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Human Cytochromes P450: Evolution and cDNA-Directed Expression

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As the first step in the process of carcinogenesis, most chemical carcinogens require metabolic activation by cytochromes P450 for conversion to highly reactive electrophiles that bind covalently to DNA. Studies in rodents suggest that low or high levels of expression of a single P450 can determine susceptibility or resistance to chemically induced cancer. Although rodent systems have been used to explore the molecular basis of chemical carcinogenesis and to identify chemicals capable of damaging genes and causing cancer, it has been understood that marked species differences exist in the expression, regulation, and catalytic activities of different P450s. Thus, large efforts are underway to study the catalytic activities of human P450s directly by expression of their cDNAs in cultured cells. Two systems are being used: *a*) transient high-level P450 production in HepG2 cells for analysis of catalytic activities, and *b*) stable expression in human B-lymphoblastoid cells to study promutagen and procarcinogen activation. These studies define the relative contributions of individual P450 forms to the activation of various chemical carcinogens. The B-lymphoblastoid cDNA expression system can also be used to determine whether a chemical will be hazardous or toxic to humans. The most intriguing aspects of P450s are the occurrence of human genetic polymorphisms in P450 expression, which could be a risk factor for chemical carcinogenesis. The best-studied P450 genetic polymorphism is the debrisoquine/sparteine polymorphism which is due to mutant *CYP2D6* alleles. Four mutant alleles have been characterized that account for most of the defective *CYP2D6* genes in Caucasians. These can be detected by polymerase chain reaction assays. The expression of other P450s is currently being studied in human tissue specimens to determine whether functional polymorphisms exist with other P450 forms.

Properties, Organization, and Nomenclature of Cytochromes P450

As a group, cytochromes P450 (P450) range in size from about 48 to 60 kDa and contain a single molecule of noncovalently bound heme. All P450s use O_2 and electrons, usually from NADPH, to oxidize substrates by production of an activated oxygen. The region of the P450 primary sequence that contains the thiolate fifth ligand to the heme iron (*1*) is highly conserved (*2*). This sequence of about 26 residues could be considered a "fingerprint" for a P450 protein.

Three general classes of P450s exist: *a*) soluble forms with high substrate specificity found in bacteria; *b*) those involved in highly specific steroid hydroxylations, some of which are located in the inner mitochrondrial membrane (encoded by nuclear DNA); and *c*) those bound to the endoplasmic reticulum of the cell, which, by and large, have broad substrate specificities. Some of these P450s activate chemical carcinogens to their proximate electrophilic forms which can bind covalently to DNA.

P450s have been organized on the basis of similarities in protein sequence, and a nomenclature system has been developed (*3*). The "P450 superfamily" is subdivided into families; individual P450s within a family are defined as having ≤40% sequence similarity with a P450 in any other family. Families are further divided into subfamilies: all P450s within a subfamily are >55% similar in sequence.

P450s are named using the root symbol CYP (*Cy*tochrome *P*450), followed by an Arabic numeral designating the family number, a letter denoting the subfamily, and another Arabic numeral representing the individual gene. Thus, CYP1A1 is P450 form 1 in the A subfamily of family 1. Names of genes are written in italics, *CYP1A1*. It should be emphasized that the nomenclature system is based solely on sequence similarity among P450s and does not indicate the properties or function of individual P450s. Circumstantially, however, the steroidogenic P450s fall into families distinct from those of the foreign compound metabolizing enzymes. The latter fall almost exclusively into mammalian families, CYP1, CYP2, CYP3, and CYP4.

Because of the diverse catalytic activities of the foreign compound metabolizing P450s, it has been difficult to identify orthologous P450 counterparts among species (an orthologous P450 gene in two species is one that shared a common ancestor before evolutionary divergence of the two species); this has complicated P450 nomenclature. Of the foreign compound metabolizing P450s, only CYP1A1 and CYP1A2 have unequivocal orthologous counterparts

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in all animal species examined. Individual P450s within a subfamily are designated by different numbers: for example, rats possess CYP2D1 through CYP2D5, humans CYP2D6 through CYP2D8, and mice CYP2D9 through CYP2D13.

