

Grx5 Is a Mitochondrial Glutaredoxin Required for the Activity of Iron/Sulfur Enzymes

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Yeast cells contain a family of three monothiol glutaredoxins: Grx3, 4, and 5. Absence of Grx5 leads to constitutive oxidative damage, exacerbating that caused by external oxidants. Phenotypic defects associated with the absence of Grx5 are suppressed by overexpression of *SSQ1* and *ISA2*, two genes involved in the synthesis and assembly of iron/sulfur clusters into proteins. Grx5 localizes at the mitochondrial matrix, like other proteins involved in the synthesis of these clusters, and the mature form lacks the first 29 amino acids of the translation product. Absence of Grx5 causes: 1) iron accumulation in the cell, which in turn could promote oxidative damage, and 2) inactivation of enzymes requiring iron/sulfur clusters for their activity. Reduction of iron levels in *grx5* null mutants does not restore the activity of iron/sulfur enzymes, and cell growth defects are not suppressed in anaerobiosis or in the presence of disulfide reductants. Hence, Grx5 forms part of the mitochondrial machinery involved in the synthesis and assembly of iron/sulfur centers.

INTRODUCTION

Glutaredoxins and thioredoxins are thioloxidoreductases required for maintaining thiol/disulfide equilibrium in cell proteins (Holmgren, 1989; Carmel-Harel and Storz, 2000; Grant, 2001) and also for the activity of specific enzymes (Aslund *et al.*, 1994; Lillig *et al.*, 1999). Glutaredoxin requires the reduced form of glutathione (GSH) as an electron donor (Holmgren and Aslund, 1995). In *Saccharomyces cerevisiae*, five glutaredoxins have been identified. Grx1 and Grx2 have two cysteine residues each at their active sites and play different roles in protecting cells against oxidants such as hydrogen peroxide and menadione (Luikenhuis *et al.*, 1998). The defensive roles of Grx1 and Grx2 may overlap with those of the cytosolic Trx1 and Trx2 thioredoxins: at least one Grx1/Grx2 glutaredoxin or Trx1/Trx2 thioredoxin is required for yeast cell viability (Draculic *et al.*, 2000). Another mitochondrial thioredoxin, Trx3, has been described in *S. cerevisiae* (Pedrajas *et al.*, 1999). Besides Grx1 and Grx2, yeast cells have three monocysteine glutaredoxins: Grx3, Grx4, and Grx5 (Rodríguez-Manzanaque *et al.*, 1999). The absence of Grx3 or Grx4 does not have a dramatic effect on sensitivity to oxidants. In contrast, the absence of Grx5 re-

sults in high sensitivity to hydrogen peroxide and menadione, increased protein oxidative damage, growth defects in minimal medium, and inability for respiratory growth (Rodríguez-Manzanaque *et al.*, 1999). The Grx3, Grx4, and Grx5 proteins have been included in a large protein superfamily that contains a conserved structural domain (from amino acids 46–132 in Grx5) defined after the human PICOT protein (Isakov *et al.*, 2000). PICOT may play a negative regulatory role in protein kinase C θ -mediated activation of the transcription factors AP-1 and NF- κ B in human cells (Witte *et al.*, 2000). The N-terminal extension of Grx3 and Grx4 (not present in Grx5) shares additional sequence homology with the N-terminal moiety of human PICOT and other members of the superfamily (Isakov *et al.*, 2000; Rahlfs *et al.*, 2001).

Respiratory growth defects of *grx5* mutant cells suggest impairment of mitochondrial functions. One of the essential processes occurring in the mitochondrial matrix of yeast cells is the generation of iron/sulfur (Fe/S) clusters, which are assembled in proteins destined to mitochondrial, cytosolic, or nuclear compartments (Craig *et al.*, 1999; Lill *et al.*, 1999; Lill and Kispal, 2000). These clusters are especially sensitive to oxidants (Keyer and Imlay, 1996) and liberate free iron that could further reactive oxygen species (ROS) production (Cadenas, 1989). Biogenesis of Fe/S clusters is a conserved process from bacteria to higher eukaryotes (Lill *et al.*, 1999; Lill and Kispal, 2000), although in humans biosynthetic complexes for Fe/S cluster assembly are also found in the cytosol (Tong and Rouault, 2000). In *S. cerevisiae*, synthesis and assembly of Fe/S clusters involves Nfs1 cysteine

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Table 1. Strains used in this work

Strain	Relevant phenotype	Comments
CML235	<i>MATa ura3-52 leu2Δ1 his3Δ200</i>	Wild type (Rodríguez-Manzanique <i>et al.</i> , 1999)
CML236	As CML235 but <i>MATα</i>	Wild type
MML19	<i>MATa grx5::kanMX4</i>	Deletion of <i>GRX5</i> in CML235
MML246	<i>MATa/MATα GRX5/grx5::kanMX4</i>	Diploid from a cross CML236 × MML19
MML248	<i>MATa/MATα GRX5/grx5::kanMX4 trx3::CaURA3</i>	Deletion of <i>TRX3</i> in MML246
MML264	<i>MATa grx5::kanMX4 trx3::CaURA3</i>	Spore from MML248
W303-1A	<i>MATa ura3-1 ade2-1 leu2-3,112 trp1-1 his3-11,15</i>	Wild type
W303-1B	As W303-1B but <i>MATα</i>	Wild type
MML100	<i>MATa grx5::kanMX4</i>	Deletion of <i>GRX5</i> in W303-1A
MML235	<i>MATα [pMM54(GRX5-3HA)]::LEU2</i>	Integration of linear pMM54 in W303-1B
MML240	<i>MATa grx5::kanMX4 [pMM54(GRX5-3HA)]::LEU2</i>	Spore from a cross MML100 × MML235
MML266	<i>MATα [pMM96(grx5-Δ8-3HA)]::LEU2</i>	Integration of linear pMM96 in W303-1B
MML271	<i>MATα (pMM98[grx5-Δ23-3HA])::LEU2</i>	Integration of linear pMM98 in W303-1B
MML289	<i>MATα grx5::kanMX4</i>	Spore from a cross W303-1B × MML100
MML290	<i>MATa grx5::kanMX4 (pMM96 [grx5-Δ8-3HA])::LEU2</i>	Spore from a cross MML100 × MML266
MML298	<i>MATa yfh::kanMX4</i>	Spore from a cross MML100 × MML281
MML300	<i>MATa yfh::kanMX4 grx5::kanMX4</i>	Spore from a cross MML100 × MML281
MML312	<i>MATa (pMM117[tTA tetO-GRX5])::URA3 tetR'-Ssn6::LEU2</i>	Integration of linear pMM117 in CML240 (Belli <i>et al.</i> , 1998)
MML313	<i>MATa grx5::kanMX4 (pMM117[tTA tetO-GRX5])::URA3 tetR'-Ssn6::LEU2</i>	Spore from a cross MML289 × MML312
MML345	<i>MATa aft1-Δ5::URA3 grx5::kanMX4</i>	Spore from a cross W303-1B × MML348
MML348	<i>MATa aft1-Δ5::URA3</i>	Several backcrosses from CML126 (Casas <i>et al.</i> , 1997) to introduce the <i>aft1-Δ5</i> mutation in the W303 background

desulfurase (Kispal *et al.*, 1999; Li *et al.*, 1999), the ferric ion-binding proteins Isu1 and Isu2 (Garland *et al.*, 1999; Schilke *et al.*, 1999), the Yah1 ferredoxin (Barros and Nobrega, 1999; Lange *et al.*, 2000), the Arh1 ferredoxin reductase (Lacour *et al.*, 1998; Manzella *et al.*, 1998), the molecular chaperones Ssq1 (Hsp70-type) and Jac1 (Hsp40 or J-type; Schilke *et al.*, 1996; Strain *et al.*, 1998; Schilke *et al.*, 1999; Lutz *et al.*, 2001; Voisine *et al.*, 2001), the homologous proteins Isa1 and Isa2 (Jensen and Culotta, 2000; Kaut *et al.*, 2000; Pelzer *et al.*, 2000), and the functionally uncharacterized protein Nfu1 (Schilke *et al.*, 1999). Exporting Fe/S for extramitochondrial proteins requires the mitochondrial ABC transporter Atm1 (Kispal *et al.*, 1999). Mutations in yeast genes involved in Fe/S cluster assembly cause iron accumulation in the mitochondria, mitochondrial DNA damage, and respiratory metabolism failure (Craig *et al.*, 1999; Lill and Kispal, 2000). Similar phenotypes are observed in null mutants for *YFH1* (Babcock *et al.*, 1997; Foury and Cazzalini, 1997), the yeast homologue for the human frataxin gene, with a possible role in mitochondrial iron homeostasis (Foury, 1999; Radisky *et al.*, 1999; although other functions have also been suggested for *YFH1* [Ristow *et al.*, 2000]). All of these observations suggest a relationship among Fe/S cluster biogenesis, mitochondrial metal homeostasis, and oxidative damage.

