A soluble NH_2-terminally truncated catalytically active form of rat cytochrome P450 2E1 targeted to liver mitochondria[1]

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Abstract The role of the NH_2-terminus of cytochrome P450 2E1 (CYP2E1) in intracellular targeting was investigated. Two NH_2-terminal CYP2E1 mutants, Δ(2–29)2E1, lacking the transmembrane domain, and N^{++}2E1, having Ala2Lys and Val3Arg substitutions, were generated and expressed in the H2.35 mouse hepatoma cell line. In cells transfected with both constructs, a 40 kDa fragment of CYP2E1 (Δ2E1) was found to be localized to mitochondria as evidenced from immunofluorescence microscopy and subcellular fractionation studies. Δ2E1 was shown to be a soluble protein localized inside the mitochondria, displayed catalytic activity when reconstituted with adrenodoxin and adrenodoxin reductase, and was also present in mitochondria isolated from rat liver. It is concluded that in the absence of the hydrophobic NH_2-terminal sequence, a putative mitochondrial import signal is exposed which targets CYP2E1 to this organelle where it is further processed.

Key words: Mitochondrial import; Chlorzoxazone; cDNA transfection; Adrenodoxin; Ethanol; Oxidative stress; Cytochrome P450 2E1

1. Introduction

Cytochrome P450s (P450s) are a superfamily of enzymes known to metabolize a wide variety of endogenous and exogenous compounds. Most of the eukaryotic P450s are membrane bound enzymes localized in either the endoplasmic reticulum (ER) membrane or inside mitochondria [1,2]. The highly hydrophobic NH_2-terminal domain present in ER localized P450s serves as a non-cleavable stop-transfer signal sequence for the cotranslational insertion and anchoring of the protein in the ER membrane [3,4]. This hydrophobic NH_2-terminus appears to be important in not only membrane binding but also in ER retention [5,6], although other parts of the P450 enzyme have also been shown to cause retention [7]. In contrast to microsomal P450s, mitochondrial P450s such as CYP11A1 (P450scc) and CYP11B (P45011β) [8] are imported into the mitochondria posttranslationally due to their amphipathic NH_2-terminal signal sequence [9]. After removal of the signal sequence, these P450s become associated with the mitochondrial inner membrane by as yet unknown mechanisms.

The ethanol inducible form of cytochrome P450 (CYP2E1) is predominantly present in the ER although CYP2E1 has also been detected in the Golgi apparatus [10], lysosomes [11] and on the outside of the plasma membrane [12]. CYP2E1 has been suggested to be involved in gluconeogenesis because of its ability to metabolize ketone bodies such as acetone [13]. In addition, CYP2E1 metabolizes a wide variety of small hydrophobic xenobiotics including well-known carcinogens and other toxic compounds [13], causes oxidative stress through the production of reactive oxygen species and has been implicated in the development of alcoholic liver disease [14].

In the past decade, many laboratories have considered the possibility of designing a soluble form of microsomal P450 which could be instrumental in resolving the three-dimensional structure of mammalian microsomal P450s. However, these attempts have been unsuccessful to date and focused on the removal of the hydrophobic NH_2-terminal transmembrane domain of P450, which is important not only in ER targeting, binding and retention, but also in the assembly of a catalytically active P450 and in protein stability [15,16].

In the present study we investigated the role of the NH_2-terminus of rat CYP2E1 in intracellular targeting and membrane association in a hepatoma cell line. NH_2-terminally mutants of CYP2E1 were shown to be imported into mitochondria and to be present in a soluble truncated form.

2. Materials and methods

2.1. Expression vectors

Full length rat CYP2E1 cDNA was cloned into the mammalian expression vector pCMV5 [17]. The CYP2E1 cDNA lacking the coding sequence for amino acids 2–29 was generated by PCR amplification using *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA), primer 1a (5′-GACGAATTCATGTGGAACCTGCCCCCAGGA-3′) and reverse primer 1b (5′-GACTCTAGATCATGAACGGGGAATGAC-3′). The cDNA for N^{++}2E1 containing the Ala2Lys and Val3Arg substitutions was created by PCR amplification using CYP2E1 cDNA, the mutated primer 2a (5′-GACGAATTCATGAAGAGGCTTGGCATCACCATTGCC-3′, bases encoding Lys and Arg are underlined) and primer 1b. The resulting cDNAs were cloned into the pCMV5 expression vector. The correct sequence of the inserts was confirmed by DNA sequencing using the ABI Prism Dye Terminator Cycle Sequencing kit from Perkin-Elmer (Norwalk, CT, USA).

