Nuclear versus cytosolic activity of the yeast Hog1 MAP kinase in response to osmotic and tunicamycin-induced ER stress

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ABSTRACT

We examined the physiological significance of the nuclear versus cytosolic localization of the MAPK Hog1p in the ability of yeast cells to cope with osmotic and ER stress. Our results indicate that nuclear import of Hog1p is not critical for osmoadaptation. Plasma membrane-anchored Hog1p is still able to induce increased expression of *GPD1* and glycerol accumulation. This is a key osmoregulatory event, although a small production of the osmolyte coupled with the nuclear import of Hog1p is sufficient to provide osmoresistance. On the contrary, the nuclear activity of Hog1p is dispensable for ER stress adaptation.

Keywords: HOG pathway; osmotolerance; unfolded proteins stress; *GPD1,2*; glycerol

1. Introduction

The osmosensing HOG MAPK pathway consists of two discrete signalling branches, some of which can lead to the phosphorylation and activation of Hog1p [1, 2], the ortholog to mammalian p38. Osmostress-induced phosphorylation of Hog1p triggers its nuclear accumulation [3] and the later transcriptional induction of many genes [4, 5], a response that was thought to be essential for viability. However, a recent research by Westfall et al. [6] questioned the need of a Hog1p-nuclear function. Instead, Hog1p would play a key cytosolic role in establishing the metabolic conditions to ensure high glycerol levels [6]. Consistent with this idea, metabolic regulation rather than *de novo* enzyme synthesis was reported to be the most important mechanism to regulate the glycerol flux upon osmotic stress [7]. Nevertheless, the alteration of the gene expression pattern under these conditions might be still important for long-term cell survival, as a number of mutants in the transcriptional machinery render cells osmosensitive [7-10]. Thus, the importance of the nuclear versus cytosolic function of Hog1p and their contribution to the adaptive response of yeast cells to osmotic stress remains obscure. Whether the MAPK regulates cytosolic events in response to other environmental cues where Hog1p plays a function also needs to be clarified.

In *Saccharomyces cerevisiae*, accumulation of unfolded proteins in the endoplasmic reticulum (ER) triggers the “so-called” unfolded protein response (UPR), a conserved signalling pathway that drives the transcription of genes such as chaperones and folding enzymes [11-13]. In addition, cell viability under ER stress conditions appears to be influenced by the HOG pathway [14], although the role of Hog1p appears to be independent of the canonical UPR signalling system [15, 16]. The MAPK controls the transcriptional induction of *GPD1*, the gene for glycerol synthesis [17], and overexpression of *GPD1* increased tunicamycin tolerance of yeast cells. Remarkably, this function was not mediated by the over-phosphorylation of the MAPK or its nuclear import [15], features that have been also observed in other stress situations [18-20].

Here, we have investigated the nuclear versus cytosolic function of Hog1 in two different cellular responses, the adaptation to osmotic and to tunicamycin-induced ER stress. Our results add new aspects concerning the essential role of Hog1 in the accumulation of glycerol and expression of Hog1-dependent genes.

2. Materials and Methods

2.1. Media and Culture Conditions

Yeast cells were cultured at 30°C in defined media, YPD (1% yeast extract, 2% peptone, 2% glucose) or SCD (0.17% yeast nitrogen base without amino acids [DIFCO], 0.5% ammonium sulfate, 2% glucose) supplemented with the appropriate drop-out mixtures (ForMedium, England). Plate phenotype experiments were made by diluting the cultures to OD₆₀₀ = 0.8 and spotting (3 µl) 10-fold serial dilutions. Unless indicated, colony growth was inspected after 2-4 days of incubation at 30°C.

