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Prokaryotic and eukaryotic monothiol glutaredoxins are able to perform the functions of Grx5 in the biogenesis of Fe/S clusters in yeast mitochondria

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Abstract **The *Saccharomyces cerevisiae* monothiol glutaredoxin Grx5 participates in the mitochondrial biogenesis of iron–sulfur clusters. Grx5 homologues exist in organisms from bacteria to humans. Chicken (cGRX5) and human (hGRX5) homologues contain a mitochondrial targeting sequence, suggesting a mitochondrial localization for these two proteins. We have compartmentalized the *Escherichia coli* and *Synechocystis* sp. homologues, and also cGRX5 and hGRX5, in the mitochondrial matrix of a yeast *grx5* mutant. All four heterologous proteins rescue the defects of the mutant. The chicken *cGRX5* gene was significantly expressed throughout the embryo stages in different tissues. These results underline the functional conservation of Grx5 homologues throughout evolution.**

Keywords: Glutaredoxin; Iron–sulfur cluster; Mitochondria; Redox regulation; Glutathione

1. Introduction

Iron–sulfur (Fe–S) clusters are involved in essential processes such as respiration, photosynthesis and nitrogen fixation [1,2]. They also act as oxidative stress sensors associated with transcription regulators, or carry out a purely structural role as protein cysteine ligands [2]. Their formation in vivo requires the sequential participation of a complex set of proteins. In bacteria these are located at the cytosol. Although some debate still exists, it seems that in eukaryotic cells Fe–S cluster biogenesis mostly, if not exclusively, occurs at the mitochondrial matrix [3–6]. The importance of Fe–S clusters is illustrated by the existence of three different biosynthetic systems for them in bacteria. Of these, the Isc system is widely present among bacterial groups and participates in the formation of Fe–S clusters for a broad spectrum of proteins.

Most components of the Isc system have homologues in the yeast *Saccharomyces cerevisiae* and other eukaryotes. In *S. cerevisiae*, Fe–S clusters are assembled at the mitochondrial matrix independently of their final destination at the cytosol, nucleus or the mitochondria [3,5]. A complex formed by the Isu1 and Isu2 proteins acts as a scaffold upon which the clusters are assembled [7]. Once formed, the transfer of the Fe–S complex from the Isu1/Isu2 scaffold to the apoprotein requires the participation of a number of proteins, among them the monothiol glutaredoxin Grx5 [7,8]. Glutaredoxins are thiol transferases that employ reduced glutathione (GSH) as a substrate and catalyze glutathionylation and deglutathionylation reactions on cysteinyl groups in proteins [9,10]. According to the presence of two or one cysteine residue at the glutaredoxin active site, they have been divided into two categories: dithiol and monothiol glutaredoxins, respectively [11]. Dithiol glutaredoxins catalyze the reduction of thiol disulfides involving two cysteinyl groups and also of mixed disulfides between a protein cysteine residue and a GSH group (14). In contrast, the monothiol glutaredoxin mechanism of action exclusively involves the deglutathionylation of mixed disulfides [12]. In *S. cerevisiae* two dithiol glutaredoxins coexist (Grx1 and Grx2) plus three monothiol glutaredoxins (Grx3, Grx4 and Grx5) [13,14]. In the absence of mitochondrial Grx5, yeast cells are unable to maturate Fe–S proteins and consequently do not carry out respiratory metabolism. Furthermore, they accumulate intracellular iron and are hypersensitive to stress by external oxidants [8,13]. Grx3 and Grx4 are located at the nucleus and probably carry out different functions to Grx5 [15,16].

Both dithiol and monothiol glutaredoxins exist in many aerobic bacterial species and in lower and higher eukaryotes [17–20]. Analysis of the human genome reveals the existence of a gene coding for a single domain protein homologous to yeast Grx5 (*hGRX5*). A similar situation occurs in chicken and other multicellular eukaryotes, where the existence of Grx5 homologues has also been revealed from genomic sequencing. Despite their widespread presence throughout the evolutive scale, few functional studies exist on both prokaryotic and eukaryotic monothiol glutaredoxins, other than those on yeast Grx5. The parasite *Plasmodium falciparum* contains a glutaredoxin with structure and enzymatic characteristics similar to those of yeast Grx5, although its function is currently unknown [21]. The yeast Grx5 homologue in *Escherichia coli*, named Grx4, has recently been described as an essential protein that lacks in vitro activity on substrates characteristic of dithiol glutaredoxins [22]. A recent report demonstrates the role of the zebra fish Grx5 homologue in Fe/S cluster synthesis and heme formation, its absence causing hypochromic

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Abbreviations: Grx, glutaredoxin; GSH, reduced glutathione; ORF, open reading frame; *t*-BOOH, *tert*-butyl hydroperoxide; Trx, thioredoxin

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anaemia in embryos [23]. In the present work, we have studied the ability of various different prokaryotic and eukaryotic monothiol Grx proteins to substitute the biological function of *S. cerevisiae* Grx5. Our results show that diverse monothiol glutaredoxins can carry out the function of Grx5 in yeast mitochondria, therefore supporting the functional conservation of this family of proteins.

