Osmoregulation and protein expression in a *pbs2Δ* mutant of *Saccharomyces cerevisiae* during adaptation to hypersaline stress

Noreen Akhtar*, Anders Blomberg, Lennart Adler

Department of General and Marine Microbiology, Lundberg Laboratory, Medicinaregatan 9C, 413 90 Göteborg, Sweden

Received 21 December 1996

Abstract **We deleted the *PBS2* gene encoding the MAP kinase activator of the osmosignaling HOG pathway in *Saccharomyces cerevisiae* and examined the effects on the kinetics of the osmoregulatory glycerol response and protein induction during adaptation to 0.7 M NaCl. Changes in protein expression as analyzed by two-dimensional polyacrylamide gel electrophoresis (2D PAGE) demonstrated that for the 29 proteins showing a 6-fold induction in wild-type cells during adaptation to NaCl stress, all displayed a decreased and delayed response in *pbs2Δ* cells. Of the seven proteins that were identified, two were previously not known to be under HOG pathway control: Ald6p, an isoform of aldehyde dehydrogenase and Dak1p, a putative dihydroxyacetone kinase. The presence of a remaining significant induction in *pbs2Δ* cells for about half of the examined proteins indicates existence of alternative osmosignaling pathway(s). Northern analysis of the salt induced transcription of *GPD1* and *GPP2*, encoding the cytosolic glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase involved in the osmostress induced glycerol production, demonstrated an about 20-fold *PBS2*-dependent transient activation, in agreement with the previously reported transient nature of the signal transduced by the HOG pathway.**

Key words: Yeast; Osmoregulation; *PBS2*; 2D-PAGE

1. Introduction

Water availability is of fundamental importance for all living organisms. Changes in the water activity call for rapid cellular adaptation for survival and growth. Hypertonic stress results in outflow of water from the cell resulting in desiccation. To counteract such dehydration, production and accumulation of osmolytes are induced, leading to readjustment of cell volume and turgor pressure [1]. In *Saccharomyces cerevisiae*, the main osmolyte is glycerol and enhancement of glycerol synthesis is a key event in the osmoregulatory response. This response is controlled by the osmosensing and signaling HOG (*h*igh *o*smolarity *g*lycerol) pathway that transmits a signal via a MAP (*m*itogen *a*ctivated *p*rotein) kinase module to target genes important for the osmostress response [2,3]. Two putative osmosensors are involved in sensing osmolarity changes in the surroundings [4,5]. One of these, Sln1p, forms together with Ypd1p [6] and the response regulator homolog Ssk1p [4] a sensor complex with similarity to bacterial 'two-component' signal transducers, which negatively regulates the HOG pathway. An alternative input originates from the *SHO1* encoded osomosensor which by an unknown mechanism activates the MAP kinase activator Pbs2p [5]. Exposure of cells to increased external osmolarity induces a rapid activation of the HOG pathway [2] which results in enhanced glycerol production via increased transcription of *GPD1* [7,8] and *GPP2/HOR2* [9,10] encoding isoenzymes for NAD^+-dependent *sn*-glycerol-3-phosphate dehydrogenase and DL-glycerol-3-phosphatase, respectively. Other genes that are activated by high osmolarity via the HOG pathway are the *CTT1* gene, encoding catalase T that is important for survival after severe osmotic stress [11], and *HSP12* gene [12] encoding a heat shock protein with an unknown function. However, not all genes that are upregulated by hyperosmotic stress are under the control of the HOG pathway, examples being the *DDR48* [13] and *HOR7* [10] genes that encode proteins with unknown functions.

To obtain a global view of how osmotic stress affects protein expression in *S. cerevisiae*, two-dimensional gel electrophoresis (2D PAGE) of whole cell extracts has proved useful [14]. Cells shifted to 0.7 M NaCl revealed a transient change in the protein expression pattern of up to 150 different proteins. Of this impressive number of protein responders, 18 proteins displayed 8-fold increase in their relative rate of synthesis. Three of these proteins were tentatively identified as Gpd1p, Hsp104p and Ctt1p. Proteins in the glycolytic pathway were, on the other hand, slightly repressed in cells exposed to saline media. Steady state growing cells adapted to saline conditions did not display the same amount of dramatic changes in the protein expression pattern; only 13 proteins showed a change that was equal to or larger than 2-fold [15]. These observations clearly indicate that the transition physiology is different and far more dynamic than that of fully adapted cells.

In the present work we report on osmoregulatory defects and the protein expression pattern of a *pbs2Δ* mutant during adaptation to saline conditions. It is demonstrated that inhibition of osmosignaling at the Pbs2p level affects the rate of synthesis of a large number of proteins.

2. Material and methods

2.1. Strains, media and growth conditions

S. cerevisiae strain U451 (MAT a, *ade2, can-100°, leu2, trp1*^{a}, *ura3*), a w303-1A derivative, kindly provided by Dr Hans Ronne, and the *pbs2Δ::LEU2* mutant derived from this strain, were maintained on YPD agar (1% yeast extract, 2% peptone, 2% glucose, 2% agar) supplemented with adenine (120 mg/l). Strains were routinely grown either in YPD or YNB (Yeast Nitrogen Base w/o amino acids) (Difco) with glucose (2%) supplemented with appropriate amino acids and nucleotides in standard amounts [16] and buffered to pH 5.9 with 10 g succinate and 6 g NaOH per l. The osmosensitivity was assessed in media supplemented with NaCl, KCl or sorbitol. Solid media contained 2% agar in addition to other ingredients. Liquid cultures were

*Corresponding author. Fax: (46) (31) 7732599.
E-mail: Noreen.Akhtar@gmm.gu.se

PII S0014-5793(97)00048-3

incubated on a rotatory shaker (110 rpm) at 28°C. Growth was monitored by determining the optical density at 610 nm in 1 cm cuvettes.

