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Erv1p from *Saccharomyces cerevisiae* is a FAD-linked sulfhydryl oxidase

Jeung-Eun Lee[a], Götz Hofhaus[a], Thomas Lisowsky[b,*]

[a] *Institut für Biochemie und Biologisch-Medizinisches Forschungszentrum, Heinrich-Heine-Universität Düsseldorf, Universitätsstraße 1, 40225 Düsseldorf, Germany*
[b] *Botanisches Institut, Heinrich-Heine-Universität Düsseldorf, Universitätsstraße 1, 40225 Düsseldorf, Germany*

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Abstract The yeast *ERV1* gene encodes a small polypeptide of 189 amino acids that is essential for mitochondrial function and for the viability of the cell. In this study we report the enzymatic activity of this protein as a flavin-linked sulfhydryl oxidase catalyzing the formation of disulfide bridges. Deletion of the amino-terminal part of Erv1p shows that the enzyme activity is located in the 15 kDa carboxy-terminal domain of the protein. This fragment of Erv1p still binds FAD and catalyzes the formation of disulfide bonds but is no longer able to form dimers like the complete protein. The carboxy-terminal fragment contains a conserved CXXC motif that is present in all homologous proteins from yeast to human. Thus Erv1p represents the first FAD-linked sulfhydryl oxidase from yeast and the first of these enzymes that is involved in mitochondrial biogenesis.

Key words: Yeast *ERV1*; Sulfhydryl oxidase; FAD; Disulfide bond; Dimer

1. Introduction

The yeast *ERV1* gene is the best characterized representative of a gene family found in a large number of lower and higher eukaryotes [1–3] and in the genomes of some double-stranded DNA viruses [4–6]. The gene encodes a small protein of 22 kDa that has a complex influence on different aspects of mitochondrial biogenesis [2,3,7] and is essential for the survival of the yeast cell [1]. Recently, it was shown that the protein is essential for a normal mitochondrial morphology and for stable maintenance of these organelles in the cell [7,8]. Mammalian homologues of Erv1p exist in man, mouse and rat. The homologous mammalian *ALR* (augmenter of liver regeneration) proteins of about 25 kDa have been characterized as important hepatic growth factors [9–11]. One characteristic feature of these proteins is the highly divergent amino-termini that are responsible for different subcellular distributions [5–8]. In contrast, the carboxy-terminal domains are highly conserved and can be functionally interchanged between yeast and human cells [8,12]. In accordance with its essential function in yeast the *ERV1* protein is found in the cytosol and inside mitochondria [7,8]. No enzymatic function has been assigned to this class of proteins yet.

One indication for a possible enzymatic function is the finding that a growth factor of human fibroblast cells, the Q6 protein, contains a domain with faint but significant homologies to the conserved domain of Erv1p and Alrp [6]. With 582 amino acids the Q6 protein is much larger than Erv1p and recently it was shown that during metazoan evolution Q6 was created by the fusion of different protein domains [6]. One of those domains of Q6 contains a CXXC motif that is the redox-active site of a flavin-linked sulfhydryl oxidase [13]. A similar motif is found in the conserved domains of yeast Erv1p and human Alrp [6,8,11].

In this communication we report that purified yeast Erv1p has the enzymatic activity of a flavin-linked sulfhydryl oxidase [13–15]. The carboxy-terminal domain with the conserved CXXC motif binds FAD and contains the enzymatic activity. Erv1p is only distantly related to human Q6 as the two proteins have adapted to different functions during evolution. The most important differences between Erv1p and Q6 concern the rate of enzyme activity, subcellular localization and the molecular weight. The assignment of a sulfhydryl oxidase activity to Erv1p is important to define its molecular targets inside yeast cells and to elucidate the role of the homologous mammalian Alrp in hepatic cell growth.

2. Materials and methods

2.1. Polymerase chain reaction (PCR experiments)

The complete yeast *ERV1* gene and a shorter fragment encoding the carboxy-terminus were amplified by standard protocols [16] using the *Taq* polymerase kit (Roche Mannheim), the yeast cDNA clone for *ERV1* [3] and the following primers: primer 1: 5′-AATACAAAGCCATATGAAAGCAATAGATAAAATG-3′ (first ATG of *ERV1*/*Nde*I); primer 2: 5′-CTTCAGAGCATATGCCTGGCTCAAGAACATAC-3′ (second ATG of *ERV1*/*Nde*I); primer 3: 5′-GATTGCTCGAGTTCGTCCCAGCCGTCCTTCCA-3′ (stop codon of *ERV1*/*Xho*I). The restriction sites *Nde*I (primers 1 and 2) and *Xho*I (primer 3) are underlined. These restriction sites have been introduced into the primers for cloning the PCR fragments into the hexahistidyl tag vector pET-24a(+) (Novagen).

