

## ANTIPEPTIDE ANTIBODIES TARGETED AGAINST SPECIFIC REGIONS OF RAT CYP2D1 AND HUMAN CYP2D6

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### ABSTRACT:

Four peptides (pep<sub>23-33</sub>, pep<sub>26-36</sub>, pep<sub>283-297</sub>, and pep<sub>409-419</sub>) corresponding to unique sequences in rat cytochrome P450 (CYP) 2D1 and/or human CYP2D6 were selected for production of anti-peptide antibodies. Rat liver microsomal protein was recognized by antiserum to all four peptides in ELISAs; however, antisera against pep<sub>23-33</sub> and pep<sub>26-36</sub> proved not usable for any other applications. Western blots of microsomal protein from a cell line specifically expressing human CYP2D6 revealed that antisera to pep<sub>283-297</sub> and pep<sub>409-419</sub> recognized a single protein band of the same molecular size as CYP2D6. Antisera to pep<sub>283-297</sub> and pep<sub>409-419</sub> recognized a rat microsomal protein presumed to be CYP2D1, because it comigrated with human CYP2D6 and had an apparent molecular size of 52 kDa. An unknown protein of ~85 kDa was also recognized by pep<sub>409-419</sub>. Recognition of microsomal protein(s) by antisera to

pep<sub>283-297</sub> and pep<sub>409-419</sub> was blocked by pep<sub>283-297</sub> or a bovine serum albumin-pep<sub>409-419</sub> conjugate, respectively. Antiserum to pep<sub>283-297</sub> was used to analyze sex and strain differences in liver microsomes prepared from Sprague-Dawley, Fischer 344, and Dark Agouti male and female rats. Sprague-Dawley and Fischer 344 rats expressed similar amounts of CYP2D1, but expression in Dark Agouti rats was significantly lower. The antiserum did not detect a sexual dimorphism in any of the strains. A significant correlation between anti-peptide<sub>283-297</sub> immunoreactivity and  $V_{max}$  for dextromethorphan O-demethylation existed in female rat strains; however, this relationship did not exist in male rat strains. These data suggest antisera against pep<sub>283-297</sub> and pep<sub>409-419</sub> are useful in studying expression of rat CYP2D1 and human CYP2D6.

The CYP<sup>1</sup> enzymes are a large family of proteins responsible for the oxidative metabolism of a wide variety of xenobiotics, as well as many endogenous compounds, including steroids, prostaglandins, bile acids, and fatty acids (1, 2). Within the CYP family, the CYP2D subfamily is particularly important because a genetic polymorphism exists for human CYP2D6. This renders 8–10% of Caucasians deficient in their ability to metabolize many clinically important drugs, including cardiovascular and neuroleptic drugs (3; for review, see ref. 4). This CYP2D6-deficient group has an ineffective oxidation pathway that results in the poor metabolizer phenotype and leads to unexpected drug toxicities and interactions (5, 6).

Because the rat CYP2D1 isozyme has overlapping substrate specificity and shares 97% homology with the human CYP2D6 isoform, several rat strains are used as animal models for the human poor and extensive metabolizer phenotypes. In particular, the female DA rat is an animal model for the poor metabolizer phenotype, whereas the SD and F344 rats are often used as models for the extensive metabolizer

phenotype (7, 8). Nevertheless, there are still significant differences in substrate specificities between the rat and human isoforms, and the strain differences in rat CYP2D1 are not fully understood. In particular, a thorough understanding of expression and specificity of these isozymes is still lacking.

Although specific antibodies for CYP isozymes can play an important role in characterizing enzyme function and form, an easily accessible and reproducible source of antibodies against the CYP2D family is not available. This is partially because CYP isozymes are difficult to purify to homogeneity, and the quantity of purified protein is often a limiting factor for antiserum or monoclonal antibody development. In addition, antibodies generated against the purified CYP enzymes are often not specific (9–11).

The availability of isoform sequence data has made it possible to target antibodies against localized regions of CYP isozymes. This has been accomplished either by using fusion proteins that contain isozyme-specific CYP regions (e.g. ref. 12) or by using synthetic peptide sequences unique to particular isozymes (e.g. refs. 13 and 14). There are several advantages of using this so-called anti-peptide antibody approach for generation of specific CYP antibodies. First, the antigenic site is precisely known. Second, the quantity of peptide antigen is not a limiting factor. In addition, antibodies directed against specific regions of CYP enzymes can reveal meaningful topological information (15–17).

Consequently, the purpose of this study was to prepare anti-peptide antibodies specific for rat CYP2D1 and human CYP2D6. After determining the utility and specificity of the anti-peptide antibodies, we also studied the relationship between CYP2D1 activity, as measured by dextromethorphan O-demethylation, and CYP2D1 expression in male and female SD, F344, and DA rats.

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<sup>1</sup> Abbreviations used are: CYP, cytochrome P450; DA, Dark Agouti; SD, Sprague-Dawley; F344, Fischer 344; MBS, *m*-maleimidobenzoic acid *N*-hydroxy succinimide ester; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin; PBS, phosphate-buffered saline; TBS, tris-buffered saline;  $K_M$ , Michaelis constant;  $V_{max}$ , maximum initial velocity; LKM1, liver-kidney-microsome type 1 antibody.

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TABLE 1

*Rat and human proteins showing the highest degree of homology with rat CYP2D1 and human CYP2D6 peptides<sup>a</sup>*

CYP2D Peptide and Source of Sequence	Related CYP2D Family Members	Sequences and Comparisons with Related Isoforms
Pep <sub>23-33</sub>  Human CYP2D6	Human CYP2D6	M H R R Q R W A A R Y
	Human CYP2D7 <sup>b</sup>	- - - H - - - - -
	Human CYP2D8 <sup>b</sup>	- - - H - - - - -
	Rat CYP2D3	- - - - - T S - -
	Rat CYP2D4	- - - - - T S - -
	Rat CYP2D1	- - - H - - - T S - -
	Rat CYP2D5	- - - H - - - T S - -
Pep <sub>26-36</sub>  Rat CYP2D1	Rat CYP2D1	M H R R H R W T S R Y
	Rat CYP2D3	- - - - Q - - - - -
	Rat CYP2D4	- - - - Q - - - - -
	Rat CYP2D5	- - - H Q - - - - -
	Human CYP2D6	- - - - Q - - A A - -
	Human CYP2D7 <sup>b</sup>	- - - H Q - - A A - -
	Human CYP2D8 <sup>b</sup>	- - - H Q - - A A - -
Pep <sub>283-297</sub>  Human CYP2D6 <sup>c</sup> Rat CYP2D1	Human CYP2D6/rat CYP2D1	E K A K G N P E S S F N D E N
	Rat CYP2D5	- - - - - - - - - -
	Rat CYP2D2	Q - - - - - - - - -
	Rat CYP2D3	- - - - - - - - A -
	Rat CYP2D4	G Q - - - * - - - - -
	Human CYP2D7 <sup>b</sup>	A Q - - - * - - - - -
	Human CYP2D8 <sup>b</sup>	A Q - - - * - - - - -
Pep <sub>409-419</sub>  Rat CYP2D1	Rat CYP2D1	E T V W E K P H R F H
	Rat CYP2D2	- - - - - L - - -
	Rat CYP2D3	- - - - - L - - -
	Rat CYP2D4	- - - - - L - - -
	Rat CYP2D5	- A - - - - L - - -
	Human CYP2D6	- A - - - - F - - -
	Human CYP2D7 <sup>b</sup>	- A - - - - F - - -
	Human CYP2D8 <sup>b</sup>	- A - - - - F - - -

