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Identification of a Human Liver Cytochrome P-450 Homologous to the Major Isosafrole-Inducible Cytochrome P-450 in the Rat

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SUMMARY

The rat 3-methylcholanthrene-inducible family of liver cytochromes P-450 contains two proteins (P-450c and P-450d) that are immunochemically related, possess 68% total sequence homology, and are induced by a number of toxic or carcinogenic compounds. To determine whether equivalent isozymes of hepatic cytochrome P-450 are expressed in humans, as they are in several mammalian species, we performed immunoblot analyses on microsomes prepared from 14 human liver specimens and found that each one contained a 52.5-kDa protein (termed HLd) that reacted with antibodies specific for rat P-450d. In addition, one specimen contained a 54-kDa protein (termed HLc) that reacted with antibodies specific for rat P-450c. HLd was purified through the use of immunoaffinity chromatography and was found to be 56% homologous to rat P-450d and 61% homologous to the equivalent isozyme in the rabbit (P-450 LM4) through their first 18 NH_2-terminal amino acids. Finally, levels of immunoreactive HLd varied more than 10-fold among these patients but were unrelated to the patients' drug treatments, smoking habits, or amount of immunoreactive HLp, a human liver cytochrome P-450 related to the glucocorticoid-inducible family of rat cytochromes P-450. We conclude that, in man, there is a cytochrome P-450 family composed of two isozymes (HLc and HLd) that are immunochemically and structurally related to the 3-methylcholanthrene-inducible family observed in several other species.

The hepatic cytochromes P-450 are members of a gene superfamily of microsomal hemoproteins that catalyze the oxidative metabolism of a large number of foreign and endogenous lipophilic compounds (1, 2). One of the cytochrome P-450 families, referred to as the MC-inducible family, consists of a pair of immunochemically related isozymes that appear to be represented in several mammalian species (3, 4). For example, liver microsomes from rats treated with MC, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, or β-naphthoflavone contain induced levels of P-450c and P-450d (5); the former protein is homologous to rabbit P-450 LM6 and mouse P_1-450, the latter is homologous to rabbit P-450 LM4 and mouse P_3-450 (3, 6). Cytochrome P-450d (as well as equivalent isozymes in the rabbit and mouse) is present in untreated animals and is preferentially induced by isosafrole, whereas P-450c is present at very low levels in livers of untreated rats and is the predominant isozyme in the liver following treatment with inducers such as MC (5, 7). Recently, it has become apparent that trans-species homology among some isozymes of cytochrome P-450 extends to humans. Isozyme-specific antibodies and cDNA probes to the mRNA of mammalian cytochromes are being used to facilitate investigations of human cytochromes P-450. For example, Distlerath *et al.* (8) used an antibody preparation directed against rat liver debrisoquine 4-hydroxylase to monitor the purification of the human liver cytochrome P-450 isozyme responsible for debrisoquine 4-hydroxylation. Watkins *et al.* (9) recently purified HLp, a human liver cytochrome P-450 that is structurally, immunochemically, and functionally similar to the steroid-inducible rat cytochrome P-450p.

Jaiswal *et al.* (10) recently utilized mouse P_1-450 and P_3-450 cDNAs to obtain a full length human cDNA that encodes a protein homologous to mouse P_1-450. However, no evidence was obtained for a human equivalent of mouse P_3-450 (which is homologous to rat P-450d and rabbit P-450 LM4). The

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ABBREVIATIONS: MC, 3-methylcholanthrene; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

authors speculated that humans may have diverged from other mammals prior to the time that a single ancestral gene evolved into a two-member family and that the functions of human P_1-450 thus might be divided between mouse P_1-450 and P_3-450 (10).

In the present study, we utilized polyclonal and monoclonal antibodies against rat P-450c and P-450d to identify, quantitate, and purify P-450 HLd, the human isozyme homologous to rat P-450d, rabbit P-450 LM4, and mouse P_3-450. From these results, we conclude that human liver contains both members of the gene family that are MC inducible in rats, mice, and rabbits.

