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A Glutathione Reductase Mutant of Yeast Accumulates High Levels of Oxidized Glutathione and Requires Thioredoxin for Growth

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A glutathione reductase null mutant of *Saccharomyces cerevisiae* was isolated in a synthetic lethal genetic screen for mutations which confer a requirement for thioredoxin. Yeast mutants that lack glutathione reductase (*glr1*Δ) accumulate high levels of oxidized glutathione and have a twofold increase in total glutathione. The disulfide form of glutathione increases 200-fold and represents 63% of the total glutathione in a *glr1*Δ mutant compared with only 6% in wild type. High levels of oxidized glutathione are also observed in a *trx1*Δ, *trx2*Δ double mutant (22% of total), in a *glr1*Δ, *trx1*Δ double mutant (71% of total), and in a *glr1*Δ, *trx2*Δ double mutant (69% of total). Despite the exceptionally high ratio of oxidized/reduced glutathione, the *glr1*Δ mutant grows with a normal cell cycle. However, either one of the two thioredoxins is essential for growth. Cells lacking both thioredoxins and glutathione reductase are not viable under aerobic conditions and grow poorly anaerobically. In addition, the *glr1*Δ mutant shows increased sensitivity to the thiol oxidant diamide. The sensitivity to diamide was suppressed by deletion of the *TRX2* gene. The genetic analysis of thioredoxin and glutathione reductase in yeast runs counter to previous studies in *Escherichia coli* and for the first time links thioredoxin with the redox state of glutathione in vivo.

INTRODUCTION

The equilibrium between the reduced and disulfide form of protein thiols is influenced by both the intrinsic chemistry of the protein and by the extrinsic redox state of their cellular environment. Thioredoxins (Holmgren, 1985; Buchanan *et al.*, 1994), glutaredoxins (Holmgren, 1989; Wells *et al.*, 1993), and glutathione (Meister, 1989; Meister, 1995) contain redox-active thiols and along with antioxidants (Halliwell, 1994) foster a predominantly reducing environment (Gilbert, 1995). In addition, specific functions attributed to thioredoxins include the redox control of both enzyme (Holmgren, 1985; Buchanan *et al.*, 1994) and transcription factor activation (Schenk *et al.*, 1994), defending against oxidative damage (Chae *et al.*, 1994; Kuge and Jones, 1994), supplying the reducing equivalents for deoxyribonucleotide synthesis (Miranda Vizuete *et al.*, 1994), and promoting the proper folding of proteins (Lyles and Gilbert, 1991; Lundstrom *et al.*, 1992; Lundstrom and Holmgren, 1995). Many of these functions are also assigned to glutathione and/or glutaredoxin (Meister, 1989; Wells *et al.*, 1993; Aslund *et al.*, 1994).

Almost exclusively these diverse functions derive from in vitro studies on isolated proteins. The importance of thioredoxin in vivo is unclear. Ideally, a genetic approach would allow the study of the interplay between thioredoxin and all cellular processes within the context of a living cell. However, genetic research on thioredoxin has been hampered by an apparent broad redundancy in the thiol-based redox systems. The extensive genetic analysis of *E. coli* offers striking and perplexing cases of unexpected mutant phenotypes. For example, mutants containing null mutations in thioredoxin, thioredoxin reductase, glutaredoxin, glutathione synthetase, and glutathione reductase are viable and grow at normal rates (Apontoweil and Berends, 1975; Fuchs and Warner, 1975; Russel and Model, 1985; Tuggle and Fuchs, 1985; Perham, 1987; Russel and Holmgren, 1988; Russel *et al.*, 1990). Among double mutants tested, mutants lacking both glutathione reductase and thioredoxin reductase

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are viable, but grow poorly (Perham, 1987), and a double mutant lacking thioredoxin and glutaredoxin grows well on rich media, but is not viable in low sulfate medium in liquid culture (Miranda Vizuete *et al.*, 1994). In addition, two results which have challenged our basic understanding of the thiol-based redox systems are the observations that the redox status of glutathione is normal in mutants lacking glutathione reductase (Tuggle and Fuchs, 1985) and that disulfide bond formation of cytoplasmic proteins may be regulated by thioredoxin reductase through a mechanism that is inexplicably independent of thioredoxin (Derman *et al.*, 1993).

Unlike *E. coli*, the yeast *S. cerevisiae* appears to be more susceptible to perturbations of thioredoxin and glutathione. Again, thioredoxin is not essential, but deletion of the two thioredoxin genes *TRX1* and *TRX2* has a profound effect on cell growth (Muller, 1991, 1994, 1995). The rate of DNA synthesis decreases by threefold, the cells are methionine/cysteine auxotrophs, cell size increases, and the generation time in rich medium lengthens from 90 to 120 min. Remarkably, the levels of deoxyribonucleotides are normal, thus refuting the simple hypothesis that slow reduction of ribonucleotide reductase is limiting DNA synthesis in the thioredoxin double mutants (Muller, 1994). The active site cysteines of thioredoxin are crucial for optimum DNA synthesis, but the target of thioredoxin reduction is unknown (Muller, 1995).

Also in contrast to *E. coli*, it was recently shown that glutathione is essential for growth of yeast. Mutants with a deletion of *GSH1* (encoding γ-glutamylcysteine synthetase) require glutathione supplementation to support growth (Wu and Moye Rowley, 1994). However, like *E. coli*, mutants lacking glutathione reductase grow at normal rates (Collinson and Dawes, 1995).

