THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 9, pp. 6118–6126, February 26, 2010

The Thioredoxin-Thioredoxin Reductase System Can Function *in Vivo* as an Alternative System to Reduce Oxidized Glutathione in *Saccharomyces cerevisiae*[*] [S]

Received for publication, September 6, 2009, and in revised form, November 8, 2009 Published, JBC Papers in Press, December 1, 2009, DOI 10.1074/jbc.M109.062844

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Cellular mechanisms that maintain redox homeostasis are crucial, providing buffering against oxidative stress. Glutathione, the most abundant low molecular weight thiol, is considered the major cellular redox buffer in most cells. To better understand how cells maintain glutathione redox homeostasis, cells of *Saccharomyces cerevisiae* were treated with extracellular oxidized glutathione (GSSG), and the effect on intracellular reduced glutathione (GSH) and GSSG were monitored over time. Intriguingly cells lacking *GLR1* encoding the GSSG reductase in *S. cerevisiae* accumulated increased levels of GSH via a mechanism independent of the GSH biosynthetic pathway. Furthermore, residual NADPH-dependent GSSG reductase activity was found in lysate derived from *glr1* cell. The cytosolic thioredoxin-thioredoxin reductase system and not the glutaredoxins (*Grx1p*, *Grx2p*, *Grx6p*, and *Grx7p*) contributes to the reduction of GSSG. Overexpression of the thioredoxins *TRX1* or *TRX2* in *glr1* cells reduced GSSG accumulation, increased GSH levels, and reduced cellular glutathione E_h'. Conversely, deletion of *TRX1* or *TRX2* in the *glr1* strain led to increased accumulation of GSSG, reduced GSH levels, and increased cellular E_h'. Furthermore, it was found that purified thioredoxins can reduce GSSG to GSH in the presence of thioredoxin reductase and NADPH in a reconstituted *in vitro* system. Collectively, these data indicate that the thioredoxin-thioredoxin reductase system can function as an alternative system to reduce GSSG in *S. cerevisiae in vivo*.

Aerobic organisms maintain a reducing intracellular environment to facilitate appropriate functioning of numerous processes, including the proper folding of proteins and maintenance of their activity. Cellular mechanisms that maintain redox homeostasis are crucial, because they provide a buffer against conditions that may perturb the redox environment of cells and/or induce oxidative stress (1, 2). Changes to redox state such as exposure to reactive oxygen species (ROS) can lead to detrimental effects including oxidation of sulfhydryl groups in proteins that can result in loss of activity (3). Given the importance of redox homeostasis, it is vital to understand the mechanisms involved in maintaining normal redox homeostasis. The high abundance of glutathione (1–10 mM) in cells and its low redox potential (−240 mV) make the glutathione system a major intracellular redox buffer in most cells (1, 4, 5). Previous studies have indicated that cells lacking Cu,Zn-superoxide dismutase, genes involved in glutathione homeostasis or those involved in the pentose phosphate pathway can alter intracellular the glutathione redox balance (6–9), demonstrating the complexity of redox systems. Therefore, understanding the factors that affect cellular glutathione homeostasis contributes importantly to our overall understanding of how cells maintain intracellular redox homeostasis.

In *Saccharomyces cerevisiae*, reduced glutathione (GSH)[3] biosynthesis occurs in the cytosol via two ATP-dependent steps. The first is catalyzed by γ-glutamylcysteine synthetase encoded by *GSH1* (10), which catalyzes condensation of glutamate and cysteine. Glutathione synthetase encoded by *GSH2* (11) catalyzes the addition of glycine to γ-glutamylcysteine to form GSH (12). GSH is an important antioxidant, because yeast strains altered in their GSH redox state are sensitive to oxidant-induced stress (13–16).

Several cellular processes use the reducing power of GSH including glutathione peroxidases, which mediates the reduction of hydrogen peroxide to water, generating stoichiometric quantities of oxidized glutathione (GSSG) in the process. GSSG can be recycled to GSH by glutathione reductase using reducing equivalents supplied by NADPH (2). *GLR1* encodes the only known GSSG reductase in *S. cerevisiae*. Mutants deleted for *GLR1* are viable, overaccumulate GSSG, and are hypersensitive to oxidants (17, 18). Besides glutathione, cells have other redox-active molecules such as the thioredoxins and glutaredoxins that participate in oxidative stress defense.

Thioredoxins are low molecular weight thiol-disulfide oxidoreductases with a conserved CGPC active site and are involved in the reduction of enzymes that are oxidized to form a disulfide (19). These enzymes include 3′-phosphoadenosine 5′-phosphosulfate (PAPS) reductase required for sulfur assimilation and ribonucleotide reductase (RNR) required for maintaining dNTPs for DNA synthesis (3). *S. cerevisiae* contains two cytoplasmic thioredoxins encoded by *TRX1* and

* This work was supported by grants from the Australian Research Council and the Cancer Institute New South Wales.
[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.
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[3] The abbreviations used are: GSH, reduced glutathione; GSSG, oxidized glutathione; HA, hemagglutinin.

TABLE 1
***S. cerevisiae* strains used in this study**

Strain	Genotype	Source
W303	*MAT***a** *ura3–52 leu2–3,112 trp1–1 ade2–1 his3–11*	Chris M. Grant
JL3	*gsh1::LEU2* in W303	Grant *et al.* (14)
CY7	*glr1::TRP1* in W303	Grant *et al.* (17)
ST201	*gsh1::LEU2 glr1::TRP1* in W303	This study
ST203	*glr1::TRP1 GSH1*-HA::*kanMX* in W303	This study
ST204	*grx6:: natMT1 grx7:: hphNT1 glr1:: TRP1* in W303	This study
ST208	*grx1::LEU2 grx2::HIS3 glr1::TRP1* in W303	This study
ST209	*grx1::LEU2 grx2::HIS3 grx6:: natMT1 grx7:: hphNT1 glr1:: TRP1* in W303	This study
ST301	*trx1::hphNT1 glr1::TRP1 in* W303	This study
ST302	*trx2::hphNT1 glr1::TRP1 in* W303	This study
BY4743	*MAT***a***/MATα his3Δ1/ his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lysΔ0 ura3Δ0/ura3Δ0*	EUROSCARF
BY4743 *gsh1*)	*gsh1::kanMX* in BY4743	EUROSCARF
BY4743 (*glr1*)	*glr1::kanMX* in BY4743	EUROSCARF
BY4743 (*hgt1*)	*hgt1::kanMX* in BY4743	EUROSCARF

TRX2. Mutants deleted for both *TRX1* and *TRX2* are defective in sulfate assimilation, consistent with the role of the two thioredoxins as hydrogen donors for PAPS reductase (20). Trx2p is involved in oxidative stress defense because the *trx2* mutant is sensitive to H_2O_2 (21, 22). Thioredoxin reductase encoded by *TRR1* reduces oxidized thioredoxins directly. Mutants deleted for *TRR1* are sensitive to hydrogen peroxide and are auxotrophic for methionine (23, 24).

