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Mitogen-Activated Protein Kinase Hog1 Is Essential for the Response to Arsenite in *Saccharomyces cerevisiae*▽

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Here we describe, for the first time, that budding yeast mitogen-activated protein kinase Hog1 and its upstream activators Pbs2 and Ssk1 are essential for the response to arsenite. Hog1 is rapidly phosphorylated in response to arsenite and triggers a transcriptional response that involves the upregulation of genes essential for arsenite detoxification.

Saccharomyces cerevisiae sets up a battery of mechanisms in response to arsenic that involve transcription factors (Yap1 and Arr1/Acr1/Yap8) (20), cell membrane transporters (Arr3/Acr3) (21), vacuole transporters that transfer arsenite-conjugated glutathione into the vacuole (Ycf1) (7), and an arsenate reductase (Arr2/Acr2) that reduces arsenate to arsenite before it can be a substrate of the transporters (15). However, it is still unknown if any of the mitogen-activated protein kinase (MAPK) pathways in *Saccharomyces cerevisiae* are involved in the response to arsenite.

To study the function and importance of MAP kinase signaling in the response to arsenite in *Saccharomyces cerevisiae*, we monitored the sensitivity to arsenite of strains deficient in the MAPKs Hog1, Fus3, Kss1, and Slt2 as well as some of their upstream regulators, such as Ste7 and Bck1. As shown in Fig. 1, all the mutants and the wild type were able to grow at similar rates in rich medium (yeast extract-peptone-dextrose [YPD]). However, strains deficient in Hog1 (*hog1*Δ) did not grow in media containing sodium arsenite, indicating that Hog1 activity is necessary for the proper response of *Saccharomyces cerevisiae* to this metalloid.

This sensitivity could be explained by the toxicity caused by sodium in *hog1*Δ mutants (8). However, the presence of sodium in the media did not affect the growth of strains lacking Hog1 (Fig. 1), confirming that the toxicity of sodium arsenite in *hog1*Δ mutants was produced by As(III).

Some of the physiological effects caused by arsenite are thought to be produced by its capacity for producing reactive oxygen species (ROS). We monitored the sensitivity of the previously described mutants to hydrogen peroxide, a well-known ROS producer. As shown in Fig. 1, all the mutants and the wild type showed similar sensitivity to hydrogen peroxide, indicating that arsenite produced its toxic effects in the cell through a mechanism that cannot be exclusively explained by its ROS production capacity.

These results showed that Hog1 is required to respond to a

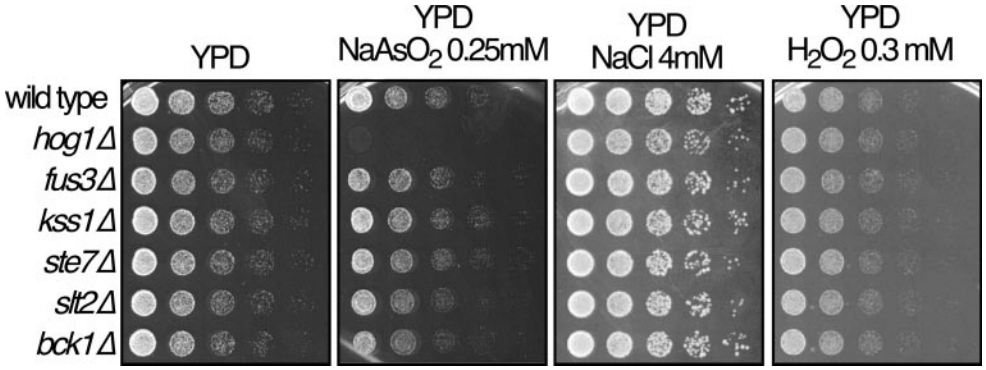

FIG. 1. Hog1 is essential for survival under arsenite stress. Serial dilutions (1:5) of the wild type and several mutants (*hog1*Δ, *fus3*Δ, *kss1*Δ, *ste7*Δ, *slt2*Δ, and *bck1*Δ) were plated on rich medium plates (YPD) in the presence of sodium arsenite, sodium chloride, and hydrogen peroxide. All of the strains used in this report were in the genetic background of the BY series, obtained from EUROSCARF. Pictures were taken after the plates were incubated for 2 to 3 days at 24°C.

