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RESEARCH ARTICLE

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Trehalose accumulation induced during the oxidative stress response is independent of *TPS1* mRNA levels in *Candida albicans*

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Abstract Growing cells of the *Candida albicans* trehalose-deficient mutant *tps1/tps1* were extremely sensitive to severe oxidative stress exposure (H_2O_2). However, their viability was not affected after saline stress or heat-shock treatments, being roughly equivalent to that of the parental strain. In wild-type cells, these adverse conditions induced the intracellular accumulation of trehalose together with activation of trehalose-6P synthase, whereas the endogenous trehalose content and the corresponding biosynthetic activity were barely detectable in the *tps1/tps1* mutant. The addition of cycloheximide did not prevent the marked induction of trehalose-6P synthase activity. Furthermore, the presence of H_2O_2 decreased the level of *TPS1* mRNA expression. Hence, the conspicuous trehalose accumulation in response to oxidative stress is not induced by increased transcription of *TPS1*. Our results are consistent with a specific requirement of trehalose in order to withstand a severe oxidative stress in *C. albicans*, and suggest that trehalose accumulation observed under these conditions is a complex process that most probably involves post-translational modifications of the trehalose synthase complex.

Keywords *Candida albicans* · *TPS1* gene · mRNA levels · Trehalose · Oxidative stress

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Introduction

The massive storage of trehalose has been consistently proven as a rapid mechanism of cell protection against a variety of potentially lethal injuries [3, 18, 20, 22]. A complex of two enzymes is involved in trehalose synthesis, i.e. trehalose-6P synthase (Tps1p) and trehalose-6P phosphatase (Tps2p), whereas its physiological degradation is catalyzed by trehalases (acid and neutral enzymes, Ath1p and Nth1p, respectively). Therefore, the net content of trehalose under both physiological and stress conditions depends on the balance between the synthetic and hydrolyzing capacities [3, 20]. This non-reducing disaccharide acts on plasma membranes by replacing water molecules in the polar head groups of phospholipids [9]. In addition, trehalose functions as a chaperone, being able to suppress the formation of large aggregates of denatured proteins. Later on, rapid trehalose degradation provides the energy necessary for the correct renaturation of such proteins after stress recovery [19].

Changes in the oxidative environment may be an important factor in the course of in vivo infections by pathogenic opportunistic fungi [14]. In turn, the generation of reactive oxygen species (ROS) and other oxidants inside the host's body that occurs mainly during phagocytosis is a defensive response, essential in the further destruction of the pathogen [17]. For this reason, study of the mechanisms by which pathogens resist severe oxidative stress is important in order to understand the progress of productive infections. *Candida albicans* is an opportunistic fungus whose clinical incidence has most dramatically increased during the last few years, due to the extension of the immunocompromised human population [10]. In this yeast, the production of trehalose contributes to pathogenesis, since disruption of *TPS1* and *TPS2* genes reduces virulence in mice [21, 25, 26]. We have previously investigated whether *TPS1* and *TPS2* genes trehalose accumulation plays a protective role against ROS in *C. albicans* and showed that the double homozygous *tps1/tps1* mutant, deficient in trehalose synthesis, was very sensitive to severe H_2O_2 exposure [1]. Here, we report that this highly-susceptible phenotype is restricted to oxidative agents, and not to other kind of potentially lethal stress treatments. The increased activity of H_2O_2-triggered Tps1p appears to be largely independent of increased expression of *TPS1* mRNA.

Materials and methods

Yeast and bacterial strains, culture conditions, and oxidative stress treatments

Candida albicans strains CAI.4 (*ura-3::imm-434/ura-3::imm-434*) (*TPS1*) and its isogenic trehalose-deficient derivative (*tps1/tps1*) [25] were used in this study. Yeast cell cultures were grown at 28 °C by shaking in a medium consisting of 2% peptone, 1% yeast extract and 2% galactose (YPgal). The strains were maintained by periodic subculturing in solid YPgal.