2.2. Cell culture and transient transfection

H2.35 cells (mouse SV-40 transformed hepatocytes) were purchased from the American Type Culture Collection (Rockville, MD, USA). H2.35 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Rockville, MD, USA) containing 1.0 g/l glucose supplemented with 4% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.2 μM dexamethasone and maintained at 37°C in a humidified atmosphere of 5% CO_2. H2.35 cells were

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Abbreviations: P450, cytochrome P450; ER, endoplasmic reticulum; Adx, adrenodoxin; AdR, adrenodoxin reductase; NaDOC, sodium deoxycholate; PBS, phosphate buffered saline

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transfected with a mixture of plasmid DNA and the cationic lipid DMRIE-C (Life Technologies) and harvested 24–30 h after the addition of the DNA/DMRIE-C mix.

2.3. Western blot analysis

Cells were harvested, sonicated in homogenization buffer (50 mM HEPES pH 7.4 containing 0.25 M sucrose and 0.5 mM EDTA) and centrifuged at $3000\times g$ for 10 min. The post-nuclear supernatant was mixed with SDS-PAGE sample buffer, proteins were separated on SDS-PAGE, transferred to nitrocellulose membranes, blocked and incubated with antibodies as described previously [10].

2.4. Subcellular fractionation

Transfected cells were washed with phosphate buffered saline (PBS), collected by scraping and homogenized into 2 ml homogenization buffer (50 mM HEPES pH 7.4 containing 0.25 M sucrose, 1 mM EDTA, 1 μg/ml antipain, 10 μg/ml leupeptin and 1 mM PMSF). The homogenate was centrifuged at $3000\times g$ for 10 min and 2 ml post-nuclear supernatant was layered on top of a discontinuous sucrose gradient and centrifuged as described [18]. Fractions (1 ml) were collected from bottom to top after puncturing the bottom of the tube. Protein was determined after acid precipitation according to Peterson [19] and samples were analyzed by Western blot.

2.5. Membrane association and topology

Δ(2–29)2E1 transfected cells were homogenized and the post-nuclear supernatant was prepared as described above. The mitochondrial fraction was prepared by centrifugation of the post-nuclear supernatant at $10\,000\times g$ for 15 min. Membrane association of Δ2E1 was investigated by sodium deoxycholate (NaDOC) treatment. The mitochondrial fraction was resuspended in 50 mM Tris-HCl buffer pH 7.4 containing 100 mM KCl and 5 mM $MgCl_2$ at a protein concentration of 1 mg/ml and incubated in the presence of 0.05% NaDOC for 30 min on ice. The particulate and soluble fractions were separated by centrifugation at $10\,000\times g$ for 15 min and both fractions were analyzed by Western blot.

Membrane topology was determined by the protease protection assay. Briefly, the mitochondrial fraction was diluted in 50 mM Tris-HCl buffer pH 8.0 containing 10 mM $CaCl_2$ and 150 mM NaCl to a protein concentration of 1 mg/ml. The samples were incubated with proteinase K (83 μg/ml) in the presence or absence of 0.05% NaDOC at 37°C for 30 min. The reaction was terminated by the addition of PMSF (final concentration 10 mM) and the samples were analyzed by Western blot.