2. Materials and methods

2.1. Strains and plasmids

S. cerevisiae strains are described in Table 1. Plasmid pMM221 [16] contains the sequence coding for the mitochondrial targeting signal of Grx5 followed by a polylinker region and a 3HA/His_6 tag, under the control of the doxycycline-regulatable $tetO_2$ promoter [24]. Plasmid pMM227 contains the *S. cerevisiae GRX3* gene cloned in pMM221, in frame with the upstream and downstream coding sequences present in the vector [16]. Plasmid pMM509 contains the coding sequence of *E. coli* Grx4 from aa +2 to the last coding nucleotide, that was cloned between the *Not*I and *Bgl*II sites of the pMM221 polylinker, in frame with the Grx5 mitochondrial targeting sequence and the 3HA/His_6 tag of the vector. Plasmid pMM521 contains the *Synechocystis* sp. GrxC coding sequence from aa +1 to the last coding nucleotide, PCR-amplified from a pQE80-derived clone containing the entire GrxC ORF (a gift from F.J. Florencio, University of Sevilla). It was subcloned between the NotI and PmeI sites of pMM221. cDNA coding for the human homologue of yeast Grx5 (hGRX5) was from the American Type Culture Collection (IMAGE clone no. 6066312, from a human testis cDNA library). The region coding from aa +32 to the last coding nucleotide was subcloned between the *Not*I and *Pme*I sites of pMM221, in frame with adjacent coding sequences in the vector, resulting plasmid pMM540. cDNA coding for the chicken Grx5 homologue (cGRX5) was amplified from chicken liver RNA (a gift from R. Soler, University of Lleida), from the first codon to the stop codon, and cloned in pBluescript SK+ (plasmid pMM626). The coding sequence from aa +33 to the last nucleotide preceding the stop codon was then subcloned into pMM221, using the *Not*I and *Pme*I polylinker sites, in frame with adjacent coding sequences of the vector, resulting plasmid pMM636. Plasmid pMM54 [8] contains a *GRX5-3HA* construction under its own promoter.

2.2. Growth conditions

S. cerevisiae cells were grown as described in Ref. [16]. Expression from the $tetO_2$ promoter was modulated with different doxycycline concentrations. Samples for further analyses were taken from cells growing exponentially at 30 °C for at least 10 generations.

2.3. In Silico analysis

Yeast Grx5 homologues were searched using BLASTP (NCBI, www.ncbi.nlm.nih.gov/BLAST). Multiple protein sequence alignments were done with ClustalW [25] (European Bioinformatic Institute tools, www.ebi.ac.uk). The MITOPROT program [26] was employed to predict mitochondrial targeting sequences and cleavage sites.

2.4. In situ hibridization

Paraformaldehyde-fixed samples were cryoprotected with 20% sucrose at 4 °C. Cryostat sections (16 μm-thick) were mounted on silane coated slides air-dried and stored at −80 °C. A plasmid derived from pBluescript SK+ that contained the *cGRX5* first exon coding sequence (directionally cloned between the *Hin*dIII–*Bam*HI vector sites) was used to synthesize the antisense and sense (control) riboprobes, in the presence of dig RNA labelling mix digoxigenin UTP (Roche, Mannheim, Germany). Further steps were carried out by standard procedures [27]. Embryos were staged according to the Hamburger and Hamilton stage series [28].

2.5. Other methods

Mitochondria were purified and subfractionated [29] from exponential yeast cultures in lactate medium. Conditions for Western analyses were as in Ref. [16]. Standard genetic methods were employed [8,16]. Aconitase and malate dehydrogenase were assayed using the methods described in Ref. [30]. Enzyme assay extracts from cells growing exponentially in YPGalactose medium were prepared in 0.1 M Tris buffer, pH 8.0, plus protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 0.2 mM tosylsulphonyl phenylalanyl chloromethyl ketone, and 2 μM pepstatin, final concentrations), using glass beads to break the cells.

3. Results

Among prokaryotic organisms, most proteobacteria and cyanobacteria species whose genome has been sequenced contain genes coding for Grx5 homologues [18,19]. In order to determine whether bacterial homologues of Grx5 were able to carry out the functions of the yeast protein, we focused our attention on *E. coli* Grx4 and *Synechocystis* sp. GrxC proteins. *E. coli* Grx4 is an essential protein whose biological role remains elusive [22]. *Synechocystis* sp. GrxC is the only monothiol glutaredoxin present in this cyanobacterium and its biological role is also unknown. Homology between yeast Grx5 and bacterial Grx4 and GrxC extends throughout the entire protein sequence (Fig. 1), except that Grx5 contains a 29 aa N-terminal extension that targets the protein to the

