Yeast Erv2p Is the First Microsomal FAD-linked Sulfhydryl Oxidase of the Erv1p/Alrp Protein Family*

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Saccharomyces cerevisiae Erv2p was identified previously as a distant homologue of Erv1p, an essential mitochondrial protein exhibiting sulfhydryl oxidase activity. Expression of the ERV2 (essential for respiration and vegetative growth 2) gene from a high-copy plasmid cannot substitute for the lack of ERV1, suggesting that the two proteins perform nonredundant functions. Here, we show that the deletion of the ERV2 gene or the depletion of Erv2p by regulated gene expression is not associated with any detectable growth defects. Erv2p is located in the microsomal fraction, distinguishing it from the mitochondrial Erv1p. Despite their distinct subcellular localization, the two proteins exhibit functional similarities. Both form dimers in vivo and in vitro, contain a conserved YPCXXC motif in their carboxyl-terminal part, bind flavin adenine dinucleotide (FAD) as a cofactor, and catalyze the formation of disulfide bonds in protein substrates. The catalytic activity, the ability to form dimers, and the binding of FAD are associated with the carboxyl-terminal domain of the protein. Our findings identify Erv2p as the first microsomal member of the Erv1p/Alrp protein family of FAD-linked sulfhydryl oxidases. We propose that Erv2p functions in the generation of microsomal disulfide bonds acting in parallel with Ero1p, the essential, FAD-dependent oxidase of protein disulfide isomerase.

ERV1† is an essential gene of the yeast Saccharomyces cerevisiae (1). The encoded protein Erv1p is required for maintenance of intact mitochondria in the cell (2). Recently, Erv1p was demonstrated to function as a sulfhydryl oxidase (3). This enzymatic activity is associated with the carboxyl-terminal part that harbors a YPCXXC motif and noncovalently bound FAD. Yeast contains a distant homologue of Erv1p termed Erv2p (4). The sequence similarities are restricted to the carboxyl-terminal part of the proteins, including a highly conserved YPCXXC motif. The functions of Erv1p and Erv2p seem to be nonredundant, because overexpression of Erv2p complemented neither a conditional mutant of ERV1 nor a deletion mutant of this gene (4).

Recently, a few other proteins have been identified containing a domain with homology to the carboxyl-terminal portion of Erv1p. This part is the hallmark of the new Erv1p/Alrp protein family and comprises 80–100 amino acid residues. It contains a CXCC motif that is responsible for disulfide bond formation activity. In mammals, members of this protein family have been characterized as important growth factors. The human ALR (augmenter of liver regeneration) gene encodes a hepatotrophic growth factor (5–8), the carboxyl-terminal part of which can functionally replace the corresponding yeast Erv1p sulfhydryl oxidase domain (8, 9). The human and chicken Q6 inhibitors of cell growth have a function in the reversible silencing of the division of fibroblasts and also contain a domain homologous to Erv1p and Alrp (10). The chicken Q6 protein is the enzymatically best-characterized sulfhydryl oxidase of the Erv1p/Alrp protein family (10–13). Other members are encoded by the genome of double-stranded DNA viruses (4, 14, 15). Recently, the viral protein E10R has been identified as a component of a new pathway of cytosolic disulfide bridge formation (16).

Remarkably, these enzymes are found in different subcellular compartments. Yeast Erv1p was identified mostly inside mitochondria (2, 9); the viral E10R protein executes its function in the cytosol (16); and proteins of the quiescin Q6 family are excreted from cells (10, 11). The sequence similarities and the conserved YPCXXC motif in the carboxyl-terminal domain indicate that Erv2p is a novel member of the Erv1p/Alrp protein family. However, the subcellular localization and enzymatic function of Erv2p have not been determined hitherto.

