Identification and Characterization of a Mitochondrial Targeting Signal in Rat Cytochrome P450 2E1 (CYP2E1)*

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Cytochrome P450 2E1 (CYP2E1) lacking the hydrophobic NH₂-terminal hydrophobic transmembrane domain is specifically targeted to mitochondria, where it is processed to a soluble and catalytically active form (Δ2E1) with a mass of about 40 kDa. Small amounts of Δ2E1 were also observed in mitochondria isolated from rat liver, indicating that this form of CYP2E1 is also present in vivo. In the present study the mitochondrial targeting signal was identified and characterized by the use of several NH₂-terminally truncated and mutated forms of CYP2E1 that were expressed in the mouse H2.35 hepatoma cell line. Two potential mitochondrial targeting sequences were identified in the NH₂ terminus of CYP2E1. Deletion of the first potential mitochondrial targeting sequence located between amino acids 50 and 65, as in Δ(2–64)2E1, still resulted in mitochondrial targeting and processing, but when, in addition to the first, the second potential mitochondrial targeting sequence located between amino acids 74 and 95 was also deleted, as in Δ(2–95)2E1, the mitochondrial targeting was abolished. Mutation of the four positively charged Arg and Lys residues present in this sequence to neutral Ala residues resulted in the abrogation of mitochondrial targeting. Deletion of a hydrophobic stretch of amino acids between residues 76 and 83 also abolished mitochondrial targeting and import. Once imported in the mitochondria, these constructs were further processed to the mature protein Δ2E1. It is concluded that mitochondrial targeting of CYP2E1 is mediated through a sequence located between residues 74 and 95 that positively charged residues as well as a hydrophobic stretch present in the beginning of this sequence are essential for this process.

The majority of the mitochondrial proteins are encoded from nuclear DNA, synthesized in the cytosol as precursors and postranslationally targeted to the mitochondria (1–3). Many of the proteins that are destined for import into the mitochondrial matrix contain a signal sequence in their NH₂ terminus that directs these proteins to the mitochondria (4), although these signal sequences are also found in the COOH terminus of the protein (5, 6). Despite the fact that there is no general consensus for mitochondrial matrix targeting sequences, many of these sequences were demonstrated to be rich in positively charged and hydrophobic amino acid residues and usually are able to form an amphiphilic secondary structure (7, 8). The mitochondrial matrix targeting sequence initially interacts with the import receptor (Tom20) that is part of the translocase of the outer membrane (TOM) complex present in the mitochondrial outer membrane (9, 10). The surface of Tom20, which is rich in negatively charged amino acid residues, is thought to recognize and bind the positively charged residues present in these signal sequences; however, additional binding forces such as hydrophobic interactions also play an important role (10). After binding, the protein is then allowed to cross the lipid bilayer through the general translocation pore formed by the TOM complex (11). Proteins destined for the matrix are then transferred to the translocase of the inner membrane (TIM) complex and translocated into the matrix space (12, 13), where the NH₂-terminal signal sequence of the protein is proteolytically removed by proteases present in the matrix (14, 15).

The NH₂-terminal hydrophobic transmembrane domain of microsomal cytochrome P450s (P450s) was shown not only to be responsible for the cotranslational targeting in a signal recognition particle (SRP)-dependent manner to the endoplasmic reticulum (ER) membrane (16, 17), but also to be responsible for anchoring and retention in the ER membrane (18, 19). Not all P450 enzymes are retained in the ER membrane by the same mechanisms; as was recently demonstrated, CYP2C1 is retained by static retention preventing exit from the ER, while CYP2E1 is retained by a retrieval mechanism allowing it to be recycled back from post-ER compartments (20, 21). In contrast to microsomal P450s, mitochondrial P450s are, like most of the nuclear encoded mitochondrial proteins, postranslationally targeted to mitochondria by means of their amphipilic NH₂-terminal signal sequence (22, 23).

Cytochrome P450 2E1 (CYP2E1) is the alcohol-inducible member of the P450 family and has been suggested to be involved in gluconeogenesis because of its ability to metabolize ketone bodies such as acetone (24, 25). In addition, a wide variety of small hydrophobic xenobiotics including many well known carcinogenic and toxic compounds are metabolized by CYP2E1 (24). CYP2E1 is also known for its ability to cause oxidative stress that could ultimately lead to apoptosis (26) and to alcoholic liver disease (27, 28).

