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REVIEW

Recent advances in P450 research

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ABSTRACT
P450 enzymes comprise a superfamily of heme-containing proteins that catalyze oxidative metabolism of structurally diverse chemicals. Over the past few years, there has been significant progress in P450 research on many fronts and the information gained is currently being applied to both drug development and clinical practice. Recently, a major accomplishment occurred when the structure of a mammalian P450 was determined by crystallography. Results from these studies will have a major impact on understanding structure-activity relationships of P450 enzymes and promote prediction of drug interactions. In addition, new technologies have facilitated the identification of several new P450 alleles. This information will profoundly affect our understanding of the causes attributed to interindividual variations in drug responses and link these differences to efficacy or toxicity of many therapeutic agents. Finally, the recent accomplishments towards constructing P450 null animals have afforded determination of the role of these enzymes in toxicity. Moreover, advances have been made towards the construction of humanized transgenic animals and plants. Overall, the outcome of recent developments in the P450 arena will be safer and more efficient drug therapies. *The Pharmacogenomics Journal* (2001) **1,** 178–186.

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The first report of an unknown carbon monoxide binding pigment in rat liver microsomes was in 1958. Four years later, the hemoprotein nature of the microsomal pigment was elucidated and named cytochrome P450 because of its unique 450-nm optical absorption peak which occurred when the cytochrome was in its reduced carbon monoxide bound form. It was a year later, 1963, when the function of P450 as a monooxygenase in the metabolism of 17-hydroxyprogesterone was discovered.[1] Since that time, an enormous amount of literature concerning P450 has accumulated.

From the 1960s to the present, research in the P450 field has involved identification of their catalytic properties by testing thousands of compounds. In the 1970s, regulatory mechanisms and multiplicity of P450 enzymes began to be elucidated, and in the 1980s and 1990s, cloning and identification of additional genes occurred. To date, about 100 P450 genes have been identified in mammalian species. Each of these enzymes appears to be variously expressed in different organs or cell types and can change during the development of a species. In addition to the mammalian enzymes, numerous and interesting P450 genes are also being described in microbes, fungi, plants and insects.[2]

Cytochrome P450 (CYP) is now regarded as a superfamily of heme-containing enzymes that have diversified from a single ancestral protein to many different forms that are widely distributed in nature. The mammalian P450 gene family dates back about a billion years when it evolved from CYP51 (lanosterol 14α-

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demethylase) of *Ascomycete fungi*.[3] However, it is generally believed that the xenobiotic metabolizing enzymes in families 1–4 evolved later, about 600 million years ago. This was a time when animals became terrestrial and P450s in these families evolved due to the interaction between plants and animals.[4]

P450 enzymes are targets for recent research because they are involved in a vast array of biological processes and catalyze chemicals by a variety of oxidative mechanisms. The result of these oxidations may be activation or inactivation of a substrate. Almost any moderately to highly hydrophobic organic chemical is likely to be a substrate for a P450.[2] These chemicals include not only exogenous compounds such as drugs, pollutants, pesticides and natural products, but also many endogenous chemicals including steroids, eicosanoids, and other important regulatory molecules.

Because CYP genes play key roles in so many areas, the need to understand the diversity, functions and regulation of these enzymes continues. Studies on CYP which have shown a remarkable expansion in the past 40 years, have mainly focused on mammalian P450s participating in the metabolism of xenobiotic compounds, particularly drugs, and the biosynthesis and metabolism of steroid hormones and other lipids.[1] Although many new aspects of P450 research have been forthcoming including the elucidation of receptors involved in xenobiotic regulation of certain CYPs, this review will limit its focus to three areas of investigation including new advances in P450 structure, on describing polymorphisms within the P450 superfamily, and finally, the use of transgenic animals for characterizing P450 functions.

P450 STRUCTURE

P450s are a complex gene superfamily comprised of over 70 families. They function to metabolize xenobiotics and to synthesize biologically active compounds such as steroids, prostaglandins and arachidonate metabolites. Although all P450s have a similar structural core, each has a preferred set of substrates and a unique mechanism of substrate recognition. The method in which P450s recognize their substrates has largely eluded investigators in the field for years, thereby making it difficult to fully determine their function. In order to better understand the functionality of individual CYPS, it has become important to determine their structure.

Understanding P450 structure has been extremely difficult especially for the mammalian enzymes because of their hydrophobicity. Thus, only the structures of the soluble enzymes have been determined. These studies began in 1985 with the publication of the first crystalline structure of P450cam. Since then, several soluble bacterial P450s have been crystallized and their structures determined including a recently crystallized enzyme, CYP51 from *Mycobacterium tuberculosis*.[5] Of the bacterial enzymes, P450cam (CYP101) and P450BM3 (CYP102) are probably most well understood.[6] Between these two bacterial P450s, there is about 20% sequence identity and 47% similarity.

