Genetic analysis of the adaptation of *Saccharomyces cerevisiae* to high osmolarity

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To my parents

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Abstract. The osmoregulatory response of baker's yeast *Saccharomyces cerevisiae* was studied by using mainly a genetical approach. The yeast cells respond to hyper-osmotic stress by an enhanced production and accumulation of glycerol. Synthesis of glycerol is carried out by a cytoplasmic glycerol 3-phosphate dehydrogenase (GPD) and a glycerol 3-phosphatase (GPP). The GPP activity was shown to be due to two isoenzymes, Gpp1p and Gpp2p. Production of Gpp2p was induced by hyper osmotic stress and controlled by the osmo signaling high osmolarity glycerol (HOG) pathway. The *GPP1* and *GPP2* genes were identified by using a reverse genetics approach.

The *PBS2* gene encoding a MAP kinase activator of the HOG pathway was cloned by complementation of the *osg2* mutant isolated in a screen for osmosensitive glyceroldefective mutants. The effect of a *PBS2* deletion on protein expression during adaptation to high salinity was analyzed by two dimensional polyacrylamide gel electrophoresis. Of the 29 highly NaCl-responsive proteins, all showed induction that was dependent on *PBS2*. The kinetics of the protein induction and the transcriptional activation of the *GPP2* and *GPD1* genes, indicate the HOG pathway is mainly important for a rapid and transient response after transfer of cells to increased osmolarity.

The *SGD1* gene, implied in the regulation of glycerol synthesis, was cloned as a suppressor of the *osg3* defect. The protein encoded by *SGD1* is essential, deletion of the gene is lethal. The predicted amino acid sequence contains a strong nuclear localization signal and a leucine zipper motif. High dosage of *SGD1* relieves sensitivity of HOG pathway mutants to salt stress. This effect involves increased glycerol production and enhanced expression of the osmostress induced *GPD1* gene. *SGD1* appears to be subject to negative autoregulation; increased gene dosage leads to reduced levels of *SGD1* transcript. The results suggest that Sgd1p functions as a repressor of *GPD1* (and possibly *GPP2*) expression in a HOG pathway dependent fashion.

The *SOT1* gene involved in tolerance to Na+ stress was isolated as a suppressor of the *osg4* mutant. Deletion of the *SOT1* gene rendered the cells specifically sensitive to Na^+ and *sot1* cells display increased intracellular Na+/K+ ratios at high external salinities. The protein encoded by the *SOT1* gene exhibits homology to tumor suppressor proteins known to interact with the cytoskeleton.

Keywords: Glycerol, osmoregulation, salt tolerance, *GPP1*, *GPP2*, *PBS2*, *SGD1*, *SOT1*, HOG pathway, 2D-PAGE

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This thesis is based on following papers, which will be referred to by their Roman numerals.

I. Norbeck, J., Påhlman, A-K., Akhtar, N and L. Adler. (1996) Purification and characterization of two isoenzymes of DL-glycerol-3-phosphatase from *Saccharomyces cerevisiae*: Identification of the corresponding *GPP1* and *GPP2* genes and evidence for osmotic regulation of Gpp2p expression by the osmosesing mitogen-activated protein signal tranduction pathway. *J. Biol. Chem.* **271**:13875-13881.

II. Akhtar, N., Blomberg, A and L. Adler. (1996) Osmoregulation and protein expression by a *pbs2Δ* mutant of *Saccharomyces cerevisiae* during adaptation to hypersaline stress. Submitted.

III. Akhtar, N., Larsson, K and L. Adler. (1996)*SGD1* encodes an essential protein that negatively regulates the expression of the *GPD1* encoded NAD^+-dependent glycerol 3-phosphate dehydrogenase in a High Osmolarity Glycerol MAP kinase pathway-dependent way. Manuscript.

IV. Larsson, K., Akhtar, N and L. Adler. (1996) *SOT1*, a gene with homology to a group of tumour suppressor genes, is necessary for salt-tolerance in *Saccharomyces cerevisiae*. Manuscript.

CONTENTS

1. Introduction

Environmental stresses exerted on the living yeast cell such as temperature shifts, variations in nutrient availability, desiccation or lowered water activity, require rapid acclimatisation for survival (Mager and Ferreira, 1993, Piper, 1993, Blomberg and Adler, 1993). Most organisms possess specific adaptive mechanisms to counter the insult of the stress conditions. Access to water is fundamental to all cells and increased concentration of solutes leads to a decrease in free water available to the cells. Lowered water concentration results in an outflow of water from the cell, leading to shrinkage and, for walled cells, loss of turgor. Furthermore, if the stress solute is NaCl, the normal cellular functions are impaired due to the toxicity of the Na^+ ions. The most prominent strategy undertaken to counter such solute stress, is exclusion of the stress solute (e.g. Na^+ ions) and the accumulation of osmotically active molecules in the cytoplasm, thereby increasing the osmotic pressure of the cell. This adaptive response causes water to re-enter the cell, and the lost cell volume and turgor pressure can be regained. The osmotically active molecules accumulated in the cell must be non-toxic and compatible with the cellular machinery, therefore termed compatible solutes. Only a limited spectrum of organic molecules can act as compatible solutes, such as glycerol in yeast and proline in plants (Csonka, 1989, Kinne, 1993). In this thesis, the main focus will be the osmoregulatory response in yeast. Yeast cells induce production and accumulation of polyols when subjected to hyperosmotic stress (Blomberg and Adler, 1993). Baker's yeast, *Saccharomyces cerevisiae*, specifically accumulates glycerol as an osmolyte and its role in osmoregulation is now well established. Thus, while the physiological response of yeast to osmotic stress has been of a long-standing interest, only in recent years the attention has been devoted to mechanisms at the molecular level. Particularly the discovery of a signal transduction pathway triggered by osmotic stress and similar in structure to signaling pathways found in mammalian cells has brought the field of osmoregulation to the limelight (Brewster *et al.*, 1993). There are many advantages in choosing *S. cerevisiae* for studies in osmoregulation. It is generally regarded as a highly tractable model organism for eukaryotic cells. Tools for molecular and genetic analysis of *S. cerevisiae* are well-developed and the cells are fast growing compared to other higher eukaryotes. Cellular processes are often evolutionary conserved, thus fruitful inferences to higher eukaryotes can often be made by studying yeast. Finally, the recent sequencing of the complete *S. cerevisiae* genome by a unique world-wide collaboration of scientists has opened new perspectives for future research in yeast molecular biology. A basic functional analysis of the hitherto non-characterised genes has been initiated after completion of the sequencing. Thus, it might soon be possible to obtain clones and deletion mutants in ones field of interest from a central resource, thereby saving significant amounts of time and work.

The aim of this thesis was to isolate and characterise genes involved in the osmoregulatory glycerol response of *S. cerevisiae* by isolation and molecular complementation of osmotically sensitive mutants. Since mutations in genes not primarily involved in osmoregulation can also

lead to an osmosensitive phenotype, we attempted to isolate mutants having specific defects in glycerol production and accumulation. This was accomplished by using a density gradient centrifugation where cells failing to accumulate glycerol to normal levels could be separated from wild type cells. In the process of determining the phenotypes of the mutants, the activities of the enzymes involved in glycerol production were determined. This lead to the discovery of an osmotically induced activity of a specific phosphatase involved in glycerol production. In paper I we report purification, characterisation of two iso-enzymes of DL-glycerol 3-phosphatase and identification of the corresponding genes. The expression of one of the genes, the *GPP2* gene, is regulated by the osmosensing signal transduction pathway, called the HOG pathway (Brewster *et al.*, 1993). The second paper (paper II) deals with the effect on protein expression in a mutant defective in the HOG pathway, analysed by two-dimensional gel electrophoresis. The salt-instigated response of protein synthesis is affected to various degrees in the signaling mutant. We found several proteins that are completely dependent on intact HOG pathway while other display a partially affected response. The third paper (paper III) is concerned with cloning and characterisation of an essential gene, named *SGD1* (*s*uppressor of *g*lycerol *d*efect), suppressing one of the osmosensitive mutants isolated. Interestingly, this gene is also able to suppress mutants defective in the signal transduction pathway when present on high copy number plasmid. This effect involves a negative control of the genes involved in glycerol production and is mechanistically dependent on autoregulation of *SGD1* expression. In the fourth paper (paper IV), cloning of a gene specifically involved in tolerance to Na^+, is described. This gene was previously isolated as a suppressor of the *osg4* mutant The hypothetical protein encoded by this gene has significant similarity to tumour suppressor proteins identified in *Drosophila*, mouse and *homo*. Thus, the evaluation of the protein function might provide clues to the function of its mammalian counterparts.

2. Osmotic stress

2.1. Osmosis

Movement of water molecules across biological membranes is essentially unrestricted as the membranes are highly permeable to water. The direction of the flow is determined by the biophysical need of the cell to achieve thermodynamic water potential equilibrium with the environment. This diffusion of water across membranes is called osmosis. The water potential of the cell is dependent on the turgor pressure exerted by the cell wall and the intracellular osmotic pressure determined by the solute concentration of the cytosol (Blomberg and Adler, 1992). When the internal water potential is lower than the surroundings, *i.e.* the cell is exposed to hyptonic stress, water strives to enter the cell increasing the cell volume. The cell wall present in many organisms counteracts this influx of water by generating a counteracting turgor

pressure. During hypertonic stress conditions, on the other hand, water is lost from the cell to the environment. The consequent decrease in cell volume and turgor pressure initiates mechanisms that strive for recuperation of the cell from the stress exerted. The mechanisms by which the cell adjusts the individual components of its intracellular water potential to adapt to the changes of the ambient water potential is termed osmoregulation (Blomberg and Adler, 1992).

