Structure-Function Analysis of Yeast Grx5 Monothiol Glutaredoxin Defines Essential Amino Acids for the Function of the Protein*

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Grx5 defines a family of yeast monothiol glutaredoxins that also includes Grx3 and Grx4. All three proteins display significant sequence homology with proteins found from bacteria to humans. Grx5 is involved in iron/sulfur cluster assembly at the mitochondria, but the function of Grx3 and Grx4 is unknown. Three-dimensional modeling based on known dithiol glutaredoxin structures predicted a thioredoxin fold structure for Grx5. Positionally conserved amino acids in this glutaredoxin family were replaced in Grx5, and the effect on the biological function of the protein has been tested. For all changes studied, there was a correlation between the effects on several different phenotypes: sensitivity to oxidants, constitutive protein oxidation, ability for respiratory growth, auxotrophy for a number of amino acids, and iron accumulation. Cys60 and Gly61 are essential for Grx5 function, whereas other single or double substitutions in the same region had no phenotypic effects. Gly115 and Gly116 could be important for the formation of a glutathione cleft on the Grx5 surface, in contrast to adjacent Cys117. Substitution of Phe50 alters the β-sheet in the thioredoxin fold structure and inhibits Grx5 function. None of the substitutions tested affect the structure at a significant enough level to reduce protein stability.

Glutaredoxins are thiol oxidireductases that catalyze redox reactions involving reduced glutathione as a hydrogen donor for the reduction of protein disulfides (dithiol mechanism of action) or glutathione-protein-mixed disulfides (monothiol mechanism of action) (see Refs. 1 and 2 for review). Previously described glutaredoxins are small proteins (about 10 kDa) with a conserved active site that includes two cysteine residues (Cys-Pro-Tyr-Cys). Site-directed mutagenesis (3–5) has demonstrated that both cysteine residues in the active site are required for the dithiol reaction. In contrast, the amino-terminal cysteine is sufficient to catalyze the deglutathionylation of the reduced glutathione-mixed disulfides that are formed under oxidative stress conditions (5).

Three-dimensional structures of oxidized and reduced forms of viral, bacterial, and mammalian glutaredoxins and also of reduced glutathione-glutaredoxin complexes have been identified using x-ray crystallography (6, 7) or nuclear magnetic resonance spectroscopy (8–14). These studies have revealed which residues, apart from those at the active site, are important for stable interactions between glutathione and the glutaredoxin molecule (10, 13, 14). Dithiol glutaredoxins are members of the thioredoxin superfamily (15, 16) along with at least five other classes of proteins that interact with cysteine-containing substrates (thioredoxins, DsbA, protein disulfide isomerases, glutathione S-transferases, and glutathione peroxidases). This superfamily shares a structural motif (called the thioredoxin fold or αβα fold) formed by a four or five-stranded β-sheet (with parallel and antiparallel strands) surrounded by three or more α-helices distributed on either side of the β-sheet (15, 16). Thioredoxins share with glutaredoxins the ability to reduce disulfides, although the former directly use NADPH as hydrogen donor (1).

Dithiol glutaredoxins participate in a large number of functions in prokaryotic and eukaryotic cells, including the activation of ribonucleotide reductase (17) and 3'-phosphoadenylylsulfate reductase (18), reduction of ascorbate (19), regulation of the DNA binding activity of nuclear factors (20), and neuronal protection against dopamine-induced apoptosis (21, 22). A family of three Saccharomyces cerevisiae proteins (Grx3, Grx4, and Grx5) has been described (23) that has significant homology with dithiol glutaredoxins, preferentially at the carboxyl-terminal region of the molecules. The absence of any of these proteins leads to a decrease in cellular glutaredoxin activity, even though they do not contain the conserved active site of classic dithiol glutaredoxins. Instead, these proteins contain the conserved Cys-Gly-Phe-Ser motif at the amino-terminal region (23). This is the only cysteine residue found in Grx3 and Grx4, whereas Grx5 has an additional cysteine at the carboxyl-terminal moiety. From these data, it has been proposed that Grx3, Grx4, and Grx5 constitute a family of monothiol glutaredoxins in yeast (23). However, although there is a high degree of homology among them, these three proteins seem to carry out different cellular functions: the absence of Grx5 causes dramatic sensitivity to oxidants and growth defects in minimal medium, whereas no clear phenotypes are observed when Grx3 or Grx4 is absent. More recently, it has been shown that Grx5 is located at the mitochondria and involved in the biogenesis of iron/sulfur clusters (24). Accumulation of cellular iron when Grx5 is absent could lead to protein oxidation and sensitivity to external oxidants. Available data about Grx3 and Grx4 indicate that they are not located in the mitochondria (24).

