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Glutathione Synthetase Is Dispensable for Growth under Both Normal and Oxidative Stress Conditions in the Yeast *Saccharomyces cerevisiae* Due to an Accumulation of the Dipeptide γ-Glutamylcysteine

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Glutathione (GSH) synthetase (Gsh2) catalyzes the ATP-dependent synthesis of GSH from γ-glutamylcysteine (γ-Glu-Cys) and glycine. *GSH2*, encoding the *Saccharomyces cerevisiae* enzyme, was isolated and used to construct strains that either lack or overproduce Gsh2. The identity of *GSH2* was confirmed by the following criteria: 1) the predicted Gsh2 protein shared 37–39% identity and 58–60% similarity with GSH synthetases from other eukaryotes, 2) increased gene dosage of *GSH2* resulted in elevated Gsh2 enzyme activity, 3) a strain deleted for *GSH2* was dependent on exogenous GSH for wild-type growth rates, and 4) the *gsh2* mutant lacked GSH and accumulated the dipeptide γ-Glu-Cys intermediate in GSH biosynthesis. Overexpression of *GSH2* had no effect on cellular GSH levels, whereas overexpression of *GSH1*, encoding the enzyme for the first step in GSH biosynthesis, lead to an approximately twofold increase in GSH levels, consistent with Gsh1 catalyzing the rate-limiting step in GSH biosynthesis. In contrast to a strain deleted for *GSH1*, which lacks both GSH and γ-Glu-Cys, the strain deleted for *GSH2* was found to be unaffected in mitochondrial function as well as resistance to oxidative stress induced by hydrogen peroxide, *tert*-butyl hydroperoxide, and the superoxide anion. Furthermore, γ-Glu-Cys was at least as good as GSH in protecting yeast cells against an oxidant challenge, providing the first evidence that γ-Glu-Cys can act as an antioxidant and substitute for GSH in a eukaryotic cell. However, the dipeptide could not fully substitute for the essential function of GSH in the cell as shown by the poor growth of the *gsh2* mutant on minimal medium. We suggest that this function may be the detoxification of harmful intermediates that are generated during normal cellular metabolism.

INTRODUCTION

Glutathione (GSH) is an ubiquitous tripeptide (γ-glutamylcysteinylglycine) usually found as the most abundant intracellular thiol compound in most organisms. Roles have been proposed for GSH in many cellular processes, including amino acid transport, synthesis of proteins and nucleic acids, modulation of enzyme activity, and metabolism of xenobiotics, carcinogens, and reactive oxygen species (ROS; Meister and Anderson, 1983; Douglas, 1987; Meister, 1988; Sies, 1993; Grant and Dawes, 1996). Despite these various potential functions, GSH was found to be totally dispensable for growth in *Escherichia coli* under both normal conditions and in cells exposed to oxidative stress (Apontoweil and Berends, 1975; Greenberg and Demple, 1986). In contrast, GSH is an essential metabolite in yeast, being required as a reductant under normal growth conditions (Grant *et al.*, 1996b). In addition, strains lacking GSH or altered in their GSH redox state are sensitive to oxidative stress induced by

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peroxides and the superoxide anion, as well as to the toxic products of lipid hydroperoxides (Izawa *et al.*, 1995; Grant *et al.*, 1996a,b,c; Turton *et al.*, 1997). This is analogous to the situation in higher eukaryotes, in which depletion of GSH induced by treatment with L-buthionine(*S,R*)-sulfoximine leads to oxidative tissue damage along with the breakdown of mitochondria (Mårtensson *et al.*, 1991). Recently, GSH was also found to be essential in *Schizosaccharomyces pombe* and a role in the detoxification of endogenously derived metabolites via a GSH-conjugate pump was proposed (Chaudhuri *et al.*, 1997).

GSH synthesis proceeds via two ATP-dependent steps. The first is the formation of the dipeptide γ-glutamylcysteine (L-γ-Glu-Cys) from glutamate and cysteine, catalyzed by γ-glutamylcysteine synthetase (Gsh1). This rate limiting step in GSH synthesis is feedback inhibited by the end product GSH (Huang *et al.*, 1988; Meister, 1988). The *GSH1* gene, encoding γ-glutamylcysteine synthetase, has been cloned from yeast by complementation of a *gsh1* mutation (Ohtake and Yabuuchi, 1991) and as a high-copy suppressor of a temperature-sensitive mutation affecting mitochondrial biogenesis (Lisowsky, 1993). The predicted protein sequence of the yeast enzyme shows high homology with other eukaryotic Gsh1 sequences but only limited homology with the bacterial enzyme consistent with a high degree of conservation among eukaryotes (Ohtake and Yabuuchi, 1991).