2.2. Osmoregulation and osmotolerance

The ability to osmoregulate is inherent in all cells and of fundamental importance. The internal milieu of the cell must be kept constant for optimal function of the cellular processes, and this endeavour is designated homeostasis. Osmoregulation is thus an homeostatic mechanism, operating during growth regardless of the water potential (Blomberg, 1988). The osmotic stress response has been extensively studied in *S. cerevisiae*. The efflux of water from the cells is extremely rapid upon transfer to high osmolarity conditions, occurring within a time scale of less than a minute (Morris *et al.*, 1986). The cell shrinks due to reduction in the cell volume and turgor pressure following the water loss. Presumably the very first response is release of water from the vacuole to the cytoplasm, thereby minimising the immediate effects of dehydration (Latterich and Watson, 1993). Thereafter, during the adaptive phase, accumulation of compatible solutes commences which increases the internal osmotic pressure and allows backflow of water into the cell (Brown and Simpson, 1972, Yancey *et al.*, 1982). The loss of turgor and cell volume is supposedly sensed by specific osmosensing proteins that initiate activation of signal transducing pathways commencing induced production and/or accumulation of the compatible solute(s) (Brewster, *et al.*, 1993). The osmotolerant yeast *Zygosaccharomyces rouxii* and the less osmotolerant *S. cerevisiae* both utilise glycerol as the compatible solute, however, differing in the course leading to accumulation of glycerol (Brown, 1978, Edgley and Brown, 1983). *Z. rouxii* has a constant production of glycerol irrespective of the osmotic pressure, while the increased accumulation of glycerol upon osmotic stress results from active transport of glycerol into the cell (Van Zyl *et al.*, 1990). However, in *S. cerevisiae* the production of glycerol is induced after transfer to high osmolarity conditions, which coupled with closing of the *FPS1* encoded glycerol facilitator present in the cell membrane account for the accumulated glycerol (Brown, 1978, Luyten *et al.*, 1995). A compatible solute increases the internal osmotic potential without having harmful effects on the metabolic machinery (Low, 1985). In bacterial, plant and mammalian cells, amino acids and derivatives of amino acids are the most prevalent compatible solutes while yeast and fungi have a preference towards polyhydroxy alcohols, polyols (Blomberg and Adler, 1992, Kinne, 1993). The accumulation of compatible solutes restores to a certain level the lost water potential , however, it is not the only prerequisite for acquiring osmotolerance. The physiological state of the cell is vitally important

for surviving the osmotic stress and log phase cells of *S. cerevisiae* cells have been shown to be extremely sensitive to an osmotic upshock as compared to transition- and stationary phase cells (Mackenzie *et al.*, 1986). This phenomenon is termed water stress plating hypersensitivity. Only a few yeast species exhibit this phenomenon and all the resistant strains were shown to accumulate at least one polyol during normal growth which would partly explain the resistance (MacKenzie *et al.*, 1988). This hypersensitvity of the log phase cells is circumvented by short time conditioning in media with moderate salt concentration which leads to increased glycerol accumulation and production. However, the accumulated glycerol solely cannot account for the osmotolerance since osmotically conditioned cells washed free of glycerol still exhibit osmotolerance on high osmolarity media (Blomberg and Adler, 1989). Addition of cycloheximide prevents this acquired osmotolerance, implying that expression of new proteins is necessary to attain osmotolerance. Trehalose has been implicated as an osmotolerance factor since its levels of accumulation correlated with increased osmotolerance (MacKenzie, *et al.*,1988). Trehalose is accumulated in the transient and stationary phases and it has been proposed to serve as a protectant against various stresses such as desiccation, heat and cold shock (Attfield, 1987, Crowe *et al.*, 1984, Hottiger *et al.*, 1987). However, it is probably only one of the factors involved in osmotolerance since heat-conditioned exponential phase cells with increased levels of trehalose still exhibited hypersensitivity towards osmotic stress (Trollmo *et al.*, 1988).

3. Glycerol

3.1. Glycerol metabolism.

The principle compatible solute in baker's yeast is glycerol, the simplest of the polyhydroxy alcohols. Its synthesis encompasses conversion of dihydroxyacetone phosphate (DHAP), an intermediate metabolite in the glycolysis, to glycerol-3-phosphate (G3P) which is then dephosporylated to form glycerol (Gancedo *et al.*, 1968) (Fig. 1). The enzyme responsible for the conversion of DHAP to G3P is NAD^+-dependent *sn*-glycerol 3-phosphatedehydrogenase, encoded by two different genes, *GPD1* and *GPD2* (Albertyn *et al.*, 1994a, Eriksson *et al.*, 1995, Larsson *et al.*, 1993). The *GPD1* gene is the osmostress induced iso-gene while the Gpd2p has a distinct role during anaerobic conditions (see further) (Albertyn, *et al.*, 1994a, Ansell *et al.*, 1996, Blomberg and Adler, 1989, Larsson, *et al.*, 1993). The dephosphorylation step involves two specific glycerol 3-phosphatases, Gpp1p and Gpp2p, where the first is the constitutive form and the latter the induced form during hypertonic stress (Paper I). Glycerol can also be utilised as a carbon source by *S. cerevisiae* and is then converted to DHAP by two enzymes, glycerol kinase (Gut1p) and a mitochondrial FAD^+-dependent glycerol 3-phosphate dehydrogenase (Gut2p) (Gancedo, et al., 1968, Rønnow and Kielland-Brandt, 1993, Sprague

and Cronan, 1977) (see Fig. 1). Mutations in the genes encoding these enzymes, *GUT1* and *GUT2*, leads to failure of growth on glycerol, hence it is the main pathway for dissimilation of glycerol in *S. cerevisiae* (Pavlik et al., 1993, Rønnow and Kielland-Brandt, 1993). Recently, however, genes encoding proteins in a second glycerol degrading pathway, the dihydroxyacetone pathway, have been identified in *S. cerevisiae* (Norbeck and Blomberg,1996b) (Fig. 1). This pathway, although not confirmed to operate in *S. cerevisiae*, would turn glycerol into dihydroxyacetone by a $NADP^+$-dependent glycerol dehydrogenase (GLD) and a subsequent phosphorylation to dihydroxyacetone phosphate by a dihydroxyacetone kinase (DAK) (Adler *et al.*, 1985, Gancedo, *et al.*, 1968, Norbeck and Blomberg, 1996c). Tentative genes encoding these proteins have recently been identified, the *GCY1* and *YPR1* genes encoding the GLD protein, and *DAK1* and *DAK2* genes encoding the DAK enzyme (Norbeck and Blomberg, 1996b).

This pathway operates in other yeasts; in *S. pombe* it is used during growth on glycerol while in *Z. rouxii* it is induced during osmotic stress (Gancedo etal., 1986, Van Zyl *et al.*, 1991). The rate of synthesis of these two enzymes in S. *cerevisiae* is up-regulated in cells exposed to hyper-osmotic stress and this might be an indication of a role for glycerol degradation in osmoregulation, needed for fine-tuning of the glycerol levels inside the cell (Norbeck and Blomberg, 1996c)

3.2. Glycerol permeability

Glycerol passes across the cell membrane either by passive diffusion or by facilitated diffusion, mediated by a membrane protein, Fps1p, that belongs to the MIP channel protein family (Luyten, *et al.*, 1995, Van Aelst *et al.*, 1993). All members of the MIP proteins share six membrane spanning regions and consist mainly of glycerol/metabolite-facilitators, metabolite transport proteins and water channels (Reizer et al., 1993). The cytosolic content of glycerol can thus be regulated by the Fps1 glycerol facilitator that appears to close quickly upon osmotic stress leading to increased retention of glycerol inside the cell (Luyten, *et al.*, 1995). During normal growth conditions, most of the glycerol produced leaks out into the medium since the Fps1p channel is open. In chemostat experiments, *S. cerevisiae* cells growing in high osmolarity media accumulate up to 60% of the total glycerol produced and maintain a gradient of 600-fold against the surroundings (Ölz *et al.*, 1993). When the stress is removed, the glycerol is rapidly lost from the cell, indicating regulation of the Fps1p by reversible modification (Luyten, *et al.*,1995). Presumably the Fps1p has also an important role during hypo-osmotic shock as the survival rate of the cells decreases 50-fold in a fps1 mutant compared to the wild type upon a down-shock from 10% NaCl to distilled water (Luyten *et al.*, 1996). Recently, mutants in the *FPS1* gene have been isolated that are defective in cell fusion during mating (Philips and Herskowitz, 1996). Haploid S. *cerevisiae* cells exist as two different

sexes, a or *alfa*, that mate to form diploids. Mating involves fusion of both cells and this process involves degradation of the cell wall at the position of fusion. The role of the Fps1p is probably to adjust the intracellular osmolarity to prevent bursting of the cell. The Fps1p does not seem to play any significant role in uptake of glycerol for utilisation since no impairment of glycerol catabolism could be observed in a *fps1Δ* mutant (Luyten, *et al.*, 1995). The more osmotolerant species *D. hansenii* and *Z. rouxii* posses carrier proteins that actively transport glycerol across the cell membrane (Adler, *et al.*, 1985, Brown, 1974, Larsson et al., 1990, Van Zyl, *et al.*, 1990).This enables the cells to maintain a glycerol gradient of 1000-fold during high osmolarity conditions. Kinetic data do not indicate any presence of a glycerol transport system in *S. cerevisiae* and so far there has not been any reports identifying a transport system activley taking up glycerol in this species.