2.2. Strains and plasmids

S. cerevisiae strains used in this study are listed in Table 1. Strains containing integrated versions of *HOG1* tagged with *GFP* (Hog1-GFP-Kan^R) and plasma membrane-associated Hog1-GFP-CCAAX [6, 23] were a gift of J. Thorner. The YCplac111-based plasmids [24], pVR53 that contains the wild-type *HOG1* gene, and pVR53-NLS, carrying the *HOG1* coding sequence fused in-frame to the nuclear localization sequence from SV40 [25], a gift of P. Alepuz, were transformed into the wild-type and *hog1*Δ mutant in the W303-1A background (Table 1). The pRS414-8xCRE-*lacZ* plasmid [26] was kindly provided by K. Tatebayashi. Plasmid p416-TEFmut4-yECitrine which allows the PCR-based amplification of a promoter replacement cassette [27] was kindly provided by E. Nevoigt. PCR was carried out using the forward primer P7-GPD2, TAGCTTACGGACCTATTGCCATTGTTATTCCGATTAATCTATTGTCAGCTGAAGCTTCGTACG and the reverse primer P8c-GPD2, TGCGTTCGCTTAAGGAATGTGTATCTTGTTAATCTTCTGACAGCAAGCATTTTTCTAGAGAACTTAG. Primers for verification of the correct integration of the replacement cassette were GPD2-V1, AAGACGACGATGGCTCTG, which binds upstream *pGPD2* and Kan-S2, GTCAAGGAGGGTATTCTGG, which binds in KanMX.

2.3. Northern blot analysis

Total RNA was extracted, purified and separated as previously described [15]. Then, samples were transferred to a Nylon membrane and hybridized with non-radioactive DIG labelled probes containing sequences of *GPD1* (+43 to +835), *ACT1* (+10 to +1,075), *STL1* (-1029 to

+379) or *SNR19/UI* (-124 to +536). PCR labelling of DNA probes, membrane pre- and hybridizations, and immunological detection were performed as described [15].

2.4. *β -Galactosidase assay*

Exponentially SCD-Trp growing cells ($OD_{600} = 0.5$) were collected (15 units of OD_{600}), washed with Z buffer (60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl, 1 mM $MgSO_4$), and protein extracts were prepared and processed for galactosidase activity as previously described [15]. One unit is defined as the amount of enzyme that is able to convert 1 nmol of the substrate o-NPG per min under the assay conditions. The given values represent the mean $\pm$ SD of three independent experiments, each conducted in triplicate.

2.5. *Glycerol assay*

YPD-grown cells ($OD_{600} = 0.5$) were collected by centrifugation and resuspended in the same medium containing 2 μ g/ml tunicamycin, 0.4 M NaCl or 1.0 M sorbitol. After 3 h, aliquots (1 ml) of the cultures were boiled for 10 min, centrifuged at 15,300 x g for 10 min (4 °C), and the supernatants assayed for glycerol content. In the case of experiments with *gpd1* mutant strains (Fig. 3B), glycerol production was measured after 20 h of culture in SCD containing 0.75 M sorbitol. Glycerol content was determined as previously described [15], and is expressed as μ g of glycerol per mg of yeast cells, dry weight ($OD_{600} = 1$ equals 0.3 mg cells dry weight/ml). Increase in glycerol content was estimated after subtraction of the values measured in unstressed control samples. The values given represent the mean $\pm$ SD of three independent experiments each conducted in triplicate.

3. Results

3.1. *The cytosolic activity of Hog1p provides osmostress and ER stress protection*

Prior work by our group showed that Hog1p-dependent effects on ER stress are mediated by the basal activity of the MAPK. Thus, neither the phosphorylation of Hog1p nor its nuclear import could be observed in tunicamycin-treated cells [15]. However, a parallel study by Bicknell *et al.* [16] showed evidence of Hog1p phosphorylation upon a long-term period of stress, suggesting a nuclear function of the MAPK. Thus, we first tried to clarify whether the nuclear import of Hog1p might be essential in the response to ER stress. We investigated how tunicamycin exposure affects growth of cells in which nuclear entry of Hog1p is prevented

(Fig. 1A). For this, we used the yeast strains constructed by Westfall et al. [6], in which a Hog1-GFP-CCAAX chimera was anchored to the plasma membrane (*HOG1-CCAAX*), or native Hog1 excluded from the nucleus by knock-out of the importin- β homolog *NMD5* gene [3]. As can be seen, both experimental approaches rendered cells showing the same growth phenotype on tunicamycin plates as the wild type strain, and only the *hog1* Δ mutant strain displayed diminished resistance to the drug (Fig. 1A). Thus, the cytosolic function of Hog1p appears to provide full osmostress [6] and ER stress protection.