<sup>a</sup> Corresponding nucleic acid and protein sequences were checked for matching sequences against all other recorded sequences in the GenEMBL nucleic acid sequence and the SWISS-PROT protein sequence libraries. Dashes indicate common amino acids. Asterisks indicate amino acid deletions.

<sup>b</sup> These isoforms are pseudogenes (see ref. 19).

<sup>c</sup> The actual sequence numbers for this sequence in the human CYP2D6 isoform are amino acids 280-294. For simplicity, the rat CYP2D1 sequence number was used for antipeptide antibody designation.

### Materials and Methods

MBS, BSA, *p*-nitrophenyl phosphate, and goat antirabbit IgG (whole molecule) alkaline phosphatase conjugate were purchased from Sigma Chemical Co. (St. Louis, MO). Activated KLH and BCA protein assay reagent kits were purchased from Pierce Chemical Co. (Rockford, IL). Polyacrylamide and *N,N'*-methylene-bis-acrylamide were purchased from U.S. Biochemical Corp. (Cleveland, OH). Sephacryl S-200 and G-25 Sephadex gel filtration media were purchased from Pharmacia (Piscataway, NJ). Sodium dodecyl sulfate, Tween-20, acetonitrile, perchloric acid, and Dynatech Immulon 1 96-well microtiter plates were purchased from Fischer Scientific (Springfield, NJ). Alkaline phosphatase color development reagents 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt and *p*-nitro blue tetrazolium chloride and molecular weight markers were purchased from Bio-Rad Laboratories (Hercules, CA). Freund's complete and incomplete adjuvants were purchased from Calbiochem (San Diego, CA). Microsomes from lymphoblastoma cell lines expressing human CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, and CYP3A4 were obtained from GENTEST, Inc. (Woburn, MA). Dextromethorphan hydrobromide and dextrorphan tartrate were obtained from Research Biochemicals, Inc. (Natick, MA).

**CYP2D Sequence Analysis.** The protein sequences of rat CYP2D1 (8; accession M16654), CYP2D2 (8; accession M166550), and human CYP2D6 (18; accession M33388) were analyzed for hydrophilicity, surface probability, and antigenic index using the sequence analysis software package from the

Genetics Computer Group, Inc. (Madison, WI). Four peptides (pep<sub>23-33</sub>, pep<sub>26-36</sub>, pep<sub>283-297</sub>, and pep<sub>409-419</sub>) corresponding to unique or common sequences in rat CYP2D1 and/or human CYP2D6 were selected (table 1 and fig. 1). The corresponding nucleic acid or amino acid sequences of these peptides were checked for matching sequences against all other recorded sequences in the GenEMBL nucleic acid sequence library or the SWISS-PROT protein sequence data library, respectively (see table 1).

**Peptide Synthesis and Coupling to Carrier Proteins.** The four peptides shown in table 1 were synthesized using a Milligen 9050 synthesizer using 9-fluorenylmethyloxycarbonyl chemistry by Dr. Dave Klapper of the Department of Microbiology and Immunology at the University of North Carolina (Chapel Hill, NC). The peptides were synthesized with an extra aminoterminal cysteine for use in covalent coupling to the carrier protein. For immunization, peptides were coupled to maleimide activated KLH according to the manufacturer's instructions. Free peptide was removed by gel filtration on a Sephacryl S-200 column or by dialysis. Complimentary BSA-peptide conjugates were synthesized for use in screening the sera for antipeptide antibodies following the method of Liu *et al.* (19). Briefly, BSA (10 mg) was dissolved in 1 ml PBS (pH 7.4) and a 100  $\mu$ l aliquot of MBS (25  $\mu$ g/ml) in dimethylformamide was added. The reaction was conducted at room temperature for 30 min. Activated BSA was separated from free MBS by gel filtration on Sephadex G-25. The lyophilized peptide was then added directly to the activated BSA at a ratio of 1:4 (w/w). After stirring the reaction mixture for 3

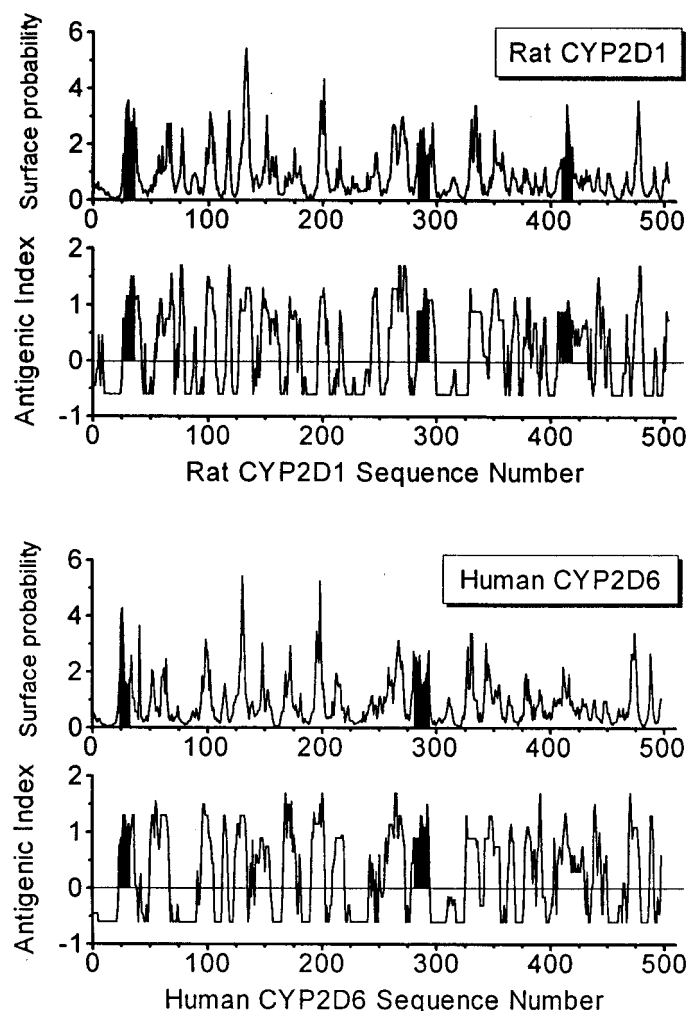


FIG. 1. Plot of the data obtained from surface probability and antigenic index analyses of rat CYP2D1 (upper two plots, respectively) and human CYP2D6 (lower two plots, respectively).

