THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 50, Issue of December 10, pp. 51923–51930, 2004

*Printed in U.S.A.*

# Nuclear Monothiol Glutaredoxins of *Saccharomyces cerevisiae* Can Function as Mitochondrial Glutaredoxins*

Received for publication, September 7, 2004
Published, JBC Papers in Press, September 28, 2004, DOI 10.1074/jbc.M410219200

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**Glutaredoxins are thiol oxidoreductases that regulate protein redox state. In *Saccharomyces cerevisiae*, Grx1 and Grx2 are cytosolic dithiol glutaredoxins, whereas Grx3, Grx4, and Grx5 are monothiol glutaredoxins. Grx5 locates at the mitochondrial matrix and is needed for iron/sulfur cluster biogenesis. Its absence causes phenotypes such as inactivation of iron/sulfur enzymes and sensitivity to oxidative stress. Whereas Grx5 contains a single glutaredoxin domain, in Grx3 and Grx4 a thioredoxin-like domain is fused to the glutaredoxin domain. Here we have shown that Grx3 locates at the nucleus and that the thioredoxin-like domain is required for such location. We have addressed the functional divergence among glutaredoxins by targeting Grx2/3/4 molecules to the mitochondrial matrix using the Grx5 targeting sequence. The mitochondrial forms of Grx3 and Grx4 partially rescue the defects of a *grx5* null mutant. On the contrary, mitochondrially targeted Grx2 does not suppress the mutant phenotype. Both the thioredoxin-like and glutaredoxin domains are needed for the mitochondrial activity of Grx3, although none of the cysteine residues at the thioredoxin-like domain is required for rescue of the *grx5* phenotypes. We have concluded that dithiol glutaredoxins are functionally divergent from monothiol ones, but the latter can interchange their biological activities when compartment barriers are surpassed.**

Glutaredoxins (Grx)[1] are small (~10 kDa) thiol disulfide oxidoreductases that catalyze glutathione (GSH)-disulfide redox reactions (1, 2). They reduce protein disulfides through a dithiol mechanism that involves two cysteine residues in the active site of the glutaredoxin molecule, or they deglutathionylate GSH-protein mixed disulfides through a monothiol mechanism of action involving a single cysteine at the active site (2). In contrast to glutaredoxins, thioredoxins employ NADPH directly as hydrogen donor. Besides glutaredoxins and GSH, the glutaredoxin system also includes glutaredoxin reductases that regenerate GSH from glutathione disulfide. Glutaredoxins have a number of biological roles. These include activation of ribonucleotide reductase (1, 3) and 3′-phosphoadenylylsulfate reductase (4), reduction of ascorbate (5), GSH-mediated reduction of dihydrolipoamide (6), regulation of the DNA binding activity of nuclear factors (7), neuronal protection against dopamine-induced apoptosis (8) and excitotoxic mitochondrial damage (9), and regulation of signal cascades that protect against oxidative stress (10, 11). Glutaredoxins play an important role in protecting against protein oxidative damage by regulating the glutathionylated state of essential sulfhydryl groups (12). To carry out the above functions, glutaredoxins are present in various different cell compartments. In human cells, Grx1 glutaredoxin is cytosolic (13), whereas a second glutaredoxin (Grx2) has two isoforms that are, respectively, located at the nucleus and the mitochondria (14, 15). In plants, glutaredoxins have also been described at the cytosol and mitochondria (16). The existence of cross-talk between the glutaredoxin and thioredoxin systems is illustrated by the fact that human Grx2 is able to deglutathionylate GSH-protein mixed disulfides through thioredoxin reductase as an alternative to GSH (17).

The thioredoxin and glutaredoxin systems are also present in yeasts (18–20). *Saccharomyces cerevisiae* contains both a cytosolic and a mitochondrial thioredoxin system (21, 22) whose functions may overlap with those of the glutaredoxin system (23). *S. cerevisiae* contains two glutaredoxins (Grx1 and Grx2) with conserved CPYC motifs in their active sites (24). Both are cytosolic, though a minor fraction of Grx2 molecules is located at the mitochondria (25). A single glutathione reductase (Grr1) shares a cytosolic and mitochondrial location (26). In addition to the dithiol glutaredoxins, *S. cerevisiae* cells contain three monothiol glutaredoxins (Grx3, Grx4, and Grx5) with the motif CGFS in their active sites (27, 28). Grx5 is located at the mitochondrial matrix and participates in the biogenesis of iron/sulfur (Fe/S) clusters (29, 30). The absence of Grx5 causes the inactivation of enzymes containing Fe/S clusters, intracellular iron accumulation, hypersensitivity to external oxidants, high levels of protein oxidation, auxotrophy for a number of amino acids, and low growth rates in rich medium (27, 29). A number of proteins also become hyperglutathionylated in null *grx5* mutants (31). All of these phenotypes are probably a consequence of disruption of the Grx5 function in the biogenesis of Fe/S clusters because most of the alterations are shared by other mutants affected in the biogenesis of the clusters (32, 33). Grx3 and Grx4 molecules are larger than Grx5 because they contain a thioredoxin-like domain that is N-terminal located relative to the glutaredoxin domain, with a linking region between the two domains (28, 34).

Yeast monothiol glutaredoxins are members of a large group of proteins present in organisms ranging from bacteria to higher eukaryotes (28, 35). The human member is the PICOT

* This work was supported in part by grants from the Spanish Ministerio de Ciencia y Tecnología (BMC2001-1213-C02-01) and from the Generalitat de Catalunya (2001SGR00305). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a predoctoral grant from the Generalitat de Catalunya.

§ Supported by a Ramón y Cajal contract from the Ministerio de Ciencia y Tecnología.

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[1] The abbreviations used are: Grx, glutaredoxin; GSH, reduced glutathione; GFP, green fluorescent protein; Trx, thioredoxin.

