

Saccharomyces cerevisiae Hog1 Protein Phosphorylation upon Exposure to Bacterial Endotoxin*

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The yeast Hog1 protein is both functionally and structurally similar to the mammalian p38, belonging to the same family of mitogen-activated protein (MAP) kinases and responding to extracellular changes in osmolarity. Since p38 mediates lipopolysaccharide (LPS) effects in mammalian cells, we now tested the responsiveness of Hog1 upon exposure of the yeast *Saccharomyces cerevisiae* to bacterial LPS. In the presence of *Escherichia coli* LPS (100 ng/ml) and an endotoxically active, hexaacylated, synthetic lipid A (compound 506; 100 ng/ml), Hog1 becomes phosphorylated with a maximum of phosphorylation between 3 and 6 h, whereas a tetraacylated, inactive form of lipid A (compound 406) did not cause any modification in the phosphorylation state of Hog1. A triple labeling immunocytochemical study showed that phosphorylated Hog1 translocates into the nucleus after a 90-min incubation and becomes sparsely located in the cytoplasm. The translocation of the phospho-Hog1 is preceded by an increased expression of the *HOG1* gene and concomitant with the expression of the Hog1 target gene, *GPD1*. We also observed that cells unable to synthesize Hog1 do not resist LPS as efficiently as wild-type cells. We conclude that the yeast *S. cerevisiae* is able to respond to the presence of Gram-negative bacteria endotoxin and that Hog1 is involved in this response.

In response to stress, cells have developed a variety of mechanisms to give a specific and adaptive response. The stress responses rely on the recognition of the specific stress through a cellular sensor followed by a signal transduction pathway that often involves one or more chains of enzymatic activities, leading to several coordinated intracellular actions, which ultimately results in a modification of gene expression and in the production of proteins aimed to alleviate the consequences of stress. The cellular response to stress generally involves mitogen-activated protein kinase (MAPK)² cascades, which are common and highly conserved signaling modules found in both higher and lower eukaryotic cells (1). A prototype of the stress-activated protein kinases (SAPK) family is the yeast Hog1 MAP

kinase, which specifically responds to increased extracellular osmolarity and is essential for cell survival under these conditions (Ref. 2, and for review, see Refs. 3 and 4). *Saccharomyces cerevisiae* detects and responds to high extracellular osmolarity through the high osmolarity glycerol (HOG) MAPK pathway (5, 6). Upon phosphorylation by the dual specific kinase Pbs2, Hog1 is translocated into the nucleus (7, 8), where it directly targets several transcription factors leading to changes of gene expression (9–13). A portion of activated Hog1 is apparently also present in the cytoplasm to mediate post-transcriptional and translational effects (14–16), to achieve osmoadaptation. Lately, it was described that Hog1 modulates the activity of membrane transporters, *i.e.* acting directly in cytoplasmic targets (17). Until recently, it was generally accepted that Hog1 was just involved in the response to increased extracellular osmolarity, but recent reports showed that it is also activated by heat stress (18), oxidative stress (16, 19, 20), and citric acid stress (21).

The Hog1 homologue in mammalian cells is p38, which is structurally related, sharing a Thr-Gly-Tyr (TGY) dual phosphorylation motif (22). Furthermore, p38 has the ability to rescue a *S. cerevisiae* *hog1Δ* mutant in a hyperosmotic environment (22, 23) or under oxidative stress (19). There is also a functional homology between yeast Hog1 and p38 since p38 is a central mediator of the hyperosmotic (Ref. 24, and for review, see Ref. 25) and of the oxidative (26, 27) stress response in mammalian cells. It has also been reported that the human MTK1 can complement the MAPKKK mutants, *ssk2Δ ssk22Δ* (28).

However, it is still unknown whether the functional homology between Hog1 and p38, in *S. cerevisiae*, can be extended to other stress situations besides osmolarity and oxidative stress. In particular, it is well established that one of the main roles of p38 is the regulation of the inflammatory response (27, 29). The prototypical activator of cells of the immune and inflammatory systems is lipopolysaccharide (LPS), the component of the outer leaflet of the cell wall of Gram-negative bacteria. LPS activates macrophages, a pivotal cell type in LPS response, triggering the production and release of proinflammatory cytokines and nitric oxide (29) through the activation of the p38 kinase pathway (30, 31). The inflammatory activity of LPS is due to the lipid moiety of the molecule, termed lipid A, and usually referred as the bacterial endotoxin. Situations where lipid A, an amphiphilic compound, is exposed result in the immunostimulation of the host (31). Thus, the present study was designed to test whether the p38 MAP kinase yeast homologue, Hog1, is

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² The abbreviations used are: MAPK, MAP kinase; MAP, mitogen-activated protein; MAPKKK, MAPK kinase kinase; SAPK, stress-activated protein kinases; HOG pathway, high osmolarity glycerol pathway; LPS, bacterial lipopolysaccharide; PBS, phosphate-buffered saline.

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also activated upon exposure of *S. cerevisiae* to bacterial endotoxin.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—The *S. cerevisiae* strains used in this study were wild-type W303-1A (Mata *ade 2-1 can 1-100 his 2-11,15 leu 2-3,112 trp1-1 ura3-1*), provided by Professor Johan Thevelein (Katholieke University of Leuven, Belgium), and its derivative YSH 444 (*hog1Δ:: TRP1*) mutant, obtained from Professor Stefan Hohmann (32). Yeast cells were grown at 30 °C in yeast nitrogen base (YNB) medium supplemented with the appropriate selective amino acids and bases and 2% glucose. Growth was assessed by measuring the optical density at a wavelength of 640 nm ($A_{640\text{ nm}}$). The cells were grown to midlog phase, divided, and incubated with 100 ng/ml (33) *Escherichia coli* LPS (Sigma), lipid A compound 506 or 406 (Peptides International, Louisville, KY) or with NaCl 0.8 M (as a control) (34).