3.3. The role of glycerol in redox-regulation

Apart from being an osmolyte, glycerol plays an important role in the redox-regulation of the cell. In fermenting cells, NADH produced by glyceraldehyde-3-phosphate dehydrogenase is re-oxidised to NAD^+ by alcohol dehydrogenase, thus in principal keeping a redox balance. However, intermediates in the lower part of glycolysis are constantly removed for biosynthetic purposes that results in an excess of reducing equivalents. This surplus of NADH can be re-oxidised in the mitochondria by respiration during aerobic conditions, albeit the mitochondrial functions are partly repressed during growth on glucose. Thus, in order to maintain the intracellular redox balance, an alternative route is utilized involving NADH dependent conversion of DHAP to G3P and subsequent dephosphorylation of G3P to glycerol (Van Dijken and Scheffers, 1986). Shift from aerobic to anaerobic conditions induces expression of the *GPD2* gene, one of the iso-genes encoding glycerol 3-phosphatedehydrogenase, resulting in increased glycerol production (Ansell *et al.*, 1996, Eriksson *et al.*, 1995). Deletion mutants for the *GPD2* gene exhibit poor growth and induced accumulation of NADH under anoxic conditions, thus showing the vital importance of the glycerol formation for maintained redox balance during these conditions (Ansell, *et al.*, 1996). However, the absence of the *GPD2* enzyme is partially compensated by the *GPD1* enzyme during anaerobic conditions. If the cells are subjected to osmotic stress under the same conditions, the loss of *GPD2* function is completely compensated. If, on the other hand, the *GPD1* gene is also deleted together with the *GPD2* gene, the cells are not able to grow anaerobically, confiming the assumption that the *GPD1* enzyme in part counteracts absence of the GPD2 enzyme. The expression of the *GPD2* gene is not regulated by *ROX1* that encodes a transcriptional regulator of hypoxic genes, indicating the existence of an alternative redox-signalling pathway under operation during these conditions (Ansell, et al., 1996).

3.4. The Gpd1p during osmotic stress.

Glycerol production is up-regulated in response to hypertonic conditions and this increase is partially mediated by an osmosensing signalling pathway, the high osmolarity glycerol (HOG) pathway (Brewster *et al.*, 1993). The NAD^+ dependent glycerol 3-phosphate dehydrogenase is regarded as the key enzyme in glycerol synthesis and the *GPD1* gene is responsible for the increased synthesis of glycerol 3-phosphate dehydrogenase during osmotic stress (Albertyn *et al.*,1994a, Blomberg and Adler, 1989, Larsson *et al.*, 1993). The enzyme encoded by the *GPD1* gene is a 43 kDa protein and share a 47-50% homology to other eukaryotic GPDs from *Drosophila*, mouse and rabbit. It has an N-terminal extension (a feature shared with the Gpd2p enzyme) that is unique among related GPD enzymes, only the yeast species *S. pombe* possesses a similar sequence. The physiological function of this sequence is currently not known. The Gpd1p enzyme is indispensable for growth in salinities above 1 M NaCl, however, at lower salinities the basal activity of the Gpd2p enzyme is sufficient to rescue the glycerol defect in a *gpd1* mutant (Albertyn, *et al.*, 1994a, Ansell, *et al.*, 1996, Luyten, et al., 1995). A *gpd1Δgpd2Δ* double mutant does not produce any detectable glycerol and is extremely osmosensitive; it does not grow in salinities above 0.5 M NaCl (Ansell, *et al.*, 1996).

3.5. The Glycerol 3-phosphatase

The dephosphorylation step of glycerol 3-phosphate to glycerol was previously assumed to be carried out by a non-specific phosphatase, however, Tsuboi and Hudson, (1956) described a partially purified phosphomonoesterase that was highly specific towards G3P at pH 6.5. In paper I we show that the activity of a phosphatase carrying out the dephosphorylation of G3P increased in osmotically stressed cells. The increase in the phosphatase activity correlated well with increased osmolarity and addition of cycloheximide prevented this increase, indicating the necessity of *de novo* synthesis of this enzyme. In mutants lacking the acid phosphatases (encoded by *PHO5*, *PHO10* and *PHO11* genes) and the alkaline phosphatase (encoded by *PHO8*), the glycerol 3-phosphatase activity still persisted to the same levels as the wild type cells, thus confirming the existence of a hitherto unknown phosphatase. The dephosphorylation reaction was carried out using different substrates closely related to G3P. In crude extracts, less than 9% of the acitivity observed on G3P was exerted on glycerol 2-phosphate, glycerate 3-phosphate, glycerate 2,3-diphosphate, glucose 6-phosphate and fructose 6-phosphate. The same pattern was observed in mutants lacking the major phosphatases and from this we conclude that a specific glycerol 3-phosphatase carried out the dephosphorylation step in the formation of glycerol. The molecular weight of the phosphatase was determined to 30 kDa and attempts to purify the enzyme revealed, surprisingly, the existence of two forms of the glycerol

3-phosphatase enzyme. In eluted extracts from non-stressed cells (after gel filtration and ion exchange chromatography), the activity profile exhibited a single peak while in eluates from cells grown at high salinity, two peaks could be detected. After purification of the two proteins, N-terminal sequences were determined from peptides generated by in-gel trypsination. The sequences obtained were then used for a search in the SwissProtein Data Bank which identified two open-reading frames. The form I of the phosphatase, designated GPP1 (glycerol 3-phosphatase 1), corresponded to the putative gene YIL053w on chromosome IX and the form II, GPP2, was identified as the ORF encoded by the YER062c gene on chromosome V. The amino acid sequence revealed over 90% homology between the two genes and search for additional homologues identified two genes with 35% homology, the *DOG1* and *DOG2* genes (Randez-Gil *et al.*,1995, Sanz *et al.*, 1994). The *DOG1* gene encodes a phosphatase that dephosphorylate the non-metabolizable glucose analogue 2-deoxyglucose (Randez-Gil, *et al.*, 1995). The *DOG2* gene product is 92% homologous to the Dog1p and the physiological role of these enzymes is not yet characterised. The two glycerol 3-phosphatases form together with the Dog1p and Dog2p a new family of low molecular weight phosphatases. Both forms of the glycerol 3-phosphatase were tentatively mapped on two-dimensional polyacrylamide gels based on theoretical Mr and pattern of expression and subsequently confirmed by N-terminal sequencing. The Gpp1p was constitutively expressed in the absence of osmotic stress and as abundant as actin (Act1p) while the synthesis rate of Gpp2p in 1.4 M NaCl was induced to the same levels as Gpp1p, from non-detectable levels in unstressed cells. Purified forms of both enzymes were highly specific to glycerol 3-phosphate and showed no stereospecific preference, with essentially the same activities on the racemic substrate (DL-forms) of G3P as the L-form. The V_{max} and K_m values were quite similar in both forms. The activity of the phosphatases was tested in *hog1Δ* and *pbs2Δ* mutant strains that are defective in the HOG signaling pathway that triggers the osmotic stress response (Brewster, *et al.*, 1993). The mutant strains failed to induce the GPP activity in 0.7 M NaCl and in the *hog1Δ* strain, the second peak corresponding to Gpp2p seen during purification of the enzymes was absent. These results clearly indicate that the Gpp2p expression is regulated by the HOG pathway. This assumption is further confirmed by data published by (Hirayama et al., 1995) where they report cloning of cDNAs for hyper-osmolarity-responsive genes of *S. cerevisaie*. One of these genes, *HOR2*, was identified by us as the gene encoding the Gpp2p. The *HOR2* transcript was strongly induced by osmotic stress conditions and in a *hog1Δ* mutant, the induction was reduced considerably. However, some induction still persists in mutants defective in the signaling pathway, indicating the involvement of other mechanisms acting on the osmotically triggered up-regulation of *HOR2/GPP2* transcription. Other genes targeted by the HOG pathway exhibit the same type of induction pattern with partial dependency on the pathway during osmotic stress (see further).

4. Signaling pathways

Extreme changes in the external osmolarity influence the cell in a dramatic way and to overcome this, several mechanisms must be in operation and co-ordinately regulate the cellular events following the osmotic shock. Regulation is achieved by stimulation of signaling pathways that transduce the stress information and target genes or proteins involved in the response. To this date, two specific osmosensing signal transduction pathways in *S. cerevisiae* have been detected, the PKC1 pathway responding to hypotonic stress and the HOG pathway responding to hypertonic stress (Brewster, *et al.*, 1993, Davenport *et al.*, 1995). Furthermore, the RAS-cAMP pathway has recently received considerable attention as a negative regulator of the general stress response that is activated by various stresses such as heat shock, oxidative stress, DNA damage and high osmolarity (Marchler *et al.*, 1993, Marquez andSerrano, 1996, Schüller *et al.*, 1994, Varela *et al.*, 1995a). Apart from the HOG pathway functioning during high osmolarity conditions, at least one additional mechanism essential for induction of osmo-specific genes is postulated by the data accumulated in our and other laboratories. In addition, when the stress solute in question is NaCl, the Ca^{2+} dependent calmodulin/calcineurin pathway contributes to the regulation modes. Both the PKC1 and HOG pathway involve protein kinases with homologues found in higher eukaryotes, designated MAP kinases (*m*itogen *a*ctivated *p*roteinkinase) or ERKs (*e*xtra-cellular signal *r*egulated *k*inase) (for a recent review, see Treisman, 1996). A series of protein kinases, working in a specific order, trigger the signal received by phosphorylation to cellular targets. The most upstream kinase of such a module is a MAP kinase kinase kinase (MAPKKK) that acts on the kinase downstream, a MAP kinase kinase (MAPKK) or a MAP kinase activator which in turn phosphorylates the MAP kinase (MAPK). The MAPKKK and MAPK are serine/threonine kinases, while the MAPKK is a dual-specificity kinase that phosphorylates tyrosine residues in addition to serine/threonine. The phosphorylated form of MAPK often translocates to the nucleus where it regulates the activity of one or more transcription factors (Treisman, 1996). This theme of propagating an external signal via a series of kinases is conserved among different species, and in a few cases MAP kinases from mammalian cells have successfully suppressed *S. cerevisiae* mutants defective in components in a MAP kinase signaling pathway (Galcheva-Gargova *et al.*, 1994, Han *et al.*, 1994). To this date, five different MAPK pathways have been discovered in *S. cerevisiae*, influencing a wide variety of different cellular processes, like the mating response, pseudohyphal development, osmosensing, cell wall synthesis and regulation of sporulation (Herskowitz, 1995).