2.2. Purification of the yeast Erv1p-6His proteins from Escherichia coli

The two PCR constructs encoding the complete protein and a carboxy-terminal fragment of Erv1p were expressed in *E. coli* strain BL21 (Clontech). The presence of the hexahistidyl tag at the carboxy-terminus of the proteins allowed rapid purification with nickel agarose according to the standard protocols for the isolation under native conditions in NaCl–phosphate buffer (50 mM NaCl, 50 mM KH_2PO_4, 10 mM imidazole, pH 7.5) (Qiagen). Proteins were bound to nickel agarose and eluted with 50 mM NaCl, 50 mM KH_2PO_4, 200 mM imidazole, pH 7.5. Purification to homogeneity was verified by SDS–polyacrylamide gels and by antibody tests. In a final step the proteins were dialyzed against 50 mM Tris–HCl pH 8.0.

2.3. Spectroscopy of yeast Erv1p-6His

The dialyzed proteins were directly used for spectroscopy. The visible spectra of these protein solutions were recorded with an S-10 diode array photometer (Zeiss). Under identical conditions a reference of 15 μM purified FAD (Sigma) was measured.

To test the reversible reduction of protein-bound FAD a 600 μl

*Corresponding author. Fax: (49)-211-81 12881.
E-mail: lisowsky@uni-duesseldorf.de

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sample of 25 μM Erv1p in elution buffer (200 mM imidazole, 50 mM phosphate pH 7.5, 200 mM NaCl) was completely reduced with a few grains of Na-dithionite. The visible spectra were recorded in a J&M diode array photometer for 30 min. The oxidation of dithionite was followed at 315 nm and FAD was recorded at 460 nm.

2.4. Preparation of reduced lysozyme as a substrate for Erv1p

The reduction of lysozyme was done as described elsewhere [15] by dissolving 20 mg lysozyme (Serva) in 1 ml of degassed 100 mM Tris buffer containing 6 M guanidine hydrochloride and 0.3 mM EDTA, followed by the addition of dithiothreitol (DTT) to a final concentration of 6 mM. The solution was incubated at pH 8.0 overnight at 37°C and then adjusted to pH 3.5 with glacial acetic acid. DTT was removed by a Sephadex G25 column equilibrated with deoxygenated 8 M urea containing 0.1% acetic acid (v/v) and 0.3 mM EDTA.

2.5. Enzyme assay for sulfhydryl oxidase

Yeast *ERV1* proteins of the long and short form corresponding to 600 pmol bound FAD were diluted in 1.2 ml measurement buffer (2 M urea in 100 mM potassium phosphate buffer pH 7.5 containing 1 mM EDTA) together with reduced lysozyme that corresponds to 55 nmol reduced thiol groups. The initial content of thiol groups was determined from a sample withdrawn before the addition of the protein. Aliquots of 200 μl were withdrawn at different time intervals and determined for their thiol content. For this purpose samples were diluted with 790 μl of measurement buffer and then 10 μl DTNB (Ellman's reagent [17]) was added to a final concentration of 10 μM. After 2 min the extinction at 412 nm was measured and the thiol content was calculated using an extinction coefficient of 13.6 mM^{-1} cm^{-1}.

2.6. Isolation of native Erv1p-GFP from yeast cells

In previous experiments [7,8] Erv1p was expressed as a fusion protein with GFP from an episomal plasmid transformed into yeast. For the preparation of native protein this yeast strain was grown to early logarithmic phase in glucose minimal medium. From 5 ml culture medium the cells were collected in an Eppendorf tube and disrupted with glass beads in NaCl–phosphate buffer (200 mM NaCl, 50 mM KH_2PO_4, pH 7.5) using a Brown homogenizer. After high speed centrifugation the clear supernatant was used for the experiments. Aliquots with 50 μg of total protein were applied to SDS–polyacrylamide gels.

