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Critical amino-terminal segments in insertion of rat liver cytochrome P450 3A1 into the endoplasmic reticulum membrane

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Abstract. An in vitro transcription-translation assay was used to study the membrane topology of rat liver cytochrome P450 3A1. N-terminus deletion mutants were constructed to assess the importance of N-terminal regions in the stable incorporation of the protein into the microsomal membranes. Wild-type nascent cytochrome P450 bound to microsomes as an integral membrane protein through its hydrophobic N-terminal segments, uncleaved by signal peptidase. Deletion of the most N-terminal hydrophobic segment (positions 7–26) had a dramatic effect on endoplasmic reticulum membrane integration. Confirming the essential role of this stretch in P450 3A1 membrane targeting, proteolysis-resistant membrane-associated peptides were observed in all the in vitro translated mutants containing that segment. It is concluded that the membrane topogenesis of P450 3A1 is determined mainly by the amino-terminal hydrophobic segment.
Key words. Cytochrome P450; membrane topology; endoplasmic reticulum; in vitro translation; rat liver.

The hepatic forms of cytochrome P450 are membrane-resident haemoproteins which are synthesized in membrane-bound polysomes and co-translationally translocated into the endoplasmic reticulum (ER) [1]. These enzymes, together with NADPH-P450 reductase (E.C.1.6.2.4) and cytochrome b_5 are components of the P450-monooxygenase system, which is responsible for the metabolism of a wide variety of structurally diverse substrates. Cytochrome P450 stands for a large number of related enzymes encoded by a gene superfamily that comprises at least 36 families, 12 of which exist in all mammals [2].

Knowledge of the membrane topology of the components of the monooxygenase system is important for understanding structural and functional interactions between these enzymes. Therefore, numerous studies have been performed on the membrane topology of the components of the monooxygenase system. It is reported that NADPH-P450 reductase is inserted in the membrane via a 6-kDa N-terminal peptide, probably spanning the membrane twice [3]. On the other hand, cytochrome b_5 does not seem to contain a transmembrane spanning segment, but rather appears to be integrated by a membrane-embedded segment which has the C-terminus and the N-terminus exposed on the same side of the membrane [4, 5]. It appears most likely that liver P450 haemoproteins are anchored to the ER membrane by sequences located at the N-terminus, exposing the bulk of the protein to the cytoplasm [6, 7]. Based on experimental data obtained for several different forms of liver cytochrome P450, two topological models have been proposed [8–10]. One of them postulates the existence of a single transmembrane peptide anchor encompassing the first 25 residues, whereas the other proposes the existence of a transmembrane hairpin loop.

In the present paper, a study is reported on the membrane topology of rat liver P450 3A1 using an in vitro transcription-translation assay of wild-type and N-terminal truncated mutants. Our results demonstrate that P450 3A1 is inserted into the microsomal membranes through the first N-terminally-located hydrophobic domain.

Materials and methods

Rat liver CYP3A1 full-length cDNA [11] (EMBL access no. X64401) was subcloned into pBluescript II SK (Stratagene), after deletion of the extra ATG located 66 nucleotides upstream of the translation initiation codon. The resulting CYP3A1 cDNA, containing the 1512 bp coding region plus the 5′ (8 bp) and the 3′ (28 bp) adjacent noncoding sequences, was inserted in the vector downstream of the T7 promotor.

Site-directed mutagenesis was performed using the polymerase chain reaction (PCR). Oligonucleotides were synthesized on a Pharmacia Gene Assembler Plus, and were subsequently purified by gel electrophoresis. PCR was carried out using a Braun Thermocycler 60, for 30 cycles, in a medium containing 10 mM Tris-HCl, pH

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                       10        20        30        40        50        60
                   -    -                   +    ++      +                 +
P450 3A1     MDLLSALTLE TWVLLAVVLV LLYGFGTRTH GLFKKQGIPG PKPLPFFGTV LNYYMGLWKF

3A1Δ7-26     MDLLSA---- ---------- ------TRTH GLFKKQGIPG PKPLPFFGTV LNYYMGLWKF

3A1Δ7-35     MDLLSA---- ---------- ---------- -----QGIPG PKPLPFFGTV LNYYMGLWKF

3A1Δ42-58    MDLLSALTLE TWVLLAVVLV LLYGFGTRTH GLFKKQGIPG P--------- --------KF
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Figure 1. N-terminal sequences of wild-type and mutant cytochrome P450 proteins. Potential transmembrane sequences as deduced from Nelson and Strobel [8] are underlined. Positively and negatively charged residues are indicated in the wild-type sequence (histidine is assumed to be uncharged).