2.6. Immunofluorescence microscopy

Cells grown and transfected on glass coverslips were washed three times in PBS, fixed in 2% formaldehyde in PBS for 10 min and permeabilized with 0.2% Triton X-100 in PBS for 10 min. After blocking in 10% fetal bovine serum in PBS for 2.5 h, the cells were incubated with anti-CYP2E1 antibodies (1:5000 dilution) in 3% bovine serum albumin (BSA) in PBS for 90 min followed by FITC conjugated goat anti-rabbit antibodies (1:500 dilution) in 3% BSA in PBS for 90 min. For double immunostaining, FITC conjugated goat anti-rabbit and TRITC conjugated goat anti-mouse antibodies were used to visualize CYP2E1 and mitochondrial Hsp70 (mHsp70, Affinity Bioreagents Inc., Goldon, CO, USA) related immunoreactivity respectively. Stained cells were carefully mounted with a drop of Vecta-Shield (Vector Laboratories, Burlingame, CA, USA) on a glass slide. The glass slides were viewed under an Olympus BX60 microscope equipped with Olympus PM20 camera (Tokyo, Japan).

2.7. Catalytic activity

The catalytic activity of Δ2E1 was determined by monitoring the formation of 6-hydroxy-chlorzoxazone essentially as described [10]. Mitochondria from cells transfected with Δ(2–29)2E1 and empty plasmid were isolated and disrupted by sonication (three bursts of 10 s with 30 s intervals) on ice. The incubation mixture consisted of 100 μg sonically disrupted mitochondria, 50 μM chlorzoxazone, a NADPH generating system (0.2 mM NADPH, 2.0 mM glucose 6-phosphate and 3 U/ml glucose 6-phosphate dehydrogenase), 1 nmol adrenodoxin (Adx), 0.1 nmol adrenodoxin reductase (AdR), kindly supplied by Prof. Rita Bernhardt, University of Saarbrücken, Germany, and 50 mM phosphate buffer pH 7.4 in a final volume of 0.25 ml. After 30 min the reaction was terminated by the addition of orthophosphoric acid and an internal standard (0.04 μg acetaminophen) was added. After extraction, the samples were analyzed by HPLC equipped with an amperometric detector.

2.8. Isolation of rat liver mitochondria

Untreated male Sprague-Dawley rats were anesthetized and killed by decapitation. Livers were perfused with PBS, removed from the animal and mitochondria were prepared according to Boyer et al. [20] and additionally purified by sedimentation through a discontinuous sucrose gradient [21]. Mitochondria purified in this way were shown to be heavily enriched in mitochondrial marker protein (20-fold enrichment of mHsp70) and essentially devoid of microsomal contamination (less than 0.5% of NADPH cytochrome P450 reductase).

3. Results

3.1. Transient expression and characterization of the CYP2E1 mutants

The two NH_2-terminal CYP2E1 mutants, Δ(2–29)2E1 and N^{++}2E1, together with wt2E1 (Fig. 1A shows the NH_2-terminal amino acid sequences) were transfected into H2.35 cells. As expected, wt2E1 produced the full length CYP2E1 protein with an apparent molecular weight of 52 kDa (Fig. 1B), whereas in cells transfected with empty plasmid (mock) no protein was observed, demonstrating that this cell line does not express significant amounts of endogenous CYP2E1. In cells transfected with N^{++}2E1, a band with similar mobility as wt2E1 was observed together with a minor band around