To test further this idea, we investigated how increased nuclear residence of Hog1p affects yeast sensitivity to both NaCl- and tunicamycin-induced ER stress. For this, we used a Hog1p variant in which a generic nuclear import signal (SV40-NLS) was tagged at the carboxyl terminus of the MAPK [25]. As shown, expression of such a protein kinase provided partial protection against osmostress, although *hog1* Δ mutant cells still displayed a clear osmosensitive phenotype as compared with the wild-type (Fig. 1B). Furthermore, nuclear concentration of Hog1p did not alleviate the growth defect of the mutant strain on tunicamycin (Fig. 1B).

3.2. *Hog1* is excluded from the nucleus in *HOG1-CCAAX* cells

We wonder whether the nuclear translocation of Hog1 and the effects on transcription that it normally causes were still possible in *HOG1-CCAAX* cells. Previous research by Westfall et al. [6] showed that the Hog1-GFP-CCAAX chimera was mainly tethered to the plasma membrane, although it seems possible that a portion of the recombinant protein could still shuttles between the cytoplasm and the nucleus. To clarify this point, we followed the expression of *STL1* in cells of the wild-type, *hog1* Δ and *HOG1-CCAAX* strain. *STL1*, which encodes a glycerol proton symporter [28] belongs to a class of yeast genes that exhibit very strong induction in wild-type cells but little in the *hog1* Δ mutant [5]. We were unable to detect induction of *STL1* in response to tunicamycin (data not shown), a result that could be explained by the lack of nuclear import of Hog1 under this condition [15]. Unlike, the transcript was up-regulated in wild-type cells by 45 min after exposure to 0.4 M NaCl (Fig. 2A), confirming the massive nuclear import of the MAPK upon osmotic stress [29, 30]. Moreover, the expression was completely abolished in either *hog1* Δ or *HOG1-CCAAX* cells (Fig. 2A). Similar results were observed when the expression mediated by Hog1p and the Hog1-GFP-CCAAX chimera was assayed by using an 8xCRE-*lacZ* reporter (Fig. 2B), which contains eight tandem repeats of the *ENAI*-derived CRE sequence [26]. Like *STL1*,

expression of the ATP-driven ion pump-encoding gene *ENA1* [31], has been reported to be strictly Hog1-dependent [5].

3.3. Plasma membrane-anchored Hog1 is still able to provide increased *GPD1* expression

We examined the tunicamycin-induced expression of *GPD1*, the gene for glycerol production. Unlike *STL1* or *ENA1*, the expression of *GPD1* requires, in addition to Hog1, the activity of several transcription factors, including Msn1p, Msn2p, Msn4p, and Hot1p [32]. Consistent with all of this, loss of Hog1 caused a strong reduction of *GPD1* expression, although a transcriptional response was still evident (Fig. 3A). As expected, *HOG1-CCAAX* cells also exhibited a diminished response. However, the relative levels of *GPD1* mRNA were still about twice higher in this strain than in cells lacking the MAPK. Consistent with this, *HOG1-CCAAX* cells also showed increased accumulation of glycerol as compared with those of the *hog1Δ* mutant (Fig. 3B). This was even more evident in cells exposed to NaCl or sorbitol, conditions that result in higher glycerol production. For example, *HOG1-CCAAX* and *hog1Δ* mutant cells exposed to sorbitol accumulated about 65% and 30% of glycerol, respectively, compared to the wild-type (Fig. 3B). Altogether, our results suggest that glycerol accumulation under the physiological conditions tested is mainly explained by transcriptional up-regulation. Our results also show that the nucleus excluded-Hog1 form is still able to provide increased transcription of *GPD1*, as well as a remarkable glycerol accumulation.