The final step in GSH synthesis is the ligation of glycine with L-γ-Glu-Cys catalyzed by GSH synthetase (Gsh2). Genes encoding Gsh2 have been identified in several bacterial and eukaryotic species (Gushima *et al.*, 1984; Mutoh *et al.*, 1991; Peters *et al.*, 1992; Habenicht *et al.*, 1993; Rawlins *et al.*, 1995; Ullmann *et al.*, 1996). However, prior to this study the *Saccharomyces cerevisiae* gene encoding Gsh2 had not been identified. A putative *GSH2* gene was proposed from the phenotype of a mutant isolated in a search for strains deficient in GSH biosynthesis. This phenotype included resistance to methylglyoxal, whose mutagenic properties are dependent on the presence of GSH, and reduced GSH synthetase activity (Ohtake *et al.*, 1990). Although this phenotype was consistent with a partial defect in *GSH2*, the identity of the gene was not further characterized, and there was no data on the effect of a complete null mutation. Herein, we report the identification of the yeast gene *GSH2*, encoding GSH synthetase, and the first characterization of eukaryotic strains that either completely lack or overexpress GSH synthetase.

MATERIALS AND METHODS

Yeast Strains and Media

The *S. cerevisiae* strains used in this study were CY4 (*MAT***a** *ura3–52 leu2–3 leu2–112 trp1–1 ade2–1 his3–11 can1–100*) and its isogenic derivatives CY8 (*gsh1::LEU2*) and CY4p (petite) described previously (Grant *et al.*, 1996b) and CY97 (*gsh2::HIS3*) described below.

Strains were grown in rich YEPD medium (2% [wt/vol] glucose, 2% [wt/vol] bactopeptone, 1% yeast extract) or minimal SD medium (0.17% [wt/vol] yeast nitrogen base without amino acids, 5% [wt/vol] ammonium sulfate, 2% [wt/vol] glucose) supplemented with appropriate amino acids and bases. For growth on nonfermentable carbon sources, YEPGE contained 3% (vol/vol) glycerol and 1% ethanol. Media were solidified by the addition of 2% (wt/vol) agar.

Cloning and Disruption of GSH2

The *GSH2* gene was isolated by PCR amplification of total yeast DNA with oligonucleotides specific for *GSH2* sequences. A 2524-bp fragment was amplified by using oligonucleotides that hybridized 747 bp upstream of the putative ATG start codon (5′-CACTTTGGATCCTTCATCATCGCCG-3′) and 301 bp downstream of the TAG stop codon (5′-CTAAAGGAATTCAGTAAGAAACAGG-3′). The 5′ oligonucleotide introduced a *Bam*HI restriction site (underlined) and the 3′ oligonucleotide introduced an *Eco*RI restriction site (underlined) to facilitate cloning into the polylinker region of plasmid pRS426 (Christianson *et al.*, 1992). The resulting construct was called pG520 and was verified by DNA sequence analysis.

A null allele of *GSH2* (strain CY97) was generated in strain CY4 by a one-step polymerase chain reaction (PCR) amplification protocol that replaced the entire *GSH2* ORF with the yeast *HIS3* gene (Baudin *et al.*, 1993). The oligonucleotides used for the PCR disruption of *GSH2* were GSH2-D1 and GSH2-D2 whose sequences are 5′-GCCACTTCAAGCAATTATAGGAAGAAAGCACTACTCCTATAAAATAGCGCTAGGAGTCACTGCCA-3′ and 5′-ATCTTCCTAGCATCTATGTGTATAGTACATGTACACCTAGTAAAGGGAAAGCGCGCCTCGTTCAG-3′, respectively. The underlined regions correspond to *HIS3* sequences. To rule out any possibility of having picked a fast-growing variant of the *gsh2* disruption strain from the transformation plate, strain CY97 was crossed to a wild-type strain, and after sporulation of the resulting diploid, spores were isolated and six *gsh2* segregants were identified (*HIS3*). All six strains displayed the same phenotypes as CY97 for growth in the absence of GSH and in the presence of H_2O_2 as described in RESULTS AND DISCUSSION.

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. Cells were grown to stationary phase in YEPD, and 10-μl aliquots of each culture, diluted to A_{600} values of 3.0 and 0.3, were spotted onto appropriate plates. Sensitivity was determined by comparison of growth to the wild-type strain after 2–3 d. Dose–response curves were obtained by growing cells to exponential phase ($1–2 \times 10^7$ cells/ml) in YEPD medium at 30°C and treating them with 4 mM H_2O_2 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 growth.

Determination of GSH Levels and Gsh1 and Gsh2 Enzyme Activities

Total GSH was determined by a microtiter plate assay method described previously (Vandeputte *et al.*, 1994). The Gsh1 and Gsh2 enzyme assays were performed according to previously published protocols (Kistler *et al.*, 1990; Ohtake *et al.*, 1990). For the determination of Gsh1 activity, formation of the dipeptide γ-Glu-Cys was measured by using an amino acid analyzer (Beckman, Fullerton, CA) and is expressed as nmoles of γ-Glu-Cys formed per minute per milligram of protein. Gsh2 activity is expressed as nmoles of GSH formed per minute per milligram of protein.

High-Performance Liquid Chromatography (HPLC) and Electrochemical Analysis of γ-Glu-Cys and GSH

Preparation of yeast cells for analysis of low molecular weight thiols was based on a method previously described for tissue extracts (Harvey *et al.*, 1989). Cell pellets were broken in ice-cold 0.6 N perchloric acid containing 2 mM EDTA by vortex mixing with glass beads. After cell breakage, proteins were precipitated by incubating on ice for 15 min and removed by centrifugation in a Microfuge for 15 min at 4°C. A 200-μl aliquot of supernatant was adjusted to pH 3.5 with 5 M NaOH, and the final sample was diluted in mobile phase prior to HPLC and electrochemical analysis.