Shaded regions represent the peptides chosen for anti-CYP2D production (see table 1 for amino acid sequences of the peptides).

hr at room temperature, the conjugates were purified on Sephacryl S-200. A portion of the activated BSA was not coupled to peptide for use in testing for antilinker group antibodies.

**Rabbits and Immunization Schedule.** Female New Zealand White rabbits were purchased from Myrtle's Rabbitry (Thompson Station, TN) and allowed to recover from shipping for a minimum of 1 week. Preimmune serum was collected before immunizations. Two rabbits/peptide conjugate were immunized subcutaneously at multiple injection sites with 200  $\mu$ g KLH-peptide antigen in 1 ml PBS emulsified with an equal volume of Freund's complete adjuvant. Rabbits were boosted at 4-week intervals with 100  $\mu$ g antigen in 1 ml PBS emulsified with 1 ml of Freund's incomplete adjuvant. Blood was collected 1 week after each booster immunization for preparation of immune serum. All animal protocols in these studies were conducted in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

**Preparation of Liver Microsomes.** Female and male SD rats were purchased from Charles River (Wilmington, MA). F344 and DA rats were purchased from Bantin and Kingman (Freemont, CA). All animals were ~14 weeks of age at the time of microsome preparation. Liver microsomes were prepared by standard techniques as previously described (20). Protein concentrations were determined using the Pierce BCA protein assay kit and CYP content was determined as described by Omura and Sato (21).

**ELISA.** Antisera were screened for peptide specific antibodies using the corresponding BSA-peptide conjugates by ELISA. Ninety-six-well microtiter plates were coated with 500 ng/well BSA-peptide conjugate and MBS-activated BSA in 0.1 M carbonate buffer (pH 9.6) for 3 hr at 37°C. The plates were washed 5 times with PBS containing 0.1% Tween-20 (v/v) and stored for up to 2 weeks at 4°C. Various dilutions of anti-peptide antisera were prepared in PBS containing 0.1% Tween-20, and 100  $\mu$ l aliquots were added to the microtiter plate wells in triplicate. The plates were incubated overnight at 4°C. After washing the plates as previously described, a 100  $\mu$ l aliquot of goat antirabbit IgG alkaline phosphatase conjugate (1:30,000) was added to each well and incubated for ~2 hr at room temperature. The plates were again washed, and a 100  $\mu$ l aliquot of *p*-nitrophenol phosphate (30 mg/ml in 10 mM diethanolamine containing 0.5 mM MgCl $\cdot$ 6H $_2$ O) was added to each well. After a 1-hr color development, the amount of chromogenic product formation was determined using an ELISA plate reader at 405 nm. The sigmoidal fitting function of the Origin software package (Microcal, Inc., Northampton, MA) was used to determine the best-fit line for the absorbance at 405 nm vs. log antibody dilution. The antibody dilution that produced 50% of maximal response in each ELISA experiment was defined as the titer.

For testing each of the anti-peptide antiserum against CYP2D1 in rat microsomal protein, microsomal preparations (500 ng/well) were coated on microtiter plates and tested as described for BSA-peptide conjugates.

**Western Blots.** To determine if the anti-peptide antibodies could specifically recognize CYP2D1 and CYP2D6, rat liver microsomal proteins (25  $\mu$ g/lane) and microsomes prepared from the h2D6 lymphoblastoma cell line specifically expressing CYP2D6 (5  $\mu$ g/lane, GENTEST, Woburn, MA) were subjected to SDS-PAGE on an 8% polyacrylamide gel for 2 hr at ~100 V. Microsomes (5  $\mu$ g/lane) prepared from lymphoblastoma cell lines expressing CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2E1, and CYP3A4 were also used as negative controls. The proteins were transferred to nitrocellulose filters at 85 V for 45 min at 4°C using prechilled 25 mM Tris, 192 mM glycine, and 20% v/v methanol (pH 8.3) transfer buffer. The nitrocellulose filters were incubated for 2 hr in BSA blocking buffer (3% w/v BSA in TBS containing 0.05% v/v Tween-20) to reduce nonspecific binding and then washed twice for 5 min in TBS containing 0.05% Tween-20. The antisera were diluted in antibody buffer (1% w/v gelatin in TBS containing 0.02% w/v sodium azide) and added to the filters at a final titer of 1:4,000 (anti-pep $_{283-297}$ ) or 1:8,000 (anti-pep $_{409-419}$ ) for detection of rat CYP2D1 or 1:1,000 (anti-pep $_{283-297}$ ) or 1:2,000 (anti-pep $_{409-419}$ ) for human CYP2D6. The filters were incubated overnight at room temperature. In selected experiments, antibody binding was blocked by adding peptide (final concentration 1  $\mu$ M) or BSA-peptide conjugate (final concentration 10  $\mu$ g/ml) to the primary antibody solution. Filters were washed as previously described and a goat antirabbit IgG alkaline phosphatase conjugate (1:10,000) was added. After incubation at room temperature for ~2 hr, the filter was washed and protein bands were detected with Bio-Rad color development reagents following the manufacturer's directions. Approximate protein molecular weights were determined based on their relative electrophoretic mobility in comparison with biotinylated molecular weight markers.

To determine if the anti-peptide antibodies could detect sex and strain differences in expression of CYP2D1, microsomes prepared from adult male and female SD, F344, and DA rats ( $N = 3$ /group) were analyzed in a single Western blot assay as described using anti-pep $_{283-297}$  serum (1:4,000). Anti-pep $_{409-419}$  antiserum was not tested due to the cross-reacting 85 kDa protein (see Results). The relative amount of anti-pep $_{283-297}$  reactive protein was measured using a Bio-Rad GS-670 Imaging Densitometer. As part of the validation of this assay, we first established there is a linear relationship between the amount of rat liver microsomal protein (range of 1–50  $\mu$ g/lane) applied to the gel and the relative band intensities. The correlation coefficient for this relationship is 0.987 ( $p < 0.05$ ).

