force for water. Adaptation to altered osmolarity is an active process based on sensing osmotic changes and developing of appropriate cellular responses to maintain cellular activity. A central role in osmoadaptation is the accumulation of chemically inert osmolytes, mainly glycerol in *S. cerevisiae* ((Brown 181-242); (Yancey et al. 1214-22)).

In osmostress two different aspects need to be considered: survival of sudden changes in the external osmolarity, and the acquisition of tolerance to this high external osmolarity. The underlying molecular mechanisms for survival of a hyperosmotic shock and adaptation to high osmolarity are probably distinct but overlapping: cells adapted to moderately high osmolarity survive a severe osmotic shock better than nonadapted cells ((Norbeck and Blomberg 121-37); (Schuller et al. 4382-89); (Mager and Varela 253-58)).

---

Some years ago, it was discovered the involvement in osmoadaptation of a mitogen-activated protein kinase (MAP kinase) cascade called the HOG pathway, a conserved eukaryotic signal transduction module ((Brewster et al. 1760-63); (Gustin et al. 1264-300)). Today it is known that many basic principles of osmoadaptation are conserved across eukaryotes, and therefore yeasts and the HOG pathway are an ideal model system for study these processes.

### ***Osmotic Signalling Pathways***

Changes in medium osmolarity have been shown to affect different signalling pathways in *S. cerevisiae*: the cAMP-dependent protein kinase pathway, the phosphatidylinositol-3,5-biphosphate pathway and the most important the HOG pathway.

Protein kinase A (cyclicAMP [cAMP]-dependent protein kinase) has been shown to affect expression of genes upon an osmotic upshift (Norbeck and Blomberg 121-37). Protein kinase A mediates a general stress response that is observed under essentially all stress conditions, such as heat shock, nutrient starvation, high ethanol levels, oxidative stress, and osmotic stress ((Marchler et al. 1997-2003); (Ruis and Schuller 959-65)). Therefore, protein kinase A probably does not respond directly to osmotic changes. It is not well understood how the activity of protein kinase A is controlled by stress.

More recently, it has been observed that an osmotic shock stimulates production of phosphatidylinositol-3,5-bisphosphate, which could serve as a second messenger in an osmotic signalling system (Dove et al. 187-92). However its actions have not been established yet.

---

And finally, there is the HOG pathway. The HOG pathway is the best-characterized system implicated in osmostress. This pathway is activated within less than 1 min by osmotic upshift (Brewster et al. 1960-63) and cells lacking the pathway or unable to activate it can not survive in high osmolarity medium. Therefore, the HOG pathway coordinates a significant part of the cellular response of yeast cells to high osmolarity.

## **MAPK Pathways**

MAP kinases have been implicated in different signalling processes apart from osmostress. In fact, MAP kinase (MAPK) cascades are common signalling modules found in both higher and lower eukaryotic cells. Conservation of these cascades between yeast and humans is indicated by the fact that individual kinases in the yeast pathway can be replaced by the corresponding human enzymes (de Nadal, Alepuz, and Posas 2003).

These signalling pathways play essential roles in the response to environmental signals or hormones, growth factors, cytokines, controlling cell growth, morphogenesis, proliferation, and stress responses. ((Banuett 1994; Chang and Karin 1994); (Gustin et al. 1994); (Kultz and Burg 1994); (Kyriakis and Avruch 1994); (Ligterink and Hirt 1994)).

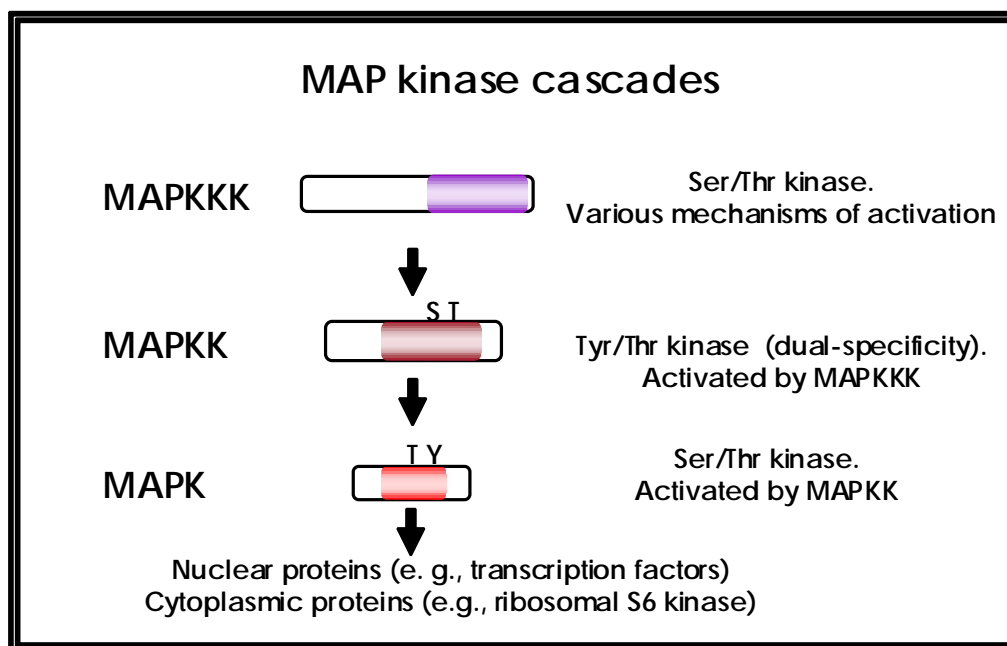
### ***Components of a MAPK Signalling Pathway: the Central Core***

All the MAPK cascades are composed of three consecutively activated tiers of kinases: a MAP kinase, a MAP kinase kinase (MAPKK) or MEK, and a MAPKKK

---

(MAPKKK) or MEKK. The MAPKKK phosphorylates and thereby activates the MAPKK on serine and threonine within a conserved part at the N-terminal lobe of the kinase domain. Subsequently, the MAPKK phosphorylates the MAP kinase on a threonine (sometimes serine) and tyrosine residue, which are located adjacent to each other separated by a single amino acid (Thr/Ser-X-Tyr). This phosphorylation site is located in the activation loop of the catalytic domain; dual phosphorylation on threonine and tyrosine is needed for activation of the MAP kinase. MAP kinase pathways are negatively controlled by protein phosphatases acting on both the MAPKK and the MAP kinase (serine-threonine phosphatases) or only on the MAP kinase (tyrosine phosphatases) (Keyse 1986-92). Typically, phosphorylation stimulates transfer of the MAP kinase from the cytosol to the nucleus, where it phosphorylates targets on serine/threonine followed by a proline. However, a portion of activated MAP kinase is apparently also present in the cytoplasm to mediate cytoplasmic events (Reiser, Ruis, and Ammerer 1991, 1147-61).





**Figure1: MAPK pathways: schematic organization.** All the MAPK cascades are composed of three consecutively activated tiers of kinases: a MAP kinase, a MAPKK and a MAPKKK. The MAPKKK phosphorylates and activates the MAPKK on serine and threonine at the N-terminal lobe of the kinase domain. Subsequently, the MAPKK phosphorylates the MAP kinase on a threonine (sometimes serine) and tyrosine residue.

### *Other Signalling Components of MAPK Signaling Pathways*

How does the signal arrive to the MAPKKK? In general MAPKKKs consist of an N-terminal regulatory and a C-terminal catalytic kinase domain. The regulatory domain locks the C-terminal kinase domain in the inactive state. Activation may occur by phosphorylation through an upstream protein kinase or through interaction with other proteins. The activation mechanisms and sensor systems upstream of MAP kinase pathways are diverse and include receptor-tyrosine kinase (in animal systems), G-protein-coupled receptors, phosphorelay systems, and others. Different MAP kinase pathways form interacting signalling systems. For instance, one MAPKK may control several different MAP kinases, as is observed for example in yeasts. Thus, different pathways within the same

---

organism often share kinases. Especially in higher eukaryotes, but even in *S. cerevisiae*, this situation results in highly complex network systems of signalling pathways.

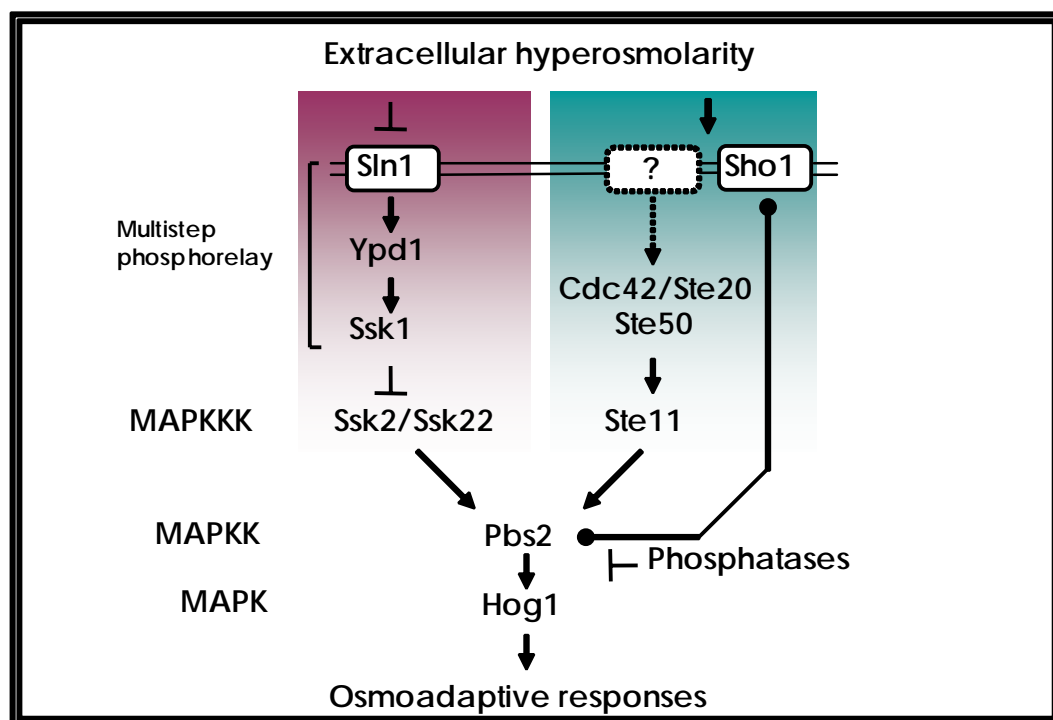
Scaffolding proteins could contribute to the signal specificity by insulating different MAPK modules. In the yeast HOG pathway, binding of Ste11 MAPKKK to Pbs2 MAPKK restricts Ste11 to activating only Pbs2, and not other MAPKKs ((Harris et al. 1815-24); (Posas and Saito 1702-05)). Although scaffolds play an important role in signalling specificity, other mechanisms may exist for signal specificity, for example MAPK substrate specificity and negative feedback loops involving, for example, protein phosphatases (Chang and Karin 37-40).

Targets of a MAPK cascade can be divided in two major groups, transcription factors and other effectors molecules (kinases, phosphatases,...) ((Alepuz, Cunningham, and Estruch 91-98); (Andrews and Stark 2685-93); (Rouse et al. 1027-37)).

## **The HOG Pathway**

The HOG pathway is the best-understood osmoresponsive system in eukaryotes and one of the best understood MAP kinase pathways. As other MAPK pathways, the central core of the yeast HOG pathway comprises a layer of MAPKKs (Ssk2, Ssk22 and Ste11) that are responsible for the activation of the MAPKK Pbs2. Once activated, Pbs2 phosphorylates and activates the Hog1 MAPK (Brewster et al. 1760-63).

Hog1 is a relative of the p38 and c-Jun N-terminal kinase (JNK) families of stress-activated protein kinases (SAPKs), specifically responds to increased extracellular osmolarity and is required for cell survival under these conditions. Its activation results in the initiation of the osmstress response and adaptation. This compresses the metabolic regulation, cell cycle adaptation and gene expression regulation.



**Figure2. The HOG Pathway.** The HOG pathway is a MAPK kinase pathway. It is constituted by a central core of MAPK kinases and two osmosensing branches Sln1 and Sho1. The signal converges at the MAPKK Pbs2. Once Hog1 has been activated different osmoadaptive responses are unleashed.

---

## ***Components of the HOG Pathway***

The HOG pathway is activated predominantly by two mechanisms that lead to the activation of either the Ssk2 and Ssk22 or the Ste11 MAPKKKs. These mechanisms are respectively, the Sln1 branch and the Sho1 branch (Maeda, Takekawa, and Saito 554-58).

All available data suggest that the Sho1 and Sln1 osmosensing branches function independently and upstream of the MAPK. The molecular mechanism(s) by which osmosensors detect osmotic changes remains unknown. They could detect changes in the physicochemical properties of the solvent due to altered water concentration or water structure; alternatively, they may sense mechanical stimuli that may occur as a consequence of the changes in water activity ((Gustin et al. 1264-300); (Wood 230-62)). However, it is commonly accepted that osmosensors operate at the cell surface as integral membrane proteins, although other possibilities could exist.

### ***Sln1 Osmosensor***

The Sln1 branch involves a “two-component” osmosensor, composed of the Sln1-Ypd1-Ssk1 proteins that conduces the signal to the redundant MAPKKKs Ssk2/22.

The Sln1 is a transmembrane protein organized into four distinct regions: an N-terminal section with two transmembrane domains, a linker region, a histidine kinase domain and a receiver domain (Ota and Varshavsky 566-69). Histidine kinases and receiver domains form so-called two-component systems, which

---

are the prototype sensing and signalling units of prokaryotes ((Saito 2497-509); (Stock, Robinson, and Goudreau 183-215)). Histidine kinase signalling systems are much less frequent in eukaryotic organisms. In fact, Sln1 is the only sensor histidine kinase in the *S. cerevisiae*.

There is strong evidence that Sln1 directly senses osmotic changes, but the nature of the signal it is not know. Whatever it is, the signal is transduced using a phopho-relay mechanism involving the Ypd1 and Ssk1 proteins.

Surprisingly, Sln1 is a negative regulator of the HOG signalling pathway, and deletion of *SLN1* is lethal because of pathway overactivation (Maeda, Wurgler-Murphy, and Saito 242-45). In other words, the Sln1 histidine kinase is activated by hypo-osmolarity (cell swelling) and inhibited by hyperosmolarity (cell shrinking). Under low osmolarity, Sln1 autophosphorylates itself, this phosphate is then transferred to the receiver domain and subsequently to a specific His on Ypd1 protein. Finally, the phosphate is transferred to an Asp on Ssk1 ((Janiak-Spens, Sparling, and West 6673-78; Janiak-Spens et al. 411-17); (Janiak-Spens and West 136-44); (Posas et al. 865-75)). The phosphorylated Ssk1 is inactive.

Upon an hyperosmotic shock, the histidine kinase activity from Sln1 drops transiently leading to the Ssk1 dephosphorylation, and consequently to its activation ((Maeda, Takekawa, and Saito 554-58); (Maeda, Wurgler-Murphy, and Saito 242-45); (Posas and Saito 1385-94); (Posas, Takekawa, and Saito 175-82; Posas et al. 865-75)). The activated Ssk1 binds to the N-terminal regulatory domain of Ssk2 leading to Ssk2 autophosphorylation and activation. The MAPKK Pbs2 has been shown to be a direct substrate for phosphorylated Ssk2 (Posas and Saito 1385-94).

### *Sho1 Osmosensor*

Pbs2 activation can also be achieved by a second, independent mechanism that involves the transmembrane protein Sho1, the MAPKKK Ste11, the Ste11-binding protein Ste50, the Ste20 p21-activated kinase (PAK) and the small GTPase Cdc 42 (Posas, Witten, and Saito 5788-96). Activation of Pbs2 by Ste11 requires the interaction of Pbs2 with Sho1 ((Reiser, Salah, and Ammerer 620-27); (Raitt, Posas, and Saito 4623-31)).

Sho1 is a protein of four transmembrane domains within the N-terminal part, a linker domain, and a SH3 domain for protein-protein interaction ((Posas and Saito 1702-05); (Raitt, Posas, and Saito 4623-31)). Functional homologs of Sho1 have been isolated from other yeasts but homologs from higher eukaryotes have not been reported. Sho1 is not an osmosensor itself (Raitt, Posas, and Saito 4623-31). However, the Sho1 branch certainly mediates HOG pathway activation upon an osmotic upshock, therefore an unidentified osmosensor should exist.

Sho1 is located at places on the cell surface where growth and cell expansion occur ((Raitt, Posas, and Saito 4623-31); (Reiser, Salah, and Ammerer 620-27)). It is possibly that the cell has to monitor osmotic changes very closely to these areas. Sho1 could function as a protein that directs signal transduction complexes to such areas. It should also be noted that anchoring of Sho1 to the cell surface is necessary for signalling, but not its specific localization.

---

It has been speculated that the Sho1 branch monitors (mainly) osmotic changes during cell growth and expansion, while the Sln1 branch (mainly) senses osmotic changes in the environment.

Activation of the Sho1 branch involves rapid and transient formation of a protein complex at the cell surface ((Raitt, Posas, and Saito 4623-31); (Reiser, Salah, and Ammerer 620-27)). The complex formed appears to consist of at least Sho1 and Pbs2. These two proteins interact via a proline-rich region of the N terminus of Pbs2 and a SH3 domain located in the hydrophilic C terminus of Sho1 ((Maeda, Takekawa, and Saito 554-58); (Posas and Saito 1702-05)). The strength with which the SH3 domain binds the Pbs2 motif correlates with the ability to activate the HOG pathway and survival at high osmolarity (Seet and Pawson R708-R710). In addition, the complex probably also contains, not necessarily at the same time, the PAK Ste20 (Raitt, Posas, and Saito 4623-31), the Rho-like G-protein Cdc42 ((Raitt, Posas, and Saito 4623-31); (Reiser, Salah, and Ammerer 620-27)) and the MAPKKK Ste11 ((O'Rourke and Herskowitz 2874-86); (Posas and Saito 1702-05)), as well as Ste50, which is required for Ste11 function ((Bulavin et al. 6845-54); (O'Rourke and Herskowitz 2874-86); (Posas, Witten, and Saito 5788-96)). Lately, Lim WA laboratory has described a region in the C terminus of Sho1 that binds Ste11 independently of Pbs2. Therefore, Sho1 has at least two separable interaction regions: one that binds Ste11 and mediates its activation, and one that binds Pbs2, directing Ste11 to act on Pbs2 (Zarrinpar et al. 825-32).

The initial signalling event it is unknown. Since Sho1 does not seem to function as a sensor itself (Raitt, Posas, and Saito 4623-31), additional proteins are probably

---

required. Sho1 then binds Pbs2 recruiting it to the cell surface. In addition, there are recruited Cdc42 plus the interacting PAK kinase(s) Ste20 and the MAPKKK Ste11. The assembly of the complex may then lead to activation of the PAK Ste20, phosphorylation of Ste11, and subsequently phosphorylation of Pbs2. Phosphorylation and activation of Pbs2 leads to dissociation of the complex.

### **Sln1 vs Sho1 Branch**

It has been proposed that different sensitivities of the two branches may allow the cell to respond over a wide range of osmolarity changes (Maeda, Takekawa, and Saito 554-58). In an *ssk2ssk22* double mutant, which completely relies on the Sho1 branch, stimulation of Hog1 tyrosine phosphorylation requires at least 300 mM NaCl, becomes visible after about 2 min, and reaches a maximum at 5 min. In contrast, in a *sho1* mutant, which relies on the Sln1 branch only, Hog1 phosphorylation is already apparent with 100 mM NaCl and is maximal after 1 min with 300 mM NaCl. These data suggest that Sln1 is more sensitive than the sensor of the Sho1 branch. It also appears that the Sho1 branch operates in an on-off fashion, while the Sln1 branch shows an approximately linear dose response up to about 600 mM NaCl.

Other less well-characterized osmosensing mechanisms could also be feeding signals into the HOG pathway (Van Wuytswinkel et al. 382-97). Since mammalian cells do not seem to have specific stress sensors similar to Sln1, determination of the sensor mechanism coupled to Sho1 could help to decipher the molecular identity of mammalian osmosensors.



---

Moreover a third osmosensing branch working in parallel with the Sho1 branch was proposed. This branch would consist in the osmosensor membrane protein Mbs2 (O'Rourke and Herskowitz 4739-49). The authors of this work based their assumption in the fact that there is some residual signalling, which is *STE11* dependent, still occurring in the absence of Sho1, and that this signalling requires Mbs2 activity. However an *ssk1 sho1 msb2* strain is more osmosensitive than an *ssk1 sho1 MBS2*, the biological importance of Mbs2 is not quite clear.

### ***Signaling Through the HOG Pathway***

Pbs2 is activated by phosphorylation on Ser514 and Thr518 by any of the three MAPKKs Ssk2/Ssk22 and Ste11. Phosphorylation of the substrate of Pbs2, the Hog1 MAP kinase, occurs in the cytosol. Dual phosphorylation on the conserved Thr174 and Tyr176 activates the MAP kinase Hog1 ((Brewster et al. 1760-63); (Schuller et al. 4382-89)). The timing and the period of the response depends on the severity of the shock. When an osmotic shock is low, such as 0.4 M NaCl, Hog1 phosphorylation peaks within 1 min and disappears within about 30 min. With a more severe osmotic shock, for instance, 1.4 M NaCl, Hog1 phosphorylation peaks at about 30 min and remains high for several hours before it decays ((Vandenbol, Jauniaux, and Grenson 153-59).

Phosphorylation causes a rapid and marked concentration of Hog1 in the nucleus, while under normal conditions Hog1 is distributed between the cytosol and the nucleus ((Ferrigno et al. 5606-14); (Reiser, Ruis, and Ammerer 1147-61)). Nuclear concentration of Hog1-GFP can be observed within less than 1 min after a hyperosmotic shock. This effect is specific, since a range of other stress

---

conditions do not cause Hog1 phosphorylation and do not mediate nuclear translocation ((Ferrigno et al. 5606-14); (Reiser, Ruis, and Ammerer 1147-61); (Schuller et al. 4382-89)). Phosphorylation on both Thr174 and Tyr176 of Hog1 by Pbs2 is necessary and sufficient for nuclear concentration, since mutation of one or both of these sites makes the subcellular localization of Hog1 unresponsive to osmotic shock ((Ferrigno et al. 5606-14); (Reiser, Ruis, and Ammerer 1147-61)). The transport into the nucleus is dependent on the phosphorylation status of Hog1 but not of its protein kinase activity (Ferrigno et al. 5606-14). Nuclear export however, is dependent on its kinase activity and coincides with its dephosphorylation (Reiser, Ruis, and Ammerer 1147-61).

Concentration of Hog1 in the nucleus requires Gsp1 (Ferrigno et al. 5606-14), a Ran G-protein needed for nuclear import of proteins containing nuclear localization signals (Oki et al. 624-34). Nuclear import of Hog1 also requires the karyopherin-beta Nmd5, while several other known nuclear import factors do not seem to be required (Ferrigno et al. 5606-14).

Several proteins affect residence of Hog1 in the nucleus for example, phosphatases. Ptp2 and Ptp3 function as nuclear and cytoplasmic anchors for Hog1 respectively (Mattison and Ota 1229-35). Therefore, deletion of *PTP2* diminishes Hog1 nuclear accumulation and overexpression prolongs the period of nuclear residence, while the opposite was observed for *PTP3* (Mattison et al. 7651-60).

Transcription factors are implicated in the MAPK nuclear retention too. Deletion of the genes encoding the transcription factors Msn2, Msn4 (Reiser, Ruis, and Ammerer 1147-61), Hot1, and Msn1 (Rep et al. 5474-85) has been shown to

reduce the period of Hog1 nuclear localization ((Reiser, Ruis, and Ammerer 1147-61); (Rep et al. 5474-85)).

Interestingly, the more severe the osmotic shock, the longer it takes until phosphorylated, active Hog1 is translocated into the nucleus, an observation at odds with the apparent need to respond even more rapidly to severe stress (Vandenbol, Jauniaux, and Grenson 153-59). Also, the HOG-dependent transcriptional response is delayed under such conditions, as apparent from time courses of mRNA levels of HOG-dependent genes after osmotic shock ((Rep et al. 715-27); (Vandenbol, Jauniaux, and Grenson 153-59)). This suggests that some adaptation must occur before Hog1 can be activated and transferred to the nucleus and that translocation is specifically blocked in an unknown way until this process is completed.

Both phosphorylation of Hog1 and nuclear localization are transient effects. Depending on the severity of the osmotic shock, Hog1 remains phosphorylated and located in the nucleus for several minutes or even up to a few hours ((Brewster et al. 1760-63); (Maeda, Wurgler-Murphy, and Saito 242-45); (Mattison and Ota 1229-35); (Tamas et al. 159-65) ; (Vandenbol, Jauniaux, and Grenson 153-59)). There is a good correlation between the period of Hog1 phosphorylation and its apparent nuclear localization ((Ferrigno et al. 5606-14); (Mattison and Ota 1229-35); (Reiser, Ruis, and Ammerer 1147-61)), which could indicate a causal relationship between nuclear export and dephosphorylation. Hog1 nuclear accumulation suggests that part of the actions under the control of the MAPK take place in this subcellular compartment. However, there is not

doubt that activated Hog1 also mediates regulatory effects outside the nucleus. Therefore, either a portion of activated Hog1 remains in the cytosol.

### ***Signal Regulation: Phosphatases***

The appearance of phosphorylated Hog1 is a transient event ((Jacoby et al. 17749-55); (Maeda, Wurgler-Murphy, and Saito 242-45); (Tamas et al. 159-65); (Vandenbol, Jauniaux, and Grenson 153-59)). Therefore, the pathway is controlled by specific feedback mechanisms. One of these feedback mechanisms proposed involves protein phosphatases.

Two phosphotyrosine phosphatases (Ptp2 and Ptp3) as well as three phosphoserine/threonine phosphatases (Ptc1 to Ptc3) genetically interact with the HOG pathway; overexpression of any of these phosphatases suppresses the lethality caused by inappropriate activation of the HOG pathway ((Jacoby et al. 17749-55); (Maeda, Wurgler-Murphy, and Saito 242-45); (Mattison and Ota 1229-35); (Ota and Varshavsky 2355-59) ; (Warmka et al. 51-60); (Wurgler-Murphy et al. 1289-97)). In the case of Ptp2, Ptp3, and Ptc1, there is great direct evidence that they affect the HOG pathway and act upon Hog1.

### ***PTPs***

Overexpression of *PTP2* and *PTP3* suppresses inappropriate activation of the HOG pathway by deletion of *SLN1* or constitutive activation of *Sln1*, *Ssk2*, or *Pbs2*, suggesting that they indeed target the MAP kinase ((Jacoby et al. 17749-55); (Wurgler-Murphy et al. 1289-97)). As expected, overexpression of *PTP2* and *PTP3* diminishes the amount of tyrosine-phosphorylated Hog1. Ptp2 and Ptp3

---

interact directly with Hog1 ((Jacoby et al. 17749-55); (Wurgler-Murphy et al. 1289-97)). In *ptp2* and *ptp2 ptp3* mutants, tyrosine phosphorylation of Hog1 upon osmotic shock is stronger and more prolonged. Moreover, in the double mutant is also observed without osmotic shock ((Jacoby et al. 17749-55); (Wurgler-Murphy et al. 1289-97)). Since even in the *ptp2 ptp3* double mutant the level of tyrosine-phosphorylated Hog1 is still responsive to osmotic shock, other dephosphorylation mechanisms must exist ((Jacoby et al. 17749-55); (Wurgler-Murphy et al. 1289-97)).