All P450s have a similar three-dimensional structure with a similar three-dimensinal structure with a conserved structural core[6] (Figure 1). Moreover, a region that is associated with substrate binding and conserved throughout the P450 family is the center portion of the I helix.[7] The microbial P450s contain four β sheets and approximately 13 α helices with an insertion termed the 'J' helix. The conserved structural core consists of a four-helix bundle, comprising helices D, E, I, and L and helices J and K. Helix I contains a highly conserved threonine with an acidic residue just N-terminal to this which is positioned over the pyrrole ring B in the active site. Helix K contains the absolutely conserved Glu-X-X-Arg motif that may be involved in stabilizing the core structure. Helix L forms part of the heme-binding region. There are two sets of structurally conserved β sheets: β sheet 1 containing five strands and β sheet 2 with two strands. These sheets help form the hydrophobic substrate access channel. As is true for all P450s, there is a structurally conserved consensus sequence on the proximal face of the heme containing the absolutely conserved cysteine residue. This residue forms the 5th ligand of the heme iron and is the reason for the characteristic 450-nm Soret absorbance found in the carbon monoxide bound protein. All the P450 structures contain a coil termed the 'meander' which is highly conserved in structure and is also located on the proximal face of the protein.[6]

Because there had not been a crystal structure of a mammalian P450, in 1992 Gotoh,[8] using the structure of CYP101 identified regions that he believed were involved in substrate recognition designating them with an SRS (Substrate Recognition Site) number. These SRS regions were extrapolated to the mammalian microsomal P450s (Figure 1). While most of the SRS regions were actually believed to be in the active site/heme pocket and involved in substrate orientation, helix A, the F–G loop and β strands 1–1, and 1–2 appear to be involved in substrate recognition. The variable regions believed to be associated with substrate binding are helices A, B, B′, F, and G and their adjacent loops. Loops B-B′ and B′-C line the active site (SRS-1) and helices F and G and their loops form part of the access channel and the ceiling of the active site (SRS-2 and SRS-3). The β turn at the end of the β sheet 4 protrudes into the active site (SRS-6) as does the region at the N-terminus of β strand 1–4 (SRS-5). As previously mentioned for the microbial enzymes, the other region associated with substrate binding and conserved throughout the P450 family is the center portion of helix I (SRS-4).[6,7]

Recently a major milestone was achieved with the first crystallization and structural analysis of a microsomal eucaryotic P450.[9] The difficulty in preparing crystalline structures of a microsomal P450 was overcome by engineering a more soluble enzyme. CYP2C5, a progesterone hydroxylase, was modified to increase solubility and promote crystallization. Removal of the N-terminal trans domain and the addition of a 4-residue histidine tag to the C-terminus resulted in P450 constructs that were more soluble and fully active. Other mutations between amino acid residues 201 to 210 were also made to diminish membrane interaction and aggregation of CYP2C5.[10] This engineered P450 was used to produce dif-