Glutaredoxins are small proteins that act as thiol oxidoreductases involved in the reduction of protein disulfides or glutathione-protein mixed disulfides (25). In *S. cerevisiae*, the dithiol glutaredoxins are encoded by *GRX1* and *GRX2* and contain CPYC at their active site (26). Cells lacking *GRX1* are sensitive to superoxide generating agents and hydroperoxides, whereas cells lacking *GRX2* are sensitive to hydroperoxides but not superoxides, indicating that the functions of *GRX1* and *GRX2* are only partially overlapping (26), and the two genes are differentially regulated (27).

S. cerevisiae contains five monothiol glutaredoxins. Grx3p, Grx4p, and Grx5p contain the CGFS motif in their active site. Grx3p and Grx4p regulate the nuclear localization of the Aft1p transcription factor involved in iron homeostasis (28, 29), whereas Grx5p participates at the late stages of the biosynthesis of Fe/S clusters in the mitochondrion (30, 31). *GRX6* and *GRX7* encode two monothiol glutaredoxins that contain active site sequences CSYS and CPYS, respectively. Both Grx6p and Grx7p are localized in the Golgi, but are not involved in oxidative protein folding (32). Cells lacking *GRX6* and/or *GRX7* were shown to be sensitive to oxidants hydrogen peroxide and diamide in one study (32), but showed no obvious phenotype, except for mild resistance to the glycosylation inhibitor tunicamycin in another (33). The biological role of Grx6p and Grx7p remains unknown.

Although the thioredoxins and glutaredoxins are similar in structure and have overlapping functions (22), they are regulated in a different manner. The oxidized disulfide form of thioredoxin is reduced directly by thioredoxin reductase using NADPH as the electron donor, whereas glutaredoxin is reduced by glutathione (GSH), and the oxidized glutathione (GSSG) is in turn reduced by glutathione reductase using electrons donated by NADPH (25). Glr1p is highly specific for the reduction of GSSG. Alternatively, while thioredoxins have been found to reduce a broad spectrum of substrates, GSSG was generally not considered to be a good substrate for thioredoxins (34, 35). However, cells deleted for *TRR1* over-accumulate both GSH and GSSG (18). Furthermore, the *trr1 glr1*, and the *trx1 trx2 glr1* mutants are inviable (18, 36), indicating a potential link between these two systems.

To further understand how cells maintain intracellular glutathione redox balance, we investigated the mechanisms of how cells respond to increased levels of GSSG *in vivo*. Surprisingly, we found that cells deleted for *GLR1*, encoding the only known glutathione reductase in *S. cerevisiae*, are able to convert GSSG to GSH. This led us to investigate possible alternative mechanisms that can reduce GSSG in cells.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—The *S. cerevisiae* strains used (Table 1) were derived from BY4743 (Open Biosystems) or from W303 as described previously (14). Single gene deletion strains in the W303 background were generated using PCR to amplify the disrupted allele and flanking region (~200–400 bp) from the strains available from the *S. cerevisiae* Genome Deletion Project (37) or using the appropriate plasmids from the PCR tool kit (38). The amplified DNA product was transformed into W303 with selection on YEPD medium containing 220 μg/ml geneticin (for *KanMX* selection, Invitrogen), 100 μg/ml nourseothricin (for *natMT1* selection, Sigma) or 300 μg/ml hygromycin (for *hphNT1* selection, Invitrogen). Transformation was performed using the lithium acetate-based method (39). Each deletant was confirmed by PCR analysis on genomic DNA isolated from the respective strains. Chromosomal HA-tagging of *GSH1* was performed using the PCR-based targeted gene insertion method described (38). The resulting PCR fragment was transformed into W303 to generate strains containing an in-frame *GSH1* allele with a C-terminal chromosomal HA tag. Multiple mutants were generated by mating and sporulation using standard yeast genetic methods.

The N-terminally HA-tagged galactose-inducible *TRR1*, *TRX1*, and *TRX2* constructs were generated using the Gateway system (Invitrogen) according to the manufacturer's instructions. Plasmids carrying the respective genes from the *S. cerevisiae* ORF collection (40) were shuffled into pDonr-221 using the BP reaction. The entry clones obtained were then used to introduce the respective genes into pAG416GAL1-ccdB (41) using the LR reaction. The resulting clones were sequenced for verification.

Growth Conditions—Yeast strains were grown in YEPD medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, or synthetic defined medium (SD) containing 0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, and supplemented with minimal auxotrophic requirements. For induction of genes under the control of the *GAL1* promoter, cells were grown for 2 days in SD medium and re-inoculated to synthetic defined medium containing 2% (w/v) galactose (SD-GAL) instead of glucose. Auxotrophic supplements were added as follow: 76 mg/liter of L-tryptophan, L-isoleucine, and L-valine, 260 mg/liter L-leucine, 380 mg/liter L-histidine, 26 mg/liter uracil, and 18 mg/liter adenine. Appropriate supplements were omitted for the purpose of selecting plasmids.

Western Blot Analysis—Cells were grown as stated, and ~40 ml of cells were harvested and lysed in lysis buffer (0.1 M Tris-HCl, pH 8, 20% (v/v) glycerol, 2 mM phenylmethylsulfonyl fluoride, 1% SDS). Protein content of cell lysate was determined and adjusted to 2 μg/μl in sample-loading buffer (5% (v/v) β-mercaptoethanol, 40% (v/v) glycerol, Tris-HCl, pH 6.8). Samples were boiled for 10 min, and 30 μg of each sample were loaded onto a 4–12% NuPAGE gel (Invitrogen). Anti-HA was diluted to 1:1000, and anti-Pgk1p was diluted to 1:5000.

β-Galactosidase Assay—Cells were grown to exponential phase (A_{600} ~ 0.5), harvested, and analyzed for β-galactosidase activity as described (42). β-Galactosidase activity was expressed as units of ONPG (*O*-nitrophenyl-β-D-galactopyranoside) hydrolyzed (nmol) per min over total protein (mg), and the extent of induction was the ratio of the β-galactosidase activity of the treated sample to that of the untreated sample. For each strain, three independent transformants were assayed.