The observation that expression of *PTP2* and *PTP3* is stimulated after osmotic shock in a HOG-dependent manner suggested that the phosphatases are part of a feedback loop ((Jacoby et al. 17749-55); (Wurgler-Murphy et al. 1289-97)). However, this transcriptional effect cannot be responsible for the rapid decline of the phosphorylation state of Hog1, because the increase in phosphatase activity due to increased production of the enzyme occurs after the level of Hog1 phosphorylation has started to decrease (Wurgler-Murphy et al. 1289-97). Instead, some observations suggest that the phosphatases might not perform as specific regulators. The negative feedback loop via Hog1-dependent stimulation of *PTP2* and *PTP3* expression would mean that the pathway inactivates itself in an autoregulatory mode. However an autonomous negative feedback loop would ignore the success of the response. On the other hand, dephosphorylation of Hog1 requires its catalytic activity (Wurgler-Murphy et al. 1289-97). This suggests that Hog1 activates the phosphatases to stimulate its own deactivation.

---

More studies have to be done to clarify how the phosphates action over the cascade is controlled.

### *PTCs*

Among the serine/threonine phosphatases, Ptc1 seems to be the only implicated in the deactivation of the HOG pathway. In mutants lacking both Ptc1 and Ptp2, the inappropriate HOG pathway overactivation causes a growth defect. No other combination of deletion mutations between the two Ptps and the three Ptcs causes similar effects (Warmka et al. 51-60). Deletion of *PTC1* causes constitutive dual phosphorylation of Hog1 which is hardly responsive to osmotic shock (Warmka et al. 51-60). Perhaps Ptp2 preferentially dephosphorylates Hog1 that already is dephosphorylated on Thr174.

### **Physiological Roles of Hog1**

Hog1 phosphorylation results in its activation. At the same time, Hog1 phosphorylation led to its rapid accumulation in the nucleus. As it has been mentioned, Hog1 nuclear accumulation indicates that part of the actions under the control of the MAPK take place in this compartment.

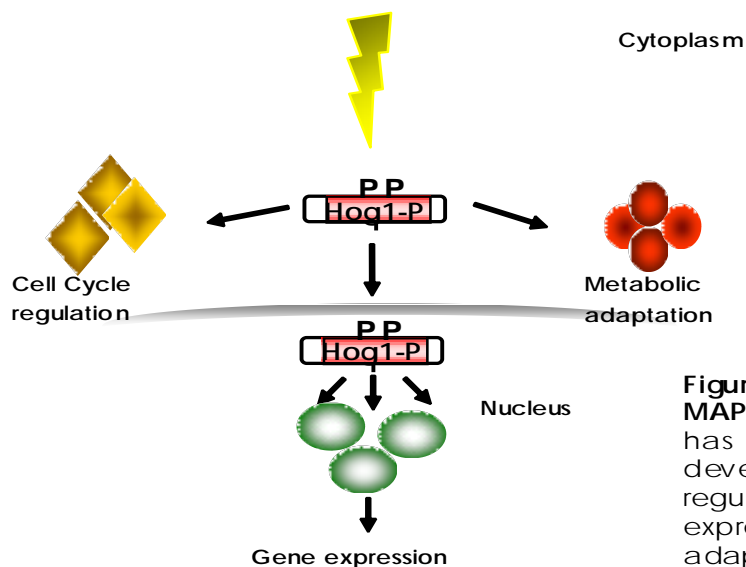
One of these actions is regulation of the gene expression. In *S. cerevisiae*, genome-wide transcription studies have shown that a large number of genes (~7%) show changes in their expression levels after a mild osmotic shock and that the Hog1 MAPK plays a central role in much of this global gene regulation. These osmostress-regulated genes are implicated mainly in carbohydrate metabolism, general stress protection, protein production and signal

---

transduction. This global change in transcription could account, at least in part, for the metabolic adjustments required for osmostress adaptation (Hohmann 300-72).

On the other hand, Hog1 activation results in the adaptation of the cell cycle. Sustained activation of the Hog1 MAPK led to cell growth arrest even in the absence of stress suggesting a possible role of the MAP kinase in the control of the cell cycle. It have been described that hyperactivation of the HOG pathway resulted in accumulation of cells in the G1/S and G2/M transitions, similar to the observed for osmotically stressed cells. Actually, G1 arrest upon osmostress is mediated by Hog1(Escote et al. 997-1002). Hog1 regulates progression at the G1 phase by a dual mechanism that involves downregulation of cyclin expression and direct targeting of the CDK-inhibitor protein Sic1. The MAPK interacts physically with Sic1 *in vivo* and *in vitro*, and phosphorylates a single residue at the carboxyl terminus of Sic1, which, in combination with the downregulation of cyclin expression, results in Sic1 stabilization and inhibition of cell-cycle progression (Escote et al. 997-1002).

These results indicate that Hog1 imposes a delay in these critical phases. The arrest could be a necessary mechanism for proper adaptation to a new conditions and cell survival.



**Figure 3. Functions of the MAPK Hog1.** Once Hog1 has been activated it develops different functions regulating cell cycle, gene expression and metabolic adaptation.

Hog1 is also implicated in metabolic adaptation and translation.

For instance, the activation of the HOG-MAPK pathway leads to an activation of 6-phosphofructo-2-kinase (PFK2) which is accompanied by a complex phosphorylation pattern of the enzyme that increase its enzymatic activity. This change in the activity is the result of phosphorylation of the enzyme mediated by protein kinases from the HOG-MAPK pathway. The activation of PFK2 leads to an activation of the upper part of glycolysis, which is a precondition for glycerol accumulation, one of the main osmoadaptive process. Yeast cells containing *PFK2* accumulate three times more glycerol than cells lacking *PFK2*, which are not able to grow under hypertonic stress.

Hog1 is implicated in translation too. One example is the regulation of the kinase Rck2. Rck2 is a yeast Ser-Thr protein kinase homologous to mammalian calmodulin kinases. This kinase requires phosphorylation for activation. Hog1 is



---

able to phosphorylate Rck2. In fact, Rck2 phosphorylation is transiently increased during osmostress or in mutants with a hyperactive HOG pathway (Bilsland-Marchesan et al. 3887-95). Once Rck2 has been phosphorylated by Hog1, it acts over the translation elongation factor 2, and this is important for attenuation of protein synthesis in response to osmotic stress (Teige et al. 5625-30).

### ***Transcription***

As it has been mentioned, increases in extracellular osmolarity results in changes in the expression of a large number of genes.

Better understanding of the yeast response to osmotic stress could be achieved from the use of DNA microarrays to perform a genome-wide analysis of the transcriptional response under this type of stress.

In the last few years, there has been a total of six independent studies in which yeast cells were exposed to osmotic stress and the global transcriptional response has been analyzed ((Causton et al. 323-37); (Gasch et al. 4241-57); (Posas et al. 17249-55); (Rep et al. 8290-300); (Yale and Bohnert 15996-6007); (O'Rourke and Herskowitz 532-42)). From all them, it can be concluded: first, that transcriptional responses after osmotic stress depends on the conditions tested (such as time of exposure to salt and osmolite concentration); second, that Hog1 controls three major aspects of the response to increased osmolarity: it determines the magnitude of gene induction, determines the duration of gene regulation, and limits activation of other MAPK cascades. And finally, it is important to understand that responses to ion stress require the activity of several pathways and that a single gene can receive different inputs

---

(Antonsson et al. 16821-28). For instance, the expression of the *ENA1* ATPase is regulated by both a calcium signalling pathway and the HOG signalling pathway (Aoyama et al. 4868-74).

Different classifications have been proposed to organize genes whose transcription change upon an osmotic stress: by biological function, type of response or time of response (Posas et al. 17249-55). Attending to the onset of transcription and biological function it have been proposed the next list of highly induced genes after exposure to 0.4 M NaCl for 10 min (Posas et al. 17249-55): genes encoding proteins involved in carbohydrate metabolism, components of the protein biosynthesis machinery, proteins related to ion homeostasis, proteins required for signal transduction and finally genes encoding unknown proteins.

A significant proportion of the induced genes is also controlled by other stress conditions. In many cases this reflects a general stress response mediated by Msn2 and Msn4 proteins. There are at least two explanations for the large overlap between stress responses: different stress conditions can often occur in nature simultaneously, and therefore any type of stress stimulates a broad response; and the specific stress disturbs cellular functions leading to another type of stress. For instance, osmotic stress may interfere with electron transport leading to the production of reactive oxygen species. In this case osmotic shock would indirectly induce an oxidative stress inducing genes implicated in oxidative protection. Genes encoding for proteins required for oxidative protection and for the heat shock response are induced very rapidly upon osmotic shock.

---

Induction of a number of genes after osmotic shock is mediated at least in part by the Hog1 signal transduction pathway (Bjorkqvist et al. 128-32). The analysis of the contribution of the HOG pathway it has been done comparing the level of induction of responsive genes in wild type cells and in a strain lacking the MAP kinase gene. Analysis of the data indicated that in many cases the response in *hog1* cells was different from that observed in wild type cells. However, from a quantitative point of view, the changes ranged from a weak effect to a virtual loss of the response. This indicates that the Hog1 pathway is certainly involved in the transcriptional response of most salt-responsive genes, but in many cases, it is not the unique relevant signalling pathway. The level of dependence on Hog1 differs upon the stress conditions.

On the other hand, the group of HOG-dependent genes contains many of the most strongly induced and most highly expressed osmoregulated genes, and the majority of the genes have been characterized previously.

In addition, in salt medium a set of genes was strongly expressed in the *hog1* mutant than in the wild type. The majority of these genes are known to be involved in the mating of yeast cells; their expression is stimulated by mating pheromone. Stimulated expression of these genes results on the inappropriate stimulation of the pheromone response pathway which share components ((Akada, Yamamoto, and Yamashita 267-74); (Albertyn, Hohmann, and Prior 12-18)) with the HOG pathway.

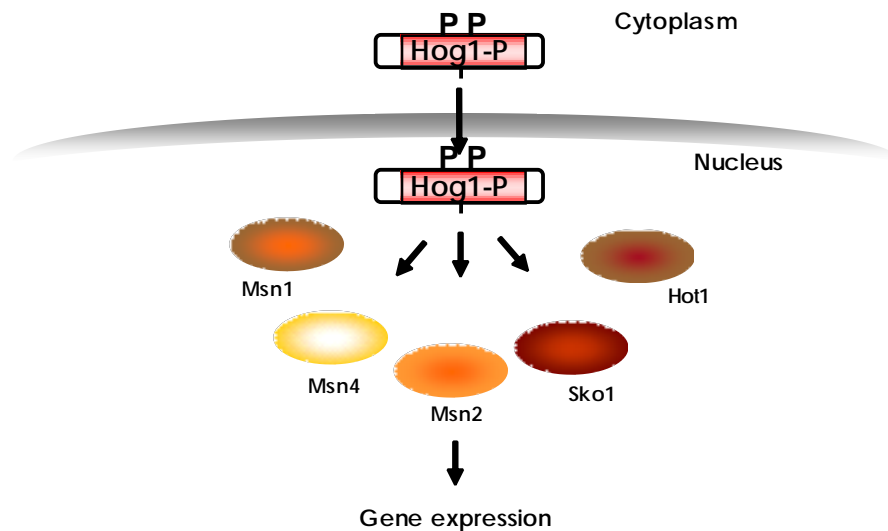
## **Transcription Factors Under the Control of the MAPK**

The Hog1 kinase mediates its effects through at least five different transcription factors: the redundant zinc finger proteins Msn2 and Msn4 (Schuller et al. 4382-89); Hot1 (which does not belong to a known family of transcription factors) (Rep et al. 5474-85); the bZIP protein Sko1 (Proft et al. 1123-33), and the MADS box protein Smp1 (de Nadal, Casadome, and Posas 229-37).

At the beginning of this work, Hog1 transcription effectors were unclear. During the last years intensive research have resulted, not only in the identification on new transcription factors (for instance Smp1, whose description is included in this work), but also in the characterization of the mechanisms underlying those factors that were known at that moment. For instance, Sko1 was first described as a repressor of gene expression, however lately, it has been described with activator properties depending on stress (Proft and Struhl 1307-17).

In addition to the conventional MAPK role in regulating transcription factor activity by direct phosphorylation of transcription factors, it has been report that Hog1 can associate with the chromatin at promoter regions of a target genes. Activated Hog1 is recruited to osmotic-inducible promoters through interaction with specific transcription factors (Alepuz et al. 767-77). Furthermore, recent results support a model in which promoter-localized Hog1 stimulates transcription by phosphorylation of specific components of the RNA pol II holoenzyme (Alepuz et al. 2433-42). Additionally, Hog1 has been reported to bind to Sko1-dependent promoter through its interaction with Sko1, and both

proteins are required for the recruitment of the SAGA histone acetylase and SWI/SNF nucleosome-remodeling complexes in response to osmostress necessary for transcription (Proft and Struhl 1307-17).



**Figure 4. Transcription factors under the control of the MAPK Hog1.** At the beginning of this work there were Known five transcription factors under the control of the MAPK Hog1.

## ***MSN2/4***

Msn2/Msn4 are a pair of apparently redundant transcription factors that bind to stress response elements (STREs) ((Aoyama et al. 4868-74); (Aramburu, Rao, and Klee 237-95); (Aravind and Ponting 458-59)). These elements are characterized by the core sequence CCCCT in either orientation and are usually found in two or more copies in front of Msn2/Msn4 target genes ((Ayscough and Drubin 927-30); (Baker 3774-84)). These appear to control a general stress response.

---

Both transcription factors shuttle between the nucleus and the cytoplasm, by a variety of mechanisms that are thought to involve the protein kinase A, Hog1, and TOR kinase signal transduction pathways, depending on the environmental conditions (Schmitt and McEntee, 1996); (Beck and Hall, 1999); (Rep et al. 715-27)). Upon stress, Msn2/Msn4 are phosphorylated and translocated to the nucleus ((Barbet et al. 25-42); (Batiza, Schulz, and Masson 23357-62)). Since in osmotic shock conditions, Msn2 and Hog1 are both localized in to the nucleus, they could interact in this compartment ((Alonso-Monge et al. 717-30); (Amoros and Estruch 1523-32); (Barbet et al. 25-42)). One of the most accepted model points out the protein kinase A mediating translocation of Msn2 (and Msn4) to the nucleus under any type of stress conditions. Once in the nucleus, further stress-specific processes, for instance mediated by Hog1, could confer full transcriptional activity to Msn2.

Msn2/Msn4 control a subset of the Hog1-dependent genes. Most of these genes contain several STREs, however some of the genes whose osmotic induction was strongly affected by deletion of *MSN2* and *MSN4* do not appear to contain STRE elements at all.

### ***MSN1***

Msn1 is distantly related to Hot1 and has been shown to affect the expression of *GPD1* and *CTT1* after osmotic shock (Ansell et al. 2179-87). However, the relationship of Msn1 to the HOG pathway has not yet been investigated.

### ***HOT1***

---

Hot is one of the best characterized transcription factors under the control of Hog1. Hot1 physically interacts with Hog1. Its binding to DNA and subsequent transactivation activity are regulated by the kinase ((Rep et al. 8290-300); (Alepuz et al. 2433-42)).

Analysis of the mechanism by which the Hot1 controls Hog1-mediated osmostres gene expression have shown that the transcription factor interacts with Hog1, and this interaction is critical for recruitment of the MAPK to Hot1 dependent promoters and essential for their transcriptional induction upon stress (Alepuz et al. 767-77).

Although Hot1 is phosphorylated by the Hog1, this direct phosphorylation of Hot1 by the MAPK is indeed not critical for the regulation and activation *per se*. Thus activation of the gene expression by Hot1 must revolve around a mechanism other than phosphorylation of the activator by the MAPK (Alepuz et al. 767-77). Actually, it serves as an anchor for the MAPK to recruit the RNA pol-II enzyme (Alepuz et al. 2433-42).

Deletion of *HOT1* partially suppresses the lethality caused by over activation of the HOG pathway. This indicates that Hot1 is partially mediating the HOG-dependent responses (Rep et al. 5474-85).

### ***SKO1***

Sko1 is a protein that belongs to the ATF/CREB family of AP1-related transcription factors (ATF) ((Nehlin, Carlberg, and Ronne 5271-78); (Vincent and Struhl 5394-405)), which in mammalian cells are known as cAMP response element (CRE)-binding (CREB) proteins (De Cesare and Sassone-Corsi 343-69). Such factors

---

possess a bZIP domain, i.e., a leucine zipper for dimerization, and a basic transcription activation domain. Many ATF/CREBs can form dimers not only with themselves but also with other members of the same family, depending on how the binding site is organized. Sko1 is largely localized in the nucleus. Its localization is not affected by mild osmotic stresses (Pascual-Ahuir et al. 37373-78), but after a 1M NaCl stress, Sko1 is localized at the entire cell.

Sko1 is a target of different signalling pathways: such as protein kinase A ((Pascual-Ahuir et al. 37373-78); (Proft et al. 1123-33)), and the HOG pathway. One or both signalling pathways act on Sko1 activity for the proper expression of different subset of genes.

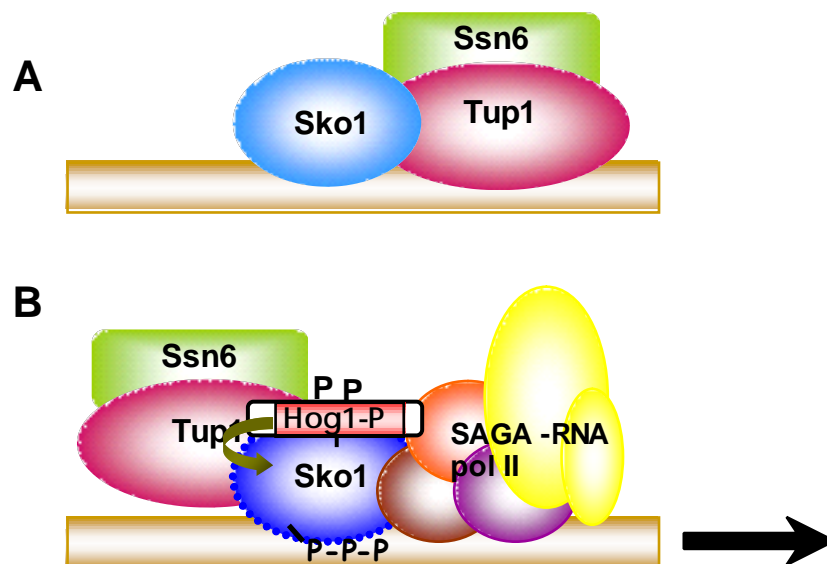
The nature of the genes regulated by Sko1 is diverse. Sko1 controls expression of a Na export plasma membrane pump called *ENA1*, *HXT1* (an hexose transporter gene. Described in this work), and other genes whose function it is not known, such as *GRE2* and *HAL1*. The promoter regulation of *HAL1* and *GRE2* seem to be simpler than that of *ENA1* and *HXT1*, where stimulated expression under salt stress is mediated exclusively via the CRE site(s) (Sko1 DNA binding sites) (Proft and Serrano 537-46)). In contrast in *ENA1* and *HXT1* promoters, Sko1 is implicated in coordination with other transcription factors and signalling pathways to regulate properly gene expression.

The Hog1 dependent molecular mechanism for Sko1 activity was described some time ago. Sko1 is phosphorylated directly by Hog1 at multiple sites within the N-terminal region (Proft et al. 1123-33). Recently, it has been described that upon an osmotic shock, the complex of phosphorylated Sko1p-Ssn6-Tup1-Hog1, in *GRE2*, *HAL1* promoters, recruits SWI/SNF and SAGA chromatin modifying



complexes which promote RNA polymerase II binding and transcriptional activation (Proft and Struhl 1307-17). The ability of Sko1-Ssn6-Tup1 complex to switch between a transcriptional activator and an active repressor may be a general mechanism for the cell to achieve a rapid change of transcriptional activity.

In addition, PKA is also able to phosphorylate Sko1 and this phosphorylation seems to increase the repressor activity of Sko1.



**Figure 5. Osmotic-regulated transcriptional activation of the *GRE2* gene by the transcription factor Sko1.** (A) During basal conditions Sko1 recruits Tup1-Ssn6 complex and repress expression. (B) After an osmotic stress, Hog1 phosphorylates Sko1 becoming an activator and recruiting SAGA and SWF/SNF complex and RNAPol II complex.



## RESULTS

## PAPERS

### *Paper 1:*

De Nadal E, Casadome L, Posas F. "Targeting the MEF2-like transcription factor Smp1 by the stress-activated Hog1 mitogen-activated protein kinase. *Molecular and Cellular Biology*. 2003 Jan; 23(1): 229–37.

### *Paper 2:*

Tomas-Cobos L, Casadome L, Mas G, Sanz P, Posas F. "Expressions of the HXT1 low affinity glucose transporter requires the coordinated activities of the HOG and glucose signalling pathways." *Journal of Biological Chemistry*. 2004 May; 279(21): 22010-9.

## Targeting the MEF2-Like Transcription Factor Smp1 by the Stress-Activated Hog1 Mitogen-Activated Protein Kinase

Eulàlia de Nadal, Laura Casadomé, and Francesc Posas\*

Cell Signaling Unit, Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra,  
Barcelona E-08003, Spain

Received 21 June 2002/Returned for modification 5 August 2002/Accepted 2 October 2002

Exposure of *Saccharomyces cerevisiae* to increases in extracellular osmolarity activates the stress-activated Hog1 mitogen-activated protein kinase (MAPK), which is essential for cell survival upon osmotic stress. Yeast cells respond to osmotic stress by inducing the expression of a very large number of genes, and the Hog1 MAPK plays a critical role in gene transcription upon stress. To understand how Hog1 controls gene expression, we designed a genetic screen to isolate new transcription factors under the control of the MAPK and identified the MEF2-like transcription factor, Smp1, as a target for Hog1. Overexpression of *SMP1* induced Hog1-dependent expression of osmosensitive genes such as *STL1*, whereas *smp1Δ* cells were defective in their expression. Consistently, *smp1Δ* cells displayed reduced viability upon osmotic shock. In vivo coprecipitation and phosphorylation studies showed that Smp1 and Hog1 interact and that Smp1 is phosphorylated upon osmotic stress in a Hog1-dependent manner. Hog1 phosphorylated Smp1 in vitro at the C-terminal region. Phosphorylation of Smp1 by the MAPK is essential for its function, since a mutant allele unable to be phosphorylated by the MAPK displays impaired stress responses. Thus, our data indicate that Smp1 acts downstream of Hog1, controlling a subset of the responses induced by the MAPK. Moreover, Smp1 concentrates in the nucleus during the stationary phase, and the lack of *SMP1* results in cells that lose viability in the stationary phase. Localization of Smp1 depends on *HOG1*, and consistently, *hog1Δ* cells also lose viability during this growth phase. These data suggest that Smp1 could be mediating a role for the Hog1 MAPK during the stationary phase.

Mitogen-activated protein kinase (MAPK) cascades are common signaling modules found in both higher and lower eukaryotic cells. Activation of a MAPK results in modification of a set of target proteins, often transcription factors, that allow the generation of appropriate cellular responses to an external stimulus. Stress-activated protein kinases (SAPKs) are a subset of MAPKs activated by environmental and genotoxic stresses (reviewed in references 9 and 20). A prototype of the SAPK family is the yeast p38-related MAPK, Hog1, which specifically responds to increased extracellular osmolarity and is required for cell survival under these conditions.

Activation of the yeast Hog1 MAPK induces diverse osmo-adaptive responses, such as regulation of gene expression. Genome-wide transcriptional analyses showed that a great number of genes are regulated by osmotic stress in a *HOG1*-dependent manner. Among the genes under the control of Hog1 are genes that encode proteins implicated in carbohydrate metabolism, general stress protection, protein production, and signal transduction (reviewed in reference 8). One mechanism by which SAPKs, and MAPKs in general, modulate gene expression is by direct modification of transcription activators. In *Saccharomyces cerevisiae*, only four transcription factors, Sko1, Hot1, and the redundant Msn2 and Msn4, have been proposed to be controlled by the Hog1 MAPK. These factors are unrelated, and the mechanisms by which Hog1

regulates their function may differ from one to another. Hot1, Msn2, and Msn4 activate transcription, whereas Sko1 represses and activates different subsets of osmotic-inducible and Hog1-regulated genes (15, 17, 18). Sko1 is an ATF/CREB factor that represses genes under nonstress conditions by the recruitment of the general corepressor complex Cyc8-Tup1. In response to osmotic stress, Sko1 is phosphorylated by Hog1, thereby relieving repression (15, 16). Msn2 and Msn4 are generic stress factors controlled by PKA and Hog1 by an unknown mechanism. Hot1 physically interacts with Hog1, and its binding to DNA and subsequent transactivation activity are regulated by Hog1 kinase activity (1, 19). Global gene expression analyses carried out to dissect the specific roles for each transcription factor have revealed that each of these factors can account for a limited effect on global gene expression by regulation of a small subset of the osmostress-inducible genes (3, 17). Evaluation of the subset of osmostress-responsive genes controlled by each factor revealed that the activators reported to be under the control of Hog1 were not sufficient to explain the impact of Hog1 on gene expression.

The mechanism by which Hog1 regulates gene expression is not completely understood. In particular, there remained the possibility that additional transcription factors were required for gene expression upon stress. Therefore, we undertook a genetic screen to identify new activators under the control of the MAPK. Thus, we found that Smp1, a member of the MEF2C family of transcription factors, is involved in Hog1 signaling. Interestingly, it was reported previously that in metazoan cells, the MEF2C family of transcription factors can be targeted by the mammalian p38 MAPK (11). We show here

\* Corresponding author. Mailing address: Cell Signaling Unit, Facultat de Ciències de la Salut i de la Vida, Universitat Pompeu Fabra (UPF), C/Doctor Aiguader 80, Barcelona E-08003, Spain. Phone: 34-93-542 2848. Fax: 34-93-542 2802. E-mail: francesc.posas@ceps.upf.es.

that Hog1 and Smp1 interact *in vivo*, and we have identified the relevant Hog1 phosphorylation sites in Smp1 and analyzed their effect on stress-regulated gene expression *in vivo*. Furthermore, we report that Smp1 may play an important role not only in osmotic stress responses, but also in a new function for the Hog1 MAPK required for cell survival in the stationary phase.

## MATERIALS AND METHODS

**Yeast strains.** The following yeast strains were used: L40 (*MATa ura3 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ*), TM141 (*MATa ura3 leu2 ura1 his3*), TM233 (*MATa ura3 leu2 ura1 his3 his2 hog1::TRP1*), YEN44 (*MATa ura3 leu2 ura1 his3 smp1::HIS3*), YEN46 (*MATa ura3 leu2 ura1 his3 his2 hog1::TRP1 smp1::HIS3*), and YEN114 (*MATa ura3 leu2 ura1 HOG1-HA×6-HIS3*). Integration of the *STL1-LacZ* reporter construct (PEN05) in wild-type, *hog1Δ*, and *smp1Δ* strains yielded YEN2 (*MATa leu2 ura1 his3 STL1-LacZ URA3*), YEN7 (*MATa leu2 ura1 his3 his2 hog1::TRP1 STL1-LacZ URA3*), and YEN48 (*MATa leu2 ura1 his3 smp1::HIS3 STL1-LacZ URA3*). Genomic disruptions were made by long flanking homology PCR-based gene disruption.

**Buffers and media.** Buffer A consists of 50 mM Tris-HCl (pH 7.5), 15 mM EDTA, 15 mM EGTA, 2 mM dithiothreitol (DTT), 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamide, and 5 μg of leupeptin per ml. Kinase buffer consists of 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 10 mM MgCl<sub>2</sub>. Buffer B consists of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 2 mM DTT, 1% Triton X-100, 1 mM PMSF, 1 mM benzamide, and 5 μg of leupeptin per ml. Kinase buffer consists of 50 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, and 2 mM DTT. Phosphatase inhibitor mixture contains 10 mM NaF, 1 mM sodium pyrophosphate, and 10 mM β-glycerophosphate. Sodium dodecyl sulfate (SDS) loading buffer consists of 50 mM Tris-HCl (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol. Yeast extract-peptone-dextrose (YPD) medium contains, per liter, 10 g of yeast extract, 20 g of peptone, and 20 g of dextrose. Selective medium contains 1.7 g of yeast nitrogen base (Difco) per liter, 5 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> per liter, 20 g of dextrose per liter, and supplements (100 mg each of the amino acid[s] uracil or adenine, as appropriate, except where indicated). X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) solid medium contains selective medium buffered with MES [2-(N-morpholino)ethanesulfonic acid] at pH 7 plus 0.1 mg of X-Gal per ml. All yeast growth was at 30°C.