In this work we demonstrate that Grx5 is a mitochondrial glutaredoxin required for the activity of Fe/S-containing enzymes and that its absence affects iron homeostasis and causes osmotic stress at the cell.

MATERIALS AND METHODS

Yeast Strains and Plasmids

The yeast strains are described in Table 1. The following plasmids contain the genes indicated in parenthesis plus their own promoter

and terminator sequences (without adjacent complete open reading frames [ORFs]) cloned in the multicopy vector Yeplac181 (Gietz and Sugino, 1988): pMM62 (*GRX5*), pMM70 (*SSQ1*), pMM72 (*ISA1*), and pMM74 (*ISA2*). Plasmids pCM316, pCM317, and pCM318 contain the complete *GRX4*, *GRX3*, and *GRX5* ORFs, respectively (without further sequences), cloned in the multiple cloning site of pCM190 (Garí *et al.*, 1997) under the control of the doxycycline-regulated *tetO₇* promoter. pMM54 is a Ylplac128 (Gietz and Sugino, 1988) derivative with *GRX5* under its own promoter, tagged at the 3'-end with three hemagglutinin (HA) epitopes in tandem. pMM117 is a derivative of Ylplac211 (Gietz and Sugino, 1988) containing the doxycycline-regulated tTA activator (Garí *et al.*, 1997) and the *GRX5* ORF (plus terminator sequences) under the control of the *tetO₇* promoter.

Growth Conditions and Determination of Cell Parameters

Cells were grown at 30°C in YPD, YPG (as YPD but with 3% glycerol instead of dextrose), SD medium (0.67% yeast nitrogen base, 2% glucose, and auxotrophic requirements), or SC medium (the same as SD plus drop-out mixture [Kaiser *et al.*, 1994]). Specific supplements were omitted from the SC medium when required. For growth in anaerobic conditions, inoculated plates were incubated in an anaerobiosis chamber. Cell numbers (in formaldehyde-fixed samples) and mean cell volumes were determined in a Coulter Z2 counter (Beckman Coulter, Fullerton, CA). 4',6-Diamidino-2-phenylindole staining was done as described by Kaiser *et al.* (1994).

Gene Disruptions and Other Genetic Methods

Standard methods were used for DNA manipulation, transformation, crosses between yeast strains, sporulation, and tetrad analyses. SC medium with appropriate supplements was used to select transformants on *grx5* mutant cells. The wild-type *GRX5* allele was disrupted in the W303 background using the *kanMX4* cassette, as previously described (Rodríguez-Manzanique *et al.*, 1999). A similar approach was used to construct a null mutant in *TRX3*, using a

polymerase chain reaction-amplified cassette with the *CaURA3* marker from pAG60 (Goldstein *et al.*, 1999). Oligonucleotides for amplification of the disruptant cassettes were designed to disrupt most of the targeted gene upon transformation with the amplified DNA.

Isolation of *grx5*Δ Suppressors

Exponentially growing MML19 cells were transformed with a yeast genomic DNA library in the multicopy plasmid YEp13. The transformation mixture was then plated on SD agar plates, which were incubated for 6 d at 30°C. Wild-type CML235 cells were transformed in parallel as a control to quantify transformation efficiency. Two independent transformation experiments were carried out with ~60,000 transformants on wild-type cells. Plasmids were recovered from growing *grx5* transformants, amplified in *Escherichia coli*, and retransformed on MML19 cells to confirm their ability to suppress the transformation defect of *grx5* mutants. Finally, we recovered nine plasmids that gave stable transformation on *grx5* cells. Restriction fragment analysis and hybridization with a *GRX5* probe demonstrated that the plasmids corresponded to four different clones. One (three separate isolates) contained *GRX5*, and the other three clones contained inserts from other chromosomal locations: pMM44 (one isolate), pMM45 (two isolates), and pMM46 (three isolates). Partial sequencing of insert ends was carried out to reveal the genes included in each suppressor plasmid.

Construction of *GRX5* Derivatives with Deletions at the Mitochondrial Targeting Sequence

Plasmid pMM54 (*GRX5-3HA*) was used as a template to generate two constructions with deletions in the *GRX5* coding sequence, using the ExSite approach (Weiner and Costa, 1995). The resulting pMM96 plasmid contains a deletion that spans from base pairs +4 to +27 (*grx5-Δ8*), and pMM98 has a deletion spanning from base pairs +4 to +72 (*grx5-Δ23*). Linearized (*EcoRV* digestion) plasmids pMM96 and pMM97 were stably integrated at the chromosomal *LEU2* locus of W303-1B cells and generated strains MML266 and MML271, respectively.

Sensitivity to Menadione

Cells growing exponentially in YPD medium at 30°C (2×10^7 cells/ml) were added with menadione. Plasmid-transformed cultures were grown in SD medium in selective conditions. In this case, cells were transferred to YPD medium for 4 h before sensitivity analyses. After various treatment times, 1:5 serial dilutions were made, and drops were spotted onto YPD plates. Growth was recorded after 2 d of incubation at 30°C.

Western Blot Analyses

Western blot analyses were done according to the method of Belli *et al.* (1998). 12CA5 anti-HA mAb (Roche Diagnostics, Mannheim, Germany) was used at a 1:5000 dilution. An anti-lipoic acid antibody was used at a 1:50,000 dilution to detect protein-bound lipoic acid (Cabiscol *et al.*, 2000). Anti-Aco1 aconitase (1:2000 dilution, from R. Lill), anti-succinate dehydrogenase (1:1000 dilution, from B. Lemire), and anti-cytochrome *b*₂ (1:1000 dilution, from E. Valentin) antibodies were also used.

Isolation of Mitochondrial Fractions

Mitochondria were purified as described by Luttk *et al.* (1998). Zymolyase 20T (ICN Biochemicals, Cleveland, OH) was used at 3 mg/g of cells (dry weight). Spheroplasts were broken (eight strokes) with a Dounce homogenizer. Mitochondria (pellet) were separated from the postmitochondrial (supernatant) fraction, resuspended in a hypotonic solution (20 mM HEPES plus 1 mM phenylmethylsulfo-

nyl fluoride), and centrifuged in a microfuge (12,000 rpm, 10 min) at 0°C. The resulting pellet and supernatant were respectively considered as the intermembrane space and matrix fractions.

Identification of the Signal Peptide Cleavage Site for Grx5

Four grams of cells grown in YPG medium were resuspended in 50 mM Tris buffer, pH 7.5, plus 20 mM NaCl and disrupted in a French Press (SLM Aminco). After centrifugation (12,000 rpm, 30 min), the crude supernatant was applied to an ionic exchange column (DEAE 15HR, Waters, Milford, MA). Proteins were eluted with a linear gradient (20–400 mM NaCl in Tris-HCl buffer, pH 7.5), and fractions containing Grx5 were identified by Western blot using specific polyclonal antibodies. Proteins in the fractions were separated by two-dimensional electrophoresis. First dimension was performed in a Protean Isoelectric Focusing Cell (Bio-Rad, Hercules, CA) using 17-cm IPG strips (Bio-Rad Ready Strip, pH range 3–11). Second dimension was performed according to the denaturing discontinuous buffer system of Laemmli. Proteins were transferred to a polyvinylidene difluoride membrane using a semidry system, and the spot corresponding to Grx5 (identified by Western blot in a duplicate membrane with the above antibodies) was N-terminal sequenced by Edman degradation using a Beckman LF3000 sequencer equipped with a phenylthiohydantoin derivative analyzer (System Gold, Beckman).

