Different signalling pathways contribute to the control of *GPD1* gene expression by osmotic stress in *Saccharomyces cerevisiae*

Martijn Rep,[1] Jacobus Albertyn,[2] Johan M. Thevelein,[1] Bernard A. Prior[2]† and Stefan Hohmann[1,3]

Author for correspondence: Stefan Hohmann. Tel: +46 31 773 2595. Fax: +46 31 773 2599.
e-mail: hohmann@gmm.gu.se

1 Laboratorium voor Moleculaire Celbiologie, Katholieke Universiteit Leuven, Kardinaal Mercierlaan 92, B-3001 Heverlee, Flanders, Belgium

2 Department of Microbiology and Biochemistry, University of the Orange Free State, PO Box 339, Bloemfontein 9300, South Africa

3 Department of Cell and Molecular Biology/Microbiology, Göteborg University, Box 462, SE-405 30 Göteborg, Sweden

Yeast cells respond to a shift to higher osmolarity by increasing the cellular content of the osmolyte glycerol. This response is accompanied by a stimulation of the expression of genes encoding enzymes in the glycerol production pathway. In this study the osmotic induction of one of those genes, *GPD1*, which encodes glycerol-3-phosphate dehydrogenase, was monitored in time course experiments. The response is independent of the osmolyte and consists of four apparent phases: a lag phase, an initial induction phase, a feedback phase and a sustained long-term induction. Osmotic shock with progressively higher osmolyte concentrations caused a prolonged lag phase. Deletion of *HOG1*, which encodes the terminal protein kinase of the high osmolarity glycerol (HOG) response pathway, led to an even longer lag phase and drastically lower basal and induced *GPD1* mRNA levels. However, the induction was only moderately diminished. Overstimulation of Hog1p by deletion of the genes for the protein phosphatases *PTP2* and *PTP3* led to higher basal and induced mRNA levels and a shorter lag phase. The protein phosphatase calcineurin, which mediates salt-induced expression of some genes, does not appear to contribute to the control of *GPD1* expression. Although *GPD1* expression has so far not been reported to be controlled by a general stress response mechanism, heat-shock induction of the *GPD1* mRNA level was observed. However, unregulated protein kinase A activity, which strongly affects the general stress response, only marginally altered the mRNA level of *GPD1*. The osmotic stimulation of *GPD1* expression does not seem to be mediated by derepression, since deletion of the *SSN6* gene, which encodes a general repressor, did not significantly alter the induction profile. A hypo-osmotic shock led to a transient 10-fold drop of the *GPD1* mRNA level. Neither the HOG nor the protein kinase C pathway, which is stimulated by a decrease in external osmolarity, is involved in this effect. It was concluded that osmotic regulation of *GPD1* expression is the result of an interplay between different signalling pathways, some of which remain to be identified.

Keywords: osmotic stress, signal transduction, high osmolarity glycerol (HOG) pathway, protein kinase A, yeast

INTRODUCTION

The ability to adapt to altered osmolarity of the surrounding growth medium is of fundamental importance for uni- and multicellular organisms. A strategy in osmoadaptation probably employed by all cell types is the production and accumulation of compatible osmolytes to adjust the intracellular osmolarity to that

†**Present address:** Department of Microbiology, University of Stellenbosch, Private Bag XI, 7602 Matieland, South Africa.

Abbreviations: HOG, high osmolarity glycerol; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; PKC, protein kinase C; STRE, stress response element.

of the growth medium (Yancey *et al.*, 1982). Such osmolytes are species- and stage-specific and comprise ions, sugars, sugar alcohols and amino acids and their derivatives. We have studied the response of the yeast *Saccharomyces cerevisiae* to osmotic stress as a eukaryotic model system for molecular mechanisms in osmoadaptation. This organism employs glycerol as osmolyte (Blomberg & Adler, 1992; Brown & Edgley, 1980; Hohmann, 1997).

Glycerol is produced in two enzymic steps from the glycolytic intermediate, dihydroxyacetone phosphate (Gancedo *et al.*, 1968; Prior & Hohmann, 1997). The two enzymes are each encoded by two highly homologous isogenes: *GPD1* and *GPD2* encode glycerol-3-phosphate dehydrogenase and *GPP1* and *GPP2* encode glycerol-3-phosphatase (Albertyn *et al.*, 1994; Ansell *et al.*, 1997; Eriksson *et al.*, 1995; Larsson *et al.*, 1993; Norbeck *et al.*, 1996). The expression of these genes appears to be differentially regulated in response to altered growth conditions. Expression of *GPD2* and *GPP1* is stimulated under anaerobic conditions, when glycerol production becomes essential for redox regulation (Ansell *et al.*, 1997; A.-K. Påhlman & L. Adler, unpublished results). Transcription of *GPD1* and *GPP2* is stimulated by an increase in external osmolarity (Albertyn *et al.*, 1994; Ansell *et al.*, 1997; Hirayama *et al.*, 1995; Hohmann, 1997; Norbeck *et al.*, 1996).

The molecular mechanisms by which alterations in external osmolarity mediate responses in gene expression are of major interest. In yeast, two well studied signal transduction pathways are known to be rapidly stimulated by changes in osmolarity (Banuett, 1998; Hohmann, 1997). The high osmolarity glycerol (HOG) response pathway consists of two putative transmembrane osmosensors, Sho1p and Sln1p, and a branched mitogen-activated protein kinase (MAPK) cascade with Hog1p as the sole terminal MAPK (Brewster *et al.*, 1993; Maeda *et al.*, 1994, 1995; Posas & Saito, 1997, 1998; Posas *et al.*, 1996). Phosphorylation of Hog1p is stimulated by increased external osmolarity within about 1 min (Brewster *et al.*, 1993; Maeda *et al.*, 1994, 1995; Siderius *et al.*, 1997). The phosphorylation state of Hog1p is controlled not only by upstream kinases but also by the protein phosphatases Ptp2p and Ptp3p (Jacoby *et al.*, 1997; Wurgler-Murphy *et al.*, 1997). The protein kinase C (PKC) pathway also consists of a MAPK cascade with Slt2/Mpk1p as the terminal MAPK (Irie *et al.*, 1993; Lee *et al.*, 1993; Lee & Levin, 1992; Levin & Errede, 1995; Levin *et al.*, 1990; Martin *et al.*, 1993). Phosphorylation of Slt2/Mpk1p has been shown to be rapidly stimulated by a decrease in external osmolarity by unknown sensing mechanisms (Davenport *et al.*, 1995). In addition to these osmo-sensing pathways, there is a signalling route specifically required for the adaptation to high salt but not to general osmotic stress (Danielsson *et al.*, 1996; Hirata *et al.*, 1995; Mendoza *et al.*, 1994). This pathway includes the protein phosphatase calcineurin, which consists of a catalytic and a regulatory subunit (Stark, 1996). The catalytic subunit is redundantly encoded by the genes *CNA1/CMP1* and *CNA2/CMP2* (Cyert *et al.*, 1991; Liu *et al.*, 1991) while the regulatory subunit is encoded by *CNB1* (Cyert & Thorner, 1992).

Yeast cells not only have specific stress response mechanisms but also respond to stress in general (Ruis & Schüller, 1995; Siderius & Mager, 1997). The general stress response is at least partly controlled by protein kinase A (PKA) and mediated redundantly by transcription factors Msn2p and Msn4p which bind to so called stress response elements (STREs) in the promoters of many genes (Belazzi *et al.*, 1991; Boy-Marcotte *et al.*, 1998; Görner *et al.*, 1998; Mager & de Kruijff, 1995; Marchler *et al.*, 1993; Martinez-Pastor *et al.*, 1996; Siderius & Mager, 1997).