Proteins homologous to yeast monothiol glutaredoxins exist in all types of organisms from bacteria to humans (23, 25, 26). The human homologue (PICOT1, protein kinase C-interacting cousin of thioredoxin; YPD, 1% yeast extract, 2% peptone, and 2% dextrose.

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a negative regulator of protein kinase C-6 in the pathway leading to activation of the activator protein 1 and nuclear factor κB transcription factors (27). The conserved region has been termed PICOT homology domain, and in Grx5, it corresponds to the majority of the peptide (23, 25). Human PICOT, yeast Grx3 and Grx4, and other eukaryotic homologous proteins possess amino-terminal extensions of PICOT homology domain. These extensions have signatures characteristic of thioredoxins or dithiol glutaredoxins that do not encompass the oxidoreductase active site (25). All these observations support the differential roles displayed by monothiol glutaredoxins regardless of their structural similarities.

In this work, we show that Grx5 defines a ubiquitous family of proteins whose members are present in most types of organisms and are characterized by the presence of a thioredoxin fold structure. We also demonstrate the essential biological roles of a number of conserved amino acid residues, such as a cysteine located at the previously proposed active site in the amino-terminal region and a pair of glycines in the carboxy-terminal region.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and Amino Acid Replacements**—CML235 (MATa ura3-52 leu2-1 his3Δ200) was used as wild-type strain. MML19 is an isogenic Δgrx5::kanMX derivative of CML235 (23). MML61 was obtained from the latter by chromosomal integration of the YIpplac211 vector (integrative, LEU2 marker) (28). MML161 was constructed similarly, although a YIpplac211-derived plasmid (pMM25) with GRX5 expressed under its own promoter was integrated at LEU2. Other strains listed in Table I resulted from integration of pMM25-derived plasmids carrying the indicated point mutations at the mutant leu2 locus of MML19.

The following plasmids contain the cloned GRX5 open reading frame (with the type of mutation in the translation product indicated in parentheses) without further upstream or downstream sequences, under the control of the dicyclic-regulatable tetO2 promoter in plasmid pCM190 (29): pCM319 (wild-type GRX5), pMM176 (F50E), pMM113 (C60S), pMM155 (G61V), pMM127 (G115V), and pMM112 (G116V). These plasmids were then transformed into strain CML236 (30), which carries the dicyclic-inducible tetR-SSN6 repressor gene, to determine the stability of the Grx5 wild-type protein and the amino acid-substituted derivatives.

Point mutations in the GRX5 open reading frame that yielded the different amino acid replacements were constructed by the ExSite method (31), using either pMM25 or pCM319 DNA as a template. Oligonucleotides for PCR amplification were designed in such a way that the alterations that did not alter the translation product was introduced near to the desired point mutation and used as a marker for it. Successful introduction of the mutations was confirmed by DNA sequencing.

**Growth Conditions and Determination of Sensitivity to Menadione**—Cells were usually grown at 30 °C in rich YPD medium. Plasmid-bearing transformants were grown in synthetic complete medium (32) without the selective auxotropic requirement. Plates of synthetic defined medium (0.67% yeast nitrogen base, 2% glucose, and auxotropic requirements) were used to test mutant growth. Cells growing exponentially in YPD medium at 30 °C (about 2 × 10^6 cells/ml) were treated with menadione (10 mM) to determine sensitivity to it. After treatment, 1.5 serial dilutions were made, and drops were spotted onto YPD plates.

Growth was recorded after 2 days of incubation at 30 °C. Growth analysis was performed on plates incubated at 30 °C. Growth analysis was performed on plates incubated at 30 °C.

