The osmo-inducible *gpd1*$^+$ gene is a target of the signaling pathway involving Wis1 MAP-kinase kinase in fission yeast

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Abstract **The *gpd1*$^+$ gene of *Schizosaccharomyces pombe* encodes an isozyme of NADH-dependent glycerol-3-phosphate dehydrogenases that is involved in glycerol synthesis, whose expression is induced upon an upshift of the medium osmolarity. We provide evidence that this osmotic induction of *gpd1*$^+$ in *S. pombe* is under the control of a MAP-signaling pathway involving the *wis1*$^+$ gene-product, which is a homologue of MAP-kinase kinases. The results suggested that the *gpd1*$^+$ gene is a downstream target of the osmosensing signaling that is transmitted through Wis1, thereby defects of either of these genes result in the similar phenotype, namely, osmosensitive for growth, because of the failure in accumulation of the intracellular osmoprotectant, glycerol.**

Key words: Osmoregulation in fission yeast; Glycerol production; MAP-signaling pathway; Wis1 kinase

1. Introduction

Exposure of cells to high environmental osmolarity leads to dehydration, and decrease in cell viability. Accordingly, the ability of cells to adapt to external osmotic stress is a fundamental biological process that protects the organisms against fluctuation in the water activity and solute content of their environment. In fact, many types of both prokaryotic and eukaryotic cells have developed mechanisms to adapt to severe osmotic changes of their environment (often called osmoregulation) [1,2]. Recently, such an osmoregulation has attracted much attention with special emphasis on the molecular mechanism underlying signal transdution in response to osmotic stimuli.

In the case of eukaryotic cells, recent studies on a budding yeast *Saccharomyces cerevisiae* have begun to provide us with insight at the molecular level into signal transduction and gene regulation in response to external osmotic stimuli. For example, a MAP (mitogen-activated protein) kinase cascade was suggested to be involved in the osmosensing signal transduction [3–5]. Interestingly, a reminiscent of prokaryotic two-component signal transduction system was also suggested to be linked closely to the signaling cascade [6–8]. In this context, little information is available with regard to such an osmoregulation in the evolutionary divergent yeast *Schizosaccharomyces pombe*, which is another model yeast in terms of the current molecular microbiology. In this respect, we recently identified two *S. pombe* genes (*gpd1*$^+$ and *gpd2*$^+$), each encoding an isozyme of glycerol-3-phosphate dehydrogenases (GPD1 and GPD2, respectively) [9]. One of them (*gpd1*$^+$) was demonstrated to be crucially responsible for the osmoprotective accumulation of intracellular glycerol in *S. pombe*, thereby the defect of this particular gene results in osmosensitive for growth under osmotically stressful conditions [9]. It was further demonstrated that the expression of *gpd1*$^+$ is induced at the level of transcript in response to an osmotic upshift [9].

The *wis1*$^+$ gene of *S. pombe* encodes a homologue of MAP-kinase kinases [10]. This gene was originally identified as a dosage-dependent regulator of mitosis [10], and recently implicated as a genetic suppressor for the lethality due to *Δptc1*/*Δptc3*, each of which encodes a protein phosphatase 2C (PP2C) in *S. pombe* [11]. A *Δwis1* mutant exhibits pleiotropic phenotypes, namely, an elongated morphology, a rapid reduction of viability upon entry into stationary phase, thermosensitivity and osmosensitivity for growth [10,11]. It was recently proposed that *wis1*$^+$ may be involved in the osmoregulation in *S. pombe* [11,12]. A crucial question then arose as to whether or not the osmotically responsive gene, *gpd1*$^+$, is under the control of Wis1 MAP-kinase kinase, thereupon we address this particular issue in this study.

2. Materials and methods

2.1. Strains and media

S. pombe strains, PR109 (h$^-$ *leu1-32 ura4-D18*) and JM544 (h$^-$ *leu1-32 ura4-D18 wis1::ura4*$^+$) were used [11,12]. These strains were grown in YPD medium composed of 1% yeast extract, 2% polypeptone and 2% glucose, or in SD medium composed of 0.67% yeast nitrogen base without amino acids (Difco), supplemented with 2% glucose and other necessary growth requirements in standard amounts [13].