The osmotic induction of several genes has been reported to depend on the HOG pathway or on PKA or on a combination of both (Albertyn *et al.*, 1994; Hirayama *et al.*, 1995; Márquez & Serrano, 1996; Norbeck *et al.*, 1996; Schüller *et al.*, 1994; Varela *et al.*, 1995). In this work we have studied in time course experiments the control of the mRNA level of the *GPD1* gene after an increase or a decrease in external osmolarity. This analysis reveals that the HOG pathway plays an important, albeit not exclusive, role in osmotic induction of *GPD1* expression, while PKA only contributes marginally, if at all, to osmotic induction. It also appears that osmotic control of *GPD1* expression is the result of an interplay between different signalling pathways, some of which remain to be identified.

METHODS

Plasmid and yeast strain constructions. The yeast strains used in this study are listed in Table 1. The construction of the *hog1Δ::TRP1* deletion mutant has been described previously (Albertyn *et al.*, 1994; Brewster *et al.*, 1993). In some strains the entire coding region of *HOG1* was deleted by a PCR approach (Eberhardt & Hohmann, 1995). The *SLT2/MPK1* gene was deleted in W303-1A as described by Lee *et al.* (1993) and *PTP2* was deleted according to Maeda *et al.* (1993). To delete *PTP3* the gene plus its flanking regions were amplified by PCR and cloned into pUC18. A *ptp3Δ::URA3* deletion construct was made by replacing a *Bgl*II fragment, covering the region from −22 (relative to the translational start codon) to +2738 (49 bp before stop codon), by a *Bam*HI fragment with the *URA3* gene from YDpU (Berben *et al.*, 1991). The strains YMR 88 (*ptp2Δ::LEU2 ptp3Δ::URA3*) and YMR 90 (*hog1Δ::TRP1 ptp2Δ::LEU2 ptp3Δ::URA3*) are spores derived from a cross between YMR 86 (*ptp3Δ::URA3*) and YSH 854 (*ptp2Δ::LEU2 hog1Δ::TRP1*). Disruptions of *BCY1*, *TPK1* and *TPK2* were transferred from their original background (SP1) into W303-1A by transformation with DNA fragments containing the respective disruption alleles. These were amplified by PCR from chromosomal DNA of strains RS13-58A-1 (the *tpk2::HIS3* allele) and RS13-7C-1 (the *bcy1::LEU2* and *tpk1Δ::URA3* alleles; Nikawa *et al.*, 1987). The mutations were combined with each other and with a *hog1Δ::TRP1* allele from YSH 445 (*MATα hog1Δ::TRP1*) through several rounds of crossing and sporulation to obtain strains YMR 70 (*tpk1Δ::URA3 tpk2::HIS3*), YMR 72 (*tpk1Δ::URA3 tpk2::HIS3 bcy1::LEU2*) and YMR 93 (*tpk1Δ::URA3 tpk2::HIS3 bcy1::LEU2 hog1Δ::TRP1*). The *ssn6Δ* strain YMR 125 was constructed from MAP6 (kindly

Table 1. Strains used

Strain	Genotype	Source or reference
W303-1A	*MATa leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2 mal0*	Thomas & Rothstein (1989)
YSH 849	(W303-1A) *mpk1Δ::TRP1*	This work
YSH 444	(W303-1A) *hog1Δ::TRP1*	Albertyn *et al.* (1994)
YSH 445	(W303-1A) *MATα hog1Δ::TRP1*	This work
DHT23-1B	(W303-1A) *cna1/cmp1Δ::URA3 cna2/cmp2Δ::HIS3*	Nakamura *et al.* (1993)
YSH 816	(W303-1A) *ptp2Δ::LEU2*	This work
YMR 86	(W303-1A) *ptp3Δ::URA3*	This work
YMR 88	(W303-1A) *ptp2Δ::LEU2 ptp3Δ::URA3*	This work
YMR 90	(W303-1A) *hog1Δ::TRP1 ptp2Δ::LEU2 ptp3Δ::URA3*	This work
YSH 7.50-2B	(W303-1A) *MATα hog1Δ::TRP1 ptp2Δ::LEU2*	This work
YMR 70	(W303-1A) *tpk1Δ::URA3 tpk2::HIS3*	This work
YMR 73	(W303-1A) *tpk1Δ::URA3 tpk2::HIS3 bcy1::LEU2*	This work
YMR 92	(W303-1A) *tpk1Δ::URA3 tpk2::HIS3 bcy1::LEU2 hog1Δ::TRP1*	This work
SP1	*MATa leu2 his3 trp1 ura3 ade8 can1 SUC GAL mal0*	Toda *et al.* (1985)
RS13-58A-1	(SP1) *tpk1^{w1} tpk2::HIS3 tpk3::TRP1 bcy1::LEU2*	Nikawa *et al.* (1987)
RS13-7C-1	(SP1) *tpk1D::URA3 tpk2^{w1} tpk3::TRP1 bcy1::LEU2*	Nikawa *et al.* (1987)
W303-1A ADE2	*MATa leu2-3/112 ura3-1 trp1-1 his3-11/15 can1-100 GAL SUC2 mal0*	Markus Proft, Valencia
MAP6	(W303-1A ADE2) *ssn6::loxp-KAN-loxp*	Markus Proft, Valencia
YMR 125	(W303-1A ADE2) *ssn6::loxp*	This work

provided by M. Proft, Valencia) by removing the *KAN* marker using the pSH47 plasmid encoding the Cre recombinase (Guldener *et al.*, 1996). The W303-1A ADE2 strain background is a direct derivative of W303-1A lacking the *ade2* marker.

Media and growth conditions. Cells were grown on a rotary shaker at 30 °C in YP medium (2% peptone, 1% yeast extract) supplemented with a carbon source as indicated. Synthetic medium (SM) consisting of amino-acid-free yeast nitrogen base (Difco) and 2% glucose was described by Sherman *et al.* (1983). For stress induction experiments, cells were pregrown in YP medium containing 2% glucose (YPD) without osmotic adjustment (water activity 0·998 a_w) to mid-exponential phase (OD_{600} between 0·7 and 1·5). For osmotic support, 0·5 M sorbitol was added to the growth medium of the *slt2/mpk1Δ* mutant. Osmotic stress was applied by adding NaCl to the cultures resulting in final concentrations of 0·5 M NaCl (3%; 0·980 a_w), 0·85 M NaCl (5%; 0·970 a_w) or 1·4 M NaCl (8%; 0·954 a_w). Alternatively, cells were sedimented and resuspended in YPD medium supplemented with 0·95 M sorbitol (0·980 a_w, equivalent to 0·5 M NaCl) or 1·5 M sorbitol (0·970 a_w, equivalent to 0·85 M NaCl). For osmotic downshock experiments cells were grown to mid-exponential phase in YPD medium supplemented with 0·85 M NaCl plus 0·5 M sorbitol (0·962 a_w). Cells were sedimented and resuspended in YPD supplemented with 0·5 M sorbitol but lacking NaCl.

For heat stress, medium amounting to one-third of the culture volume was pre-heated to 66 °C and added to the culture to give a final temperature of 42 °C. Cells were then incubated in a water bath at 42 °C and samples taken at the times indicated. For entry into a non-proliferative phase, cells pregrown to either mid-exponential phase or stationary phase were reinoculated in fresh medium and growth was monitored until cells stopped dividing. For growth on a non-fermentable carbon source or for nitrogen starvation, cells were pregrown in YP medium containing 8% glucose, sedimented and resuspended in either YP medium containing 3% ethanol for 6 h (growth on a non-fermentable carbon source) or in SM without ammonium sulphate containing 8% glucose (for nitrogen starvation). Nitrogen starvation was conducted for 24 h; there was still glucose present in the growth medium after this period. For glucose starvation experiments, cells were pregrown in YPD medium until exponential phase and resuspended in YP medium for 6 h.

