RESEARCH COMMUNICATION

Two isoforms of *Saccharomyces cerevisiae* glutaredoxin 2 are expressed *in vivo* and localize to different subcellular compartments

José R. PEDRAJAS*, Pablo PORRAS†, Emilia MARTÍNEZ-GALISTEO†, C. Alicia PADILLA†, Antonio MIRANDA-VIZUETE‡ and J. Antonio BÁRCENA†[1]

*Departamento de Biología Experimental, Facultad de Ciencias Experimentales, University of Jaén, 23071-Jaén, Spain, †Departamento de Bioquímica y Biología Molecular, Campus de Rabanales, edificio C-6, 1ª planta, University of Córdoba, 14071-Córdoba, Spain, and ‡Karolinska Institute, Department of Biosciences at Novum, Center for Biotechnology, S-14157 Huddinge, Sweden

Glutaredoxin (Grx)2 from *Saccharomyces cerevisiae* is a member of the two-cysteine (dithiol) subfamily of Grxs involved in the defence against oxidative stress in yeast. Recombinant yeast Grx2p, expressed in *Escherichia coli*, behaves as a 'classical' Grx that efficiently catalyses the reduction of hydroxyethyl disulphide by GSH. Grx2p also catalyses the reduction of GSSG by dihydrolipoamide with even higher efficiency. Western blot analysis of *S. cerevisiae* crude extracts identifies two isoforms of Grx2p of 15.9 and 11.9 kDa respectively. The levels of these two isoforms reach a peak during the exponential phase of growth in normal yeast extract/peptone/dextrose ('YPD') medium, with the long form predominating over the short one. From immunochemical analysis of subcellular fractions, it is shown that both isoforms are present in mitochondria, but only the short one is detected in the cytosolic fraction. On the other hand, only the long form is prominent in microsomes. Mitochondrial isoforms should represent the processed and unprocessed products of an open reading frame (YDR513W), with a putative start codon 99 bp upstream of the *GRX2* start codon described thus far. These results indicate that GRX2 contains two in-frame start codons, and that translation from the first AUG results in a product that is targeted to mitochondria. The cytosolic form would result either by initiation from the second AUG, or by differential processing of one single translation product.

Key words: glutathione, lipoamide, oxidative stress, thioltransferase, yeast.

INTRODUCTION

Glutaredoxins (Grxs) are small proteins (approx. 10 kDa) that are highly conserved throughout evolution and which catalyse GSH–disulphide oxidoreductions [1]. The first function assigned to Grx was as an electron donor in the reduction of an intramolecular disulphide in ribonucleotide reductase [2] by means of a dithiol mechanism [3]. The active site sequence (Cys-Pro-Tyr-Cys) responsible for this activity is conserved in most species. However, only one of these cysteine residues is involved in other important reactions also catalysed by Grx *in vitro*, such as the GSH-dependent reduction of protein–glutathionyl mixed disulphides (protein-SSG), i.e. the so-called 'deglutathionylase' or 'dethiolase' activity [4–6]. Since oxidative conditions induce the accumulation of protein-SSG [7,8], Grx might have a role in the defence of cells against reactive oxygen species by means of its dethiolase activity. Evidence for a protective role of Grx under oxidative conditions has been reported from biochemical [5,6,9,10] and genetic [11,12] approaches.

Two genes encoding Grxs have been characterized in *Saccharomyces cerevisiae*, *GRX1* and *GRX2*, which share a high degree of similarity with other known Grxs from different species [12,13]. Grx1p and Grx2p appear to have different roles in yeast, i.e. *GRX1* mutants are sensitive to oxidative stress caused by superoxide anion, as induced by menadione (2-methyl-1,4-naphthoquinone), whereas *GRX2* mutants are sensitive to stress induced by hydrogen peroxide; in addition, Grx2p accounts for most of the GSH-dependent oxidoreductase activity in yeast cells [12]. Moreover, *GRX1* and *GRX2* show different expression patterns under various stress conditions, although both genes possess and are regulated by stress-response elements [14].

A group of three additional genes, named *GRX3*, *GRX4* and *GRX5*, having homology with *grx* have also been identified in yeast [11]. The proteins encoded by these genes show a marked difference with other Grxs in that they only have one cysteine in the active site instead of two. This might be a substantial property that would limit the action of this subfamily of Grxs to processes that occur via a monothiol mechanism. Mutants lacking any of these three proteins showed a marked decrease (approx. 60%) in Grx activity, as determined by the standard assay in crude extracts, and were more prone to protein oxidative damage and more sensitive to osmotic stress than the wild-type. In addition, the *grx2grx5* mutant was non-viable, indicating that Grx2p and Grx5p can functionally substitute for each other; the triple mutation *grx3grx4grx5* was also lethal [11].

A new mammalian Grx has been characterized recently that specifically localizes in the mitochondria and the nucleus, and is expressed in two isoforms, one of them with an N-terminal extension that functions as a mitochondrial targeting signal [15,16]. From an analysis of the genomic DNA sequence of

Abbreviations used: Grx, glutaredoxin; HED, 2-hydroxyethyl disulphide; IPTG, isopropyl β-D-thiogalactoside; ORF, open reading frame; protein-SSG, protein–glutathionyl mixed disulphide; YPD, yeast extract/peptone/dextrose medium.

[1] To whom correspondence should be addressed (e-mail bb1barua@uco.es).