Materials and Methods

Goat anti-rabbit IgG, peroxidase-rabbit antiperoxidase, and peroxidase-conjugated rabbit anti-mouse IgG were obtained from Miles Scientific (Naperville, IL). Nitrocellulose paper was purchased from Bio-Rad Laboratories (Richmond, CA), and 3,3′-diaminobenzidine tetrahydrochloride was purchased from Pfaltz and Bauer (Stamford, CT). Sepharose CL-4B was obtained from Pharmacia (Piscataway, NJ). All other materials were of reagent grade or better.

Human liver specimens. Specimens were obtained at surgery under protocols approved by the Committee for the Conduct of Human Research at the Medical College of Virginia. All patients had normal serum transaminase and bilirubin levels and each received atropine prior to general anesthesia. Patient code numbers refer to individual livers with some specimens having appeared in another study (9). There were 14 samples examined, patient 5 not being available for this study. *Patients 1–8* have been described in detail elsewhere (9). *Patient 9* was a 36-year-old white male organ donor and was receiving no medications prior to hospitalization. The patient received 200 mg of diphenylhydantoin, 200 mg of dexamethasone, 60 mg of amobarbital, an acetaminophen suppository, ampicillin, and codeine during the 24 hr prior to surgery. *Patient 10* was a 36-year-old white female who underwent elective removal of a hepatic hemangioma and was not taking medications prior to hospitalization. Three hr before surgery she received 2 mg of lorazepam, 8 mg of morphine sulfate, and 1 g of cephazolin. *Patient 11* was a 38-year-old white female organ donor. She was hospitalized for 13 days and received total daily doses of 300 mg of diphenylhydantoin, 16 mg of dexamethasone, and 24,000,000 units of penicillin G. Two days before her death, she was given 20 mg of furosemide, 800 mg of sulfamethoxazole, 160 mg of trimethoprim, and 120 mg of gentamycin. *Patient 12* was a 46-year-old white male who underwent elective left hepatic lobectomy for metastatic colon cancer. He was a heavy drinker and was taking no medications at the time of admission. *Patient 13* was a 47-year-old white male who underwent elective left hepatic lobectomy for colon cancer metastases. The patient's medical history included a prior problem with alcoholism. As part of our study protocol, he received a total of 12.5 g of triacetyloleandomycin over 7 days before surgery. In addition, he received prochlorperazine, 10 mg, on three occasions but not during the 3 days prior to surgery. The patient also received the preoperative medications of cefazolin (1 g), meperidine (50 mg), and hydroxyzine (25 mg). *Patient 14* was a 50-year-old white male who was admitted for surgical evaluation of a mass in the right lobe of the liver. The patient was healthy and was on no medications but complained of pruritis. The patient's laboratory tests suggested the possibility of minimal liver dysfunction in that there were slight (less than 2-fold) elevations in the serum activities of an alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase. However, at surgery, the liver appeared normal and histologic examination of a wedge biopsy of grossly normal liver revealed only nonspecific, periportal inflammation. The patient received 15 mg of flurazepam on the night before surgery. *Patient 15* was a 39-year-old white male who underwent elective hepatic lobectomy for metastatic colon cancer. His only medications prior to surgery were 1 g of cefazolin, 75 mg of meperidine and 75 mg of hydroxyzine pamoate.

All liver specimens were transported from the operating room on ice. The microsomal fraction from the livers was prepared by differential centrifugation according to the method of van der Hoeven and Coon (11). The isolated microsomes were stored at −70°. Protein concentrations were measured colorimetrically (12).