2.7. Standard techniques

E. coli strains DH5-α [18] and BL21 (Clontech) were used for cloning experiments and amplification of plasmid DNA. Plasmid DNA was isolated from *E. coli* by alkaline lysis [19]. Purification, restriction enzyme digestion, ligation and analysis of yeast DNA or PCR products on agarose gels were performed as described [20]. Nucleotide sequences of the DNA constructs were controlled by the biochemical method that uses T7 polymerase, [α-^{35}S]dATP and the appropriate primers [21].

3. Results

3.1. Yeast Erv1p contains a FAD moiety

Sulfhydryl oxidases from chicken egg white and the human Q6 growth regulator [13] depend on a FAD molecule for their enzyme activity [14,15]. Therefore it was tested whether purified Erv1p contains FAD. For this purpose full-length Erv1p and a shorter fragment (Fig. 1) were expressed in *E. coli* with a hexahistidyl tag attached to the carboxy-terminus and purified to homogeneity. Fig. 2 shows the nearly identical visible spectra of the short and the long form of Erv1p. Those spectra are typical for an FAD moiety and exhibit the distinct differences from free FAD [15]. The absorbance maximum of free FAD at 450 nm is shifted by about 10 nm in the protein. The shoulder at 480 is also shifted and appears more pronounced for protein-bound FAD. The FAD content of the proteins was calculated from the OD_{460} values using an extinction coefficient of 10 mM^{-1} cm^{-1}. After determination

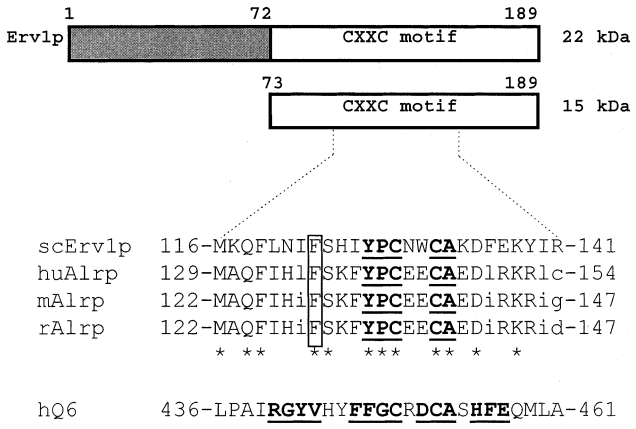

Fig. 1. Domain structure of yeast Erv1p. The protein contains 189 amino acids. The amino-terminal part with the first 72 amino acids (gray bar) has no sequence homologies with any other protein. The carboxy-terminal domain (white bar) from amino acid 73 to 189 has about 40% amino acid homology with mammalian Alrp [12] and contains a conserved CXXC motif. Sequence alignments of this region are shown for yeast (sc) Erv1p, human (h), mouse (m) and rat (r) Alrp. Identical amino acids found at the same position in all four proteins are marked by asterisks. Amino acids that are different between the human, mouse and rat protein are written in small letters. Numbers at the end of the sequences give the position of the amino acids in the proteins. The conserved amino acids associated with the CXXC motif are underlined and written in bold letters. The boxed phenylalanine residue is present in all proteins and is essential for a functional yeast Erv1p [2,3]. The domain of the human Q6 protein (hQ6) that contains the cysteine pair of the redox-active site is included in this figure. Important amino acids that define the consensus motif for Q6 proteins are written in bold letters and underlined [13].

of the protein concentration it was calculated that about 0.8 FAD molecules per monomer unit of protein are found. This value suggests the presence of one FAD molecule per monomer unit. Treatment of the protein with 6 M urea completely removed the FAD molecule, demonstrating that it is firmly attached but not covalently linked to the protein. Finally, it was tested whether the bound FAD of Erv1p can be reversibly reduced with dithionite and oxygen. Addition of dithionite to a sample of Erv1p completely reduced the FAD component (for details see Section 2). After excess of dithionite was oxidized by air, the FAD component was completely reoxidized in less than 1 min. Compared to free FAD the redox reactions of Erv1p with dithionite and oxygen were about 10 times slower and not linear. Both the alterations of the spectra and the slower kinetics of the redox reactions are clear indications that the FAD is bound to the protein [14,15].