In this study, we identify Erv2p as a constituent of the microsomal fraction. Erv2p exhibits an FAD-dependent sulfhydryl oxidase activity that is associated with a conserved YPCXXC motif in the carboxyl-terminal region. Thus, Erv2p resembles the enzymatic function of mitochondrial Erv1p (3) but seems to be involved in a specific disulfide bond formation pathway in microsomes. Erv2p may act in parallel to Ero1p, an FAD-dependent essential enzyme involved in disulfide bond formation in protein disulfide isomerase of the endoplasmic reticulum (17). However, both the molecular function of these two enzymes and their protein targets appear to be distinct.

EXPERIMENTAL PROCEDURES

Yeast Strains and Cell Growth—For the subcellular localization experiments the S. cerevisiae strain W303 (MATa, ura3-1, ade2-1, trp1-1, his3-11, 15, leu2-3, 112) was used as wild type. Deletion of the ERV2 gene was performed by a PCR-based method using the histidine auxotrophic marker (18, 19). Exchange of the endogenous promoter of the ERV2 gene for a galactose-inducible promoter (strain Gal-ERV2) was
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performed as described previously (20, 21). PCR fragments corresponding to the coding region (nucleotides 1–536) and the 5′ upstream region (nucleotides −1 to −600) of Erv2p were cloned into the BamHI-HindIII or HindIII-Aval restriction sites of the pYE51 vector carrying the GAL10 promoter. Cells were grown as detailed previously using rich (YP) or minimal media (22–24) and lactate medium (25) containing the required carbon sources.

**PCR Experiments**—The yeast ERV2 gene was amplified from cloned genomic DNA (4) by standard protocols (26) using the listed primers. Restriction sites used for cloning are given in bold letters.

For expression of Erv2p in *Escherichia coli* with a hexahistidinyl tag at the carboxyl-terminal domain of Erv2p, and a shorter fragment encoding the carboxyl-terminal domain were amplified from the genomic copy of Erv2p (4). DNA fragments were cloned into the hexahistidinyl-tag vector pET-24a (+) (Novagen). Primers were as follows: primer 1 (ATG of ERV2/NdeI), 5′-GGACGCAATGTAAGAAACAGATGCTAAAG-3′; primer 2 (ATG of ERV2/NdeI), 5′-ACGACACG-CATATGGCATGATGGCAAGATGAC-3′; and primer 3 (stop codon of ERV2/HindIII), 5′-CTCTAGAGTTCTACGCTGTTTAACCCCTCTCTCTTCTGTC-3′.

The disruption cassette to generate the Δerv2 strain was amplified by the following primers: primer 4 (Del-ERV2-1), 5′-GGTGGACACATGATGGCAAGATGCTAAAG-3′; primer 5 (Del-ERV2-3), 5′-GGATTATAGATATATCATCTGTCCTGTTTTACGATGTTCGATGTCG-3′; and primer 6 (Del-ERV2-2), 5′-CATATGAAACAGATGCTAAAG-3′; and primer 7 (ERV2/HindIII), 5′-AACCTTACATACGCTGTTTAACCCCTCTCTCTTCTTCTGTC-3′.

For amplification of PCR fragments corresponding to the coding region of ERV2 (nucleotides 1–536), the following primers were used: primer 8 (ERV2-HindIII), 5′-AAGCTTTACCTGCGAGTTCAAAATG-3′; and primer 9 (ERV2/Aval), 5′-CTCGAGACGTTCTGTTCTGCAATT-3′.

**Purification of the Yeast Erv2p-6His Proteins from *E. coli***—The two PCR constructs encoding the complete protein and a carboxyl-terminal fragment of Erv2p were expressed in *E. coli* strain BL21 (CLONTECH). Only the shorter protein fragment was successfully expressed in *E. coli*, because induction of expression of full-length Erv2p-6His resulted in cell lysis. The 16-kDa carboxyl-terminal fragment was termed C-Erv2p-6His. The presence of the hexahistidinyl tag at the carboxyl terminus of this protein allowed rapid purification with nickel-nitrioltriacetic acidagarose according to the standard protocol for the isolation under native conditions in NaCl-phosphate buffer (50 mM NaCl, 50 mM KH₂PO₄, 10 mM imidazole, pH 7.5; Qiagen). Proteins were bound to nickel-nitrioltriacetic acid-agarose and eluted with 50 mM NaCl, 50 mM KH₂PO₄, and 200 mM imidazole, pH 7.5. Purification to homogeneity was verified by SDS-polyacrylamide gels. In a final step, the proteins were dialyzed against 50 mM Tris-HCl pH 8.0 and used for spectroscopy with a 10-diode-array photometer (Zeiss).