**Analytical Methods**—Protein carbonyl levels in crude cell extracts were quantified according to the dinitrophenylhydrazine derivatization method (23). Total iron content was determined by atomic absorption spectroscopy.

**RESULTS**

**Yeast Grx5 Is a Member of a Ubiquitous Family of Proteins Sharing the PICOT Domain**—Yeast Grx5 has been characterized as a monothiol glutaredoxin-like protein whose amino acid sequence displays extensive homology (particularly at what have been designated its amino-terminal and carboxyl-terminal regions) with a family of proteins whose members are present in all living organisms from bacteria to humans (23, 25). The carboxyl-terminal region also has significant homology with classic dithiol glutaredoxins (23). To extend these initial studies, the Institute for Chemical Research and Swiss Protein Databases were searched for proteins with the highest homology with Grx5 (E value cutoff, 1 × e^-10), using FASTA analysis. We only considered proteins that retained the putative active site CGFS sequence in the amino-terminal region (23) for comparison. The 35 protein sequences with the highest similarity score with Grx5 were then aligned using the ClustalW program (Fig. 1). A putative Grx5 homologue from Candida albicans, as deduced from the genome sequence of the latter organism, was also included for comparison. Extensions at the amino-terminal and carboxyl-terminal ends (that are present only in some of the family members (see below)) were omitted for the alignment. The existence of two amino-terminal and carboxyl-terminal regions with extensive homology (23) (separated by a less well-conserved region with a slightly variable length) was confirmed in this extended study.

Most multicellular eukaryotic members of the Grx5 family have large amino-terminal extensions. This is also the case for the S. cerevisiae Grx3 and Grx4 glutaredoxins and for one of the two sequences in fission yeast (Fig. 1). This amino-terminal extension includes a highly conserved duplication of the region shown in Fig. 1 in the cases of human and rat species and in one of the two mouse species (Q6JLZ2M) (Ref. 25 and this study). Interestingly, the Arabidopsis thaliana protein Q8ZPH2A (but not other members of the same protein family in this plant species) contains three conserved domains in tandem, but only the most carboxyl-terminal of these is shown in the Fig. 1 alignment. On the other hand, S. cerevisiae Grx5, the C. albicans Grx5 homologous protein, the other fission yeast protein, and all bacterial members of the family have shorter versions of the protein without amino-terminal extensions.

The domain shown in the alignments is almost totally coincident with the PICOT homology domain region named after the human Grx5 homologue (25, 27). Our study shows that this domain may be shared by proteins from prokaryotes (both Archaea and bacteria) and eukaryotes. These proteins may have divergent functions and different cellular locations.

**A Thioredoxin Fold Structure Is Predicted for Grx5 Glutaredoxin**—Grx5 has sequence similarity with dithiol glutaredoxins, mostly at the carboxyl-terminal moiety (23). The three-
dimensional structure of a number of dithiol glutaredoxins was already known, from either x-ray crystallography or NMR spectroscopy studies. Structures in the Protein Data Bank were used to construct a three-dimensional model for Grx5. Two protein structures, pig liver thioltransferase (1KTE) (7) and pig liver glutaredoxin (3GRX) (14), yielded useful models for Grx5. Amino acid sequences of these two proteins show 28% identity to Grx5. Models based both on the structure of phage T4 glutaredoxin (1ABA) (6), which is considered to provide a valid representation of the Grx5 protein structure. The proposed model shows that the similarity between Grx5 and other proteins from the superfamily extends to the amino-terminal moiety of the molecule.

Cys\textsuperscript{60}, but Not Cys\textsuperscript{117}, Is Essential for the Biological Activity of Grx5—Grx5 contains two cysteine residues at positions 60 and 117. The first is part of a conserved sequence common to all members of the family (Ref. 23; Fig. 1). It is exposed at the surface of Grx5 between a \( \beta \)-strand and an \( \alpha \)-helix region (Fig. 3). Cys\textsuperscript{117} is only present in certain family members (Fig. 1), although many dithiol glutaredoxins also contain a cysteine residue in an equivalent position (23). This cysteine is the first \( \alpha \)-helix region is named according to the nomenclature for thioredoxin fold structure (15). Abbreviations for organisms were as follows: Sc, S. cerevisiae; Ca, C. albicans; Sp, Schizosaccharomyces pombe; Nm, Neisseria meningitidis; Hs, Homo sapiens; Lm, Leishmania major; H. influenzae; Ec, E. coli; Vc, Vibrio cholerae; Rp, Rickettsia prowazekii; S. pombe, S. cerevisiae Grx5.

