Stimulation of the yeast high osmolarity glycerol (HOG) pathway: evidence for a signal generated by a change in turgor rather than by water stress

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Abstract The *Saccharomyces cerevisiae* HOG pathway controls responses to osmotic shock such as production of the osmolyte glycerol. Here we show that the HOG pathway can be stimulated by addition of glycerol. This stimulation was strongly diminished in cells expressing an unregulated Fps1p glycerol channel, presumably because glycerol rapidly equilibrated across the plasma membrane. Ethanol, which passes the plasma membrane readily and causes water stress by disturbing the hydration of biomolecules, did not activate the HOG pathway. These observations suggest that stimulation of the HOG pathway is mediated by a turgor change and not by water stress per se.

Key words: Osmotic stress; High osmolarity glycerol pathway; Mitogen-activated protein kinase; Glycerol; Transmembrane transport

1. Introduction

The HOG (high osmolarity glycerol) pathway mediates a significant part of the response of yeast cells to a hyperosmotic shock [1–3], since it is required for the stimulated expression of more than 100 genes [4]. The osmo-induced genes include *GPD1* [5–8] and *GPP2* [7–10], which encode enzymes involved in the production of glycerol, the main osmolyte accumulated by yeast cells [11,12]. Increased intracellular glycerol levels are thought to counteract water loss by diminishing the water activity of the cytosol, thereby leading to water uptake [13–15].

Central to the HOG pathway is a mitogen-activated protein kinase (MAPK) cascade composed of three MAPKKK (Ssk2p, Ssk22p and Ste11p), a MAPKK (Pbs2p) and a MAPK (Hog1p) [1–3]. Upon stimulation, Hog1p is phosphorylated on T174 and Y176 and translocated into the nucleus [16–21]. Two branches upstream of Pbs2p are controlled by different plasma membrane proteins, Sho1p and Sln1p, which are thought to function as osmosensors [22,23]. Sho1p controls Pbs2p via Ste20p, Ste50p and Ste11p [23–25] while Sln1p passes a signal via Ypd1p, Ssk1p and Ssk2p/Ssk22p to Pbs2p [2,22,26,27].

While phosphotransfer and subcellular localisation of the signalling proteins have been intensively studied, relatively little is known about the physical parameters that stimulate the HOG pathway. Since sorbitol or NaCl can trigger Hog1p phosphorylation, the chemical nature of the osmoticum appears to be irrelevant [16,22]. However, any osmoticum causes at least two different effects, namely cell shrinkage [15] and a diminished water concentration in the cell [28]. Cell shrinkage, i.e. loss of turgor, is thought to twist the membrane bilayer [13], which alters the activity of certain plasma membrane proteins such as that of mechanosensitive channels [29,30]. Water loss affects hydration of biomolecules and leads to molecular crowding, thereby potentially altering protein conformation and activity. Unfolded proteins are known to stimulate cellular responses, such as the heat shock response [31,32]. We refer to these two types of effects caused by osmotic shock as turgor stress and water stress, respectively.

In this work we address the question whether turgor or water stress (or both) triggers the HOG pathway by using two different compounds, glycerol and ethanol. Ethanol is thought to pass the lipid bilayer readily and therefore it is not expected to cause turgor stress [32,33]. However, ethanol diminishes the water activity of the cytosol and partially replaces water, thereby disturbing the hydration of biomolecules and causing water stress [32,33]. Glycerol, on the other hand, is a compatible solute and hence does not cause water stress [34]. Glycerol does not seem to pass the lipid bilayer of the yeast plasma membrane readily, although this had been assumed for many years [35,36].

In fact, intracellular glycerol concentrations are not only controlled at the level of production but also by regulated transmembrane transport through the glycerol facilitator Fps1p [12,36,37]. While the main physiological role of Fps1p appears to be the export of glycerol from the cell upon a hypo-osmotic shock, Fps1p can transport glycerol in both directions [35,36]. The transport activity of Fps1p is rapidly regulated by osmotic shock [36,37]. Closing of Fps1p upon a hyper-osmotic shock ensures intracellular glycerol accumulation. Deletion of the N-terminal regulatory domain leads to constitutive, unregulatable glycerol transport activity [36,37]. Hence, the use of different Fps1p alleles provides an experimental tool to manipulate glycerol transport across the yeast plasma membrane.

In this report we show that addition of glycerol to yeast cells stimulates the HOG pathway as evidenced by dual phos-

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phorylation of Hog1p and by enhanced *GPD1* expression. Apparently the cell tries to counteract high external glycerol by increased intracellular glycerol. However, in cells expressing a constitutive Fps1p glycerol transport channel, this effect was strongly diminished presumably because glycerol rapidly equilibrates across the plasma membrane and hence does not cause a loss of turgor. Ethanol did not stimulate the HOG pathway at all. These observations suggest that the HOG pathway is specifically stimulated by a change in turgor and not by water stress per se.