Table 1
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 *MAT*α	Wild type
MML100	*MAT***a** *grx5::kanMX4*	Ref. [8]
MML235	*MAT*α [pMM54(*GRX5-3HA*)]::*LEU2*	Integration of linear pMM54 in W303-1B
MML240	*MAT***a** *grx5::kanMX4* [pMM54(*GRX5-3HA*)]::*LEU2*	Ref. [8]
MML241	*MAT*α *grx5::kanMX4* [pMM54(*GRX5-3HA*)]::*LEU2*	Spore from a cross MML100 × MML235
MML289	*MAT*α *grx5::kanMX4*	As MML100, but derived from W303-1B
MML443	*MAT***a** [pMM227(*GRX3-3HA*)]::*LEU2*	Ref. [16]
MML454	*MAT***a** *grx5::kanMX4* [pMM227(*GRX3-3HA*)]::*LEU2*	Ref. [16]
MML455	*MAT*α *grx5::kanMX4* [pMM227(*GRX3-3HA*)]::*LEU2*	Spore from a cross MML241 × MML443
MML673	*MAT*α [pMM509(*grx4-3HA*)]::*LEU2*	Integration of linear pMM509 in W303-1B
MML681	*MAT*α [pMM521(*grxC-3HA*)]::*LEU2*	Integration of linear pMM521 in W303-1B
MML706	*MAT*α *grx5::kanMX4* [pMM521(*grxC-3HA*)]::*LEU2*	Spore from a cross MML100 × MML681
MML707	*MAT*α *grx5::kanMX4* [pMM521(*grx4-3HA*)]::*LEU2*	Spore from a cross MML100 × MML673
MML725	*MAT***a** [pMM540(*hGRX5-3HA*)]::*LEU2*	Integration of linear pMM540 in W303-1A
MML731	*MAT*α *grx5::kanMX4* [pMM540(*hGRX5-3HA*)]::*LEU2*	Spore from a cross MML289 × MML725
MML760	*MAT*α [pMM636(*cGRX5-3HA*)]::*LEU2*	Integration of linear pMM636 in W303-1B
MML771	*MAT***a** *grx5::kanMX4* [pMM636(*cGRX5-3HA*)]::*LEU2*	Spore from a cross MML100 × MML760

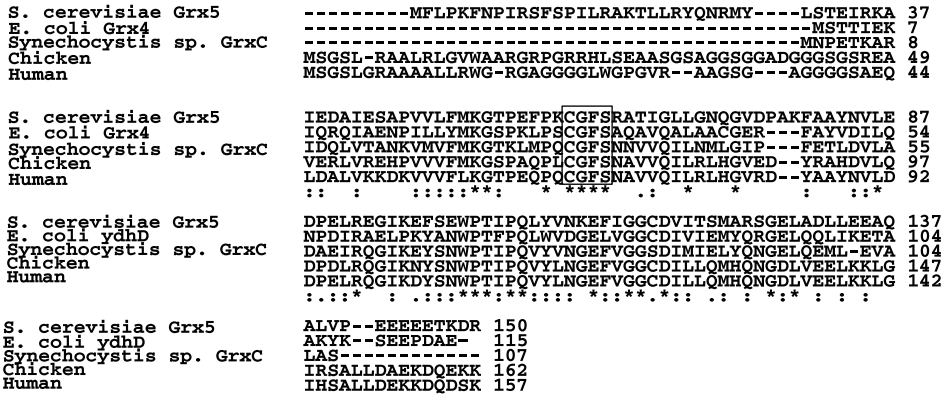

Fig. 1. Sequence of various different monothiol glutaredoxins. ClustalW multiple alignment analysis of the following monothiol glutaredoxins (SwissProt accession number into parenthesis): *S. cerevisiae* Grx5 (Q02784), *E. coli* Grx4 (P37010), *Synechocystis* sp. GrxC (P73056), *Gallus gallus* cGRX5 (Q5ZK23), *Homo sapiens* hGRX5 (Q86WY3). Asterisks mark positions with identical residues. The conserved active site sequence CGFS is boxed.

mitochondrial matrix [8]. The CGFS region essential for the monothiol glutaredoxin activity [18] is present in the three proteins.