Evolution of P450s and Species Differences

P450s are believed to have evolved from a common ancestor about 3 billion years ago (*4*). The earliest P450s probably existed to metabolize steroids such as cholesterol in order to maintain membrane integrity, and several P450s continued through the course of evolution to function in steroid biosynthesis and metabolism. Only more recently (during the last 100–500 million years) have P450s taken on the role of ridding the organism of foreign compounds. Numerous gene duplications have occurred during the past 200 million years; most importantly, new P450 genes were formed in certain species and some lost in other species. This process is best illustrated by examining the complexity of the *CYP2D* genes in rodents and humans (*5*). Rats and mice possess five genes, all of which appear to be normal, whereas humans have one active gene and two pseudogenes. The rodent genes have also undergone numerous "homogenizing" gene conversion events, so that no orthologous counterpart of any single rat gene can be identified in mice. Other events, in addition to gene conversion, that occur in a species-specific manner, possibly due to environmental influences, have resulted in catalytic activities and regulatory circuits that can be unique to a given animal.

It is not currently understood what environmental factors give rise to the battery of P450s in an animal or, at a more basic level, how and why foreign compound metabolizing P450s have arisen. One possibility is that these enzymes evolved as a defense against toxins found in the food chain, particularly in plants, in which a large number of toxins has been developed, probably as a defense against predators. New P450s thus appeared in response to plant toxins or new plant varieties or as a means of exploiting new sources of vegetation. Conversely, when a plant source is no longer available, certain P450s might disappear from the gene pool. Indeed, the entire *CYP2D* locus appears to be vanishing in humans (*5*).

The consequences of P450 evolution are species differences and genetic polymorphisms. Species differences in P450s have meant that better focus and direct examination of human enzymes and less reliance on data from rodents are required to predict the adverse effects of chemicals. P450 polymorphisms have had a direct impact on the development of drugs in the pharmaceutical industry, because certain established P450 forms clearly exhibit genetic differences and are known to metabolize and gen-

Table 1. cDNA-expressed human cytochromes P450.

P450	Tissue	Rodent ortholog	cDNA expression	Substrates[a]
CYP1A1	Many[b]	Yes	Yeast (*7*)	Benzo[*a*]pyrene
CYP1A2	Liver	Yes	Vaccinia (*8*)	2-Acetylaminofluorene
			Lymphoblastoid (*9*)	Aflatoxin B_1
				Heterocyclic arylamines
CYP2A6[c]	Liver		Vaccinia (*10*)	Coumarin
	Lung		Lymphoblastoid (*9*)	Aflatoxin B_1
				N-Nitrosodiethylamine
CYP2B7	Liver		Vaccinia (*11*)	7-Ethoxycoumarin
				Aflatoxin B_1
CYP2C8	Liver		Vaccinia (*12*)	Tolbutamide
			Yeast (*13*)	
CYP2C9	Liver		Vaccinia (*12*)	Tolbutamide
CYP2C10	Liver		Yeast (*13*)	Tolbutamide
CYP2C17	Liver		COS (*14*)[d]	Mephenytoin (racemic)
CYP2C18	Liver		COS (*14,15*)	Mephenytoin (racemic)
CYP2C19	Liver		COS (*14*)	Mephenytoin (racemic)
CYP2D6	Liver		COS (*16*)	Debrisoquine
	Kidney		Vaccinia (*17*)	Bufaralol
			Lymphoblastoid (*18*)	
CYP2E1	Liver	Yes	COS (*19*)	Ethanol
			Vaccinia (*20*)	*N*-Nitrosodimethylamine
			Lymphoblastoid (*21*)	
CYP2F1	Lung	Yes	Vaccinia (*22*)	Naphthylamine
CYP3A3	Liver		Vaccinia (*23*)	Nifedipine
			Lymphoblastoid (*9*)	Aflatoxin B_1
CYP3A4	Liver		COS (*24*)	Nifedipine
			COS (*25*)	Aflatoxin B_1
			Vaccinia (*23*)	
CYP3A5	Liver		Vaccinia (*23*)	Nifedipine
				Aflatoxin B_1
CYP4B1	Lung	Yes	Vaccinia (*26*)	

[a]Partial list of preferred substrates for each P450; a more complete compilation is given by Guengerich and Shimada (*6*).
[b]CYP1A1 is not appreciably expressed in the absence of inducers such as polycyclic aromatic hydrocarbons or dioxins.
[c]Formerly called CYP2A3.
[d]COS is a monkey-kidney-derived cell line.

erally inactivate therapeutic agents. The extent of P450 polymorphisms in humans is also being evaluated with a view to diagnostic analysis of mutant human P450 genes, to investigate whether the presence or absence of a P450 involved in activation or inactivation of a carcinogen results in an increased risk of or protection against cancer.