2. Materials and methods

2.1. Strains, plasmids and growth conditions

The *Saccharomyces cerevisiae* strains used in this study were wild-type W303-1A (*MATa leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2 mal0*) [38] and the isogenic *fps1Δ::LEU2* mutant [39]. YEp*FPS1* is a 2μ plasmid with *URA3* as selection marker derived from YEp*lac*195 [40] containing the *FPS1* gene on a 3.8 kb *Sal*I/*Hin*dIII fragment [39]. YEp*fps1-Δ1* (also a YEp*lac*195 derivative) encodes an *FPS1* allele, where amino acids 13–230 have been deleted [37]. This allele mediates unregulated glycerol transport.

Yeast transformations were performed by the lithium acetate method [41] and plasmids were selected and propagated in *Escherichia coli* TOP10F′.

Yeast cells were routinely grown on a rotary shaker at 30°C in yeast nitrogen base (YNB) medium [42] containing 2% glucose as a carbon source. To generate turgor and/or water stress, NaCl, glycerol or ethanol were added from concentrated stock solutions directly to the cell suspension. Samples for Northern and Western blot analysis were taken simultaneously at the time points indicated in the figures.

2.2. Hog1p phosphorylation

Cells were pre-grown in YNB medium supplemented with 2% glucose to an OD_{600nm} of about 1.0. The cells were then concentrated four-fold by sedimentation and resuspension in a smaller volume of the same medium. NaCl (0.8 M), glycerol (0.8 M) or ethanol (1.6 M, final concentrations) was added to the cell suspension. Samples of 1 ml were taken at the time points indicated in the figures, sedimented, resuspended in 50 μl of two-fold concentrated SDS loading buffer (100 mM Tris–HCl, pH 6.8, 200 mM dithiothreitol, 4% sodium dodecyl sulfate (SDS), 0.2% bromophenol blue, 20% glycerol) and boiled at 95°C for 10 min. 7.5 μl of each sample was separated by SDS–PAGE and blotted onto nitrocellulose filters (HybondC extra, Amersham). Filters were blocked with 2% skimmed milk (Difco) in PBST (8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l Na_2HPO_4, 0.24 g/l KH_2PO_4, pH 7.4 and 0.05% (v/v) Tween 20).

Hog1p phosphorylated on both T174 and Y176 was detected by an antibody specific to phosphorylated p38 MAPK (9211S, New England Biolabs). An antibody raised against phosphorylated and unphosphorylated Hog1p was used as a loading control (yC-20, Santa Cruz Biotechnology). The antisera were applied at 1:1000 (phosphorylated Hog1p) and 1:5000 (total Hog1p) dilutions. After washing the filters in PBST, the membranes were incubated for 1 h with secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit IgG 1:5000 or alkaline phosphatase-conjugated rabbit anti-goat IgG 1:15 000) in PBST. The membranes were then incubated for detection with 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate and 75 mg/ml nitroblue tetrazolium.

2.3. Northern analysis

Cells were prepared as for determination of Hog1p phosphorylation. Total RNA was isolated at the time points indicated in the figures and separated by electrophoresis as described previously [43]. Blots were hybridised with ^{32}P-labelled PCR fragments of *GPD1*, *HSP12*, *CTT1* and *IPP1* in buffer containing 7% SDS, 0.5 M sodium phosphate buffer, pH 7.0 and 1 mM EDTA. The signal was quantified using a phosphor imager (Fuji, BAS-1000).

2.4. Reproducibility of data

Experiments were generally performed in duplicate or triplicate giving consistent results. The relative mRNA levels differed between experiments by no more than 20%. Data from representative experiments are shown.

