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Hybrid cytochromes P-450 identify a substrate binding domain in P-450IIC5 and P-450IIC4

(progesterone/deoxycorticosterone/expression/COS-1 cells/mutagenesis)

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ABSTRACT **The cytochrome P-450 superfamily of enzymes catalyzes the oxidative metabolism of innumerable lipophilic compounds (e.g., drugs, carcinogens, steroids). Although the three-dimensional structure of a soluble bacterial P-450 (P-450cam) has been solved, little is known about the structures of the membrane-bound mammalian P-450s. Thus, the structural features of these enzymes that determine their multisubstrate specificity are unknown. In this report, we identify a segment of the primary structure of the structurally similar but functionally distinct cytochromes P-450IIC5 and P-450IIC4, which determines the apparent affinity of these cytochromes for the conversion of progesterone into the mineralocorticoid deoxycorticosterone. P-450IIC5 exhibits a >10-fold lower apparent K_m than P-450IIC4 for progesterone 21-hydroxylation. Chimeric cDNAs were constructed and expressed in COS-1 cells, which encode hybrids between these enzymes. The hybrid enzymes were assayed for catalytic activity and compared to the parental proteins. A segment of P-450IIC5 was identified that conferred the lower K_m of P-450IIC5 to P-450IIC4. Sequential reduction of the length of the exchanged segments led to a hybrid enzyme with a high affinity derived largely from P-450IIC4, which contains three amino acid residues derived from P-450IIC5 clustered between positions 113 and 118. This suggests that this region is part of a substrate binding domain. This region maps by alignment of amino acid sequences to a residue of P-450cam, which has been implicated in substrate binding, suggesting that these segments of the primary structure serve a similar functional role in these two distantly related proteins.**

It is not known how the ability to efficiently metabolize structurally distinct substrates is encoded in the amino acid sequence of individual forms of cytochromes P-450 (P-450)‡ or how the genetic diversity of P-450 genes determines the catalytic diversity of this family of enzymes. Here we identify a domain of a P-450 primary structure that contributes to the determination of these catalytic properties. Our approach to the identification of this domain exploits the structural similarity and functional diversity of P-450IIC4 and P-450IIC5. Because of the high degree of structural similarity between these two proteins, it appeared likely that the interchange of portions of these cDNAs would yield functional enzymes when the chimeric genes were expressed. We anticipated that the hybrid enzymes would exhibit some or all of the properties characteristic of the two parent enzymes.

P-450IIC5 is unusual among the hepatic P-450s in that it catalyzes with a low K_m the conversion of progesterone by 21-hydroxylation to the mineralocorticoid deoxycorticosterone at rates similar to those of the adrenal progesterone 21-hydroxylase (2). P-450IIC4 exhibits a high amino acid sequence identity with P-450IIC5 (3) but no catalytic functions have been identified for this protein. We show here that microsomes prepared from transfected COS-1 cells that express P-450IIC4 catalyze progesterone 21-hydroxylation with a 10-fold or greater K_m than that of P-450IIC5. From cDNAs encoding these isozymes, we constructed and expressed chimeric cDNAs that defined a small segment of the primary structure of P-450IIC5 that confers the higher efficiency for progesterone 21-hydroxylation to the previously inefficient catalyst P-450IIC4.

MATERIALS AND METHODS

The coding regions of the P-450IIC5 cDNA, formerly designated p1–8 (4), and of the P-450IIC4 cDNA, formerly designated p1–88 (3), were inserted into the vector pBluescript (Stratagene) and the mammalian expression vectors pSVL (Pharmacia) and pCMV I (M. Stinski, University of Iowa) by standard techniques (5). Higher levels of expression were obtained by transient transfection of COS-1 cells with constructs in pCMV I than in pSVL. The constructs derived from p1–8 and p1–88 are designated pCMVIIC5 and pCMVIIC4, respectively.