Thiols were separated by using an Isco model 2350 HPLC system with a Spherisorb octadecyl silane (2) 5-μm HPLC steel column (4.5 × 250 mm). Isocratic elution was performed with 0.1 M KH_2PO_4, 0.35% (vol/vol) acetonitrile (Dudman *et al.*, 1996) at a flow rate of 0.9 ml/min. GSH and γ-Glu-Cys were measured by electrochemical detection with a Coulochem II (ESA, Bedford, MA) detector (E = +550 mV).

RESULTS AND DISCUSSION

Identification of GSH2 That Encodes GSH Synthetase

Analysis of the sequence of chromosome XV revealed an open reading frame of 492 codons (YOLO49w, GenBank accession no. Z74791) whose putative protein product exhibited significant similarity to eukaryotic GSH synthetases. The predicted protein sequence (named Gsh2) shares 37–39% identity and 58–60% similarity over the entire sequence with GSH synthetases from rat, *Schizosaccharomyces pombe*, and *Arabidopsis thaliana* (Figure 1). Like other eukaryotic GSH synthetases, the yeast amino acid sequence shows limited homology to that of *E. coli*. The yeast protein shares 44% similarity and 18% identity with the bacterial enzyme but this does not include the proposed ATP or γ-glutamylcysteine binding sites (Yamaguchi *et al.*, 1993; Fan *et al.*, 1995; Hara *et al.*, 1995). The yeast sequence contains the highly conserved glycine-rich domains (Figure 1) that have been suggested to play an essential role in the catalytic activity of GSH synthetases (Ullmann *et al.*, 1996). However, the cysteine residue (Cys-302 in the *Arabidopsis* sequence), which is conserved in all other eukaryotic enzymes and may play a role in substrate binding (Oppenheimer *et al.*, 1979; Ullmann *et al.*, 1996), is absent from the yeast sequence. Similar to the other eukaryotic Gsh2 enzymes, hydropathy analysis of the yeast Gsh2 indicated a pattern typical of a soluble protein.

GSH2 Encodes GSH Synthetase

To confirm the identity of the putative *GSH2* gene, it was amplified by PCR and cloned into the multicopy vector pRS426 (see MATERIALS AND METHODS). The resulting plasmid pG520 (mcGSH2) was transformed into the wild-type yeast strain CY4, and Gsh2 activity was measured as the ATP-dependent ligation of glycine to γ-glutamylcysteine to form GSH. Gsh2 activity was elevated by greater than 10-fold in cells containing multiple copies of *GSH2* compared with the vector alone (Table 1, compare mcGSH2 with pRS426). This increased Gsh2 activity resulting from the overexpression of *GSH2* and the high degree of homology to other GSH synthetases confirms that *GSH2* encodes the yeast GSH synthetase. We next examined the effect of increased *GSH2* copy number on cellular GSH levels.

Overexpression of GSH2 Does Not Effect Cellular GSH Levels

Previous studies have shown that γ-glutamylcysteine synthetase (Gsh1) catalyzes the first and rate-limiting step in GSH synthesis and is feedback inhibited by GSH (Huang *et al.*, 1988; Meister, 1988). To examine the roles of Gsh1 and Gsh2 in yeast, a multicopy vector containing *GSH1*, encoding Gsh1 (mcGSH1), was introduced into the wild-type yeast strain CY4 and GSH levels were examined. Increased *GSH1* gene dosage resulted in an approximately 66% increase in intracellular GSH levels (Table 1, compare mcGSH1 with YEp351). To confirm that Gsh1 was overexpressed in the mcGSH1 transformant, Gsh1 activity was measured. Multiple copies of *GSH1* were found to result in an eightfold increase in Gsh1 activity (Table 1, compare mcGSH1 with YEp351). Thus, despite the increased Gsh1 activity, there was only a modest increase in cellular GSH levels.

We next examined the effect of overexpression of *GSH2* on GSH levels. In contrast to *GSH1*, multiple copies of *GSH2* were found to have no effect on GSH levels (Table 1, compare mcGSH2 with pRS426), despite an approximately 10-fold increase in Gsh2 activity (Table 1). To examine whether cooverexpression of *GSH1* and *GSH2* leads to increased levels of GSH, yeast cells were transformed with the mcGSH1 and mcGSH2 plasmids. GSH levels were again elevated in cells containing mcGSH1, but there was little or no effect of mcGSH2 even in the presence of mcGSH1 (Table 1). These results indicate that the cellular levels of GSH are tightly regulated and are consistent with Gsh1 being the rate-limiting step in GSH biosynthesis in yeast.

