One Single In-frame AUG Codon Is Responsible for a Diversity of Subcellular Localizations of Glutaredoxin 2 in Saccharomyces cerevisiae

Glutaredoxins belong to a family of small proteins with glutathione-dependent disulfide oxidoreductase activity involved in cellular defense against oxidative stress. The product of the yeast GRX2 gene is a protein that is localized both in the cytosol and mitochondria. To throw light onto the mechanism responsible for the dual subcellular distribution of Grx2 we analyzed mutant constructs containing different targeting information. By altering amino acid residues around the two in-frame translation initiation start sites of the GRX2 gene, we could demonstrate that the cytosolic isoform of Grx2 was synthesized from the second AUG, lacking an N-terminal extension. Translation from the first AUG resulted in a long isoform carrying a mitochondrial targeting presequence. The mitochondrial targeting properties of the presequence and the influence of the mature part of Grx2 were analyzed by the characterization of the import kinetics of specific fusion proteins. Import of the mitochondrial isoform is relatively inefficient and results in the accumulation of a substantial amount of unprocessed form in the mitochondrial outer membrane. Substitution of Met→Ala, the second translation start site, to Val resulted in an exclusive targeting to the mitochondrial matrix. Our results show that a plethora of Grx2 subcellular localizations could spread its antioxidant functions all over the cell, but one single Ala to Gly mutation converts Grx2 into a typical protein of the mitochondrial matrix.

Reversible oxidation of disulfide bonds is catalyzed by thiol-disulfide oxidoreductases namely thioredoxin (Trx) and glutaredoxin (Grx). These are small proteins belonging to the “thioredoxin fold” structural family, with an active site containing the dithiol motif CXXC, although some members are monothiolic (6, 8–12). The functions of both systems overlap to a certain extent, although the major difference is that Grxs have a glutathione binding site and are capable of reducing GSH protein-mixed disulfides (6, 13, 14). Mitochondrial isoforms of each member of both systems have been characterized in yeast (15–17) and mammals (18–20). Three Grx subfamilies have been distinguished (21). In the yeast Saccharomyces cerevisiae, the proteins Grx1 and Grx2 belong to subfamily 1 (22). Grx3, Grx4, and Grx5 are monothiolic members of the second subgroup. Grx3 and Grx4 possess an additional Trx domain and are constituents of subfamily 3 (12). These five Grxs also differ in regard to their subcellular localization. Grx1 is cytosolic, Grx3 and Grx4 are nuclear (17, 23), Grx5 is mitochondrial (24), and Grx2 has a dual localization in the cytosol and mitochondria (16). Grx2 stands out among other Grxs for its efficiency in transferring reducing equivalents from reduced lipoamide to oxidized glutathione (25). Grx5 is essential for the functional assembly of iron-sulfur centers (24) and Grx3 and Grx4 need their Trx domain for nuclear targeting and for Grx-like activity (17).

A major aspect of the understanding of protein functions in eukaryotic cells is represented by the analysis of the mechanisms by which proteins are targeted and distributed to their subcellular localizations (26). Most mitochondrial proteins are encoded by nuclear DNA and are processed by a specific machinery (“translocases”) consisting of several sets of proteins, whose functions involve specific binding, translocation, maturation, and membrane insertion (27). Proteins targeted to the mitochondrial matrix bear a presequence, which is recognized by Tom20 and Tom22 at the outer membrane. Proteins cross this membrane through a general insertion pore that is formed by the TOM (translocase of the outer membrane) complex. Transfer through the outer membrane is followed by the insertion into the inner membrane via the TIM23 (translocase of the inner membrane) complex, in a membrane potential-dependent manner. Subsequently, most of the polypeptide chains of mitochondrial matrix proteins are transported into the matrix compartment by the action of the import motor complex (28). Once in the matrix, the presequence is eventually cleaved by matrix processing peptidase (MPP).

The abbreviations used are: Trx, thioredoxin; Grx, glutaredoxin; DHFR, dihydrofolate reduce- tase; MPP, mitochondrial processing peptidase; PGK, phosphoglycerate kinase; PK, proteinase K; TOM, translocase of the outer membrane; TSS, translation start site; UTR, untranslated region; MES, 4-morpholinethanesulfonic acid; WT, wild type; Bis-Tris, 2-[bi(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; MOPS, 4-morpholinepropanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; ER, endoplasmic reticulum.
An increasing number of proteins have been identified that reside and function in more than one cellular compartment. They employ a variety of strategies to reach such dual destinations (29). Grx2 represents an example for the localization in multiple subcellular compartments, in this case the cytosol and mitochondria. Localization of human Grx2 in mitochondria has been shown to be due to alternative splicing of one single gene (20). This variant Grx2 has de-glutathionylase and glutathione S-transferase activity and can be reduced by TrxR, a member of the Trx system what constitutes an interesting link between both "redoxin" systems (30). It also attenuates apoptosis by preventing cytochrome c release in HeLa cells (31). In contrast to the mammalian protein, Grx2 from the yeast S. cerevisiae has a dual subcellular localization but originates from one single transcript (16). Only a very few other proteins with a similar behavior have been identified to date (29). The dual localization of yeast Grx2 is related to the presence of an in-frame translation start site (TSS) upstream of the canonical one, which produces a potential mitochondrial N-terminal targeting sequence (16). Spreading of the protein over subcellular compartments is apparently the consequence of post-transcriptional phenomena taking place during and/or after translation.