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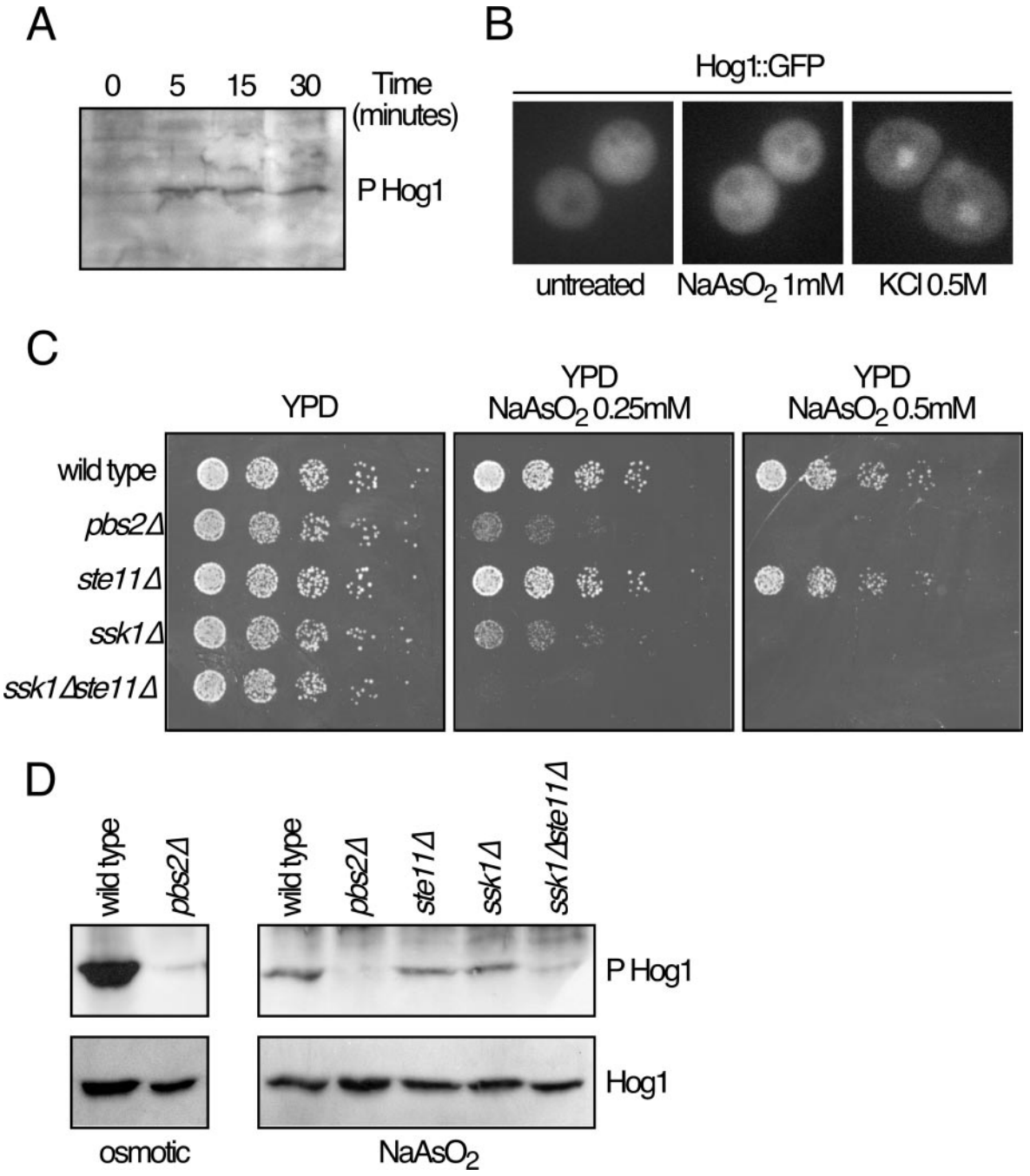