3. Results

3.1. Addition of glycerol to yeast cells stimulates an osmotic stress response

We added glycerol to a final concentration of 0.8 M to yeast cells and monitored HOG pathway signalling and expression of the stress-induced genes *GPD1*, *HSP12* and *CTT1*. *HSP12* and *CTT1* are induced by many stress conditions [44–46] and induction is dependent on the redundant transcription factors Msn2p and Msn4p [46–48]. *GPD1* is induced by osmotic shock, heat shock and oxidative stress [5,7,49]; induc-

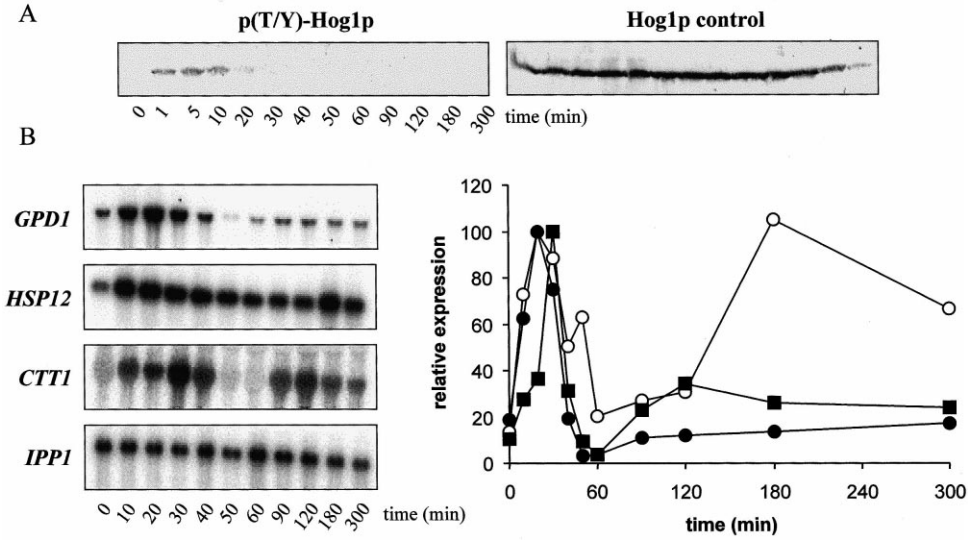

Fig. 1. Addition of glycerol to yeast cells stimulates the HOG pathway and expression of osmo-responsive genes. A: Western blot analysis of cell lysates before and after addition of glycerol to a final concentration of 0.8 M. Phosphorylation of Hog1p was monitored using an antibody specific to p38 MAPK phosphorylated on both tyrosine and threonine and an anti-Hog1p antibody as a loading control. B: Northern blot analysis of total mRNA. The graphs represent quantification of the mRNA levels of *GPD1* (●), *HSP12* (○) and *CTT1* (■) relative to those of *IPP1*. The highest relative mRNA level for each gene was set at 100.