With this in mind we have performed experiments to establish the molecular mechanisms that contribute to the multiple subcellular locations of yeast Grx2. We have introduced specific mutations into the coding sequence of GRX2 to ascertain whether the cytosolic isofrom arises from translation initiation at the second AUG (32), by retrograde movement from the mitochondria (33, 34), or by other unknown mechanisms. To characterize the biogenesis of the mitochondrial isofroms, we have determined the submitochondrial localization of Grx2. The targeting properties of the N-terminal extension, generated by translation initiation from the first AUG, was determined by the analysis of the in vitro import behavior of specifically designed fusion proteins containing different segments of Grx2. The analysis of subcellular distribution and import kinetics of the Grx2 mutants showed that specific properties of the N-terminal extension were responsible for the specific distribution of Grx2 to multiple cellular compartments. The characterization of its subcellular localization will provide the basis for the definition of the cellular functions of the multiple Grx2 isoforms.

### MATERIALS AND METHODS

**Strains—**S. cerevisiae strains used are listed in Table 1. Cells were grown on YPG (1% (w/v) yeast extract, 2% (w/v) bactopeptone, 3% (w/v) glycerol), YPD (1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (w/v) dextrose), or SD-Leu-His (0.7% (w/v) yeast nitrogen base without amino acids, 2% (w/v) dextrose), or SD-Gal-His (0.7% (w/v) yeast nitrogen base without amino acids, 2% (w/v) dextrose), 50 mM MES, pH 6.0, 0.03% (w/v) histidine, and 0.03% (w/v) leucine). The Escherichia coli strain used for general transformation procedures was HB101, except for the Su9-Grx2 and PreGrx2-DHFR constructs, where One-Shot cells (Invitrogen) were used.

**Molecular Biology—**The overlapping fragments method (35) was used to perform site-directed mutagenesis of GRX2, cloned into the pGEM-Te Vector System (Promega) as indicated in a previous study (16). The following primers were used (mutated sequences are underlined): F5, 5’-TCTACTCCAAAATGTTGATCCCGAG-3’; M1V, 5’-CTGGTATATATTGGTTCGGAGACC-3’; M35V, 5’-CTACTCCAAAATGTTGATCCCGAGAG-3’. The mutated fragments were cloned into the yEP-352 vector and transformed into the MML-44 yeast strain, in which the GRX2 gene was deleted (Δgrx2), using the lithium acetate method (36). The Su9-Grx2, Su9-STPK-Grx2, and PreGrx2-DHFR chimeras were produced using the following primers: Su9-Start-f, 5’-CACTCTGAGGATCCCCCTAGTATCTCC-3’; Su9-End-NdeI-f, 5’-ACCAATATGGCCTCCGGAAG-3’; grx2-Start-NdeI-f, 5’-GCTTCTCTCACTATGGTATCC-3’; grx2-End-r, 5’-TGGGATATATATTGGTTATCACG-3’. PreGrx2-Start-f, 5’-GGGAATTTCCTAATTGTTTATAC-3’; PreGrx2-End-r, 5’-CTTGTTCGGATATTAG-3’. DHFR-NdeI-f, 5’-TCTTCCGACGCTCATATGGTATCC-3’; DHFR-NdeI-r, 5’-TGAGACTCCAATCTTTGTCCGAC-3’. The primary anti-Grx2 antibodies were used at a dilution of 1:1000 and developed with the enhanced chemiluminescence (ECL2) system (Amersham Biosciences). Antibodies were obtained as described previously (16). The membranes were visualized with a chemiluminescence reader (Syngene).

For fluorescent analysis by confocal microscopy, parental and mutant strains of S. cerevisiae were cultured in YPG medium to 1.5–2.5 × 10⁶ cells/ml.