The osmosensitive *osg2-2* mutant was isolated as previously described [8,17]. The osmotolerance of the *osg2-2* strain and the *pbs2Δ::LEU2* mutant was examined by following growth in 2.8 l Fernbach flasks containing 500 ml YNB medium supplemented with 0 M or 0.7 M NaCl. The flasks were inoculated to OD_{610}=0.05 from precultures of 20 ml YNB medium grown for 2 days.

E. coli strains DH5α and TG1 [18,19] were used as bacterial hosts for plasmids and were grown at 37°C in LB medium, supplemented with ampicillin when needed.

2.2. Genetic techniques

Plasmid DNA was isolated from *E. coli* by either using the alkaline lysis method [20] or the Wizard mini prep system (Promega, USA). Other recombinant DNA techniques were carried out according to a conventional laboratory manual [19,20]. Transformation of yeast cells was performed using the lithium acetate method [21].

A *S. cerevisiae* genomic library cloned into YCp 50 plasmid [22], was used to isolate fragments complementing the *osg2-2* defect. Transformants were selected on YNB plates lacking uracil and tested for complementation of the salt-sensitive phenotype on plates containing 1.4 M NaCl. Plasmid DNA from positive clones was isolated, amplified in *E. coli* and used to retransform the *osg2-2* mutant cells to confirm complementation. The fragments were restriction mapped and subcloned into the shuttle vector pRS316 [23] to localize the complementing gene.

Part of the complementing gene was sequenced directly from the pRS316 vector. The sequence was determined by the Sequenase kit, Version 2.0 (US Biochemicals) based on the dideoxy chain termination method.

The sequence obtained was searched against the EMBL database using the GCG sequence analysis software package. To construct a deletion mutant of the *PBS2* gene, the *Bam*HI-*Sac*I fragment containing the *PBS2* gene was cloned into modified pBluescript (Stratagene, USA) where the *Eco*RV-*Sma*I sites were removed. An internal part of the gene was deleted by cutting with the *Spe*I-*Eco*RV, which removes 1978 nucleotides from the coding sequence, and this segment was replaced with a *Nhe*I-*Sma*I fragment containing the *LEU2* gene from plasmid YDp-L [24]. The plasmid was linearized with *Apa*I-*Sac*I and transformed into the wild-type strain U451 and plated on YNB-agar lacking leucine. Obtained transformants were tested for salt sensitivity by replica plating onto YNB plates containing 1.4 M NaCl. The deletion was confirmed by Southern hybridisation. Total yeast DNA was cut with *Cla*I and transferred to a nylon membrane filter (Boehringer Mannheim) from a 0.7% (w/v) agarose gel. A 3.2 kb long *Dra*I-*Sac*I fragment of the *PBS2* gene was used as a probe. The filter was incubated with DIG (Boehringer Mannheim, Germany) labeled DNA probe overnight to allow hybridisation and the hybridisation was subsequently visualised with the Luminogen AMPPD detection kit (Boehringer Mannheim, Germany).

2.3. Preparation of cell-free extracts for enzyme activity determination

Cells grown to the density of OD_{610}=1.0 in 500 ml YNB medium in Fernbach flasks with or without 0.35 M NaCl were pelleted, washed with TRED (10 mM triethanolamine, 1 mM EDTA, 1 mM dithiothreitol, pH 7.5) buffer and disrupted with glass beads as previously described [25]. The obtained protein extract was desalted by passage through a Sephadex G-25 column (PD-10, Pharmacia, Sweden). Protein concentration was determined using the Coomassie dye binding technique (Bio-Rad, USA)

2.4. Enzyme assays and glycerol analysis

The specific activity of NAD^+-dependent glycerol-3-phosphate dehydrogenase was determined in crude cell extracts according to the method of André et al. [25]. The glycerol-3-phosphatase was assayed as previously described [8] using the methods of Sussman and Avron [26].

For measurements of glycerol production during osmotic stress, the cells were grown to mid-log phase in YNB medium followed by mixing with an equal amount of fresh medium to give the NaCl concentrations of 0 or 0.7 M. Samples were taken at immediately after and 1, 2, 4 and 6 h after the shift. The total and intracellular levels of glycerol were determined as described [27] using a commercial glycerol analysis kit (Boehringer Mannheim, Germany).

2.5. Radiolabeling of protein for two-dimensional gel electrophoresis (2D PAGE)

Cells were grown to OD_{610}=0.8 in 2.8 l Fernbach flasks containing 500 ml YNB medium and subjected to osmotic stress by the addition of 200 ml YNB medium containing NaCl to give a final concentration of 0.7 M NaCl. Cell samples of 5 ml were withdrawn immediately before addition of salt and at T=20, 40, 120 and 240 min after shift to 0.7 M NaCl. The samples were transferred to Falcon tubes and labeled with 75 μCi of [^{35}S]methionine (15 μCi/μl, 1270 Ci/mmol, Amersham Corp., USA) by incubation at 30°C in a roller drum (35 rpm) for 20 min. Incorporation was terminated by the addition of 150 μl cycloheximide (14 mg/ml). After incubation, the cells were pelleted by centrifugation and frozen in liquid nitrogen.

2.6. Sample preparation for 2D-PAGE

For preparation of protein extracts the frozen cell pellets were resuspended in 100 μl of sample buffer containing 0.3% (w/v) sodium dodecyl sulfate (SDS; Bio-Rad, USA), 5% (v/v) β-mercaptoethanol (Bio-Rad, USA), and 50 mM Tris (Sigma, USA) buffer, pH 8.0. Disruption of the cells was achieved by addition of 0.1 g of acid washed glass beads (diameter 0.5 mm) followed by alternate vortexing four times for 30 s, with intermittent cooling on ice for at least 1 min. The disrupted cell samples were heated at 98°C for 5 min and placed on ice. The samples were then treated with 20 μl of RNAse A (0.25 mg/ml) and DNase I (1 mg/ml) in 0.5 M Tris and 50 mM $MgCl_2$, pH 7.0 for 10 min at 0°C. Glass beads and cell debris were pelleted by centrifugation (15 000×g, 10 min). The protein concentration of the supernatant was determined using a protein assay kit (Sigma-diagnostics, USA, Cat. P5656) which is based on a modified procedure of Lowry. Incorporated radioactivity was determined by counting in a scintillator as described [14].

