High-osmolarity signalling in *Saccharomyces cerevisiae* is modulated in a carbon-source-dependent fashion

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High-osmolarity-induced expression of the small heat-shock gene *HSP12* is regulated by the HOG (high-osmolarity glycerol) pathway and PKA (protein kinase A). To analyse the regulatory input of both signal transduction pathways, high-salt-induced *HSP12* expression in different genetic backgrounds on glucose-, ethanol- and glycerol-based culture media was examined. Upon exposure to high-osmolarity stress, the kinetics of induction of *HSP12* in cells growing on the non-fermentable carbon sources are strikingly different from those on glucose. Derepression of *HSP12* gene expression under non-stress conditions was observed in cells growing on non-fermentable carbon sources. High-salt challenge resulted in a lower induction of the *HSP12* mRNA levels in ethanol-grown cells as compared to glucose-grown cells, whereas in glycerol-grown cells hardly any high-salt induction of *HSP12* mRNA levels could be detected. Analysis of signalling through the HOG pathway suggested that glycerol may influence the activity of this signalling route, possibly via negative feedback. Furthermore, the cellular level of PKA activity was found to have a great impact on stress-responsive gene transcription. On the basis of the data obtained it was concluded that modulation of PKA activity plays a major role in the stress response. A glucose-dependent, PKA-regulated cellular component is postulated to affect high-osmolarity-induced *HSP12* expression.

Keywords: stress response, signal transduction, protein kinase A (PKA), high-osmolarity glycerol (HOG) pathway, *HSP12*

INTRODUCTION

Being a unicellular organism, *Saccharomyces cerevisiae* is subject to an ever-changing environment which may challenge its growth potential. To survive, yeast cells must cope with alterations in e.g. temperature, osmolarity, redox conditions and availability of nutrients. To overcome these stress conditions, a set of cellular responses is triggered leading to the immediate repair of cellular damage and adaptation to the encountered situation. Adapted cells are not only able to resume growth, but are also more protected when exposed to a new stress situation (Trollmo *et al.*, 1988; Varela *et al.*, 1992). This protection is not only limited to a second challenge of the same kind of stress, but also to other

types of stress, indicating that the stress response consists of both specific and general elements (Coote *et al.*, 1991; Trollmo *et al.*, 1988; Varela *et al.*, 1992). Insight into the molecular mechanisms that enable yeast to sense and respond to adverse growth conditions has increased enormously over the last few years with the characterization of stress-responsive genes and signal transduction pathways mediating their expression (for an extensive review, see Hohmann & Mager, 1997).

The responses of yeast to an increase in the osmolarity of the medium comprise both activation of specific and general stress-induced signal transduction pathways. The high-osmolarity glycerol (HOG) mitogen-activated protein (MAP) kinase pathway was characterized as one of five MAP kinase pathways in yeast (Brewster *et al.*, 1993; Levin & Errede, 1995). Increasing the osmolarity of the medium triggers the activation of the pathway via two putative high-osmolarity receptors, Sln1p and Sho1p (Maeda *et al.*, 1994, 1995). Sln1p is the sensor of

Abbreviations: HOG, high-osmolarity glycerol; MAP kinase, mitogen-activated protein kinase; PKA, protein kinase A; STRE, general stress-responsive element.

a three-component histidine kinase system with Ypd1p and Ssk1p as response regulators (Posas *et al.*, 1996). Activation by high osmolarity releases a block upon the two MAPKK kinases Ssk2 and Ssk22 after which Pbs2p (MAPK kinase) can stimulate the Hog1p kinase by a characteristic dual phosphorylation. Interestingly, the activity of Pbs2p can also be regulated directly by Sho1p. The Sho1p induction of Pbs2p activity is thought to involve interaction of the proteins via an SH3-like domain (Maeda *et al.*, 1995). The activation of Hog1p is a prerequisite for the increase in internal glycerol concentration to compensate for the loss of water during high-osmolarity stress (Brewster *et al.*, 1993). Expression of the gene encoding the key enzyme in glycerol production, *GPD1*, is controlled by the activity of the HOG pathway (Albertyn *et al.*, 1994; Larsson *et al.*, 1993). However, the mechanism linking Hog1p activation to the downstream responses is unknown.

The small heat-shock protein gene *HSP12* is also expressed upon high-osmolarity challenge (Varela *et al.*, 1995). This gene belongs to a vast group of so-called general stress-responsive genes. Like *GPD1*, high-osmolarity-induced expression of *HSP12* is regulated by the activation of Hog1p. Although the function of Hsp12 in yeast is still unknown, it seems likely that it contributes to the stress adaptation in general, because it is also expressed after heat shock, oxidative stress and under nutrient-limiting conditions (Varela *et al.*, 1995). A second input of signal transduction into the regulation of *HSP12* expression is mediated by PKA. *HSP12* mRNA levels are increased when PKA activity is lowered, as has been shown by examining PKA-mutant yeast strains.