Chimeric genes were constructed from these cDNAs in pBluescript or pSVL by exchange of restriction fragments, and each was inserted into pCMV I for expression in COS-1 cells by transient transfection. Constructs were verified by restriction mapping. Chimeras A, E, and G were further characterized by dideoxynucleotide sequencing across the junctions where the interchanges were made. In some cases, a new restriction site was introduced to match a site in one of the parent genes by site-directed mutagenesis. This led to the introduction of a *Sty* I site in P-450IIC5 by changing A to C at nucleotide 370 and a *Dra* III restriction site in P-450IIC4 by changing T to C at nucleotide 528. The latter introduced a serine found in P-450IIC5 in place of the Leu-162 of P-450IIC4. This mutation is present in constructs using splice site 3 (D, E, and F in Fig. 4). Site-directed mutagenesis was based on the phosphorothionate method (6, 7) with the following modifications. The single-stranded template was rescued from pBluescript with VCS M13 helper phage (Stratagene) according to the manufacturer's instructions. Double-stranded phosphorothionate DNA was nicked with *Pst* I. In addition, an $Asn^{118} \rightarrow$ Lys mutation was introduced into IIC4 by changing nucleotide 397 from G to C. Mutants were identified by restriction mapping and confirmed by dideoxynucleotide sequencing (8).

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‡P-450, a generic term for one or more forms of cytochrome P-450, individual forms of P-450 are designated according to a proposed uniform system of nomenclature (1). The common usage names are rabbit cytochrome P-450 1 for P-450IIC5 and P-450cam for P-450CIA1.

COS-1 cells were kindly provided by M. Waterman (University of Texas Southwestern Medical Center). The cells were transfected by the DEAE-dextran procedure (9, 10). DNA for transfection was purified in a CsCl step gradient (11). Cells were harvested in phosphate-buffered saline with a rubber policeman or were assayed for progesterone metabolism 36–48 hr after transfection.

Microsomes were prepared from COS-1 cells by homogenization in 1 ml of 0.25 M sucrose/1 mM EDTA with 12 strokes in a Potter–Elvehjem glass/glass homogenizer (Kontes) at ≈200 rpm. The homogenate was centrifuged at 10,000 × *g* for 5 min, and microsomes were sedimented from the supernatant at 150,000 × *g* for 20 min at 4°C in a TL100 tabletop ultracentrifuge. Microsomes were resuspended in 50 mM potassium phosphate, pH 7.4/0.2 mM EDTA/20% (vol/vol) glycerol.

For immunoblots, 50 μg of microsomal protein was applied to each well of a SDS/10% polyacrylamide gel (12), transferred to nitrocellulose (13), and reacted sequentially with the monoclonal antibody 2F5 (14), rabbit anti-mouse IgG (Sigma), and ^{125}I-labeled protein A (Amersham). Monoclonal antibody 2F5 reacts with both P-450IIC5 and P-450IIC4 (3). The protein bands were visualized by autoradiography.

Cellular progesterone 21-hydroxylase activity was determined by supplementing the culture medium with 2 or 20 μM [4-^{14}C]progesterone (56 Ci/mmol; 1 Ci = 37 GBq; Amersham). After 60 min, 21-hydroxyprogesterone levels were assayed in the medium by TLC and autoradiography as described (2).

Microsomal progesterone 21-hydroxylase activity was determined by a radiometric TLC assay as described (2) with the following modifications: the assay was performed at 37°C in 0.1 ml of 50 mM potassium phosphate (pH 7.4) containing 5–20 μg of microsomal protein and an NADPH-regenerating system consisting of 5 mM isocitrate, 0.05 unit of isocitrate dehydrogenase, 5 mM $MgCl_2$, and 1 mM NADPH. The substrate was added as methanolic solution with the final concentration of methanol being 1%. The reaction was initiated by the addition of the NADPH-regenerating system. The reaction catalyzed by microsomes from cells transfected with pCMVIIC5 was linear with protein (up to 50 μg per assay) and time (0–60 min). At the end of the 60-min incubation period, unlabeled progesterone (4 nmol) and 21-hydroxyprogesterone (2 nmol) were added, and the products were extracted with 1 ml of chloroform. The chloroform phase was evaporated and analyzed by TLC (1).