Bacterial Grx4 and GrxC were expressed in the mitochondria of a yeast Δ*grx5* mutant, using pMM221 [16] derivatives. Subfractionation studies indicated that almost the totality of the Grx4 or GrxC protein expressed in the Δ*grx5* cells was targeted to the mitochondria, where it was located at the matrix, as occurs with yeast Grx5 (Fig. 2A). A minor proportion of GrxC and a major one of Grx4 at their respective mitochondrial fractions exhibited a lower electrophoretic mobility than expected for mature processed forms. They probably correspond to non-processed molecules with the mitochondrial targeting sequence, which nevertheless are internalized into the matrix mitochondria. These lower mobility forms are also observed in Western analyses of total cell extracts (Fig. 2D). Comparing their mobility with that of mature Grx5 and considering the respective amino acid lengths of the respective molecules (Fig. 1), this supports the notion that a fraction of Grx4 and GrxC expressed in yeast is not processed. *E. coli* Grx4 partially rescued the inability of yeast Δ*grx5* cells to grow in respiratory conditions (YPD-glycerol medium) (Fig. 2B), while *Synechocystis* sp. GrxC rescued the respiratory defects of the mutant with an efficiency comparable to that of nuclear Grx3 glutaredoxin (Fig. 2B). Bacterial Grx4 and GrxC were almost as efficient as yeast Grx3 in rescuing the hypersensitivity of a Δ*grx5* mutant to external oxidants such as *tert*-butyl hydroperoxide (*t*-BOOH) or diamide (Fig. 2C). The ratio of the activities of the two mitochondrial enzymes aconitase (containing Fe–S clusters) and malate dehydrogenase (without Fe–S clusters) provides a measure of the efficiency of the Fe–S assembly in mitochondrial proteins [16]. We determined this ratio in Δ*grx5* cells expressing Grx4 or GrxC in their mitochondria (Fig. 2D). Cyanobacterial GrxC was more efficient than *E. coli* Grx4 in its ability to synthesize active aconitase; in fact, relative aconitase levels in cells expressing GrxC were almost similar to those in cells expressing yeast Grx3 at their mitochondria (Fig. 2D). Switching off expression of the Grx genes from the *tet* promoter abolished aconitase activity (Fig. 2D and box). Malate dehydrogenase activity did not significantly vary from one condition to another (data not shown). It is important to stress that in the absence of doxycycline, that is, in the same conditions as in the growth pattern experiments shown in Fig. 2B and C, the levels of both processed and unprocessed Grx4 and GrxC were at least comparable to those of Grx5 in wild type yeast cells (Fig. 2D, box). To summarize, bacterial monothiol glutaredoxins are able to complement mutant phenotypes of yeast cells lacking Grx5, with cyanobacterial GrxC being more efficient in this respect than *E. coli* Grx4.

BLASTP search of the human genome revealed the existence of a gene coding for a putative product that displays a large degree of homology with *S. cerevisiae* Grx5 (Fig. 1). The gene is located at human chromosome 14 (position 14q32.13) and is called C14orf87 (GenBank Accession No. BC047680). Based on the homology of its putative product with other monothiol glutaredoxins with a single Grx domain, the name *hGRX5* is proposed for this gene. Although the hGRX5 protein displays homology with the PICOT protein which is also present in human cells, the latter is a monothiol glutaredoxin of the Trx–Grx type [19,20]. A mitochondrial targeting sequence is predicted for hGRX5, with a cleavage site after position +31. We therefore subcloned the *hGRX5* cDNA corresponding to the predicted mature form of the protein in pMM221 and studied the ability of hGRX5 to rescue the defects of the yeast Δ*grx5* mutant. The human protein was efficiently compartmentalized in the mitochondrial matrix of the mutant (Fig. 3A) and in these conditions complemented the defects of the mutant with respect to: growth under respiratory conditions (Fig. 3B), sensitivity to external oxidants such as *t*-BOOH or diamide (Fig. 3C), and synthesis of active aconitase (Fig. 3D). Malate dehydrogenase levels were similar in all conditions tested (data not shown). These results point to the conservation between human hGRX5 and Grx5 regarding their functionality in the yeast mitochondria.

A similar BLASTP search using yeast Grx5 as bait revealed the presence of a gene located at chromosome 5 of the chicken genome, coding for a Grx5 homologue (named LOC423440, GenBank Accession No. AJ720261). We propose calling this gene *cGRX5*. Its predicted product displays extensive homology with hGRX5 and also with other monothiol glutaredoxins (Fig. 1). A mitochondrial targeting se-