Determination of Intracellular Glutathione Levels—Reduced (GSH) and oxidized glutathione (GSSG) were estimated as described previously (14, 43). Cells were grown in SD medium (40 ml) to an A_{600} of 1 (~2×10^7 cells/ml) and harvested by centrifugation (10,000 × *g*, 20 s), washed in 3 ml of ice-cold PBS buffer, resuspended in 350 μl of ice-cold 8 mM HCl containing 3% (w/v) 5-sulfosalicilic acid and disrupted using a mini-bead beater (1-min high speed, 4 °C). The lysate was clarified by centrifugation (5 min, 10,000 × *g*, 4 °C), and the supernatant used to determine total glutathione. GSSG was determined by reacting GSH in samples with 2-vinylpyridine prior to the assay. Levels of GSH and GSSG were calculated as described in Ref. 43.

Estimation of Intracellular Redox Environment—Intracellular values of the GSSG/2GSH half-cell redox potential (glutathione E_h') were determined as an indicator of the cellular redox environment (1). These were calculated from intracellular concentrations of GSH and GSSG using the Nernst equation: $E_h' = E_0 - 2.303(RT/nF)\log_{10}[(GSH)^2/(GSSG)]$. Glutathione E_h' values are expressed in mV; E_0 is the standard potential for reduced glutathione (−0.24 mV) at pH 7, R is the gas constant (8.31 J mol^{-1} K^{-1}), T is the absolute temperature (303 K), n is the number of electrons transferred (2), and F is the Faraday constant (96,406 J V^{-1} mol^{-1}). To estimate intracellular glutathione concentration, cell volumes were estimated from cell major (*a*) and minor (*b*) axes using the formula (44): $V = \pi a^2 b/6$. Cell dimensions were determined microscopically at ×100 magnification using a graticule calibrated against a slide micrometer grating. For each strain, at least 100 cells were measured and the average cell volume determined.

Analysis of Glutathione Reductase Activity in Cell Lysates—Cells were harvested according to the procedure used for Western blot analysis. Cell lysates were clarified by ultracentrifugation (100,000 × *g*, 4 °C, 20 min), and proteins present in each soluble fraction were concentrated using an Amicon Ultra centrifugal filters (3 kDa cutoff; Millipore) at 4 °C. Cell lysates were mixed with an equal volume of ice-cold phosphate-buffered saline and re-concentrated using the above columns. This process was repeated twice to reduce the levels of small molecular weight compounds including GSH and GSSG. Lysates prepared in this manner were assayed immediately. Glutathione reductase activity was determined by monitoring oxidation of NADPH (340 nm). Complete (total) reaction mixtures contained 20 μl of cell lysate in phosphate-buffered saline and NADPH (600 μM). Reactions were initiated by addition of 1 mM GSSG, and reduction of GSSG was estimated by monitoring GSSG-dependent NADPH oxidation, which was reflected by a decrease in absorbance at 340 nm. The background level of NADPH oxidation was also determined by monitoring NADPH oxidation by the corresponding cell lysate in the absence of added GSSG. GSSG-dependent oxidation of NADPH was calculated by subtracting the values obtained for GSSG-independent NADPH oxidation from the value obtained for the "complete" reaction mixture. Activity was normalized to the protein concentration present in each sample and expressed as nmol of NADPH oxidized per minute per mg of protein (nmol min^{-1} mg^{-1}).

Reduction of GSSG by Purified Trx1p or Trx2p and Trr1p in Vitro—Plasmids expressing six-histidine residue-tagged versions of Trx1p (pBAD-YTRX1) and Trr1p (pBAD-YTRR1) were a kind gift from Barry Rosen (45). Trx2p was amplified by PCR and cloned into the pBAD expression vector (Invitrogen). Histidine-tagged proteins were purified by Ni^{2+}-nitrilotriacetic acid chromatography and protein purity checked on SDS-PAGE gels. Glutathione reductase (Glr1p) derived from *S. cerevisiae* was purchased from Sigma. Reduction of GSSG by Glr1p or Trr1p was measured *in vitro* with purified proteins. Reaction mixes contained NADPH (600 μM) and either Glr1p (6.67 nM), or Trr1p (0.25 μM) and Trx1p (1.5 μM), or, Trr1p (0.25 μM) and Trx2p (1.5 μM). Reactions were started by addition of 1 mM GSSG and reduction of GSSG to GSH estimated by following the decrease in oxidation of NADPH (340 nm).

RESULTS

Treatment of Cells with Extracellular GSSG Leads to Accumulation of Intracellular GSH—To investigate if perturbation in cellular glutathione redox environment can lead to a change in cellular E_h', intracellular GSSG concentration was increased by adding GSSG to the medium, and the changes in intracellular glutathione concentration and cellular E_h' of the glutathione redox couple monitored over time.

In wild-type and *glr1* cells, addition of 100 μM GSSG extracellularly led to an increase in intracellular glutathione concentration from 1 to 3 mM in 20 min and reaching a maximum of 6 mM after 2 h of treatment (Fig. 1*A*). Increased intracellular glu-

