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The Yeast *Saccharomyces cerevisiae* Contains Two Glutaredoxin Genes That Are Required for Protection against Reactive Oxygen Species

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Glutaredoxins are small heat-stable proteins that act as glutathione-dependent disulfide oxidoreductases. Two genes, designated *GRX1* and *GRX2*, which share 40–52% identity and 61–76% similarity with glutaredoxins from bacterial and mammalian species, were identified in the yeast *Saccharomyces cerevisiae*. Strains deleted for both *GRX1* and *GRX2* were viable but lacked heat-stable oxidoreductase activity using β-hydroxyethylene disulfide as a substrate. Surprisingly, despite the high degree of homology between Grx1 and Grx2 (64% identity), the *grx1* mutant was unaffected in oxidoreductase activity, whereas the *grx2* mutant displayed only 20% of the wild-type activity, indicating that Grx2 accounted for the majority of this activity in vivo. Expression analysis indicated that this difference in activity did not arise as a result of differential expression of *GRX1* and *GRX2*. In addition, a *grx1* mutant was sensitive to oxidative stress induced by the superoxide anion, whereas a strain that lacked *GRX2* was sensitive to hydrogen peroxide. Sensitivity to oxidative stress was not attributable to altered glutathione metabolism or cellular redox state, which did not vary between these strains. The expression of both genes was similarly elevated under various stress conditions, including oxidative, osmotic, heat, and stationary phase growth. Thus, Grx1 and Grx2 function differently in the cell, and we suggest that glutaredoxins may act as one of the primary defenses against mixed disulfides formed following oxidative damage to proteins.

INTRODUCTION

Glutaredoxin from *Escherichia coli* was first discovered as a small, heat-stable protein required for the glutathione-dependent synthesis of deoxyribonucleotides catalyzed by ribonucleotide reductase (Holmgren, 1976). Glutaredoxin 1 is a 9-kDa protein that acts as a reduced glutathione (GSH)-dependent disulfide oxidoreductase by virtue of the two cysteine residues in its active site (Holmgren and Aslund, 1995). Later studies in mutants that lacked both glutaredoxin and thioredoxin revealed that *E. coli* actually contains three glutaredoxins (Grx1–3), with glutaredoxin 3 also able to function in ribonucleotide synthesis (Aslund *et al.*, 1994). In contrast, glutaredoxin 2 was proposed to be the first member of a novel class of glutaredoxins that lack activity as hydrogen donors for ribonucleotide reductase (Vlamis-Gardikas *et al.*, 1997). Glutaredoxins have subsequently been identified and isolated from various eukaryotes, including human, bovine, pig, yeast, and rice (Minakuchi *et al.*, 1994; Holmgren and Aslund, 1995). The structure of these proteins has been highly conserved throughout evolution, particularly in the region of the active site (Wells *et al.*, 1993; Holmgren and Aslund, 1995). However, despite extensive structural analysis, little is known regarding the biochemical function of these eukaryotic glutaredoxins in vivo.

There appears to be considerable functional overlap between the glutaredoxin and thioredoxin systems. Similar to glutaredoxin, thioredoxin is a small, heat-stable protein that contains two redox-active cysteines in its active site and can serve as an electron donor for ribonucleotide reductase. The oxidized disulfide form

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of thioredoxin is reduced by NADPH and thioredoxin reductase, an enzyme that is a member of the FAD-containing pyridine disulfide oxidoreductase class of proteins. In contrast, the glutaredoxin system consists of NADPH, GSH, and glutathione reductase with electrons being transferred from NADPH to glutaredoxin via GSH (Holmgren, 1990). Utilization of GSH results in its conversion to the disulfide form, and it is regenerated in an NADPH-dependent reaction catalyzed by glutathione reductase (Grant and Dawes, 1996). Glutaredoxin shows no activity with thioredoxin reductase, and similarly, thioredoxins are not reduced by GSH and glutathione reductase (Holmgren, 1979). In addition to their function in ribonucleotide reduction, glutaredoxins and thioredoxins have proposed roles in many cellular processes, including reduction of dehydroascorbate, protein folding and regulation, and sulfur metabolism (Holmgren, 1989; Wells *et al.*, 1993).

The yeast *Saccharomyces cerevisiae* contains two genes encoding thioredoxins, designated *TRX1* and *TRX2*, which are dispensable under normal growth conditions (Gan, 1991; Muller, 1991). However, deletion of *TRX1* and *TRX2* affects the cell cycle, resulting in a prolonged S phase and a shortened G_1 phase, which does not occur as a result of alterations in the levels of deoxyribonucleotides (Muller, 1991, 1995). In addition, a *trx1 trx2* double mutant cannot grow in the absence of methionine or cysteine, presumably because of a defect in sulfate assimilation, indicating that thioredoxin is the only hydrogen donor for 3′-phosphoadenosine 5′-phosphosulfate reductase in yeast (Muller, 1991). Thioredoxins are also required to maintain the redox balance of GSH, and loss of *TRX1* and *TRX2* results in elevated levels of oxidized glutathione (GSSG), indicating a link between thioredoxin and GSH with the redox status of the cell (Muller, 1996). In yeast, a single thioltransferase (glutaredoxin) has been identified and purified, which was later cloned, sequenced, and designated *TTR1* (Gan *et al.*, 1990; Gan, 1992). Here, we show that yeast actually contains two genes encoding glutaredoxins, and that their gene products are required for protection during conditions of oxidative stress. This is the first in vivo demonstration of a requirement for glutaredoxins in protection against reactive oxygen species.

MATERIALS AND METHODS

Yeast Strains and Media

The *S. cerevisiae* strains used in this study were CY4 (Grant *et al.*, 1996b) and its isogenic derivatives Y70 (*grx1*), Y100 (*grx2*), and Y117 (*grx1 grx2*) described below.

Strains were grown in rich YEPD medium [2% (wt/vol) glucose, 2% (wt/vol) bactopeptone, and 1% (wt/vol) yeast extract] or minimal SD medium [0.17% (wt/vol) yeast nitrogen base without amino acids, 5% (wt/vol) ammonium sulfate, and 2% (wt/vol) glucose (Sherman *et al.*, 1974)] supplemented with appropriate amino acids and bases: 2 mM leucine, 4 mM isoleucine, 1 mM valine, 0.3 mM histidine, 0.4 mM tryptophan, 0.15 mM adenine, and 0.2 mM uracil. For growth on nonfermentable carbon sources, YEPGE contained 3% (vol/vol) glycerol and 1% (vol/vol) ethanol. Media were solidified by the addition of 2% (wt/vol) agar.