For oxidative stress challenges, cultures grown in YPgal until the exponential phase ($OD_{600}=0.5$–1.0) were divided into several identical aliquots, and treated with different H_2O_2 concentrations (or maintained without H_2O_2 as a control). The samples were incubated at 28 °C for 1 or 2 h. In some experiments, cycloheximide was added to a final concentration of 50 µg/ml. Viability was determined after appropriate dilution of the samples with sterile water by plating in triplicate on solid YPgal. Between 30 and 300 colonies were counted per plate. The percentage of survival was normalized to control samples (100% viability).

Preparation of permeabilized cells and cell-free extracts, and enzymatic assays

At the indicated times, aliquots were harvested, washed, and resuspended at known densities (usually 10–15 mg/ml, wet weight) in 10 mM 4-morpholine-ethanesulfonic acid buffer (MES), pH 6.0. Cellular permeabilization for measurement of Ath1p or cell-free extracts preparation to determine Nth1p and Tps1p activities were carried out as described previously [5].

Ath1p was assayed by incubating 50 µl of permeabilized cells (0.5–1.0 mg wet weight) as enzymatic source with 200 µl of 200 mM trehalose prepared in 100 mM sodium acetate, pH 5.6. The assay for Nth1p contained 50 µl of cell-free extracts (25–100 µg of protein) and 200 µl of 200 mM trehalose prepared in 25 mM MES pH 7.1, 125 µM $CaCl_2$. The reactions were incubated at 37 °C for 15–30 min and stopped by heating in a water bath at 100 °C for 5 min. The glucose released was measured using the glucose oxidase-peroxidase method. One unit of trehalase is defined as the amount of enzyme that hydrolyzes 1 µmol of trehalose (2 µmol glucose) per minute. Specific activity is expressed either as milliunits per mg of wet weight (Ath1p) or as milliunits per mg of protein (Nth1p). Tps1p was measured at 40 °C in the supernatants of cell-free extracts as described elsewhere [5]. Specific activity is expressed as milliunits per mg of protein.

mRNA expression of *TPS1* gene induced by H_2O_2

Total RNA was extracted as described in [7] using the Gibco TRIzol reagent. The RNA samples were heated at 65 °C for 15 min, fractionated on 1.5% agarose gels containing 2.2 M formaldehyde, and transferred to a nylon membrane. The membrane was stained with 0.02% methylene blue in 0.3 M sodium acetate, pH 4.3, and then washed with water to visualize the ribosomal RNA. The membrane was destained with 1% SDS and hybridized in a buffer containing 50 mM Na_2HPO_4 (pH 7.2), 1 mM EDTA, 1% BSA and 7% SDS. A 1.1-kb *Eco*RI–*Xho*I fragment from plasmid pOZ33 [10], comprising 150 bp of the promoter and the first 1,050 bp of the coding region of the gene, was used as probe for *C. albicans TPS1*. The probe was labeled as described in Feinberg and Vogelstein [12] using the Pharmacia labeling kit. The intensity of each band was quantified using the Scion Image software for Windows, and normalized with the intensity of the corresponding rRNA control. For comparison, each value was referred to the corresponding time zero (value of the mRNA levels in control cells maintained at 28 °C), which was arbitrarily assigned to 100.

Other analyses

Intracellular trehalose was extracted from 20–50 mg yeast samples in 1 ml boiling water and the concentration measured with commercial trehalase (Sigma) following the method described by Blázquez et al. [6], except that glucose was estimated by the glucose oxidase-peroxidase procedure. Parallel controls were run to correct for the basal levels of glucose.

Growth was monitored by measuring the optical density of cultures at 600 nm or by direct cell counting with a hemocytometer; at least 200 cells were counted each time. A linear correlation between the two parameters was obtained. Protein was determined by the method of Lowry et al. [16] with bovine serum albumin as standard.