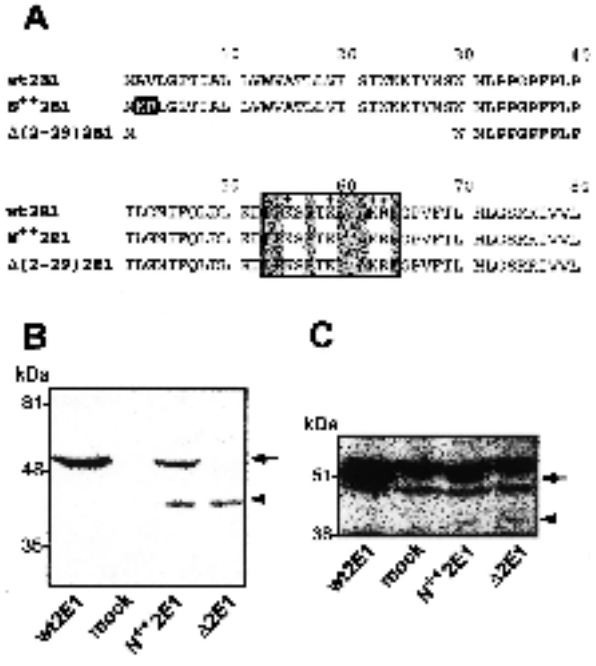

Fig. 1. Transient expression of wt2E1, N^{++}2E1 and Δ(2–29)2E1 in H2.35 cells. A: NH_2-terminal amino acid sequence of the constructs used. Amino acids which were substituted are represented in a black box and amino acids which were deleted are represented by an empty space. The putative mitochondrial targeting signal is boxed, with the hydrophobic amino acids (localized on one side of the α-helix) in gray boxes, hydrophilic residues in white and positively charged amino acids indicated with +. B: The post-nuclear supernatants isolated from H2.35 transfected with either wt2E1, empty plasmid (mock), N^{++}2E1 or Δ(2–29)2E1 were analyzed by using CYP2E1 specific antibodies. C: Post-nuclear supernatants analyzed by antibodies specifically recognizing the COOH-terminal five amino acids of CYP2E1. The arrow indicates the full length CYP2E1 and the arrowhead indicates the 40 kDa band Δ2E1.

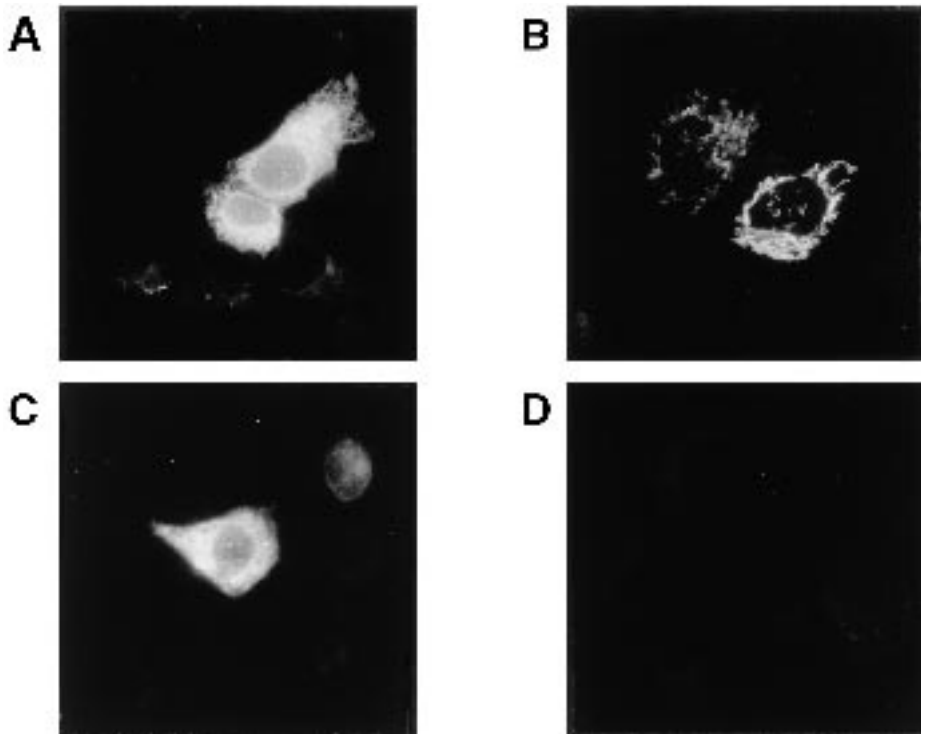

Fig. 2. Immunofluorescence microscopy of hepatoma cells expressing wt2E1, Δ2E1 and N^{++}2E1. H2.35 cells were grown and transfected with (A) wt2E1, (B) Δ(2–29)2E1, (C) N^{++}2E1 and (D) empty plasmid on glass coverslips and after fixation and permeabilization were incubated with CYP2E1 specific antibodies followed by FITC conjugated anti-rabbit IgG as described in Section 2.

