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MAP Kinase-Mediated Stress Relief that Precedes and Regulates the Timing of Transcriptional Induction

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Summary

In yeast, hyperosmotic stress causes an immediate dissociation of most proteins from chromatin, presumably because cells are unprepared for, and initially unresponsive to, increased ion concentrations in the nucleus. Osmotic stress activates Hog1 MAP kinase, which phosphorylates at least two proteins located at the plasma membrane, the Nha1 Na^+/H^+ antiporter and the Tok1 potassium channel. Hog1 phosphorylation stimulates Nha1 activity, and this is crucial for the rapid reassociation of proteins with their target sites in chromatin. This initial response to hyperosmolarity precedes and temporally regulates the activation of stress-response genes that depends on Hog1 phosphorylation of transcription factors in the nucleus. Thus, a single MAP kinase coordinates temporally, spatially, and mechanistically distinct responses to stress, thereby providing very rapid stress relief that facilitates subsequent changes in gene expression that permit long-term adaptation to harsh environmental conditions.

Introduction

All living cells adapt to environmental stress by dramatically changing their patterns of gene expression. There are many distinct forms of cellular stress as well as a large variety of molecular strategies to defend against the different stresses. Typically, stress-induced alterations in gene expression are rapid, highly selective, and specifically designed to alleviate the particular form of cellular insult, although yeast cells also have a general stress response that is activated upon a wide variety of conditions (Gasch et al., 2000; Causton et al., 2001). The paradigm for a stress response involves: (1) a cellular sensor that recognizes the specific stress, (2) a signal transduction pathway that often involves one or more distinct enzymatic activities, (3) modification of specific transcriptional activator and repressor proteins by the ultimate product of the signal transduction pathway, and (4) direct alteration of gene expression, thereby resulting in the production of proteins that directly relieve the stress. In the yeast *Saccharomyces cerevisiae*, considerable knowledge exists about how signal transduction pathways stimulate the expression of distinct subsets of "stress genes".

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The response to stress often involves mitogen-activated protein kinase (MAPK) cascades, which are among the most intensively studied signal transduction pathways in eukaryotes. All eukaryotes contain several discrete MAPK pathways that are stimulated by distinct environmental changes (Garrington and Johnson, 1999; Davis, 2000; Chen et al., 2001). For example, the five *S. cerevisiae* MAPK pathways mediate responses to hyperosmotic stress, heat, and hypotonic stress, α factor stimulation, and nutrient starvation (Gustin et al., 1998; Posas et al., 1998; Elion, 2000). Upon stress, distinct kinases are sequentially activated by phosphorylation, culminating with the generation of an enzymatically active MAP kinase. Activated MAP kinases directly phosphorylate DNA binding transcriptional activators and repressors, thereby changing their functional properties and hence directly altering gene expression at the level of transcriptional initiation. MAP kinases also phosphorylate a wide variety of proteins that are not transcriptional regulators (Chen et al., 2001). However, none of these substrates have been directly linked to the relief of environmental stress.

In *S. cerevisiae*, the Hog1 MAP kinase is the ultimate signaling component of the high osmolarity glycerol (HOG) pathway that mediates the response to hyperosmotic stress (Brewster et al., 1993). Activation of the HOG pathway leads to a massive and rapid transcriptional response, in which the expression of several hundred genes is induced within 5–10 min (Posas et al., 2000; Rep et al., 2000). The mechanisms of transcriptional induction by Hog1 are surprisingly complex. Upon stress, Hog1 is activated and translocated to the nucleus (Ferrigno et al., 1998), whereupon it interacts with and phosphorylates at least three different specific transcription factors—Sko1, Hot1, and Smp1 (Alepuz et al., 2001; Proft et al., 2001; de Nadal et al., 2003). Hog1 directly interacts with Hot1- and Sko1-dependent promoters, suggesting that it's part of transcriptional-activation complexes (Alepuz et al., 2001; Proft and Struhl, 2002). Hog1-mediated phosphorylation of Hot1 leads to increased DNA binding, and hence transcriptional activation of target promoters (Alepuz et al., 2001). In contrast, Hog1 phosphorylation of Sko1 converts the Sko1-Cyc8-Tup1 repressor complex into a transcriptional activator (Proft and Struhl, 2002). Hog1 also appears to directly recruit the RNA polymerase (Pol) II machinery to stress promoters, although the relevant substrate(s) is unknown (Alepuz et al., 2003). Lastly, Hog1 may regulate translation efficiency during osmostress by phosphorylating the Rck2 protein kinase (Bilsland-Marchesan et al., 2000; Teige et al., 2001).

Here, we demonstrate several unexpected features of the hyperosmotic stress response including a direct function for Hog1 that precedes its well-known role in transcriptional activation. First, we show that most chromatin-associated proteins dissociate from the DNA template within the first minute after osmotic stress, presumably due to increased ionic strength in the nucleus. Second, we identify a very rapid, Hog1-dependent mechanism that permits reassociation of these proteins

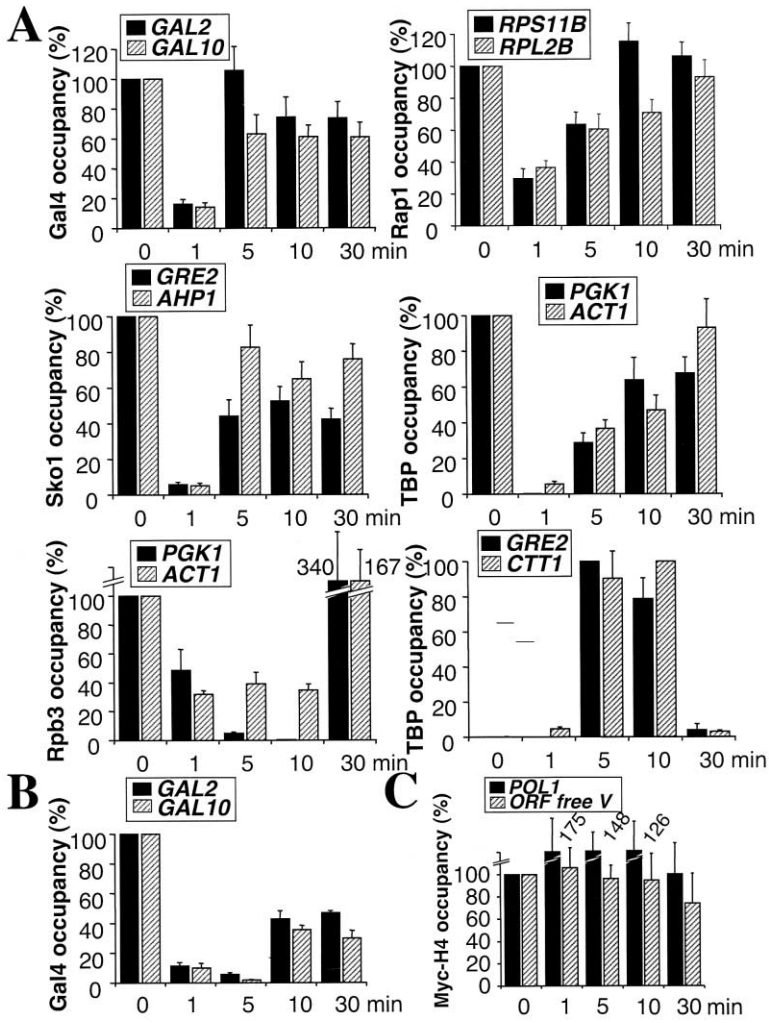

Figure 1. Hyperosmotic Stress Immediately Inhibits the In Vivo Association of Transcription Factors and Initiating RNA Polymerase II with Chromatin

(A) In vivo association with the indicated promoters of Gal4, Rap1, Sko1, TATA binding protein (TBP), and RNA polymerase II subunit Rpb3 in strains W303-1A or MAP37 expressing $(HA)_3$-tagged Sko1 that were osmotically stressed by 0.4 M NaCl for the indicated times. The fold immunoprecipitation efficiency over the *POL1* coding sequence control in unstressed cells (100%) is 38 (78) for Gal4 at *GAL2* (*GAL10*), 4 (8) for Rap1 at *RPS11B* (*RPL2B*), 20 (8) for $(HA)_3$-Sko1 at *GRE2* (*AHP1*), 12 (6) for TBP at *PGK1* (*ACT1*), and 5 (4) for Rpb3 at *PGK1* (*ACT1*). Maximal occupancy of TBP at *GRE2* (*CTT1*) corresponds to 10 (11)-fold over the *POL1* coding sequence control.