Deletion analysis of the promoter of *HSP12* (Varela *et al.*, 1995) and of other genes induced by multiple stresses such as *CTT1* (Marchler *et al.*, 1993) and *DDR2* (Kobayashi & McEntee, 1993), revealed a general stress-responsive *cis*-acting element (STRE) to be involved in mediating the general stress signal. STRE sequences are present in the promoter of many stress-response genes in variable amounts and orientation (Ruis & Schüller, 1995; Siderius & Mager, 1997). Whether this reflects a way to fine-tune stress-induced expression is not known. Two proteins, Msn2p and Msn4p, containing zinc-finger DNA-binding domains which bind to STRE sequences were shown to mediate the general stress-response signal (Martinez-Pastor *et al.*, 1996; Schmitt & McEntee, 1996).

As described in Siderius & Mager (1997), the only signal which is known to influence the response to a variety of stress conditions in yeast is related to PKA activity. The Ras/PKA pathway has been shown to regulate both growth-related metabolism, e.g. the upshift of ribosomal protein gene expression when shifting to glucose as a carbon source (Griffioen *et al.*, 1994), as well as expression of stress-responsive genes (Marchler *et al.*, 1993; Varela *et al.*, 1995). For this reason PKA activity might be considered as a switch mechanism that yeast cells can use to stop growth and initiate the stress response when necessary. The way PKA activity is regulated is poorly understood. Activation of PKA

involves the release of the catalytic subunits Tpk1, 2 and 3p from their regulatory subunit Bcy1p, as occurs after an increase in the cAMP level in the cell. On the other hand, PKA activity is supposed to be regulated via a cAMP-independent fashion through the fermentable-growth-medium (FGM) pathway (Thevelein, 1994). It has previously been demonstrated that general stress-responsive transcription is under negative regulation by cAMP. Whether the underlying mechanism resides in the modulation of the affinity of Bcy1p for the catalytic subunits or of their activity is elusive.

To investigate the role of the specific and general signal transduction pathways in the response of yeast to high-osmolarity stress, we analysed the kinetics of *HSP12* expression in various signalling mutants. To further elucidate the role of PKA activity in stress-induced signal transduction we have also examined the high-osmolarity response in yeast cells grown on media with glucose, ethanol or glycerol as sole carbon source. We show that the net output of stress-induced signal transduction varies greatly depending on the carbon source utilized.

METHODS

Strains and growth conditions. Yeast strains used in this study are listed in Table 1. Yeasts were cultured in YP medium supplemented with glucose (2%, w/v), ethanol (1%, v/v) or glycerol (3%, v/v) or YNB supplemented with the same carbon sources as well as the appropriate amino acids. Standard culturing conditions were at 30 °C on a rotary shaker (220 r.p.m.) unless indicated otherwise. *HSP12* promoter-*GUS* fusions ($\Delta 2$ and $\Delta 32$, containing 561 and 234 bp of the 5' flanking region of the *HSP12* start codon, respectively) were inserted in the *URA3* locus in the different mutants used in this study and in their isogenic wild-type. Proper integration and copy number were determined by Southern blotting. For the Hog1p phosphorylation analysis, the EG123 strain was transformed with a pMA3a plasmid carrying the *HOG1* gene (pFJ0) (Varela *et al.*, 1995).

High-osmolarity-stress experiments and RNA isolation. Cells cultured overnight were diluted to an OD₆₆₀ of 0.1 (Beckman, DU-6z spectrophotometer) and grown until the early-exponential phase (OD₆₆₀ of 0.2) and then subjected to high-osmolarity stress (0.7 M NaCl) by addition of a concentrated (5 M) salt solution. Samples were taken at the time-points indicated in the figures by quickly freezing the cells in liquid nitrogen. After thawing the samples in cold water, cells were washed in 1 ml H₂O and resuspended in 400 µl lysis buffer (TES, 10 mM Tris/HCl, pH 7.5; 10 mM EDTA; 0.5% SDS). An equal volume of acid phenol was added and cells were incubated at 65 °C for 60 min with occasional brief vortexing. Samples were then cooled on ice and spun in the microfuge. Acid/phenol extraction was repeated, followed by a chloroform extraction, keeping the samples on ice and centrifugation at 4 °C. Sodium acetate was added (3 M, pH 5.3, 40 µl) and RNA was precipitated with 1 ml ice-cold 100% ethanol. The RNA pellet was washed in 70% ice-cold ethanol and resuspended in 25 µl H₂O.