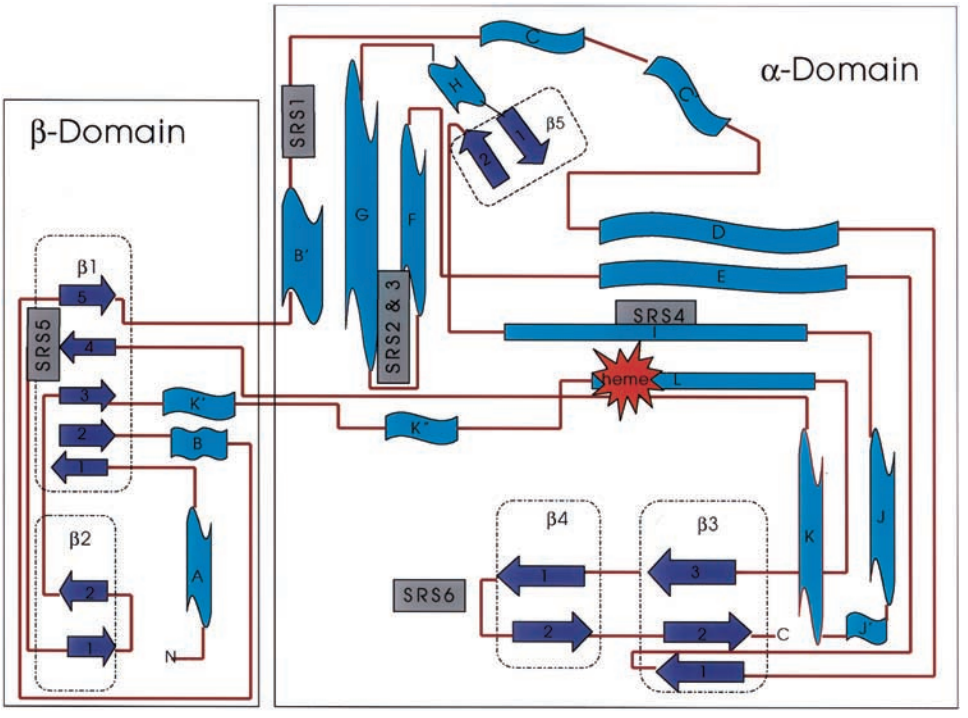

Figure 1 A cartoon illustrating the secondary structure of P450 enzymes. The turquoise banners represent helices, blue arrows represent the β-sheets, and the brown lines represent the random coils connecting the helices and strands. The approximate SRS regions are represented by a gray square. The sizes of the elements are not in proportion to their actual length. The figure is a modified representation of the two domains and was adapted from Peterson and Graham.[6]

fraction quality crystals that have yielded the first mammalian P450 structure.[9]

Once the structure of CYP2C5 had been determined, differences between the microbial P450, CYP102, and CYP2C5 were identified.[9] The topological elements that define the active site of CYP2C5 are the same as those seen in microbial P450s, but the spatial arrangement differs. Only SRS-4 located in the center of helix I where it crosses the heme is highly conserved. The relative positions of the remaining SRSs are more varied among P450 structures and their locations in CYP2C5 differ considerably from those of CYP102. There are significant structural differences between the substrate binding site of this P450 and those of soluble microbial P450s as the spatial organization is poorly conserved.

The most divergent topological regions between CYP2C5 and CYP102 are the N-terminal β sheet domain and the F–G loop. The N-terminal domain of CYP2C5 is rotated relative to the heme-binding site when compared to the CYP102 structure.[9] However, the spatial organization of the heme-binding site of CYP2C5 is similar to the soluble microbial P450s. Also similar to microbial P450s is the axial ligand at the fifth coordination site of the heme iron which is provided by Cys^{432}. Coordination of the heme iron by a cysteine plays a central role in the capacity of P450s to catalyze the scission of the dioxygen bound to the 6th coordination site of the heme iron.

Examination of the crystalline structure revealed that the orientation of CYP2C5 relative to the membrane, positions the electrostatic dipole of the P450 to maximize the attraction between the enzyme and reductase.[9]. The entrance to the putative substrate access channel between the F–G loop and the N-terminal β sheet system is located in this membrane attachment surface. This suggests that lipophilic substrates and products could enter and exit directly from the bilayer. An alternative substrate access channel is suggested by the relatively loose packing of the B–C loop with helices G and I. This alternate route may allow substrates to enter along helix I and would allow metabolites with increased water solubility to exit into the cytoplasm. All of the residues that contact or nearly contact the docked progesterone in CYP2C5 are in the six predicted SRSs.[9] Results from these studies suggest mechanisms for the interaction between mammalian P450s and their substrates.

More recently, a crystal structure was obtained for a ligand-binding domain of the human nuclear pregnane X receptor (PXR) or steroid and xenobiotic receptor (SXR).[11] In addition, the same ligand-binding domain complexed to the cholesterol-lowering agent, SR12813 was crystallized. This receptor plays an integral role in the regulation of the drug metabolizing P450, CYP3A4, by modulating its expression in response to certain xenobiotics. Thus determining its structure can provide information regarding which xenobiotics may affect expression of CYP3A4. From the crystalline structure, it was determined that a small number of polar residues are spaced throughout the smooth hydrophobic ligand-binding pocket of hPXR. The unique

composition of the ligand pocket allows hPXR to bind a diverse set of chemicals and also permits a single ligand to dock in multiple orientations.[11] These results provide important insights into how hPXR interacts with xenobiotics.