2.2. Glycerol assay

Glycerol was analysed enzymatically with a commercial glycerol assay kit (F-kit from Boehringer-Mannheim), as described previously [14]. Expornentially growing cells in YPD medium were collected and re-suspended in fresh YPD medium, or the same medium containing 1.2 M NaCl and then incubated for one hour at 30°C. Amounts of glycerol was determined by the kit and calculated as an absolute amount of glycerol (μmol) per a certain number of cells giving one A_{600} unit of optical density (measured by a spectrophotometer, Shimadzu UV-160).

2.3. Northern hybridization analysis

For Northern hybridization analysis, expornentially growing cells in YPD medium were collected and re-suspended in fresh YPD medium, or the same medium containing 1.2 M NaCl, and then incubated for one hour at 30°C. A total RNA fraction was prepared from the cells, according to the method of Elder et al. [15]. After denaturation with formamide-formaldehyde, RNA (10 μg) was analyzed on a 1.4% agarose gel containing formaldehyde, followed by alkali-blotting onto Hybond-N$^+$ (Amersham Int.). Hybridization was carried out with ^{32}P-labelled probes, each specifically encompassing the *gpd1*$^+$ or *gpd2*$^+$ coding sequence [9], at 65°C for two hours in Rapid-hyb buffer, as recommended by the supplier (Amersham Int.).

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Abbreviations MAP, mitogen activated protein; GPD, NADH-dependent glycerol-3-phosphate dehydrogenase.

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3. Results

A *Δwis1* strain exhibits the phenotype of osmosensitive for growth [11,12]. First of all, thus, we asked if this osmosensitive phenotype of *Δwis1* can be suppressed by introduction of *gpd1*$^+$ on a multicopy plasmid. As shown in Fig. 1, a wild-type strain (PR109) with respect to *wis1*$^+$ was able to grow on SD plates containing 1.5 M sorbitol, while its *Δwis1* derivative (JM544) was not, exhibiting the osmosensitivity for growth on the high osmolarity plate. However, this defect for growth was suppressed, provided that JM544 was transformed either with pHAI184 or pHAI186, which respectively carries *gpd1*$^+$ or *gpd2*$^+$ [9]. Essentially the same results were obtained, even when SD plates containing 0.5 M NaCl was used as a high osmolarity medium (data not shown). These results were best interpreted as meaning that a presumed overproduction of glycerol-3-phosphate dehydrogenase (GPD), due to the introduction of the muticopy genes of *gpd1*$^+$ or *gpd2*$^+$, resulted in an accumulation of the osmoprotective solute (intracellular glycerol), thereby the *Δwis1* cells were protected against the high osmolarity stress. This interpretation is based on the idea that the *Δwis1* mutant may be defective in the osmoresponsive intracellular accumulation of glycerol, thereby resulting in the phenotype of osmosensitive for growth.

We then examined this idea by directly asking whether or not the *Δwis1* mutant is defective in the production of glycerol, particularly in its intracellular accumulation, in response to the medium osmolarity. To examine this, both the wild-type and Δwis1 cells were grown in YPD medium, and then transferred into the same fresh medium, supplemented with 1.2 M NaCl. After incubation for one hour, the production and intracellular accumulation of glycerol was measured for these cells (Fig. 2). In the case of the wild-type cells, an accumulation of both total and intracellular glycerol was observed upon the upshift to the high osmolarity medium. In the case of the *Δwis1* cells; however, the level of production of total glycerol was significantly lower than that in the wild-type cells (panel A). The intracellular accumulation of glycerol in response to the hyper-osmotic stress was largely abolished, if not completely (panel B). These results suggested that the *wis1*$^+$ gene-product is somehow required, if not absolutely, for the de novo production of glycerol in response to the medium osmolarity.