Northern blotting techniques. Total RNA was isolated at the time points indicated in the figures and separated by electrophoresis according to the method described by de Winde *et al.* (1996). Blots were hybridized in buffer containing 7% SDS, 0·5 M sodium phosphate buffer, pH 7·2, and 1 mM EDTA. The signal was quantified using a phosphorimager (Fuji, BAS-1000). The probes used were generated by PCR from chromosomal DNA of W303-1A. The *GPD1* probe includes sequences from −527 to +95 relative to the start codon (this excludes sequences homologous to *GPD2*). Probes for *CTT1* (−14 to +1007), *HSP12* (−139 to +493) and *SSA3* (+3 to +980) were kindly provided by Dr de Winde (Leuven, Belgium). Probe fragments were labelled with High Prime (Boehringer Mannheim).

As a loading control we have used an *IPP1* probe, including sequences from +16 to +846 relative to the start codon. *IPP1* encodes inorganic pyrophosphatase and the protein level was found to be entirely unaffected by osmotic shifts (Norbeck & Blomberg, 1997; A. Blomberg & J. Norbeck, personal communication). Commonly, *ACT1*, which encodes actin, has been used as a loading control. Comparison of the *IPP1* and the *ACT1* signals showed that the *ACT1* level responds significantly to both hyper- and hypo-osmotic shock while that of *IPP1* remains constant (data not shown).

Reproducibility of the data. Experiments were generally performed at least in duplicate or triplicate with consistent results, i.e. the differences between wild-type and mutants were highly reproducible. The relative mRNA levels differed from experiment to experiment by no more than 20%. The results from representative experiments are shown.

RESULTS

Osmotic induction of *GPD1* expression is transient and more severe osmotic stress leads to a progressive delay of the response

We have monitored the mRNA level of *GPD1* in time course experiments after a shift to higher osmolarity. When using either 0·5 M NaCl or 0·95 M sorbitol, which both give the same water activity of 0·980 a_w, the induction curves were almost superimposable (Figs 1 and 2). Thus, the response is independent of the solute used. The response appeared to consist of different phases starting with an approximately 20-fold induction within 30 min. Subsequently, the mRNA level dropped to about the same level as before osmolyte addition, slowly increased again and then stayed four- to fivefold higher than before induction, even after prolonged incubation (Figs 1 and 2 and data not shown).

When higher osmolyte concentrations were used, such as 0·85 M NaCl and 1·5 M sorbitol (0·970 a_w), the response was delayed and the highest *GPD1* mRNA level was observed after about 2 h instead of 30–45 min at 0·980 a_w (Figs 1 and 2). The maximal mRNA level during the initial induction was higher than at the lower solute concentration. After prolonged incubation the *GPD1* mRNA level was 10-fold higher as compared to before the shift to higher osmolarity (data not shown).

When even higher salt concentrations (1·4 M, 0·954 a_w) were applied, the response was shifted further to later time points, but also the induction profile differed. There was no apparent rapid induction but instead a slow increase to an about fivefold higher *GPD1* mRNA level. Thus, the profile of osmotic induction of *GPD1* expression is dependent on osmolyte concentrations.

The HOG pathway is involved in the timing and determines the amplitude of the response

The *hog1*Δ mutation affected *GPD1* expression in different ways (Figs 1 and 2). First, the mRNA level of *GPD1* was diminished both before and after osmotic

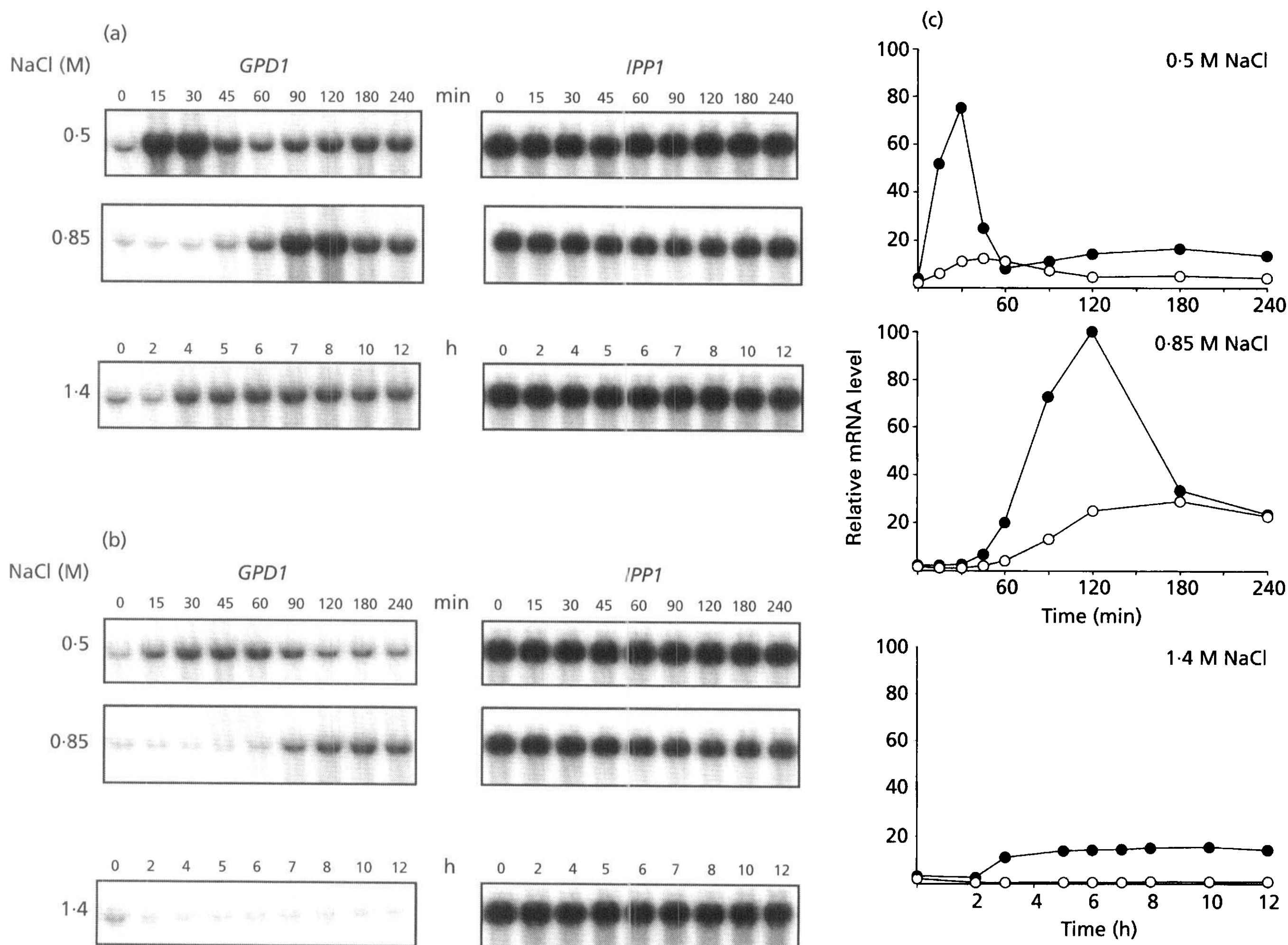

Fig. 1. *GPD1* mRNA levels after a shift to higher NaCl concentrations in the wild-type (a) and the *hog1*Δ mutant (b). (c) Graphs represent quantification of the mRNA levels relative to those of *IPP1* for the wild-type (●) and the *hog1*Δ mutant (○). The highest relative mRNA level was set to 100.