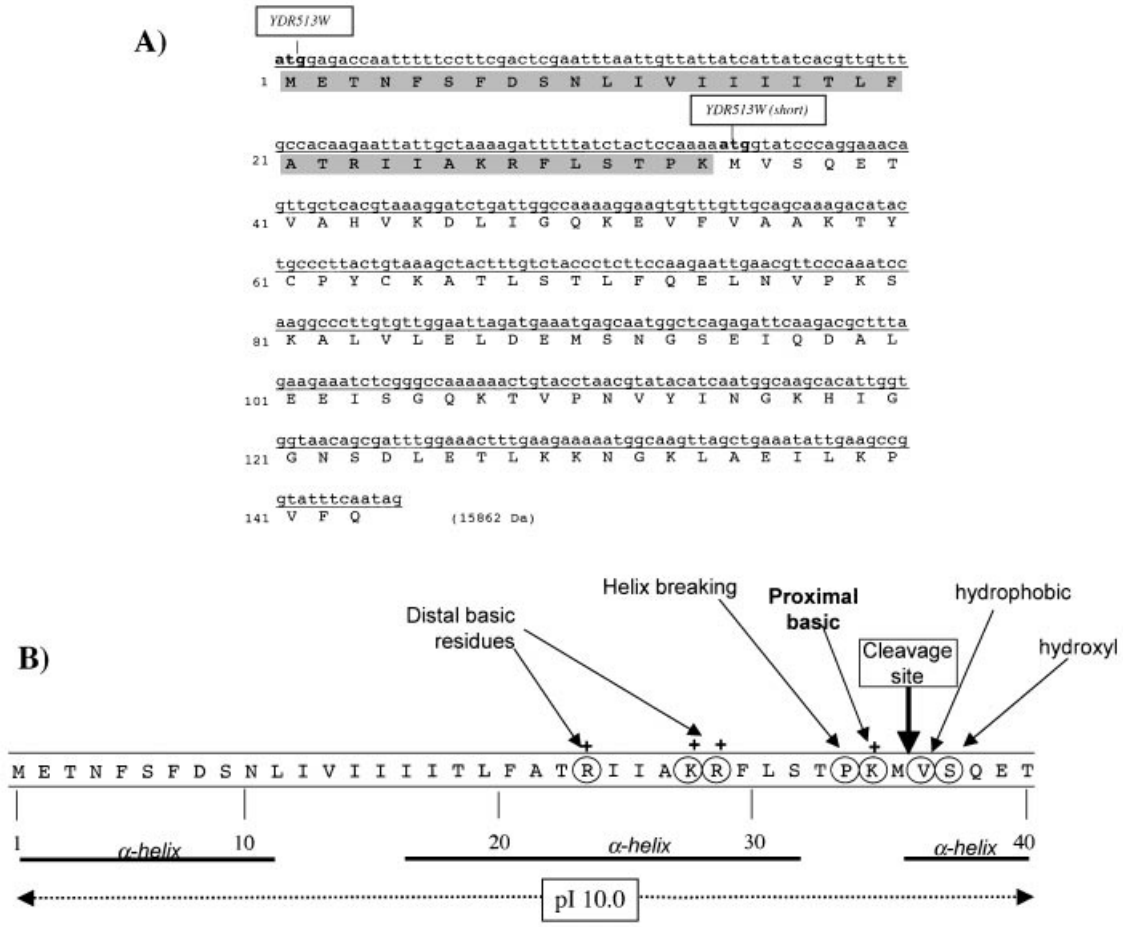

Figure 1 Sequence analysis of Grx2p from *Saccharomyces cerevisiae*

(**A**) The upper line shows a stretch of genomic DNA sequence of *S. cerevisiae* comprising two start codons that define ORFs YDR513W and YDR513W (short), as indicated. The N-terminal putative pre-sequence is shown on a grey background, and the calculated molecular mass of the protein is indicated at the end of the sequence. (**B**) Analysis of the N-terminal extension. Structural requirements that define this sequence as a precursor recognized and cleaved by matrix-processing peptidase are indicated as described by Ito [29]. The number and position of distal basic residues vary among known extension peptides, whereas the most frequent proximal basic residue is arginine. A helix–linker–helix structure is required for effective cleavage of precursors. Predicted secondary structure is also indicated underneath.

S. cerevisiae, we have observed that the *grx2* gene coding for Grx-2 (thioltransferase) [13] has a potential in-frame start codon 99 bp upstream of the initial methionine that has been described to date, which would define a different open reading frame (ORF) (YDR513W; see Figure 1). YDR513W would code for a protein longer than Grx2p, with an N-terminal extension of 34 residues. To check whether this peptide constitutes a mitochondrial signal, we have studied the subcellular localization of Grx2p.

We show here that Grx2p can be targeted to mitochondria or to the cytosol, and a mechanism that accounts for this differential localization is proposed and discussed.

EXPERIMENTAL

Materials

All reagents employed were of analytical grade and were purchased from Sigma, unless otherwise specified.

Strains and growth conditions

The yeast strains FY1679 (*MAT***a** *ura3–52 his3Δ200*) and CML235 (*MAT***a** *ura3–52 leu2Δ1 his3Δ200*) were used as the wild-type. *grx2* (MML44) and *grx5* (MML19) mutants were obtained from the wild-type CML235, as described previously [11], and were kindly provided by Dr E. Herrero (University of Lleida, Lleida, Spain). Yeast cells were grown at 30 °C in yeast extract/peptone/dextrose (YPD) medium. *Escherichia coli* strain BL21(DE3) was employed as a host for DNA cloning.