Antibodies against rat cytochrome P-450 isozymes. Polyclonal antibodies against purified rat P-450c and P-450d were raised in rabbits, and the IgG fractions were purified as described (5, 13). Each antibody fraction was immunoabsorbed with solubilized microsomes from phenobarbital-treated rats bound to Sepharose 4B to remove any possible nonspecific antibodies (5, 13). Since rat P-450c and P-450d are immunochemically related to each other, anti-P-450c and anti-P-450d were further absorbed with partially purified preparations of the heterologous proteins (4). The resulting antibodies, anti-P-450c(-d) and anti-P-450d(-c), are monospecific for the antigen of immunization (4). Monoclonal antibodies were produced against rat P-450c as described (14). Three of the nine monoclonal antibodies (CD2, CD3, and CD5) react equally well with rat P-450c and P-450d, whereas the remaining six monoclonal antibodies are specific for rat P-450c (14).

Immunoblot analysis. Immunoblot analyses were performed as previously described (15). Briefly, microsomal proteins were separated by electrophoresis in 1.5-mm $NaDodSO_4$-polyacrylamide (10%) slab gels and then electrophoretically transferred to nitrocellulose sheets. The sheets were incubated overnight at 25° in phosphate-buffered

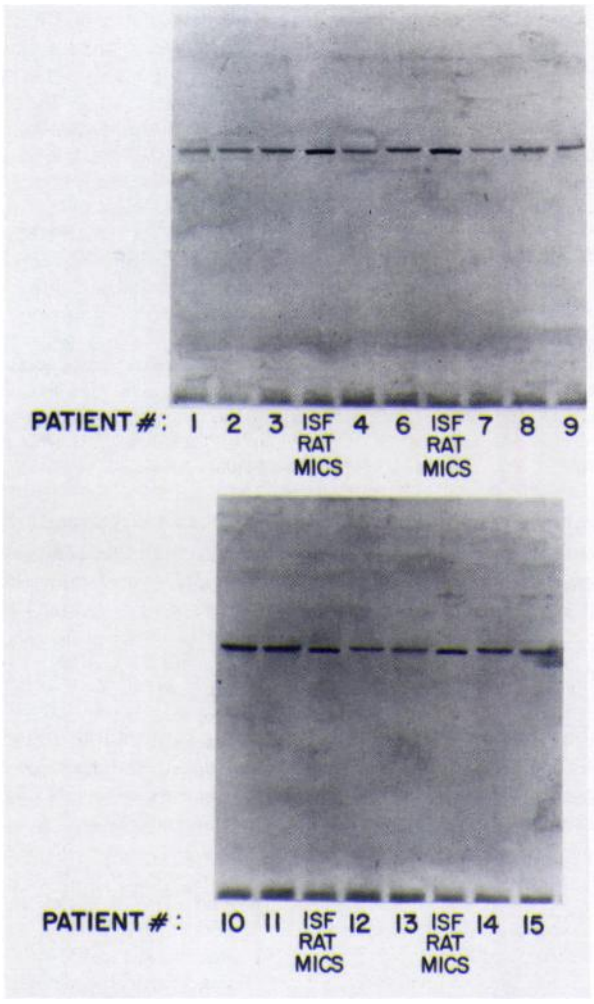

Fig. 1. Qualitative immunoblots of human liver microsomal proteins immunoreactive with anti-P-450d(-c). Human liver microsomes (50 μg) from the indicated patients or liver microsomes from isosafrole (*ISF*)-treated rats (5 μg) were immunoblotted as described in Materials and Methods using anti-P-450d(-c) (4).