If thioredoxin does play a central role in the thiol chemistry of the cell, are there conditions in which thioredoxin is essential for growth? To answer this question, a genetic screen searched for mutations in yeast that would be lethal in combination with mutations in the two thioredoxin genes.

MATERIALS AND METHODS

Yeast Strains and Genetic Methods

All yeast strains used in this study were derived from W303 (Wallis *et al.*, 1989) and EMY55 (*MAT***a***/MATα, trx1Δ::TRP1/TRX1, trx2Δ::LEU2/TRX2, ade3Δ/ADE3, LYS2/lys2Δ::HIS3, ade2/ade2, trp1-1/trp1-1, leu2-3, 112/leu2-3,112, ura3-1/ura3-1, his3-11/his3-11, can1-100/can1-100*) (Muller, 1992). EMY60 is the standard wild-type strain (*MAT***a***, TRX1, TRX2, ade2-1oc, ade3Δ-100, trp1-1, leu2-3,112, lys2Δ::HIS3, ura3-1, his3-11, can1-100*). EMY61(*trx1Δ::TRP1, TRX2*), EMY62(*TRX1,trx2Δ::LEU2*), and EMY63(*trx1Δ::TRP1, trx2Δ::LEU2*) are isogenic with EMY60 except as noted. EMY55-7D is isogenic with EMY63 except it is *Matα*. EMY56-4B is isogenic with EMY55-7D except the *trx1Δ::TRP1* allele is replaced with *trx1Δ::LYS2*.

EMY97 is (*MAT***a***/MATα, trx1Δ::TRP1/TRX1, trx2Δ::LEU2/TRX2, thd1-78/thd1-78, ade3Δ/ade3Δ, lys2Δ::HIS3/lys2Δ::HIS3, ade2/ade2, trp1-1/trp1-1, leu2-3,112/leu2-3,112, ura3-1/ura3-1, his3-11/his3-11, can1-100/can1-100*). EMY97 was created following three out-crosses to remove the *thd1-78* mutation from the mutagenized background of EMY63(pLI833):thd1-78. Since the *thd1-78* mutation did not have a known phenotype alone, it was followed in genetic crosses and tetrad analysis by one of two methods. In diploids that were heterozygous *TRX1/trx1Δ, TRX2/trx2Δ, THD1/thd1-78*, the *thd1-78* mutation was assumed to segregate with the wild-type thioredoxin genes in products of sporulation that yielded four viable spores where two were *trx1Δ, trx2Δ* and two were *TRX1, TRX2*. In diploids that were *trx1Δ/trx1Δ, trx2Δ/trx2Δ, THD1/thd1-78* and contained a plasmid-bearing *TRX1*, following sporulation and standard tetrad analysis, the *thd1-78* segregants were recovered in haploids that required the plasmid. The plasmids used were either the 2-μm based pLI833 (see below) or pLI840, a centromere-based plasmid that carries *TRX1* and *URA3* (Muller, 1995). In all tetrad analyses, the *thd1-78* mutation segregated as a recessive mutation in a single gene.

EMY101 (*MAT***a***/MATα,trx1Δ::LYS2/TRX1,trx2Δ::LEU2/TRX2, glr1Δ::TRP1/glr1Δ::TRP1, ade3Δ/ade3Δ, lys2Δ::HIS3/lys2Δ::HIS3, ade2/ade2, trp1-1/trp1-1, leu2-3,112/leu2-3,112, ura3-1/ura3-1, his3-11/his3-11, can1-100/can1-100*) was created as follows. EMY60 was transformed with a *Sac*I–*Not*I digest of pLI66 containing *glr1Δ::TRP1*. A Trp$^+$ transformant was mated with EMY56-4B to yield EMY100 (*TRX1/trx1Δ::LYS2, TRX2/trx2Δ::LEU2, glr1Δ::TRP1/GLR1*). EMY100 was sporulated to yield EMY100-9A (*Matα, trx1Δ::LYS2, TRX2, glr1Δ::TRP1*) and EMY100-9C (*Mat***a***, TRX1, trx2Δ::LEU2, glr1Δ::TRP1*). EMY101 is EMY100-9A mated with EMY100-9C.

EMY100-5A (*Mat***a***, glr1Δ::TRP1, TRX1, TRX2*), EMY100-6D (*Mat***a***, trx1Δ::LYS2, TRX2, glr1Δ::TRP1*), and EMY100-9C (*Mat***a***, TRX1, trx2Δ::LEU2, glr1Δ::TRP1*) are all derivatives of EMY100.

Standard yeast genetic methods were used for the analysis of strains and crosses (Sherman *et al.*, 1986). The compositions of synthetic dextrose minimal medium (SD), yeast peptone dextrose rich broth medium (YPD), and selective media for transformants are described elsewhere (Sherman *et al.*, 1986). SD complete (SDC) was SD with the addition of 0.2% casamino acids (Difco, Detroit, MI), 50 μg/ml adenine, 25 μg/ml uracil, and 100 μg/ml tryptophan.