FIG. 2. Hog1 phosphorylation and localization. (A) Hog1 phosphorylation (P Hog1) in response to arsenite. Western blotting analysis of yeast extracts (13) showed that Hog1 is phosphorylated after 0.5 mM sodium arsenite treatment for the indicated times. Hog1 phosphorylation was detected using specific anti-phosphorylated Hog1 protein. (B) Hog1 localization. Hog1-deficient cells were transformed with plasmid pRS-Hog1-GFP (5). Hog1::GFP protein expressed from this plasmid was detected after treatment with 0.5 M KCl or 1 mM sodium arsenite for 10 min. (C) Mutants in the Hog1 pathway show different sensitivities to sodium arsenite. Serial dilutions of the wild type and *pbs2*Δ, *ste11*Δ, *ssk1*Δ, and *ssk1*Δ*ste11*Δ mutant strains were plated in rich media containing several concentrations of sodium arsenite. Pictures were taken after 2 to 3 days at 24°C. (D) Phosphorylation of Hog1 in different mutants. Strains described for panel C were treated with KCl (0.8 M) for 10 min (osmotic) or sodium arsenite (1 mM) for 10 min ($NaAsO_2$). Phosphorylation and abundance of Hog1 were monitored by Western blotting using specific polyclonal antibodies (9079; Santa Cruz).

new stimulus, namely sodium arsenite, distinct from hyperosmotic shock, hydrogen peroxide, or cold shock responses (16, 19).

The sensitivity of *hog1*Δ cells to arsenite indicated that the presence of the MAPK Hog1 was required for the correct biochemical response to this insult. If signaling through Hog1 is essential for its function in these conditions, Hog1 should be activated by phosphorylation of residues Thr174 and Tyr176,