Although CYP2E1 is predominantly located in the membrane of the ER, it has also been demonstrated to be present in lysosomes (29), peroxisomes (30), Golgi apparatus (31), and on the outer surface of the plasma membrane (32, 33). Previously

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The abbreviations used are: TOM, translocase of the outer membrane; ER, endoplasmic reticulum; P450, cytochrome P450; SRP, signal recognition particle; TIM, translocase of the inner membrane; mHsp70, mitochondrial heat shock protein 70; Adx, adrenodoxin; AdR, adrenodoxin reductase.
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TABLE I

Sequences of the oligonucleotides used as polymerase chain reaction primers

| Primer | Sequence 5’ to 3’ |
|--------|------------------|
| 1a     | GACGAACTTACGTTGACGCCTTCGCCACAGGA |
| 1b     | GACGAACTTACGTTTTATGCTGCTCTGATGCTAC |
| 1c     | GACGAACTTACGTATGTTGTTGCCAGTGCAGGAGT |
| 1d     | GACGAACTTACGTTACAGGAGACAGGAGGAGGAGGAGG |
| 1e     | GACGAACTTACGTTACGTAGGCTTACGATGAGGAGGAGG |
| 1f     | GACGAACTTACGTTACGTAGGCTTACGATGAGGAGGAGG |
| 1g     | GACGAACTTACGTTACGTAGGCTTACGATGAGGAGGAGG |
| 1h     | GACGAACTTACGTTACGTAGGCTTACGATGAGGAGGAGG |
| 1i     | GACGAACTTACGTTACGTAGGCTTACGATGAGGAGGAGG |
| 2a     | GACTCTAGATCATGAACGGGGAATGAC |
| 2b     | TGTAATCCCAGCAATTTGGGA |

The nucleotides coding for the bases that were mutated are represented in a boldface type, and the recognition sites for the restriction enzymes are depicted in italics.

we reported that an NH₂-terminally truncated form of CYP2E1 was specifically targeted to the mitochondria (34). Deletion or mutation of the NH₂-terminally hydrophobic transmembrane domain of CYP2E1 resulted in the mitochondrial localization of the protein when these constructs were transiently expressed in a mouse hepatoma cell line. It was demonstrated that this mitochondrially localized form of CYP2E1 was a NH₂-terminally truncated form of CYP2E1 of ~40 kDa named Δ2E1, which was shown to be soluble, catalytically active, and localized inside the mitochondria. In addition, Δ2E1 was present in low levels in vivo in mitochondria isolated from rat liver.

In the present investigation, the mitochondrial targeting sequence of CYP2E1 was identified by constructing several NH₂-terminal deletion mutants of CYP2E1, which lacked one or both putative mitochondrial targeting sequences that are present in the NH₂ terminus of CYP2E1. Moreover, the role of the positively charged and hydrophobic amino acid residues present in this sequence in the mitochondrial targeting was investigated by mutational analysis.

EXPERIMENTAL PROCEDURES

Expression Vectors—The CYP2E1 cDNAs encoding the truncated CYP2E1 variants Δ(2-29)2E1, Δ(2-64)2E1, Δ(2-76)2E1, Δ(2-95)2E1, and Δ(2-95)2E1 were generated by polymerase chain reaction amplification using Pfu DNA polymerase (Stratagene, La Jolla, CA), the appropriate sense primer containing an ATG codon, primers 1a, 1b, 1c, 1d, and 1e, respectively (Table I); the wild-type antisense primer 2a; and CYP2E1 cDNA as a template. The resulting cDNAs were cloned in between the two unique BsgI restriction sites located outside the cloning box; 235 bases 3′ of the XbaI site, and CYP2E1 cDNA also has a unique BglI restriction site at position 193 (corresponding to amino acid position 65) that incorporated the mutations, primer 1f for RK30 (corresponding to amino acid position 65) that incorporated the mutations were generated by polymerase chain reaction amplification using the sense primer containing a BglI site and the appropriate primers, primer 1h for R75A and primer 1i for RR30, the antisense primer that anneals just after the BglI site of the vector primer 2b, and pCMV-CYP2E1 as a template. The resulting cDNA fragments were cloned in between the two BglI sites of pCMV5 vector still containing the cDNA coding for the first amino acids of the construct. The correct sequences of all inserts were confirmed by DNA sequencing using the ABI PRISM® dye terminator cycle sequencing kit from PerkinElmer Life Sciences.

Cell Culture, Transient Transfection, and Western Blotting—H2.35 cells were grown and transfected as described (33, 34). Western blot analysis was performed as described (31) and immunoreactive bands were visualized using the Fuji Las-1000 luminescent image analyzer (Fujifilm, Sweden).