Other Methods

Analysis of protein carbonylation after derivatization of carbonyl groups with dinitrophenylhydrazine was carried out as described by Cabiscol *et al.* (2000). Enzymatic activities were assayed by the following standard methods: aconitase, citrate synthase, malate dehydrogenase (Robinson *et al.*, 1987), and succinate dehydrogenase (Munujos *et al.*, 1993). Activities were expressed in units (nanomoles per minute) per milligram of cell protein. Extracts were prepared in 0.1 M Tris buffer, pH 8.1, plus 2 mM EDTA using glass beads to disrupt the cells. Nonmitochondrial citrate synthase is not stable at this pH (Liao *et al.*, 1991). Whole cell and mitochondrial iron were determined under reducing conditions (Fish, 1988), with bathophenanthroline sulfonate (BPS) as chelator and after acid digestion of cells or mitochondria with 3% nitric acid. Mitochondrial and postmitochondrial iron were also determined according to the method of Tageras *et al.* (1980) after incubation of the samples with 10 mM 2-(*N*-morpholino)ethanesulfonic acid-KOH buffer, pH 4.5, plus 1% SDS (1 h, 95°C). No significant differences were observed among results with both methods. Heme covalently bound to cytochrome *c* was detected as described by Vargas *et al.* (1993), using the Supersignal detection system (Pierce Chemical, Indianapolis, IN). To purify yeast Grx5 glutaredoxin, the entire *GRX5* ORF was cloned in-frame in pGEX-4T1 (Amersham Pharmacia Biotech, Piscataway, NJ) to generate a GSH *S*-transferase (GST)-Grx5 fusion protein. The construct was expressed in *E. coli* cells. GST-Grx5 was purified from bacterial cell extracts using GSH-Sepharose 4B columns (Amersham Pharmacia). After thrombin cleavage, Grx5 was separated from GST and contaminants by preparative electrophoresis, using a Bio-Rad 491 PrepCell. Polyclonal anti-Grx5 antibodies were raised in rabbits and purified from rabbit serum in a protein A-Sepharose CL-4B column (Amersham Pharmacia).

RESULTS

Defects in *grx5* Mutants Are Suppressed by Genes Involved in Fe/S Cluster Assembly

Given the defective growth phenotype of null *grx5* mutants in minimal SD medium, we isolated transformants (on MML19 cells) from a multicopy genomic DNA library that were able to grow in such conditions. Three different clones,

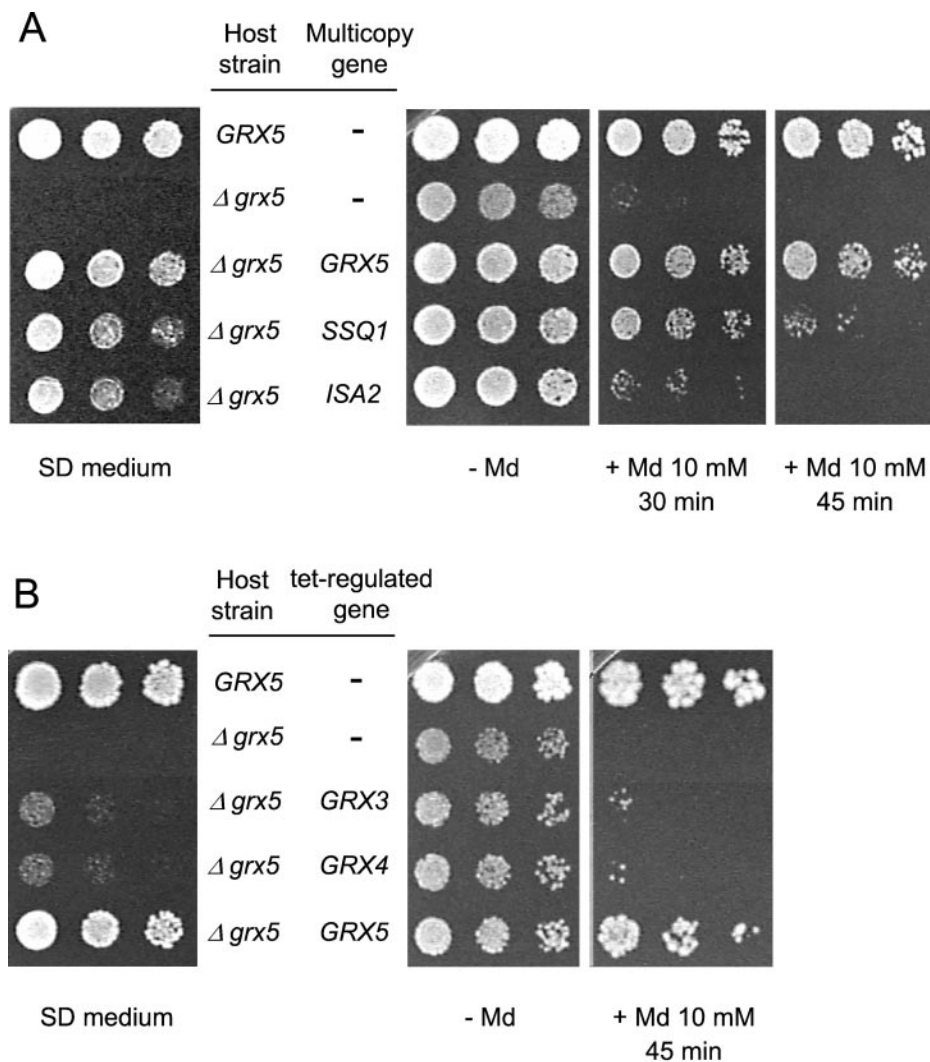
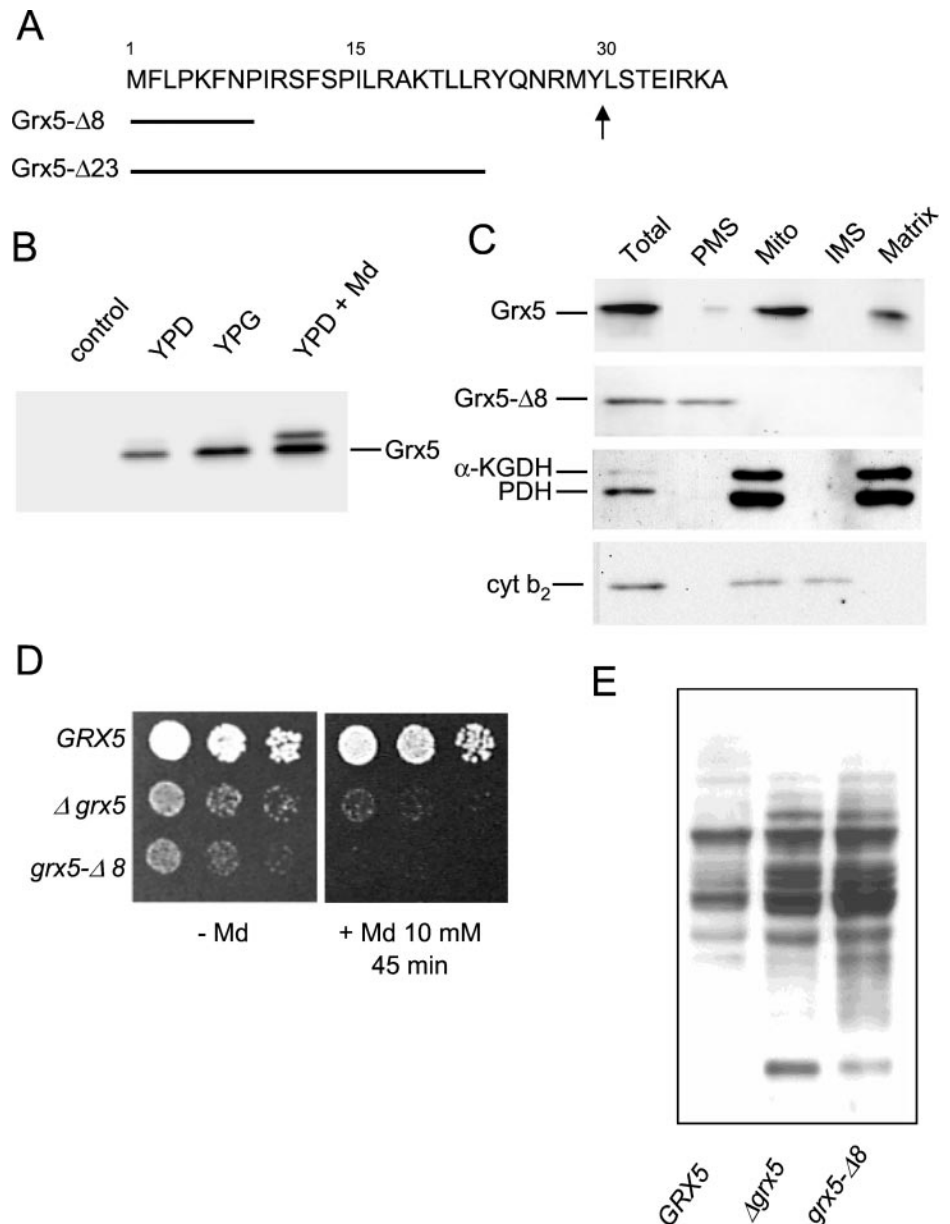