2.7. 2D-PAGE

The 1st dimension was run using immobilin strips as described by Norbeck and Blomberg [28]. The strips were rehydrated overnight in rehydration solution (8 M urea, 1% Nonidet P-40, 1% Pharmalyte 3-10A, 10 mM dithiothreitol (DTT) and 0.001% bromphenol blue). A total amount of 20 μg protein was loaded onto each strip and the proteins were focused overnight at 3500 V, 1 mA. The gel strips were soaked in 1D gel equilibration buffer (36.6 g/l Tris base, 11.9 g/l Tris-HCl, 3% SDS, 10 mM DTT and bromphenol blue) and covered with agarose (0.5% w/v) in 25% Tris slab gel buffer (130.8 g/l Tris base, 66.3 g/l Tris-HCl). The strips were mounted on 12.5% 2D gels and run for 4–5 h at maximum 1600 W/gel. The gels were then dried and exposed to Phosphoimager plates (Molecular Dynamics, USA) for 3–4 days. Images were transferred to a Sparc 1 system (SUN Microsystems, USA) and processed by the 2D software PDQuest (PDI, USA). Gels were quantitatively analyzed by normalizing to the total dpm count of all resolved proteins in a gel and expressed as ppm. Statistical analysis was performed utilizing functions within the PDQuest software. Proteins displaying significant changes in quantity between samples were selected by a Student t-test on log transformed values. Each analysis involved at least two independent experiments.

2.8. Northern blotting

For isolation of RNA cells were grown in YNB medium to the OD_{610}=0.8–1.0 and an equal amount of NaCl containing YNB medium was added to give a final salt concentration of 0.7 M NaCl. Samples of 10 ml were taken immediately before addition of salt and then at T=20, 40, 60 and 120 min after the salinity shift. RNA was isolated by using standard procedures and subjected to gel electrophoresis, blotted to nylon filter and hybridized as previously described [29].

The sequences of the oligonucleotides used for probing were 5′-TGTACTATTGGAGCGAAAACTTCT-3′ for *GPD1*, 5′-CTTGCTCATTGATCGGATATCCTAA-3′ for the *GPP2* probe and 5′-AATCGATTCTCAAAATGGCGTGAGG-3′ for the *ACT1* probe, kindly provided by Ines Eberhardt (K.U. Leuven, Belgium).

All oligonucleotides were end-labeled with [γ-^{32}P]dATP, using 5 U polynucleotide kinase (Gibco BRL, Life Technology, USA). The filters were developed and analysed on a Phosphoimager (Molecular Dynamics, USA).

3. Results

3.1. Phenotype of the osg2-2 mutant and cloning of a gene complementing the osg2-2 mutation

In search for genes involved in the osmoregulatory glycerol response of *S. cerevisiae* we isolated a set of *osg* mutants falling into four complementation groups as described by Larsson et al. [8]. One of the mutants, the *osg2-2* mutant, displayed osmosensitivity on high osmolarity media adjusted with NaCl, sorbitol or KCl and defective induction of the NAD^+-dependent glycerol 3-phosphate dehydrogenase activity and glycerol production in response to hyperosmotic stress (data not shown). A DNA fragment, cloned in the YCp50 vector [22], complementing the osmosensitivity defect was isolated and further subcloned into the low copy number plasmid pRS316 [23]. The complementing subclone was partially sequenced and search in the databases revealed identity with the previously isolated *PBS2* gene [30]. Since *PBS2* is demonstrated to encode a MAP kinase activator involved in the osmosignaling HOG pathway [2], we decided to characterize more broadly its role in the cellular response to hyperosmotic stress.

3.2. Phenotype of the pbs2 deletion mutant

A deletion of the *PBS2* gene was constructed as described in Section 2. The deletion was confirmed by Southern analysis and the osmosensitive phenotype was assessed by culturing cells on media adjusted to high osmolarity with various solutes (data not shown). Growth curves revealed that the *pbs2*Δ mutant was still able to grow in moderate salinities, although more slowly with a generation time of about 8 h compared to 3 h for the wild-type cells during the exponential phase in YNB supplemented with 0.7 M NaCl. The mutant also displayed the characteristic aberrant cell morphology shown by *pbs2*Δ mutants at high osmolarity [31]. The osmoregulatory glycerol response was impaired in the mutant which shows a lower and more slowly induced total glycerol production than in wild-type cells on transfer to medium containing 0.7 M NaCl (Fig. 1A,B). The intracellular accumulation of glycerol only reach levels that are 20–40% of those of the wild-type (Fig. 1C) even after prolonged incubation, in agreement with the observation by Brewster et al. [2] where the intracellular levels were measured after incubation of cells at high salinity for 1 h. The defective osmoregulatory glycerol response by the mutant was reflected in lower basal activities as well as de-

