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 OSMOSENSING MITOGEN-ACTIVATED PROTEIN KINASE SIGNAL TRANSDUCTION PATHWAY*

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The existence of specific DL-glycerol-3-phosphatase (EC 3.1.3.21) activity in extracts of Saccharomyces cerevisiae was confirmed by examining strains lacking non-specific acid and alkaline phosphatase activities. During purification of the glycerol-3-phosphatase, two isoforms having very similar molecular weights were isolated by gel filtration and anion exchange chromatography. By microsequencing of trypsin-generated peptides the corresponding genes were identified as previously sequenced open reading frames of unknown function. The two genes, GPP1 (YIL053W) and GPP2 (YER062C) encode proteins that show 95% amino acid identity and have molecular masses of 30.4 and 27.8 kDa, respectively. The intracellular concentration of Gpp2p increases in cells subjected to osmotic stress, while the production of Gpp1p is unaffected by changes of external osmolarity. Both isoforms have a high specificity for DL-glycerol-3-phosphate, pH optima at 6.5, and KM in the range of 3-4 mM. The osmotic induction of Gpp2p is blocked in cells that are defective in the HOG-mitogen-activated protein kinase pathway, indicating that GPP2 is a target gene for this osmosensing signal transduction pathway. Together with DOG1 and DOG2, encoding two highly homologous enzymes that dephosphorylate 2-deoxyglucose-6-phosphate, GPP1 and GPP2 constitute a new family of genes for low molecular weight phosphatases.

Maintenance of water homeostasis is fundamental to most cells. Exposure to hyperosmotic conditions initiates responses serving to maintain an osmotic gradient across the cell membrane, appropriate for turgor and cell volume control. A common mechanism behind this adaptation involves intracellular accumulation of one or more nonpolar osmolytes. Among eukaryotic cells this response is best understood for the yeast Saccharomyces cerevisiae, which on exposure to dehydration stress initiates increased production and intracellular accumulation of glycerol (1, 2). Glycerol is produced through reduction of dihydroxyacetone phosphate to glycerol-3-phosphate and subsequent dephosphorylation to glycerol. The responsible enzymes are a NAD⁺-dependent glycerol-3-phosphate dehydrogenase (GPD) and a poorly characterized glycerol-3-phosphatase. Transfer of cells to hyperosmolar conditions leads to increased production of GPD (3) due to transcriptional activation of one of the isogenes (GPD1) for the enzyme (4-6). In addition, membrane permeability to glycerol is diminished (7), presumably mainly due to inhibition of the facilitator that transports glycerol across the membrane (8). Under strongly hyperosmotic conditions, S. cerevisiae can accumulate glycerol up to molar concentrations (9). The osmotic induction of GPD1 appears to be controlled via an osmosensing pathway (6), involving cell surface osmosensors encoded by SHO1 (10) and a two-component system encoded by SLN1/SSK1 (11, 12). These detection systems are linked to a cascade of protein kinases encoded by SSK2, SSK22, PBS2, and HOG1, forming a mitogen-activated protein (MAP) kinase signaling pathway (10, 12, 13).

Osmotic control of glycerol production might also be exerted at the level of dephosphorylation of glycerol-3-phosphate. The early work by Tsuboi et al. (14, 15) indicated the existence of a phosphatase in S. cerevisiae having a specific role in glycerol production. These researchers described a partially purified phosphomonoesterase from bakers’ yeast that displayed a high specificity toward glycerol-3-phosphate at pH 6.5. Using conditions that repress the ortho-phosphate repressible phosphatases and strains lacking various nonspecific phosphatases, we here confirm the existence of a phosphatase activity, specific for DL-glycerol-3-phosphate in extracts of S. cerevisiae. To functionally characterize this enzyme and gain further insight into its role in cellular osmoregulation we took on purification of the glycerol-3-phosphatase and unexpectedly detected two molecular forms of the enzyme, one of which shows constitutive expression, and the other being induced by increased extracellular osmolarity. By partial amino acid sequencing of tryptic digests of the two molecular forms, the corresponding genes, previously reported as open reading frames without function, were identified. We propose different roles for the two glycerol-3-phosphatase isoenzymes that belong to a previously unrecognized family of low molecular weight phosphomonoesterases.