3.2. The yeast Erv1p protein has a sulfhydryl oxidase activity

The identification of bound FAD and the presence of a CXXC motif (see Fig. 1) in yeast Erv1p with some similarities to the redox-active site of sulfhydryl oxidases [13] were further indications for a comparable enzyme activity of the yeast protein. To verify this hypothesis full-length Erv1p and the shorter fragment (Fig. 1) were investigated for the ability to introduce disulfide bonds into protein. Completely reduced lysozyme was used as a substrate after dilution in mildly denaturing buffer to make all thiol groups accessible for the enzyme. The reactions were started by adding the purified yeast Erv1p proteins to the substrate mixture. At different time points aliquots were withdrawn and assayed for their

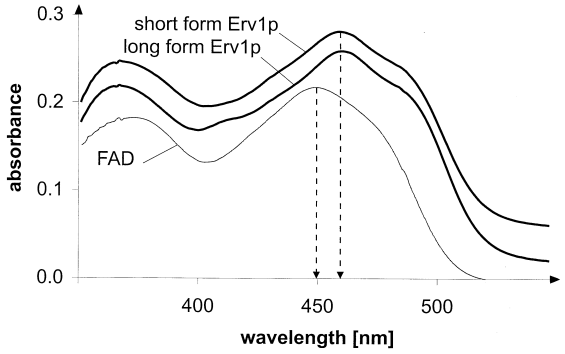

Fig. 2. Spectroscopy of the purified 22 kDa and 15 kDa fragments of yeast Erv1p. The absorbance in the range from 350 nm to 550 nm was measured. Solid lines represent the spectra for the long (22 kDa) and short (15 kDa) forms of yeast Erv1p and the thin line a reference sample with 15 μM pure FAD. The Erv1p spectra are characteristic for an FAD moiety, but show distinct differences from free FAD. The absorbance maximum in the range of 450 nm is shifted about 10 nm in the protein sample. Dashed lines with arrows mark the position of the maximum for protein-bound and free FAD. The shoulder in the spectra at 480 nm is also shifted and appears more pronounced in protein-bound FAD [14,15] (for details see Section 2).

thiol content [17]. For the full-length yeast Erv1p of 22 kDa and the carboxy-terminal fragment of 15 kDa a linear oxidation of the substrate was observed (see Fig. 3). The turnover numbers for the enzymes were 8.6 min^{-1} for the full-length protein and 7.8 min^{-1} for the carboxy-terminal fragment.

3.3. *Erv1p isolated from yeast forms a dimer under non-reducing conditions*

In previous experiments a Erv1p-GFP fusion protein was used to investigate the subcellular localization of the protein

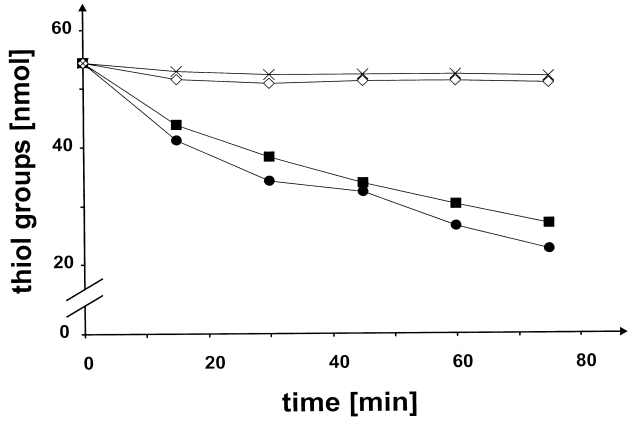

Fig. 3. Sulfhydryl oxidase assay with full-length Erv1p and a 15 kDa carboxy-terminal fragment. Hexahistidyl-tagged proteins were expressed in *E. coli* and purified to homogeneity by nickel agarose. Reduced lysozyme corresponding to 55 nm thiol groups was incubated with Erv1p. The concentration of the short and long forms of Erv1p was adjusted to 50 pmol bound FAD for each measured sample. Oxidation of thiol groups was measured spectroscopically at 412 nm after addition of 10 μM DTNB by determining the decrease of extinction at the listed time points. The values for the 22 kDa protein are marked by ■ and for the 15 kDa fragment by ●. Identical samples without protein (×) or with equal amounts of pure FAD alone (◇) were incubated under the same conditions (for details see Section 2).