**Preparation of Reduced Lysozyme and Enzyme Assays for Erv2p**—The reduction of lysozyme and the spectroscopic determination of disulfide bridge formation were done as described earlier (3).

**Miscellaneous Methods**—The following published methods were used: manipulation of DNA and PCR (27), transformation of yeast cells (28–29), isolation of yeast mitochondria (25, 30) and postmitochondrial supernatant (20), purification of mitochondria by Nycodenz density gradient centrifugation (31), and preparation of microsomal fraction and cytisol (32). Antibodies against Erv2p were raised using the carboxyl-terminal peptide CRRVSLEEAKQHG after coupling to maleimide-activated ovalbumin (Pierce; Ref. 32). The strains Δerv2 and Gal-ERV2 served to verify the antibody specificity. *E. coli* strain DH5-α (33) was used for cloning experiments and amplification of plasmid DNA.

**RESULTS**

**Depletion of Erv2p Is Not Associated with Detectable Growth Defects**—To initiate the functional analysis of Erv2p, we constructed a yeast strain in which the entire ERV2 gene was deleted (Δerv2 cells). The mutant cells did not display any detectable growth defects when grown on glucose or glycerol-medium (minimal medium and complete results not shown). Identical results were obtained using a strain allowing the depletion of the Erv2p protein by regulated gene expression with a galactose-inducible promoter (Gal-ERV2 cells). This excluded the possibility that a phenotypical consequence of the defect in Erv2p was suppressed during construction of the Δerv2 cells. Hence, in striking contrast to the essential ERV1, inactivation of ERV2 is not associated with any detectable growth defects.

**Erv2p Cofractionations with Microsomes**—The subcellular localization of Erv2p was investigated by fractionation experiments using an antibody raised against the carboxyl-terminal 14 amino acid residues of the protein. In extracts derived from wild-type cells, a protein of 22 kDa was recognized by the Erv2p-directed antiserum (Fig. 1A). This finding is in good agreement with the calculated molecular mass of 22.1 kDa for Erv2p. No immunoreactive protein in the 22-kDa region was detectable in cultures derived from the Gal-ERV2 strain grown in the absence of galactose (Fig. 1A) or in Δerv2 cells (cf. Fig. 5A), demonstrating the specificity of the antiserum for Erv2p. The majority of Erv2p was found in the postmitochondrial supernatant, whereas only a minor portion of Erv2p was associated with the mitochondrial fraction (Fig. 1A).

Because isolated mitochondria are usually contaminated
The amount of Erv2p in purified mitochondria was at least 10-fold reduced, whereas mitochondrial Tim44p was not depleted (Fig. 1B). The depletion of Erv2p in purified mitochondria was virtually identical to the reduction of the endoplasmic reticulum protein Sbh1p. These results suggest that in contrast to Erv1p, Erv2p is not a mitochondrial protein. In accordance, Erv2p was enriched in the microsomal membrane fraction, which was nearly free of contaminating mitochondrial protein as verified by immunostaining analysis for Tim44p of the inner membrane (Fig. 1B). Erv2p appeared to be exclusively associated with a particulate fraction, because the protein was detected in the pellet of a high-speed centrifugation of the post-mitochondrial supernatant (Fig. 1C). In contrast, the cytosolic protein Pgk1p was recovered in the supernatant. In conclusion, our fractionation studies identified Erv2p as a protein associated with microsomes.