(B) Gal4 association with the *GAL2* and *GAL10* promoters in W303-1A cells that were osmotically stressed by 0.6 M sorbitol for the indicated times.

(C) In vivo association of myc-tagged histone H4 with the indicated chromosomal regions in strain UCC1111myc-H4 that was osmotically stressed by 0.4 M NaCl for the indicated times.

with chromatin within a few minutes. This Hog1-dependent function precedes, and indeed is required for, Hog1-dependent activation of gene expression. Third, we show that this pretranscriptional response to osmotic stress involves direct modulation of ion transport by Hog1-mediated phosphorylation of the Nha1 Na^+/H^+ antiporter (and to a lesser extent the Tok1 potassium channel). Thus, our results indicate that a single MAP kinase can mediate temporally and mechanistically distinct responses to stress, and that MAP kinases can mediate very rapid stress relief in a manner that precedes and facilitates the long-term change in gene expression patterns.

Results

Hyperosmotic Stress Results in a Very Rapid Dissociation of Most Chromatin-Associated Proteins, but Not of Histones or Elongating RNA Polymerase II

Although maximal Hog1-dependent activation of osmotic stress genes often occurs between 5–10 min (Posas et al., 2000; Rep et al., 2000), little is known about earlier stages of the response to hyperosmolarity. Here, we use chromatin immunoprecipitation (ChIP) to measure binding of DNA binding transcriptional regulatory proteins Gal4, Rap1, and HA-tagged Sko1 to their recognition sequences in various promoters before and during a sudden increase in osmolarity (Figure 1A). Sko1 is a direct target of Hog1 kinase that is responsible for induction of many osmotically regulated genes, whereas Gal4 and Rap1 are unrelated to osmotic stress. As expected, the binding of all three factors is readily detectable when the cells were grown in normal growth medium. Unexpectedly, a moderate hyperosmotic shock (0.4 M NaCl) severely reduces the binding of all three transcription factors within the first minute of stress exposure. Similarly, association of the TATA binding protein (TBP) or the Rpb3 subunit of Pol II with highly active, stress-independent promoters (e.g., *PGK1* and *ACT1*) is drastically decreased within one minute of osmotic stress (Figure 1A).

To distinguish whether the above stress is caused to the toxic effect of Na^+ or the osmotic effect that causes the loss of water from the cell interior, we performed similar experiments on cells subjected to osmotic stress with 0.6 M sorbitol. Association of Gal4, Rap1, Sko1, TBP, and Rpb3 to their target sites in chromatin is drastically and rapidly reduced in a manner similar to that of

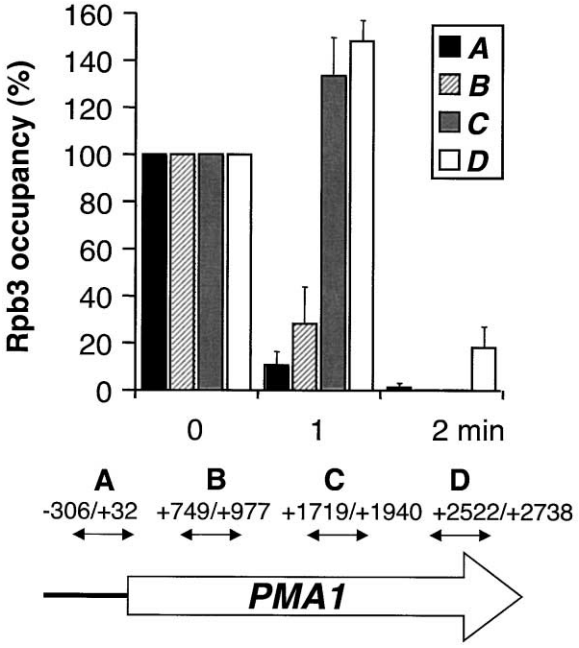

Figure 2. Elongating RNA Polymerase II Does Not Dissociate, but Continues to Travel along the Gene upon Hyperosmotic Stress

Association of RNA polymerase II subunit Rpb3 with the *PMA1* promoter and different locations within the *PMA1* open reading frame in W303-1A cells that were osmotically stressed by 0.4 M NaCl for the indicated times. The fold immunoprecipitation efficiency in unstressed cells was arbitrarily set to 100% and corresponds to 6 (4) over the *POL1* coding sequence control at locations A and B (C and D).

NaCl-stressed cells (data for Gal4 is shown in Figure 1B). Thus, the immediate loss of transcription factors from chromatin is caused by hyperosmotic stress, which most likely increases ionic strength in the nucleus.

Two observations indicate that the apparent loss of protein occupancy in the first minute of osmotic stress reflects true dissociation from the chromatin template and not decreased crosslinking in the presence of increased salt (or some other artifact of the ChIP assay). First, in a strain whose sole source of histone H4 is a myc-epitope-tagged version of the protein, there is no significant decrease in histone H4 binding at two different genome locations under the same NaCl stress conditions (Figure 1C). Indistinguishable results were obtained under several conditions where the efficiency of histone crosslinking to DNA was limited by reducing the time of crosslinking or by lowering the formaldehyde concentration (data not shown). Second, although Pol II occupancy at the *PMA1* promoter is severely reduced within the first minute of osmotic stress, Pol II occupancy at several locations within the downstream portion of *PMA1* coding region is unaffected (Figure 2). The distinct behavior of Pol II at promoters and coding regions excludes the possibility of a crosslinking artifact, and it indicates that promoter-associated, but not elongating Pol II, is affected in the early stage of osmotic stress.

Interestingly, Pol II occupancy at all regions of *PMA1* coding region is virtually eliminated at 2 min after osmotic stress, even though Pol II association at the 3′ portion of the mRNA-coding region (Figure 2, regions C and D) at 1 min after stress is comparable to that in unstressed cells. The pattern of Pol II occupancy across the *PMA1* locus strongly resembles the pattern observed for the last round of transcriptional elongation that occurs immediately after Gal4-dependent initiation is blocked (Mason and Struhl, 2003). Thus, under conditions when most proteins dissociate from chromatin, elongating Pol II remains bound, but new Pol II initiation events are inhibited.

The immediate dissociation of most proteins, but not histones or elongating Pol II, upon osmotic stress in vivo is remarkably similar to that occurring in vitro when chromatin is treated with 0.4 M NaCl. Indeed, treatment of chromatin (or isolated nuclei) with moderate concentrations of salt is the standard method to extract nonhistone proteins and transcription factors, but not elongating Pol II. Thus, the rapid protein dissociation in response to hyperosmotic stress is due to increased nuclear ion concentrations above a critical level. Furthermore, the results strongly suggest that yeast cells are unprepared for, and initially unresponsive to, the influx of salt and the loss of water from the cell, and that the resulting increase in ionic strength causes molecular mayhem in the nucleus.

A Very Rapid Hog1-Dependent Response to Osmotic Stress that Precedes Transcriptional Activation

Although many proteins dissociate from chromatin immediately upon osmotic stress, they reassociate with the chromatin template at the correct location within a few minutes (Figure 1A). Gal4 and Sko1 binding levels recover to near wild-type levels within about 5 min, whereas recovery to wild-type levels of Rap1 binding takes about 10 min. TBP binding at nonregulated genes (*PGK1* and *ACT1*) is evident at 5 min after the osmotic stress, although maximal levels take somewhat longer. Pol II association with these promoters is even more delayed; at 10 min after osmotic stress, Pol II levels are near background at the *PGK1* promoter and are 3-fold below levels in unstressed cells at the *ACT1* promoter.