Plasmids

pLI833 was constructed as follows. The *ADE3* gene from a *Bam*HI-*Sal*I fragment of DK202 (Davis, 1992) was inserted into the *Xba*I site of pBluescript IISK− (Stratagene) by blunt end ligation after filling in the ends to give pLI831. *TRX1* on a *Bam*HI-*Sal*I fragment from pLI809 (Muller, 1991) was ligated into *Bam*HI-*Sal*I-digested pLI831 to yield pLI832. The *Not*I-*Sal*I fragment of pLI832, containing *TRX1* adjacent to *ADE3* in the same orientation, was ligated to *Eag*I-*Sal*I-digested pTD29 (Geiser *et al.*, 1993). Plasmid pTD29 in based on YEp24 and carries *LYS2*.

pLI1036 is based on YCp50 and contains a 10.2-kb chromosomal insert as shown in Figure 2. The *Bam*HI-*Bam*HI fragment of pLI1036 was removed to yield pLI1077. The 2.3-kb *Hin*DIII-*Xba*I fragment, the 2.2-kb *Eco*RI (vector)-*Kpn*I fragment, and the 6.5-kb *Kpn*I-*Eco*RI fragment were cloned into pRS316 (Sikorski and Hieter, 1989) to yield pLI1065, pLI1048, and pLI1042, respectively.

pLI66 was created as follows. The *GLR1* gene of pL1065 was moved into pGEM5Zf(−) (Promega, Madison, WI) on a *Not*I-*Sal*I fragment to yield pLI65. The *TRP1* gene on an *Eco*RI fragment from pLI823 (Muller, 1991) was ligated into the *Eco*RI site of pBluescript II KS+. *TRP1* on a *Kpn*I-*Bam*HI fragment then replaced the internal *Kpn*I-*Bam*HI region of *GLR1* in pLI65 to yield pLI66.

Isolation and Identification of THD1

EMY63(pLI833) was mutagenized to 1% survival with ethylmethane sulfonate and plated on YPD with 2.5 μg/ml adenine. The low

adenine deepened the red color but was not necessary to see the color effect.

The *THD1* gene was cloned by transforming EMY63(pLI833): thd1-78 with a yeast genomic library in YCp50 (Muller, 1991), and plasmid DNA was isolated from transformants that sectored. Sixteen of 18 encoded TRX1 or TRX2 and allowed growth of EMY63(pLI833):thd1-78 on SD-met plates. Two of the 18 contained the identical plasmid, pLI1036. The DNA sequence of a 718-bp region of the pLI1065 insert was determined and found to be identical to the published sequence of *GLR1* (Collinson and Dawes, 1995).

To establish that *thd1-78* and *glr1*Δ were allelic tetrad analysis of LMS2(pLI840) (*trx1*Δ*::TRP1/trx1*Δ*::LYS2, trx2*Δ*::LEU2/TRX2, thd1-78/glr1*Δ*::TRP1, trp1-1/trp1-1, leu2-3,112/leu2-3,112, ura3-1/ura3-1, lys2*Δ*::HIS3/lys2*Δ*::HIS3, his3-11/his3-11, ade2-1oc/ade2-1oc, ade3*Δ*-100/ade3*Δ*-100, can1-100/can1-100*) was performed. All *trx2*Δ progeny required pLI840 (i.e., *TRX1*) as assayed on 5-fluoro-orotic acid (5-FOA) plates (Sherman *et al.*, 1986).

High-Performance Liquid Chromatography (HPLC) Analysis of Cellular Thiols

Low-molecular-weight thiols were extracted, labeled with monobromobimane, and analyzed with HPLC following methods used to analyze the glutathione content of numerous microorganisms (Fahey and Newton, 1987; Newton *et al.*, 1992). However, this is the first time they were applied to *Saccharomyces cerevisiae*. The high sensitivity of a Waters HPLC system permitted the scaling down of the previous methods. Cells were harvested by rapid filtration (1 ml of exponential culture) onto 0.2 μm, 13-mm Nylaflo membranes (Gilman) and washed with 4 ml of dH_2O. Within 1 min the filtered cells underwent one of three treatments. 1) For analysis of cellular thiols, the filter was transferred to a microfuge tube containing 0.2 ml of extraction buffer mB [50% acetonitrile, 0.25 mM monobromobimane (mBBr), 20 mM Tris, (pH 8.0), 5 mM diethylenetriaminepentaacetic acid] and incubated at 60°C for 10 min. (A 10-fold increase in mBBr did not change the recovery of derivatized thiols.) The reaction was stopped with 2 μl of 5 M methanesulfonate, 200 μl of dH_2O was added, the filter was removed, and solid debris was pelleted by centrifugation. The supernatant was extracted twice with 0.4 ml of dichloromethane to remove acetonitrile and excess mBBr. A 60-μl aliquot of the 300-μl sample was analyzed with HPLC. 2) As a control, cells were treated as described in 1), except 0.5 mM *N*-ethylmaleimide (freshly prepared) replaced the mBBr in the extraction buffer, and, after a 10-min incubation at 60°C to block free thiols, 5 μl of 10.0 mM mBBr in acetonitrile was added and the sample was incubated an additional 10 min at 60°C. A 60-μl aliquot of the 300-μl sample was analyzed with HPLC. 3) For analysis of cellular disulfides, cells were treated as described in 2), except after the incubation with *N*-ethylmaleimide, 10.0 μl of 21 mM dithiothreitol was added to reduce disulfides to thiols. After a 10-min incubation at 60°C, 50 μl of 10.0 mM mBBr in acetonitrile was added. The extract was incubated for 10 min at 60°C and the reaction stopped with 3 μl of 5 M methanesulfonic acid. The volume was increased with 387 μl of dH_2O, the filter and debris were removed, and the sample was extracted twice with 0.67 ml of dichloromethane. A 100-μl aliquot of the 500-μl sample was analyzed with HPLC. Before all treatments an aliquot of the growing culture was fixed with 3.7% formaldehyde, sonicated, and cell number determined with a Coulter Multisizer II. Samples were kept under low light at all times. HPLC was performed with a Waters 717plus Autosampler, the 474 Scanning Fluorescence Detector, and a Waters Symmetry C_{18} (4.6 × 250-mm) column with a Waters Sentry C18 guard column. Buffer A consisted of 0.15% trifluoroacetic acid in water and buffer B was HPLC-grade methanol. With a flow rate of 1.2 ml/min, the linear gradients were 15–23% B, 0–30 min; 100% B, 30.01–35 min; and 15% B, 35.01- 45.00 min. HPLC was automated and chromatograms were analyzed with Waters Millenium software. The standard curve was linear from 10 to 2000 picomoles of glutathione. As a control, 80 μM reduced GSH in 50 μl of dH_2O was processed following these protocols and the analysis yielded 94% recovery with only 1.8% in the disulfide form.