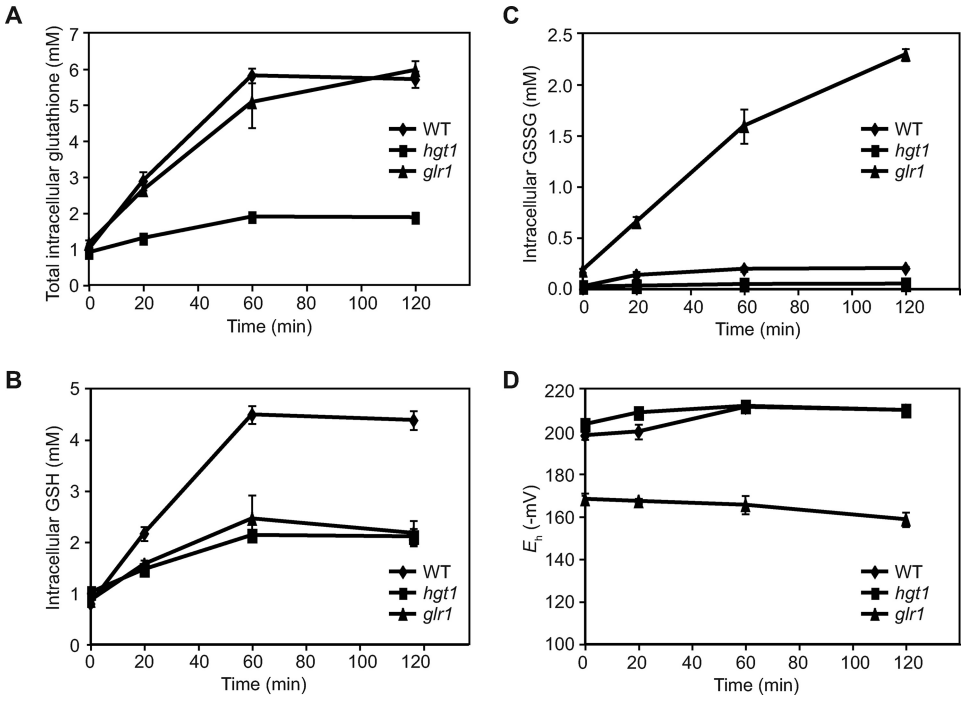

FIGURE 1. **Effect of extracellular GSSG on intracellular glutathione redox homeostasis.** The wild-type, *hgt1* and *glr1* cells were grown to exponential phase (A_{600}, 0.5) in SD medium exposed to 100 μM GSSG, and intracellular glutathione was determined over the indicated time course. *A*, total intracellular glutathione; *B*, GSH; and *C*, GSSG of the indicated strains were determined. *D*, cellular glutathione E_h' was determined over the time course. *Error bars* indicate the S.E. of four samples from two independent experiments.

tathione accumulation was dependent on the high affinity glutathione transporter Hgt1p/Opt1p (encoded by *YJL212c*), because cells lacking *HGT1* exhibited a minimal increase in intracellular glutathione upon GSSG treatment (Fig. 1*A*). Upon GSSG treatment, an accumulation of total glutathione in wild-type and *glr1* cells was therefore largely due to uptake of GSSG by Hgt1p. Previous studies found that Hgt1p is the sole high affinity GSH transporter in *S. cerevisiae* (46). Here it was demonstrated that Hgt1p could also transport GSSG into cells, because a minor increase in glutathione level was observed in the *hgt1* mutant treated with GSSG. This indicates the presence of another less efficient route for GSSG uptake in *S. cerevisiae*.

Upon GSSG treatment, the glutathione accumulated by wild-type cells was predominantly in the reduced form GSH (Fig. 1*B*). This increase in GSH was likely to be due to the uptake of GSSG, followed by conversion of GSSG to GSH by GSSG reductase Glr1p, because *glr1* cells accumulated predominantly GSSG upon GSSG treatment (Fig. 1*C*). Interestingly, the level of GSH in the *glr1* mutant increased by up to 2-fold after exposure to GSSG for 1 h (Fig. 1*B*). Cells deleted for *GLR1* have a more oxidizing intracellular environment as reflected from the E_h' determination (Fig. 1*D*). Although exposure of wild-type and *glr1* cells to GSSG led to a change in intracellular GSH and GSSG concentration (Fig. 1, *B* and *C*), cellular E_h' remained relatively constant over the time of treatment. These data indicate that in the presence of excess GSSG, cells increase the level of GSH possibly to maintain cellular E_h'. Increased GSH accumulation in the *glr1* cells treated with GSSG could have been due to elevated GSH synthesis and/or reduction of GSSG by a Glr1p-independent mechanism.

Treatment of Cells with Extracellular GSSG Leads to Accumulation of GSH in a Gsh1p- and Glr1p-independent Manner—The first committed step for *de novo* synthesis of GSH is catalyzed by γ-glutamylcysteine synthetase encoded by *GSH1* (10). To determine whether elevated GSH accumulation in the *glr1* cells treated with GSSG was associated with the expression of *GSH1*, *GSH1* expression was monitored in the *glr1* mutant over time using a *GSH1*::*lacZ* construct. The protein level of Gsh1p was also monitored using Western blot analysis of a *glr1* strain harboring a chromosomally HA-tagged *GSH1* under the control of its native promoter (Fig. 2, *A* and *B*).

The transcript level of *GSH1* and the level of Gsh1-HAp did not increase in *glr1* cells after exposure to GSSG for 60 min (Fig. 2, *A* and *B*), despite a significant increase in intracellular concentration of GSSG and GSH (Fig. 1). From these data, it is unlikely that transcriptional regulation of *GSH1* is involved but it is not possible to exclude that elevated GSH accumulation in *glr1* cells treated with GSSG may have been due to post-translational regulation of Gsh1p activity. To explore this issue and to determine whether increased GSH in *glr1* cells treated with GSSG was dependent on GSH synthesis *per se*, *GSH1* was deleted in the *glr1* mutant.

The *gsh1 glr1* mutant is auxotrophic for glutathione but was found to be capable of utilizing GSSG as a sole source of glutathione (Fig. 2*D*). In exponential phase, *gsh1 glr1* cells grown in medium supplemented with 25 μM GSH accumulated intracellular GSSG to a similar level as that of *glr1* cells (Fig. 2*C*). The *gsh1 glr1* mutant treated with GSSG accumulated total glutathione (Fig. 2*E*), and GSSG increased by more than 4-fold relative to untreated cells at time 0 (Fig. 2*G*). Surprisingly, upon GSSG treatment the *gsh1 glr1* mutant also displayed an accumulation of intracellular GSH (Fig. 2*F*). To exclude any possibility of GSH contamination being present in the GSSG stock solution used for this experiment, a separate GSSG stock solution was treated with 2-vinylpyridine to derivatize any GSH that may have been present at trace levels. Comparable data were obtained for GSSG pretreated with 2-vinylpyridine (Fig. 2, *E* and *F*). The lower level of GSH and total glutathione, but similar level of GSSG in the *gsh1 glr1* mutant may indicate that cells degrade excess GSSG, and/or in the *gsh1 glr1* mutant uptake of GSH cannot keep pace with oxidation of GSH to GSSG followed by degradation of GSSG. These data indicate that the increase in GSH level observed in the *glr1* mutant treated with GSSG was due to the presence of a Glr1p-independent system capable of reducing GSSG to GSH.

