Amino-Terminal Sequence and Structure of Monoclonal Antibody Immunopurified Cytochromes P-450

Kuo-Chi Cheng,[‡] Sang Shin Park,[‡] Henry C. Krutzsch,[§] Preston H. Grantham,[§] Harry V. Gelboin,[‡] and Fred K. Friedman*,[‡]

Laboratory of Molecular Carcinogenesis and Laboratory of Experimental Carcinogenesis, National Cancer Institute, Bethesda, Maryland 20205

Received September 17, 1985; Revised Manuscript Received December 9, 1985

ABSTRACT: Six hepatic cytochromes P-450 were isolated from 3-methylcholanthrene-treated animals by immunopurification with monoclonal antibodies. The purified cytochromes P-450 include 57- and 56-kDa polypeptides from Sprague-Dawley rats, 57- and 56-kDa polypeptides from $C_{57}BL/6$ mice, a 56-kDa polypeptide from DBA/2 mice, and a 53-kDa polypeptide from guinea pigs. These isozymes were structurally compared by peptide mapping using both sodium dodecyl sulfate–polyacrylamide gel electrophoresis and high-pressure liquid chromatography and by amino acid and NH_2-terminal sequence analyses. The 57-kDa polypeptides from rats and mice have similar but nonidentical peptide maps and amino acid compositions and are about 80% homologous in their NH_2-terminal amino acid sequence. The 56-kDa polypeptides from rats and both mice strains have very similar peptide maps and amino acid compositions and identical NH_2-terminal sequences. The NH_2-terminal sequence of the mice 56-kDa polypeptides corresponds to that reported for the mouse P_1-450 isozyme except that we identified two additional residues, proline and serine, at the NH_2 terminus in the 57-kDa polypeptide from $C_{57}BL/6$ mice that were not deduced from the cDNA sequence of the mouse P_1-450 isozyme. The guinea pig 53-kDa polypeptide has a distinct peptide map relative to the other polypeptides studied and an NH_2-terminal sequence with only partial homology to the 56- and 57-kDa polypeptides from rats and mice. This report shows the varying degree of structural relatedness among the isozymes examined and demonstrates the suitability and advantage of immunopurified cytochromes P-450 for sequencing and structural studies.

The cytochromes P-450 of the mixed function oxidase system metabolize a wide range of endogenous and exogenous compounds, including chemical carcinogens, drugs, steroids, and prostaglandins (Conney, 1967; Gillette et al., 1972; Gelboin, 1980). The capability to metabolize such a diverse array of substrates results from a multiplicity of cytochrome P-450 isozymes that have unique yet overlapping substrate specificities (Lu & West, 1980). Further insight into cytochrome P-450 multiplicity, regulation, and phylogenetic relationships would be achieved upon purification and characterization of the isozymes present in various species and tissues under different physiological and environmental conditions, such as exposure to chemical inducers of particular isozymes. Progress toward purification of a large number of isozymes has been hindered by the difficulties encountered in using conventional purification procedures which are lengthy, may result in relatively low yields, and often are not applicable to the purification of cytochromes P-450 present in low concentrations.

Monoclonal antibodies (MAbs)[1] have recently been introduced as a tool for the study of cytochrome P-450 multiplicity (Park et al., 1980, 1982a,b, 1984; Boobis et al., 1981; Reubi et al., 1984). In our laboratory, we have prepared panels of MAbs to a variety of cytochromes P-450 (Park et al., 1980, 1982a,b, 1984). We have previously reported on the use of MAbs in phenotyping cytochrome P-450 dependent catalytic activities in tissues from humans (Fujino et al., 1982; Friedman et al., 1985a) and animals (Fujino et al., 1984), in developing radioimmunoassays for cytochrome P-450 (Song et al., 1984, 1985), and for immunopurification of cytochromes P-450 from different animal tissues (Friedman et al., 1983; Cheng et al., 1984a).

In this report we present structural analyses of MAb-immunopurified cytochromes P-450. Six hepatic cytochrome P-450 isozymes immunopurified from 3-methylcholanthrene-treated animals have been analyzed with respect to amino acid composition, NH_2-terminal amino acid sequence, and peptide patterns formed after proteolytic digestion. These isozymes exhibit varying degrees of structural homology. The newly determined NH_2-terminal sequence of a guinea pig cytochrome P-450 shows its partial homology to the rat and mice isozymes. The NH_2-terminal sequence of an immunopurified mouse isozyme corresponds to that derived from the cDNA sequence of the mouse P_1-450 isozyme except that we detected two NH_2-terminal residues not deduced from the cDNA sequence. The ease and efficiency of MAb-directed immunopurification strongly suggest its future utility for isolating cytochromes P-450 from a variety of biological sources for subsequent sequencing and structural studies.

Experimental Procedures

Preparation of Monoclonal Antibodies and Immunoaffinity Matrix. Production of hybridoma cells was essentially as previously described (Park et al., 1982b). Monoclonal antibodies were prepared from ascites fluid by precipitation with 1.6 M ammonium sulfate. The MAbs used, 1-7-1 and 1-31-2, were prepared to MC-induced rat liver cytochrome P-450. The

* Author to whom correspondence should be addressed.
‡ Laboratory of Molecular Carcinogenesis.
§ Laboratory of Experimental Carcinogenesis.