4.1. The High Osmolarity Glycerol pathway

An important step towards understanding the events taking place at molecular level during osmotic stress was taken by uncovering the existence of an osmosensing and signalling pathway (Fig. 2). Two genes encoding putative MAPKK and MAPK homologues were cloned by complementing osmotically sensitive mutants and shown to influence glycerol production and accumulation in S. *cerevisiae* (Brewster, *et al.*, 1993). The gene encoding the MAPKK is identical to a previously cloned gene, *PBS2*, and mutations in this gene renders the cells sensitive to the antibiotic polymyxin B (Boguslawski, 1992, Boguslawski and Polazzi, 1987). The MAPK is encoded by the *HOG1* gene and this protein becomes tyrosine-phosphorylated in cells subjected to osmotic stress in a Pbs2p-dependent manner (Brewster, *et al.*, 1993). The Hog1p-phosphorylation is rapidly induced, within a time course of 1 min after transfer to high osmolarity media, and exhibits a transient response with considerably reduced phosphorylation after 20 min. Following this discovery, the upstream components of this pathway, named HOG pathway (*h*igh *o*smolarity *g*lycerol) were soon identified. Two osmosensors are involved in mediating the signal to the pathway (Maeda *et al.*, 1995, Maeda *et al.*, 1994). One is closely related to the "two-component"systems functioning as signal-transducers in bacteria, however, in the yeast case recent evidence points to the involvement of three proteins rather than two (Posas *et al.*, 1996). The second osmosensor is presumed to directly interact with the Pbs2p protein kinase and no homologues to this protein have yet been identified. The "two-component" related system embodies a membrane-bound protein, Sln1p, a newly identified protein Ypd1p and a response regulator, Ssk1p, that transmits the signal to the a functionally redundant pair of MAPKKKs Ssk2p and Ssk22p (Posas, *et al.*, 1996). During low osmolarity conditions, the Sln1p is auto-phosphorylated on a histidine residue in the kinase domain with subsequent transfer of the phosphate group to an aspartate residue in the receiver domain of the protein. The phosphate group is then transferred to a histidine on Ypd1p and finally passed on to an aspartate residue in Ssk1p (Maeda, *et al.*, 1995, Maeda, *et al.*, 1994, Posas, *et al.*,1996). The phosphorylated form of Ssk1p is inactive, hence under these conditions no signal is transmitted to the HOG pathway (Maeda, *et al.*,1994). In high osmolarity conditions the un-phosphorylated form of Ssk1p predominates, as auto-phosphorylation of the Sln1p is inhibited and consequently the Ssk2p and Ssk22p are phosphorylated with an activation of the HOG pathway as a result (Maeda, *et al.*, 1995). The Sln1p-Ypd1p-Ssk1p complex thus functions as a negative regulator of the pathway. Deletion of the *SLN1* gene is lethal to the cells and this lethality is overcome by over-expression of the tyrosine phosphatases Ptp2p and Ptp3p which act on the Hog1p (Flanagan *et al.*, 1996, Maeda, *et al.*, 1994). Moreover, over-expression of the *PTC1* and *PTC3* genes encoding serine/threonine phosphatases of the PP2C type acting on Pbs2p, suppresses the lethality of *sln1Δ* (Maeda *et al.*, 1993). Deleting the *PBS2* and *HOG1* genes also leads to suppression of the lethality caused by *SLN1* deletion (Maeda, *et al.*, 1995). In other words, constitutive activation of the HOG pathway is lethal to the cells, however, the

rational behind this lethality remains yet to be evaluated. Surprisingly, deletion of the *SSK2* and *SSK22* genes does not lead to osmosensitivity and induction of Hog1p phosphorylation by osmotic stress persisted in these mutants (Maeda, *et al.*, 1995). This pointed towards the existence of other mechanisms stimulating Hog1p phosphorylation, possibly converging at the level of the Pbs2p. Screening for mutations rendering *ssk2Δ/ssk22Δ* mutants osmosensitive led to the cloning of the second osmosensor, the Sho1p that contains four putative transmembrane domains (Maeda, *et al.*, 1995). An SH3 domain in the Sho1p is presumed to interact directly with a putative proline-rich SH3 binding domain in thePbs2p and activate the kinase by an unknown mechanism. Experiments where Hog1p-phosphorylation was detected in *ssk2Δssk22Δ* and *sho1Δssk2Δ* mutants revealed that the Sln1p/Ypd1p/Ssk1p pathway responds to lower osmolarity, peaking at 0.1 M NaCl and in a more rapid manner than the Sho1p, within 1 minute of the osmotic shock (Maeda, *et al.*, 1995). The Sho1p mediates activation at a slightly higher osmolarity, with no phosphorylation at 0.2 M NaCl while reaching maximum already at 0.3 M NaCl and responding 2 min after the shock. In the wild type cells the maximum level of Hog1p phosphorylation was reached at 0.3 M NaCl and increasing the osmolarity had no further effect on the phosphorylation. The presence of two distinct osmosensors responding to different osmolarity ranges and showing different time responses might increase the sensitivity of the system towards external osmolarity and increase the flexibility of the response. The rapid and transient response of the HOG pathway indicates that the role of the pathway is important in triggering the response while other, yet to be identified mechanisms, are involved in the sustained response in fully adapted cells. The expression of the *GPD1* gene is partly regulated by the HOG pathway upon osmotic stress (Albertyn, *et al.*, 1994a, Eriksson *et al.*,1996b). Deletion of *HOG1* causes a decreased level of induction, however, it is not totally abolished, indicating the existence of another pathway targeting the *GPD1* gene during high osmolarity stress.

Transcription factors that are targeted by Hog1p remain yet to be identified although a protein interacting with Hog1p has been recently isolated in a two-hybrid screen (Holzmueller *et al.*, 1996). Deletion of the corresponding gene gives a clear phenotype only in a genetic background deleted for the Mig1p-like transcription factors encoded by *MSN2* and *MSN4*, which are implicated in transcriptional activation of several general stress-responding genes (Martinez-Pastor *et al.*, 1996). Genes involved in the general stress response are up-regulated by different stresses like high osmolarity, heat stress, oxidative stress, nutrient starvation and weak acid stress. *CTT1* encoding Catalase T, *HSP104* encoding a heat shock protein, *DDR2* encoding a protein involved in the DNA damage response and *UB14* encoding polyubiquitin, belong to this class of stress responsive genes (Marchler, *et al.*, 1993, Schüller, *et al.*, 1994). These genes contain an element having the sequence motif CCCCT (or the complemetary AGGGG) in their promoter region. This element, termed STRE (*st*ress *r*esponsive *e*lement), is important for the stress mediated transcriptional activation of these genes and Msn2p and Msn4p appear to bind to STREs (Ruis and Schüller, 1995, Martinez-Pastor *et al.*, 1996). The up-regulation of these

genes during osmotic stress is stimulated by the HOG pathway and occurs *via* STREs (Ruis and Schüller, 1995). However, STREs are unlikely to be the only elements targeted by the HOG pathway since genes regulated by this pathway have been isolated that completely lack such elements in the promoter region. The *ALD2* and *DAK1* promoters lack STREs albeit still exhibit HOG pathway triggered induction (Miralles and Serrano, 1995, Norbeck, 1996b; paper II). Deletion of the gene for the above mentioned Hog1p-interacting protein in a *msn2Δmsn4Δ* background led to complete lack of induction of the *CTT1* promoter by high osmolarity conditions while deletion of this gene alone (whose identity is not yet revealed by the researchers) caused only minor changes in the expression of the promoter (Holzmueller *et al.*, 1996). This indicates the necessity of all three proteins in optimal induction of the general stress response. This is further confirmed by the observation that the global protein expression pattern, as analysed by two dimensional polyacrylamide gel electrophoresis (2D PAGE), was not detectably altered in a *msn2Δmsn4Δ* double mutant when grown in high osmolarity media (H. Alipour and A. Blomberg, unpublished).

Apart from being osmosensitive, the *hog1Δ* and *pbs2Δ* mutants exhibit an aberrant cell morphology when incubated in high osmolarity media (Boguslawski, 1992). When cells are shifted to increased osmolarity, the actin cytoskeleton is disassembled and after adaptation to the new conditions, the skeleton is re-organised (Chowdhury *et al.*, 1992). However, in the *pbs2Δ* and *hog1Δ* mutants the re-positioning of the cytoskeleton following an osmotic upshock is defective, leading to the observed morphology (Brewster and Gustin,1994). The HOG pathway mutants fail to restore the filaments to the original bud and instead starts budding at a new site, giving rise to multi-budded cells. The biochemical basis of this observation has yet to be elucidated. The HOG pathway might communicate (cross-talk) with other pathways involved in controlling cell morphology which is suggested by the observation that mutations in the *STE12* gene, encoding the transcription factor involved in the yeast mating response, partially rescues the osmosensitive and aberrant morphology phenotype of *hog1Δ* and *pbs2Δ* mutants (O'Rourke and Herskowitz, 1996). This suppression occurred in 0.5 M NaCl and 1.0 M sorbitol, although not at higher osmolarities. The glycerol levels were not affected in the *ste12Δ* mutant, implying that the osmosensitivity of the *hog1Δ* and *pbs2Δ* mutants at lower osmolarities is not due to a defective osmoregulatory glycerol response but some other mechanism is evidently involved. Furthermore, the Sln1p has recently been shown to regulate the activity of the essential Mcm1p transcription factor in a manner independent of the downstream components of the HOG pathway, suggesting the involvement of the Sln1p in other cellular processes than control of the protein kinase cascade (Yu *et al.*, 1995).