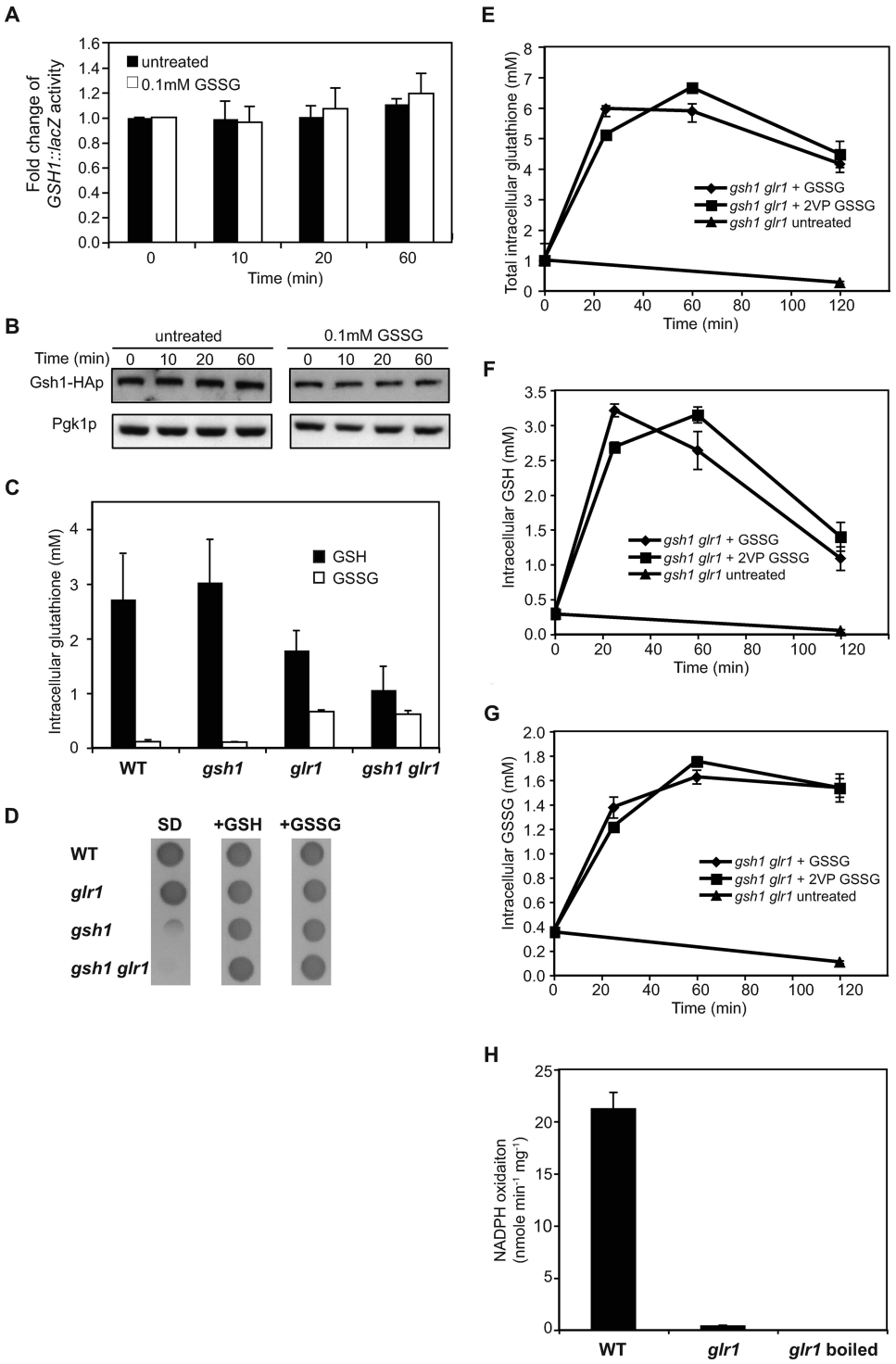

FIGURE 2. **Extracellular GSSG leads to an increase in intracellular GSH in a *GSH1*- and *GLR1*-independent manner.** *A*, exponentially growing *glr1* cells transformed with the *GSH1*::*lacZ* construct were treated with 100 μM GSSG and assayed for β-galactosidase activity. *B*, chromosomal copy of *GSH1* in the *glr1* mutant was tagged with HA. Exponentially growing cells were treated with 100 μM GSSG over the indicated time, and the cell lysate analyzed for Gsh1-HAp using anti-HA antibodies. Pgk1p was detected using anti-Pgk1p antibodies as a loading control. *C*, indicated mutants were grown to A_{600} 0.5 in SD medium supplemented with 25 μM GSH. Cells were harvested and analyzed for intracellular GSH and GSSG levels. *D*, indicated strains were grown for 2 days and A_{600} adjusted to 1.0 before 5 μl of the diluted cultures were spotted onto SD plates containing no glutathione, 25 μM GSH, or 25 μM GSSG. *E–G*, cells of *gsh1 glr1* were treated with 100 μM GSSG (*diamond*), 100 μM GSSG treated with 2-vinyl-pyridine (*square*), or untreated (*triangle*). *E*, intracellular total glutathione; *F*, GSH; and *G*, GSSG were determined at the indicated time. *Error bars* indicate the S.E. of four samples of two independent experiments. *H*, GSSG reductase activity in *glr1* cell lysate. Wild-type and *glr1* cells were grown to exponential phase, and the cell lysate of each strain was assayed for GSSG reductase activity.

Presence of Residual GSSG Reductase Activity in the glr1 Mutant—To further examine the Glr1p-independent GSSG reductase activity *in vitro*, whole cell lysates of the wild-type and *glr1* strains were assayed for their ability to reduce GSSG using an NADPH oxidation assay.

The cell lysate derived from *glr1* cells displayed some residual GSSG-dependent oxidation of NADPH (~2% of wild-type cells; Fig. 2*H*), indicating the presence of residual GSSG reductase activity. This residual Glr1p-independent GSSG reductase activity was NADPH-dependent because a GSSG-dependent oxidation of NADH was not observed (data not shown). Cell lysates derived from *glr1* cells that were subjected to heating (100 °C, 10 min) did not exhibit residual GSSG reduction activity (Fig. 2*H*), indicating that the residual Glr1p-independent GSSG reductase activity was heat labile and may be due to the presence of a protein in cells, other than Glr1p, with the capacity to reduce GSSG to GSH.

The *in vivo* data and the total cell lysate analysis (Fig. 2) indicate that *S. cerevisiae* has an alternative system to Glr1p that reduces GSSG in a NADPH-dependent manner. Although the residual GSSG reductase activity was relatively low, based on the *in vitro* assay, it still provided sufficient activity to reduce GSSG *in vivo*, as observed in the *gsh1 glr1* cells (Fig. 2). We surmised that there are two Glr1p-independent systems that could possibly reduce GSSG to GSH in *S. cerevisiae*, both of which are dependent on NADPH. These are the glutaredoxin and the thioredoxin systems.