Western Blot Analysis—Protein extracts were obtained using the urea/SDS method as described previously (35). Samples were boiled at 95 °C for 10 min and then separated by 10% SDS-polyacrylamide gel electrophoresis. The proteins were transferred onto polyvinylidene difluoride membranes (0.45 μm , from Amersham Biosciences), which were blocked for 1 h at room temperature in Tris-buffered saline medium (200 mM Tris and 150 mM NaCl, pH 7.6) containing 0.1% Tween 20 (TBS-T) with 5% bovine serum albumin. Dual phosphorylation of Hog1 was detected using a rabbit antibody against the dually phosphorylated (Thr-174 and Tyr-176) form of p38 (Cell Signaling), 1:250 in TBS-T with 3% bovine serum albumin in which the membranes were incubated overnight at 4 °C. After three washing periods with TBS-T, the membranes were incubated with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Amersham Biosciences; 1:10,000) during 1 h at room temperature. After washing three times with TBS-T and incubation with enhanced chemifluorescence (ECF, Amersham Biosciences) for 5 min, the membranes were analyzed using the VersaDoc 3000 (Bio-Rad). The membranes were then stripped and reprobed for total Hog1 protein. Briefly, the membranes were incubated for 1 h at room temperature with 0.1 M glycine, pH 2.3, to strip the previous antibodies and probed using a goat anti-C-terminal Hog1p antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:500) as described above, using an alkaline phosphatase-conjugated anti-goat secondary antibody (Calbiochem, UK; 1:5,000).

Immunocytochemistry—10 ml of cells were harvested by centrifugation, resuspended in 4% paraformaldehyde at room temperature for 15 min, and washed twice with PBS (140 mM NaCl, 3 mM KCl, 20 mM NaH_2PO_4 , 15 mM KH_2PO_4 , pH 7.4). Cells were spheroplasted by resuspension in phosphate buffer (1.2 M sorbitol, 100 mM KH_2PO_4 , pH 7.5), with 0.17% β -mercaptoethanol and 1 mg/ml zymolyase 100T (Seikagaku, Japan) and incubated at 30 °C with gentle shaking for 45 min. Spheroplasts were washed once with PBS and resuspended in phosphate buffer. Then, the spheroplasts were added to the coverslips, previously coated with poly-L-lysine, allowed to adhere for 1 h at 37 °C, and blocked with PBS with 3% bovine serum albumin and 0.1% Tween 20 (blocking solution) for 30 min. The spheroplasts

were then incubated for 2 h with the primary antibodies, rabbit anti-phospho-p38 MAP kinase (Cell Signaling; 1:100) and goat anti-Hog1 (Santa Cruz Biotechnology; 1:400), prepared in the blocking solution. The coverslips were washed three times with PBS and incubated, in the dark, for 2 h, with the secondary antibodies (Alexa Fluor 488-labeled donkey anti-rabbit and Alexa Fluor 594-labeled donkey anti-goat antibodies, Molecular Probes). As a control, spheroplasts were incubated only with secondary antibodies, without previous incubation with primary antibodies. The coverslips were then washed twice with PBS and incubated with 15 $\mu\text{g}/\mu\text{l}$ Hoechst 33342 (Molecular Probes) for 5 min, washed three times with PBS, and mounted on Prolong antifade (Molecular Probes). The coverslips were analyzed on a Zeiss Axiovert 200 inverted fluorescence microscope, with a $\times 100$ objective equipped with a cooled CCD camera (Roper Scientific, Tucson, AZ) and analyzed with MetaFluor 5.0 software. The final figures were prepared using Paint Shop Pro 7 without further manipulation.

Gene Expression—Total mRNA was extracted from yeast cells (5×10^7 cells/ml) in control conditions (no stress) or after exposure to 100 ng/ml LPS, using an RNeasy mini kit (Qiagen, Izasa, Portugal) according to the manufacturer's instructions for yeast cells. mRNA samples (3 μg) were reverse transcribed using Transcriptor first-strand cDNA synthesis kit for reverse transcription-PCR (Roche Diagnostics, Lisbon, Portugal). After denaturation at 65 °C for 10 min in a Whatman Biometra thermocycler, in the presence of 60 μM random hexamer primer, mRNA samples were incubated with 20 units of Protector RNase inhibitor, 1 mM dNTPs, and 10 units of transcriptor reverse transcriptase, at 25 °C for 10 min and 55 °C for 30 min.

The real-time PCR reactions were performed in a LightCycler 2.0 (Roche Diagnostics), using a LightCycler® Fast Start DNA MasterPlus SYBR Green I kit (Roche Diagnostics). The PCR reaction was based on the SYBRGreen method, according to the manufacturer's instructions, which relies on primer specificity and requires no detection probes. The primers used in the real-time LC protocols were for *HOG1*, forward primer 5'-GATGCCTTGGCTCATCCTTA-3' and reverse primer 5'-ACTGTATGGCCTGGTTACCG-3'; for *GPD1*, forward primer 5'-TTTTTGCCCCGATCTGTAGC-3' and reverse primer 5'-CGTTGTTACCCAGCCTAGA-3'; and for the reference gene *18S*, forward primer 5'-CGGCTACCACATCAAGGAA-3' and reverse primer 5'-GCTGGAATTACCGCGGCT-3'. These primers were designed using OLIGO6® software (Cambio, Cambridge, UK). The selected sets of primers were checked against GenBank™ using BLAST to confirm their gene specificity. Every test run included RNase-free water as a negative control. The amplification and detection were performed using the following parameters: a preincubation at 95 °C for 10 min for FastStart TaqDNA polymerase activation followed by 45 cycles of amplification: denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension at 72 °C for 17 s. After the 45 cycles, melting curve analysis was performed to confirm product specificity. The melting curve analysis was achieved by heating the mixture up to 95 °C and then cooling to 65 °C for 15 s and then slowly (0.1 °C/s) heating back to 95 °C. Raw data were analyzed by relative quantification using the $2^{-\Delta\text{CT}}$ method (36).