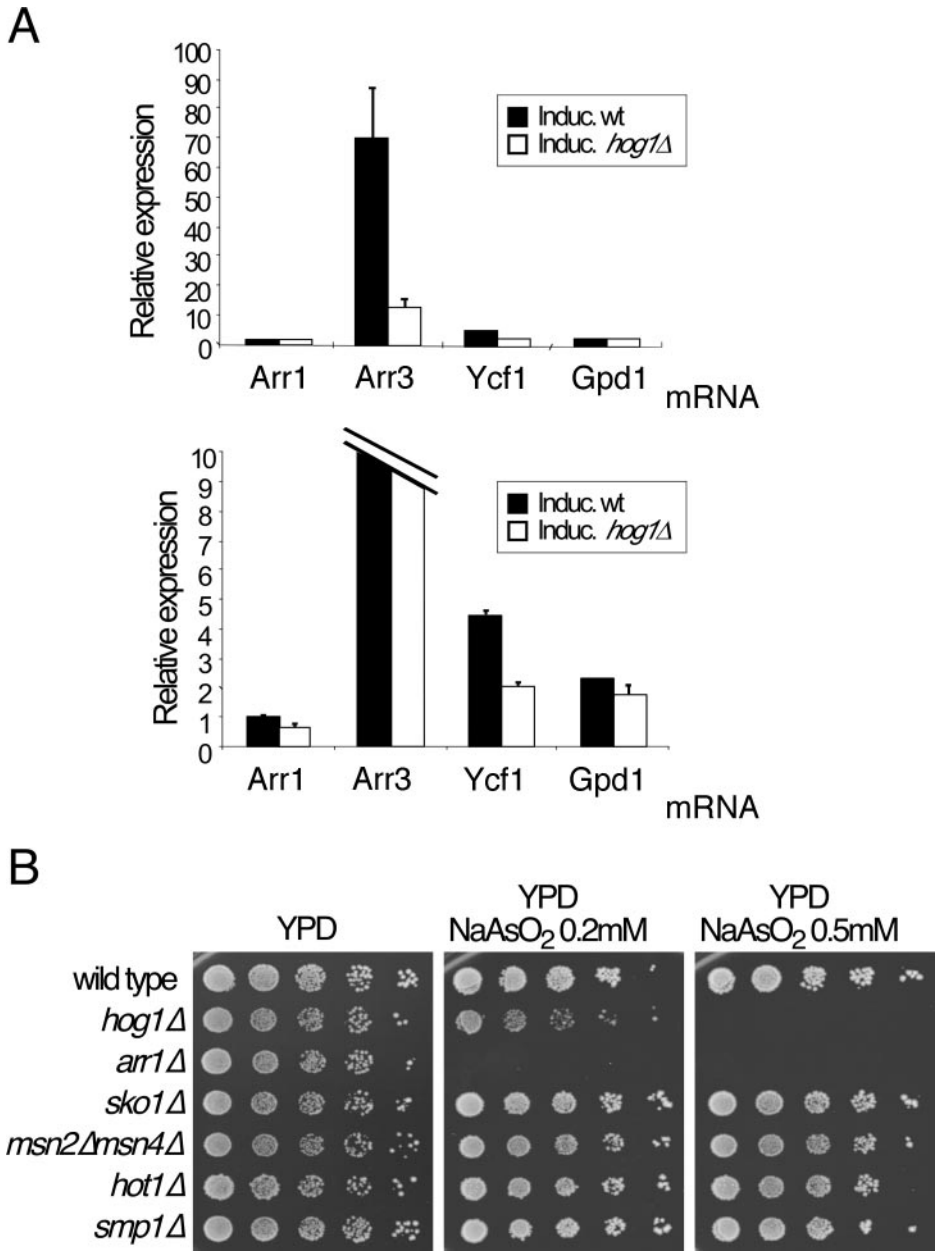

FIG. 3. Hog1-dependent gene expression in response to sodium arsenite. (A) Wild-type and *hog1*Δ strains were treated with 1 mM sodium arsenite for 1 h. After total RNA extraction, Arr1, Arr3, Ycf1, and Gpd1 mRNAs were quantified using quantitative reverse transcription-PCR (RT-PCRQ). Bars show the ratio of treated/untreated for each strain. Two graphs are shown for better observation of scale differences between experiments. Standard deviation of three experiments is shown. (B) Transcription factors and sensitivity to arsenite. Serial dilutions of the wild type and *hog1*Δ, *arr1*Δ, *sko1*Δ, *msn2*Δ*msn4*Δ, *hot1*Δ, and *smp1*Δ mutants were plated in rich media and media containing sodium arsenite. Pictures were taken after 2 to 3 days at 24°C. The double mutant *msn2*Δ*msn4*Δ was kindly provided by Raul García. wt, wild type.

which is required for Hog1 activation. Using commercial antibodies raised against phosphorylated human p38 (17852-R; Santa Cruz), we were able to detect phosphorylation of Hog1 protein in response to sodium arsenite (Fig. 2A) that appeared soon after arsenite treatment (5 min).

In response to high osmolarity, Hog1 phosphorylation is accompanied by translocation to the nucleus, increasing the abundance of the kinase in this cellular compartment (5). Using a *HOG1*::*GFP* allele, we monitored the localization of Hog1 in yeast cells after sodium arsenite treatment. As shown in Fig. 2B, Hog1 relocalization did not occur as much as observed in hyperosmotic stress-treated cells, indicating that the phosphorylation was not enough to trigger the accumulation of Hog1 protein in the nucleus. Nevertheless, it is possible that small amounts of Hog1 phosphorylation and Hog1 relocation to the nucleus are enough to trigger a complete response to arsenite.

Hog1 is activated through a series of phosphorylation events involving the MAPK kinase Pbs2 and two different MAPK kinase kinase (MAPKKK) branches involving, on the one hand,

the MAPKKK Ste11, and on the other hand, the MAPKKKs Ssk2/Ssk22. To test whether any of those pathways was involved in the cellular response to arsenite, we monitored the sensitivity to sodium arsenite of several yeast strains carrying mutations in components of the Hog1 pathway. As shown in Fig. 2C, *pbs2*Δ mutants were highly sensitive to arsenite in a way similar to that of *ssk1*Δ and *hog1*Δ mutants (Fig. 1), while mutants in the MAPKKK Ste11 did not show any sensitivity to arsenite. Interestingly, the sensitivity of the double mutant *ssk1*Δ*ste11*Δ to sodium arsenite was higher than that of *pbs2*Δ or *ssk1*Δ.