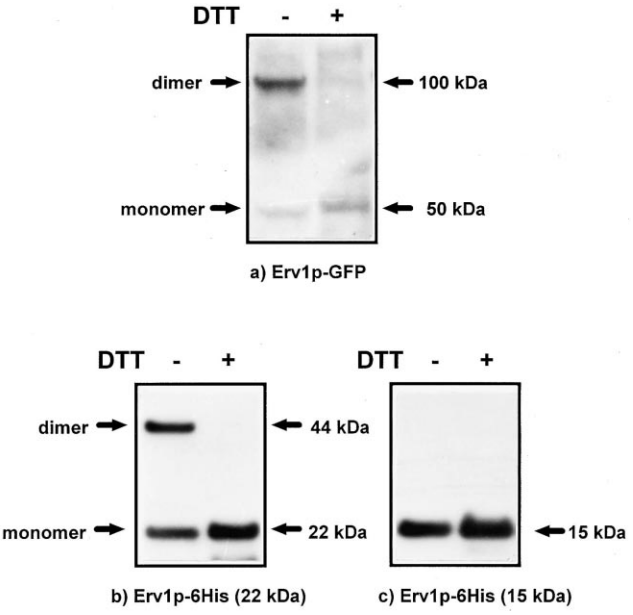

Fig. 4. Investigation of dimer formation for Erv1p. a: Erv1p is isolated as a dimer from yeast cells. Aliquots of 50 μg total protein extracts from yeast cells expressing the fusion protein Erv1p-GFP were separated in a 4–12% SDS–polyacrylamide gel with (+) or without (−) 10 mM DTT in the sample buffer. Arrows indicate the position of bands with a molecular weight of about 100 kDa (dimer) or 50 kDa (monomer). Protein bands were visualized by a monoclonal antibody against GFP and chemiluminescence. The same analysis was done with the long form (b) and the short form of Erv1p-6His (c) isolated from *E. coli*. Aliquots of 50 ng highly purified Erv1p-6His (22 kDa or 15 kDa) were used for this purpose. Arrows point to the position of bands with a molecular weight of about 44 kDa (dimer) or 22 kDa (monomer) and 15 kDa (monomer). Only the long form of Erv1p can form dimers under these conditions. Protein bands were visualized by antibodies against the hexahistidyl tag using chemiluminescence.

[7,8]. Total protein extracts from the yeast cells expressing this fusion protein were prepared under native conditions without reducing agents in the buffer. Aliquots of these total protein extracts containing Erv1p-GFP were separated in SDS–polyacrylamide gels with or without reducing agent in the loading buffer of the sample. Fig. 4a shows the protein pattern after Western blot and immunodetection with an antibody specific for GFP. For the sample without any reducing agent a major protein band of 100 kDa is detected that corresponds to a homodimer and in addition a minor band of 50 kDa that represents the monomer. Addition of reducing agent to the sample results in the detection of a single band of 50 kDa for the monomer.

3.4. *The amino-terminus of Erv1p is essential for dimer formation*

Full-length Erv1p and the carboxy-terminal fragment were purified from *E. coli* under native extraction conditions and both proteins were tested for the ability to form dimers. The results are included in Fig. 4b,c. The long form of Erv1p-6His isolated from *E. coli* gave a comparable pattern of protein bands to Erv1p-GFP from yeast cells. Under non-reducing conditions the dimer of 44 kDa and the monomer of 22 kDa are both present. In contrast to this finding the 15 kDa carboxy-terminal fragment of Erv1p-6His shows no detectable formation of any dimer complex in the sample without reduc-

ing agent, indicating that the intact amino-terminus is essential for the formation of dimers.

4. Discussion

In this communication Erv1p is identified as a FAD-linked sulfhydryl oxidase by its ability to oxidize thiol groups in a protein substrate, the presence of a FAD moiety in the carboxy-terminal domain and the formation of dimers in vivo and in vitro. The bound FAD is reversibly reducible with dithionite and oxygen indicating a functional involvement in the enzymatic activity [15]. The full-length *ERV1* protein and the truncated 15 kDa fragment both contain FAD and exhibit significant sulfhydryl oxidase activities. Comparable enzyme activities of both Erv1p forms indicate that the amino-terminus is not required for the catalytic reaction. The finding that the 15 kDa fragment of Erv1p exists as a monomer demonstrates that the amino-terminus is essential for dimer formation but that dimerization is not essential for the function in disulfide formation. Dimers of Erv1p may be essential for the function in yeast as previous experiments demonstrated that the amino-terminus is indispensable for an active Erv1p in vivo [3,8].