4.2. The protein kinase C pathway

The second osmosensing signaling pathway hitherto discovered in *S. cerevisiae* also involves an integral module of MAP kinases that is regulated by the protein kinase Pkc1p (Levin and Bartlett-Heubusch, 1993, Davenport *et al.*, 1995) (Fig 3). The yeast protein kinase C is a homologue to Ca^{2+}-stimulated protein kinases in mammalian cells that are involved in regulation of processes like cell growth, proliferation and differentiation (Watanabe *et al.*, 1994). The yeast *PKC1* gene is essential for growth and loss of Pkc1p function results in a lysis phenotype due to a defect in the cell wall assembly (Levin and Bartlett-Heubusch, 1993). The MAP kinase pathway regulated by Pkc1p is comprised of the MAPKKK Bck1p, two functionally redundant MAPKKs, Mkk1p/Mkk2p, and the MAP kinase Mpk1p, also known as Slk1p (Costigan *et al.*, 1992, Irie *et al.*, 1993, Lee *et al.*,1993, Lee and Levin, 1992, Mazzoni *et al.*, 1993). The Pkc1p probably effects other targets than this pathway since the lysis defect in mutants in the MAP kinase cascade is only manifested at 37° C while in the *pkc1*Δ mutant it is present at all temperatures. Two protein phosphatases, Ppz1p and Ppz2p, have been proposed to function in this branched pathway since *ppz1Δ* and *ppz2Δ* mutants exhibit the same lysis defect as mutants in the MAP kinase pathway, and a *ppz1Δppz2Δmpk1*Δ triple mutant shows an extreme phenotype similar to a *pkc1*Δmutant (Lee *et al.*, 1993). Additionally, a protein of unknown function, Bck2p, is implicated in the branched pathway, the role of this component is, however, not clear (Lee, *et al.*, 1993). Apart from the lysis defect, deletion of genes in the *PKC1* pathway causes sensitivity to starvation and heat-shock and results in defective glycogen accumulation and meiosis. Furthermore, bud formation is not ceased upon entry into the stationary phase. Thus it is quite clear that the PKC1 pathway target more than one cellular process. Consequently, a few proteins have been identified that interact or affect the activity of the Pkc1p. The essential GTP-binding Rho1p, involved in polarised cell growth, have been shown to interact with Pkc1p and deletion of the *SST4* gene, encoding a phosphatidylinositol kinase, produces a lysis defect resembling the deficiency in a *pkc1*Δ mutant (Kamada *et al.*, 1996, Nonaka *et al.*, 1995,Yoshida *et al.*, 1994). However, genetic data indicate that these proteins have other additional targets than the Pkc1p, thus making the overall picture quite complex.

A few candidate transcription factors for the *PKC1* pathway have been identified, the Rlm1p containing a conserved domain of the MADS box family transcription factors and the two HMG1-like (high mobility group) chromatin associated proteins Nhp6ap/Nhp6bp (Costigan *et al.*, 1994,Watanabe *et al.*, 1995). The *KCS1* gene encoding a protein resembling the basic leucine zipper transcription factors was identified as a gene in which mutations suppressed a *pkc1* allele that promoted increased rates of recombination, thus adding yet another phenotype to a *pkc1* mutant (Huang and Symington, 1995). The target genes for the mentioned transcription factors have yet to be identified, however, the *BGL2* gene, that codes for a ß-glucanase involved in cell wall degradation, is evidently negatively regulated by the *PKC1* pathway (Shimizu *et al.*, 1994). Deletion mutants in the *PKC1* pathway are usually stabilised by adjusting the osmolarity with 1 M sorbitol pointing to a possible involvement of the pathway

in tolerance to changes in osmolarity. Down-shock experiments showed increased phosphorylation of the MAP kinase Mpk1p that was dependent on upstream components while increased osmolarity led to a decrease in Mpk1p phosphorylation (Davenport, *et al.*, 1995). As previously mentioned, the Hog1p is tyrosine phosphorylated in the opposite fashion with decreased osmolarity resulting in reduced phosphorylation. Evidently the *PKC1* pathway and the HOG pathway function independently of each other, nevertheless data regarding tyrosine phosphorylation of Hog1p indicate a possible cross-talk between these two pathways. The basal level of tyrosine phosphorylation of the Hog1p was lower in mutants in the *PKC1* pathway and was not altered when the cells were exposed to hypo-osmotic shock (Davenport, *et al.*, 1995). This suggests that the pathway affects signalling through the HOG pathway.

4.3. The Ras-adenylate cyclase pathway

The Ras-adenylate cyclase pathway (or RAS-cAMP pathway) is implicated in negative regulation of the general stress responsive genes (Ruis and Schüller, 1995) (Fig. 4). It is commonly recognised as a pathway involved in nutrient sensing during switch from respiratory to fermentative growth and is transiently induced upon addition of glucose to the medium (Thevelein, 1992, Thevelein, 1994). The Cdc25p stimulate an exchange of GDP to GTP on the Ras1p and Ras2p, which are yeast homologues to human oncogene RAS proteins (Barbacid, 1987), and renders the proteins active. The Ira1p and Ira2p negatively regulate the RAS proteins by inducing the intrinsic GTPase activity residing in Ras1p and Ras2p. Active RAS proteins in turn activate the enzyme adenylate cyclase (Cyr1p) which catalyses formation of cAMP from ATP. The function of cAMP is to act as a signal molecule that stimulates protein kinase A (PKA) activity by dissociating the regulatory subunit of PKA from the catalytic subunit. The regulatory subunit, Bcy1p, binds as a dimer to the catalytic subunit which is encoded by the *TPK1*, *TPK2* and *TPK3* genes. Very few substrate proteins of PKA have hitherto been identified, among them fructose 1,6-bisphosphatase, the transcription factor Adr1p and phosphatidyl serine synthase. Furthermore, PKA exerts a feed-back inhibition on cAMP synthesis by an unknown mechanism. Some basal activity of RAS-cAMP pathway is required for progress through the START site in the G1 phase of the cell cycle (Wittenberg and Reed,1996). An overactive Ras pathway evokes sensitivity towards heat shock and nutrient starvation, and in general a reduced stress resistance (Thevelein, 1994). It also leads to poor growth on non-fermentable carbon sources, failure to sporulate, and low levels of the storage carbohydrates glycogen and trehalose, while decreased activity has essentially the opposite effect. Heat shock genes are constitutively expressed and sporulation occurs readily in rich media in strains with reduced PKA activity. In such cells transcription of genes regulated by STREs such as *CTT1*, *HSP12* and *ENA1* is enhanced (Marchler, *et al.*, 1993, Marquez and Serrano, 1996, Varela, *et al.*, 1995a). In cells with high PKA activity, such as a *bcy1* mutant,

the transcription of these genes is dramatically reduced, verifying negative regulation exerted by PKA. Protein expression studies on cells subjected to osmotic stress further confirm PKA as a general negative regulator of stress genes. In exponentially growing cells, the basal levels of proteins normally induced during osmotic stress were elevated in a mutant with low PKA activity while high PKA activity increased expression of proteins repressed during high osmolarity conditions (Norbeck and Blomberg, 1996d). Furthermore, increased PKA activity was strongly correlated with decreased osmotolerance, substantiating the negative regulating role of the RAS-cAMP pathway in stress resistance.

5. Protein expression during osmotic stress

Studies on the protein expression changes induced by high osmolarity conditions, as analysed by 2D PAGE, have revealed several important mechanisms operating in the cell during osmotic stress. Transfer of cells of the strain Y41 to high osmolarity media engenders extensive alterations in the protein expression pattern that are essentially transient (Blomberg, 1995). Approximately 150 of the resolved proteins exhibited changes in the expression when exposed to 0.7 M NaCl, of which 18 where highly NaCl responsive with eight-fold or more increases in the rate of protein synthesis. A variety of complex regulatory modes could be discerned, from induction to repression with different pattern of time response of induction/repression and declination. When the same strain was analysed during steady state growth in 0.7 M NaCl, only 13 proteins displayed significant alterations in synthesis rate (Norbeck and Blomberg, 1996a). This further confirmed the transient nature of the response observed in the cells transferred to 0.7 M NaCl. Furthermore, a *S. cerevisiae* strain exhibiting a higher degree of halotolerance (SKQ2n), than the strain studied in the above experiments (Y41) displayed notable changes in 73 different proteins during steady state growth in 1.4 M NaCl, while the Y41 strain essentially responded as in 0.7 M NaCl (Norbeck and Blomberg,1996b). Thus, it is possible that the less profound and transient response of the changes in protein expression on transfer of the Y41 strain to high salinity is responsible for its lower degree of osmotolerance.

5.1. Protein expression in a *pbs2Δ* mutant

The *PBS2* gene (Boguslawski and Polazzi, 1987) was isolated in our laboratory by complementation of an osmotically sensitive glycerol defective (*osg*) mutant (Larsson, *et al.*, 1993). The *osg* mutants were isolated in a mutant screen where cells showing defective accumulation of glycerol when subjected to hyperosmotic stress, were enriched in a density gradient. The defective glycerol accumulation results in a higher buoyant density compared to wild type cells and, hence, the defective cells can be separated from the normal ones in a density

gradient (Morales *et al.*, 1990). This screen yielded mutants that fell into four different complementation groups, *osg1-osg4*. The *osg1* group comprised mutants in the *GPD1* gene and the *osg2* group consisted of *pbs2* mutants. The *osg3* and *osg4* groups led to the identification of novel genes with unknown function and will be dealt with later on in this thesis.