TABLE I
*Strains employed in this work*

| Strain | Relevant phenotype | Comments |
|---|---|---|
| W303–1A | *MAT***a** *ura3–1 ade2–1 leu2–3,112 trp1–1 his3–11,15* | Wild type |
| W303–1B | As W303–1A but *MATα* | Wild type |
| MML100 | *MAT***a** *grx5*::*kanMX4* | Ref. 29 |
| MML240 | *MAT***a** *grx5*::*kanMX4* [pMM54(*GRX5–3HA*)]::*LEU2* | Ref. 29 |
| MML241 | *MATα grx5*::*kanMX4* | As MML100, but derived from W301–1B |
| MML443 | *MAT***a** [pMM227(*GRX3–3HA*)]::*LEU2* | Integration of linear pMM227 in W303–1A |
| MML446 | *MAT***a** [pMM229(*GRX4–3HA*)]::*LEU2* | Integration of linear pMM229 in W303–1A |
| MML458 | *MAT***a** [pMM225(*GRX2–3HA*)]::*LEU2* | Integration of linear pMM225 in W303–1B |
| MML454 | *MAT***a** *grx5*::*kanMX4* [pMM227(*GRX3–3HA*)]::*LEU2* | Spore from a cross MML241 × MML443 |
| MML456 | *MAT***a** *grx5*::*kanMX4* [pMM229(*GRX4–3HA*)]::*LEU2* | Spore from a cross MML241 × MML446 |
| MML465 | *MAT***a** *grx5*::*kanMX4* [pMM225(*GRX2–3HA*)]::*LEU2* | Spore from a cross MML100 × MML458 |
| MML488 | *MAT***a** pMM338::*LEU2* | Integration of linear pMM338 in W303–1B |
| MML490 | *MAT***a** pMM340::*LEU2* | Integration of linear pMM340 in W303–1B |
| MML492 | *MAT***a** pMM342::*LEU2* | Integration of linear pMM342 in W303–1B |
| MML500 | *MAT***a** *grx5*::*kanMX4* pMM338::*LEU2* | Spore from a cross MML100 × MML488 |
| MML502 | *MAT***a** *grx5*::*kanMX4* pMM340::*LEU2* | Spore from a cross MML100 × MML490 |
| MML504 | *MAT***a** *grx5*::*kanMX4* pMM342::*LEU2* | Spore from a cross MML100 × MML492 |
| MML507 | *MAT***a** [pMM344(*GRX3-C72S-3HA*)]::*LEU2* | Integration of linear pMM344 in W303–1B |
| MML509 | *MAT***a** [pMM349(*GRX3-C211S-3HA*)]::*LEU2* | Integration of linear pMM349 in W303–1B |
| MML514 | *MAT***a** *grx5*::*kanMX4 a* [pMM349(*GRX3-C211 S-3HA*)]::*LEU2* | Spore from a cross MML100 × MML509 |
| MML516 | *MAT***a** *grx5*::*kanMX4 a* [pMM344(*GRX3-C72S-3HA*)]::*LEU2* | Spore from a cross MML100 × MML507 |
| MML563 | *MAT***a** [pMM387 (*GRX3-C2S-3HA*)]::*LEU2* | Integration of linear pMM387 in W303–1B |
| MML566 | *MAT***a** *grx5*::*kanMX4 a* [pMM387(*GRX3-C2S-3HA*)]::*LEU2* | Spore from a cross MML100 × MML563 |
| MML607 | *MAT***a** pMM221::*LEU2* | Integration of linear pMM221 in W303–1A |
| MML614B | *MAT***a** *grx5*::*kanMX4* pMM221::*LEU2* | Spore from a cross MML241 × MML607 |

protein, a negative regulator of protein kinase θ in the pathway that controls c-Jun N-terminal kinase (36). This translocates from the cytosol to the nucleus in response to oxidative stress (37). The mouse PICOT homologue is differentially expressed at stages of mouse embryogenesis concomitant with neural tube closure (38).

Some functional interactions may exist between monothiol and dithiol glutaredoxins in *S. cerevisiae*. However, they do not fully substitute each other because the overexpression of Grx3 or Grx4 does not rescue the above defects of *grx5* cells (29). It has recently been shown that Grx4 is located at the nucleus and that it interacts with the Bud32 protein kinase (39). In the current work we have demonstrated that Grx3 is also located at the nucleus and that its thioredoxin-like domain is required for nuclear targeting. We have also shown that when either Grx3 or Grx4 are targeted to the mitochondria they can substitute for Grx5 function, unlike dithiol glutaredoxins. Therefore, differential compartmentalization determines separate biological functions for the members of the monothiol glutaredoxin family.

## EXPERIMENTAL PROCEDURES

*Yeast Strains and Plasmids*—The yeast strains employed in this work are described in Table I. Plasmid pMM221 was based on the *LEU2* integrative vector YIplac128 (40), with the doxycycline-controlled tTA transactivator and $tetO_2$ promoter regions from pCM188 (41), the mitochondrial targeting sequence of *GRX5* from pCM318 (29), and the 3HA/$His_6$ tag from pCM265 (42), plus a polylinker created with adequate complementary oligonucleotides. Plasmid pMM225 contains the *GRX2* coding sequence from the nucleotide at position +103 to the last coding nucleotide, which is in-frame with the 3HA/$His_6$ tag. It was cloned between the NotI and PstI sites of the vector pMM221. Similarly, plasmids pMM227 and pMM229, respectively, contain the *GRX3* and *GRX4* coding sequences from the second to the last coding codon, cloned between the NotI and PstI sites of pMM221. Plasmids pMM338, pMM340, and pMM342 derive from pMM221 by, respectively, cloning the sequences of *GRX3* coding from amino acid 2 to 133 (including the Trx domain), 2 to 198 (Trx domain plus linker), or 199 to 285 (Grx domain) between the NotI and PstI sites of the vector. pMM344, pMM349, pMM387, and pMM451 are pMM227 derivatives with the following respective point mutations in the Grx3 sequence: C72S, C211S, C2S, and C18A. Plasmid pMM453 also derives from pMM227 and contains the three point mutations C2S, C18A, C72S in the coding sequence for Grx3. GFP fusions to different regions of Grx3 were constructed under the control of the *MET25* promoter in pUG35 (from W. H. Hegemann, Institute of Microbiology, Duesseldorf, Germany), using the EcoRI and HindIII vector sites. pMM423 and pMM421, respectively, contain the *GRX3* coding sequences from amino acid 1 to 133 and 199 to 285, in-frame with the GFP sequence at the C terminus. Plasmid pMM425 is a pUG35 derivative with the whole *GRX3* sequence C-terminal tagged with GFP.

*Growth Conditions*—YPD medium (1% yeast extract, 2% peptone, 2% dextrose) was usually employed. YPGly and YPGal media are similar to YPD except that they, respectively, contain 3% glycerol and 2% galactose instead of dextrose. Synthetic S.D. medium contains 0.67% yeast nitrogen base, 2% glucose, and the auxotrophic requirements. Doxycycline was added to the medium at the indicated concentrations to modulate expression from the $tetO_2$ promoter. Cells were exponentially grown at 30 °C under the required conditions for at least 10 generations before samples were taken for analysis.

*Genetic Methods*—Standard methods were employed for DNA manipulation, transformation, crosses between yeast strains, and tetrad analyses. PCR-amplified *S. cerevisiae* genomic DNA was used for cloning with appropriate oligonucleotides for amplification that created the required restriction sites. Point mutants to yield the different amino acid replacements were constructed by the ExSite method (43). In this case, oligonucleotides for PCR amplifications were designed in such a way that a restriction site not altering the translation product was introduced as a marker near the desired point mutation. Introduction of the mutations was confirmed by DNA sequencing. Integrative plasmids were made linear by EcoRV digestion before transformation of the wild type cells to yield the respective derivatives.