EXPERIMENTAL PROCEDURES

Strains, Media, and Growth Conditions—The genotypes of the S. cerevisiae strains used are shown in Table I. Strains were routinely grown in a medium composed of 1% yeast extract, 2% Bacto peptone, and 2% glucose (YPD) or in a synthetic yeast nitrogen base (YNB)

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Two dl-Glycerol-3-phosphatases from S. cerevisiae

**Table I** Yeast strains used

| Name  | Genotype                        | Source or reference |
|-------|---------------------------------|---------------------|
| YS18  | MATα his3 leu2 ura3-35 can1       | A. Hinnen           |
| YMR4  | MATα his3 leu2 ura3-35 can1       | A. Hinnen           |
| SK2m  | MATαADE2+ Ade2/ ade2-1 his5       |                     |
| YPH102a| ura3 leu2 his3A ade2 lys2         | (36)                |
| J BY10| ura3 leu2 his3A ade2 lys2 trp1     | (13)                |
| J BY13| ura3 leu2 his3A ade2 lys2 trp1    | (13)                |
| SH613 | SUC2 mal4 gal2 CUP1              | S. Harashima        |
| SH3544| YPH102a MATα pho8::URA3 ura3 s3 apo1::ura311 |                     |
| SH3598| YPH102a MATα pho8::URA3 ura3 s3 apo1::ura311 |                     |

**RESULTS**

Evidence for Specific Glycerol-3-phosphatase Activity in Crude Extract—To facilitate the search for a specific glycerol-3-phosphatase in crude extracts of *S. cerevisiae*, efforts were made to minimize the presence of various nonspecific alkaline and acid phosphatases. The acid phosphatases of *S. cerevisiae* encoded by the PHOS, PHO10, and PHO11 genes as well as the alkaline phosphatase encoded by the PHO8 gene are all repressed at the transcriptional level by inorganic phosphate (22, 23). To curb formation of these enzymes, cell cultures were grown with 10 mM inorganic phosphate. Enzyme activity measurements in extracts from such cultures indicated negligible contribution to the observed glycerol-3-phosphatase activity from residual activity of the repressible acid (Pho5p) and alkaline (Pho8p) phosphatases, since mutants lacking the corresponding genes (strain YMR4 and SH3544, respectively) demonstrated activities similar to those of the wild type strains (Table II). Neither was the analyzed glycerol-3-phosphatase activity notably influenced by the absence of the nonrepressible
was observed for cells grown at 0.7 M NaCl (data not shown), and phosphate monoesters used for the substrate specificity test.

Determination of relative molecular masses by gel filtration and by gradient gel electrophoresis. To characterize the glycerol-3-phosphatase, the molecular mass of the enzyme was estimated from the mobility of the enzyme standard on gel filtration (Table II).

The results show that the total cellular activity of glycerol-3-phosphatase increased with increased extracellular salinity (Fig. 1). Culturing cells with various concentrations of NaCl resulted in a corresponding increase of the enzyme activity showing specificity for glycerol 3-phosphate.

Effect of Extracellular Osmolarity on Glycerol-3-phosphatase Activity—Cells were cultured with various NaCl concentrations. The observed increase in activity was specific for glycerol 3-phosphate; no increased hydrolysis of the other organic phosphate monoesters used for the substrate specificity test was observed for cells grown at 0.7 M NaCl (data not shown), indicating that the demonstrated regulation is specifically exerted on the enzyme activity showing specificity for glycerol 3-phosphate.

To examine whether protein synthesis is required to achieve this increase in activity, cells were shifted from basal medium to medium containing 0.7 M NaCl in the presence or absence of cycloheximide. In the absence of inhibitor, enzyme activity increased to levels 1.5 and 1.7 times the original value after incubation for 60 and 110 min, respectively. This increase was completely prevented by the presence of 100 μg/ml of cycloheximide (data not shown), indicating that the increase in glycerol-3-phosphatase activity requires protein synthesis.

Osmotic stress-induced glycerol production in S. cerevisiae requires the HOG1 and PBS2 genes, encoding MAP kinase homologues of the osmosensing and signaling HOG pathway.