In paper II we report HOG pathway controlled protein expression in *S. cerevisiae* upon transfer to high osmolarity conditions, as analysed by 2D PAGE. Exponentially growing wild type and *pbs2*Δ mutant cells were shifted to 0.7 M NaCl and changes in protein expression were followed by labelling the cells with ^{35}S-methionine. Analysis of the protein pattern revealed a grey-scale of dependency on intact HOG pathway for osmotic stress induced protein synthesis. Roughly fifty proteins displayed increased rate of synthesis in the wild type cells with at least two-fold induction levels at 20-40 min. after salinity shift. We focused on the 29 proteins that were at least 6-fold induced during adaptation to high salinity and we classified these proteins into four different classes. One class comprised a group of 6 proteins that were strongly dependent on an intact Pbs2p function for their expression, they remained below, or only slightly above, detection level in *pbs2Δ* cells both at low and high salinity. Another class consisted of 8 proteins that displayed a detectable and absolute requirement of Pbs2p for their induction following osmotic stress. No induction occurred in *pbs2* cells exposed to NaCl stress. The only protein identified in this class was the Ald6p, that catalyses reduction of acetaldehyde to acetic acid with concomitant NADH formation. This reaction is reportedly induced during adaptation to high osmolarity stress (Blomberg and Adler, 1989) and is thought to supply the cell with adequate amounts of NADH for the increased glycerol synthesis required for osmoregulation. Thus, considering the physiological function the regulation exerted by the HOG pathway appears rational. A third class contained proteins that displayed an induction by increased salinity that was partly dependent on Pbs2p. These proteins exhibited a delayed induction that reached considerably lower levels in *pbs2* cells than in wild type cells. In this class involving 11 proteins, two enzymes involved in glycerol metabolism could be identified Gpd1p and the newly identified, dihydroxyacetone kinase, Dak1p (Norbeck and Blomberg,1996b). Finally a group of 4 proteins was discerned that characteristically showed osmotic induction that was only slightly *PBS2* dependent. To this group belonged Tdh1p, an iso-form of glyceraldehyde 3-phosphate dehydrogenase, (Norbeck and Blomberg, 1996c) and the heat shock proteins Hsp104p and Ssa3p, the latter being an Hsp70 protein. The latter proteins harbour STREs or STRE like elements in their promoters (Schüller *et al.*, 1994; Boorstein and Craig, 1990, Norbeck *et al.*, 1996d) which serve as targets for both HOG pathway and protein kinase A control (Shuller and Ruis, 1995). The protein kinase A pathway exerts a negative control on these elements and the strong response remaining in the HOG pathway-blocked *pbs2Δ* mutant might at least partly stem from the decrease in protein kinase activity that is associated with transfer to increased osmolarity (Marquez and Serrano, 1996).

The pattern of induction of the Gpd1p was further examined by RNA blot analysis of the *GPD1* following osmotic stress in 0.7 M NaCl. The *GPP2* mRNA was also detected to explore whether it was regulated in a similar manner to the *GPD1* transcript. Both transcripts displayed the same decreased rate of induction in mutants deleted for *PBS2*. This induction peaked at 60 min after transfer to 0.7 M NaCl as compared to 20 min. in the wild type cells. The maximum levels of transcript were also about 5 times lower in the mutant than in the wild type. Interestingly, the induction of both *GPP2* and *GPD1* transcripts showed a stronlgy transient behaviour in wild type cells, in agreement with the transient duration of the HOG pathway signal after activation by hyper osmotic stress (Brewster *et al.*, 1993). The HOG pathway thus appears to be responsible for the immediate induction observed in the wild type cells, while the sustained steady state induction seems to rely on other signaling mechanisms. Promoter analysis of the *GPD1* gene, using a *GPD1* promoter-CAT gene fusion (Eriksson *et al.*, 1996), unveiled two different promoter regions responsible for the osmotic induction; one being dependent on the HOG pathway the other being completely HOG independent, receiving a signal from a hitherto unidentified pathway. However, the promoter data from the CAT analysis did not display the transient response observed in the Northern analysis. This discrepancy might be due to a higher stability of the CAT transcript and might imply a second level of regulation exerted on mRNA stability. In summary, it appears that the HOG pathway affects the expression of all the strongly NaCl responsive proteins that were detected by our 2D PAGE analysis. Several of the proteins displayed an absolute requirement of the HOG pathway for their NaCl induced upregulation while a large group were dependent on the pathway to various extents, foremost for a rapid response to the stress insult.

6. The *SGD1* gene

The *osg3-1* mutant displayed a glycerol defect only at salinities above 0.7 M NaCl, while in 0.7 M NaCl the induction could not be distinguished from wild type cells (paper III). Activity measurements conducted for the enzymes involved in glycerol production confirmed the observed glycerol phenotype, with impaired induction of GPD and GPP activities at high salinity. Finally, Northern analysis of the *GPD1* transcript verified that the poor GPD activity was at least partly dependent on a defective transcriptional activation of the *GPD1* gene in the *osg3-1* mutant at high salinity. The expression of the *DDR48* gene is induced by high salinity in a HOG pathway-independent fashion (Miralles and Serrano, 1995). Hence, it was possible that the high salinity defect of the *osg3-1* mutant was due to a deficiency in this pathway. However, the *osg1-3* mutant showed a normal induction of the *DDR48* gene on exposure to salt stress (paper III). Attempts to clone the gene corresponding to the *osg3-1* mutation resulted in two set of clones containing the same overlapping DNA fragments. These clones complemented the osmosensitive phenotype. Subcloning and sequencing identified a putative ORF sequenced by

the *Saccharomyces* genome project with unknown function on chromosome XII. Efforts to link the cloned gene to the *osg3-1* mutation failed from which we concluded that the gene is a suppressor of the mutation. Therefore the gene was designated *SGD1*, *s*uppressor of *g*lycerol *d*efect, as it suppresses the high salinity glycerol defect.

Searches in various databases failed to detect any homologues to the hypothetical protein. A sequence analysis of Sgd1p revealed several overlapping putative nuclear localisation signals (Kalderon *et al.*, 1984, Robbins *et al.*, 1991), which would indicate that the Sgd1p is localised in the nucleus. The sequence also contained a leucine zipper region (Landschulz *et al.*, 1988), however, without the DNA binding basic region that normally is found just upstream the leucine zipper in the transcription factors characterized by these two features (Busch and Sassone-Corsi, 1990, Neuberg *et al.*, 1989). Nevertheless, this zipper region would indicate that the Sgd1p has the potential of participating in protein-protein interactions. In addition, there is also a stretch of acidic amino acids in the Sgd1p carboxy terminal end, similar to those found in transcriptional activators (Ma and Ptashne, 1987, Seipel *et al.*, 1994). These features taken together might be indicative of a role of Sgd1p in transcriptional regulation. Since deletion of *SGD1* gene is lethal to the cells, one can assume a more general role of the *SGD1* gene product than the presumed function in glycerol production. Mutants defective in the HOG pathway, the *pbs2*Δ and *hog1*Δ mutants (Brewster, *et al.*, 1993), transformed with the *SGD1* gene on a multicopy plasmid (*SGD1*-2μ) regained the ability to grow in high osmolarity media. The basis for this suppression appears to be linked to the increased glycerol production in the HOG mutants. No effect on glycerol production could be discerned in wild type cells transformed with *SGD1*-2μ. In the *pbs2*Δ mutant, the presence of *SGD1*-2μ caused a strongly enhanced *GPD1* expression, GPD and GPP activity and glycerol production while in the *hog1*Δ mutant the effects were similar but not as strong as in the *pbs2Δ* background. The salt induced glycerol production in a *pbs2*Δ*hog1*Δ double mutant harbouring *SGD1*-2μ was similar to that of the corresponding *hog1Δ* mutant, indicating that high dosage of *SGD1* suppressed the osmoregulatory defects of mutants with a blocked HOG pathway more strongly if Hog1p is present. RNA blot analysis of *SGD1* transcripts indicated that high dosage of the gene leads to suppressed transcription, suggesting negative autoregulation as was recently described for the yeast DnaJ homologue *SIS1* (Zhong *et al.*, 1996). Thus, it appears that it is down-regulation of the *SGD1* expression that causes the increased glycerol production of the *pbs2*Δ and *hog1*Δ mutants containing the *SGD1*-2μ plasmid. This observation points towards a repressing role of the Sgd1p on *GPD1* expression (and possibly also on *GPP2* expression). Since this down regulation of *SGD1* expression gives an effect in the HOG mutants but not in the wild type cells, it indicates that the repression is subject to some form of HOG pathway control. Furthermore, since the effects are stronger in the *pbs2Δ* mutant than in the *hog1Δ* or *pbs2Δhog1Δ* double mutant, Hog1p might play a direct or indirect role for the Sgd1p mediated repression. An alternative interpretation is that Sgd1p is targeted by a parallel pathway and that

the effects seen are due to a cross-talk between the HOG pathway and this unidentified pathway.

7. Other cellular processes involved in the osmotic homeostasis

Apart from the osmoregulatory response, other cellular processes are required for adaptation to and survival during high osmolarity conditions. Proper function of the vacuole is essential for survival in high osmolarity media (Banta *et al.*, 1988, Latterich and Watson, 1991). A mutant defective in the *SSV1* gene required for vacuolar biogenesis, protein-sorting and osmo-homeostasis lost viability within ten seconds following osmotic shock (Latterich and Watson, 1993). Although the mechanism behind this shock sensitivity is not fully understood, presumably the immediate flow of water from the vacuole to the cytoplasm is impaired in this mutant having a defective vacuole. These observations indicate an important role of the vacuole for the immediate shock tolerance of the cell to osmotic stress, before commencement of other adaptive responses such as glycerol production. Maintenance of a correct positioning of the cytoskeleton is another important factor for survival after osmotic shock. Following osmotic shock, the actin cytoskeleton is reversibly disassembled and failure of re-organising the skeleton leads to osmosensitivity (Chowdhury, *et al.*, 1992). Certain temperature sensitive mutants in the single essential *ACT1* gene cause an osmosensitive phenotype at permissive temperature (Novick and Botstein, 1985). Extragenic suppressor of the osmosensitive phenotype of these actin *ts*-mutants were identified by Chowdhury *et al.* (1992), one of the these suppressor genes *RAH3* was assumed to encode an actin-binding protein.