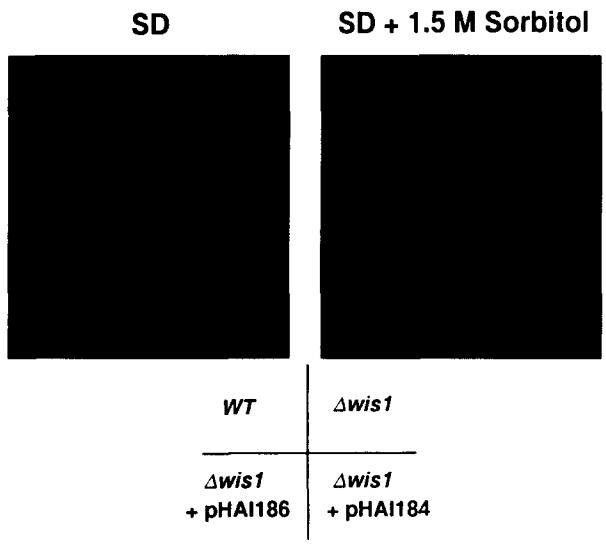

Fig. 1. Examination of osmosensitivity for growth of *S. pombe*. The *Δwis1* strain (JM544) was transformed by each plasmid carrying *gpd1*$^+$(pHAI184) or *gpd2*$^+$(pHAI186). Then, the indicated cells were streaked on SD agar plate or SD agar plate suppremented with 1.5 M sorbitol, and then incubated at 30°C. After 4 days, the plates were photographed.

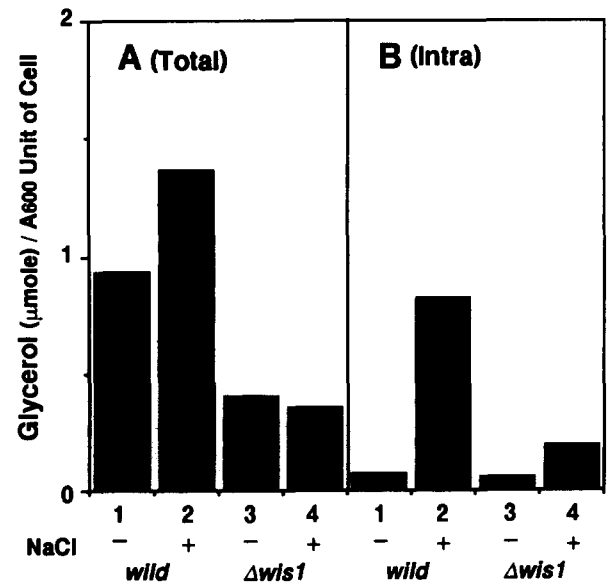

Fig. 2. Glycerol production in *S. pombe* in response to osmotic upshift. Total (panel A) and intracellular (panel B) glycerol, produced by the wild type (PR109, lanes 1 and 2) and the *Δwis1* mutant (JM544, lanes 3 and 4) were measured for the cells grown for one hour in either YPD (lanes 1 and 3) or YPD suppremented with 1.2 M NaCl (lanes 2 and 4).

These results prompted us to suppose that the expression of *gpd1*$^+$ is directly dependent upon the function of *wis1*$^+$ (that of *gpd2*$^+$ might be so too). This was thus examined by Northern hybridization analyses for the *gpd1*- and *gpd2*-mRNA (Fig. 3). Total RNA fractions were isolated from the same set of cells as those prepared for Fig. 2, and then subjected to Northern hybridization analyses with appropriate probes, each sepcific for *gpd1*$^+$ or *gpd2*$^+$ (Fig. 3, upper and middle panels, respectively). Both the *gpd1*- and *gpd2*-mRNA were expressed at a detectable level even in the cells growing in YPD medium (lane 1). Upon shift to the high osmolarity medium, the *gpd1*-mRNA increased markedly within one hour, while the level of the *gpd2*-mRNA appeared not to be significantly affected (lane 2). In the *Δwis1* background, however, the level of the *gpd1*-transcript remarkably decreased regardless of the medium osmolarity (lanes 3 and 4), while that of the *gpd2*$^+$-transcript was affected only slightly. These results indicated that the Wis1 protein is required crucially, if not absolutely, for the osmotic-induction of *gpd1*$^+$, while the expression of *gpd2*$^+$ is most likely independent on *wis1*$^+$.

4. Discussion

In fission yeast, here the *gpd1*$^+$ gene was demonstrated to be a most downstream target of the putative MAP-signaling cascade involving *wis1*$^+$, whose gene-product is a homologue of MAP-kinase kinases. Recently, Millar et al. showed that the *pyp2*$^+$ gene encoding protein tyrosine phosphatase is also under