**Yeast Erv2p Contains Noncovalently Bound FAD—** Yeast Erv1p belongs to a new class of sulfhydryl oxidases (3) that use a special YPCXXC motif in the carboxyl-terminal domain (Fig. 2) and FAD as a cofactor (11–13). Because the sequence similarities between Erv1p and Erv2p are highest for the carboxyl-terminal domains, we tested whether this domain of Erv2p exhibits the same enzymatic function as Erv1p. For this purpose, Erv2p was expressed in *E. coli* with a hexahistidinyl tag attached to the carboxyl terminus. In contrast to Erv1p, the full-length protein of Erv2p was toxic for *E. coli* under all investigated conditions. Induction of expression of full-length Erv2p in *E. coli* immediately resulted in cell lysis. Therefore, a carboxyl-terminal fragment was expressed that starts with methionine at position 70 (Fig. 2). This fragment contains the conserved YPCXXC motif of Erv2p and was successfully expressed and purified to homogeneity by chromatography on nickel-nitrilotriacetic acid-agarose. The 16-kDa carboxyl-terminal protein fragment of Erv2p was termed C-Erv2p-6His. Solutions of C-Erv2p-6His were analyzed by SDS-PAGE in the presence or absence of reducing agents in typical for flavin-containing proteins (11–13). Determination of the protein concentration revealed that ~0.8 FAD per molecule of C-Erv2p-6His were bound. Treatment of C-Erv2p-6His with 6 M urea completely removed the FAD moiety without resulting in major differences in the absorption, confirming that free and protein-bound FAD do not differ significantly in their extinction coefficients (results not shown). Together, these data demonstrate that C-Erv2p-6His carries FAD in a 1:1 stoichiometry. The cofactor is firmly attached but not covalently linked to the protein. Similar findings were made for yeast Erv1p (3) and mammalian Alrp (34).

**Yeast Erv2p Has a Sulfhydryl Oxidase Activity—** The protein fragment C-Erv2p-6His was investigated by using the standard enzyme assay for sulfhydryl oxidases and compared with full-length Erv1p. Reduced lysozyme was used as a substrate (3). Reactions were started by adding the purified yeast proteins to the substrate mixture. At different time points, aliquots were withdrawn and analyzed for their thiol content (35). C-Erv2p-6His was able to oxidize the substrate in a time-dependent manner. Increasing amounts of protein gave a corresponding increase in enzyme activity (Fig. 4). The enzymatic turnover number (4.5/min) of C-Erv2p-6His was comparable with that of Erv1p (6.2/min; Ref. 3). Taken together, the carboxyl-terminal domain of Erv2p exhibits FAD-linked sulfhydryl oxidase activity.

**Erv2p Forms Dimers under Nonreducing Conditions—** Extracts from wild-type and erv2 yeast cells were prepared under nonreducing conditions. Aliquots were separated by SDS-PAGE in the presence or absence of reducing agents in the loading buffer of the sample (Fig. 5). Immunostaining analysis with the Erv2p-reactive antibody revealed a 44-kDa protein in the absence of reducing agent, suggesting that Erv2p forms a homodimer (Fig. 5A). Addition of reducing agent to the sample resulted in the detection of a single band of 22 kDa, corresponding to the monomeric form of Erv2p. Only the wild-type strain displayed the Erv2p-specific protein bands. Comparable analyses with a recombinant C-Erv2p-6His fragment demonstrated that this fragment also formed dimers under nonreducing conditions (Fig. 5B). This result indicates that the carboxyl-terminal domain of Erv2p, in addition to the enzymatic sulfhydryl oxidase activity, harbors the information for dimerization.

**FIG. 2. Domain structure of yeast Erv1p and Erv2p.** The amino-terminal domains of ~70 amino acids (gray bars) share no significant sequence similarities with each other or with any other protein. The carboxyl-terminal regions (white bars) contain ~20% of amino acid residues at identical positions. The region around the conserved YPCXXC motif is aligned for yeast Erv1p and Erv2p. *Numbers* at the end of the sequences give the positions of the amino acid residues in the proteins. In addition, Erv1p contains a pair of cysteine residues (C-C) in the amino-terminal region that is not present in Erv2p. In contrast, Erv2p has 2 cysteine residues (C-C) in the carboxyl-terminal domain that are not found in Erv1p. Arrow, phenylalanine residue that is essential for functional yeast Erv1p (3).
DISCUSSION