P450 POLYMORPHISMS

Due to the recent sequencing of the human genome, a great deal of activity has occurred in the identification of new genes and allelic variants. Examination of the genome revealed that only about 3% is coding sequence and the frequency of polymorphisms in these regions is about 1 in 1000 bases.[12] Genetic polymorphisms are defined as allelic variants that occur with a frequency of 1% or greater within the human population.[13] Many polymorphisms will be silent, but about 68% of the possible mutations will result in an amino acid substitution.[3] Geneticists consider any allele that persists in a population at a frequency of <1 in 10^6 as having a reason for existing which can include selective pressures such as diet or environmental constituents.[14]

In order for organisms to handle selective pressures such as chemicals in the environment, the xenobiotic or drug metabolizing enzymes evolved and with that evolution came a great deal of heterogeneity in xenobiotic metabolizing capabilities. Genetic heterogeneity has been increasingly recognized as a significant source of variation in drug response.[15] This variation is largely due to inherited differences that can influence the pharmacokinetics and hence pharmacology of drug substrates. The effects can be profound toxicity or reduced efficacy of drugs metabolized by such polymorphic enzymes.[12] A new area of investigation, pharmacogenomics, has emerged that is applicable to the enzymes involved in the metabolism of xenobiotics and the molecular events associated with polymorphisms. Pharmacogenomics has become a popular field that will eventually explain why drugs work better in some individuals than they do in others.[16]

The molecular genetic basis for inherited traits in metabolic enzymes began to be elucidated in the late 1980s with the initial cloning and characterization of a polymorphic human gene encoding the drug metabolizing enzyme, debrisoquine hydroxylase (CYP2D6).[12] Since that time, many polymorphisms have been defined for the drug metabolizing enzymes. Of the enzymes exhibiting polymorphisms, the CYPs or P450 enzymes have been best characterized and play a significant role in individual differences in response to therapeutics. Many P450-dependent drug-metabolism reactions are performed by polymorphic enzymes.[4] Indeed, of the different human P450 genes identified thus far, about 60% of these exhibit polymorphic sites that affect the protein sequence. Some are known and have been associated with the ability to metabolize drugs. Other polymorphic sites will be outside the coding region and may influence gene expression.[3] A continuously updated website (http://www.imm.ki.se/CYPalleles/) provides a major mechanism for viewing newly identified P450 alleles and relevant references.

Rapid progress in identifying polymorphisms has been made possible by new techniques including allele-specific hybridization coupled with real-time PCR or micro array analysis.[17] Currently, allele identification by DNA-chip analysis is expensive and few reports describe the use of this technology for screening single-nucleotide polymorphisms (SNPs). However, real-time PCR is being used with more frequency. In this assay, the hybridization probes are combined with the traditional forward and reverse primers. Generally, hybridization is performed with two different oligonucleotides that hybridize to two adjacent internal sequences during amplification. One probe is labeled with a reporter fluorochrome and the other with a quencher fluorochrome. After hybridization, the probes are in close proximity and the emitted fluorescence is quantitated. Differentiation of different alleles is performed by determining the melting curves after PCR. PCR products are then subjected to PCR direct sequencing. Using real time PCR and generation of the melting profiles of an allele-specific fluorescent probe, a polymorphism was identified at codon 432 in the CYP1B1 gene, named CYP1B1*2.[18] Geneotype frequency was determined to be 0.4 for the CYP1B1*2 allele.

In addition, allele-specific hybridization coupled with PCR can be used to screen many samples at a given time. For example, CYP2D6 was amplified from DNA isolated from 22 samples of human liver. The allelic frequencies of the more common CYP2D6*4 and *5 variants were found to be 22% and 9%, respectively.[19] In another study, the CYP2E1 alleles, c1 and c2 were analyzed in 102 blood samples of Japanese volunteers.[20] The frequencies of the normal and mutant alleles were found to be 0.83 and 0.17, respectively. With these technologies, the number of known defective P450 alleles is increasing at a rapid pace. The catalytic activity of the mutant P450s can be divided into three classifications, those where the enzyme is absent producing no activity, those with an enzyme exhibiting diminished activity, and a third group containing P450s that possess enhanced metabolic capabilities (Table 1).

Completely inactive alleles have been found for many P540s including CYP2D6, CYP2C19, and CYP2A6 (Table 1). The homozygous presence of such alleles leads to a total absence of enzyme and an impaired ability to metabolize drugs specific for these enzymes, hence the poor metabolizer (PM) phenotype. More than 30 different defective CYP2D6 alleles and about 70 CYP2D6 variants have been identified.[4] Of these, the six most common defective alleles will predict the PM phenotype.[21]

Genetic polymorphisms exhibiting inactive alleles are also associated with CYP2C19, the *S*-mephenytoin hydroxylase. The CYP2C19 polymorphism affects as many as 20% of Asians but only 3% of Caucasians. Indeed, a marked difference occurs in the frequency of the PM phenotype between Caucasians and Orientals.[22] However, there are few differences between Caucasians and Blacks in the frequency of the CYP2C19 PM phenotype. Therefore, certain racial differences in expression of CYP2C19 can affect drug elimination and response. At least nine different defective CYP2C19 alleles have been identified.[23–27] Of these CYP2C19*2 and CYPC19*3 contain mutations that create an aberrant splice site and a premature stop codon and are the most common