3.4. Glycerol accumulation is not essential for osmoadaptation but requires the nuclear function of Hog1p

We tried to clarify the exact need of nuclear activity of Hog1p in the response of yeast cells to either osmotic or ER stress. We compared the growth of *gpd1* cells containing Hog1p or the Hog1-GFP-CCAAX chimera on NaCl-, sorbitol- or tunicamycin-containing YPD medium (Fig. 4A). In these strains, the production of glycerol depends exclusively on *GPD2*, the homolog of *GPD1*, which unlike this, is not regulated by the MAPK Hog1p [33]. Consistent with all of this, the glycerol accumulation in these strains was around 10-fold lower (Fig. 4B, lower graph), as compared with that found in cells expressing a *GPD1* gene (Fig. 3B), but similar between *gpd1Δ HOG1* and *gpd1Δ HOG1-CCAAX* cells (Fig. 4B). As expected, the *gpd1* mutation had no major effects on growth of the *HOG1* strain under mild osmostress conditions provided by 0.4 M NaCl or 0.75 M sorbitol (Fig. 4A), a result that is in agreement with previous reports [6, 17]. Similar results were found when cells were grown in liquid

SCD containing 0.75 M sorbitol (Fig. 4B, upper graph). However, knock-out of *GPD1* combined with membrane-anchoring of Hog1p caused a pronounced growth defect under osmostress conditions (Fig. 4A and 4B). Finally, the phenotypic response on tunicamycin of *HOG1-CCAAX* cells lacking *GPD1* did not differ from that shown by cells of the *gpd1Δ HOG1* strain (Fig. 4A).

Finally, we explored the impact of unregulated Gpd2p activity on growth under osmotic and ER stress conditions. The native *GPD2* promoter was replaced by a weaker mutated *TEF1* promoter [27] in *gpd1Δ HOG1* and *gpd1Δ HOG1-CCAAX* cells. In full agreement with this, the glycerol production in these strains was lower than that observed in cells in which the *GPD2* gene is driven by its own promoter (Fig. 4B, lower graph). Despite of this, *pTEF1-GPD2* transformants in a *gpd1Δ HOG1* background still showed osmotolerance, while the corresponding *HOG1-CCAAX*-counterparts displayed no apparent growth (Fig. 4A) or increased osmosensitivity (Fig. 4B, upper graph). On the other hand, the response to tunicamycin did not show major differences between *pTEF1-GPD2*-derivatives of *gpd1Δ HOG1* and *gpd1Δ HOG1-CCAAX* (Fig. 4A).

4. Discussion

Consistent with the lack of Hog1 phosphorylation and nuclear import by tunicamycin exposure [15], cells in which Hog1p is tethered to the plasma membrane, or excluded from the nucleus by knock-out of the importin-β homologue *NMD5*, were found to fully adapt to ER stress. Changes in the mRNA levels of *STL1* or expression of a reporter driven by CRE sequences from *ENAI* [26] in wild-type cells exposed to the drug, were also undetected. Transcription of these genes is strictly Hog1-dependent [5]. Although a role of Hog1p in promoting expression of *GPD1* and glycerol accumulation was demonstrated, it seems clear that the cytosolic activity of the MAPK is the sole responsible of its function in response to ER stress induced by tunicamycin exposure.