Cloning and Disruption of GRX1 and GRX2

The *GRX1* and *GRX2* genes were isolated by PCR amplification of total yeast DNA with oligonucleotides specific for *GRX* sequences. For *GRX1*, a 1617-bp fragment was amplified using oligonucleotides that hybridized 735 bp upstream of the putative ATG start codon (5′-GCCTCGAGAGATGAACAGATCCAAG-3′) and 549 bp downstream of the TAG stop codon (5′-ACTACTCGTGTTCATCTTGGACA-3′) respectively. A 1246 bp fragment was cloned into the polylinker region of plasmid pRS426 (Christianson *et al.*, 1992) using an *Xho*I restriction site introduced by the 5′ oligonucleotide (underlined) and a naturally occurring *Eco*RI site. The resulting construct was called pG501 and was verified by DNA sequence analysis. For *GRX2*, a 1436 bp fragment was amplified using oligonucleotides that hybridized 941 bp upstream of the putative ATG start codon (5′-GTTGCACAAAGATATCGATAACCCG-3′) and 163 bp downstream of the TAG stop codon (5′-CGGAATTCAGCGGGTCTCATTGGT-3′), respectively. The amplicon was cloned into the polylinker region of plasmid pRS424 (Christianson *et al.*, 1992) using an *Eco*RI restriction site introduced by the 3′ oligonucleotide (underlined) and a naturally occurring *Cla*I site. The resulting construct was called pL4 and was verified by DNA sequence analysis.

The *GRX1* disruption construct pG507 was made by insertion of a 1.6-kb *Bam*HI fragment containing the yeast *LEU2* gene, isolated from plasmid YDp-L (Berben *et al.*, 1991), into the *Bgl*II restriction site in pG501, which lies 53 bp downstream from the *GRX1* ATG start codon. The 2.8-kb *Pst*I fragment from pG507 was used to direct homologous recombination at the *GRX1* locus, creating strain Y100 (*grx1*). A null allele of *GRX2* (strain Y100) was generated in strain CY4 by a one-step PCR amplification protocol that replaced the entire *GRX2* open reading frame (ORF) with the yeast *HIS3* gene (Baudin *et al.*, 1993). The oligonucleotides used for the PCR disruption of *GRX2* were GRX2-D1 and GRX2-D2, the sequences of which were 5′-TTTGCCACAAGAATTATTGCTAAAAGATTTTTATCTACTCCAAAAAGCGCTAGGAGTCACTGCCA-3′ and 5′-TATATATATGTAAATATTATGAAGGGGATATTAGCGTAATTTAAAGGAAAGCGCGCCTCGTTCAG-3′ respectively. The underlined regions correspond to *HIS3* sequences. The *grx1 grx2* double mutant strain (Y117) was generated by disrupting *GRX1*, using plasmid pG507, in strain Y100.

Sensitivity to Oxidants

Sensitivity to H_2O_2, *tert*-butyl hydroperoxide, cumene hydroperoxide, menadione, and diamide was determined by spotting strains onto YEPD plates containing various concentrations of oxidants (Grant *et al.*, 1997). Cells were grown to stationary phase in YEPD, and 10-μl aliquots of each culture, diluted to an A_{600} of 3.0 and 0.3, were spotted onto appropriate plates. Sensitivity was determined by comparison of growth with the wild-type strain after 3 d. Dose-response curves were obtained by growing cells to exponential phase (1–2 $\times$ 10^7 cells/ml) in SD medium at 30°C and treating with 4 mM H_2O_2 or 18 mM menadione for 1 h. Aliquots of cells were diluted in fresh YEPD medium at 20-min intervals and plated in triplicate on YEPD plates to obtain viable counts after 3 d of growth.

β-Galactosidase Assays

The *GRX1::lacZ* fusion construct was made by inserting the 744-bp *Pst*I-*Bgl*II fragment of pG501 into the same sites of YIp358R (Berben *et al.*, 1991). The resulting fusion construct, designated pL1, contains the ATG codon and 17 codons from the *GRX1* coding region inserted in frame with the *lacZ* gene. The *GRX2::lacZ* fusion construct was made by insertion of a 1-kb PCR fragment amplified using

oligonucleotides 5-GTTGCACAAAGAATTCGATAACCCG-3′ and 5′-CCTTGGATCCGGGAACGTTCAATTC-3′ into YIp357 (Berben *et al.*, 1991). The amplicon was cloned into the polylinker region of YIp357 using *Eco*RI and *Bam*HI restriction sites introduced by the oligonucleotides (underlined). The resulting fusion construct, designated pL2, contains the ATG codon and 44 codons from the *GRX2* coding region inserted in frame with the *lacZ* gene.

For the determination of β-galactosidase activity, transformants were assayed essentially as described previously (Rose and Botstein, 1983). Cells were grown to early exponential phase (A_{600} = 1) at 30°C before treatment with various stress conditions. Cells were exposed to 0.3 mM H_2O_2, 18 mM menadione, or 1.5 mM diamide for 1 h. Heat shock conditions were 39°C for 30 min, and osmotic shock conditions were 0.4 M sodium chloride for 30 min. β-Galactosidase activity is expressed as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per microgram of total protein.

RNA Analysis

For Northern analysis, cell cultures were grown to an A_{600} of 1.0, and RNA was extracted by the method of Schmitt *et al.* (1990). Total RNA (10 μg) was separated by electrophoresis in a 1% formaldehyde gel. Nitrocellulose filters were probed for *GRX1* using a 744-bp *Pst*I-*Bgl*II DNA fragment from pG501 and for *GRX2* using the 1-kb PCR fragment used in the construction of pL2. Loading controls were probed with a 1.7-kb *Hin*dIII fragment of the *PDA1* gene (Wenzel *et al.*, 1995). Quantitation of transcript levels was made using a Molecular Dynamics (Sunnyvale, CA) PhosphorImager.

Determination of Thiol Levels and Glutathione Reductase Activity

Glutathione reductase activity was determined by the method of Casalone *et al* (1988) and is expressed as nanomoles of NADPH oxidized per minute per milligram of protein. Total glutathione, GSH, and GSSG were determined by a microtiter plate assay method (Vandeputte *et al.*, 1994). Free thiol groups were measured using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; Rice-Evans *et al.*, 1991) and are expressed as micromoles per milligram of total protein.

