Genomic Cloning and Protein Expression of a Novel Rat Brain Cytochrome P-450 CYP2D18* Catalyzing Imipramine N-Demethylation*

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We have previously reported the isolation of two cDNA clones, designated 2d-29 and 2d-35, which have identical open reading frames and code for a novel brain cytochrome P-450 (P-450) belonging to the CYP2D subfamily, and noted that the mRNA of clone 2d-35 seems to be expressed in the brain but not in the liver (1). Although the deduced amino acid sequence of these clones differs from that of the liver CYP2D4 by only 5 amino acids distributed in the C-terminal region, this new P-450 cDNA clone contained a unique 5′-extension, and we posit in this report by analysis of a genomic clone that this 5′-untranslated sequence is derived from a gene distinct from that of CYP2D4. Thus, this novel P-450 was named P-450 2D18 according to the recommended nomenclature (2). The expressibility of this cDNA was confirmed by in vitro translation using a reticulocyte system, and protein expression was performed using COS-M6 cells. Immunoblot analysis showed a cross-reacting band of the predicted size range with anti-P-450 2D6 antiserum, which was not seen in control cells. Furthermore, the CYP2D18-expressed COS cell lysate showed N-demethylation activity toward imipramine, whereas another brain P-450 CYP4P6-expressed COS cell lysate showed 10-hydroxylation activity. This is the first report that associates an individual P-450 isozyme in brain with a particular metabolic alteration of the antidepressant imipramine.

Cytochrome P-450 is a general term for heme-thiolate enzymes, which exist in large quantities primarily in the liver and catalyze the metabolism of xenobiotics, carcinogens, steroids, and fatty acids. Among the P-450 superfamily members, much attention is paid to subfamily 2D P-450, which shows much polymorphisms (3–5), because possible relationships between members of this subfamily of isozymes and disorders of the central nervous system have been suggested (5, 6). For instance, it has been shown that the polymorphism in the 4-hydroxylation of debrisoquine, a substrate of CYP2D2, is associated with susceptibility to Parkinson’s disease (5) and that 4-hydroxylation of 1,2,3,4-tetrahydroisoquinoline, a possible candidate for causing Parkinson’s disease, is catalyzed by subfamily 2D P-450 in rat liver (7). It was also demonstrated that the human isozyme CYP2D6 participates in the metabolism of imipramine, a commonly used antidepressant (8). However, the vast majority of these studies were performed using liver microsomes or liver isoforms of P-450. Thus, whether subfamily 2D P-450 catalyzes these metabolisms in brain is not clear. In our laboratory, we have been interested in the brain P-450 monooxygenase system, and our studies have revealed evidence for the existence and functionality of multiple forms of P-450 (9–13). Recently, Sequeira and Strobel demonstrated brain microsomal catalytic activities toward imipramine and showed differences in the effects of the inducers on liver and brain imipramine metabolism (14). Thereafter, we cloned from a rat brain cDNA library two cDNA clones, termed 2d-29 and 2d-35, which have an identical open reading frame and code for a novel brain P-450 belonging to the CYP2D subfamily. The difference between the two clones is that cDNA clone 2d-29 has a foreshortened 3′-untranslated sequence with a poly(A) tail. We further showed that the mRNA of the longer clone, 2d-35, was expressed in the brain but not in the liver (1). Although they code for a P-450 protein, which differs from CYP2D4 (15, 16) by only 5 amino acids in the C-terminal region, clones 2d-29 and 2d-35 have unique 5′-untranslated sequences.

In order to investigate whether or not this brain CYP2D is a distinct form, we screened a rat genomic library resulting in the demonstration shown in this report that the 5′-untranslated sequence of this novel brain P-450 2D is derived from a gene distinct from CYP2D4, suggesting that the expression of this P-450 is regulated differently. Thus, this P-450 was named CYP2D18 according to the recommended nomenclature (2). Subsequently, we expressed the protein product of CYP2D18 using COS-M6 cells and performed an analysis of the catalytic capabilities of the expressed recombinant protein.

EXPERIMENTAL PROCEDURES

Materials—A rat genomic library (catalog number RL1023) was purchased from Clontech (Palo Alto, CA); [α-32P]dATP (3,000 mCi/mmol) and [α-32P]dCTP (3,000 mCi/mmol) were from ICN (Irvine, CA); [35S]methionine (1,000 Ci/mmol) was from Amersham Corp.; pure nicotinamide membrane and Ntynar filters were from Schleicher & Schuell; Qiagen Lambda Kit was from QIAGEN (Chatsworth, CA); TNT 2 CYP refers to a cytochrome P-450 gene(s) and cDNA(s), which is the nomenclature recommended by Nelson et al. (2).
Brain Cytochrome P-450 2D18

T3-coupled reticulocyte lysate system was from Promega (Madison, WI), and the 35S-labeled [32P]ATP was from Amersham (Arlington Heights, IL). The full-length cDNA was subcloned into the EcoRI fragment of the ECORI vector pBLCosM6 by the method of Towbin et al. (19). The expression vector pCDNAI and COS-M6 cells were generous gifts from Dr. Tetsu Kamitani (University of Texas Medical School).