### TABLE 1

| Strain    | Relevant genotype                | Reference | Comments                          |
|-----------|----------------------------------|-----------|-----------------------------------|
| YPH499    | MATa ade2-101 his3-D200 leu2-Δ1 try1-Δ63 lys2-801 | (57)      | Wild strain                       |
| CML235    | MATa ura3-52 leu2-Δ1 his3-Δ200 | (12)      | Wild strain                       |
| MML44     | MATa grx2::LEU21                | (12)      | Obtained by deletion from CML235  |

**Source**

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In Vitro Imports—Yeast mitochondria were isolated from YPH499 cells grown on YPG medium (final A660 nm: 1.5–2.5), according to published methods (39, 40), resuspended in SE buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH, pH 7.2) to a final concentration of 10 mg of protein/ml and stored at −80 °C. Radiolabeled Grx2 was synthesized by in vitro transcription in rabbit reticulocyte lysate (Amersham Biosciences) in the presence of [35S]methionine after in vitro transcription using SP6 polymerase (Promega) (40, 41). Specific primers for each mutant form of Grx2 were used to produce the respective radiolabeled precursor proteins. Import into isolated mitochondria was performed in import buffer (3% (w/v) fatty-acid free bovine serum albumin, 80 mM KCl, 5 mM MgCl2, 10 mM MOPS-KOH, pH 7.2, 10 mM KP) containing 2 mM NADH and 2 mM ATP. Mitochondria (25 μg of protein/100-μl reaction) were added, and the import reaction was started by adding 10 μl of reticulocyte lysate. After incubation for 2–30 min at 25 °C, the import reaction was stopped by addition of 50 μM valinomycin. Samples were then divided into two sets of 45-μl volume, and one of them was incubated with 100 μg/ml proteinase K (PK) for 15 min at 0 °C. Mitochondria were subsequently re-isolated and washed once with SE buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) to deactivate the PK. Mitochondrial proteins were separated by Tricine-SDS-PAGE (16.5% acrylamide) and analyzed by autoradiography. The imported proteins were quantified using the program ImageQuaNT (General Electric).

Submitochondrial Fractionation—Mitochondria for subcellular fractionation were obtained as indicated in the import experiments section above. Samples were prepared by resuspending 25 μg of mitochondria in 100 μl of SE buffer and subjected to the following treatments. PK treatment over whole mitochondria was performed by adding 5 μg of PK and incubating for 15 min at 4 °C, and the reaction was stopped by addition of 2 μl of 100 mM PMSF. Mitochondria were then pelleted by 12,000 × g centrifugation for 15 min, resuspended in 100 μl of SE buffer and trichloroacetic acid precipitated. Disruption of the membranes was performed by adding 5 μl of 10% (v/v) Triton X-100 and incubating for 10 min at 4 °C with vigorous shaking. Elimination of the outer membrane (swelling) was performed by applying a mild osmotic shock to mitochondrial samples. To remove lightly associated proteins from the inner mitochondrial membrane, mitoplasts obtained by mild osmotic shock could only originate by translation initiation from the internal AUG. This demonstrated that the second AUG was a functional TSS at least when it was at the 5′-end of mRNA in the absence of native 5′-UTR. To approximate the in vivo genetic environment, we generated mutant M1V that possessed the presequence, but its first start codon was rendered non-functional by mutation of AUG to GUG. In this case, the second AUG functioned as an internal TSS in the absence of an upstream AUG, producing a short isoform of Grx2 lacking the mitochondrial targeting peptide. Again, this short form predominantly localized to the cytosol. It is worth mentioning that in this mutant a trace of Grx2 was consistently found in the mitochondria, likely as a consequence of non-canonical residual translation initiation at GUG as has been shown to occur occasionally in yeast (44). Mutant FS carried an additional nucleotide (A) just before the second AUG to change the reading frame so that the context around 5′-UTR AUG remained unmodified, but its function as TSS would produce an aberrant undetectable mitochondrial protein. In this mutant, a cytosolic isoform was markedly present that could only originate by translation initiation from the internal AUG. Because the translation from the second TSS (Met18) was very important for the expression of the cytosolic form of Grx2, we introduced a point mutation to generate mutant M35V, destroying the TSS and essentially abolishing expression of the short cytosolic form. Interestingly, when the internal AUG was suppressed, we were not able to detect Grx2 in the cytosol but observed an exclusive mitochondrial localization. Moreover, we did not observe a trace of the long isoform, thus indicating complete processing of the immature Grx2, a situation that was clearly different to wild-type cells. To directly visualize the subcellular distribution of Grx2 and to corroborate the conclusions obtained by the subcellular fractionation analysis of the mutant Grx2 constructs, we performed immunofluorescence double labeling experiments. Preparations of selected mutants were analyzed by confocal microscopy using antibodies directed against Grx2 and the control proteins porin (mitochondria) and PGK (cytosol) (Fig. 1B). Co-localization of Grx2 with a cytosolic marker was very promi-
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TABLE 2

Description of DNA nucleotide and protein amino acid sequences of GRX2 mutant constructs

Most of the sequence between both ATG sites was not modified and is omitted to highlight modified sites near the ATG codons. Translation start sites are underlined; altered codons and the corresponding amino acid are in bold type; mutated nucleotides and amino acids are typed in italics. fs stands for frame-shift in mutant FS. Light grey colored letters indicate either mature DHFR or Su9 target peptide.