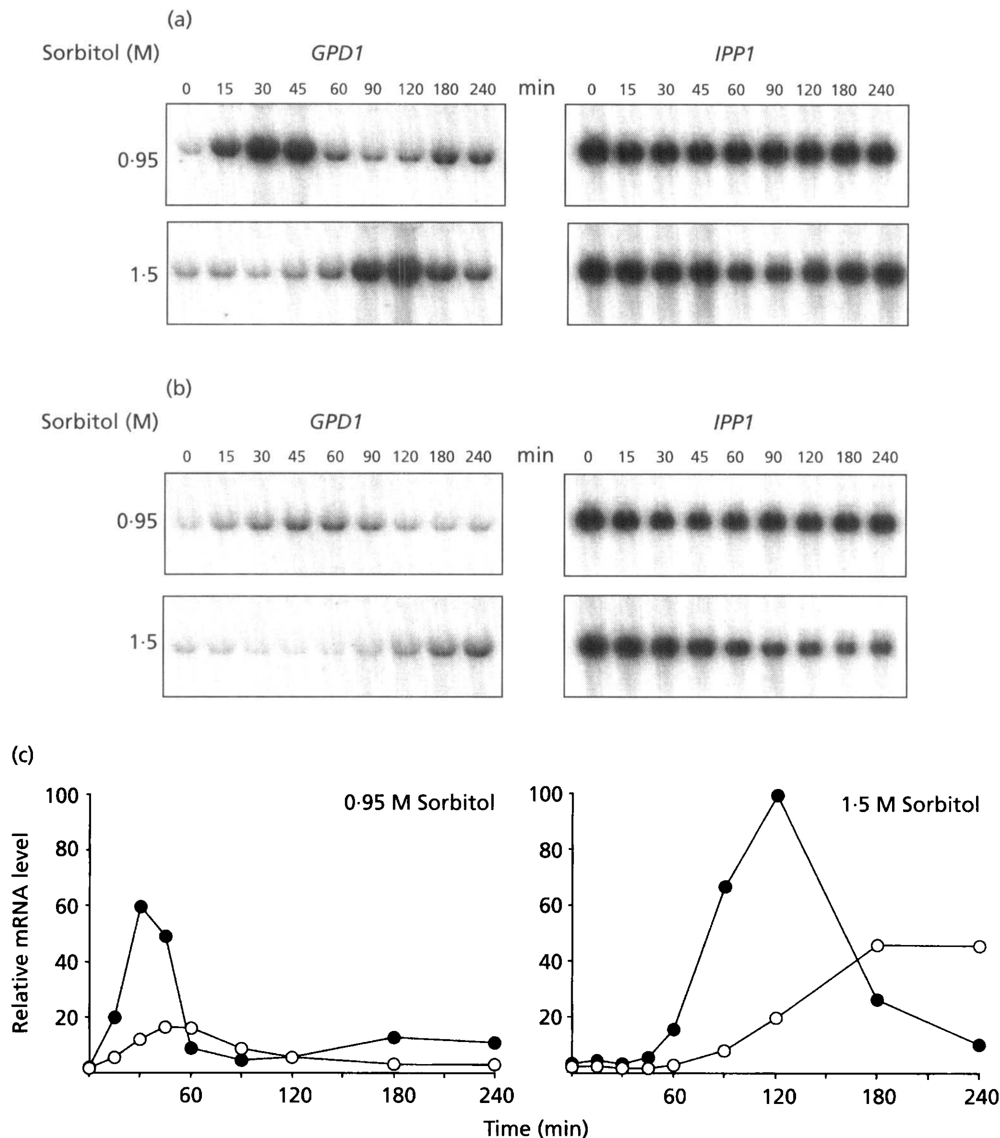

Fig. 2. *GPD1* mRNA levels after a shift to higher sorbitol concentrations in the wild-type (a) and the *hog1*Δ mutant (b). Sorbitol at 0·95 M results in the same water activity of 0·980 a_w as 0·5 M NaCl, and 1·5 M sorbitol causes the same osmotic stress as 0·85 M NaCl (0·970 a_w). (c) Graphs represent quantification of the mRNA levels relative to those of *IPP1* for the wild-type (●) and the *hog1*Δ mutant (○). The highest relative mRNA level was set to 100.

shock. In addition, maximal induction was consistently shifted to later time points at the lowest and the intermediate solute concentration. At 1·4 M NaCl (0·954 a_w) the *hog1*Δ mutant was unable to increase the *GPD1* mRNA level within 12 h after the shift. The lack of induction is not due to cell death (data not shown) and the *IPP1* mRNA level remained constant during the entire experiment.

Although expression of *GPD1* was strongly affected in the *hog1*Δ strain, we note that the mutant was able to mount a response after a shift to 0·980 and 0·970 a_w. Maximal induction was diminished from about 20- to 25-fold in the wild-type to about eightfold in the *hog1*Δ mutant at 0·980 a_w and from about 40- to 20-fold at 0·970 a_w. After prolonged incubation at 0·970 and 0·980 a_w, the *GPD1* mRNA level was about half that of the wild-type and four- and eightfold higher as compared to before the upshift.

Since the *hog1*Δ mutant is osmosensitive (Albertyn *et al.*, 1994; Brewster *et al.*, 1993) some of the effects observed in that strain, such as the delay in the response, could be due to secondary effects of the mutation and not to a specific involvement of the HOG signalling cascade. Therefore, we did the same time course experiment at 0·85 M NaCl with mutations in the *PTP2* and *PTP3* genes. These two genes encode protein phosphatases and deletion of *PTP2* and *PTP3* increases the phosphorylation state (Jacoby *et al.*, 1997; Wurgler-Murphy *et al.*, 1997) and hence most probably the activity of Hog1p. Deletion of *PTP2* and of *PTP2* plus

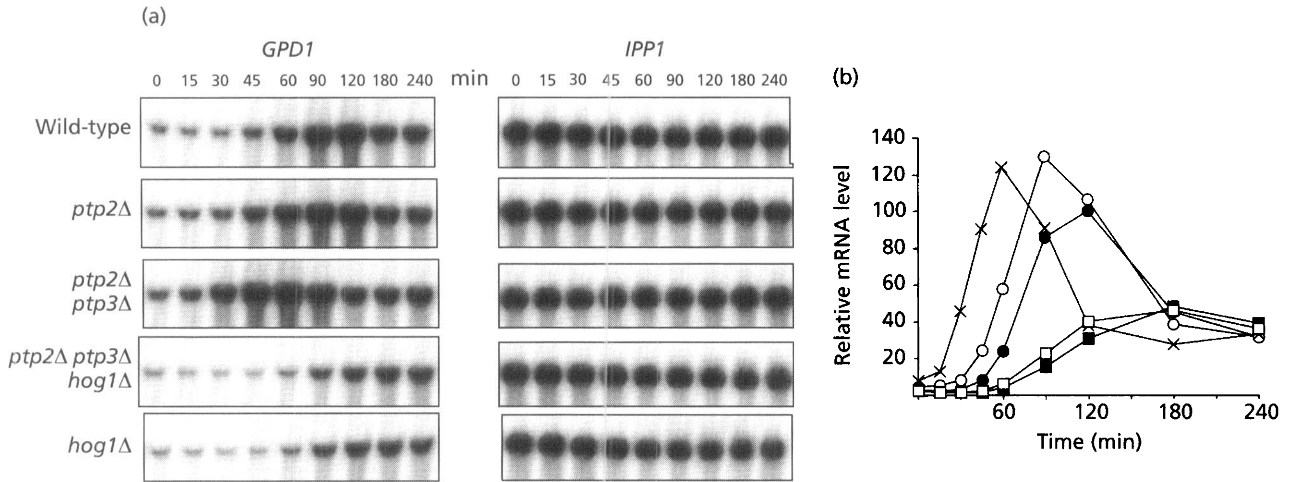

Fig. 3. (a) Osmotic induction of *GPD1* mRNA levels by 0·85 M NaCl in mutants lacking the protein kinase Hog1p and/or the protein phosphatases Ptp2p and Ptp3p, which control Hog1p. (b) The graph represents relative mRNA levels for the wild-type (●) and the *ptp2Δ* (○), *ptp2Δ ptp3Δ* (×), *ptp2Δ ptp3Δ hog1Δ* (■) and *hog1Δ* (□) mutants. The highest relative mRNA level in the wild-type was set to 100.