RESULTS

Progesterone 21-Hydroxylase Activity of P-450IIC5 and P-450IIC4. To compare the enzymic characteristics of P-450IIC5 and P-450IIC4, the corresponding cDNAs were inserted into the pCMV I vector and expressed in COS-1 cells by transfection. When the cell culture medium was supplemented with progesterone at 20 μM, cells transfected with either P-450IIC5 or P-450IIC4 exhibited progesterone 21-hydroxylase activity (Fig. 1). At lower substrate concentrations (2 μM), cells transfected with P-450IIC4 showed only weak activity but those transfected with P-450IIC5 are still highly active (60- to 80-fold above untransfected cells; Fig. 1). This suggests that both cDNAs direct the expression of catalytically active proteins and that these enzymes differ in their apparent K_m for the substrate progesterone.

Microsomes were isolated from transfected cells and characterized for the expression of protein and enzyme activity. Immunoblotting with the monoclonal antibody 2F5 (14) indicated that proteins that comigrated with purified P-450 1 were expressed in microsomes from cells transfected with each of the constructs but not in mock-transfected cells (Fig. 2). When the 21-hydroxylation of progesterone catalyzed by

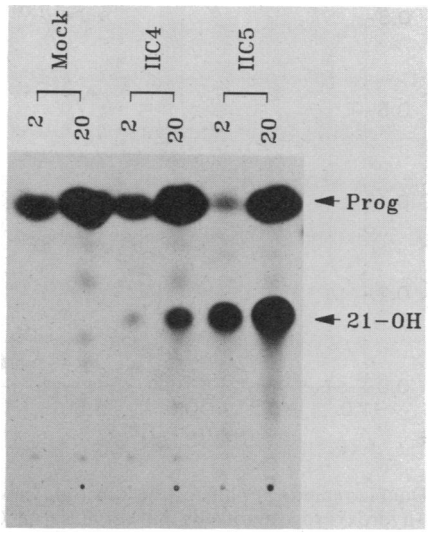

FIG. 1. Progesterone 21-hydroxylation in transfected and mock-transfected COS-1 cells at 2 and 20 μM substrate concentrations. Cells were transfected in 10-cm-diameter culture dishes with pCMV1 vector (mock) or the P-450IIC4 (IIC4) or P-450IIC5 (IIC5) cDNAs cloned in pCMV1. Thirty-six hours after transfection, the medium was removed and replaced by 2 ml of medium containing 2 μM or 20 μM [4-^{14}C]progesterone as indicated on top. After a 1-hr incubation at 37°C the medium was removed and analyzed as described. Arrows labeled Prog and 21-OH indicate the mobilities of unmetabolized substrate and of the metabolite 21-hydroxyprogesterone (deoxycorticosterone), respectively. An autoradiogram of the thin-layer chromatogram is shown. Typically, 3–6 nmol of product is formed in 60 min at 37°C by cells in a 10-cm dish transfected with P-450IIC5 under the conditions shown for a 2-μM initial substrate concentration (30–60% substrate conversion).

microsomes isolated from the transfected cells was assessed, the microsomes containing P-450IIC5 exhibited an apparent K_m of 1.8 μM (1.6 and 2.0 μM in two independent transfections), which is close to the value of 1.7 μM reported for the purified enzyme (15). In contrast, the apparent K_m for progesterone 21-hydroxylation of the expressed P-450IIC4 could not be precisely determined but was clearly >20 μM (Fig. 3). V_{max} values varied for different experiments and appeared to reflect the efficiency of expression as judged by immunoblotting. Values of 42 and 25 pmol·mg^{-1}·min^{-1} were esti-

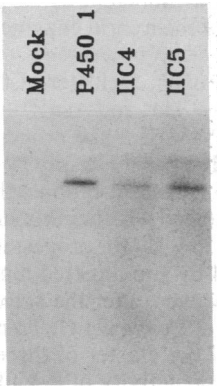

FIG. 2. Immunoblot of microsomes prepared from COS-1 cells and of purified rabbit P-450 1. Microsomal protein (50 μg) from mock-transfected COS-1 cells (Mock), from cells transfected with pCMVIIC4 (IIC4) and pCMVIIC5 (IIC5) DNAs and 1 pmol of purified rabbit P-450IIC5 (P450 1) were separated in a SDS/10% polyacrylamide gel, transferred to nitrocellulose, and reacted sequentially with the monoclonal antibody 2F5, rabbit anti-mouse antibody, and ^{125}I-labeled protein A as described. An autoradiogram of the blot is shown.