Cloning and expression of recombinant yeast Grx2p

Two primers were designed (yGrx2-*Nde*I; 5′-CTACTCCA-CAT**ATG**GTATCCCAGGAAACAG-3′, forward, and yGrx2-*Bam*HI; 5′-TTAGCGGGATCCAAA**CTA**TTGAAATACC-3′, reverse) to clone the *GRX2* gene according to the sequence obtained from the database (*Saccharomyces* Genome Data Bank, locus GLRX). The forward primer contained the initiation

codon (shown above in bold letters) and an *Nde*I site (underlined), and the reverse primer contained the stop codon (also shown above in bold letters) and a *Bam*HI site (underlined). The primers were used to amplify *S. cerevisiae* genomic DNA by PCR (35 cycles at 96 °C for 1 min, 50 °C for 1 min and 68 °C for 1.5 min) with Expand Long Template System (Roche Molecular Biochemicals). The PCR product was cloned into the pGEM-Te Vector System (Promega) and sequenced. The amplified fragment was subcloned into the pET-15b expression vector (Novagen) fused at the N-terminus to a 20-amino-acid peptide containing a polyhistidine tag and a thrombin-cleavage site. The construct, named pET-15b-Grx2(s), was verified by PCR using the above primers, and *E. coli* cells were transformed. Single colonies were selected and inoculated in Luria–Bertani medium containing 1 μg/ml ampicillin, and were grown at 37 °C until a D_{600} of 0.5 was attained; the recombinant protein was induced with 0.5 mM isopropyl β-D-thiogalactoside (IPTG), and the cells were allowed to continue growing at 37 °C for 4 h. Collection, lysis and crude extract preparation was performed as described previously [17]. Histidine-tagged (His-)Grx2p was purified from the extract by chromatography on a TALON™ Metal Affinity Resin column (Clontech), and the histidine tag was subsequently removed by controlled incubation with thrombin, as described previously [18].

Assay of enzymic activities

Grx activity was determined spectrophotometrically by measuring the reduction of 0.5 mM 2-hydroxyethyl disulphide (HED) (Aldrich Chemicals) by 0.5 mM GSH in the presence of NADPH and 0.5 unit of yeast glutathione reductase (Sigma G-3664) at 25 °C, monitoring the disappearance of NADPH at 340 nm [19]. One unit is defined as the oxidation of 1 μmol of NADPH/min. Peroxidase activity was determined by substituting HED with peroxide substrates, as indicated and where appropriate.

Grx-dependent reduction of 0.5 mM GSSG by 0.64 mM dihydrolipoamide was measured spectrophotometrically in the presence of 0.36 mM NADH and 1.7 units of lipoamide dehydrogenase (Sigma L-2002), following the disappearance of NADH at 340 nm. One unit of dihydrolipoamide:SSG reductase activity is defined as the oxidation of 1 μmol of NADH/min. Organelle-marker enzymes were assayed according to previously published procedures: cytochrome *c* oxidase [20] was used as a mitochondrial marker, glucose-6-phosphate dehydrogenase [21] was a cytosolic marker, and NADPH:cytochrome *c* reductase was used as a microsomal marker [22].

Preparation and purification of polyclonal antibodies against yeast Grx2p

Recombinant Grx2p was used to immunize New Zealand White rabbits as described by Harboe and Ingild [23]; it was immobilized on to CNBr-activated Sepharose-4B (Amersham Biosciences) and packed in a column. Immune sera were loaded and recycled several times; after washing with 2 M NaCl, elution was performed with 0.5 M formic acid, followed by rapid neutralization with 2 M Trizma.

Subcellular fractionation of yeast cells

Cells of *S. cerevisiae* grown in YPD medium up to the stationary phase were harvested and subjected to subcellular fractionation, as described previously [24]. Spheroplasts were prepared by incubation with lyticase (0.5 mg/g of cells) in 20 mM potassium phosphate, pH 7.4, containing 1.2 M sorbitol. Spheroplasts were harvested by centrifugation, diluted in 20 mM buffer A [Mes/KOH (pH 6.0)/0.6 M sorbitol/0.5 mM PMSF] and disrupted in a Dounce homogenizer. The crude mitochondrial fraction was separated by differential centrifugation with buffer A, and finally collected at 12000 ***g*** and suspended in buffer A. The purity of this mitochondrial preparation was increased further by ultracentrifugation in 14–18 % (w/v) Nycodenz (Sigma). Centrifugation at 100000 ***g*** for 1.5 h generated the cytosolic fraction, which was dialysed against 20 mM Tris/HCl, pH 8.0/0.1 M NaCl, and the microsomal fraction, which was resuspended in 50 mM potassium phosphate buffer, pH 7.4/0.1 M NaCl.

SDS/PAGE and Western blotting

SDS/PAGE was performed with homogeneous 15 % (w/v) acrylamide gels, followed by Coomassie Blue staining or electrophoretic semi-dry transfer to nitrocellulose membranes. The membranes were processed by the method of Towbin et al. [25]. The primary antibodies were used at 1:500 dilution, and developed with the enhanced chemiluminescence (ECL®) system (Amersham Biosciences). The amount of Grx in the blots was calculated densitometrically using a Syngene gel documentation system and pure Grx as a standard.

Protein concentration was determined spectrophotometrically (Bio-Rad) [26] using ovalbumin as a standard.