In this communication, we report on the identification of Erv2p, the first member of the Erv1p/Alrp sulfhydryl oxidases in the endoplasmic reticulum. The enzymatic activity of Erv2p is associated with the conserved carboxyl-terminal part of the protein, which consists of ~100 amino acid residues. This domain is present in a number of other proteins and is the characteristic hallmark of the new Erv1p/Alrp protein family involved in the introduction of disulfide bridges in protein substrates. Members of this family include mammalian Alrp and vertebrate quiescin Q6 (5, 6, 8, 10, 11). For the chicken Q6 sulfhydryl oxidase, the enzyme mechanism is well elucidated (12, 13). This enzyme depends on noncovalently bound FAD, uses O\textsubscript{2} for oxidation, and contains a C\textsuperscript{XX}C motif that is functionally involved in the redox reaction (13). All currently known members of this protein family are involved in distinct disulfide bond formation pathways localized outside the endoplasmic reticulum (3, 11–13, 16). This is remarkable because most of the oxidative protein folding normally takes place in the endoplasmic reticulum (36–38). So far, the only other FAD-linked disulfide bond-forming enzyme of the endoplasmic reticulum is Ero1p (17). Erv2p and Ero1p share no sequence homologies and display several distinct features. Ero1p is a membrane-bound protein of 65 kDa that performs an essential task in protein folding (17, 36, 37). In addition, Ero1p functionally interacts with protein disulfide isomerases and the homologous protein Mpd2p, another class of disulfide bridge-forming enzymes in microsomes (17). Therefore, the molecular mechanisms underlying the generation of disulfide bonds by Ero1p and sulfhydryl oxidases of the Erv1p/Alrp type are radically different.

An interesting homologue of Erv2p in yeast is the essential protein Erv1p, which is localized mostly in mitochondria (2, 9). The characteristics of both yeast proteins are remarkably similar. Erv1p and Erv2p can oxidize thiol groups in a protein substrate and contain noncovalently bound FAD and a special YPCXXC motif in the carboxyl-terminal domain. The enzyme turnover numbers of C-Erv2p-6His and Erv1p with lysozyme as an in vitro substrate are nearly identical and are both substantially lower than that of native chicken egg white sulfhydryl oxidase (3, 12). This suggests that Erv1p and Erv2p may not serve as generic sulfhydryl oxidase enzymes but, rather, may be specific for a narrow range of substrates. This notion is consistent with the fact that glutathione and dithiothreitol are not used as substrates by both proteins.2 Both yeast proteins form dimers in vivo that are stable in nonreducing SDS gels, arguing for covalent disulfide bridges between the monomers. However, current evidence suggests that Erv1p and Erv2p use different strategies for dimer formation. The carboxyl-terminal fragment of Erv2p still efficiently forms dimers even when the amino-terminal part of the protein is removed, whereas the amino-terminal domain of Erv1p is indispensable for dimer formation (3). This indicates that the cysteine residues used for dimer formation are localized in different positions of the two proteins. Inspection of the two protein sequences indeed identifies a pair of cysteine residues in the amino-terminal part of Erv1p (Fig. 2), whereas Erv2p lacks cysteine residues within the first 70 residues. Instead, Erv2p contains additional cysteine residues near the carboxyl terminus that are not found in Erv1p (4).

The different compartmentation of Erv1p and Erv2p is of particular interest. Although Erv1p was found mostly in mitochondria (2, 9), our subcellular fractionation experiments demonstrate that Erv2p is not a mitochondrial component but,

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FIG. 3. The purified 16-kDa fragment of yeast Erv2p (C-Erv2p-6His) contains a noncovalently bound FAD. The absorbance spectrum was recorded in the range of 350–550 nm using the purified 16-kDa fragment of yeast Erv2p (thick line) and pure FAD (thin line). Arrows, absorption maxima of protein-bound and free FAD (3, 11–13).
rather, highly enriched in the microsomal preparations. The small portion of Erv2p that is still associated with the mitochondrial fraction corresponds well with the amount of contaminating microsomes. This residual fraction of Erv2p may well reflect the tight association of a fraction of the smooth endoplasmic reticulum with mitochondria (39). In conclusion, our data indicate that most if not all of Erv2p is localized inside microsomes.