**Plasmids.** The *STL1-LacZ* reporter construct PEN05 was generated by cloning the *STL1* promoter (base pairs -824 to +4) by PCR into Yip358R (*CEN URA3*) (12). Plasmid PEN45 carries a 3.4-kbp *EcoRI*/*SacI* fragment containing *SMP1* in a multicopy vector YEp-lac181. p426GAG1 (*P<sub>GAG1</sub>-GST URA3<sup>+</sup>* 2 μm) is a gift from M. Takekawa (unpublished). The *P<sub>GAG1</sub>-GST* allows the expression of GST-fusion proteins via the yeast *P<sub>GAD1</sub>* promoter. Full-length wild-type and mutant *SMP1* genes were cloned into plasmid p426GAG1. Full-length *SMP1* and *SMP1ΔMADS* (which contains a 90-amino-acid terminal deletion of the MADS box domain) were cloned into the *SacI* site of the pBTM116 plasmid to fuse them to the LexA binding domain. pACT11-HOG1 was obtained by fusion of the full-length *HOG1* PCR product with the *GAL4* activation domain in pACT11. The bacterial expression plasmid pGEX-4T (Pharmacia) allows the expression of GST-tagged proteins in *Escherichia coli*. Full-length wild-type and several truncated *SMP1* alleles were cloned into the *EcoRI* site of the pGEX-4T plasmid by PCR. Construction of *SMP1* site-directed mutants was carried out by PCR-directed mutagenesis. Each mutation was verified by DNA sequencing. The *SMP1-m4* was sequenced to verify that it contained only the desired mutations. The m1 mutant contains a double amino acid substitution, namely, Ser365 and Ser376 to Ala, while m2 contains the Ser348 and the Ser357 amino acids mutated to Ala. The m4 contains a quadruple amino acid substitution, namely, Ser348, Ser357, Thr365 and Ser376 to Ala. Those mutant alleles were cloned into pGEX-4T for bacterial expression and into p426GAG1 (*P<sub>GAG1</sub>-GST URA3<sup>+</sup>* 2 μm) for yeast expression. The *SMP1*-GFP plasmid contains the *SMP1* gene fused to the green fluorescent protein gene (*GFP*) in a multicopy plasmid pRS426.

**Isolation of *STL1-LacZ* reporter activators.** Wild-type yeast strain TM141 carrying an integrated *STL1-LacZ* reporter (PEN05) was transformed with a yeast genomic library in the multicopy plasmid YEp13. Colonies were grown in synthetic media containing X-Gal for 5 to 6 days. From approximately 30,000 colonies, 20 positive clones were selected by their ability to induce *STL1* and thus to produce β-galactosidase. Plasmids were isolated, and their *HOG1* dependence was evaluated in a *hog1Δ* strain containing the same reporter construct. Plasmids from *HOG1*-dependent positives were isolated and partially sequenced. Candidate genes were subcloned and assayed as before.

**Two-hybrid analysis.** The two-hybrid analysis was carried out essentially as described previously (5) by using pACT11 and pBTM116 as the activation domain plasmid and LexA as the DNA-binding domain plasmid. The plasmids LexA-SMP1 and LexA-ΔMADSMP1 were cotransformed with pACT-HOG1 using the L40 reporter strain. Positive clones were selected and further tested for β-galactosidase activity as follows. Cells (~5 × 10<sup>6</sup>) were spotted onto YPD plates, incubated for 5 h at 30°C, and replicated onto nitrocellulose membranes. β-Galactosidase activity was visualized *in situ* by using X-Gal as described elsewhere (5).

**In vivo coprecipitation assays.** In vivo interaction of GST-SMP1 with Hog1 was determined by GST pull-down experiments. Mid-log-phase cells were grown in the presence of 2% galactose for 4 h and subjected or not subjected to a brief osmotic shock (0.4 M NaCl, 10 min). Yeast extract (750 μg in a mixture of buffer A plus 150 mM NaCl plus phosphatase inhibitor) was incubated with 50 μl of glutathione-Sepharose beads overnight at 4°C. The beads were washed extensively with buffer A plus 150 mM NaCl, resuspended in loading buffer, and separated by SDS-polyacrylamide gel electrophoresis (PAGE). Immunoblotting was done by using anti-HA monoclonal antibody 12CA5 (Roche) and anti-GST monoclonal antibody (Pharmacia) together with ECL reagent (Pharmacia).

**In vivo SMP1 phosphorylation assays.** Wild-type and mutant GST-SMP1 proteins were purified as described above but in the presence of buffer A without EGTA and EDTA. Bead-bound proteins were incubated with 40 U of calf-intestinal alkaline phosphatase (Roche) in 50 μl of alkaline phosphatase buffer for 60 min at 30°C. After alkaline phosphatase treatment, the beads were washed with several column volumes and Smp1 was detected by immunoblotting by using an anti-GST monoclonal antibody.

**Purification of GST proteins in *E. coli* and in vitro kinase assays.** GST fusion proteins encoding Pbs2(EE), Hog1, and full-length or truncated Smp1 were constructed by using pGEX-4T (Pharmacia), expressed in *E. coli* DH5, and purified by using glutathione-Sepharose beads (Pharmacia) in buffer B as described previously (14). Phosphorylation of Smp1 by Hog1 was monitored by the following in vitro kinase assay. One microgram of recombinant GST-HOG1 from *E. coli* was activated by phosphorylation by using 0.5 μg of GST-PBS2(EE) in the presence of kinase buffer and ATP. After 15 min at 30°C, 5 μg of wild-type or mutant versions of Smp1, purified from *E. coli*, was added to the previous mixture together with [γ-<sup>32</sup>P]ATP (0.2 μCi/μl). The mixture was then incubated for 5 min at 30°C, and the reactions were terminated by the addition of 2× SDS loading buffer. The labeled proteins were resolved by SDS-PAGE and detected by autoradiography.

**β-Galactosidase assays.** The transformed yeast strains were grown selectively until mid-log phase in the appropriate selective liquid media and then diluted in YPD for 3 h. Logarithmically growing cells (optical density at 660 nm, 0.5 to 0.8) were treated or not treated with 0.4 M of NaCl for 35 min and permeabilized by ethanol-toluene treatment, and β-galactosidase activity was determined as described previously (6).

**Cell viability assays.** Cell viability was scored essentially as described previously (7). Yeast cells were grown in YPD or minimal medium to an optical density of 0.8 and subjected or not subjected to an osmotic shock (1 M NaCl for 60 min). Viability was determined by adding propidium iodide (PI) at 0.004 mg/ml and measuring the number of PI-positive cells by using a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.). Cell viability was also determined by adding Phloxin B at 0.5 mg/ml to the untreated or treated cells as before and counting the stained cells by visual microscopy.

**GFP fluorescence microscopy.** GFP was visualized without fixation by using a Nikon E-600 with an ORCA II CCD camera (Hamamatsu). Images were taken at 100× magnification and converted to Photoshop format version 4.0 (Adobe Systems).

## RESULTS

**The MEF2C-related Smp1 transcription factor regulates HOG-dependent gene expression.** Osmotic stress gene expression is strongly defective in *hog1Δ* cells (see above); however, deletion of the transcription factors described as being under the control of the MAPK has only a limited effect on osmotic stress gene expression. Thus, it was predicted that other transcription factors must exist under the control of the Hog1 MAPK. To identify new Hog1-regulated transcription factors, we conducted a screening by which we isolated genes whose overexpression was able to induce *STL1* expression. The *STL1* gene

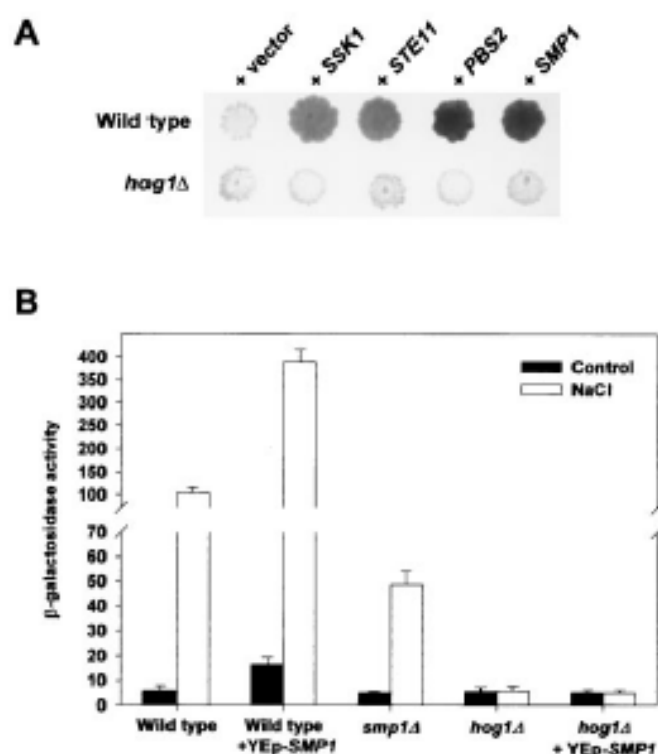


FIG. 1. Smp1 transcription factor modulates *HOG1*-mediated *STL1* gene expression. (A) Yeast multicopy plasmids capable of inducing *STL1-LacZ* expression in wild-type cells were isolated, and their dependence on *HOG1* was assayed in wild-type cells (YEN2) and *hog1Δ* cells (YEN7). A representative filter  $\beta$ -galactosidase assay demonstrating induction of *STL1-LacZ* by several positive clones from the screening is shown. (B) Smp1 modulates expression of *STL1-LacZ*. Wild-type, *hog1Δ*, and *smp1Δ* strains containing the *STL1-LacZ* reporter system (strains YEN2, YEN7, and YEN48) were transformed with a control plasmid or a multicopy plasmid expressing *SMP1*.  $\beta$ -Galactosidase activity was assayed in cells that were grown to mid-log phase and that were subjected (open bars) or not subjected (control; filled bars) to hyperosmotic stress (0.4 M NaCl for 35 min).  $\beta$ -Galactosidase activity is given in nanomoles per minute per milligram and is the result of the measurement in quadruplicate of results for two independent transformants.

encodes a putative sugar transporter, and it is one of the genes that is most strongly induced in response to osmotic stress and is completely under the control of Hog1 (13).

Yeast cells containing an integrated *STL1-LacZ* reporter construct were transformed with a multicopy genomic library, and positive clones were selected by their ability to induce *STL1* and, therefore, to produce  $\beta$ -galactosidase on X-Gal-containing plates (described in Material and Methods). Thus, 20 positive clones were identified from approximately 30,000 colonies. Plasmids were isolated from positive clones and tested again for their ability to induce *STL1* in both wild-type and *hog1Δ* strains containing the *STL1-LacZ* reporter. Only clones that induced *STL1* in a *HOG1*-dependent manner were partially sequenced. From the genomic clones, candidate genes were subcloned into a multicopy plasmid and tested as above.

As expected, some of the clones capable of inducing our reporter system encoded upstream components of the HOG pathway, namely *STE11*, *PBS2*, and *SSK1*, that are known to be able to induce activation of Hog1 by overexpression (Fig. 1A).

In addition to those upstream components of the HOG pathway, we identified elements that could be acting at the level of transcription regulation. We thus identified some genes encoding components of the basic transcription machinery, such as *SRB9* and *SRB4* (data not shown) and a gene encoding the Smp1 protein, a member of the MEF2 transcription factor family (Fig. 1A).

To determine the importance of *SMP1* in regulation of gene expression, we quantified *STL1* expression in liquid  $\beta$ -galactosidase assays. As shown in Fig. 1B, cells overexpressing *SMP1* induced expression of the *STL1* reporter gene to approximately fourfold-higher levels than did wild-type cells under both nonstress and stress conditions. Overexpression of *SMP1* in *hog1Δ* cells did not have any effect on *STL1* expression. Furthermore, deletion of *SMP1* resulted in reduced *STL1* expression (Fig. 1B). Similar results were obtained when *STL1* expression was analyzed by Northern blotting (data not shown). Deletion of the gene that encodes for the Hot1 transcription factor had an even more extensive impact on *STL1* expression than did deletion of the *SMP1* gene, indicating that Hot1 plays a major role in the regulation of *STL1* (data not shown).

The role of Smp1 on gene expression was also assayed by analyzing the expression of other stress-responsive genes known to be controlled by the Hog1 MAPK (13, 17). We created reporter genes containing the *CWP1*, *ALD3*, and *HXT1* gene promoters fused to the LacZ reporter system. Similar to the observations with *STL1*, expression of *CWP1* was induced by overexpression of *SMP1*, whereas deletion of *SMP1* partially reduced its expression upon stress. No *SMP1*-dependent differences on gene expression were observed when the *HXT1* and *ALD3* gene reporters were used, although these reporters were induced under osmotic stress in a *HOG1*-dependent manner (data not shown). Thus, the genetic screen yielded a clone, *SMP1*, encoding a transcription factor of the MEF2 family, capable of regulating a subset of stress-responsive genes under the control of the Hog1 MAPK.

**Smp1 is phosphorylated after osmotic stress in a Hog1-dependent manner.** When yeast cells are exposed to osmotic stress, the Hog1 MAPK is rapidly phosphorylated and activated (10) (Fig. 2B). To determine whether Smp1 is regulated in response to osmotic stress, we tested whether Smp1 was also phosphorylated upon osmotic stress. Wild-type and *hog1Δ* strains were subjected to a brief osmotic shock, and GST-tagged Smp1 protein was monitored by Western blotting by using anti-GST antibodies. The GST-Smp1 fusion protein was fully functional as tested for its ability to induce *STL1-LacZ* expression (data not shown). As shown in Fig. 2A, when subjected to osmotic stress, the mobility pattern of Smp1 was altered. The mobility change of Smp1 was induced by phosphorylation, because when extracts from osmotic-stressed cells were treated with alkaline phosphatase, the mobility pattern could be reversed (Fig. 2A). Interestingly, Smp1 phosphorylation was rapidly induced upon stress but the kinetics lagged behind those observed for the phosphorylation of Hog1. This kinetic relationship is consistent with Smp1 being dependent on Hog1 (Fig. 2B). Moreover, phosphorylation of Smp1 in response to osmotic stress depends on Hog1, since Smp1 from *hog1Δ* cells did not undergo the mobility shift observed in wild-type cells. Thus, these results showed that Smp1 is rapidly

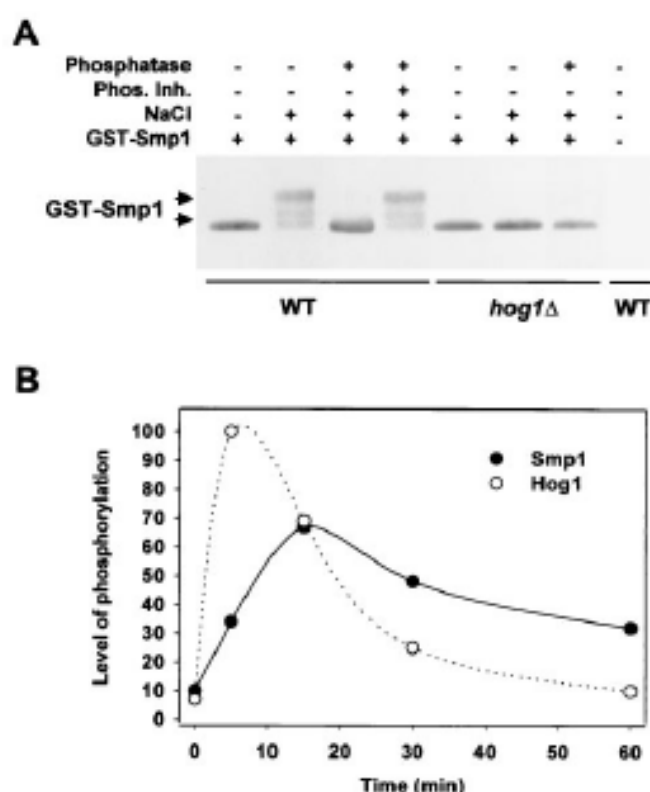


FIG. 2. In vivo phosphorylation of Smp1 upon osmotic stress depends on Hog1. (A) GST-SMP1 was expressed under the  $P_{GAL1}$  promoter in wild-type (TM141) and *hog1Δ* (TM233) cells. Yeast cells were grown in the presence of galactose for 4 h and subjected (+) or not subjected (-) to a brief osmotic shock (0.4 M NaCl for 10 min), and the extracts were treated (+) or not treated (-) with 10 U of alkaline phosphatase or phosphatase inhibitors (Phos. Inh.). Extracts were separated by SDS-PAGE, and GST-tagged Smp1 was detected by the use of polyclonal GST-specific antibodies. The control strain (without GST-SMP1) was the wild-type strain (TM141). (B) Time course of Hog1 and Smp1 phosphorylation upon osmotic stress. Cells expressing GST-SMP1 in a wild-type strain were subjected to osmotic stress (0.4 M NaCl), and cells were collected at different times. Phosphorylation of Smp1 and Hog1 was monitored by Western blotting by using anti-GST antibodies and anti-phospho-p38 MAPK (Thr180/Tyr182) antibodies (New England BioLabs), respectively. The level of phosphorylation was measured from scanned films by using Quantity One software (Bio-Rad).

phosphorylated upon hyperosmotic shock in vivo and that this modification depends on the Hog1 MAPK.

**Hog1 interacts with the putative transcription activator domain of Smp1.** To obtain direct evidence for the interaction of Smp1 and Hog1, we tested by two-hybrid analysis whether these proteins interacted. Smp1 or a mutant containing a deletion on the MADS box and MEF2 domains (amino acids 1 to 90) were fused to the LexA-DNA binding domain, and their interaction with the full-length Hog1 fused to the GAL4 activator domain was tested. Figure 3A shows a typical result and indicates that Smp1 interacts with Hog1 through its C-terminal domain and not through the MADS and MEF2 domains.

The interaction between Hog1 and Smp1, shown by the two-hybrid data, was confirmed by in vivo coprecipitation experiments. Yeast YEN114 cells (which express HA-tagged Hog1 from their own genomic locus) were transformed with a

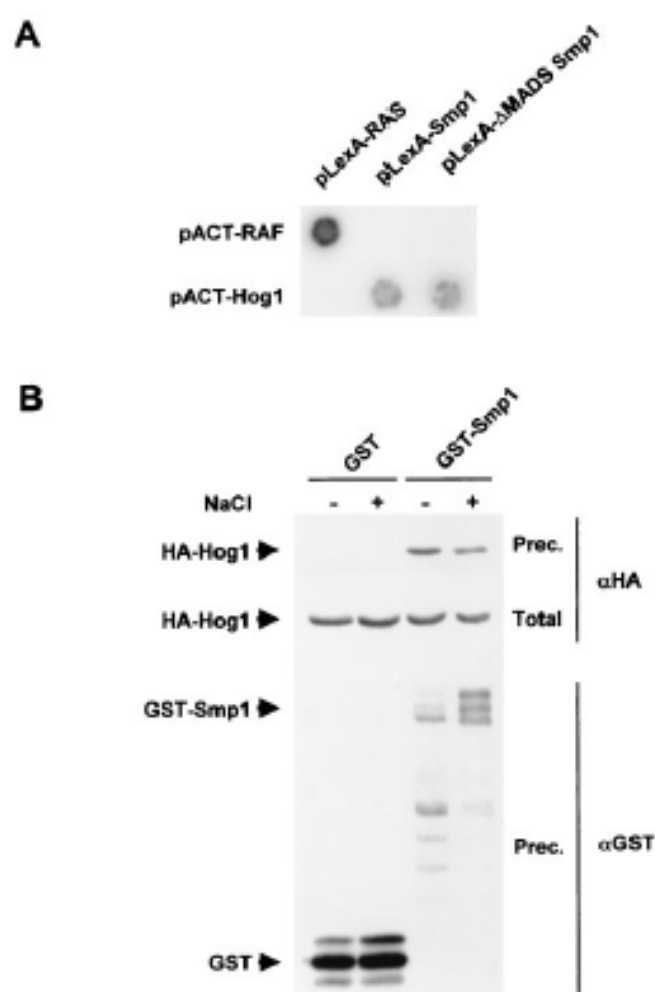


FIG. 3. In vivo binding of Hog1 to Smp1. (A) Interaction as shown by two-hybrid analysis of Hog1 and Smp1. The wild-type version and a mutant that lacks the MADS and MEF2 domains ( $\Delta$ MADS Smp1) fused to the LexA DB were expressed with the full-length HOG1 fused to the GAL4 activator domain. A representative filter  $\beta$ -galactosidase assay demonstrating interactions between Hog1 and Smp1 is shown. Proteins encoded by the control plasmids pLexA-RAS<sup>V12</sup> and pACT-RAF, which are known to interact with each other, are shown for comparison. (B) Smp1 coprecipitates with Hog1. Strain YEN114 (which expresses HA-tagged Hog1 from the wild-type locus) was transformed with a plasmid expressing GST or GST-SMP1 under the  $P_{GAL1}$  promoter. Cells were grown in the presence of galactose, and samples were taken before (-) or 10 min after (+) the addition of NaCl to a final concentration of 0.4 M. GST proteins were affinity purified through a glutathione-Sepharose matrix; the presence of HA-Hog1 in the precipitates was probed by immunoblotting with anti-HA (indicated by  $\alpha$ HA at right), and GST-containing proteins (indicated by  $\alpha$ GST at right) were detected by using antibodies against GST. Total, <10% of the input protein; Prec., total amount of Hog1 or GST precipitated.

plasmid that expresses a GST-tagged Smp1 from the  $GAL1$  promoter. Cells were subjected to a brief osmotic shock, and Smp1 was precipitated by using glutathione-Sepharose beads. The presence of Hog1 in the precipitates was probed with an anti-HA monoclonal antibody. As shown in Fig. 3B, Smp1 was able to coprecipitate Hog1 irrespective of the environmental conditions. Thus, these in vivo binding assays confirmed the conclusion of the two-hybrid analysis indicating that Hog1 binds to the Smp1 transcription factor.

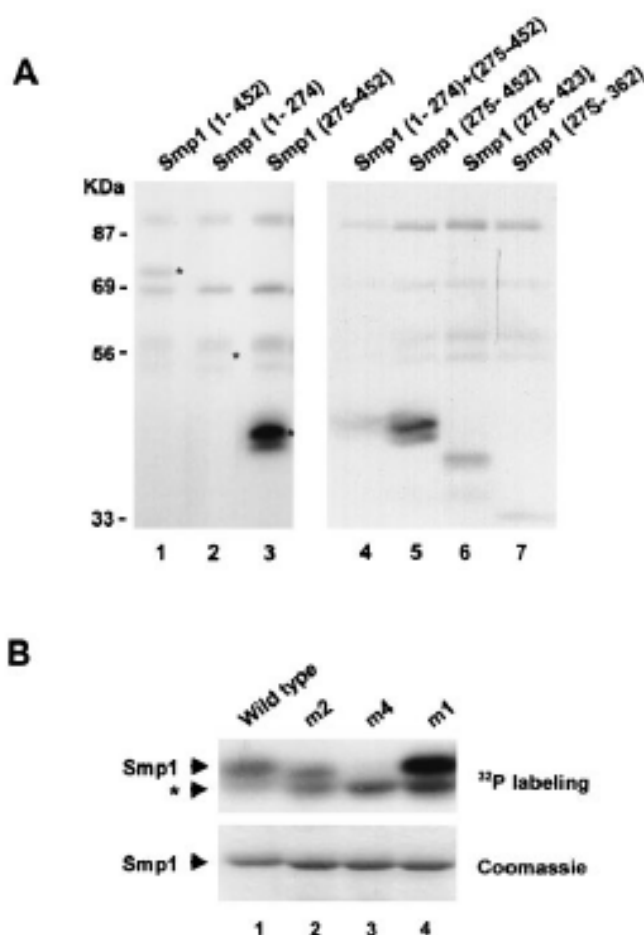


**Phosphorylation of multiples sites at the C terminus of Smp1 by Hog1 affects Smp1 function.** Smp1 is phosphorylated upon osmotic stress in a Hog1-dependent manner and interacts physically with the MAPK. We then tested whether Smp1 phosphorylation was carried out directly by the Hog1 MAPK by using purified proteins in an *in vitro* kinase assay. For this purpose, Hog1 and a constitutively activated version of Pbs2 [PBS2(EE)] were purified as GST-fusion proteins from *E. coli*. In the first step of the reaction, Hog1 was activated by phosphorylation in the presence of PBS2(EE) and ATP (2). Then Smp1, purified from *E. coli* as a GST-tagged protein, and [ $\gamma$ - $^{32}$ P]ATP were added to the reaction. As shown in Fig. 4A, lane 1, full-length Smp1 was phosphorylated by Hog1 (see the asterisk), whereas when a catalytically impaired Hog1 (hog1 KN) was used instead of the wild-type Hog1, no phosphorylation of Smp1 was observed (data not shown), thus suggesting that Smp1 is a direct substrate for the MAPK Hog1.

To map the phosphorylation site(s) for Hog1 in Smp1, we created several truncated *SMP1* alleles and expressed them as GST-tagged proteins in *E. coli*. After purification, the same amounts of the pure proteins were subjected to *in vitro* phosphorylation by activated Hog1 (as described above). A truncated form of Smp1 containing the N-terminal region (amino acids 1 to 274) was unable to be phosphorylated by Hog1, whereas a C-terminal region (amino acids 275 to 452) was strongly phosphorylated by the MAPK (Fig. 4, lanes 2 and 3). Thus, phosphorylation of Smp1 by Hog1 occurs in a region which is coincident with the Hog1 binding domain. It is worth noting that when both N-terminal and C-terminal fragments were present in the same assay, the amount of phosphorylation of the C-terminal fragment was strongly reduced (Fig. 4, lane 4).

To further determine the phosphorylation sites in Smp1, we created several truncated versions of the C-terminal domain (amino acids 275 to 452) and expressed them as GST-tagged proteins in *E. coli*. Truncated forms were purified and assayed as described previously. A 30-residue C-terminal deletion had little effect on Smp1 phosphorylation, whereas deletion of the last 90 residues had a stronger effect on phosphorylation (Fig. 4, lanes 6 and 7). Four sequences corresponding to the consensus phosphorylation site for MAPKs (Ser-Pro/Thr-Pro) are present in the region comprised of residues 275 to 423. We created point mutant versions to replace Ser and Thr residues with Ala, and we tested them for phosphorylation by Hog1. Single mutations of Ser 348, Ser 357, Thr 365, and Ser 376 did not alter Smp1 phosphorylation (data not shown). We then constructed several combinations of mutant sites and tested them by phosphorylation. Mutation of both Thr 365 and Ser 376 (m1) did not change the level of phosphorylation, whereas mutation on both Ser 348 and Ser 357 (m2) slightly decreased the phosphorylation of Smp1 (Fig. 4B). Smp1 phosphorylation was only abolished in the Smp1-m4 mutant, which contains mutations in all four sites (Fig. 4B).

