Original Article

Requirement of peroxiredoxin on the stationary phase of yeast cell growth

Toshihiko Watanabe, Hayato Irokawa, Ayako Ogasawara, Kenta Iwai and Shusuke Kuge

Department of Microbiology, Tohoku Pharmaceutical University, 4-4-1 Komatsushima, Aoba-ku, Sendai,
Miyagi 981-8558, Japan

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ABSTRACT — Toxic chemicals often induce reactive oxygen species (ROS). Although one of the most abundant ROS-sensitive proteins is in the peroxiredoxin (Prx) family, the function of Prx proteins is poorly understood because they are inactivated under high concentrations of hydrogen peroxide. Like mammalian cells, the model eukaryote *Saccharomyces cerevisiae* possesses multiple Prx proteins. Among the five Prx family proteins, Tsa1 and Ahp1 have the highest and second-highest expression levels, respectively. Here, we focused on a previously uncharacterized phenotype resulting from Tsa1 loss: impaired growth during the late exponential phase. We overexpressed catalase (*CTT1*) and Ahp1 in cells with disruptions in *TSA1* and its homologue, *TSA2* (*tsa1/2Δ* cells), and we found that neither Ctt1 nor Ahp1 overexpression suppressed the impaired cell growth at the stationary phase, although the ROS levels were successfully suppressed. Furthermore, the cell cycle profile was not altered by Tsa1/2 loss, at least in the late exponential phase; however, the glucose consumption rate slowed in the late exponential phase. Our results suggest that ROS levels are not responsible for the growth phenotype. Tsa1 might have a specific function that could not be replaced by Ahp1.

Key words: Peroxiredoxin, Tsa1, Ahp1, Yeast, Glucose metabolism, Reactive oxygen species

INTRODUCTION

Peroxiredoxin (Prx) is a family of thioredoxin (Trx)-dependent peroxidases found in species ranging from *Escherichia coli* to humans (Wood *et al.*, 2003b). Prx proteins reduce hydrogen peroxide (H_2O_2) using electrons from NADPH in the Trx-dependent redox system of thiol-disulfide exchange between catalytic cysteines. Enzymatic characterization indicates that Prx proteins can efficiently compete with other peroxidase catalases and glutathione peroxidase at low H_2O_2 concentrations (Fourquet *et al.*, 2008) and at the physiological level of H_2O_2 produced during aging (Molin *et al.*, 2011). In contrast, Prx proteins' peroxidase activity is inactivated by the overoxidation of a catalytic cysteine to sulfinic acid at higher H_2O_2 concentrations (Wood *et al.*, 2003a). Because this characteristic is unique to eukaryotic Prx proteins, it has been suggested that Prx proteins determine the level at which H_2O_2 reacts with other signaling molecules and that eukaryotic cells have acquired this function by evolution (Wood *et al.*, 2003a). Prx dimers and decamers

are formed under physiological conditions (Wood *et al.*, 2003b). The decamer form and two dodecamers of a major yeast Prx, Tsa1, were induced in response to oxidative stress and heat stress; these multimeric forms are likely to be induced by the overoxidation of the catalytic cysteine (Jang *et al.*, 2004). Interestingly, Tsa1 multimerization is associated with a gain in chaperone function, which reduces the levels of proteins aggregated by heat stress, and this effect is required for heat shock resistance (Jang *et al.*, 2004).

It is conceivable that Tsa1 has a physiological function even in unstressed cells because Tsa1 is partially oxidized in unstressed cells (Okazaki *et al.*, 2007) and appears to control the cellular reactive oxygen species (ROS) level (see below). In fact, life extension is associated with the antioxidant (peroxiredoxin) function of Tsa1 during the long-term stationary phase (Molin *et al.*, 2011). Another apparent phenotype resulting from Tsa1 loss is impaired growth during the late exponential phase in rich medium containing glucose (Wong *et al.*, 2004). We focused our attention on this phenotype to examine whether the ROS

level, which is determined by the antioxidant function of Tsa1, is responsible for growth during the late exponential phase.