RESULTS

Isolation and Identification of THD1

Although extensive genetic analysis of thioredoxin has been performed in *E. coli*, the only conditions in which thioredoxin is required for growth is a thioredoxin and glutaredoxin double deletion mutant growing in low sulfate medium in liquid culture (Miranda Vizuete *et al.*, 1994). To isolate a mutant of yeast with an absolute and more general requirement for thioredoxin, an unbiased genetic screen was undertaken (Figure 1A). The screen is based on the color of EMY63(pLI833) colonies growing on rich medium. In this strain, the chromosomal thioredoxin genes are deleted and the only source for thioredoxin is the plasmid pLI833. Colonies of EMY63(pLI833) are predominantly white with red-pigmented sectors (Figure 1B). The red is a consequence of the *ADE3* gene on the plasmid pLI833 in combination with an *ade2* mutation in the chromosome. Under rich growth conditions, pLI833 is readily lost as the cells divide since none of the genes on pLI833 are required. Thus, only a fraction of the descendants of any cell retain the plasmid and these form the red sectors. The strategy for isolating mutants that required thioredoxin for growth was to search for mutant colonies that were solid red because they required the *TRX1* gene born on pLI833.

Following ethylmethane sulfonate mutagenesis, 120,000 colonies were screened and 24 solid red colonies were chosen for study. Of these, 2 belonged to the *THD1* (*th*ioredoxin *d*ependent) complementation group represented by the EMY63(pLI833):thd1-78 mutant, 3 belonged to a second complementation group that did not have thioredoxin dependence, and 19 had integrated pLI833. The *thd1-78* mutation gave rise to solid red colonies (Figure 1B) and segregated in genetic crosses as a recessive mutation in a single gene. Tetrad analysis of the diploid EMY97 (*trx1*Δ*/TRX1, trx2*Δ*/TRX2, THD1/thd1-78*) established an absolute requirement for either *TRX1* or *TRX2* (Figure 1C). Spores carrying the *thd1-78* mutation in combination with the *trx1*Δ and *trx2*Δ deletions germinated and underwent one or two divisions before lysis 3–4 days later. This result does not preclude a role for thioredoxin or glutathione reductase in germination, since pools from the parent could supply activity in the germinating spores.

To identify the *THD1* gene, I screened a yeast genomic library for plasmids that would complement the solid red phenotype of the EMY63(pLI833):thd1-78 mutant and restore the original sectored phenotype. The plasmids recovered from the library encoded