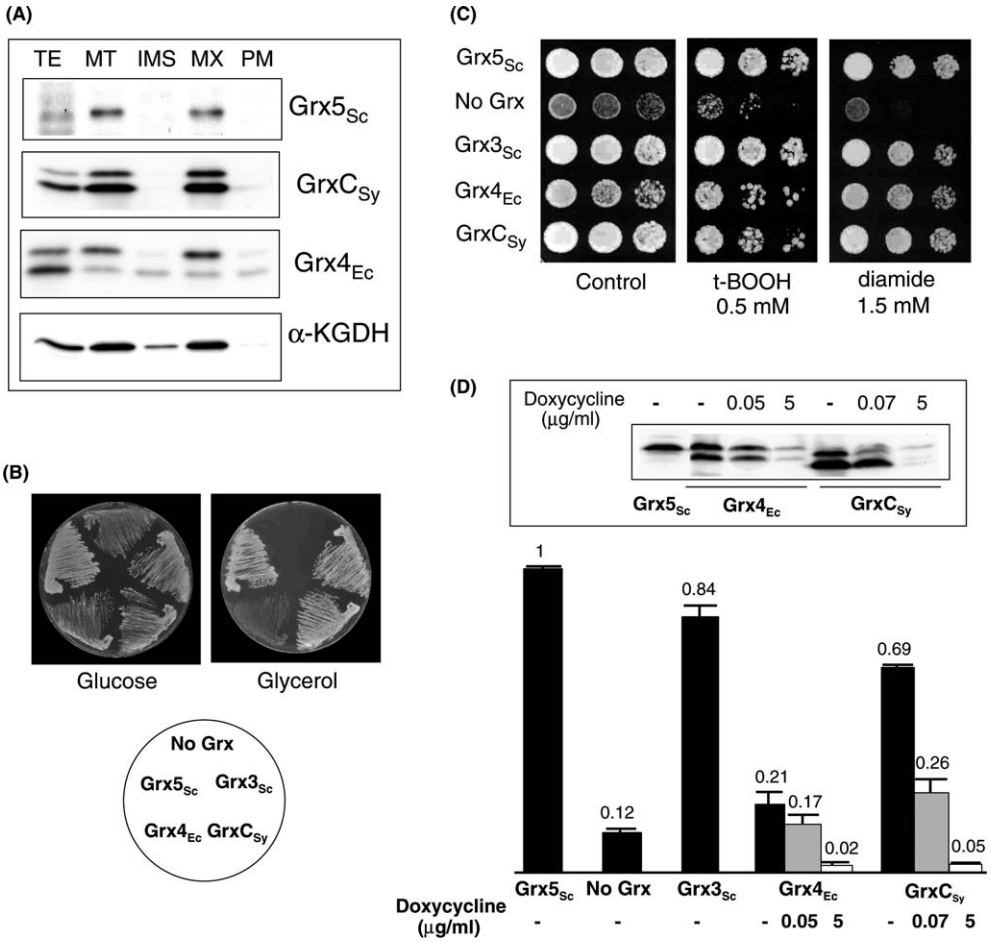

Fig. 2. Rescue of the *S. cerevisiae* Δ*grx5* mutant defects by the mitochondrial forms of *E. coli* Grx4 and *Synechocystis* sp. GrxC. (A) Derivatives of pMM221 are able to compartmentalize *E. coli* Grx4 (pMM509, integrated in strain MML673) or *Synechocystis* sp. GrxC (pMM521, integrated in strain MML681) in the mitochondrial matrix of *S cerevisiae* cells. Cells were exponentially grown in lactate medium at 30 °C to an absorbance (600 nm) of about 0.6 before mitochondrial isolation and subfractionation. Strain MML235 expressing a Grx5-3HA construct under its own promoter [8] was employed as control. TE, total cell extract; MT, mitochondrial fraction; IMS, intermembrane space; MX, matrix; PM, post-mitochondrial fraction. Twenty micrograms of protein was loaded in the TE lines, and 5 μg 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 α-ketoglutarate dehydrogenase (α-KGDH). (B) Growth on glucose (YPD plates) or glycerol (YPGly plates), after 3 days at 30 °C, of strains with a chromosomal Δ*grx5* background expressing no mitochondrial glutaredoxin (MML289) or the mitochondrial forms of *S. cerevisiae* Grx5 ($Grx5_{Sc}$, MML241), *S. cerevisiae* Grx3 ($Grx3_{Sc}$, MML455), *E. coli* Grx4 ($Grx4_{Ec}$, MML707) or *Synechocystis* sp. GrxC ($GrxC_{Sy}$, MML706). (C) Sensitivity to *t*-BOOH or diamide of the strains described in (B), after 3 days at 30 °C on YPD plates. (D) Ratio between aconitase and malate dehydrogenase activities in exponential cells at 30 °C in YPGalactose medium. The same strains were employed as in (B). Doxycycline was used at the indicated concentrations in the growth medium to modulate expression from the $tetO_2$ promoter. Values are normalized with respect to strain MML241 that expresses yeast Grx5 under its own promoter (unit value). Boxed panels: Western blot analyses of HA-tagged Grx in total cell extracts from the strains expressing the indicated Grx.

quence is also predicted for cGRX5, with a cleavage site after aa +32. Total liver RNA was then used to clone the cDNA expanding the entire *cGRX5* coding sequence plus the stop codon, without flanking regions. This was followed by subcloning of the region coding for the predicted mature protein in pMM221. This allowed 3HA tagging plus mitochondrial matrix targeting of cGRX5 (Fig. 3A). Using the same strategy as above, it was demonstrated that cGRX5 rescues the defects of the *S. cerevisiae grx5* mutant with respect to growth in glycerol medium, sensitivity to oxidants and the activity of the Fe/S enzyme aconitase (Fig. 3). However, restoration of aconitase levels was not as efficient as in the case of the human species. Malate dehydrogenase remained at similar levels in the strains tested (data not shown).