**Dextromethorphan Assays.** Liver microsomes from male and female SD, F344, and DA rats ( $N = 3$ /group) were studied to determine *in vitro* metabolism of dextromethorphan. The NADPH regenerating system consisted of 8 mM glucose-6-phosphate, 1 unit/ml glucose-6-phosphate dehydrogenase, 4 mM MgCl $_2$ , and the addition or absence (for the controls) of 0.5 mM NADP $^{+}$ . To each duplicate 1.5 ml reaction tube, 25  $\mu$ l of the NADPH regenerating system, 100  $\mu$ l of dextromethorphan at concentrations from 1 to 500  $\mu$ M, and an appropriate aliquot of metabolic incubation buffer were added. This mixture

was preincubated at 37°C for 5 min before the addition of the microsomal protein. The microsomal protein was added to start the reaction at a final concentration of 1 mg/ml in a total reaction volume of 750  $\mu$ l. The reaction mixture was incubated at 37°C for 20 min. Immediately following the incubation, the metabolic reaction was stopped by the addition of 75  $\mu$ l of HClO<sub>4</sub>. After vortexing, the tubes were centrifuged at 14,000g for 4 min to precipitate the protein. The supernatant fluid was removed and added to an autoinjector vial in preparation for the HPLC analysis.

The HPLC procedure was a modification of the method of Kronbach *et al.* (22). The HPLC column was a 3.9 mm  $\times$  150 mm Phenyl-Nova Pak steel column with a guard column containing the same packing material (Waters, Milford, MA). A linear gradient from 20–45% CH<sub>3</sub>CN:phosphate buffer (0.1 M potassium phosphate, monobasic adjusted to pH 4.0 with phosphoric acid) was run over 1 min followed by isocratic conditions of 45% CH<sub>3</sub>CN:phosphate buffer for the next 8 min. The Waters HPLC system consisted of Millennium chromatography software, a model 600E multisolute delivery system, a model 717 autoinjector, and a model 470 scanning fluorescence detector. The fluorescence detector was set at 270 nm for excitation and 312 nm for emission. Analytical recoveries and identity of dextromethorphan and the dextrophan metabolite were determined by comparison with an exact amount of authentic external standards of dextromethorphan and dextrophan injected at the start of each analytical run.

**Data Analysis.** Kinetic data for determination of  $K_M$  and  $V_{max}$  values for dextrophan formation were analyzed according to the graphical methods of Lineweaver-Burke (1/V vs. 1/S), as described in the text by Segel (23). Statistical analysis of the data from each rat strain and sex group was performed by a two-way analysis of variance followed by a Student-Newman-Keuls multiple range test. Statistical significance was defined at a level of  $p < 0.05$ . Linear regression analysis was used for determining the relationship between dextromethorphan  $V_{max}$  and CYP2D1 relative intensity on Western blot. All statistical analyses were performed using SigmaStat for Windows (Jandel Scientific Software, San Rafael, CA).

## Results

**Selection of Peptides.** Table 1 shows the four peptides selected for production of anti-CYP2D1 and anti-CYP2D6 antibodies. The first step in selection of these peptides involved identification of regions having high surface probability and antigenic index (fig. 1). We then determined which of these regions had amino acid sequences unique to CYP2D1 and/or CYP2D6. Table 1 shows rat and human proteins identified in the data base search as having the highest degree of homology to the selected peptides. Pep<sub>23–33</sub> was found to be a region unique to human CYP2D6. It had 90.9% homology with the related human CYP2D7 and CYP2D8, which are pseudogenes (19). Pep<sub>26–36</sub> and pep<sub>409–419</sub> corresponded to unique amino acid sequences present in rat CYP2D1. There was, however, some homology in these regions between related CYP2D forms (see table 1). Pep<sub>26–36</sub> had >80% homology with rat CYP2D3, CYP2D4, and CYP2D5. Rat CYP2D2 had only 45% homology in this region. Pep<sub>409–419</sub>, although unique to CYP2D1, had ~91% homology with four other rat CYP2D isoforms. Pep<sub>283–297</sub> corresponded to a common sequence found in CYP2D1 and CYP2D6. This sequence was also found in rat CYP2D5, but not in the other related rat CYP2D forms.

**ELISA of BSA-Peptide Conjugates and Rat Liver Microsomal Proteins.** All immunized rabbits produced high titer antibodies to their corresponding BSA-peptide conjugate (results not shown). The response against linker groups in activated BSA (not coupled to peptide) or with preimmune serum was either very weak or not present. Pep<sub>23–33</sub> produced the lowest titers of 1:48,000 and 1:58,000 in the two rabbits immunized with this peptide. The results obtained with pep<sub>26–36</sub> were the most variable with titers of 1:64,000 and 1:420,000 in the two rabbits. The titers against pep<sub>283–297</sub> were 1:55,000 and 1:90,000. Pep<sub>409–419</sub> elicited the highest titers of 1:170,000 and 1:250,000. Based on preliminary ELISA and Western

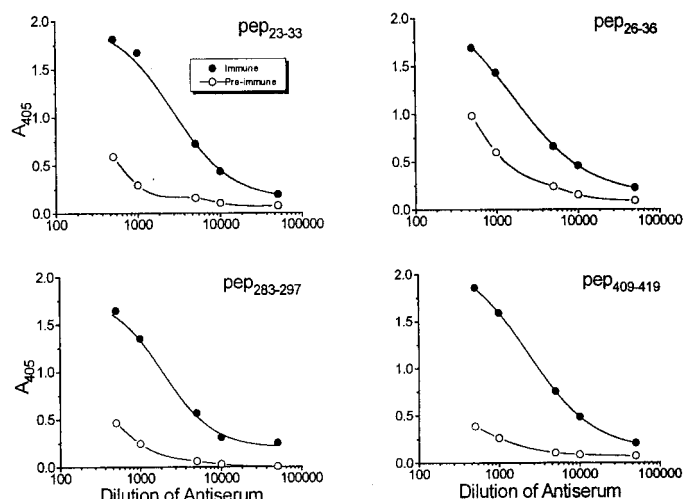


Fig. 2. ELISA results from the analysis of rat liver microsomal protein with the four anti-CYP2D antiserum.

Microtiter plates were coated with 500 ng/well of microsomal protein prepared from adult male SD rats and tested with preimmune (open circles) or antipeptide antiserum (closed circles) at varying dilutions. A representative antiserum from each immunization pair is shown.

blot results, the serum collected after the first boost from a single rabbit was chosen for further study.

The results from ELISA titers of rat liver microsomal protein using a representative animal for each peptide are shown in fig. 2. Antisera to pep<sub>23–33</sub>, a sequence unique to human CYP2D6, produced a strong reaction against rat liver microsomal protein. Antiserum to pep<sub>26–36</sub> recognized rat liver microsomal protein(s) in an ELISA, but the preimmune serum for this peptide also showed a moderately strong reaction. Antisera to pep<sub>283–297</sub> and pep<sub>409–419</sub> both produced a strong reaction against rat microsomal protein, with titers of 1:3,000 and 1:5,000, respectively. The preimmune sera for these peptides also showed low reactivity or nonspecific binding at the more concentrated dilutions, but this reactivity was trivial at higher dilutions of the antiserum.