Figure 1. Suppression of phenotypic defects of null *grx5* mutants. (A) CML235 wild-type (*GRX5*) cells and MML19 ($\Delta grx5$) cells nontransformed or transformed with the multicopy plasmids pMM62 (*GRX5*), pMM70 (*SSQ1*), or pMM74 (*ISA2*) were tested for growth on SD medium plates (left) and for sensitivity to 10 mM menadione (Md) treatment at 30°C (right). Serial dilutions of exponential cultures (treated with menadione for the indicated times) were plated on YPD plates. (B) CML235 and MML19 cells transformed with the pCM190 (*tetO₇* promoter)-derived plasmids pCM317 (*GRX3*), pCM316 (*GRX4*), or pCM318 (*GRX5*) were tested for growth on SD medium plates (left) and for menadione sensitivity (right) in overexpression conditions (minus doxycycline).

whose inserts were characterized by DNA sequencing, were redundantly isolated in two independent experiments. Plasmid pMM44 contains two complete ORFs, *SSQ1* and *ARC18*. Plasmid pMM45 contains *ROX1*, *UBA3*, *ISA2*, *HOS1*, and *SPE3*, and pMM46 contains *RPS22B*, *YLR368w*, *SSQ1*, and *ARC18*. We focused our attention on *SSQ1* and *ISA2*, two genes involved in Fe/S cluster assembly at the mitochondria (see INTRODUCTION). To confirm that *SSQ1* and *ISA2* were really suppressors of *grx5* defects, both genes and their respective promoter and terminator sequences were cloned in the multicopy plasmid YEplac181 and then used to transform a *grx5* null mutant. This mutant was also transformed with a construction carrying *GRX5* under its own promoter in YEplac181. Transformants were obtained in all three cases (~50% transformation efficiency for *SSQ1* and 30% for *ISA2* relative to *GRX5*). Multicopy plasmids containing *SSQ1* or *ISA2* allowed *grx5* cell growth in SD medium (Figure 1A, left). In addition, the *SSQ1* plasmid suppressed better the sensitivity of *grx5* cells to menadione than did the *ISA2* plasmid (Figure 1A, right). Although *ISA2* is highly homologous in sequence to *ISA1*, overexpression of the latter was

not capable of suppressing the *grx5* phenotypes (Rodríguez-Manzanique, Tamarit, Bellí, Ros, and Herrero, unpublished results). We therefore conclude that, when overexpressed, some (but not all) of the genes involved in the Fe/S cluster assembly are capable of counteracting the absence of *GRX5*. This suggests a functional relationship between Grx5 glutaredoxin and Fe/S cluster assembly at the mitochondria. As with mutants in the Fe/S assembly machinery and with yeast frataxin mutants (Babcock *et al.*, 1997; Jensen and Cullotta, 2000; Kaut *et al.*, 2000), *grx5* cells are unable to use glycerol as a sole carbon source (Rodríguez-Manzanique *et al.*, 1999). In contrast with other phenotypes shown in Figure 1A, *grx5* cell respiratory ability was not directly rescued by transformation with the *GRX5* gene. To the contrary, a plasmid carrying *GRX5* only rescued growth ability on glycerol in a chromosomal *grx5* background after this mutant had been crossed with wild-type cells transformed with the *GRX5* plasmid, followed by sporulation of the resulting diploid. This is consistent with *grx5* cells having extensively accumulated mutations in mitochondrial DNA, thereby causing the respiratory defect.

Figure 2. Grx5 is a mitochondrial glutaredoxin. (A) N terminus Grx5 amino acid sequence. Arrow marks where the precursor form is processed, as determined by N terminus sequencing of mature Grx5. The length of the two constructed signal peptide deletions (Grx5- Δ 8 and Grx5- Δ 23) is indicated. (B) Western blot immunodetection of 3HA-tagged Grx5 in extracts from exponential cultures of MML240 cells in YPD and YPG medium at 30°C and in YPD medium plus menadione (Md, 30 min). Left lane (control) corresponds to W303-1A cell extracts. The same amount of total cell protein (40 μ g) was run in each lane. (C) MML235 and MML266 (Grx5- Δ 8) cells grown exponentially in YPG medium at 30°C were fractionated, and the resulting fractions were analyzed by Western blot. Anti-HA antibodies were used to detect Grx5 (MML235 fractions) and Grx5- Δ 8 (MML266 fractions), and anti-lipoic acid antibodies were used to detect the matrix markers pyruvate dehydrogenase (PDH) and α -ketoglutarate dehydrogenase (α -KGDH). Cytochrome b_2 (cyt b_2) was used as an intermembrane space (IMS) marker. Results for pyruvate dehydrogenase, α -ketoglutarate dehydrogenase, and cytochrome b_2 are shown only for MML266; similar results were observed for MML235. Ten micrograms of protein were loaded in each lane for "Total" cell extracts and postmitochondrial supernatant (PMS) fractions, and 3 μ g were loaded for the mitochondrial (Mito), intermembrane space, and matrix fractions. (D) Sensitivity to menadione in strains MML240 (*GRX5*), MML100 (Δ *grx5*), and MML290 (*grx5*- Δ 8) growing exponentially at 30°C in YPD medium. (E) Protein carbonylation in extracts from exponentially growing MML240, MML100, and MML290 cells in YPD medium at 30°C.



We also tested whether *GRX3* or *GRX4* (the other two members of the same gene family) suppressed the phenotypes of a *grx5* mutant when overexpressed from the doxycycline-regulatable *tet* promoter (Garí *et al.*, 1997). *Grx3* and *Grx4* only slightly overcame the sensitivity to menadione or the growth defect in SD medium of cells deficient in *Grx5* (Figure 1B), suggesting that *Grx5* performs different functions from the other two members of the family.

Grx5 Is a Mitochondrial Glutaredoxin

Genetic interactions between *Grx5* and mitochondrial proteins implicated in the biogenesis and protein assembly of Fe/S clusters suggested that *Grx5* could be a mitochondrial glutare-

doxin. PSORT analysis predicts a mitochondrial location for *Grx5* due to mitochondrial targeting signatures at its N-terminal region (Pon and Schatz, 1991). To confirm this, we raised antibodies against *Grx5* protein that had been expressed in *E. coli* and then purified. We used these antibodies to purify the mature form of the overexpressed (from the *tet* promoter) *Grx5* protein in *S. cerevisiae*. N terminus sequencing of the protein spot isolated from a two-dimensional gel showed that mature *Grx5* begins with the sequence LSTEIRKA. Hence, the mature product lacks the first 29 amino acids predicted from the proposed *GRX5* ORF (Figure 2A). Remarkably, *GRX3* and *GRX4* sequences have no homology with these N-terminal *Grx5* residues (Rodríguez

guez-Manzanque *et al.*, 1999), and PSORT analysis predicts no mitochondrial location for either Grx3 or Grx4.