*In vivo* phosphorylation assays carried out as described previously in wild-type cells transformed with a wild-type GST-tagged Smp1 or the mutant Smp1-m4 protein demonstrated that mutation of the four sites in the Smp1-m4 protein eliminated most of the mobility shift due to phosphorylation of Smp1 in response to osmotic stress (Fig. 5A). Taken together, these results indicate that there is a specific cluster of four



**FIG. 4.** Hog1 phosphorylates the C-terminal domain of Smp1. (A) *In vitro*-activated Hog1 phosphorylates Smp1. Various Smp1 fragments were tested for their ability to be phosphorylated by an *in vitro*-activated Hog1. The positions of the Smp1 fragments included in the constructs are indicated in parentheses. Recombinant tagged proteins were purified from *E. coli* and subjected to phosphorylation by activated Hog1 as described in Materials and Methods. Phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography. Asterisks indicate the positions of Smp1 proteins detected by Coomassie staining. (B) Mutation of Smp1 Ser348, Ser357, Thr365, and Ser376 to Ala abolishes Hog1 phosphorylation. The wild-type Smp1 fragment (amino acids 275 to 452) and various Smp1 site-directed mutants were tested for Hog1 phosphorylation as described in the legend for panel A. The Smp1 mutants and their corresponding mutations (indicated in parentheses) are as follows: m2 (Ser348 and Ser357 to Ala), m4 (Ser348, Ser357, Thr365, and Ser376 to Ala), and m1 (Thr365 and Ser376 to Ala). After phosphorylation, the proteins were resolved by SDS-PAGE, and phosphorylated proteins were detected by autoradiography (upper panel). GST-tagged Smp1 proteins were detected by Coomassie blue stain (lower panel). The asterisk indicates a contaminant phosphorylation not corresponding to Smp1.

phosphorylation sites for Hog1 that is located within 28 amino acids in the C terminus of Smp1.

To assess the role of Hog1 phosphorylation in Smp1, we studied the effect of the mutation of the phosphorylation sites in gene expression. Wild-type and *smp1Δ* cells were transformed with a vector containing full-length *SMP1* or the *SMP1-m4* mutant allele (unphosphorylatable by Hog1) under the wild-type promoter, and the effect on transcription was measured by expression of the *STL1::LacZ* reporter assay as

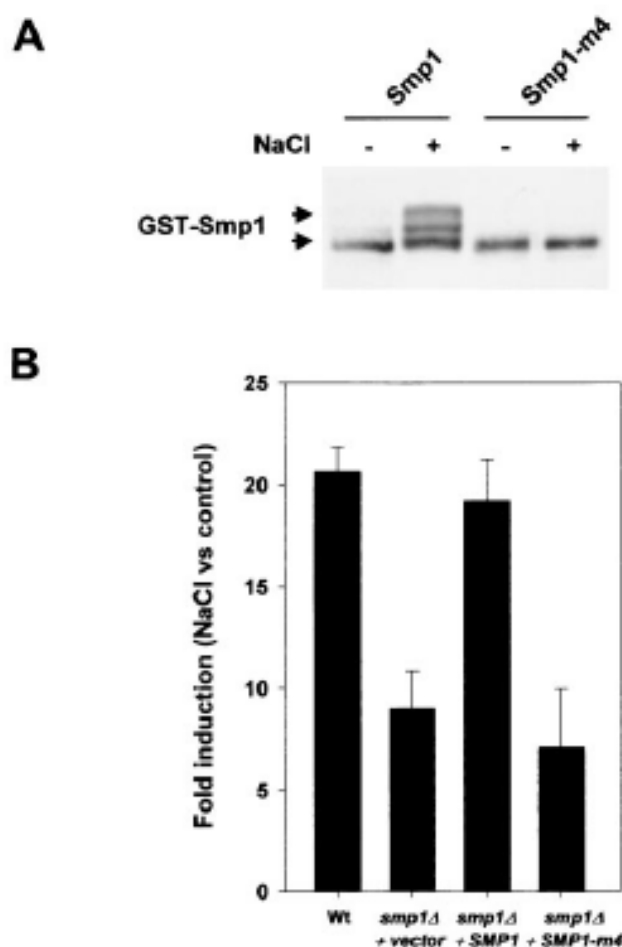


FIG. 5. Smp1 phosphorylations are required for Smp1 transcriptional activity. (A) Mutation of Ser348, Ser357, Thr365, and Ser376 to Ala abolishes *in vivo* Hog1 phosphorylation. Full-length Smp1 and the quadruple-mutant protein Smp1-m4 (mutations: Ser348, Ser357, Thr365, and Ser376 to Ala) were tested for Hog1 phosphorylation as described in the legend for Fig. 2A. After phosphorylation, the proteins were resolved by SDS-PAGE and detected by immunoblotting by using anti-GST polyclonal antibodies. (B) Hog1 phosphorylation modulates Smp1 function. A wild-type strain and the *smp1Δ* mutant strain (YEN44) containing the reporter *STL1::lacZ* were transformed with a centromeric plasmid expressing wild-type *SMP1* or the quadruple-mutant *SMP1-m4* (unphosphorylatable by Hog1). The cells were subjected to a brief osmotic shock, and  $\beta$ -galactosidase activity was assayed as described in the legend for Fig. 1B.  $\beta$ -Galactosidase activity is given as fold induction of control versus that of NaCl-treated cells and is the result of the measurement in quadruplicate of results for two independent transformants.

before. As shown previously, deletion of *SMP1* resulted in a decrease of *STL1* expression upon osmotic stress. Expression of wild-type *SMP1* restored the levels of *STL1* expression to wild-type levels upon stress, whereas expression of the *SMP1-m4*, which is no longer phosphorylatable by Hog1, was completely unable to restore *STL1* expression in *smp1Δ* cells (Fig. 5B). Smp1 alleles carrying simultaneous mutations of Ser 348 and Ser 357 (m2) or Thr 365 and Ser 376 altered expression of *STL1* only to a minor extent (*STL1* expression was 76 and 90% of that of the wild-type allele, respectively). These results indicated that the Hog1 phosphorylation plays an important role in the regulation of Smp1 transcriptional activity.

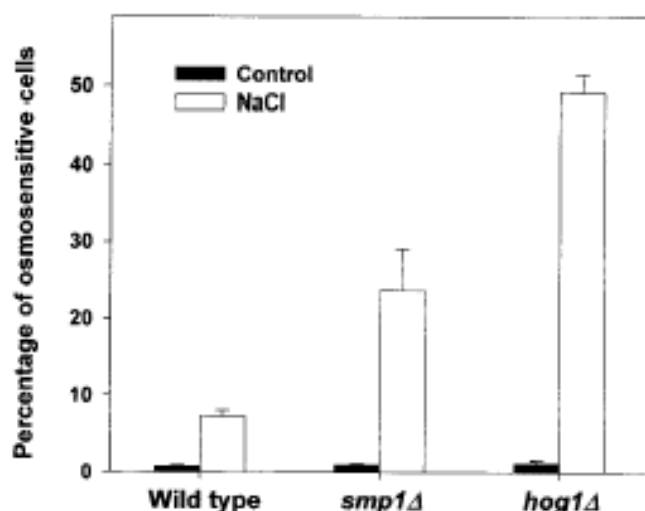


FIG. 6. Cell survival upon osmotic stress is reduced in *hog1Δ* and *smp1Δ* strains. To analyze the role of Smp1 in osmotic stress adaptation, we measured the incorporation of PI, as described in Materials and Methods, as a measure of cell viability in wild-type, *hog1Δ* mutant, and *smp1Δ* mutant strains. Yeast cells were grown in YPD and subjected (open bars) or not subjected (filled bars) to an osmotic shock (1 M NaCl for 60 min). Viability was determined by the addition of PI and measurement of the percentage of PI-positive cells by using a flow cytometer. The results are the measurements in duplicate from six independent experiments. The data were confirmed by Phloxin B staining and visual microscopy (see Materials and Methods).

Smp1 controls part of the Hog1-mediated osmotic stress responses. Smp1 regulates the expression of several stress-responsive genes. To understand the role of Smp1 in osmotic stress adaptation, we aimed to determine the importance of the presence of Smp1 to the generation of osmotic stress responses. Deletion of *SMP1* did not cause an obvious osmosensitivity phenotype on plates containing sorbitol or NaCl (data not shown). This was similar to the results observed with cells containing deletions of any of the known transcription factors under the control of the MAPK. However, it was previously reported that *hot1Δ* cells were more sensitive than wild-type cells to a severe osmotic shock (19). Thus, we tested whether *smp1Δ* cells were viable after an osmotic shock. Exponentially growing wild-type, *smp1Δ*, or *hog1Δ* cells were subjected to an osmotic shock (1 M NaCl for 60 min), and viability was determined as described in Materials and Methods. As depicted in Fig. 6, *hog1Δ* cells were highly sensitive to an osmotic shock compared to the wild-type cells. Interestingly, *smp1Δ* cells were also sensitive to an osmotic shock, although less sensitive than *hog1Δ* cells. Similarly to the results observed for the *STL1-LacZ* expression, a wild-type allele of *SMP1* was able to suppress *smp1Δ* osmosensitivity, whereas the *SMP1-m4* mutant allele was unable to restore cell viability. Double-mutant *hog1Δ smp1Δ* cells were as sensitive as *hog1Δ* cells (data not shown). Thus, these data indicated that Smp1 plays a role in the generation of osmotic stress responses, most likely by the regulation of a subset of the osmotic stress-responsive genes controlled by the Hog1 MAPK.

**Both Smp1 and Hog1 are relevant for cell viability in stationary phase.** To study the mechanism of Smp1 action, we analyzed the cellular distribution of Smp1. For this purpose,

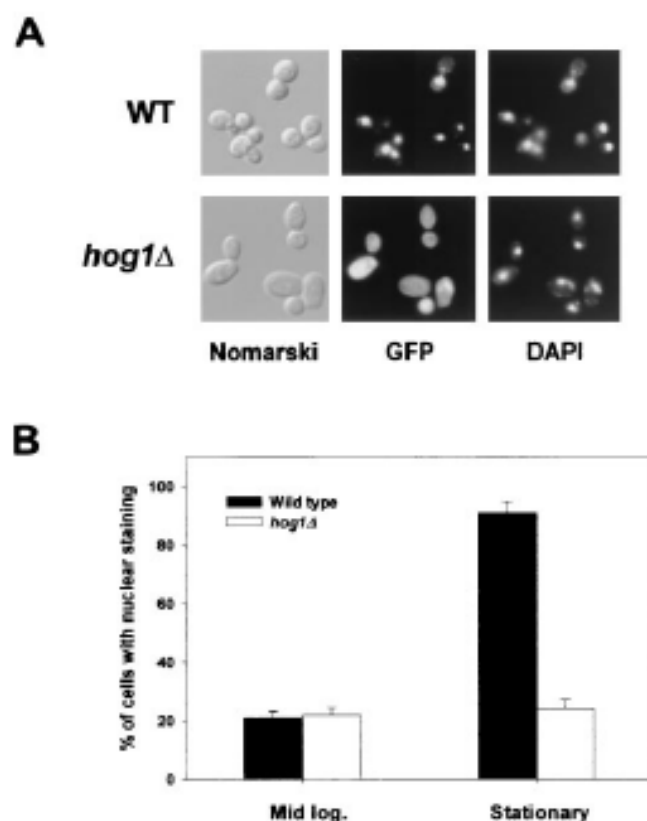


FIG. 7. Smp1 nuclear localization depends on Hog1. The wild-type and the *hog1Δ* mutant strains were transformed with a plasmid carrying a GFP-tagged Smp1 expressed under its own promoter. The cells were grown and Smp1-GFP was detected by fluorescence microscopy as described in Materials and Methods. (A) Smp1 localizes into the nuclei of wild-type cells in the stationary phase. Cells of the indicated types carrying Smp1-GFP were grown to the stationary phase, and images were taken. Representative images showing the localization of Smp1-GFP are presented. The positions of the nuclei were determined by DAPI staining. (B) Quantitative data were obtained from wild-type or *hog1Δ* cells growing at the mid-logarithmic (Mid log.) or stationary phase of growth. The data represent the results of three independent experiments which included counting 300 cells each.

we fused *SMP1* to the N terminus of the GFP and expressed the fusion in yeast under its own promoter. The Smp1-GFP fusion protein was fully functional as tested for *STL1-LacZ* expression (data not shown). Microscopic examination of cells expressing Smp1-GFP revealed that Smp1 localizes throughout the cytoplasm and nuclei of unstressed cells. When cells carrying Smp1-GFP were exposed to a brief osmotic shock (0.4 M NaCl for 5 min), Smp1 did not change its distribution significantly. However, strong nuclear localization was observed in most of the wild-type cells when they were grown in stationary phase (Fig. 7). Nuclear localization of Smp1-GFP was abolished by elimination of Hog1 activity, either by gene deletion or by impairing its catalytic activity (Fig. 7). However, it is worth noting that distribution of the nonphosphorylatable Smp1-m4 protein fused to GFP was similar to that observed in the wild type (data not shown). These results suggested that Hog1 could be playing a role during stationary phase by indirectly controlling Smp1 localization.

It was reported that deletion of the Hog1 homologue Spc1/

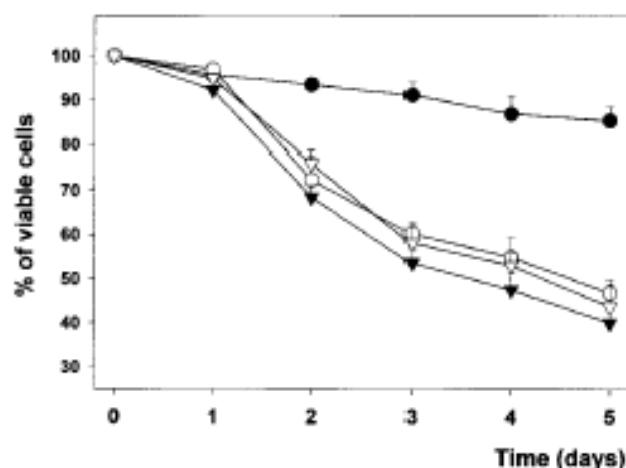


FIG. 8. Hog1 and Smp1 are required for cell viability in stationary phase. Wild-type (●), *hog1Δ* (▽), *smp1Δ* (○), or *hog1Δ smp1Δ* (▼) cells were grown in minimal medium to stationary phase and allowed to grow for the indicated period of time. Viability was determined by adding PI and measuring the percentage of PI-positive cells by using a flow cytometer. The results are the measurements in duplicate from four independent experiments. The data were confirmed by Phloxin B staining and visual microscopy (see Materials and Methods).

Sy1 MAPK from *Schizosaccharomyces pombe* resulted in cells with less viability during the stationary phase (22). To study whether Hog1 and Smp1 were playing a role during this phase of growth, we monitored the cell viabilities of wild-type, *smp1Δ*, and *hog1Δ* cells during 5 days of culture in stationary phase as before. As shown in Fig. 8, wild-type cells maintained cell viability throughout the culture period, whereas *smp1Δ* and *hog1Δ* cells lost viability during growth in the stationary phase. Similar results were obtained with the W303 strain background (data not shown). Furthermore, *smp1Δ hog1Δ* double-mutant cells did not show differences compared to *hog1Δ* cells. It is worth noting that whereas a plasmid containing wild-type *SMP1* was able to restore the cell viability of a *smp1Δ* strain, a plasmid containing the *SMP1-m4* allele (encoding the Smp1 version unphosphorylatable by Hog1) was not able to complement *smp1Δ* mutation (data not shown). Thus, these data suggest that apart from controlling osmotic stress responses, Smp1 plays an important role for cell survival during growth in the stationary phase and that this role could be mediated by the MAPK through regulation of Smp1 localization.

## DISCUSSION

Yeast cells respond to increases in osmolarity in the extracellular environment by activating the stress-activated MAPK Hog1. A major outcome of the activation of the Hog1 MAPK is the regulation of gene expression. One of the most common mechanisms by which SAPKs regulate gene expression is by modification of specific transcription factors (9), and several transcription regulators have been proposed to be controlled by the Hog1 MAPK. However, due to their DNA binding specificities and the profile of gene induction upon stress shown by DNA microarrays, they cannot account for the regulation of all of the genes under the control of Hog1. In

addition, a mutant strain carrying deletions in *hot1*, *msn1*, *msn2*, and *msn4* is not osmosensitive (19).

From these considerations, we suspected that additional transcription factors were required for the osmotic stress-induced regulation of gene expression by the MAPK Hog1. In this report, we describe a genetic screen in which we identified Smp1, a MEF2-like protein, as one such factor. The results of this study demonstrate that both overexpression and deletion of *SMP1* result in altered expression of osmosensitive genes such as *STL1* and *CWP1* but not of others, such as *ALD3* or *HXT1*. It was reported that Smp1 has MEF2-related DNA-binding specificities (4), and a conserved sequence similar to the predicted DNA binding site for Smp1 was consistently present on the promoters of the osmosensitive *STL1* and *CWP1* but not *HXT1* and *ALD3* genes (Fig. 1 and data not shown).

Several lines of evidence suggest that Smp1 is actually a direct substrate for the MAPK. In vivo studies suggested that Hog1 was able to interact with Smp1 and that Smp1 was phosphorylated upon stress in a *HOG1*-dependent manner. The relationship of Smp1 and Hog1 in vivo are further supported by the in vitro evidence that Hog1 phosphorylates Smp1 directly. Four independent phosphorylation sites were mapped within 28 amino acids. Similarly, phosphorylation of Sko1 by Hog1 was restricted to three phosphorylation sites clustered within 19 amino acids (15). The introduction of several phosphates in a small pocket of amino acids might result in conformational changes required for switching the function of those transcriptional regulators. In vitro phosphorylation studies also showed that full-length Smp1 is phosphorylated less efficiently by Hog1 than by the C-terminal domain alone (Fig. 3, lanes 1 and 3). This observation raised the possibility that a region of Smp1 could be limiting the access of the MAPK to the phosphorylation sites. Consistent with this observation was the fact that simultaneous incubation of the C-terminal domain with an N-terminal domain (which is not phosphorylated by Hog1) resulted in a dramatic decrease of C-terminal phosphorylation (Fig. 4, lane 4). This might suggest that Smp1 must be in a preactivated state (i.e., bound to DNA or interacting with other factors) to be accessible for phosphorylation and activation by the MAPK.

Phosphorylation of Smp1 by Hog1 is important for Smp1 function. Upon osmotic stress, Smp1 is strongly phosphorylated, and mutation of the phosphorylation sites to Ala results in an Smp1 that is unable to regulate gene expression (Fig. 5). In yeast, there exists a second MEF2-related protein, Rlm1, that is under the control of the Slr2/Mpk1 MAPK. Interestingly, Mpk1 phosphorylates Rlm1 in a region similar to that found for Smp1, and this phosphorylation results in an increase of its transcriptional activity (4, 23). Thus, two independent MAPK signaling pathways could be controlling the two MEF2-related factors by a similar mechanism. In mammals, regulation of MEF2A and MEF2C factors has been shown to be under the control of the p38 MAPKs, among other kinases (reviewed in references 9 and 11). Phosphorylation of the transcription activator domain of these factors by p38 stimulates MEF2 activity, which is analogous to the mechanism proposed for the yeast Smp1.

Apart from the role of Smp1 in the regulation of a subset of osmosensitive genes under the control of the Hog1 MAPK

(and thus a role in osmoadaptation), we found that *smp1Δ* cells lose viability in the stationary phase, as occurs with *hog1Δ* cells (Fig. 8). This phenotype is reminiscent of that observed upon deletion of the Spc1/Sly1 MAPK in *Schizosaccharomyces pombe* (21, 24), and although the molecular mechanism(s) of this deficiency remains uncharacterized, the formal possibility exists that this could be caused by the lack of some specific transcript required for this phase of growth. As shown in Fig. 7, Smp1 concentrates into the nucleus when cells enter into the stationary phase, and this could be of relevance for the induction of specific genes. In *hog1Δ* cells, Smp1 nuclear accumulation is clearly diminished, and thus this could impede the normal function of Smp1. Because *smp1Δ* and *smp1Δ hog1Δ* cells display similar viability, it is likely that the deficiency observed in *hog1Δ* cells could be caused by the improper function of Smp1 rather than other mechanisms.

Taken together, our results show that Smp1 is a direct target for the Hog1 MAPK and that two different levels of regulation can be controlling the activity of this transcription factor. Under stress, Hog1 phosphorylates the putative transcription activation domain stimulating Smp1 activity, and, when entering into the stationary phase, Hog1 is required for proper localization of the transcription factor.

#### ACKNOWLEDGMENTS

We thank Mercè Carmona for her technical assistance.

This work was supported by grants PM99-0028 from the Direcció General de Investigació Científica i Tècnica (Ministry of Science and Technology, Spain), Distinció de la Generalitat de Catalunya per a la Promoció de la Recerca Universitària, Joves Investigadors DURSI (Generalitat de Catalunya), and the EMBO Young Investigator Program to F.P. L.C. is the recipient of an F.P.U. predoctoral fellowship from the MEC, Spain.

The first two authors contributed equally to this work.

#### REFERENCES

1. Alepuz, P. M., A. Jovanovic, V. Reiser, and G. Ammerer. 2001. Stress-induced map kinase Hog1 is part of transcription activation complexes. *Mol. Cell* 7:767–777.
2. Bilsland-Marchesan, E., J. Arino, H. Saito, P. Sunnerhagen, and F. Posas. 2000. Rck2 kinase is a substrate for the osmotic stress-activated mitogen-activated protein kinase Hog1. *Mol. Cell. Biol.* 20:3887–3895.
3. Causton, H. C., B. Ren, S. S. Koh, C. T. Harbison, E. Kanin, E. G. Jennings, T. I. Lee, H. L. True, E. S. Landier, and R. A. Young. 2001. Remodeling of yeast genome expression in response to environmental changes. *Mol. Biol. Cell* 12:323–337.
4. Dodou, E., and R. Treisman. 1997. The *Saccharomyces cerevisiae* MADS-box transcription factor Rlm1 is a target for the Mpk1 mitogen-activated protein kinase pathway. *Mol. Cell. Biol.* 17:1848–1859.
5. Durfee, T., K. Bedner, P. L. Chen, S. H. Yeh, Y. Yang, A. E. Kilburn, W. H. Lee, and S. J. Elledge. 1993. The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes Dev.* 7:555–569.
6. Gaxiola, R., I. F. de Larrinoa, J. M. Villalba, and R. Serrano. 1992. A novel and conserved salt-induced protein is an important determinant of salt tolerance in yeast. *EMBO J.* 11:3157–3164.
7. Groot, P. W. J., et al. 2001. A genomic approach for the identification and classification of genes involved in cell wall formation and its regulation in *S. cerevisiae*. *Comp. Funct. Genomics* 2:124–142.
8. Holmann, S. 2002. Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol. Mol. Biol. Rev.* 66:300–372.
9. Kyriakis, J. M., and J. Avruch. 2001. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol. Rev.* 81:807–869.
10. Maeda, T., M. Takekawa, and H. Saito. 1995. Activation of yeast Pbs2 MAPKK by MAPKKs or by binding of an SH3-containing osmosensor. *Science* 269:554–558.
11. McKinsey, T. A., C. L. Zhang, and E. N. Olson. 2002. MEF2: a calcineurin-dependent regulator of cell division, differentiation and death. *Trends Biochem. Sci.* 27:40–47.
12. Myers, A. M., A. Tzagoloff, D. M. Kinney, and C. J. Lusty. 1986. Yeast shuttle

- and integrative vectors with multiple cloning sites suitable for construction of lacZ fusions. *Gene* 48:299–310.
13. Posas, F., J. R. Chambers, J. A. Heyman, J. P. Hoeffler, E. de Nadal, and J. Arino. 2000. The transcriptional response of yeast to saline stress. *J. Biol. Chem.* 275:17249–17255.
  14. Posas, F., S. M. Wurgler-Murphy, T. Maeda, E. A. Witten, T. C. Thai, and H. Saito. 1996. Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorylation mechanism in the SLN1-YPD1-SSK1 “two-component” osmosensor. *Cell* 86:865–875.
  15. Proft, M., A. Pascual-Ahuir, E. de Nadal, J. Arino, R. Serrano, and F. Posas. 2001. Regulation of the Sko1 transcriptional repressor by the Hog1 MAP kinase in response to osmotic stress. *EMBO J.* 20:1123–1133.
  16. Proft, M., and K. Struhl. 2002. Hog1 kinase converts Sko1-Cyc8-Tup1 repressor complex into an activator that recruits SAGA and SWI/SNF in response to osmotic stress. *Mol. Cell* 9:1–20.
  17. Rep, M., M. Krantz, J. M. Thiele, and S. Hohmann. 2000. The transcriptional response of *Saccharomyces cerevisiae* to osmotic shock. Hot1p and Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway-dependent genes. *J. Biol. Chem.* 275:8290–8300.
  18. Rep, M., M. Proft, F. Remize, M. Tamas, R. Serrano, J. M. Thiele, and S. Hohmann. 2001. The *Saccharomyces cerevisiae* Sko1p transcription factor mediates HOG pathway-dependent osmotic regulation of a set of genes encoding enzymes implicated in protection from oxidative damage. *Mol. Microbiol.* 40:1067–1083.
  19. Rep, M., V. Reiser, U. Gartner, J. M. Thiele, S. Hohmann, G. Ammerer, and H. Ruis. 1999. Osmotic stress-induced gene expression in *Saccharomyces cerevisiae* requires Msn1p and the novel nuclear factor Hot1p. *Mol. Cell Biol.* 19:5474–5485.
  20. Robinson, M. J., and M. H. Cobb. 1997. Mitogen-activated protein kinase pathways. *Curr. Opin. Cell Biol.* 9:180–186.
  21. Shiozaki, K., and P. Russell. 1996. Conjugation, meiosis, and the osmotic stress response are regulated by Spc1 kinase through Atf1 transcription factor in fission yeast. *Genes Dev.* 10:2276–2288.
  22. Toone, W. M., S. Kuge, M. Samuels, R. A. Morgan, T. Toda, and N. Jones. 1998. Regulation of the fission yeast transcription factor Pap1 by oxidative stress: requirement for the nuclear export factor Crm1 (exportin) and the stress-activated MAP kinase Sty1/Spcl. *Genes Dev.* 12:1453–1463.
  23. Watanabe, Y., G. Takaue, M. Hagiwara, K. Irie, and K. Matsumoto. 1997. Characterization of a serum response factor-like protein in *Saccharomyces cerevisiae*, Rlm1, which has transcriptional activity regulated by the Mpk1 (Slf2) mitogen-activated protein kinase pathway. *Mol. Cell Biol.* 17:2615–2623.
  24. Wilkinson, M. G., M. Samuels, T. Takeda, W. M. Toone, J. C. Shieh, T. Toda, J. B. Millar, and N. Jones. 1996. The Atf1 transcription factor is a target for the Sty1 stress-activated MAP kinase pathway in fission yeast. *Genes Dev.* 10:2289–2301.

## Expression of the *HXT1* Low Affinity Glucose Transporter Requires the Coordinated Activities of the HOG and Glucose Signalling Pathways\*

Received for publication, January 20, 2004  
Published, JBC Papers in Press, March 10, 2004, DOI 10.1074/jbc.M400902004

Lidia Tomás-Cobos<sup>‡§¶</sup>, Laura Casadomé<sup>‡¶\*</sup>, Glòria Mas<sup>¶\*</sup>, Pascual Sanz<sup>‡ §§</sup>,  
and Francesc Posas<sup>‡</sup>

From the <sup>‡</sup>Instituto de Biomedicina de Valencia, Jaime Roig 11, 46101 Valencia and the <sup>¶</sup>Cell Signaling Unit, Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, 08003 Barcelona, Spain

Expression of the *HXT1* gene, which encodes a low affinity glucose transporter in *Saccharomyces cerevisiae*, is regulated positively in response to glucose by the general glucose induction pathway, involving the Snf3/Rgt2 membrane glucose sensors, the SCF-Grr1 ubiquitination complex and the Rgt1 transcription factor. In this study we show that, in addition to the glucose signaling pathway, regulation of *HXT1* expression also requires the HOG pathway. Deletion of components in the glucose signaling pathway or in the HOG pathway results in impaired *HXT1* expression. Genetic analyses showed that, whereas the glucose signaling pathway regulates *HXT1* through modulation of the Rgt1 transcription factor, the HOG pathway modulates *HXT1* through regulation of the Sko1-Tup1-Ssn6 complex. Coordinated regulation of the two signaling pathways is required for expression of *HXT1* by glucose and in response to osmotic stress.