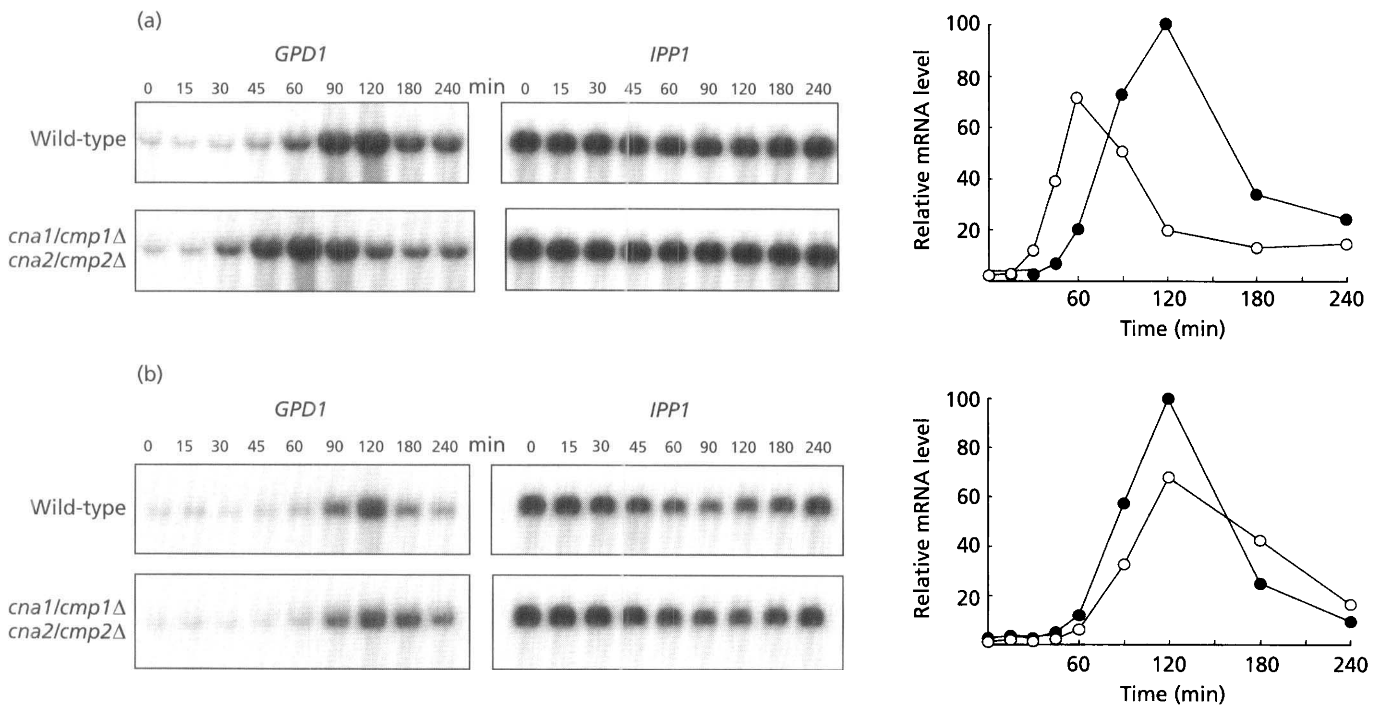

Fig. 4. Osmotic induction of *GPD1* mRNA levels by 0·85 M NaCl (a) and 1·5 M sorbitol (b) in the wild-type (●) and a *cmp1Δ cmp2Δ* mutant (○) which lacks the catalytic subunit of the protein phosphatase calcineurin. The highest relative mRNA level in the wild-type was set to 100.

PTP3 led to a somewhat higher basal and induced level of the *GPD1* mRNA and also shifted the response to earlier time points (Fig. 3). This observation is consistent with a direct involvement of the HOG pathway in controlling *GPD1* expression. This is further confirmed by the fact that deletion of *HOG1* in a *ptp2Δ ptp3Δ* double mutant resulted in exactly the same induction profile as in the *hog1Δ* single mutant, as expected if

PTP2 and *PTP3* mediated their effect on *GPD1* expression solely via Hog1p. The *ptp2*Δ and *ptp2*Δ *ptp3*Δ mutations did not lead to an increased maximal induction since the basal level was already 1·5- and 2·5-fold higher as compared to wild-type, respectively.

The calcineurin protein phosphatase does not appear to mediate salt-induced expression of *GPD1*

The calcineurin protein phosphatase has been shown to be required for the induction of expression of the *PMR2/ENA1* gene by high salt concentrations but not by a general osmolarity upshift (Márquez & Serrano, 1996). Mutants lacking the two genes for the catalytic subunit of the phosphatase are viable but display a complex phenotype, including salt sensitivity (Danielsson *et al.*, 1996; Mendoza *et al.*, 1994, 1996). We therefore tested the effect of the *cna1/cmp1*Δ *cna1/cmp2*Δ mutation on salt-induced expression of *GPD1* at 0·85 M NaCl. The induction profile was altered to some extent, i.e. maximal induction was diminished by about 30% and shifted to an earlier time point (Fig. 4a). This may point to some involvement of the calcineurin pathway. However, a similar reduction of maximal *GPD1* induction was seen with sorbitol as osmolyte (Fig. 4b), even though sorbitol does not stimulate the calcineurin pathway (Márquez & Serrano, 1996). Together with the absence of a salt-specific component in the induction of *GPD1* expression in wild-type or *hog1*Δ mutant (Figs 1 and 2), this leads us to believe the small reduction of *GPD1* expression in the mutant does not point to a specific involvement of this pathway.

We have performed a similar time course experiment after a shift to 0·85 M NaCl with a *slt2/mpk1*Δ mutant lacking the terminal protein kinase of the PKC pathway. This pathway can be stimulated by a hypo- but not by a hyperosmotic shock (Davenport *et al.*, 1995). Neither basal nor induced *GPD1* mRNA levels nor the induction profile was altered in this mutant (data not shown), confirming an earlier report that *GPD1* expression is not affected by mutations in the PKC pathway (Hirayama *et al.*, 1995).

Induction of *GPD1* expression is not mediated by the cAMP-PKA pathway

The expression of several yeast genes is induced by any type of stress, i.e. by a general stress response, which is mediated by so-called STREs in the promoter of the target genes (Kobayashi & McEntee, 1993; Mager & de Kruijff, 1995; Marchler *et al.*, 1993; Martinez-Pastor *et al.*, 1996; Ruis & Schüller, 1995; Siderius & Mager, 1997; Varela *et al.*, 1995). There are three such elements in the *GPD1* promoter at positions −330, −186 and −34. To investigate if expression of *GPD1* is stimulated by other stress types, we have performed Northern blotting analysis after a shift to higher temperature (Fig. 5), after entry into the non-proliferative growth phase (pre-stationary phase), during nitrogen starvation and during growth on a non-fermentable carbon source, all conditions known to stimulate expression of genes controlled by STREs. Only heat shock caused about a fivefold stimulation of the *GPD1* mRNA level (Fig. 5). The induction was equally rapid as that of *CTT1*. Apparently, expression of *GPD1* can also be controlled by stress conditions other than high osmolarity. We have used 18S rRNA in this experiment as standard for the quantification, since both *IPP1* and *ACT1* mRNA (data not shown) decrease dramatically during the time course, perhaps due to a general reduction of transcription of non-stress genes under these conditions. Although 18S rRNA also decreases initially, it correlates better with the total RNA loaded.