We tested whether it was possible to detect a 3HA-tagged version of Grx5 expressed under its own promoter. This tagged form fully complemented all the *grx5* mutant defects in the MML240 strain, obtained after crossing the *grx5* mutant with a wild strain carrying an integrative plasmid with the *GRX5-3HA* construction. A major form of the expected mobility in Western blots was detected in extracts from exponential cells grown on both glucose and glycerol (Figure 2B). A larger minor form was also observed, especially in cells grown in YPD medium after menadione treatment. Although this treatment did not up-regulate *GRX5* expression (Rodríguez-Manzanque *et al.*, 1999), the total amount of immunodetectable Grx5 protein almost doubled compared with untreated cells (Figure 2B). This increase points to some kind of posttranscriptional regulation of Grx5 levels in oxidative conditions. We then used the tagged version of Grx5 to determine its cellular location. Cell fractionation studies demonstrated that wild-type Grx5 is located at the mitochondria (Figure 2C). After causing outer membrane disruption under hypotonic conditions, mitochondrial subfractionation showed that Grx5 entirely colocalized with two matrix lipophilic acid-modified proteins, pyruvate dehydrogenase and α -ketoglutarate dehydrogenase (Figure 2C). From these results, we hypothesized that the deletion of a number of amino acids at the N terminus would cause Grx5 to remain at the cytoplasm. Two shorter forms of Grx5 were constructed, one lacking the first eight amino acids (Grx5- Δ 8) and the other lacking the first 23 residues (Grx5- Δ 23; Figure 2A). We obtained the same results with both, although here we only show results corresponding to the eight-residue deletion. The shorter version of Grx5 remained exclusively at the cytoplasm (Figure 2C), thus confirming the importance of N terminus residues for adequate targeting of Grx5 at the mitochondria.

We then addressed the possibility that this version of Grx5, present at the cytosol, could also protect cells against oxidative stress. We therefore constructed a strain that produced only the cytosolic Grx5- Δ 8 version. This strain was hypersensitive to menadione (Figure 2D) and showed constitutive carbonylation levels even higher than those of cells containing no Grx5 (Figure 2E). We conclude that Grx5 is located in the mitochondria and that the abnormal presence of Grx5 at the cytosol does not complement the phenotypes that result from the absence of mitochondrial Grx5. The moderately dominant negative effect of the *grx5- Δ 8* allele leaves open the possibility that the mutant protein interferes with some cytosolic mechanism involved in oxidative stress defense.

The Absence of Grx5 Causes Inactivation of Mitochondrial Fe/S Enzymes

The defects associated with the absence of *GRX5* are common to mutants in mitochondrial proteins involved in the biosynthesis/assembly of Fe/S clusters (Lill and Kispal, 2000, and references therein). These defects and the genetic interactions between *GRX5* and genes responsible for the biogenesis of Fe/S clusters led us to investigate the involvement of Grx5 in the biogenesis and/or repair of such clusters. We therefore measured the activity of mitochondrial enzymes containing Fe/S clusters in a condi-

tional mutant in which *GRX5* expression under the *tet* promoter was doxycycline regulated. For this purpose, the wild-type *GRX5* allele was deleted and a *tet-GRX5* construction was integrated at the *LEU2* locus. The resulting strain (MML313) grew on glycerol in the absence of doxycycline (*GRX5* expressed), but upon addition of the antibiotic (*GRX5* transcription immediately repressed), growth became arrested in ~12 h. 4',6-Diamidino-2-phenylindole staining confirmed that mitochondria retained the DNA after 24 h of inhibition of *GRX5* expression, which is in accordance with a [*rho*⁻] phenotype. The activity of two mitochondrial enzymes with Fe/S clusters (aconitase and succinate dehydrogenase) decreased dramatically when *GRX5* was not expressed (<15% activity after 24 h; Figure 3A). In contrast, the activity of two mitochondrial enzymes not depending on Fe/S clusters (citrate synthase and malate dehydrogenase) remained unchanged. Western blot analyses of aconitase and succinate dehydrogenase demonstrated that the amounts of the two proteins were not affected by inhibition of *GRX5* expression (Figure 3B), indicating that the changes in activity can be attributed to impairment of formation of mature molecules.

The inhibition of Fe/S enzyme activities could give a clue to the *grx5* mutant growth defects in minimal SD medium. When comparing growth of wild-type and *grx5* cells in defined SC medium that lacked each of the 20 amino acids, we observed that *grx5* cells were unable to grow (or grew poorly) when deprived of leucine, lysine, or glutamic acid (Figure 3C). However, the absence of any other amino acid did not affect growth. The growth defect was rescued when the mutant cells were transformed with a centromeric plasmid expressing *GRX5* (Rodríguez-Manzanque, Tamarit, Bellí, Ros, and Herrero, unpublished results). Thus, auxotrophy for these three amino acids could explain defective growth of the mutant in minimal medium. All three amino acids require Fe/S enzymes for their biosynthesis. Glutamate biosynthesis requires mitochondrial aconitase (Gangloff *et al.*, 1990), and the inactivation of this Fe/S enzyme also explains the glutamate requirement of *isa1* and *isa2* mutants (Jensen and Culotta, 2000). Lysine auxotrophy probably results from the inactivation of the mitochondrial Fe/S enzyme homoaconitase, which is involved in its synthesis (Bhattacharjee, 1985; De Freitas *et al.*, 2000). Lysine auxotrophy also occurs in *isa* mutants (Jensen and Culotta, 2000). Leucine biosynthesis requires the cytoplasmic Fe/S enzyme isopropyl malate isomerase, Leu1 (Kohlhaw, 1988). Inactivation of this enzyme has been described for a number of mutants altered in the assembly of Fe/S cluster proteins (Kispal *et al.*, 1999; Kaut *et al.*, 2000; Lange *et al.*, 2000). All of these data support the relationship between the growth defects observed in *grx5* cells in minimal medium and the inactivation of Fe/S cluster-containing enzymes implicated in amino acid biosynthesis.

Cells without Grx5 Are Not Defective in the Holoforms of Heme-containing Proteins

Although heme groups and Fe/S clusters have different biosynthetic pathways, both are also synthesized within the mitochondria, require a source of reduced iron, and are sensitive to oxidative stress (Kranz *et al.*, 1998; Lange *et al.*,

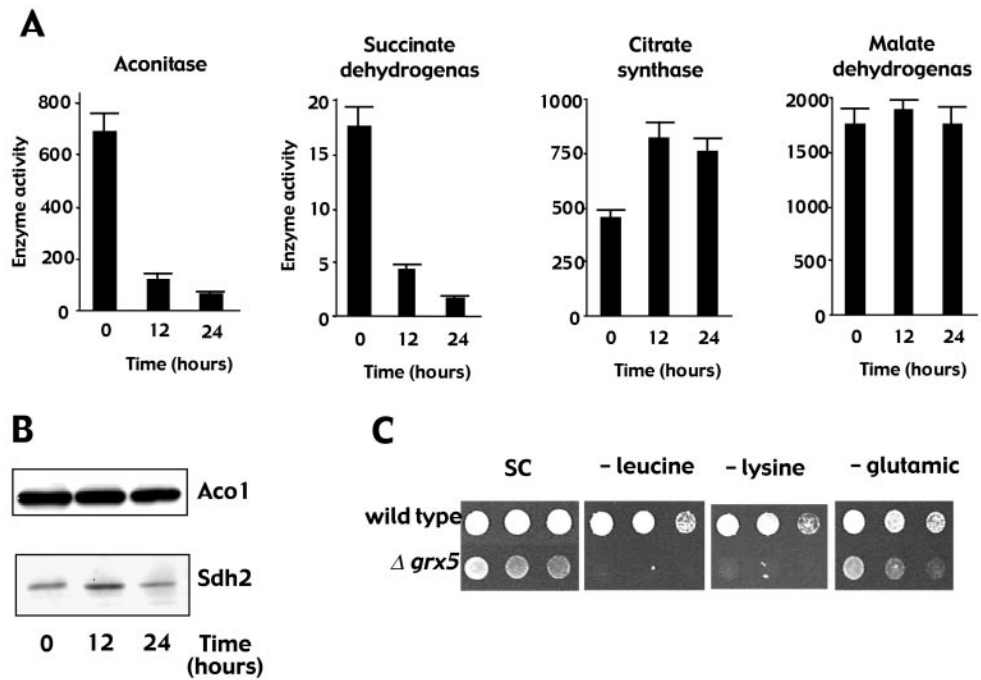


Figure 3. Lack of Grx5 negatively affects Fe/S enzyme activity. (A) MML313 cells growing exponentially in YPG medium at 30°C (1×10^7 cells/ml) were added at time 0 with doxycycline (2 μ g/ml) to inhibit *GRX5* expression. Enzyme activity in cell extracts was determined at the indicated times. (B) Western blot analysis of aconitase (Aco1) and succinate dehydrogenase (Sdh2) in the same samples as in A. (C) Growth of wild-type CML235 and mutant MML19 ($\Delta grx5$) cells containing the integrative *LEU2* plasmid YIplac128 (Gietz and Sugino, 1988) in SC medium with all the supplements or deprived of leucine, lysine, or glutamic acid.