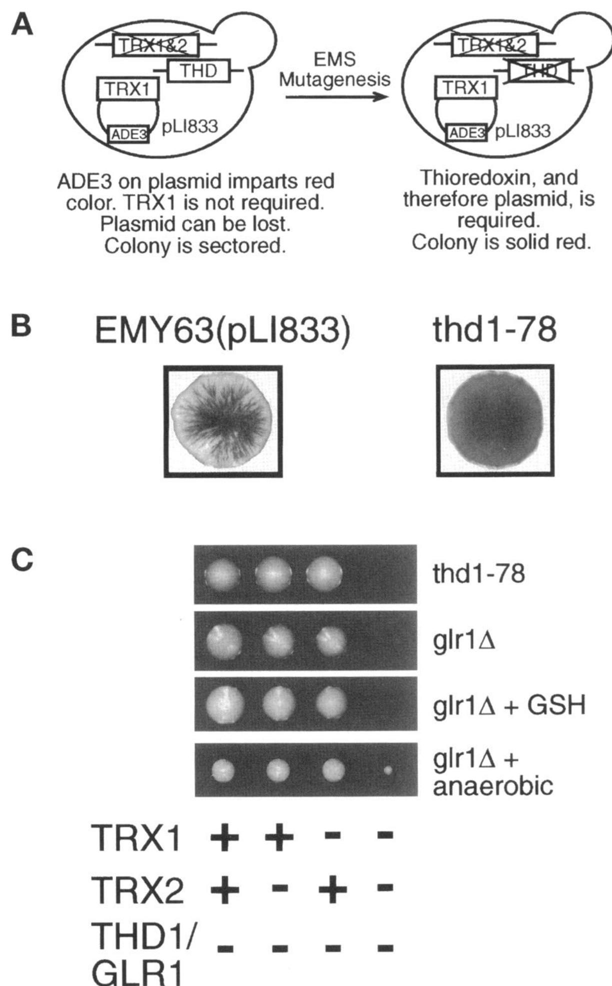

Figure 1. Isolation of thioredoxin-dependent mutants. (A) Strategy for obtaining *thd* mutations. EMS, ethylmethane sulfonate. (B) Red/white colony sectoring assay. EMY63(pLI833) and EMY63(pLI833): thd1-78 plated on YPD medium with low adenine. EMY63(pLI833) shows the typical red/white sectoring, whereas EMY63(pLI833): thd1-78 is solid red. (C) Tetrad analysis confirming thioredoxin dependence. EMY97 was sporulated and 24 tetrads dissected onto YPD medium. All (28/28) *trx1*Δ, *trx2*Δ, *thd1-78* segregants were not viable. A representative tetrad is shown in the top panel. EMY101 was sporulated and plated under various conditions. Of 40 tetrads dissected onto YPD (for example second panel), all 41 of 41 *trx1*Δ, *trx2*Δ, *glr1*Δ segregants were not viable. Of 22 tetrads dissected onto YPD containing an additional 10 mM GSH, all 29 of 29 *trx1*Δ, *trx2*Δ, *glr1*Δ segregants were not viable (e.g., third panel). Of 48 tetrads dissected onto YPD and then incubated in a BBL Gaspak anaerobic chamber, 53 of 55 *trx1*Δ, *trx2*Δ, *glr1*Δ segregants grew as a microcolony (e.g., bottom panel). Cells were incubated at 30°C.

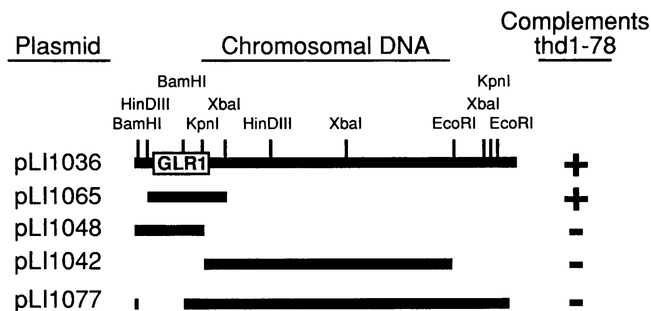

Figure 2. Cloning and identification of *THD1*. EMY63(pLI833): thd1-78 was transformed with a yeast genomic library. Complementation of thd1-78 was scored by the ability of plasmids to allow sectoring of EMY63(pLI833):thd1-78. Only the chromosomal insert is shown for all plasmids. Construction of the plasmids is described in MATERIALS AND METHODS.

either the expected thioredoxin genes or *GLR1* encoding glutathione reductase (Figure 2). At the time of these experiments, the *GLR1* gene was not identified, but the isolation of the *GLR1* gene was recently reported (Collinson and Dawes, 1995) and was located on chromosome 16 as part of the yeast genome project. Deletion analysis revealed that only the region encoding *GLR1* was required to complement *thd1-78* (Figure 2). Several additional experiments verified that *THD1* is *GLR1*. Using the cloned *GLR1* gene, a *glr1*Δ*::TRP1* null mutant was created which, like the *thd1-78* mutant, had an absolute requirement for either *TRX1* or *TRX2* (Figure 1C). Linkage analysis confirmed that *thd1-78* was an allele of *GLR1* (see MATERIALS AND METHODS). Finally, cell extracts of the *thd1-78* mutant had no detectable glutathione reductase activity as assayed by the method described by Massey and Williams (1965). Deletion mutants of *GLR1* were also found to be viable and have no glutathione reductase activity (Collinson and Dawes, 1995); however, no further characterization of the glr1Δ was previously reported.

It was concluded that cells unable to actively reduce glutathione require thioredoxin for viability. The requirement cannot be bypassed by supplementation of the YPD growth medium with reduced 10 mM glutathione (Figure 1C). (YPD medium contained 30 ± 10 μM glutathione, all in the disulfide form.) Only anaerobic conditions were permissive for growth (Figure 1C). This growth was feeble but emphasizes that oxygen plays a role in the lethality of the triple *trx1*Δ, *trx2*Δ, *glr1*Δ mutant.

Redox State of Glutathione in the Thioredoxin and Glutathione Reductase Mutants

Glutathione reductase mutants of *E. coli* maintain normal levels of reduced glutathione by an undetermined process (Tuggle and Fuchs, 1985). Perhaps in yeast thioredoxin preserves glutathione reduction, thus explaining the requirement for thioredoxin in the glutathione reductase mutants. To test this hypothesis, the glutathione content of the glutathione reductase and thioredoxin mutants was examined.

In contrast to *E. coli*, glutathione reductase is the primary enzyme that dictates the redox status of glutathione in yeast. The disulfide form of glutathione represented 63% of the total glutathione in the *glr1*Δ mutant compared with only 6% in the wild-type cells (Figure 3 and Table 1). The oxidized glutathione content increased 20-fold, whereas the reduced form decreased a modest 37%. The total glutathione content increased from ~190 picomoles/10^6 cells for the wild-type to ~300 picomoles/10^6 cells for the *glr1*Δ and *glr1*Δ/*trx*Δ mutants. The regulation of the total glutathione content may provide an additional mechanism for controlling the reduced/disulfide ratio, since glutathione is synthesized in the reduced form (Meister, 1989, 1995).

Although not the dominant factor, thioredoxin clearly assists glutathione reductase in establishing the high ratio of reduced:oxidized glutathione. In the thioredoxin double mutant, the level of the disulfide form of glutathione increased to 22% of the total glutathione (Figure 3 and Table 1). The absence of either thioredoxin in conjunction with the *glr1*Δ mutation increased the disulfide form to 70%, reproducibly higher than the 63% caused by the *glr1*Δ mutation alone (Table 1). The glutathione content of the thioredoxin mutant was elevated to 688 picomoles/10^6 cells, but because the thioredoxin double mutant is larger, this value represents approximately the same average cellular concentration as the *glr1*Δ and *glr1*Δ/*trx*Δ mutants (Table 1).