As the immediate dissociation of many proteins from chromatin in response to osmotic stress reflects increased ion concentrations in the nucleus, the recovery response most likely involves the export of those ions, thus enabling the transcription factors to reassociate with their chromosomal recognition sites. This strongly suggests that there is a bona fide stress response that is mediated by a specific signaling event. Given the role of the HOG pathway, we examined whether Hog1 MAPK kinase is important for the rapid adaptation to NaCl stress. As shown in Figure 3A, cells that lack Hog1 show a significant delay in regaining normal levels of Gal4 or Rap1 binding to chromatin during NaCl stress. For example, Gal4 completely reassociates with the *GAL2* promoter in wild-type cells within 5 min, while *hog1* cells showed drastically reduced Gal4 binding even 10 min after stress treatment. Similar observations were made at osmostress responsive genes like *GRE2* or *AHP1* where Sko1 reassociation after salt shock is significantly delayed in the absence of Hog1 function (data not shown). Thus, Hog1 is critical for the immediate stress response upon NaCl shock.

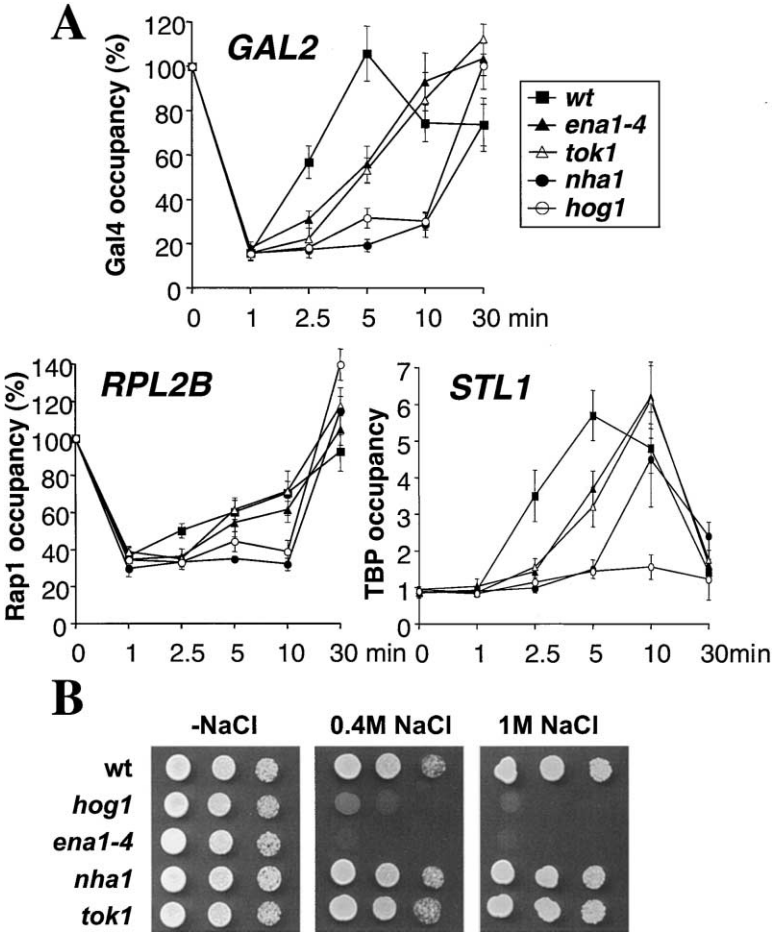

Figure 3. Hog1 MAPK and Nha1 Na^+/H^+ Antiporter Are Important for the Immediate Adaptation to NaCl Stress

(A) Association of Gal4 with the *GAL2* promoter, Rap1 with the *RPL2B* promoter, and TBP at the *STL1* promoter in wild-type (W303-1A), *ena1-4* (SKY697), *tok1* (MAP75), *nha1* (MAP72), and *hog1* (MAP32) cells subjected to hyperosmotic stress by 0.4 M NaCl for the indicated times. In the case of Gal4 and Rap1, the fold immunoprecipitation efficiency over the *POL1*-coding sequence control of unstressed cells was arbitrarily set to 100%. TBP binding is represented as the fold immunoprecipitation efficiency over the *POL1*-coding sequence control.

(B) Growth of the indicated strains in medium containing the indicated amount of NaCl.

The DNA binding proteins tested regain some or all of their chromatin association within the first 5 min of stress, therefore indicating a rapid Hog1-dependent cellular adaptation. However, maximal TBP recruitment to promoters of osmoinducible genes (e.g., *GRE2* and *CTT1*, which are shown in Figure 1A) occurs 5–10 min after exposure to NaCl. Translation of the induced RNAs and significant levels of biological function of the induced defense proteins will occur at even later times, suggesting that the initial Hog1-dependent response to osmotic stress precedes the Hog1-dependent transcriptional response. Furthermore, osmotic stress and Hog1-dependent phosphorylation converts Sko1 from a Cyc8-Tup1-dependent repressor into a transcription activator (Proft and Struhl, 2002) even though Sko1 dissociates from its target genes during the initial hyperosmotic shock. It is obvious that rapid reassociation of Sko1 must precede and therefore be independent of Sko1-dependent transcriptional induction. Thus, Hog1 plays two mechanistically and temporally distinct roles in the response to osmotic stress. It is required first for the rapid adaptive response that permits proteins to reassociate with chromatin, and subsequently for the transcriptional response that involves phosphorylation of activator proteins such as Sko1, Hot1, and Smp1.

Nha1 Is Important for the Fast Recovery from NaCl Stress

To identify plasma membrane transporters that might contribute to the immediate relief from NaCl shock, we focused at the Na^+ and K^+ extrusion systems (Serrano and Rodriguez-Navarro, 2001). The Ena1 Na^+ extrusion ATPase is by far the most important extrusion pump when yeast cells are grown in the presence of NaCl. However, Ena1 is not a likely candidate for the immediate response to hyperosmotic shock, because its expression is repressed under nonstress conditions (Marquez and Serrano, 1996; Proft and Serrano, 1999). The Nha1 Na^+/H^+ antiporter exchanges protons from the outer medium by Na^+ from the cytosol and is a constitutively expressed protein (Prior et al., 1996; Banuelos et al., 1998). The outwardly rectifying Tok1 K^+ channel is the only described extrusion system specific for K^+ in yeast (Ketchum et al., 1995).

We used *ena1-4*, *nha1*, and *tok1* deletion mutants to unravel the role of the respective transporters in the immediate salt response in the manner described above for the *hog1* deletion strain. In the *nha1* strain, there is a pronounced delay in Gal4 and Rap1 reassociation that is comparable to the effect observed in the *hog1* strain (Figure 3A). Both *ena1-4* and *tok1* strains show a much more moderate delay in the reassociation of Gal4 and Rap1 to chromatin. Thus, the function of the Nha1 Na^+/H^+ antiporter is of critical importance for the immediate recovery from a NaCl shock. Tok1 and Ena1 play a detectable, but lesser role in this immediate adaptive response, and we suspect that the minor effect of Ena1 reflects imperfect repression of the *ENA* genes. In contrast, long-term growth at elevated NaCl concentrations does not require *NHA1* or *TOK1*, but it is critically depen-

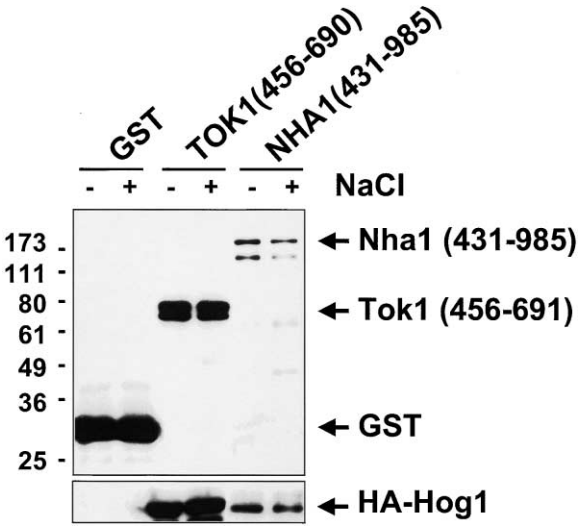

Figure 4. Hog1 Kinase Interacts with the C-Terminal Hydrophilic Domains of Nha1 Na^+/H^+ Antiporter and Tok1 K^+ Channel In Vivo

Coprecipitation of $(HA)_3$-Hog1 by GST-Nha1(431–985) and GST-Tok1(456–691) from yeast cell free extracts. Cells were briefly (5 min) treated or not with 0.4 M NaCl as indicated. GST-containing proteins (upper image) and $(HA)_3$-Hog1 (lower image) were detected by Western blotting using antibodies against GST or the HA epitope.