We also tested the level of Hog1 activation after arsenite stress in several mutants (Fig. 2D). While the phosphorylation of Hog1 was undetectable in *pbs2*Δ and *ssk1*Δ*ste11*Δ mutants, *ssk1*Δ mutants showed a phosphorylation similar to that of the wild type and *ste11*Δ mutant, indicating that there is not a direct correlation between the amount of Hog1 phosphorylation and the sensitivity of *S. cerevisiae* to arsenite and that Ssk1 could have a role in arsenite response independent of its function on Hog1 phosphorylation.

Alternatively, it was also possible that Hog1 response could be hampered by Skn7. Ssk1 and Skn7 are both regulated by the histidine phosphotransferase Ypd1. Skn7 has functions that counteract the Hog1 pathway (10–12). In fact, its deletion has a synthetic negative effect with the deletion of Ptc1, a phosphatase that negatively regulates Hog1 phosphorylation (10). As demonstrated by the kinetic studies performed by Janiak-Spens et al. (9), in regular conditions phosphotransfer from Ypd1 to Ssk1 is strongly favored over phosphotransfer to Skn7. Therefore, we hypothesized that elimination of Ssk1 could indirectly hyperactivate Skn7 and promote its counter-Hog1 effects.

One of the main functions of MAPK pathways is the regulation of transcriptional events in response to specific stimuli. We studied the abundance of several mRNAs in the wild type and *hog1*Δ mutants in response to sodium arsenite by quantitative reverse transcription-PCR, as described previously (6). We monitored the expression of four mRNAs: the transcription factor Arr1, essential for the upregulation of several genes involved in the response to arsenite (2, 14); the plasma membrane transporter Arr3; the vacuole transporter Ycf1; and Gpd1, the product of a glycerol-3-phosphate dehydrogenase gene, essential in the response to hyperosmotic stress and regulated by Hog1 activity (1).

As shown in Fig. 3A, the Arr3 mRNA is highly induced in response to arsenite treatment, while Arr3 induction in *hog1*Δ mutants is much lower. A smaller but significant difference was observed in Ycf1 mRNA expression. This defective induction of Arr3 and Ycf1 expression is consistent with the high sensitivity of *hog1*Δ mutants to sodium arsenite.

The fact that the abundance of Arr3 and Ycf1 mRNAs depended on Hog1 led us to consider the possibility of transcriptional regulation of those genes by Hog1. To gain insight into the regulatory mechanism, we analyzed the sensitivity of strains deficient in the transcription factors Sko1, Msn2/4, Hot1, and Smp1, all regulated by Hog1. We also checked the arsenite resistance of a mutant lacking Arr1, which is responsible for the transcription of Arr3 and Ycf1 in response to arsenite.

As shown in Fig. 3B, *arr1*Δ and *hog1*Δ are sensitive to different concentrations of arsenite. However, the rest of the mutants assayed (*sko1*Δ, *msn2*Δ*msn4*Δ, *hot1*Δ, and *smp1*Δ) did not show any growth defect in plates containing up to 1 mM sodium arsenite (Fig. 3B and data not shown). This result is consistent with a mechanism of Hog1 regulation of arsenite response that is independent of Sko1, Msn2/4, Hot1, and Smp1, which highlights a possible novel mechanism of Hog1-dependent transcriptional regulation.

The results described here have many similarities with those obtained in *Schizosaccharomyces pombe* and mammalian cells (3, 4, 17, 18). The fact that mammals, *S. cerevisiae*, and *S. pombe* use similar MAP kinase pathways to respond to arsenite indicates that the study of arsenite toxicity in those two yeasts can also render interesting results for the knowledge of the biochemical mechanisms of action of arsenite in humans.

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