GSH-dependent Disulfide Oxidoreductase Activity

Glutaredoxin activity was measured by the reduction of the mixed disulfide formed between β-hydroxyethylene disulfide (HED) and GSH (Holmgren and Aslund, 1995). Cell-free extracts were prepared by breaking cells with glass beads using a Minibead beater (Biospec Scientific, Bartlesville, OK) for 30 s at 4°C. Where indicated, extracts were heat treated at 85°C for 5 min to inactivate enzymes such as glutathione reductase, thioredoxin reductase, and other interfering, non-heat–stable activities (Holmgren, 1976). This was confirmed by the fact that the heat-treated extracts were entirely dependent on exogenous glutathione reductase for activity. The components of the glutaredoxin system, NADPH (0.4 mM), GSH (1 mM), and glutathione reductase (6 μg/ml), as well as HED (0.7 mM) were added to a reaction volume of 1 ml in 0.1 M Tris-Cl (pH 7.4). A mixed disulfide between HED and GSH is formed within 2 min, and the reaction was started by the addition of 10–100 μl of cell extracts. The reaction was followed by the decrease in A_{340} attributable to the oxidation of NADPH.

RESULTS

Identification of GRX1 Encoding Glutaredoxin 1

Analysis of the sequence of yeast chromosome III revealed an ORF of 110 codons (YCL35c, GenBank accession number x59720), the putative protein product of which exhibited significant similarity to known glutaredoxins. The predicted protein (named Grx1) shares 40–52% identity and 61–76% similarity over the entire sequence with glutaredoxins from *E. coli*, rice, and humans (Figure 1). In addition, *GRX1* is homologous to the previously identified yeast gene *TTR1*, encoding thioltransferase 1 (Gan *et al.*, 1990; Gan, 1992). *TTR1* shares 64% identity and 85% similarity with *GRX1* (Figure 1), and we propose renaming *TTR1* as *GRX2*, in accordance with the standard nomenclature suggested by Holmgren and Aslund (1995) for the thioltransferase class of proteins.

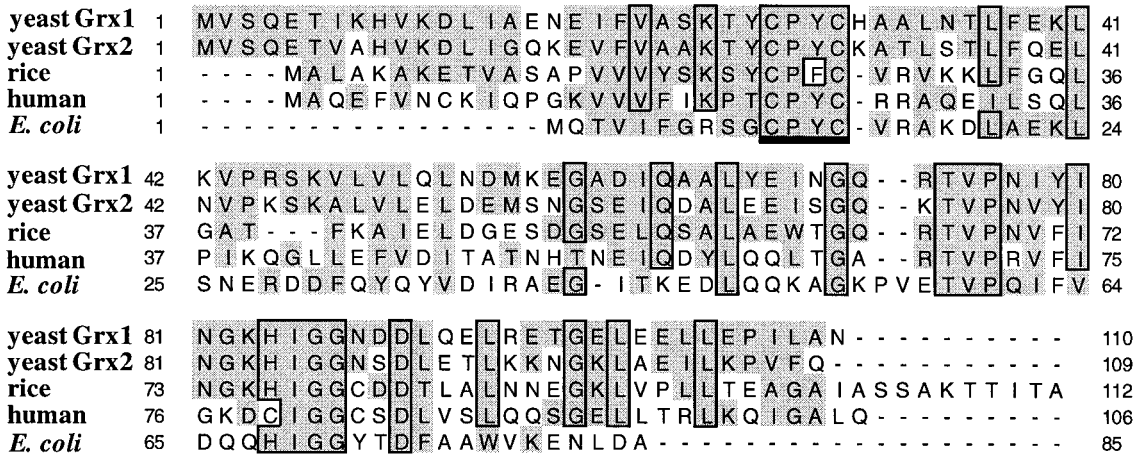

Figure 1. Comparison of the predicted amino acid sequence of *GRX1* and *GRX2* with glutaredoxins from rice, human, and *E. coli.* Amino acid sequences were aligned for maximal homology, with dashes used to denote gaps introduced for maximal alignment. Identical amino acid residues are boxed, and conserved residues are shaded. Alignments were performed using the program ClustalW, version 1.4 (Thompson *et al.*, 1994) and displayed using the graphic program seqVu 1.01 (J. Gardner, The Garvan Institute, Sydney, New South Wales, Australia). The active site sequence (CPYC) is underlined.

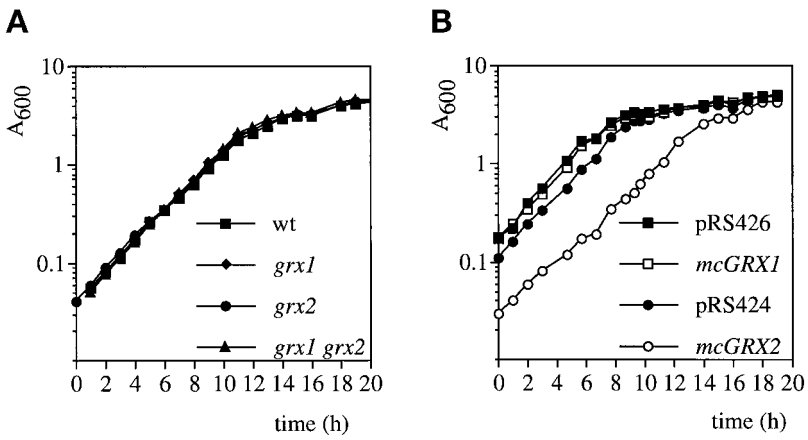

Figure 2. Growth of glutaredoxin strains. (A) Strains CY4 (wt), Y70 (*grx1*), Y100 (*grx2*), and Y117 (*grx1 grx2*) and (B) CY4 transformed with pRS426, mc*GRX1*, pRS424, and mc*GRX2* were grown in minimal medium (SD), and growth was monitored by optical density at 600 nm (A_{600}). Strains were inoculated to the same cell density to start each experiment (our unpublished results).

The active site of glutaredoxins contains two redox-active cysteine residues that are conserved in *GRX1* and *GRX2* (positions 27 and 30 in yeast numbering). Unlike microbial and plant glutaredoxins, animal glutaredoxins contain an additional half-cysteine pair in the pentapeptide Cys^{79}-Ile-Gly-Gly-Cys^{83} (human numbering) (Hopper *et al.*, 1989) that are not conserved in the yeast proteins. Interestingly, the intervening tripeptide (Ile-Gly-Gly) is conserved in plant and microbial glutaredoxins, as well as in *GRX1* and *GRX2*, and the C-terminal cysteine has been replaced by a conserved histidine residue (Figure 1; Hopper *et al.*, 1989). Conserved regions are also found at positions 74–76 and 84–87 (yeast numbering), which as well as the region around the active site have been proposed to play a role in interactions with other proteins, including ribonucleotide reductase (Xia *et al.*, 1992).