40 kDa (Fig. 1B, arrowhead). In Δ(2–29)2E1 transfected cells, no protein with the predicted size around 50 kDa could be detected. However, as observed for N^{++}2E1, a CYP2E1 immunoreactive protein of about 40 kDa was seen which implied the presence of a truncated form of CYP2E1 (Δ2E1). Anti-peptide antibodies raised against the terminal five amino acids of the COOH-terminus of human CYP2E1 [22] recognized wt2E1, the full length N^{++}2E1 (Fig. 1C, arrow) and also Δ2E1 (Fig. 1C, arrowhead). This demonstrated that the COOH-terminus of Δ2E1 was identical to the COOH-terminus of wt2E1 and that Δ2E1 was an NH_2-terminally truncated CYP2E1 form.

3.2. Intracellular localization of Δ2E1

The intracellular distribution of the expressed wt2E1, N^{++}2E1 and Δ2E1 was examined by indirect immunofluorescence microscopy (Fig. 2). Cells transfected with wt2E1 displayed a reticular staining pattern throughout the cytoplasm typically observed for ER proteins, confirming the expected ER localization for wt2E1 (Fig. 2A). In N^{++}2E1 transfected cells, the same reticular staining pattern was observed indicating that the majority of the protein is associated with the ER (Fig. 2C). Cells transfected with Δ(2–29)2E1 displayed an immunofluorescent staining being confined to worm-like structures mainly localized in the perinuclear region of the cell (Fig. 2B). This staining pattern is observed for proteins associated with the mitochondria, suggesting a mitochondrial localization for Δ2E1. Once again, no significant staining could be observed in cells transfected with empty plasmid which demonstrated that these cells are devoid of significant amounts of endogenous CYP2E1 (Fig. 2D). In order to further establish the mitochondrial localization of Δ2E1,

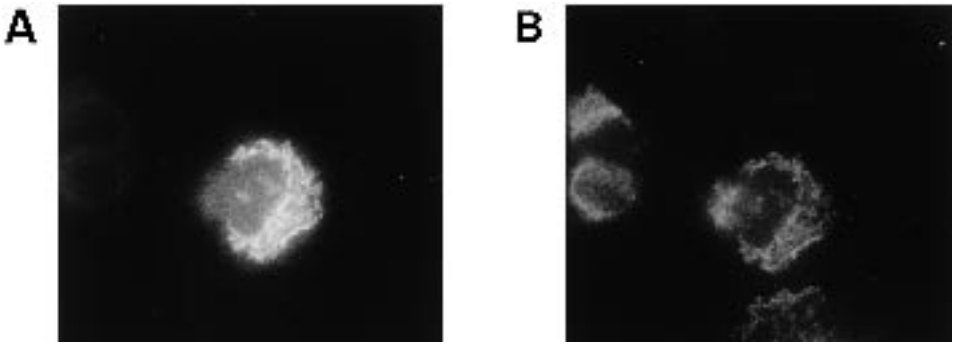

Fig. 3. Association of Δ2E1 with mitochondria revealed by immunofluorescent microscopy. Δ(2–29)2E1 transfected cells were double stained with (A) CYP2E1 specific antibodies visualized by FITC conjugated anti-rabbit IgG and (B) mitochondrial Hsp70 antibodies visualized by TRITC conjugated anti-mouse IgG. As seen, both proteins are localized to the same structures.