8. The response of yeast to salt stress

The response of *S. cerevisiae* to NaCl stress involves to two components, adaptation to ion (Na^+) toxicity and osmotic stress (Blomberg and Adler, 1993). The influx of Na^+ occurs via K^+ carriers encoded by the *TRK1* and *TRK2* genes (Gaber *et al.*, 1988, Ko and Gaber, 1991, Rodriguéz-Navarro and Ramos, 1984). During salt stress conditions, the K^+ transport system switches from a low affinity mode to a high affinity mode that is mainly determined by the Trk1p (Haro *et al.*, 1993). This shift enables the cells to better distinguish between Na^+ and K^+, thereby minimising the uptake of Na^+. In addition to the effects caused by high osmolarity on cell volume and turgor, Na^+ ions are inhibitory to enzyme activity. To circumvent this inhibition, the Na^+ ions are actively exported from the cytosol by a P-type ATPase encoded by the *ENA1* gene which is the first gene of a cluster containing up to five highly homologous *ENA* repeats (Garciadeblas *et al.*, 1993, Haro *et al.*, 1991, Wieland *et al.*, 1995). *ENA1* is regulated by the HOG pathway and the PKA pathway during low salinity stress (Marquez and

Serrano, 1996) while in higher salt concentrations the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin influences the transcription of this gene (Mendoza *et al.*, 1994). Calcineurin consists of a catalytic subunit encoded by the two genes *CNA1* and *CNA2* (Cyert *et al.*, 1991, Liu *et al.*, 1991) and a regulatory subunit encoded by *CNB1* (Cyert and Thorner, 1992). Binding of the regulatory subunit to the catalytic subunit is required for the phosphatase activity (Cohen, 1989). The Ca^{2+}-binding protein calmodulin is essential for calcineurin function as it influences binding of Cnb1p to the Cna1p/Cna2p complex (Cyert and Thorner, 1992). Cells mutated in the regulatory subunit fail to induce *ENA1* gene expression and, furthermore, the shift of the K^+ transporter to a high affinity state is inhibited (Mendoza, *et al.*, 1994). The *ENA1* gene product is also activated post-transcriptionally by calmodulin (Wieland, *et al.*, 1995). Thus, the calcineurin/calmodulin pathway is highly important in determining the NaCl tolerance of the cell. Another protein implicated in the regulation of *ENA1* gene is a 14-3-3 homologue, Bmh1p. In mammalian cells 14-3-3 proteins interact with regulatory proteins and modulate their action (Aitken, 1995). Deletion of the two genes *BMH1* and *BMH2* encoding the two yeast homologues, causes sensitivity towards Na^+ and its analogue Li^+ (Varela, 1995b) In the *bmh1Δ* mutant the induction of *ENA1* gene expression upon transfer to high salinity is abolished, indicating an involvement of the Bmh1p in the regulation of the *ENA1* gene. In addition to the negative regulation of the *ENA1* expression by the PKA pathway, the two serine/threonine phosphatases Ppz1p and Ppz2p functions as negative modulators of *ENA1* expression (Posas *et al.*, 1995). Deletion of the genes encoding these proteins renders the cells more NaCl tolerant. The mechanism behind this control is not yet evaluated, however, this effect is not seen in *mpk1Δ* background, implying a link to the PKC1 pathway. Other genes involved in salt tolerance of *S. cerevisiae* are the *HAL1-HAL3* genes which, when over-expressed, improve the salt tolerance of the cells (Gaxiola *et al.*, 1992, Gläser *et al.*, 1993, Kron *et al.*, 1995, Murguia *et al.*, 1995). The *HAL1* gene is upregulated by high NaCl concentrations and overexpression of this gene increase intracellular K^+ accumulation which might be responsible for the increased salt tolerance (Gaxiola, *et al.*, 1992). The other two genes, *HAL2* and *HAL3*, encoding Met22p and Sis2p, are not induced by salt stress (Murguia, *et al.*, 1995). The Met22p is involved in methionine biosynthesis and encodes an enzyme that is sensitive to Na^+ inhibition. It is assumed that enhanced expression of *HAL2* improve growth under salt stress by overcomming methionine limitation (Gläser *et al.* 1993). The Sis2p has been implied in cell cycle control and regulates *ENA1* expression in addition to calcineurin (Ferrando *et al.*, 1995).

8.1. The *SOT1* gene

The *SOT1* gene, *so*dium *t*olerance gene, was cloned by complementing the *osg4* mutant (paper IV). The *osg4* mutant displayed overproduction of glycerol and sensitivity only to NaCl, thus it

was not a true osmosensitive mutant. Genetic analysis revealed that this mutant contained two mutations, one responsible for the salt sensitivity and the second for the enhanced glycerol production. Complementation of the salt sensitive phenotype resulted in the cloning of the *SOT1* gene. Deletion of the *SOT1* gene rendered the cells sodium sensitive albeit not to the sodium analogue Li^+. A characteristic feature of the *sot1Δ* mutant is increased accumulation of Na^+ and a markedly increased Na^+/K^+ ratio at high NaCl concentration. Addition of KCl to the high salinity medium restored growth of the *sot1Δ* mutant. The Na^+/K^+ ratio is important for the salt tolerance of the cell, Na^+ becoming strongly toxic as this ratio is increased (Camacho *et al.*, 1981). Northern analysis of the *SOT1* transcript did not reveal a salt instigated response, the expression remained constitutively low. Multicopy expression of *SOT1* under the control of its own promoter did not increase NaCl tolerance of cells having various types of mutations affecting salt tolerance. Analysis of the hypothetical protein encoded by the *SOT1* gene revealed homology to a group of tumour suppressor proteins found in higher eukaryotes. These tumour suppressor proteins are characterised by at least two WD repeats which are present at equivalent positions in the Sot1p. The WD repeats are often found in regulatory proteins and are thought to assist in protein-protein interactions and multimerisation (Neer *et al.*, 1994). The tumour suppressor proteins found in *Drosophila* (encoded by the *D-lgl* gene) and humans (encoded by the *hugl* gene) interact with myosin II, the non-muscle heavy-chain myosin that is linked to the cytoskeleton and promote the contact between the actin network and the plasma membrane (Strand *et al.*, 1994, Strand *et al.*, 1995). Furthermore, these proteins are detected in large complexes that include actin and a serine kinase. It is therefore of considerable interest that the *SOT1* gene according to recent information in the *Saccharomyces* Genome Database is identical to a gene, *SRO7*, cloned as a muticopy suppressor of a *rho3* defect. *RHO3* is a member of the *ras* superfamily genes and is required for the maintenance of cell polarity in cells having initiated bud formation (Matsui and Toh-E, 1992). Since the cytoskeletal structure is essential to align cell structures parallel to the mother-bud axis and target secretory vesicles to the growing bud (Chant, 1994), Sot1p/Sro7p might be associated with this function. A location of Sotp/Sro7p close to or inside the plasma membrane can be reconciled with its interference with ion homeostasis through interaction with transporters.

9. Final remarks and perspectives

In this thesis, various aspects of the process of adaptation to osmotic stress and salt stress have been studied from mainly a genetic point of view. Analysis of osmosensitive mutants led to the investigation of different cellular processes involved in the adaptive process, from signaling to the production of the molecules essential for the final response. Glycerol is the key compatible solute accumulated in *S. cerevisiae* during osmotic stress and the osmoregulatory glycerol response has therefore been the primary process studied when characterising the osmosensitive mutants. The identification of two glycerol-specific phoshatases, Gpp1p and Gpp2p (paper I), revealed that the second step in the biosynthetic pathway to glycerol is also encoded by genes subject to HOG pathway controlled expression. Studies of protein expression during the adaptation in a *pbs2Δ* mutant defect in the signaling pathway (paper II), revealed that the HOG pathway is mainly important for the immediate response of a large number of osmotically induced proteins. We also demonstrated that a number of unidentified proteins are completely dependent on the HOG pathway for induction by salt stress, indicating that this pathway controls cell functions not yet discovered. More sensitive methods to identify proteins on 2D gels that are now available, combined with the accessibility of the complete sequence of the *S. cerevisiae* genome opens up for interesting possibilities of identifying and studying further novel proteins(genes under HOG pathway control. The protein encoded by the *SGD1* gene (paper III) is another intriguing gene product identified by complementation of an osmosensitive mutant. An unusual feature of this gene is an apparent autoregulation. The function of Sgd1p is not fully identified although obtained data points towards a HOG controlled repressing role in the regulation of glycerol production. Since no component downstream the HOG1 encoded MAP kinase has yet been identified, despite intense efforts of several laboratories, the *SGD1* gene is a highly interesting candidate for further studies. Finally the identification of *SOT1* involved in sodium tolerance (paper IV) unexpectedly brought our interest into the interesting field of cancer research. The similarity of Sot1p to tumour suppressor proteins found in higher eukaryotes opens up for using the genetic tractability of *S. cerevisiae* for studying in molecular detail the function of these proteins.