Immunofluorescent Microscopy—After transfection, the H2.35 cells were fixed and processed for immunostaining as described (33, 34). Transfected proteins were detected by using CYP2E1-specific antibodies and visualized by anti-rabbit fluorescein isothiocyanate-conjugated antibodies. Cells were double-immunostained by sequential incubation with CYP2E1-specific rabbit polyclonal antibodies, which were visualized by anti-rabbit fluorescein isothiocyanate-conjugated antibodies, followed by incubation with mitochondrial heat shock protein 70 (mHsp70)-specific monoclonal antibodies (Affinity Bioreagents Inc., Golden, CO), which were visualized by anti-mouse tetramethyl rhodamine isothiocyanate-conjugated antibodies. Cells were viewed under a Nikon Eclipse E600 fluorescence microscope equipped with a camera using the 60× oil immersion objective.

Catalytic Activity—The catalytic activity of several of the CYP2E1 variants was determined by monitoring the hydroxylation of chlorozoxazone (31, 34). The post-nuclear supernatant of cells transfected with Δ(2-29)2E1, Δ(2-64)2E1, Δ(2-95)2E1, or empty plasmid was prepared, and organelles were disrupted by sonication (three bursts of 10 s with 30-s intervals) on ice. Mitochondria were isolated from cells transfected with empty plasmid or Δ(2-29)2E1 and disrupted by sonication as described previously (34). The incubation mixture consisted of 250 μg of sonic disrupted post-nuclear supernatant or 100 μg of sonic disrupted mitochondria, 50 μM chlorozoxazone, a NADPH generating system (0.2 mM NADPH, 2.0 mM glucose 6-phosphate, and 3 units/ml glucose-6-phosphate dehydrogenase) and 50 μM phosphate buffer, pH 7.4, in a final volume of 250 μl in the presence or absence of 1.0 nmol of adrenodoxin (Adx), 0.1 nmol of adrenodoxin reductase (AdR), kindly supplied by Prof. Rita Bernhardt (University of Saarbrücken, Germany). After 30 min of incubation at 37 °C, the reaction was terminated by the addition of orthophosphoric acid, and samples were extracted and analyzed on an Anol Varian ProStar HPLC system (Varian, Walnut Creek, CA) equipped with an amperometric detector (Bioanalytical Systems Inc, West Lafayette, IN).

RESULTS

The mitochondrial targeting of a NH₂-terminally truncated form of CYP2E1, Δ(2-29)2E1, was investigated by constructing several NH₂-terminal deletion mutants of CYP2E1 (see Fig. 1). Examination of the first 100 amino acids of CYP2E1 revealed that there are two amino acid stretches that could serve as potential mitochondrial targeting signals (the underlined sequences shown in Fig. 1). Both these sequences are rich in positively charged and hydrophobic amino acids and are potentially able to form an amphiphilic secondary structure, features that are characteristic for a mitochondrial targeting signal.

H2.35 cells transfected with Δ(2-29)2E1 were analyzed by Western blotting and a NH₂-terminally truncated form of CYP2E1 of around 40 kDa named Δ2E1 (Fig. 2) was detected
Subcellular localization by immunofluorescent microscopy confirmed the mitochondrial localization of Δ2E1 (Fig. 3). Indeed, the staining pattern observed using CYP2E1-specific antibodies (Fig. 4, upper left panel) was identical to that observed using antibodies recognizing the mitochondrial protein, mHsp70 (Fig. 4, upper right panel). To identify the targeting sequence that was responsible for the mitochondrial targeting and import, two constructs were designed that lacked one or both potential targeting sequences.

Δ(2–64)E1, in which the first potential targeting signal (amino acids 51–64) was deleted, was transfected into H2.35 cells, which were analyzed by Western blotting. In addition to Δ2E1, a protein with the expected full length of the construct was observed (Fig. 2), and both these proteins were recovered in the mitochondrial fraction (data not shown). Immunofluorescent microscopy demonstrated that cells transfected with Δ(2–64)E1 displayed a similar staining pattern to that observed for Δ(2–29)E1-transfected cells (Fig. 3) and was shown to colocalize with mHsp70 (Fig. 4), confirming its mitochondrial localization. These results clearly demonstrated that deletion of the first 64 amino acids of CYP2E1 did not affect the mitochondrial targeting and that the region between amino acids 51 and 64 was not responsible for directing Δ(2–29)E1 to the mitochondria.