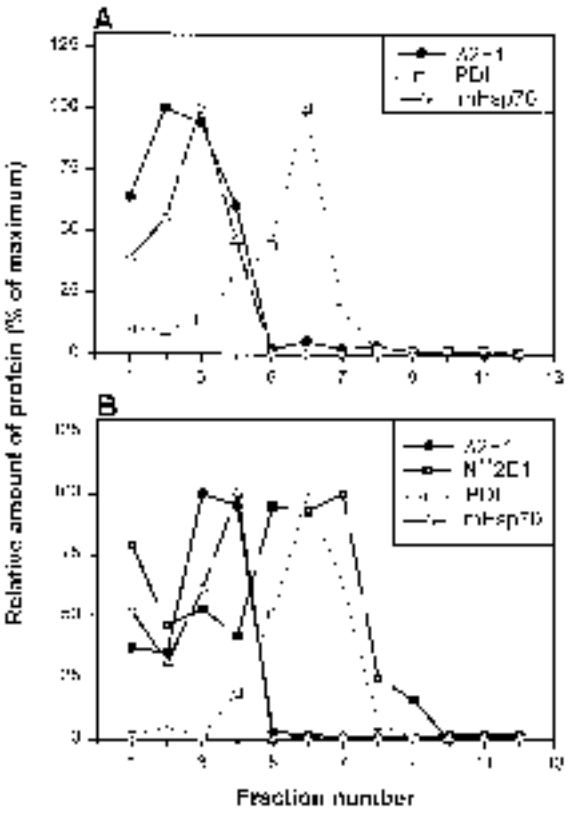

Fig. 4. Subcellular distribution of Δ2E1 and N^{++}2E1 in H2.35 cells. A: Cells transfected with Δ(2–29)2E1 express Δ2E1 which is recovered in the same fractions as the mitochondrial marker mHsp70. B: Cells transfected with N^{++}2E1 express both full length N^{++}2E1 which is recovered in the fractions containing the ER marker protein disulfide isomerase (PDI) and Δ2E1 which is recovered in the mitochondrial fractions. The fraction containing the highest amount of each protein was set at 100% and the protein content of the other fractions was related to this value. Relative amounts of protein were quantified by densitometric analysis. Fractions were harvested from the bottom (fraction number 1) to the top (fraction number 12) of the gradient.

Δ(2–29)2E1 transfected cells were double immunostained with a monoclonal antibody recognizing mHsp70, a protein residing in the mitochondria, and with a CYP2E1 specific antiserum. As shown in Fig. 3, the staining pattern observed with the CYP2E1 specific antiserum recognizing Δ2E1 (Fig. 3A) was identical to that observed with the mHsp70 specific antibodies (Fig. 3B), thereby revealing the mitochondrial localization of this protein.

The intracellular distribution of Δ2E1 was also assessed by subcellular fractionation of cells transfected either with Δ(2–29)2E1 or N^{++}2E1 on discontinuous sucrose gradients. Fig. 4A shows that in cells transfected with Δ(2–29)2E1, Δ2E1 was recovered solely in the fractions which also contained the mitochondrial marker. In N^{++}2E1 transfected cells, the full length N^{++}2E1 was recovered in fractions where the ER marker was present, which confirmed its ER localization, and was well separated from Δ2E1 present in the mitochondrial fractions (Fig. 4B).

3.3. Membrane association and topology of Δ2E1

The membrane association of Δ2E1 was investigated by treatment of the mitochondrial fraction obtained from Δ(2–29)2E1 transfected cells with 0.05% NaDOC, known to release soluble proteins into the soluble fraction but not membrane bound proteins. Nearly all Δ2E1 was recovered in the soluble fraction (S) (Fig. 5A). The soluble mHsp70 was used as a control and was also shown to be present in the soluble fraction (Fig. 5A). NaDOC treatment of the microsomal fraction of cells transfected with wt2E1 resulted in the recovery of integral membrane proteins such as wt2E1 and NADPH cytochrome P450 reductase in the particulate fraction (data not shown). In order to confirm that Δ2E1 was indeed a soluble protein, mitochondria were disrupted by sonication (three bursts of 15 s each with 30 s intervals) [8]. The membranous and soluble fractions were separated by centrifugation and samples were analyzed by Western blotting. About 85% of the Δ2E1 present in the mitochondria was recovered in the soluble fraction, indicating that, as observed in the NaDOC experiments, Δ2E1 was a soluble protein.

The mitochondrial pellet of cells expressing Δ2E1 was also treated with proteinase K in order to determine the topology of Δ2E1 in the mitochondria (Fig. 5B). In non-permeabilized mitochondria Δ2E1 was not digested by proteinase K, however when 0.05% NaDOC was added all Δ2E1 was digested. The mitochondrial matrix protein mHsp70 displayed identical behavior. These observations demonstrate that Δ2E1 is a soluble protein and is located inside the mitochondria.