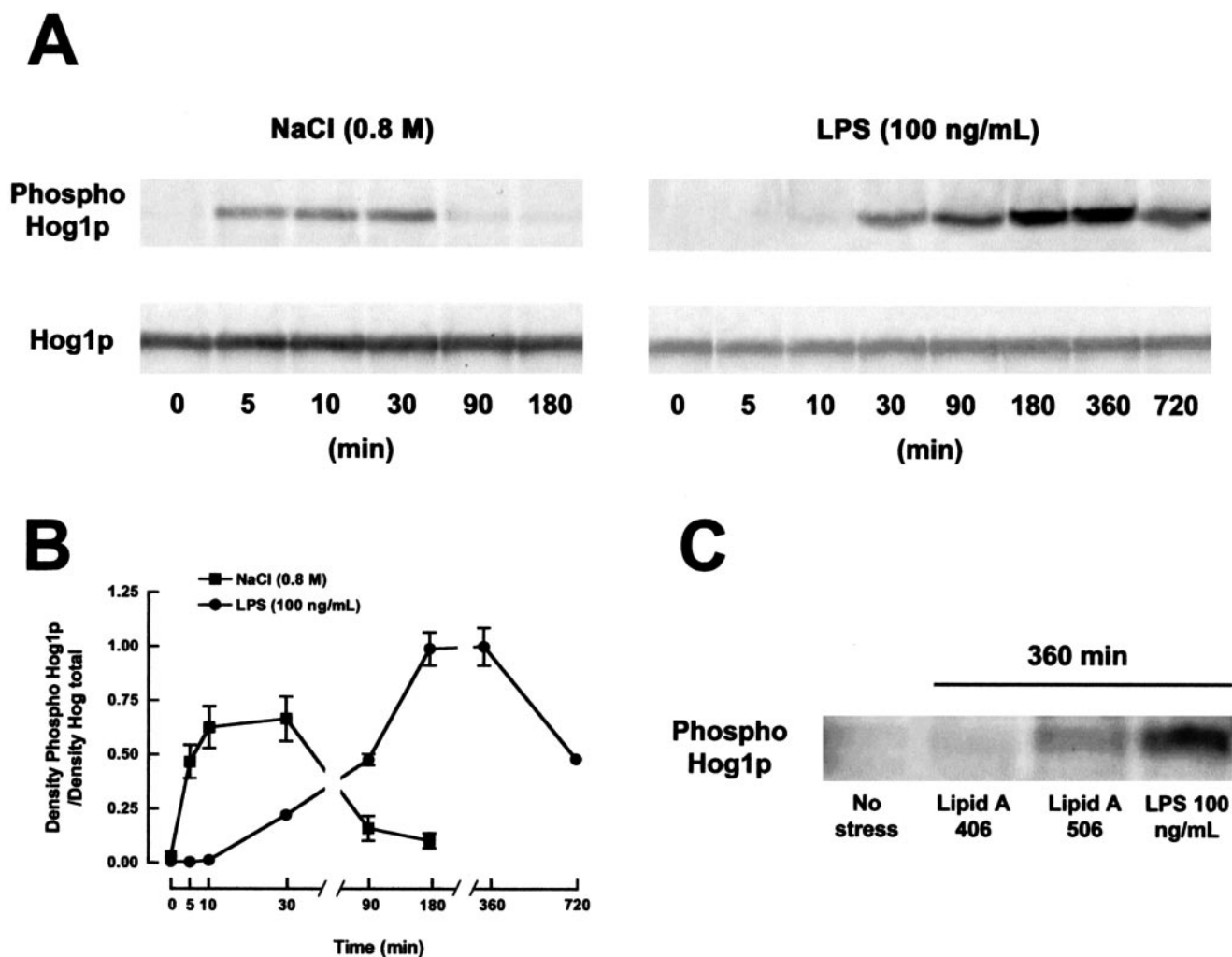


FIGURE 1. Hog1 protein is phosphorylated upon LPS exposure. *A*, representative Western blot showing the dual phosphorylation (*Phospho*) of Hog1 upon hyperosmotic stress (positive control blots on the *left*) and LPS exposure (blots on the *right*). Soluble protein extracts were prepared from cells incubated with 0.8 M NaCl and cells incubated with 100 ng/ml LPS at the time points indicated in the figure. The dually phosphorylated form of Hog1 was detected using a previously validated anti-phospho-p38 antibody. The same Western blot membranes were reprobbed against total Hog1 (*lower blots*), using a polyclonal anti-C-terminal Hog1 antibody, to calculate the ratio of density phospho-Hog1/density Hog1. *B*, illustrates the different time course phosphorylation of Hog1 in cells exposed to osmotic stress when compared with those exposed to LPS. The incubation with compound 506 (a proinflammatory, hexaacetylated, synthetic *E. coli* like-lipid A) or with a commercial *E. coli* LPS (100 ng/ml) followed the same kinetics (results not shown), with a maximum of phosphorylation at 360 min of incubation (*C*), whereas compound 406 (the inactive, tetraacylated, form of lipid A) did not cause any modification. The values are shown as the means \pm S.E. of three different Western blots from three different experiments.

Viability Assay—The sensitivity to LPS was evaluated in pregrown cells at 30 °C until an $A_{640\text{ nm}}$ of 0.8–1. The cells were then divided and incubated without or with 100 ng/ml LPS or, as a control, with 0.8 M NaCl. To quantify the number of viable cells during the incubation period, 100- μ l aliquots were taken and plated into Petri dishes. The number of colonies was counted after 3 days at 30 °C. Under these conditions, each colony represents a viable cell. For the spotting experiments, 10-fold serial dilutions of an exponentially growing culture were spotted onto YNB agar plates supplemented without or with 100 ng/ml LPS or as a control, with 0.8 M NaCl. Plates were incubated 48 h at 30 °C before being photographed.