| Constructs/strains | DNA and predicted protein sequences |
|--------------------|-------------------------------------|
| WT                 | ... M E ... P K M V S Q ...         |
|                    | ..TTC ATG GAG... CCA AAA ATG GTA TCC CAG... |
| Δ1-34              | ... M V S Q ...                      |
|                    | ..- - - - - - ATG GTA TCC CAG...     |
| M1V                | ... P K M V S Q ...                  |
|                    | ..TTC GTG GAG... CCA AAA ATG GTA TCC CAG... |
| FS                 | ... P K / fs M V S Q ...            |
|                    | ..TTC ATG GAG... CCA AAA / A ATG GTA TCC CAG... |
| M35V               | ... P K V S Q ...                    |
|                    | ..TTC ATG GAG... CCA AAA ATG GTA TCC CAG... |
| Su9-Grx2           | ... D G H M V S Q ...                |
|                    | ... Y S S T P K M V S Q ...          |
|                    | ... Y S S T P K M V S Q ...          |
| Su9-STPK-Grx2      | ... Y S S T P K M V S Q ...          |
|                    | ... TAC TCT TCC ACC CCC AAA ATG GTA TCC CAG... |
| PreGrx2-DHFR       | ... P K M V R P ...                  |
|                    | ... TTP ATG GAG... CCA AAA ATG GTT CGA CCA... |

In cells expressing the full precursor proteins (WT and yEP-GRX2) or in cells expressing the protein devoid of presequence (Δ1–34). However, in the M35V mutant, no cytosolic staining was observed, whereas full co-localization with a mitochondrial marker was detected. The results obtained by immunofluorescence were in full agreement with those derived from the subcellular fractionation experiments described above. We conclude that the concurrent expression from two in-frame AUG start codons in the GRX2 gene resulted in the biosynthesis of a cytosolic short form and a long form containing a mitochondrial presequence.

Immature Grx2 Is Associated to the Outer Membrane, whereas the Mature Form Is in the Matrix—Surprisingly, the large immature mitochondrial form of Grx2 that was observed during expression of the full-length GRX2 gene in vivo was absent in the mutant M35V. This indicated that Met35 may be responsible for an incomplete processing of Grx2 by the import machinery. To assess a possible physiological role of the large immature form associated with mitochondria we analyzed the submitochondrial localization of Grx2 (Fig. 2A). We isolated mitochondria from wild-type yeast cells and subjected them to a subfractionation protocol. As control proteins we used the soluble matrix protein mitochondrial Hsp70 (mtHsp70) and the integral inner membrane protein Tim23. The long, immature Grx2 was found to be associated with the outer membrane, exposing most of its polypeptide chain to the cytosol as deduced from its sensitivity to PK in intact mitochondria. In contrast, the fully processed form of Grx2 was resistant to external proteases even after opening of the mitochondrial outer membrane by osmotic swelling, a behavior identical to the matrix protein mtHsp70. This indicated that the processed Grx2 most likely is a soluble matrix protein. Interestingly, immature Grx2 was bound tightly enough to the outer membrane to resist high salt washing and to be retained in the membrane phase after carbonate extraction. Similar to the membrane protein Tim23, the immature form of Grx2 behaved like an integral membrane protein, in this case of the outer mitochondrial membrane. These results showed that, in addition to the cytosolic isoform generated by alternative translation initiation, Grx2 seemed to be located in two separate submitochondrial compartments, matrix and outer membrane.

What might be the physiological significance of the presence of the immature Grx2 as an integral membrane protein? We asked if the membrane association of Grx2 was a specific mitochondrial phenomenon or related to a general ability to interact with cellular membranes. To answer this question we further refined our subcellular fractionation protocol to distinguish other membrane cellular compartments (Fig. 2B). The subcellular distribution pattern was surprisingly different between WT and M35V Grx2. We found the immature Grx2 also associated to the membrane of the rough endoplasmic reticulum. In contrast, the mutant M35V had a unique localization to the mitochondrial matrix. Taken together, Grx2 distributed via three isoforms of different sizes to widespread locations from mitochondria to cytosol and endoplasmic reticulum, the short form in the cytosol, the long, immature form associated with the ER, and the outer mitochondrial membrane, whereas the fully imported form was processed and localized in the mitochondrial matrix.

In Vitro Import Experiments—Because the high degree of variability in the subcellular targeting of wild-type Grx2 was not anticipated, we set
out to analyze the mitochondrial import properties in more detail to gain some insight into the mechanisms involved. The efficiency of the targeting by the presequence and the influence of the mature part on import were analyzed by

in vitro
import experiments using isolated mitochondria from S. cerevisiae. Wild-type Grx2 or the respective mutant forms (Table 2) were synthesized as radiolabeled precursor proteins by in vitro transcription/translation using rabbit reticulocyte lysate. In addition to the modifications of Grx2 described above we

