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Effects of amino-terminus truncation in human cytochrome P450IID6 on its insertion into the endoplasmic reticulum membrane of *Saccharomyces cerevisiae*

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A truncated form of cytochrome P450IID6 deprived of 22 NH_2-terminal amino acids residues (P450IID6Δ1–22) was found in both the cytosol and the microsomal fraction of the yeast, *Saccharomyces cerevisiae*. A reduced CO difference spectrum of this form was characterized by the absence of absorption at 448 nm and weak absorption at 420 nm. Another peculiarity of P450IID6Δ1–22 expression was its reduced content in the yeast cells compared to that of P450IID6, with the intracellular levels of the corresponding mRNAs being the same. We suggest that the deleted form of P450IID6, i.e. lacking 22 NH_2-terminal amino acid residues, is not inserted properly in the endoplasmic reticulum membrane: it does not take up the proper conformation to enable normal heme binding and is degraded in the yeast cells.

Cytochrome P450IID6; Amino-terminus; Endoplasmic reticulum membrane; Yeast; Heterologous expression

1. INTRODUCTION

Cytochrome P450IID6, like other members of the cytochrome P450 family, is an integral part of the endoplasmic reticulum. According to the latest findings, these proteins seem to be cytoplasmic with a short NH_2-terminus spanning the membrane [1]. Most probably, the latter fulfils two functions [2–4]. First, it plays the part of an uncleavable signal sequence by providing a means for signal recognition particle-mediated insertion of the growing polypeptide chain into the membrane. Second, it serves as a stop–transfer signal which arrests co-translational translocation of the polypeptide chain through the membrane such that it is predominantly exposed to the cytoplasm.

To verify this suggestion cDNAs for truncated forms of P450s lacking 22–30 amino-terminal amino acid residues were constructed and expressed in bacterial and yeast cells [5–8]. These truncated P450s exhibited catalytic activity independent of whether they were membrane bound [5,6] or soluble [8].

Thus the functions of the amino-terminal amino acid residues in cytochrome P450 topogenesis are still obscure. In this work we have made an effort to clarify the role of the amino-terminus of human cytochrome P450IID6 in its insertion into the endoplasmic reticulum membrane of the yeast, *Saccharomyces cerevisiae*.

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2. MATERIALS AND METHODS

The nucleotide sequence encoding cytochrome P450IID6 was prepared from a plasmid containing the CYP2D6 gene with a modified 5′ region by the PCR method [9]. These procedures will be described in detail elsewhere. The truncated version of the CYP2D6 gene lacking the codons for 22 NH_2-terminal amino acids (CYP2D6Δ1–66) was constructed by the PCR method, too. To this end the initial nucleotide sequence was cloned in the pGEM4 plasmid and used as a template for amplification. The PCR copy was prepared using the CGGATCCATGCACCGGCGC primer with twelve 3′-terminal nucleotides identical to the 67–79 region of the CYP2D6 gene and seven 5′-terminal nucleotides designed to form a *Bam*HI restriction site in the PCR copy, as well as the standard GTCGACTCTAGAG primer that is part of the pGEM4 polylinker region. Taking into account the large size of the DNA fragment under copying, the amplification (30 cycles: 94°C, 0.5 min; 55°C, 2 min; 75°C, 3 min) was carried out with Pfu DNA polymerase (Stratagene, USA) with a high 3′–5′ endonuclease activity and a low level of errors during DNA replication. The PCR product was cleaved by *Bam*HI and *Eco*RI endonucleases, cloned in pGEM4 and sequenced according to [10]. The clones bearing the CYP2D6Δ1–66 gene free of side mutations were selected. The CYP2D6 and CYP2D6Δ1–66 genes were re-cloned into the pYeDP1/8–2 shuttle vector under the control of the inducible GAL10-CYC1 promoter [11] at the *Bam*HI/*Eco*RI restriction sites as described [12]. The resulting constructions were used to transform *Saccharomyces cerevisiae* strain 2805 (a MAT pep4::His3 prb 1-δ can1 Gal2 his3δ ura3-52) by electroporation [13]. The conditions for promoter induction were given in [11].

Synthesis of mRNAs specific for P450IID6 and P450IID6Δ1–22 was followed by Northern hybridization [14]. The yeast microsomal fraction was isolated as in [11]. Immunoblotting was performed ac-

cording to [15] using monoclonal anti-P450IID6 antibodies. The content of P450IID6 in the microsomal fraction was evaluated from reduced CO difference spectra recorded with a Hitachi 557 spectrophotometer automatically correcting the base-line. The extinction coefficient for cytochrome P450 was taken equal to 91 $mM^{-1} \cdot cm^{-1}$. The protein concentration in the microsomal fractions was measured in the presence of 1% sodium cholate by the method of Lowry [16].