RESULTS

Hog1 Protein Is Phosphorylated upon Exposure to Bacterial Lipopolysaccharide—To determine whether the exposure of yeast cells to bacterial LPS results in the activation of Hog1

protein, we quantified the activation of Hog1 by measuring the formation of its activated form, the dually phosphorylated form (Thr-174/Tyr-176) of Hog1, using a selective antibody (34). Phosphorylation of Hog1 was investigated by Western blot analysis of cells incubated with 100 ng/ml LPS, a concentration able to trigger p38 activation/phosphorylation in human neutrophils (33). After 30 min of incubation with LPS, it was possible to detect the phosphorylated form of Hog1 (Fig. 1A). The levels of phosphorylated Hog1 then increased continuously up to 180–360 min (Fig. 1A), when the maximal phosphorylation was observed. The levels of phosphorylated Hog1 continuously decreased up to 12 h of incubation with LPS (Fig. 1A). As a positive control, we evaluated the degree of phosphorylation of Hog1 under osmotic stress (0.8 M NaCl) (34). As observed in previous studies (34), after 5 min of exposure to 0.8 M NaCl, Hog1 became phosphorylated, reaching a transient maximal phosphorylation at 10–30 min and remaining detectable until

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90 min (Fig. 1A). To ascertain that the increase in phosphorylated Hog1 levels does not represent a possible increase in Hog1 expression but rather of its phosphorylation, we followed the total levels of Hog1. As illustrated in the reprobed membranes using an anti-C-terminal Hog1p antibody, we found an increase in the density of Hog1 upon exposure to LPS (Fig. 1A). Thus, we calculated the ratio between the relative density of phosphorylated Hog1 and total Hog1 for the cells exposed to LPS or NaCl 0.8 M (Fig. 1B) to adequately compare the level of phosphorylation induced by hyperosmolarity and by LPS, irrespective of changes in the total levels of Hog1. As illustrated in Fig. 1B, hyperosmolarity induced a faster Hog1 phosphorylation (reaching a maximum of phosphorylation at 10–30 min) than that observed upon incubation with LPS (reaching a maximum of phosphorylation at 180–360 min), which displayed a delay of 30 min. This ratio analysis also indicated that the degree of phosphorylation was higher in cells exposed to LPS than in cells incubated with NaCl 0.8 M (Fig. 1B). Regardless of the phosphorylation kinetics, taken together, these data clearly show that LPS exposure induces Hog1 phosphorylation.

Since there are reports showing that commercial LPS contains several bioactive contaminants (37, 38) that could confound the interpretation of our observed LPS-induced effects, we went on to study whether lipid A (the LPS component that ultimately binds the TLR4 receptor and induces activation of p38) is able to stimulate, in yeast, the phosphorylation of Hog1p. We used two different kinds of lipid A, a proinflammatory, hexaacylated, synthetic *E. coli*-like lipid A, the compound 506 (39, 40) and a tetraacylated lipid A that antagonizes the inflammatory response, the compound 406, which is endotoxically inactive (41–43). We observed that only compound 506, but not its endotoxically inactive analogue, compound 406, results in an active phosphorylation of the Hog1 protein (Fig. 1C). The methodology used by us in the preparation of suspensions of both forms of lipid A results in the formation of pure aggregates of each form of lipid A, according to the results described by Mueller *et al.* (39). Also, according to these authors, the stimulation of mononuclear cells with pure aggregates results in a lower production of tumor necrosis factor- α , *i.e.* activation, whereas a mixture of several compounds results in a higher stimulation, a result mimicked by suspensions of natural LPS. In our experiments, we also found that incubation with similar concentrations of compound 506 and *E. coli* LPS, during 6 h, results in a lower level of Hog1 phosphorylation in the presence of the synthetic lipid A (Fig. 1C).

The difference between the two forms of lipid A now described by us also excludes the hypothesis that the effect observed upon exposure to LPS or lipid A could be due to a disturbance of the cell membrane by hydrophobic substances since the biologically inactive synthetic lipid A compound 406 does not induce Hog1 phosphorylation. Besides, compound 406 incorporates more efficiently with phospholipid cell membranes than other amphiphilic substances, a process mediated by the LPS-binding protein (40). Thus, these findings suggest that the effects triggered by LPS are not due to a nonspecific but are likely to be receptor-mediated, although this still remains to be fully established.

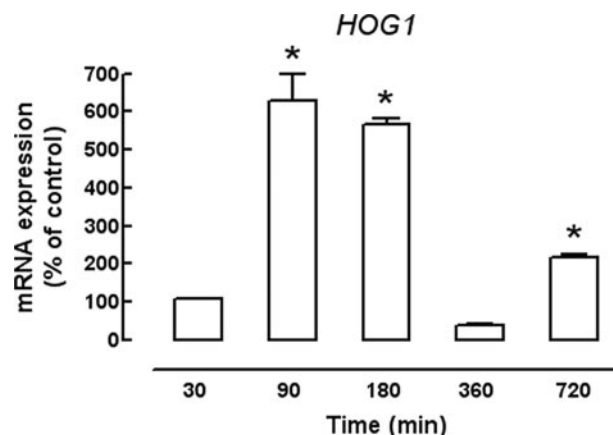


FIGURE 2. **HOG1 mRNA levels are up-regulated upon LPS exposure.** Yeast cells were exposed to 100 ng/ml LPS during 12 h. Samples were taken at 30, 90, 180, 360, and 720 min of incubation, and total mRNA was extracted and reverse-transcribed to cDNA. The variation of *HOG1* mRNA levels was assayed by SYBR Green real-time PCR in reactions normalized to contain initial equal amounts of cDNA using the reference gene *18S*. *HOG1* mRNA levels were maximally increased 6-fold at 90 min, returning to control levels at 360 min of LPS exposure. The results are mean \pm S.E. in percentage of control (set as 100%) of three experiments from three independent preparations. *, $p < 0.01$ versus control (100%).

