An Inhibitory Monoclonal Antibody Binds in Close Proximity to a Determinant for Substrate Binding in Cytochrome P450IIC5*

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We used the expression of chimeric proteins and point mutants to identify amino acids of the hepatic progesterone 21-hydroxylase P450IIC5 which are part of an epitope recognized by an inhibitory monoclonal antibody and which affect substrate binding. Three amino acids of P450IIC5 at positions 113, 115, and 118 were introduced into P450IIC4, which is 95% identical to P450IIC5. The resultant chimeric protein acquired binding of the monoclonal antibody 1F11, which is highly specific and inhibitory for P450IIC5. Point mutants in P450IIC4 showed that two of the three changes, T115S and N118K, contribute to the epitope recognized by this antibody. The T115S mutant bound the antibody weakly (Kₐ >30 nM) whereas the N118K mutant bound the antibody as tightly as P450IIC5 (Kₐ ~0.7 nM). Thus, residues 115 and 118 are located on the surface of these enzymes, and the Lys/Asn difference at amino acid 118 is largely responsible for the high degree of discrimination which this antibody exhibits between P450IIC5 and P450IIC4. The valine to alanine mutation at position 113 conferred to P450IIC4 a lower apparent Kₐ for progesterone 21-hydroxylation. Because antibody binding was not affected by this mutation, it is tempting to speculate that this residue is buried in the protein where it exerts its effect on the catalytic activity by interaction with the substrate or alters the positions of residues of the active site. The close proximity of the epitope at positions 115 and 118 to Ala113 suggests that the inhibitory monoclonal antibody interferes with substrate binding.

The mammalian enzymes of the cytochrome P450 (P450)
superfamily metabolize a multitude of substrates and only few details are known about how this ability is encoded in the primary and tertiary structures of these proteins. This is largely due to the lack of direct three-dimensional structural information for the mammalian P450 enzymes. We have used previously the expression of chimeric P450s to define segments in the primary structures of P450IIC4 and P450IIC5 which determine substrate binding (1). Epitopes of antibodies which are directed against functional proteins are expected to lie on the surface of the molecule and therefore have the potential to yield information about the three-dimensional organization of the protein. Thus, the identification of epitopes provides information regarding surface residues of the protein. Antipeptide antibodies that react with functional proteins and that are directed toward specific segments of the protein provide similar information, and they have been useful in the elucidation of the topology of P450 enzymes (2, 3). In this report, we describe the mapping of amino acids of the epitope recognized by a monoclonal antibody, 1F11, to obtain structural and functional information about the two progesterone 21-hydroxylases P450IIC5 and P450IIC4. When compared with P450IIC4, P450IIC5 catalyzes progesterone 21-hydroxylation with a >10-fold lower apparent Kₐ. The 1F11 antibody is highly specific for and inhibitory of P450IIC5 (4), but it does not react with P450IIC4. We have expressed chimeric enzymes derived from these two P450s and point mutants of P450IIC4 to identify not only residues on the surface of these proteins which contribute to the epitope recognized by the 1F11 monoclonal antibody, but also to identify a specific residue which modulates the apparent Kₐ for progesterone 21-hydroxylation.

MATERIALS AND METHODS

The monoclonal antibodies 1F11 and 2F5 were developed against P450 1 (P450IIC5) which had been purified from rabbit liver as reported earlier (4). Chimeric cDNAs were constructed in pBluescript by use of helper phage R408 (Stratagene, La Jolla, CA) or M13K07 (Pharmacia LKB Biotechnology Inc.). Site-directed mutagenesis was performed with the phosphorothionate method (5), using reagents supplied by Amersham Corp.