**FIG. 1.** ClustalW alignment of proteins displaying homology to \( S.\) cerevisiae Grx5. Amino-terminal and carboxyl-terminal extensions were not considered for the alignment. Protein species are named according to their Swiss-Prot accession number, except for ml10053, which corresponds to the Institute for Chemical Research accession number. The numbers in parentheses indicate the position of the first amino acid residue shown. The top row indicates the predicted secondary structure. The four predicted \( \alpha \)-helix regions are named according to the nomenclature for thioredoxin fold structure (15). Abbreviations for organisms were as follows: Sc, S. cerevisiae; Ca, C. albicans; Sp, Schizosaccharomyces pombe; Nm, Neisseria meningitidis; Hs, Homo sapiens; Lm, Leishmania major; H. influenzae; Ec, E. coli; Vc, Vibrio cholerae; Rp, Rickettsia prowazekii; S. pombe, S. cerevisiae Grx5.
Increased iron concentration in and the constitutive carbonylation of cell proteins (23, 24). Described above, including the accumulation of cellular iron determined whether the mutations that caused an increase in an indicator of oxidative damage to protein (24). Thus, we with a rise in the number of protein carbonyl groups, which is an indicator of oxidative damage to protein (24). Thus, we determined whether the mutations that caused an increase in

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**A** Number of Conserved Residues Are Important for Grx5 Activity—Besides Cys$^{60}$ and Cys$^{117}$, other residues are also conserved in the Grx5 sequence as revealed by comparison with the other family members. We introduced a number of single and double point mutations into Grx5 (Fig. 4A; Table I) that changed the amino acid residues in the putative active site region. Changes were also introduced in other amino acids that were presumably important for maintaining the three-dimensional structure of Grx5. Thus, according to the proposed model (Fig. 3), the F50E mutation could alter a β-strand that is part of the active site cleft of Grx5. The G115V and G116V changes alter a glycine pair that is conserved in all dithiol and monothiol glutaredoxins (23). This pair is probably important for the proper orientation of $\alpha_3$ relative to $\alpha_1$ and Cys$^{60}$ (Fig. 3). Of the single and double amino acid changes in the conserved CFGS region, only the G61V change caused biological inactivation of Grx5 (Fig. 4C). In contrast, the F50E and the single G115V and G116V mutations annulled the biological activity of Grx5. Less bulky side chains were also used for Gly$^{61}$ or Gly$^{116}$ substitutions. In both cases, introduction of an alanine residue maintained the wild-type phenotype, whereas serine disrupted the biological activity of the protein (Fig. 4C). We concluded that some but not all of the conserved residues in the Grx5 family are essential for the activity of the protein.

Loss of Grx5 causes other phenotypic effects besides those described above, including the accumulation of cellular iron and the constitutive carbonylation of cell proteins (23, 24). Increased iron concentration in grx5 cells has been associated with a rise in the number of protein carbonyl groups, which is an indicator of oxidative damage to protein (24). Thus, we determined whether the mutations that caused an increase in