3.4. Rat liver mitochondria

In order to investigate if Δ2E1 was present in vivo, mito-

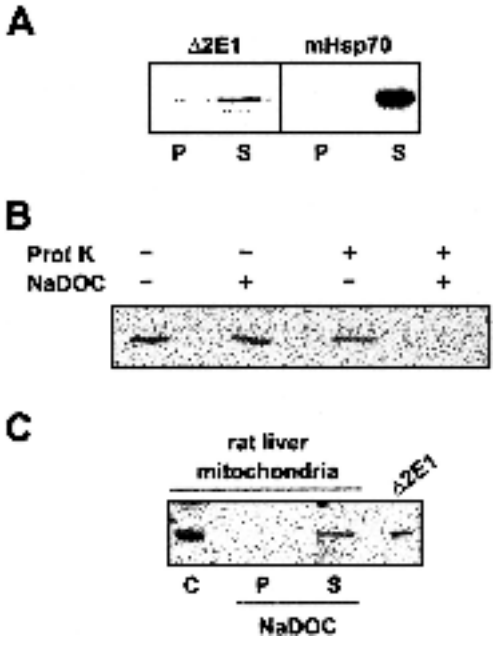

Fig. 5. Intramitochondrial localization and membrane association of Δ2E1. A: The mitochondrial fraction of Δ(2–29)2E1 transfected cells was incubated in the presence of 0.05% NaDOC and the particulate (P) and soluble (S) fractions were isolated and analyzed for both Δ2E1 and the soluble mitochondrial protein mHsp70. B: The mitochondrial fraction of cells transfected with Δ(2–29)2E1 was incubated with or without proteinase K (Prot K) and NaDOC as indicated. After digestion, samples were analyzed for Δ2E1. The results revealed that Δ2E1 was localized inside the mitochondria. C: Presence of Δ2E1 in mitochondria isolated from rat liver. Δ2E1 was recovered solely in the soluble fraction (S) after NaDOC treatment of mitochondria from livers isolated from control rats (con). Δ2E1 expressed in H2.35 cells was added as a standard in the last lane.

Table 1
Catalytic activities of mitochondria isolated from H2.35 cells transfected with empty plasmid (mock) and with Δ(2–29)2E1

	pmol 6-hydroxy-chlorzoxazone/min/mg protein	
	−Adx/AdR	+Adx/AdR
Mock	0.23	0.50
Δ2E1	0.37	2.09

Sonically disrupted mitochondria were assayed in the presence (+) or absence (−) of Adx and AdR for the 6-hydroxylation of chlorzoxazone as described in Section 2. Results shown are the average of two determinations and represent three independent transfections.

chondria, essentially devoid of ER contamination, were isolated from control rat liver. The rat liver mitochondrial fraction was examined by Western blot using the CYP2E1 specific antiserum. As shown in Fig. 5C, the mitochondrial fraction purified from rat liver contained a band which was identical to Δ2E1 obtained from Δ(2–29)2E1 transfected cells. Moreover, Δ2E1 present in rat liver mitochondria was also recovered in the soluble fraction (S) after NaDOC treatment. This demonstrated that Δ2E1 is also present in rat liver mitochondria as a soluble protein.

3.5. Catalytic activity of Δ2E1

The catalytic activity of Δ2E1 was determined by monitoring the formation of 6-hydroxy-chlorzoxazone in mitochondria disrupted by sonication and reconstituted with Adx and AdR. Mitochondria isolated from Δ2E1 expressing cells displayed significantly higher catalytic activity than mitochondria isolated from mock transfected cells when reconstituted with both Adx and AdR (Table 1). When Adx and AdR were omitted from the incubation mixture only low levels of 6-hydroxy-chlorzoxazone could be detected, indicating that the catalytic activity of Δ2E1 is strongly supported by these two mitochondrial electron transport proteins. Similar results were obtained in three independent transfection experiments.

4. Discussion

Although it has been described that CYP2E1 is mainly localized in the ER membrane, small amounts of CYP2E1 are also found in the Golgi apparatus, in lysosomes and on the outside of the plasma membrane. Here, we describe the presence of an NH_2-terminally truncated soluble form of CYP2E1, Δ2E1, in yet another cellular compartment, the mitochondria. Δ2E1 was also shown to be catalytically active and to be present in mitochondria isolated from rat liver.