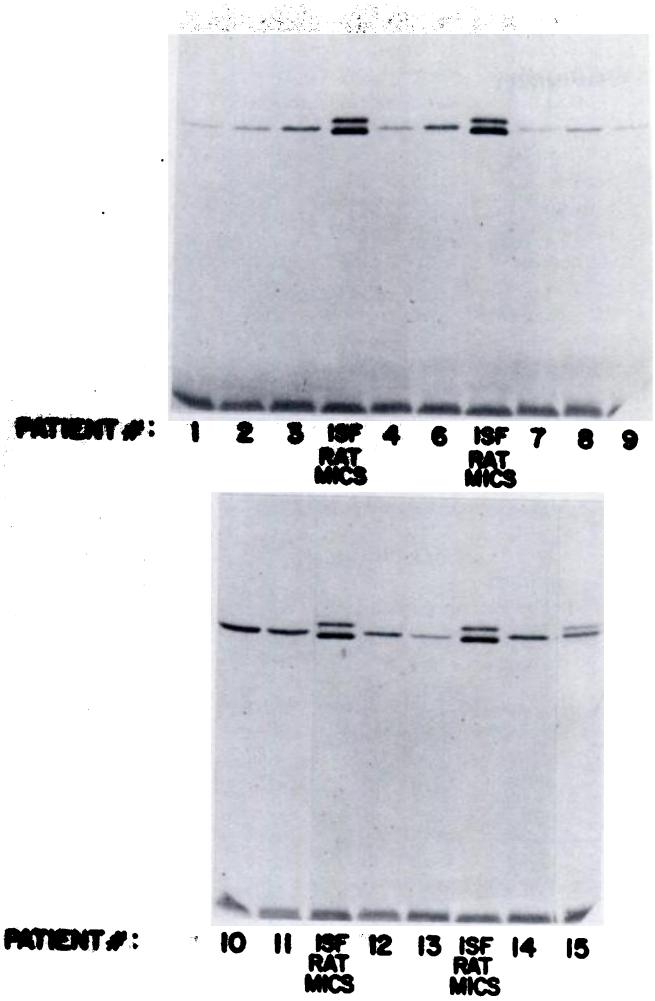

Fig. 2. Qualitative immunoblots of human liver microsomal proteins immunoreactive with a mixture of three monoclonal antibodies (CD2, CD3, and CD5) that react with both rat P-450c and P-450d. Human liver microsomes (50 μg) from the indicated patients or liver microsomes from isosafrole (*ISF*)-treated rats (5 μg) were immunoblotted as described in Materials and Methods using the monoclonal antibodies.