[1] Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography; MAb, monoclonal antibody; SV8 protease, *Staphylococcus aureus* V8 protease; MC, 3-methylcholanthrene; DMAA, *N*,*N*-dimethyl-*N*-allylamine.

coupling of monoclonal antibodies to CNBr-activated Separose 4B (Pharmacia) was carried out as described previously (Cheng et al., 1984a).

Preparation of Microsomes. Liver microsomes from male Sprague-Dawley rats were prepared by differential centrifugation (Lu & Levin, 1972). The same procedure was used to prepare liver microsomes from guinea pigs (male, 180–200 g, NIH) and DBA/2 and C_{57}BL/6 mice (male, 20–25 g). All animals were treated with MC (ip, 30 mg/kg, dissolved in corn oil) for 3 consecutive days prior to sacrifice. Isolated microsomes were finally suspended in 10 mM phosphate buffered 0.9% saline (pH 7.4) (PBS) containing 25% glycerol and stored at −70 °C.

Immunoaffinity Purification. Fifty milligrams of liver microsomes was suspended in PBS containing 25% glycerol to a final concentration of 1 mg/mL. The microsomes were solubilized by the addition of Emulgen 911 (10%) to a final concentration of 0.2%. The mixture was stirred for 30 min at 4 °C. This step resulted in almost 100% solubilization of microsomal proteins. The solubilized preparations from rats or C_{57}BL/6 mice were first loaded on a Sepharose–MAb 1-31-2 column (1.5 × 5 cm), and the effluent from this column was further loaded on a Sepharose–MAb 1-7-1 column (1.5 × 5 cm). The solubilized preparations of DBA/2 mice and guinea pigs were loaded directly on a Sepharose–MAb 1-7-1 column. After loading, each column was washed sequentially with the following two buffers: 40 mL of PBS (pH 7.4) containing 25% glycerol and 0.2% Emulgen 911 and 40 mL of PBS (pH 5.0) containing 25% glycerol and 0.1% Emulgen 911. The tightly bound material was eluted with 40 mL of 30 mM sodium phosphate buffer (pH 3.0) containing 25% glycerol and 0.1% Emulgen 911. The eluant was adjusted to neutral pH and dialyzed against 20 mM sodium phosphate buffer, pH 7.2, containing 25% glycerol and 0.2% sodium cholate. The dialyzed protein was concentrated on a small hydroxylapatite column, dialyzed against 25 mM sodium phosphate buffer, pH 7.2, containing 25% glycerol, and stored at −70 °C. Protein concentration of the cytochrome P-450 was determined by the method of Lowry et al. (1951).

Peptide Mapping. Peptide maps were prepared by essentially the same procedure as described by Cleveland et al. (1977). In brief, 10 μg of cytochrome P-450 was denatured by the addition of 0.2% SDS and boiled for 2 min. The pH of the sample was then adjusted by the addition of 0.1 M sodium phosphate buffer, pH 7.2, when *Staphylococcus aureus* V8 protease (SV8 protease) (Sigma) was used or by 0.1 M sodium phosphate buffer, pH 8.0, when α-chymotrypsin (Sigma) and endoproteinase Lys-C (Boehringer Mannheim) were used. The amounts of protease used were 0.4 μg for SV8 protease and α-chymotrypsin and 1.0 μg for endoproteinase Lys-C, and digestion time was 1 h for all four proteases. After digestion, the reaction was stopped by boiling for 2 min in 1% SDS. The peptides were separated by SDS–PAGE on gradient slab gels of 12.5–20% polyacrylamide according to Laemmli (1970).

High-pressure liquid chromatography of tryptic and α-chymotryptic digestions of cytochromes P-450 was performed in the following manner. The digestion mixtures, in a final volume of about 0.2 mL, contained 100 μg of cytochrome P-450, 25 mM sodium phosphate buffer, pH 8.0, 4 M urea, 25% glycerol, and 4 μg of protease. The mixture was incubated at 30 °C for 24 h and the reaction stopped by addition of 0.2% trifluoroacetic acid. Seventy-five micrograms of the digest was then applied to a Spectra-Physics Model 8995 HPLC equipped with a Zorbax ODS column (Du Pont, 4.6 × 250 cm). The peptides were eluted by combination of two solvents programmed as follows: 0–4 min with 0.1% aqueous trifluoroacetic acid (solvent A); 4–90 min from 100% solvent A to 35% solvent A and 65% of 0.1% trifluoroacetic acid in acetonitrile (solvent B); 90–95 min from 35% solvent A and 65% solvent B to 100% solvent B. Solvent flow rate was maintained at 1 mL/min. Eluted peptides were monitored by their absorption at 220 nm with a Waters Model 450 detector, and output was recorded on a Spectra-Physics SP4270 integrating recorder. All reagents used were HPLC grade (Fisher).

Amino Acid Analysis. The amino acid composition of cytochrome P-450 was determined essentially as described (Tarr, 1986) by the University of Michigan Protein Sequencing Center (Ann Arbor, MI). Cytochrome P-450 was precipitated by the addition of an equal volume of acetone, washed with water, and then dissolved in 6 N HCl. The sample was sealed under vacuum and subjected to hydrolysis for 24 h at 105 °C. Cysteine was determined as cysteic acid after performic acid oxidation of the protein. Tryptophan was determined spectrophotometrically (Beaven & Holiday, 1952).