Table 1 CYP alleles and enzyme activity

No Activity (No Enzyme Expressed)	Decreased Activity	Increased Activity
CYP2A6*2, 2A6*4A, 2A6*4B, 2A6*4D, 2A6*5, CYP2A6*3	CYP2A6*6, 2A6*7, 2A6*9	
CYP2C19*2A, 2C19*2B, 2C19*3, 2C19*4, 2C19*5A, 2C19*5B, 2C19*6, 2C19*7, 2C19*8,		
CYP2D6*3A, 2D6*4A, 2D6*4B, 2D6*4C, 2D6*4D, 2D6*4K, 2D6*4X2, 2D6*5, 2D6*6A, 2D6*6B, 2D6*6C, 2D6*7, 2D6*8, 2D6*11, 2D6*12, 2D6*13, 2D6*14, 2D6*15, 2D6*16, 2D6*19, 2D6*20, 2D6*38	CYP2D6*2A, 2D6*9, 2D6*10A, 2D6*10B, 2D6*17, 2D6*18, 2D6*36, 2D6*41	CYP2D6*2XN CYP2D6*1XN
	CYP1A2*1C	CYP1A2*1F
	CYP2E1*2	CYP2E1*1D
	CYP2C9*2, 2C9*3	
CYP3A5*3, CYP3A5*5, CYP3A5*6		CYP3A5 *1
		CYP2B6*6

mutant alleles (Table 1). However, additional mutations must be present in Caucasians given that the known alleles do not account for all PMs identified.

Five different defective CYP2A6 alleles have also been described thus far including CYP2A6*2 which encodes an inactive enzyme resulting from a Leu^{160}His substitution, and CYP2A6*3 probably generated through gene conversion between CYP2A6 and CYP2A7, a pseudogene, causing an inactive product[28] (Table 1). The allele frequency is ~1–3% for CYP2A6*2 in Caucasians whereas a deletion of the CYP2A6 gene appears to be very common among Asian populations.[4] In addition, a novel single nucleotide polymorphism (SNP) that alters stability and activity of CYP2A6, CYP2A6*8, in Japanese has recently been described.[29] A SNP at T^{1412}C resulted in Ile^{471}Thr substitution. The frequency of this polymorphism was high, 15.7%, and caused a reduction in stability of the heterologously expressed enzyme. Moreover, the mutant exhibited coumarin hydroxylase activity but lacked the ability to C-oxidize nicotine. SNPs in other P450s that affect expression have recently been found. CYP3A5*3 and CYP3A5*6 exhibit SNPs that cause alternative splicing and protein truncation resulting in the absence of CYP3A5 from tissues of certain individuals.[30]

In addition to defective CYP genes, there are also alleles that cause diminished or altered enzyme activity.[4] The most common CYP2D6 allele in the Chinese is CYP2D6*10, Pro^{34}-Ser substitution, resulting in an enzyme that exhibits an impaired folding capacity and the expression of a functional enzyme is diminished. The most frequent CYP2D6 variant in Black African (Zimbabwean) and Afro-American populations appears to be CYP2D6*17. The enzyme exhibits a five-fold higher K_m for codeine. An additional CYP possessing mutant alleles with altered activity is CYP2C9.[31] Two variant CYP2C9 enzymes are known and both exhibit amino acid substitutions. CYP2C9*2 encodes an enzyme with an Arg^{144}Cys mutation that displays impaired functional interactions with P450 reductase. The other variant, CYP2C9*3 yields an enzyme with an Ile^{359}Leu that has a lower affinity for many substrates (Table 1). Additional CYP2A6 genes have been found with dimished activity including CYP2A6*6,[32] CYP2A6*7,[29] and CYP2A6*9.[33] CYP2C8 was also found to exhibit polymorphisms that

resulted in altered kinetic parameters. CYP2C8*2 and *3 have higher K_m and V_{max} values for the 6 α-hydroxylation of paclitaxel when compared to wild-type CYP2C8, CYP2C8*1.[34]