RESULTS

Sequence analysis of yeast Grx2

Analysis of genomic DNA of *S. cerevisiae* in the vicinity of the *GRX2* gene shows an in-frame start codon 99 bp upstream of the canonical ATG, which defines ORF YDR513W (Figure 1A). Translation of this new ORF would produce a 15.9 kDa protein similar to the yeast Grx2 described to date, bearing a 34-residue N-terminal extension peptide. Prediction of subcellular localization using TragetP [27] identifies residues 1–34 as a signal peptide. This signal peptide bears a highly hydrophobic stretch between residues 9 and 26, and is rich in basic residues, with a theoretical pI of 10.0 (Figure 1), which are usual features of peptides bound for targeting and translocation to mitochondria [28]. Interestingly, this pre-sequence has acidic residues, but despite this unusual property the signal peptide meets most of the requirements to serve as a substrate of mitochondrial-processing peptidase [29] (Figure 1B). These include a set of three basic distal residues that are located on the same side of an α-helix, thus allowing interaction with the α subunit of mitochondrial-processing peptidase; a helix–linker–helix structure encompassing the breaking peptide bond that would be between Met^{35} and Val^{36}; a hydrophobic and a hydroxy-group-containing pair of residues at positions 1 and 2 N-terminal of the cleavage site; and a proximal basic residue at position -2 relative to the cleavage site (arginine in the consensus sequence, but in this case occupied by lysine).

cDNA cloning, purification and characterization of recombinant Grx2p

Grx2 expressed as a His-tagged derivative was purified from bacteria by affinity chromatography on a TALON™ column. It was subsequently subjected to controlled digestion by thrombin to remove the histidine tag, as described in the Experimental section. The purity and size of the protein was verified by SDS/PAGE (Figure 2A). Recombinant Grx2p was tested for its ability to catalyse thiol–disulphide oxidoreduction reactions by means of the usual standard assay of Grx, and was compared

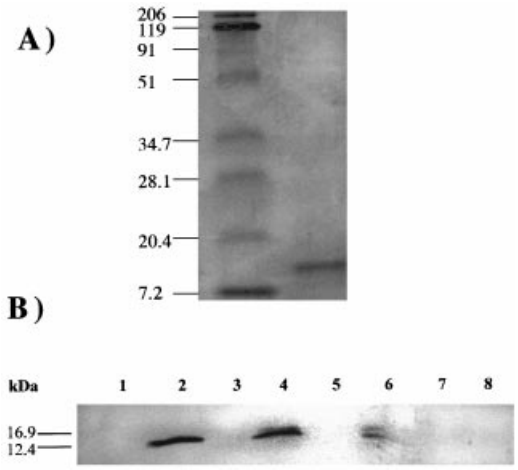

Figure 2 SDS/PAGE of purified recombinant Grx2p and Western blot analysis detection in crude extracts

(**A**) After IPTG induction, the protein was purified as polyHis-Grx2 and digested with thrombin under controlled conditions to eliminate the polyhistidine tag. Lane 1, Bio-Rad pre-stained molecular-mass markers; lane 2, Grx2p. Protein was stained with Coomassie Blue. (**B**) Samples from cells of *S. cerevisiae* grown in YPD medium to the stationary phase were collected by centrifugation, suspended in electrophoretic sample buffer and subjected to Western blot analysis with anti-Grx2 antibodies, as described in the Experimental section. Lanes 1 and 8, unstained molecular-mass markers; lane 2, 0.1 μg of yeast Grx2p; lane 3, 0.15 μg of yeast Grx5p; lanes 4–6, 20 μl of crude extracts from wild-type, *grx2* and *grx5* mutant strains respectively; lane 7, 0.1 μg of human recombinant Grx1.

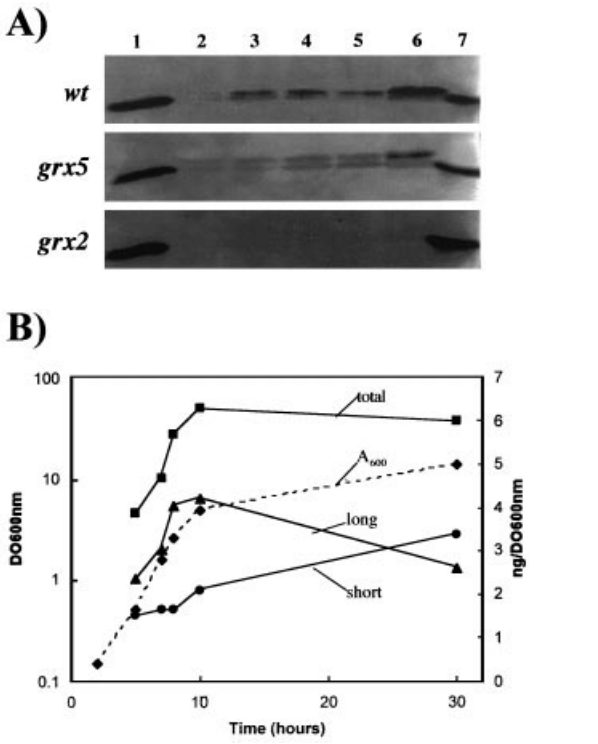

Figure 3 Expression of Grx2p along the growth of *S. cerevisiae* in YPD medium

(**A**) Aliquots (1 ml) were withdrawn from the growth medium at intervals along the growth curve, and were analysed by Western blotting, as indicated in the Experimental section: lanes 1 and 7, 0.1 μg of Grx2p; lanes 2–6, samples of 20 μl taken at equivalent points from early exponential to late stationary growth phases for all three strains. (**B**) A growth curve of wild-type (wt) *S. cerevisiae* and the concentration of long and short isoforms of Grx2p determined by quantitative densitometry from the blots expressed as ng/DO600nm. ◆ - - ◆, absorbance (DO) at 600 nm; ▲–▲, Grx2p long isoform; ●–●, Grx2p short isoform; ■–■, total Grx2p.