Results

Correlation between trehalose content and cell viability in response to several stress treatments

Budding yeast cells growing in exponential phase on glucose or other fermentable sugars rapidly store large amounts of trehalose when they are suddenly submitted to a variety of nutritional or environmental stresses [3, 20, 22]. We investigated whether this protective role of endogenous trehalose was also operative in cultures of the opportunistic pathogen *C. albicans*. As shown in Table 1, proliferating cultures of the wild-type strain CAI.4 underwent a conspicuous increase in their intracellular storage of trehalose when subjected for 2 h to a set of well-established stress challenges (i. e. heat shock at 42 °C; saline stress with 300 mM NaCl, or oxidative stress with 50 mM hydrogen peroxide). Trehalose accumulation was associated with both a moderate activation of the Tps1p and the concomitant inactivation of the hydrolytic Nth1p (Table 1). In contrast, the basal disaccharide content remained practically unmodified upon identical challenges in exponential *tps1/tps1* cultures, Tps1p activity being barely detectable in this mutant (Table 1). This result confirms that *C. albicans* contains only a single gene coding for functional Tps1p activity [5, 25].

When the degree of cell killing was recorded in parallel using the same samples, both strains displayed a similar high percentage of survival after being challenged with thermal or saline stress (approximately 100% survival, Fig. 1A, B). However, *tps1/tps1* cultures suffered a dramatic loss of cell viability following

Table 1 Effect of different stress treatments on the trehalose content and the enzymatic activities of trehalose-6P synthase and neutral trehalase in *Candida albicans*. Exponential yeast cells from wild-type (CAI.4) and *tps1/tps1* strains were grown at 28 °C in YPgal medium and subjected to different types of stress for 2 h. For other details, see Fig. 1. Numbers in *parentheses* represent the relative activity normalized to the control for each parameter, taking the control treatment as 1.0

Treatment	Trehalose[a]		T-6P synthase[b]		Neutral trehalase[c]	
	CAI.4	*tps1/tps1*	CAI.4	*tps1/tps1*	CAI.4	*tps1/tps1*
Control	2.88 (1.0)	2.1	16.2 (1.0)	< 2.0	17.5 (1.0)	14.3 (1.0)
H_2O_2(50 mM)	11.6 (4.0)	1.2	39.4 (2.4)	< 2.0	6.6 (0.4)	7.8 (0.5)
NaCl (300 mM)	5.90 (2.0)	2.2	27.3 (1.7)	< 2.0	16.2 (0.6)	15.5 (1.1)
42 °C	24.3 (8.4)	3.2	72.7 (4.5)	< 2.0	31.5 (1.8)	20.6 (1.4)

[a]nmol trehalose/mg wet wt
[b]nmol trehalose/min/mg protein
[c]nmol glucose/min/mg protein

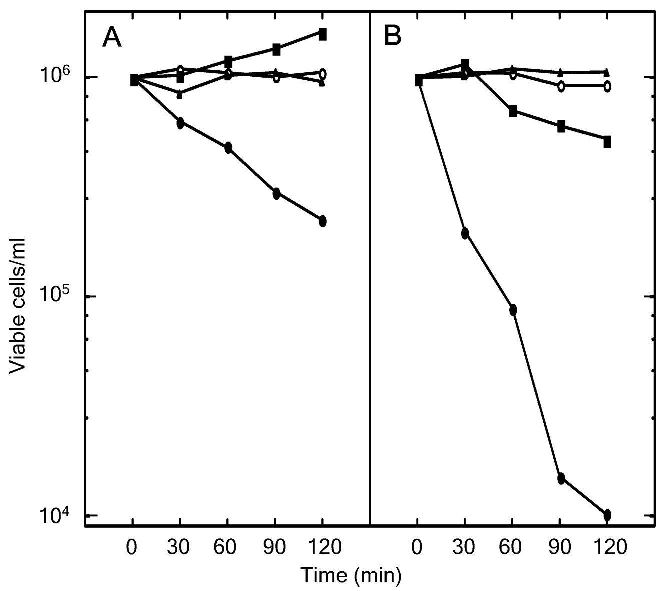

Fig. 1 Effects of stress treatments on cell viability in **A** wild-type (CAI4) and **B** *tps1/tps1* strains of *Candida albicans*. Yeast cells in exponential phase were adjusted to a cellular density of 1.0–1.2×10^6 cells/ml and treated for 120 min with 50 mM H_2O_2 (*filled circles*), 300 mM NaCl (*triangles*) or heat-shocked at 42 °C (*squares*). Untreated control samples (*open circles*) were maintained at 28 °C. Error bars were omitted for the sake of clarity, but the standard deviation was lower than 12%

exposure to oxidative agents (Fig. 1B), whereas the parental CAI.4 cells exhibited only a weak sensitivity to 50 mM H_2O_2 (Fig. 1A). These results are also consistent with previous observations concerning the requirement of an intact *TPS1* gene in *C. albicans* in order to withstand severe oxidative stress [1].