Yeast cells are able to adjust cellular metabolism, gene expression, and growth in response to environmental stimuli. For example, the presence of glucose, the most preferable carbon source, is able to elicit a complex metabolic response based in two major levels: i) allosteric modification of different enzymes and ii) regulation of gene expression. Transcriptional regulation varies from inhibition of expression (glucose repression) to activation of transcription (glucose induction) (see Refs. 1–4 for reviews). Some of the genes induced in response to glucose encode for glycolytic enzymes, ribosomal proteins, and glucose transporters. Expression of the low affinity glucose transporter *HXT1* has been used as a model to study the process of transcriptional activation by glucose (3). Genetic and biochemical studies have defined several components that are involved in the regulation of *HXT1* expression. Glucose availability in the surrounding media is assessed by the membrane glucose sen-

sor proteins Snf3 and Rgt2. This signal is then transmitted to the SCF-Grr1 ubiquitination complex (5, 6), which finally modulates the activity of Rgt1, a transcription factor that belongs to the Cys<sub>2</sub>-Zinc cluster protein family, which acts as a transcriptional repressor in the absence of glucose (3, 7). Additional components of the glucose induction pathway are Std1 and Mth1, two proteins that modulate negatively *HXT1* expression (3, 8); recent studies indicate that Std1 and Mth1 may interact with the C-terminal tails of the glucose sensors Rgt2 and Snf3 and with Rgt1 (9–11) and that the SCF-Grr1 complex is involved at least in the inactivation of Mth1, mediating in this way the glucose-induced dissociation of Rgt1 from *HXT1* promoter and its activation (12). Moreover, data from several laboratories suggest the existence of an additional uncharacterized transcription factor, different from Rgt1, that regulates *HXT1* gene expression (7).

Exposure of yeast cells to increases in extracellular osmolarity results in the activation of the Hog1 MAPK<sup>1</sup> pathway. Activation of the Hog1 MAPK induces diverse osmo-adaptive responses such as regulation of gene expression. Genome-wide transcriptional analyses showed that a great number of genes are regulated by osmotic stress in an *HOG1*-dependent manner. Among these, there are genes that encode proteins implicated in carbohydrate metabolism, general stress protection, protein production, and signal transduction (reviewed in Ref. 13). Several transcription factors have been reported to lie downstream of the MAPK, regulating different subsets of osmotic stress-responsive genes by different mechanisms. The general stress response transcription factors Msn2/Msn4 and the transcriptional regulator Hot1 are important for the recruitment of the Hog1 MAPK to stress-inducible promoters (14, 15). On the other hand, modification of Smpl1, a member of the MEF2 family of transcription factors, by Hog1 is important to modulate its transcriptional activity (16). Sko1, a member of the ATF-CREB family, inhibits transcription of several osmotic stress-inducible genes through recruitment of the general co-repressor complex Tup1-Ssn6 (17–19). Sko1 is phosphorylated by the Hog1 MAPK upon stress, and this is crucial to switch Sko1-Tup1-Ssn6 from a repressor to an activator complex (20, 21).

In this work we show that regulation of *HXT1* expression is achieved by two independent transcription factors, Rgt1 and Sko1, controlled by the glucose induction and HOG signaling pathways, respectively. Thus, induction of *HXT1* gene expression in response to glucose and in response to osmotic stress

\* This work was supported in part by the Spanish Ministry of Science and Technology (MCYT) (Grant BMC2002-00208 to P. S.) and by the Ministry of Science and Technology (Grants PM99-0028 and BMC2003-00321), Distinció de la Generalitat de Catalunya per a la Promoció de la Recerca Universitària, Joves Investigadors DURSI (Generalitat de Catalunya), and the EMBO YIP program (to F. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Both authors contributed equally to this work.

¶ Supported by a fellowship from the MCYT (Spain).

\*\* Recipients of a Formación Personal Universitario pre-doctoral fellowship from the Ministry of Education and Culture (Spain).

§§ To whom correspondence should be addressed. Tel.: 34-96-339-1760; Fax: 34-96-339-0800; E-mail: sanz@ibv.csic.es.

<sup>1</sup> The abbreviations used are: MAPK, mitogen-activated protein kinase; MNNG, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine; X-gal, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside; CHIP, chromatin immunoprecipitation.



TABLE I  
Strains used in this study

Strain	Genotype	Reference
TM141 (wild type)	MATa <i>his3 leu2 trp1 ura3</i>	(16)
TM233	MATa <i>hog1Δ::TRP1</i> derivative of TM141	(16)
<i>snf1Δhog1Δ</i>	MATa <i>snf1Δ::KanMX hog1Δ::TRP1</i> of TM141	This study
TM260	MATa <i>pbs2Δ::LEU2</i> derivative of TM141	(42)
FP50	MATa <i>ste11Δ::HIS3 ssk2Δ::LEU2 ssk22Δ::LEU2 his3 leu2 ura3</i>	(42)
FP57	MATa <i>sho1Δ::TRP1 ste11Δ::HIS3</i> derivative of TM141	(42)
TM257	MATa <i>ssk2Δ::LEU2 ssk22Δ::LEU2 leu2 trp1 ura3</i>	(42)
W303-1A (wild type)	MATa <i>ode2 his3 leu2 trp1 ura3 can1</i>	(43)
<i>snf1Δ</i>	MATa <i>snf1Δ::KanMX</i> derivative of W303	This study
<i>msn2Δmsn4Δ</i>	MATa <i>msn2Δ::HIS3 msn4Δ::TRP1</i> derivative of W303	(44)
<i>rgt1Δ</i>	MATa <i>rgt1Δ::KanMX</i> derivative of W303	This study
<i>rgt1Δhog1Δ</i>	MATa <i>rgt1Δ::KanMX hog1Δ::TRP1</i> derivative of W303	This study
<i>tup1Δ</i>	MATa <i>tup1Δ::KanMX</i> derivative of W303	(18)
<i>tup1Δhog1Δ</i>	MATa <i>tup1Δ::KanMX hog1Δ::TRP1</i> derivative of W303	This study
<i>sho1Δ</i>	MATa <i>sho1Δ::KanMX</i> derivative of W303	(18)
<i>sho1Δhog1Δ</i>	MATa <i>sho1Δ::KanMX hog1Δ::TRP1</i> derivative of W303	This study
<i>sho1Δrgt1Δ</i>	MATa <i>sho1Δ::KanMX rgt1Δ::URA3</i> derivative of W303	This study
MSY401 (wild type)	MATa <i>his3 leu2 trp1 ura3</i>	(9)
MSY441	MATa <i>snf3Δ::hisG rgt2Δ::HIS3</i> derivative of MSY401	(9)
MSY192	MATa <i>std1Δ::HIS3 mth1Δ2</i> derivative of MSY401	(9)
<i>std1Δmth1Δhog1Δ</i>	MATa <i>std1Δ::HIS3 mth1Δ2 hog1Δ::TRP1</i> derivative of MSY401	This study
ENY.WA-1A (wild type)	MATa <i>his3 leu2 trp1 ura3</i>	(45)
ENY.cat80-8b	MATa <i>grr1 (cat80-24)</i> derivative of ENY.WA-1A	(45)
LC99	MATa <i>ura3 leu2 his3 rgt1::KAN Ylp368R-HXT1 (LEU2)</i>	This study
LC91	MATa <i>ura3 leu2 trp1 his3 rgt1::KAN Ylp368R HXT1 (URA3)</i>	This study

(provided glucose was present) requires the coordinated activity of two independent signaling pathways that converge at the promoter level of *HXT1*.

#### EXPERIMENTAL PROCEDURES

**Strains and Genetic Methods.**—*Saccharomyces cerevisiae* strains used in this study are listed in Table I. *snf1Δ::KanMX*-mutated alleles were obtained by gene disruption using a BamHI fragment from plasmid pUC-*snf1Δ::KanMX* (11). *hog1Δ::TRP1*-mutated alleles were obtained by gene disruption using plasmid pDGH16 (22). *rgt1Δ::URA3* alleles were obtained by gene disruption using plasmid pUC-*rgt1Δ::URA3* (see below). All mutants were confirmed by PCR analysis using specific oligonucleotides. Standard methods for genetic analysis and transformation were used. Yeast cultures were grown in synthetic complete (SC) medium lacking appropriate supplements to maintain selection for plasmids, supplemented with different carbon sources.

**Plasmids.**—Centromeric plasmid pC-HXT1-lacZ (LEU2) was described previously (23). The *HXT1* expression cassette (*HXT1* promoter fused to *Escherichia coli lacZ* gene) was subcloned into plasmids pRS313(HIS3), pRS314(TRP1), and pRS316(URA3) (24). Plasmids pEG202-Rgt1 (LexA-Rgt1) and the corresponding empty vector pEG202 were described in a previous study (11). Plasmid pSH18-18 (GlexAop-lacZ) was described previously (25). To perform promoter analysis, PCR-generated DNA fragments containing several regions of the *HXT1* promoter up to the ATG were cloned into Ylp358R(URA3) or Ylp368R(LEU2) (26). To analyze internal promoter regions, different stretches from the 5' upstream region were amplified by PCR and inserted into the CYC1-lacZ reporter construct pJS205 (27).

Plasmid pUC-*rgt1Δ::URA3* was constructed in the following way. Plasmid pUC-Rgt1 (11) was digested with BglII and dephosphorylated with calf intestinal phosphatase. In this way we removed a central 2806-bp region of *RGT1*, leaving 444 and 335 bp at 5' and 3' ends, respectively, as flanking regions. A BamHI fragment from plasmid YDp-U (28), containing the *URA3* selection marker, was subcloned into the BglII sites of the former plasmid to give pUC-*rgt1Δ::URA3*, which was digested with BamHI and SalI to obtain a linear fragment that was used in the disruption experiments.

**Enzyme Assays.**—Cells growing exponentially in 2% raffinose plus 0.05% glucose were pulsed with either 0.4 M NaCl (final concentration), 2% glucose (final concentration) or a combination of 0.4 M NaCl and 2% glucose (final concentration). At times 0 and 60 min, aliquots were taken from the cultures, and the  $\beta$ -galactosidase activity was assayed in permeabilized cells and expressed in Miller units as in Ref. 29. Values are means from three to four independent transformants (S.D. < 15% in all cases). *TUP1*-deficient strains flocculate and, thus,  $\beta$ -galactosidase activity was assayed in yeast extracts as in a previous study (30) and expressed in Miller units/mg of protein. Invertase activity was assayed

in whole cells as described in a previous study (31).

**Immunoblot Analysis.**—Preparation of protein extracts was essentially performed as described (30). The extraction buffer was 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 1 mM dithiothreitol, 10% glycerol, 1 mM EDTA, 5 mM sodium pyrophosphate, 50 mM NaF and contained 2 mM phenylmethylsulfonyl fluoride and Complete protease inhibitor mixture (Roche Applied Science). Anti-phospho-p38 MAPK (Cell Signaling Technology) polyclonal antibodies were used to follow Hog1 phosphorylation.

**Isolation of *HXT1*-LacZ Reporter Repressors.**—LC91 (MATa *ura3 leu2 trp1 his3 rgt1::KAN Ylp358R HXT1-URA3*) and LC99 (MATa *ura3 leu2 his3 rgt1::KAN Ylp368R-HXT1-LEU2*) were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) as described in a previous study (32). Briefly, cells were grown in YPD (yeast extract, peptone, dextrose) at 30 °C to  $A_{600}$  of 0.3, washed in Tris-maleate buffer (pH 6.0), and resuspended in 1/5 of the original volume in the same washing buffer. Then, cells were incubated with a solution of 30  $\mu$ g/ml MNNG in 10 mM sodium acetate buffer (pH 5.0) for 60 min at 30 °C. After washes with 1% sodium thiosulfate, cells were grown in YPD, at 30 °C for 4 h. Mutagenized cells were plated on minimal medium plates containing X-gal (~1000 colonies/plate). After incubating at 30 °C for 4 days, positive clones were isolated. Mutant cells were then classified into complementation groups. Three mutants that represented the larger complementation groups were transformed with a yeast YCp50-genomic library. Positive clones were selected by their ability to block *HXT1* expression. Plasmids that complemented the corresponding mutations were isolated and sequenced.

**Chromatin Immunoprecipitation Assays.**—Chromatin immunoprecipitation PCR assays were performed as described previously (14). In all ChIP experiments, yeast cultures were grown in raffinose to early log phase ( $A_{600}$  0.6–1.0) before cells were exposed to 2% glucose or osmotic stress.

#### RESULTS

**The HOG Pathway Regulates *HXT1* Gene Expression by Glucose and Osmotress.**—Expression of the *HXT1* low affinity glucose transporter is regulated by glucose availability, being inhibited when glucose levels are scarce and activated in the presence of the sugar (see Ref. 3 for review). As shown in Fig. 1, cells growing exponentially in 2% raffinose showed very low levels of *HXT1* expression (measured as a transcriptional fusion of the *HXT1* promoter to the *lacZ* gene, encoding  $\beta$ -galactosidase enzyme; see "Experimental Procedures"). After a pulse of 2% glucose, expression of *HXT1* was induced, in agreement with what it has been reported previously (see Ref. 3 for review). However, *HXT1* induction rate was higher when cells

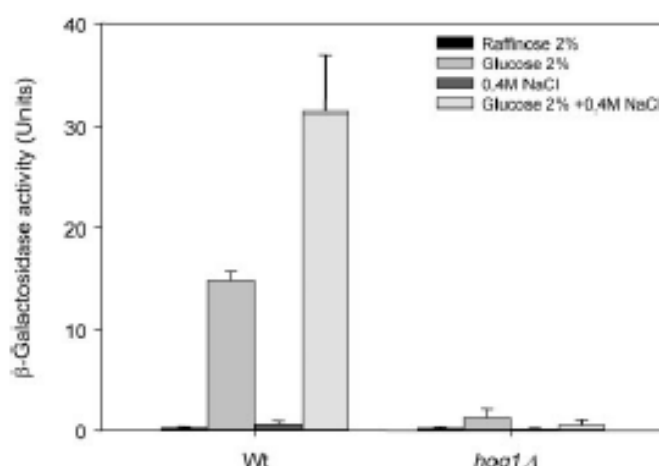


FIG. 1. The Hog1 MAPK plays a major role in the induction of *HXT1* expression by glucose. Wild type (TM141) and *hog1Δ* (TM233) cells were transformed with plasmid pC-HXT1-lacZ. Transformants were grown to mid-logarithmic phase in selective SC-2% raffinose plus 0.05% glucose medium.  $\beta$ -Galactosidase activity was assayed in cells 60 min after a pulse of either 2% glucose, 0.4 M NaCl, or 0.4 M NaCl plus 2% glucose. Values are mean  $\beta$ -galactosidase activities from four to six transformants (bars represent S.D.).

were subjected simultaneously to 2% glucose plus 0.4 M NaCl (Fig. 1). Similar results were obtained when 1 M sorbitol was used instead of NaCl (data not shown). These results were in agreement with data from microarray analyses that indicated that *HXT1* expression was enhanced after treatment with 0.4 M NaCl (33), 1 M NaCl (34), or 1 M sorbitol (35) in the presence of glucose. It is worth noting that no induction of *HXT1* expression was observed if cells were subjected only to osmotic stress in the absence of glucose (Fig. 1).

To determine if the HOG pathway was responsible for over-induction of *HXT1* expression in response to glucose plus osmotic stress, we analyzed *HXT1-lacZ* expression in a *hog1Δ* mutant strain (Fig. 1). To our surprise, *HXT1* expression was not induced even by glucose alone, indicating that the Hog1 protein kinase was required not only to overinduce *HXT1* expression by glucose plus osmotic stress but also to regulate *HXT1* expression by glucose. The absence of induction by glucose in a *hog1Δ* mutant was not due to a delay in the rate of induction, because when cells were grown overnight in 2% glucose, *hog1Δ* mutant cells still showed very reduced levels of *HXT1* expression in comparison to wild type cells (data not shown). A recovery in the induction of *HXT1* by glucose was obtained if *hog1Δ* mutants were transformed with a plasmid carrying a wild type Hog1 kinase, but not with a plasmid with a catalytically inactive form (Hog1KS-KN) (data not shown), indicating that the activity of the Hog1 kinase was necessary to allow induction of *HXT1* expression by glucose. In contrast to *HXT1*, expression of *HXT2*, encoding an intermediate-affinity glucose transporter that is repressed by glucose, was not affected in a *hog1Δ* strain (data not shown), indicating that the action of Hog1 was specific on *HXT1* expression.

Snf1 protein kinase activity affects negatively *HXT1* expression (11). To rule out the possibility that the absence of Hog1 kinase could stimulate the activity of the Snf1 kinase and then inhibit *HXT1* expression, we studied the activity of Snf1 protein kinase in a *hog1Δ* mutant by analyzing the regulation of the expression of *SUC2* (a glucose repressed gene) and found that it was similar to wild type (Table II). More importantly, induction of *HXT1* expression by glucose in a double *hog1Δsnf1Δ* mutant was similar to the *hog1Δ* mutant (Table II). These results indicated that the defect in the induction of *HXT1* by glucose in *hog1Δ* cells was not related to the activa-

TABLE II

*Snf1* protein kinase is not activated in a *hog1Δ* mutant

Wild type (TM141), *snf1Δ*, *hog1Δ* and double *snf1Δ hog1Δ* mutant cells were transformed with plasmid pC-HXT1-lacZ. Transformants were grown to mid-logarithmic phase in selective SC-4% glucose medium; then, invertase and  $\beta$ -galactosidase activities were measured as described under "Experimental Procedures." Values for invertase are means from three different transformants (S.D. < 10% in all cases), and values for  $\beta$ -galactosidase are means from four to six transformants (S.D. < 15% in all cases).

Strain	( <i>HXT1-lacZ</i> ) $\beta$ -galactosidase	( <i>SUC2</i> ) invertase
	units	
Wild type	100.4	<1
<i>snf1Δ</i>	114.8	<1
<i>hog1Δ</i>	10.4	<1
<i>snf1Δ hog1Δ</i>	13.2	<1

tion of Snf1 protein kinase and that the Hog1 MAPK played a crucial role in the regulation of *HXT1* induction by glucose.

**Osmostress Caused by Extracellular Glucose Results in Hog1 Activation and Induction of *HXT1* Gene Expression**—To analyze whether only the Hog1 MAPK or the integrity of the HOG pathway was needed for the induction of *HXT1* by glucose, we followed *HXT1* expression in mutants on several components of the HOG pathway. As shown in Fig. 2A, deletion of the *PBS2* MAPK kinase or simultaneous deletion of the three MAPK kinase kinases of the HOG pathway, *STE11*, *SSK2*, and *SSK22*, abolished induction of *HXT1* by glucose. Thus, the integrity of the main core of the HOG pathway is required for *HXT1* induction by glucose.

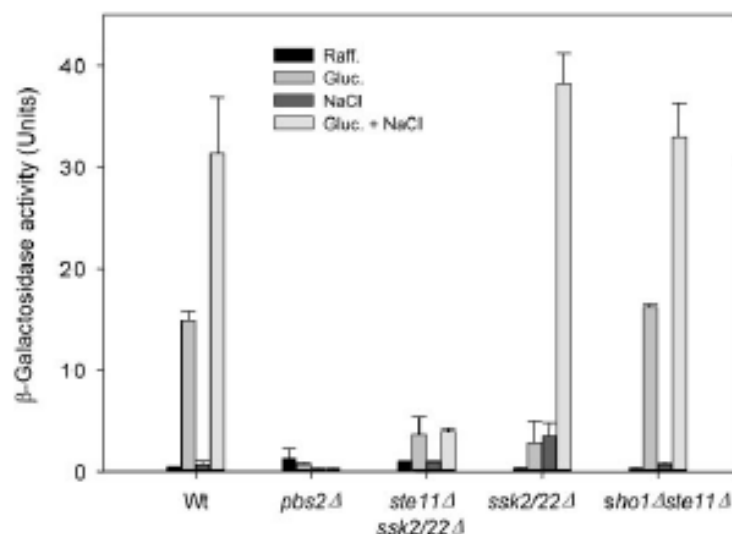
Two upstream sensing mechanisms activate the core of the HOG pathway, the Sln1 "two-component" osmosensor and a second mechanism that involves the Sho1 transmembrane protein (36). Mutations in the Sho1 branch (double *sho1Δste11Δ* mutant) did not alter *HXT1* expression (Fig. 2A). However, mutants in the Sln1 branch of the HOG pathway (*ssk2Δssk22Δ* or *ssk1Δ* (data not shown)) showed a clear defect in the induction of *HXT1* by glucose (Fig. 2A). It is worth noting that, on the later strains, induction of *HXT1* was similar to wild type only when both glucose and NaCl were added (Fig. 2A). These results indicate that induction of *HXT1* expression by glucose is mediated by the Sln1 branch of the HOG pathway.

Activation of the Hog1 MAPK by phosphorylation has been described to occur in response to osmotic stress (see the introduction). To test whether glucose "per se" or the osmotic stress caused by the addition of 2% glucose to the medium was responsible for Hog1 activation, we followed Hog1 phosphorylation in response to the addition of sugar. As shown in Fig. 2B, addition of 2% (110 mM) glucose or 2% galactose to raffinose growing cells induced Hog1 phosphorylation to the same extent as treatment with 110 mM NaCl. Moreover, addition of higher concentrations of glucose or galactose led to higher levels of Hog1 phosphorylation (data not shown). As expected, phosphorylation of Hog1 by sugar occurred in wild type cells but not in *pbs2Δ* cells (Fig. 2B). Time course experiments showed that phosphorylation of Hog1 by glucose was transient (Fig. 2C), as it has been described for NaCl (13). Thus, Hog1 activation is caused by an increase in extracellular osmolarity caused by the addition of sugar, not necessarily restricted to glucose.

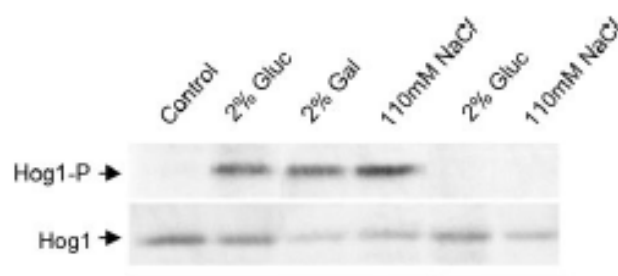
Because the presence of glucose was always necessary to stimulate *HXT1* expression and because in the absence of an active HOG pathway no induction of *HXT1* was observed (Figs. 1 and 2), we suggest the possibility that the addition of 2% glucose to raffinose growing cells would elicit two different signals, one that would be transmitted through the glucose induction pathway (see below) and another, where glucose would act as an osmolyte that would activate the Sln1 branch of



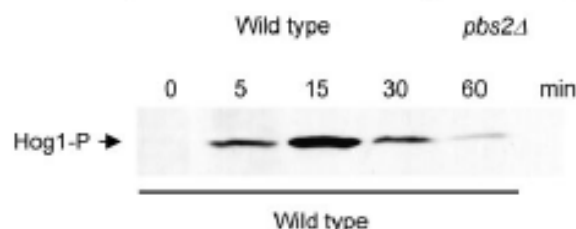
A



B



C



**FIG. 2. Activation of the Hog1 MAPK by glucose involves the Sln1 osmosensor.** *A*, induction of *HXT1* gene expression by glucose requires the Sln1 branch of the HOG pathway. Wild type (TM141) and several mutants of the HOG pathway, *pbs2Δ* (TM260), *ste11Δssk2Δssk22Δ* (FP50), *ssk2Δssk22Δ* (TM257), and *sho1Δste11Δ* (FP57), containing appropriated centromeric p*HXT1-lacZ* plasmids, were grown to mid-logarithmic phase in selective SC-2% raffinose plus 0.05% glucose medium. β-Galactosidase activity was assayed in cells 60 min after a pulse of either 2% glucose or 0.4 M NaCl plus 2% glucose. *B*, high sugar concentration results in activation of the Hog1 MAPK. Wild type (TM141) or *pbs2Δ* (TM260) cells were grown as in *A* and subjected to 2% glucose (Gluc), 2% galactose (Gal), or 110 mM NaCl. After 10 min, Hog1 phosphorylation (Hog1-P) was detected by immunoblot analysis using antibodies anti-phospho-p38 MAPK; anti-Hog1 was used as loading control. *C*, transient phosphorylation of Hog1 by external glucose. Wild type (TM141) cells, grown as in *B*, were subjected to 2% glucose for the indicated period of time and phosphorylated Hog1 (Hog1-P) was detected by immunoblot analysis.

the HOG pathway, more sensitive to osmotic changes in the environment (13).

**Induction of *HXT1* Expression upon Glucose Plus Osmotic Stress Depends on the Integrity of the Glucose Signaling Pathway**—Induction of *HXT1* by glucose depends on the glucose signaling pathway (3). Then, we wanted to test whether integrity of the glucose signaling pathway was required to allow overinduction *HXT1* in response to glucose plus osmotic stress. Inactivation of the membrane glucose sensors Snf3 and Rgt2, and the SCF-Grr1 ubiquitination complex abolished *HXT1* expression by both glucose and osmotic stress (Fig. 3A). In contrast, deletion of the *MTH1* and *STD1* genes, known regulators of Rgt1 transcriptional repressor (8), resulted in constitutive expression of *HXT1*. However, in the double *std1Δmth1Δ* mutant, osmotic stress but not glucose, was able to induce *HXT1* expression at even higher levels in a Hog1-dependent manner (Fig. 3A). Thus, integrity of the main core of the glucose signaling pathway (Snf3/Rgt2 and SCF-Grr1) is essential to allow overinduction of *HXT1* in response to glucose plus osmotic stress. If repressing properties of Rgt1 are avoided (*std1Δmth1Δ* mutants), then the HOG pathway may overinduce *HXT1* expression in response to osmotic stress.

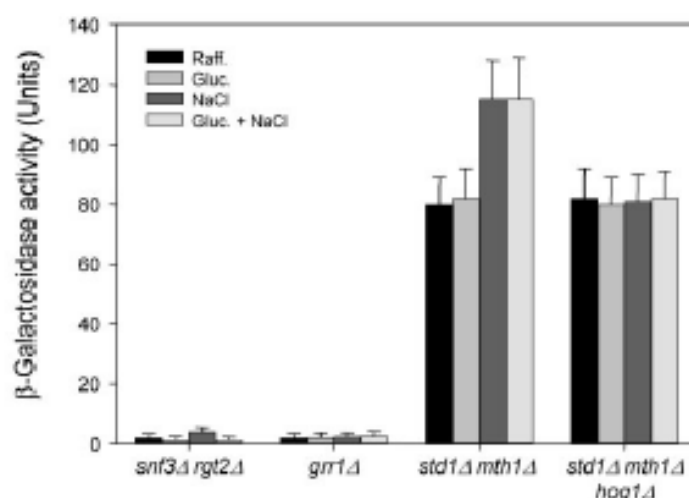
Deletion of *RGT1* repressor resulted in a mild deregulation of

*HXT1* expression in absence of glucose and no further induction by glucose (Ref. 3 and Fig. 3B). In contrast, a clear induction of *HXT1* expression was observed by NaCl alone or by glucose plus NaCl (Fig. 3B). These effects were dependent on the presence of Hog1 kinase, because in the double *rgt1Δhog1Δ* mutant no induction of *HXT1* was observed under any condition (Fig. 3B). Thus, in the absence of the Rgt1 transcriptional repressor, activation of the HOG pathway by osmotic stress leads to full *HXT1* induction, even in the absence of glucose.

An alternative explanation for the results presented so far was that the function of Rgt1 could be regulated directly by the Hog1 kinase. However, this was unlikely, because when we tested the transcriptional properties of a LexA-Rgt1 fusion, these were similar in both wild type and *hog1Δ* mutant (Table III).