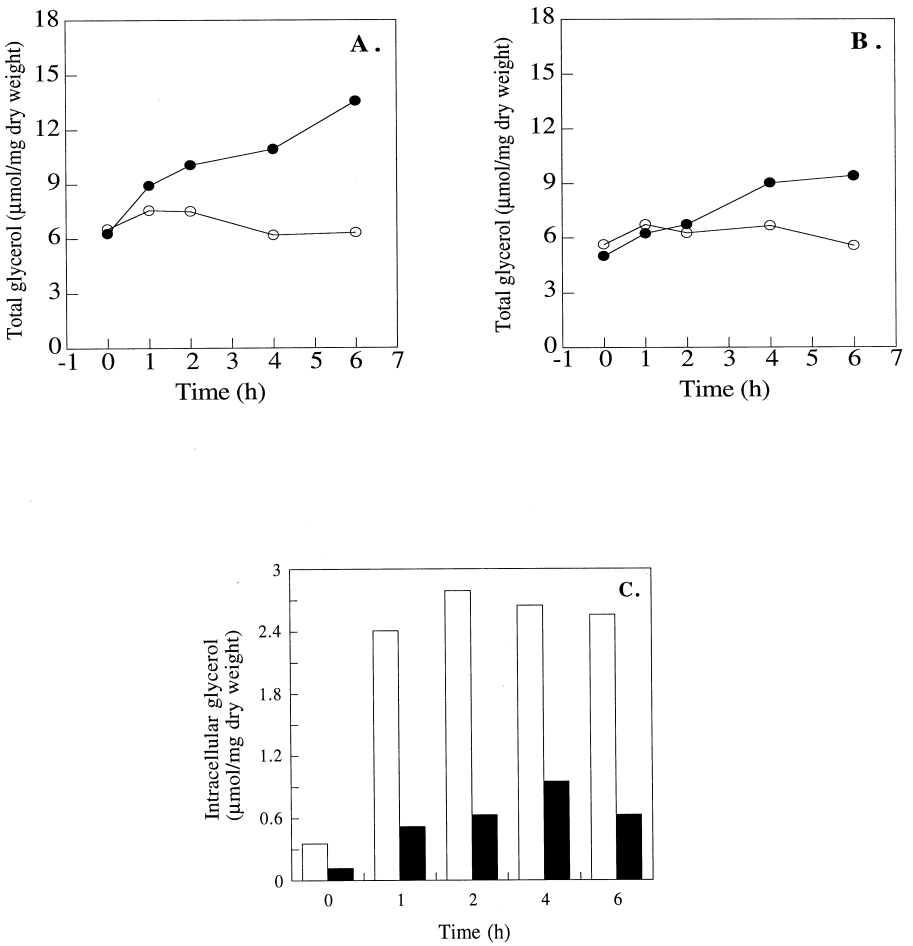

Fig. 1. Total glycerol produced by wild-type (A) and *pbs2*Δ mutant (B) cells following transfer to YNB medium containing 0.7 M NaCl. Cells were grown to mid-log phase in YNB medium and an equal volume of fresh medium containing no salt or NaCl to give a final concentration of 0.7 M was added. Values are the mean of two independent experiments. (○) Cells transferred to 0 M NaCl; (●) cells transferred to 0.7 M NaCl. (C) Intracellular glycerol levels of wild-type (open bars) or *pbs2* (shaded bars) cells after transfer to medium containing 0.7 M NaCl. The intracellular glycerol levels were determined from two independent experiments.

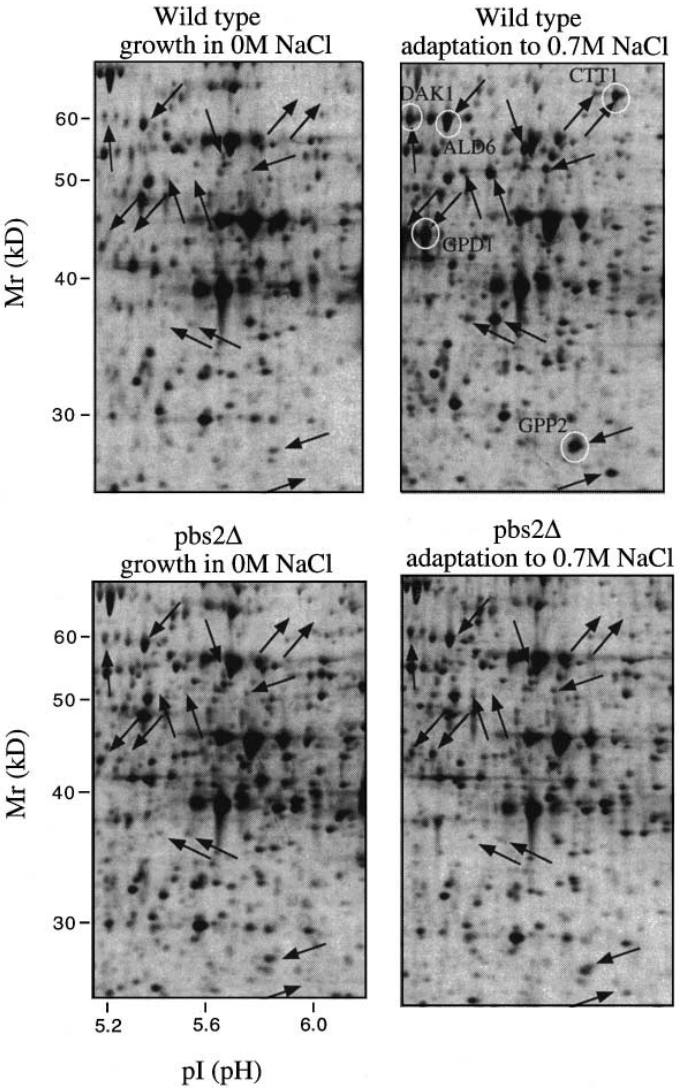

Fig. 2. 2D PAGE analysis of [^{35}S]methionine-labeled proteins of wild-type (upper panels) and *pbs2*Δ cells (lower panels) growing exponentially in basal YNB medium (left panels) and 20–40 min after transfer to YNB medium containing 0.7 M NaCl (right panels). The arrows mark the position of proteins that are 6-fold induced by high salinity in wild-type cells and show defective induction in the *pbs2*Δ mutant. Circles mark the positions of a few of the identified proteins. Only the central region of the gels is displayed.

creased induction by high osmolarity of the two enzyme activities involved in glycerol production the NAD^+-dependent glycerol-3-phosphate dehydrogenase (GPD) and glycerol-3-phosphatase (GPP) (data not shown).