NH_2-Terminal Sequence Determination. Amino acid sequencing was carried out on a Beckman 890 sequencer using a 0.1 M Quadrol program. Four to twenty nanomoles of protein was dialyzed for 24 h against 500 mL of glass double distilled water containing 0.05% SDS and 0.5 mL of 0.4 M DMAA in pyridine/water (3/2) (Pierce), followed by another 24-h dialysis against 500 mL of water containing 0.5 mL of a 0.4 M DMAA solution. The sample was lyophilized and then dissolved in 0.4 mL of 62.5% trifluoroacetic acid solution prior to sequencing. Anilinothiazolinone amino acids were manually converted to phenylthiohydantoin derivatives by treatment with 0.2 mL of 1 N HCl at 80 °C for 5 min. The phenylthiohydantoin amino acids were identified and quantitated with a Waters Model 710B HPLC equipped with a WISP autoinjector and a Du Pont Zorbax ODS column (6.2 × 80 cm) at 66 °C. All reagents used for sequencing were sequencer grade from Beckman.

RESULTS

Preparation of Immunopurified Proteins. We have isolated immunochemically related hepatic microsomal cytochromes P-450 from several animals by a simple and efficient immunopurification procedure using MAbs 1-31-2 and 1-7-1, which were prepared to MC-induced rat liver cytochrome P-450 (Cheng et al., 1984a,b). These immunopurified proteins are electrophoretically homogeneous as judged by SDS–PAGE (Cheng et al., 1984b) and gradient SDS–PAGE (Figure 1A) and include the 56- and 57-kDa polypeptides from both Sprague-Dawley rats and C_{57}BL/6 mice, a 56-kDa polypeptide from DBA/2 mice, and a 53-kDa polypeptide from guinea pigs.

Peptide Mapping by SDS–PAGE. To further characterize the structural relatedness of these six immunopurified proteins, their proteolytic digests were analyzed by SDS–PAGE (Figure 1). We established optimal conditions for partial proteolysis with SV8 protease, α-chymotrypsin, and endoproteinase Lys-C, for which the peptide maps did not change significantly from 1 to 2 h of digestion.

Figure 1B presents a typical peptide pattern following digestion by SV8 protease, which specifically cleaves proteins at aspartic and glutamic acids. The peptide map of the 57-kDa polypeptide of Sprague-Dawley rats (track 1) is remarkably similar to that of C_{57}BL/6 mice (track 3). Most of the resolved peptides from rats have mobilities similar to those of mice, although the relative intensities of Coomassie blue staining may differ. This suggests that the proteolytically

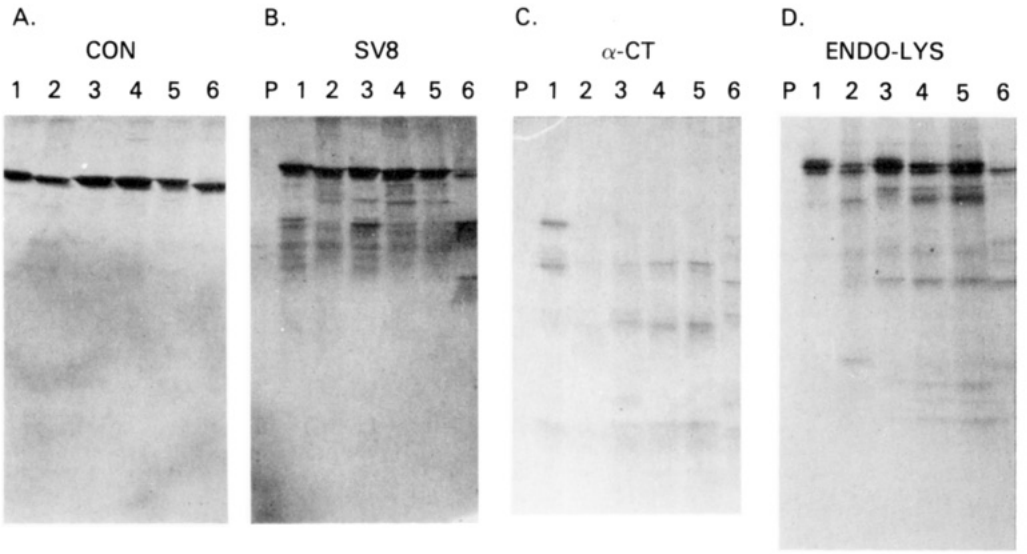

FIGURE 1: Peptide mapping on SDS–PAGE. (A) Untreated cytochromes P-450. Tracks: 1, rat 57-kDa polypeptide; 2, rat 56-kDa polypeptide; 3, $C_{57}BL/6$ mouse 57-kDa polypeptide; 4, $C_{57}BL/6$ mouse 56-kDa polypeptide; 5, DBA/2 mouse 56-kDa polypeptide; 6, guinea pig 53-kDa polypeptide. (B) Cytochromes P-450 digested with SV8 protease. Tracks: P, protease alone; 1, rat 57-kDa polypeptide; 2, rat 56-kDa polypeptide; 3, $C_{57}BL/6$ mouse 57-kDa polypeptide; 4, $C_{57}BL/6$ mouse 56-kDa polypeptide; 5, DBA/2 mouse 56-kDa polypeptide; 6, guinea pig 53-kDa polypeptide. (C) Cytochromes P-450 digested with α-chymotrypsin; samples are in the same order as in (B). (D) Cytochromes P-450 digested with endoproteinase Lys-C; samples are in the same order as in (B).

sensitive sites, while located at the same position, are digested at different rates. Likewise, the peptide maps of the 56-kDa polypeptide (track 2) from Sprague-Dawley rats are also very similar to that of 56-kDa polypeptides from $C_{57}BL/6$ (track 4) and DBA/2 mice (track 5). Although some of the peptides derived from the 57-kDa polypeptides have mobilities similar to those from the 56-kDa polypeptides, the overall peptide patterns for these two polypeptides are definitely different. This result clearly indicates that the 56-kDa polypeptides are not merely posttranslational cleavage products of the 57-kDa polypeptides. On the other hand, the 53-kDa polypeptide of guinea pigs (track 5) exhibits a very different peptide map, indicative of a primary structure that is distinct from the other five polypeptides.