Grx1p, Grx2p, Grx6p, and Grx7p Do Not Contribute to Reduction of GSSG in Cells Deleted of GLR1—Recently, it was demonstrated that mammalian glutaredoxin 2 (Grx2) can use thioredoxin reductase to reduce several substrates, including GSSG. This indicates that apart from the conventional glutathione reductase pathway, the glutaredoxin-thioredoxin reductase path-

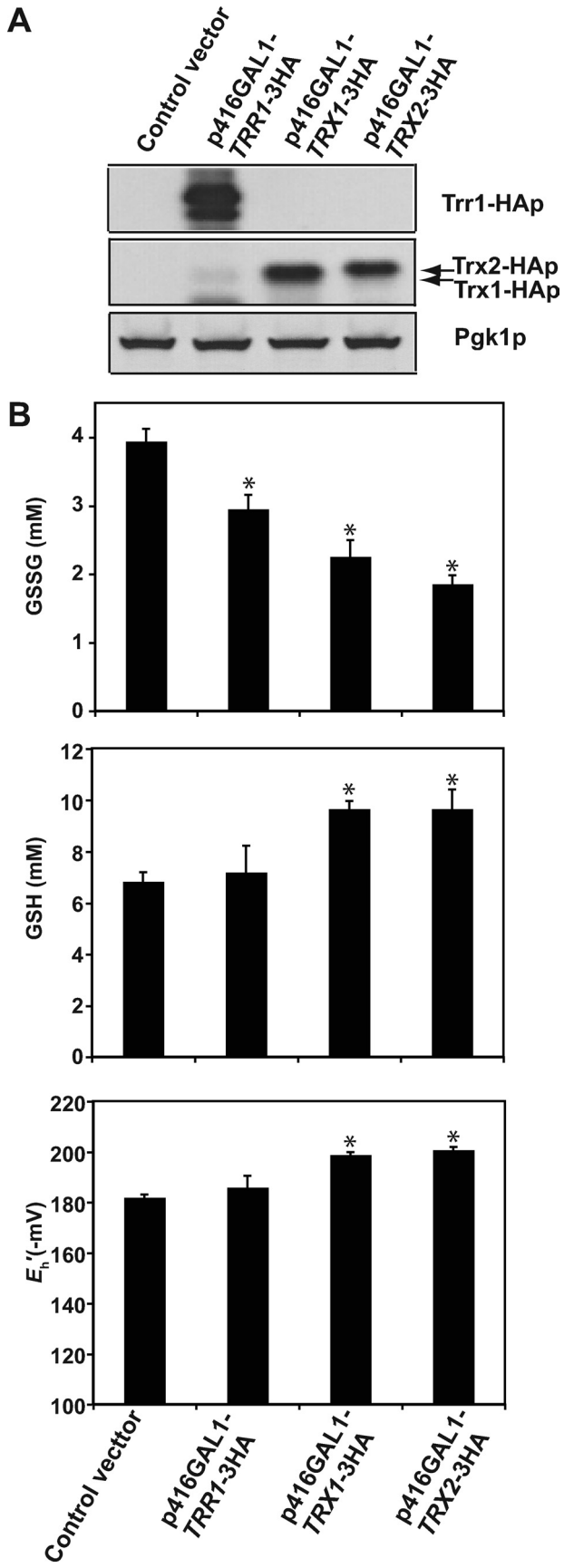

FIGURE 3. **The thioredoxin-thioredoxin reductase system can reduce GSSG *in vivo*.** *A* and *B*, overexpression of *TRR1*, *TRX1*, and *TRX2* in *glr1* cells reduces intracellular GSSG. The *glr1* mutant was transformed with pGAL1-TRR1–3XHA, pGAL1-TRX1–3XHA, or pGAL1-TRX2–3XHA. The transformed cells were grown to stationary phase in SD minus uracil medium and re-inoculated into SD-GAL minus uracil medium, and the cultures were allowed to grow for 18 h to A_{600} 0.5–1.0 before harvesting. *A*, Western blot analysis (*top*) of cell lysate using anti-HA antibody. *B*, intracellular concentration of GSSG, GSH, and glutathione E_h' of the indicated strains. *Error bars* indicate S.E. from six replicates from two independent experiments. *Asterisks* (*) indicate significant differences (p value <0.05) compared with the *glr1* mutant harboring the control vector.

way may be able to act as an alternate system to regenerate GSH from GSSG. Grx2 has a CSYC motif at its active site, and mutation of the second cysteine residue to serine (CSYC to CSYS) does not abolish Grx2 activity (47). In *S. cerevisiae*, *GRX6* encodes a monothiol glutaredoxin containing the CSYS motif. Furthermore it was shown that glutaredoxins Grx1p, Grx2p, Grx6p, and Grx7p displayed activity toward the artificial disulfide substrate hydroxyethyl disulfide (HEDS) (26, 32), indicating that these proteins may be able to reduce GSSG. Therefore, to investigate the possible involvement of these glutaredoxins in reducing GSSG, their respective genes were deleted in the *glr1* strain background. Monothiol glutaredoxins Grx3p, Grx4p, and Grx5p were not included in these analyses because they do not contain the CSYS motif and do not display activity toward HEDS (48, 49).

The *grx6 grx7 glr1*, *grx1 grx2 glr1*, and *grx1 grx2 grx6 grx7 glr1* cells did not show any obvious growth defect in SD medium (supplemental Fig. S1), and cell lysates of these strains still retained GSSG reductase activity at a level comparable to that of the *glr1* strain (supplemental Fig. S1) indicating that the Grx1p, Grx2p, Grx6p, and Grx7p do not contribute significantly to the residual reductase activity observed in *glr1* cells.

The Thioredoxin-Thioredoxin Reductase System Can Reduce GSSG to GSH—Cells deleted for *TRR1* or *TRX1* and *TRX2* overaccumulate oxidized as well as reduced glutathione (18, 36). Furthermore, the *trr1 glr1* and the *trx1 trx2 glr1* mutants are inviable (18, 50), indicating that there is a close relationship between the thioredoxin and the glutathione systems. It was recently demonstrated that Trx1p of *S. cerevisiae* is able to reduce GSSG *in vitro* (51), indicating a potential role of the thioredoxin in reducing GSSG to GSH *in vivo*. To investigate the possible role of the thioredoxin-thioredoxin reductases in GSSG reduction *in vivo*, the *TRX1*, *TRX2*, or *TRR1* genes were separately placed under the control of the *GAL1* promoter, overexpressed in the *glr1* mutant, and cell lysates were analyzed for GSSG reductase activity, intracellular GSH/GSSG levels, and the cellular glutathione E_h' were estimated.

Overexpression of *TRR1*, *TRX1*, and *TRX2* was each verified using Western blot analysis (Fig. 3*A*). Overexpression of *TRR1*, *TRX1*, or *TRX2* led to a significant (p value < 0.05) reduction in cellular GSSG level, and there was a significant increase (p value < 0.05) in GSH levels when *TRX1* and *TRX2* were overexpressed (Fig. 3*B*). The *glr1* cells overexpressing *TRX1* or *TRX2* displayed a more reducing environment as indicated by the glutathione E_h' (Fig. 3*B*, *bottom panel*).