Separation of RNA was performed as described by Varela *et al.* (1995). DNA probes were labelled using the random-priming method (Prime-a-Gene labelling system; Promega). Signal quantification was performed by phosphorimaging (Phosphorimager 425; Molecular Dynamics). The *PGK1*

Table 1. *S. cerevisiae* strains used

Strain	Genotype
EG123	MAT α <i>leu2-3 his4 trp1 ura3-52 can1</i>
W303IA	MAT α <i>leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can 1-100 GAL SUC mal0</i>
Δ <i>msn2/4</i>	MAT α <i>leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can 1-100 GAL SUC mal0 msn2::HIS3 msn4::TRP1</i>
YPH 102	MAT α <i>ura3 leu2 his3 ade2 lys2</i>
JB13	MAT α <i>ura3 leu2 his3 ade2 lys2 hog1::TRP1</i>
SPI	MAT α <i>leu2 his3 trp1 ade8 can1 ura3</i>
S13-3A	MAT α <i>leu2 his3 trp1 ade8 can1 ura3 tpk2::HIS3 tpk3::TRP1 bcy::LEU2</i>
S18-D	MAT α <i>leu2 his3 trp1 ade8 can1 ura3 tpk1^{w1} tpk2::HIS3 tpk3::TRP1</i>
RS13-58A-1	MAT α <i>leu2 his3 trp1 ade8 can1 ura3 tpk1^{w1} tpk2::HIS3 tpk3::TRP1 bcy::LEU2</i>

mRNA level was used as a loading control for cells grown on glucose. Because *PGK1* mRNA levels decreased after high-salt challenge of cells grown on non-fermentable carbon sources, the 25S rRNA level was used as additional loading control.

Western blotting and antibody staining. Tyrosine phosphorylation of Hog1p was examined in the EG123 strain carrying a *HOG1* overexpression plasmid (pFJ0). Total protein samples were isolated as described by Davenport *et al.* (1995). Proteins were separated on 12.5% polyacrylamide gels and blotted onto nitrocellulose. The phosphotyrosine content of Hog1p was examined using the anti-phosphotyrosine monoclonal antibody PY20 (Transduction Laboratories). Anti-Hog1p antibody was kindly provided by Dr M. Gustin (Rice University, Houston, USA). Antibody binding was visualized using enhanced chemiluminescence (ECL; Amersham) after binding of a horseradish peroxidase-conjugated second antibody.

RESULTS

PKA activity and the HOG pathway regulate the kinetics of *HSP12* expression after high-osmolarity stress

We have previously demonstrated that the *HSP12* gene transcription is activated by the HOG pathway and repressed under conditions of high PKA activity (Varela *et al.*, 1995). To discriminate between derepression and induction of the *HSP12* expression, we were interested to know how the activity of PKA and HOG signalling are integrated in the regulation of *HSP12* promoter activity. For this purpose we analysed the kinetics of induction of *HSP12* mRNA levels after subjecting cells to high-osmolarity stress (0.7 M NaCl) in high- and low-PKA mutants as well as in the Δ *hog1* strain. As shown in Fig. 1(a) and quantified in Fig. 1(b), *HSP12* mRNA levels reached a maximum 90 min after the addition of salt in the wild-type. When comparing the response in the PKA mutants, a shift of the maximum *HSP12* mRNA levels to an earlier time-point is evident. From the analysis of mutant strains with either one of the *TPK* gene products, it is clear that all three *TPK* gene products can be involved in the repression of *HSP12* gene expression (M. Siderius, unpublished). Therefore, there is a good correlation between the potential PKA activity (wild-type > *TPK1*, *tpk2,3* > *tpk1^w*, *tpk2,3*) and the earlier timing of maximal *HSP12* mRNA levels after salt stress. Furthermore,

although at low levels, the high-PKA mutant (*tpk2*, *tpk3*, *bcy1*) still responded to high osmolarity with an increase in *HSP12* mRNA level, as quantified in Fig. 1(c). The absolute levels of *HSP12* mRNA were only affected significantly in the high-PKA strain. To analyse the input of the HOG pathway, high-osmolarity-induced *HSP12* mRNA levels were examined in the Δ *hog1* strain. The maximum *HSP12* mRNA levels in response to high salt in this mutant appeared at a later time-point as compared to the wild-type (Fig. 2). Throughout this paper, we followed the assumption that the changes in mRNA levels are mainly a reflection of transcriptional regulation. It can not be excluded however that post-transcriptional events (e.g. mRNA stability) contribute to the observed effects.