Expression of HOG1 Gene Is Increased upon Exposure to LPS—The observation that the total levels of Hog1 increased during exposure to LPS (Fig. 1A, right panel) led us to study whether LPS modified the relative levels of expression of the *HOG1* gene. After a 90-min exposure to 100 ng/ml LPS, the levels of *HOG1* mRNA were increased 6-fold when compared with the control (without incubation with LPS), as evaluated by SYBR Green real-time PCR (Fig. 2). This increased level of expression of *HOG1* was maintained until a 3-h incubation with LPS (Fig. 2). However, at 6 h of incubation, the relative expression of this gene decreased to control levels (Fig. 2). These results show that upon LPS exposure, in addition to an increase in Hog1 phosphorylation, there is an up-regulation of the expression of *HOG1*. Furthermore, the comparison of the kinetics of the LPS-induced changes in *HOG1* mRNA and of phosphorylated Hog1 indicates that the up-regulation of *HOG1* mRNA levels precedes the maximal levels of phosphorylated Hog1.

Hog1p Is Translocated to the Nucleus upon LPS-induced Phosphorylation—Upon activation by hyperosmotic stress, Hog1 translocates into the nucleus, from where it coordinates the expression of numerous genes (7, 8), controlling the activity of specific transcriptional activators and repressors (9, 10, 43–45). Therefore, we decided to examine whether the Hog1 protein is translocated to the nucleus upon exposure to LPS. To monitor the subcellular localization of the inactive/dephosphorylated and the active/phosphorylated forms of Hog1 protein, we performed a triple labeling immunocytochemical assay. We labeled phosphorylated Hog1 (green) and total Hog1 (red) using the same antibodies used in the Western blot analysis. To evaluate the nuclear localization of the phosphorylated form of the Hog1 protein, we labeled yeast genomic DNA using the fluorescence probe Hoechst 33342 (blue). Triple co-localization (white) shows the presence of phosphorylated Hog1 in the nucleus. We performed this immunocytochemical assay in unstressed yeast cells, in yeast cells exposed to 100 ng/ml LPS