with human Grx1. Grx2p efficiently catalysed the reduction of HED by GSH (59.4 ± 6.6 units/mg; $k_{cat} = 713$ min^{-1}), although with a lower efficiency than that of human Grx1 (180 ± 21.8 units/mg) and rat Grx1 (137.8 ± 39.6 units/mg) under equivalent conditions, but higher than that of human Grx2 (37 ± 1 units/mg [16]; 3 units/mg [15]). Reduction of GSSG by dihydrolipoamide was catalysed by yeast Grx2p with high efficiency (71.5 ± 23.9 units/mg; $k_{cat} = 858$ min^{-1}). A recombinant truncated form of Grx5p that lacks 18 residues at the N-terminus was also produced, and proved to be completely incapable of catalysing any of the reactions tested above (results not shown). Since Grx5 and Grx2 have been assigned protective antioxidant roles [11], and it has been shown that Grx acts as an electron donor for human plasma GSH peroxidase [30], we also tested for the reduction of three peroxides: cumene hydroxide, t-butyl hydroxide and hydrogen peroxide. None of the proteins showed any trace of peroxidase activity.

Recombinant Grx2p was used to obtain polyclonal antibodies from rabbits and to purify them by immunoaffinity chromatography. The affinity-purified anti-Grx2p preparation was used to study the expression of the protein in cells of *S. cerevisiae* by Western blotting (Figure 2B). Anti-Grx2 antibodies recognize two bands in Western blots of *S. cerevisiae* cells: one larger (15.9 kDa) than recombinant Grx2p, and one that coincides with the same molecular mass (11.9 kDa). The proteins are expressed in wild-type and *grx5* strains in the stationary phase; as expected, the *grx2* mutant is negative with anti-Grx2, demonstrating that both bands are products of *GRX2*. Therefore yeast Grx1p, which is present in all three strains, does not cross-react with the antibodies. The same lack of cross-reactivity was also true for Grx5p and human Grx1 (Figure 2B).

We have also monitored the levels of both isoforms along the normal growth curve in YPD medium, and have found that both proteins reach a peak during the exponential phase, but their relative proportions vary, the long form being predominant at the exponential phase (Figure 3).

Subcellular localization of Grx2

Cells of *S. cerevisiae* grown to stationary phase were collected and subjected to subcellular fractionation, as described in the Experimental section. Preparations of cytosolic and mitochondrial fractions were obtained by differential centrifugation, and cross-contamination between them was unequivocally discarded, as judged by their levels of marker enzymes (Table 1). As expected, a subfraction of the endoplasmic reticulum, the so-called mitochondria-associated membrane ('MAM') [31], is co-isolated with mitochondria. The microsomal fraction showed 'membrane leakage' from other subcellular fractions.

A Western blot analysis was performed with affinity-purified anti-Grx2 antibodies and, from the results shown in Figure 4, we conclude that both isoforms of Grx2p are localized in mitochondria. The aforementioned two bands observed in crude extracts of *S. cerevisiae* are present in the mitochondrial fraction, whereas only the short form is detected in the cytosolic fraction, and only the long isoform is predominantly detected in microsomes.

Table 1 Enzymic activities of subcellular fractions of *S. cerevisiae*

The activities were measured by the methods described in the Experimental section. The data presented are the means ± S.D. for independent experiments ($n = 3$). NADPH:cyt *c* R'ase, NADPH:cytochrome *c* reductase; G6PDH, glucose-6-phosphate dehydrogenase; Cyt *c* oxidase, cytochrome *c* oxidase; Lip, lipoamide.

	Specific activity (m-units/mg)					
				Grx		
Subcellular fraction	NADPH:cyt *c* R'ase	G6PDH	Cyt *c* oxidase	HED*	Lipoamide†	Lip/HED‡
Cytosol	5 ± 4	68 ± 1	0	98 ± 4	32 ± 4	0.3
Mitochondria	28 ± 14	0	46 ± 13	28 ± 5	28 ± 0.1	1
Microsomes	18 ± 8	10 ± 3	13 ± 4	5 ± 0.2	67 ± 3	13.4

* Standard assay for Grx (i.e. the rate of HED reduction by GSH).
† Rate of GSSG reduction by dihydrolipoamide.
‡ The ratio of both activities.

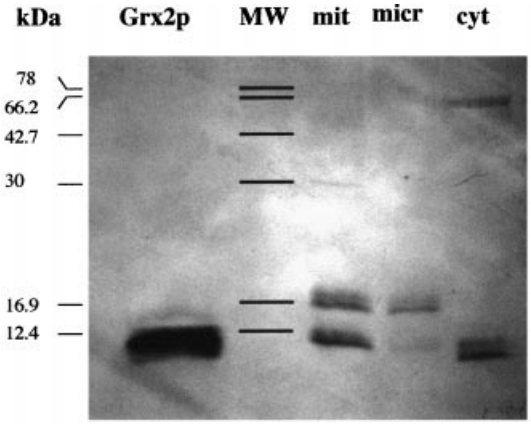

Figure 4 Localization of Grx2p in subcellular fractions of *S. cerevisiae*

Mitochondrial (mit), cytosolic (cyt) and microsomal (micr) fractions were obtained from cells of *S. cerevisiae* grown to the stationary phase, as described in the Experimental section. Protein (25 μg) from each fraction was loaded on to SDS/polyacrylamide gels, and analysed by Western blotting. Grx2p (50 ng) was included as a reference; MW, molecular-mass standards.

DISCUSSION

Grx2p, the product of ORF YDR513W (short) from *S. cerevisiae*, has been cloned, and its subcellular distribution has been studied by Western blotting. In doing so, we have detected two isoforms of different sizes, expressed at the same time in yeast cells.