Oxidation of debrisoquine by the microsomal fractions was performed in a reaction mixture containing 2.4 mM debrisoquine, 4.8 mM NADPH and 0.56 μM microsomal P450IID6 in 0.1 M potassium phosphate buffer (pH 7.4) at 37°C. The reaction products were extracted with methylene chloride and analyzed by high pressure liquid chromatography using a Supelco C18 5μ column (250 × 4.6 mm) and a Varian Star-9000 high pressure chromatograph supplied with a fluorescent detector (λ_{ex} = 220 nm; λ_{em} > 300 nm).

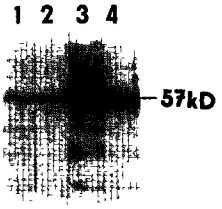

Fig. 2. Western blot analysis of P450IID6 and P450IID6Δ1–22 in the transformed yeast cells. Lanes: 1, 60 μg of post-microsomal supernatant protein from 2805(pYeDP/CYP2D6Δ1–66); 2, 15 μg of microsomal protein from 2805(pYeDP/CYP2D5Δ1–66); 3, 15 μg of microsomal protein from 2805(pYeDP/CYP2D6); 4, 30 μg of microsomal protein from human hepatocytes.

3. RESULTS AND DISCUSSION

3.1. *Synthesis of mRNAs for cytochromes P450IID6 and P450IID6Δ1–22 in* Saccharomyces cerevisiae

Total RNA was isolated from the transformed yeast cells growing for 24–36 h and analyzed by the Northern hybridization method using ^{32}P-labeled cDNA for P450IID6 as a probe. Fig. 1 shows that mRNA, hybridized with the specific probe, was accumulated in the yeast strains bearing plasmids YeDP (CYP2D6) and YeDP(CYP2D6Δ1–66). The length of mRNA, synthesized in these strains, was about 2,000 bp. Thus, cDNAs encoding both full-size P450IID6 and its shortened form, lacking the NH_2-terminal membrane insertion signal, were transcribed in the yeast cells. Judging from the intensities of the bands in the autoradiographs, as well as from radioactivities of the corresponding zones of the gels, evaluated by liquid scintillation counting, the transcription of both cDNAs was maintained at nearly the same level.

3.2. *Characterization of P450IID6 synthesized in yeast cells*

To characterize P450IID6 synthesized in yeast cells microsomal and post-microsomal fractions were isolated and subjected to SDS-electrophoresis in 10% polyacrylamide gels followed by immunoblotting with monoclonal antibodies against P450IID6. As is seen from Fig. 2, the microsomal fraction of yeast transformed with the plasmid containing the cDNA for P450IID6 contains a protein reacting to the anti- P450IID6 antibodies. The mobility of this protein during SDS-PAGE was identical to that of P450IID6 detected by the same method in human liver microsomes (Fig. 2), and corresponded to a molecular mass of 57 kDa in line with literature data [17].

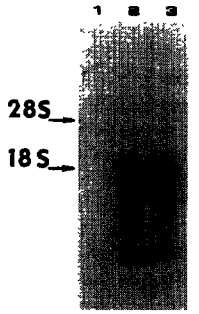

Fig. 1. Northern blot analysis of P450IID6 and P450IID6Δ1–22 mRNAs. The total nucleic acid fraction (10 μg) prepared from 2805(pYeDP) (lane 1), 2805(pYeDP/CYP2D6) (lane 2) and 2805(pYeDP/CYP2D6Δ1–66) (lane 3) were analyzed by 1% agarose gel electrophoresis, transferred to nitrocellulose filters, and then hybridized. The arrows indicate the positions of the yeast 18 S and 28 S RNA bands used as markers.

Fig. 3 shows the CO difference spectrum of the microsomal fraction with a pronounced peak at 448 nm specific for all native cytochrome P450s. Absorbance at this wave length corresponded to 160 pmol of P450IID6 per mg of microsomal protein, i.e. 0.9% of the total microsomal protein.

As could be expected on the basis of the spectral data, P450IID6, incorporated into the yeast microsomal membranes, was able to catalyze oxidative hydroxylation of its specific substrate, namely debrisoquine. Fig. 4 represents the results of analysis of the reaction mixtures by HPLC. Comparing chromatographic patterns for the cases when the mixture contained (dotted lines) and did not contain (solid lines) P450IID6 in the yeast microsomes, it can be seen that the arrow-marked peak corresponds to the product of debrisoquine conversion. A similar pattern was observed when human liver microsomes were substituted for microsomes from yeast transformed with YeDP(CYP2D6) (not shown).