7.4, 50 mM KCl, 2 mM $MgCl_2$, the nucleotide triphosphates at a concentration of 0.1 mM each, 1 μg/ml of wild-type CYP3A1-pBluescript II SK, primer oligonucleotides at concentrations of 0.3–1.4 nM, and 2.5–5 U/100 μl of Taq polymerase (Perkin-Elmer Cetus). The PCR product was subsequently treated with the appropriate restriction enzymes, and the digestion product was gel-purified and ligated to the wild-type cDNA-pBluescript previously digested with the same restriction enzyme. The recombinants obtained were checked by sequencing the mutated region using a Sequenase (United States Biochemical) or a Pharmacia T_7 sequencing kit.

Constructs were linearized using EagI or EcoRI, which cut at unique sites located in the 3′ noncoding region of the cDNA. Subsequently, transcription and capping were carried out using the mCAP mRNA Capping Kit (Stratagene). The size of the transcribed mRNA was checked by agarose gel electrophoresis. In vitro translation was carried out using rabbit reticulocyte lysate, pretreated with nuclease from *Micrococcus* [12]. The reaction mix (final volume 24 μl) contained: 0.5 μg RNA, 1 mM ATP, 0.2 mM GTP, 20 mM creatine phosphate, 70–80 mM KCl, 0.5 mM dithiothreitol, 0.5 mM spermidine, 0.5 mM CTP, 1.7 μg calf liver tRNA, 50 μM of each amino acid (except methionine) and 50 μM [^{35}S]-methionine (1000 Ci/mmol, Amersham International). Where indicated, 5 μl dog pancreas microsomes (Amersham) were included in the translation mixture. Reactions were carried out for 60 min at 30 °C.

Neosynthesized proteins were analyzed by SDS-polyacrylamide gel (PAGE) as described by Laemmli [13]. After electrophoresis, gels were treated with Amplify (Amersham) and dried, and autoradiography was performed at −80 °C.

Association of polypeptides with microsomal membranes was determined using alkaline extraction [14]. In short, 20 μl of the translation mix was incubated for 30 min at 0 °C with 4 ml of 0.1 M Na_2CO_3 (pH 11). The mixture was centrifuged for 2 h at 45,000 rpm in a Beckman SW 60 Ti rotor. Polypeptides were precipitated with 10% trichloroacetic acid (plus 50% acetone in samples containing Triton), and analyzed by gel electrophoresis.

Membrane-associated polypeptides were analyzed by the four-lane protease assay [15]. This test is based on the fact that microsomal membranes are impermeable to proteinase K and therefore only those proteins exposed to the cytoplasmic side of the membrane are digested [15]. A volume of 5 μl of translation mix was incubated with 0.1–0.2 mg/ml proteinase K at 0 °C for 2 h in the presence or absence of 1% Triton X-100. After proteolysis inhibition with with 2 mg/ml of phenylmethylsulfonyl fluoride, polypeptides were analyzed by gel electrophoresis using 7.5 or 10% gels. Low molecular mass peptides (1–15 kDa) were analyzed on 22% gels containing 6 M urea [16].

Results

In order to investigate the mode of interaction of the amino-terminal hydrophobic regions of P450 3A1 with the ER membrane, deletion mutants were constructed. Figure 1 shows the N-terminal sequences of the wild-type and the various truncated mutants which have deletions of either the first or the second hydrophobic segment (Δ7–26 and Δ42–58) and also of the first