Ultra-rapid metabolism is caused by the occurrence of duplicated, multiduplicated or amplified genes.[4] At present, alleles with 2, 3, 4, 5 and 13 gene copies in tandem have been reported for CYP2D6 and the number of individuals carrying multiple gene copies of this enzyme is highest in Ethiopia and Saudi Arabia where up to a third of the population (29%) displays this genotype (Table 1). Another example of an enzyme with an allele that exhibits greater metabolic capabilities when compared to its wild-type counterpart is CYP3A5. This P450 is polymorphically expressed at high levels in a minority of Caucasians. This polymorphism is due to SNPs in the promoter region of the CYP3A5 gene and individuals with the CYP3A5*1 allele constitutively express large amounts of the enzyme[30] (Table 1). The occurrence of this P450 is also affected by race. CYP3A5 was more frequently expressed in African Americans (60%) than in Caucasians (33%). This P450 represents 50% of the hepatic CYP3A content in certain individuals expressing the enzyme, suggesting that CYP3A5 is important in interindividual and interracial differences in reactions catalyzed by the CYP3A enzymes. A SNP was also found in another P450, CYP2B6, which resulted in enhanced catalytic activity due to a substitutions from $Gln^{172}His$. The frequency of this allele was 19.9% in the Japanese population.[35]

USE OF GENETICALLY ALTERED ANIMALS TO STUDY P450 FUNCTION

The role of human CYPs in metabolism of xenobiotics including therapeutic agents has been studied *in vitro* through the use of inhibitory antibodies or specific inhibitors and in animals by CYP-specific inducers. Recent progress in molecular biology has facilitated the engineering of animal models that do not express CYPs or receptors involved in the regulation of P450s.[36–40] These null mutants have enabled direct studies of the *in vivo* role of P450 enzymes in the toxicity, metabolism and carcinogenesis of xenobiotics.

The first report of a CYP null mouse was in 1995 describing the Cyp1a2 knockout animals[41] and since that time many other mouse lines have been developed. These null mouse lines are generally lacking one of the xenobiotic-metabolizing CYPs that may be associated with adverse drug reactions, but have not been linked to serious developmental or physiological defects or pathological phenotypes. The construction of these mouse lines has led to the conclusion that xenobiotic-metabolizing CYPs may only be required for metabolism of foreign chemicals[36] and that they are not essential for mammalian development or physiological homeostasis.[37] Moreover, those CYPs with conserved activity and regulation across species, CYP1A1, 1A2, 1B1, and 2E1, have been good candidates for null mouse construction.[37] These null mouse lines provide relevant information on the functions of their human counterparts.

Recently a Cyp1a1 knockout mouse line was constructed[42] and when hepatic enzyme activities associated with Cyp1a1 were assessed in microsomes from control and TCDD-treated Cyp1a1 (–/–) animals, a significant loss of aryl hydrocarbon hydroxylase activity (AHH) and ethoxyresorufin O-deethylase activity (EROD) was noted, when compared with microsomes from treated and untreated wild-type mice. Because Cyp1a1 plays a key role in the metabolic potentiation of many environmental carcinogens, mutagens and toxins, this mouse line is invaluable for carcinogenesis and toxicity studies. Similarly, Cyp1a2 plays a role in metabolic activation of many xenobiotics making this P450 important in carcinogenesis studies. Thus, mouse lines have also been constructed that lack Cyp1a2.[43,44]

CYP1B1 shares many properties with CYP1A1 such as the metabolic activation of polycyclic aromatic hydrocarbons (PAHs). In addition to activating polycyclic aromatic hydrocarbons, CYP1B1 functions as a physiological regulator in that a deficiency in the enzyme has been linked to congenital glaucoma in humans. The exact role of CYP1B1 in this disorder is not yet known. Because catalytic activity and expression patterns between mouse and humans are fairly well conserved, a Cyp1b1 null mouse has recently been constructed.[45] These mice are almost completely protected from acute bone marrow cytotoxicity initiated by 7,12-dimethylbenz[*a*]anthracene (DMBA). In addition, the preleukemic effects of DMBA were ablated and these animals did not exhibit appreciable amounts of DMBA-DNA adducts in bone marrow when compared to wild-type mice. Cyp1b1-null mice that were intragastrically exposed to DMBA revealed an attenuated incidence of lymphomas when compared to wild-type mice.[46] Such observations suggest that tissue-specific expression of CYP1B1 may contribute to cancer susceptibility in humans.

CYP2E1 metabolizes a large number of low-molecular weight chemicals such as industrial solvents and chemicals of toxicological and carcinogenic significance. This P450 is constitutively expressed and induced in liver and other tissues by treatment with ethanol and other small molecular weight compounds. A Cyp2e1-null mouse line has been established[47] and used to investigate the role of CYP2E1 in toxicity associated with benzene and acetaminophen. Myelotoxicity due to benzene exposure is a consequence of metabolism by CYP2E1, thus the null mice were considerably more resistant to benzene-induced cytotoxicity than wild-type mice.[48] Similar results were obtained when acetaminophen was the substrate. This analgesic is also metabolized to a reactive intermediate by CYP2E1.[49] Mice lacking Cyp2e1 were more resistant to acetaminophen-mediated hepatotoxicity when compared to wild-type mice, confirming that CYP2E1 is responsible for mediating toxicity from this drug. These results are in good agreement with *in vitro* studies, which demonstrate that microsomes from animals treated with ethanol exhibit greater formation of the reactive acetaminophen metabolite.