1999). Heme deficiency in yeast has also been found associated with mutations such as *atm1* (Kispal *et al.*, 1997), *jac1* (Voisine *et al.*, 2001), and *yfh1* (Foury, 1999), although this could be the result of alterations in iron homeostasis. Therefore, we analyzed the presence of the mature form of cytochrome *c* in cell extracts from MML313 cells (*tetO₇-GRX5*) grown in YPG medium before and after the addition of doxycycline. Holo-cytochrome *c* was detected through the peroxidase activity displayed by the heme groups. The content of holo-cytochrome *c* did not decrease even after 48 h of repression of *GRX5* expression (Figure 4). Although cytochrome *c* is rather stable in normal growth conditions (half-life of ~ 7 h [Pearce and Sherman, 1995]), a moderate to strong effect of Grx5 absence on heme synthesis would have been detected during the time course of the experiment. Thus, Grx5 is not essential for the biosynthesis of heme-containing proteins in mitochondria.

Iron Accumulates in the Cells in the Absence of Grx5

We next analyzed whether, as with other mutants in Fe/S cluster biogenesis, the absence of Grx5 caused iron accumulation in the cells. In the *grx5* mutant growing exponentially in YPD medium, an almost sixfold increase in total cell iron was observed with respect to wild-type cells (Figure 5A). This was paralleled by a decrease in aconitase activity but not in the activity of the non-Fe/S enzyme citrate synthase (Figure 5A). Inhibition of aconitase in the *grx5* cells grown on glucose was not as dramatic as that observed in cells conditionally expressing *GRX5* on glycerol (Figure 3A). The iron accumulation was attributable to the absence of Grx5. In fact, in MML313 (*tetO₇-GRX5*) cells cultured in YPG medium under the same conditions as in Figure 3A, inhibition of *GRX5* expression by doxycycline induced iron accumulation in the cell (Figure 5B).

A yeast frataxin mutant (*yfh1*) preferentially accumulates iron at the mitochondria, whereas cytosolic iron becomes depleted (Babcock *et al.*, 1997; Radisky *et al.*, 1999). On the contrary, when non-*GRX5*-expressing cells were subfractionated into mitochondrial and postmitochondrial fractions, iron was shown to hyperaccumulate in both fractions (Figure 5C). Control analyses showed that the postmitochondrial fraction was not contaminated by mitochondrial enzymes (citrate synthase and α -ketoglutarate dehydrogenase; Rodríguez-Manzanaque, Tamarit, Bellí, Ros, and Herrero, unpublished results). Under our conditions, mitochondrial iron represents 23–28% of total cell iron in both the absence and presence of *GRX5* expression (as calculated from the data in Figure 5C).

Inactivation of Fe/S Enzymes in the Absence of Grx5 Is Not a Consequence of Iron Accumulation in the Cell

Grx5 could be directly responsible for maintaining iron homeostasis. In that case, inactivation of Fe/S enzymes in *grx5* cells could be caused by the generation of ROS due to the presence of high levels of iron. Alternatively, Grx5 could be directly implicated in the biogenesis of Fe/S-protein complexes. In the latter case, increased iron levels in both cytosol and mitochondria would be a consequence of impairment of the formation of such complexes in the absence of the glutaredoxin. We used two approaches to test the direct involvement of Grx5 in Fe/S-enzyme biogenesis independently of the iron levels existing in the cell. First, we used the iron chelator BPS to create conditions in which internal iron levels in *grx5* cells were similar to those in wild-type cells. In such conditions, aconitase activity remained greatly diminished in the mutant, in contrast to the non-Fe/S enzyme malate dehydrogenase (Figure 6A). It should be noted that,

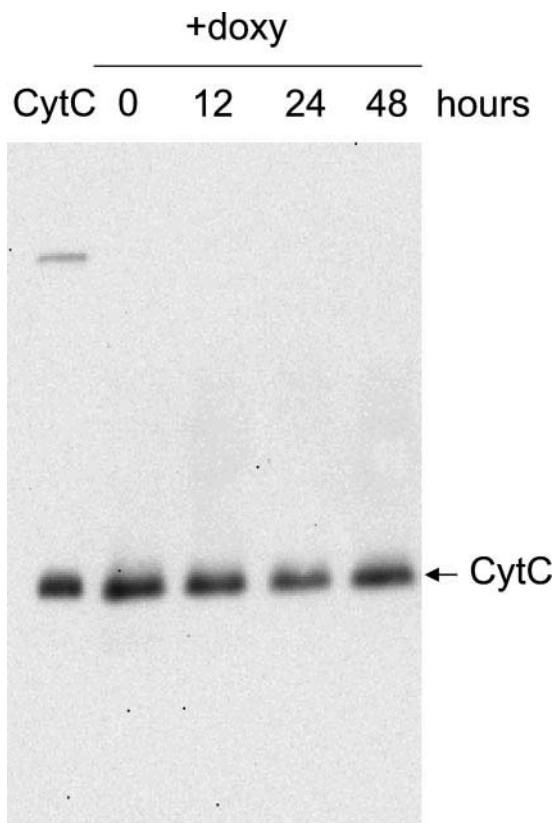


Figure 4. Effect of Grx5 depletion on the amount of heme covalently bound to cytochrome *c*. Expression of *GRX5* was interrupted (time 0) by the addition of doxycycline (2 µg/ml) to MML313 cells growing exponentially in YPG medium at 30°C. Proteins from whole cell lysates (samples taken at the indicated times after antibiotic addition) were separated by nonreducing SDS-PAGE, blotted onto a polyvinylidene difluoride membrane, and analyzed for heme-carrying proteins. In these conditions, heme bound to cytochrome *c* was the most prominent band detected (marked with an arrow). As a standard, 0.1 µg of cytochrome *c* from bovine heart was loaded on the left-most line.

in this particular genetic background, reduction of aconitase activity in *grx5* cells compared with wild-type cells is even higher than in the W303 background in the same growth conditions (compare Figures 6A and 5A).

Second, based on the fact that Aft1 is a transcriptional factor involved in the expression of genes responsible for the high-affinity iron transport system (Yamaguchi-Iwai *et al.*, 1995; Casas *et al.*, 1997), we constructed a single *aft1* and a double *aft1 grx5* mutant. As expected, *aft1* cells had reduced intracellular iron levels, and, probably as a consequence of this, activity of iron-dependent enzymes such as aconitase was also reduced (Figure 6B). The absence of Grx5 did not lead to iron accumulation in the *aft1 grx5* cells, indicating that increased levels of the metal in *grx5* cells requires Aft1-dependent iron transport. Importantly, the double mutant displayed additional reduction of aconitase activity compared with *aft1* cells, in conditions where intracellular iron remained low (Figure 6B), thus confirming that the primary

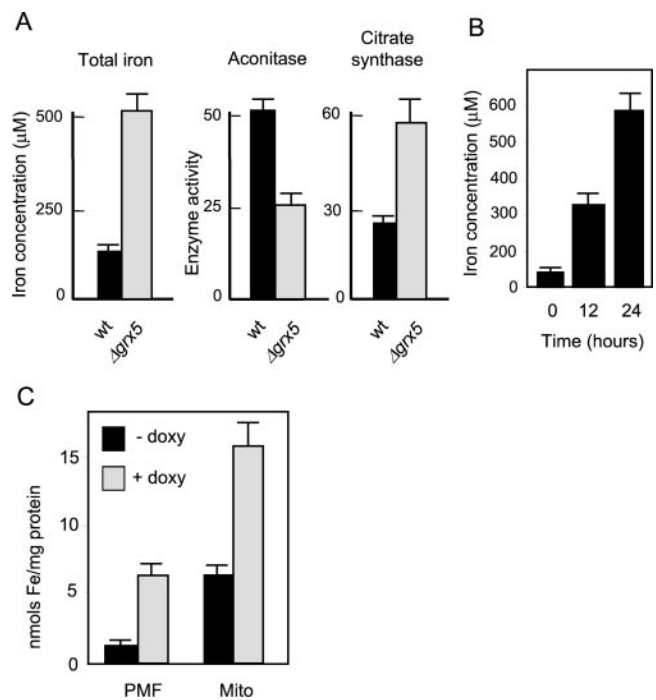


Figure 5. Iron accumulates in the cell in the absence of Grx5. (A) W303-1A (wt) and MML100 ($\Delta grx5$) cells were grown exponentially in YPD medium at 30°C to determine iron concentration in the cell and also aconitase and citrate synthase activities in total cell extracts. (B) MML313 cells growing exponentially in YPG medium at 30°C were added (time 0) with doxycycline (2 µg/ml), and total cell iron concentration was determined at different intervals after antibiotic addition. (C) Distribution of iron (relative to total protein in the fraction) between postmitochondrial fraction (PMF) and mitochondria (Mito), in MML313 cells untreated (–doxy) or treated for 24 h with doxycycline at 2 µg/ml (+doxy).

consequence of the absence of Grx5 is not the accumulation of iron.