3.3. *General effects on global protein expression by increased extracellular salinity*

To examine the effect of a *PBS2* deletion on global protein expression, cultures of wild-type and *pbs2*Δ cells growing exponentially in YNB medium were subjected to a shift in salinity from 0 to 0.7 M NaCl. A portion of each culture was labeled with [^{35}S]methionine before transfer to establish the basal pattern of protein synthesis and similarly labeled samples were taken at intervals after the salinity shift. Each experiment was repeated independently at least once and the resultant series of samples were analyzed by protein separation by two-dimensional gel electrophoresis (2D PAGE) and analyzed by computer-aided quantification of individual proteins. Since it was previously observed [14] that the most marked changes in protein expression occur 20–40 min after transfer to 0.7 M NaCl, we have compared the salt stress response of the *pbs2*Δ mutant with that of the wild-type cells, focusing on the changes during the first hour after the shift. Among the different patterns previously observed, the most conspicuous was that of highly NaCl induced proteins, being the main interest of the present report.

Of the 29 proteins that displayed a 6-fold induction in the wild-type cells during the first 20–40 min of adaptation to increased salinity, all proved to be dependent on an intact Pbs2p function for their response, although the extent of this dependence varied for individual proteins (Fig. 2).

3.4. *Pbs2p-dependent expression of the highly NaCl-induced proteins*

With respect to their Pbs2p dependency, the strongly NaCl-induced proteins could be tentatively divided into four categories (Fig. 3). For the class I proteins comprising 4 members, inhibition of the HOG pathway at the Pbs2p level had only a

REGULATORY CLASS	KINETIC PROFILE	PROTEINS IN CLASS (Mr/pI)	MAX SYNTHETIC RATE (ppm)
I	 HSP104p	p98.7/5.4 (Hsp104p)	2275
		p73.0/4.9 (Ssa3p)	3487
	 Ssa3p	p33.1/8.3 (Tdh1p)	1900
		p23.5/5.8	4491
	 Tdh1p		
II	 Dak1p	p99.4/7.8[f]	1341
		p63.3/6.0	2626
		p59.7/5.2 (Dak1p)	2309
		p53.9/5.7	7195
		p51.3/5.7	1640
		p43.9/5.3[a] (Gpd1p)	13144
		p43.8/5.2[a] (Gpd1p)	7893
	 Gpd1p	p33.3/9.0	5097
		p31.2/8.8	1602
		P26.5/6.0	1520
		P27.6/6.7	1406
III	 Ald6p	p99.1/8.2[f]	1193
		p59.3/5.3[d]	3077
		p58.4/5.4[d] (Ald6p)	18565
		p57.1/6.1	816
		p52.3/6.4	2944
		p36.3/5.5[b]	813
		p34.6/6.4	4621
		p27.7/5.8	4702
IV	 Ctt1p	p74.9/5.8[e]	1520
		p74.9/5.8[e]	980
		p63.0/5.9 (Ctt1p)	699
		p52.1/5.5[c]	6816
		p51.5/5.5[c]	966
		p36.6/5.5[b]	6998

a, b, c, d, e, f) Identical letters indicate 2D spots which are putative charge modifications of the same protein.

Fig. 3. Histograms displaying NaCl induced responses of selected proteins in wild-type and *pbs2*Δ cells. All proteins were induced 6-fold in wild-type cells following transfer to YNB medium containing 0.7 M NaCl. The proteins are divided into four classes (class I–IV) dependent on their response in the *pbs2*Δ mutant. For each class only the response of the proteins with known identity is displayed, for the proteins with unknown identity their estimated M_r and p*I* are given. The ppm number indicates the maximum radioactivity incorporation of the protein relative to the totally incorporated activity. The leftmost first bar (wild-type) and the third (*pbs2*Δ) bar in each diagram depict control values for cells growing exponentially without salt. The other bars represent, from left to right, labeling for 20–40 min (wild-type and *pbs2*Δ) and for *pbs2*Δ cells, 40–60 min after transfer to 0.7 M NaCl.

moderate effect on the rate and extent by which the proteins were induced by salt stress. Three proteins in this group had a previously determined identity: Tdh1p, an isoform of glyceraldehyde-3-P dehydrogenase [28] and the heat shock proteins Hsp104p and Ssa3p [32]. The second set of proteins encompassing 11 members are characterized by a considerably delayed and decreased induction by salt stress in cells lacking Pbs2p. This group of proteins included two osmostress-induced enzymes: Gpd1p and Dak1p. The identities of these proteins were previously established from sequencing of pep-