Yeast Strains Deleted for GSH2 Are Viable

A total gene deletion of *GSH2* was generated in the haploid wild-type strain CY4, by replacement of the entire *GSH2* open reading frame with the yeast *HIS3* gene. CY97, the resulting strain, was viable, indicating that *GSH2* is not an essential gene for normal aerobic growth. This phenotype was the result of a single mutation, because six independent spore segregants from the cross to a wild-type strain (see MATERIALS AND METHODS) behaved similarly with respect to growth on the range of media described and in re-

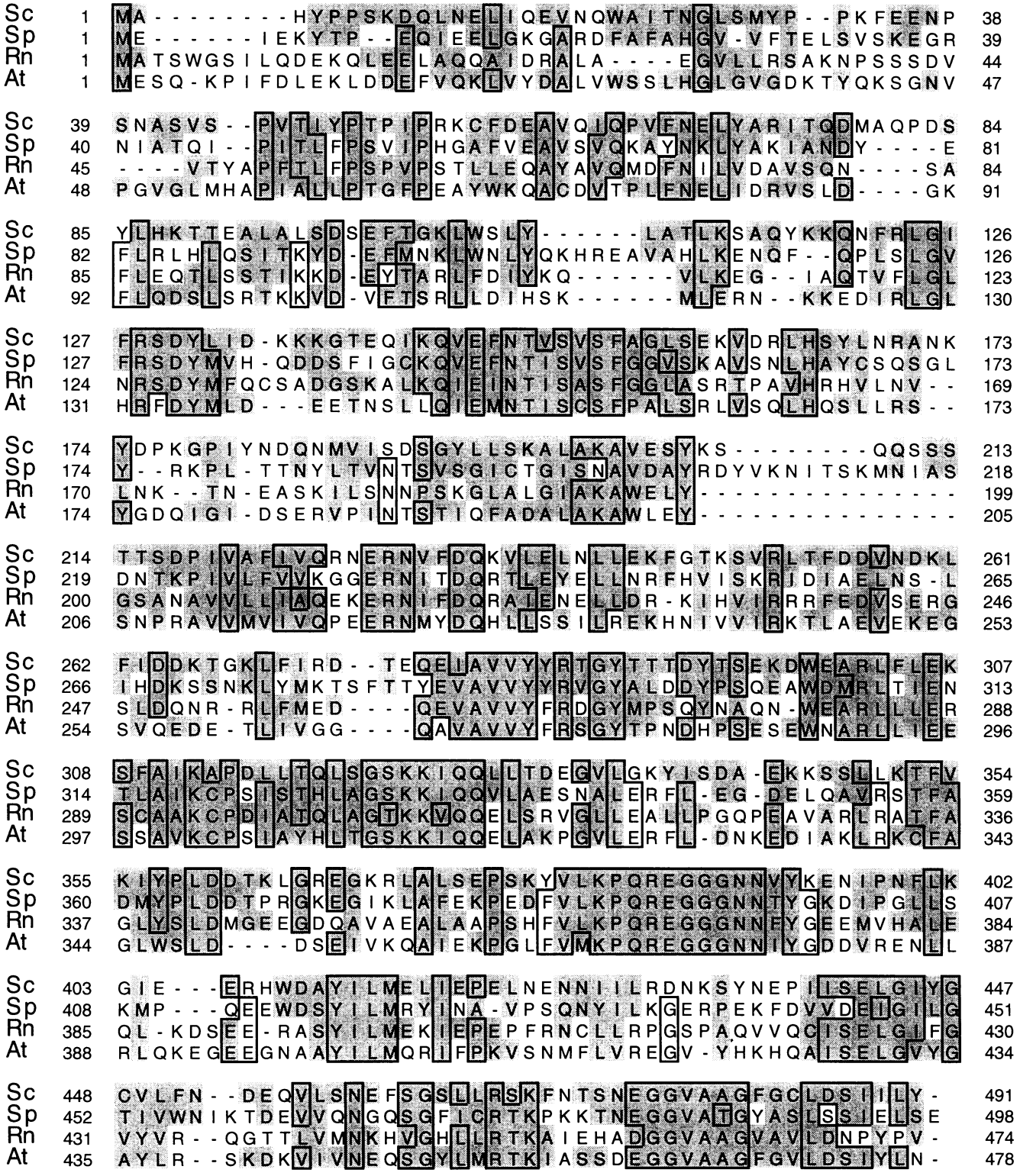

Figure 1. Comparison of the predicted amino acid sequence of yeast Gsh2 with representative eukaryotic GSH synthetases. Gsh2 from *S. cerevisiae* (Sc) was compared with *S. pombe* (Sp), *Rattus norvegicus* (Rn), and *A. thaliana* (At). The sequences are aligned for maximal homology with dashes used to denote gaps introduced for maximal alignment. Identical amino acid residues are boxed and conserved residues are shaded. The conserved glycine-rich regions (for details see text) are between residues 381–394 and residues 478–484 (Sc numbering). The

Table 1. Gsh1 and Gsh2 enzyme activities and GSH content of strains overexpressing *GSH1* and *GSH2*