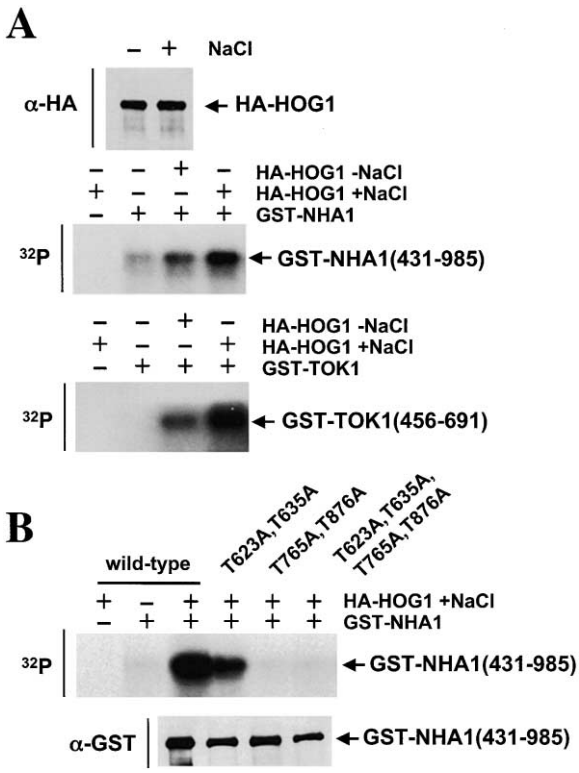

Figure 5. Hog1 MAPK Phosphorylates the Nha1 and Tok1 C-Terminal Domains In Vitro

(A) Immunopurified $(HA)_3$-Hog1 from either unstressed or stressed (0.4 M NaCl for 5 min) yeast cells (MAP51) was incubated with purified GST-Nha1(431–985) (from *hog1* mutant cells MAP32) or purified GST-Tok1(456–691) (from *E. coli*) in the presence of ^{32}P-ATP. $(HA)_3$-Hog1 was detected by Western blotting using an antibody against the HA epitope (upper image), and phosphorylated GST-Nha1 or GST-Tok1 proteins were detected by autoradiography (lower images).

(B) Hog1 phosphorylates Nha1 at T765 and T876 in vitro. Kinase assays with salt activated $(HA)_3$-Hog1 and wild-type or the indicated mutant versions of GST-Nha1(431–985) were performed as in (A).

dent on *ENA1-4* (Figure 3B), which is transcriptionally induced by osmotic stress and Hog1-dependent phosphorylation of Sko1 (Proft and Serrano, 1999).

We additionally tested whether the immediate salt response had an influence on the kinetics of the transcriptional induction by measuring the induced recruitment of TBP at the osmoinducible *STL1* gene. This addresses the level of transcriptional initiation and avoids potential problems related to RNA stability. As for Gal4 or Rap1, we observed delayed TBP recruitment in *nha1*, *ena1-4*, and *tok1* deletion mutants, with the delay being most pronounced in the *nha1* mutant (Figure 3A). However, in accord with the long-term growth assays, increased TBP association is observed in an *nha1* strain, but not in a *hog1* strain, indicating that Nha1 is specifically involved in the immediate response to osmotic stress.

Hog1 MAPK Interacts with and Phosphorylates Nha1 Na^+/H^+ Antiporter and Tok1 K^+ Channel

Given that Hog1 MAPK, the Nha1 Na^+/H^+ plasma membrane antiporter, and (to a lesser extent) the Tok1 K^+ channel are involved in the fast adaptation to salt stress, we examined whether Hog1 physically interacts with Nha1 and Tok1 in vivo. Both the Nha1 and Tok1 proteins contain highly hydrophobic N-terminal transmembrane domains and hydrophilic C-terminal domains. As it is unlikely that a MAPK would phosphorylate a transmembrane domain, we fused the hydrophilic C-terminal regions of Nha1 (amino acids 431–985) or Tok1 (amino acids 456–691) to GST and expressed the fusion proteins in cells that also expressed HA-tagged Hog1 from its natural chromosomal locus. Purification of the GST-Nha1 or GST-Tok1 fusion proteins, but not GST alone, by affinity chromatography resulted in the specific copurification of HA-Hog1 (Figure 4). The Hog1 interactions with the Nha1 and Tok1 C-terminal domains are not altered when the cells were treated with 0.4 M NaCl. Thus, Hog1 MAPK interacts, directly or indirectly, with the soluble parts of at least two ion transport proteins, the Na^+ specific Nha1 antiporter and the K^+ specific Tok1 channel.

We next addressed whether Hog1 directly phosphorylates Nha1 and Tok1 using immunoprecipitated HA-epitope-tagged Hog1 from either nonstressed or salt-stressed yeast cells. As substrates, we added either affinity purified GST-Nha1 from a *hog1* deletion yeast strain or bacterially expressed GST-Tok1. In both cases we used the C-terminal hydrophilic domains that interacted with Hog1 in vivo. As shown in Figure 5A, both the Nha1(431–985) and the Tok1(456–691) C-terminal regions are efficiently phosphorylated by Hog1 in vitro. Phosphorylation was more efficient when the Hog1 MAPK was purified from NaCl-treated cells, consistent with a greater pool of active Hog1 (Brewster et al., 1993). Taken together, our results show that Hog1 kinase interacts with and phosphorylates the C-terminal regions of the Nha1 and Tok1 ion extrusion proteins.

Hog1 Phosphorylation of Nha1 at Thr765 and Thr876 Is Critical for the Immediate Response to Osmotic Stress

Given the critical role of Nha1 in the immediate response to salt stress, we focused on whether Hog1-mediated phosphorylation of Nha1 is critical for this adaptive response. MAPKs are proline-directed serine/threonine kinases, and all natural substrates that have been identified for Hog1 are phosphorylated at one or multiple SP or TP motifs (Bilsland-Marchesan et al., 2000; Proft et al., 2001; Teige et al., 2001; de Nadal et al., 2003). Nha1 has four TP motifs (positions 623, 635, 765, 876), all of which are located in the C-terminal region that is bound and phosphorylated by Hog1. We therefore individually mutated all four threonines to alanines in the context of GST-Nha1(431–985) and combined them to double mutants Nha1^{T623A,T635A} or Nha1^{T765A,T876A}, or the fully mutated Nha1^{T623A,T635A,T765A,T876A}. As shown in Figure 5B, mutation of T623 and T635 has no significant effect on phosphorylation by Hog1 in vitro. In contrast, Hog1 phosphorylation of Nha1 is reduced to background levels when T765 and T876 are mutated. When tested individually, both T765A and T876A single mutants are readily phosphorylated by Hog1 (data not shown). These results indicate that Hog1 phosphorylates Nha1 at positions T765 and T876.

To functionally link Nha1 phosphorylation by Hog1 to the rapid adaptation to NaCl stress, we constructed a strain containing an Nha1-$(HA)_3$ epitope-tagged version of the full-length protein. Immunological detection of the Nha1-HA fusion reveals increased electrophoretic mobility that is caused by a brief NaCl shock and is completely dependent on Hog1 (Figure 6A). This alteration in Nha1 mobility is caused by phosphorylation, because it is reversed when the protein extracts are treated with alkaline phosphatase (Figure 6A).