Thus, the cDNA for P450IID6 when expressed in yeast cells, gives the intact protein localized mainly in the endoplasmic reticulum membrane and capable of transforming specific substrates by coupling with endogenous NADPH:cytochrome P450 reductase.

3.3. *Characterization of P450IIDΔ1–22 synthesized in yeast cells*

In this case fractionation of the transformed cells followed by SDS-PAGE and immunoblotting demonstrated a protein reacting to the anti-P450IID6 antibodies and characterized by molecular weight as close to that of P450IID6 (Fig. 2). Two specific details should be mentioned: (1) the total intracellular content of

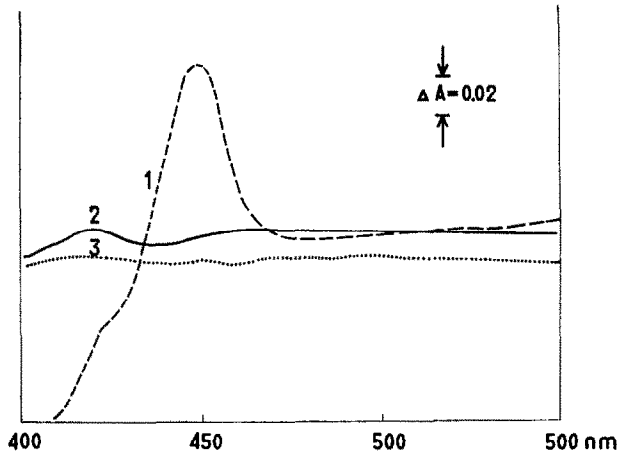

Fig. 3. Fe^{2+} vs. Fe^{2+} CO difference spectra of microsomes and total cell homogenate. The differential absorption spectra (sodium dithionite reduced carbon monoxide) of a yeast microsome suspension (in 100 mM KH_2PO_4, pH 7.4) and total cell homogenate were recorded: 1, microsomes from 2805(pYeDP/2CYP2D6); 2, microsomes from 2805(pYeDP/CYP2D6Δ1–66); 3, total cell homogenate from 2805(pYeDP/CYP2D6Δ1–66).

P450IIDΔ1–22 was approximately one order of magnitude lower than that of P450IID6, (ii) the intracellular localization of P450IID6Δ1–22 was dual, i.e. both microsomal and cytosolic, since an essential fraction of the protein was detected in the post-microsomal supernatant. As mentioned above, the content of mRNAs for P450IID6 and P450IID6Δ1–22 were similar if not identical. Therefore the reduced content of P450IID6Δ1–22 is likely to have resulted from its enhanced degradation which in turn was due to aberrant insertion into the endoplasmic reticulum membrane.

In contrast to P450IID6, P450IID6Δ1–22 synthesized in the yeast cells did not show any absorbance at 450 nm, either in the microsomal or cytosolic fractions (Fig. 3). A very weak peak at 420 nm was observed.

The above data suggest that either P450IID6Δ1–22 cannot normally bind the heme residue or this protein is completely denatured during the procedure of subcellular fractionation. The former statement seems to be more reasonable, especially in the light of the data of Clark and Waterman [18] who have shown that a shortened form of cytochrome P45017α, lacking 17 NH_2-terminal amino acid residues, cannot be expressed in a catalytically active and heme-containing form in COS-1 cells.

By and large, the data on the role of the NH_2-terminal membrane insertion signal in the incorporation of the cytochrome P450 polypeptide chain into membranes and in its ability to accept the heme-containing form are rather controversial.

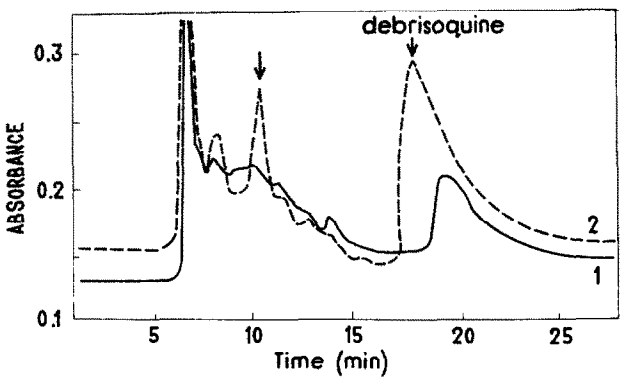

Fig. 4. HPLC analysis of the products of debrisoquine oxidation by the isolated microsomes. 1, extract of the reaction mixture after the reaction with microsomes from 2805(pYeDP); 2, extract of the reaction mixture after the reaction with microsomes from 2805(pYeDP/CYP2D6).

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