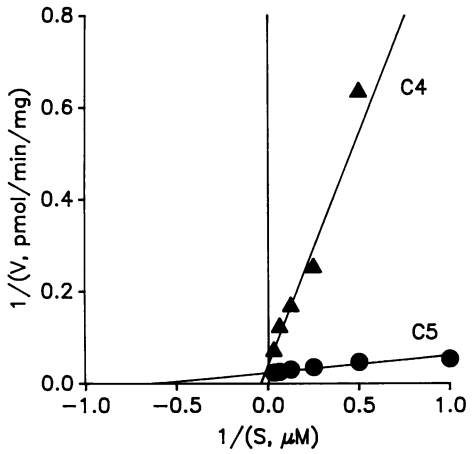

FIG. 3. Double-reciprocal plots of the substrate-dependent formation of 21-hydroxyprogesterone by microsomes from COS-1 cells transfected with pCMVIIC5 (●; K_m = 1.6 μM, V_{max} = 42 pmol·min^{-1}·mg^{-1}) or pCMVIIC4 (▲; K_m = 25 μM, V_{max} = 25 pmol·min^{-1}·mg^{-1}). Lines show the estimates obtained by nonlinear minimization of residuals from untransformed values assuming Michaelis–Menten kinetics.

mated for P-450IIC5 and P-450IIC4, respectively, from the experiment shown in Fig. 3. Based on these experiments, we conclude that P-450IIC5 and P-450IIC4 differ largely in their apparent K_m for progesterone 21-hydroxylation.

Identification of Segments of P-450IIC5 That Confer a Low K_m for Progesterone to P-450IIC4. Chimeric genes were constructed and expressed to identify segments of the primary structure that contain determinants for the apparent K_m for progesterone 21-hydroxylation. Hybrid enzymes with high affinity were tentatively identified by their ability to form deoxycorticosterone at rates similar to P-450IIC5 at a 2-μM concentration of progesterone. The activity of P-450IIC4 is roughly 10-fold lower than that of P-450IIC5 at this substrate concentration. The chimeras were constructed in a step-wise fashion as outlined in Fig. 4. Each step was designed to reduce by about one-half the number of amino acid changes resulting from the transfer of a segment of pCMVIIC5 to pCMVIIC4. Restriction sites common to both cDNAs were used when possible. When necessary, restriction sites found in one of the pair were introduced into the other by site-directed mutagenesis to provide appropriate sites. The activities of the hybrid enzymes are summarized in Fig. 4.

Based on this approach, a cluster of three substitutions—Val^{113} → Ala, Ser^{115} → Thr, and Asn^{118} → Lys—proved to be sufficient to confer P-450IIC5-like progesterone 21-hydroxylase activity to P-450IIC4 in this assay system (construct G in Fig. 4). The Asn^{118} → Lys substitution is the least conservative of the three, and we tested whether this substitution alone was sufficient to confer a low K_m for progesterone 21-hydroxylase activity to P-450IIC4 by site-directed mutagenesis. This substitution did not, however, alter the activity of P-450IIC4.

Kinetic Properties of Construct G. To more precisely characterize the effect of the cluster of three amino acid substitutions on the catalytic activity of P-450IIC4 toward progesterone, we examined the kinetic properties of progesterone 21-hydroxylation in microsomal fractions from cells transfected with the "minimal" hybrid G. The apparent K_m for progesterone 21-hydroxylation in microsomes of cells transfected with construct G was 3 μM (2 and 4 μM in independent determinations) and the maximal velocity was 35 pmol·mg^{-1}·min^{-1}. When compared with P-450IIC4, the values for construct G indicate a significant increase in affinity toward the substrate progesterone, mediated by the cluster of three