The broad and overlapping substrate specificities of P450s can confound identifying specific functions of individual CYPs. Thus, targeting one P450 for homologous recombi-

nation may result in a mouse in which another P450 substitutes for the role of the deleted enzyme. Such a response raises the possibility that deletion of several P450s in one mouse line could give insight into the contribution of these enzymes to the metabolism of a single substrate. To date, only one double-null mouse line, in which both Cyp1a2 and Cyp2e1 were disrupted, has been constructed.[50] These mice were highly resistant to acetaminophen toxicity when compared to wild-type, or mice lacking either Cyp1a2 or Cyp2e1, indicating that both CYP2E1 and CYP1A2 contribute to the metabolism and ultimate toxicity associated with acetaminophen.[40] Thus, double-null mice can provide an *in vivo* model for studying the combined function of CYPs in chemically-induced toxicity.

Several receptors involved in the regulation of P450s have been targeted for disruption. The aryl hydrocarbon receptor (AhR) is a transcriptional regulatory protein, which binds to DNA response elements known as DRE (dioxin response element). Because the [*Ah*] gene battery comprises at least six and probably many more genes, the AhR controls constitutive expression of several xenobiotic-metabolizing enzymes. AhR-null mice have been constructed by several laboratories[43,51,52] and these animals exhibited immune system impairment and hepatic fibrosis confirming that AhR has a developmental and physiological role. Activation of the AhR by polycyclic aromatic hydrocarbons (PAHs) and associated compounds is associated with disruption of multiple hormone systems resulting in developmental and reproductive impairment.[53] This may explain why mice lacking the AhR exhibit developmental alterations. When used to assess toxicity, these AhR-null mice were resistant to the acute toxicity from high levels of TCDD that induced pathological responses in the liver of wild-type mice.[53,54] In addition, treatment of null and wild-type mice with benzo[*a*]pyrene resulted in tumor formation in either skin or liver of control animals, but mice lacking the AhR possessed no apparent tumors.[51] These mice present an important tool for determining the toxic effects associated with dioxin and PAH exposure.

In response to peroxisome proliferators such as fibric acid derivatives, the nuclear receptor peroxisome proliferator-activated receptor α(PPARα) regulates transcription of genes encoding peroxisomal, mitochondrial, and some P450 enzymes involved in fatty acid oxidation. PPARα null mice have been constructed.[55,56] Upon treatment with the known peroxisome proliferator clofibrate, no proliferator pleiotropic response was observed indicating that PPARα is involved in this response.[55] Thus, mice lacking PPARα are refractory to induction of genes encoding fatty acid metabolizing enzymes, such as Cyp4a, peroxisome proliferation and carcinogenesis.[55] Moreover, short-term starvation of these PPARα-null mice caused hepatic steatosis, myocardial lipid accumulation, and hypoglycemia, a phenotype similar to that of humans with genetic defects in mitochondrial fatty acid oxidation enzymes.[56] Thus, PPARα has a critical role in the transcriptional regulatory response to fasting and the PPARα-null mice may be useful as a model for abnormalities of human fatty acid utilization. Unfortunately, this mouse model cannot be used to study the effects of inducers on the human CYP4A subfamily as it has recently been demonstrated that human hepatic CYP4A is not inducible by peroxisome proliferators. The reason for this refractoriness may be because there are insufficient levels of PPARα in human liver.[57]

The orphan nuclear receptor, pregnane X receptor (PXR)/steroid and xenobiotic receptor (SXR)42 is important in xenobiotic-mediated induction of the CYP3A genes[58] and CYP2C8.[59] To more fully understand the role of this receptor in the regulation of CYP3A and species differences associated with inducibility of this gene family, PXR null and humanized PXR mouse lines were constructed.[60] The loss of PXR did not alter basal expression of mouse Cyp3a. However, the Cyp3a gene was no longer inducible by prototypical inducers such as dexamethasone and PCN, indicating a role for this receptor in mediating xenobiotic induction of Cyp3a. This mouse line was also used to examine whether PXR played a role in induction of Cyp3a by phenobarbital, a known inducer of the CYP3A gene family. In mice lacking PXR, Cyp3a was induced by phenobarbital.[61] Interestingly, the phenobarbital-mediated induction of Cyp3a was accompanied by induction of a second P450, Cyp2b10, and this enzyme was consistently higher in the PXR null mice than wild-type. Cyp2b10 is also up-regulated by xenobiotics such as phenobarbital and chemically related compounds by the constitutive androstane receptor (CAR). Results from these experiments suggest that CAR also regulates Cyp3a expression. To more fully understand species differences, transgenic animals lacking mouse PXR, but containing human PXR were examined for Cyp3a inducibility by inducers known to enhance expression of human CYP3A. Human PXR caused constitutive up-regulation of the mouse Cyp3a gene and was responsive to human-specific inducers such as rifampicin.[60] In wild-type mice, rifampicin does not induce Cyp3a. These results demonstrate that the species origin of the receptor rather than the structure of Cyp3A genes, dictates the pattern of Cyp3a inducibility.