### Table II

| Strain | Relevant genotype | Glycerol-3-phosphatase activity (nmol min⁻¹ mg protein⁻¹) |
|--------|-------------------|--------------------------------------------------------|
| YS 18  | Parent strain     | 68.0 ± 3.8                                             |
| YMR4   | pho5,3-ura31      | 70.2 ± 3.9                                             |
| SH 611 | Parent strain     | 73.1 ± 0.5                                             |
| SH 3544| pho8::URA3        | 67.6 ± 5.0                                             |
| SH 3598| pho3-1 pho13::URA3| 62.5 ± 0.3                                             |

### Table III

| Substrate                  | Crude extract | Gpp1p | Gpp2p |
|----------------------------|---------------|-------|-------|
| dl-glycerol 3-phosphate    | 100           | 100   | 100   |
| Glycerol 2-phosphate       | 8.6           | 2.7   | 0.8   |
| Glycerate 3-phosphate      | 3.0           | 0.01  | 0.2   |
| Glycerate 2,3-diphosphate  | 2.8           | 0.4   | 1.0   |
| Glucose 6-phosphate        | 3.7           | 0.5   | 0.5   |
| Fructose 6-phosphate       | 3.7           | 0.1   | 1.0   |
| 2-Deoxyglucose 6-phosphate | ND*           | 0.2   | 0.0   |

*ND*, not determined.
Mono-Q ion chromatography (1.4 M NaCl (form II) was eluted at slightly lower salt concentration. The extracts of cells grown at high salinity, an additional form peak (form I) with extracts of nonstressed cells, whereas with NaCl gradient. The phosphatase activity emerged as a single
tase in extracts of
(13). Examination of the specific activity of glycerol 3-phosphatase in extracts of pbs2a and hog1a mutants cultured at elevated salinities demonstrated that either defect in the signaling pathway caused a complete inhibition of the osmotic induction of the glycerol 3-phosphatase activity (Fig. 3).

Purification of Glycerol 3-phosphatase—To purify the glycerol-3-phosphatase we employed gel filtration and anion exchange chromatography. As a first step, extracts of cells grown in YNB medium and the same medium containing 1.4 M NaCl were subjected to Superdex 200 gel filtration (Fig. 4, A and B). The presence of a shoulder in the activity profile of eluate from extracts of cells grown at high salinity suggested two forms of the enzyme in salt grown cells. This assumption was reinforced by the subsequent Mono-Q ion chromatography step (Fig. 4, C and D) in which the proteins were eluted by a linear 0–0.15 M NaCl gradient. The phosphatase activity emerged as a single peak (form I) with extracts of nonstressed cells, whereas with extracts of cells grown at high salinity, an additional form (form II) was eluted at slightly lower salt concentration. The active Mono-Q fractions from cells cultured in 1.4 M NaCl media were further resolved by rechromatography on the same column, this time eluted with a combined pH and KCl gradient (see “Experimental Procedures” and Fig. 5A). By this step form I, hereafter called Gpp1p, was purified to electrophoretic homogeneity as revealed by silver-stained SDS gel electrophoresis (Fig. 5B), while the recovery of activity of form II by this procedure was insignificant. Enzyme activity data demonstrated that Gpp1p was not appreciably affected by changed salinity, while the second molecular form, hereafter called Gpp2p, increased strongly with salinity from an activity below detection level in nonstressed cells (Fig. 4, C and D). A quantitative summary of the purification of the two isoforms as performed with extracts of cells grown at 1.4 M NaCl, is shown in Table IV.

N-terminal Sequence Analysis of Gpp1p and Gpp2p—Gpp1p was excised from SDS gels and deaved by in-gel trypsinization. The generated peptides were separated on reverse phase HPLC, and selected fractions were subjected to N-terminal sequencing. The eight peptides examined revealed the sequences shown in Table V. When these peptides were compared with sequence data of the Swiss Protein Data Bank, full identity was established with an open reading frame recognized by the systematic sequencing of chromosome IX. The corresponding gene (YIL053W) which was designated GPP1, encodes a
protein of 271 amino acids having a molecular mass of 30.4 kDa.

SDS-polyacrylamide gel electrophoresis and trypsinization of Gpp2p, using the most active fractions from the Mono-Q chromatography (Fig. 4D), yielded seven sequences that showed striking similarity to Gpp1p, although some of the sequences displayed a slight but distinct sequence deviation from the corresponding regions of Gpp1p (Table V, unique amino acids underlined). Data base search revealed full identity with the primary structure of a gene (YER062C) identified by sequencing of chromosome V. This gene encodes a protein of 250 amino acids having a molecular mass of 27.8 kDa, which is slightly lower than for Gpp1p. This gene was designated GPP2.

Comparison of GPP1 and GPP2—Sequence comparisons between the GPP1 and GPP2 using the Gap and Best Fit program from the Genetics Computer Group demonstrated strong homology, the inferred amino acid sequences showing 95% identity (Fig. 6). The only identified proteins to which Gpp1p and Gpp2p show significant identity (30–35%) are two phosphomonoesterases encoded by DOG1 and DOG2 (24, 25).