Comparison of the Growth Phenotypes of the Glutathione Reductase and Thioredoxin Mutants

To further explore the relationship between the thioredoxin and glutathione reductase, the phenotype of the *glr1*Δ mutant was examined and compared with the thioredoxin mutants.

DNA synthesis is protracted in the thioredoxin double mutant, increasing the duration of the S-phase by threefold and shortening G_1 (Muller, 1991). Currently, the impeded step in replication is not known. Ribonucleotide reduction appears to be adequate since the deoxynucleotide triphosphate levels are normal (Muller, 1994; Muller *et al.*, 1994). The accumulation of cells in the S-phase during asynchronous growth of the thioredoxin mutant is easily visualized with flow cytometry (Figure 4A). The timing of cell cycle events in the *glr1*Δ mutant in contrast was indistinguishable from the wild type (Figure 4A). The generation time of the *glr1*Δ mutant was also the same as the wild type. Thus, the redox status of glutathione had no effect on progression through the cell cycle.

The thioredoxin double mutant cannot assimilate sulfate as the sole source of sulfur (Muller, 1991). This result suggested that thioredoxin is the only hydrogen donor to 3′-phosphoadenosine 5′-phosphosulfate reductase and that unlike *E. coli* (Russel *et al.*, 1990; Miranda Vizuete *et al.*, 1994) glutathione/glutaredoxin does not contribute. In support of this conclusion, growth of the *glr1*Δ mutant is indistinguishable from the wild type on minimal defined media (Figure 4B).

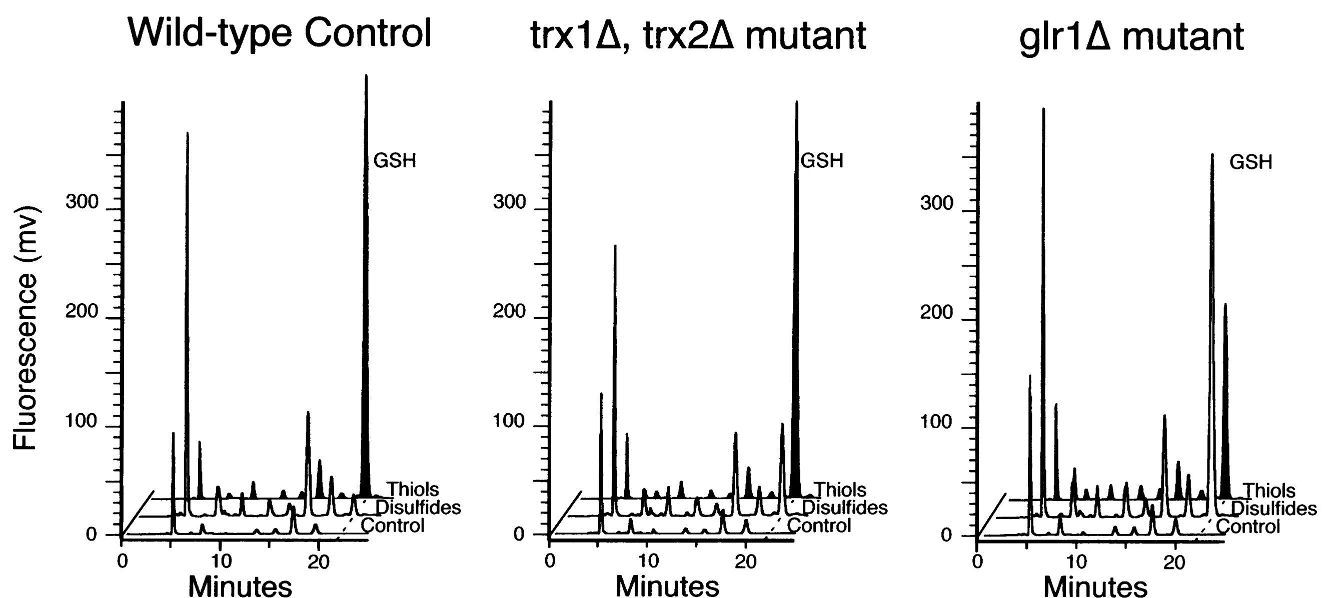

Figure 3. HPLC chromatograms of monobromobimane derivatives of cellular thiols. EMY60 (wild type), EMY63 (*trx1*Δ, *trx2*Δ), and EMY100-5A (*glr1*Δ) were analyzed as described in MATERIALS AND METHODS.

Table 1. Glutathione content of thioredoxin and glutathione reductase mutants

Strain	Genotype	Glutathione content (picomoles/10^6 cells) GSH	GSS	Total glutathione (average concentration, mM)	% disulfide
EMY60	wild-type	175 ± 16	10.7 ± 1.5	5.5	5.8
EMY61	trx1Δ	202 ± 29	13.1 ± 1.4	6.3	6.1
EMY62	trx2Δ	175 ± 10	11.2 ± 1.9	5.5	6.0
EMY63	trx1Δ, trx2Δ	572 ± 54	116 ± 12	8.4	22
EMY100-5A	glr1Δ	110 ± 6.3	191 ± 14	9.7	63
EMY100-6D	trx1Δ, glr1Δ	84 ± 8.6	206 ± 25	9.4	71
EMY100-9C	trx2Δ, glr1Δ	92 ± 9.1	209 ± 24	9.7	69

All strains are isogenic except for the relevant mutations shown. Glutathione content was measured as described in Figure 3 and is the average of at least five independent determinations. Total glutathione is the average cellular concentration based on cell size measurements determined with a Coulter Multisizer II. The cell volume was 3.4×10^{-14} l for wild-type and thioredoxin single mutants, 8.2×10^{-14} l for EMY63 and 3.1×10^{-14} l for *glr1Δ* mutants.