### Western Blot Analysis of SD Rat Liver Microsomal Protein.

Western blot experiments with antisera against pep<sub>23–33</sub> and pep<sub>26–36</sub> revealed cross-reactivity with several unknown proteins (results not shown); therefore, these antisera were not considered useful for our applications. Antisera to pep<sub>283–297</sub> and pep<sub>409–419</sub> both detected a protein of approximately the same molecular weight ( $M_r \sim 52$  kDa) as rat CYP2D1 (fig. 3). In addition, antiserum against pep<sub>283–297</sub> may have recognized a slightly larger, but unresolved protein that could be one of the other related CYP2D forms. Antiserum against pep<sub>409–419</sub> recognized an additional protein band at ~85 kDa. The identity of this protein is not known. Recognition of proteins in Western blot by antiserum to pep<sub>283–297</sub> could be blocked by inclusion of 1  $\mu$ M pep<sub>283–297</sub> in the primary antibody solution. Inclusion of 10  $\mu$ g/ml BSA-pep<sub>409–419</sub> in the primary antibody solution blocked recognition of proteins by antiserum to pep<sub>409–419</sub>. The ability of pep<sub>283–297</sub> or BSA-pep<sub>409–419</sub> to block binding of their respective antiserum indicates highly specific binding.

**Western Blot Analysis of Human CYP2D6.** Antisera to pep<sub>283–297</sub> and pep<sub>409–419</sub> detected CYP2D6 ( $M_r \sim 52$  kDa) in microsomes prepared from the h2D6 lymphoblastoma cell line specifically expressing CYP2D6 (fig. 4). This protein band comigrated with the rat liver microsomal protein band recognized by these antisera. The specific recognition of CYP2D6 was blocked by the inclusion of 1  $\mu$ M pep<sub>283–297</sub> or 10  $\mu$ g/ml BSA-pep<sub>409–419</sub> conjugate, respectively,

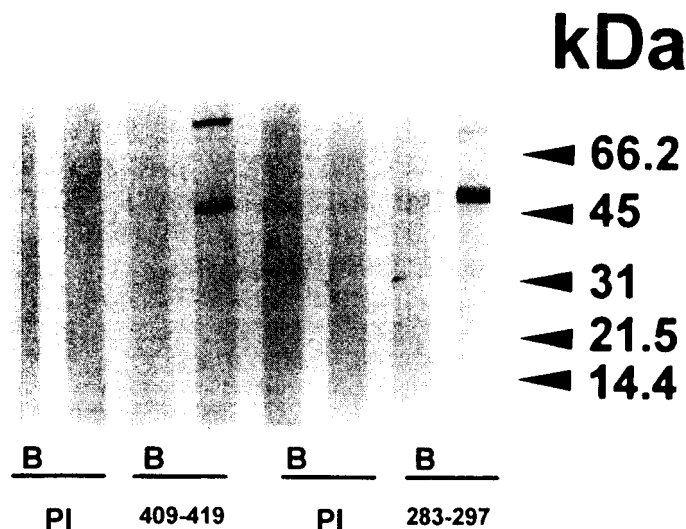


FIG. 3. Western blot analysis of adult male SD rat liver microsomal protein (25  $\mu$ g/lane).

Lanes labeled PI were probed with preimmune serum, lanes labeled 409–419 were probed with anti-pep<sub>409–419</sub> serum, and lanes labeled 283–297 were probed with anti-pep<sub>283–297</sub> serum. In lanes labeled B (blocked), BSA-conjugated peptide or free peptide was included in the primary antibody incubation as an inhibitor of antibody binding (see *Materials and Methods*).

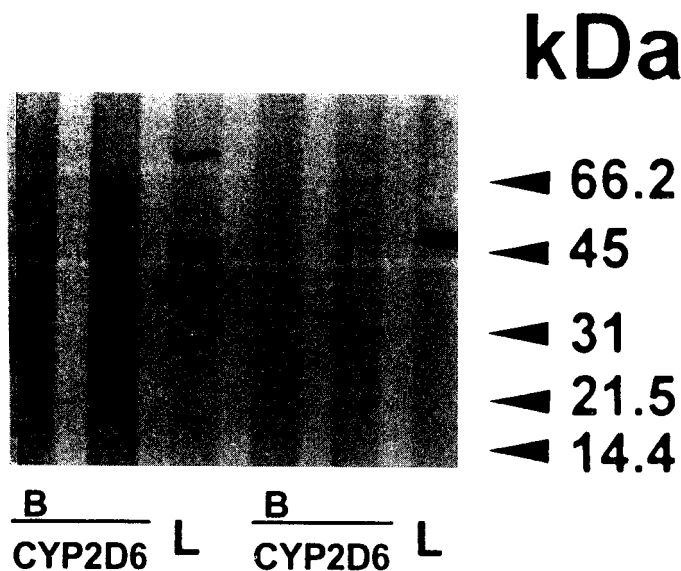


FIG. 4. Western blot analysis showing comigration of GENTEST CYP2D6 microsomal protein and the rat liver microsomal protein recognized by anti-pep<sub>409–419</sub> and anti-pep<sub>283–297</sub> serum.

Microsomes prepared from the GENTEST h2D6 lymphoblastoma cell line that specifically expresses CYP2D6 (5  $\mu$ g/lane) are in lanes labeled CYP2D6, and rat liver microsomes (25  $\mu$ g/lane) are in lanes labeled L. Lanes 1–3 were probed with antiserum-to-pep<sub>409–419</sub>, and lanes 4–6 were probed with antiserum-to-pep<sub>283–297</sub>. Lanes labeled B included BSA-conjugated peptide or free peptide in the primary antibody incubation as an inhibitor of antibody binding (see *Materials and Methods*).

in the primary antibody incubation solution. Antisera to pep<sub>283–297</sub> and pep<sub>409–419</sub> were also tested using microsomes prepared from lymphoblastoma cell lines specifically expressing human CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2E1, or CYP3A4. There was no recognition of protein in any of these preparations by either of these antipeptide antibodies (data not shown).