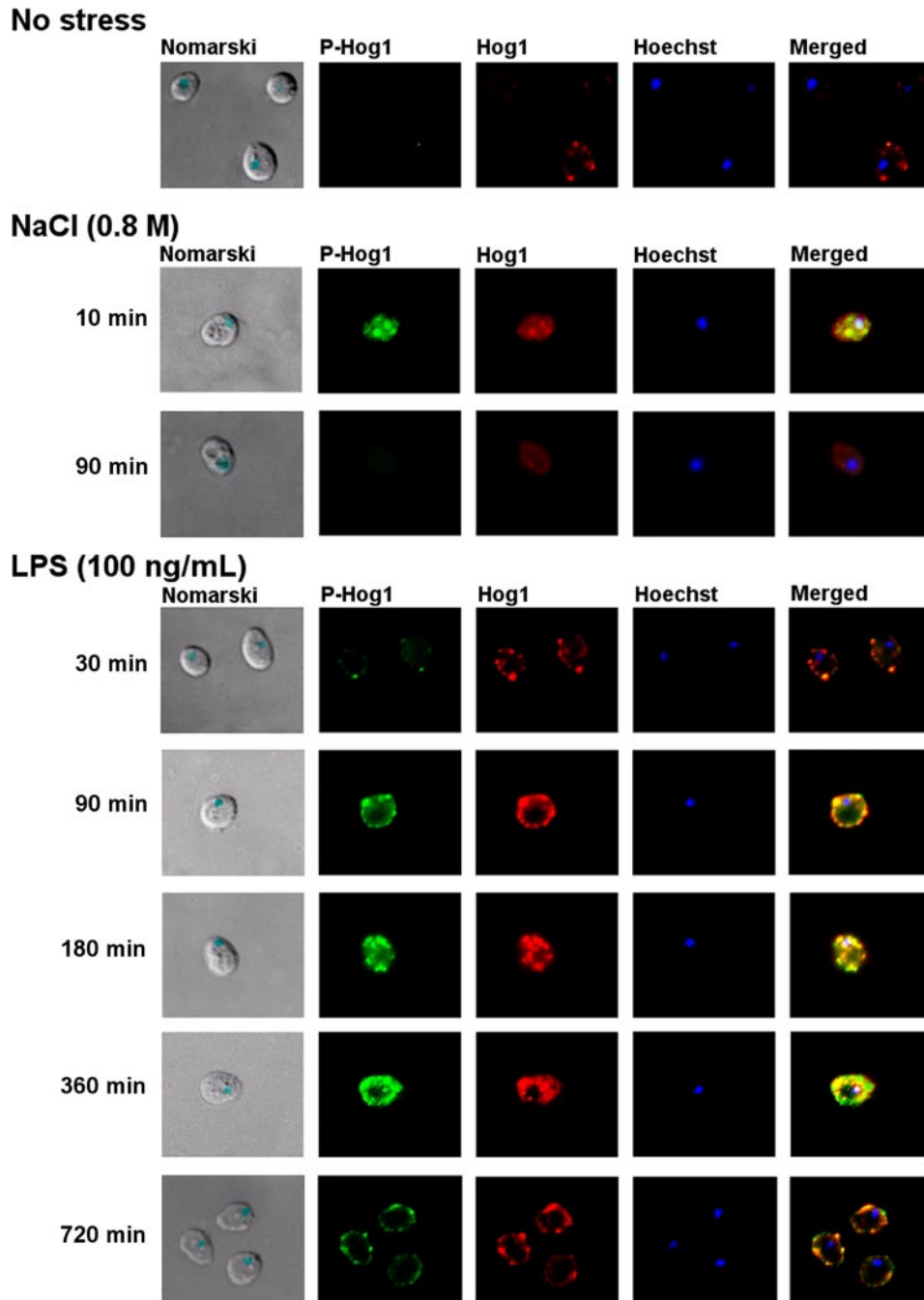


FIGURE 3. LPS exposure triggers a transient phosphorylation of Hog1 protein and its translocation to the nucleus. A triple labeling immunocytochemical assay was performed in control cells (*No stress*), in cells subjected to high osmolarity (*i.e.* exposed to 0.8 M NaCl), and in cells exposed to 100 ng/ml LPS for the time periods indicated. The immunocytochemical identification of phosphorylated Hog1 (*p-Hog1*, second column of panels from the left; stained green) was achieved using a selective antibody against the dually phosphorylated form of p38 MAP kinase. The immunocytochemical identification of Hog1 was achieved using a polyclonal antibody against the C-terminal of Hog1 protein (*middle column* of panels; stained red), and for the identification of the cell nucleus, yeast genomic DNA was labeled using the fluorescence probe Hoechst 33342 (*second column* of panels from the right; stained blue). Merged images (*right panels*) illustrate that there is a nuclear localization in addition to a cytosolic localization of phosphorylated Hog1 at 90 min, 3 h, and 6 h of incubation with LPS (triple co-localization yielding white). Note that LPS exposure only triggered the nuclear localization of the phosphorylated form of Hog1 after 30 min with a maximal effect at 3–6 h, whereas high osmolarity induced localization of phospho-Hog1 peaked at 10 min (maintained until a 30-min incubation, results not shown) and disappeared at 3 h. The images shown are representative of five experiments producing qualitatively identical results.

for different time periods, or in yeast cells exposed to 0.8 M NaCl, as a positive control (Fig. 3).

As described previously for hyperosmotic stress conditions (7) and now demonstrated by us using an immunocytochemis-

try assay with specific antibodies for both total and phospho-Hog1, after phosphorylation, Hog1 transiently accumulates in the nucleus, with a time course matching its phosphorylation status (compare the effects of hyperosmotic stress in Figs. 1 and 3). Likewise, exposure to LPS (100 ng/ml) for 30 min led to an increase in the levels of the phosphorylated form of Hog1, which was solely located in the cytoplasm. After 90 min of incubation with LPS, the phosphorylated form of Hog1 was predominantly localized in the cytoplasm, although there was already some phospho-Hog1 located in the nucleus (Fig. 3). Only between 3 and 6 h of incubation with LPS was it possible to find a clear accumulation of phosphorylated Hog1 protein in the nucleus, albeit some phosphorylated Hog1 remained in the cytoplasm. After 12 h of incubation with LPS, the levels of phosphorylated Hog1 decreased, in agreement with the data obtained in the Western blot analysis (Fig. 1). The remaining phosphorylated Hog1 was restricted to the cytoplasm, which indicates that its nuclear localization is a transient event. This suggests that upon exposure to LPS, Hog1 is translocated into the nucleus in a transient manner that correlates with its time course of phosphorylation, as observed in yeasts under hyperosmotic stress.

Exposure to LPS Increases the Expression of GPD1—One of the main strategies used by yeasts in osmoadaptation is the production and accumulation of the compatible osmolyte, glycerol (reviewed in Ref. 4). To achieve it, there is an increased expression of genes encoding enzymes in the glycerol production pathway. One of these genes is *GPD1*, which encodes glycerol-3-phosphate dehydrogenase. The HOG pathway plays a central, albeit not exclusive, role in osmotic induction of *GPD1* expression (10, 46). Following our observation that

Hog1 is phosphorylated in the presence of LPS, we decided to evaluate whether *GPD1* expression is also increased upon LPS exposure. For that purpose, total mRNA was extracted from yeast cells and reverse-transcribed to cDNA. The variation of

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GPD1 mRNA levels were assayed by SYBR Green real-time PCR in reactions normalized to contain initial equal amounts of cDNA using the reference gene *18S*. After a 90-min exposure to LPS, the expression of *GPD1* was increased 6-fold, reaching a maximal increase of 9-fold at 3 h of incubation when compared with the control (no LPS) (Fig. 4). After 6 h of incubation with

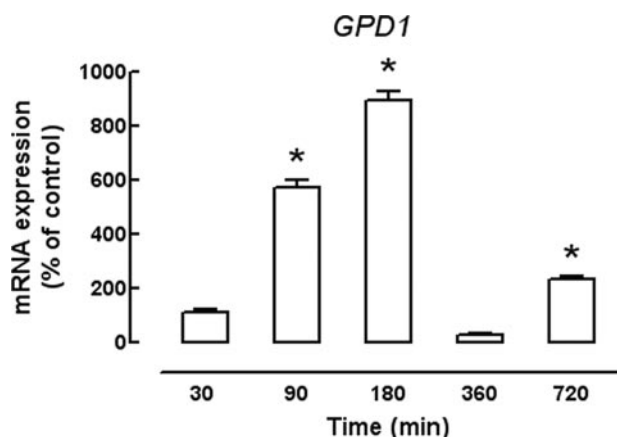


FIGURE 4. ***GPD1* mRNA levels are up-regulated upon LPS exposure.** Yeast cells were exposed to 100 ng/ml LPS during 12 h. Samples were taken at 30, 90, 180, 360, and 720 min of incubation, and total mRNA was extracted and reverse-transcribed to cDNA. The variation of *GPD1* mRNA levels was assayed by SYBR Green real-time PCR in reactions normalized to contain initial equal amounts of cDNA using the reference gene *18S*. *GPD1* mRNA level up-regulation was evident at 90 min and reached its maximum at 180 min of incubation with LPS. The results are mean \pm S.E. in percentage of control (set as 100%) of three experiments from three independent preparations. *, $p < 0.01$ versus control (100%).