Two-dimensional PAGE Analysis of Gpp1p and Gpp2p—From the expression pattern and the theoretical Mr, the positions of Gpp1p and Gpp2p were tentatively localized on analytical two-dimensional PAGE gels. To confirm the identity, the located protein spots were excised from preparative gels, and internal peptides were generated and sequenced. The obtained amino acid sequences (Table V) unequivocally identified the candidate spots as the GPP1 and GPP2 encoded proteins. The
The HOG Pathway Controls Osmotic Induction of GPP2—To further examine the effect of the HOG pathway on osmotic induction of the D,L-glycerol-3-phosphatase activity, enzymes were purified from extracts of a hog1Δ and a parent strain that had been both incubated for 1 h at 0.7 M NaCl. The parent strain displayed clear Gpp2p and Gpp1p peaks after Mono-Q chromatography, while the hog1Δ mutant showed roughly the same amount of Gpp1p as the parent but insignificant Gpp2p enzyme (Fig. 9).

Table IV shows the amino acid sequences of peptides generated from the two isoforms of glycerol-3-phosphatase, resolved by protein chromatography or two-dimensional PAGE. Numbers refer to the amino acid positions in the predicted protein sequences of the YIL053W (GPP1) and YER062C (GPP2) genes. Underlined residues mark positions of unique amino acids.

| Purification of Gpp1p and Gpp2p from extracts of S. cerevisiae SKQ grown at 1.4 M NaCl |   |   |   |   |   |
|---|---|---|---|---|
| Fraction | Volume | Protein | Specific activity | Total Activity | Purification |
| Cell-free extract | 2 | 27520 | 108 | 2973 | 1 |
| Superdex 200 | 11 | 181.50 | 7063 | 1282 | 65 |
| Mono-Q | 1 | 257.2 | 24029 | 618 | 222 |
| Mono-Q II | 1 | 8.55 | 9400 | 80 | 87 |
| GPP1 | 1 | 4.97 | 12602 | 60 | 112 |
| GPP2 | 1 | 4.97 | 12602 | 60 | 112 |
| Hog2p | 1 | 4.97 | 12602 | 60 | 112 |
| Hog1Δ | 1 | 4.97 | 12602 | 60 | 112 |

*Activity not detectable.*

Table V shows the amino acid sequences of peptides generated from the two isoforms of glycerol-3-phosphatase, resolved by protein chromatography or two-dimensional PAGE. Numbers refer to the amino acid positions in the predicted protein sequences of the YIL053W (GPP1) and YER062C (GPP2) genes. Underlined residues mark positions of unique amino acids.

| Constitutive form (GPP1) |   |   |   |   |   |
|---|---|---|---|---|
| Purified by protein chromatography |   |   |   |   |   |
| INAALFDV(V)GX | 32-44 | DXPXDH | 40-45 |
| FPDADEEYYVNM | 86-97 | TFDIAIK | 58-64 |
| WAVATXG | 132-138 | FAPDXNH | 65-71 |
| WFDILK | 146-151 | (WIAVAT)XG | 111-117 |
| XRPEFYITA | 152-161 | YFITAND | 136-142* |
| OGKPHPEPYLV | 166-176 | OGKPHPEPYLV | 145-155 |
| VGIATTFDDA | 215-225 | VG | 223-226 |
| VGEYNAEDX | 244-252 |   |   |
| Extracted from preparative two-dimensional PAGE |   |   |   |   |   |
| FPDFADDEYYV | 86-96 | VNAALFDVGD | 12-23 |
| YGHEXIIEVP | 107-115 | FAPDFAH | 65-71 |
| VVFEDAPA | 195-208 | WFEH | 125-128 |
| XFIATND | 146-142* | VVVFEDAPA | 174-182 |
| VG | 223-223 |

*Sequence is preceded by E in YIL053W and would therefore not provide a site for trypsin.*