Carbon sources affect the output of stress-induced signalling

To further analyse the contribution of presumed changes in PKA activity in the high-salt-induced derepression of *HSP12* gene expression, we examined the response to 0.7 M NaCl of cells grown on glucose, ethanol or glycerol. The observation that *HSP12* gene expression was derepressed in cells growing on non-fermentable carbon sources under non-stress conditions (Fig. 3; *t* = 0) might, at least partly, reflect a lower PKA activity as compared to glucose-grown cells. This assumption is supported by the carbon-source-mediated regulation of enzymes that were shown to be PKA targets, e.g. the glycolytic enzyme Pfk26p which is inactive in cells growing on ethanol and is activated in a PKA-dependent way when shifting to glucose as carbon source (François *et al.*, 1984; Boles *et al.*, 1996). Alternatively, loss of glucose repression of the *HSP12* gene expression could also explain part of the derepression on non-fermentable carbon sources. In addition to the analysis of mutant strains, examining salt-induced *HSP12* mRNA levels on non-fermentable carbon sources may give complementary information on the role of PKA in the stress response of yeast.

When yeast cells grown on non-fermentable carbon sources were exposed to high-osmolarity stress, growth arrest occurred comparable to the response on glucose-based medium (results not shown). This growth arrest

Fig. 1. High-salt-induced increase of *HSP12* mRNA in PKA mutants. (a) Northern analysis of high-salt (0.7 M NaCl) -induced mRNA levels of *HSP12* and *PGK1*. (b) Quantification of *HSP12* mRNA level relative to the 25S rRNA in the wild-type (□); *tpk2, tpk3* (◇); *tpk1^W, tpk2, tpk3* (○) and *tpk2, tpk3, bcy1* (△). (c) Quantification of *HSP12* mRNA level relative to the 25S rRNA in the *tpk2, tpk3, bcy1* strain.

Fig. 2. High-salt-induced increase of *HSP12* mRNA level in $\Delta hog1$ strain. (a) Northern analysis of high-salt (0.7 M NaCl) -induced mRNA levels of *HSP12* and *PGK1*. (b) *HSP12* mRNA levels relative to *PGK1* mRNA levels in the wild-type (□) and $\Delta hog1$ strain (■).

was reflected by the decrease of mRNA levels for ribosomal protein S33, which accompanied the response to high-osmolarity stress (Fig. 3a). In contrast to the fairly constitutive presence of *PGK1* mRNA on glucose-based medium, high-osmolarity conditions on ethanol- or glycerol-based media led to a marked decrease of *PGK1* mRNA level. After exposure to 0.7 M NaCl, induction of *HSP12* gene expression varied greatly in cells grown on glucose-, ethanol- or glycerol-based media. Comparing the induction levels and kinetics, the induced *HSP12* mRNA levels are lower on ethanol-based medium as compared to glucose (Fig. 3a, b). The lower level of induction is combined with a delay of the response in a W303IA background, but not in SPI (Fig. 3c, d). When glycerol was used as carbon source, the non-stress level of *HSP12* mRNA was found to be higher as compared with that on ethanol-based medium. Furthermore, when we analysed the high-salt-induced expression of *HSP12* on glycerol-based medium, hardly any increase in mRNA level was observed. Apparently, *HSP12* mRNA levels are influenced by factors that are dependent on the type of carbon source.

Signalling through the HOG pathway after high-osmolarity challenge is diminished in glycerol-grown cells

Activation of the HOG MAP kinase pathway after a high-osmolarity stress challenge results in the production of glycerol as osmolyte to compensate for the loss of water (Hohmann, 1997). The strong reduction of high-salt-induced *HSP12* mRNA levels in cells that were grown with glycerol as sole carbon source could be a reflection of negative feedback on the HOG pathway activity by glycerol or one of its derivatives. To examine

Fig. 3. High-salt-induced increase of *HSP12* mRNA levels on glucose-, ethanol- or glycerol-based media. (a) Northern analysis of mRNA levels after high-salt (0.7 M NaCl) exposure of SPI cells grown with either glucose, ethanol or glycerol as carbon source. (b) Quantification of relative (to 25S rRNA) level of *HSP12* mRNA in strain SPI on glucose (□), ethanol (△) or glycerol (○). (c, d) Induction of *HSP12* mRNA levels relative to the $t = 0$ level in strains SPI (c) and W303IA (d).

