Severe reduction of superoxide dismutase activity in the yeast *Saccharomyces cerevisiae* with the deletion or overexpression of *GTS1*

Abudokirim Abudugupura, Zhaojun Xua, Kazuhiro Mitsua, Harumi Hisakib, Nobuo Uedab, Tatsumi Amemiyaa, Kunio Tsurugia, a, b

a Department of Biochemistry 2, University of Yamanashi, Faculty of Medicine, 1110 Shinokato, Yamanashi 409-8898, Japan

b Department of Biochemistry, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi, Tokyo 173-0002, Japan

Received 24 February 2003; received in revised form 22 April 2003; accepted 23 April 2003

First published online 23 May 2003

Abstract

We report herein that the level of reactive oxygen species (ROS) observed using dihydrorhodamine is much higher in either *GTS1*-deleted (*gts1*Δ) or *GTS1*-overexpressing (TMp*GTS1*) transformants than in the wild-type and that the levels of protein carbonyls are increased and the glutathione levels are decreased in both transformants. Consistently, the activities of superoxide dismutases (SODs) in both *gts1*Δ and Tmp*GTS1* were severely weakened, while the protein levels of both Cu/Zn-SOD and Mn-SOD were not so changed. As the intracellular copper levels were significantly increased in both transformants, we hypothesized that, in either *gts1*Δ or Tmp*GTS1* cells, the imbalanced homeostasis of copper induced an accumulation of ROS which caused inactivation of SODs further increasing ROS levels.

1. Introduction

The gene *GTS1* was originally isolated from a yeast cDNA library using oligonucleotides encoding three copies of the Gly-Thr repeat found in the clock-related gene period [1], and thus named *GTS1* [2]. Although the repeat was translated as Ala-Gln in the *GTS1* product Gts1p [3], experiments using transformants carrying different copies of *GTS1* showed pleiotropic effects on yeast phenotypes, including the timing of budding and sporulation in a gene dosage-dependent manner [2,4]. In contrast, the growth rate of either *GTS1*-deleted (*gts1*Δ) or *GTS1*-overexpressing (TMp*GTS1*) transformants was decreased in the presence of cytotoxic metals and drugs [5], and the life span of both transformants was shortened [4]. These results suggest that some disturbed timing caused by mutations in the *GTS1* dosage affects the cellular response to various stresses. Supporting this notion, we found that both *gts1*Δ and Tmp*GTS1* affect the ultradian oscillation of energy metabolism in aerobic continuous cultures, leading to the uncoupling of oscillations of cellular responses to various forms of stress, including oxidative agents [6]. However, the mechanism by which *GTS1* affects anti-oxidative activity of yeast has not been clarified.

It is widely recognized that free radicals or reactive oxygen species (ROS) are involved in speeding the aging process or shortening life span. In yeast, disruption of copper/zinc superoxide dismutase (Cu/Zn-SOD) encoded by *SOD1*, which accounts for 80–90% of the total SOD activities during the growth on glucose [7,8], results in a severe shortening of life span [9]. Thus, in the present study, we examined whether the changes in *GTS1* dosage affect the cellular tolerance of endogenous oxidative agents, finding that in both *gts1*Δ and Tmp*GTS1*, endogenous ROS levels are increased and SOD activity is severely decreased.

2. Materials and methods

2.1. Yeast strains and culture conditions

A haploid strain of *Saccharomyces cerevisiae*, W303-1A (MATa Suc2 ade2 can1 his3 trp1 lue2 ura3), was used in
the present study. The GTS1-deleted (gts1Δ) cells were constructed by disrupting the gene as described previously [2]. The GTS1-overexpressing (TMpGTS1) cells were constructed by transformation with the multicopy plasmid YEp24 carrying GTS1 with its own promoter [6]. Cells were cultured at 30°C in a synthetic medium consisting of 0.67% yeast nitrogen base without amino acids, 2% glucose, and 20 μg each of adenine and essential amino acids per ml. When the turbidity of cultures reached an absorbance of 0.8–1.0 at 600 nm (OD₆₀₀), cells were harvested and used in every experiment.

2.2. Treatment with dihydrorhodamine

Intracellular ROS were detected with dihydrorhodamine 123 (Sigma-Aldrich, Deisenhofen, Germany) [10]. Dihydrorhodamine 123 (5 μg) was added to 1 ml cell suspension (1×10⁶ cells ml⁻¹) and incubated at 30°C for 2 h. Cells were then observed under a fluorescence microscope.