Screening of Rat Genomic Library—A probe for screening the rat genomic library was prepared by PCR using P-450 2D18 cDNA (a template with a pair of primers as follows; 5'-AGTGGATCCCTCTGAGAACATTTGAT-3' and 5'-CGCGAATTCTAGTGGATCCTCCTCTGAGAAGTCTGGAGCCT-3') and labeled by the method of Laemmli (18), and immunoblot analysis was carried out according to the method of Towbin et al. (19) with slight modification.

RESULTS

Isolation of the CYP2D18 Gene and Southern Blot Analysis—In order to determine whether our brain subfamily 2D P-450 is derived from a gene distinct from liver CYP2D4, 1,800,000 plaques from a rat genomic library were screened with a 503-bp PCR product designated 29S1A2 as a probe. The PCR product probe covers the 5'-untranslated sequence and the N-terminal region of the novel clone (Fig. 1C). Two rounds of screening, 6 positive clones were obtained. Fig. 1 shows the Southern blot analysis of these 6 positive clones hybridized with the common probe, 29S1A2 (Fig. 1A) and a CYP2D18-specific oligonucleotide, 29S2 (Fig. 1B). In lanes 2 and 6 of Fig. 1 (A and B), bands of approximately 3.5 kb that hybridized with both the common probe 29S1A2 and the CYP2D18-specific oligonucleotide probe were observed, whereas in the other lanes, only hybridization bands with the common probe were detected. The clones that hybridized only with the common probe apparently reflect the CYP2D4 gene or another CYP2D gene of high similarity with 2D4. These results reveal that the unique 5'-untranslated sequence of the novel brain P-450 is derived from a gene distinct from CYP2D4. Thus, this P-450 was named CYP2D18 according to the recommended nomenclature (2).

Sequence Analysis of the 5'-Upstream Region of the CYP2D18 Gene—The nucleotide sequence of the 5'-upstream region of CYP2D18 gene is shown in Fig. 2. The putative transcription start site, indicated as +1 in Fig. 2, was determined by S1 mapping analysis (Fig. 3). The 5'-flanking sequence of CYP2D18 is quite different from that of CYP2D4, which was reported to have “a slightly atypical TATA sequence and no CCAAT sequence” (16). The 5'-flanking sequence of CYP2D18 has no putative TATA sequence nor CAAT box close to the transcription start site, and it has typical TATA-less features such as having a very GC-rich sequence close to the transcription start site and other putative transcription start sites shown by weaker protection bands seen in Fig. 3B. Two glucocorticoid responsive element “half-sites” (TGTTCT) (20) were found separated by 200 bp at nucleotides −564 and −766, although further study is needed to show whether these sequences are related to the regulation of CYP2D18 expression or not.

RNA Mapping with S1 Nuclease—A strand-specific DNA probe was synthesized using an antisense sequence within the unique 5'-untranslated sequence of CYP2D18 as a primer. The template used was a deletion clone of the EcoRI fragment of the CYP2D18 gene generating a 0.4-kb DNA probe (Fig. 3A). As shown in Fig. 3B, the lanes containing brain mRNA, liver
mRNA, and liver total RNA showed protection bands of 128 bp in size. Weaker and broader bands were also detected 4–5 bp above the 128-bp bands. Due to a low expression level of 2D18 in brain as previously shown in Northern blot analysis (1), we used 5 μg of mRNA and 50 μg of total RNA for hybridization and a longer exposure time for autoradiography to obtain visible protection bands. The observation that the intensity of bands in the lanes containing liver samples is the same or rather weaker than that of brain mRNA suggests the existence of CYP2D18 in liver at a low expression level.

In Vitro Translation of CYP2D18 cDNA Clones—Prior to expressing the recombinant protein product in mammalian cells, in vitro transcription/translation reactions were performed using the TNT T3-coupled reticulocyte system with CYP2D18 cDNA, 2d-29, and 2d-35, as templates without further modification. The data of Fig. 4A show bands of the predicted size range (50 kDa) on SDS-PAGE in both lanes containing cDNA 2d-29 and 2d-35.