Chicken is a useful model to study gene expression during embryogenesis. We therefore studied *cGRX5* mRNA expression by in situ hybridization using tissues from embryos at different stages of development. Expression was observed at different regions in embryos at stage 24 (4-days-old) (Fig. 4). In 16-day embryos (stage 42 of development), a strong expression signal was observed in many tissues. In particular, various different types of nervous system cells (such as motoneurons in the spinal cord, Purkinje cells in cerebellum and granular cells in the visual cortex) displayed a strong signal (Fig. 5). Significant expression was also observed in muscle, kidney and lung cells (Fig. 5), in liver and in heart, among other tissues. We therefore conclude that *cGRX5* expression is required in different tissue cells during embryogenesis.

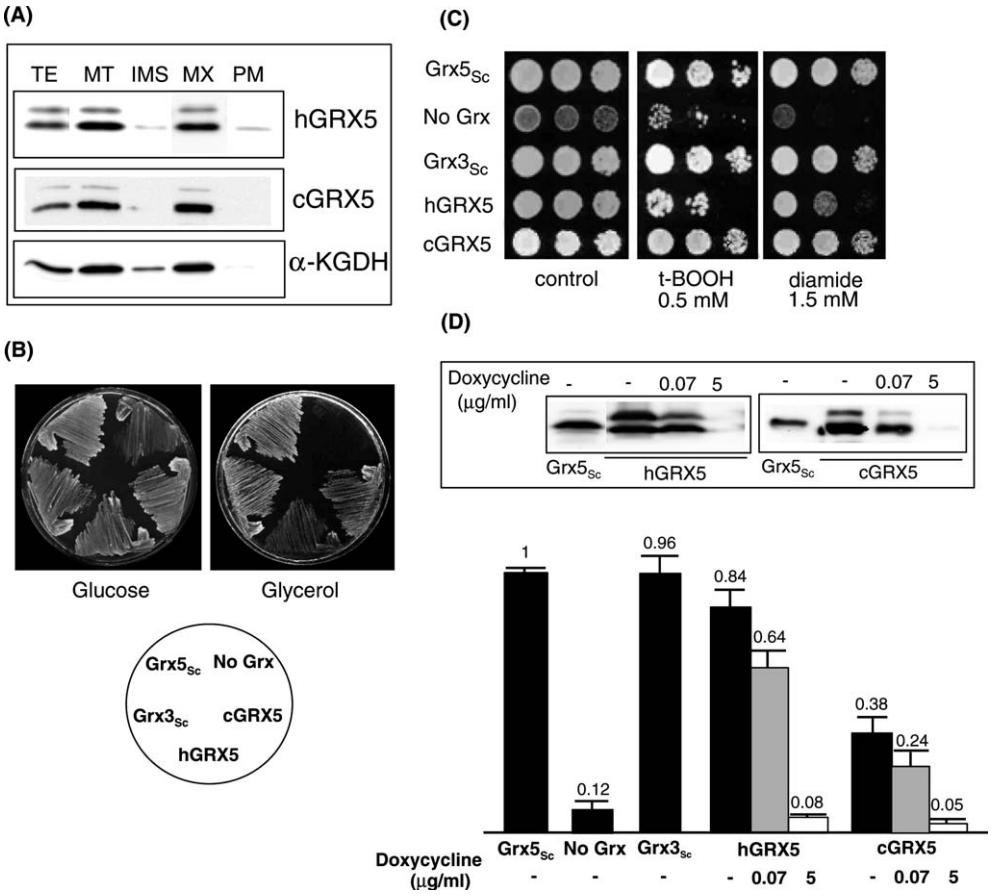

Fig. 3. Rescue of the *S. cerevisiae* Δ*grx5* mutant defects by the mitochondrial forms of human hGRX5 and chicken cGRX5. (A) Derivatives of pMM221 are able to compartmentalize hGRX5 (pMM540, integrated in strain MML725) or cGRX5 (pMM636, integrated in strain MML760) in the mitochondrial matrix of *S. cerevisiae* cells. Experimental conditions were as in Fig. 2A. (B) Growth on glucose or glycerol, after 3 days at 30 °C, of strains with a Δ*grx5* background expressing no mitochondrial glutaredoxin (MML289) or the mitochondrial forms of *S. cerevisiae* Grx5 ($Grx5_{Sc}$, MML241), *S. cerevisiae* Grx3 ($Grx3_{Sc}$, MML455), human hGRX5 (MML731) or chicken cGRX5 (MML771). (C) Sensitivity to *t*-BOOH or diamide of the strains described in (B), after 3 days at 30 °C on YPD plates. (D) Ratio between aconitase and malate dehydrogenase activities in exponential cells at 30 °C in YPGalactose medium. The same strains were employed as in (B). Doxycycline was used at the indicated concentrations in the growth medium to modulate expression from the $tetO_2$ promoter. Values are normalized with respect to strain MML241 that expresses yeast Grx5 under its own promoter (unit value). Boxed panels: Western blot analyses of HA-tagged Grx in total cell extracts from the strains expressing the indicated Grx.