Strain[a]	Enzyme activity[b]		GSH (nmol/10^{-7} cells)[b]
	Gsh1 (nmol/min/mg)	Gsh2 (nmol/min/mg)	
YEp351	1.7 ± 0.4	0.5 ± 0.2	1.9 ± 0.1
mcGSH1	13.6 ± 0.5	0.5 ± 0.1	3.2 ± 0.9
pRS426	ND[d]	0.9 ± 0.1	1.6 ± 0.1
mcGSH2	ND	10.2 ± 1.8	1.8 ± 0.1
YEp351/pRS426	2.3 ± 0.6	1.0 ± 0.3	1.5 ± 0.3
YEp351/mcGSH2	2.7 ± 0.5	9.0 ± 1.5	1.8 ± 0.5
mcGSH1/pRS426	12.5 ± 2.9	1.0 ± 0.2	2.8 ± 0.3
mcGSH1/mcGSH2	15.5 ± 1.3	9.0 ± 1.5	3.5 ± 0.5

[a] Enzyme assays and GSH content were measured in cell extracts prepared from strain CY4 transformed with plasmids YEp351, mcGSH1 (YEp351 containing *GSH1*), pRS426 and mcGSH2 (pRS426 containing *GSH2*), and pairs of plasmids as indicated.

[b] Values shown are the means (±SE) of at least three independent determinations.

[c] Value shown for mcGSH1 is the mean (±SE) of eight determinations.

[d] ND, not determined.

sponse to oxidative stress (see below). The *gsh2* mutant showed wild-type growth rates on rich glucose-based medium and on nonfermentable carbon sources, such as glycerol and ethanol. This is in contrast to *gsh1* mutants, which are unable to grow on nonfermentable carbon sources, reflecting the importance of GSH for mitochondrial function (Kistler *et al.*, 1986; Lisowsky, 1993; Grant *et al.*, 1996b; Schmidt *et al.*, 1996). In addition, strains deleted for *GSH1*, required exogenous GSH for growth under nonstress conditions on minimal medium (Grant *et al.*, 1996b). Similarly, *gsh2* mutants were dependent on exogenous GSH for wild-type growth rates (Figure 2). However, in contrast to *gsh1* mutants, *gsh2* mutants were able to form small colonies on minimal medium in the absence of GSH consistent with a limited amount of growth. The dipeptide γ-Glu-Cys was also able to restore the growth of both the *gsh1* and *gsh2* mutants on minimal medium, indicating that it can be taken up by cells and replace the essential function of GSH (Figure 2).

γ-Glu-Cys Levels Are Elevated in a Strain Deleted for GSH2

Levels of γ-Glu-Cys and GSH were compared in the wild-type and *gsh1* and *gsh2* mutants by means of HPLC with electrochemical detection, and representative traces are shown in Figure 3. The dipeptide γ-Glu-Cys was undetectable in the wild-type strain and GSH was present at 0.72 ± 0.14 nmol/10^7 cells. As might be expected in a strain lacking the enzyme for the first step in GSH biosynthesis, the *gsh1* mutant contained no detectable γ-Glu-Cys or GSH. The *gsh2* mutant also lacked GSH but contained high levels of γ-Glu-Cys of 3.45 ± 0.15 nmol/10^7 cells. A new peak was also visible in the *gsh2* mutant (approximately 5.5 min; Figure 3) that may represent another low molecular weight thiol that is elevated in this mutant. The absence of GSH and presence of γ-Glu-Cys in the *gsh2* mutant provide further evidence that *GSH2* encodes GSH synthetase. Studies in mammalian cells have shown that GSH levels are tightly regulated and that Gsh1 is feedback inhibited by the end product GSH (Huang *et al.*, 1988; Meister, 1988). Because γ-Glu-Cys accumulated to a fivefold molar excess over GSH, Gsh1 may not be feedback inhibited by γ-Glu-Cys. This prediction will require further enzymatic studies to confirm.

GSH2 Is Dispensable for Growth under Conditions of Oxidative Stress

We next examined whether the *gsh2* mutant was affected in its resistance to ROS. Accordingly, wild-type cells and petite *gsh1* and *gsh2* mutants were tested for growth on plates containing various concentrations of hydrogen peroxide, cumene hydroperoxide, *tert*-butyl hydroperoxide, diamide, and menadione (Figure 4). Surprisingly, the *gsh2* mutant showed a higher level of resistance to H_2O_2 and *tert*-butyl hydroperoxide than the *gsh1* mutant, which was sensitive to these peroxides (Figure 4). It was previously found that GSH was not required for resistance to the aromatic peroxide cumene hydroperoxide (Grant *et al.*, 1996b), and similarly, the *gsh2* mutant grew as well as the wild-type

Figure 1 (cont). protein sequence alignment was made by using the program "Clustal W version 1.4" (Thompson *et al.*, 1994) and displayed by using the graphic program "seqVu version 1.01" (J. Gardner, The Garvan Institute, Sydney).