Immunoblot Analysis of CYP2D18-expressed COS-M6 Cell Lysate—In order to show that the protein product expressed in COS cells is a member of the P-450 2D subfamily, immunoblot analysis was performed using anti-P-450 2D6 (21) polyclonal antibody. Transfection of COS-M6 cells was performed with the expression vector pcDNA carrying the CYP2D18 cDNA insert. Cells transfected with expression vector only were prepared as a control, and cells expressing CYP4F4 and CYP4F6, also isolated from the brain cDNA library (22), were likewise prepared for comparison. As shown in Fig. 4B, protein immunoblot analysis of the CYP2D18-expressed cell lysate with anti-P-450 2D6 antibody gave a cross-reacting band with a molecular mass of 50 kDa, which was identical to the size shown in the in vitro translation study, whereas neither control cells nor CYP4F4-expressed cells showed reaction bands with anti-P-450 2D6 antibody.

Catalytic Activity of P-450 2D18 toward Imipramine Metabolism—The catalytic activity toward imipramine (14) was determined using control cells and CYP2D18- and CYP4F6-expressed cell lysates as described above. As shown in Fig. 5B, CYP2D18-expressed cell lysate showed significant catalytic activity toward imipramine N-demethylation. On the other hand, we were able to detect significant imipramine 10-hydroxylation activity using a CYP4F6-expressed cell lysate (Fig. 5C). The calculated catalytic activities obtained by subtracting the background values of control COS cells from the values of CYP2D18- or CYP4F6-expressed COS cell lysate were 2.1 pmol/min/mg protein for N-demethylation by CYP2D18-expressed COS cells and 2.7 pmol/min/mg protein for 10-hydroxylation by CYP4F6-expressed COS cell lysate. These data show a metabolite specificity of the two isoforms resulting in either N-demethylation or hydroxylation.

DISCUSSION

Because P-450 isoenzymes are intensively studied in liver, antibodies and protein or nucleotide sequence data from liver...
isoforms are usually employed to investigate the P-450 monoxygenase systems in extrahepatic tissues such as brain, in which P-450 content and mRNA expression level seem to be lower. In our laboratory we have cloned from a rat brain cDNA library new P-450 isozymes including \(\text{CYP4F4}, \text{CYP4F5}, \text{CYP4F6}\) (22), and \(\text{CYP2D18}\) (1). Among them, the family 2D P-450 is a point of focus due to a possible correlation between this subfamily of isoenzymes and disorders of the central nervous system (5–7).

Komori has reported the isolation from a rat brain cDNA library of a partial cDNA clone of P-450 2D4 (23). Wyss et al. have recently published that they have cloned \(\text{CYP2D4}\) cDNA from rat brain mRNA by reverse transcription PCR and that they have detected \(\text{CYP2D4}\) protein in a brain P-450 fraction by immunoblot analysis (24). In our hands, all the positive clones sequenced during the cloning of \(\text{CYP2D18}\) cDNA from a rat brain library had the 2D18-specific 5' extension except one partial clone without an N-terminal sequence, suggesting that reliable distinction between \(\text{CYP2D4}\) and 2D18 is best obtained using the 5'- or 3'-untranslated region unique sequence. Thus, the weight of the evidence from isolation of these clones reflects the expression of \(\text{CYP2D18}\) in brain. Furthermore, our Northern blot analysis showed that a strong hybridization band in the lane containing the liver sample was shorter in size than a weak hybridization band in brain (data not shown), indicating

**Fig. 3.** RNA mapping with S1 nuclease. A, a strand-specific DNA probe internally labeled with \([γ-\text{32P}]\text{dATP}\) was synthesized using an oligonucleotide bearing an antisense sequence in the 5'-unique extension of \(\text{CYP2D18}\) cDNA as a primer and a deletion clone of \(\text{CYP2D18}\) gene as a template, generating a 0.4-kb probe. B, the probe was then hybridized with rat brain mRNA and total RNA, and rat liver mRNA and total RNA, followed by S1 nuclease digestion. The protected bands of 128 bp in size are indicated by the arrow.

**Fig. 4.** In vitro translation of cDNA clones, 2d-29 and 2d-35 (A) and immunoblot analysis of \(\text{CYP2D18}\)-expressed COS-M6 cell lysate with polyclonal antibody against human \(\text{CYP2D6}\) (B). A, the TNT-T3-coupled reticulocyte lysate system (Promega) was used. 0.5 \(\mu\)g of cDNAs cloned into pBluescript SK- were used without modification as templates for the transcription/translation reaction. The \(\text{35S}\)-labeled protein products were analyzed by 10% SDS-PAGE followed by autoradiography. Lane 1, luciferase positive control DNA; lane 2, 2d-29; lane 3, 2d-35. B, 50 \(\mu\)g of protein of each sample were used. Lane 1, lysate of COS cells transfected with vector only; lane 2, \(\text{CYP4F4}\)-expressed COS cell lysate; lane 3, \(\text{CYP2D18}\)-expressed COS cell lysate (96 h after transfection); lane 4, \(\text{CYP2D18}\)-expressed COS cell lysate (72 h after transfection).