Peptide mapping using α-chymotrypsin is shown in Figure 1C. The peptide pattern generated from the 57-kDa polypeptide of rats (track 1) by α-chymotrypsin resembles that of the 57-kDa polypeptide of $C_{57}BL/6$ mice (track 3). Digestions of the 56-kDa polypeptides from rats (track 2) and mice (tracks 4 and 5) all generate three major peptides, the mobilities of which are similar to three corresponding major peptide products of digestion of the 57-kDa polypeptides. The peptide pattern of the guinea pig 53-kDa polypeptide generated by α-chymotrypsin (track 5) is again distinct from those of the other five polypeptides.

Digestion of the 57- and 56-kDa polypeptides of rats by endoproteinase Lys-C generated few peptides (Figure 1D, tracks 1 and 2). The peptides generated from the 56- and 57-kDa polypeptides of both strains of mice (tracks 3, 4, and 5) have very similar mobilities. The 53-kDa polypeptide of guinea pigs again displayed a unique peptide pattern (track 5).

Peptide Mapping by HPLC. The structural relatedness of the six immunopurified cytochromes P-450 was further examined by peptide mapping using HPLC. The procedure detects major as well as subtle structural differences between isozymes, since HPLC can separate peptides with a single amino acid substitution. The HPLC patterns for tryptic and α-chymotryptic peptides under our established optimal conditions were reproducible and unchanged with a prolonged incubation time. Typical elution patterns for the tryptic peptides of cytochromes P-450 are presented in Figure 2. HPLC resolved more than 40 major peptides from each cytochrome P-450. Although the overall elution pattern of the 57-kDa polypeptide of rats (Figure 2B) did not appear to match that of the other polypeptides, upon close examination we found that 11 out of 40 major peaks of the 57-kDa rat polypeptide had retention times (31.3, 43.6, 45.4, 45.8, 46.2, 47.0, 51.2, 52.8, 66.5, 69.4, and 71.1 min) and intensities

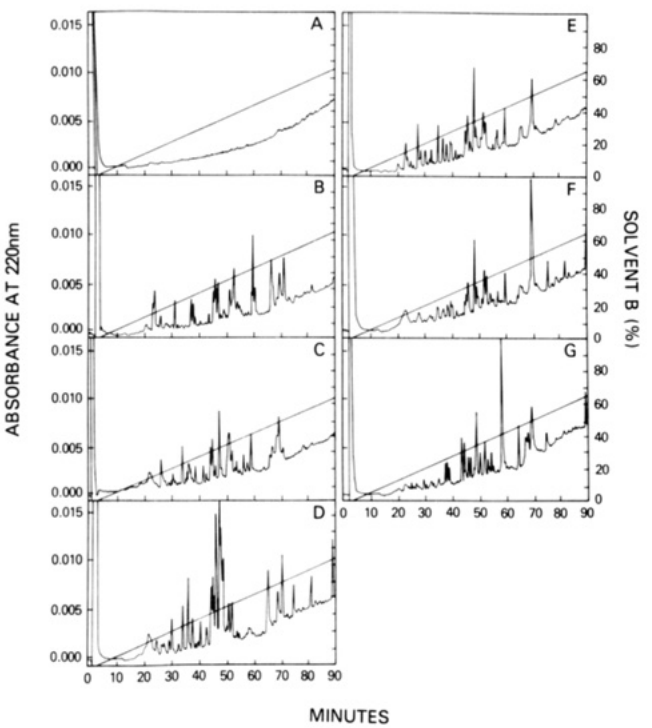

FIGURE 2: Elution patterns of tryptic fragments of cytochromes P-450 on HPLC: (A) blank containing 3.75 μg of trypsin; (B) 75 μg of tryptic digest of rat 57-kDa polypeptide; (C) 75 μg of tryptic digest of rat 56-kDa polypeptide; (D) 75 μg of tryptic digest of $C_{57}BL/6$ mouse 57-kDa polypeptide; (E) 75 μg of tryptic digest of $C_{57}BL/6$ mouse 56-kDa polypeptide; (F) 75 μg of tryptic digest of DBA/2 mouse 56-kDa polypeptide; (G) 75 μg of tryptic digest of guinea pig 53-kDa polypeptide. Elution of peptide was performed with a gradient as shown. Peptides were monitored by their absorption at 220 nm.

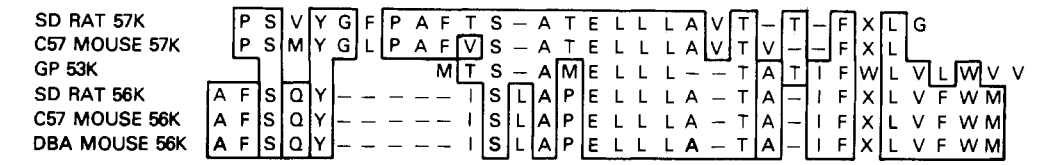

FIGURE 3: Sequence homology of immunopurified cytochromes P-450. The amino acid residues are denoted by their single-letter abbreviations.