MATERIALS AND METHODS

Yeast strains, media, transformation and reagents

Isogenic strains of *Saccharomyces cerevisiae* derived from BY4742 (*MAT α his3 Δ 1 leu2 Δ 0 lys2 Δ 0 ura3 Δ 0*) were used in this study. We used BY4742 as the wild-type strain. To disrupt *TSA1* and *TSA2*, we used *hphMX* and *kanMX* (or *URA3*) selection markers, respectively, as described previously (Goldstein and McCusker, 1999). BY4742 and the selection marker plasmids were obtained from EUROSCARF (Frankfurt, Germany). The yeast cells were transformed using the lithium acetate method, essentially as described by Ito *et al.* (1983). The cells were grown in a synthetic medium (1.67% Bacto™ yeast nitrogen base without amino acids, DB Difco, NY, USA) supplemented with 0.04 mg/ml adenine, 0.02 mg/ml uracil, amino acids and 2% glucose (SDM), as described previously (Dunn and Wobbe, 1997). To select and maintain the plasmid DNA, we omitted one of the nutrients (for the selection marker) from the medium. The cells growing in the exponential phase were washed and diluted in an appropriate medium to a cell density of $A_{600} = 0.001$ and cultured at 30°C. We determined the glucose levels in the medium using an F-kit D-Glucose (Roche Diagnostics, Basel, Switzerland).

Construction of plasmids

CTT1 (nt -965 to +633 of the ORF) and *AHP1* (nt -970 to +673) were PCR-amplified and cloned into the multicopy plasmid pRS425 (*LEU2* selection marker). Similarly, *TSA1* (nt -254 to +308 of ORF) was cloned into the multicopy plasmids pRS426 (*URA3*) and pRS423 (*HIS3*). The pRS42x (Christianson *et al.*, 1992) series was provided by the National Bio-Resource Project (NBRP) of the MEXT, Japan.

Measurements of intracellular ROS, cell viability, and mitochondrial activity by flow cytometry

We used dihydroethidium (DHE), rhodamine 123 and propidium iodide (PI) to determine intracellular ROS, mitochondrial activity and cell viability, respectively. Stock solutions were prepared as follows: 1.9 mg/ml DHE (WAKO pure chemical, Osaka, Japan) and 2.0 mg/ml rhodamine 123 (WAKO) were dissolved in DMSO, and 1 mg/ml PI (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in ethanol. We mixed the 0.5 ml of yeast cell cultures ($A_{600} = 1$) with 5 μ l of each stock solution and

incubated the samples for 10 min at room temperature. After the cells were washed with the growth medium, we observed the fluorescence in each group of cells (10,000 cells) with a FACSCalibur (Becton, Dickinson and Company, NJ, USA; BD) using FL1 for rhodamine 123; FL3 for PI and DHE. We determined the relative averages of the DHE fluorescence. Cell viability was determined by counting the cells that excluded PI. The fluorescent levels of the dead cells were determined as described previously (Ocampo and Barrientos, 2011).

Cell cycle analysis

The cells were harvested at each time point, washed and fixed with 70% ethanol for 1 hr on ice. They were then collected by centrifugation, suspended in 50 mM sodium citrate (pH 7.0) containing 0.5 mg/ml RNase A and 5 μ g/ml PI (prepared in ethanol to 1 mg/ml) and incubated overnight at 4°C. We analyzed PI-stained cells using a FACSCalibur (BD) on the FL3 mode. We determined the cell cycle profile of the wild-type and *tsa1/2 Δ* cells ($N = 4$) using the Modifit software (BD).

Statistical analysis

Multiple independent replicates (at least $n = 3$, except as indicated) were performed for each experiment, and the data are presented as the means of three independent experiments with the standard error of the mean (S.E.M.). We used Student's *t* tests for statistical analyses between two samples.