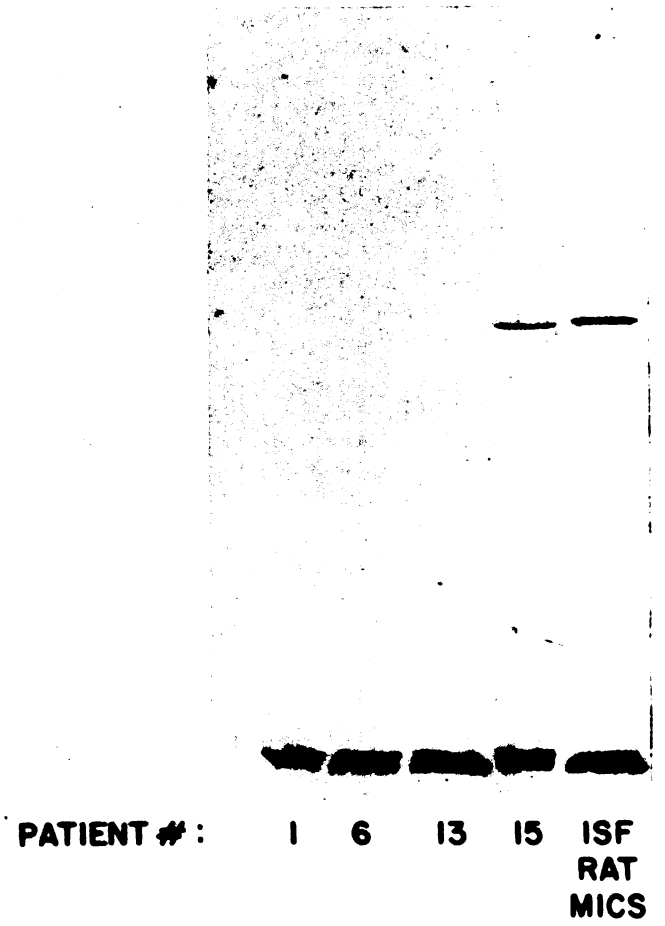

Fig. 3. Qualitative immunoblots of human liver microsomal proteins immunoreactive with a mixture of six monoclonal antibodies against rat P-450c (14). Human liver microsomes (50 μg) from the indicated patients or liver microsomes from isosafrole (*ISF*)-treated rats (5 μg) were immunoblotted as described in Materials and Methods using a mixture of monoclonal antibodies specific for P-450c (14).

saline containing 10% dialyzed calf serum and 3% bovine serum albumin and then reacted with rabbit polyclonal antibodies followed sequentially with goat anti-rabbit IgG, peroxidase-rabbit antiperoxidase, and, finally, with 3,3′-diaminobenzidine tetrahydrochloride in 0.006% hydrogen peroxide. When mouse monoclonal anti-P-450c antibodies (14) were used as the primary antibody, the second antibody was peroxidase-conjugated rabbit anti-mouse IgG. HLp was immunoquantitated on blots of the human liver microsomes developed with the monoclonal antibody 13-7-10 (16) graciously supplied by Dr. P. Kremers (Universite de Liege, Belgium) using purified HLp (9) as the standard. Quantitative immunoblot analyses were performed at protein concentrations within the complete transfer range (up to 35 μg). The density and total area of the immunostained bands were determined as previously described (15). In preliminary analyses, dilution of a given microsomal sample produced densitometric readings which were directly proportional to the amount of applied protein.

Immunoaffinity purification. Anti-rat cytochrome P-450d (1000 mg of IgG) was covalently bound to CNBr-activated Sepharose CL-4B (45 ml) with 94% efficiency by the method of Thomas *et al.* (13). The remaining active groups on the Sepharose were blocked with ethanolamine, and noncovalently bound antibody was removed with the low and high pH buffers described previously (13). The column was equilibrated with 50 mM potassium phosphate buffer (pH 7.4) containing 100 mM KCl, 20% glycerol, 0.2% Lubrol PX, 0.5% sodium cholate, 0.1 mM EDTA, and 0.1 mM dithiothreitol. Human liver microsomes from patient 11 (500 mg of protein) were solubilized in the column equilibration buffer containing 0.1 mM phenylmethylsulfonyl fluoride. The solubilized human liver microsomes (15 mg of protein/ml) were loaded onto the immunoaffinity column at a flow rate of 0.5 ml/min, and absorbance of the effluent at 280 nm was monitored continuously. The column was then washed sequentially with one column volume each of equilibration buffer, equilibration buffer containing 0.5 M KCl, equilibration buffer without sodium cholate, and equilibration buffer without sodium cholate, EDTA, and KCl. The antibody-bound protein was then eluted with 100 mM glycine-HCl buffer (pH 3.1) containing 20% glycerol, 0.1% Lubrol PX, and 0.1 mM EDTA. The fractions with absorbance at 280 nm were pooled, dialyzed against 40 mM sodium phosphate (pH 7.0) containing 20% glycerol, 0.01 mM EDTA, and 0.1 mM dithiothreitol, and concentrated by ultrafiltration (Amicon PM-10 membrane). The immunoaffinity-purified protein was precipitated with acetone (17) and redissolved in 200 μl of hexafluoroacetone trihydrate, and the NH_2-terminal sequence was determined by automated Edman degradation with a spinning cup sequencer as previously described (18).