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**DISCUSSION**

Higher eukaryotes have cytosolic dithiol glutaredoxins, which are required for maintaining the reduced status of protein thiol groups and for the activity of specific proteins (1, 2, 17–22). Furthermore, a mitochondrial dithiol glutaredoxin has recently been described in human cells (38, 39). Its existence reveals the importance of glutathione as a hydrogen donor for protein disulfide groups not only in the cytoplasm, but also in other cellular compartments. S. cerevisiae cells contain two dithiol glutaredoxins (Grx1 and Grx2) that are located at the cytosol and are highly homologous to other prokaryotic and eukaryotic members of the family (40, 41). However, no typical dithiol glutaredoxin seems to exist in yeast mitochondria. A family of three proteins (Grx3, Grx4, and Grx5) has recently been described in yeast. They all have glutaredoxin signatures...
but contain a single cysteine residue at the conserved putative active site (23). Based on this and the fact that both single mutants and combinations of double mutants display reduced glutaredoxin activity in cell extracts, we classified them as monothiol glutaredoxins. Grx5 is mitochondrially located and is involved in the maturation of Fe/S cluster-containing proteins at the organelle matrix (24). Defects in grx5-null mutants are common to mutants in other genes involved in Fe/S cluster assembly (24, 42) and include sensitivity to oxidants, auxotrophy for amino acids whose biosynthesis requires Fe/S-containing enzymes, respiratory defects, and iron accumulation. The function of yeast Grx5 is different from that of its human homologue, the PICOT protein, which has been proposed as a modulator of the protein kinase C-\(\text{H9258}\) pathway (25, 27). Yeast Grx3 and Grx4 are not mitochondrial, and their absence does not cause the phenotypes observed in grx5 cells. On the other hand, sequence alignment reveals that Grx3 and Grx4 are closer relatives to PICOT than Grx5. This raises the possibility, which has yet to be investigated, of a functional relationship between Grx3/Grx4 and the PICOT protein. Taken together, the above observations show a spatial and functional separation between yeast monothiol glutaredoxins.

Here we have centered our attention on the Grx5 structure-function relationship. From studies with dithiol glutaredoxin mutants in which one of the two Cys residues in the active site was eliminated, it has been concluded that monothiol glutaredoxins are active against mixed disulfides involving glutathione and protein sulfhydryls (5, 43). A three-dimensional model of Grx5, based on the known structure of a number of dithiol glutaredoxins, is proposed. Grx5 has an obvious thioredoxin fold structure. Cys60 (in the conserved P\(\text{XCGFS}\) region) lies opposite the Gly 115 and Gly 116 residues conserved in both monothiol and dithiol glutaredoxins. As in the dithiol molecules (14, 44), this glycine pair forms a loop that could confer flexibility for the appropriate positioning of \(\text{H9251}\) relative to \(\text{H9251}\) (Fig. 3). Thus, the two \(\text{H9251}\) and \(\text{H9251}\) regions form the glutathione cleft with the \(\text{H9252}\)-sheet at the bottom. From structural studies involving dithiol glutaredoxins, we can also deduce that other conserved residues in Grx5 are important for the stabilization of glutathione at the active site groove and its interaction with Cys60. Asp118 is present at the \(\text{H9251}\) region of Grx5 and is conserved in both glutaredoxin families (Ref. 23 and this work). It has been proposed that this residue establishes an ionic interaction with the \(\text{H9251}\)-amino group of the glutamic acid residue of glutathione (3, 13, 14). Lys20 of Grx5 is also conserved in both types of glutaredoxins, and its amino group could interact electrostatically with the \(\text{H9251}\)-carboxylate of the carboxyl-terminal glycine of glutathione (13, 14). Stabilization of glutathione in the cleft of...
The secondary structure elements at the amino acid substitutions introduced in the GRX5 gene (MML161) or the null CML235. The strains listed were derived from MML19 by insertion of the corresponding plasmids. Plasmids (YIplac211 vector or derivatives with wild-type or GRX5 mutations) were integrated at the chromosomal LEU2 locus after transformation with DNA that had been linearized by digestion at the single EcoRV site within the plasmid LEU2 gene. The amino acid replacements introduced in the GRX5 translation product are indicated. The secondary structure elements at the amino acid substitution positions are inferred from the model in Fig. 3.

| Strain     | Plasmid | Point mutation | Inferred secondary structure at position |
|------------|---------|----------------|------------------------------------------|
| MML160    | YIplac211 | No insert     |                                          |
| MML161    | pMM25   | Wild-type     | GRX5                                    |
| MML163    | pMM27   | C60S           | Coil                                    |
| MML165    | pMM28   | C117S          | Helix                                   |
| MML219    | pMM76   | F62S           | Helix                                   |
| MML221    | pMM77   | F58V           | Coil                                    |
| MML223    | pMM78   | K59Q           | Coil                                    |
| MML225    | pMM79   | G116V          | Coil                                    |
| MML273    | pMM88   | P58V K59Q     | Coil                                    |
| MML274    | pMM90   | P58V F62S     | Coil, helix                             |
| MML276    | pMM92   | G115V          | Coil                                    |
| MML322    | pMM124  | G61V           | Helix                                   |
| MML374    | pMM174  | F50E           | Beta                                    |
| MML421    | pMM202  | G115A          | Coil                                    |
| MML423    | pMM203  | G115S          | Coil                                    |
| MML425    | pMM204  | G61A           | Helix                                   |
| MML427    | pMM205  | G61S           | Helix                                   |