Also, addition of cycloheximide simultaneously with the oxidative treatment did not prevent the intracellular accumulation of trehalose (Table 2), and only caused a marked inhibitory effect on Nth1p (Table 2), whereas the corresponding enzymatic activities of Tps1p and Ath1p remained virtually unmodified (Table 2). These data suggest that H_2O_2-induced changes in trehalose metabolism take place in the absence of de novo protein synthesis. However, they should be analyzed with caution, since the addition of cycloheximide alone enables yeast cells to acquire some protection from hydrogen peroxide [8].

Changes in *C. albicans* *TPS1* mRNA levels caused by H_2O_2 stress

The H_2O_2-induced activation of Tps1p activity and the subsequent increase of endogenous trehalose prompted us to study *TPS1* mRNA expression by Northern blot analysis using the *TPS1* coding region as probe [25]. As

Table 2 Effect of cycloheximide on trehalose content and enzymatic activities involved in trehalose metabolism in exponential cultures of *C. albicans* subjected to oxidative stress. Exponential yeast cells from CAI.4 and *tps1/tps1* strains grown in YPgal were exposed for 1 h to 50 mM H_2O_2 in the absence or presence of 50 µg cycloheximide/ml. Trehalose and the enzymatic activities were measured as described in Materials and methods. Numbers in *parentheses* represent the relative activity normalized to the control for each parameter, taking the control treatment as 1.0

Treatment	Trehalose[a]		Trehalose-6P synthase[b]		Neutral trehalase[c]		Acid trehalase[d]	
	CAI.4	*tps1/tps1*	CAI.4	*tps1/tps1*	CAI.4	*tps1/tps1*	CAI.4	*tps1/tps1*
Control	2.8 (1.0)	1.2	15.9 (1.0)	< 2.0	19.2 (1.0)	20.7 (1.0)	7.5 (1.0)	9.4 (1.0)
H_2O_2	8.3 (3.0)	1.9	28.3 (1.8)	< 2.0	11.1 (0.6)	10.4 (0.5)	2.1 (0.3)	4.8 (0.5)
H_2O_2 + Cycloheximide	7.7 (2.7)	1.6	22.5 (1.4)	< 2.0	9.3 (0.5)	8.4 (0.4)	3.5 (0.5)	5.7 (0.6)

[a]nmol trehalose/min/mg wet weight
[b]nmol trehalose/min/mg protein
[c]nmol glucose/min/mg protein
[d]nmol glucose/min/mg wet weight

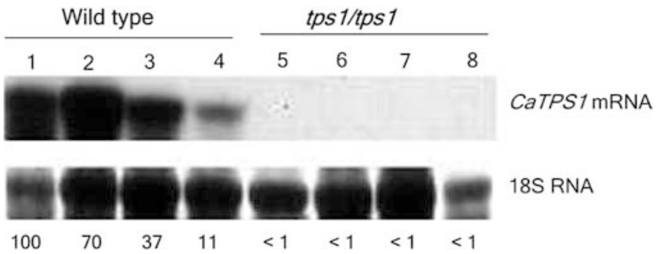

Fig. 2 Northern analysis of *C. albicans TPS1* (*CaTPS1*) mRNA during H_2O_2 treatment. Total RNA was extracted from the wild-type strain CAI.4 or the *tps1/tps1* mutant grown in YPgal at 28 °C (*lanes 1, 5*), or after incubation for 1 h at 37 °C in the absence of H_2O_2 (*lanes 2, 6*), or in the presence of 10 mM (*lanes 3, 7*) or 50 mM (*lanes 4, 8*) H_2O_2. *CaTPS1* mRNA was detected by Northern analysis using a specific probe (*upper row*). *Bottom row* shows methylene blue staining of 18S rRNA. The numbers below each lane represent quantification of the intensity of the *TPS1* mRNA bands