*Other Methods*—Mitochondria were purified and fractionated from cell cultures in lactate medium as described in Ref. 44. Western blot analyses were done as in Ref. 29, with 12CA5 anti-HA monoclonal antibody (Roche Diagnostics) at a 1:5,000 dilution and anti-lipoic acid antibody at 1:50,000 dilution. Aconitase and malate dehydrogenase were assayed following the methods described in Ref. 45. Extracts from cells growing exponentially in YPGal medium were prepared in 0.1 M Tris buffer, pH 8.0, plus protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 0.2 mM tolylsulfonyl phenylalanyl chloromethyl ketone, 2 μM pepstatin A, final concentrations), using glass beads to break the cells. Microscopic localization of GFP-labeled proteins in living cells was done by standard techniques (46).

## RESULTS

*The Thioredoxin-like Domain of Grx3 Is Required for Targeting the Protein to the Nucleus*—The three monothiol glutaredoxins of *S. cerevisiae* share a Grx domain that displays partial homology with dithiol glutaredoxins and contains the single cysteine active site (27, 28) (Fig. 1*A*). This conserved domain has also been termed PICOT after its presence in the human PICOT protein (34). In addition, Grx3 and Grx4 contain

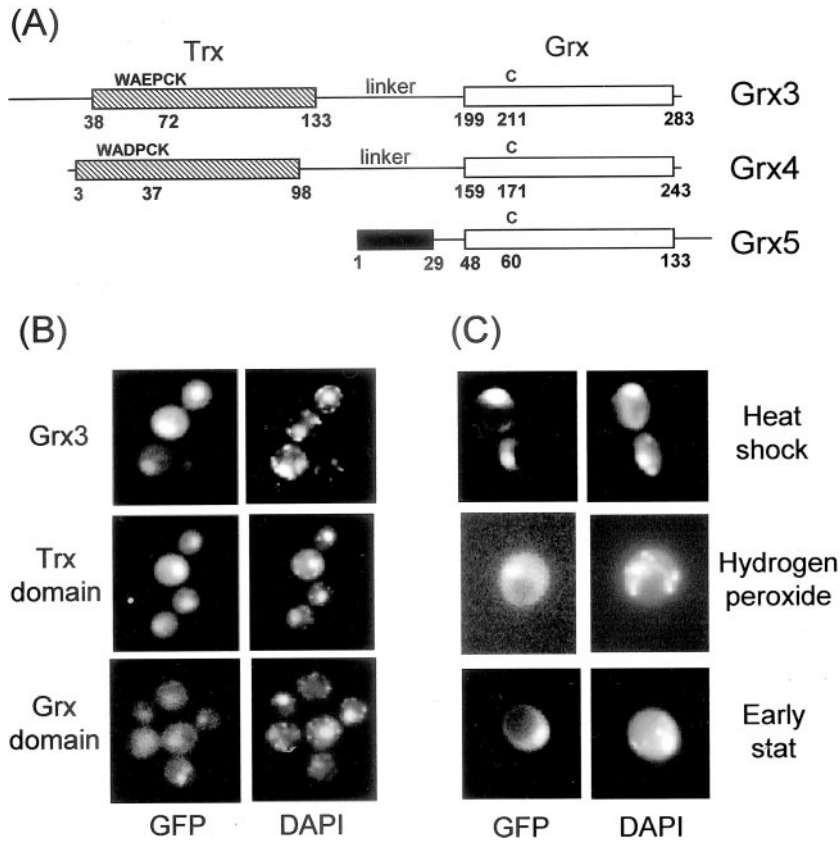


FIG. 1. **Nuclear localization of Grx3.** *A*, domains of the monothiol glutaredoxins Grx3, Grx4, and Grx5. The thioredoxin-like (*Trx*) and glutaredoxin (*Grx*) domains are indicated with the positions of the initial and final amino acids at each domain (see Ref. 34 for more details). The conserved sequences at the Trx domain of Grx3 and Grx4 that are reminiscent of the thioredoxin active site are also shown. The position of the cysteine at the active site of the Grx domain is indicated. The *black box* corresponds to the mitochondrial targeting sequence of Grx5. *B*, fluorescence micrographs of exponentially growing cells in S.D. medium without methionine at 30 °C expressing the whole Grx3 sequence (pMM425) or the Trx (pMM423) or Grx (pMM421) domains of Grx3, tagged at the C terminus with GFP. Cells were also 4′,6-diamidino-2-phenylindole (DAPI)-stained; images are shown in parallel. *C*, exponentially growing cells in S.D. medium at 30 °C expressing Grx3·GFP (pMM425) were subjected to heat shock (30 min at 38 °C), hydrogen peroxide treatment (0.5 mM, 1 h), or cultured until early stationary phase (72 h at 30 °C). GFP fluorescent and DAPI-stained cells are shown.

an N-terminal extension that is highly homologous to thioredoxin sequences (Trx domain in Fig. 1*A*). This domain is not present in Grx5, which instead contains a mitochondrial targeting sequence (29). The Trx domain contains a WAD/EPCK sequence that is reminiscent of the authentic thioredoxin active site motif WCGPCK (47). The Trx domain is conserved in many members of the Grx3/Grx4 protein family in other eukaryotic organisms (28, 35). A non-conserved linker region is present between the Trx and Grx domains (Fig. 1*A*). It has recently been shown that Grx4 is located at the nucleus (39). Using a C terminus GFP-tagged construction under the control of the *MET25* promoter, we have observed that Grx3 is also targeted to the nucleus in conditions of maximum expression from the promoter (Fig. 1*B*). When the expression level of the Grx3·GFP fusion protein was reduced by adding intermediate methionine concentrations (up to 25 $\mu$M) to the growth medium, Grx3 was still concentrated at the nucleus (not shown), discarding any possible abnormal location caused by overexpression. We concluded that Grx3 and Grx4 are both nuclear glutaredoxins, which suggests that their functions could be redundant.

To determine the region of Grx3 responsible for its nuclear localization, the Trx and Grx domains were separately tagged with GFP in the same *MET25*-based vector as above. Cells expressing the Trx·GFP construction showed a nuclear fluorescence pattern, whereas those with the Grx·GFP construction showed diffuse fluorescence (Fig. 1*B*). This indicates that the Trx domain (but not the Grx domain) contains a nuclear location signal for Grx3 and is able to be directed to the nucleus on its own. Analysis of the Trx region amino acid sequence did not reveal either a simple or bipartite canonical nuclear localization signal (48). We addressed the possibility of Grx3 location being regulated by external stresses in a similar way to the external conditions-controlled nuclear location of several transcription factors that mediate stress responses (Msn2/4, Yap1, Aft1) (19, 49, 50). However, neither heat shock, hydrogen peroxide-mediated oxidative stress, nor entry into the post-diauxic stage of the growth curve affected the nuclear location of Grx3 (Fig. 1*C*). We have therefore concluded that Grx3 has a constitutive nuclear location.