Modification of the Intracellular Redox Potential Does Not Suppress the *grx5* Growth Defects

Glutaredoxins have been assigned a role as general reductants of disulfide bonds in cell proteins (Prinz *et al.*, 1997; Carmel-Harel and Storz, 2000). In *E. coli*, inactivation of the glutaredoxin and/or thioredoxin systems alters the thiol-disulfide equilibrium, which can be reversed in anaerobic conditions or by external reductants such as dithiothreitol (DTT; Prinz *et al.*, 1997). We hypothesized that the growth defects in the absence of Grx5 could be caused by the alteration of the redox potential at the mitochondria and, consequently, by the inhibition of oxidation-sensitive Fe/S-containing proteins. To address this problem, wild-type and *grx5* cells were cultured in SD minimal medium in anaerobic conditions, a situation that should compensate, at least in part, for the effect of the *grx5* mutation on the thiol-disulfide equilibrium. However, the mutant was unable to grow on SD plates in anaerobiosis (Figure 7A). While growing in anaerobiosis in SC medium, *grx5* cells still accumulated high

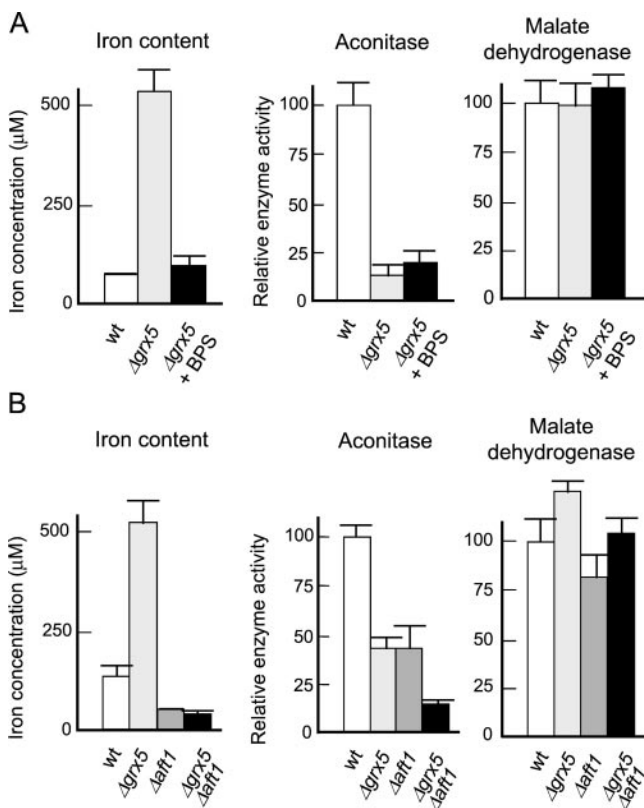


Figure 6. Activity of Fe/S enzymes is inhibited in *grx5* cells independently of intracellular iron concentration. (A) CML235 (wt) or MML19 ($\Delta grx5$) cells were grown exponentially in YPD medium (also in the presence of 80 μ M BPS in the case of MML19 cells), and total cellular iron concentration and aconitase and malate dehydrogenase activity were determined. (B) The same parameters as in A were determined in exponential YPD cultures at 30°C of W303-1A (wt), MML100 ($\Delta grx5$), MML348 ($\Delta aft1$), and MML345 ($\Delta grx5 \Delta aft1$) cells.

amounts of iron compared with wild-type cells (Figure 7A). As a second approach, cells were cultured on SD plates to which with different amounts of DTT were added, in conditions where this reductant has been shown to be active on yeast cells *in vivo* (Holst *et al.*, 1997). DTT was unable to suppress the growth defects of the *grx5* mutant at concentrations up to 4 mM (Figure 7B). Higher DTT concentrations were partially inhibitory of growth of wild-type cells in SD medium, whereas in complete medium *grx5* cells did not show higher sensitivity to DTT than wild-type cells (Rodríguez-Manzanaque, Tamarit, Bellí, Ros, and Herrero, unpublished results). We conclude that the *grx5* defective growth is not primarily due to the alteration of the intracellular redox potential, thus supporting the direct participation of Grx5 glutaredoxin in Fe/S cluster biogenesis.

DISCUSSION

Yeast cells contain both dithiol (Grx1, Grx2) and monothiol (Grx3, Grx4, Grx5) glutaredoxins. The two types of glutaredoxins coexist in many species from bacteria to humans

(Rodríguez-Manzanaque *et al.*, 1999), but specific roles for monothiol glutaredoxins have not previously been established. Grx5 is the yeast glutaredoxin whose absence causes the most dramatic effects on the oxidative damage to cell proteins, sensitivity to external oxidants, and general growth defects (Rodríguez-Manzanaque *et al.*, 1999). We have shown that Grx5 is located at the mitochondrial matrix and that its absence has a negative effect on the activity of mitochondrial proteins with Fe/S clusters but not on heme-containing proteins. Mitochondrial matrix location has also been demonstrated for other proteins that participate in Fe/S center protein assembly (reviewed by Lill and Kispal, 2000). The functional relationship between Grx5 and Fe/S cluster assembly was confirmed by the fact that overexpression of genes participating in Fe/S cluster assembly partially suppressed various *grx5* cell phenotypes. Of those, *SSQ1* codes for a Hsp70-type chaperone that might stabilize apo-proteins for Fe/S cluster coordination (Lill and Kispal, 2000) or even participate directly in the recognition/transfer step of clusters from Isu proteins to receptor polypeptides (Silberg *et al.*, 2001). The other partial suppressor of *grx5* mutants is *ISA2*. IscA, its product homologue in *E. coli*, complexes with ferredoxin to transfer iron and sulfide to form [2Fe-2S]-ferredoxin (Ollagnier-de-Choudens *et al.*, 2001). The fact that ferredoxin is also a component of the Fe/S cluster synthesis machinery in yeast suggests the hypothesis that multiprotein complexes form part of such a machinery.

The function of Grx5 in the assembly of Fe/S centers is not apparently related to that proposed for the human homologue PICOT, which would regulate signaling through the protein kinase C θ pathway (Isakov *et al.*, 2000; Witte *et al.*, 2000). Although Grx5 contains the PICOT homology domain, it lacks the N-terminal extension (with a thioredoxin or glutaredoxin-like module) present in Grx3 and Grx4 and also in other members of the PICOT superfamily (Isakov *et al.*, 2000). This, coupled with the differential compartmentalization, supports the hypothesis that the PICOT domain could be shared by various oxidoreductases, which contain a CKSF motif in the domain (Rodríguez-Manzanaque *et al.*, 1999) but serve different biological functions.