Results

We developed immunoblots of liver microsomes from 14 human samples with anti-P-450d(-c) and found, in each case, that only a single 52.5-kDa protein was visualized (Fig. 1). As

TABLE 1
Immunoquantitation of HLd and HLp

Patient number	Parameter			
	HLd[a]	HLp[b]	Smoking habits	Medications[c]
1	67	288	Unknown	Unknown
2	42	275	Non-smoker (reformed smoker)	Erythromycin-base Neomycin
3	71	150	Unknown	Vitamin K
4	66	650	Unknown	Dexamethasone Phenobarbital Diphenylhydantoin
6	84	200	Unknown	Hydrocortisone Cimetidine
7	55	175	Non-smoker	None
8	45	563	Light smoker 3 Pk. Yr.[d]	Dexamethasone Phenobarbital Diphenylhydantoin
9	85	313	Unknown	Dexamethasone Diphenylhydantoin Amobarbital
10	98	225	Non-smoker	None
11	77	525	Unknown	Dexamethasone Diphenylhydantoin Furosemide Sulfamethoxazole Trimethoprim Gentamycin
12	33	100	25 Pk. Yr.	None
13	11	763	37 Pk. Yr.	Triacetyloleandomycin
14	100	100	Non-smoker	Flurazepam
15	34	ND[e]	Unknown	None

[a] HLd was immunoquantitated as described in Materials and Methods using 25 μg of each microsomal preparation and setting the value obtained for specimen 14 (470 arbitrary units) as 100%.
[b] HLp was immunoquantitated as described in Materials and Methods and is expressed with respect to the value obtained for specimen 14 (80 pmol/mg of microsomal protein).
[c] Medications received on day of surgery not included.
[d] Pk. Yr. is defined as a package of cigarettes per day for 1 year.
[e] ND, not determined.

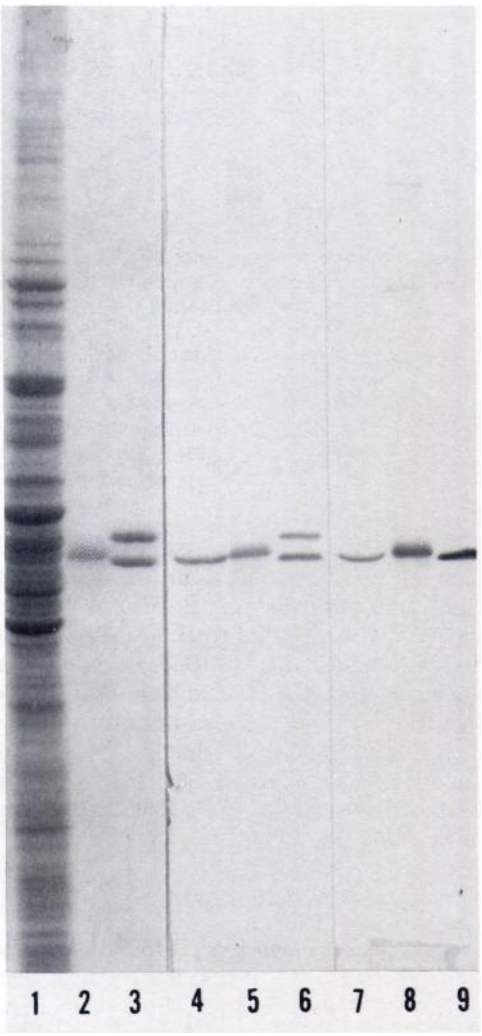

Fig. 4. Coomassie blue-stained NaDodSO$_4$ gel (*tracks 1–3*) and immunoblots (*tracks 4–9*) of human liver microsomes (patient 11), immunoaffinity-purified P-450 HLd and purified rat P-450c and P-450d probed with anti-P-450d (*tracks 4–6*) or rat anti-P-450d(-c) (*tracks 7–9*). Tracks *1, 4* and *7* contain human liver microsomes (20 μg of protein), tracks *2, 5, and 8* contain immunoaffinity-purified P-450 HLd, and tracks *3, 6,* and *9* contain a mixture of rat P-450c and rat P-450d. NaDodSO$_4$-gel electrophoresis, immunoblotting, and staining were performed as previously described (14).