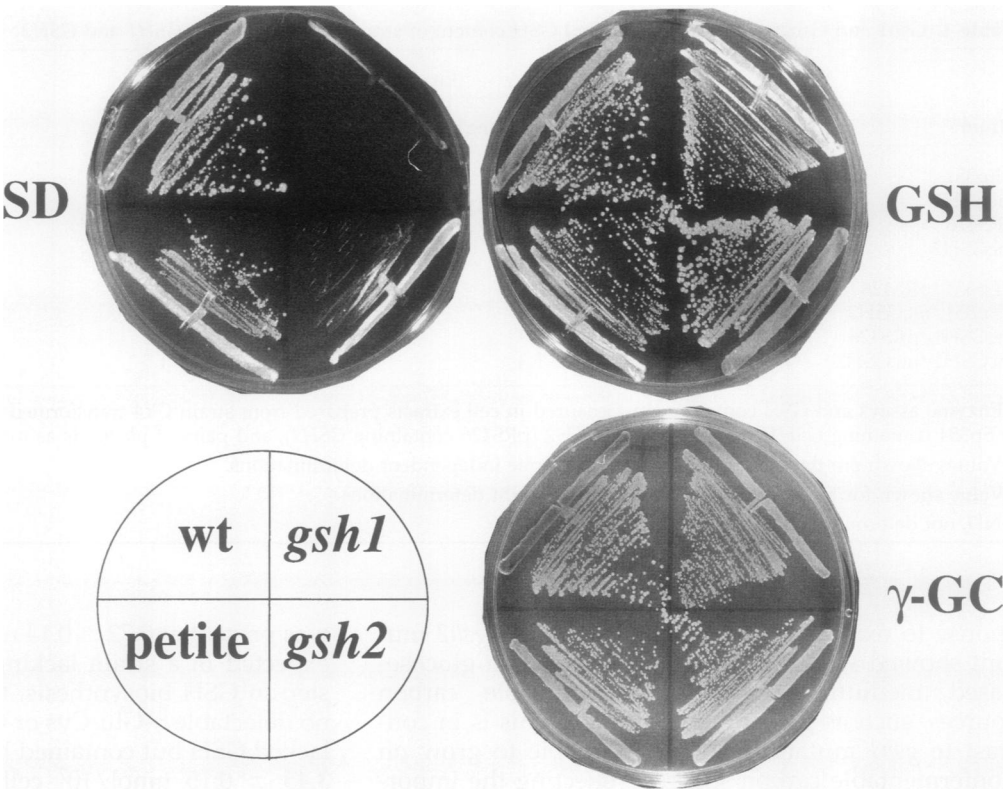

Figure 2. Exogenous GSH is required for growth of strains deleted for *GSH1* and *GSH2*. Strains CY4 (wt), CY4p (petite), CY8 (*gsh1*), and CY97 (*gsh2*) were streaked for single colonies on minimal medium (SD) and SD supplemented with 1 mM GSH or 1 mM γ-glutamylcysteine (γ-GC). Plates were incubated for 3 d at 30°C.

strain on plates containing 0.2 mM cumene hydroperoxide. On higher concentrations of cumene hydroperoxide (0.25 mM), there was no growth of any of the strains tested. Diamide is a thiol-specific oxidant that can readily oxidize GSH (Kosower and Kosower, 1995) and has been shown to induce the expression of *TRX2* and *GLR1* in yeast (Kuge and Jones, 1994; Grant *et al.*, 1996a). Strains lacking GSH (*gsh1* and *gsh2*) were found to be resistant to diamide (Figure 4) and could grow on concentrations of diamide as high as 5 mM, whereas the highest permissible concentration for the wild-type strain was 1.5 mM. Recently, a *trx2* mutant was also found to be resistant to diamide, but it is unclear how this relates to cellular thiol levels because GSH was unaffected in this strain (Muller, 1996). Mutants lacking *GSH2* were more resistant to the superoxide anion generated by the redox-cycling agent menadione (1,4-naphthoquinone) than the wild-type strain (Figure 4). Strains affected in mitochondrial function (petite and *gsh1*) were resistant to menadione at concentrations as high as 1 mM, which may reflect the mode of superoxide anion generation that is dependent on a redox-cycling mechanism in the presence of oxygen (Hassan and Fridovich, 1979).

To quantitatively compare the oxidant sensitivity of the *gsh1*, *gsh2*, and petite mutants, a dose–response curve to hydrogen peroxide was generated. Strains were grown to exponential phase (A_{600} = 1) and

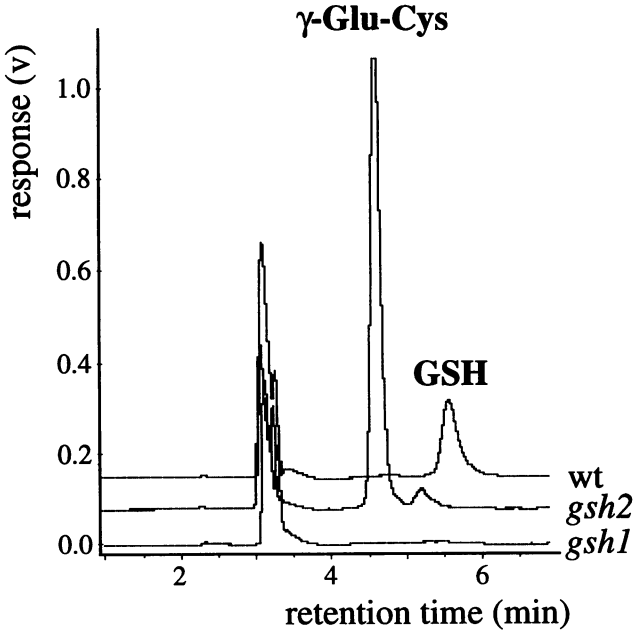

Figure 3. HPLC-electrochemical profiles of low molecular weight thiols from wild-type cells and *gsh1* and *gsh2* mutants. Chromatograms were prepared as described in MATERIALS AND METHODS. Peaks corresponding to GSH and γ-Glu-Cys are marked.