Table I: Amino Acid Compositions of Immunopurified Cytochromes P-450

amino acid	residues per cytochrome P-450 polypeptide[a]					
	rat		$C_{57}BL/6$ mouse		DBA/2 mouse,	guinea pig,
	57 kDa	56 kDa	57 kDa	56 kDa	56 kDa	53 kDa
Ala	26	28	25	28	31	25
Cys[b]	9	5	8	8	8	9
Asx	47	45	46	46	51	41
Glx	47	46	45	47	48	45
Phe	32	34	31	34	30	31
Gly	39	36	39	34	39	35
His	18	13	14	13	12	10
Ile	28	27	25	27	27	32
Lys	30	33	30	33	35	30
Leu	58	51	59	51	52	52
Met	8	5	10	8	12	8
Pro	28	33	31	33	31	29
Arg	33	37	37	31	32	34
Ser	38	33	39	33	30	32
Thr	24	25	27	25	23	21
Val	34	41	41	40	42	32
Tyr	12	13	14	13	12	12
Trp[c]	2	4	4	4	3	2

[a] The number of residues of each amino acid is rounded off to the nearest integer. [b] Determined as cysteic acid (Tarr, 1986). [c] Determined spectrophotometrically (Beaven & Holiday, 1952).

similar to those of the 57-kDa polypeptide of $C_{57}BL/6$ mice (Figure 2D). The elution pattern of the rat 56-kDa polypeptide (Figure 2C) only partially resembled that derived from the 56-kDa polypeptides of $C_{57}BL/6$ mice and DBA/2 mice (Figure 2E,F). In particular, the peptide patterns of these three polypeptides between retention times of 42 and 54 min were nearly identical. On the other hand, the 56-kDa polypeptides of the two mice strains exhibited almost identical peptide maps. The pattern of the 53-kDa polypeptide of guinea pigs did not resemble either of the other cytochromes P-450.

HPLC elution patterns of the α-chymotryptic fragments of the immunopurified cytochromes P-450 were also obtained (Figure S1 of the supplementary material; see paragraph at end of paper regarding supplementary material). The profiles of the 57-kDa polypeptides of rats and $C_{57}BL/6$ mice resembled neither each other nor that of the 56-kDa polypeptides. In contrast, the 56-kDa rat polypeptide partially resembled the 56-kDa polypeptides of $C_{57}BL/6$ and BDA/2 mice in their elution patterns. The 53-kDa polypeptide of guinea pigs again displayed a peptide pattern distinct from that of the other five polypeptides.

Amino Acid Composition. Table I presents the amino acid composition of the six immunopurified cytochromes P-450. The compositions of these polypeptides were similar; all contain 40–50% hydrophobic amino acids. The 57-kDa rat polypeptide had an amino acid composition most similar to that of the 57-kDa polypeptide of $C_{57}BL/6$ mice, except for slight differences in the histidine and valine content. The 56-kDa polypeptides of rats and both mice strains had similar amino acid compositions. Comparison of the amino acid composition of the 57-, 56-, and 53-kDa polypeptides reveals significant differences in their amino acid content, especially for histidine, isoleucine, leucine, valine, and serine.

Amino Acid Sequences. NH_2-terminal sequence data were obtained for each of the immunopurified proteins (Figure S2 of the supplementary material). With our sample treatment and sequencing procedures all six proteins exhibited an unblocked and unique NH_2-terminal amino acid. The initial yields for coupling and cleavage of the NH_2-terminal residue

Table II: Amino-Terminal Sequences of Cytochromes P-450 Determined by Edman Degradation or Deduced from Nucleotide Sequences of Cytochrome P-450 cDNAs