Yeast Strains Deleted for GRX1 and GRX2 Are Viable

A strain carrying a disruption of *GRX1* was generated by insertion of the yeast *LEU2* gene within the coding region of *GRX1* in the haploid wild-type strain CY4 (see Materials and Methods). A strain containing a total gene deletion of *GRX2* was generated by replacement of the entire *GRX2* ORF with the yeast *HIS3* gene. Finally, a double mutant strain lacking both *GRX1* and *GRX2* was generated (*grx1 grx2*). The resulting strains were all viable, indicating that *GRX1* and *GRX2* are not essential for normal aerobic growth. In addition, the glutaredoxin mutants showed wild-type growth rates on rich glucose-based medium (YEPD) and on nonfermentable carbon sources such as glycerol. The glutaredoxin mutants were also able to grow at the same growth rate on minimal medium (SD), indicating that sulfate can be assimilated as the sole source of sulfur (Figure 2A). The effect of overexpressing *GRX1* or *GRX2* on the growth of wild-type cells (CY4) was examined by growing cells containing multicopy *GRX1* (mc*GRX1*) or *GRX2* (mc*GRX2*) on minimal SD medium (Figure 2B). mc*GRX1* did not affect the growth of CY4 (compare pRS426 with mc*GRX1*), whereas mcGRX2 resulted in a marked lag phase and reduced growth rate (compare pRS424 with *mcGRX2*). In addition, when the growth of these strains was compared by streaking for single colonies on SD plates, cells containing mc*GRX2* grew extremely slowly, forming small pinpoint colonies with occasional faster-growing revertants.

Grx1 and Grx2 Display GSH-Disulfide Oxidoreductase Activity

The oxidoreductase activity of glutaredoxins can be measured by their ability to reduce the mixed disulfide formed between GSH and HED (Holmgren and Aslund, 1995). Briefly, the assay contains HED, GSH, and glutathione reductase, and the reaction rate is followed by the oxidation of NADPH. Both the wild-type and *grx1* mutant strains displayed similar GSH-dependent oxidoreductase activities during stationary phase growth (Table 1). In contrast, the *grx2* and *grx1*

Table 1. GSH-dependent disulfide oxidoreductase activity in glutaredoxin mutants

Strain	Stationary phase	Heat-treated[a] stationary	Heat-treated[a] exponential
wt	442 ± 40	118 ± 1	47 ± 10
grx1	435 ± 29	134 ± 4	46 ± 4
grx2	88 ± 22	21 ± 2	8 ± 2
grx1 grx2	66 ± 13	22 ± 5	ND

Glutaredoxin activity is given as nanomoles of NADPH oxidized per minute per milligram of protein. Data are the means of triplicate experiments. ND, not detectable.

[a] Extracts were heat treated at 85°C for 5 min (for details see text).

grx2 double mutants displayed only 20 and 15% of the wild-type activity. To ensure specificity of the assay for glutaredoxins, extracts were heat treated at 85°C for 5 min to inactivate enzymes such as glutathione reductase, thioredoxin reductase, and other interfering, non-heat–stable activities (Holmgren, 1976). This treatment should not affect glutaredoxins or thioredoxins, which are heat stable, and thioredoxin shows no activity in the HED assay because of its dependence on thioredoxin reductase. Exponential phase wild-type cells displayed GSH-dependent disulfide oxidoreductase activity (47 $nmol \cdot min^{-1} \cdot mg^{-1}$), which was elevated twofold in stationary phase cells (Table 1). The heat-stable oxidoreductase activity was unaffected in the *grx1* mutant during both exponential and stationary phase growth. In contrast, the activity fell to 17 and 18% of the wild-type levels in the *grx2* mutant during exponential and stationary phase growth, respectively. In the *grx1 grx2* double disruption strain, oxidoreductase activity was undetectable during exponential phase growth, and a low 18% activity was detectable during stationary phase growth. These results indicate that Grx2 accounts for the majority of GSH-dependent oxidoreductase activity in yeast.

To determine whether the differences in GSH-disulfide oxidoreductase activity could be accounted for by differential expression of *GRX1* and *GRX2*, transcript levels were measured by Northern blot analysis (Figure 3). The level of the *GRX1* transcript was approximately half that of the *GRX2* transcript in a wild-type strain, which is not sufficient to account for the differences in oxidoreductase activity observed. Interestingly, when *GRX2* was deleted, the level of the *GRX1* transcript increased by approximately threefold, which again did not correlate with the measured oxidoreductase activity. In agreement with the transcript analysis, the relative expression levels of *GRX1::lacZ* and *GRX2::lacZ* fusion constructs (see Figure 6) indicated that differences in expression of *GRX1* and *GRX2* could not account for the differences in oxidoreductase activity observed.

To address the question of whether Grx1 lacked GSH-disulfide oxidoreductase activity, assays were performed on strains containing multicopy *GRX1* (mc*GRX1*) or *GRX2* (mc*GRX2*). In wild-type cells grown to stationary phase, mc*GRX1* and mc*GRX2* resulted in an approximate twofold to threefold increase in heat-stable oxidoreductase activity (Table 2, compare pRS426 with mc*GRX1* and pRS424 with mc*GRX2*). Similarly, mc*GRX1* and mc*GRX2* resulted in a fourfold to sixfold increases in oxidoreductase activity during exponential phase growth. Because the increase in activity in the strain containing mc*GRX1* may have arisen because of an indirect effect on Grx2, activity was next determined in the *grx1 grx2* double mutant, which is devoid of glutaredoxin activity (Table 2). Again, both mc*GRX1* and mc*GRX2* resulted in significant increases in glutaredoxin activity; however, mc*GRX2* resulted in a fivefold greater increase compared with mcGRX1. These results indicate that both Grx1 and Grx2 possess GSH-dependent disulfide oxidoreductase activity. To examine the differences between Grx1 and Grx2 further, we next examined the response of *grx1* and *grx2* mutants to various stress conditions during which mixed disulfides are likely to be formed.