To further examine the relative contribution of Trx1p and Trx2p in reduction of GSSG *in vivo*, the *glr1*, *trx1 glr1*, and *trx2 glr1* mutants were treated with extracellular GSSG and examined for intracellular glutathione concentration. The *trx1 glr1*

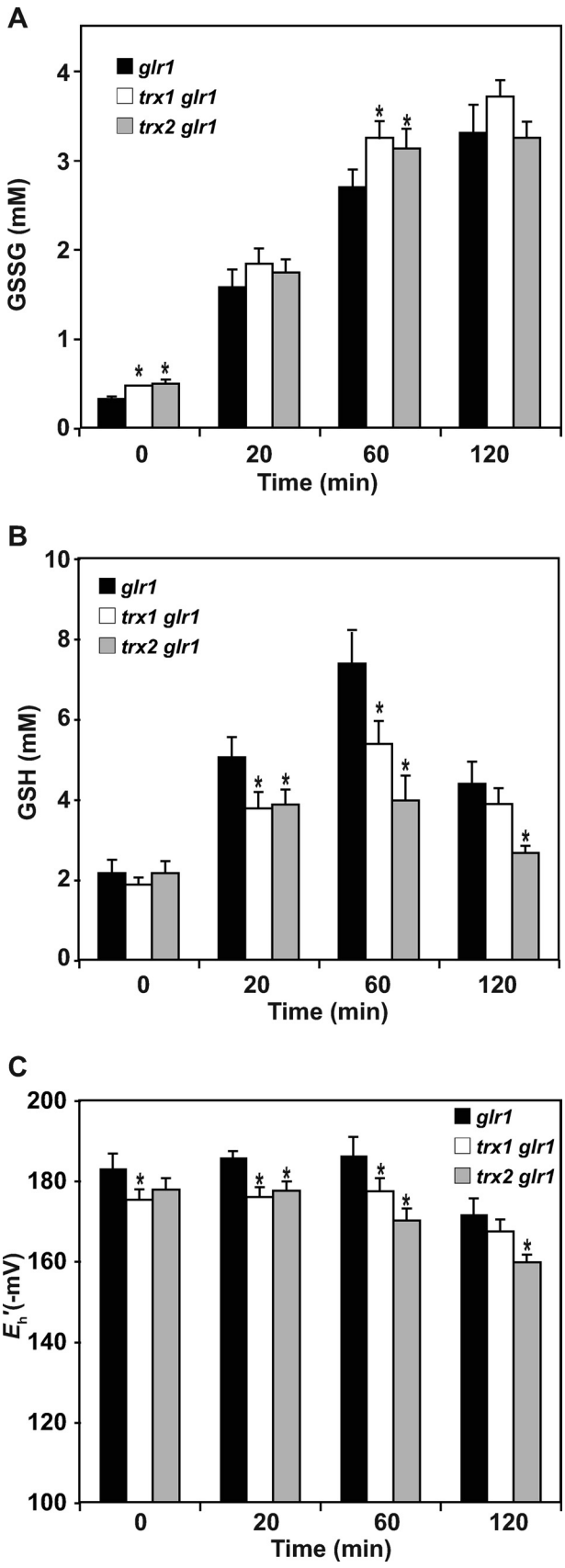

FIGURE 4. **The *trx1 glr1* and *trx2 glr1* strains have a decreased capacity to convert GSSG to GSH compared with the *glr1* strain *in vivo*.** The indicated strains were grown to exponential phase (A_{600}, 0.5), in SD medium treated with 100 μM GSSG, and intracellular glutathione was determined over the indicated time. *A*, GSSG and *B*, GSH of the indicated strains were determined. *C*, cellular glutathione E_h' was determined over the indicated time. *Error bars* indicate the S.E. of six biological replicates from two independent experiments. *Asterisks* (*) indicate significant differences (p value < 0.05) compared with the *glr1* mutant at the indicated time.

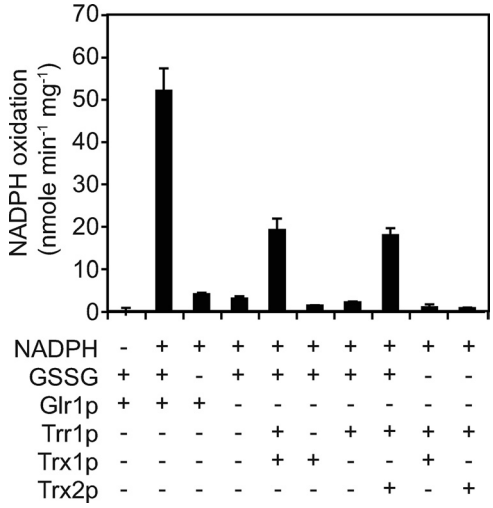

FIGURE 5. **Trx1p and Trx2p can facilitate reduction of GSSG to a similar level *in vitro*.** Purified proteins were reconstituted with the indicated compounds, and the rate of NADPH oxidation to $NADP^+$ was monitored at 340 nm. *Error bars* indicate the S.D. of three replicates.

and the *trx2 glr1* mutant had a small but significantly higher level of intracellular GSSG relative to the *glr1* mutant in untreated conditions (*Time 0*, Fig. 4*A*). Upon GSSG treatment, the *trx1 glr1* and the *trx2 glr1* mutant accumulated slightly more GSSG at 60 min (Fig. 4*A*) and displayed less GSH accumulation compared with the *glr1* mutant at 20 and 60 min (Fig. 4*B*). Furthermore, *glr1* cells deleted for *TRX1* or *TRX2* displayed a more oxidizing environment as determined using the glutathione E_h' compared with the *glr1* single mutant (Fig. 4*C*).

To investigate the relative efficacy of Trx1p and Trx2p in GSSG reduction, *in vitro* reduction of GSSG was examined in the presence of purified Trr1p, NADPH, Trx1p, or Trx2p. To compare the efficacy of GSSG reduction by the thioredoxin-thioredoxin reductase system with Glr1p, purified Glr1p was also examined.

The data from Fig. 5 indicate that Trr1p cannot reduce GSSG without the presence of Trx1p or Trx2p. The Trx1p-Trr1p and Trx2p-Trr1p combinations reduced GSSG with similar efficacy (~18–19 nmol/min/mg), although at a much lower rate compared with Glr1p (52 nmol/min/mg). The respective V_{max} (μmol/min/mg) and K_m (mM) values for GSSG reduction were as follows: Trx1p (V_{max} = 101.4; K_m 2.6), Trx2p (V_{max} = 101; K_m = 2.5), and Glr1p (V_{max} = 118; K_m 0.08). These data indicate that the thioredoxin-thioredoxin system is able to reduce GSSG, although Glr1p is much more efficient in doing so.