FIGURE 1. Subcellular localization of mutant Grx2 isoforms. A, mitochondrial (m) and cytosolic (c) fractions were prepared from extracts of YPG-grown cells transformed with the indicated plasmids and then analyzed by Western blotting with antibodies anti-Grx2 (upper panel) and anti-porin, a mitochondrial marker (lower panel). Glucose-6-phosphate dehydrogenase activity, a cytosolic marker, was measured in all fractions (lower histogram). Protein content was normalized in all lanes at 25 μg of total protein. B, visualization of WT and mutant Grx2 isoforms in S. cerevisiae cells by double labeling fluorescence microscopy. Parental and mutant strains of S. cerevisiae were cultured in YPG medium, collected, and fixed in glass slides for double immunostaining with specific antibodies to Grx2 and to either porin or PGK, as mitochondrial and cytosolic markers, respectively. Anti-Grx2 was revealed with fluorescein (green fluorescence) and organelar markers with rhodamine (red fluorescence). yEP-GRX2 is the plasmid carrying the whole intact sequence to detect any possible differential behavior between genomic and vectorial expression of GRX2. Strain Δgrx2 was transformed with the empty vector to test any possible influence of the vector itself. Other names and abbreviations are explained in Table 2. In every panel, individual staining is shown together with the merged images of both stainings. Orange to yellow hue indicates colocalization.
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A

![Diagram A](image1)

B

![Diagram B](image2)

**FIGURE 2.** Submitochondrial localization of native and mutant isoforms of Grx2. A. *S. cerevisiae* cells grown on YPG medium were processed for isolation of the mitochondrial fraction (no treat.). The obtained preparation was then subjected to different treatments to analyze subfractions as indicated under "Materials and Methods." Triton X-100 to disrupt the membranes (Triton X-100), swelling by osmotic shock to eliminate the outer membrane (swelling), salt washed to remove lightly associated membrane proteins (salt wash), and carbonate treatment to extract and pellet the membranes (carb. extr.). As indicated, samples were digested with PK (PK+) to eliminate all exposed proteins. P, pellet; S, supernatant. Subfractions were analyzed by Western blot and immunodecoration with anti-Grx2 antibodies (upper panel) or with anti-mtHsp70 (middle panel) and anti-Tim23 (lower panel) as matrix and inner membrane markers, respectively. For more details see text. B, further subcellular fractionation was achieved to distinguish several membrane compartments as described under "Materials and Methods." Each subcellular fraction was analyzed by Western blot and immunodecoration with anti-Grx2 antibodies and organellar markers. mito, mitochondria; cyto, cytosol; rER, rough endoplasmic reticulum; sER, smooth endoplasmic reticulum. PGK, phosphoglycerate kinase; Sec61, rough endoplasmic reticulum membrane marker; Tim23, inner mitochondrial membrane marker. p, precursor form; m, mature form. A double band in the cytosolic fraction of WT (c1 and c2) might be due to some sort of modification of the short form whose nature is out of the scope of this study. Multiple distribution of wild-type Grx2 was observed versus one single localization of M35V.

We constructed fusion proteins to separately analyze the effects of presequence and the mature part of Grx2 on the import reaction (Fig. 3A).

When we translated the wild-type mRNA of GRX2, we observed the generation of two translation products (p and star symbol, Fig. 3B, lane 7), similar to the *in vivo* situation. The long form (p), corresponding to the first TSS, carried mitochondrial targeting information as was demonstrated by the specific association of the precursor form with the mitochondrial surface *in vitro*. This precursor protein also entered the mitochondria in a membrane potential-dependent manner and was processed by the matrix-processing peptidase to a shorter mature form (m) (Fig. 3B, lanes 1–6). Based on these criteria, the long form of Grx2 behaved like a typical protein of the mitochondrial matrix. The overall import process, however, was rather inefficient with just a small fraction of the precursor fully imported into the matrix compartment. A relatively large amount of precursor protein was found attached to the mitochondrial surface but not inserted into the inner membrane. We conclude that a part of the Grx2 precursor deviated of the import pathway and gave rise to the species associated with the mitochondrial outer membrane.

Similar to the experiments after expression *in vivo*, the mutant protein devoid of the second TSS, M35V, showed an efficient import and processing reaction in the mitochondrial matrix (Fig. 3B, lanes 8–13). A smaller polypeptide (star symbol), corresponding to the cytosolic form of Grx2, was not observed in the translation reactions of M35V due to the removal of the second TSS (Fig. 3B, lane 14). In contrast to the behavior of the wild-type Grx2, the precursor form of M35V showed a reduced accumulation at the outer membrane but was imported more efficiently into the matrix compartment, indicated by a relatively higher amount of the mature form after protease treatment. These results indicate that the presence of Met35 alone is not only responsible for the generation of the cytosolic form of Grx2 but also influences the targeting properties of the Grx2 precursor during the mitochondrial import process. The long form of Grx2, produced from the first TSS, therefore, contains sufficient targeting information to ensure an efficient transport to the mitochondrial matrix under *in vivo* conditions, but the presence of a Met residue at position 35 somehow markedly diminishes the efficiency of the process.