**Fig. 5.** High performance liquid chromatograms of 10-hydroxylimipramine and desipramine from in vitro metabolism of imipramine by control COS cell lysate (A), \(\text{CYP2D18}\)-expressing COS cell lysate (B), and \(\text{CYP4F6}\)-expressing COS cell lysate (C). Reaction conditions are described under “Experimental Procedures.”
the major expression of CYP2D4 or other members of the CYP2D subfamily in liver, because 2D18 cDNA is longer by a 0.15-kb unique extension than those forms as reported by Matsunaga et al. (16). However, as shown in the S1 mapping results, CYP2D18 is expressed in brain and at a very low level in liver as well. Also the results of our Northern blot analysis are quite consistent with those published by Wynn et al. (24) in which two hybridization bands corresponding in size to cDNA 2d-29 and 2d-35 were shown in lanes containing brain mRNA.

The sequence of the 5’-flanking region and exon 1 as shown in Fig. 2 demonstrates that the 5’-unique extension of CYP2D18 is derived from a gene distinct from the CYP2D4 gene whose sequence was reported by Matsunaga et al. (16). That the Matsunaga sequence for CYP2D4 is correct was confirmed by a partial cDNA sequence for CYP2D4 reported earlier by Ishida et al. (15). On the other hand, our previous report (1) demonstrated that two cDNA clones of CYP2D18 (i.e., 2d-29 and 2d-35) have identical coding sequences that are distinct and separate from that of CYP2D4. These points confirm the sequence differences between CYP2D4 and CYP2D18. If those differences were those of allelic variants, it would not be very likely that the two genes would have such different 5’-upstream sequences.

In order to investigate the differential expression of CYP2D18 and 2D4, another S1 nuclease protection assay using a probe bearing a unique 5’-extension of 2D18 and a common sequence was performed, demonstrating the existence of 2D18 both in liver and brain. On the other hand, it seems that the expression level of CYP2D4 was too low to detect the protection bands in either brain or liver (data not shown). These data are both in liver and brain. On the other hand, it seems that the two genes would have such different 5’-upstream sequences in either brain or liver (data not shown). These data are consistent with the low expression levels of CYP2D4 in liver reported by Matsunaga et al. (16).

CYP2D4 was first cloned as a partial cDNA from rat liver (15), and the intron-exon structure was determined by the cloning of genomic DNA (16). Neither the purification of CYP2D4 protein nor a full functional study of this form has been performed previously. Therefore, further studies on the characterization of this particular form of P-450 would seem in order to define further the role of 2D4 in liver.

Recently, several studies have defined the aromatase cytochrome P-450 gene structure as consisting of nine exons, II-X, encoding the protein sequence and multiple exons I containing the 5’-untranslated sequences (25–28). Each exon I with a putative promoter region is transcribed in a tissue-specific manner, and alternative splicing results in forming mRNA with the same protein encoding sequence. A possible explanation of the CYP2D18 unique 5’-untranslated sequence is that this unique 5’-extension exists upstream of the reported 2D4 gene (16) and is spliced to the 5’-end of the conserved protein encoding sequence. However, the alternative splicing hypothesis cannot explain the nucleotide differences in the C-terminal region.

A CYP2D18-protein product expressed in COS-M6 cells showed both a cross-reacting band with anti-P-450 2D6 antibody and a significant catalytic activity toward imipramine N-demethylation. Inhibition studies of brain microsomal catalytic activity toward imipramine metabolism using quinidine, a subfamily 2D P-450 inhibitor, provide evidence that both hydroxylation and N-demethylation can be mediated by subfamily 2D P-450 or some other quinidine-inhibitable enzyme (29). In order to elucidate these discrepancies, we are now expressing a protein product in Escherichia coli for large scale purification of this unique form of brain P-450.

In summary, we have demonstrated that CYP2D18 cloned from a brain library is a unique member of the 2D P-450 subfamily expressed in rat brain and is a form distinct from CYP2D4 by genomic cloning. Moreover our data provide a basis for an explanation of the relation between gene structure and regulation of CYP2D18 in relation to CYP2D4. It is possible that CYP2D18 and CYP2D4 may have a tissue-specific distribution. The CYP2D18 protein product expressed in COS-M6 cells cross-reacts with anti-P-450 2D6 antibody, demonstrating its membership in the 2D subfamily, and shows a significant catalytic activity toward imipramine N-demethylation. This report constitutes the first direct evidence that a novel specific form of P-450 participates in the metabolism of tricyclic antidepressants in brain.

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