Discussion

This work reports the presence of two isozymes of D,L-glycerol-3-phosphatase (EC 3.1.3.21) in S. cerevisiae. Peptide sequences obtained from purified isozymes demonstrated that the enzymes responsible for this activity are encoded by two distinct but highly identical genes, here named GPP1 and GPP2. These genes were previously sequenced as part of the systematic sequencing of the yeast genome but were reported as open reading frames without known function. The predicted amino acid sequences of GPP1 and GPP2 are 95% identical and show about 35% identity with that of DOG1, which was isolated by its ability to confer resistance to 2-deoxyglucose (24). This nonmetabolizable glucose analogue elicits glucose repression, and this effect is counteracted by overexpression of the DOG1 gene product which has a 2-deoxyglucose-6-phosphatase activity. Interestingly, Dog1p has a "twin" homologue encoded by the recently identified DOG2 gene (25), showing 92% amino acid identity to that of DOG1. The high similarity within each of the two gene pairs points to an origin by gene duplication and dispersal. The true physiological function of Dog1p and Dog2p is not yet established (24, 25). However, they clearly appear to have a role different from Gpp1p and Gpp2p, since these enzymes do not dephosphorylate 2-deoxyglucose 6-phosphate (Table IV) and Dog1p and Dog2p show insignificant activity on glycerol 3-phosphate (25, 26). Together these four...
enzymes constitute a previously unrecognized family of low molecular weight phosphomonoesterases, having molecular masses of approximately 30 kDa. Gel filtration and gradient gel electrophoresis under nondenaturing conditions indicate that Gpp1 and Gpp2 are catalytically active as monomers. Hence, the glycerol-3-phosphatase isozymes are clearly different from the nonspecific alkaline and acid phosphatases, which have alkaline preferences are primarily confined to the vacuole or peroxisomes (10, 12). According to this model, changes in extracellular water potential. Exposed to hyperosmotic stress, the cells increase glycerol production and glycerol accumulation. A contribution from Gpp2p to this response is indicated by the finding that this isoform, which is the only one overlapping with the osmosensors, has its main location in the cytosol, where a strict specificity would be essential to avoid interference with the plethora of phosphorylated metabolites in the metabolic network. In contrast, the nonspecific phosphatases of acid and alkaline preferences are primarily directed to the vacuole or peroxisomes of the cell (32).

Glycerol has dual functions in S. cerevisiae; it serves as a nontoxic redox sink during fermentation (35) and as an osmoregulator during hypersaline stress (2). Osmoregulation in S. cerevisiae is achieved by varying the internal glycerol concentration to adjust the intracellular osmolality in response to changes of the extracellular water potential. Exposed to hyperosmotic stress, the cells increase glycerol production and glycerol accumulation. A contribution from Gpp2p to this response is indicated by the finding that this isoform, which is the only one overlapping with the osmosensors, has its main location in the cytosol, where a strict specificity would be essential to avoid interference with the plethora of phosphorylated metabolites in the metabolic network. In contrast, the nonspecific phosphatases of acid and alkaline preferences are primarily directed to the vacuole or peroxisomes of the cell (32).

Recent evidence indicates that the signaling mechanisms behind the glycerol response are dependent on a MAP kinase module involving the PBS2 and HOG1 gene products (13). These kinases appear to be activated via membrane-localized osmosensors (10, 12). According to this model, changes in extracellular osmolality generate an internal signal that is transmitted to the MAP kinase cascade. One of the targets of this signaling is GPD1, encoding glycerol-3-phosphate dehydrogenase, the enzyme constituting the first step in the glycerol biosynthetic pathway (5, 6). The rapid induction of GPD1 expression after a step increase in salinity is blocked in pbs2Δ (3) and hog1Δ (6) signaling-defective mutants. It would seem logical that the osmoregulated Gpp2p enzyme would be controlled...
coordinately with Gpd1p via the HOG-MAP kinase pathway. Indeed, such a coordinated control appears to be exerted, as evidenced by the finding that the Gpp2p activity does increase insignificantly or only slightly in an osmosignaling-defective hog1Δ mutant that is cultured at elevated salinities (Fig. 9). In analogy with the osmostimulated expression of the GPD1 one would expect that the osmotic control is due to transcriptional induction of the GPP2 gene. Hirayama et al. (37) very recently reported the cloning of seven hyperosmolarity-responsive (HOR) genes from S. cerevisiae by a differential screening method. Two of these genes, HOR2 and HOR7, were of unknown function. When inspecting the sequences of these genes, we observed that the HOR2 gene is identical to GPP2. Interestingly, Northern blot analysis showed that the induction of the HOR2/GPP2 is induced by increased osmolarity and that this induction is inhibited in a hog1Δ strain. Hirayama et al. (37) also noted that HOR2 has a close homologue called RHR2, which is not subject to osmotic regulation. Since RHR2 is induced by increased osmolarity and that this induction is inhibited in a hog1Δ strain, we are also grateful to Dr. S. Hohmann for constructive criticism of the manuscript.

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