When compared with other Grx2s from human, rat and mouse species, yeast Grx2p shows a 29–30.5% similarity. The mature protein devoid of pre-sequence has a pI of 5.76, more acidic than the majority of mammalian Grxs, but retains all the structural features that define a two-cysteine (dithiol) subfamily of Grxs [32], including the catalytic site, the glutathione-binding site and structurally important hydrophobic domains (Figure 5). However, different from other Grx2s, yeast Grx2p has a conserved proline residue at the active site instead of a serine [15,16].

Recombinant Grx2p also displays all the catalytic properties of Grxs [33]. Its catalytic efficiency in the standard assay was lower than that of human and rat Grx1, despite its 100% identity for the active-site motif Cys-Pro-Tyr-Cys, but higher than that of human Grx2, in which the active-site proline residue is substituted with a serine. Lack of glutathione peroxidase activity is a striking property of Grx2p in the light of a recent report [34] that Grx1p catalyses peroxide reduction by GSH. These data support further the proposed functional differences between both yeast dithiol Grxs [14].

On the other hand, yeast Grx2 is very efficient as a dihydrolipoamide GSSG oxidoreductase, which might be of interest for a mitochondrial enzyme. In fact, the ratio of this activity to the standard GSH/HED oxidoreductase activity is higher in the mitochondrial than in the cytosolic fraction. Lipoic acid is a redox coenzyme of several enzymes and multienzyme complexes localized in mitochondria. A link between lipoic and glutathione pools in the mitochondria would have interesting consequences in the context of antioxidant defence, and Grx could provide the connecting role.

The 11.9 kDa isoform detected in the cytosolic fraction would represent the shortened product of YDR513W (Figure 1), i.e. the 'classical' yeast Grx (thioltransferase) initially described and studied by Gan [13], with resemblance to Grx1 from other organisms, which lack any organelle-targeting pre-sequence. However, translation from the second AUG has to be confirmed by further experiments.

The hydrophobicity of the pre-sequence might be responsible for membrane leakage and the presence of the long isoform in the microsomal fraction. It would be of interest to ascertain whether the membrane-associated form is actually an immature species trapped in the mitochondrial import machinery, or whether it has specific roles in membranes.

Nuclear-encoded mitochondrial proteins are synthesized as larger precursor molecules that, upon import into mitochondria, are subject to proteolytic cleavage, The two Grx2p isoforms present in mitochondria should correspond to the processed and unprocessed products of a transcript encoding an N-terminal mitochondrial target peptide. ORF YDR513W fulfils this requirement, as described above (see Figure 1). The size of the long isoform detected in crude extracts and in mitochondrial and microsomal subcellular fractions agrees with the size of a putative peptide resulting from translation of YDR513W.

The presence of two forms of Grx2 in the mitochondrial fraction would be due to differences in the rates of membrane translocation and proteolytic processing of one single translation product by matrix peptidases. The rationale for this explanation is on the basis of similarities with yeast fumarase, for which a fraction of the protein folds outside of the mitochondria into an import-incompetent state which, upon cleavage, is released by retrograde movement into the cytosol [28]. The rate of proteolytic cleavage might be intrinsically slow for Grx2p because of the presence of lysine at the −2 position relative to the cleavage site,

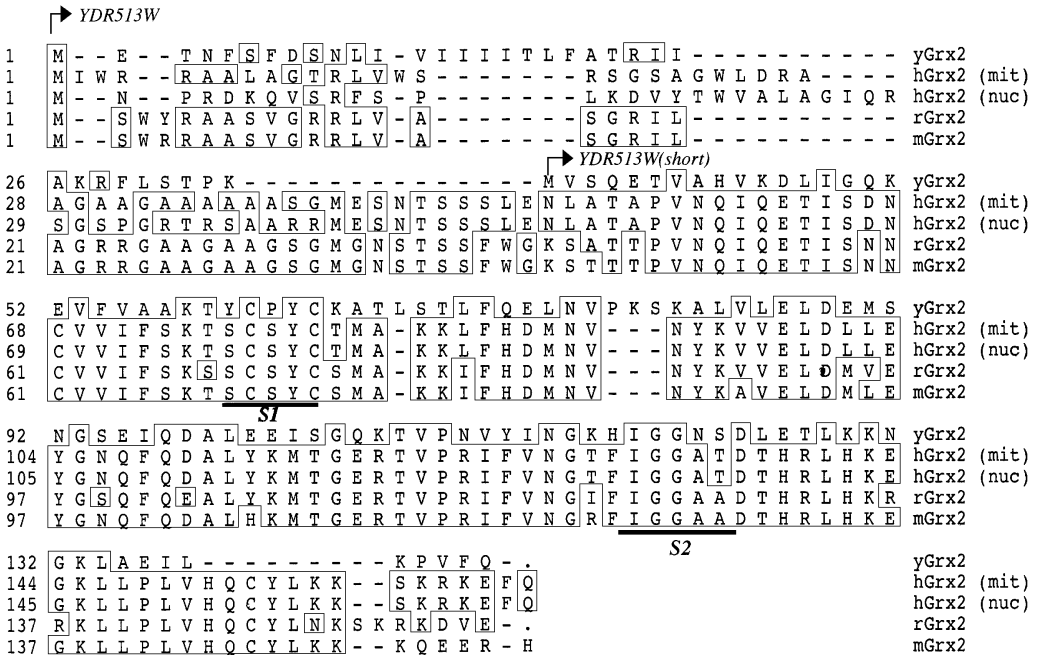

Figure 5 Amino acid sequence alignment of yeast Grx2 with human, rat and mouse Grx2 (hGrx2, rgrx2 and yGrx2 respectively)

Mitochondrial (mit) and nuclear (nuc) hGrx2s are shown [16]. Residues conserved in at least three of the aligned sequences are boxed. S1 indicates the active site and S2 the glutathione-binding site. Translation initiation sites of ORFs YDR513W and YDR513W (short) are indicated by arrows.

instead of arginine, since it is known that substitution of this arginine in model peptides greatly decreases the efficiency of the protease [29]. Slow processing would then allow for Grx2p to fold before import, and would thus return to the cytosol. Only the fraction of Grx2p that is cleaved before folding would be imported into the matrix. This is our working hypothesis for further research.