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1 The abbreviations and conventions used in this article are: The generic term "P450" is used to indicate a cytochrome P-450. Individual forms of P450 are designated according to the uniform system of nomenclature described by Nebert et al. (26) with the following exceptions: The common name, P450cam, is used for P450CI. Two forms of mouse P450IIA (14) which had not been assigned designations in the last published listing (26) are designated as P450₁₄ and P450₁₅, to distinguish the steroid 15α-hydroxylase and the coumarin hydroxylase, respectively. Mutations are designated by indicating the one-letter abbreviation for the residue that was replaced, its position in the sequence, and the one-letter designation of the new residue in the indicated order.
COS1 cells were grown in T75 or T225 flasks in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (GIBCO), modified Eagle's medium-nonsense amino acids (GIBCO), and 50 units/ml each of penicillin and streptomycin (GIBCO). The cells were transfected by the DEAE-dextran method (6). Microsomal fractions were prepared from cells 48-72 h after transfection by differential centrifugation as described (1). The 21-hydroxylation of progesterone was determined in vivo at 48 h after transfection by supplementation of the culture medium for 1 h with 2 μM [14C]progesterone, followed by extraction and quantification of the 21-hydroxyprogesterone as described earlier (1). Microsomal fractions from transfected cells were transferred onto a nylon filter using a dot blot apparatus (Schleicher & Schuell). Free binding sites were blocked with a blocking solution consisting of 2% nonfat dry milk, 0.05% Tween 20, 0.01% sodium azide, 0.15 M NaCl, and 0.01 M sodium phosphate, pH 7.4. The filter was then cut into strips which were incubated separately in 15-ml centrifuge tubes with 2 ml of microsomal fraction which had been diluted to the appropriate concentrations in blocking solution. After four washes with blocking solution, the strips were incubated with [3H]-labeled sheep-anti-mouse IgG (Amersham) and washed extensively in blocking solution. Bound label was visualized on the filter by autoradiography after it was reassembled. Individual dots were then cut from the strips, and the radioactivity bound to each was determined using a γ-counter. These values were fit with either a four-parameter logistic equation or the following equation,

$$B = \frac{B_{max}[Ab]}{K_d + [Ab]} + C$$

where $B$ is the amount of radioactivity bound to the sample (counts/min), $B_{max}$ is the estimate for maximum binding (counts/min), $K_d$ is the total molar concentration of antibody, $K_d$ is the estimated dissociation constant, and $C$ is an estimate for a constant amount of nonspecific binding (counts/min). Both methods yield indistinguishable estimates of $K_d$ by nonlinear regression using Sigma Plot 4.0 (Jandel Scientific, Corte Madera, CA).

Immunoblotting was performed by electrophoretically transferring 20 μg of microsomal protein separated on a 10% polyacrylamide gel containing 1% sodium dodecyl sulfate (7) onto nitrocellulose (8). Free binding sites were blocked with blocking solution, and the proteins were detected with 2F5, a monoclonal antibody which reacts with both P450IIIC4 and P450IIIC5 (9). This antibody recognizes an epitope that is spatially distinct from that which binds the IF1 monoclonal antibody and, in contrast to the latter, it detects P450IIIC5 on immunoblot. Monoclonal antibody 2F5 was detected using autoradiography with either [125I]-labeled goat-anti-mouse IgG or with a sheep-anti-mouse IgG conjugated to horseradish peroxidase and a luminescent substrate (ECL, Amersham).

Immunoinhibition was determined by incubating COS1 microsomes for 15 min at 22 °C in a volume of 90 μl containing 10 μM progesterone with or without 2 pmol of monoclonal antibody 1F11. The reaction was started by addition of 10 μl of a NADPH-regenerating system (10), and the sample was incubated for 60 min at 37 °C.

The substrate dependence of progesterone 21-hydroxylation was determined as described earlier (1). Briefly, 25 μg of microsomal protein were incubated in a final volume of 100 μl with varying amounts of [14C]progesterone and a NADPH-regenerating system for 30 min. After extraction with chloroform and thin layer chromatography, progesterone and 21-hydroxyprogesterone were quantified by scintillation counting. The values for apparent $K_d$ and $V_{max}$ were estimated by nonlinear fitting of the Michaelis-Menten equation to the experimentally determined rates.