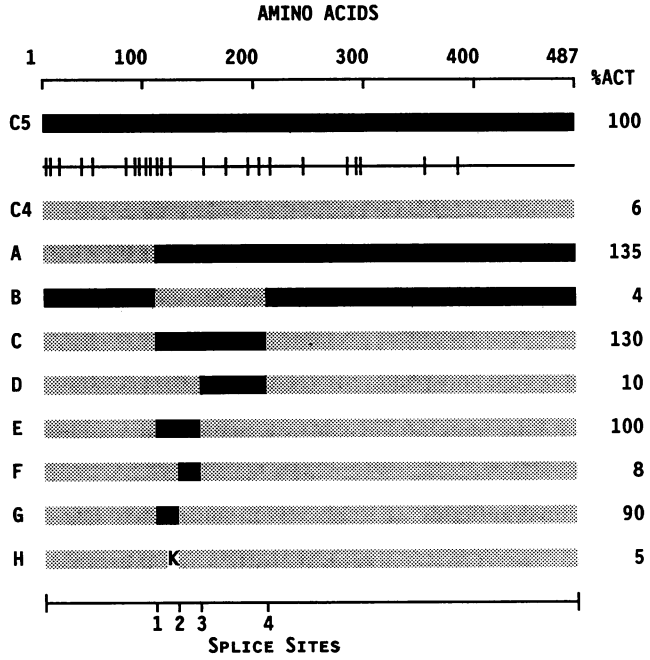

FIG. 4. Structure and progesterone 21-hydroxylase activities of P-450IIC5, P-450IIC4, and the hybrid proteins expressed in COS-1 cells. Progesterone 21-hydroxylase activity of hybrids between P-450IIC4 and P-450IIC5 was determined for cultured cells incubated with 2 μM progesterone. The left column identifies each hybrid according to the nomenclature used in the text. Hybrids are represented by solid and stippled segments corresponding to each of the parent proteins P-450IIC5 (C5) or P-450IIC4 (C4), respectively. Numbers at the bottom refer to the following splice sites used for the constructions: 1, *Sty* I restriction site in P-450IIC4, a silent mutation introduced this site into P-450IIC5; 2, *Bsp* HI; 3, a *Dra* III restriction site corresponding to a site in P-450IIC5 was introduced into P-450IIC4, this yields a Leu^{162} → Ser point mutation with no increased 21-hydroxylase activity; 4, *Sty* I. The four splice sites correspond to codons 110, 128, 162, and 210. The distribution of the 25 differences of amino acid sequence is shown by vertical lines between the parent proteins. Left column shows the progesterone 21-hydroxylase activity of cells transfected with chimeric genes or the parent plasmids expressed as a percentage of the activity (% ACT) of P-450IIC5 (3–6 nmol per plate per 60 min). The initial concentration of progesterone was chosen to maximize differences between low K_m and high K_m enzymes. The results of two or more experiments are summarized.

substitutions for amino acids 113, 115, and 118 in P-450IIC4. The maximal velocity of hybrid G is similar to values obtained for P-450IIC5. Immunoblotting indicates that hybrid G is expressed at similar concentrations as P-450IIC5 in COS-1 cell microsomes but these results cannot discriminate whether the small difference in V_{max} reflects a difference in enzyme concentration or an intrinsic property of the protein.

DISCUSSION

P-450IIC5 exhibits a >10-fold lower apparent K_m than P-450IIC4 for progesterone 21-hydroxylation. Identification and sequential reduction of amino acids of P-450IIC5 required to confer a low K_m for progesterone 21-hydroxylase activity to P-450IIC4 identified the final hybrid G (Fig. 4), which contains three amino acids from P-450IIC5 clustered at positions 113–118. When compared with P-450IIC4, this hybrid has a greatly enhanced affinity for progesterone. Therefore, we conclude that region 113–118 contains the major determinant of the differences in the affinity of P-450IIC5 and P-450IIC4 toward progesterone. This suggests that region 113–118 is (part of) a substrate binding domain in P-450IIC5.