LPS, the *GPD1* mRNA levels were lower and similar to the control levels. The time course of LPS-induced increase of *GPD1* mRNA levels matches the LPS-induced increase in Hog1 phosphorylation and translocation to the nucleus (90 min–3 h) (compare the time course of LPS effects in Figs. 3 and 4), meaning that immediately after being translocated to the nucleus, phospho-Hog1 controls the initiation of transcription of *GPD1* mRNA. This strongly suggests that upon LPS exposure, Hog1 is not only phosphorylated and translocated to the nucleus but is also in a catalytically active state, up-regulating *GPD1* mRNA levels.

***hog1*Δ Mutant Is LPS-sensitive**—To evaluate whether LPS exposure affects the growth of yeast cells and whether the Hog1 protein is involved in this effect, serial dilutions of mid-exponential-phase cultures of wild-type *S. cerevisiae* and *hog1*Δ mutant were spotted onto YNB agar plates in the presence of 100 ng/ml LPS. Growth of wild-type *S. cerevisiae* was not affected either in the presence of 100 ng/ml LPS or in the presence of 0.8 M NaCl. As a control, it was confirmed that the growth of *hog1*Δ mutant was inhibited when this strain was spotted onto 0.8 M NaCl-YNB agar plates (Fig. 5A), according to the key role of Hog1 in osmoadaptation (2). Likewise, the growth of *hog1*Δ mutant was also inhibited upon exposure to 100 ng/ml LPS (Fig. 5A). In a viability assay, pregrown yeast cells, wild-type and *hog1*Δ mutant, were incubated in YNB medium without or with 100 ng/ml LPS. Although the viability of the wild-type strain was not affected during the 12-h incubation period (Fig. 5B), the capacity of growth of the *hog1*Δ mutant decreased during this period (Fig. 5B). These results clearly suggest that the Hog1 protein plays a key role in the response to stress caused by exposure to bacterial lipopolysaccharide.

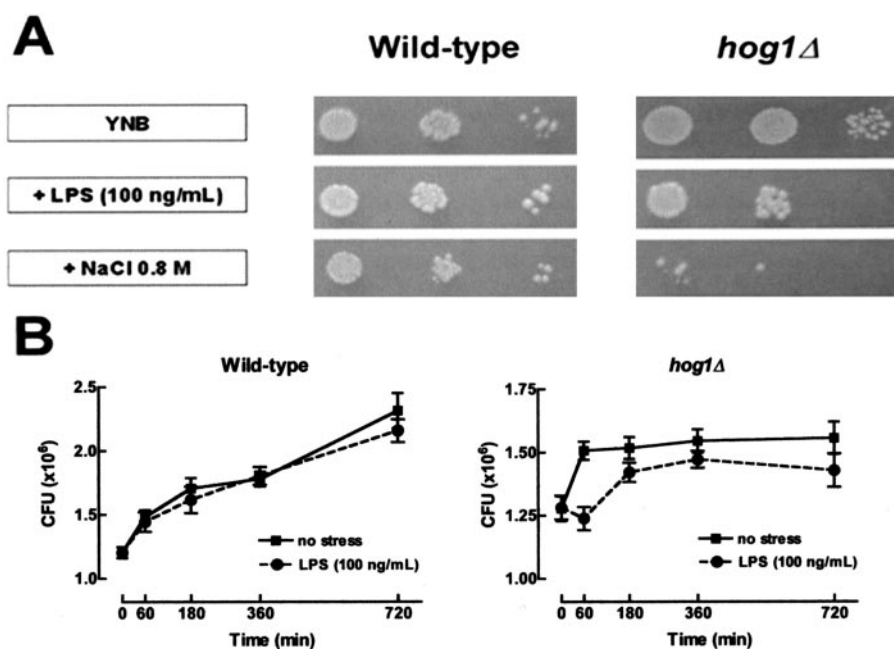


FIGURE 5. **LPS exposure inhibits the growth of *hog1*Δ mutant.** By using a spotting assay (A), the growth pattern of *S. cerevisiae* W303 and *hog1*Δ mutant was evaluated in control conditions (YNB medium) and in the presence of 100 ng/ml LPS or in the presence of 0.8 M NaCl. W303 growth was not modified either in the presence of LPS or in hyperosmolar medium (left photographs). In contrast, *hog1*Δ displayed LPS-sensitivity, although at a lesser extent when compared with that observed in hyperosmolarity stress. The photographs displayed are representative of five experiments yielding qualitatively identical results. The viability of the two strains to LPS (B) was assessed in cells pregrown at 30 °C until an $A_{640\text{nm}}$ of 0.8–1.0 and resuspended in the same medium without or with 100 ng/ml LPS. At the time points indicated, 100- μ l aliquots were taken and spread into Petri dishes. After 3 days at 30 °C, the number of colonies was counted. The results are mean \pm S.E. of five independent experiments.

DISCUSSION

Previous studies focusing in the response to extracellular changes in osmolarity showed a physiological analogy between mammalian p38 stress kinase and the yeast Hog1 protein, as expected from their extensive sequence homology (22). The present study extends the physiological homology between yeast Hog1 and mammalian p38 protein to the context of the response to bacterial endotoxin, a prototypical trigger of inflammatory responses in mammalian systems (29). Thus, we report that *S. cerevisiae* respond to LPS exposure by increasing the expression and the degree of phosphorylation of the Hog1 protein, an effect mimicked by an endotoxically active synthetic lipid A but not by a chemically similar but biologically inactive synthetic lipid A. Once phos-

phorylated, Hog1 is then translocated into the nucleus. Furthermore, we showed that Hog1 plays a key role for the yeast adaptative response to LPS challenge since the growth of a *hog1*Δ mutant was inhibited by exposure to LPS in contrast to a wild strain. These results show that, as occurred for hyperosmotic stress, there is a functional analogy between yeast Hog1 and the mammalian p38 in response to LPS exposure.