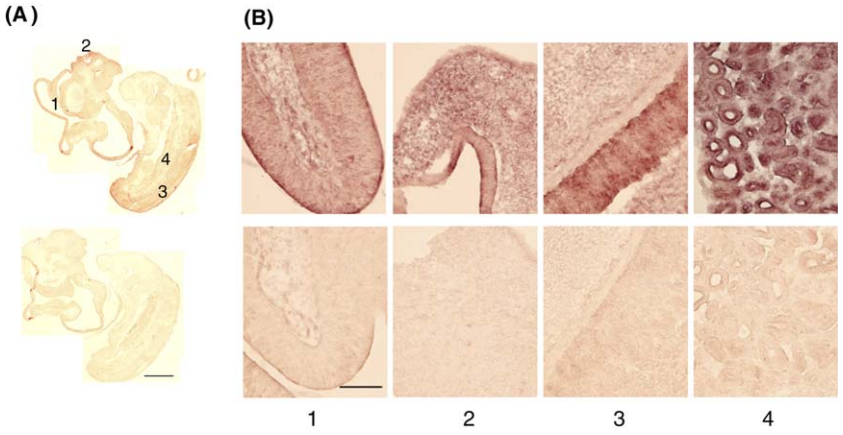

Fig. 4. *cGRX5* expression in chick embryos at stage 24 (4 days) by in situ hybridization. (A) Sagittal sections incubated with *cGRX5* antisense (upper panel) or sense (lower panel) riboprobes. (B) Different and equivalent regions of both sections photographed at higher magnification. Upper images show expression at the level of the meso-metencephalic fold (1), hindbrain (2), tail region with the most marked structure corresponding to the spinal cord (3), and mesonephros (4). Lower images show staining in the same regions with the sense riboprobe. Scale bars: 1 mm in (A), 0.1 mm in (B).

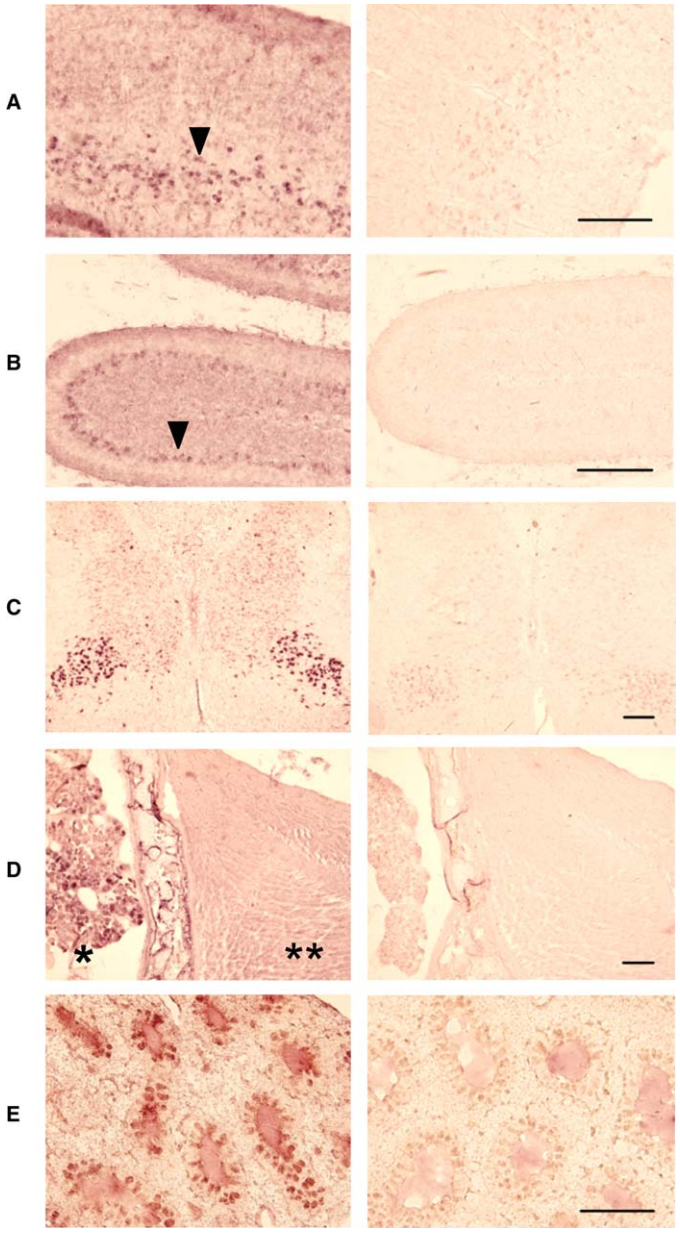

Fig. 5. Expression of *cGRX5* at late stages of chick embryogenesis. Images were taken from sections of different tissues of chick embryos at stage 42 (16 days) of development. Left and right panels correspond, respectively, to samples hybridized with the *cGRX5* antisense and sense riboprobes. (A) Visual cortex with the arrow pointing to granular cells; (B) cerebellum with the arrow pointing to Purkinje cells; (C) spinal cord showing strong staining in motorneurons; (D) kidney (*) and muscle (**) tissue; (E) lung tissue. Scale bars: 200 μm.