**RESULTS**

**Differential Binding of the Monoclonal Antibody 1F11 to P450IIIC4, P450IIIC5, and Chimera G**—We have shown previously that P450IIIC5 and P450IIIC4 metabolize progesterone to deoxycorticosterone by 21-hydroxylation when the proteins are expressed from their corresponding cDNAs in COS1 cells (1). The greater-fold higher $K_d$ of P450IIIC4 for progesterone can be lowered to the apparent $K_d$ of P450IIIC5 in a chimeric enzyme which carries three amino acids of P450IIIC5 at positions 113, 115, and 118 in P450IIIC4 (1). This chimera is referred to as chimera G.

To compare the binding of the 1F11 monoclonal antibody to chimera G with that to P450IIIC5 and P450IIIC4, we used a filter-binding assay to determine the reactivity of the 1F11 monoclonal antibody with microsomal fractions prepared from COS1 cells, expressing each protein following transfection with an expression plasmid harboring cDNAs encoding the individual proteins. Fig. 1 shows that the 1F11 monoclonal antibody binds tightly to chimera G indicating that the three amino acid differences, which determine the apparent $K_d$ toward progesterone, also transfer onto P450IIIC4 a crucial determinant of the epitope recognized by the 1F11 monoclonal antibody. This binding affinity is similar to that of the 1F11 monoclonal antibody with P450IIIC5 (Fig. 2), whereas no binding to IIC4 is detectable.

Monoclonal Antibody 1F11 Is a Potent Inhibitor of Chimera G—The efficient binding of the 1F11 monoclonal antibody to hybrid G suggested that this antibody also would inhibit

![Fig. 1. Concentration-dependent binding of monoclonal antibody 1F11 to P450 enzymes expressed in COS1 cells. A, microsomal fractions were prepared from COS1 cells transfected with expression vectors encoding P450IIIC4 (C4), P450IIIC5 (C5), chimera G (Chi G), which contains three amino acids from P450IIIC5 at positions 113, 115, and 118 in P450IIIC4, P450IIIC4-V115A (V115A), P450IIIC4-T115S (T115S), P450IIIC4-N118K (N118K), or transfected without DNA (Mock). 10 μg of microsomal protein were applied onto a nylon filter with a dot blot apparatus. The filter was then cut into vertical strips and each strip was incubated with monoclonal antibody 1F11 at the indicated concentrations. The 1F11 antibody was then detected with a [125I]-labeled sheep anti-mouse IgG, the strips were reassembled in the original order and exposed to x-ray film. B, Western blot of the samples in A with the monoclonal antibody 2F5. Ala, Ser, and Lys refer to the V115A, T115S, and N118K mutants of P450IIIC4, respectively. 20 μg of microsomal protein were denatured and separated on a 10% polyacrylamide gel containing sodium dodecyl sulfate. The P450s were detected with the monoclonal antibody 2F5, a sheep anti-mouse antibody conjugated to horseradish peroxidase, and subsequent luminescence detection by exposure to x-ray film for 30 s. Monoclonal antibody 2F5 reacts with both P450IIIC4 and P450IIIC5.
The monoclonal antibody 1F11 is highly specific for P450IIC5 and does not react measurably with P450IIC4. The
FIG. 3. Catalytic activities and expression levels of point mutants of P450IIC4 and of the parent proteins expressed in COS1 cells. A, the culture medium of COS1 cells was supplemented with 2 μM [14C]progesterone 2 days after transfection with DNA encoding P450IIC4 (C4), P450IIC5 (C5), P450IIC4-V113A (V113A), P450IIC4-T115S (T115S), P450IIC4-N118K (N118K), or without DNA (Mock). After 1 h at 37 °C the products were extracted and analyzed by TLC and autoradiography (27). P denotes the mobility of progesterone. DOC indicates the mobility of the metabolite 11-deoxycorticosterone (21-hydroxyprogesterone). The two lanes for both the V113A and T115S mutants represent results obtained with two independent clones selected in the mutagenesis experiment and confirmed by sequence analysis.