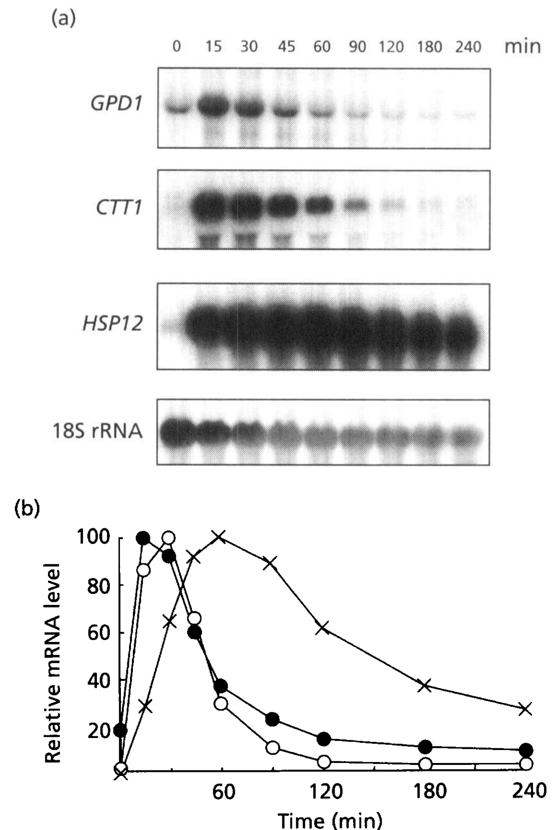

Fig. 5. mRNA levels, after a shift to 42 °C, of *GPD1* (●), *CTT1* (○) which encodes cytosolic catalase T and *HSP12* (×) which encodes a small heat-shock protein. (a) Northern blot, (b) quantification. Since the mRNA level of *IPP1* as well as that of *ACT1* steadily dropped after heat shock, 18S rRNA was employed as standard. The highest relative level of each mRNA was set to 100.

The general stress response is strongly influenced by PKA, which controls the localization of the STRE-binding protein Msn2p (Belazzi *et al.*, 1991; Görner *et al.*, 1998; Marchler *et al.*, 1993; Martinez-Pastor *et al.*, 1996; Ruis & Schüller, 1995). High PKA activity leads to low expression of STRE-controlled genes and low PKA activity leads to high expression of such genes. We wished to investigate the role of the cAMP-PKA pathway

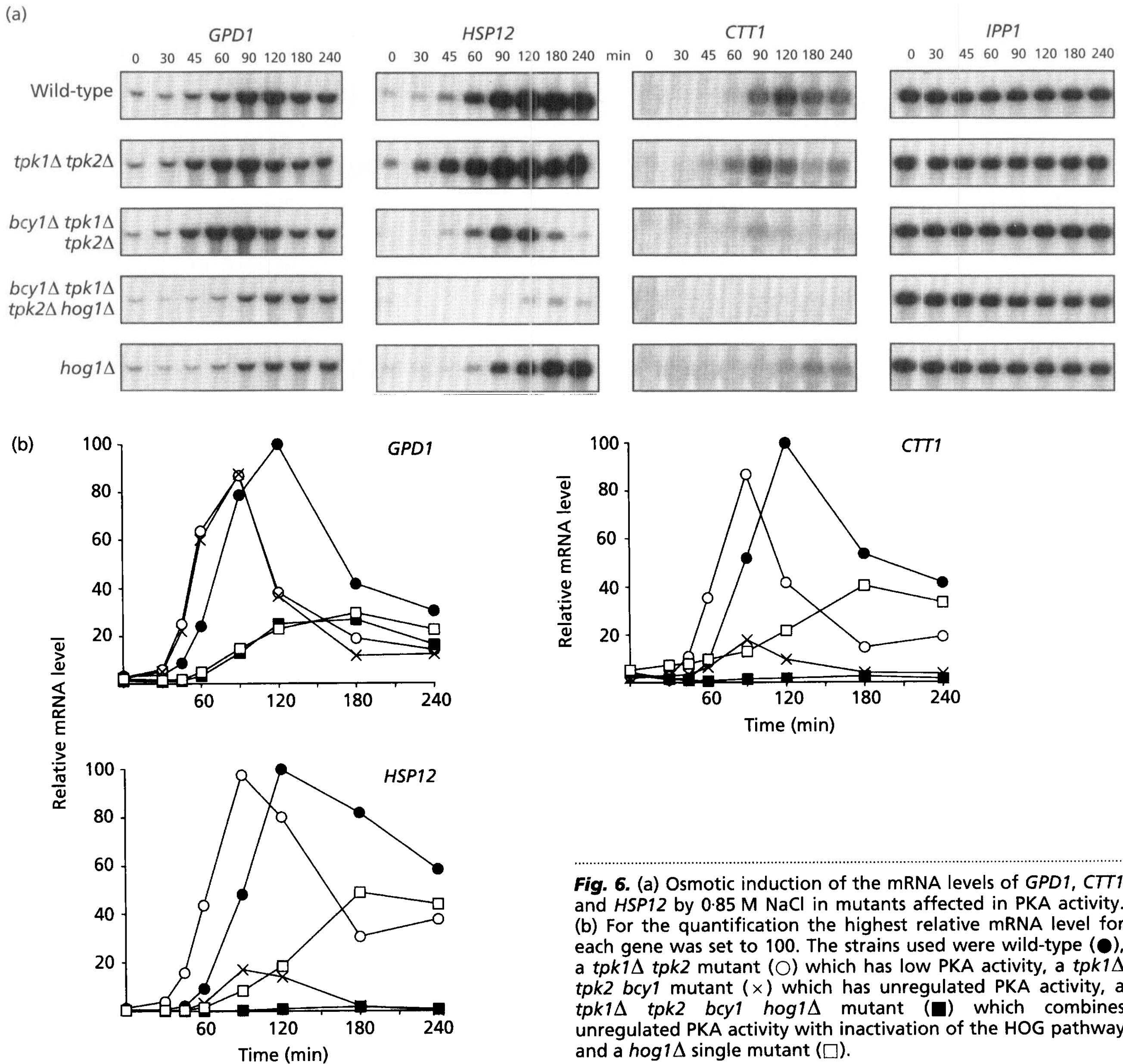

Fig. 6. (a) Osmotic induction of the mRNA levels of *GPD1*, *CTT1* and *HSP12* by 0·85 M NaCl in mutants affected in PKA activity. (b) For the quantification the highest relative mRNA level for each gene was set to 100. The strains used were wild-type (●), a *tpk1Δ tpk2* mutant (○) which has low PKA activity, a *tpk1Δ tpk2 bcy1* mutant (×) which has unregulated PKA activity, a *tpk1Δ tpk2 bcy1 hog1Δ* mutant (■) which combines unregulated PKA activity with inactivation of the HOG pathway and a *hog1Δ* single mutant (□).

in control of *GPD1* expression. To this end, we used a mutant, *tpk1Δ tpk2Δ bcy1Δ*, that lacks the cAMP-binding subunit of PKA. *TPK3*, the only gene encoding a catalytic subunit of PKA in this strain, is expressed at a very low level (Mazon *et al.*, 1993). Therefore, this strain does not show the characteristic phenotypes of strains with high, unregulated, PKA activity (like stress sensitivity) but just lacks cAMP-PKA signalling. The *tpk1Δ tpk2Δ* strain was used to assess the effect of low PKA activity.

Low PKA activity did not cause higher basal expression of any of the three genes but the induction was shifted to a slightly earlier time point in all cases (Fig. 6). This weak effect of low PKA activity on STRE-controlled genes contrasts with earlier findings (Marchler *et al.*, 1993; Martinez-Pastor *et al.*, 1996). Possibly, the activity of the *TPK3* gene, although low, is still higher than that of the more generally used TPK-attenuated alleles. Maximal induction was unaffected. Unregulated PKA activity caused strongly diminished levels of *CTT1* and *HSP12* mRNA while the profile of *GPD1* induction was identical to that observed in the mutant with low PKA activity. Hence, PKA activity does not appear to control *GPD1* expression or at least not in the same way as expression of *CTT1* and *HSP12*.