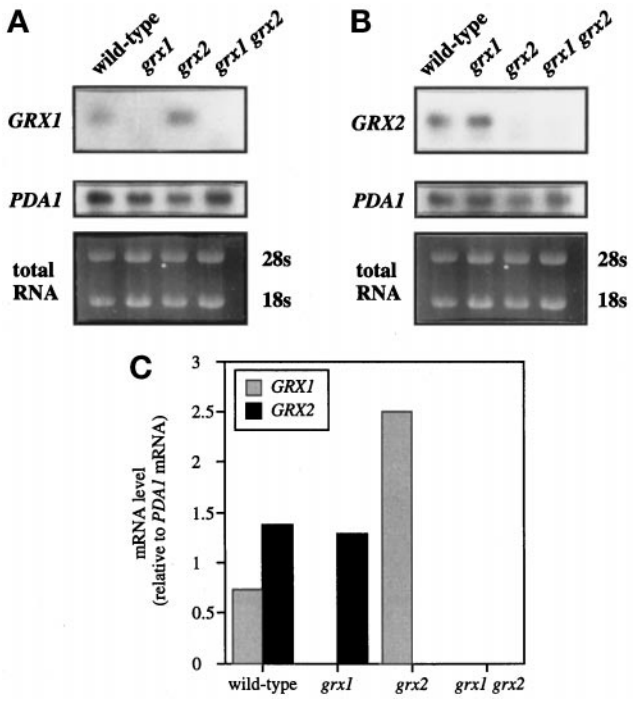

Figure 3. Transcript analysis of *GRX1* and *GRX2*. Total RNA was isolated from wild type, *grx1*, *grx2*, and the *grx1 grx2* double mutant grown to exponential phase and subjected to Northern blot analysis. The resulting blots were probed with *GRX1* (A) or *GRX2* (B) and *PDA1* as a loading control. mRNA hybridization signals were quantified for the representative experiments shown in A and B using a PhosphorImager, and the ratio of *GRX1* or *GRX2* mRNA to *PDA1* mRNA is shown.

Table 2. Glutaredoxin activity in strains overexpressing *GRX1* and *GRX2*

Strain	Wild-type exponential	Wild-type stationary	*grx1 grx2* stationary
pRS426	27 ± 4	152 ± 12	6 ± 0.4
mcGRX1	107 ± 4	486 ± 18	115 ± 19
pRS424	37 ± 7	184 ± 50	13 ± 5
mcGRX2	215 ± 11	411 ± 55	573 ± 114

Glutaredoxin activity is given as nanomoles of NADPH oxidized per minute per milligram of protein. Data are the means of triplicate experiments.

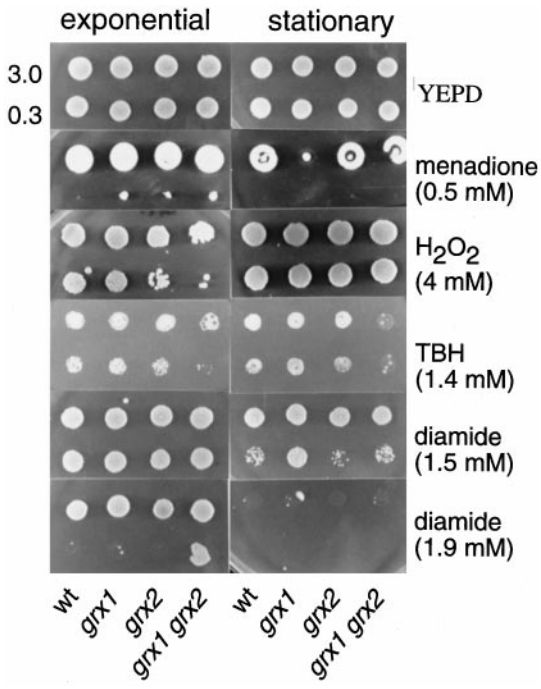

Figure 4. Strains deleted for *GRX1* or *GRX2* are sensitive to reactive oxygen species. Sensitivity to menadione, hydrogen peroxide, *tert*-butyl hydroperoxide, and diamide was determined by spotting strains on YEPD plates containing various concentrations of oxidants. Cultures of wild-type, *grx1, grx2,* and *grx1 grx2* double mutant strains were grown to exponential phase (A_{600} = 1) and into stationary phase in YEPD medium, adjusted to an A_{600} of 3.0 and 0.3 before spotting 10-μl aliquots onto appropriate plates. Plates were incubated at 30°C for 3 d before scoring growth.

Strains Lacking GRX1 or GRX2 Are Sensitive to Conditions of Oxidative Stress

The glutaredoxin mutants were unaffected in resistance to a variety of stress conditions, including heat, heavy metal, and osmotic stress. However, loss of glutaredoxins resulted in altered sensitivity to oxidative stress induced by various reactive oxygen species (ROS). Specifically, exponential and stationary phase cells were tested for growth on plates containing various concentrations of menadione, hydrogen peroxide, *tert*-butyl hydroperoxide, and diamide (Figure 4). When added extracellularly, menadione (1,4-naphthoquinone) generates superoxide radicals through a redox-cycling mechanism (Hassan and Fridovich, 1979). No difference in sensitivity to menadione was seen for exponential phase cells exposed to various concentrations of menadione (Figure 4); however, the *grx1* mutant was sensitive to 0.5 mM menadione during stationary phase. In contrast, exponential phase *grx2* and *grx1 grx2* double mutants were sensitive to 4 mM hydrogen peroxide, whereas the *grx1* mutant and all stationary phase cells were unaffected by this oxidant. In addition, the *grx1 grx2* double mutant was sensitive

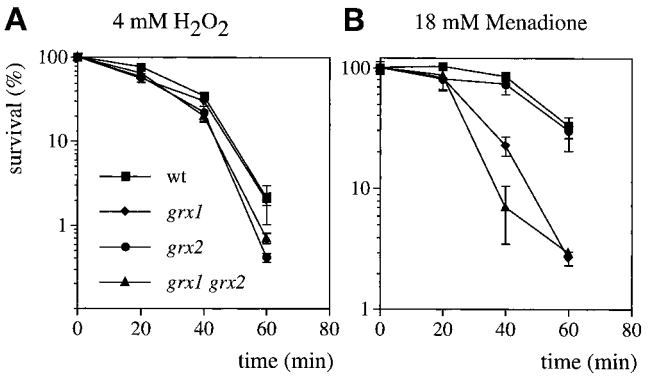

Figure 5. Sensitivity to hydrogen peroxide and menadione. Wild-type, *grx1, grx2,* and *grx1 grx2* double mutant strains were grown to exponential phase in SD medium and treated with 4 mM H_2O_2 (A) or 18 mM menadione (B). Cells were diluted and plated in triplicate onto YEPD medium to monitor cell viability at 20-min intervals. Percent survival is expressed relative to the untreated control cultures.