*Grx3 and Grx4, but Not Grx2, Rescue the Defects of Cells Lacking Grx5*—The fact that overexpression of native Grx3 or Grx4 glutaredoxins does not rescue the defects of a null Δ*grx5* mutant (29) could be a consequence of their different cellular compartmentalizations with respect to Grx5. We wondered what would happen in a situation in which Grx3 or Grx4 was targeted to the mitochondria in the absence of Grx5. We were also interested in determining the ability of a mitochondrially targeted form of the dithiol glutaredoxin Grx2 to substitute for Grx5 function when overexpressed. Grx2 is mostly located at the cytosol, although a minor fraction could be mitochondrial (25). With this objective, we constructed a vector (Fig. 2*A*) in which the desired gene was fused to the coding sequence of the mitochondria targeting signal of the Grx5 protein. In this vector (pMM221), expression of the HA-tagged chimeric protein was controlled by a $tetO_2$ promoter that also made it possible to modulate its expression according to doxycycline concentration. pMM221 was employed to clone the *GRX2*, *GRX3*, and *GRX4* sequences. In the case of *GRX2*, the sequence cloned began at the codon coding for methionine at position 35 in the open reading frame. In other words, the resulting construct consisted of the Grx5 mitochondrial targeting sequence fused to the Grx2 form that predominantly compartmentalizes at the cytosol (25). In the case of the two monothiol glutaredoxin genes, the initial ATG codons in the respective open reading frames were omitted in the pMM221-based constructions.

Yeast cells transformed with either of the respective constructions in the pMM221 vector were able to direct Grx2, Grx3, or Grx4 to the mitochondria. When identical amounts of total mitochondrial protein were analyzed from cultures grown in the absence of doxycycline, the levels of the HA-tagged forms

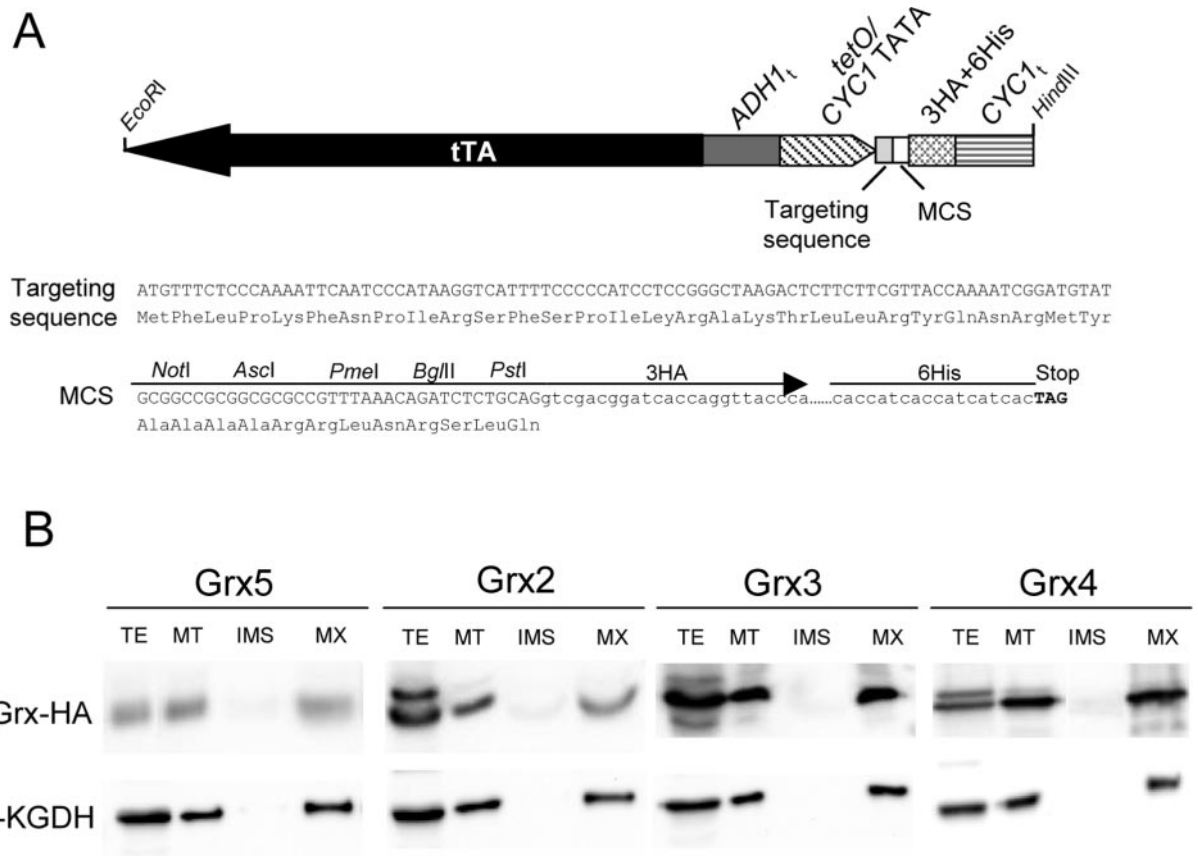


FIG. 2. **Plasmid pMM221 compartmentalizes proteins in the mitochondrial matrix through the Grx5 mitochondrial targeting sequence.** *A*, the plasmid is based on pCM318 (29), which contains the *GRX5–3HA-His*$_6$ region under the tTA-mediated control of the *tetO*$_2$ promoter. From this plasmid, the *GRX5* open reading frame (except the sequence coding for the initial 29 amino acids corresponding to the mitochondrial targeting sequence) was replaced by a synthetic multiple cloning site (*MCS*) generated with complementary oligonucleotides. The whole expression cassette (EcoRI-HindIII fragment) was then moved to the *LEU2* integrative vector YIplac128. For more details on the tTA-*ADH1* terminator *tetO*$_2$ and 3HA-His$_6$ regions, see Refs. 41 and 42, respectively. *B*, derivatives of pMM221 are able to compartmentalize Grx2 (pMM225, integrated in strain MML458), Grx3 (pMM227, strain MML443), and Grx4 (pMM229, strain MML446) at the mitochondrial matrix. Cultures of the respective strains were exponentially grown in lactate medium at 30 °C to an *A* (600 nm) of about 0.6 before mitochondrial isolation and subfractionation. Strain MML240 expressing a Grx5–3HA protein under its own promoter was employed as a control. *TE*, total cell extract; *MT*, mitochondrial fraction; *IMS*, intermembrane space; *MX*, matrix. Ten micrograms of protein were loaded in the TE lanes, whereas 1 $\mu$g was loaded in the other lanes. Anti-HA antibodies were used in the Western blot analyses to detect the HA-tagged proteins in the respective fractions, whereas anti-lipoic acid antibodies were used to detect the matrix marker $\alpha$-ketoglutarate dehydrogenase ($\alpha$-*KGDH*). Coomassie Blue staining confirmed that submitochondrial fractions were not significantly cross-contaminated (not shown).

of the above glutaredoxins were considerably higher than the level of endogenous Grx5-HA (Fig. 2*B*). This confirmed that *tetO*$_2$ is a strong promoter (41) and is responsible for protein overexpression even when it is present in a single copy integrative plasmid such as pMM221. Importantly, all the mitochondrial glutaredoxin molecules were compartmentalized at the matrix, as occurs with Grx5 (Fig. 2*B* and Ref. 29). This compartmentalization pattern was the same as that of the matrix lipoic-containing protein $\alpha$-ketoglutarate dehydrogenase. The respective mobilities of the Grx-HA bands in the mitochondrial fraction corresponded to the expected sizes of the mature proteins once the Grx5 targeting sequence had been processed. In some cases, a lower mobility band was present in minor amounts in the total extract fraction (Fig. 2*B*); this probably corresponded to the non-processed precursor form of the mitochondrial molecules.