RESULTS

Prx is required for maximum growth during the stationary phase in rich glucose medium

Tsa1 and Tsa2 are homologous Prx proteins that exist in the cytoplasm and have similar peroxidase activity. Although *TSA1* is the most abundant Prx (more than 90% of total Prx; Park *et al.*, 2000; Tachibana *et al.*, 2009), the expression of *TSA2* (approximately 1% of total Prx) is induced in a *tsa1 Δ* genetic background and compensates for the lost cellular antioxidant activity (Wong *et al.*, 2002). Thus, we examined *tsa1 Δ tsa2 Δ* double knock-out cells (*tsa1/2 Δ*) to explore the function of Tsa1. Previous reports have indicated that the loss of *TSA1* does not affect exponential growth (Wong *et al.*, 2002), but the growth rate of *tsa1 Δ* cells was demonstrated to be lower than that of wild-type cells during the late exponential and stationary phases in a nutrient-rich glucose medium (YPD; Wong *et al.*, 2004). As shown in Fig. 1A, we found a similar phenotype for the *tsa1/2 Δ* mutant in synthetic glucose medium supplemented with amino acids (SDM);

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Fig. 1. Maximum growth during the early stationary phase and physiological ROS levels are impaired by the loss of Tsa1 and Tsa2 (Tsa1/2), and the overexpression of catalase reduced the ROS levels but did not compensate for the growth retardation of *tsa1/2Δ* cells. Wild-type and *tsa1/2Δ* cells were transformed with a multicopy plasmid carrying the *CTT1* gene and cultured in SDM (-Leu). **A.** We plotted the growth curves ($N = 4$; measured by scattering at 600 nm) of wild-type cells (WT) carrying the empty vector (diamonds) and pRS425-CTT1 (triangles) and of *tsa1/2Δ* cells carrying the empty vector (squares) and pRS425-CTT1 (crosses). **B** and **C.** ROS levels and cell viability were determined ($N = 4$) during the exponential (25 hr) and early stationary (42 hr) phases. The cells were treated with either DHE or PI. The ROS levels and cell viability are indicated as the relative fluorescence of the DHE-treated cells (**B**) and the number (%) of PI-negative cells (**C**), respectively. The bars indicate wild-type cells carrying the empty vector (1) and pRS425-CTT1 (2) and *tsa1/2Δ* cells carrying the empty vector (3) and pRS425-CTT1 (4). NS* and NS** indicate that the two values are not significantly different ($P = 0.75$ and $P = 0.09$, respectively). The data are represented as the mean $\pm$ S.E.M.

the loss of Tsa1/2 significantly decreased the growth rate during the late exponential phase (from 30 hr to 42 hr).

We first investigated whether the antioxidant function of Tsa1/2 is responsible for the growth phenotype. DHE is commonly used as a ROS-sensitive probe (Peshavariya *et al.* 2007). As shown in Fig. 1B, the ROS level increased in wild-type cells during the exponential phase (25 hr), and it further increased later in the stationary phase (42 hr). Consistent with the fact that Tsa1 and Tsa2 are antioxidants (Wong *et al.*, 2002; Iraqi *et al.*, 2009), the ROS level in the *tsa1/2Δ* cells was higher than that observed

in the wild-type cells throughout the growth phases (Fig. 1B). In contrast, the cell viability was not impaired by the loss of Tsa1/2 during the early stationary phase (42 hr; Fig. 1C).

Suppression of ROS by the overexpression of catalase and another Prx, Ahp1, does not rescue the growth phenotype in *tsa1/2Δ* cells

To examine the possible effects of ROS accumulation on the growth of *tsa1/2Δ* cells during the early stationary phase, we sought to reduce ROS levels by overexpress-

ing an antioxidant enzyme, catalase (*CTT1*). Although a multicopy plasmid carrying the *CTT1* gene successfully suppressed the ROS level (Fig. 1B) to that of wild-type levels in the exponential phase (25 hr) and the early stationary phase (42 hr), the impaired growth of *tsa1/2Δ* cells was not restored (Fig. 1A). Prx was more effective as an antioxidant than was catalase under low ROS levels (Fourquet *et al.*, 2008). Thus, we examined the overexpression of another Prx, Ahp1, which is an atypical-type Prx and has the second highest expression level (4% of the total Prx; Park *et al.*, 2000; Tachibana *et al.*, 2009) of the Prx family members. We promoted the expression of Ahp1 and altered the ROS level using an exogenously introduced *AHP1* multicopy plasmid. As shown in Fig. 2, the endogenous Ahp1 level was induced to 2.6 times its level in wild-type cells by *Tsa1/2* loss, whereas exogenous *AHP1* was successfully overexpressed at 3.2 times its level in wild-type cells in *tsa1/2Δ* cells (A), and the resulting ROS level was significantly suppressed (at 31 hr) and similar to that of wild type (at 43 hr) (B). However, the reduced growth of *tsa1/2Δ* cells during the early and late stationary phases was not restored by the increased level of Ahp1 (Fig. 2C); instead, the growth of wild-type and *tsa1/2Δ* cells was suppressed during the early and late stationary phases (Fig. 2C). As a control, we demonstrated that the expression of *Tsa1* restored the growth phenotype of *tsa1/2Δ* cells (Fig. 2D).