to *tert*-butyl hydroperoxide during both exponential and stationary phase growth (Figure 4). Diamide is a thiol-specific oxidant that can readily oxidize GSH (Kosower and Kosower, 1995), and stationary phase cells that lack *TRX2*, *GSH1* or *GSH2* show increased resistance to this oxidant (Muller, 1996; Grant *et al.*, 1997). Similarly, the *grx1* mutant was more resistant to 1.5 mM diamide than the wild-type strain during stationary phase. Interestingly, stationary phase cells were more sensitive to diamide than exponential phase cells and were unable to grow at higher concentrations. The *grx1 grx2* double mutant was found to be resistant to 1.9 mM diamide during exponential phase growth (Figure 4). Hence, *GRX1* and *GRX2* appear to have different functions in the defense against diamide, depending on the growth phase. In exponential phase cells, the lack of both glutaredoxins resulted in resistance to diamide, whereas in stationary phase cells, the deletion of *GRX1* alone led to resistance.

To compare the oxidant sensitivity of the glutaredoxin mutants quantitatively, dose-response curves to hydrogen peroxide and menadione were generated. In these experiments, wild-type cells and the *grx1, grx2,* and *grx1 grx2* double mutant strains were grown to exponential phase in minimal media and then treated with 4 mM H_2O_2 or 18 mM menadione for 1 h during which cell viability was monitored (Figure 5). For the H_2O_2 treatment, both the *grx2* and the *grx1 grx2* double mutants were more sensitive compared with the wild-type strain and the *grx1* mutant (Figure 5A). In contrast, *grx1* and the *grx1 grx2* double mutant were sensitive to menadione (Figure 5B). These results indicate that loss of *GRX1* confers sensitivity to the superoxide anion, whereas loss of *GRX2* confers sensitivity to H_2O_2. Plate test experiments indicated that the *grx1* mutant was only sensitive to menadione dur-

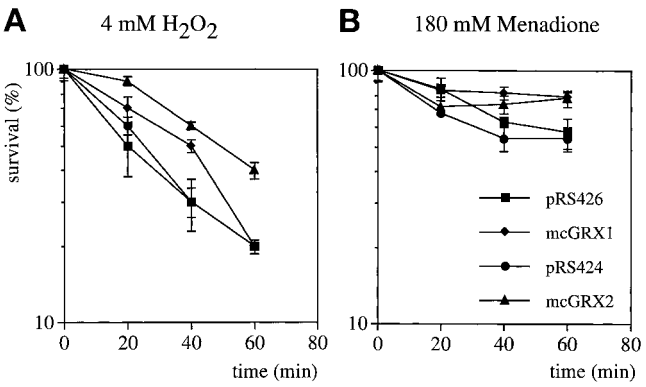

Figure 6. Overexpression of *GRX1* and *GRX2* increases resistance to oxidants. Wild-type strains containing pRS426, mc*GRX1*, pRS424, and mc*GRX2* were grown to exponential phase in SD medium and treated with 4 mM H_2O_2 (A) or 180 mM menadione (B). Cells were diluted and plated in triplicate onto YEPD medium to monitor cell viability at 20-min intervals. Percent survival is expressed relative to the untreated control cultures.

ing stationary phase, whereas the dose-response curves indicated that both *grx1* and the *grx1 grx2* double mutant were sensitive during exponential phase growth. This presumably reflects differences in the two assays, because in the case of the plate test, cells were exposed to the oxidant throughout their growth, whereas for the dose-response experiment, cells were exposed to the oxidant for just 1 h.

We next examined the effect of overexpressing *GRX1* and *GRX2* on resistance to hydrogen peroxide and menadione. These results were complicated by the fact that multicopy vectors alone appeared to increase resistance to ROS, presumably because of induction of cellular stress responses. Overexpression of *GRX2* resulted in increased resistance to 4 mM hydrogen peroxide throughout the 1 h time course (Figure 6A, compare *mcGRX2* with pRS424). In contrast, *mcGRX1* resulted in a somewhat elevated resistance to H_2O_2 during the first 40 min but fell to normal wild-type levels after 60 min (Figure 6A, compare mc*GRX1* with pRS426). Yeast cells containing multicopy vectors (pRS426 and pRS424) were extremely resistant to menadione, and treatment with 180 mM resulted in ~45% loss of viability (Figure 6B). mc*GRX1* and mc*GRX2* resulted in increased levels of resistance to menadione, with 78% survival after the 1 h treatment. Thus, overexpression of both *GRX1* and *GRX2* increased resistance to oxidative stress induced by hydrogen peroxide and the superoxide anion. Because the activity of glutaredoxin is dependent on glutathione, and alterations in glutathione levels and redox state have been shown to affect resistance to oxidative stress (Grant *et al.*, 1996a–c), we examined whether the differences in oxidant sensitivity of the glutaredoxin mutants were attributable to differences in GSH metabolism.

Loss of GRX1 or GRX2 Does Not Affect Glutathione Redox State or Glutathione Reductase Activity

Glutathione reductase (Glr) is the primary enzyme that controls the redox state of GSH and is required for protection against oxidative stress (Grant *et al.*, 1996a). Glr activity is regulated in response to ROS (Grant *et al.*, 1996a) but was unaffected in the glutaredoxin mutants, indicating that there was no increased demand for the enzyme in these strains (Table 3). Cellular redox state, as measured by the reaction of DTNB with free thiol groups, was also unaffected in the glutaredoxin mutants (Table 3). Likewise, total GSH levels, as well as the ratio of GSH to GSSG was similar in the wild-type and glutaredoxin mutants (Table 3). These results indicate that the loss of glutaredoxin activity did not affect GSH metabolism, and hence their oxidant sensitivity was not as a result of alterations in GSH redox balance in the cell. Given the requirement for *GRX1* and *GRX2* in the cellular response to oxidative stress, we examined whether the expression of the two glutaredoxin genes was regulated in response to stress conditions.