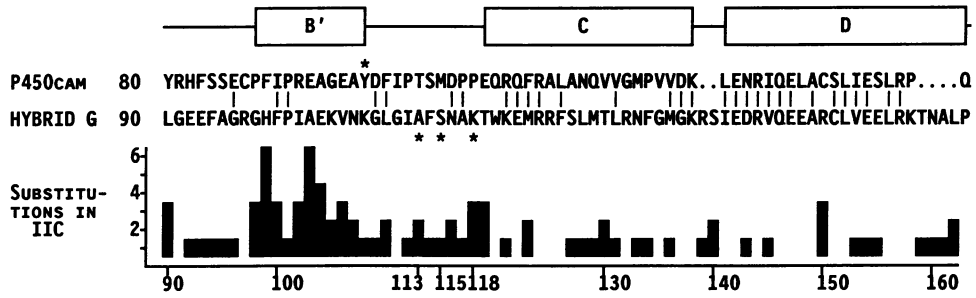

FIG. 5. Amino acid sequence alignment between hybrid G and P-450cam. Tyr^{96} of P-450cam and Ala^{113}, Ser^{115}, and Lys^{118} of hybrid G, which were derived from P-450IIC5, are indicated by asterisks. Amino acids similar in both sequences based on the evolutionary distance as measured by Dayhoff and normalized by Gribskov and Burgess (21) are matched by vertical lines. The helical domains of P-450cam deduced from the x-ray structure (16) are shown above the P-450cam sequence; gaps in the sequence are shown as dots. The frequency of substitutions among 10 class IIC P-450s is shown by the histogram below the sequence of hybrid G. The single-letter code for amino acids is used.

The region 113–118 maps closely to Tyr^{96} of P-450cam when the primary structures of P-450IIC5 and P-450cam are aligned (Fig. 5). Although the overall sequence similarity between these two proteins is low, a region of high sequence similarity is found near the three substitutions that confer efficient progesterone 21-hydroxylase activity to P-450IIC4. This point of reference corresponds to helix D in P-450cam (16). The Tyr^{96} of P-450cam appears from the x-ray structure to hydrogen bond with the substrate camphor (16), and mutants in which this residue was altered implicate Tyr^{96} as a determinant of camphor binding to P-450cam (17). Moreover, Phe^{98} and Phe^{87} of P-450cam are also in close proximity to the camphor molecule. This suggests that this region of P-450cam serves a function similar to that of the region defined in this article of the distantly related P-450IIC5 (113–118).

The segment of the amino acid sequence defined in this study complements other segments of the linear structures of P-450 enzymes that have been implicated in the determination of the substrate selectivities of other forms of cytochrome P-450. The carboxyl-terminal 375 amino acids of P-450 LM6 (rabbit P-450IA1) and P-450 LM4 (rabbit P-450IA2) (18) and the central third of P-450c (rat P-450IA1) and P-450d (rat P-450IA2) (19) have been implicated as determinants of substrate selectivity from studies of hybrid enzymes. In addition, Imai (20) characterized substrate-induced spectral changes for hybrid proteins expressed in yeast from chimeric genes derived from P-450 pHP3 (P-450IIC14) and P-450 pHP2-1 (P-450IIC2) and identified a region of these proteins, amino acid residues 211–262, as a determinant of substrate binding. However, this determinant alone was not able to confer catalytic activity to the hybrid proteins (20).

The present study has defined a specific region of the primary structures of P-450IIC5 and P-450IIC4 that determines the difference in apparent K_m for progesterone 21-hydroxylation exhibited by these enzymes. If this region is a determinant of substrate specificity among P-450s in general, it is interesting to note that this portion of the amino acid sequence is highly variable among class IIC P-450s (Fig. 5) and, thus, may contribute to their catalytic diversity. In addition, the generation of structurally distinct catalytically active, hybrid enzymes could lead to the identification of P-450s with substrate selectivities that are distinct from either of the parent enzymes. The application of this approach to the generation of additional catalytically active hybrid enzymes should lead to a more complete understanding of structural determinants of the catalytic diversity of cytochrome P-450 enzymes.

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