Induction of *CTT1* and *HSP12* expression after a shift to 0·85 M NaCl was delayed and diminished in a *hog1Δ* strain in very much the same way as observed for *GPD1*. This behaviour has been reported previously for *HSP12* (Siderius *et al.*, 1997). In the quadruple mutant having unregulated PKA activity and lacking *HOG1* (*tpk1Δ tpk2 bcy1 hog1Δ*), there was no apparent induction of

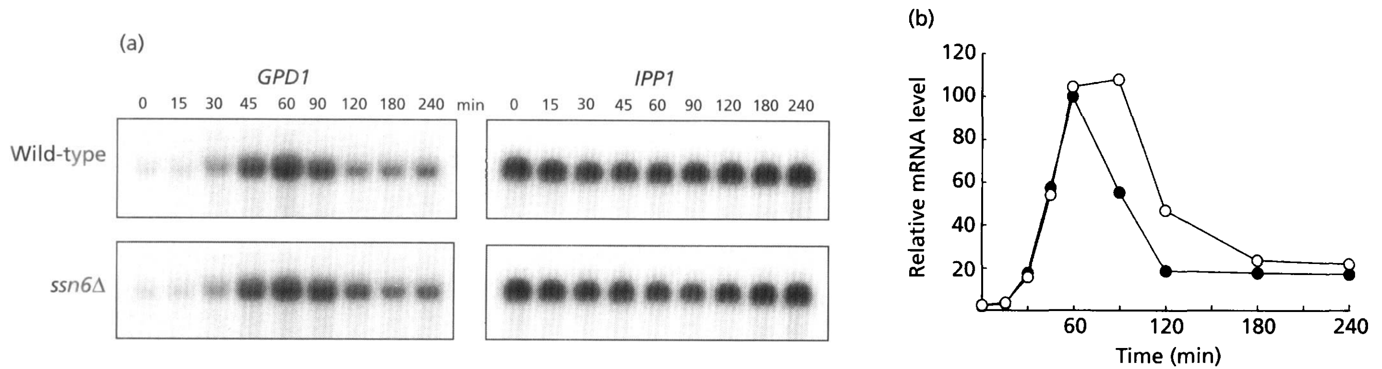

Fig. 7. Osmotic induction of *GPD1* mRNA levels by 0·85 M NaCl in the wild-type (●) and a *ssn6Δ* mutant (○) deficient in the general repressor complex Ssn6p-Tup1p. (a) Northern blot, (b) quantification. The highest relative mRNA level in the wild-type was set to 100.

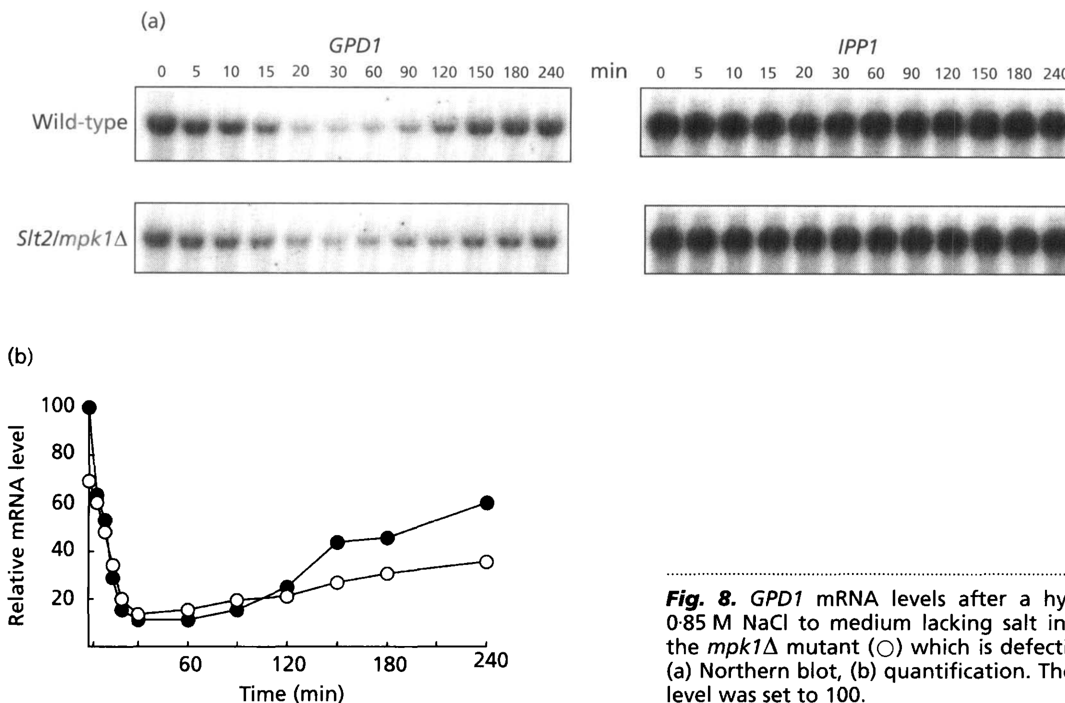

Fig. 8. *GPD1* mRNA levels after a hypo-osmotic shock from 0·85 M NaCl to medium lacking salt in the wild-type (●) and the *mpk1Δ* mutant (○) which is defective in the PKC pathway. (a) Northern blot, (b) quantification. The highest relative mRNA level was set to 100.

CTT1 and only a very minor increase in the level of *HSP12* mRNA. The induction profile of *GPD1* was essentially the same as in the *hog1Δ* single mutant. Thus, also in combination with the *hog1Δ* mutation, unregulated PKA activity neither affected the *GPD1* mRNA level nor its osmotic induction. In contrast, the osmotic response of *CTT1* seems to depend exclusively and that of *HSP12* mainly (i.e. about 98 %) on the combined activity of the HOG and the PKA pathways.

Induction of *GPD1* does not involve a depression mechanism employing *SSN6*

It has been reported that osmotic induction of some genes, including *GPD1*, is mediated by derepression and that repression under non-stress conditions is due to the Ssn6p-Tup1p general repressor complex (Keleher *et al.*, 1992; Márquez *et al.*, 1998; Roth, 1995). In contrast to these recent findings (Márquez *et al.*, 1998) we have found no evidence for repression of *GPD1* transcription under non-stress conditions by Ssn6 (Fig. 7). The induction profile in wild-type and *ssn6Δ* cells was very similar. Only the feedback phase is prolonged in the mutant with *GPD1* mRNA levels returning to an only slightly elevated level as compared to wild-type after 3 h. It should be noted that the response to 0·85 M NaCl of the strains used in this experiment is much more rapid than that of the other strains used in this study. This may be due to the absence of the *ade2* mutation in the W303-1A derived background (Table 1). The *ade2* mutation is known to affect growth phenotypes and may influence the extent of pre-adaptation to stress.

The *GPD1* mRNA level drops after a hypo-osmotic shock and this effect is independent of the PKC pathway

We have monitored the *GPD1* mRNA level after a drop in external osmolarity. For this, cells were pregrown in medium containing 0·85 M NaCl and shifted to medium lacking NaCl. Cells were fully adapted to high osmolarity before the hypo-osmotic shock and hence the mRNA level was well below the fully induced level observed in the transient induction phase after a hyperosmotic shock. Hypo-osmotic shock caused a 10-fold drop of the mRNA level within 30 min (Fig. 8). Subsequently, the *GPD1* mRNA level slowly increased again to about half of the initial level. A very similar profile was observed when sucrose was used as an osmolyte instead of NaCl (data not shown).

The same experiment was also performed with a *slt2/mpk1Δ* mutant, which lacks the terminal protein kinase of the PKC pathway. The profile and the maximal drop in the mRNA level was essentially the same as in the wild-type, except for a slower subsequent increase and a somewhat lower level (70%) before the shift (Fig. 8). Thus, the PKC pathway does not mediate the transient drop of the *GPD1* mRNA level after a hypo-osmotic shock. In a similar experiment with the *hog1Δ* mutant we could also not find evidence for the involvement of the HOG pathway in the response of *GPD1* expression to a drop in external osmolarity (data not shown).