Table 3. Glutathione reductase activity and cellular redox state in glutaredoxin mutants

Strain	Glr activity (nmol·min^{-1}·mg^{-1})	Thiol content[a] (μmol/mg)	Total GSH[b] (nmol/10^7 cells)	GSSG[c] (pmol/10^7 cells)	GSSG (%)
wt	152 ± 8	243 ± 63	3.2 ± 0.1	47.6 ± 3.4	1.5
grx1	158 ± 18	212 ± 11	3.0 ± 0.1	43.4 ± 1.4	1.5
grx2	135 ± 20	210 ± 17	2.5 ± 0.6	50.4 ± 10.5	2
grx1 grx2	149 ± 0	227 ± 18	3.0 ± 0.1	51.8 ± 9.1	1.7

Data are the means of duplicate experiments.

[a] Total thiol content includes both protein and GSH.

[b] Total GSH is the equivalent of GSH + 2GSSG.

[c] Percent GSSG is the portion of GSSG present in the cell relative to the amount of total GSH.

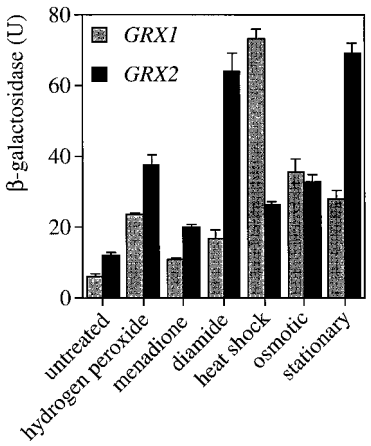

Figure 7. Expression of both *GRX1* and *GRX2* is increased under a variety of stress conditions. *GRX1::lacZ* and *GRX2::lacZ* fusion constructs were assayed for β-galactosidase activity during exponential phase growth (untreated) and after treatment with 0.3 mM hydrogen peroxide, 9 mM menadione, 1.5 mM diamide, and 39°C heat shock and after entry into stationary phase. Values shown are the means of at least three independent determinations.

GRX1 and GRX2 Expression Is Increased during Conditions of Stress

To test whether glutaredoxin expression responded to stress conditions, chimeric genes were constructed containing the *lacZ* gene fused to the *GRX1* or *GRX2* promoter. These fusion constructs were transformed into yeast, and expression was measured under a variety of stress conditions, including H_2O_2, superoxide, diamide, heat, and osmotic and stationary phase growth (Figure 7). Both *GRX1* and *GRX2* reporter constructs were expressed at relatively low levels during early exponential phase, with a slightly higher expression of *GRX2* compared with *GRX1* in agreement with the transcript analysis (Figure 3). Expression of both genes was induced in a growth phase–dependent manner, with a fivefold to sixfold increase as the cells progressed into stationary phase. In addition, expression of both *GRX1::lacZ* and *GRX2::lacZ* was induced by all of the stress conditions examined. There were, however, differences in the levels of induction, with *GRX1* expression induced 12- and 6-fold under heat shock and osmotic shock conditions, respectively, compared with the 2-fold induction under both conditions for GRX2.

DISCUSSION

Glutaredoxin was originally identified as an electron donor for ribonucleotide reductase (Holmgren, 1979), and this remains its best characterized function. Nevertheless, not all glutaredoxins serve as hydrogen donors for ribonucleotide reductase, as shown for glutaredoxins from rabbit bone marrow and pig liver, and the recently characterized Grx2 from *E. coli* (Hopper *et al.*, 1989; Vlamis-Gardikas *et al.*, 1997). Similarly, it was shown that mouse fibroblasts depleted of glutaredoxin were not affected in growth rate, DNA synthesis, or the size of the deoxyribonucleotide pool (Spyrou and Holmgren, 1996). It remains to be established whether yeast Grx1, Grx2, or both can function in the reduction of ribonucleotides, but it seems likely given that the level of deoxynucleotide triphosphates in a mutant lacking both thioredoxin genes are unaltered, indicating the existence of an alternate hydrogen donor for ribonucleotide reductase in addition to thioredoxin in yeast (Muller, 1994). Confirmation of a role for the yeast glutaredoxins in deoxyribonucleotide synthesis will have to await purification of the yeast ribonucleotide reductase, which is an extremely unstable protein (Lammers and Follmann, 1984; Harder and Follmann, 1990). The activity with ribonucleotide reductase depends on a redox-active disulfide in the active site of glutaredoxin, whereas the activity as an oxidoreductase is only dependent on the N-terminal active site cysteine (Holmgren and Aslund, 1995). Results presented here indicate that both Grx1 and Grx2 display GSH-dependent disulfide oxidoreductase activity. However, despite the high degree of homology between these two proteins, Grx2 accounted for the majority of this oxidoreductase activity in the cell. The difference in activity did not arise as a result of differential expression of the two genes and may indicate, therefore, that Grx1 and Grx2 have different functions in yeast. In this view, subtle differences in primary or secondary structure would account for the differences in activity, either through effects on catalytic activity or on interactions with other components of the glutaredoxin system.

In this present study, eukaryotic glutaredoxins were shown to be required in vivo for protection against ROS. Grx1 was found to function in protection against the superoxide anion, whereas Grx2 was required for resistance to H_2O_2. It is unclear why the two glutaredoxins were required for protection against different forms of ROS, although it is consistent with the observation that the physiological mechanisms underlying adaptive responses to hydrogen peroxide and the superoxide anion are different (Jamieson, 1992; Flattery-O'Brien *et al.*, 1993). The superoxide anion is a ubiquitous by-product of aerobic metabolism, which is itself relatively unreactive, but can serve as a precursor of highly reactive and deleterious ROS, including the hydroxyl free radical $HO^{\bullet}$ (Halliwell, 1991). In contrast, hydrogen peroxide is both freely diffusible and fairly reactive and may generate a different spectrum of cellular damage to the superoxide anion. In addition, it can generate the hydroxyl radical via the Fenton reaction. Detoxification of H_2O_2 is mediated by both catalase, which catalyses its breakdown to H_2O_2 and O_2, and by glutathione peroxidases, which use GSH as a reductants. In yeast, GSH-mediated reac-

tions appear to be the primary means of detoxifying this ROS (Grant and Dawes, 1996); however, catalases are required for the detoxification of H_2O_2 during stationary phase (Izawa *et al.*, 1996). Similarly, *GRX2* was required for H_2O_2 resistance during exponential phase growth but not during stationary phase. Stationary phase cells are generally more resistant to conditions of oxidative stress because of the increased synthesis of various antioxidants, including catalase (Izawa *et al.*, 1996), and this may compensate for the lack of *GRX2* during this growth phase. Organic peroxides such as *tert*-butyl hydroperoxide are thought to be detoxified by glutathione-dependent systems, because they are too bulky to be substrates for catalase (Grant and Dawes, 1996), and accordingly, the strain lacking *GRX1* and *GRX2* was sensitive during both exponential and stationary phases.