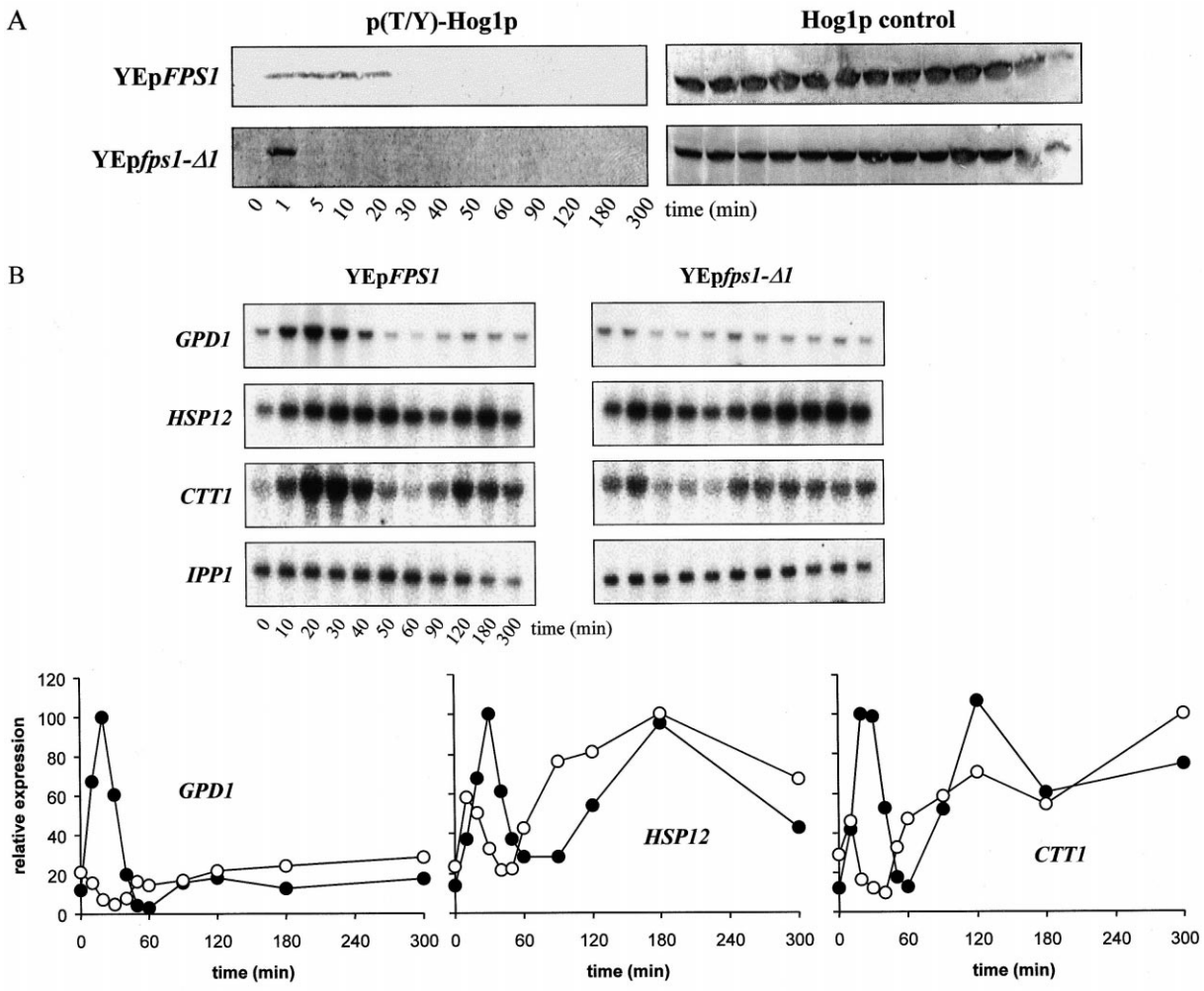

Fig. 2. Stimulation of the HOG pathway by glycerol in a strain expressing a constitutively open Fps1p glycerol channel is strongly diminished. The *fps1*Δ mutant was transformed with a plasmid carrying wild-type *FPS1* and with a plasmid carrying the constitutive channel encoded by the *fps1-Δ1* allele. A: Dual phosphorylation of Hog1p was monitored as in Fig. 1. B: Northern blot analysis of total mRNA. The graphs represent quantification of the mRNA levels of *GPD1*, *HSP12* and *CTT1* relative to those of *IPP1* for the *fps1*Δ strain transformed with *FPS1* (●) and *fps1-Δ1* (○). The highest relative mRNA level was set at 100.

tion by osmotic shock is independent of Msn2p and Msn4p [8]. Induction of all three genes by osmotic shock requires to a different extent the HOG pathway [8].

Glycerol addition rapidly stimulated Hog1p phosphorylation and phosphorylated Hog1p remained detectable for 20 min (Fig. 1A), as was previously observed after addition of 0.4 M NaCl [16,19,21]. Glycerol addition also rapidly stimulated the expression of the three stress-responsive genes (Fig. 1B). The induction profile for *GPD1* was very similar to that previously observed for addition of 0.5 M NaCl [7,8]: a rapid induction with a peak after about 20–30 min, a subsequent drop to about the same level as before the shock and a slow increase to a new steady-state level. The rapid induction of *HSP12* and *CTT1* was also very similar to the one observed previously with NaCl. In contrast to salt addition, however, the response was biphasic leading to a second strong induction after about 2–3 h.

3.2. Expression of a constitutively open Fps1p diminishes the glycerol response

We then did the same experiment with cells expressing a truncated version of the Fps1p glycerol channel, which mediates constitutive glycerol transport. For this, we transformed cells lacking *FPS1* (an *fps1*Δ mutant) with a plasmid containing the full-length wild-type *FPS1* gene or a plasmid carrying a truncated *FPS1* (*fps1-Δ1*), which we have previously constructed and characterised [36,37]. When glycerol was added to *fps1*Δ cells transformed with full-length *FPS1*, Hog1p phosphorylation and induction of the genes *GPD1*, *HSP12* and *CTT1* were very similar to those observed with untransformed wild-type cells (Figs. 2A,B and 1). However, in cells expressing the truncated, constitutively active Fps1p, Hog1p phosphorylation was only detectable in the 1 min sample (Fig. 2A). The expression of *GPD1* was not stimulated at all in these cells (Fig. 2B). The expression of both *HSP12* and *CTT1* was rapidly stimulated after glycerol addition (Fig. 2B) but the first phase of induction was shorter and less pronounced than in cells transformed with full-length *FPS1* or in untransformed wild-type cells (Figs. 1B and 2B). The second induction phase of *HSP12* and *CTT1* was similar in all three experiments (Figs. 1B and 2B).