Besides the inactivation of enzymes with Fe/S clusters such as aconitase or succinate dehydrogenase, mutants deficient in *GRX5* share a number of phenotypes with others affected in the Fe/S cluster synthesis. These deficiencies include inability to grow in respiratory conditions and iron accumulation in their mitochondria. The last of these may be responsible for the oxidative damage observed in various cellular macromolecules when Fe/S cluster assembly is disrupted because of iron-mediated ROS formation. We observed additive effects on growth rate and on protein oxidative damage between mutants in *GRX5* and in other glutaredoxin genes (Rodríguez-Manzanaque *et al.*, 1999) and also between *grx5* and *sod1* mutations (Rodríguez-Manzanaque, Tamarit, Bellí, Ros, and Herrero, unpublished observations). This could be the consequence of the inability to repair iron-mediated macromolecular damage (both in mitochondria and cytosol) in Grx5-depleted cells in the absence of other glutaredoxins or of cytosolic superoxide dismutase. In either case, compartmentalization studies and sequence analysis indicate that Grx5 is the only dithiol plus monothiol glutaredoxin that is mitochondrially located and suggests that it does not directly share functions with Grx1–4. Inabil-

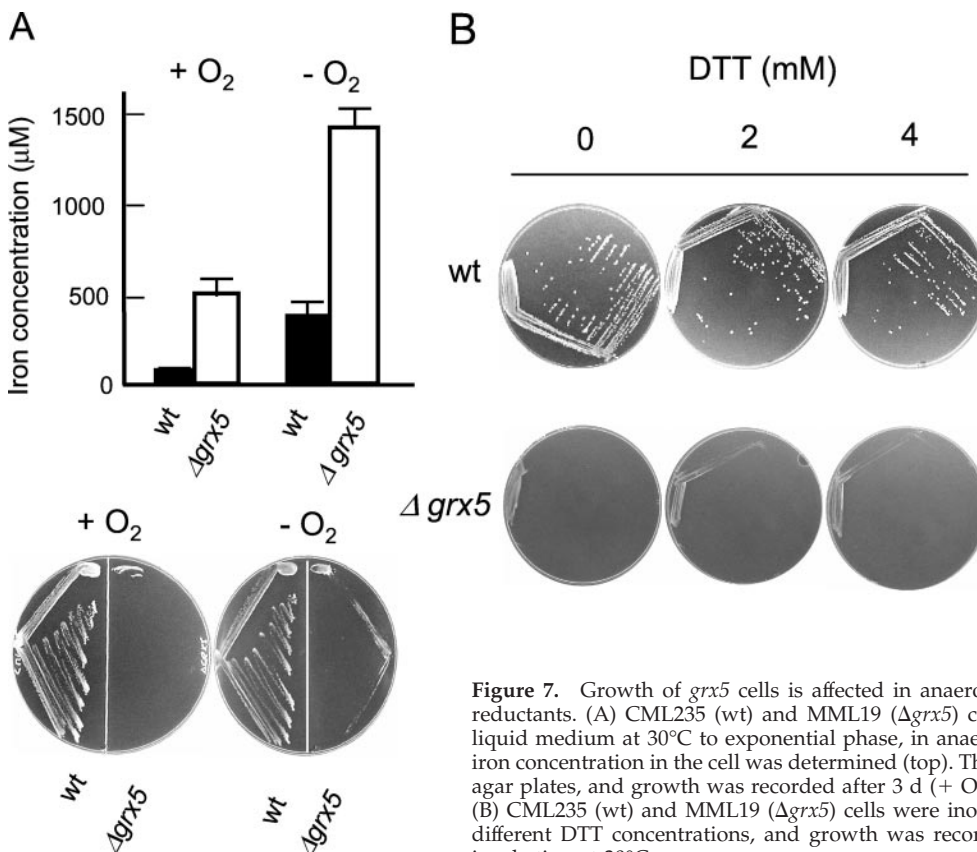


Figure 7. Growth of *grx5* cells is affected in anaerobiosis or in the presence of external reductants. (A) CML235 (wt) and MML19 ($\Delta grx5$) cells were grown exponentially in SC liquid medium at 30°C to exponential phase, in anaerobic or aerobic conditions, and total iron concentration in the cell was determined (top). The same strains were inoculated on SD agar plates, and growth was recorded after 3 d (+ O₂) or 4 d (-O₂) of incubation at 30°C. (B) CML235 (wt) and MML19 ($\Delta grx5$) cells were inoculated on SD agar plates containing different DTT concentrations, and growth was recorded after 3 d (wt) or 4 d ($\Delta grx5$) of incubation at 30°C.

ity to suppress *grx5* defects in conditions of *GRX3/4* overexpression confirms this suggestion. Similarly, a double *grx5 trx3* mutant is no more sensitive to oxidants or growth defects than a single *grx5* mutant (Rodríguez-Manzanaque, Tamarit, Belli, Ros, and Herrero, unpublished observations), thus arguing in favor of completely separate functions for Grx5 and the mitochondrial Trx3 thioredoxin. However, the relatively mild phenotypes of *grx5* cells compared with some other mutants in Fe/S cluster synthesis point to partial functional redundancy between Grx5 and other thiol oxidoreductases in the cell.

In Grx5-deficient cells, iron accumulation occurs at similar levels in mitochondria and extramitochondrial fractions, whereas in frataxin mutants iron accumulates exclusively at the mitochondria at the expense of cytosolic iron (Babcock *et al.*, 1997; Foury and Cazzalini, 1997; Radisky *et al.*, 1999). Although these differences argue against Grx5 acting in parallel with Yfh1 frataxin in the maintenance of iron homeostasis in the cell, it was still possible that extensive iron accumulation in *grx5* cells was a direct consequence of the absence of Grx5. Consequently, alterations in Fe/S enzyme activity could have been the result of the sensitivity of Fe/S clusters to high levels of ROS generated at increased iron concentrations. However, we can discard this possibility because the reduction of intracellular iron levels to almost normal or even lower than normal concentrations does not suppress the inactivation of Fe/S enzymes in *grx5* cells. Similar conclusions have been reached for Jac1 function

(Voisine *et al.*, 2001). These facts, together with the inability to suppress the *grx5* growth phenotypes in anaerobiosis or by the addition of external reductants, support a direct participation of Grx5 in Fe/S cluster assembly. The question remains as to whether the disruption of Fe/S cluster assembly could cause such high levels of mitochondrial iron and, in the case of *grx5* cells, cytosolic iron. Although the process of iron transport across the cytoplasmic membrane of yeast cells has been elucidated (Askwith and Kaplan, 1998; Eide, 1998), the mechanism of iron entry into the mitochondria remains uncertain (Lange *et al.*, 1999). A protein containing Fe/S clusters might participate in the regulation of mitochondrial iron assimilation, perhaps acting as an iron sensor that could act upstream of the nuclear transcription factor Aft1. This would explain the high levels of this metal found at the mitochondria in the absence of normal Fe/S cluster assembly at the organelle.

Although knowledge of the gene products involved in the maturation of Fe/S proteins at the yeast mitochondria has improved in recent years, the specific role of individual proteins and the biochemistry of the process remain obscure. Assembly of Fe/S centers in the apoprotein requires reduction of disulfide bridges between cysteine residues for coordination of Fe atoms (Beinert *et al.*, 1997). In the case of SoxR (a transcriptional regulator of *E. coli* involved in oxidative stress response whose activity depends on the redox state of a Fe/S cluster present in it [Hidalgo *et al.*, 1997]), GSH reductase and thioredoxins are required for the *in vivo*

response of SoxR to oxidants (Ding and Demple, 1998). Grx5 could play a similar role in yeast mitochondria, although in this case a monothiol mechanism for disulfide bridge reduction should be postulated. This would require a mixed disulfide intermediary between one of the cysteine residues and GSH that would be attacked by the monothiol glutaredoxin (Bushweller *et al.*, 1992). A variant of this hypothesis may be formulated from the observation that GSH and other monothiols are able to disassemble Fe/S clusters through GSH-derived reactive free radicals. The latter could form inactivating mixed disulfides with the cysteine residues responsible for iron chelation (Ding and Demple, 1996). Grx5 could be required to repair such toxic disulfides, therefore restoring the ability to assemble the clusters on the sulfhydryl groups of the apoproteins. Finally, Grx5 could play a role during the Nfs1-catalyzed desulfuration of cysteine. This reaction has been studied in the *Azotobacter vinelandii* homologue NiFS and involves formation of a persulfide between sulfur and Cys329 of NiFS (Zheng *et al.*, 1994). In yeast, Grx5 could be involved in the cleavage of this persulfide, leading to the release of sulfur and regeneration of reduced Nfs1. More studies are needed to determine the biochemical role of Grx5 in the formation of Fe/S clusters and to confirm whether Grx5 is part of a mitochondrial matrix multiprotein complex responsible for such a process.

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