2.3. Determination of the enzyme activity, protein carbonyl content and GSH level

Activities of SODs were determined using the Bioxytech SOD-525 kit (Oxis Health Products, Portland, OR, USA) according to the procedures described in the manual. The method is based on the SOD-mediated increase in the rate of autoxidation of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[£uorene as a substrate in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm [11]. Cell lysate (~4 mg protein) in 0.9% NaCl was centrifuged at 10,000 rpm for 60 min in a microfuge at 0°C and the supernatant was used to determine the activity of cytosolic Cu/Zn-SOD. The precipitate was re-suspended in the saline and used to determine the activity of mitochondrial Mn-SOD. Relative activities of SODs in gts1Δ and TMpGTS1 are shown as a percentage of those in the wild-type.

The protein carbonyl content was determined spectrophotometrically using 2,4-dinitrophenylhydrazine (DNPH) as described previously [12,13]. Cell lysate (~4 mg protein) was incubated with 0.4 ml 0.2% DNPH in 2 N HCl for 1 h at 37°C. After extraction with 10% trichloroacetic acid (w/v) and with ethanol/ethyl acetate (1:1, v/v), the precipitate was dissolved in 6 M guanidine hydrochloride and the absorbance at 366 nm was measured. Results are expressed as nmol DNPH incorporated per mg of protein calculated using a molecular absorption coefficient of 21,000 M⁻¹ cm⁻¹ at 366 nm for aliphatic hydrazones.

The reduced GSH level was measured by spectrophotometry using the Bioxytech GSH-400 kit (Oxis Health Products).

2.4. Western blotting

The protein levels of Cu/Zn- and Mn-SODs, and actin as a control were measured by Western blotting using anti-human Cu/Zn-SOD and Mn-SOD (SOD-100, Stressgen, Victoria, Canada), and anti-actin antibodies. Western blot analysis was performed as described previously [2] and blots were stained by the ECL detection system (Amersham, Amersham, UK).

2.5. Measurement of intracellular metal levels

About 10¹⁰ cells washed three times with ultra-pure water prepared by the Milli-Q water purification system (Millipore, Bedford, MA, USA) were burnt to ashes in a melting pot using an electric oven (500 W, 8 min). The amounts of the metals copper, iron, zinc and manganese were measured using a flame atomic absorption spectrometer (Perkin-Elmer, AAnalyst 300, Boston, MA, USA).

3. Results

3.1. Intracellular levels of ROS and oxidized materials in the GTS1 dosage transformants

When intracellular ROS levels in gts1Δ and TMpGTS1 were determined using dihydrorhodamine, they were found to be increased in both transformants, being higher in gts1Δ than TMpGTS1, compared with the wild-type cells (Fig. 1). Light microscopic observation showed that the levels of ROS were almost the same among the cells in either gts1Δ or TMpGTS1 (Fig. 1B,C) and that the morphology of cells shown by Nomarski imaging was apparently normal containing central vacuoles (Fig. 1E,F). In the wild-type, in contrast, only large and granular or shrinking cells accumulated ROS (Fig. 1A,D). As yeast cells increase in size as they age, it is likely that the highly stained cells in the wild-type are senescent cells. When the intensities of fluorescence in small and round cells were determined by an image analyzer, those of gts1Δ and TMpGTS1 were increased approximately 100- and 70-fold compared with the wild-type, respectively. These results suggest that either deletion or overexpression of GTS1 increased the intracellular ROS levels.

To evaluate the levels of ROS in gts1Δ and TMpGTS1, we treated the wild-type cells with various concentrations (0.001–1.0 mM) of menadione, which is known as a superoxide generator, in the presence of dihydrorhodamine. We found that the evaluation is hardly possible because the intensities of fluorescence were greatly variable among the menadione-treated cells while the proportions of heavily stained small cells increased with the concentration of added menadione (data not shown). Among the cells treated with 1.0 mM menadione for 2 h, about 30% of small and round cells were heavily stained with the fluorescence (Fig. 1G,H). As the cells showed a survival rate of about 80%, it is likely that the heavily stained cells were dying. A similar result was obtained when cells were
treated with hydrogen peroxide instead of menadione (data not shown).

To examine whether the increase of ROS in the *GTS1* dosage transformants was great enough to damage cellular components, the protein carbonyl content, as an indicator of the intracellular level of oxidized proteins [12], and the reduced GSH levels were determined (Fig. 2). Among the cells examined, the level of protein carbonyls was most increased and the reduced GSH most decreased in *gts1Δ*, followed by *TMPGTS1*, suggesting that the *GTS1* dosage transformants, especially *gts1Δ*, were damaged by ROS roughly in proportion to apparent ROS levels.

### 3.2. Activities of SODs and their protein levels

As SODs are the major enzymes involved in removing superoxide anion, a major ROS, the enzymic activities were compared among the wild-type and *GTS1* dosage transformants (Fig. 3). The activities of Cu/Zn-SOD were decreased five- to eightfold compared with the wild-type, being lower in *gts1Δ* than *TMPGTS1*, while the activities of Mn-SOD in both transformants were decreased to a lesser extent compared with those of Cu/Zn-SOD (Fig. 3).