Species	Residue 1	5	10	15	20	25
Sprague-Dawley Rats						
57K	H_2N-Pro-Ser-Val-Tyr-Gly-Phe-Pro-Ala-Phe-Thr-Ser-Ala-Thr-Glu-Leu-Leu-Leu-Ala-Val-Thr-Thr-Phe- X -Leu-Gly					
56K	H_2N-Ala-Phe-Ser-Gln-Tyr-Ile-Ser-Leu-Ala-Pro-Glu-Leu-Leu-Leu-Ala-Thr-Ala-Ile-Phe- X -Leu-Val-Phe-Trp-Met					
$C_{57}BL/6$ Mice						
57K	H_2N-Pro-Ser-Met-Tyr-Gly-Leu-Pro-Ala-Phe-Val-Ser-Ala-Thr-Glu-Leu-Leu-Leu-Ala-Val-Thr-Val-Phe- X -Leu- X					
56K	H_2N-Ala-Phe-Ser-Gln-Tyr-Ile-Ser-Leu-Ala-Pro-Glu-Leu-Leu-Leu-Ala-Thr-Ala-Ile-Phe- X -Leu-Val-Phe- X -Met					
DBA/2 Mice						
56K	H_2N-Ala-Phe-Ser-Gln-Tyr-Ile-Ser-Leu-Ala-Pro-Glu-Leu-Leu-Leu-Ala-Thr-Ala-Ile-Phe- X -Leu-Val-Phe-Trp-Met					
Guinea Pigs						
53K	H_2N-Met-Thr-Ser-Ala-Met-Glu-Leu-Leu-Leu-Thr-Ala-Thr-Ile-Phe-Trp-Leu-Val-Leu-Trp-Val-Val- X -Ile-Phe- X					
Long Evans Rats						
P-450c[a]	H_2N-Pro-Ser-Val-Tyr-Gly-Phe-Pro-Ala-Phe-Thr-Ser-Ala-Thr-Glu-Leu-Leu-Leu					
P-450d[b]	H_2N-Ala-Phe-Ser-Gln-Tyr-Ile-Ser-Leu-Ala-Pro-Glu-Leu-Leu-Leu-Ala-Thr-Ala-Ile-Phe					
Sprague-Dawley Rats						
MC-P-450[c]	Pro-Ser-Val-Tyr-Gly-Phe-Pro-Ala-Phe-Thr-Ser-Ala-Thr-Glu-Leu-Leu-Leu-Ala-Val-Thr-Thr-Phe-Cys-Leu-Gly					
$C_{57}BL/6$ Mice						
P_1-450[d]	Met-Tyr-Gly-Leu-Pro-Ala-Phe-Val-Ser-Ala-Thr-Glu-Leu-Leu-Leu-Ala-Val-Thr-Val-Phe-Cys-Leu-Gly					
$C_{57}BL/6$ Mice						
P_3-450[e]	Met-Ala-Phe-Ser-Gln-Tyr-Ile-Ser-Leu-Ala-Pro-Glu-Leu-Leu-Leu-Ala-Thr-Ala-Ile-Phe-Cys-Leu-Val-Phe-Trp-Met					

[a] Sequence is taken from Botelho et al. (1979) and Haniu et al. (1984). [b] Sequence is taken from Botelho et al. (1982). [c] Sequence is deduced from cDNA sequence of Yabusaki et al. (1984). [d] Sequence is deduced from cDNA sequence of Kimura et al. (1984a). [e] Sequence is deduced from cDNA sequence of Kimura et al. (1984b).

varied from 10% to 40%, and the repetitive yield of coupling and cleavage usually reached 95%. The first 25 cycles yielded positively identifiable amino acids, which are listed in Table II. Sequence homology among the immunopurified proteins is illustrated in Figure 3.

Discussion

Monoclonal antibodies have proven useful in several different phases of cytochrome P-450 research (Park et al., 1980, 1982a,b, 1984; Boobis et al., 1981; Fujino et al., 1982, 1984; Friedman et al., 1983, 1985a,b; Cheng et al., 1984a; Reubi et al., 1984; Song et al., 1984, 1985). In this study six cytochromes P-450 were purified to electrophoretic homogeneity from different tissues and species by a simple MAb-directed immunopurification procedure. The structural relationships of these immunopurified cytochromes P-450 were examined by peptide mapping using SDS–PAGE, HPLC, and amino acid composition and amino acid sequence analyses.

Peptide mapping has proven useful in comparative structural analysis of related proteins, including the cytochromes P-450 (Guengerich et al., 1982; Koop et al., 1982; Koop & Coon, 1984). In this study, peptide mapping using SDS–PAGE and HPLC demonstrated structural homology among the 56-kDa polypeptides of rats and both mice strains and between the 57-kDa polypeptides of rats and $C_{57}BL/6$ mice. A more limited structural homology between 56- and 57-kDa polypeptides was also observed. The 53-kDa polypeptide from guinea pigs appeared the most structurally distinct of the six polypeptides examined.

The NH_2-terminal sequences of the 57-kDa polypeptides of rats and $C_{57}BL/6$ mice both commenced with proline and displayed extensive sequence homology (Figure 3). Sequence differences were found in only 4 out of the first 25 residues, which were at residues 3 (Val/Met), 6 (Phe/Leu), 10 (Thr/Val), and 21 (Thr/Val) (Table II). The sequence of the 57-kDa polypeptide of Sprague-Dawley rats was the same as the amino acid sequence deduced from the rat cDNA nucleotide sequence of MC-induced cytochrome P-450 (Yabusaki et al., 1984). This sequence was inconsistent at several positions with that described for purified cytochrome P-450c from Long-Evans rats (Botelho et al., 1979). The latter, however, has subsequently been corrected (Haniu et al., 1984) and is now entirely identical with the sequence we present in this paper.

The NH_2-terminal sequence of the 57-kDa polypeptide of $C_{57}BL/6$ mice was the same as that of the mouse P_1-450 isozyme as recently deduced from its cDNA nucleotide sequence (Kimura et al., 1984a,b) with the exception that the immunopurified protein had an additional Pro-Ser dipeptide at the NH_2 terminus. The nucleotide coding sequence therefore actually commences six nucleotides upstream from that previously reported (Kimura et al., 1984a). This result thus demonstrates that, in addition to the obvious utility of MAb-based immunopurification for sequence studies of proteins of unknown sequence, it may also be applied as a useful method for confirmation of cDNA-derived sequence data as well as for the establishment of reading frames for translating nucleotide to amino acid sequences.