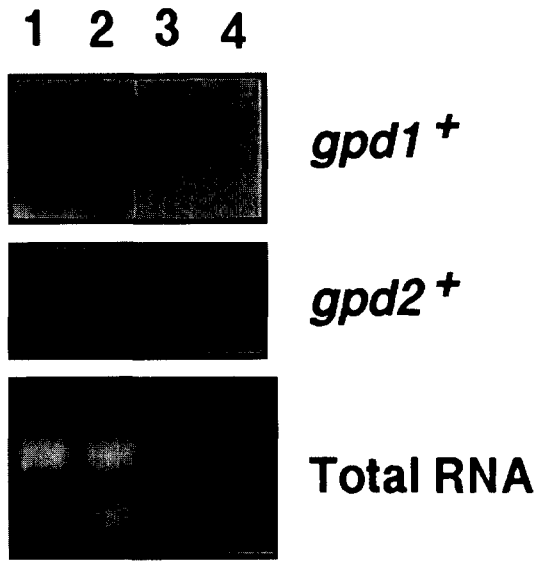

Fig. 3. Northern hybridization analysis. Total RNAs were isolated from the wild type (lanes 1 and 2) or *Δwis1* (lanes 3 and 4) cells, which were grown in either YPD (lanes 1 and 3) or YPD suppremented with 1.2 M NaCl (lanes 2 and 4) for 1 h. These were subjected to Northern hybridization analysis with appropriate probes each sepcific for *gpd1*$^+$ (upper panel) or *gpd2*$^+$ (middle panel). In lower panel, the ethidium bromide stained agarose gel is shown as control for the amounts of RNA loaded.

the control of *wis1*$^+$ [12]. To our knowledge, these are only the instances of targets, so far, identified for this particular MAP-signaling cascade in *S. pombe*. Furthermore, our finding (i.e. the regulation of *gpd1*$^+$ by *wis1*$^+$) has direct relevance to *S. pombe* physiology. As the physiological relevance, defects of either genes, *Δgpd1* or *Δwis1*, resulted in the same phenotype as to the osmosensitivity for growth, because of the failure in intracellular accumulation of the osmoprotectant, glycerol (Figs. 1 and 2). This supports the view that Wis1 MAP-kinase kinase is critically involved in the osmoregulation of *S. pombe*.

The *wis1*$^+$ gene was originally identified as a multicopy suppressor of the *cdc* phenotype caused by the triple mutations, *cdc25-22*, *wee1-50*, *win1-1* [10]. It was thus implicated as a mitotic regulator in *S. pombe*. Recently, the *wis1* locus was identified as the one, mutations in which suppress the lethality of a *Δptc1 Δptc3* strain [11]. It was thus implicated as a mediator of signaling cascade through phosphorylation/dephosphorylation of proteins. In any case, the loss of *wis1*$^+$ function does not prevent vegetative growth and division, although *wis1*$^-$ cells show an elongated morphology, and exhibit pleiotropic features including a rapid reduction of viability onset the stationary phase, a thermosensitivity for growth, and an osmosensitivity for growth [10,11]. Among these phenotypes of *wis1*$^-$ cells, its osmosensitivity is now reasonably explained by the view described above in this study. It is worth mentioning that the thermosensitive phenotype and elongated morphology of *wis1*$^-$ cells were not suppressed by the introduction of *gpd1*$^+$ gene on a multicopy plasmid (data not shown). Therefore, it is clear that *wis1*$^+$ is involved not only in the osmoregulation but also other cellular processes.

Wis1 appears to be a counterpart of PBS2 MAP-kinase kinase of *S. cerevisiae* in terms of both their catalytic and physiological function. Pbs2p was suggested to be involved in the HOG-signaling pathway, that was implicated in the osmo-inducible accumulation of glycerol in budding yeast [4]. Hog1p is a member of the MAP kinase family. Two other genes (*SLN1* and *SSK1*), which were also implicated in the osmoregulation, are particularly of interest, because they encode a pair of proteins (sensory-kinase and response-regulator, respectively), that belong to the members of the prokaryotic two-component family [6,7]. This prokaryotic type of signaling system was suggested to function at the upstream of the HOG-cascade [7]. Sln1p is a candidate for an osmosensor, and Ssk1p seems to be a regulator for MAP-kinase kinase kinases (Ssk2p and Ssk22p) [16]. In *S. pombe*, however, it is not known at present what type of signal sensing mechanism for external osmotic stimuli is involved in the osmoregulation. Moreover, the complex mechanisms by which osmotic signals are subsequently transduced, leading to an adjustment of gene expression, remains largely elusive, although Millar et al. have recently reported a new gene of *S. pombe*, *sty1*$^+$, which appears to be the homologue of HOG1 [12]. In any case, a link between the *wis1*$^+$ and osmo-inducible *gpd1*$^+$ gene, demonstrated in this study, should provide us with a clue to address these relevant issues.

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