Δ(2–95)E1, in which in addition to the first potential mitochondrial targeting signal, also the second potential targeting sequence between amino acids 75 and 95 was deleted, was expressed in H2.35 cells. A protein with a slightly lower mobility than Δ2E1 was observed by Western blotting (Fig. 2), which corresponds to the expected full-length size of the construct. Immunofluorescent microscopy demonstrated that Δ(2–95)E1 was not associated with the mitochondria and instead displayed a diffuse staining pattern throughout the whole cell, most likely representing a cytosolic localization of this protein (Fig. 3). These data demonstrated that the region between amino acids 74 and 95 was responsible for the mitochondrial targeting and that this region served as a mitochondrial targeting signal.

Previously we showed that Δ2E1 was catalytically active in the presence of the mitochondrial electron carrier proteins Adx and AdR (34). The post-nuclear supernatant of cells transfected with Δ(2–29)E1, Δ(2–64)E1, and Δ(2–95)E1 was assayed for catalytic activity (Fig. 5). As expected, cells transfected with Δ(2–29)E1 displayed catalytic activity in the presence of both Adx and AdR. Additionally, cells transfected with Δ(2–64)E1 had significant chlorzoxazone hydroxylation activity with levels well over those of cells transfected with empty plasmid (mock). When incubated in the absence of Adx and AdR, no significant increase in catalytic activity was observed, indicating that these truncated CYP2E1 variants were not able to couple with the endogenous NADPH cytochrome P450 reductase. Despite the extensive deletion in Δ(2–95)E1, some residual catalytic activity could still be detected, although the observed levels were very low. The expression levels of these
three truncated proteins were found to be very similar (see Fig. 2), which indicated that only a part of the Δ(2–95)2E1 expressed was catalytically active. Mitochondria isolated from cells transfected with Δ(2–29)2E1 displayed a 3-fold increase in catalytic activity over mitochondria isolated from mock transfected cells (Fig. 5B), indicating that the catalytic activity is associated with the mitochondria as was also previously reported (34).

The involvement of the positively charged residues that are present in the mitochondrial targeting sequence identified above, Arg at positions 75 and 76 and Lys at positions 84 and 87, was studied by mutating all four these residues to uncharged Ala residues, mutant RK−AA. Western blot analysis revealed that only the full length of the construct was expressed, the transfected cells expressed similar subcellular distribution as for RR−AA was observed. Additionally, substitution of Arg-75 with Ala (R75A) had no effect on the mitochondrial targeting and import was not affected by these mutations (data not shown). In addition, Western blot analysis revealed that these transfected cells expressed Δ2E1, indicating that mitochondrial targeting and import was not affected by these mutations (data not shown). Additionally, substitution of Arg-75 with Ala (R75A) had no effect on the mitochondrial targeting and processing, the transfected cells expressed Δ2E1 (Fig. 2) and displayed the typical mitochondrial staining pattern (Figs. 3 and 4). These results indicated that the two Arg residues at position 75 and 76 and Lys at positions 84 and 87.

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Fig. 4. Mitochondrial localization of Δ(2–29)2E1, Δ(2–64)2E1, Δ(2–76)2E1, K−A, R75A, and RR−AA. H2.35 cells transfected with the various CYP2E1 constructs were immunostained with CYP2E1-specific antibodies, which were visualized with anti-rabbit fluorescein isothiocyanate-conjugated antibodies (left panels), and with the mitochondrial protein mHsp70-specific antibodies, which were visualized with anti-mouse tetramethyl rhodamine isothiocyanate-conjugated antibodies (right panels).

Fig. 5. Catalytic activity of the NH2-terminally truncated CYP2E1 variants. A, the post-nuclear supernatant isolated from H2.35 cells transfected with empty plasmid (Mock), Δ(2–29)2E1, Δ(2–64)2E1, and Δ(2–95)2E1 was assayed in the absence (solid bars) or presence (gray bars) of Adx and AdR for the formation of 6-hydroxychlorzoxazone (6-OH-CZN) as described under “Experimental Procedures.” B, mitochondria isolated from H2.35 cells transfected with empty plasmid (mock) and Δ(2–29)2E1 were assayed in the presence of Adx and AdR for the formation of 6-hydroxychlorzoxazone. The levels measured in the mitochondria isolated from mock transfected cells was arbitrarily set at 100%. Data are represented as mean ± S.E. The numbers in parentheses at the bottom of the graph indicate the number of individual determinations. Significant differences are indicated by an asterisk (p < 0.05).
tions 75 and 76 alone were not essential for the mitochondrial targeting and import of Δ(2–29)2E1.