We also observed that nuclear Hog1p is dispensable for coping with osmostress. This is in agreement with the previous study by Westfall et al. [6], although our results add an important aspect. These authors assumed that increased glycerol production upon osmostress in *HOG1-CCAAX* cells responds to Hog1p-mediated regulation of cytosolic targets, most likely the glyceraldehyde-3-phosphate dehydrogenase isoenzymes Tdh1p, Tdh2p and Tdh3p. Evidences that these enzymes are substrate of Hog1p were presented [6], confirming previous

reports [34]. Nevertheless, the activity of Hog1p over these enzymes is not sufficient to explain the increased rate of glycerol production [6] and thus, the physiological significance of this regulation remains unclear. Unlike this, our observations suggest that Hog1p excluded from the nucleus is still able to increase the expression of *GPD1* with respect to that found in cells lacking the MAPK, and to provide glycerol at sufficient levels for osmoadaptation. Whether the transcriptional activation of *GPD1* in *HOG1-CCAAX* cells depends on the cytosolic activity of Hog1p over well-known regulators of this gene, needs to be further explored. Westfall et al. [6] showed that deletion of *HOT1*, *MSN1*, *MSN2*, *MSN4* or *SKO1* does not render *HOG1-CCAAX* cells osmosensitive. Nevertheless, a previous work by Rep et al. [32] revealed that a quadruple yeast mutant *hot1 msn1 msn2 msn4* still displays a 5-fold induction of the *GPD1* mRNA level (wild type, 13-fold). Thus, we cannot discard a role of plasma membrane-anchored Hog1p in transmitting the osmostress signal to the nucleus through the modification of these or additional factors.

Recently, Babazadeh et al. [35] demonstrated by using an elegant approach that *hog1Δ* cells in which expression of glycerol biosynthetic genes is provided by another MAPK pathway (*pFUS1-GPD1/GPP2*) show osmoadaptation. They concluded that glycerol biosynthesis is one of the most critical factors for Hog1-dependent osmoadaptation [35]. The results of Westfall et al. [6], as well as ours, also confirmed that glycerol accumulation is a key osmoregulatory event. Indeed, glycerol accumulation in cells where Hog1p is excluded from the nucleus was just around 35% lower to that found in the wild-type strain, likely explaining the high osmotolerance of *HOG1-CCAAX* cells. However, our results also suggest that glycerol production may be not as essential as it was previously assumed [6, 35] if the nuclear activity of Hog1 is intact. Indeed, yeast cells carrying a native *HOG1* gene, but in which glycerol production was compromised by knock-out of *GPD1* showed osmotolerance similar to the wild-type, a result that confirms previous reports [6, 17]. On the contrary, diminished production of glycerol combined with lack of nuclear import of Hog1p resulted in osmosensitivity. Moreover, osmotolerance in *HOG1 gpd1* cells was longer maintained when the native *GPD2* promoter was replaced by a weak mutated *TEF1* promoter. Hence, our approach suggest that Hog1p plays at least two fundamental roles, glycerol production and transcriptional regulation, and that yeast cells can overcome osmotic stress if one of these two critical roles remains functional. More than 50-osmotically regulated genes depend on Hog1p [36] and it seems that they indeed play, individually or collectively, an important function in the adaptation and growth under these conditions.

Competing interests

The authors have declared that no competing interests exist.

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Figure legends

Fig. 1. Phenotypic characterization of yeast strains containing different versions of the *HOG1* gene. (A) Growth was assayed in cells of the SO329 wild-type strain (wt), *hog1* Δ and *nmd5* Δ mutant strains, and derivatives containing a protein fusion of Hog1-GFP (*HOG1*) or a Hog1-GFP-CCAAX (*HOG1-CCAAX*) chimera, which is anchored to the plasma membrane [6]. (B) Cells of the wild-type W303-1A strain and its corresponding *hog1* Δ mutant, were transformed with plasmids YCplac111 (empty plasmid, Control), pVR53, which contains the wild-type *HOG1* gene (*HOG1*) or pVR53-NLS, carrying the *HOG1* coding sequence fused in-frame to the nuclear localization sequence from SV40 (*HOG1*-NLS) [25], and growth of transformants was tested in the presence of NaCl or tunicamycin. In all cases, cultures were grown in YPD at 30°C until exponential phase and adjusted to OD₆₀₀ = 0.8. Then, serial dilutions (10^{-3}) of the cultures were spotted (3 μ l) onto YPD plates lacking or containing tunicamycin or NaCl as indicated, and incubated at 30°C for 2-4 days, respectively. Representative experiments are shown.