We next constructed full-length Nha1-HA fusions in which threonines at positions 765 and 876 were both changed to alanine, aspartic acid, or valine. Importantly, none of the point mutated Nha1 proteins show any mobility shift after salt treatment, and their electrophoretic mobilities coincide with the phosphorylated form of the wild-type protein (Figure 6B). These observations demonstrate that Hog1 phosphorylates Nha1 at T765 and T876 in vivo upon salt shock. Further, they suggest that the wild-type Nha1 protein has a conformation that is distinct from proteins that are mutated or phosphorylated at T765 and T876.

Finally, we directly examined the importance of T765, T876 phosphorylation for the immediate response to salt stress. The dissociation-reassociation kinetics of Gal4 at the *GAL2* promoter and of Rap1 at the *RPL2B* promoter upon NaCl shock were measured in strains that carry either $(HA)_3$-epitope-tagged Nha1, Nha1^{T765A,T876A}, Nha1^{T765D,T876D}, or Nha1^{T765V,T876V} (see Figure 6C). In this experiment, Gal4 and Rap1 reassociation in the wild-type and full-length Nha1-$(HA)_3$ strains is slightly slower than observed in the experiment of Figure 3A. The reduced reassociation is due to higher ionic concentrations (and slower growth rate) in SD medium (used for Figure 6C) than in YPD medium (used for Figure 3A). Strikingly, in all mutant strains, the dramatic loss of Gal4 binding within one minute of salt stress does not occur or is largely suppressed (Figure 6C). Thus, the Nha1 proteins with mutations at positions T765 and T876 behave as gain-of-function derivatives for the fast adaptation to NaCl stress. This is consistent with the observation that the electrophoretic mobility of the mutant proteins is indistinguishable from the Hog1-phosphorylated form of wild-type Nha1. Additionally the Gal4 reassociation defect of *hog1* mutants can be suppressed by expressing a constitutively active Nha1 mutant transporter (Figure 6E). Taken together, these results indicate that phosphorylation of Nha1 at T765, T876 by Hog1 MAPK is critical for the immediate response to NaCl stress. Further, they suggest that the activity of Nha1 in reducing ion concentrations is stimulated by phosphorylation or mutation of T765 and T876, and hence that the two threonine residues are important for maintaining a relatively inactive form of Nha1.

We tested the Na^+ extrusion activities of the different Nha1 versions directly using a plate assay. The cation sensitive mutant strain *ena1-4*Δ *nha1*Δ has been successfully used to assay for Nha1 activity in vivo (Kinclova et al., 2001; Kamauchi et al., 2002). The lack of the main Na^+ extrusion pump Ena1-4 renders Nha1 the crucial Na^+ export system to allow for growth on NaCl-containing plates. When expressed in the *ena1-4*Δ *nha1*Δ background, the wild-type and all mutant Nha1 versions confer similar NaCl resistance (Figure 6F). However, in an *ena1-4*Δ *nha1*Δ*hog1*Δ strain, expression of the Nha1 wild-type protein leads only to poor survival on NaCl plates, while the mutated transporters confer greater resistance to NaCl. These results show that Nha1 activity in vivo depends on the function of Hog1 MAPK and that mutation of the Hog1 phosphorylation sites T765 and T876 increases the Nha1 salt-pumping activity.

The Immediate Response to Osmotic Stress Influences the Kinetics of Transcriptional Induction

As Nha1 is selectively involved in the initial response to hyperosmotic stress, we examined null and constitutively activated *nha1* alleles for their effects on the transcriptional induction of *STL1* after treatment with 0.4 M NaCl by measuring TBP occupancy (Figure 6D). Strains expressing wild-type Nha1 (untagged or HA-tagged) display the expected transient induction of *STL1*, with TBP occupancy being induced significantly at 5 min, maximally at 10 min, and poorly at 30 min after stress. Loss of Nha1 results in a delayed induction response, because TBP occupancy at *STL1* is below that of the wild-type strain at the 5 and 10 min time points, and it continues to increase even 30 min after salt stress. In contrast, strains expressing the constitutively active Nha1 derivatives display a precocious response, because *STL1* induction peaks at 5 min after salt treatment and is already diminishing at the 10 min time point. Importantly, the maximal level of TBP occupancy is roughly comparable in all four strains, but this maximal level occurs at different times depending on Nha1 function. Thus, the kinetics of the initial response to hyperosmotic stress governs the kinetics of the transcriptional activation profile and hence the biological response.

Discussion

Hyperosmotic Stress Causes Immediate and Widespread Dissociation of Proteins from DNA: Implications for the Transcriptional Response

Within one minute after cells are subjected to hyperosmotic stress, all transcriptional regulatory proteins tested

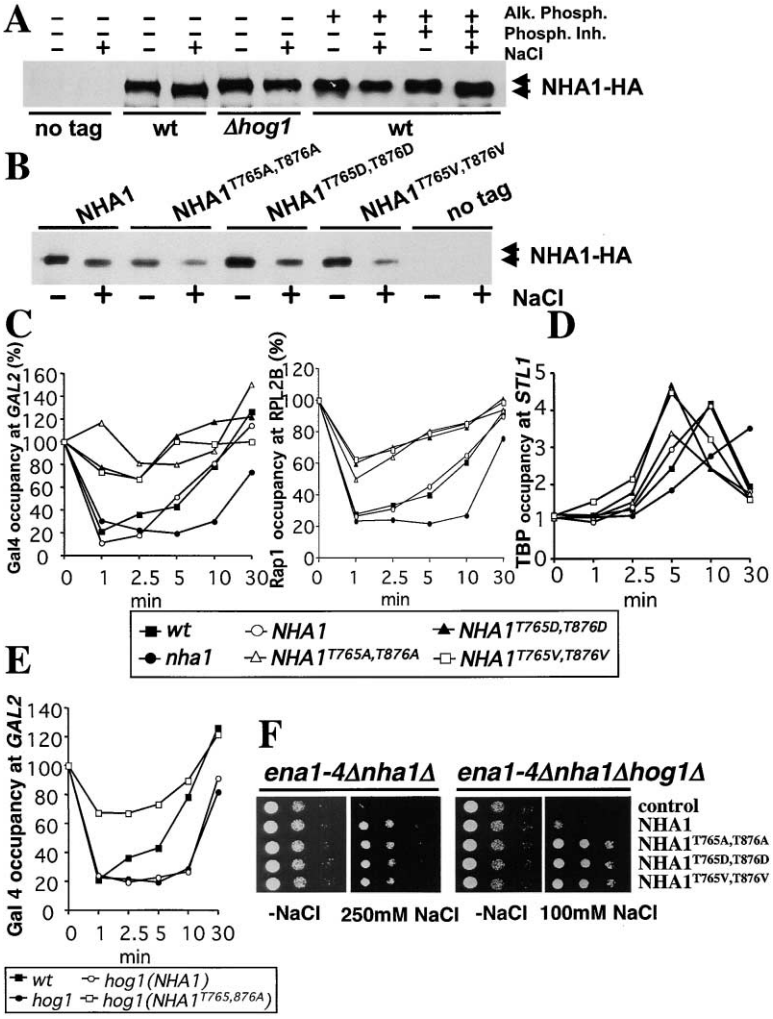

Figure 6. Hog1 Phosphorylation of Nha1 at Positions T765 and T876 Is the Critical Step in the Immediate Adaptation to NaCl Stress

(A) Nha1 is phosphorylated in vivo upon salt stress dependent on Hog1. Wild-type cells (W303-1A) transformed with the control plasmid (YCplac33, no tag) or $(HA)_3$-Nha1 expression plasmid were subjected or not to brief hyperosmotic shock (0.4 M NaCl for 5 min). $(HA)_3$-Nha1 was additionally expressed in *hog1* mutant strain MAP32. $(HA)_3$-Nha1 was detected in solubilized membrane preparations by Western blotting using an antibody against the HA epitope. Extracts were treated or not with alkaline phosphatase in the presence or absence of phosphatase inhibitors as indicated.

(B) The Nha1 mutant proteins are not phosphorylated by Hog1 and have an altered electrophoretic mobility. $(HA)_3$-Nha1 wild-type or mutant proteins were expressed in yeast *nha1* mutant cells (MAP72) that were treated or not with 0.4 M NaCl for 5 min.