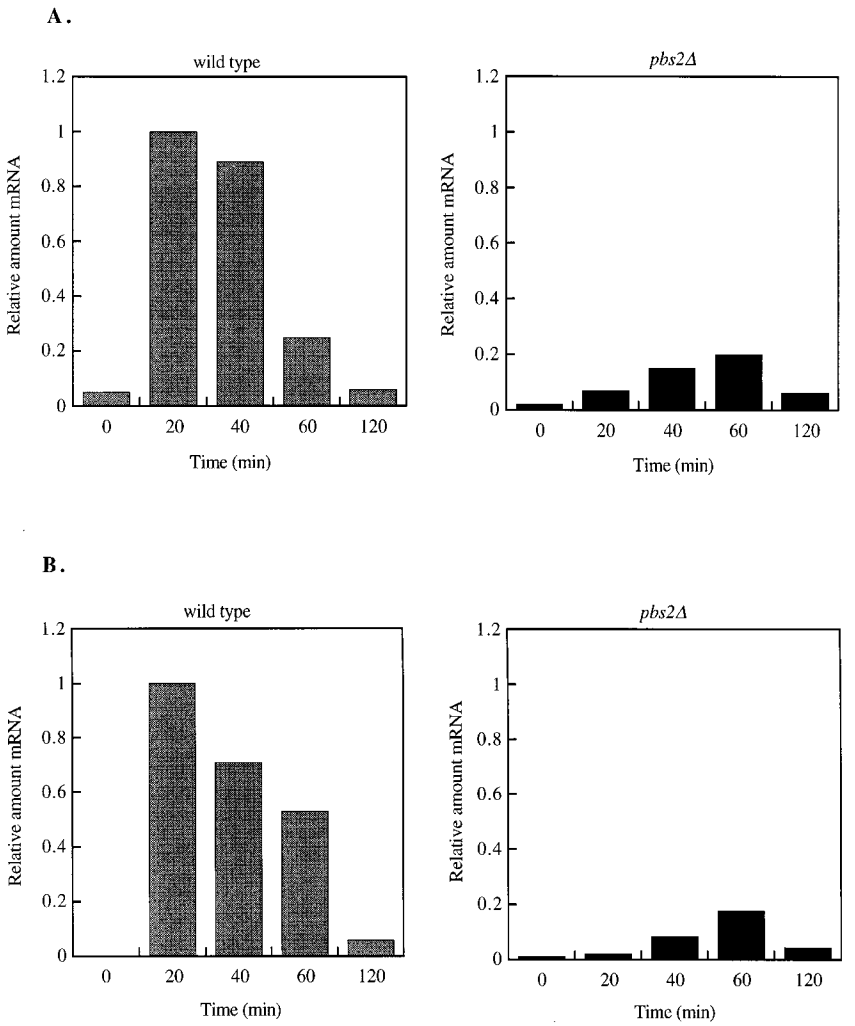

Fig. 4. Analysis of mRNA levels of *GPD1* (A) and *GPP2* (B) in wild-type and *pbs2*Δ cells after transfer to YNB medium containing 0.7 M NaCl. Samples were taken at the indicated time points and the quantities obtained were normalized to the control *ACT1* mRNA value. The bars show the relative amount of each band with the highest amount of each gene set to a value of 1.

tides generated by in-gel trypsination [15,28,33]. *GPD1* encodes an osmostress induced isoform of GPD [7,8], and *DAK1* encodes a putative dihydroxyacetone kinase [33]. The glycerol 3-phosphatase encoded by *GPP2* which is induced by high osmolarity in a HOG dependent fashion [9] could not be properly quantified on the present gels due to insufficient resolution from a nearby spot. However, previous results [9] and the profile obtained indicate that Gpp2p responds similarly to the proteins of the Gpd1p/Dak1p group. The third set of proteins is constituted by 8 members which show an induction by salt stress that appeared completely dependent on an intact HOG pathway. Virtually no induction of these proteins was noted during the period of observation (0–60 min) in *pbs2*Δ mutants exposed to salt stress. Still after 120 min no significant induction was observed (data not shown). Only one of the proteins in this group has an experimentally verified identity: Ald6p, an isoform of aldehyde dehydrogenase [33]. The final group including 6 proteins remains unclassified since we could not correctly assess the degree of induction in the *pbs2*Δ cells; the level of expression stayed below the detection limit in basal medium and at 0.7 M NaCl (Fig. 3). This group accommodates only one identified member, Ctt1p, a protein that is strongly induced by high osmolarity [11].

3.5. *Pbs2p-dependent repression at 0.7 M NaCl*

An opposite mode of response detected in wild-type cells on shift to saline conditions is repression of protein synthesis. We detected about 40 proteins that were down-regulated 2-fold at 20–40 min after shift to 0.7 M NaCl. One of the proteins in this group was identified as corresponding to the FUN (function unknown) gene YM9718.15 [28]. The repression of this protein was completely HOG pathway dependent, no repression occurred at high salinity in the *pbs2*Δ mutant (data not shown).

3.6. *The rapid, HOG pathway, dependent induction of Gpd1p is preceded by transient synthesis of mRNA*

As demonstrated above, the rapid salt-induced synthesis in wild-type cells of the Gpd1p enzyme involved in the osmoregulatory glycerol response, was clearly inferior in the *pbs2*Δ mutant. The induction profile for this glycerol producing enzyme was considerably delayed in the *pbs2*Δ cells, and the

level reached was significantly lower than in the wild-type cells. To relate protein expression to changes in transcriptional activity, we analyzed by Northern blot transcript levels of the *GPD1* gene together with those of the *GPP2* gene. Fig. 4A illustrates that in the wild-type the amount of *GPD1* mRNA increases rapidly to reach at least 20-fold increased levels 20 min after transfer to increased salinity, followed by a decrease to about 4-fold enhanced levels after 60 min. In the *pbs2*Δ cells the amount of transcript reached an optimum (at least 10-fold increase) only after 60 min after which levels decreased. A pattern very similar to that of *GPD1* mRNA was observed for *GPP2* transcripts in both wild-type and *pbs2*Δ cells (Fig. 4B). The results clearly demonstrate a strongly delayed induction of the transcriptional activity in the *pbs2*Δ mutant. The results also indicate that the rapid induction of Gpd1p synthesis in wild-type cells after shift to increased salinity (Fig. 3, class II) might be explained by transcriptional regulation alone. The rapid increase in transcript levels obviously represents a HOG mediated overshoot production of mRNA that is later adjusted to levels in equilibrium with the requirements of adapted cells. These observations are consistent with the moderately increased (3.6-fold) levels of Gpd1p reported for cells growing exponentially at 0.7 M NaCl [15]. The mRNA levels reached after the adaptation period appear insignificantly or only slightly HOG pathway dependent (Fig. 4).