The NH_2-terminal sequence of the 56-kDa polypeptides of rats and $C_{57}BL/6$ and DBA/2 mice was completely conserved (Table II). This sequence was identical with that of cytochrome P-450d of Long-Evans rats (Botelho et al., 1982) as well as with the amino acid sequence deduced from the cDNA sequence of P_3-450 from $C_{57}BL/6$ mice (Kimura et al., 1984b). Although the sequence of this isozyme from DBA/2 mice has not previously been determined, the data we obtained with the DBA/2 mouse 56-kDa polypeptide suggest a high degree of homology with the other 56-kDa polypeptides.

Purification or sequence determination of a guinea pig cytochrome P-450 has not previously been reported. While the NH_2-terminal sequence of the guinea pig 53-kDa polypeptide was the most distinct of the six immunopurified polypeptides, some sequence homology was nevertheless observed. For example, NH_2-terminal residues 2–16 of the guinea pig polypeptide were about 70% homologous (10 out of 15 residues) to residues 10–24 of the rat 57-kDa polypeptide, and a later segment (residues 11–19) of the 53-kDa polypeptide exhibited extensive homology (7 out of 9 residues) to the 56-kDa polypeptide (residues 15–25).

Varying degrees of sequence homology are evident among the six immunopurified cytochromes P-450 (Figure 3). The polypeptides of rats and $C_{57}BL/6$ mice that have the same M_r are highly homologous to one another in their NH_2-terminal sequences. For the two 57-kDa polypeptides only four substitutions are found in the first 25 residues, while this region for the three 56-kDa polypeptides is completely conserved. Using a peptide sequence (Ala-X-Glu-Leu-Leu-Leu) shared by all six cytochromes P-450 as a marker, we have found a significant homology (50%) between the NH_2-terminal sequences of 56- and 57-kDa polypeptides. More strikingly, using the same marker, we have noticed that the NH_2-terminal sequence of guinea pig 53-kDa polypeptide is also homologous to that of these 57- and 56-kDa polypeptides. This common peptide sequence in the first NH_2-terminal residues is a unique feature of MC-inducible cytochromes P-450. However, clusters of hydrophobic amino acids within this region are also a common feature of phenobarbital-inducible and constitutive cytochromes P-450 (Cheng & Shenkman, 1983; Waxman & Walsh, 1983), as they perhaps serve to anchor the NH_2-terminal residues in the membrane.

We previously demonstrated (Cheng et al., 1984a) that the 57-kDa polypeptides of rats and $C_{57}BL/6$ mice contribute most of the aryl hydrocarbon hydroxylase activities in the liver microsomes. Furthermore, for DBA/2 mice, which are relatively noninducible for this activity, the 57-kDa polypeptide was not detected. Our structural characterization indicates that the 57-kDa polypeptides of rats and $C_{57}BL/6$ mouse are thus structural as well as functional counterparts. Likewise, the 56-kDa polypeptide in rat may be the counterpart to the 56-kDa polypeptides of both strains of mice, whereas the 53-kDa guinea pig polypeptide, which bears a lesser degree of structural homology to the other five polypeptides, does not have a counterpart isozyme of similar molecular weight in rats or mice.

A major advantage of the simple MAb-based immunopurification of cytochromes P-450 is its rapidity. This procedure is thus less likely to result in undesirable proteolysis of the protein which may occur during a lengthy, multistep purification. A rapid immunopurification thus is more likely to detect NH_2-terminal amino acids that in some cases can be "clipped" in isozymes during conventional purifications (Fujita et al., 1984).

We have shown that monoclonal antibodies are a powerful tool for identification of counterpart isozymes and structurally related proteins. On the basis of a single-step immunopurification procedure, one can thus isolate MAb-specific proteins from tissue microsomes. The agreement between certain of the sequences we determined and established cytochrome P-450 sequences demonstrated the specificity of the MAb-based procedure for purification of cytochrome P-450, rather than some unrelated protein with an epitope common to that

in cytochrome P-450. Further sequencing and structural analyses of MAb-immunopurified isozymes would be valuable for studying the divergence of structure as well as of the functional domains of cytochromes P-450. The use of MAbs toward this goal is especially advantageous. A minimum number of MAbs can be used to obtain a much larger number of cytochromes P-450, owing to the presence of common MAb-specific epitopes on isozymes that are otherwise structurally distinct. For example, in this study two MAbs were used to purify six distinct cytochromes P-450 from different species and strains. A large number of cytochromes P-450 may therefore be easily obtained from different tissues, strains, and species for sequencing studies. Also, in addition to isolation of cytochromes P-450 from animals, the MAbs used in this study also interact with cytochrome P-450 in human tissues (Fujino et al., 1982; Song et al., 1985) and are thus potentially useful for purifying human cytochromes P-450.

This report further demonstrates the utility of MAbs in different phases of cytochrome P-450 research. MAbs have been previously utilized in phenotyping cytochrome P-450 dependent catalytic activities (Fujino et al., 1982, 1984) and in radioimmunoassay (Song et al., 1984, 1985) and immunopurification (Friedman et al., 1983, 1985b; Cheng et al., 1984a). Thus, the MAb-directed methodologies are very powerful tools both for analysis of cytochromes P-450 and for development of an epitope-based classification system for these isozymes. A library of MAbs would thus serve to prepare an atlas of cytochromes P-450 in different tissues, strains, and species. This information, along with subsequent derivation of their primary structures, will provide a very useful database on the genetics, evolution, and developmental aspects of cytochromes P-450.

Supplementary Material Available

HPLC elution patterns of α-chymotryptic digests of immunopurified proteins (Figure S1) and amino acid sequence data (Figure S2) (5 pages). Ordering information is given on any current masthead page.