(C) Phosphorylation of Nha1 is important for the immediate response to osmotic stress. In vivo association of Gal4 with the *GAL2* promoter and Rap1 with the *RPL2B* promoter in the wild-type strain W303-1A harboring the control plasmid YCplac33 and *nha1* mutant strain (MAP72) transformed with either the control plasmid YCplac33 or the $(HA)_3$-Nha1 (wild-type or indicated mutant) expression plasmids that were treated with 0.4 M NaCl for the indicated times. The fold immunoprecipitation over the *POL1* coding sequence control for the unstressed cells is arbitrarily set to 100% for each strain.

(D) The immediate response to osmotic stress governs the kinetics of the transcriptional response. TBP occupancy at the *STL1* promoter in the wild-type strain W303-1A harboring the control plasmid YCplac33 and *nha1* mutant strain (MAP72) transformed with either the control plasmid YCplac33 or the $(HA)_3$-Nha1 (wild-type or mutant) expression plasmids that were treated with 0.4 M NaCl for the indicated times. The immunoprecipitation efficiency is calculated as the fold over the *POL1*-coding sequence control.

(E) $Nha1^{T765A,T876A}$ rescues the defect of *hog1* mutants in the immediate salt response. Gal4 occupancy at the *GAL2* promoter in wild-type cells (W303-1A) with control plasmid YCplac33 or *hog1* mutant (MAP32) with control plasmid YCplac33, or the $(HA)_3$-Nha1 (wild-type) or $(HA)_3$-Nha1 (T765A, T876A) expression plasmid. Cells were treated with 0.4 M NaCl for the indicated times. The fold immunoprecipitation over the *POL1*-coding sequence control for the unstressed cells is arbitrarily set to 100%.

(F) Nha1 mutant proteins behave as constitutively active transporters. NaCl sensitivity plate assay using strains MAP76 (*ena1-4 nha1*) and MAP 78 (*ena1-4 nha1 hog1*) transformed with the empty plasmid (YCplac33; control) or the $(HA)_3$-Nha1 (wild-type or mutant) expression plasmids. Cells were directly spotted on SD-ura plates containing or not the indicated NaCl concentrations. Growth was monitored after two days (-NaCl) or five days (+NaCl).

dissociate from the chromatin template (Figures 7A and 7B). The observed dissociation reflects physiological reality, and not inefficient formaldehyde crosslinking, because crosslinking of histones and elongating Pol II is not affected by these osmotic stress conditions, and the rapid dissociation of transcriptional regulatory factors does not occur in strains containing the Nha1 derivatives containing mutations at threonines 765 and 876. Although we do not know the exact ion concentrations in the nucleus during the first minutes of hyperosmotic stress, the pattern of protein dissociation strongly resembles that of salt-treated chromatin in vitro, and hence is indicative of increased nuclear ion concentrations above a critical level. In this regard, chromatin immunoprecipitation provides a qualitative assessment of salt concentration in the nucleus as well as a physiological assay to define a very rapid stress response. We conclude that yeast cells are unprepared for, and initially unresponsive to, the massive import of Na^+ and loss of water from the cytosol. Furthermore, the molecular mayhem caused by hyperosmotic stress must be reversed to permit subsequent binding of transcription factors and induction of the appropriate genes.

Transcriptional profiling experiments indicate that most genes are unaffected by osmotic stress (Posas et al., 2000; Rep et al., 2000). However, we show that TBP occupancy and hence transcription from most (and perhaps all) genes is drastically inhibited during the first few minutes of osmotic stress. Moreover, under conditions of high activation, TBP and Pol II occupancies at the nonstress genes tested are very low and the return to normal levels can take as long as 10–30 min by which time cells have largely adapted to increased osmolarity. Thus, during the critical adaptation phase, a very signifi-

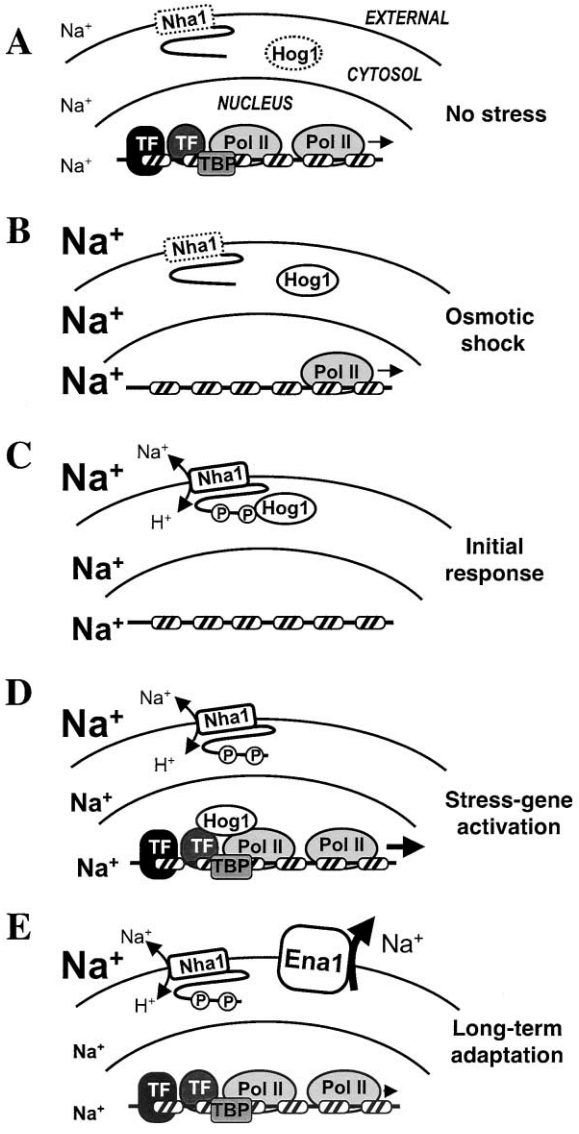

Figure 7. Hog1 Mediates a Sequential Adaptation to Hyperosmotic Stress Involving Early Modulation of Ion Transport and Subsequent Stimulation of Gene Expression

(A) Nonstress conditions. Nha1 antiporter at the plasma membrane and Hog1 kinase are in their "low-activity" forms (dotted circles), and transcription factors (TF), TBP, and Pol II in the nucleus are bound to chromatin (histones indicated as ovals with two oblique black bars) and transcribing genes (arrow). A subpopulation of Hog1 may associate with Nha1 at the plasma membrane (data not shown)

(B) Osmotic shock. Hyperosmotic stress results in elevated NaCl concentrations throughout the cell (large bold Na^+), thereby causing dissociation of transcription factors, TBP, and initiating Pol II from DNA. Histone and elongating Pol II remain associated under these conditions, and the last round(s) of transcription continues. Hog1 kinase is activated (solid circle) via the HOG MAPK cascade.

(C) Initial stress response. Hog1 kinase phosphorylates (P) Nha1 on T765 and T876, thereby stimulating Nha1 activity and extruding Na^+ (arrows) from the cell (note smaller font for Na^+ in the cytoplasmic and nuclear compartments). Elongating Pol II that did not dissociate upon the initial osmotic stress has finished the last rounds of synthesis (except perhaps at very long genes).

(D) Stress-gene activation. Continuing Nha1-dependent extrusion (note even smaller font for Na^+ in the cytoplasmic and nuclear compartments) permits reassociation of transcription factors and assembly of active transcription complexes. Transcriptional activation occurs preferentially on osmotic-inducible promoters (e.g., *ENA1*).

(E) Induction of *ENA1* results in high levels of the Ena1 Na^+ extrusion ATPase, which efficiently reduces intracellular NaCl levels (thick arrow; note very small font for Na^+ in the cytoplasmic and nuclear compartments), thereby resulting in long-term adaptation to hyperosmolarity and abrogation of the Hog1-dependent response.

cant proportion of transcriptional initiation events are directed specifically toward stress-response genes and against unrelated genes. This dramatic and transient reprogramming is not detected by conventional expression profiling, because mRNAs are relatively stable during the crucial few minutes. The gene-specific delay in returning to normal levels of TBP occupancy as well as differences in RNA half-lives is likely to account for the heretofore unexplained observation that about 20% of yeast genes appear to be downregulated by osmotic stress.