Fig. 4. Western analysis of tyrosine phosphorylation of Hog1p after high-salt challenge. Total cell extracts (40 µg) of yeast exposed to 0.7 M NaCl for times indicated on glucose-, ethanol- or glycerol-based media. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose. Phosphotyrosine content was detected with an anti-phosphotyrosine monoclonal antibody, PY20 (1:1000). The arrow indicates the position of Hog1p. Presence of Hog1p was detected with an anti-Hog1p polyclonal antibody.

the effect of the different carbon sources on the activation of signal transduction through the HOG pathway, we analysed tyrosine phosphorylation of Hog1p by Western analysis in total cell extracts. We used a *HOG1* overexpression strain to optimize the anti-phosphotyrosine labelling without affecting the phosphorylation kinetics (Brewster *et al.*, 1993). Hog1p is transiently phosphorylated on tyrosine after high-salt challenge, with a maximal phosphotyrosine level after 1 min (Fig. 4). When glycerol was used as carbon source, the response was more transient when compared with the response of glucose-grown cells (no signal at $t = 10$ min) and at a slightly lower level. Decreased salt-

induced expression of the *HSP12* gene on glycerol-based medium thus correlates with, and may at least be partly caused by, a decreased activation of Hog1p.

Stress response on non-fermentable carbon sources is modulated by PKA activity

The effect of glycerol on signalling through the HOG pathway did not explain the difference between high-salt-induced expression of *HSP12* on ethanol-based media and that on glucose. Non-fermentable carbon source conditions probably also affect the stress response in another way. To investigate the effect of PKA activity

Fig. 5. High-salt-induced *HSP12* mRNA levels in PKA mutants grown in ethanol- or glycerol-based media. (a) Northern analysis of *HSP12* and *PGK1* mRNA levels after high-salt (0.7 M NaCl) exposure of the low- (*tpk1^W*, *tpk2*, *tpk3*) and high- (*tpk2*, *tpk3*, *bcy1*) PKA strains grown on medium with ethanol as carbon source. (b) Northern analysis of high-salt-induced mRNA levels with the low- and high-PKA strains grown on glycerol-based medium.

on general stress signalling, we examined the *HSP12* response on non-fermentable carbon sources in PKA mutants (Fig. 5). Using the low-PKA mutant (*tpk1^W*, *tpk2*, *tpk3*), *HSP12* mRNA levels were higher on non-fermentable carbon sources under control ($t = 0$) as well as stress-induced conditions. This indicated that depression of *HSP12* gene expression on ethanol- or glycerol-based growth media is incomplete. The additional induction of *HSP12* mRNA levels after high-salt shock was diminished in the low-PKA mutant on ethanol-based medium comparable to the kinetics on glycerol-based medium.

The most dramatic effects on *HSP12* gene expression were observed in the high-PKA (*tpk2*, *tpk3*, *bcy1*) strain: under both normal growth and stress conditions, *HSP12* mRNA could not be detected. Catalytic activity of only Tpk1p (in the absence of the regulatory subunit Bcy1p) thus sufficed to overrule all responses on non-fermentable carbon sources. In contrast to the experiments performed with cells cultured in glucose-containing medium, no residual induction of *HSP12* mRNA levels was apparent.

PKA regulation of *HSP12* gene expression is mediated by Msn2/4p

PKA activity (Marchler *et al.*, 1993; Varela *et al.*, 1995) and the STRE-binding proteins Msn2/4p have been implicated in signal transduction underlying the general stress response of *S. cerevisiae* (Martinez-Pastor *et al.*, 1996). Deletion of the *MSN2* and *MSN4* genes results in decreased resistance of *S. cerevisiae* to a variety of severe stress conditions as well as loss of expression of general stress responsive genes (Martinez-Pastor *et al.*, 1996).

Fig. 6. High-salt-induced *HSP12* mRNA levels in the $\Delta msn2/4$ strain. (a) Northern analysis of *HSP12* and *PGK1* mRNA levels after high-salt (0.7 M NaCl) exposure of the $\Delta msn2/4$ strain grown on glucose-based medium. (b) Quantification of relative (to 25S rRNA) level of *HSP12* mRNA in the wild-type (□) and $\Delta msn2/4$ strain (■).