Registry No. Cytochrome P-450, 9035-51-2.

References

Beaven, G. H., & Holiday, E. R. (1952) *Adv. Protein Chem. 7*, 343–386.

Boobis, A. R., Slade, M. B., Stern, C., Lewis, K. M., & Davis, D. S. (1981) *Life Sci. 29*, 1443–1448.

Botelho, L. H., Ryan, D. E., & Levin, W. (1979) *J. Biol. Chem. 254*, 5635–5640.

Botelho, L. H., Ryan, D. E., Yuan, P.-M., Kutny, R., Shively, J. E., & Levin, W. (1982) *Biochemistry 21*, 1152–1155.

Cheng, K.-C., & Shenkman, J. B. (1983) *J. Biol. Chem. 258*, 11738–11744.

Cheng, K.-C., Gelboin, H. V., Song, B. J., Park, S. S., & Friedman, F. K. (1984a) *J. Biol. Chem. 259*, 12279–12284.

Cheng, K.-C., Krutzsch, H., Grantham, P., Park, S. S., Gelboin, H. V., & Friedman, F. K. (1984b) *Biochem. Biophys. Res. Commun. 123*, 1201–1208.

Cleveland, D. W., Fischer, S. G., Kirchner, M. W., & Laemmli, U. K. (1977) *J. Biol. Chem. 252*, 1102–1106.

Conney, A. H. (1967) *Pharmacol. Rev. 19*, 315–366.

Gelboin, H. V. (1980) *Physiol. Rev. 60*, 1107–1165.

Gillette, J. R., Davis, D. C., & Sasame, H. A. (1972) *Annu. Rev. Pharmacol. 12*, 57–84.

Guengerich, F. P., Dannan, G. A., Wright, S. T., Martin, M. V., & Kaminsky, L. S. (1982) *Biochemistry 21*, 6019–6030.

Friedman, F. K., Robinson, R. C., Park, S. S., & Gelboin, H. V. (1983) *Biochem. Biophys. Res. Commun. 116*, 859–865.

Friedman, F. K., Robinson, R. C., Song, B. J., Park, S. S., Crespi, C. L., Marletta, M. A., & Gelboin, H. V. (1985a) *Mol. Pharmacol. 27*, 652–655.

Friedman, F. K., Robinson, R. C., Song, B. J., Park, S. S., & Gelboin, H. V. (1985b) *Biochemistry 24*, 7044–7048.

Fujino, T., Park, S. S., West, D., & Gelboin, H. V. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3682–3686.

Fujino, T., West, D., Park, S. S., & Gelboin, H. V. (1984) *J. Biol. Chem. 259*, 9044–9050.

Fujita, V. S., Black, S. D., Tarr, G. E., Koop, D. R., & Coon, M. J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4260–4264.

Haniu, M., Ryan, D. E., Iida, S., Lieber, C. S., Levin, W., & Shively, J. E. (1984) *Arch. Biochem. Biophys. 235*, 304–311.

Kimura, S., Gonzalez, F. J., & Nebert, D. W. (1984a) *J. Biol. Chem. 259*, 10705–10713.

Kimura, S., Gonzalez, F. J., & Nebert, D. W. (1984b) *Nucleic Acids Res. 12*, 2917–2928.

Koop, D. R., & Coon, M. J. (1984) *Mol. Pharmacol. 25*, 494–501.

Koop, D. R., Morgan, E. T., Tarr, G. E., & Coon, M. J. (1982) *J. Biol. Chem. 257*, 8472–8480.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lowry, O. H., Rosebrough, N. L., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Lu, A. Y. H., & Levin, W. (1972) *Biochem. Biophys. Res. Commun. 46*, 1334–1339.

Lu, A. Y. H., & West, S. B. (1980) *Pharmacol. Rev. 31*, 277–295.

Park, S. S., Persson, A. V., Coon, M. J., & Gelboin, H. V. (1980) *FEBS Lett. 42*, 1798–1808.

Park, S. S., Cha, S.-J., Miller, H., Persson, A. V., Coon, M. J., & Gelboin, H. V. (1982a) *Mol. Pharmacol. 21*, 248–258.

Park, S. S., Fujino, T., West, D., Guengerich, F. P., & Gelboin, H. V. (1982b) *Cancer Res. 42*, 1798–1808.

Park, S. S., Fujino, T., & Gelboin, H. V. (1984) *Biochem. Pharmacol. 33*, 2070–2081.

Reubi, I., Griffin, K. J., Raucy, J., & Johnson, E. F. (1984) *J. Biol. Chem. 259*, 5887–5892.

Song, B. J., Fujino, T., Park, S. S., & Gelboin, H. V. (1984) *J. Biol. Chem. 259*, 1394–1397.

Song, B. J., Friedman, F. K., Park, S. S., Tsokos, G. C., & Gelboin, H. V. (1985) *Science (Washington, D.C.) 228*, 490–492.

Tarr, G. E. (1986) in *Microcharacterization of Polypeptides: A Practical Manual* (Shiveley, J. E., Ed.) Humana, Clifton, NJ (in press).

Waxman, D. J., & Walsh, C. (1983) *Biochemistry 22*, 4846–4855.

Yabusaki, Y., Shimizu, M., Murakami, H., Nakamura, K., Oeda, K., & Ohkawa, H. (1984) *Nucleic Acids Res. 12*, 2929–2938.