DISCUSSION

We have studied the control of the *GPD1* mRNA level by changes in external osmolarity in time course experiments. This experimental setup has allowed us to define different phases in the response and we could show that the solute concentration but not the type of solute affects these phases. By doing the same type of experiments with mutants defective in four different stress-inducible pathways, we have confirmed our previous observation that the HOG pathway plays a central role in osmotic induction of *GPD1* expression. However, there is a significant HOG-independent osmotic response. Since none of the three other pathways studied seems to be involved, the signalling pathway(s) mediating the HOG-independent response remain to be identified. We also show that a hypo-osmotic shock causes a strong, transient drop in the *GPD1* mRNA level, which is independent of the PKC pathway.

Osmotic induction of *GPD1* expression

The response of the *GPD1* mRNA level to a shift to higher external osmolarity occurs in at least four distinct phases and their timing, amplitude and occurrence depend on solute concentration but are independent of the nature of the osmolyte. We define those phases as a lag phase, an initial induction phase, a feedback phase and a sustained induction phase.

At the lowest solute concentrations applied (0·980 a_w) the mRNA level increased instantaneously while after a shift to 0·970 a_w there was a lag phase of 30–45 min before the *GPD1* mRNA level increased. At the highest salt concentration the lag phase was as long as 2–3 h. Thus, some adaptation mechanism appears to be required after the osmotic shift before the expression of the *GPD1* gene is stimulated. Since the lag phase is even longer in the *hog1Δ* mutant and since Hog1p phosphorylation is stimulated rapidly after an osmotic upshift (Maeda *et al.*, 1994, 1995), it is possible that the HOG pathway is involved in this adaptation process. The observation that the lag phase is much shorter (15 min instead of 45 min) in a *ptp2Δ ptp3Δ* strain, in which Hog1p is hyperphosphorylated, is consistent with an involvement of the HOG pathway in the early recovery from osmotic shock.

This initial induction phase is clearly affected by inactivation of the HOG pathway, consistent with the observation that Hog1p phosphorylation upon osmotic shock is rapid and transient (Maeda *et al.*, 1994, 1995). The maximal level of *GPD1* mRNA is only about 15–20% of wild-type and the induction is delayed. However, the amplitude of this delayed *GPD1* induction at 0·980 and 0·970 a_w is still 6–10-fold and 15–20-fold, respectively, revealing an important HOG-independent component in *GPD1* induction. This component may involve the sustained induction of *GPD1*, since steady-state *GPD1* levels increase with osmolarity in a *hog1Δ* mutant, reaching roughly half of the wild-type levels (data not shown).

After the initial induction the *GPD1* mRNA level drops again rapidly to almost the same level as before the osmotic shift. This feedback phase is best observed at 0·980 a_w and takes about the same time as the initial induction, i.e. about 30 min. The molecular nature of this feedback mechanism is unknown. It also occurs in the *hog1Δ* (Figs 1 and 2) and the *slt2/mpk1Δ* (data not shown) mutants and is hence at least in part independent of the HOG and the PKC pathways. We are employing time course experiments in which we monitor different events in osmotic adaptation in parallel to better understand the basis for the feedback mechanism.

As best defined at 0·980 a_w, the feedback phase is followed by a slow increase in the *GPD1* mRNA level to a constant higher mRNA level. The final level correlates with the solute concentration, i.e. the higher the solute concentration, the higher the mRNA level in fully adapted cells (Ansell *et al.*, 1997). The induction profile at the highest salt concentration (1·4 M NaCl; 0·954 a_w) can be interpreted such that the initial induction phase and hence the feedback phase are missing and that the observed response is only due to this sustained induction phase. The sustained *GPD1* mRNA level at this high salt concentration appears to be lower than that at 0·970 a_w. The correlation between lower water activity and higher *GPD1* levels does not appear to hold at these extreme conditions.

Since all these phases are also observed when the *GPD1* promoter is fused to a *lacZ* reporter gene and *lacZ*

mRNA levels are taken as a measure for the promoter activity of *GPD1*, we believe that the phases are all due to transcriptional events (unpublished observations).

Signalling pathways in osmotic induction of *GPD1* expression

Out of the four stress-inducible signalling pathways studied in this work only the HOG pathway contributes significantly to the control of *GPD1* expression. Deletion of the genes encoding the catalytic subunit of the calcineurin protein phosphatase, which controls ion homeostasis in yeast (Serrano, 1996; Serrano *et al.*, 1997), caused a 30% reduction of maximal *GPD1* levels upon osmotic shock. Since this happens both with NaCl and sorbitol, we believe that this marginal alteration is an unspecific effect of the mutation. As with the mutant with low PKA activity, the earlier response in NaCl may be a result of 'pre-adaptation' to stress, i.e. the mutations would mimic a mild stress situation.

The observation that altered PKA activity did not affect the response of *GPD1* expression to increased osmolarity is in agreement with recent data, showing that there is no significant effect of the deletion of the genes *MSN2* and *MSN4* on the induction of *GPD1* expression (M. Rep, U. Holzmüller, V. Reiser, J. M. Thevelein, S. Hohmann, G. Ammerer & H. Ruis, unpublished results). These two genes encode the transcription factors that mediate the stress response controlled by PKA (Martinez-Pastor *et al.*, 1996). This also suggests that under osmotic stress the STREs in the *GPD1* promoter are either not functional or their function is compensated by other mechanisms. Since expression of *GPD1* can be induced by heat shock and since there is no evidence for heat shock response elements in the *GPD1* promoter, the STREs might be functional under such conditions.

As discussed above, the impact of the HOG pathway appears to be different for the different phases. The level of *GPD1* expression seems to be lower under all conditions. Thus, the HOG pathway also seems to be active under normal growth conditions and not only after a shift to higher osmolarity. This is consistent with recent observations of the effects of a *HOG1* deletion on glycerol transport and a synthetic growth defect of *hog1*Δ with other mutations (Tamás *et al.*, 1999; M. Rep, J. M. Thevelein & S. Hohmann, unpublished results). Future work will address the question if the different phases and the distinct involvement of the HOG pathway in these phases have a basis in different molecular mechanisms.

Such mechanisms do not appear to involve derepression through the Ssn6/Tup1 complex (Cooper *et al.*, 1994; Keleher *et al.*, 1992; Márquez *et al.*, 1998; Roth, 1995). Deletion of *SSN6* does not have a major impact on either the basal level of *GPD1* mRNA or its induction profile. We do not know why our results in this respect differ from the findings of Márquez *et al.* (1998). It might be due to the fact that we have conducted a time course experiment while in that study mRNA was isolated from cells grown to mid-exponential phase in medium with or without 1 M NaCl.

A significant part of the osmotic response of *GPD1* expression is mediated by a HOG-independent mechanism. Our analysis excludes the PKA, calcineurin and PKC pathways as being responsible or involved in this response. Since the pathways studied here are to our knowledge the only yeast signalling routes stimulated by an increase in osmolarity or by the salt concentration of the growth medium, a novel signalling route appears to control the HOG-independent response. This pathway responds to osmolarity and not only to salt, since the HOG-independent response could be triggered by both salt and sorbitol.

Response to a hypo-osmotic shock

When cells growing on high-salt medium were shifted to medium without salt, we observed a transient drop in the *GPD1* mRNA level. Since neither the PKC pathway nor the HOG pathway (Fig. 8 and data not shown) seem to mediate this response; also, in this case, the signalling pathway(s) involved remains to be identified. It is possible that the pathway mediating the hypo-osmotic effect on the *GPD1* mRNA level is the same one that controls the feedback phase of osmotic induction, but the genetic tools to study this possibility are presently not available.

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