The protein levels of Cu/Zn-SOD and Mn-SOD were measured to determine whether SOD expression was inhibited in the *GTS1* dosage transformants (Fig. 4). Western blot analysis showed that the protein levels of Cu/Zn- and Mn-SOD were not significantly changed in both transformants compared with the wild-type (Fig. 4). Furthermore, Northern blot analysis using polymerase chain reaction-amplified oligonucleotides encoding specific sequences of *SOD1* and *SOD2* as probes showed that the mRNA levels of Cu/Zn- and Mn-SOD were not significantly changed in either transformant compared with the wild-type (data not shown). Thus, these results suggest that the decrease in enzymic activities of the SODs in the *GTS1* dosage transformants was not caused by the reduced expression of the enzymes.

### 3.3. Metal levels

As Cu/Zn-SOD and Mn-SOD require metals as essential components, intracellular levels of Cu, Fe, Zn, and Mn were determined to examine whether the decrease in enzymic activities is caused by a shortage of intracellular metals. Unexpectedly, no metals measured were decreased in the *GTS1* dosage mutants; rather, the Cu levels were significantly increased (Fig. 5). The Cu levels of *TMPGTS1* and *gts1Δ* were increased five- and 3.5-fold compared with the wild-type, respectively. On the other hand, the levels of Fe, Zn and Mn were not significantly changed (data not shown).

### 4. Discussion

We found in the present study that either inactivation or overexpression of *GTS1* induces an accumulation of ROS and a reduction in the enzymic activities of SODs. Furthermore, based on the comparison of protein carbonyl contents and reduced GSH levels, *gts1Δ* is more affected by endogenous oxidative stress than *TMPGTS1* in proportion to apparent ROS levels. We found that the levels of endogenous ROS in either *gts1Δ* or *TMPGTS1* were nearly homogeneous among the cells while the accumulation of ROS in response to exogenous oxidants was heterogeneous in the wild-type, being high in granular cells.

![Fig. 1. Intracellular levels of endogenous ROS examined using dihydrorhodamine 123 in the wild-type (A,D,G,H), *gts1Δ* (B,E) and *TMPGTS1* (C,F) cells treated with (G,H) and without 1.0 mM menadione (A-F). Cells were observed by Nomarski imaging (A-C,G) and fluorescent microscopy (D-F,H). Arrowheads in G indicate the small cells which were heavily stained with dihydrorhodamine in H.](image-url)
We reported previously that dying cells in the process of programmed cell death named autophagic death became granular reflecting the accumulation of autophagosomes [14,15]. Therefore, it is likely that only dying cells in response to exogenous oxidants accumulate ROS in the wild-type while live cells rapidly remove them. In contrast, the gts1Δ and TmpGTS1 cells are likely to be continuously exposed to a high level of ROS induced by the changes in GTS1 dosage.

So far, just why the activities of SODs are decreased in GTS1 dosage transformants remains to be explained, but the present results provide some clues regarding this problem. One possibility is that the enzymes are impaired after translation, as the mRNA and protein levels of Cu/Zn-SOD and Mn-SOD were not significantly changed in either transformant compared to the wild-type. Further, the decrease in SOD activity was not caused by a shortage of metals as catalytic cofactors in cells, as the metal levels were not decreased but rather Cu levels were significantly increased. Metals are essential for life as structural or catalytic cofactors but at the same time they are highly toxic, as they can nonenzymatically catalyze ROS generation [16,17]. It is therefore possible that the elevation of Cu levels causes an accumulation of ROS in GTS1 dosage transformants, inducing the modification of proteins, although the possibility that decreased SOD activity might be the cause of apparent ROS susceptibility is not addressed.

We previously found that Gts1p binds to the cytoplasmic portion of P-type ATPases (ABC transporters), including Ycf1p (yeast cadmium factor), and that either deletion or overexpression of GTS1 decreases cellular resistance to cadmium [5]. In yeast, the homeostasis of Cu is thought to be regulated by the CCC2 gene encoding...
a P-type ATPase [18], a human protein associated with Menkes/Wilson’s disease [19,20]. In yeast, Ccc2p locates in the Golgi apparatus and is involved in export of Cu through a secretory pathway [21,22]. Furthermore, it has been reported that the incorporation of Cu into target proteins is mediated by at least two copper chaperones, Atx1p and Lys7p [23,24], and that Atx1p functions in association with Ccc2p [25]. Thus, defective Cu delivery to SOD could be the reason for lowered SOD activity. Furthermore, either deletion or overexpression of GTS1 affects ultradian oscillations of energy metabolism in continuous aerobic cultures [6]. Thus, it is possible that disturbance of the energy metabolism oscillation affects ATP-dependent activation of P-type ATPase. Anyway, further studies should be done to test these possibilities.

References