Fig. 2. The Hog1-GFP-CCAAX chimera is tethered to the plasma membrane. (A) Cells of the indicated strains were treated with 0.4 M NaCl and at the indicated times, aliquots were withdrawn and processed for Northern blot analysis of *GPD1* mRNA. A probe containing a sequence from the yeast gene *SNR19* (*U1*) was used as loading control. A representative experiment is shown. (B) The activity of a 8xCRE-*lacZ* reporter [26] was assayed in the indicated strains exposed to 0.4 M NaCl for 45 min. Data represent the mean value $\pm$ SD of three independent experiments.

Fig. 3. Plasma membrane-anchored Hog1p is still able to up-regulate *GPD1* expression and increased glycerol accumulation. (A) RNA samples from cells of the indicated strains exposed to 2 μ g/ml of tunicamycin for 30 and 60 min were analyzed for *GPD1* mRNA levels by Northern blot. Values of mRNA levels were normalized with respect to the *ACT1* corresponding values and are represented as percentages of the maximal induction. A representative experiment is shown. Independent experiments revealed similar induction kinetics and mRNA values. (B) The indicated strains were assayed for glycerol production after 3 h of culture in YPD medium containing 2 μ g/ml tunicamycin, 0.4 M NaCl or 1.0 M sorbitol. Values are expressed as μ g of glycerol per mg of yeast cells, dry weight (dw), and represent the mean $\pm$ SD of three independent experiments.

Fig. 4. Effects of unregulated glycerol production and nuclear activity of Hog1p on the sensitivity of yeast cells to tunicamycin and osmotic stress. (A) Growth of wild-type (wt) and *gpd1Δ* mutant strains containing a protein fusion of Hog1-GFP (*HOG1*) or a Hog1-GFP-CCAAX (*HOG1-CCAAX*) chimera native [6] was assayed on NaCl-, sorbitol- or tunicamycin-containing medium. Strains containing in addition a replacement of the native promoter of *GPD2* by a mutated promoter of the unregulated *TEF1* yeast gene (*pTEF1-GPD2*) were also analysed. (B) The *gpd1Δ*-derivative strains mentioned above were analysed for growth (upper graph) and glycerol accumulation (lower graph). Cultures were grown in YPD at 30°C until exponential phase ($OD_{600} = 0.5$). Then, cells were harvested, washed and transferred to liquid SCD containing 0.75 M sorbitol and the growth at 30°C was monitored by measuring the OD_{600} . A representative experiment is shown. Glycerol was measured after 20 h of transfer. Assays were carried out as described in the Materials and Methods section. Values are expressed as μg of glycerol per mg of yeast cells, dry weight (dw), and represent the mean $\pm$ SD of three independent experiments.

Table 1. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Reference or source
W303-1A	<i>MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 GAL mal SUC2</i>	[21]
W303 <i>hog1</i> Δ	<i>MATa hog1Δ::kanMX3</i>	C. Pallotti
SO329	<i>MATa trp1-1 leu2-3,112 ura3-52 his4 can1 FUS1::lacZ::LEU2</i>	[22]
PW014	SO329 <i>hog1::URA3</i>	[23]
PW350	SO329 <i>nmd5::URA3</i>	[6]
PW355	SO329 <i>hog1::ura3::HOG1-GFP-CCAAX</i>	[6]
PW357	SO329 <i>HOG1-GFP-Kan^R</i>	[6]
PW369	PW355 <i>gpd1::URA3</i>	[6]
PW401	SO329 <i>gpd1::URA3</i>	[6]
<i>gpd1 pTEF1-GPD2</i>	PW401 <i>pGPD2::kanMX-pTEF1mut4</i>	This study
CCAAX <i>gpd1 pTEF1-GPD2</i>	PW369 <i>pGPD2::kanMX-pTEF1mut4</i>	This study

Figure 1

Figure 2

Figure 3

Figure 4