**Regulation of *HXT1* Expression by the HOG and Glucose Signaling Pathways Is Exerted at Different Sites on the *HXT1* Promoter**—As shown above, induction of *HXT1* by glucose and osmotic stress requires the activation of two independent signaling pathways, glucose induction and HOG pathways. To identify sequences in the upstream control region of *HXT1* that are important for regulation by any of these pathways, we investigated the expression of a set of segments of the *HXT1* pro-

A



B

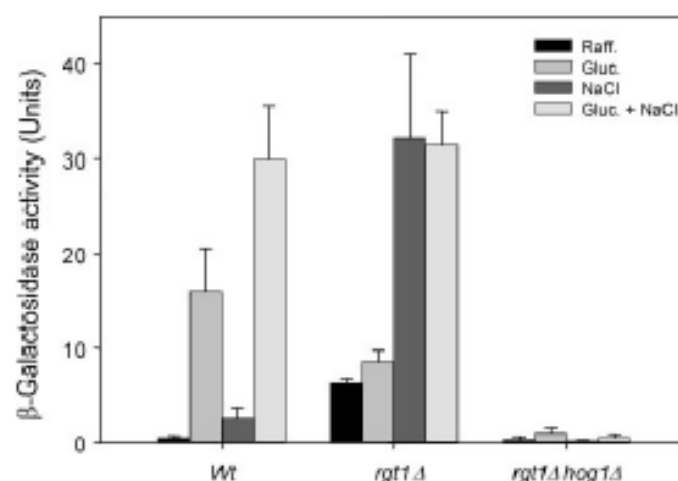


TABLE III

Transactivating properties of *Rgt1* are not affected in a *hog1Δ* mutant

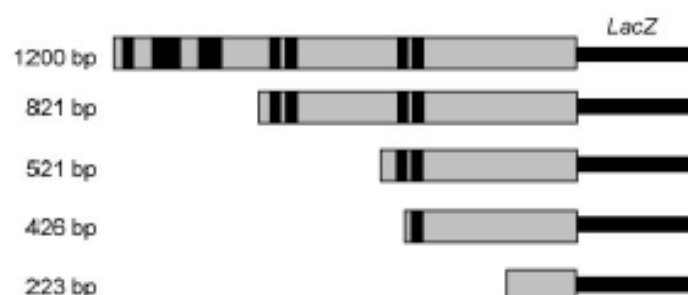
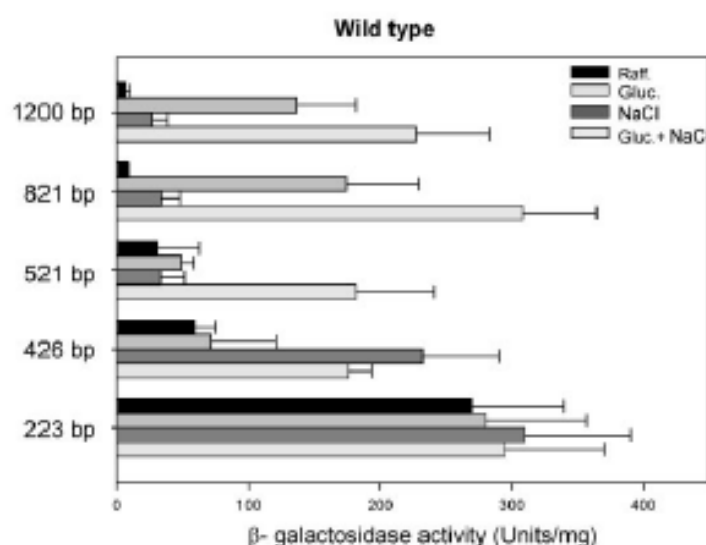
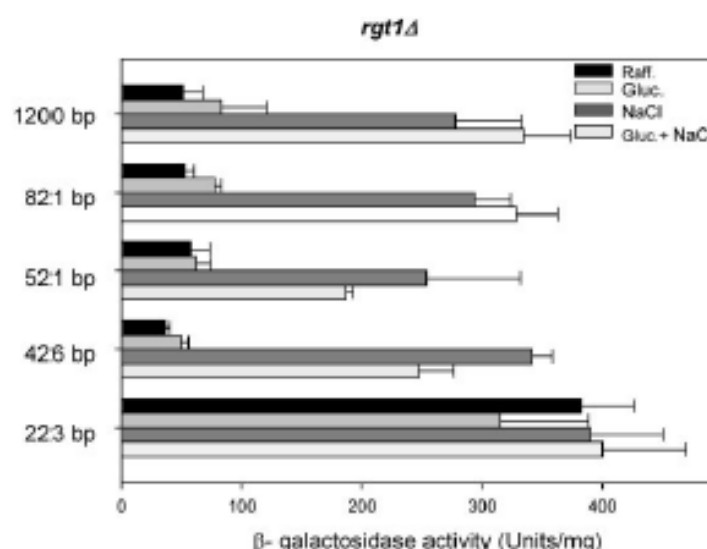
Wild type (TM141) and *hog1Δ* (TM233) cells were transformed with plasmid pSH18-18 (containing 6lexAop-lacZ) and either plasmid pEG202 (LexA) or pEG202-Rgt1 (LexA-Rgt1). Transformants were grown to mid-logarithmic phase in selective SC-4% glucose medium. Values are mean  $\beta$ -galactosidase activities from four to six transformants (S.D. < 15% in all cases).

	(6lexAop-lacZ) $\beta$ -galactosidase	
	LexA	LexA-Rgt1
	units	
Wild type	<1	1940
<i>hog1Δ</i>	<1	2133

motor fused to the *lacZ* gene, in cells growing in raffinose and then pulsed with glucose, NaCl, or glucose plus NaCl, as above (Fig. 4). Insertion of a fragment of ~200 bp (from -223 to ATG) to the Yip358R reporter vector gave high levels of  $\beta$ -galactosidase activity in any of the conditions tested, whereas insertion of larger fragments (-1200 to ATG or -821 to ATG) resulted in strong repression under basal conditions (raffinose-growing cells) and strong induction in response to glucose or to glucose plus NaCl (Fig. 4B). These results indicated that reg-

ulation of *HXT1* expression consists mainly of a derepression process. Because we observed a similar derepression pattern when we assayed a fragment containing from -821 to ATG in comparison to full-length *HXT1* promoter (from -1200 to ATG), we suggest that the fragment comprised between -821 to -223 contained the main regulatory elements of *HXT1*.

Further deletion analysis showed that a fragment containing from -521 to ATG was not induced by glucose, indicating that in the -821 to -521 region there must be sequences related to the induction of *HXT1* by glucose. We also observed that this -521 to ATG fragment was not induced by NaCl alone, but it was fully induced by glucose plus NaCl (Fig. 4B). Interestingly, deletion of *RGT1* allowed full induction of this -521 to ATG fragment by NaCl in the absence of glucose (Fig. 4C), what indicated that *Rgt1* was still able to block osmotic stress induction of this fragment in wild type cells. A fragment containing from -426 to ATG, which showed higher basal expression in raffinose and no glucose induction, suggesting a lack of *Rgt1* repression, showed strong induction by NaCl in the absence of glucose in both wild type and *rgt1Δ* strains (Fig. 4, B and C). These results supported the idea that *Rgt1* was blocking osmotic stress induction of *HXT1* by interacting with a promoter region

**A****B****C**

**FIG. 4. Regulation of *HXT1* expression by glucose or osmotic stress is exerted at different sites on the *HXT1* promoter.** *A*, schematic diagram of the *HXT1* promoter. Putative Rgt1 binding sites (spaced CGG pairs) are depicted in black. *B*, segments from the *HXT1* upstream region indicated on the left were inserted into Ylp358R or Ylp368R *lacZ* reporter plasmids (see "Experimental Procedures"). Constructs were introduced into wild type (W303-1A) (*B*) or *rgt1Δ* (*C*) cells, which were grown in raffinose and then pulsed with either 2% glucose, 0.4 M NaCl, or 0.4 M NaCl plus 2% glucose. β-Galactosidase activity (units/mg) was measured in cell extracts, 60 min after the pulses, and it is the result of the measurement in triplicate of three independent transformants (bars represent S.D.).

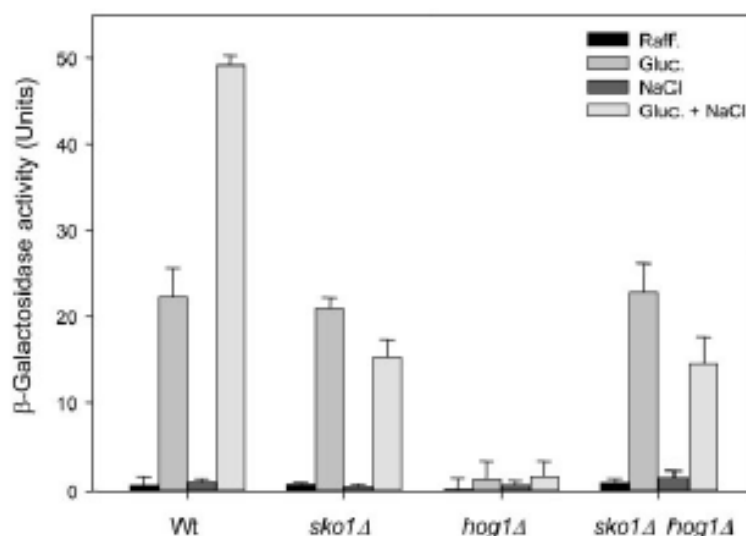
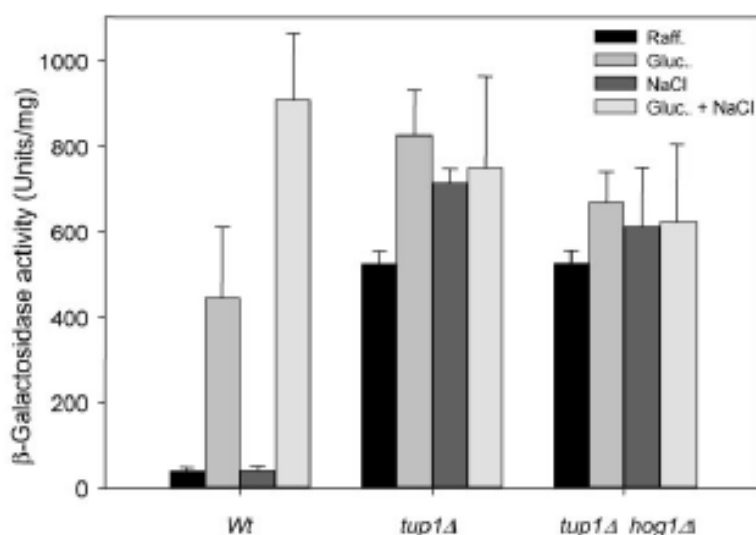
located between -521 and -426 and that the HOG pathway affected another putative repressor that interacted with a promoter region located between -426 and -223.

***Sko1 Transcription Factor Regulates *HXT1* Expression under the Control of the HOG Pathway***—As just mentioned, analysis of the *HXT1* promoter suggested the presence of an uncharacterized transcription factor regulated by HOG pathway that repressed *HXT1* expression. Inspection of the *HXT1* pro-

motor did not yield any sequence known to be regulated by specific transcription factors other than stress response elements. STRE elements are known to be binding sites for Msn2 and Msn4 transcription factors (37). However, when we tested *HXT1* expression in yeast cells deficient in both *MSN2* and *MSN4* genes, we observed a similar pattern of *HXT1* expression, compared with the wild type strain (data not shown).

To identify the additional repressing factor that regulates

**FIG. 5. The Sko1-Tup1-Ssn6 complex controls *HXT1* expression under the control of the Hog1 MAPK.** A, Hog1 regulates *HXT1* expression through the Sko1 transcriptional factor. Wild type (W303-1A), *sko1Δ*, *hog1Δ*, and *sko1Δhog1Δ* strains (A), and wild type (W301-1A), *tup1Δ* and *tup1Δhog1Δ* cells (B) were transformed with the appropriate centromeric pHXT1-lacZ plasmids. Transformants were treated and analyzed as in Fig. 1. In A: values are mean  $\beta$ -galactosidase activities from four to six transformants expressed in Miller units. In B:  $\beta$ -galactosidase activity (units/mg) was measured in cell extracts; values are mean  $\beta$ -galactosidase activities from four to six transformants (bars represent S.D.).

**A****B**

*HXT1* expression, we conducted a mutant screening on the basis of the assumption that simultaneous inactivation of *RGT1* and the unknown transcriptional repressor would render *HXT1* expression constitutively activated. Briefly, *rgt1Δ* cells growing on raffinose and containing an integrated *HXT1-lacZ* reporter construct were mutagenized with MNNG, and positive clones were selected by their ability to induce *HXT1* expression and, therefore, to produce  $\beta$ -galactosidase on X-gal-containing plates (described under "Experimental Procedures"). In this way, 30 positive clones were identified from ~55,000 colonies. Recessive mutants were selected and classified into a number of complementation groups. Three of the largest complementation groups were identified as *ssn6*, *tup1*, and *sko1* mutants by complementation cloning.

We then tested the effect of the deletion of *SKO1* in cells carrying the centromeric *HXT1-lacZ* reporter construct. As shown in Fig. 5A, deletion of *SKO1* resulted in cells able to induce *HXT1* expression in response to glucose, but no further induction of *HXT1* expression was observed by the combined action of glucose plus osmotic stress. Moreover, a double *sko1Δ hog1Δ* mutant strain showed the same pattern of expression as

the *sko1Δ* strain, indicating that the lack of expression of *HXT1* in a *hog1Δ* strain in response to glucose (Fig. 1) was caused by the inability of this strain to release Sko1 repression. Therefore, Sko1 mediates Hog1 regulation of *HXT1* expression.

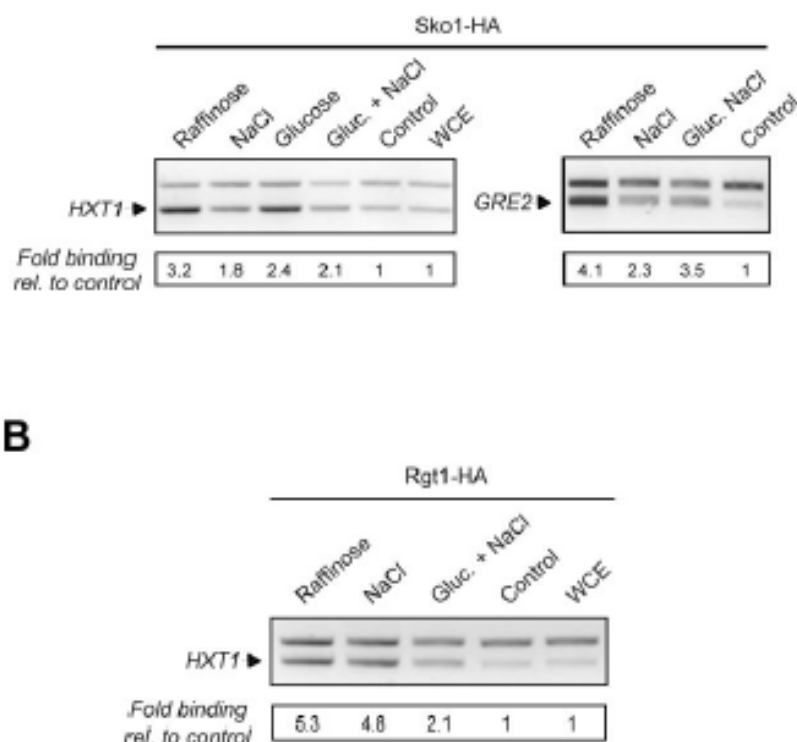
Apart from *sko1* mutants, we identified in our screening mutations in *TUP1* and *SSN6* genes. It is known that the Tup1-Ssn6 general co-repressor complex interacts with Sko1 to repress transcription of osmotic stress-regulated genes (17, 18). In addition, it is also known that the Tup1-Ssn6 complex interacts with Rgt1 to repress transcription of *HXT1* in low glucose conditions (11, 38). Consistent with these observations, mutations in *TUP1* or *SSN6* resulted in constitutive expression of *HXT1* that was not significantly enhanced by addition of glucose or NaCl (Fig. 5B; data not shown for the *ssn6Δ* mutant). As expected, deletion of *HOG1* in a *tup1Δ* strain did not affect *HXT1* expression. Therefore, our data suggest that two transcriptional repressors, Sko1 and Rgt1, are controlling *HXT1* gene expression by their binding to the Tup1-Ssn6 complex.

**Sko1 Controls *HXT1* Transcription by Direct Binding to the Promoter**—Chromatin immunoprecipitation (ChIP) analyses have shown that the Hog1 MAPK is actively recruited to os-

A

FIG. 6. Both Rgt1 and Sko1 are bound to the *HXT1* promoter under low glucose conditions as detected by ChIP analysis. Strains containing genomic tags of Sko1-HA (A) or Rgt1-HA (B) were grown in the presence of raffinose, and samples for ChIP analyses were taken before (Raffinose) or after 15 min of a pulse of 2% glucose (*Gluc*), 0.4 M NaCl (*NaCl*), or 2% glucose plus 0.4 M NaCl (*Gluc + NaCl*). Immunoprecipitations were performed by using mouse anti-HA monoclonal antibodies. PCR was performed with primers spanning the promoter region of *HXT1*, *GRE2*, and control oligonucleotides spanning the *GAL1* gene region. The exact primer sequences are available upon request. Control lanes show DNA amplified from extracts from cells without tagged protein (*Control*), or prior to immunoprecipitation (*WCE*, represents whole cell extract diluted 1:500). Data represents -fold increase over control without tag. Quantification was performed using Quantity One software from Bio-Rad.

B



mostress responsive promoters (14, 21). Consistently, our ChIP analyses showed that Hog1 was also recruited to *HXT1* promoter in response to osmotic stress (data not shown). To test whether Sko1 was also present at the *HXT1* promoter, we also utilized ChIP analysis. As shown in Fig. 6A, Sko1 was present at the *HXT1* promoter in cells growing in raffinose. Addition of NaCl resulted in a decrease of Sko1 binding, which was more pronounced than the one observed by glucose treatment. Binding of Sko1 to *HXT1* promoter had the same properties as the binding of the repressor to the *GRE2* promoter, a gene known to be regulated by Sko1 (Fig. 6A) (21).

ChIP analyses from several groups have described the presence of Rgt1 at the *HXT1* promoter in the absence of glucose and its release in response to a pulse of glucose (7, 12, 39). Consistent with these results, we found Rgt1 present at the *HXT1* promoter in cells growing in raffinose, but its binding was not affected by osmotic stress (Fig. 6B). As expected, binding of Rgt1 to the *HXT1* promoter was diminished in the presence of glucose plus NaCl (Fig. 6B). Taking all these results together, we suggest that in cells growing in low glucose conditions, Sko1 and Rgt1 are present at the *HXT1* promoter and co-repress gene transcription.

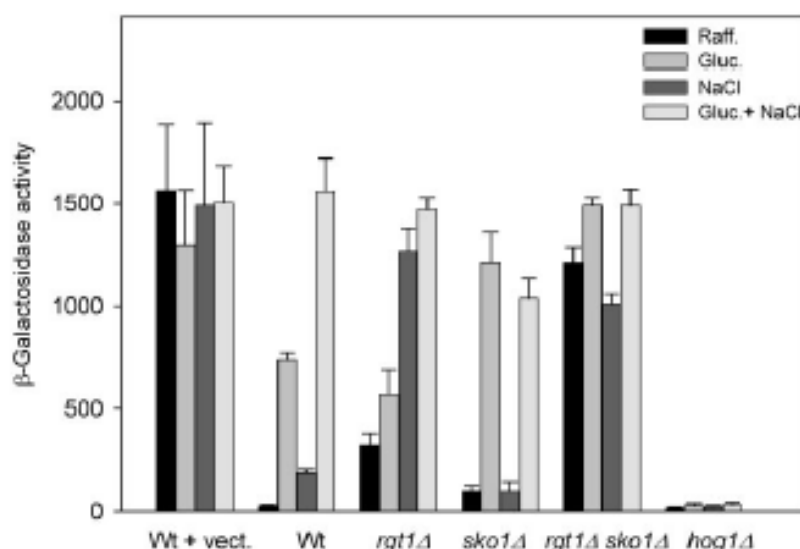
**Regulation of *HXT1* Expression Is Mediated by the Coordinated Regulation of Rgt1 and Sko1 Transcriptional Activities—**Analysis of the *HXT1* promoter (see above) showed that a small region between -521 to -223 contained possible Rgt1 and Sko1 regulatory elements that could be critical to understand the relationship between the HOG and glucose signaling pathways in the regulation of *HXT1* expression. To analyze this relationship at the promoter level, we investigated a promoter fragment of *HXT1* containing from -521 to -220 in a *CYC1-lacZ* reporter vector under the same growth conditions as above. As shown in Fig. 7, this 301-bp fragment was able to repress transcription of the *CYC1-lacZ* system in low glucose medium and derepressed transcription in response to glucose or to glucose plus NaCl, similarly to what we observed when we

used the full-length promoter in a wild type strain (Fig. 1). Rgt1 was still able to play a negative role in the regulation of this fragment in low glucose, because deletion of *RGT1* increased expression under this condition. Interestingly, osmotic stress, but not glucose, fully induced expression of the reporter in an *rgt1Δ* strain, indicating that, when there is no Rgt1, the release of Sko1 by the activation of the HOG pathway results in full expression of the reporter. Consistently, the lack of Sko1 results in defective derepression by NaCl and no overinduction of the reporter by the combined action of glucose plus osmotic stress. In addition, the simultaneous deletion of *RGT1* and *SKO1* led to constitutive expression of the reporter construct under any condition. Therefore, Rgt1 and Sko1 acted independently but coordinately to regulate expression of *HXT1* in response to glucose and osmotic stress. Our results also suggest that full *HXT1* expression requires the activity of both glucose induction and HOG signaling pathways to eliminate both repressing activities, Rgt1 and Sko1.

#### DISCUSSION

Yeast cells are able to adjust cellular metabolism, gene expression, and growth in response to environmental stimuli. In this sense, *S. cerevisiae* can deal with an extremely broad range of sugar concentrations and can metabolize glucose, its most preferable carbon source, from higher than 1.5 M (as in drying fruits) down to micromolar concentrations. To be adapted to any environmental sugar condition, yeast have developed an unusual diversity of glucose transporter proteins (17 different Hxt's) with specific individual properties and kinetics. *S. cerevisiae* has from low affinity glucose transporters such as *HXT1* and *HXT3* ( $K_m$  from 50 to 100 mM), that function when there is a good supply of sugar, to intermediate affinity transporters such as *HXT2* and *HXT4* ( $K_m$  around 10 mM), and high affinity transporters such as *HXT6* and *HXT7* ( $K_m$  around 1 mM), that function when the amount of the sugar is becoming scarce. Expression of all these transporter genes is tightly regulated at

FIG. 7. The *HXT1* promoter is regulated coordinately by the HOG and the glucose signaling pathways. An empty vector pJS205 (vect.) or a vector containing the region of the promoter of *HXT1* that comprises from -521 to -220 into pJS205 was transformed into several yeast strains. Transformants were treated and analyzed as in Fig. 4.  $\beta$ -Galactosidase activity (units/mg) was measured in cell extracts, 60 min after the pulses, and it is the result of the measurement in triplicate of three independent transformants (bars represent S.D.).



the transcriptional level by the amount of substrate in the environment. Thus, the expression of *HXT1*, a low affinity glucose transporter, is induced in the presence of glucose, whereas the expression of *HXT2* (intermediate affinity) and *HXT6* (low affinity) glucose transporters is repressed by the presence of the sugar (see Refs. 3, 40, 41 for review).

In this report, we show that full induction of *HXT1* expression requires the coordinated action of two independent signaling pathways, the glucose signaling and HOG signaling pathways. A plausible interpretation of this result could be that by increasing the expression of *HXT1* by hyperosmotic conditions, yeast could provide more substrate (glucose) for the synthesis of the osmoprotectant glycerol (see Ref. 13 for review on glycerol biosynthesis) to cope with the osmotic stress conditions. Activation of the glucose signaling pathway is mediated by the transmembrane glucose sensors Snf3 and Rgt2. On the other hand, activation of the HOG pathway can be mediated by two independent sensing systems: the two-component sensor that involves the Sln1 histidine kinase and the Sho1 sensing system (36). It has been shown that both systems are capable of leading to Hog1 activation in response to changes in the extracellular osmolarity, however, they seem to react slightly different. The Sln1 sensor is able to sense small changes in the environment and induce progressive Hog1 activation, whereas the Sho1 sensing system induces full response but only once a threshold level of osmotic stress in the environment is reached (22). The different sensitivity of the two osmosensing systems was already studied under laboratory conditions, but the physiological meaning of this different sensitivity has not been completely understood. Here, we show that small changes in extracellular sugar concentration, which result in small changes in extracellular osmolarity, are sufficient to induce Sln1-mediated Hog1 activation, whereas these changes are not high enough to induce the Sho1-sensing system (Fig. 2). This different sensitivity of the two signaling systems might have a significant physiological role, because if under specific conditions only a partial activation of Hog1 MAPK is required, a fine tuning mechanism would avoid full induction of adaptive responses that might be too energy-consuming for the cell.

Activation of the glucose signaling pathway by the presence of glucose leads to regulation of the Rgt1 transcriptional repressor. However, regulation of Rgt1 is not sufficient to induce gene expression by glucose without simultaneous activation of the HOG pathway. We also present strong evidence that the action of the HOG pathway is conducted via the Hog1 MAPK and the Sko1 transcriptional repressor. Our results also sug-

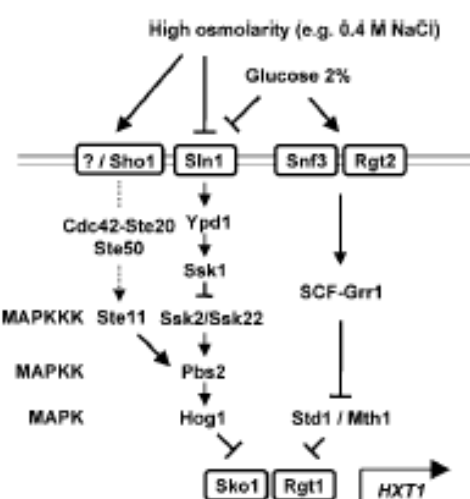


FIG. 8. Schematic diagram of the glucose signaling pathway and HOG pathways that regulate *HXT1* expression. The arrows do not necessarily indicate direct interactions (see text for details).

gest that both repressors, Rgt1 and Sko1, interact with different regions of the *HXT1* promoter. We suggest that Rgt1 interacts, at least, with a promoter region located between -521 and -426. In fact this region is included in the fragment that was used to demonstrate a direct interaction of Rgt1 with *HXT1* promoter by either DNA binding (38) or ChIP (7) analyses (fragment from -648 to -361). This region contains a spaced CGG pair sequence ( $^{-480}\text{CCG-X}_{27}\text{-}^{-450}\text{CCG}$ ) that fulfils the requirements of the consensus sequence identified to be necessary for Rgt1 binding (39). However, additional sites for Rgt1 binding must exist, because a promoter fragment containing only from -521 to ATG was not able to be properly induced by glucose. Because we have demonstrated that a promoter region from -821 to ATG contains all the regulatory regions of *HXT1*, we suggest that additional Rgt1 binding sites must be located in this -821 to -521 region. In fact we identified several spaced CGG pairs in this region ( $^{-805}\text{CCG-X}_{37}\text{-}^{-772}\text{CCG}$ ;  $^{-766}\text{CCG-X}_{27}\text{-}^{-736}\text{CCG}$ ). Thus, the -821 to ATG fragment would contain at least three spaced CGG pairs, in agreement with the described requirements for proper Rgt1 binding (39).