However, high-salt-induced increase in *HSP12* mRNA levels is not completely abolished in the *msn2/4* deletion strain (Fig. 6). A portion of the high-salt-induced increase in *HSP12* mRNA levels might be derived from either residual Msn2/4p-independent input of the HOG pathway or another high-osmolarity-induced signal. Additional deletion of the *PBS2* gene in the $\Delta msn2/4$

Fig. 7. High-salt-induced *HSP12* promoter-GUS fusions in high-PKA mutant and $\Delta msn2/4$ strains. (a) Schematic representation of the $\Delta 2$ - and $\Delta 32$ -GUS constructs. Putative Abf1- and Rap1-binding sites in the *HSP12* promoter are depicted, TATA boxes are shown as boxed Ts, heat-shock elements are denoted by HSE and STRE sequences are depicted as arrowheads. (b) Relative (to the *PGK1* mRNA level) *GUS* mRNA levels of the $\Delta 2$ - and $\Delta 32$ -GUS fusion constructs under non-stressed (□) and high-salt-induced (■) conditions. (c) Northern analysis of high-salt-induced *HSP12*, $\Delta 2$ - and $\Delta 32$ -GUS mRNA levels in the high-PKA (*tpk2, tpk3, bcy1*) and $\Delta msn2/4$ strains.

strain was previously shown to abolish the *HSP12* response to 0.4 M NaCl (Martinez-Pastor *et al.*, 1996). Although a direct link between PKA activity and the Msn2/4p has not yet been reported, stress-induced transport of the Msn2p to the nucleus appears to be correlated with PKA activity (H. Ruis, personal communication). By analysing different *HSP12* promoter-GUS fusion constructs in both the high-PKA mutant and $\Delta msn2/4$ strain we found further indications that Msn2/4p are likely to mediate the PKA effect on *HSP12* promoter activity. In the promoter deletion studies that were reported by Varela *et al.* (1995), the proximal 561 bp fragment ($\Delta 2$) of the *HSP12* promoter was shown to contain all elements necessary for full function (Fig. 7a, b). Deletion of the 5' part of the promoter up to the -234 position ($\Delta 32$) resulted in a truncated promoter (still containing two STREs) that was constitutively derepressed and slightly induced by high-osmolarity stress (Fig. 7a, b). Because derepression of $\Delta 32$ could be due to loss of a promoter element regulated by PKA, we analysed regulation of the $\Delta 32$ -GUS constructs in the high-PKA strain as well as in the $\Delta msn2/4$ strain (Fig. 7c). The $\Delta 2$ -GUS promoter construct was included in these analyses as the control for the full promoter activity. The $\Delta 2$ -GUS construct generally was regulated comparably to and following the kinetics of the endogenous *HSP12* gene, although high-salt induction resulted in a somewhat more transient response, perhaps reflecting the instability of *GUS* mRNA (Fig. 7c). In contrast, the $\Delta 32$ -GUS construct was regulated in a remarkable fashion. In the high-PKA strain, exposure to 0.7 M NaCl resulted in an initial increase in $\Delta 32$ -GUS mRNA level, followed by a decrease of mRNA level inversely parallel to the increased levels of $\Delta 2$ -GUS mRNA and that of the endogenous *HSP12* gene. Analysing the $\Delta 32$ -GUS construct in the $\Delta msn2/4$ strain showed the same kinetics. The observed kinetics of the $\Delta 32$ -GUS mRNA level was only observed in these strains upon high-osmolarity shock and not in a response to e.g. heat shock (not shown). These results

Fig. 8. High-salt-induced *HSP12* mRNA levels in the $\Delta msn2/4$ strain grown in ethanol- or glycerol-based media. (a) Northern analysis of *HSP12* and *PGK1* mRNA levels after high-salt (0.7 M NaCl) exposure of the $\Delta msn2/4$ strain grown on medium with ethanol as carbon source. (b) Northern analysis of high-salt-induced mRNA levels in the $\Delta msn2/4$ strain grown on glycerol-based medium.

further suggest that the Msn2/4p and PKA function in a common signalling pathway, regulating promoter activity of the *HSP12* gene.

PKA activity regulates the stress response at least at two different levels

To analyse at which level the non-fermentable carbon sources affected PKA input in the regulation of *HSP12* gene expression in high-osmolarity conditions, we examined *HSP12* expression after exposure of cells to 0.7 M NaCl in $\Delta msn2/4$ strain grown on ethanol- or glycerol-based media. If the influence of carbon sources

on the regulation of stress-induced *HSP12* gene expression would be mediated by a change in PKA activity, we would expect that *HSP12* expression in the $\Delta msn2/4$ strain is comparable to that in the high-PKA strain. In contrast to the high-PKA strain however, the *HSP12* gene appeared to be still responsive to high-osmolarity shock in the $\Delta msn2/4$ strain on both ethanol- and glycerol-based media (Fig. 8a, b). Although diminished, *HSP12* mRNA levels apparently follow the pattern of the induction in wild-type cells. However, the non-induced levels ($t = 0$) of *HSP12* mRNA were strongly diminished when compared to wild-type on ethanol or glycerol. Therefore, Msn2/4p appears to be involved in the derepression of *HSP12* gene expression when yeast is growing under non-fermenting conditions.