a control, liver microsomes from isosafrole-treated rats (5) were analyzed on the same blot. As expected, the isozyme-specific antibody recognized only one rat protein (P-450d) which migrated slightly faster than the immunoreactive human protein (Fig. 1). When immunoblots of the same human specimens were developed with a mixture of three monoclonal antibodies directed against a single epitope shared by rat P-450c and P-450d (14), a 52.5-kDa protein was visualized in each of the 14 patient samples, whereas both P-450c and P-450d were seen in the lanes containing rat liver microsomes (Fig. 2). The only exception was patient 15 in whom a second protein (54 kDa) was detected (Fig. 2). Strong evidence that this second human protein is related to rat P-450c was obtained by developing immunoblots with a mixture of six monoclonal antibodies directed against five epitopes on rat P-450c (14) (Fig. 3). These antibodies recognized only the 54-kDa protein in patient 15, only P-450c in rat liver microsomes, and no proteins in the microsomes from patients (1, 6, and 13) which had exhibited only the 52.5-kDa band on the previous blot (see Fig. 2). Similar results were obtained when an identical blot was developed with polyclonal antibodies made specific for rat P-450c by absorption against purified P-450d (anti-P-450c(-d)) (4) (data not shown). These results indicate that all 14 human liver specimens contained a protein immunochemically related to rat P-450d (hereafter referred to as HLd) and that only one of these specimens (from patient 15) contained a protein related to rat P-450c (hereafter referred to as HLc).

Quantitative analysis of immunoblots developed with anti-P-450d(-c) indicated that patient 14 had the highest amount of HLd. Among the other samples, relative amounts of immunoreactive HLd varied widely, over nearly a 10-fold range (11%–100% of the level of patient 14) (Table 1). The levels of HLd appeared to correlate with neither the medications the patients received nor their smoking habits. Indeed, even though polycyclic aromatic hydrocarbons induce rat P-450d, the patient with the highest level of HLd, patient 14, was a non-smoker, whereas the lowest levels of HLd were found in two smokers (patients 12 and 13). However, the interval between the last cigarette consumed and excision of the liver is not known for all cases. The levels of HLd were also unrelated to the amounts of immunoreactive HLp in the same patients (expressed relative to the amount of HLp in specimen 14) (Table 1). However, as we reported previously on a smaller number of patients, the

Cytochrome	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
HLd	Ala	Leu	Ser	Gln	Ser	Val	Pro	Phe	Ser	Ala	Thr	Glu	Leu	Leu	Leu	Ala	Ser	Ala
LM_4	Ala	Met	Ser	Pro	Ala	Ala	Pro	Leu	Ser	Val	Thr	Glu	Leu	Leu	Leu	Val	Ser	Ala
P-450d	Ala	Phe	Ser	Gln	Tyr	Ile	Ser	Leu	-	Ala	Pro	Glu	Leu	Leu	Leu	Ala	Thr	Ala
HLc		(Met)	Leu	Phe	Pro	Ile	Ser	Met	Ser	Ala	Thr	Glu	Phe	Leu	Leu	Ala	Ser	Val

Fig. 5. NH_2-terminal amino acid sequences of P-450 HLd and related proteins. The NH_2-terminal sequence for P-450 HLd was determined as described in Materials and Methods. The yield for the NH_2-terminal alanine was 76%. The sequences for rabbit P-450 LM4 (19), rat P-450d (20), and P-450 HLc (human P_1-450) (10) are shown for comparison, and are aligned to maximize homology to HLd. A gap is introduced into P-450d at position 9 to maximize homology to HLd. The NH_2-terminal sequence of mouse P_3-450 is identical to that of rat P-450d (21). Since the sequence of P-450 HLc is derived from cDNA (10), it is not known whether the NH_2-terminal Met is present in the mature protein.

amounts of HLp were highest in patients who had received those medications known to induce P-450p (but not P-450d) in the rat (dexamethasone, antiseizure drugs, triacetyloleandomycin) (9).