Future directions that are already beginning to be realized with the recent development of a humanized PXR mouse line, include additional lines that express human P450s in a background devoid of the corresponding mouse P450s. A humanized mouse line would provide an animal model for the *in vivo* expression of human CYPs and would enable determination of the role of specific human CYPs in chemical metabolism, toxicity, and carcinogenesis. A transgenic mouse line expressing the human CYP1A2 in the pancreas was established.[62] The level of CYP1A2 expressed in pancreatic microsomes from transgenic mice was comparable to that of the endogenously expressed CYP1A2 in liver. In terms of activity, the transgenic pancreas exhibited one-third to one-half of estrone oxidation by mouse liver microsomes from wild-type animals. Humanized mice containing CYP4B1 in the liver were also constructed.[63] Hepatic microsomes from these mice catalyzed the ω-hydroxylation of laurate and also activated 2-aminofluorene. These activities were not observed in control mice. Additionally, a transgenic mouse line expressing CYP3A7, the human fetal form of

CYP3A, has been developed.[64] Collectively, these mouse lines provide useful tools to study human CYPs and their relation to chemical toxicity, carcinogenesis and teratogenesis.

In addition to transgenic animals, humanized transgenic plants have been established.[65,66] Plants containing human CYPs can be used for phytoremediation of soil and groundwater pollution. For example, plants containing CYP2E1 exhibit a profound increase in metabolism of a common contaminant, tricholethylene (TCE), up to 640-fold above null plants.[66] These transgenic plants could be used for efficient remediation of many sites contaminated with halogenated hydrocarbons. Plants containing other CYPs such as, CYP1A1, CYP2B6 or CYP2C19 are also useful for breeding of herbicide-tolerant crops as well as for phytoremediation of environmental contaminants.[65]

CONCLUDING REMARKS

The present review describes some of the most recent and important advances in the P450 field. Many other areas of P450 research not discussed here, have progressed and provided exciting and important new information. Technological advances have facilitated our understanding of mechanisms of regulation, toxicity and carcinogenesis. New P450 enzymes are currently being identified that exhibit dual roles; they metabolize xenobiotics and have important physiological functions. In the drug metabolism area a great deal of progress has been aimed at identifying new polymorphic sites which could be of clinical and toxicological importance because these polymorphisms are correlated with the incidence of drug-induced toxicity and environmentally related cancers. In addition, genetic tests are being developed that will gradually replace probe drugs as the primary tool for screening populations for CYP polymorphisms. Microchip array technology is underway to assay the P450 profile of an individual in a single hybridization. Knowledge of this profile would characterize a person's risk of adverse drug reactions and possible predisposition to disease.

The first crystallization of a mammalian P450 that occurred recently will significantly advance structure-activity investigations. It may now be possible to determine how a substrate will interact with the active site of a given P450 enzyme. The technology recently developed for crystallizing a mammalian P450 can also provide insights that will facilitate the crystallization of additional enzymes. Perhaps one day we may understand how a single enzyme such as CYP3A4, can metabolize so many diverse compounds. Also described here is the current use of mice with disrupted P450 genes. These mice are providing appropriate *in vivo* models for investigating the role of specific P450s in chemically-mediated toxicity and carcinogenicity. They will be particularly useful in the future towards understanding the metabolic basis of increased or decreased human risks to chemical carcinogenesis due to polymorphisms in CYP enzymes. Another area that may potentially evolve from CYP gene targeting is the bioengineering of additional mice that expresses human CYPs, phase II enzymes, or drug transporters. These animal models will provide an *in vivo* system more similar to the human situation that will be beneficial to assessing the role of each protein in drug chemical elimination. While *in vitro* systems now play a major role in determining xenobiotic metabolism, *in vivo* models, such as the transgenic mouse, are increasingly being evaluated with the hope of better predicting human responses to therapeutic agents.

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