We also suggest that Sko1 interacts with a promoter region located between -426 and -223. However, we did not find any consensus Sko1-CRE site (TGACGTCA) in this region. Because the *HAL1* promoter contains a degenerated CRE site (TTACG-

TAA) that binds Sko1 functionally (19), we looked for degenerated sequences resembling the CRE<sub>HAL1</sub> site and found one related sequence -41<sup>8</sup>ATACGTAA-40<sup>8</sup>. We mutagenized this site to ATATTTAA to test its functionality, but we only observed a slight increase in the induction of *HXT1* by glucose in comparison to the wild type promoter. Consistently, a *hog1Δ* mutant containing this mutated promoter improved only slightly the induction of *HXT1* by glucose (data not shown). These results indicated that either this site was not fully functional or that there were additional cAMP-response element-like sites in the sequence where Sko1 was able to bind.

Our ChIP analyses data indicate that there is a positive interaction of Sko1 with the *HXT1* promoter in low glucose conditions. The addition of NaCl decreases the binding of Sko1 to the *HXT1* promoter and improves the binding of the Hog1 MAPK, similarly to what it has been described for other osmotic stress-inducible genes (20, 21). Rgt1 also binds to *HXT1* promoter in low glucose conditions, but addition of NaCl does not affect its binding. Because Rgt1 binding is only decreased by glucose (7, 12, 39), we suggest that the addition of glucose to raffinose growing cells would have a dual effect. On one hand, it would release Rgt1 from the promoter and, on the other hand, acting glucose as an osmolyte, it would activate the HOG pathway and would release Sko1 from the promoter, allowing in this way the derepression of *HXT1*. Consistent with this suggestion we have found that the addition of higher concentrations of glucose (4%) or the combined action of 2% glucose plus 0.4 M NaCl improved *HXT1* expression.

It has been described that Sko1 inhibits transcription of several osmotic stress-inducible genes through recruitment of the general co-repressor complex Tup1-Ssn6 (17–19). Sko1 is phosphorylated by the Hog1 MAPK upon stress, and this is crucial to switch Sko1-Tup1-Ssn6 from a repressor to an activator complex (20, 21). At the same time, it is known that the Tup1-Ssn6 complex interacts with Rgt1 and plays a major role in repressing expression of *HXT1* under low glucose conditions (11, 38). Therefore, the Tup1-Ssn6 co-repressor complex seems to play a dual role in the regulation of *HXT1* expression. On one hand, it helps Sko1 to repress transcription under non-osmotic stress conditions and, on the other hand, it helps Rgt1 to repress transcription in the absence of glucose. Consistent with these suggestions, mutations in *TUP1* or *SSN6* resulted in constitutive expression of *HXT1* that was not significantly enhanced by the addition of either glucose, NaCl, or both.

Taking all the results together, we propose the following model for *HXT1* gene regulation (Fig. 8). Under normal conditions (low glucose and no osmotic stress), *HXT1* promoter would be repressed by two independent repressors, Rgt1 and Sko1. In response to glucose addition, two different pathways would activate *HXT1* gene expression. Glucose would directly activate the glucose signaling pathway, which would mediate regulation of the Rgt1 repressor, and the osmotic stress caused by the addition of glucose would result in activation of the Hog1 MAPK that would result in regulation of the Sko1 repressor by the MAPK. Thus, the activity of two independent signaling

pathways would converge in the regulation of *HXT1* expression by glucose and osmotic stress.

**Acknowledgments**—We are very grateful to Dr. Francisco Estruch, Dr. Karl-Dieter Entian, Dr. Markus Proff, and Dr. Lynne Yenush for strains and plasmids; Eulàlia de Nadal for helpful advice; and M. Carmona for technical assistance.

## REFERENCES

- Gancedo, J. M., and Gancedo, C. (1997) In *Yeast Sugar Metabolism* (Zimmermann, F. K., and Entian, K. D., eds) pp. 359–377, Technomic Publishing Co., Basel, Switzerland.
- Gancedo, J. M. (1998) *Microbiol. Mol. Biol. Rev.* **62**, 334–361.
- Ozcan, S., and Johnston, M. (1999) *Microbiol. Mol. Biol. Rev.* **63**, 554–569.
- Rolland, F., Winderickx, J., and Thérèse, J. M. (2001) *Trends Biochem. Sci.* **26**, 310–317.
- Li, F. N., and Johnston, M. (1997) *EMBO J.* **16**, 5629–5638.
- Kishi, T., Sano, T., and Yamao, F. (1998) *Mol. Gen. Genet.* **257**, 143–148.
- Mosley, A. L., Lakshmanan, J., Aryal, B. K., and Ozcan, S. (2003) *J. Biol. Chem.* **278**, 10322–10327.
- Lakshmanan, J., Mosley, A. L., and Ozcan, S. (2003) *Curr. Genet.* **44**, 19–25.
- Schmidt, M. C., McCartney, R. R., Zhang, X., Tillman, T. S., Schimo, H., Wolf, S., Almerie, C., and Watkins, S. C. (1999) *Mol. Cell Biol.* **19**, 4561–4571.
- Lafuente, M. J., Gancedo, C., Jaumaux, J. C., and Gancedo, J. M. (2000) *Mol. Microbiol.* **35**, 161–172.
- Tomas-Cobas, L., and Sanz, P. (2002) *Biochem. J.* **368**, 657–663.
- Flick, K. M., Spolevov, N., Kalashnikova, T. I., Guaderrama, M., Zhu, Q., Chang, H. C., and Wittenberg, C. (2003) *Mol. Biol. Cell* **14**, 3230–3241.
- Hohmann, S. (2002) *Microbiol. Mol. Biol. Rev.* **66**, 300–372.
- Alepuz, P. M., Jovanovic, A., Reiser, V., and Ammerer, G. (2001) *Mol. Cell* **7**, 767–777.
- Alepuz, P. M., de Nadal, E., Zapater, M., Ammerer, G., and Posas, F. (2003) *EMBO J.* **22**, 2433–2442.
- de Nadal, E., Casacko, L., and Posas, F. (2003) *Mol. Cell Biol.* **23**, 229–237.
- Marquez, J. A., Pascual-Ahuir, A., Proff, M., and Serrano, R. (1998) *EMBO J.* **17**, 2543–2553.
- Proff, M., and Serrano, R. (1999) *Mol. Cell Biol.* **19**, 537–546.
- García-Giménez, M. A., and Struhl, K. (2000) *Mol. Cell Biol.* **20**, 4340–4349.
- Proff, M., Pascual-Ahuir, A., de Nadal, E., Arino, J., Serrano, R., and Posas, F. (2001) *EMBO J.* **20**, 1123–1133.
- Proff, M., and Struhl, K. (2002) *Mol. Cell* **9**, 1367–1377.
- Maeda, T., Wurgler-Murphy, S. M., and Saito, H. (1994) *Nature* **368**, 242–245.
- Mayordomo, I., and Sanz, P. (2001) *Yeast* **18**, 1309–1316.
- Sikorski, R. S., and Hieter, P. (1989) *Genetics* **122**, 19–27.
- Kuchin, S., Treich, I., and Carlson, M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 7916–7920.
- Myers, A. M., Tzagoloff, A., Kinney, D. M., and Lusty, C. J. (1986) *Gene (Amst.)* **45**, 299–310.
- Schuller, H. J., Hahn, A., Trester, F., Schutz, A., and Schweizer, E. (1992) *EMBO J.* **11**, 167–174.
- Berben, G., Dumont, J., Gilliquet, V., Bolle, P. A., and Hilger, F. (1991) *Yeast* **7**, 475–477.
- Ludin, K., Jiang, R., and Carlson, M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 6245–6250.
- Sanz, P., Alms, G. R., Haystead, T. A., and Carlson, M. (2000) *Mol. Cell Biol.* **20**, 1321–1328.
- Goldstein, A., and Lampen, J. O. (1975) *Methods Enzymol.* **42**, 504–511.
- Posas, F., Witten, E. A., and Saito, H. (1998) *Mol. Cell Biol.* **18**, 5788–5796.
- Posas, F., Chambers, J. R., Heyman, J. A., Hoeffler, J. P., de Nadal, E., and Arino, J. (2000) *J. Biol. Chem.* **275**, 17248–17255.
- Yale, J., and Bohnert, H. J. (2001) *J. Biol. Chem.* **276**, 15996–16007.
- Gasch, A. P., Spellman, P. T., Kao, C. M., Carmel-Harel, O., Eisen, M. B., Storz, G., Botstein, D., and Brown, P. O. (2000) *Mol. Biol. Cell* **11**, 4241–4257.
- de Nadal, E., Alepuz, P. M., and Posas, F. (2002) *EMBO Rep.* **3**, 735–740.
- Estruch, F. (2000) *FEMS Microbiol. Rev.* **24**, 469–486.
- Ozcan, S., Leong, T., and Johnston, M. (1999) *Mol. Cell Biol.* **19**, 6419–6426.
- Kim, J. H., Polish, J., and Johnston, M. (2003) *Mol. Cell Biol.* **23**, 5208–5216.
- Kruckeberg, A. L. (1996) *Arch. Microbiol.* **166**, 283–292.
- Bokes, E., and Hollenberg, C. P. (1997) *FEMS Microbiol. Rev.* **21**, 85–111.
- Posas, F., and Saito, H. (1997) *Science* **276**, 1702–1705.
- Thomas, B. J., and Rothstein, R. (1989) *Cell* **58**, 619–630.
- Amores, M., and Estruch, F. (2001) *Mol. Microbiol.* **39**, 1523–1532.
- Schuller, H. J., and Entian, K. D. (1991) *J. Bacteriol.* **173**, 2045–2052.

## DISCUSSION



---

## Targeting the MEF2-Like Transcription Factor Smp1 by the Stress-Activated Hog1 Mitogen-Activated Protein Kinase

MOLECULAR AND CELLULAR BIOLOGY

Jan. 2003, p. 229–237 Vol. 23, No. 1

### Summary

Yeast cells respond to increases in osmolarity in the extracellular environment activating the stress-activated MAPK Hog1. Hog1 activation results in the regulation of gene expression. One of the most common mechanisms by which Hog1 and other SAPKs regulate gene expression is by modification of specific transcription factors (Kyriakis and Avruch 807-69); Sko1, Msn2, Msn4 and Hot1 are some of the transcription regulators that have been proposed to be controlled by the Hog1 MAPK. However, other transcription regulators must exist since these regulators show different DNA binding specificities and a profile of gene induction that can not explain the regulation of the genes that modified its expression in a Hog1 dependent manner after an osmotic stress. Moreover a *hot1*, *msn1*, *msn2*, and *msn4* mutant strain is not osmosensitive (Rep et al. 5474-85).

All these data suggest that additional unidentified transcription factors under the control of the MAPK Hog1 were regulating gene expression in osmostress. Our goal was to identify some of these transcription factors.

### Identification of Smp1 as an Osmostress Regulator of the Gene Expression

A genetic screen was performed to look for unknown transcription factors. We searched for clones from a multicopy genomic library able to induce the

---

expression of Hog1 dependent genes in non stress conditions. One of the genes identified encoded for the MEF2-like protein Smp1.

Overexpression of *SMP1* resulted in the increase of the expression of the *STL1* gene. On the other hand, *smp1* cells showed a decreased in expression of *STL1*. The same pattern was observed for the osmoresponsive gene *CWP1*. In fact Smp1 regulation of *CWP1* has been reported in other works (Lamb and Mitchell 677-86). No altered pattern of expression was observed for other genes such *ALD3* or *HXT1*. Therefore, the activity of Smp1 seems to affect the expression to a subset of osmostress genes.

Further analysis of the promoter of these genes showed a conserved sequence similar to the predicted DNA binding site for Smp1 (Dodou and Treisman 1848-59) on *STL1* and *CWP1* but not *HXT1* and *ALD3* genes. These data support the previous finding about the regulation of both genes by this new transcription factor.

### **Smp1 is a Direct Substrate of the Hog1 MAPK**

It is commonly accepted that after an osmotic stress, Hog1 is activated and it is able to phosphorylate other proteins. Sko1 is a clear example of this situation. Under nonstress conditions, Sko1 is repressing some osmostress genes. After an osmotic shock, Hog1 phosphorylates Sko1, and the transcription factor changes its repression activity and becomes an activator (Proft and Struhl 1307-17). To determine if Smp1 showed a similar pattern of activation, we studied whether Smp1 was modified upon an osmotic stress. Indeed, Smp1 was modified after an osmotic upshift. Moreover, the nature of this modification consisted in a phosphorylation since treatment of protein extracts with phosphatases reverted

---

the mobility shift observed by western blot. The obvious next step was to study if Smp1 and Hog1 interacted. For that purpose, two hybrid analysis and *in vivo* coimmunoprecipitation assays were performed. Both tests suggested that Smp1 and Hog1 interact. *In vitro* phosphorylation confirmed Smp1 as a direct substrate of Hog1.

Smp1 phosphorylation sites were mapped by subsequent analysis of fragments of Smp1. At the end, four sites within 28 aminoacids in the C terminus of Smp1 were identified. This location is similar to that found in the Smp1 yeast homolog Rlm1. In this transcription factor its activator domain is located in the C-terminal domain (Dodou and Treisman 1848-59). Moreover, it was proposed that this region could be phosphorylated by the MAPK Mpk1/Stl2 inducing its transcription activity (Dodou and Treisman 1848-59).

In addition, this arrangement resembles the three phosphorylation sites for Sko1 clustered within 19 amino acids (Proft et al. 1123-33). Phosphorylation of several closed aminoacids could modulate a certain conformational change that could alter the protein activity. In other words, phosphorylation of the transcription factor by the kinase would produce a conformational change of the transcription factor altering its transcriptional activity.

More intriguing were the different levels of phosphorylation that were observed among the different Smp1 fragments. A full-length Smp1 was less phosphorylated than the C terminal domain of the protein. A possible explanation is that a region of Smp1 could be limiting the access of the MAPK to the phosphorylation sites.

In accordance with this observation, simultaneous incubation of the C-terminal domain and the N terminal domain of Smp1 resulted in a decreased of C-terminal phosphorylation. It is plausible that other activator mechanisms may act over Smp1 before the MAPK could modify the transcription factor.

### **Smp1 Phosphorylation by the Hog1 MAPK is Important for its Biological Function**

Previous experiments had demonstrated that Smp1 was phosphorylated by Hog1 upon an osmotic stress. The next step was to clarify if this phosphorylation had a biological meaning. With that objective, a Smp1 mutant was designed in which the four phosphorylation sites were mutagenized to Ala, so this mutant allele of Smp1 was unable to be phosphorylated. This Smp1 mutant was analyzed for its transcriptional activity. The altered protein was unable to regulate gene expression suggesting that proper phosphorylation was necessary for optimal function of the transcription factor. Therefore Smp1 function is compromised when Smp1 is not phosphorylated by Hog1. This mechanisms is not exclusive from Smp1, other MEF2 transcription factors show the same mechanism of activation. For example Rlm1. As Smp1, Rlm1 transcriptional activity depends on its phosphorylation by the MAPK Slt2/Mpk1. Moreover its phosphorylation site is very similar to Smp1 phosphorylation site ((Dodou and Treisman 1848-59); (Watanabe et al. 2615-23)).

In mammals, regulation of MEF2A and MEF2C factors has been shown to be under the control of the p38 MAPKs, among other kinases ((Kyriakis and Avruch 807-69); (McKinsey, Zhang, and Olson 40-47)). Phosphorylation of the

---

transcriptional activator domain of these factors by p38 stimulates MEF2 activity, which is analogous to the mechanism proposed for the yeast Smp1.

These data suggest that Smp1 would follow a well established mechanism of activation, similar to that observed for homologous transcription factors in yeast and in mammals.

In addition, phosphorylation regulation is the most common known mechanisms of regulation of the MAPK Hog1. As Smp1, Sko1 activity is modulated by phosphorylation upon an osmotic stress, becoming an activator.

### **Hog1 Controls the Process for Cell Viability in Stationary Phase Through Smp1 Activity**

Localization studies of Smp1-GFP fusion protein showed that no detectable changes were observed by osmotic stress. Smp1 was distributed between the cytoplasm and the nucleus of the cell as in non stress conditions. However, in cells growing on stationary phase, Smp1 was accumulated in the nucleus. This accumulation was not observed in *hog1* mutant cells. This suggested that Smp1 was implicated in osmoadaptation process in addition to some process of the stationary phase. Moreover, this predicted function developed in the stationary growth would be dependent on the Hog1 MAPK, since the presence of the MAPK was necessary for the proper localization of the transcription factor. Further analysis showed a lost of cell viability in *smp1* and *hog1* cells upon entry into stationary phase. Double mutant cells, *smp1 hog1* cells showed no increased in the lost of viability, indicating that Hog1 is required for some specific process for this phase. This process would be mainly under the control of Smp1, since no additional effect is observed in the double mutant. The lost of viability

---

of *hog1* cells is not surprised since a similar phenotype has been observed for the Spc1/Styl MAPK, the Hog1 homolog in *Schizosaccharomyces pombe* ((Shiozaki and Russell 2276-88); (Wilkinson et al. 2289-301)). Although the molecular mechanism(s) of this deficiency it is unclear, it has been proposed that the lost of transcription of some genes compromised cell viability.

All these data suggest that Smp1 is a direct target of the Hog1 MAPK. Hog1 activation results in Smp1 phosphorylation, essential for gene expression regulation of a subset of genes. Apart from the role in the osmoadaptation process, Smp1 would be implicated in some adaptation process of the stationary phase. In this situation phosphorylation from Hog1 is not relevant, but its presence determinates proper Smp1 localization in the nucleus.

### **Summary and Perspective**

This work describes a new transcription factor under the control of the Hog1 MAPK. Although its relationship with the MAPK is well established (direct substrate of the MAPK, well mapped phosphorylation sites and a possible biological function described) its transcription function needs to be clarify. The subset of genes whose transcription is affected must be identified (in this report we propose two genes but it is easy to predict that other genes under the control of Smp1 may exist). Microarrays could be a powerful technique to determine the cluster of genes affected.

At the same time, it seems quite clear that, although Smp1 helps to explain another portion of the Hog1 gene regulation, other transcriptional regulators

---

must exist. May be a new genetic screening with different reporter genes or a different strategy would be necessary to find this regulators.

## Expression of the *HXT1* Low Affinity Glucose Transporter Requires the Coordinated Activities of the HOG and Glucose Signalling Pathways

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 279, No. 21, Issue of May 21, pp. 22010–22019, 2004

### Summary

Increases in extracellular osmolarity results in a transient induction of the expression of stress protective genes. Among them there are genes encoding proteins involved in carbohydrate metabolism, these include genes responsible for the synthesis of glycerol : such as *GPD1* and *GPD2*, but also genes encoding plasma membrane sugar transporters, such as *STL1*, *HXT10*, *HXT1*, and *HXT5* (Posas et al. 17249-55). The induction of these transporters is Hog1 dependent.

Hxts are hexose transporters. *Saccharomyces cerevisiae* has 20 *HXTs* genes ((Bisson et al. 259-308); (Boles and Hollenberg 85-111); (Kayano et al. 15245-48),(Kruckeberg 283-92)). These Hxts proteins belong to the major facilitator superfamily (MFS) of transporters ((Marger and Saier, Jr. 13-20); (Pao, Paulsen, and Saier, Jr. 1-34)) and transport their substrates by passive, energy-independent facilitated diffusion, with glucose moving down a concentration gradient (Bisson et al. 259-308).

The way by *HXT* genes are transcriptionally regulated in response to glucose correlate with the affinity of the specific transporter for the hexose. For instance *HXT1* transcription is induced only by high concentrations of glucose (it encodes a low-affinity transporter).

Briefly, glucose induction of *HXT1* (and from *HXT1* to *HXT4*) gene is due to a repression mechanism mediated by the Rgt1 repressor. Rgt1 inhibits expression



of the *HXT* genes in the absence of glucose. Glucose concentrations are sensed by Snf3 and Rgt2 (Ozcan, Dover, and Johnston 2566-73), two glucose transporter-like proteins that serve as glucose sensors for low and high concentrations of glucose, respectively. Through an undetermined mechanism, the signal arrives to the Grr1 protein. Grr1 is required for glucose inhibition of Rgt1 function, and the Std1 and Mth1 proteins are implicated in this inhibition ((Tomas-Cobos et al. 22010-19); (Flick et al. 3230-41); (Ozcan et al. 5520-28)).

Moreover, maximal *HXT1* expression requires a high glucose-induced mechanism whose components were not known at the beginning of this work (OZCAN, JOHNSTON). Coupling of this regulatory pathway with the Rgt1-mediated mechanism causes *HXT1* to be expressed only in cells growing on high levels of glucose (Ozcan and Johnston 554-69). From the microarrays data arised the hypothesis that may be the HOG pathway could be implicated in the induction of *HXT1*.

### **The HOG Pathway Regulates *HXT1* Gene Expression on Glucose and Osmostress**

Initial experiments tried to reproduce these microarray data. Certainly, *HXT1* gene expression was regulated by glucose availability, being inhibited in the absence of glucose and activated in the presence of the sugar (Ozcan and Johnston 554-69). Cells growing on raffinose showed very low levels of *HXT1* expression. After a pulse of glucose, expression of *HXT1* was induced. *HXT1* induction rate was higher when cells were subjected simultaneously to glucose plus NaCl. Similar results were obtained when 1 M sorbitol was added. Then full induction of *HXT1* expression could be increased after the addition of glucose

---

plus salt, showing that there is another pathway taking part in its expression. As the NaCl and sorbitol are the responsible of this increase, it seemed reasonable to think that the pathway acting over *HXT1* expression was the HOG signalling pathway. Moreover, it suggested that there was another molecular mechanism (under the control of the HOG pathway) regulating *HXT1* expression apart from those belonging to the glucose signalling pathway.

The activity of the HOG pathway increase less *HXT1* expression than glucose induction. However, the presence of the pathway was absolutely necessarily since no induction of *HXT1* was observed in any of the induction conditions in *hog1* mutant cells. It could be concluded that the HOG pathway it is essential for *HXT1*, not only in osmosstress conditions, but also for the induction caused by glucose.

Correspondingly, Hog1 phosphorylation was observed after the addition of 2% of glucose or 2% of galactose to cells growing on raffinose to the same extend as treatment with 110 mM NaCl. This phosphorylation was not observed in *pbs2* cells. Thus, Hog1 activation was caused by an increase in extracellular osmolarity caused by the addition of sugar, not necessarily restricted to glucose. Moreover, increased concentration of glucose concentration, resulted in an increased in Hog1 phosphorylation. Therefore, the severity of the osmotic stress (in this case caused by the glucose) determined the level of Hog1 phosphorylation.

Two upstream sensing mechanisms activate the core of the HOG pathway, the Sn1 branch and the Sho1 branch (36). Mutations in the Sho1 branch (double

---

*sho1ste11* mutant) did not alter *HXT1* expression. However, mutants in the Sln1 branch of the HOG pathway, for instance *ssk2 ssk22*, showed a clear defect in the induction of *HXT1* by glucose.

It has been reported that the Sln1 sensor is able to sense small changes in the environment and induce progressive Hog1 activation, whereas the Sho1 sensing system functions more in an on-off fashion only once a threshold level of osmotic stress in the environment is reached (22). This suggests that small changes in extracellular sugar concentration, which result in small changes in extracellular osmolarity, are sufficient to induce Sln1-mediated Hog1 activation whereas these changes are not high enough to induce the Sho1-sensing system.

This different sensitivity of the two signalling systems might have a significant physiological role, because if under specific conditions only a partial activation of Hog1 MAPK is required, a fine tuning mechanism would avoid full induction of adaptive responses that might be too energy-consuming for the cell.

### **Induction of *HXT1* Expression upon Glucose plus Osmotic Stress Depends on the Integrity of the Glucose Signalling Pathway**

The HOG pathway activity was not sufficient for gene expression of *HXT1* without the activation of the glucose signalling pathway, and it seems reasonable, since expression of a glucose transporter would not be necessary in the absence of the hexose.

As it has been described, activation of the glucose pathway by the presence of glucose leads to regulation of the Rgt1 transcriptional repressor. However, regulation of Rgt1 was not sufficient to induce gene expression by glucose

---

without simultaneous activation of the HOG pathway. Looking carefully at *HXT1* expression on different mutants from the glucose pathway, it could be observed that glucose repression activity impeded Hog1 effects on *HXT1* expression. Therefore, the glucose signalling pathway must release Rgt1 from the promoter to observe the HOG pathway effects.

Therefore, two independent pathways act together for the appropriate expression of the hexose transporter. Moreover, both pathways must be functional since the absence of signalling from one pathway blocks the expression of *HXT1*.

The glucose signalling elements acting over *HXT1* were well described, but the clue element implicated in *HXT1* expression and under the control of Hog1 remained unknown. A genetic screening and a promoter analysis identified Sko1 as the element we were looking for, and Tup1 and Ssn6 complex repressor as the general repressor machinery implicated in the repression of *HXT1*.

### **Sko1 Transcription Factor Regulates *HXT1* expression**

To identify this new element, we designed a genetic screening. We assumed that the inactivation of the Rgt1 repressor and the unknown element would let to the constitutive activation of the *HXT1* gene. We subjected *rgt1* cells with the *HXT1::LacZ* reporter gene integrated, to MNNG treatment and we plated these cells on X-gal plate. Blue cells (those that had inactivation of both repressor elements) were transformed with a monocopy genomic library and those clones

---

that were able to recover the phenotype were sequenced and identify as *Sko1*, *Tup1* and *Ssn6*.

*Sko1* is a transcriptional repressor regulated by *Hog1* (Proft et al. 1123-33). After an osmotic stress, *Sko1* is phosphorylated by *Hog1* and converts the repressor into an activator ((Proft and Struhl 1307-17)).

Deletion of *SKO1* resulted in cells able to induce *HXT1* expression in response to glucose, but no further induction was observed after the addition of NaCl. Moreover, a double *sko1 hog1* mutant strain showed the same pattern of expression as the *sko1* strain, indicating that *hog1* cells were unable to release *Sko1* from the promoter, blocking *HXT1* expression.

By chromatin immunoprecipitation (ChIP) assays *Sko1* was detected at the *HXT1* promoter. Addition of NaCl resulted in decreased *Sko1* signal, indicating that there was a decrease in *Sko1* binding to the promoter. This reduction was accompanied by the improvement of the *Hog1* MAPK binding, as it has been described for other osmostress inducible genes ((Proft et al. 1123-33); (Proft and Struhl 1307-17)).

*Rgt1* also binds to *HXT1* promoter in low glucose conditions, but addition of NaCl did not affect its binding. Because *Rgt1* binding is only decreased by glucose ((Mosley et al. 10322-27); (Flick et al. 3230-41); (Kim et al. 111-19)), we suggest that the addition of glucose to raffinose growing cells would have a dual effect. First, released of *Rgt1* from the promoter and, second in which glucose would be acting as an osmolite, activation the HOG pathway, releasing *Sko1* from the promoter, allowing *HXT1* desrepression.

---

### **Analysis of the *HXT1* promoter**

Once Sko1 and Rgt1 were defined as the transcription factors that were regulating the *HXT1* promoter, we performed an analysis of *HXT1* promoter in order to find Sko1 and Rgt1 interacting sites. This analysis showed that Rgt1 and Sko1 interacted with different regions of the *HXT1* promoter.

Rgt1 interacts, at least, with a promoter region located between 521 and 426. This region is included in the fragment that showed direct interaction of Rgt1 with *HXT1* promoter by either DNA binding (Ozcan et al. 12428-32) or ChIP (Mosley et al. 10322-27) analyses. This region contains the consensus sequence identified to be necessary for Rgt1 binding (a spaced CGG pair sequence) (Kim et al. 111-19). However, additional sites for Rgt1 binding must exist, because a promoter fragment containing only from 521 to ATG was not able to be properly induced by glucose.