DISCUSSION

In previous studies on the regulation of high-osmolarity-induced *HSP12* gene expression, the HOG pathway and PKA activity were shown to be involved in an opposite manner: the first by inducing expression, the latter by a stress-induced derepression. Here we compared the input of both pathways into the net induction of *HSP12* expression after a high-salt challenge. We noticed that not only absolute mRNA levels, but also timing of the response is affected differently in mutants of both pathways. The shift of the high-salt-induced *HSP12* response to a later time-point in the $\Delta hog1$ strain is indicative of involvement of this pathway in the early responses to high osmolarity. Indeed, after a high-osmolarity shock the phosphotyrosine level of Hog1p displays a rapid increase. Although the high-osmolarity-induced activation of the HOG pathway is rapid, the increase in mRNA level of responsive genes such as *HSP12* and *GPD1* (Albertyn *et al.*, 1994; Varela *et al.*, 1992) occurs relatively late. In the case of the *HSP12* gene this could be a reflection of the repression of expression by PKA. Indeed, in low-PKA mutants a shift of the stress-induced peak in *HSP12* mRNA levels to an earlier point in time was observed. In the $\Delta hog1$ strain, still an increase in *HSP12* mRNA levels was observed, indicative of stress-induced loss of PKA-mediated repression. Reduction of PKA activity as a response to stress might be involved in the later, adaptive, responses of *S. cerevisiae* to stress. Although there are reports of a high-salt-induced decrease of cAMP levels (Marquez & Serrano, 1996) and modulation of PKA activity by PBS2 (Boguslawski, 1992), the mechanism by which PKA activity may be lowered under high-osmolarity stress conditions is so far not understood. The intricate interplay of both PKA and HOG pathways makes it very hard to draw conclusions from the response to stress in signal transduction mutants analysed at a fixed point in time. Therefore, to analyse the response to high osmolarity under conditions in which PKA activity is expected to be lower, we chose to study this response in cells grown with ethanol or glycerol as carbon source instead of glucose. The derepression of *HSP12* gene expression on non-fermentable carbon sources under non-stress conditions might be a reflection of lowered PKA activity.

The input of PKA activity in the regulation of *HSP12* expression was also influenced on ethanol- and glycerol-based media as compared to medium containing glucose. The non-fermentable carbon sources gave rise to increased levels of *HSP12* mRNA under non-stressed conditions. Since the derepressed levels of *HSP12* mRNA were lower in the $\Delta msn2/4$ strain, derepression is probably regulated via lowered PKA activity acting through STRE-driven transcription. Derepression of the *HSP12* gene expression was not complete because in the low-PKA mutant (*tpk1^w*, *tpk2*, *tpk3*), *HSP12* mRNA levels on ethanol or glycerol were even higher. Although derepression of the *HSP12* gene expression on non-fermentable carbon sources was evident, the mRNA levels could still be induced after a high-salt challenge, even in a $\Delta hog1$ strain (M. Siderius, data not shown). Therefore, we conclude that on both fermentable and non-fermentable carbon sources, salt-induced *HSP12* mRNA expression may be determined in part by a decrease in PKA activity.

The molecular mechanism responsible for the previously observed derepression of the $\Delta 32$ -GUS construct (Varela *et al.*, 1995) is elusive. Obviously the presence of two STREs is not sufficient for maintenance of the repressed state. In addition, transcription was found to be increased twofold upon salt challenge (Varela *et al.*, 1995). Remarkably enough, opposite regulation was observed in the high-PKA and $\Delta msn2/4$ strains exposed to salt. We have no explanation for this intriguing finding. The comparable kinetics of the $\Delta 32$ -GUS expression in the high-PKA and $\Delta msn2/4$ strains, as shown in Fig. 7(c), however strongly suggest that signalling through Msn2/4p depends on regulation of PKA activity. On the other hand, if PKA input into the *HSP12* expression would only be regulated by affecting STRE-driven promoter activity, the stress-induced *HSP12* expression should be similar in the high-PKA and $\Delta msn2/4$ strains. The $\Delta msn2/4$ strain still showed a residual high-osmolarity response of *HSP12* on both ethanol- and glycerol-based media. Intriguingly, the high-PKA strain (*tpk2*, *tpk3*, *bcy1*) still showed a high-osmolarity-induced increase in *HSP12* mRNA level on glucose-based medium but no response at all on the non-fermentable carbon sources. Apparently, high PKA activity results in more severe effects on stress-induced expression than deletion of Msn2/4p, which is indicative of additional input of PKA activity in the regulation of this response.