Partial disruption of the mitochondrial targeting signal as in Δ(2–76)2E1 and Δ(2–82)2E1 resulted in lower expression levels that were difficult to detect by Western blot analysis. The few transfected cells were, however, easily detected by immunofluorescent microscopy. Some of the cells transfected with Δ(2–76)2E1, in which the NH2 terminus including Arg-75 and Arg-76 was deleted, displayed the typical mitochondrial staining pattern, while others had a more diffuse staining pattern, a pattern similar to that observed for K-A-transfected cells (Figs. 3 and 4). This was in good agreement with the results obtained above, which showed that Arg-75 and Arg-76 had only a minor effect on mitochondrial targeting. Further disruption of the mitochondrial targeting signal as in Δ(2–82)2E1 resulted in the diffuse cytoplasmic staining pattern in cells transfected with this construct, indicating that the hydrophobic region between amino acids 76 and 83 was essential for the mitochondrial targeting of Δ(2–29)2E1 (Fig. 3).

**DISCUSSION**

The hydrophobic NH2 terminus of P450s is responsible for the cotranslational targeting of the protein to the membrane of the ER (16, 17). Deletion of the hydrophobic ER targeting signal as in Δ(2–29)2E1 results in targeting of this truncated protein to the mitochondria (34). In the present study, it was demonstrated, by sequential deletion of the NH2 terminus and by mutational analysis, that the region between amino acids 74 and 95 is responsible for the mitochondrial targeting of CYP2E1. The absence of four positively charged amino acid residues as well as a stretch of hydrophobic residues at the beginning of this targeting sequence was shown to be essential for the mitochondrial targeting and import of Δ(2–29)2E1. Western blot analysis showed that Δ2E1 displayed a slightly higher mobility than Δ(2–95)2E1, suggesting that Δ2E1 is a NH2-terminally truncated form of CYP2E1 lacking approximately the first 100 amino acids.

It is well known that many of the proteins that are targeted to mitochondria contain a mitochondrial targeting signal, also called a presequence, which is usually located at the NH2 terminus of the protein (1–4). Attempts to define a general consensus sequence for these mitochondrial targeting signals have not resulted in a well-defined motif as is known for proteins targeted to other intracellular organelles such as peroxisomes (35) or the endoplasmic compartment (36). General features valid for most of the mitochondrial targeting sequences are that they are usually rich in positively charged and hydrophobic amino acid residues, they do not contain any or very few negatively charged residues, and they have the potential to form an amphiphilic secondary structure (7, 8, 37). It is believed that the positively charged residues together with the hydrophobic residues are essential for binding to the negatively charged surface of the Tom20 receptor (10, 11). Recently, however, NMR studies have shown that the initial interaction between the signal sequence of rat aldehyde dehydrogenase and the rat Tom20 receptor was entirely mediated by hydrophobic interactions (38). The initial binding was shown not to be dependent on ionic interactions, thereby reducing the role for the positively charged residues of the presequence in the initial binding. This agreed well with the observation made in the present study; the hydrophobic region at the beginning of the mitochondrial targeting signal (amino acids 77–82) of CYP2E1 is essential for targeting to and import into the mitochondria. The positive charges of the presequence were shown to be important for transport across the mitochondrial outer and inner membrane by sequential binding to several components of the TOM and TIM complex (the “acid chain hypothesis”) (11–13) and also are thought to be necessary for recognition by the mitochondrial processing peptidase (39).

Mutation of all four positively charged residues present in the mitochondrial targeting signal of CYP2E1 to uncharged Ala residues effectively prevented mitochondrial targeting and subsequent import as shown by both immunofluorescent microscopy and Western blot analysis. However, mutation of only one or both Arg residues at the beginning of this signal did not have any major effect on the mitochondrial targeting and import. Mutation of only the two Lys residues resulted in an intermediate result; although mitochondrial targeting and import was still observed, it was less efficient as compared with the construct Δ(2–64)2E1 where the two Lys residues were still present. Deletion of the NH2 terminus until just after the two Arg residues, as in Δ(2–76)2E1, still resulted in mitochondrial targeting, although less efficient, indicating that these Arg residues are not essential for mitochondrial targeting and import. These results implied that all four positively charged residues together are essential for mitochondrial targeting, and subsequent import and processing of Δ(2–29)2E1. The construct Δ(2–82)2E1, where, in addition to the two Arg residues, a hydrophobic stretch of amino acids was deleted, displayed a similar staining pattern as observed for Δ(2–95)2E1, i.e. a cytosolic localization. This strongly suggests that the hydrophobic region between Arg-76 and Lys-84 is critical for the mitochondrial targeting of Δ(2–29)2E1.