3.3. Glycerol per se does not block an osmotic stress response

It had previously been proposed that glycerol might inhibit

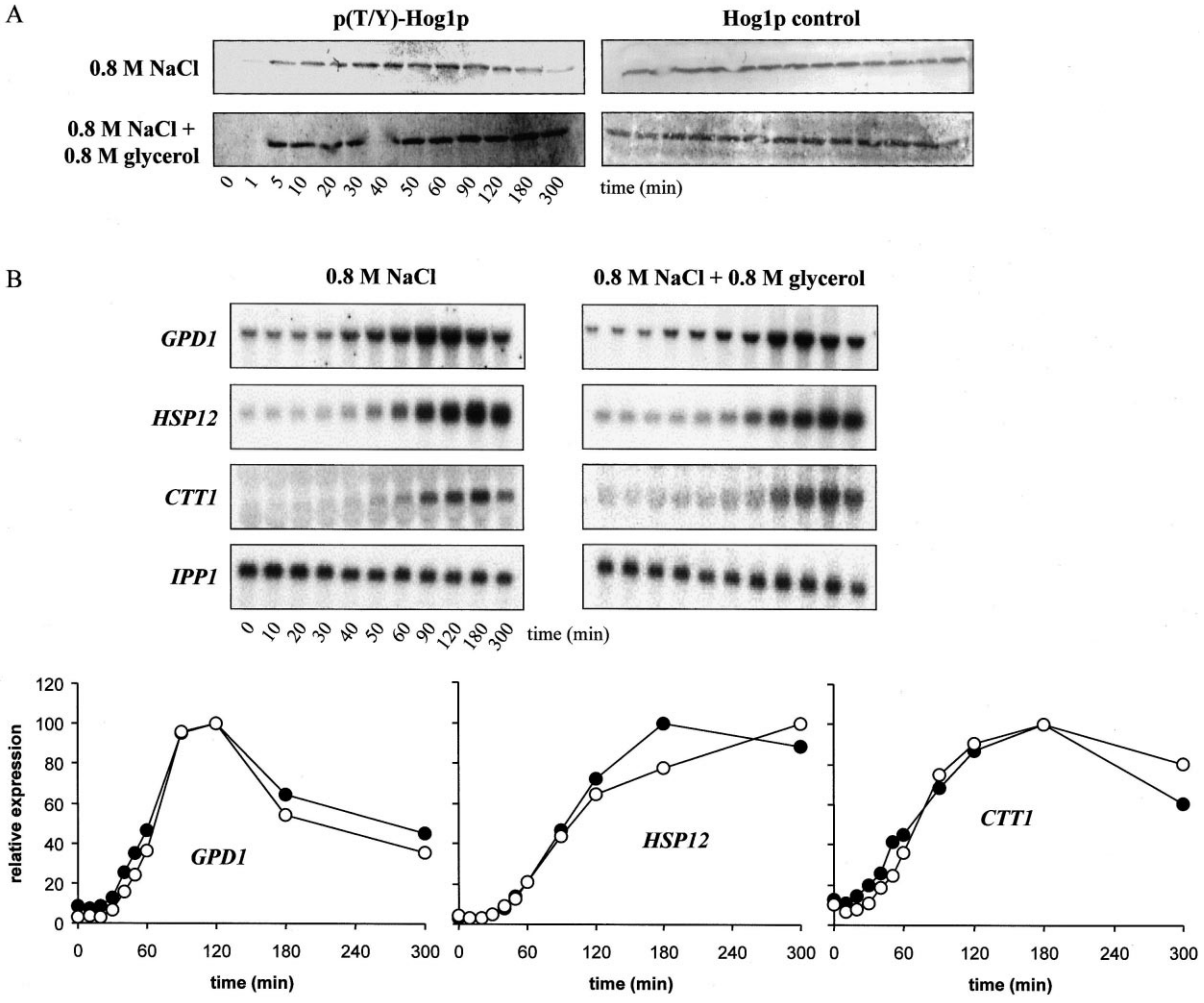

Fig. 3. Glycerol does not block the osmotic stress response. Cells were shocked by addition of 0.8 M NaCl in the absence and presence of 0.8 M glycerol (final concentrations). A: Dual phosphorylation of Hog1p was monitored as in Fig. 1. B: Northern blot analyses of the total mRNA. The graphs represent quantification of the mRNA levels of *GPD1*, *CTT1* and *HSP12* relative to those of *IPP1* for the *fps1*Δ strain transformed with the *fps1-Δ1* allele encoding the unregulated Fps1p after a shift to 0.8 M NaCl (●) and to 0.8 M NaCl plus 0.8 M glycerol (○). The highest relative mRNA level was set at 100.