Finally, given the elevated levels of oxidized glutathione and the decrease of reduced glutathione, one expected the *glr1Δ* and *trx1Δ/trx2Δ* mutants to show sensitivity to the thiol-specific oxidant diamide. The principle target of diamide is glutathione, which it rapidly converts to glutathione disulfide (Kosower and Kosower, 1995). Some proteins, including thioredoxin, may also have susceptible thiols (Kosower and Kosower, 1995).

As predicted the *glr1Δ* mutant was more sensitive than the wild type (Figure 4C). Whereas the highest permissible concentration of diamide was 1.0 mM for the wild type, the *glr1Δ* mutant could not grow above 0.6 mM. The *trx1Δ* mutation was silent and elicited no change in the response to diamide in either the wild-type or in the *glr1Δ* mutant.

Surprisingly, the deletion of *TRX2* conferred resistance to diamide. Treatment with 1.2–1.4 mM diamide, toxic concentrations for the wild type, were tolerated by the *trx2Δ* mutant. The resistance does not depend on glutathione reductase or thioredoxin-1. The *trx2Δ/trx1Δ* and the *trx2Δ/glr1Δ* double mutants also displayed a high level of resistance to diamide. The *trx2Δ* mutation was able to suppress the sensitivity produced by the *glr1Δ* mutation. There is no correlation between the total glutathione content of the cell (Table 1) and diamide resistance in the *trx2Δ* mutants.

DISCUSSION

Mutants of yeast which lack glutathione reductase require one of two thioredoxins for viability and mutants deficient for the two thioredoxins require glutathione reductase. Under aerobic growth conditions, this reciprocal requirement is absolute. Thus, the genetic screen described in this study was successful in identifying conditions in which thioredoxin was essential. In addition, characterization of the glutathione status in the glutathione reductase and thioredoxin mutants revealed a remarkable resiliency of cells to live with high levels of oxidized glutathione, and an unanticipated involvement of thioredoxin in maintaining the normally high ratio of reduced:oxidized glutathione. Of importance, these results delimit the extent of redundancy of the thiol-based redox systems in yeast.

A priori one can imagine several reasons that the triple mutant lacking both glutathione reductase and the two thioredoxins cannot live. First is an inability to synthesize deoxyribonucleotides. Both thioredoxin and glutaredoxin supply reducing equivalents to ribonucleotide reductase, the enzyme which catalyses the reduction of ribonucleotides to their corresponding deoxyribonucleotides (Holmgren, 1989). Glutaredoxin is a small redox protein which is reduced by glutathione. Glutaredoxin is present in *S. cerevisiae* (Gan, 1992), and the normal levels of deoxyribonucleotides in a thioredoxin double mutant (Muller, 1994) suggest that glutaredoxin alone is an effective reductant of ribonucleotide reductase. However, deoxyribonucleotide depletion is an unlikely cause for the synthetic lethality of the *glr1Δ*, *trx1Δ*, and *trx2Δ* mutations. Supplementation of the growth medium with reduced glutathione does not suppress the lethality of the *glr1Δ*, *trx1Δ*, and *trx2Δ* triple mutations. In addition, the reduced glutathione levels in the *glr1Δ* mutant and *glr1Δ,trx1(2)Δ* double mutants are still >2 mM. Given an ample supply of glutathione and the presence of a functional glutaredoxin, reduction of ribonucleotide reductase should be adequate.

A more likely explanation for the synthetic lethality is a toxic accumulation of oxidized glutathione in the

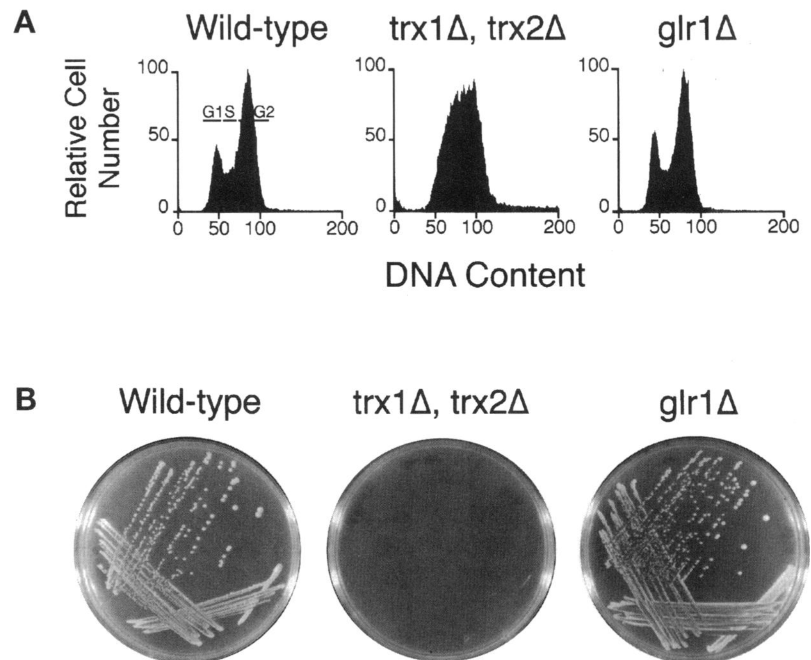

Figure 4. Growth characteristics of the glutathione reductase mutant. (A) Flow cytometry was performed on EMY60 (wild type), EMY63 (*trx1*Δ, *trx2*Δ), and EMY100-5A (*glr1*Δ) as described previously (Muller, 1991). (B) Growth of the same strains on SD-met, defined medium in which sulfate is the sole source of sulfur. (C) Diamide sensitivity of the thioredoxin and glutathione mutants. Cells were grown overnight in SDC, aliquots were sonicated, and cell concentration was determined with the Coulter Multisizer II. Five-microliter aliquots containing 10,000, 1000, or 100 cells were spotted onto SDC plates containing a range of diamide concentrations.