Alternative splicing is the mechanism that operates in mammals to target Grx2 either to the mitochondria or to the nucleus [15,16]. In *S. cerevisiae*, for which the coding DNA is not interrupted by introns, more rudimentary solutions seem to operate that consist of either overlapping ORFs and alternative AUG selection [35,36] or slow processing and retrograde movement [37,38], as might be the case for Grx2, as discussed above. An intermediate situation might function in the fission yeast *Schizosaccharomyces pombe*, the *Grx* gene of which contains three introns [39], and which also bears an 'additional' in-frame start codon 90 bp upstream of the 'main' start codon (Gene Bank *S. pombe* genomic DNA). Furthermore, the use of alternative translation initiation sites, combined with alternative splicing, might increase the complexity of the population of Grx isoforms, as has been suggested recently [15].

Whether translation occurs from both start codons or there exists one single translation product, and possible regulatory mechanisms that might be involved in controlling the proportion of cytosolic and mitochondrial isoforms, are interesting questions for further investigations that will shed more light on novel ways in which post-genomic events in eukaryotic cells are controlled.

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REFERENCES

1 Holmgren, A. (1989) Thioredoxin and glutaredoxin systems. J. Biol. Chem. **264**, 13963–13966

2 Holmgren, A. (1976) Hydrogen donor system for *Escherichia coli* ribonucleoside-diphosphate reductase dependent upon glutathione. Proc. Natl. Acad. Sci. U.S.A. **73**, 2275–2279

3 Bushweller, J. H., Aslund, F., Wuthrich, K. and Holmgren, A. (1992) Structural and functional characterization of the mutant *Escherichia coli* glutaredoxin (C14- - - -S) and its mixed disulfide with glutathione. Biochemistry **31**, 9288–9293

4 Yang, Y., Jao, S., Nanduri, S., Starke, D. W., Mieyal, J. J. and Qin, J. (1998) Reactivity of the human thioltransferase (glutaredoxin) C7S, C25S, C78S, C82S mutant and NMR solution structure of its glutathionyl mixed disulfide intermediate reflect catalytic specificity. Biochemistry **37**, 17145–17156

5 Jung, C. H. and Thomas, J. A. (1996) S-glutathiolated hepatocyte proteins and insulin disulfides as substrates for reduction by glutaredoxin, thioredoxin, protein disulfide isomerase, and glutathione. Arch. Biochem. Biophys. **335**, 61–72

6 Gravina, S. A. and Mieyal, J. J. (1993) Thioltransferase is a specific glutathionyl mixed disulfide oxidoreductase. Biochemistry **32**, 3368–3376

7 Bellomo, G., Mirabelli, F., DiMonte, D., Richelmi, P., Thor, H., Orrenius, C. and Orrenius, S. (1987) Formation and reduction of glutathione-protein mixed disulfides during oxidative stress. A study with isolated hepatocytes and menadione (2-methyl-1,4-naphthoquinone). Biochem. Pharmacol. **36**, 1313–1320

8 Seres, T., Ravichandran, V., Moriguchi, T., Rokutan, K., Thomas, J. A. and Johnston, Jr, R. B. (1996) Protein S-thiolation and dethiolation during the respiratory burst in human monocytes. A reversible post-translational modification with potential for buffering the effects of oxidant stress. J. Immunol. **156**, 1973–1980

9 Bandyopadhyay, S., Starke, D. W., Mieyal, J. J. and Gronostajski, R. M. (1998) Thioltransferase (glutaredoxin) reactivates the DNA-binding activity of oxidation-inactivated nuclear factor I. J. Biol. Chem. **273**, 392–397

10 Davis, D. A., Newcomb, F. M., Starke, D. W., Ott, D. E., Mieyal, J. J. and Yarchoan, R. (1997) Thioltransferase (glutaredoxin) is detected within HIV-1 and can regulate the activity of glutathionylated HIV-1 protease in vitro. J. Biol. Chem. **272**, 25935–25940

11 Rodríguez-Manzaneque, M. T., Ros, J., Cabiscol, E., Sorribas, A. and Herrero, E. (1999) Grx5 glutaredoxin plays a central role in protection against protein oxidative damage in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **19**, 8180–8190