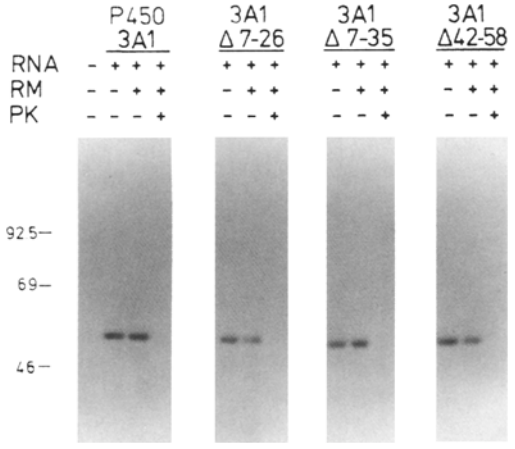

Figure 2. In vitro translation of cytochrome P450 proteins. In vitro translation was carried out as described in the 'Materials and methods'. Extra additions are indicated as RM (dog pancreas microsomes) and PK (Proteinase K). Analysis was performed by PAGE (7.5% gel). Molecular masses are indicated using Rainbow markers (Amersham).

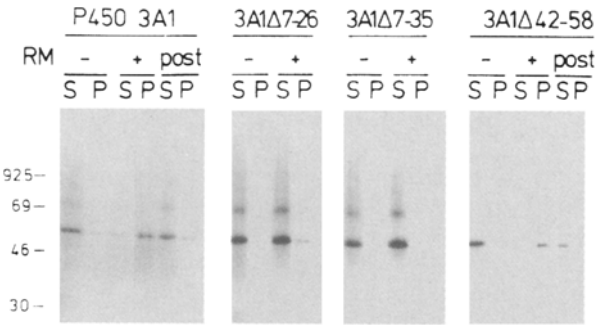

Figure 3. Membrane insertion of cytochrome P450 proteins. RNAs encoding the various constructs were translated in the presence or absence of microsomes (RM), or the membranes were added after translation had been terminated (post). Association of proteins to membranes was established by their resistance to sodium carbonate extraction at pH = 11. S indicates the supernatant and P the pellet fraction. Analysis was performed by PAGE (10%) gel. Molecular masses are indicated using Rainbow markers (Amersham).

hydrophobic segment plus the contiguous positively charged region (Δ7–35).

In vitro transcription-translation analysis of P450 3A1 yielded a protein of the expected size, i.e. 57 kDa (fig. 2). The various deletion mutants migrated slightly faster than the wild-type protein, in good agreement with the cDNA constructs. The presence of dog pancreas microsomes during translation did not alter the electrophoretic mobility of the translated proteins, indicating that P450 3A1 does not contain a cleavable signal sequence, similar to other forms of liver microsomal cytochrome P450 [17, 18]. Post-translational treatment with proteinase K caused disappearance of the in vitro synthesized proteins, regardless of the presence of microsomes in the translation mix, showing that most of the protein is accessible to proteinase K. It has long been shown that microsomes retain their closed vesicular structure after protease treatment and remain impermeable to macromolecules [15, 19, 20]. Therefore, the complete disappearance of the P450 proteins observed following proteinase K treatment indicates that the in vitro synthesized proteins are exposed to the extramicrosomal space [15, 20].

Association of the cytochrome P450 proteins to microsomal membranes was studied using alkaline carbonate extraction [14]. This treatment removes all secretory and peripheral proteins from the microsomes, yielding a microsomal pellet containing the membrane-anchored proteins and a supernatant containing all other proteins. Figure 3 shows that no sedimentation of cytochrome P-450 3A1 occurred when translation was carried out in the absence of microsomes or when microsomes were added post-translationally, indicating that nonspecific sedimentation did not occur to any significant extent. Translation in the presence of microsomes revealed that wild-type P450 3A1 and mutant 3A1Δ42–58 were membrane associated (fig. 3). Mutants lacking the amino-terminal hydrophobic segment, i.e. 3A1Δ7–26 and 3A1Δ7–35, did not integrate into the membrane.

As shown in fig. 2, proteinase K digested the major part of the cytochrome P450 protein. Since this enzyme cannot penetrate the membrane, analysis of the peptides produced by proteinase K treatment should reveal membrane-protected segments. Figure 4 shows that digestion of P450 3A1 yielded a membrane-protected peptide with an apparent molecular mass of about 2 kDa, which is similar to the expected size of a single membrane-spanning segment. Alkaline extraction of proteinase K-treated samples revealed that this peptide from wild-type cytochrome P450 3A1 was recovered predominantly in the pellet fraction (fig. 4), indicating that it is membrane associated. In the presence of Triton X-100 no proteinase K-resistant peptides were observed, confirming that protease resistance was mediated by the microsomal membranes. Mutants 3A1Δ7–26 and 3A1Δ7–35, lacking the amino-terminal-hydrophobic segment, did not produce a membrane-protected peptide, while mutant 3A1Δ42–58 behaved in a similar way to the wild-type protein (results not shown). It should be noted that proteinase K treatment yielded a second membrane-protected peptide (about 7 kDa). Since this peptide was recovered in the supernatant fraction after alkaline extraction, it probably represents an internal fragment protected from the protease by its peripheral association with the membrane.