The question then was to assess how the specific properties of the presequence and the mature part would affect the import process, resulting in multiple mitochondrial localizations. The fusion protein PreGrx2-DHFR, containing the entire Grx2 N-terminal extension, was imported into isolated mitochondria in a Δψ-dependent manner and was processed to the appropriate mature form by MPP in the matrix compartment (Fig. 4A). As was observed for the authentic Grx2 precursor, the translation reaction of PreGrx2-DHFR resulted in additional smaller products due to the weak first TSS. However, these fragments were not imported into mitochondria, as indicated by their sensitivity to the protease treatment. Hence, the Grx2 presequence was completely sufficient to target a heterologous passenger protein to the mitochondrial matrix. However, consistent with the import experiments using the wild-type Grx2, import efficiencies were relatively low compared with the standard preprotein construct Su9-DHFR, containing a strong targeting signal derived from the ATPase subunit 9 (Su9) from *Neurospora crassa*. In addition, similar to the full-length protein, we found a significant amount of the Grx2-DHFR precursor associated with mitochondria, independent of the membrane potential. This indicated that the association of the Grx2 precursor with the outer mitochondrial membrane relied most likely on the properties of the presequence. To exclude an influence of the folding state of the mature part on the import process we generated a second set of constructs that represented chimeric fusion proteins of wild-type and mutant Grx2 with the Su9 (amino acids 1–72) targeting sequence (Fig. 3A). One construct, Su9-Grx2, contained the efficient Su9 presequence preceding a polypeptide that essentially represented the short cytosolic form of Grx2. This preprotein construct showed full translocation to the matrix (Fig. 4A) and a highly efficient import kinetics (Fig. 4B). Su9-Grx2 imported ~10-fold faster than the construct PreGrx2-DHFR and over 100-fold faster than Grx2 WT (Fig. 4B). Based on this high import efficiency, we conclude that the folding state of Grx2 did not interfere with the mitochondrial import reaction.

The mature part in the construct Su9-Grx2 was identical to the short cytosolic form of Grx2. However, the processing site of MPP is located 4 amino acids upstream of the second TSS between Leu10 and Ser31 (Fig. 3A). This difference in size resulted in a small difference in the running behavior during SDS-PAGE between the smaller cytosolic and the mature mitochondrial form. Interestingly, when we included the tetrapeptide -STPK-, matching the N terminus of the mature Grx2 that was generated after processing by MPP, in the Su9-Grx2 fusion construct, the import rate was even higher. This holds true for both Su9 (1–68)-STPK-Grx2 and PreGrx2-DHFR chimeras (Fig. 4B). Finally, we analyzed the *in vivo* subcellular distribution of these chimeras (Fig. 4C).

3 P. Porras, C. A. Padilla, S. Ogueta, and J. A. Bárceña, unpublished data.
Irrespective of the presence of the STPK tetrapeptide, Grx2 fused with the Su9 presequence localized exclusively to the mitochondrial matrix in agreement with the in vitro import experiments. This behavior clearly demonstrated that the mature part is not responsible for the membrane association of the long Grx2 isoform. It should be noted that the Met residue, which occupies position 35 in WT but is located at position 73 in these chimeras, no longer perturbs the translocation process. It should also be noted that, in contrast to WT, the second TSS is not operative in Su9-Grx2, whereas the fusion of the Grx2 presequence with DHFR provokes translation from the second TSS thus resulting in a “pseudo-mature” polypeptide containing the DHFR moiety as observed in the lysate lanes (Fig. 4A).

In summary, our data demonstrate that the diversity of Grx2 subcellular localizations is largely determined by the presence of the amino acid Met₃⁵, providing both an alternative translation start site for the cytosolic form and influencing mitochondrial targeting and import efficiencies. A single point mutation in the codon encoding Met₃⁵ drastically alters Grx2 distribution, resulting in an exclusive targeting to the mitochondrial matrix. To a lesser extent, a specific basic tetrapeptide immediately after the MPP cleavage site had a slight enhancing effect on the import rate. Although the mitochondrial targeting sequence of Grx2 was principally able to target the precursor to the mitochondrial matrix, the translocation efficiency is very low compared with canonical mitochondrial targeting sequences. The slow import kinetics most likely contributed to the association of the long Grx2 isoform with both the mitochondrial and ER membranes.

**DISCUSSION**

Recent results indicated that Grx2 exhibits a dual cellular localization in the cytosol and the mitochondria of *S. cerevisiae*. An analysis of the *GRX2* gene sequence revealed the presence of two in-frame AUG codons as potential translation start sites (16). By the introduction of specific point mutations into the open reading frame of *GRX2* we analyzed the effects of the presence of putative alternative TSSs on the subcellular distribution of Grx2 isoforms and the molecular mechanisms resulting in its unusual localization pattern.