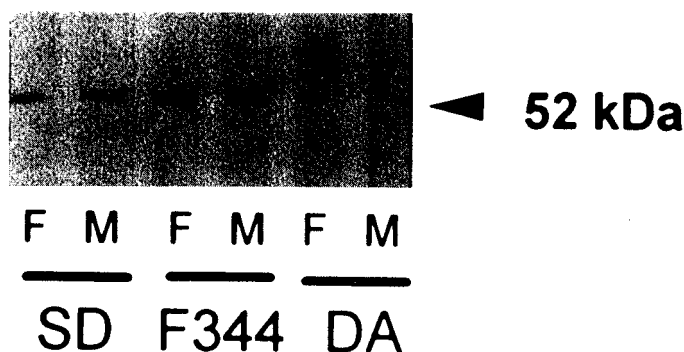


FIG. 5. A representative Western blot analysis of sex and strain differences in CYP2D expression.

Liver microsomal proteins (25  $\mu$ g/lane) prepared from a single SD female and male rat (lanes 1 and 2, respectively), F344 female and male rat (lanes 3 and 4, respectively), and DA female and male rat (lanes 5 and 6, respectively) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Antiserum-to-pep<sub>283–297</sub> was used for detection of CYP2D proteins.

**Analysis of Rat CYP2D1 Sex and Strain Differences in Protein Expression and *In Vitro* Dextromethorphan Metabolism.** Microsomes prepared from adult male and female SD, F344, and DA rats were analyzed by Western blot using antiserum to pep<sub>283–297</sub> (fig. 5, table 2). The CYP content was approximately equal for all microsomal preparations (table 2). The amount of the 52 kDa protein detected by Western blot analysis in SD rats and F344 rats was approximately equal. The relative amount of this protein in DA males and females ranged from 61–74% of the SD and F344 rats, and it was significantly lower ( $p < 0.05$ ).

The HPLC retention times of dextrophan (resulting from dextromethorphan *O*-demethylation) and dextromethorphan were  $\sim 3.5$  and 5.7 min. The least squares linear regression analysis of the Lineweaver-Burke plots of substrate concentrations from 1 to 50  $\mu$ M indicated a linear relationship for each set of data. The correlation coefficient ( $r$  value) for the individual analyses ranged from 0.92 to 0.99. The  $K_M$  values for the male SD, F344, and DA rats were  $11.1 \pm 3.0$ ,  $12.4 \pm 2.9$ , and  $9.9 \pm 0.92$   $\mu$ M, respectively. The  $K_M$  values for the females of the same three strains were  $7.3 \pm 2.0$ ,  $7.7 \pm 0.75$ , and  $5.4 \pm 1.3$   $\mu$ M, respectively. The  $K_M$  values for the females were significantly lower ( $p < 0.05$ ) than the male values. Because these  $K_M$  values were similar to the  $K_M$  values from other studies of CYP2D1 metabolism of dextromethorphan (24), no attempts were made to determine if another enzyme, with a higher  $K_M$  value, was metabolizing the drug. The  $V_{max}$  values for the same animals are shown in table 2.

As evidence that anti-pep<sub>283–297</sub> antiserum specifically recognizes CYP2D1, we plotted the individual  $V_{max}$  values for the formation of dextrophan (in nmol/min/mg; see table 2) vs. the relative intensity of the anti-pep<sub>283–297</sub> immunoreactive bands determined by Western blot ( $N = 18$ ). A least squares linear regression analysis of these data indicated there was a positive relationship, but the relationship was not strong for these data ( $r^2 = 0.394$ ,  $y = 0.0964x - 0.0894$ ). Nevertheless, the plot of these data revealed a clear distinction between the males and females of all three strains, with the males always having greater  $V_{max}$  values than the females of the same strain (table 2). Consequently, we reanalyzed the data to determine the relationship for the separate male and female populations. The analysis of the male data for  $V_{max}$  values and relative intensity of the CYP2D1 Western blot bands ( $N = 9$ ) showed a positive correlation with an  $r^2$  of 0.257 ( $y = 0.0842x + 0.00893$ ). The relationship, however, showed that the

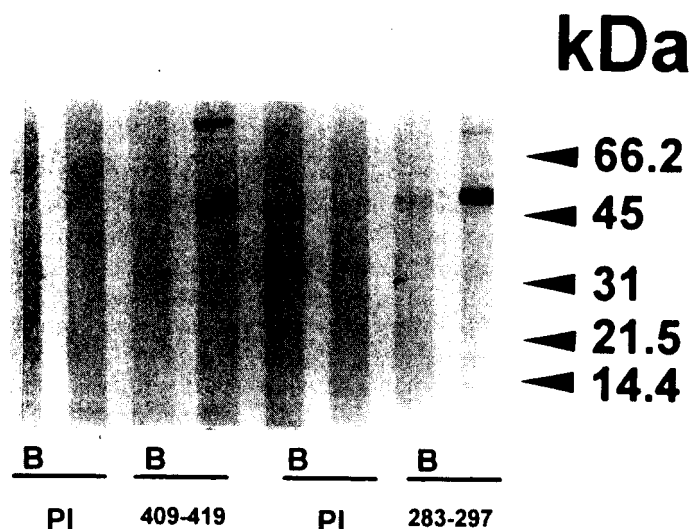


FIG. 3. Western blot analysis of adult male SD rat liver microsomal protein (25  $\mu$ g/lane).

Lanes labeled PI were probed with preimmune serum, lanes labeled 409–419 were probed with anti-pep<sub>409–419</sub> serum, and lanes labeled 283–297 were probed with anti-pep<sub>283–297</sub> serum. In lanes labeled B (blocked), BSA-conjugated peptide or free peptide was included in the primary antibody incubation as an inhibitor of antibody binding (see *Materials and Methods*).

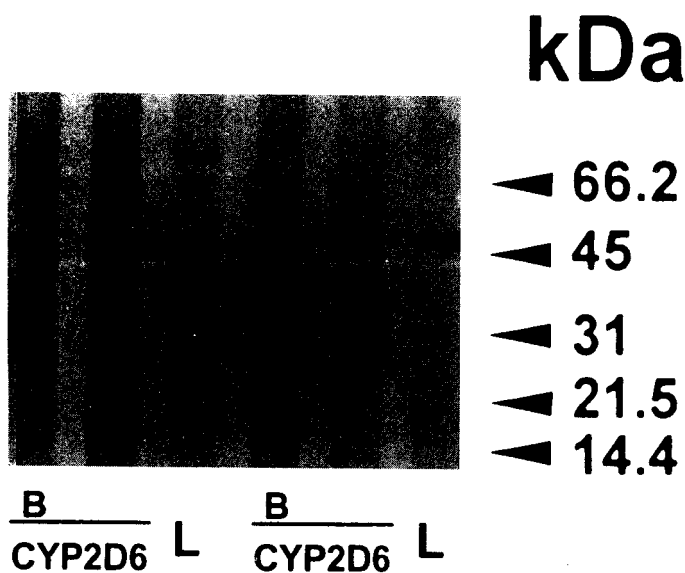


FIG. 4. Western blot analysis showing comigration of GENTEST CYP2D6 microsomal protein and the rat liver microsomal protein recognized by anti-pep<sub>409–419</sub> and anti-pep<sub>283–297</sub> serum.