Because the residual high-osmolarity-induced expression of the *HSP12* gene in the $\Delta msn2/4$ strain is no longer observed when PBS2 is also deleted (Martinez-Pastor *et al.*, 1996), it is likely that this induction is the net input of HOG pathway activation. The residual levels of high-salt-induced *HSP12* mRNA observed in the high-PKA strain on glucose are probably also due to input of the HOG pathway. The complete absence of *HSP12* mRNA on ethanol- or glycerol-based media in the high-PKA strain might be due to inhibition of the output of Hog1p activation. Although a minor decrease in tyrosine phosphorylation of Hog1p was observed on

glycerol-based medium, high-salt-induced tyrosine phosphorylation was still detectable on both ethanol and glycerol. Therefore the level of inhibition of the output of the HOG pathway signalling most likely lies downstream of the MAP kinase.

Since the HOG pathway is very closely linked to the production of glycerol (Brewster *et al.*, 1993), we tested whether the absence of a significant induction of *HSP12* mRNA level in glycerol-grown cells could be due to feedback inhibition of glycerol or one of its metabolites. In contrast with ethanol-grown cells, activation of the HOG pathway as measured by the phosphotyrosine level of Hog1p was found to be affected in the glycerol-grown cells. Reduced activation of the HOG pathway may occur for example because glycerol or glycerol-3-P is metabolized into lipid components of the plasma membrane, changing the environment of the putative high-osmolarity receptors Sho1p or Sln1p. The response to lowering of the osmolarity in the medium has been proposed to involve signalling derived from sensing membrane stretch (Kamada *et al.*, 1995). Whether a comparable mechanism is involved in regulation of the high-osmolarity response needs to be addressed in future research. Alternatively, compounds such as glycerol-3-P were proposed to affect histidine kinase activity by acting as phosphate donors (Posas *et al.*, 1996). Utilization of glycerol as sole carbon source would therefore need to result in changes in lipid composition of the plasma membrane or higher glycerol-3-P concentration in the cell. We tried to mimick the glycerol effects by culturing the yeast cells in medium containing glycerol combined with glucose or ethanol. However, no significant change in the resulting kinetics of *HSP12* gene expression as compared to that observed on glycerol was found (not shown). As uptake and metabolism of glycerol could be different under these conditions, these experiments might not be conclusive.

Concluding remarks

To summarize the data presented in this paper, we observed that stress-induced reduction of PKA activity mediates *HSP12* expression through Msn2/4p and STRE-driven promoter activity but also by affecting an unknown component(s). Exposure to osmotic stress resulted in a residual increase in *HSP12* in the high-PKA strain grown in the presence of glucose. On non-fermentable carbon sources, however, this residual response was lost. Because the $\Delta msn2/4$ strain is still responsive on ethanol or glycerol, absence of the *HSP12* gene expression in the high-PKA strain is due to absence of input of PKA into the other part of the stress response. This regulation is mediated through a component which is regulated by glucose and might control the output of Hog1p activation. Since regulation of the main glucose repression pathway seems to fit into the above-mentioned characteristics in being regulated by PKA activity and being glucose-regulated, we hypothesize that part of the high-osmolarity induction of *HSP12* gene expression might be influenced by components of this pathway. Connection of this signalling pathway with the stress

Fig. 9. Hypothetical model of the regulation of high-osmolarity-induced *HSP12* expression. Exposure of *S. cerevisiae* to high-osmolarity conditions leads to activation of *HSP12* gene expression mediated by Hog1p activation and reduction of PKA activity. PKA regulation of the *HSP12* promoter activity is mediated by Msn2/4p and another factor, X, which affects output of the HOG pathway on non-fermentable carbon sources. Considering the glucose- and PKA-mediated regulation and links of multicopy suppressors of $\Delta snf1$ with the stress response, component(s) of the main glucose repression pathway might be involved in this regulation. Factor X might be a component of a stress-response pathway upstream of the transcription machinery or could be part of the mediator complex, regulating affinity or transcriptional activity of RNA polymerase II holoenzyme.

response is not unlikely as several multicopy suppressors of the $\Delta snf1$ strain (MSN2/4, ROX3) appear to influence stress-induced gene expression (Evangelista *et al.*, 1996; Martinez-Pastor *et al.*, 1996). Interaction between glucose-regulated and stress-response signalling could occur at the level of signal transduction components and/or by influencing transcriptional activity or affinity of the RNA polymerase II holoenzyme via components of the mediator complex (these options are encapsulated in 'factor X' in the working model presented in Fig. 9). We are currently testing this working hypothesis and preliminary experiments with mutants in the main glucose repression pathway indeed indicate involvement of this pathway with the high-osmolarity response of the *HSP12* gene.

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