triple mutant. The level of oxidized glutathione already increases 10-fold in the thioredoxin double mutant and 20-fold in the glutathione reductase mutant. In combination the accumulation of oxidized glutathione will likely rise even further. An exceptionally high level of oxidized glutathione would accelerate improper disulfide bond formation and promote the addition of glutathione to protein sulfhydryls. Both effects could disrupt protein function.

Finally, a mutant lacking both thioredoxins and glutathione reductase may be overwhelmed by damage from oxygen-derived free radicals. Thioredoxin peroxidase (Chae *et al.*, 1994) and glutathione peroxidase (Galiazzo *et al.*, 1987) detoxify peroxides to water and are key elements of the protective antioxidant systems of cells (Michiels *et al.*, 1994). In the absence of thioredoxins and glutathione reductase, the normal rate of destruction of peroxides will be compromised. Cells would be particularly vulnerable to oxidative damage during the S-phase of the cell cycle, since the increase in glutathione consumption caused by reduction of glutaredoxin for deoxyribonucleotide synthesis could divert reducing power away from glutathione peroxidase. Although the growth is slow, the observation that the *glr*Δ, *trx1*Δ, *trx2*Δ triple mutant does grow anaerobically supports the hypothesis that oxygen-derived free radicals are instrumental in the loss of viability of the triple mutant growing aerobically.

This is the first time thioredoxin deficiency was shown to cause a change in the redox status of glutathione. The disulfide form of glutathione increases from 6 to 22% of the total glutathione in the thioredoxin double mutant. Thus, normally thioredoxin plays an unexpected part in maintaining the high ratio reduced:oxidized glutathione in vivo. Direct reduction of glutathione disulfide by thioredoxin is unlikely based on the negligible rate of reduction measured in vitro (Holmgren, 1979). Either the in vitro measurements underestimate the ability of thioredoxin to reduce oxidized glutathione in vivo, or the increase in

the disulfide form of glutathione in the thioredoxin mutant is an indirect effect of thioredoxin deficiency. The reductive capacity of glutathione reductase may be insufficient to maintain the normal reduced:oxidized glutathione ratio under conditions where glutathione must perform unassisted those functions normally shared with thioredoxin.

In addition, the total glutathione content increases in the *trx1*Δ, *trx2*Δ double mutant. Thus, yeast has an underlying mechanism of adjusting the level of glutathione in response to thioredoxin deficiency. A candidate for integrating thioredoxin and glutathione levels is YAP1 (Moye Rowley *et al.*, 1989; Hussain and Lenard, 1991; Schnell, *et al.*, 1992; Gounalaki and Thireos, 1994). YAP1 is a yeast transcription factor that responds to oxidative stress and regulates the transcription of *TRX2* (Kuge and Jones, 1994), *GLR1* (Collinson and Dawes, 1995), and *GSH1* (Wu and Moye Rowley, 1994). The elevated levels of oxidized glutathione in the *trx1*Δ, *trx2*Δ double mutant indicates a more oxidizing cellular environment which could trigger YAP1 activation.

Previously *TRX2* was shown to be a crucial part of the oxidative stress response to peroxides in yeast (Kuge and Jones, 1994). Elevated transcription of *TRX2*, mediated by YAP1, confers resistance to hydrogen peroxide presumably through increased activity of the thioredoxin peroxidase. In contrast, the loss of *TRX2* strengthened resistance to diamide. The mechanism for the enhanced resistance does not require thioredoxin-1, glutathione reductase, or increased levels of glutathione. Two explanations for this resistance are that the *trx2*Δ mutation induces a response that protects the cell or that diamide oxidizes thioredoxin-2 to form a deleterious product.

Reports of high levels of intracellular oxidized glutathione are rare (Deneke and Fanburg, 1989; Adams *et al.*, 1993). Under normal growth conditions only the endoplasmic reticulum, a cellular compartment that favors thiol oxidation, has ratios of oxidized:reduced glutathione similar to the glr1Δ mutants (Hwang *et al.*, 1992). In general, mammalian cells export oxidized glutathione that accumulates under stress conditions (Nicotera *et al.*, 1985; Deneke and Fanburg, 1989; Kretzschmar, *et al.*, 1992; Adams *et al.*, 1993). However, in a striking similarity to the *glr1*Δ mutants, $CD4^+$ lymphocytes isolated from symptomatic human immunodeficiency virus-positive patients had on average 65% of their total glutathione in the disulfide form, displayed a twofold increase in total glutathione, and had a modest 30% decrease in reduced glutathione (Aukrust *et al.*, 1995). Thus, the $CD4^+$ lymphocytes had all three hallmarks of the yeast *glr1*Δ mutants. Unfortunately, the glutathione reductase levels were not measured in these $CD4^+$ cell populations. The deeper recognition of the interdependence of thioredoxin and glutathione may benefit the design and interpretation of therapies which hope to ameliorate oxidative damage in human immunodeficiency and other human diseases.

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