Presently, it is unclear in which microsomal fraction Erv2p might be localized. Even though localization in the lumen of the endoplasmic reticulum seems to be most likely, it has to be noted that the protein does not harbor a carboxyl-terminal HDEL retrieval motif typical of soluble components of this compartment. The different subcellular localization of Erv2p and Erv1p might result from targeting information contained within the highly divergent amino termini (9). However, neither Erv1p nor Erv2p contains sequences that are typical for targeting to mitochondria or the endoplasmic reticulum. The unusual characteristic of subcellular targeting of both proteins is further supported by the fact that neither Erv1p nor Erv2p is apparently proteolytically processed at its amino termini. Subcellular distribution is only one function associated with the amino-terminal domains of the Erv1p/Alrp family. In some members these domains are also involved in dimer formation (3) and most likely also in substrate interactions.

To date, Erv1p is the only disulfide bond formation activity identified inside mitochondria. Even though the substrates for Erv1p are presently unknown, substrates of Erv1p seem to perform an essential function in yeast cells. In contrast, the microsomal compartment contains several other enzymes that are essential for oxidative protein folding (17, 36, 38–41). The most prominent member of these proteins is the already mentioned Ero1p (17). One important difference between these

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**FIG. 4.** The 16-kDa carboxyl-terminal fragment of Erv2p (C-Erv2p-6His) exhibits sulfhydryl oxidase activity. Reduced lysozyme corresponding to 55 nmol thiol groups was incubated with the indicated amounts of C-Erv2p-6His, full-length Erv1p, or pure FAD without added protein sample. The oxidation of thiol groups was followed spectrophotometrically at 412 nm after addition of 10 μM 5,5’-dithiobis(2-nitrobenzoic acid). The time-dependent decrease of extinction indicates the oxidation of the thiol groups.

**FIG. 5.** The carboxyl-terminal portion of Erv2p is responsible for dimer formation. A, the postmitochondrial supernatants of wild-type (wt) and Δerv2 cells were separated by SDS-PAGE in the presence or absence of 300 mM β-mercaptoethanol (βME) in the sample buffer. Erv2p was visualized by immunostaining. A band of 44 kDa corresponds to a homodimer, and a band of 22 kDa corresponds to the monomer. B, aliquots of 20 ng of C-Erv2p-6His (16 kDa) purified from E. coli were analyzed on a 4–12% SDS-polyacrylamide gel with or without 10 mM dithiothreitol in the sample buffer. The positions of the 32-kDa dimer and the 16-kDa monomer are indicated. Protein bands were visualized by antibodies specific for the hexahistidinyl tag of C-Erv2p-6His.
enzymes and FAD-dependent sulphydryl oxidases is the finding that the latter have no protein disulfide isomerase function and use oxygen in the redox reaction (12). The presence of multiple systems for disulfide bridge formation in the microsomal fraction could explain the nonessential function of Erv2p in yeast. One implication of this finding is that Erv2p has been adapted to specific but nonessential substrates inside microsomes.

The latest interesting case of a protein harboring the sulphydryl oxidase domain is the viral member of the Erv1p/Alrp protein family termed E10R (16). This enzyme was found to be essential for disulfide bond formation in a virus-encoded glutaredoxin. In turn, glutaredoxin introduces disulfide bridges into viral coat proteins that are essential for virus assembly in the cytosol of the infected cell. Thus, the E10R protein functions as a specific sulphydryl oxidase in the cytosol, a compartment that is devoid of disulfide bond-forming enzymes.

Our current data characterize Erv2p as a new member of the Erv1p/Alrp protein family with a specific function in the oxidative protein-folding pathway inside microsomes. Therefore, identification of the natural target proteins of Erv2p in yeast represents an important future goal.

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