HOG pathway signalling via a feedback mechanism [18]. Therefore, we tested whether diminished Hog1p phosphorylation and induction of gene expression observed in cells expressing the unregulatable Fps1p could be due to inhibition of HOG pathway signalling by rapidly inflowing glycerol. For this, we added 0.8 M NaCl and, simultaneously, 0.8 M NaCl and 0.8 M glycerol to *fps1*Δ cells expressing the constitutive Fps1p. Under both conditions, strong and sustained phosphorylation of Hog1p was observed (Fig. 3A). The expression of all three genes was induced and the induction profiles after addition of NaCl and NaCl plus glycerol were virtually superimposable (Fig. 3B). Hence, glycerol did not inhibit osmotic shock signalling in these experiments.

Interestingly, NaCl-stimulated Hog1p phosphorylation was more sustained in cells expressing the unregulatable Fps1p (Fig. 3A) as compared to wild-type cells (Fig. 4A). Note that the salt concentration used in these experiments (0.8 M) was higher than that employed in previous reports (0.4 M) [16,19,21]. Therefore Hog1p phosphorylation is detectable in wild-type cells for about 60 min (Fig. 4A) as compared to 20–30 min in previous reports.

3.4. Ethanol does not stimulate the HOG pathway

The data reported above suggest that glycerol-mediated stimulation of the HOG pathway and of HOG pathway-dependent responses requires the maintenance of a glycerol concentration gradient across the plasma membrane. Ethanol rapidly equilibrates across the yeast plasma membrane due to its small size and lipid-soluble nature and hence no such concentration gradient is maintained [32,33]. We added ethanol to a final concentration of 1.6 M to wild-type yeast cells. Phosphorylated Hog1p remained undetectable (Fig. 4A) and the mRNA level of *GPD1* did not increase (Fig. B. Expression of *CTT1* and *HSP12* was stimulated by ethanol but in contrast to the induction observed after glycerol addition, the mRNA level of both genes increased steadily in just a single phase (Fig. 4B). The ethanol-mediated induction of *CTT1* and *HSP12* also followed a different profile than that mediated by salt (Fig. 4B), suggesting different regulatory mechanisms. The inability of ethanol to stimulate the HOG pathway was not due to an inhibitory effect of ethanol: when 1.6 M ethanol and 0.8 M NaCl were added simultaneously to wild-type yeast cells, the HOG pathway and *GPD1* expression were strongly