Translation of *GRX2* mRNA in vivo and *in vitro* resulted in the generation of two polypeptide products. Our results indicate that this phenomenon is a direct consequence of the translation process.
FIGURE 4. In vitro import kinetics and in vivo localization of chimeric fusion proteins. A, radioactive patterns in gels from the import kinetics experiments of three representative chimeras are shown. Legends are similar to those of previous figures: time of import and functioning of membrane potential are indicated below the gels; + PK, samples digested with PK; lanes 5 and 11, whole lysates before import; p, precursor form; m, mature processed form. B, quantitative graphical representation of the import kinetics of all the chimeras tested. Import is quantified as percentage of processed protein relative to total precursor available for import. Chimeras of Su9 presequence are more efficient than chimeras with PreGrx2; for clarity, they have been separated into two graphs with different scales. All measurements have been performed three times and the data plotted are the average ± S.D. (n = 3). C, in vivo subcellular localization of chimerical preproteins. Chimeric proteins were expressed in S. cerevisiae cells cultivated in YPG medium, collected, and processed to isolate subcellular fractions. Each fraction was then subjected to Western blot analysis with anti-Grx2 antibodies and antibodies to standard mitochondrial and cytosolic markers, porin and PGK, respectively. mito, mitochondria; cyto, cytosol; rER, rough endoplasmic reticulum; sER, smooth endoplasmic reticulum.
According to the linear scanning model (32), large ribosomal subunits initiate translation at the first AUG they encounter. However, if the AUG is located in a suboptimal nucleotide sequence context, it may be missed and the ribosome would slide downstream to initiate translation at the next AUG. Another model for initiation of translation postulates direct entry of the ribosome at internal AUG triplets (internal ribosomal entry site) (45). In GRX2, the second TSS is present at an in-frame AUG, representing the amino acid residue Met35. Hence, expression of the wild-type GRX2 gene in vivo results in two polypeptides of 15.9 and 12.0 kDa. Translation from the “internal” AUG codon occurred either in the presence (FS) or in the absence (M1V) of AUG triplets within the 5'-UTR of its mRNA. When this internal TSS is rendered “terminal” by elimination of the sequence context around each AUG of GRX2 mRNA mutants and internal AUG usage agree well with the linear scanning model (Table 3). The context is rather unfavorable for AUG1 but optimal for AUG2 in WT and in most mutants. The chimeric mutant Su9-Grx2 represented an exception, because the opposite situation takes place. Interestingly, when expressed in vivo, this mutant lacks a cytosolic form of Grx2. This indicates that translation form internal AUG starts only when the ribosome finds suboptimal conditions for initiation from the 5'-UTR AUG triplet. On the other hand, in PreGrx2-DHFR a usually inactive downstream TSS in DHRF becomes fully operative when the first TSS is changed to a suboptimal context. These findings demonstrate that the main reason for the usage of AUG2 is the unfavorable context for translation from AUG1. From this data we conclude that the second AUG in GRX2 mRNA is fully functional for the ribosomal translation initiation, giving rise to the cytosolic form of Grx2.

Translation from the first AUG of GRX2 mRNA yields a precursor protein that contains mitochondrial targeting information in the form of an N-terminal extension. The putative Grx2 sequence has all the elements for recognition by the mitochondrial import machinery, that is, a high degree of hydrophobicity and a set of basic residues that determine a pI of 10.0 (46). Three pentapeptides between Ile13 and Ile28 of the precursor would configure an appropriate α-helical motif to specifically fit the hydrophobic groove of Tom20 (47), the outer membrane receptor of the import machinery. The stretch Ala21-Thr-Arg-Ile24 represents the best candidate for a receptor interaction (see Fig. 5A). It also exhibits structural components that fit quite well in proposed consensus sequence motifs models for the action of MPP (48, 49). Using in vitro import experiments we could directly show that this N-terminal extension fulfills the criteria for a mitochondrial precursor. We fused the Grx2 precursor (amino acids 1–34) to the N terminus of the cytosolic protein DHFR as a reporter. The resulting precursor protein (PreGrx2-DHFR) was specifically transported into the mitochondrial matrix in a membrane potential-dependent manner. These experiments showed that Grx2 belongs to the small group of proteins with a dual cellular localization, in this case the cytosol and mitochondria, whose targeting mechanism is based on the synthesis of two polypeptides from one transcript (50).

However, the situation concerning the subcellular localization of Grx2 might be even more complex. When compared with preproteins containing the efficient mitochondrial targeting signal derived from subunit 9 (Su9) of the F0-ATPase of N. crassa, the import kinetics was relatively slow. The relative inefficiency of the Grx2 precursor is likely due to structural features of the Grx2 signal peptide, i.e. a lower number of positively charged amino acids and the localization of the potential Tom20 recognition signal at the end of the precursor, very close to the MPP processing site. Remarkably, the precursor of Grx2 contains a long stretch of hydrophobic amino acids (Fig. 5A). This hydrophobic segment did not interfere with a mitochondrial targeting process per se, as the exclusive mitochondrial localization of the mutant M35V demonstrates. However, it is conceivable that this segment mediates the membrane association observed with the long isoform of Grx2. In this case, the precursor protein seemed to be integrated with its N-terminal end into the outer mitochondrial membrane, exposing the bulk of the protein to the cytosolic side. Generally, the exact sequence of events leading to the insertion of proteins containing a N-terminal membrane anchor sequence into the outer membrane is discussed controversially (51). At least, it seems to be clear the Tom40 insertion pore plays a major role also in this process. The import properties of Grx2 are compatible with the discussed models. However, future experiments need to address the mechanism responsible for the membrane association of Grx2.