Mitochondrially expressed Grx2 was not able to rescue the growth defects of a mutant lacking Grx5 (Fig. 3, *A* and *B*). In contrast, these defects were rescued when either Grx3 or Grx4 was targeted to the mitochondrial matrix. In fact, in rich (YP-Gal) media, these cells exhibited growth rates similar to wild type cells (Fig. 3*A*) and were able to grow in minimal S.D. medium or in respiratory conditions in which glycerol was the only carbon source (Fig. 3*B*).

To quantify the ability of the mitochondrial Grx3 or Grx4 forms to substitute for the biological function of Grx5, we determined aconitase activity (relative to malate dehydrogenase) in the respective strains. Aconitase is a mitochondrial enzyme that contains Fe/S clusters and whose activity is depleted in null *grx5* mutants (29), whereas malate dehydrogenase, which is also mitochondrial, lacks such clusters. Because the activity of mitochondrial enzymes is significantly decreased in glucose media, experiments were done in a rich medium with galactose as the carbon source. These are conditions compatible with growth competence of all the strains employed in this study. Importantly, absolute levels of malate dehydrogenase did not significantly vary from one strain to another (data not shown), which indicated the absence of any nonspecific mitochondrial dysfunction. In accordance with the previously described growth phenotypes, overexpressed mitochondrial Grx2 did not rescue the aconitase activity defect in the Δ*grx5* background (Fig. 4). However, overexpression of Grx3 or Grx4 did rescue this defect. To know whether aconitase activity was also restored when either Grx3 or Grx4 was expressed at levels equivalent to those of Grx5, we modulated the expression of the *GRX3-HA* or *GRX4-HA* constructs from the *tetO*$_2$ promoter with intermediate concentrations of doxycycline. When expressed levels of Grx3 or Grx4 were similar to those of endog-

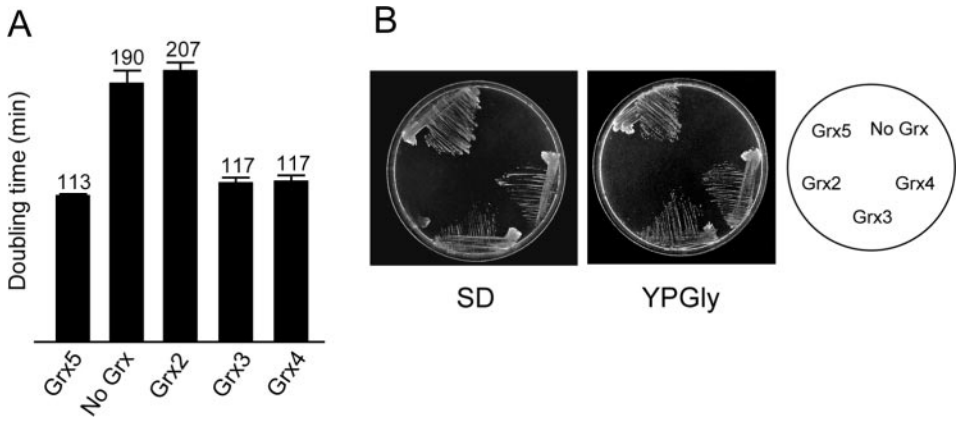


FIG. 3. **Rescue of the Δ*grx5* mutant growth defects by mitochondrial forms of glutaredoxins.** *A*, doubling times in min (*bars*, means of three experiments) were calculated from exponential cultures in liquid YPD medium at 30 °C. The strains employed carry a chromosomal null *grx5* allele and express the following mitochondrial glutaredoxin forms: MML240 (*Grx5*, endogenous promoter), MML100 (*No Grx*), MML465 (*Grx2*, pMM221-based), MML454 (*Grx3*, pMM221-based), MML456 (*Grx4*, pMM221-based). *B*, growth on S.D. or YPGly plates after 3 days at 30 °C of the following chromosomal Δ*grx5* strains expressing the indicated mitochondrial glutaredoxins: MML240 (*Grx5*), MML614B (*No Grx*), MML465 (*Grx2*), MML454 (*Grx3*), MML456 (*Grx4*).