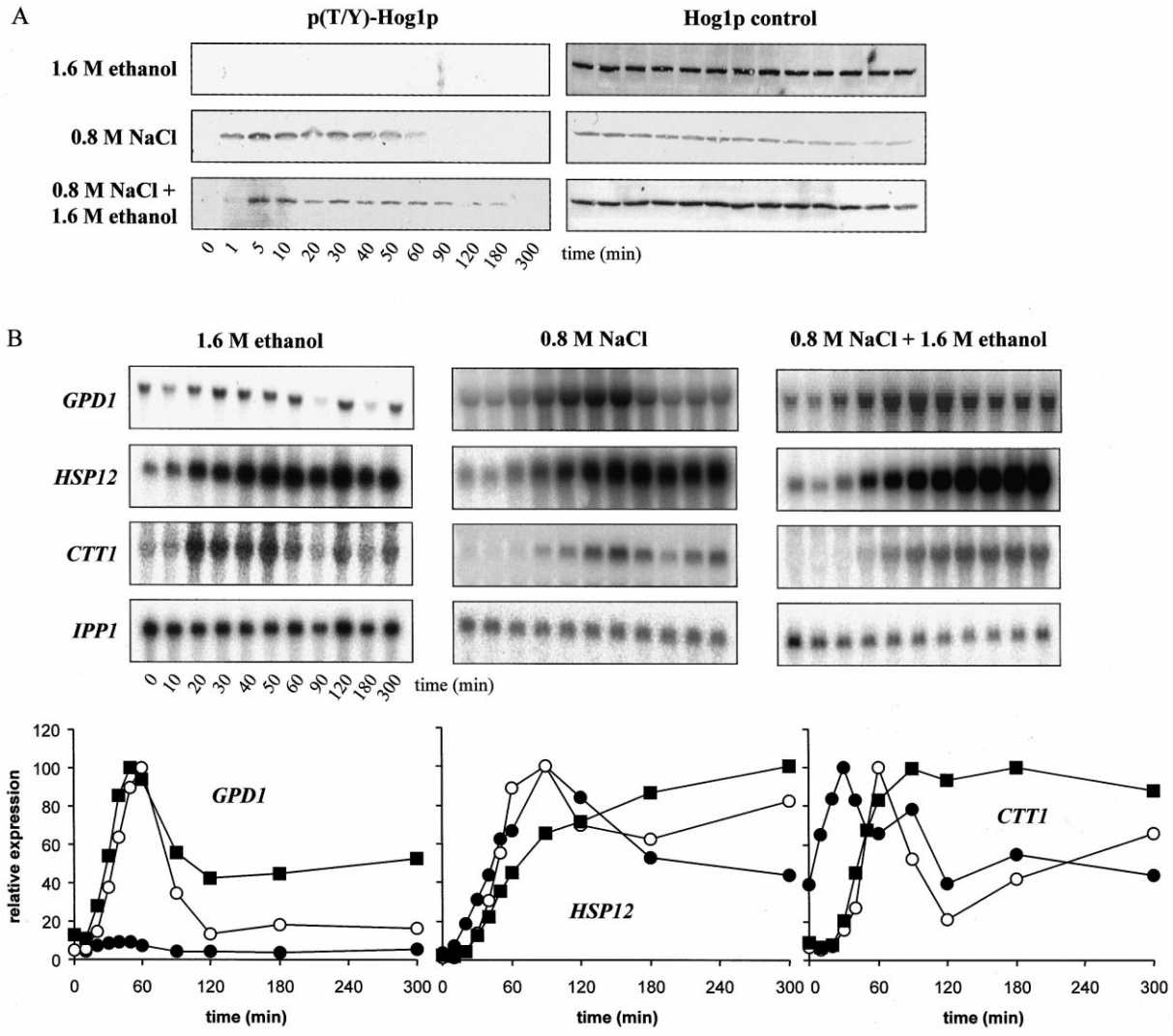

Fig. 4. The HOG pathway is not stimulated by addition of 1.6 M ethanol. A: Dual phosphorylation of Hog1p was monitored as in Fig. 1. B: Northern blot analysis of total mRNA. The graphs represent quantification of the mRNA levels of *GPD1*, *CTT1* and *HSP12* relative to those of *IPP1* after addition of 1.6 M ethanol (●), 0.8 M NaCl (○) and of 1.6 M ethanol and 0.8 M NaCl simultaneously (■). The highest relative mRNA level for each gene was set at 100.

and rapidly stimulated (Fig. 4A,B). Hog1p phosphorylation as well as induction of *GPD1* and *CTT1* expression stayed high for a longer period when cells were treated with both salt and ethanol and induction of *HSP12* followed a somewhat different time course than with NaCl alone.

4. Discussion

Yeast cells appear to respond to the addition of glycerol in very much the same way as to the addition of salt or sorbitol: the HOG pathway is stimulated, the expression of HOG pathway-dependent genes is turned on and apparently the Fps1p glycerol channel is inactivated. The occurrence of the latter effect is difficult to test directly due to high glycerol concentrations in the experiment and hence is an assumption based on the different behaviour of cells expressing a full-length or a truncated, unregulatable Fps1p (Fig. 2). In any case, it appears that yeasts cell mount a response aimed at counteracting extracellular glycerol by production and retention of internal glycerol. It should be noted that in nature it is very unlikely that yeast cells are suddenly exposed to such high amounts of glycerol.

We assume that in cells expressing the unregulatable allele of Fps1p added glycerol equilibrates across the membrane within a few minutes. Although it is very difficult to measure the internal glycerol concentration directly in the presence of high external glycerol, our previous studies on Fps1p-mediated glycerol transport support this assumption. We have demonstrated directly by glycerol transport assays that the *fps1-Δ1* allele used in our experiments mediates high, unregulated glycerol uptake [36]. Fps1p can transport glycerol in both directions and by measuring intracellular glycerol concentrations we have shown that internally accumulated glycerol equilibrates across the plasma membrane after a hypo-osmotic shock within about 3 min [35–37]. The intracellular glycerol concentration attained by yeast cells under hyper-osmotic stress is similar to that used in our experiments [11,50]. An equilibration period of about 3 min is in good agreement with Hog1p phosphorylation being detectable only within the first 1–5 min after glycerol addition in cells expressing the

unregulatable Fps1p. Taken together, the observed diminished HOG pathway activation in cells expressing the constitutive Fps1p is very likely due to rapid glycerol inflow and equilibration across the plasma membrane rather than to a specific role of Fps1p in the control of the HOG pathway. This conclusion is consistent with the finding that glycerol-induced HOG signalling is very similar in a wild-type and an *fps1*Δ mutant (data not shown). We note that even in cells with a closed Fps1p channel added glycerol is likely to equilibrate across the plasma membrane eventually: in cells lacking Fps1p internally produced glycerol is slowly exported and equilibrates upon a hypo-osmotic shock within about 60 min as compared to 3 min in wild-type cells [36,37].