The requirement for glutaredoxins in protection against ROS may reflect a specific role in the regulation of a cellular antioxidant(s), or a more general role in protection against oxidants as a result of their disulfide oxidoreductase activity. For example, glutathione peroxidases are antioxidant enzymes that catalyze the breakdown of hydroperoxides using GSH as a reductant (Grant and Dawes, 1996), and in vitro studies have shown that both the glutaredoxin and thioredoxin systems can serve as electron donors for human plasma (selenium-dependent) glutathione peroxidase (Bjornstedt *et al.*, 1994). Thus, the absence of a glutaredoxin may result in a reduced ability to regenerate active glutathione peroxidase after detoxification of ROS. In addition, the antioxidant ascorbic acid, which can detoxify hydrogen peroxide and other forms of ROS, may require glutaredoxin activity for maintenance of its function. Utilization of ascorbic acid results in its conversion to dehydroascorbate, and it is regenerated in a GSH-dependent reaction catalyzed by glutaredoxin and protein disulfide isomerase (Wells *et al.*, 1990; Meister, 1994; Park and Levine, 1996). However, it appears unlikely that Grx1 or Grx2 function in vivo as dehydroascorbate reductases, because the five-carbon analogue erythroascorbic acid is the predominant form detected in yeast (Nick *et al.*, 1986; Kim *et al.*, 1993).

Glutaredoxins catalyze the cleavage of mixed disulfides in the presence of low concentrations of GSH and may therefore protect cells by reducing any mixed disulfides formed during oxidative stress (Chrestensen *et al.*, 1995). In addition, in vitro experiments indicate that glutaredoxins can reactivate a number of oxidized enzymes by reducing the mixed disulfides formed as a result of thiol oxidation (Terada *et al.*, 1992; Terada, 1994; Yoshitake *et al.*, 1994). This repair activity is not restricted to enzymes, because glutaredoxin from human erythrocytes is able to reactivate membrane proteins and to regenerate hemoglobin from the mixed disulfide hemoglobin-*S*-*S*-glutathione (Mieyal *et al.*, 1991; Terada *et al.*, 1992). More recently, glutaredoxin has been isolated from the human ocular lens and was found to dethiolate mixed disulfides formed during oxidative stress, preventing loss of lens transparency and cataract formation (Raghavachari and Lou, 1996). Thus, yeast glutaredoxins may function in vivo to reduce mixed disulfides formed as a result of oxidative damage to proteins. Mixed disulfides may be formed by protein *S*-thiolation when protein sulfydryls are oxidized to from mixed disulfides with low–molecular weight thiols such as GSH (Thomas *et al.*, 1995). No increase in the levels of protein-bound GSH, both under normal growth conditions or after exposure to oxidative stress, was detected in the various glutaredoxin mutants (our unpublished observations). However, differences in protein-GSH conjugates that occur at the subcellular level (e.g., in the vacuole or mitochondrion) would not be detected in our whole-cell extracts, nor would conjugates to other thiols such as cysteine or homocysteine, and this remains the subject of our ongoing investigations.

Because Grx2 accounted for the majority of oxidoreductase activity in the cell, its primary function may be to detoxify any mixed disulfides formed as a result of damage caused by ROS. Grx1 could also function in the GSH-dependent disulfide oxidoreductase assay and may therefore be required in addition to Grx2 during certain stress conditions or after the formation of particular mixed disulfide substrates. In agreement with this, the expression of both *GRX1* and *GRX2* was elevated in response to various stress conditions, including hydrogen peroxide, the superoxide anion, diamide, heat shock, osmotic shock, and stationary phase. Similar levels of induction were seen for *GRX1* and *GRX2* in response to oxidative stress induced by hydrogen peroxide, menadione, and diamide. However, *GRX1* was induced 12- and 6-fold in response to heat and osmotic stress, respectively, compared with the 2-fold inductions seen for *GRX2*. Increased expression in response to multiple stress conditions is similar to the regulation seen for many stress-responsive genes, including *CTT1, DDR2, TPS2*, and *HSP12* which are controlled by a stress response element (Ruis and Schuller, 1995). In fact, the promoter regions of *GRX1* and *GRX2* were found to contain putative stress response elements (our unpublished observations), which places them among this family of stress-responsive genes. The expression of other genes forming the glutaredoxin and thioredoxin systems is also induced in response to oxidants, including *GSH1*, *GLR1*, and *TRX2*, which are regulated by the yAP-1 transcriptional activator (Kuge and Jones, 1994; Wu and Moye-Rowley, 1994; Stephen *et al.*, 1995; Grant *et al.*, 1996a,c). Thus, in response to increased ROS, yeast cells can increase the levels of both glutaredoxin and thioredoxin, and this may be one of the primary defenses against oxidative damage to proteins.

Thioredoxin 2 has also been implicated in protection against hydroperoxides, because a *trx2* mutant was sensitive to hydrogen peroxide (Kuge and Jones, 1994). In addition, the loss of both *TRX1* and *TRX2* was found to result in alterations to the cellular GSH redox balance, elevating the levels of oxidized glutathione (Muller, 1996). In contrast, loss of *GRX1* or *GRX2* did not affect the GSH redox balance, cellular thiol levels, or glutathione reductase activity. It remains to be established how great the functional overlap is between the glutaredoxin and thioredoxin systems, especially given that they appear to be balanced in *E. coli* (Miranda-Vizuete *et al.*, 1996). However, differences in the function of these two systems have already come to light in yeast, because thioredoxin was found to be essential for sulfur metabolism (Muller, 1991), whereas the glutaredoxin mutants presented here showed wild-type growth rates with sulfate as the sole source of sulfur. This is in contrast to prokaryotic systems, in which both thioredoxin and glutaredoxin serve as hydrogen donors for 3′-phosphoadenosine 5′-phosphosulfate reductase. Overexpression of *GRX2* resulted in a slow-growth phenotype, which was not related to problems in sulfur assimilation (our unpublished observations). Recently, it has been demonstrated that glutaredoxin can catalyze both the formation and reduction of mixed disulfides (Ruoppolo *et al.*, 1997), and an increase in either process may account for the slow-growth phenotype. Occasional faster-growing colonies were formed from cells transformed with mc*GRX2*, and a genetic analysis of these revertants should lead to a better understanding of glutaredoxin function in yeast.

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