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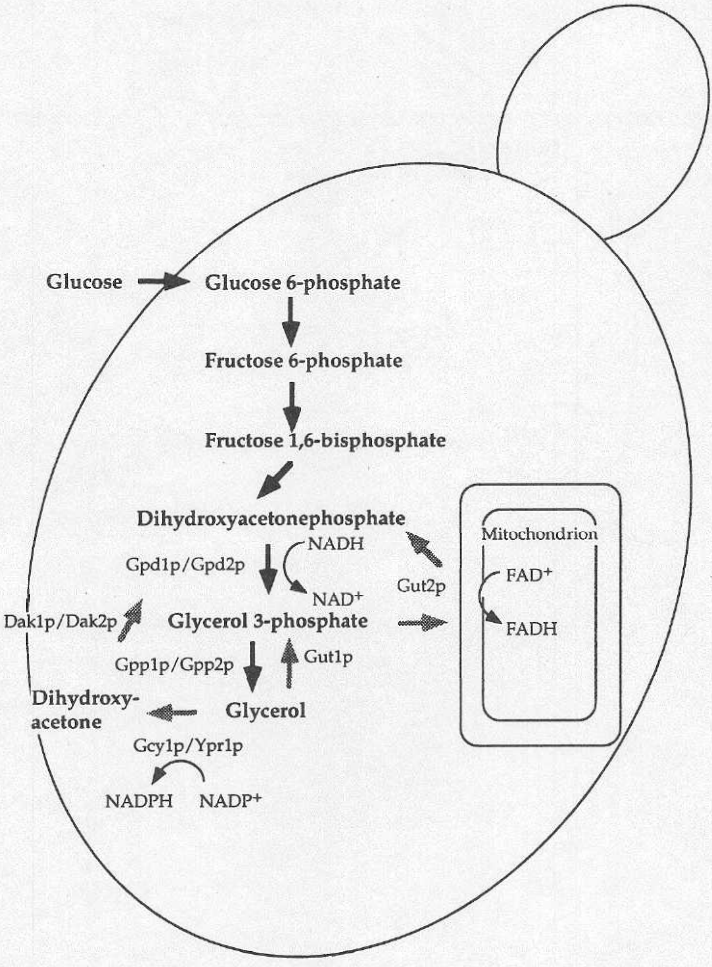

Figure 1. Glycerol metabolism in *S. cerevisiae*. Abbreviations: Gpd1p/Gpd2p-NAD^+-dependent glycerol 3-phosphate dehydrogenase; Gpp1p2/Gpp2p- glycerol 3-phosphatase; Gut1p-glycerol kinase; Gut2p-FAD^+-dependent glycerol 3-phosphate dehydrogenase; Gcy1p/Ypr1p-glycerol dehydrogenase; Dak1p/Dak2p-Dihydroxyacetone kinase.The grey arrows represent the glycerol dissimilatory pathways.

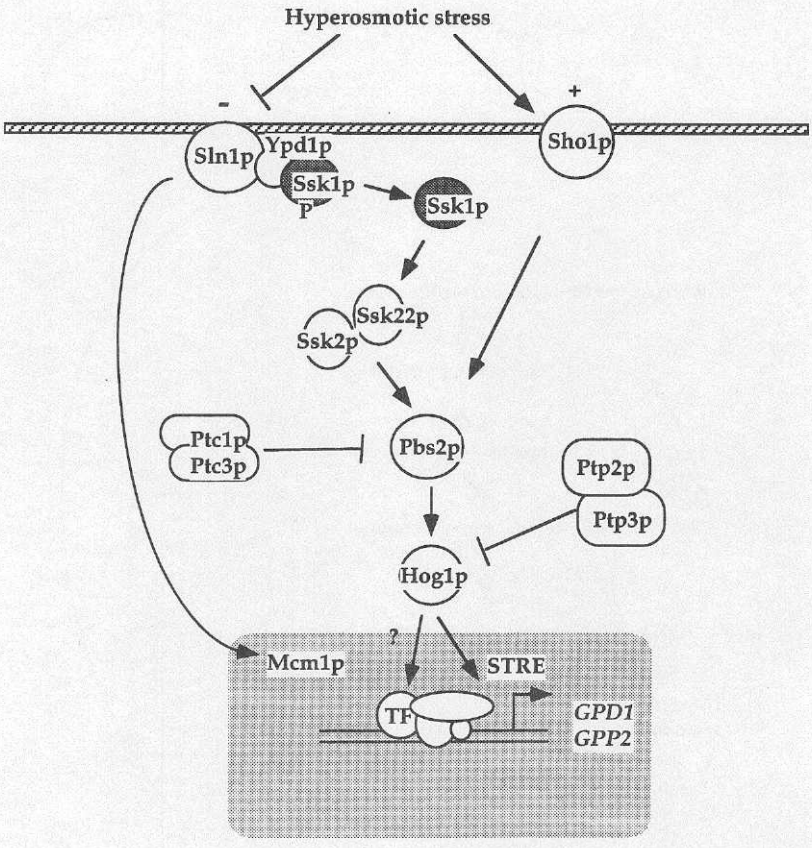

Figure 2. The components of the high osmolarity glycerol pathway. The Sln1p negatively modulates the pathway while the Sho1p has an activating role. See text for further details. Arrow-heads denote activation and T-heads show inhibition.

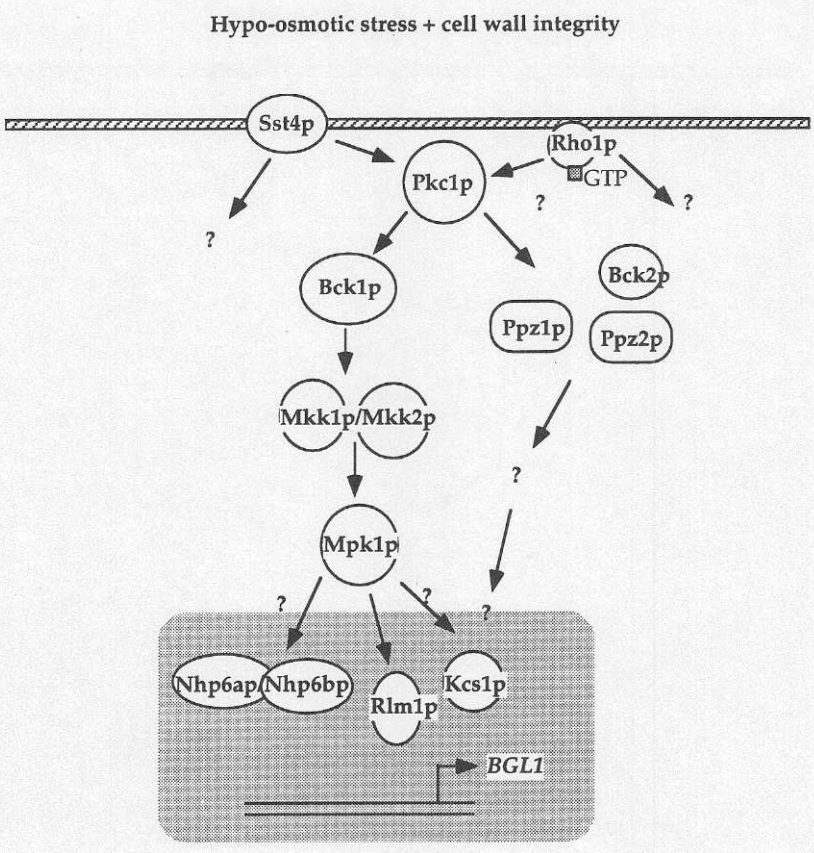

Figure 3. The second osmosensing MAP kinase pathway, regulated by the protein kinase C, is induced by hyperosmotic stress and is important for the cell wall integrity. For details of abbreviations, see text.

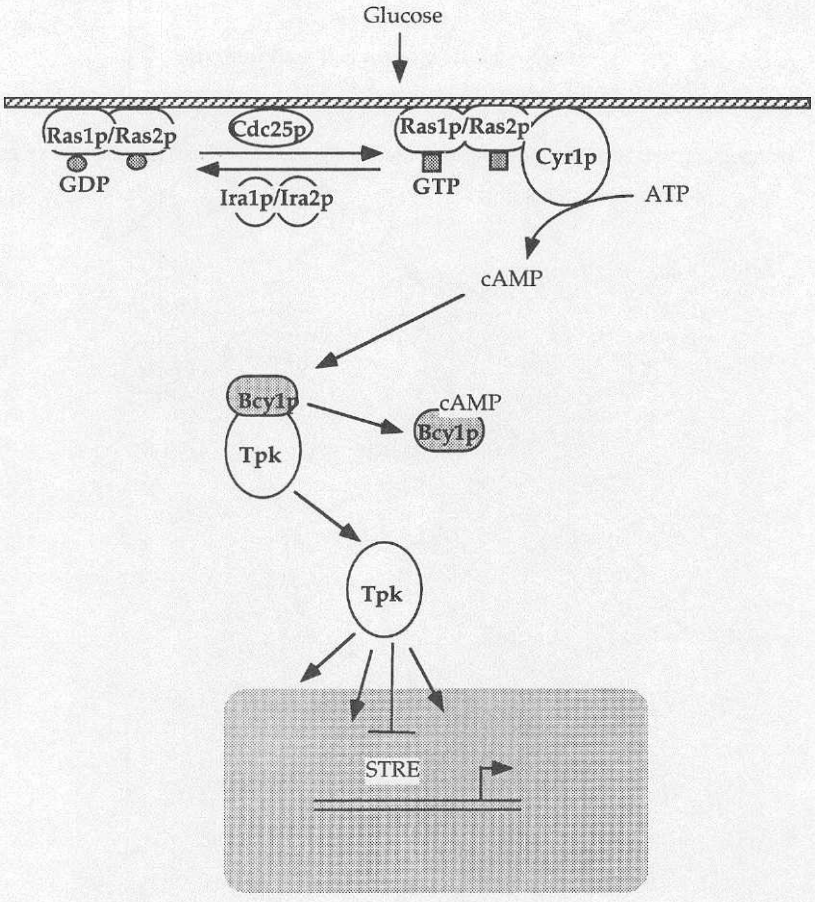

Figure 4. A overview of the RAS-cAMP pathway that is involved in nutrientsensing and negatively regulates the general stress response via the STRE-elements. For the details of the abbreviations, see text.

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