The HOG pathway is controlled by a strong feedback mechanism as demonstrated by the transient nature of Hog1p phosphorylation. The underlying mechanisms are poorly understood although stimulation of certain protein phosphatases has been implicated [22,51]. A comparison between wild-type cells (Fig. 4A) and cells expressing the unregulatable Fps1p, which lose glycerol constantly (Fig. 3A), shows that Hog1p is much more sustained and detectable for about 300 min in cells that are unable to accumulate the glycerol they produce. In wild-type cells, phosphorylated Hog1p disappears after about 60 min under the conditions employed here. A similar pattern is observed in cells that are unable to produce any glycerol, i.e. *gpd1*Δ *gpd2*Δ double mutants (unpublished data). Since expression of phosphatase genes is stimulated in these cells (unpublished data), this suggests that feedback cannot solely be the consequence of HOG-mediated stimulation of the phosphatases or their expression, but rather involves a turgor detection mechanism. In fact, dual phosphorylation, as opposed to tyrosine phosphorylation, of Hog1p and the expression profile of *GPD1* are only marginally altered in cells lacking the phosphatases Ptp2p and Ptp3p or Ptc1p ([7] and unpublished data). It has been proposed that glycerol itself may be involved in this feedback, perhaps by inhibiting specific steps in the HOG pathway [18]. Here we show that even very high glycerol concentrations do not seem to inhibit stimulation of the HOG pathway although we note that this finding does not exclude the involvement of intracellular glycerol or an intermediate in its production in feedback regulation. Such a glycerol effect could be overruled in our experiments by the simultaneous addition of NaCl. In any case, these observations suggest that the phosphorylation state of Hog1p is controlled by an interplay between constitutive dephosphorylation and osmoshock-stimulated phosphorylation by Pbs2p. This is best illustrated in glycerol-shocked cells expressing the constitutive Fps1p, where the stimulus, i.e. the glycerol concentration gradient, collapses quickly leading to dephosphorylation by an apparently pre-existing mechanism. Hence, a specific stimulation of a feedback mechanism by osmotic shock does not seem to be required.

All three stress agents used in this study stimulate expression of the general stress-responsive genes *HSP12* and *CTT1* while only salt and glycerol stimulated expression of *GPD1*. Together with the observation that salt and glycerol stimulate the HOG pathway while ethanol does not, this suggests that ethanol-mediated induction is due to a different, HOG pathway-independent mechanism. This is further supported by the observation that the time course of induction of *HSP12* and *CTT1* shows different kinetics upon simultaneous addition of salt and ethanol reflecting additive effects of the time courses observed after addition of salt or ethanol alone. While salt induction is likely due to a stimulation of the HOG pathway, ethanol-mediated induction of *HSP12* and *CTT1* is probably controlled by the general stress response pathway [45,47,52]. Interestingly, simultaneous addition of salt and ethanol leads to prolonged Hog1p phosphorylation as compared to addition of salt alone. Since ethanol does not stimulate the HOG pathway when added alone the reason for the ethanol effect in the presence of salt may be due to unspecific interference with the dephosphorylation mechanisms. The induction profile conferred by glycerol of *HSP12* and *CTT1* shows a strongly biphasic character and is different from that conferred by salt. Interestingly, simultaneous addition of salt and glycerol leads to an induction profile that is superimposable on that conferred by salt alone. The first induction phase observed with glycerol is likely due to HOG pathway stimulation since it is diminished along with Hog1p phosphorylation in cells expressing the unregulatable Fps1p. The second phase may be due to a growth retardation caused by high amounts of glycerol in the medium, probably through a general stress response mechanism. Since salt causes both an osmotic shock and growth retardation the glycerol effect is overruled by that of salt when glycerol and salt are added together.

Our observations suggest that the HOG pathway is activated by substances that do not rapidly enter the cell and hence maintain, at least for some time, a concentration gradient across the membrane. Thus it appears that the signal perceived by the putative osmosensors is indeed a change in membrane tension, i.e. turgor stress, rather than a change in the interaction with water molecules. Such a scenario has always been assumed based on the localisation of the putative sensors Sln1p and Sho1p in the plasma membrane and on studies of HOG signalling in *fps1* deletion mutants [53]. By using different glycerol concentrations and different alleles of Fps1p as well as Hog1p phosphorylation as a measure for the activity of the osmosensors it should be possible to describe the function of different osmosensor alleles quantitatively with respect to both the amplitude and the timing of stimulation. Such a system can be useful for the functional analysis of the yeast osmosensors.

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