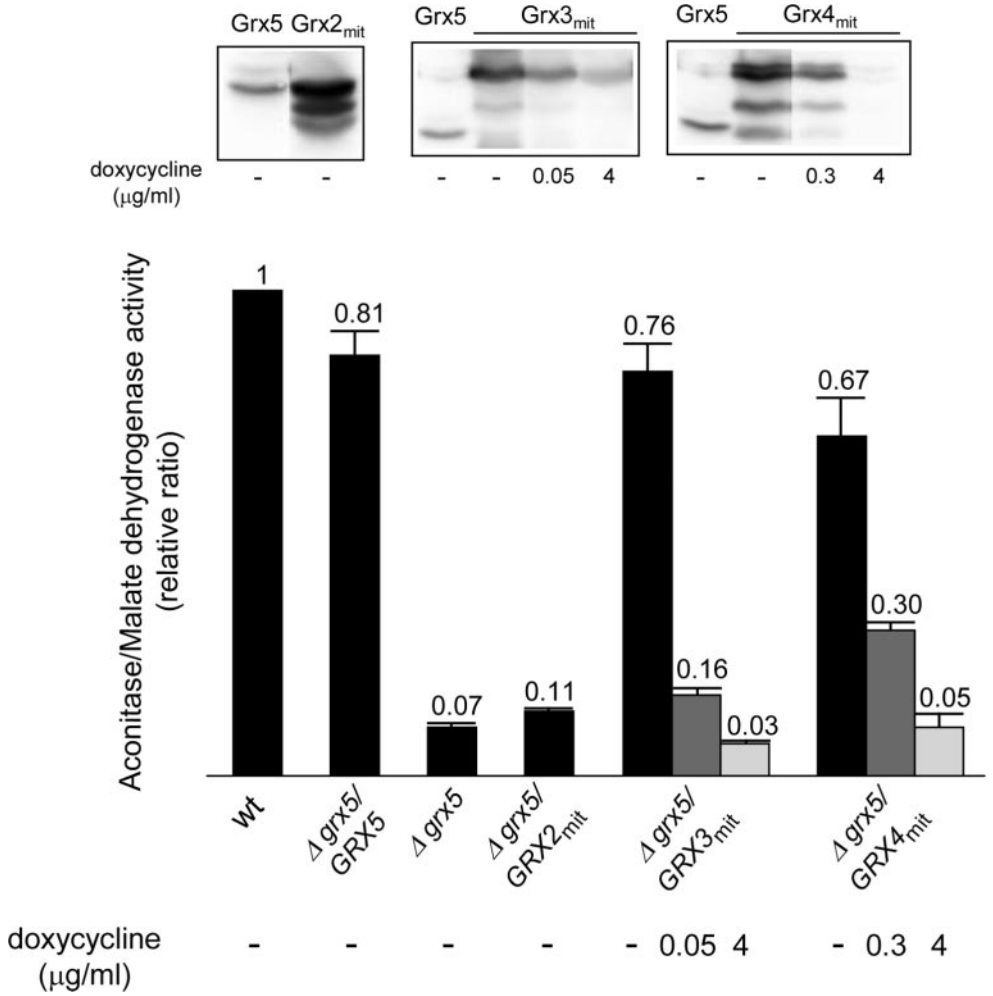


FIG. 4. **Ratio between aconitase and malate dehydrogenase activities in exponential cells at 30 °C in YPGal medium.** Strains that express different mitochondrial HA-tagged glutaredoxin forms were employed: W303–1A (*wild type*), MML240 (Δ*grx5*/*GRX5*, endogenous *GRX5* promoter), MML100 (Δ*grx5*), MML465 (Δ*grx5*/*GRX2*$_{mit}$, pMM221-based), MML454 (Δ*grx5*/*GRX3*$_{mit}$, pMM221-based), MML456 (Δ*grx5*/*GRX4*$_{mit}$, pMM221-based). Doxycycline was employed at the indicated concentrations in the growth medium to modulate expression from the *tetO*$_2$ promoter. Values are normalized with respect to the ratio in the wild type strain (unit value). *Upper panels* show the Western blot analyses of HA-tagged glutaredoxins in total cell extracts from the following strains: MML240 (*Grx5*), MML465 (*Grx2*$_{mit}$), MML454 (*Grx3*$_{mit}$), and MML456 (*Grx4*$_{mit}$).

enous Grx5 (Fig. 4, *upper panels*), aconitase activity was only partially restored in the Δ*grx5* cells. High concentration of doxycycline (4 μg/ml) almost totally switched off expression from the *tet* promoter; in these conditions, aconitase activity was comparable with that in Δ*grx5* cells (Fig. 4). The two monothiol glutaredoxins, Grx3 and Grx4, were therefore only able to completely substitute the mitochondrial Grx5 function when they were overexpressed.

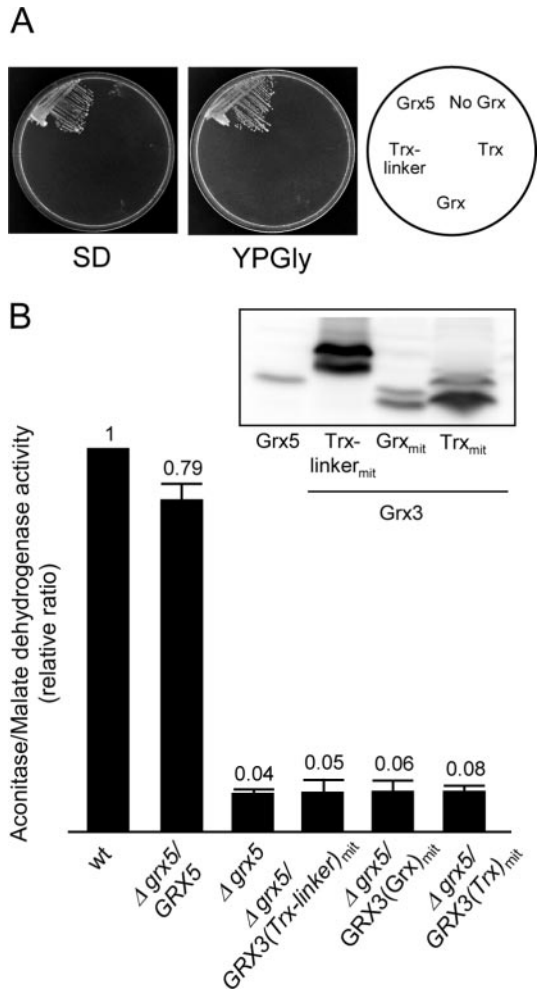


FIG. 5. **Rescue of the growth defects of a Δ*grx5* mutant by mitochondrial truncated derivatives of Grx3.** *A*, growth on S.D. or YPGly plates (3 days at 30 °C) of the following strains carrying the chromosomal Δ*grx5* allele and expressing the indicated mitochondrial glutaredoxin forms: MML240 (*Grx5*, endogenous promoter), MML614B (*No Grx*), MML500 (*Trx* domain of Grx3, pMM221-based), MML502 (*Trx plus linker* domain of Grx3), and MML504 (*Grx* domain of Grx3). *B*, ration between aconitase and malate dehydrogenase activities in exponential cells at 30 °C in YPGal medium expressing *GRX5* or truncated derivatives of *GRX3* coding for the respective mitochondrial forms: W303–1A (*wild type*), MML240 (Δ*grx5*/*GRX5*), MML100 (Δ*grx5*), MML502 (Δ*grx5*-/*GRX3(Trx-linker)*$_{mit}$), MML504 (Δ*grx5*/*GRX3(Grx)*$_{mit}$), and MML500 (Δ*grx5*/*GRX3(Trx)*$_{mit}$). Values are normalized with respect to the ratio in the wild type strain (unit value). The *upper panel* shows the Western blot analysis of the HA-tagged forms in the respective strains: MML240 (*Grx5*), MML502 (*Trx-linker*$_{mit}$), MML504 (*Grx*$_{mit}$), and MML500 (*Trx*$_{mit}$).

*The Mitochondrially Located Trx or Grx Domains of Grx3 Do Not Rescue the Δgrx5 Defects When Expressed Separately*—The partial ability of Grx3 or Grx4 to substitute for the Grx5 biological function at the mitochondria could be caused by impairment of the function of the Grx domain because of the bulky Trx domain. Alternatively, this could be the result of partial enzymatic activity of the Grx domain of Grx3 or Grx4 in contrast with the mature Grx5 protein. To distinguish between the two possibilities, we separately targeted the Trx, Trx plus linker, or Grx domains of Grx3 to the mitochondria using the pMM221 vector. The three domains were detected as overexpressed proteins (Fig. 5*B*) and became localized at the matrix (data not shown). However, neither the Trx nor the Grx regions alone nor the Trx plus linker region could complement the phenotypes caused by the absence of Grx5. Growth in minimal S.D. medium or in glycerol respiratory conditions (Fig. 5*A*) and aconitase/malate dehydrogenase activities were defective in Δ*grx5* cells expressing the truncated versions of Grx3 (Fig. 5*B*). This indicated that the Grx domain of Grx3 is not functionally equivalent to Grx5 and that the partial biological activity of Grx3 compared with Grx5 is not exclusively because of interference between the Trx and Grx domains.