To purify P-450 HLd, we applied solubilized microsomes from patient 11 to an immunoaffinity column consisting of anti-P-450d bound to Sepharose 4B. After extensive washing and elution of the column with pH 3 buffer, a protein was recovered that migrated upon NaDodSO$_4$-polyacrylamide gel electrophoresis as a single 52.5-kDa band (Fig. 4) and reacted specifically with anti-P-450d or anti-P-450d(-c) upon immunoblot analysis (Fig. 4). Amino-terminal amino acid analyses of the immunopurified HLd showed 56% homology with rat P-450d and 61% homology with rabbit P-450 LM4 through their first 18 amino acids (Fig. 5).

Discussion

In this report we have identified P-450 HLd, an isozyme of human liver cytochrome P-450 that is structurally and immunochemically related to rat P-450d. Through the first 18 NH_2-terminal amino acids the immunoaffinity-purified HLd shows 61% homology with rabbit P-450 LM4 and 56% homology with rat P-450d and mouse P_3-450. In fact, HLd shows greater similarity with rabbit P-450 LM4 (61%) than rat P-450d shares with rabbit LM4 (44%). In addition, each of the amino acid differences between P-450 HLd and rat P-450d or rabbit P-450 LM4 can be accounted for by single base substitutions within the codon for that amino acid. A comparison of the P-450 HLd NH_2-terminal sequence with 13 additional isozymes of rat P-450 revealed only 6–28% homology. When the deduced NH_2-terminal sequence of P-450 HLc (human P_1-450) (10) is aligned with P-450 HLd to maximize homology, the proteins are found to share eight common amino acids. Thus, our results indicate that P-450 HLd is immunochemically and structurally related to the major isosafrole-inducible isozyme of cytochrome P-450 (rat P-450d, mouse P_3-450, and rabbit P-450 LM4) within the MC-inducible cytochrome P-450 gene family.

Based on these results, it may be reasonably concluded that there are a minimum of two human genes and gene products related to the rodent and rabbit MC-cytochromes P-450. Nebert and colleagues (10) have identified one human gene equivalent to P-450c, and Quattrochi *et al.* (22) have obtained evidence from genomic Southern blots for the existence of a second related gene. Moreover, the latter study reported that mRNAs from both genes were expressed in one human liver. Indeed, we used the same human cDNA probe (22) and found by Northern blot analyses that our RNA samples from three human livers contain a hybridizable mRNA species (3.0 kb) related to rabbit P-450 LM4 and, hence, to P-450 HLd (data not shown). Finally, the present study reveals that all of the human livers we have examined to date contain P-450 HLd, and that one contains a second gene product (HLc) related to rat P-450c.

In a preliminary communication, Adams *et al.* (23) analyzed 31 human liver samples by immunoblots developed with antibodies against two rat MC-inducible P-450 isozymes and found that 9 contained two immunoreactive proteins. These proteins were "co-ordinately expressed" (23) in that neither was detected in the remaining 22 liver samples. If Adams *et al.* (23) measured HLd and HLc, then their results are strikingly different from ours because immunoreactive HLd is a ubiquitous isozyme in our patient population. Expression of HLd and HLc in our patients more closely resembles expression of the equivalent forms in untreated rats, mice, and rabbits (3, 24) because HLd was regularly present at variable levels and HLc was rarely detected. The basis for the variability in HLd levels is unknown. Unlike HLp (9), the levels of HLd do not appear to be influenced by any of the medications the patients received (Table 1). It is possible that dietary or other environmental factors affect the level of P-450 HLd, just as isosafrole or certain chlorinated biphenyls selectively foster the accumulation of P-450d (but not P-450c) in rats, in part, by inhibiting the degradation of P-450d (24). A final possibility is that there is genetic polymorphism in expression of HLd.

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