12 Luikenhuis, S., Perrone, G., Dawes, I. W. and Grant, C. M. (1998) The yeast *Saccharomyces cerevisiae* contains two glutaredoxin genes that are required for protection against reactive oxygen species. Mol. Biol. Cell. **9**, 1081–1091
13 Gan, Z. R. (1992) Cloning and sequencing of a gene encoding yeast thioltransferase. Biochem. Biophys. Res. Commun. **187**, 949–955
14 Grant, C. M., Luikenhuis, S., Beckhouse, A., Soderbergh, M. and Dawes, I. W. (2000) Differential regulation of glutaredoxin gene expression in response to stress conditions in the yeast *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **1490**, 33–42
15 Gladyshev, V. N., Liu, A., Novoselov, S. V., Krysan, K., Sun, Q. A., Kryukov, V. M., Kryukov, G. V. and Lou, M. F. (2001) Identification and characterization of a new mammalian glutaredoxin (thioltransferase) Grx2. J. Biol. Chem. **276**, 30374–30380
16 Lundberg, M., Johansson, C., Chandra, J., Enoksson, M., Jacobsson, G., Ljung, J., Johansson, M. and Holmgren, A. (2001) Cloning and expression of a novel human glutaredoxin (Grx2) with mitochondrial and nuclear isoforms. J. Biol. Chem. **276**, 26269–26275
17 Pedrajas, J. R., Kosmidou, E., Miranda-Vizuete, A., Gustafsson, J. A., Wright, A. P. and Spyrou, G. (1999) Identification and functional characterization of a novel mitochondrial thioredoxin system in *Saccharomyces cerevisiae*. J. Biol. Chem. **274**, 6366–6373
18 Pedrajas, J. R., Miranda-Vizuete, A., Javanmardy, N., Gustafsson, J. A. and Spyrou, G. (2000) Mitochondria of *Saccharomyces cerevisiae* contain one-conserved cysteine type peroxiredoxin with thioredoxin peroxidase activity. J. Biol. Chem. **275**, 16296–16301
19 Luthman, M. and Holmgren, A. (1982) Glutaredoxin from calf thymus. Purification to homogeneity. J. Biol. Chem. **257**, 6686–6690
20 Madden, E. A. and Storrie, B. (1987) The preparative isolation of mitochondria from Chinese hamster ovary cells. Anal. Biochem. **163**, 350–357
21 Deutsch, J. (1983) in Methods of Enzymatic Analysis, vol. 3 (Bergmeyer, H. U., ed.), pp. 190–197, Verlag Chemie, Deerfield Beach, FL
22 Zinser, E., Sperka-Gottlieb, C. D., Fasch, E. V., Kohlwein, S. D., Paltauf, F. and Daum, G. (1991) Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote *Saccharomyces cerevisiae*. J. Bacteriol. **173**, 2026–2034
23 Harboe, H. M. G. and Ingild, A. (1983) Immunization, isolation of immunoglobulins and antibody titer determination. In Handbook of Immunoprecipitation-in-Gel Techniques (Axelsen, N. H., ed.), pp. 345–351, Blackwells Scientific Publications, Oxford
24 Glick, B. S. and Pon, L. A. (1995) Isolation of highly purified mitochondria from *Saccharomyces cerevisiae*. Methods Enzymol. **260**, 213–223
25 Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
26 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254
27 Emanuelsson, O., Nielsen, H., Brunak, S. and von Heijne, G. (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J. Mol. Biol. **300**, 1005–1016
28 Pfanner, N. and Neupert, W. (1990) The mitochondrial protein import apparatus. Annu. Rev. Biochem. **59**, 331–353
29 Ito, A. (1999) Mitochondrial processing peptidase — multiple-site recognition of precursor proteins. Biochem. Biophys. Res. Commun. **265**, 611–616
30 Bjornstedt, M., Xue, J., Huang, W., Akesson, B. and Holmgren, A. (1994) The thioredoxin and glutaredoxin systems are efficient electron donors to human plasma glutathione peroxidase. J. Biol. Chem. **269**, 29382–29384
31 Achleitner, G., Gaigg, B., Krasser, A., Kainersdorfer, E., Kohlwein, S. D., Perktold, A., Zellnig, G. and Daum, G. (1999) Association between the endoplasmic reticulum and mitochondria of yeast facilitates interorganelle transport of phospholipids through membrane contact. Eur. J. Biochem. **264**, 545–553
32 Xia, B., Vlamis-Gardikas, A., Holmgren, A., Wright, P. E. and Dyson, H. J. (2001) Solution structure of *Escherichia coli* glutaredoxin-2 shows similarity to mammalian glutathione-S-transferases. J. Mol. Biol. **310**, 907–918
33 Holmgren, A. and Aslund, F. (1995) Glutaredoxin. Methods Enzymol. **252**, 283–292
34 Collinson, E. J., Wheeler, G. L., Garrido, E., Avery, A. M., Avery, S. V. and Grant, C. M. (2002) The yeast glutaredoxins are active as glutathione peroxidases. J. Biol. Chem., in the press
35 Slusher, L. B., Gillman, E. C., Martin, N. C. and Hopper, A. K. (1991) mRNA leader length and initiation codon context determine alternative AUG selection for the yeast gene MOD5. Proc. Natl. Acad. Sci. U.S.A. **88**, 9789–9793
36 Souciet, G., Menand, B., Ovesna, J., Cosset, A., Dietrich, A. and Wintz, H. (1999) Characterization of two bifunctional *Arabdopsis thaliana* genes coding for mitochondrial and cytosolic forms of valyl-tRNA synthetase and threonyl-tRNA synthetase by alternative use of two in-frame AUGs. Eur. J. Biochem. **266**, 848–854
37 Knox, C., Sass, E., Neupert, W. and Pines, O. (1998) Import into mitochondria, folding and retrograde movement of fumarase in yeast. J. Biol. Chem. **273**, 25587–25593
38 Sass, E., Blachinsky, E., Karniely, S. and Pines, O. (2001) Mitochondrial and cytosolic isoforms of yeast fumarase are derivatives of a single translation product and have identical amino termini. J. Biol. Chem. **276**, 46111–46117
39 Cho, Y. W., Kim, H. G., Park, E. H., Fuchs, J. A. and Lim, C. J. (2000) Cloning, expression and regulation of *Schizosaccharomyces pombe* gene encoding thioltransferase. Biochim. Biophys. Acta **1517**, 171–175

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