The location of the sulfhydryl oxidase activity in the 15 kDa carboxy-terminal fragment of the protein is in accordance with the finding that this domain contains a conserved CXXC motif (Fig. 1). Alignment of the consensus sequences for Erv1p/Alrp, human Q6 and the homologous chicken egg white sulfhydryl oxidase [13] shows the similarities of these CXXC motifs, but also exhibits specific differences that identify Erv1p/Alrp as a sub-class of their own. The functional importance of this domain is further stressed by a mutation in *ERV1* that changes a conserved phenylalanine residue next to this CXXC motif (see Fig. 1) thereby causing a severe defect in yeast [1,2]. Amino acids of functional importance like aromatic and charged amino acids are conserved in the neighborhood of the two vicinal cysteine residues in all proteins, but the yeast Erv1p and the mammalian Alrp CXXC motifs are still more closely related to each other than human Alrp and the human Q6 protein domain.

In spite of similar enzyme activities Erv1p and human Q6 are only distantly related indicating adaptations to different cellular functions during evolution. The most significant differences concern the subcellular localization, enzyme activity and molecular weight. The chicken enzyme and the human Q6 protein of about 600 amino acids are both secreted from cells and are discussed to have a function in the formation of the extracellular matrix whereas the 22 kDa yeast Erv1p is found in the cytosol and inside mitochondria [7,8]. The enzyme turnover number of Erv1p is about 10% of that of the native chicken enzyme [14,15] when one uses lysozyme as an in vitro substrate. The slower enzyme turnover number of yeast Erv1p might in part be explained by the heterologous expression in *E. coli* and incomplete natural folding of the protein. However, the most likely explanation is a different substrate specificity for yeast and human enzymes as lysozyme cannot be the preferred natural substrate for Erv1p. The specific function of Erv1p in mitochondrial biogenesis indicates that this enzyme has been adapted to specific targets in yeast and does not represent a general sulfhydryl oxidase for many different cellular proteins. In accordance with this hypothesis our latest experiments show that glutathione is not used as a substrate by Erv1p (unpublished results). In that respect it is important to notice that yeast Erv1p is the first sequenced sulfhydryl oxidase with a documented essential role in the biogenesis of mitochondria [1–3], especially in the maintenance of intact mitochondrial membranes and a normal mitochondrial morphology [7]. In the literature one finds some evidence for a connection between mitochondrial morphology and sulfhydryl oxidase activity [22,23], but so far no other protein or gene sequences have been determined. Most of these data are related to the investigation of sperm development in higher eukaryotes and the identification of proteins of 50–60 kDa with sulfhydryl oxidase activities [23]. Whether those proteins have any relation to Erv1p/Alrp or Q6 will remain unclear until isolated proteins and their sequences are available.

An interesting new aspect for a mitochondrial sulfhydryl oxidase activity and the formation of disulfide bonds is the recent finding of a direct correlation between the electron transport chain and the formation of disulfide bonds [24,25] in *E. coli*. But whether there also exists a functional correlation between a mitochondrial sulfhydryl oxidase activity, the mitochondrial electron transport chain and the formation of disulfide bonds in yeast remains highly speculative at the moment.

For higher eukaryotes it is important to notice that the human Alrp domain with the CXXC motif can functionally substitute the yeast domain in vivo [8,12] indicating that this domain also has the enzymatic activity of a sulfhydryl oxidase. Human Q6 protein and Alrp are both characterized as growth factors and this is in agreement with the finding that redox proteins are important means to regulate and modulate cell growth and the activity of enzymes or hormones [26,27]. For example many mammalian cytokines and growth factors have recently been identified as thioredoxin proteins [26–28].

To understand the functional similarities and differences of Erv1p/Alrp and the Q6 proteins it will be important to identify their respective natural target proteins or molecules in the living cell. Neither for Q6 nor for Erv1p are the in vivo targets known yet. We assume that in accordance with the specialized function of Erv1p and Alrp in mitochondrial biogenesis and hepatic cell growth one will also find selective in vivo target proteins or molecules like for many different proteins with thioredoxin motifs [26,27].

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