Effect of *tsa1/2* disruption on the cell cycle and carbohydrate metabolism

There were no significant differences in the cell cycle profiles between wild-type and *tsa1/2Δ* cells, at least from the exponential to the early stationary phases (Fig. 3). Next, we addressed glucose metabolism. Aerobically growing yeast cells can switch their metabolism from glucose degradation to respiration. When the glucose level falls to less than 0.2%, the cells stop dividing for several hours and then switch to respiration (Maris *et al.*, 2001). The growth limitation during these changes in carbon metabolism in the early stationary phase is called the “diauxic shift” (Dickinson and Schweizer, 1999). Our results suggested that *Tsa1* might act in the diauxic shift to promote further cell growth without obvious effects on cell viability or cell cycle progression. Thus, we examined the effect of *TSA1/2* disruption on carbohydrate metabolism. We examined the time-dependent glucose consumption during the exponential phase, the diauxic shift and the stationary phase (Fig. 4A). The overall glucose consumption rates (glucose consumption per hour per cell (A_{600})) between wild-type and *tsa1/2Δ* cells were similar; however, the consumption rate of *tsa1/2Δ* cells dur-

ing the diauxic shift tended to be lower than that of wild-type cells. In contrast, the exponential phase and the post diauxic shift were significantly higher than that of wild-type cells (Fig. 4B).

DISCUSSION

We addressed the impaired yeast growth during the late exponential phase in rich glucose medium upon *Tsa1* loss (Wong *et al.*, 2004). We examined whether the ROS level, which is determined by the antioxidant function of *Tsa1/2*, is responsible for growth during the late exponential phase. We showed that the cellular ROS level was increased by loss of *Tsa1/2*. The enhanced ROS level in *tsa1/2Δ* cells was successfully reverted by the overexpression of *Ctt1* and *Ahp1* (Figs. 1 and 2). It should be noted that the antioxidant effect of *Ahp1* was greater than that of *Ctt1* (Fig. 1B and Fig. 2B). Although we could not estimate the expression level of *Ctt1* because of the lack of an antibody, Prx, like *Ahp1*, may exhibit stronger anti-peroxide activity at low levels of ROS, as occurs under physiological conditions (Fourquet *et al.*, 2008). Although the ROS level was successfully reduced at the diauxic shift, there was no obvious effect on the growth rate at this time in *tsa1/2Δ* cells. Furthermore, no obvious effect of *Tsa1/2* loss on the cell cycle profile at the diauxic shift was observed (Fig. 3).

The glucose consumption rate in *tsa1/2Δ* cells seemed to be reduced during the diauxic shift. Because approximately half of *Tsa1* is continuously oxidized (Okazaki *et al.*, 2007) at the expense of NADPH, the intracellular NADPH level of *tsa1/2Δ* cells might be higher than that of wild-type cells. However, glucose-6-phosphate dehydrogenase activity, which catalyzes the rate-limiting step of the pentose-phosphate pathway, is inhibited by NADPH (Bautista *et al.*, 1992, 1988). The pentose phosphate pathway is required for the production of NADPH and essential molecules for cell growth, such as ribonucleotides. Thus, one possibility is that the loss of *Tsa1/2* may reduce the flow of glucose to the pentose phosphate pathway, resulting in slower cell growth under the reduced glucose condition.