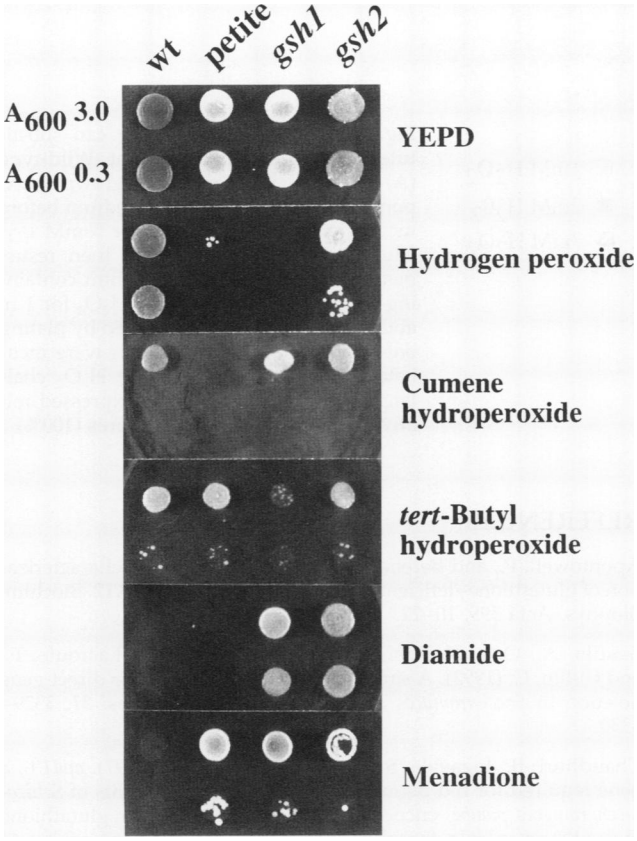

Figure 4. Sensitivity of GSH-deficient yeast cells to ROS. Sensitivity to hydrogen peroxide, cumene hydroperoxide, *tert*-butyl hydroperoxide, diamide, and menadione was determined by spotting strains onto YEPD plates containing various concentrations of oxidants. Cultures of wild-type, petite, *gsh1*, and *gsh2* strains were grown to stationary phase in YEPD and diluted to A_{600} values of 3.0 and 0.3, and 10 μl aliquots were spotted onto appropriate plates. Plates were incubated at 30°C for 2 d before scoring growth.

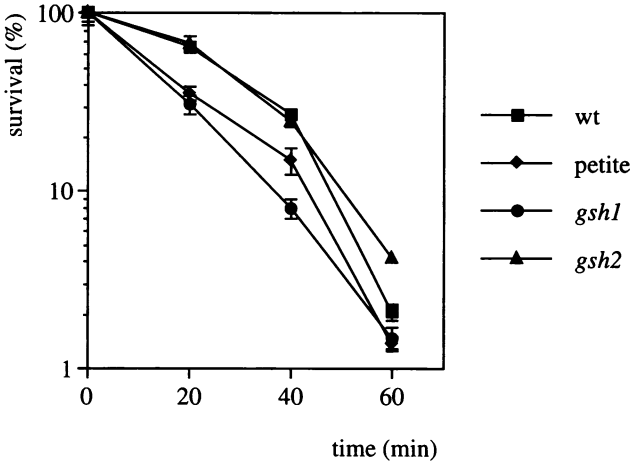

Figure 5. Sensitivity to hydrogen peroxide. Strains CY4 (wt), CY4p (petite), CY8 (*gsh1*), and CY97 (*gsh2*) were grown to exponential phase in YEPD and treated with 4 mM H_2O_2 for 1 h. Cells were diluted and plated in triplicate onto YEPD medium to monitor cell viability at 20-min intervals. Percentage survival is expressed relative to the untreated control cultures (100%).

treated with 4 mM H_2O_2 for 1 h, during which cell viability was monitored (Figure 5). The *gsh1* mutant was very sensitive to H_2O_2 compared with the wild-type strain, and some of this sensitivity could be attributed to the petite nature of the mutant (Figure 5; Grant *et al.*, 1996b). In contrast, the *gsh2* mutant was unaffected in its resistance to H_2O_2 over the 1-h treatment period.