The majority of the proteins that are posttranslationally imported into mitochondria contain an NH_2-terminal amphipathic signal sequence, which is usually removed after import [23,24]. The basic requirements for this mitochondrial targeting sequence are about 20–60 amino acid residues with several positive charges, almost no negative ones and several hydroxylated residues [25,26]. The targeting sequence is assumed to be able to form an amphipathic α-helix and through its positive charges able to bind to the negatively charged surface of the mitochondrial import machinery which is located both on the outer and inner membrane of the mitochondria [27]. It has been demonstrated that mitochondrial P450s are imported into mitochondria through their NH_2-terminal signal sequence [8,9]. There are, however, reports describing mitochondrial targeting sequences which are localized at the COOH-terminus or inside the protein [28,29].

We found that after removal of the hydrophobic NH_2-terminal transmembrane domain of CYP2E1, the resulting truncated protein is targeted to and imported into mitochondria. This indicates that removal of the hydrophobic transmembrane domain exposes a putative mitochondrial import signal. Examination of the NH_2-terminal amino acid sequence of Δ(2–29)2E1 reveals that there is a region rich in positively charged amino acids (residues 53–64) which is able to form an amphipathic α-helix and therefore could potentially serve as a mitochondrial targeting signal (Fig. 1A). We suggest that after mitochondrial import the potential targeting sequence is removed thereby generating the 40 kDa fragment Δ2E1. When two positively charged amino acids were introduced into the NH_2-terminus of CYP2E1 the same mitochondrially localized Δ2E1 could be observed, although the majority of the translated protein was the expected full length form which was located in the ER. The reason for the mitochondrial import and formation of Δ2E1 from a small fraction of N^{++}2E1 remains unclear, but a similar mechanism as postulated for the mitochondrial import of a truncated form of CYP1A1 could well be operating here [30]. It was shown that a fraction of the nascent CYP1A1 chains were able to escape ER insertion and that these chains, once localized in the cytosol, were cleaved by an unknown cytosolic endoprotease. After removal of the NH_2-terminus, a cryptic mitochondrial targeting signal was exposed and the truncated CYP1A1 was imported into mitochondria. The introduction of the two positive charges in CYP2E1 might possibly compromise ER targeting, thereby allowing for the removal of the hydrophobic transmembrane domain and subsequent exposure of the mitochondrial targeting signal.

It has been demonstrated that specific pathways exist for import and processing of mitochondrial P450s in different tissues [31]. It was shown that in vitro translated precursors of CYP11A1 and CYP11B are only imported and processed to their mature form in mitochondria originating from steroidogenic tissues. Δ2E1 was only detected when Δ(2–29)2E1 and N^{++}2E1 were transfected in a cell line from hepatocyte origin, whereas no Δ2E1 could be observed when these CYP2E1 mutants were transfected into either COS-1 cells or a fibroblast cell line (data not shown). This suggests that mitochondrial import of these NH_2-terminal CYP2E1 mutants is also dependent on the cell type used.

Δ2E1 was shown to display catalytic activity when reconstituted with the mitochondrial electron transport proteins Adx and AdR. These two enzymes were essential for generating a significant level of catalytic activity. A similar Adx/AdR dependence was reported for the catalytic activity of an NH_2-terminally truncated CYP1A1, which was also shown to display a different substrate specificity compared to microsomal CYP1A1 [32]. An altered substrate specificity of Δ2E1, although demonstrating chlorzoxazone 6-hydroxylase activity, can at this point not be excluded. The physiological implications of the presence of a catalytically active truncated CYP2E1 present inside mitochondria are unknown, but the potential ethanol dependent induction of this protein and its capability to cause oxidative stress are interesting in light of the fact that mitochondrial swelling and apoptosis are early events in alcoholic liver disease [14,33].

In conclusion, we have for the first time described a soluble

catalytically active form of ER derived P450, which could perhaps be instrumental in obtaining a crystal structure.

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