**TABLE II**

Protein carbonyl content in Grx5 mutants with different amino acid substitutions

| Grx5 amino acid substitution | Relative carbonyl content |
|------------------------------|---------------------------|
| None                         | 1.00                      |
| F50E                         | 1.48 ± 0.07               |
| C60S                         | 1.42 ± 0.03               |
| G61S                         | 1.59 ± 0.04               |
| G115V                        | 1.52 ± 0.05               |
| C117S                        | 1.55 ± 0.04               |

**FIG. 6.** Half-life of Grx5. CML276 cells transformed with plasmids that expressed the wild-type GRX5 gene or different mutant derivatives under the control of the tetO7 promoter were grown in YPD medium at 30 °C. At time 0, doxycycline (20 μg/ml) was added to repress Grx5 synthesis. A, Western blot analysis of Grx5 in CML276 cells transformed with pCM319. The same amount of total cell protein was loaded for each sample. The left-most run corresponds to CML276 cells expressing only the chromosomal GRX5 gene under its own promoter. B, quantification of Grx5 levels in samples taken at successive intervals after doxycycline addition from Western blot analyses similar to that shown in A. Transformants that expressed the indicated Grx5 forms were used. At least two independent experiments were carried out for each transformant.

E. coli Grx3 glutaredoxin could also involve ionic interaction with an Arg49 residue (14). This residue is not present in all dithiol glutaredoxins, but an equivalent Arg49 residue is present in the α2-helix of Grx5 and is conserved in all the monothiol glutaredoxins analyzed. It may therefore also contribute to glutathione stabilization. A coiled region following the α2-helix (Fig. 3) is particularly conserved among dithiol glutaredoxins, as is a 15-residue loop of monothiol glutaredoxins (10). The active cysteine in position 61, whereas a bulky side chain would impede access of the glutathione molecule to the active site. We also hypothesized that changing Phe50 for a residue such as glutamic acid would alter the β-sheet structure that delimits the glutathione cleft and would make it difficult to appropriately position glutathione.
one relative to Cys60. In fact, this was the case: the F50E change annulled the Grx5 activity.

Grx5 contains a second cysteine at position 117, which is not required for the protein biological activity. This carboxyl-terminal cysteine residue is also present at equivalent positions in many, but not all, monothiol and dithiol glutaredoxins; Grx3 and Grx4, for instance, do not possess it. Mutation of this cysteine in E. coli Grx3 has no effect on enzyme activity, and it has been proposed that the residue could have a regulatory role on the interaction of glutaredoxin with a second glutathione molecule necessary in the dithiol mechanism of action (14). There is, however, no evidence for such regulatory role in Grx5.

We have determined the half-life of the Grx5 protein using the tet promoter to conditionally express Grx5. The same result was obtained for the wild-type strain and for the different mutants, which is an argument against major alterations in the three-dimensional structure of the protein, even in the case of amino acid replacements that cause loss of activity.

The absence of Grx5 causes a number of phenotypic effects that are all closely related (24). Thus, the primary defect in the assembly of Fe/S clusters would lead to (i) an inability to synthesize a number of amino acids, (ii) respiratory growth defects, and (iii) an accumulation of iron in the cell. As a consequence of the latter, there is an accumulation of reactive oxygen species in the cells, which in turn increases the level of protein carbonyl groups and makes cells more sensitive to external oxidants. Those amino acids substitutions that affect the biological activity of Grx5 alter all the indicated phenotypes as a consequence of the latter, there is an accumulation of reactive oxygen species in the cells, which in turn increases the level of protein carbonyl groups and makes cells more sensitive to external oxidants. Those amino acids substitutions that affect the biological activity of Grx5 alter all the indicated phenotypes.

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