A Novel Function for a MAP Kinase in the Immediate Stress Response

The classical role of MAP kinases in responses to environmental stress is phosphorylation of transcription factors that directly induce the expression of genes that alleviate the stress. Here, we show that a MAPK can mediate a rapid stress response that precedes the transcriptional response. Specifically, Hog1 kinase phosphorylates the Nha1 Na^+/H^+ antiporter at Thr765 and Thr876 in response to osmotic stress, and this phosphorylation is essential for the immediate adaptation that permits proteins to reassociate with their target sites. Hog1 also phosphorylates the Tok1 K^+ channel, and Tok1 contributes to the immediate response, although to a lesser extent than Nha1. This fast and direct stress response at the level of cytoplasmic membrane transport adds a remarkable feature of MAPK function, as the same Hog1 MAPK mediates the subsequent transcriptional activation of stress-defense genes (Figures 7C and 7D).

Our results suggest that Hog1 phosphorylation activates the Na^+ extrusion activity of Nha1, thereby lowering the nuclear NaCl concentration and permitting binding of transcription factors. Although the Na^+/H^+ exchange rates of basal and activated Nha1 are unknown, the Nha1 derivatives with T765 and T876 changed to A, D, or V behave as constitutively active antiporters, because the rapid dissociation of DNA binding factors during NaCl stress is largely abolished, and the defect in the immediate stress response of a *hog1* mutant can be rescued by the mutated Nha1 proteins. Very importantly, the increased antiporter activities of the mutated Nha1 variants can be detected by a direct salt sensitivity assay. This strongly suggests that T765 and T876 are important to keep Nha1 in a "low activity" state and that activation of Nha1 can occur by Hog1 phosphorylation or mutation of these threonines. In accord with this interpretation, the long C-terminal hydrophilic extension of Nha1 is not required for its ion exchange activity (Kinclova et al., 2001), and an inhibitory region of Nha1 (Kamauchi et al., 2002) contains both Hog1 phosphorylation sites identified here.

Under nonstress conditions, the electrophoretic mo-

bility of all Nha1 mutant proteins tested differs from that of the wild-type protein. This difference in mobility corresponds to an apparent molecular weight difference of approximately 5 kDa, and it is remarkable given that the wild-type and mutant proteins differ by only two amino acid substitutions that do not involve charged residues in the case of alanine or valine. These observations strongly suggest that the "low-activity" wild-type protein and the "high activity" Nha1 mutant derivatives exist in significantly different conformations. Furthermore, the Hog1-dependent phosphorylated form of Nha1 behaves indistinguishably from the Nha1 mutant derivatives, suggesting that phosphorylation causes a comparable conformational change that activates the antiporter function of Nha1. We speculate that an intramolecular interaction within Nha1 that involves T765 and/or T876 masks the active site. Phosphorylation or mutation of T765 and T876 would disrupt this intramolecular interaction, thereby unleashing the full catalytic activity of the Nha1 antiporter.

Sequential Adaptation to Osmostress by Early Modulation of Ion Transport and Late Stimulation of Gene Expression

Our results define a sequential response to environmental stress mediated by a single MAP kinase. The first stress response involves the direct activation of the Nha1 (and probably Tok1) by Hog1 phosphorylation, presumably reducing ion concentrations in the nucleus, and thus permitting transcriptional regulatory proteins to reassociate with their genomic targets (Figure 7C). For obvious reasons, reassociation of transcription factors must precede Hog1-dependent activation of stress-response genes. As a consequence, mutations that eliminate or enhance Nha1 function cause a corresponding delay or acceleration in the transcriptional activation of Hog1- and osmotically inducible genes. In this regard, the transcriptional response to very high salt concentrations is significantly delayed, even though activated Hog1 is detected in the nucleus (Rep et al., 1999; Posas et al., 2000; Van Wuytswinkel et al., 2000). Our results suggest that the long delay reflects the extra time needed for the nontranscriptional, Nha1-dependent response to reduce ion concentrations to the levels that are compatible with transcription. In particular, the initial response and transcriptional induction in SD medium (Figures 6C and 7D) is slower than in YPD medium (Figure 3A), presumably due the higher ionic concentration in SD medium. More generally, they suggest that the duration of this initial adaptation will correlate with the strength of the saline stress and govern the kinetics of the transcriptional response.

Hog1 phosphorylation of Nha1 and Tok1 during the initial response presumably occurs near the plasma membrane, because both ion transporters contain multiple membrane-spanning domains and hence are likely to be tethered at this cellular location (Figure 7C). Hog1 copurifies with GST-Nha1 independently of osmotic stress, and the Pbs2 MAPKK serves as a scaffold for the HOG MAP kinase components and interacts with the Sho1 plasma membrane osmosensor (Posas and Saito, 1997). Therefore a fraction of Hog1 MAPK might be already physically associated with Nha1 before stress. In contrast, Hog1 phosphorylation of transcription factors requires the import of activated Hog1 into the nucleus (Ferrigno et al., 1998; Reiser et al., 1999), and Hog1 associates with Sko1- and Hot1-inducible promoters in a manner dependent on activator binding (Alepuz et al., 2001; Proft and Struhl, 2002), suggesting that phosphorylation of these transcription factors may occur at the promoter (Figure 7D). It will be of interest to decipher whether the nuclear and plasma membrane substrates are targeted by the same Hog1 molecules subsequently or by distinct subpopulations.

At the level of Na^+ export, the hyperosmotic stress response can be divided into early and late phases that are governed by different transporters (Figures 7C–7E). The Nha1 Na^+/H^+ antiporter is critical for the pretranscriptional stress response, while the Ena1 Na^+ ATPase is less important. The limited role of Ena1 is explained by repression of *ENA1* in the absence of stress, and its small contribution to the early stress response is likely due to imperfect repression. As defined by reassociation of proteins with chromatin, the pretranscriptional response is not abolished in *hog1* and *nha1* strains, but rather significantly delayed. This delayed response may be explained by the activity of Ena1 and other Na^+ export systems (in the case of *nha1*) or by low-level, Hog1-independent activity of Nha1 (in the case of *hog1*).

In contrast, Ena1 is required for growth in the presence of elevated Na^+ concentrations, whereas Nha1 and Tok1 have virtually no role in this long-term response to hyperosmotic stress. Nha1 protein levels are not subject to regulation by salt stress, indicating that activated Nha1 (and presumably Tok1) are insufficient to reduce ion concentrations to levels that are required for transcription and cell growth. Instead, the long-term response to hyperosmotic stress requires robust, Hog1-dependent induction of *ENA1*. How does the long-term response occur in an *nha1* mutant strain? We suggest a positive feedback loop consisting of: (1) low level Ena1 or other Na^+ export activity causing a slight reduction in ion concentrations, (2) limited reassociation of proteins with chromatin, (3) a small amount of Hog1-dependent activation of *ENA1*, and (4) increased Ena1 activity thereby facilitating further export of ions. Thus, yeast cells use a constitutive antiporter for the immediate Na^+ extrusion and an inducible ATPase for the long-term adaptation to Na^+ stress.

Concluding Remarks

By analogy with the initial effects of hyperosmotic stress, it is likely that other forms of environmental stress will cause sufficient mayhem to disrupt transcription and other complex biological processes that rely on multiprotein machineries. In such cases, the cell must have specific mechanisms to alleviate the initial effects of the stress to permit subsequent induction of stress-response genes. In the case of hyperosmotic stress, the initial insult is easily detectable by chromatin immunoprecipitation, whereas putative disruptions due to other forms of stress have yet to be described in molecular terms. In any event, we speculate that eukaryotic cells will often respond to stress in a biphasic manner in which the pretranscriptional phase occurs rapidly via modifications of already synthesized proteins and per-

mits the subsequent long-term response that involves induced expression of genes encoding defense proteins. In the case of hyperosmotic stress analyzed here, a single MAP kinase is the key regulator for both the pretranscriptional and the transcriptional responses. It is possible that other MAP kinases involved in diverse stress responses will also phosphorylate substrates that are required for the initial adaptation and help coordinate the early and later stages of the response to environmental stress.