*Only the Cysteine Residue at the Glutaredoxin Domain Is Required for the Biological Activity of Grx3*—Grx3 contains two cysteine residues at positions 72 (Trx domain) and 211 (Grx domain) that are conserved at equivalent positions in Grx4 and in homologues present in other organisms (28, 34). Cys-72 is in the context of the partially conserved thioredoxin active site (Fig. 1*A*). The observation that both the Trx and Grx domains are required if Grx3 is to replace the biological activity of Grx5 at the mitochondria could be explained by the need for both Cys-72 and Cys-211. If this were the case, Grx3 would, in fact, act through a dithiol mechanism of action. To test this hypothesis, we separately mutated the two cysteine residues and targeted the respective Grx3 derivatives to the mitochondria of Δ*grx5* cells. Only the C211S substitution eliminated the Grx3 activity, as well as aconitase/malate dehydrogenase activities, when we measured growth in minimal medium or under respiratory conditions (Fig. 6, *A* and *B*). Cellular fractionation analyses showed that this mutant form still localizes at the mitochondrial matrix (data not shown). This indicates that the conserved cysteine at the Trx domain does not play a significant role at the mitochondria. Two other cysteine residues that are present in the context of the Trx domain of Grx3 at positions 2 and 18 are not conserved in Grx4 or in other members of the same family. We decided to test whether either of these two residues could have an active role in Grx3, at least in the absence of Cys-72. We therefore constructed single point mutants in both of these cysteines, along with a triple C2S,C18A,C72S mutant, and expressed them as mitochondrial Grx3 forms in Δ*grx5* cells. The single C2S or C18A mutant Grx3 forms as well as the triple mutant derivative behave like the wild type Grx3 form once internalized into the mitochondria of Δ*grx5* cells when the aconitase/malate dehydrogenase activity ratio (Fig. 6*B*) or the ability for growth in minimal or glycerol medium (not shown) were determined. We concluded that cysteine at position 211 is required for the biological activity of Grx3 at the mitochondria and that the cysteine residues at the Trx region are not required for the redox function of the Grx moiety.

## DISCUSSION

Grx3, Grx4, and Grx5 are monothiol glutaredoxins that localize at different cellular compartments in *S. cerevisiae* cells. We had previously shown that Grx5 is located at the mitochondrial matrix (29); the present study has demonstrated the nuclear location of Grx3, which is similar to that of Grx4 (39). This nuclear location is not affected by a number of stresses. These glutaredoxins are an example of a protein family whose members are differentially compartmentalized, therefore pointing to divergent biological functions. Protein families may emerge from duplication events affecting genome regions of variable lengths. They are a source of functional diversification and allow organisms to adapt to their environments with greater efficiency (51). *S. cerevisiae* contains a large number of families, ranging from 2 to more than 100 gene members (52). An ancient poliploidization event, which could have occurred about 200 million years ago, may have been the origin of such a large number of gene families (53), although later, shorter, independent duplications could have contributed to the diversity of such families and also to the number and wide range of family members (54). Differential compartmentalization of the respective protein products of a gene family may play an im-

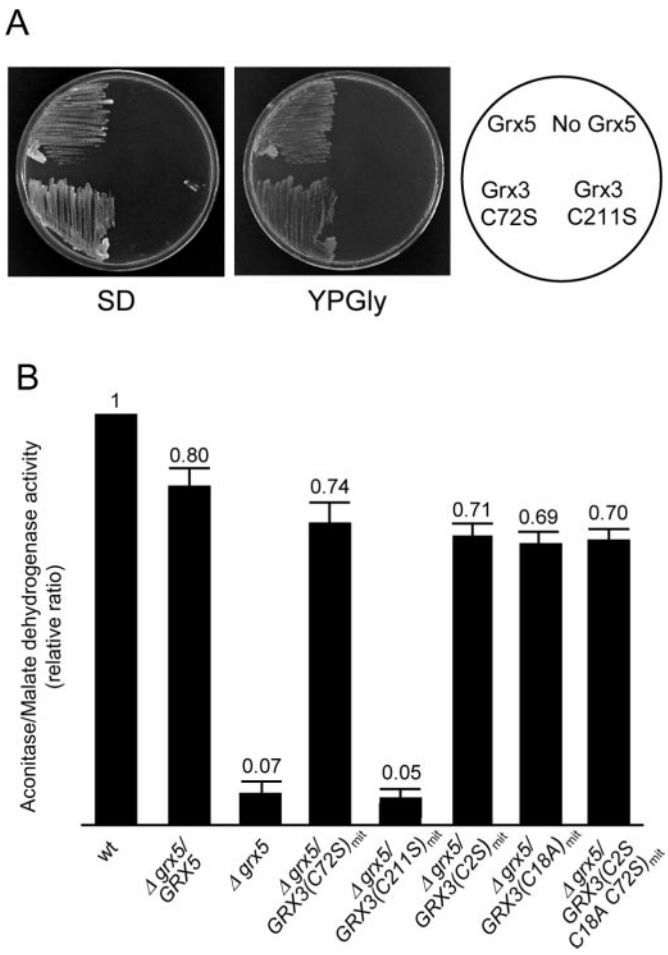


FIG. 6. **Rescue of the growth defects of a Δ*grx5* mutant by mitochondrial derivatives of Grx3 carrying point mutations.** *A*, growth on S.D. or YPGly plates (3 days at 30 °C) of the following strains carrying the chromosomal Δ*grx5* allele and expressing the indicated mitochondrial glutaredoxins: MML240 (*Grx5*), MML614B (*No Grx*), MML516 (*Grx3 C72S*), MML514 (*Grx3 C211S*). *B*, ratio aconitase/malate dehydrogenase in exponential cells at 30 °C in YPGal medium, corresponding to the following strains: W303–1A (*wild type*), MML240 (Δ*grx5*/*GRX5*), MML100 (Δ*grx5*), MML516 (Δ*grx5/GRX3(C72S)*$_{mit}$), MML514 (Δ*grx5/GRX3(C211S)*$_{mit}$), MML566 (Δ*grx5*/*GRX3(C2S)*$_{mit}$), MML642 (Δ*grx5*/*GRX3(C18A)*$_{mit}$), and MML644 (Δ*grx5/GRX3(C2S, C18A,C72S)*$_{mit}$). Values are normalized with respect to the ratio in the wild type strain (unit value).

portant role in biological adaptation to changes in external conditions. Among proteins implicated in defense against oxidative stress in *S. cerevisiae*, there are several examples of molecules with the same enzyme activity having different locations (see Ref. 19 for a review). However, the picture may be more complicated than this. For instance, Sod1 Cu,Zn-dependent superoxide dismutase is located at both the cytosol (major form) and mitochondria (55). Alternative translation from two different start codons of *GLR1* mRNA generates mitochondrial or cytosolic isoforms of Glr1 glutathione reductase (26). Also, post-translational processing may lead to cytosolic and mitochondrial forms of the dithiol glutaredoxin Grx2 (25). Therefore, a single gene can code for products with alternative locations due to transcriptional or post-transcriptional events.