The above data support the role of thioredoxin in reducing GSSG *in vivo*. A single thioredoxin is able to support the conversion of GSSG to GSH *in vivo*, because both the *trx1 glr1* and the *trx2 glr1* mutants were still able to accumulate GSH in the presence of excess GSSG, and the *in vitro* data indicate that Trx1p and Trx2p reduces GSSG at a similar efficacy.

DISCUSSION

Glutathione is the major low molecular weight redox buffer in most cells (1). Understanding the mechanism that controls the production of GSH and the recycling of GSSG in cells will provide further insight into how cellular redox homeostasis is maintained. Here, it was demonstrated that cells have robust mechanisms to maintain cellular glutathione redox homeostasis. Excess oxidized glutathione is readily converted to GSH, mainly through the activity of the glutathione reductase Glr1p. The *in vivo* data presented here also strongly indicate that excess GSSG leads to an increase in cellular GSH level, and this increase is independent of *GSH1* and can occur in the absence of glutathione reductase encoded by *GLR1.* The presence of an alternative system to reduce GSSG in *S. cerevisiae* was demonstrated, and this system is likely to be the cytosolic thioredoxin-thioredoxin reductase system.

Several pieces of evidence support the role of GSSG reduction to GSH by the thioredoxin-thioredoxin reductase system. First, it was demonstrated that overexpression of either of the cytosolic thioredoxins Trx1p or Trx2p, or the thioredoxin reductase Trr1p led to a decrease in intracellular GSSG level in the *glr1* mutant *in vivo*. Second, deletion of *TRX1* or *TRX2* in the *glr1* mutant treated with GSSG reduced the accumulation of GSH compared with the *glr1* mutant alone. Furthermore, it was previously shown that in *S. cerevisiae*, the *trx1 trx2* double mutant and the *trr1* mutant overaccumulate both oxidized and reduced glutathione. In addition, the *trr1 glr1* double mutant and the *trx1 trx2 glr1* triple mutants are inviable (18), indicating that the thioredoxin-thioredoxin reductase system overlaps with the glutathione reductase system. These data indicate that the cytosolic thioredoxin-thioredoxin reductase system can prevent the overaccumulation of GSSG in cells lacking the glutathione reductase Glr1p. The synthetic lethality associated with simultaneous disruption of the thioredoxin-thioredoxin reductase and glutathione reductase systems makes it difficult to determine the extent to which, or indeed whether or not, other systems contribute in reduction of GSSG *in vivo*. In addition the role that additional factors including GSH synthesis, GSH/GSSG degradation, transport of GSH/GSSG between cellular compartments, and/or formation/turnover of glutathione-protein mixed disulfides play in maintenance of overall cellular and organellar redox homeostasis requires further investigation.

Although thioredoxins have been shown to reduce a broad spectrum of substrates, GSSG is generally not considered to be a good substrate for thioredoxins (19, 35). It has been demonstrated that the thioredoxin-thioredoxin reductase system in the malaria parasite *Plasmodium falciparum* and fruit fly *Drosophila melanogaste*r can convert GSSG to GSH *in vitro* (52, 53). Recently the second order rate constant of GSSG reduction by thioredoxin 1 and thioredoxin reductases in *S. cerevisiae* was determined by *in vitro* reconstitution of the purified proteins (51). It was shown that purified yeast thioredoxin 1 and thioredoxin reductase can reduce GSSG with a second order rate constant of 6 mM^{-1} min^{-1}. This rate constant of the thioredoxin-thioredoxin reductase system in *S. cerevisiae* is similar to that established for the analogous system in *Escherichia coli* and comparable to that of *D. melanogaster* (10 mM^{-1} min^{-1}) (51–53). These rate constants are at least ~100–400 lower when comparing the reduction of GSSG to the reduction of insulin as a substrate by the thioredoxin-thioredoxin reductase system (19). However, given that GSSG may accumulate in the millimolar range, it was proposed that this rate is physiologically significant, and the reaction may occur *in vivo* (53). Additionally, the activity for GSSG reduction by the thioredoxins *versus* glutathione reductase measured *in vitro* using recombinant forms of the enzymes, or native forms isolated from cells may also differ to that of the native forms of the respective enzymes in intact cells. Differences in the availability of NADPH, GSSG, and/or other factors, and the presence of competing side reactions may also influence the relative contribution of the glutathione reductase and thioredoxin systems for reduction of GSSG *in vivo*. The contribution of the Trr1p/Trx1,2p system is likely to become more important when cells experience certain stress conditions (*e.g.* oxidative) where the burden on the glutathione redox system is known to increase and GSSG overaccumulation may ensue (7, 8, 14, 15). The observations that: 1) the *gsh1 glr1* mutant can utilize GSSG as the sole source of glutathione; 2) the *gsh1 glr1* strain accumulates GSH when challenged with exogenous GSSG and that overexpression of thioredoxins or thioredoxin reductase can reduce GSSG accumulation in *glr1* cells; and 3) deletion of a single thioredoxin reduces the accumulation of GSH in the presence of excess GSSG all provide further support that the thioredoxin-thioredoxin reductase system can function to reduce GSSG to GSH *in vivo*. In a recent study of *Arabidopsis*, it was demonstrated that mutants lacking an isoform of glutathione reductase still exhibit dynamic reduction of GSSG *in vivo* under oxidative stress conditions (54). It was also demonstrated that the *Arabidopsis* thioredoxins were capable of reducing GSSG *in vitro*. These findings led to the proposal that the thioredoxin-thioredoxin reductase system may function as a backup for cytosolic glutathione reductase in *Arabidopsis.* Therefore, it is likely that the thioredoxins-thioredoxin reductase system and the glutathione reductase system function to reduce GSSG not only in plants and yeast, but also in a similar manner in other species.

Acknowledgments—We thank Anita Ayer for providing the grx6 and grx7 mutants and Geoff Kornfeld for helpful suggestions.

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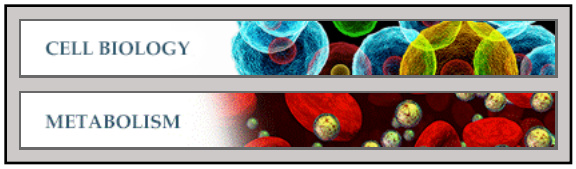
Cell Biology:

The Thioredoxin-Thioredoxin Reductase System Can Function *in Vivo* as an Alternative System to Reduce Oxidized Glutathione in Saccharomyces cerevisiae



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J. Biol. Chem. 2010, 285:6118-6126.
doi: 10.1074/jbc.M109.062844 originally published online December 1, 2009