Our results show that, apart from being distributed to cytosol and mitochondria, Grx2 is located in two different mitochondrial subcompartments. Again, there are only a few examples of a multiple localization of a single protein inside mitochondria themselves, most prominently the yeast protein Mct1 (NADH-cytochrome b5 reductase) (52), for which an incomplete import reaction is the main cause for the differential distribution inside mitochondria. In addition, we found a minor part of the immature form of Grx2 associated with membranes of the ER. Interestingly, a connection between mitochondrial and ER membrane systems has been postulated with the existence of the so-called “mitochondrial associated membrane fraction” (53). It remains to be clarified if the localization to ER membranes is a genuine property of Grx2 or due to an association with the mitochondrial associated membrane fraction.

However, if the endogenous precursor is replaced by an efficient mitochondrial targeting sequence, only full transport to the
mitochondrial matrix is observed, indicating that the C-terminal segments, representing the full cytosolic Grx2 on their own, do not interfere with the import reaction. Apart from inducing the synthesis of an additional cytosolic isoform of Grx2 as discussed above, the presence of the internal AUG has a dramatic role in Grx2 translocation and subcellular distribution. A simple Ala to Gly mutation in this codon, changing Met\(^{35}\) to Val, was sufficient both to prevent the membrane association of the immature long Grx2 isoform and to improve processing by the import machinery. Considering the cleavage site by MPP (see Fig. 5A), residue Met\(^{35}\) belongs to the mature part of mitochondrial Grx2. Hence, we could conclude that it is not the presequence alone but also additional elements near the N-terminal end of the mature protein that determine the way the precursor interacts with the import apparatus.

In summary, the following factors are responsible for the subcellular diversity of Grx2 in yeast: (i) one suboptimal first translation start site
None of these factors alone could be responsible for the subcellular pattern, but rather the conjunction of all of them was necessary for the observed complex phenotype of GRX2. The whole process, giving rise to the pleiotropic subcellular localization of Grx2 in yeast, is shown schematically in Fig. 5B. Multiple strategies for the localization of proteins in more than one subcellular compartment in eukaryotic cells have been established (26, 29). Among the different possible mechanisms, S. cerevisiae Grx2 constitutes a unique case in that it cannot be included into one single group of proteins. Its targeting process fits well into two types of mechanisms: (a) “single transcript, multiple proteins, multiple translation initiation sites” and (b) “single transcript, single protein-thwarted translation” (29).

Physiological Significance—Analysis of the isoforms in different compartments may contribute to understand the physiology of the eukaryotic ot (26). The conjunction of factors here described resulted in a balanced distribution of Grx2 between the cytosol and the mitochondrion. This complex situation would be justified by the acquisition of some physiological advantages. Like Grx2, yeast glutathione reductase (Glr1p) has also been shown to distribute between cytosol and mitochondrion (54), thus confirming the functioning of the so-called glutaredoxin system (9) in both compartments. A similar mechanism of internal AUG usage is responsible for the subcellular distribution of both components of the system. However, Grx2 exhibits a more complex cellular distribution pattern, which includes membrane compartments. It remains to be known whether membrane Grx2 function is independent of glutathione reductase.

Mammalian forms of Grx2 have been shown to play a role in mitochondrial response to oxidative stress and redox signals (31, 55, 56). We have shown that Grx2 is particularly well suited for the catalysis of electron transfer from lipoamide to oxidized glutathione as compared to other glutaredoxins (25) in agreement with a role in the maintenance of mitochondrial thiol-disulfide equilibrium. The presence of membrane-anchored Grx2 in WT cells raises the possibility of an additional membrane-specific functional role as has been recently reported for mammalian Grx2 acting on mitochondrial membrane protein thiols (56). We are currently investigating whether Grx2 has other specific roles in the context of defense against oxidative stress in intact mitochondria from WT and mutant cells.

Altogether, the results reported here add up to the multifunctionality of yeast Grx2 derived both from its four subcellular localizations, namely the cytosol, mitochondrial matrix, and the mitochondrial and ER membranes, and from its plural catalytic capacities, that is, thiol-disulfide oxidoreductase, peroxidase, and glutathione transferase. All these characteristics speak of a particular case of genetic pleiotropy: one single gene, S. cerevisiae GRX2, gives rise to a plethora of diverse phenotypes.
Subcellular Localizations of Glutaredoxin 2

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