Thus, in contrast to the *gsh1* mutant, the *gsh2* mutant was unaffected in resistance to oxidative stress conditions induced by both H_2O_2 and *tert*-butyl hydroperoxide. In addition, the *gsh2* mutant was somewhat more resistant to the superoxide anion generated by the redox-cycling drug menadione compared with the wild-type strain. This indicates that despite a similar lack of GSH as the *gsh1* mutant, the *gsh2* mutant was unaffected in resistance to ROS and in mitochondrial function. Since the *gsh2* mutant can still synthesize the dipeptide L-γ-Glu-Cys and in fact contains elevated levels compared with the wild-type strain (Figure 3), we examined whether the dipeptide could act as an antioxidant and substitute for GSH directly in the *gsh2* mutant.

γ-Glutamylcysteine Can Substitute for GSH as an Antioxidant

The ability of L-γ-Glu-Cys to act as an antioxidant and substitute for GSH was tested by preincubating cells with the dipeptide prior to challenging them with H_2O_2. Wild-type cells (CY4) and *gsh1* cells (CY8) were grown to exponential phase in minimal medium (A_{600} = 0.5) and incubated in the presence of 5 mM GSH or 5 mM L-γ-Glu-Cys for 1 h to allow for uptake of the thiol compounds. Cells were then resuspended in minimal medium containing 1–3 mM H_2O_2 for 1 h, and cell viability was determined. Preincubation with either GSH or L-γ-Glu-Cys was found to improve cell survival of both the wild-type and *gsh1* cells compared with control cells preincubated with water alone (Figure 6). Survival increased by greater than fourfold after incubation with GSH or L-γ-Glu-Cys in wild-type cells challenged with 3 mM H_2O_2 and *gsh1* cells challenged with 2 mM H_2O_2. Thus, the dipeptide appears to be as least as good as GSH in terms of its ability to serve as an antioxidant in protection against hydrogen peroxide.

These are the first findings to indicate that γ-Glu-Cys may act as an antioxidant in addition to its role as an intermediate in GSH biosynthesis. They also raise important questions regarding the evolution of the ubiquitous tripeptide GSH, because if the dipeptide γ-Glu-Cys can substitute for GSH, why synthesize

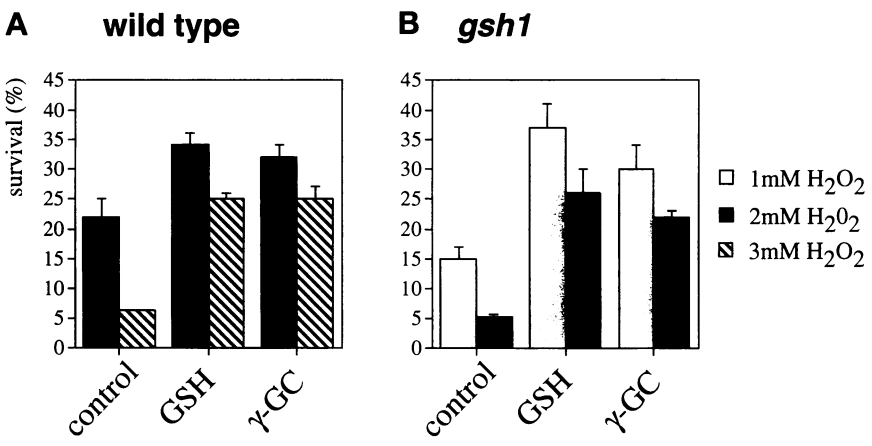

Figure 6. γ-Glutamylcysteine can substitute for GSH as an antioxidant. Wild-type (A) and *gsh1* (B) strains were grown to exponential phase in minimal medium before incubation with 5 mM GSH or 5 mM L-γ-Glu-Cys for 1 h. Cells were then resuspended in fresh minimal medium containing various concentrations of H_2O_2 for 1 h, and cell viability was determined by plating onto YEPD plates. Control cells were incubated with water prior to the H_2O_2 challenge. Percentage survival is expressed relative to untreated control cultures (100%).

GSH? The answer may be related to the role of GSH in other processes in addition to its antioxidant activity. For example, the addition of glycine to the dipeptide may promote increased stability or allow for specificity in transport and as a cofactor in enzyme and conjugation reactions. Alternate functions of GSH, in addition to a role in protection against oxidative stress, may also explain why the *gsh2* mutant is dependent on GSH for growth despite the presence of the dipeptide γ-Glu-Cys (Figure 2). In this view, γ-Glu-Cys may be able to substitute for GSH as an antioxidant but not for the other functions of GSH in the cell. Interestingly, GSH has recently been implicated in an essential role for the removal of endogenously derived metabolites in *S. pombe* (Chaudhuri *et al.*, 1997). This mechanism proceeds via conjugation to GSH and subsequent transport into the vacuole and may involve the previously identified GSH conjugate pump Ycf1 (Li *et al.*, 1996, 1997). The dipeptide γ-Glu-Cys was able to partially substitute for GSH in this detoxification pathway (Chaudhuri *et al.*, 1997), consistent with our observation that exogenously added γ-Glu-Cys could rescue the growth of GSH-deficient mutants in the absence of GSH (Figure 2). Thus, the essential role of GSH in yeast may be the conjugation and subsequent transport of toxic intermediates that arise during normal cellular metabolism. Further studies of GSH metabolism in yeast will allow for a more complete understanding of this key peptide in eukaryotes and in particular, the potential role of GSH defects in disease processes.

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