Hog1, a prototype member of the stress-activated protein kinase family, is constitutively expressed in yeast where it plays a key role in the adaptative response to different stressful conditions such as osmotic (2), oxidative (16, 19, 20), heat (18), or citric acid stress (21). The exposure of *S. cerevisiae* to high osmolarity (reviewed in Ref. 4) leads to a rapid (less than 30 min) and sustained (up to several hours depending on the severity of the hyperosmotic conditions) phosphorylation of the Hog1 protein. Upon phosphorylation, Hog1 is translocated into the nucleus (7, 8), where it directly targets several transcription factors leading to a change of gene expression (9, 10, 13). In particular, there is an increased expression of the enzymes required for the synthesis of a major osmolyte, glycerol, especially *GPD1*, the gene that codes for glycerol-3-phosphate dehydrogenase (5). Interestingly, a portion of the activated Hog1 protein is apparently also present in the cytoplasm to mediate post-transcriptional and translational effects involved in osmoadaptation (14, 15). We now confirmed the key steps of Hog1 activation and nuclear translocation in yeast cells exposed to 0.8 M NaCl. Most importantly, we report for the first time that the exposure of yeast cells to LPS also engages the phosphorylation of Hog1, its translocation to the nucleus, and the activation of *GPD1* gene expression. However, the kinetics of the LPS-induced Hog1 phosphorylation and translocation into the nucleus was slower than that induced by osmotic stress. In fact, whereas the osmotic stress-induced Hog1 phosphorylation peaked after 10–30 min, the LPS-induced Hog1 phosphorylation peaked at 180–360 min. Likewise, the osmotic stress induced translocation of phosphorylated Hog1 into the nucleus peaked at 10 min, whereas that caused by LPS peaked at 180–360 min. It has been described that several concentrations and/or different osmotically active substances have different kinetics of phosphorylation, subcellular translocation, and activation of gene expression (8, 10, 33, 47). However, this delay in the recruitment of the Hog1 by LPS may also be related to the observed ability of LPS to enhance the density of total Hog1 as a likely result of the increased levels of *HOG1* mRNA. Nevertheless, apart from this kinetic difference, the stress response to high osmolarity and to LPS exposure in yeast appears to follow a similar Hog1-dependent pathway. In fact, in contrast to other types of stressful conditions (exposure to heat, to weak organic acids, or to ethanol) that failed to induce the import of phosphorylated Hog1 into the nucleus (7, 8), we now found that LPS effectively triggers the translocation of phosphorylated Hog1 protein into the nucleus. This was directly confirmed by immunocytochemistry using a previously validated antibody against phosphorylated Hog1 in yeast (34) as well as by the ability of LPS to increase the expression of *GPD1*, which is regulated by the presence of phosphorylated Hog1 in the

nucleus (32, 48). Recently, it was described that Hog1, activated by oxidative stress, translocates to the nucleus at a lower extent than the observed under hyperosmolarity, with a phosphorylation peak at 45 min (16).

We also observed that the responses to LPS and high osmolarity were transient, suggesting that the Hog1 pathway might be controlled by feedback mechanisms, like dephosphorylation by Ser/Thr phosphatases (49) and by Tyr phosphatases (50), as suggested by others (for a review, see Ref. 4). We now observed that after 6 h of incubation with LPS, all the nuclear Hog1 shifts to the cytoplasm. Surprisingly, part of this cytoplasmic protein remains phosphorylated, even 12 h after the beginning of the exposure to LPS. This leads us to the conclusion that the control mechanisms of the HOG pathway in response to LPS exposure are different from those observed under other stress situations and confirms the hypothesis that the nuclear export of phospho-Hog1 is not strictly dependent on its dephosphorylation (51) and that, upon exposure to LPS, phosphorylated Hog1 is involved in cytoplasmic processes, kept active while LPS is present. Likewise, in *Debaryomyces hansenii*, it was observed that, under extreme osmotic conditions, Hog1 is maintained phosphorylated in the cytoplasm, and the authors related this fact as a need to resist a continuous aggression (52).

Some of the differences found between the recruitment of the HOG pathway by high osmolarity and by LPS may be related to the initial sensing devices engaged by the two types of stress conditions. In fact, the molecular determinant of the yeast high osmolarity sensor have been unraveled as two independent branch sensors, one being the “two-component” osmo-sensing complex Sln1-Ypd1-Ssk1 (53, 54) and the other being the membrane-bound protein Sho1 (55). In contrast, the pathways by which yeast signals the presence of LPS are unknown since this report provides the first demonstration that yeast can respond to LPS. In particular, it has not been defined whether yeasts are endowed with a receptor analogous to Toll-like receptors, which sense LPS in mammalian cells (31). However, the currently described ability of the hexaacylated lipid A but not of the tetraacylated lipid A to engage the Hog1 phosphorylation is highly suggestive of the involvement of a receptor since the former but not the later form of lipid A is an endotoxin acting through Toll-like receptors in mammalian cells (40). At least, the obtained results demonstrate that yeast possesses a specific mechanism to recognize for LPS (*i.e.* lipid A), similar to the one found in mammalian cells but not in *Drosophila* cells (56).

From an evolutionary point of view, this ability of yeast to sense bacterial LPS may be an important pathway for the optimization of yeast survival. In fact, yeast cells have to cope with various adverse conditions in their environment, including toxins from plant, fungi, and bacteria, which makes it logical that yeasts should have evolved mechanisms to sense and adapt to the presence of bacterial toxins. Particularly in situations of mixed infections where yeasts are both exposed to live and lysed bacteria (due to macrophage attack), such an LPS-induced/sensing mechanism would be an advantage for the survival of yeast. The presently observed inhibition of growth upon exposure to LPS in a *hog1*Δ mutant clearly shows that the recruitment of this HOG1-dependent pathway is crucial for the adap-

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tative response of yeast to LPS. Finally, the parallel between the responses of yeast Hog1 protein to LPS with the key role of mammalian p38 in inflammatory response opens the possibility of considering the use of yeasts as a basic model to study the signal transducing mechanisms underlying inflammatory responses at the cellular level and to assist in the search and screening of new potential anti-inflammatory drugs.

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