shown in Fig. 2, 10 and 50 mM H_2O_2 caused a drastic decrease in mRNA levels during mild heat shock at 37 °C in wild-type cells compared to an identical sample incubated for the same period without the oxidant (the mRNA levels dropped between seven- and nine-fold). Similar analysis with *tps1/tps1* confirmed the correct disruption of the two chromosomal copies of *TPS1*. Although at these results were initially somewhat surprising, a similar lack of correlation between enzymatic activity and Northern blot analysis was observed for the STRE-regulated gene *GGS1/TPS1* in *S. cerevisiae* [23].

Discussion

In several yeast species, a large accumulation of trehalose in response to different kinds of stress appears to be crucial to ensure proper protection of cell integrity [3, 19, 20]. In the case of the opportunistic pathogen *C. albicans*, although high levels of intracellular trehalose are also stored under several stress conditions, its protective role seems to be mainly restricted to severe oxidative challenges (Table 1, Fig. 1) [1]. Indeed, the trehalose-deficient mutant *tps1/tps1* underwent dramatic cell killing when exposed to an oxidant agent (H_2O_2 50 mM), but not after heat (42 °C) or saline (300 mM NaCl) shock (Fig. 1B). Moreover, the degree of survival was not correlated with trehalose levels, since the higher trehalose accumulation was observed during heat shock (Table 1, Fig. 1). These data indicate that intracellular trehalose content and cell protection are not always correlated phenomena [2, 18, 22, 24] and also highlight the need to study the effect of trehalose on other overlapping stress-responsive pathways.

The mechanism by which trehalose protects against oxidative stress still remains unknown. According to Singer and Lindquist [19], trehalose is likely required to stabilize proteins and phospholipids of membranes subjected to grave damage by ROS. In addition, we cannot exclude that ROS enhances the metabolic glycolytic disorder of the *tps1/tps1* mutant [25]. In this context, the significant induction of antioxidant enzymes (catalase, superoxide dismutase, and gluthatione reductase) upon H_2O_2 exposures in both strains should be mentioned (unpublished results).

Note that the marked accumulation of intracellular trehalose recorded upon H_2O_2 exposure was not prevented by the addition of cycloheximide (Table 2), neither was the enhanced expression of *TPS1* mRNA (Fig. 2). Rather, transcription of *TPS1* was blocked as long as the concentration of H_2O_2 was increased (Fig. 2). We do not have a plausible explanation for this result, but similar observations have been made in *S. cerevisiae*, where activation of *GGS1/TPS1* by a variety of stress exposures occurred in parallel with an impaired expression of the corresponding mRNA [23]. The authors suggested that their findings were not contradictory and pointed to the involvement of mechanisms such as targeted mRNA degradation as well as changes in translational efficiency and protein stability [23]. Although our data are not entirely conclusive, the reduction in *TPS1* mRNA levels during H_2O_2 exposure, and the negligible effect of cycloheximide on Tps1p activity (Table 2), suggest that H_2O_2-induced Tps1p activation might be caused by post-translational modification of the pre-existing enzyme. To date, however, a satisfactory mechanism for Tps1p regulation has not been provided. As occurs in other yeasts [3, 11, 15, 18, 20], the most obvious regulatory mechanism is reversible phosphorylation by cAMP-dependent protein kinases (PKAs). Nevertheless, previous attempts to explain the changes in Tps1p activity in terms of phosphorylation by PKAs were ruled out [4, 13]. *TPS1* has been cloned in *C. albicans*, but no consensus sequences of phosphorylation for PKAs or other kinases have been reported in the corresponding protein [25].

The protection conferred by trehalose against oxidative stress must be highlighted because it conveys a rational basis for the need for trehalose as a contributory factor of virulence during candidiasis [11, 21, 25, 26]. Our results indicate that this protection depends on both transcriptional and, very likely, post-translational regulation of the enzymatic complex implied in trehalose biosynthesis. Hence, the pathogen is endowed with a fast and efficient mechanism for infection of the host's tissues. We propose that such mechanisms could be a widespread phenomenon among other fungal pathogens.

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