Experimental Procedures

Yeast Strains and DNAs

The following yeast strains were used for chromatin immunoprecipitation (Figures 1–3, 6). W303-1A (*MATa ura3 leu2 trp1 his3 ade2*), MAP37 (*3HA-SKO1*) (Proft et al., 2001), MAP32 (W303-1A with *hog1::KAN MX*), SKY697 (W303-1A with *ena1-4::HIS3*) (Ferrando et al., 1995), MAP72 (W303-1A with *nha1::KAN MX*), MAP75 (W303-1A with *tok1::KAN MX*), UCC1111 (*MATa ade2::his3-200 leu2-0 lys2-0 met15-0 trp1-63 ura3-0 adh4::URA3-TEL(VII-L) hhf2-hht2::MET15 hhf-hht1::LEU2 pRS412(ADE2 CEN ARS)-HHF2-HHT2*), UCC1111-myc-H4 (UCC1111 with myc-tagged histone H4) (Ng et al., 2003). For coprecipitation experiments (Figure 4), GST-Nha1 and GST-Tok1 fusion proteins were expressed in yeast strain MAP51 (W303-1A with *3HA-HOG1*) (Proft and Struhl, 2002). For salt sensitivity plate assays, strains MAP76 (W303-1A with *ena1-4::HIS3 nha1::KAN MX*) and MAP 78 (W303-1A with *ena1-4::HIS3 nha1::loxp hog1::KAN MX*) were used. Deletion strains for *nha1* and *tok1* were constructed by PCR-based gene replacement.

Yeast expression plasmids harboring *GST-NHA1(431–985)* and *GST-TOK1(456–691)* were constructed by insertion of the *NHA1* or *TOK1* fragments covering the hydrophilic C-terminal domains into pYEX-4T (2 micron, *URA3*, P_{CUP1}-*GST*, Clontech). *TOK1(456–691)* was additionally inserted into expression vector pGEX-KG to allow the expression of GST-Tok1(456–691) in *E. coli*. Mutations T623, T635, T765, and T876 to A were introduced into pYEX-4T containing *NHA1(431–985)* by in vitro site-directed mutagenesis (QuikChange XL from Stratagene). A C-terminal fusion of the *NHA1* gene with 3HA epitopes was first generated by PCR-mediated tagging. The *NHA1-HA* fusion gene together with 680 nucleotides of promoter sequence was then amplified by PCR and inserted into plasmid YCplac33 (*CEN*, *URA3*). The resulting centromeric *NHA1-HA* expression plasmid was subjected to site directed mutagenesis to change T765 and T876 of Nha1 to A, D, or V, respectively.

Chromatin Immunoprecipitation

Cells were grown in YPD medium (Figures 1–3) or SD-ura medium (Figure 6C) until exponential phase and then osmotically stressed by the addition of 0.4 M NaCl or 0.6 M sorbitol. Chromatin preparation and immunoprecipitation were performed as described previously (Kuras and Struhl, 1999), except that insoluble total chromatin was separated from the soluble fraction by spinning for 2 min in a microcentrifuge. After sonication, soluble chromatin fragments were obtained by spinning for 30 min in a microcentrifuge. The following antibodies were used for immunoprecipitation: α-$Gal4_{DBD}$ (rabbit polyclonal, Santa Cruz), α-Rap1 (goat polyclonal, Santa Cruz), α-HA (12CA5 ascites), α-TBP (polyclonal) (Kuras and Struhl, 1999), α-Rpb3 (mouse monoclonal, NeoClone, Madison, WI), and α-Myc (rabbit polyclonal, Upstate Biotechnology).

Quantitative PCR analyses were performed in real time using an Applied Biosystems 7700 sequence detector, using the *POL1* coding sequence as a negative control in all experiments. Each immunoprecipitation was performed at least twice from different chromatin samples, and the immunoprecipitation efficiency was calculated in triplicate by dividing the amount of PCR product from the immunoprecipitated sample by the amount of PCR product in the input sample prior to immunoprecipitation. All values were then normalized for the *POL1* coding sequence control. In the case of myc-tagged histone H4, the immunoprecipitation efficiency was calculated as the fold immunoprecipitation over the untagged control strain. The experimental error for these experiments is $\pm$ 20%.

Coprecipitation Assays

GST-Nha1 and GST-Tok1 fusion proteins were expressed in yeast strain MAP51 (W303-1A with *3HA-HOG1*). Fusion gene expression was induced by addition of $CuSO_4$ to a final concentration of 0.5 mM for 1 hr, and GST pulldowns from cell-free extracts were performed as described previously (Pascual-Ahuir et al., 2001). GST containing proteins were detected by Western blot using α-GST polyclonal antibody (Z-5, Santa Cruz Biotechnology, 1:5000), and HA-Hog1 was detected using α-HA monoclonal antibody (12CA5, 1:10,000).

In Vitro Kinase Assays

$(HA)_3$-Hog1 was immunoprecipitated from yeast strain MAP51. Cells were grown to exponential phase and subjected or not to brief osmotic shock (0.4 M NaCl for 5 min). Whole protein lysates were prepared in buffer A (50 mM Tris-HCl [pH 7.5], 15 mM EDTA, 15 mM EGTA, 150 mM NaCl, 2 mM dithiothreitol (DTT), 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride [PMSF]) supplemented with complete protease inhibitor cocktail (Roche) and incubated with α-HA monoclonal antibody (12CA5) and protein A Sepharose CL-4B beads (Amersham) over night at 4°C. Beads were extensively washed with buffer A and finally stored in kinase buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 2 mM DTT). GST-Nha1(431–985) wild-type and point mutated versions were expressed in yeast strain MAP32 (W303-1A with *hog1::KAN MX*). GST-Tok1 was expressed in bacteria DH5α. The fusion proteins were purified by affinity chromatography using glutathione Sepharose 4B (Amersham) in buffer A + complete protease inhibitor cocktail (Roche) and eluted with 10 mM glutathione in kinase buffer. Kinase reactions contained equal amounts of GST fusion proteins together with $(HA)_3$-Hog1 in kinase buffer and γ-^{32}P-ATP (0.2 μCi/μl). Reactions were carried out for 10 min at 30°C and were stopped by the addition of 2$\times$ SDS loading buffer. Labeled proteins were resolved by SDS-PAGE and detected by autoradiography. GST containing fusion proteins were detected by immunoblotting using a polyclonal anti-GST antibody (Z-5, Santa Cruz Biotechnology).

Immunodetection of Nha1

Wild-type and the T765A, T876A mutant version of $(HA)_3$-epitope tagged Nha1 were expressed from a centromeric plasmid under the control of the native *NHA1* promoter in wild-type (W303-1A), *hog1* (MAP32), and *nha1* (MAP72) mutant cells. Cells were subjected or not to brief hyperosmotic shock (5 min, 0.4 M NaCl). Cell lysates were prepared in breaking buffer (125 mM Tris/HCl [pH 8], 120 mM KCl, 10 mM EDTA, 2 mM DTT, 1 mM PMSF) and 2 volumes of GTED20 (10 mM Tris/HCl [pH 7.6], 1 mM EDTA, 1 mM DTT, 20% glycerol) were added. Cell debris was separated by centrifugation (10 min, 1000 g). The membrane fraction was obtained by centrifugation (30 min, 30.000 g) and resuspended in GTED20 containing 2.5 mg/ml N-Hexadecyl-N,N-Dimethyl-3-Ammonio-1-Propanesulfonate (Zwittergent 3-16, Sigma) and 3 mg/ml Na-deoxycholate. Membrane proteins were solubilized for 5 min at room temperature followed by brief sonication. Solubilized proteins were obtained in the supernatant after centrifugation (10 min, 30.000 g). $(HA)_3$-Nha1 proteins were detected after SDS-PAGE (6% PA) by immunoblotting using a monoclonal anti-HA antibody.

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