Despite the fact that Δ(2–95)2E1 displayed catalytic activity, it was lower than that observed in cells transfected with Δ(2–29)2E1 and Δ(2–64)2E1, which expressed Δ2E1. In addition, it was further demonstrated that the catalytic activity is localized in the mitochondria. These data suggest that to efficiently assemble a catalytically active Δ2E1 the folding and processing machinery present in the mitochondrial matrix is required. It should, however, be emphasized that the observed activities as well as the protein expression levels are very low when compared with those observed for wild-type CYP2E1. The demonstration that the catalytic activity is associated with isolated mitochondria, together with the observation that all the mitochondrial localized CYP2E1 variants are processed to the mature Δ2E1, strongly indicates that Δ2E1 is present in the mitochondrial matrix.

The results obtained in the present study allow the construction of a model that can explain the mitochondrial targeting of CYP2E1, where binding of the SRP and subsequently the cotranslational targeting pathway has been compromised by either deletion or mutation of the hydrophobic NH2-terminal transmembrane domain. The translation of CYP2E1 is normally initiated on ribosomes present in the cytosol and binding of the SRP to the hydrophobic NH2 terminus arrests this translation as soon as the nascent chain emerges from the ribosome (16, 17, 40). Translation only commences when the arrested complex is properly docked to the translocation complex present in the membrane of the ER (40). Deletion or modification of the hydrophobic NH2 terminus of CYP2E1 compromises the binding of the SRP, resulting in the translation of the entire protein on the cytosolic ribosomes. After synthesis, these proteins are most likely bound by cytosolic chaperones, targeted to the mitochondria by the exposed mitochondrial targeting sequence located between residues 74 and 95. After interaction with the TOM and TIM complex, CYP2E1 is translocated into the matrix of the mitochondria were the presequence is removed by the mitochondrial processing peptidase resulting in the mature protein, Δ2E1.

Analysis of the secondary structure of the region of CYP2E1 between amino acids 74 and 95 revealed that the region from 83 to 95 was able to form an α-helical structure, while the
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region from 76 to 80 preferentially forms a β-sheet. The presence of the α-helical part alone is not sufficient for targeting of Δ2–29/2E1 to the mitochondria. Deletion of the hydrophobic part between residues 76 and 83 abolished the mitochondrial targeting as observed for Δ2–82/2E1, emphasizing the importance of this hydrophobic stretch in the initial targeting and binding of the protein to the mitochondria. It can be suggested that, for the α-helical structure to bind to the hydrophobic groove present in the Tom20 receptor, an initial interaction of the hydrophobic residues located between residues 76 and 83 is necessary for the binding of the presequence to the mitochondrial import receptor. After proper binding of the presequence to Tom20, the presence of the positively charged amino acid residues becomes important for the actual translocation over the outer and inner membrane of the mitochondria and also for the interaction with the proteolytic and folding machinery in the mitochondrial matrix.

A model has been proposed for the mitochondrial targeting of an NH2-terminal truncated form of CYP1A1 expressed in both β-naphthoflavone-induced rat liver and transiently in COS cells (41). By using an in vitro translation assay in the presence or absence of canine pancreatic microsomes and isolated rat liver cytosolic proteins, it was suggested that up to 25% of the nascent chains were able to escape cotranslational targeting. After proper binding of the presequence to Tom20, the presence of the positively charged amino acid residues becomes important for the actual translocation over the mitochondrial matrix. These escaped chains were processed by a cytosolic endopeptidase, resulting in the exposure of a mitochondrial targeting signal that directed the truncated CYP1A1 to the mitochondrial import receptor. After proper binding of the presequence to Tom20, the presence of the positively charged amino acid residues becomes important for the actual translocation over the outer and inner membrane of the mitochondria and also for the interaction with the proteolytic and folding machinery in the mitochondrial matrix.

In conclusion, when SRP binding to the hydrophobic NH2-terminal transmembrane domain of CYP2E1 is compromised, protein translocation is not arrested and the entire CYP2E1 protein is translocated in the cytosol. A mitochondrial targeting signal located between amino acid residues 74 and 95 is being exposed, probably after binding of the translated CYP2E1 protein with cytosolic chaperones, and the protein is targeted to the mitochondria. The mitochondrial targeting and import of Δ2–29/2E1 is strongly dependent on the presence of positively

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