B, immunoblot of microsomal fractions obtained from the cells in A. 20 μg of microsomal protein was separated on a 10% polyacrylamide-sodium dodecyl sulfate gel, blotted to nitrocellulose, and reacted sequentially with monoclonal antibody 2F5 and a 125I-labeled sheep anti-mouse IgG. An autoradiogram of the blot is shown.

exchange in P450IIC4 of the three amino acids at positions 113, 115, and 118 of P450IIC5 to form chimera G was sufficient, however, to confer immunoreactivity with the 1F11 monoclonal antibody to this enzyme, and the antibody was found to inhibit the microsomal enzyme. This indicates that

at least part of this segment is exposed at the surface of this functional protein. A similar finding was made for P450IIB1 in a previous study in which its topology was investigated with antipeptide-antibodies (2). This study showed that antibodies directed against two peptides corresponding to amino acids 108–116 and 122–131, respectively, of P450IIB1 bound to the native enzyme in an enzyme-linked immunosorbent assay. Moreover, this antibody correctly localized the enzyme in rat liver microsomes as judged by immunoelectron microscopy and enzyme-linked immunosorbent assay (2). These two peptides align to residues 107–115 and 121–130, respectively, of P450IIC5. Thus the segment of P450IIC5 which we show in this study to contribute to the epitope recognized by the 1F11 monoclonal antibody lies between these two segments of P450IIB1. It is therefore likely that residues in this region are on the surface of both P450IIB1 and P450IIC5.

Because chimera G also exhibits the apparent $K_m$ of P450IIC5 for progesterone 21-hydroxylation rather than that of P450IIC4, we asked whether the changes necessary to confer immunoreactivity are identical or independent from the changes which modulate the apparent $K_m$. Two individual mutations in P450IIC4, N118K and T115S, were able to transfer immunoreactivity with the 1F11 monoclonal antibody onto P450IIC4. This indicates, that Ser$^{118}$ and Lys$^{118}$ of P450IIC5 are part of the epitope recognized by the 1F11 monoclonal antibody and that their side chains are on the surface of the protein. The T115S change had a clearly detectable but weak effect when compared to the N118K mutation which alone conferred most of the immunoreactivity to P450IIC4. This indicates that for monoclonal antibody 1F11, Lys$^{118}$ is the major determinant which makes P450IIC5 distinguishable from P450IIC4.

The T115S and the N118K changes had no detectable effect individually on the catalytic activity of the mutants. The V113A change, however, decreased the apparent $K_m$ of P450IIC4 from >25 μM (1) to 7.5 μM. The apparent $K_m$ of the wild type P450IIC5 is 1.7 μM (1, 12). This marked decrease in $K_m$ is likely to reflect an increase in the affinity of the enzyme for the substrate resulting from either a direct interaction of Ala$^{113}$ with the substrate or by an alteration of the confor-
The bacterial protein which contains Tyr\textsuperscript{14} (16, 22), a residue which determines binding of the substrate camphor in P450cam by positioning the substrate by hydrogen bonding (23, 24). This surface loop is one of five flexible loops which are thought to control the access of the substrate to P450cam (25). Thus, the V113A mutation which affects the $K_m$, for progesterone 21-hydroxylation could affect either the binding of progesterone directly or the access of progesterone to its binding site. This suggests that despite their low identity in amino acid sequence, P450 enzymes share important topological features. The substrate-contact loop of the P450cam structure which harbors Tyr\textsuperscript{12} (23) could therefore be a general determinant of substrate specificity in P450 enzymes. Because this loop aligns to the hypervariable region of the eukaryotic P450s which we have characterized here, it is tempting to speculate that the loop structure of this region allows genetic variation to occur which alters substrate selectivity but does not disrupt the overall topology of the P450s thereby contributing to the functional diversity of closely related P450 enzymes.

This study has defined structural features of P450s IIC5 and IIC4 which are likely to be conserved in all members of class II, the largest and most diverse of the P450 gene family. Residue 113 markedly influences catalytic activity and other residues in close proximity are located at the surface where they act as epitopes for inhibitory antibodies.

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