Microsomes prepared from the GENTEST h2D6 lymphoblastoma cell line that specifically expresses CYP2D6 (5  $\mu$ g/lane) are in lanes labeled CYP2D6, and rat liver microsomes (25  $\mu$ g/lane) are in lanes labeled L. Lanes 1–3 were probed with antiserum-to-pep<sub>409–419</sub>, and lanes 4–6 were probed with antiserum-to-pep<sub>283–297</sub>. Lanes labeled B included BSA-conjugated peptide or free peptide in the primary antibody incubation as an inhibitor of antibody binding (see *Materials and Methods*).

in the primary antibody incubation solution. Antisera to pep<sub>283–297</sub> and pep<sub>409–419</sub> were also tested using microsomes prepared from lymphoblastoma cell lines specifically expressing human CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2E1, or CYP3A4. There was no recognition of protein in any of these preparations by either of these antipeptide antibodies (data not shown).

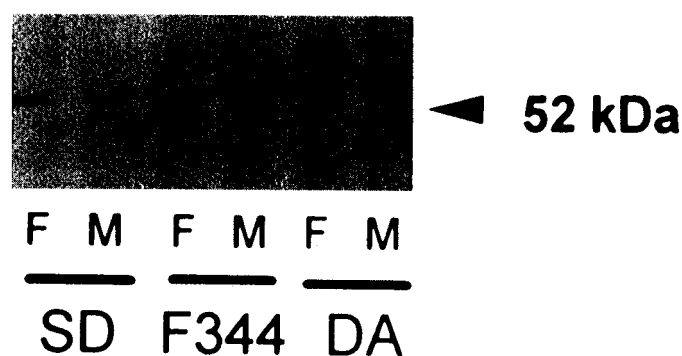


FIG. 5. A representative Western blot analysis of sex and strain differences in CYP2D expression.

Liver microsomal proteins (25  $\mu$ g/lane) prepared from a single SD female and male rat (lanes 1 and 2, respectively), F344 female and male rat (lanes 3 and 4, respectively), and DA female and male rat (lanes 5 and 6, respectively) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Antiserum-to-pep<sub>283–297</sub> was used for detection of CYP2D proteins.

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TABLE 2

Relative intensity of anti-pep<sub>283-297</sub> immunoreactive protein and  $V_{max}$  values for dextromethorphan O-demethylation in male and female rat strains<sup>a</sup>

Strain/Sex	CYP Content	$V_{max}^b$	Relative Intensity
	nmol/mg protein	nmol/min/mg	
SD males	0.90 ± 0.37	0.414 ± 0.07	3.73 ± 0.16
SD females	0.85 ± 0.19	0.316 ± 0.11	4.53 ± 0.88
F344 males	1.01 ± 0.08	0.366 ± 0.06	4.13 ± 0.99
F344 females	0.78 ± 0.10	0.231 ± 0.02	3.84 ± 0.06
DA males	0.93 ± 0.06	0.142 ± 0.01 <sup>c</sup>	2.75 ± 0.27 <sup>d</sup>
DA females	0.96 ± 0.91	0.090 ± 0.01 <sup>c</sup>	2.75 ± 0.40 <sup>e</sup>

<sup>a</sup> N = 3/group.

<sup>b</sup> Male values were significantly higher ( $p < 0.05$ ) than female values.

<sup>c</sup> Significantly different ( $p < 0.05$ ) from all other groups.

<sup>d</sup> Significantly different ( $p < 0.05$ ) from all groups, except DA females.

<sup>e</sup> Significantly different ( $p < 0.05$ ) from all groups, except DA males.

relative intensity of the protein band was not a good indicator of  $V_{max}$ . The analysis of the female data showed a very different result. There was a statistically significant ( $p < 0.01$ ), positive correlation between  $V_{max}$  values and the relative intensity of the CYP2D1 immunoreactive bands ( $r^2 = 0.92$ ,  $y = 0.118x - 0.225$ ).

### Discussion

This study describes the successful production of antibodies targeted against members of the CYP2D family using an antipeptide antibody approach. Four peptides corresponding to unique and/or common regions of rat CYP2D1 and human CYP2D6 were coupled to KLH and used to generate antibodies. All rabbits produced high titer antibodies against their corresponding BSA-peptide conjugate. In addition, all of the antipeptide antibodies recognized rat liver microsomal protein in ELISA (fig. 2).

Amino acid sequences unique to the aminoterminal regions of human CYP2D6 (pep<sub>23-33</sub>) and rat CYP2D1 (pep<sub>26-36</sub>) generated antibodies that cross-reacted with numerous proteins in rat liver microsomal preparations, as determined by Western blot (data not shown). Most of these cross-reacting proteins were in the molecular size range of CYP enzymes (*i.e.* 50–60 kDa). The cause of the cross-reactivity observed with these antisera is unknown. However, the aminoterminal region is important for signaling insertion into the endoplasmic reticulum membrane and for anchoring the CYP enzymes in the membrane (for a review, see ref. 25). Because of the cross-reactivity, these antisera were not used in further analyses.

Pep<sub>283-297</sub> was chosen as a hapten because its sequence was common to rat CYP2D1 and human CYP2D6. Consequently, the antiserum reacted with human CYP2D6 in microsomes isolated from the h2D6 lymphoblastoma cell line specifically expressing that enzyme (fig. 4). This cell line does not contain significant amounts of any other CYP enzymes (26, 27). Antiserum to this peptide also recognized a rat liver microsomal protein of approximately the same molecular weight ( $M_r \sim 52$  kDa) as rat CYP2D1 (figs. 3 and 4). These data suggested the antiserum to pep<sub>283-297</sub> was recognizing the correct protein. Recognition could be blocked by pep<sub>283-297</sub>; however, the BSA-peptide conjugate failed to block binding of the antibody. This preferential inhibition suggested that only the free (uncoupled) peptide had the correct conformation (or sufficient affinity) to inhibit the specific binding of antibody to the CYP2D protein.

Although antiserum to pep<sub>283-297</sub> correctly recognized an ~52 kDa CYP2D1-like protein, it also seemed to react with a slightly larger, but unresolved protein (figs. 3 and 4). Attempts to resolve another protein band by running the gels for longer periods of time or by using

different percentages of acrylamide did not reveal another clear protein band on Western blots. In addition, when lower amounts of protein (*i.e.* 10  $\mu$ g) were applied to the gel, this higher molecular weight band was not detected. Nevertheless, if this protein band is real, its size, recognition by the antibody, and inhibition of binding by free peptide suggest it is a related CYP2D isozyme, possibly CYP2D5 (28), because it also contains the pep<sub>283-297</sub> sequence (table 1).