4. Discussion

The *PBS2* gene encodes a MAP kinase activator involved in the osmosensing and signaling HOG pathway of *S. cerevisiae* [2]. Pbs2p has a central role in this pathway in integrating two distinct signals from the upstream osmosensors encoded by *SLN1* and *SHO1* [4,5]. While Sho1p appears to communicate directly with Pbs2p via an SH3 domain of Sho1p [5], Sln1p forms a signal transducer complex with other components [6], which feeds into Pbs2p via two redundant kinases encoded by *SSK2* and *SSK22* [5]. The *HOG1* encoded MAP kinase located downstream of Pbs2p is activated by Pbs2p dependent phosphorylation in cells exposed to hyperosmotic stress [2,5]. No direct molecular target of Hog1p has been identified so far. We cloned the *PBS2* gene by molecular complementation of *osg2-2*, and we have further characterized the effect of a *PBS2* deletion on the kinetics and capacity of the cells for osmoregulation and protein induction on exposure to hypersaline stress. We here report that for the 29 most strongly NaCl induced proteins, all showed a decreased and delayed induction in the *pbs2*Δ mutant. Although there are genetic evidence for a *PBS2* and *HOG1* independent osmosignaling pathway which is activated at high salt concentrations [13], our results clearly indicate that the HOG pathway is crucial for rapid adaptation to hyperosmotic stress by synthesis of a large number of selected proteins. Among the four classes of HOG dependent responses that were discerned for the strongly induced proteins (Fig. 3), one involved a class of proteins that were only moderately affected by deletion of *PBS2* (Fig. 3, class I). The identified proteins in this class were Tdh1p, Hsp104p and the heat inducible Hsp70 protein Ssa3p [28,32]. The osmotic induction of Tdh1p is completely abolished in a *bcy1* strain having high protein kinase A activity while the osmostress induced protein levels are strongly increased in a *tpk1*^{w} strain having low protein kinase A activity [34]. It has been demonstrated that the HOG and the protein kinase A pathways act antagonistically on the STRE (*st*ress *r*esponse *e*lement) driven osmostress induced transcription of *CTT1* [11,35]. Since the promoter of *TDH1* contains STREs [34], this gene might be subject to a similar type of regulation, as was recently reported for the STRE controlled *HSP12* gene [12]. The presence of STRE like elements in the promoter of *HSP104* [35] and *SSA3* [36] and the cognate response of the other unidentified members of this class of proteins may indicate they are also subject to STRE mediated control. An additional type of control that can not be excluded for NaCl responding proteins is that mediated via the Na^+ specific calcineurin pathway [37]. Marquez and Serrano [38] demonstrated that the *PMR2A/ENA1* gene, encoding a membrane localized ATPase involved in sodium extrusion, is subject to osmotic control via the HOG pathway at low osmolarities, while at high salt concentrations expression is subject to calcineurin mediated Na^+ specific control.

Among the proteins for which deletion of *PBS2* resulted in a strongly but not fully inhibited response were two enzymes with known identity: Gpd1p and Dak1p. Although *GPD1* was previously reported to be under HOG control [7], osmotic induction of Dak1p was not known to be HOG mediated. *DAK1* encodes a putative dihydroxyacetone kinase assumed to function in a glycerol dissimilating pathway [33]. The physiological reason for osmotic induction of Dak1p is not understood although it was suggested [33] that this pathway might serve to fine tune the glycerol pool under high osmolarity conditions.

The osmostress induced changes in expression of the Gpd1p enzyme involved in the osmoregulatory glycerol response exhibited an immediate >60-fold increased expression 20–40 min after transfer to 0.7 M NaCl (Fig. 3, class II), while showing a significantly slower response in the *pbs2*Δ mutant. These observations are consistent with the fact that glycerol production displayed only a moderate increase in the *pbs2*Δ mutant after transfer to 0.7 M NaCl (Fig. 1A). Importantly, the intracellular glycerol concentration remained considerably lower in the *pbs2*Δ cells than in wild-type cells, also for mutant cells which after 6 h of incubation started to grow (Fig. 1B). We have previously observed that the Gpd1p defective *osg1* mutant is capable of accumulating wild-type levels of glycerol at high osmolarity in exponentially growing cells despite poor glycerol production [8], indicating that the poor accumulation of the *pbs2*Δ mutation might also involve defective glycerol retention. However, the glycerol channel encoded by the *FPS1* gene seems to be closed during hyper-osmotic stress and transport via this channel does not appear to be controlled by the HOG pathway [39].

The rapid and strong induction of Gpd1p (Fig. 3, class II) after a salinity shift, and the indications of a similar response of Gpp2p (the protein was incompletely resolved on the 2D gels), prompted us to examine the kinetics of the osmostress induced transcription of these genes. Both transcripts showed a strong transient accumulation in wild-type cells at about 20 min after the salinity shift, followed by a decline to considerably lower levels (Fig. 4). This rapid and transient response is in agreement with the temporary activation of the HOG pathway by osmotic stress. The *PBS2* dependent tyrosine phosphorylation of Hog1p occurs within 1 min after transfer to 0.4 M NaCl and shows a duration of about 20 min [2]. These data indicate that the HOG pathway functions as an alarm signal

for immediate activation of the osmotically induced genes. Following this burst of activity the HOG control is gradually turned off and during later stages of adaptation the delayed HOG independent induction appears to determine transcript levels (Fig. 4, 120 min). The mechanisms behind the sustainable HOG independent response are not known. Eriksson et al. [40] recently demonstrated that the HOG independent signal targets a region of the *GPD1* promoter distinct from that accepting the HOG mediated information.

Our 2D studies also revealed a class of 8 proteins that was not induced at all following NaCl stress after deletion of *PBS2* (Fig. 3, class III). Considering the nature of the HOG signal, one would assume that these proteins fulfill functions specifically required during the early phase of adaptation to salt stress. The only identified protein in this group is Ald6p, which catalyzes the reduction of acetaldehyde to acetic acid, an activity that is observed to increase during adaptation of *S. cerevisiae* to osmotic stress [27]. A likely physiological reason for this response is to provide the cell with sufficient NADH for glycerol production during adaptive osmoregulation. An additional isoform of this family of enzymes, encoded by *ALD2/ALD5*, was previously shown to be induced by osmotic stress in a HOG and protein kinase A pathway dependent way [13].