Discussion

The results obtained in this study, based on experiments on membrane integration of the nascent wild-type apoprotein and its N-terminal truncated forms, show that

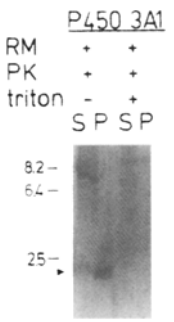

Figure 4. Proteinase K-resistant peptides from cytochrome P450 proteins. RNAs were translated as indicated before. Total translation products were subjected to proteinase K treatment, in the presence or in the absence of 1% Triton X-100 as indicated. Subsequently, membrane association was determined by the alkaline extraction method. Proteins were analyzed by electrophoresis on a 22% polyacrylamide/6 M urea gel. S indicates supernatant and P the pellet fraction. Proteinase K-resistant membrane-associ- indicated by an arrowhead.

cytochrome P450 3A1 is targeted to the ER membrane through sequences located at the N-terminus. As described for other hepatic forms of cytochrome P450 [6, 7], in P450 3A1 the short N-terminal signal-anchor peptide is not cleaved.

Truncated P450 3A1 mutants lacking the amino-terminal hydrophobic segment, i.e. 3A1Δ7–26 and 3A1Δ7–35, did not integrate into the membranes and did not give proteinase K-resistant membrane-protected peptides. For 3A1Δ7–26 it might be argued that the polycationic region preceding the second hydrophobic stretch would prevent membrane integration. The results with mutant 3A1Δ7–35, which lacked both the amino-terminal hydrophobic stretch and the contiguous polycationic region, show that this was not the case and that the second hydrophobic sequence, corresponding to residues 36–58 in P450 3A1, cannot replace the native signal-anchor peptide. Conversely, deletion of most amino acid residues in the second apolar domain, as in mutant 3A1Δ42–58, did not significantly impair in vitro anchoring of the nascent peptide into the microsomes. Therefore, our results demonstrate a major role for the first N-terminal hydrophobic segment of cytochrome P450 3A1 in the integration of the nascent protein into the ER membrane. This observation confirms the theoretical predictions of the membrane-spanning domain [10, 21], indicating that residues 7–26 of P450 3A1 constitute a single transmembrane segment.

Studies on the role of the N-terminus in membrane integration of cytochrome P450 have led to two models; either a single transmembrane peptide anchor situated in the first N-terminal hydrophobic segment, or a transmembrane hairpin loop containing the first two N-terminal hydrophobic segments [8–10]. The latter model has been supported by labelling studies with a fluorescent probe [22]. On the other hand, results of proteolysis studies could be fitted into both models [9]. Recent studies have revealed that besides N-terminal segments, other domains of the P450 proteins can be involved in the structural interaction with the ER membrane [23–25], a hypothesis not assessed in the present study on the P450 3A1 form. The results presented in this paper support the first topological model, i.e. membrane integration being mainly mediated by the first N-terminal hydrophobic segment. A similar conclusion has been drawn for P450 members from different families, such as P450 2B1 [6, 26, 27] and P450 2B4 [28, 29], in spite of the relatively low degree of structural homology with P450 3A1. Thus, most evidence from various P450s now favors a topological model where the protein is inserted into the ER membrane by a single transmembrane peptide anchor localized at the N-terminus of the protein. Whether this is a general feature of all liver microsomal P450 forms, or whether some P450s can have a membrane hairpin anchor, remains to be established.

In conclusion, the results presented in this paper demonstrate that P450 3A1 is targeted and anchored to the microsomal membrane through the first native N-terminal hydrophobic segment, which cannot be replaced in this function by an alternative hydrophobic polypeptide region of the same protein.

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