There are significant structural differences between members of the Grx3/Grx4/Grx5 family. The fusion of a thioredoxin-like domain to a glutaredoxin domain may have occurred in a eukaryotic common ancestor of *S. cerevisiae* and other yeasts (34) as an important event in the functional specialization of the Grx3/Grx4 pair (Trx-Grx structure) with respect to Grx5 (with only the glutaredoxin domain). In the present study we have shown that the thioredoxin-like domain is essential for the nuclear location of Grx3 (and probably also for that of Grx4, given the similarity between the two proteins), although no canonical nuclear localization sequences are detectable in the Trx domains of Grx3 or Grx4. This is not the first example of a nuclear glutaredoxin, as an isoform of human Grx2 (of the dithiolic class) also occurs at the nucleus (14, 15). In the case of thioredoxins, there are multiple examples of nuclear forms that, among other functions, could be involved in regulation of transcription factors (47). Although the possible regulatory role of nuclear glutaredoxins has not been determined, in particular of human Grx2, it is tempting to speculate that they also may regulate the redox state of essential cysteine residues of nuclear transcription factors via reversible glutathionylation. Related to this, it has been shown that glutathionylation of a cysteine residue from a NF-κB subunit regulates the DNA binding activity of the latter (56).

Grx3 and Grx4 glutaredoxins are an example of how fusion of different protein domains may be a source of functional diversity. In the case of enzyme activities involved in oxidative stress responses, the existence of thiol peroxidase·glutaredoxin fusion proteins has recently been demonstrated in a number of different bacteria (57, 58). In these molecules, the glutaredoxin moiety acts as a donor of reducing power for the thiol peroxidase-mediated reduction of hydroperoxides. Thioredoxin domains can also be part of fusion proteins, for instance fused to nucleoside-diphosphate kinase domains (see Ref. 59 and citations therein). The hypothetical activity of the Trx moiety in Grx3 remains, however, unclear. One of the two cysteine residues that are essential for the thiol-oxidoreductase activity of these molecules is missing from the sequence reminiscent of the thioredoxin active site. Here, we have also shown that none of the cysteine residues in the Trx domain of Grx3 is required for the activity of the molecule; this includes the above mentioned cysteine. In contrast, the single cysteine residue in the Grx domain is essential. It is also important to note that Grx4 does not contain other cysteine residues apart from one in the Grx domain active site and another in the active site-like region of the Trx domain (Fig. 1*A*), and yet it is still able to rescue the Δ*grx5* phenotypes when targeted to the mitochondria. We have therefore concluded that Grx3 (and probably Grx4 also) is really a monothiol glutaredoxin and that although the Trx domain is required for nuclear targeting of the molecule, it does not actively participate in the thiol-oxidoreductase reaction. However, the Trx domain is required for the activity of the Grx3 molecules, at least when targeted to the mitochondria, probably because it stabilizes the conformation of the Grx domain in an active state. We have made *in silico* models of the Grx3 three-dimensional structure in which the Trx domain comes into close contact with the Grx domain in a stable conformation (data not shown).

In a previous study (27), we showed the synthetic lethality of the Δ*grx2* and Δ*grx5* mutations. However, Grx2 and Grx5 do not have overlapping functions, as we have demonstrated here that Grx2 does not substitute for the absence of Grx5 even when it is overexpressed at the mitochondria. The synthetic lethality between the two mutants could be because of less direct reasons, such as the fact that yeast cells lacking Grx2 cannot cope with the general oxidation of cellular proteins that occurs in the absence of Grx5 (27). The phenotype of a Δ*grx2* mutant discards the participation of Grx2 in the mitochondrial synthesis of iron/sulfur clusters, even though a small amount of this glutaredoxin compartmentalizes at the mitochondria (25). Results from physiological studies shown here reinforce the idea based on enzymatic analyses that dithiol and monothiol glutaredoxins have different mechanisms of action (1, 60, 61).

Dithiol glutaredoxins reduce both cysteine residues forming a disulfide bond, whereas monothiol glutaredoxins act by deglutathionylating GSH-protein mixed disulfides. At the mitochondrial iron-sulfur assembly complex, Grx5 seems to act in the later steps in the biosynthetic process, probably by maintaining the essential cysteine residues of the assembly complex in a reduced state (30). This would involve the deglutathionylation of mixed disulfides affecting such cysteine residues. *In vitro* studies have shown that dithiol glutaredoxin mutants that lack the most C-terminal of the cysteine residues at the active site are still able to deglutathionylate substrates (60); however, our results indicate that the mitochondrial function related to iron/sulfur cluster assembly cannot be carried out by a dithiol glutaredoxin such as Grx2.

On the contrary, Grx3 and Grx4 are able to substitute for Grx5 at the mitochondria. This does not mean that they normally participate in the synthesis of iron/sulfur clusters, because this process occurs exclusively at the mitochondria in *S. cerevisiae* (62). However, when one of those glutaredoxins is addressed to the mitochondria, it is then able to acquire this new function in the iron/sulfur assembly complex although not as efficiently as Grx5 itself. The mitochondrial forms of Grx3 or Grx4 only totally rescue the absence of Grx5 as determined by aconitase activity levels when they are overexpressed. Surprisingly, both Trx and Grx domains are required for this function, though only the cysteine residue of the latter domain is biologically active. The molecule probably only adopts an active conformation that is sufficient to carry out the function in the iron/sulfur assembly complex when both domains are present. This is an example of molecules sharing the same enzyme activity that functionally diverge through acquisition of new protein domains and separate compartmentalization. We hypothesize that the function of Grx3/Grx4 at the nucleus has evolved from an ancient glutaredoxin participating in the biogenesis of the iron/sulfur clusters, based on (i) the similarity between the phylogenetic profiles of orthologs of Grx5 and other components of the iron/sulfur assembly complex among bacterial species and (ii) the absence of molecules with the Trx-Grx structure among bacteria and archea (34). Following this reasoning, we propose that bacterial monothiol glutaredoxins are also involved in the biogenesis of iron/sulfur clusters. Experimental studies are required to confirm this hypothesis. In addition, determining the role of nuclear monothiol glutaredoxins at their original location will help to understand their function from an evolutive point of view.

*Acknowledgments*—We thank Lidia Piedrafita for excellent technical assistance and José A. Bárcena for comments on the manuscript.

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Molecular Basis of Cell and Developmental Biology:

# Nuclear Monothiol Glutaredoxins of *Saccharomyces cerevisiae* Can Function as Mitochondrial Glutaredoxins

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*J. Biol. Chem. 2004, 279:51923-51930.*
*doi: 10.1074/jbc.M410219200 originally published online September 28, 2004*