Sko1 binding region was mapped between -426 and -223. However, we did not find any consensus Sko1-CRE site (TGACGTCA) in this region. Because the HAL1 promoter contains a degenerated CRE site (TTACGTAA) that binds Sko1 functionally (19), we looked for similar degenerated sequence. Only one similar sequence was found. However, when the effect of a mutagenized sequence was analyzed, only a slight increase in the induction of HXT1 was observed.

These results indicated that either this site was not fully functional or that there were additional cAMP-response element like sites in the sequence where Sko1 was able to bind. This region of Sko1 interaction was confirmed by ChIPs analysis.

---

## **Tup1 and Ssn6**

Two other proteins were identified in the genetic screening as regulators of *HXT1* expression: Tup1 and Ssn6.

The Tup1-Ssn6 corepressor forms one of the largest and most important gene-regulatory circuits in budding yeast. The Tup-Ssn6 complex is attract by a DNA binding protein (a transcription factor) thereby localizing it to the regulatory regions of specific genes, where it brings transcriptional repression (Rebecca L. Smith and Alexander D. Johnson 2000).

The relationship between Sko1 and the Tup1-Ssn6 complex it was described some time ago. Tup1-Ssn6 general co-repressor complex interacts with Sko1 to repress transcription of osmostress-regulated genes ((Marquez et al. 2543-53); (Proft and Serrano 537-46)). Sko1 is phosphorylated by the Hog1 MAPK upon stress, and this is necessary to switch Sko1-Tup1-Ssn6 from a repressor to an activator complex ((Proft et al. 1123-33); (Proft and Struhl 1307-17)) inducing transcription.

In addition, it is also known that the Tup1-Ssn6 complex interacts with Rgt1 to repress transcription of *HXT1* in low glucose conditions ((Tomas-Cobos and Sanz 657-63); (Ozcan et al. 12428-32)).

These relationships would explain why mutations in *TUP1* or *SSN6* resulted in constitutive expression of *HXT1* that was not significantly enhanced by addition of glucose or NaCl. Therefore two transcriptional repressors, Sko1 and Rgt1, are controlling *HXT1* gene expression by their binding to the Tup1-Ssn6 complex.

---

## **Model**

With all these evidence, we propose the following model for *HXT1* gene regulation. Under normal conditions (low glucose and no osmostress), *HXT1* promoter would be repressed by two independent repressors, Rgt1 and Sko1. Glucose addition would let to the activation of the glucose pathway and the HOG pathway, removing Rgt1 and Sko1 from the promoter with the subsequent *HXT1* gene expression.

The hexose would transmit two types of signals. From one side, it would activate the glucose signalling pathway, mediating Rgt1 repression regulation; from the other side, glucose addition would be sensed as an osmostress, resulting in Hog1 MAPK activation. As a consequence, the Sko1 repressor would be phosphorylated by the MAPK converting it in to an activator. The addition of glucose plus NaCl would increase the osmostress signal, increasing the magnitude of the signal, and subsequently gene expression.

## **Perspective**

The HOG pathway regulates the expression of a great deal of genes with a very different nature. Most of these genes are expressed in other situations (as nutrient induction) or stresses (as oxidative stress), but at the same time they seem necessary for osmotic surviving or adaptation. Then, a multiple regulation mechanism integrating both inputs is necessary. The *HXT1* is a good example of this situation. The hexose transporter is need in the presence of high concentrations of glucose and a molecular mechanism, more or less understood, regulates the appropriate expression. At the same time, the



---

glucose *per se* is an osmosstress, and a molecular mechanism under the control of Hog1 regulates either the appropriate expression of the transporter.

Glucose and HOG signalling pathways act coordinately for *HXT1* expression, establishing a rank of priorities: glucose specificity imposes its action over the effects of the HOG signalling pathway. Since a biological point of view, the increase of *HXT1* expression by hyper osmotic conditions could provide more substrate (glucose) for the synthesis of the osmoprotectant glycerol (Hohmann 300-72). This scheme could be reproducible for other genes with a very similar pattern of expression.

In addition, this would be against the individual activity of the signalling pathways of the yeast and in agreement with a more interconnected signalling view where a stimulus would be sense in different manners activating different signalling pathways leading to the appropriate adaptability of the organism.

On the other hand, *HXTs* have been studied for years. They serve as a model to understand how the glucose transporters in mammals could be regulated and expressed. Recently many papers have been trying to elucidate the different mechanisms that regulate *HXT1* expression. Other hexoses transporters, as *HXT2* are well known, but for *HXT1* although many regulators have been proposed, the specific activity of them it is still obscure. For instance, it has been desmotrated that Hxk2 is implicated in *HXT1* induction, and that Snf1 plays a role in the *HXT1* repression (Tomas-Cobos and Sanz 657-63), but how these proteins are acting it is not known.

---

At the same time, it was quite established (Ozcan and Johnston 554-69) the existence of an activator mechanisms whose nature was unknown. In this report we show that the HOG pathway through its repressor transcription factor Sko1 is implicated in the induction of the hexose transporter. And that this induction is essential for *HXT1* expression.

## **Bibliography**

## Reference List

Akada, R., J. Yamamoto, and I. Yamashita. "Screening and identification of yeast sequences that cause growth inhibition when overexpressed." Mol.Gen.Genet. 254.3 (1997): 267-74.

Albertyn, J., S. Hohmann, and B. A. Prior. "Characterization of the osmotic-stress response in *Saccharomyces cerevisiae*: osmotic stress and glucose repression regulate glycerol-3-phosphate dehydrogenase independently." Curr.Genet. 25.1 (1994): 12-18.

Alepuz, P. M., K. W. Cunningham, and F. Estruch. "Glucose repression affects ion homeostasis in yeast through the regulation of the stress-activated ENA1 gene." Mol.Microbiol. 26.1 (1997): 91-98.

Alepuz, P. M. et al. "Osmostress-induced transcription by Hot1 depends on a Hog1-mediated recruitment of the RNA Pol II." EMBO J 22.10 (2003): 2433-42.

Alepuz, P. M. et al. "Osmostress-induced transcription by Hot1 depends on a Hog1-mediated recruitment of the RNA Pol II." EMBO J 22.10 (2003): 2433-42.

Alepuz, P. M. et al. "Stress-induced map kinase Hog1 is part of transcription activation complexes." Mol.Cell 7.4 (2001): 767-77.

- 
- Alonso-Monge, R. et al. "Hyperosmotic stress response and regulation of cell wall integrity in *Saccharomyces cerevisiae* share common functional aspects." Mol.Microbiol. 41.3 (2001): 717-30.
- Amoros, M. and F. Estruch. "Hsf1p and Msn2/4p cooperate in the expression of *Saccharomyces cerevisiae* genes HSP26 and HSP104 in a gene- and stress type-dependent manner." Mol.Microbiol. 39.6 (2001): 1523-32.
- Andrews, P. D. and M. J. Stark. "Dynamic, Rho1p-dependent localization of Pkc1p to sites of polarized growth." J.Cell Sci. 113 ( Pt 15) (2000): 2685-93.
- Ansell, R. et al. "The two isoenzymes for yeast NAD<sup>+</sup>-dependent glycerol 3-phosphate dehydrogenase encoded by GPD1 and GPD2 have distinct roles in osmoadaptation and redox regulation." EMBO J 16.9 (1997): 2179-87.
- Antonsson, B. et al. "Protein kinase C in yeast. Characteristics of the *Saccharomyces cerevisiae* PKC1 gene product." J.Biol.Chem. 269.24 (1994): 16821-28.
- Aoyama, K. et al. "Spy1, a histidine-containing phosphotransfer signaling protein, regulates the fission yeast cell cycle through the Mcs4 response regulator." J.Bacteriol. 182.17 (2000): 4868-74.
- Aramburu, J., A. Rao, and C. B. Klee. "Calcineurin: from structure to function." Curr.Top.Cell Regul. 36 (2000): 237-95.

- 
- Aravind, L. and C. P. Ponting. "The GAF domain: an evolutionary link between diverse phototransducing proteins." Trends Biochem.Sci. 22.12 (1997): 458-59.
- Ayscough, K. R. and D. G. Drubin. "A role for the yeast actin cytoskeleton in pheromone receptor clustering and signalling." Curr.Biol. 8.16 (1998): 927-30.
- Baker, H. V. "Glycolytic gene expression in *Saccharomyces cerevisiae*: nucleotide sequence of GCR1, null mutants, and evidence for expression." Mol.Cell Biol. 6.11 (1986): 3774-84.
- Banuett, F. "Signalling in the yeasts: an informational cascade with links to the filamentous fungi." Microbiol.Mol.Biol.Rev. 62.2 (1998): 249-74.
- Barbet, N. C. et al. "TOR controls translation initiation and early G1 progression in yeast." Mol.Biol.Cell 7.1 (1996): 25-42.
- Batiza, A. F., T. Schulz, and P. H. Masson. "Yeast respond to hypotonic shock with a calcium pulse." J.Biol.Chem. 271.38 (1996): 23357-62.
- Bilsland-Marchesan, E. et al. "Rck2 kinase is a substrate for the osmotic stress-activated mitogen-activated protein kinase Hog1." Mol.Cell Biol. 20.11 (2000): 3887-95.
- Bisson, L. F. et al. "Yeast sugar transporters." Crit Rev.Biochem.Mol.Biol. 28.4 (1993): 259-308.

- 
- Bjorkqvist, S. et al. "Physiological response to anaerobicity of glycerol-3-phosphate dehydrogenase mutants of *Saccharomyces cerevisiae*." Appl.Environ.Microbiol. 63.1 (1997): 128-32.
- Blomberg, A. and L. Adler. "Physiology of osmotolerance in fungi." Adv.Microb.Physiol 33 (1992): 145-212.
- Boles, E. and C. P. Hollenberg. "The molecular genetics of hexose transport in yeasts." FEMS Microbiol.Rev. 21.1 (1997): 85-111.
- Brewster, J. L. et al. "An osmosensing signal transduction pathway in yeast." Science 259.5102 (1993): 1760-63.
- Brown, A. D. "Microbial water stress." Bacteriol.Rev. 40.4 (1976): 803-46.
- Brown, A. D. "Compatible solutes and extreme water stress in eukaryotic micro-organisms." Adv.Microb.Physiol 17 (1978): 181-242.
- Bulavin, D. V. et al. "Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation." EMBO J 18.23 (1999): 6845-54.
- Causton, H. C. et al. "Remodeling of yeast genome expression in response to environmental changes." Mol.Biol.Cell 12.2 (2001): 323-37.
- Chang, L. and M. Karin. "Mammalian MAP kinase signalling cascades." Nature 410.6824 (2001): 37-40.

- 
- De Cesare, D. and P. Sassone-Corsi. "Transcriptional regulation by cyclic AMP-responsive factors." Prog.Nucleic Acid Res.Mol.Biol. 64 (2000): 343-69.
- de Nadal, E., P. M. Alepuz, and F. Posas. "Dealing with osmostress through MAP kinase activation." EMBO Rep 3.8 (2002): 735-40.
- de Nadal, E., L. Casadome, and F. Posas. "Targeting the MEF2-like transcription factor Smp1 by the stress-activated Hog1 mitogen-activated protein kinase." Mol.Cell Biol. 23.1 (2003): 229-37.
- Dodou, E. and R. Treisman. "The *Saccharomyces cerevisiae* MADS-box transcription factor Rlm1 is a target for the Mpk1 mitogen-activated protein kinase pathway." Mol.Cell Biol. 17.4 (1997): 1848-59.
- Dove, S. K. et al. "Osmotic stress activates phosphatidylinositol-3,5-bisphosphate synthesis." Nature 390.6656 (1997): 187-92.
- Escote, X. et al. "Hog1 mediates cell-cycle arrest in G1 phase by the dual targeting of Sic1." Nat.Cell Biol. 6.10 (2004): 997-1002.
- Ferrigno, P. et al. "Regulated nucleo/cytoplasmic exchange of HOG1 MAPK requires the importin beta homologs NMD5 and XPO1." EMBO J 17.19 (1998): 5606-14.
- Flick, K. M. et al. "Grr1-dependent inactivation of Mth1 mediates glucose-induced dissociation of Rgt1 from HXT gene promoters." Mol.Biol.Cell 14.8 (2003): 3230-41.



- 
- Gasch, A. P. et al. "Genomic expression programs in the response of yeast cells to environmental changes." Mol.Biol.Cell 11.12 (2000): 4241-57.
- Gustin, M. C. et al. "MAP kinase pathways in the yeast *Saccharomyces cerevisiae*." Microbiol.Mol.Biol.Rev 62.4 (1998): 1264-300.
- Gustin, M. C. et al. "MAP kinase pathways in the yeast *Saccharomyces cerevisiae*." Microbiol.Mol.Biol.Rev. 62.4 (1998): 1264-300.
- Harris, K. et al. "Role of scaffolds in MAP kinase pathway specificity revealed by custom design of pathway-dedicated signaling proteins." Curr.Biol. 11.23 (2001): 1815-24.
- Hohmann, S. "Osmotic stress signaling and osmoadaptation in yeasts." Microbiol.Mol.Biol.Rev 66.2 (2002): 300-72.
- Jacoby, T. et al. "Two protein-tyrosine phosphatases inactivate the osmotic stress response pathway in yeast by targeting the mitogen-activated protein kinase, Hog1." J Biol.Chem. 272.28 (1997): 17749-55.
- Janiak-Spens, F., D. P. Sparling, and A. H. West. "Novel role for an HPt domain in stabilizing the phosphorylated state of a response regulator domain." J.Bacteriol. 182.23 (2000): 6673-78.
- Janiak-Spens, F. et al. "Differential stabilities of phosphorylated response regulator domains reflect functional roles of the yeast osmoregulatory SLN1 and SSK1 proteins." J.Bacteriol. 181.2 (1999): 411-17.

- 
- Janiak-Spens, F. and A. H. West. "Functional roles of conserved amino acid residues surrounding the phosphorylatable histidine of the yeast phosphorelay protein YPD1." Mol.Microbiol. 37.1 (2000): 136-44.
- Kayano, T. et al. "Evidence for a family of human glucose transporter-like proteins. Sequence and gene localization of a protein expressed in fetal skeletal muscle and other tissues." J.Biol.Chem. 263.30 (1988): 15245-48.
- Keyse, S. M. "Protein phosphatases and the regulation of mitogen-activated protein kinase signalling." Curr.Opin.Cell Biol. 12.2 (2000): 186-92.
- Kim, C. et al. "Functional impact of lymphangiogenesis on fluid transport after lymph node excision." Lymphology 36.3 (2003): 111-19.
- Kruckeberg, A. L. "The hexose transporter family of *Saccharomyces cerevisiae*." Arch.Microbiol. 166.5 (1996): 283-92.
- Kultz, D. and M. Burg. "Evolution of osmotic stress signaling via MAP kinase cascades." J.Exp.Biol. 201 ( Pt 22) (1998): 3015-21.
- Kyriakis, J. M. and J. Avruch. "Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation." Physiol Rev 81.2 (2001): 807-69.
- Lamb, T. M. and A. P. Mitchell. "The transcription factor Rim101p governs ion tolerance and cell differentiation by direct repression of the

- 
- regulatory genes NRG1 and SMP1 in *Saccharomyces cerevisiae*." Mol.Cell Biol. 23.2 (2003): 677-86.
- Ligterink, W. and H. Hirt. "Mitogen-activated protein (MAP) kinase pathways in plants: versatile signaling tools." Int.Rev.Cytol. 201 (2001): 209-75.
- Maeda, T., M. Takekawa, and H. Saito. "Activation of yeast PBS2 MAPKK by MAPKKs or by binding of an SH3-containing osmosensor." Science 269.5223 (1995): 554-58.
- Maeda, T., S. M. Wurgler-Murphy, and H. Saito. "A two-component system that regulates an osmosensing MAP kinase cascade in yeast." Nature 369.6477 (1994): 242-45.
- Mager, W. H. and J. C. Varela. "Osmostress response of the yeast *Saccharomyces*." Mol.Microbiol. 10.2 (1993): 253-58.
- Marchler, G. et al. "A *Saccharomyces cerevisiae* UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions." EMBO J. 12.5 (1993): 1997-2003.
- Marger, M. D. and M. H. Saier, Jr. "A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport." Trends Biochem.Sci. 18.1 (1993): 13-20.
- Marquez, J. A. et al. "The Ssn6-Tup1 repressor complex of *Saccharomyces cerevisiae* is involved in the osmotic induction of HOG-dependent and -independent genes." EMBO J 17.9 (1998): 2543-53.

---

Mattison, C. P. and I. M. Ota. "Two protein tyrosine phosphatases, Ptp2 and Ptp3, modulate the subcellular localization of the Hog1 MAP kinase in yeast." Genes Dev. 14.10 (2000): 1229-35.

Mattison, C. P. et al. "Differential regulation of the cell wall integrity mitogen-activated protein kinase pathway in budding yeast by the protein tyrosine phosphatases Ptp2 and Ptp3." Mol.Cell Biol. 19.11 (1999): 7651-60.

McKinsey, T. A., C. L. Zhang, and E. N. Olson. "MEF2: a calcium-dependent regulator of cell division, differentiation and death." Trends Biochem.Sci. 27.1 (2002): 40-47.

Mosley, A. L. et al. "Glucose-mediated phosphorylation converts the transcription factor Rgt1 from a repressor to an activator." J.Biol.Chem. 278.12 (2003): 10322-27.

Nehlin, J. O., M. Carlberg, and H. Ronne. "Yeast SKO1 gene encodes a bZIP protein that binds to the CRE motif and acts as a repressor of transcription." Nucleic Acids Res. 20.20 (1992): 5271-78.

Norbeck, J. and A. Blomberg. "The level of cAMP-dependent protein kinase A activity strongly affects osmotolerance and osmo-instigated gene expression changes in *Saccharomyces cerevisiae*." Yeast 16.2 (2000): 121-37.

- 
- O'Rourke, S. M. and I. Herskowitz. "The Hog1 MAPK prevents cross talk between the HOG and pheromone response MAPK pathways in *Saccharomyces cerevisiae*." Genes Dev. 12.18 (1998): 2874-86.
- O'Rourke, S. M. and I. Herskowitz. "A third osmosensing branch in *Saccharomyces cerevisiae* requires the Msb2 protein and functions in parallel with the Sho1 branch." Mol.Cell Biol. 22.13 (2002): 4739-49.
- O'Rourke, S. M. and I. Herskowitz. "Unique and redundant roles for HOG MAPK pathway components as revealed by whole-genome expression analysis." Mol.Biol.Cell 15.2 (2004): 532-42.
- Oki, M. et al. "Nuclear protein import, but not mRNA export, is defective in all *Saccharomyces cerevisiae* mutants that produce temperature-sensitive forms of the Ran GTPase homologue Gsp1p." Mol.Gen.Genet. 257.6 (1998): 624-34.
- Ota, I. M. and A. Varshavsky. "A gene encoding a putative tyrosine phosphatase suppresses lethality of an N-end rule-dependent mutant." Proc.Natl.Acad.Sci.U.S.A 89.6 (1992): 2355-59.
- Ota, I. M. and A. Varshavsky. "A yeast protein similar to bacterial two-component regulators." Science 262.5133 (1993): 566-69.
- Ozcan, S., J. Dover, and M. Johnston. "Glucose sensing and signaling by two glucose receptors in the yeast *Saccharomyces cerevisiae*." EMBO J. 17.9 (1998): 2566-73.

---

Ozcan, S. et al. "Two glucose transporters in *Saccharomyces cerevisiae* are glucose sensors that generate a signal for induction of gene expression." Proc.Natl.Acad.Sci.U.S.A 93.22 (1996): 12428-32.

Ozcan, S. et al. "Glucose uptake and catabolite repression in dominant HTR1 mutants of *Saccharomyces cerevisiae*." J.Bacteriol. 175.17 (1993): 5520-28.

Ozcan, S. and M. Johnston. "Function and regulation of yeast hexose transporters." Microbiol.Mol.Biol.Rev. 63.3 (1999): 554-69.

Pao, S. S., I. T. Paulsen, and M. H. Saier, Jr. "Major facilitator superfamily." Microbiol.Mol.Biol.Rev. 62.1 (1998): 1-34.

Pascual-Ahuir, A. et al. "Multiple levels of control regulate the yeast cAMP-response element-binding protein repressor Sko1p in response to stress." J.Biol.Chem. 276.40 (2001): 37373-78.

Posas, F. et al. "The transcriptional response of yeast to saline stress." J Biol.Chem. 275.23 (2000): 17249-55.

Posas, F. and H. Saito. "Osmotic activation of the HOG MAPK pathway via Ste11p MAPKKK: scaffold role of Pbs2p MAPKK." Science 276.5319 (1997): 1702-05.

Posas, F. and H. Saito. "Activation of the yeast SSK2 MAP kinase kinase kinase by the SSK1 two-component response regulator." EMBO J 17.5 (1998): 1385-94.

- 
- Posas, F., M. Takekawa, and H. Saito. "Signal transduction by MAP kinase cascades in budding yeast." Curr.Opin.Microbiol. 1.2 (1998): 175-82.
- Posas, F., E. A. Witten, and H. Saito. "Requirement of STE50 for osmostress-induced activation of the STE11 mitogen-activated protein kinase kinase in the high-osmolarity glycerol response pathway." Mol.Cell Biol. 18.10 (1998): 5788-96.
- Posas, F. et al. "Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 "two-component" osmosensor." Cell 86.6 (1996): 865-75.
- Proft, M. et al. "Regulation of the Sko1 transcriptional repressor by the Hog1 MAP kinase in response to osmotic stress." EMBO J 20.5 (2001): 1123-33.
- Proft, M. and R. Serrano. "Repressors and upstream repressing sequences of the stress-regulated ENA1 gene in *Saccharomyces cerevisiae*: bZIP protein Sko1p confers HOG-dependent osmotic regulation." Mol.Cell Biol. 19.1 (1999): 537-46.
- Proft, M. and K. Struhl. "Hog1 kinase converts the Sko1-Cyc8-Tup1 repressor complex into an activator that recruits SAGA and SWI/SNF in response to osmotic stress." Mol.Cell 9.6 (2002): 1307-17.
- Raitt, D. C., F. Posas, and H. Saito. "Yeast Cdc42 GTPase and Ste20 PAK-like kinase regulate Sho1-dependent activation of the Hog1 MAPK pathway." EMBO J 19.17 (2000): 4623-31.

- Reiser, V., H. Ruis, and G. Ammerer. "Kinase activity-dependent nuclear export opposes stress-induced nuclear accumulation and retention of Hog1 mitogen-activated protein kinase in the budding yeast *Saccharomyces cerevisiae*." Mol.Biol.Cell 10.4 (1999): 1147-61.
- Reiser, V., S. M. Salah, and G. Ammerer. "Polarized localization of yeast Pbs2 depends on osmostress, the membrane protein Sho1 and Cdc42." Nat.Cell Biol. 2.9 (2000): 620-27.
- Rep, M. et al. "Different signalling pathways contribute to the control of GPD1 gene expression by osmotic stress in *Saccharomyces cerevisiae*." Microbiology 145 ( Pt 3) (1999): 715-27.
- Rep, M. et al. "The transcriptional response of *Saccharomyces cerevisiae* to osmotic shock. Hot1p and Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway-dependent genes." J Biol.Chem. 275.12 (2000): 8290-300.
- Rep, M. et al. "Osmotic stress-induced gene expression in *Saccharomyces cerevisiae* requires Msn1p and the novel nuclear factor Hot1p." Mol.Cell Biol. 19.8 (1999): 5474-85.
- Rouse, J. et al. "A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins." Cell 78.6 (1994): 1027-37.
- Ruis, H. and C. Schuller. "Stress signaling in yeast." Bioessays 17.11 (1995): 959-65.



- 
- Saito, H. "Histidine phosphorylation and two-component signaling in eukaryotic cells." Chem.Rev. 101.8 (2001): 2497-509.
- Schuller, C. et al. "The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the *Saccharomyces cerevisiae* CTT1 gene." EMBO J 13.18 (1994): 4382-89.
- Seet, B. T. and T. Pawson. "MAPK signaling: Sho business." Curr.Biol. 14.17 (2004): R708-R710.
- Shiozaki, K. and P. Russell. "Conjugation, meiosis, and the osmotic stress response are regulated by Spc1 kinase through Atf1 transcription factor in fission yeast." Genes Dev. 10.18 (1996): 2276-88.
- Smits, G. J. et al. "Cell wall dynamics in yeast." Curr.Opin.Microbiol. 2.4 (1999): 348-52.
- Stock, A. M., V. L. Robinson, and P. N. Goudreau. "Two-component signal transduction." Annu.Rev.Biochem. 69 (2000): 183-215.
- Tamas, M. J. et al. "Stimulation of the yeast high osmolarity glycerol (HOG) pathway: evidence for a signal generated by a change in turgor rather than by water stress." FEBS Lett. 472.1 (2000): 159-65.
- Teige, M. et al. "Rck2, a member of the calmodulin-protein kinase family, links protein synthesis to high osmolarity MAP kinase signaling in budding yeast." Proc.Natl.Acad.Sci.U.S.A 98.10 (2001): 5625-30.

- 
- Tomas-Cobos, L. et al. "Expression of the HXT1 low affinity glucose transporter requires the coordinated activities of the HOG and glucose signalling pathways." J.Biol.Chem. 279.21 (2004): 22010-19.
- Tomas-Cobos, L. and P. Sanz. "Active Snf1 protein kinase inhibits expression of the *Saccharomyces cerevisiae* HXT1 glucose transporter gene." Biochem.J. 368.Pt 2 (2002): 657-63.
- Van Wuytswinkel, O. et al. "Response of *Saccharomyces cerevisiae* to severe osmotic stress: evidence for a novel activation mechanism of the HOG MAP kinase pathway." Mol.Microbiol. 37.2 (2000): 382-97.
- Vandenbol, M., J. C. Jauniaux, and M. Grenson. "Nucleotide sequence of the *Saccharomyces cerevisiae* PUT4 proline-permease-encoding gene: similarities between CAN1, HIP1 and PUT4 permeases." Gene 83.1 (1989): 153-59.
- Vincent, A. C. and K. Struhl. "ACR1, a yeast ATF/CREB repressor." Mol.Cell Biol. 12.12 (1992): 5394-405.
- Warmka, J. et al. "Ptc1, a type 2C Ser/Thr phosphatase, inactivates the HOG pathway by dephosphorylating the mitogen-activated protein kinase Hog1." Mol.Cell Biol. 21.1 (2001): 51-60.
- Watanabe, Y. et al. "Characterization of a serum response factor-like protein in *Saccharomyces cerevisiae*, Rlm1, which has transcriptional activity regulated by the Mpk1 (Slt2) mitogen-activated protein kinase pathway." Mol.Cell Biol. 17.5 (1997): 2615-23.

- 
- Wilkinson, M. G. et al. "The Atf1 transcription factor is a target for the Sty1 stress-activated MAP kinase pathway in fission yeast." Genes Dev. 10.18 (1996): 2289-301.
- Wood, J. M. "Osmosensing by bacteria: signals and membrane-based sensors." Microbiol.Mol.Biol.Rev. 63.1 (1999): 230-62.
- Wurgler-Murphy, S. M. et al. "Regulation of the *Saccharomyces cerevisiae* HOG1 mitogen-activated protein kinase by the PTP2 and PTP3 protein tyrosine phosphatases." Mol.Cell Biol. 17.3 (1997): 1289-97.
- Yale, J. and H. J. Bohnert. "Transcript expression in *Saccharomyces cerevisiae* at high salinity." J Biol.Chem. 276.19 (2001): 15996-6007.
- Yancey, P. H. et al. "Living with water stress: evolution of osmolyte systems." Science 217.4566 (1982): 1214-22.
- Zarrinpar, A. et al. "Sho1 and Pbs2 Act as Coscaffolds Linking Components in the Yeast High Osmolarity MAP Kinase Pathway." Mol.Cell 14.6 (2004): 825-32.