In contrast to this putative mechanism, the overexpression of another peroxiredoxin, *Ahp1*, which resulted in a reduced ROS level as expected, failed to reverse the growth defect in the stationary phase. Like *Tsa1*, *Ahp1* acts as a peroxidase and uses NADPH. Thus, we speculate that *Tsa1* performs a specific function that allows maximum growth in reduced-glucose conditions. One difference between *Tsa1* and *Ahp1* in the peroxidase active state is oligomer formation: *Tsa1* forms dimers and

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Fig. 2. Overexpression of Ahp1 reduces the ROS level but does not compensate for the growth limitation of *Tsa1/2* cells during the early stationary phase. Wild-type and *tsa1/2Δ* cells were transformed with a multicopy plasmid carrying the *AHP1* gene and cultured in SDM (-Leu). **A.** Western blotting of Ahp1 was performed as described previously (Iwai *et al.*, 2010). Lysates were prepared from the indicated yeast cells. The relative expression levels (Ahp1/actin) are indicated below the blot. **B.** The ROS levels were determined ($N = 3$) during the early stationary (31 hr) and stationary (43 hr) phases. The cells were treated with DHE. The ROS levels and cell viability are indicated as the relative fluorescence of DHE-treated cells. The bars indicate wild-type cells carrying the empty vector (1) and pRS425-AHP1 (2) and *tsa1/2Δ* cells carrying the empty vector (3) and pRS425-AHP1 (4). **C.** We plotted the growth curves ($N = 4$; measured by scattering at 600 nm) of wild-type cells (WT) carrying the empty vector (diamonds) and pRS425-AHP1 (triangles) and of *tsa1/2Δ* cells carrying the empty vector (squares) and pRS425-AHP1 (crosses). **D.** We plotted the growth curves ($N = 4$; measured by scattering at 600 nm) of wild-type cells (WT) carrying the empty vector (diamonds) and pRS426-TSA1 (triangles) and of *tsa1/2Δ* cells carrying the empty vector (squares) and pRS426-TSA1 (crosses). NS indicates that the two values are not significantly different ($P = 0.28$). The data are represented as the mean $\pm$ S.E.M.

Fig. 3. Cell cycle analysis of wild-type and *tsa1/2Δ* cells. We determined the cell cycle profile of wild-type and *tsa1/2Δ* cells from the 23, 28 and 46 hr time points of the growth curves (right) of the wild-type and *tsa1/2Δ* cells in SDM. There were no significant differences between wild-type and *tsa1/2Δ* cells at 28 hr (*P* values of G0/G1, S and G2/M are 0.527, 0.07 and 0.97, respectively).

Fig. 4. Effects of the loss of Tsa1/2 on the glucose consumption rate during growth. **A.** We determined time-dependent glucose levels in the medium (broken lines) and compared these levels with the growth curve (continuous lines). The growth curves (*N* = 4) of wild-type cells (upper panel, open diamonds) and *tsa1/2Δ* cells (lower panel, closed squares) and glucose levels (*N* = 4) in the medium were determined as described in the text. **B.** Changes in the glucose consumption rate from the exponential phase to the stationary phase. The bars indicate the glucose consumption rate (glucose (%)/hr/*A*₆₀₀) at each time point (the time indicates the endpoint of each calculation). The asterisks indicate significance (*: *P* = 0.03, **: *P* = 0.09, ***: *P* = 0.019). The data are represented as the mean ± S.E.M.

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decamers (Jang *et al.*, 2004), whereas Ahp1 forms only dimers (Trivelli *et al.*, 2003). In addition, a recent finding indicates that the dimer interface distinguishes Ahp1 from other typical Prxs, such as Tsa1 (Lian *et al.*, 2012). It will be interesting to determine whether the higher-order structures of Tsa1 are required for the growth phenotype. Oxidative stress and heat stress induces higher molecular weight complexes of Tsa1 that exhibits switching from peroxidase to molecular chaperone function in yeast (Jang *et al.*, 2004). However, it is unlikely that the chaperone function of the Tsa1 multimer exhibits the growth phenotype, because we found that reduction of ROS level by Ahp1 overexpression failed to reverse the growth defect in the stationary phase (Fig. 2).

Taken together, our findings suggest that in addition to peroxidase activity, Tsa1 might have an unknown function that modulates growth when the glucose level is decreased. Toxic chemicals often induce ROS and affect cellular redox status. Thus, ROS-dependent toxicity of chemicals may affect cell growth through the alteration of the redox status of Prx proteins.

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