Several other studies have examined antipeptide antibodies directed against amino acid residues between 280 and 300 from other CYP enzymes (13, 15–17, 29). Each of these studies resulted in the successful production of antibodies against the intended enzyme; however, in at least one case, the antibody reacted with other CYP isoforms (17). Antipeptide antibodies directed against residues 283–294 and 290–296 of rat CYP1A2 can inhibit enzymatic activity of this enzyme (13). Preliminary attempts to inhibit dextromethorphan metabolism (a CYP2D1 substrate) with our anti-pep<sub>283-297</sub> serum indicated that this antiserum does not inhibit CYP2D1 enzymatic activity (data not shown).

LKM1 autoantibodies are found in a group of patients with a life-threatening autoimmune hepatitis (30). Serum from these patients recognizes human CYP2D6 (31), as well as rat CYP2D1 (32). Epitope mapping studies of CYP2D6 using LKM1 autoantibodies show serum from most patients with this form of autoimmune hepatitis reacts against residues 262–270 (33, 34). However, the serum from ~25% of these patients recognizes a smaller region that corresponds to residues 283–289 (33). This region encompasses pep<sub>283-297</sub>, which we used to generate our anti-CYP2D antibodies. These studies and our data suggest that the amino acid residues from ~260–300 of CYP2D1 and CYP2D6 are exposed on the surface of the enzyme and comprise a major antigenic region.

Antiserum to pep<sub>409-419</sub>, a sequence that is unique to rat CYP2D1 (table 1), recognized a rat microsomal protein with approximately the same molecular weight as rat CYP2D1 ( $M_r \sim 52$  kDa). In addition, a protein of ~85 kDa was detected. The identity of this protein is unknown, and no matching sequences were detected in a search of the GenEMBL nucleic acid sequence library or the SWISS-PROT protein data base. Antiserum to pep<sub>409-419</sub> also recognized human CYP2D6 in microsomes prepared from the h2D6 lymphoblastoma cell line. Anti-pep<sub>409-419</sub> binding to the 52 kDa and 85 kDa proteins in rat microsomes, and binding to CYP2D6 could be blocked by BSA-pep<sub>409-419</sub> conjugate. Unconjugated pep<sub>409-419</sub> was unable to block the binding of the antibodies, suggesting unique conformational requirements for inhibition.

As described herein, pep<sub>409-419</sub> is unique to rat CYP2D1, yet antiserum directed against this peptide recognized human CYP2D6. There are two amino acid differences between rat CYP2D1 and human CYP2D6 in the region of pep<sub>409-419</sub> (table 1). Human CYP2D6 has an alanine (A) substituted in place of the threonine (T) at position 410 of rat CYP2D1. These amino acids are similar, with both having aliphatic side chains. This similarity may explain the cross-reactivity between the two forms. CYP2D6 has a phenylalanine (F) instead of the histidine (H) at position 416 of rat CYP2D1. Histidine contains a basic, hydrophilic side chain, whereas phenylalanine contains a hydrophobic side chain and would not be expected to be exposed on the surface of the protein. Because these are not similar amino acid substitutions at position 416 and the antibodies recognize both proteins, this suggests that this amino acid position has less effect on antibody binding.

Other epitope studies of CYP enzymes show that the region between amino acid residues 410–425 of CYP enzymes is an important antigenic site on the surface of these proteins. For instance, antiserum



generated against rat CYP2B1 recognizes a synthetic peptide that corresponds to amino acids 408–421 of CYP2B1 (17). In addition, the region comprising amino acid residues 410–429 of human CYP2D6 is another of the antigenic determinants of CYP2D6 recognized by anti-LKM1 antiserum (34).

The debrisoquine 4-hydroxylase deficiency in the DA rat is reported to be due to extremely low levels of CYP2D1 (35) or to a structurally altered form of CYP2D1 (8). Several additional members of the rat CYP2D family (28, 36) have been identified in addition to the previously identified CYP2D1 and CYP2D2 (8). These authors (28, 36) suggest that the DA deficiency is due to the absence of expression of CYP2D1. They found that CYP2D2, CYP2D3, and CYP2D5 are expressed in the DA rat, but have no activity toward CYP2D1 substrates (36). It is difficult to discern whether our results support the findings of Larrey *et al.* (35) or of Matsunaga *et al.* (28, 36). Although antiserum to pep<sub>283–297</sub> does detect a minor protein of approximately the same molecular weight as CYP2D1 in DA rats, it is possible that the protein being detected is another member of the CYP2D family. Indeed, a peptide sequence identical to pep<sub>283–297</sub> is found in CYP2D5 and mRNA for this isoform is detected in DA rats (27, 35). Although Matsunaga *et al.* did not detect CYP2D1 mRNA in DA rats, it is possible that if the protein is indeed expressed at very low levels (as proposed by Larrey *et al.*), the mRNA would not be detected unless amplified by polymerase chain reaction. Nevertheless, our anti-pep<sub>283–297</sub> serum does correctly identify the DA rats as deficient in CYP2D immunoreactivity.

Dextromethorphan *O*-demethylation has been identified as a CYP2D6 specific reaction (37–39). This reaction has been used in rats to help develop animal models for the human CYP2D6 polymorphism (24, 40). In our studies, linear regression analysis revealed a poor relationship between relative intensity of anti-pep<sub>283–297</sub> immunoreactive protein and the  $V_{\max}$  values for dextromethorphan *O*-demethylation in male rats. The lack of correlation for the male rats may be due to the involvement of multiple CYP forms, including male-specific forms. Indeed, the  $V_{\max}$  values for males of all strains were significantly greater ( $p < 0.05$ ) than for females (table 2). In addition, involvement of other CYP forms, including male-specific forms, has been suggested in the metabolism of other known CYP2D1 substrates (41). The regression analysis for female data demonstrated a significant positive correlation between  $V_{\max}$  values and the relative intensity of the CYP2D1 immunoreactive bands. However, this result does not exclude the possibility of involvement of other CYP isoforms in the *O*-demethylation of dextromethorphan. Certainly, high- and low-affinity components for this reaction have been identified in SD females (24).

This study presents an alternative approach for production of antibodies targeted against specific CYP enzymes. This approach is much less cumbersome than traditional methods that involve purification of the CYP enzyme from hepatic microsomal fractions. Although these antipeptide antibodies were not always exclusively specific for CYP2D1 or CYP2D6, their utility is apparent. Because the epitope against which the antibody is directed is precisely known, important topological information about related forms can be derived. Finally, these antibodies should be useful in quantification of CYP2D isozymes.

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