4. Discussion

During Fe–S cluster biogenesis in *S. cerevisiae* cells, sulfur atoms are provided by the activity of cysteine desulfurase (Nfs1) to the Isu1/Isu2 scaffold. How iron atoms are recruited and delivered to Isu1/Isu2 is not well-understood. It has recently been proposed that IscA, the bacterial homologue of yeast Isa1 and Isa2 proteins [3], could carry out the function of recruiting iron to the scaffold complex [31]. In *S. cerevisiae* mitochondria, Grx5 would be required at a step after the formation of the Fe/S clusters on Isu1/Isu2, maybe during insertion of the former into apoproteins [7]. The fact that overexpression of Isa2 partially suppresses the defects of a *grx5* mutant [8] and that a physical interaction has been detected between Grx5 and Isa1 [19] suggest some functional relationship between Grx5 and the Isa proteins. The specific role of Grx5 in the biosynthetic process is not known, although the requirement for an appropriate redox state of cysteine residues in proteins involved both in the biogenesis of the clusters on the Isu1/Isu2 scaffold and in the transfer of clusters to apoproteins seems evident. In vitro observations with components of the bacterial Isc system support the requirement for specific redox conditions for acceptor cysteine residues in the biosynthetic complexes. Thus, *E. coli* dithiolic glutaredoxins 1, 2 and 3 stimulate insertion of Fe/S clusters into the FNR apoprotein [32]. Also, delivering iron atoms from the *E. coli* IscA protein into the IscU scaffold requires the thioredoxin system [31]. By acting as a deglutathionylation enzyme, Grx5 could re-

pair mixed disulfides formed between GSH and components of the biosynthetic complex under the oxidant conditions of the mitochondrial matrix.

The biological role of prokaryotic Grx5 homologues has not been determined, although extensive studies have been performed in vitro with the essential Grx4 protein of *E. coli* [22]. In the present study Grx4 and the *Synechocystis* sp. GrxC protein are able to substitute for Grx5 function when compartmentalized in the yeast mitochondria. Not all the defects of the *grx5* mutant are however rescued at the same level, cyanobacterial GrxC performing better than *E. coli* Grx4. The increase in aconitase activity over the mutant basal levels in the cells expressing the heterologous protein was only about twofold in the case of Grx4, which could be taken as a measure of the relative efficiency of Fe–S cluster assembly in the apoprotein. However, this was sufficient to allow significant growth in glycerol medium (which requires the maturation of the Fe–S Rip1 protein present in the mitochondrial electron carrier chain) and resistance to diamide and *t*-BOOH treatment. In contrast, *Synechocystis* sp. GrxC protein and the eukaryotic homologue hGRX5 made it possible to achieve aconitase levels close to those found in wild type cells, which is indicative of an efficient complementation of the *grx5* defects. Chicken cGRX5 allowed achieving intermediate aconitase levels. Besides the cysteine residue at the active site, Grx5 contains a second cysteine at position 117. Many of the Grx5 homologues also contain a cysteine residue at an equivalent position. By site directed mutagenesis, we have shown that C117 is not essential for the biological activity of Grx5 [18]. Of the proteins tested in this study, only GrxC lacks that cysteine residue. In spite of this, this glutaredoxin is able to function at the yeast mitochondria, confirming the dispensability of the second cysteine in vivo.

Overall, the above studies point to a functional conservation between Grx5 and its bacterial and eukaryotic homologues. They are in accordance with the demonstration of the role of the zebra fish Grx5 homologue in Fe/S cluster synthesis and heme formation [24], and extend the observations to prokaryotes. Fe–S assembly mostly, if not totally, occurs at the mitochondria in eukaryotes. In accordance with this, primary sequence analysis predicts a mitochondrial localization for hGRX5 and cGRX5. Our in situ hybridization experiments demonstrate that the *cGRX5* gene is expressed in a variety of tissues, particularly in the nervous system, from early stages of chicken embriogenesis. This is accordance with the situation in zebra fish, where the Grx5 homologue is also expressed in a variety of tissues during embryogenesis [24], and altogether may reflect the importance of Fe/S clusters for functions such as respiration from early embryo stages. Although our results do not definitively prove the universal requirement for a monothiol glutaredoxin of the Grx type for mitochondrial Fe–S synthesis, they clearly demonstrate, for all the molecules tested, their ability to participate in the Fe–S biosynthetic complexes in yeast mitochondria. In other words, no functional barriers related to low molecular weight monothiol glutaredoxins have appeared along evolution.

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