The revelation of an additional group of 6 strongly NaCl induced proteins whose presence remained below detection level in *PBS2* deleted background (class IV, Fig. 3) indicates an important involvement of the HOG pathway in yet unidentified cellular processes that are directly or indirectly linked to adaptation of *S. cerevisiae* to hyperosmotic stress.

Acknowledgements: We are grateful to Eva Sköld for skilful help with the glycerol measurements. This work was supported by grants from the Swedish National Board for Natural Sciences (NFR), the Swedish National Board for Technical Development (TFR).

References

[1] Blomberg, A. and Adler, L. (1992) Adv. Microbial Phys. 33, 145–212.
[2] Brewster, J.L., De Valoir, T., Dwyer, N.D., Winter, E. and Gustin, M.C. (1993) Science 259, 1760–1763.
[3] Ruis, H. and Schüller, C. (1995) BioEssays 17, 959–965.
[4] Maeda, T., Wurgler-Murphy, S.M. and Saito, H. (1994) Nature 369, 242–245.
[5] Maeda, T., Takekawa, M. and Saito, H. (1995) Science 269, 554–558.
[6] Posas, F., Wurgler-Murphy, S.M., Maeda, T., Witten, E.A., Thai, T.C. and Saito, H. (1996) Cell 86, 865–875.
[7] Albertyn, J., Hohmann, S., Thevelein, J.M. and Prior, B.A. (1994) Mol. Cell Biol. 14, 4135–4144.
[8] Larsson, K., Ansell, R., Eriksson, P. and Adler, L. (1993) Mol. Microbiol. 10, 1101–1111.
[9] Norbeck, J., Påhlman, A.-K., Akhtar, N., Blomberg, A. and Adler, L. (1996) J. Biol. Chem. 271, 13875–13881.
[10] Hirayama, T., Maeda, T., Saito, H. and Shinozaki, K. (1995) Mol. Gen. Genet. 249, 127–138.
[11] Marchler, G., Schüller, C., Adam, G. and Ruis, H. (1993) EMBO J. 12, 1997–2003.
[12] Varela, J.C.S., Praekelt, U.M., Meacock, P.A., Planta, R.J. and Mager, W.H. (1995) Mol. Cell. Biol. 15, 6232–6245.
[13] Miralles, V.J. and Serrano, R. (1995) Mol. Microbiol. 17, 653–662.
[14] Blomberg, A. (1995) J. Bacteriol. 177, 3563–3572.
[15] Norbeck, J. and Blomberg, A. (1996a) FEMS Microbiol. Lett. 137, 1–8.
[16] Sherman, F., Fink, G.R. and Hicks, J.B. (1983) Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[17] Morales, C., André, L. and Adler, L. (1990) FEMS Microbiol. Lett. 69, 73–78.
[18] Hanahan, D. (1983) J. Mol. Biol. 166, 577–580.
[19] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) in: Molecular Cloning: a Laboratory Manual (Nolan, C., ed.) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[20] Ausubel, F.M., Brent, R., Kingston, R.E., Moor, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1994) in: Current Protocols in Molecular Biology, vol. 1–3 (Janssen, K., ed.) Wiley, Chichester.
[21] Gietz, D., St. Jean, A., Woods, R.A. and Schiestl, R.H. (1992) Nucl. Acids Res. 20, 1425.
[22] Rose, M.D., Novick, P., Thomas, J.H., Botstein, D. and Fink, G.R. (1987) Gene 60, 237–243.
[23] Sikorski, R.S. and Hieter, P. (1989) Genetics 122, 19–27.
[24] Berben, G., Dumont, J., Gilliquet, V., Bolle, R.-A. and Hilger, F. (1991) Yeast 7, 475–477.
[25] André, L., Hemming, A. and Adler, L. (1991) FEBS Lett. 286, 13–17.
[26] Sussman, I. and Avron, M. (1966) Biochim. Biophys. Acta 661, 199–204.
[27] Blomberg, A. and Adler, L. (1989) J. Bacteriol. 171, 1087–1092.
[28] Norbeck, J. and Blomberg, A. (1997) Yeast, in press.
[29] Eriksson, P., André, L., Ansell, R., Blomberg, A. and Adler, L. (1995) Mol. Microbiol. 17, 95–107.
[30] Boguslawski, G. and Polazzi, J.O. (1987) Proc. Natl. Acad. Sci. USA 84, 5848–5852.
[31] Boguslawski, G. (1992) J. Gen. Microbiol. 138, 2425–2432.
[32] Garrels, J.I., Futcher, B., Kobayashi, R., Latter, G.I., Schwender, B., Volpe, T., Warner, J.R. and McLaughlin, C.S. (1994) Electrophoresis 15, 1–15.
[33] Norbeck, J. and Blomberg, A. (1997) J. Biol. Chem. 272, in press.
[34] Norbeck, J. (1996) Protein expression of yeast during growth under osmotic stress. PhD Thesis, Göteborg University, Göteborg, Sweden (ISBN 91-628-2256-X).
[35] Schüller, C., Brewster, J.L., Alexander, M.R., Gustin, M.C. and Ruis, H. (1994) EMBO J. 13, 4382–4389.
[36] Boorstein, W.R. and Craig, E.A. (1990) EMBO J. 9, 2543–2553.
[37] Mendoza, I., Ruvio, F., Rodriguez-Navarro, A. and Pardo, J.M. (1994) J. Biol. Chem. 269, 8792–8796.
[38] Marquez, J.A. and Serrano, R. (1996) FEBS Lett. 382, 89–92.
[39] Luyten, K., Albertyn, J., Skibbe, W.F., Prior, B.A., Ramos, J., Thevelin, J.M. and Hohmann, S. (1995) EMBO J. 14, 1360–1371.
[40] Eriksson, P. (1996) Identification of the two GPD isogenes of *Saccharomyces cerevisiae* and characterization of their response to hyper-osmotic stress. PhD Thesis, Göteborg University, Göteborg, Sweden (ISBN 91-628-2168-7).