cDNA Expression of Human P450s and Risk Assessment

Human P450s can be studied directly after isolation from tissues such as liver by reconstitution with NADPH-P450 oxidoreductase and analysis of their catalytic activities in the tissue or in microsomal extracts, using immunochemical approaches. These types of investigations have led to determination of substrate specificities of individual P450 forms and the role of specific P450s in total liver microsomal metabolism of specific procarcinogens (*6*).

cDNA expression has also been used to study human P450 catalytic activities. It has been relatively straightforward to isolate human P450 cDNAs from cDNA libraries constructed from liver and lung tissue RNA, using antibodies and cDNA probes against different rodent P450 forms. Human P450s that have been isolated to date, their tissue-specific expression, whether a rodent counterpart exists, and a typical substrate are listed in Table 1. Two cDNAs, CYP1A1 and CYP1A2, were isolated from human cells and liver libraries, respectively, and apparently share enzymatic properties with their rodent counterparts. At least nine cDNAs that encode active enzymes in the CYP2 family have also been isolated. One form, CYP2F1, appears to be expressed preferentially in extrahepatic tissues. Of these cDNA-expressed forms, only CYP2E1 exhibits catalytic activities that are similar to those of rat and rabbit CYP2E1. Four distinct CYP3A P450 cDNAs were isolated from human liver, one of which is expressed only in the fetus. Only two CYP3A P450s have been described in rat, and one in rabbit. All CYP3A P450s exhibit testosterone 6β-hydroxylase activities, but activities toward other substrates may differ. To date, no P450 has been demonstrated in the CYP4 family, although they are known to exist.

Human cDNA-expressed P450s can be examined for their abilities to activate promutagens and procarcinogens using three protocols: *a*) direct assay of active metabolites by analytical separation; *b*) an Ames test assay in which human P450s are substituted for the standard Aroclor-induced rat liver extracts; and *c*) *in-situ* DNA binding assays in cells that express human P450s.

The Ames test has been used to evaluate the role of different human P450s in the activation of the cooked food-derived mutagen, 2-amino-3-methylimidazo [4,5-*f*]quinoline (IQ), by cDNA-expressed human liver P450 (*20*). CYP1A2, an enzyme expressed constitutively in liver and not in extrahepatic tissues, was the most active, followed by P450s in the CYP3 family which are expressed in several extrahepatic tissues. Food mutagens are typically carcinogens in nonliver tissue (*27*), although they are potent hepatocarcinogens in nonhuman primates (*28*). These compounds might be activated in the liver and extrahepatically by CYP1A2 and the CYP3 P450s, respectively.

Aflatoxin B_1 activation was measured by DNA binding *in situ* in an experiment in which cells that express different forms of human P450 in culture were incubated with radiolabeled carcinogens; DNA-bound carcinogen was then determined (*29*). Five human P450 forms were found to catalyze DNA binding, while another seven forms were inactive. These five forms were also active in aflatoxin B_1 mutagenesis, as measured in the Ames test; however, the conditions of the experiment did not distinguish those P450s with high affinity for aflatoxin B_1 from low-affinity forms.

Metabolic activation by human P450s of a rodent and ruminant pneumotoxin, 4-ipomeanol, was also evaluated (*30*). Surprisingly, the primary human lung P450s CYP2F1 and CYP4B1 were unable to activate this compound; in contrast, rabbit CYP4B1 and human CYP1A2, a nonpulmonary P450, were active. These data indicate a marked species difference in the catalytic activity of one P450.

Human P450s have also been expressed using a B-lymphoblastoid cell line, AHH-1, with herpes virus-based vectors. A number of P450s have been accurately expressed using this system, including CYP1A2, CYP2A6, CYP2D6, CYP2E1, and CYP3A4 (Table 1). This expressed system differs from that of *Vaccinia*, since the P450s are stably expressed. AHH-1 cells are especially useful for expressing P450s because they were originally developed as a eukaryote mutagen testing system (*31*).

Metabolic activation of aflatoxin B_1 was also studied using cell lines that express individual P450s (*9*). Cells were assayed for toxicity and mutagenicity at the hypoxanthine guanine phosphoribosyl transferase (*hprt*) locus. CYP1A2-expressing cells were the most sensitive to the toxicity and mutagenicity of aflatoxin B_1: as little as 10 ng/mL of the carcinogen in the medium produced a significant response. CYP3A4-expressing cells were also sensitive to aflatoxin B_1, although about 5- to 10-fold less than CYP1A2-containing cells. Cells that express CYP2A6 were the least sensitive, and cells with no expressed human P450 were resistant to the compound at a level of 1 μg/mL. These data suggest that CYP1A2 has the highest activation activity for aflatoxin B_1 and is probably the P450 form that activates the carcinogen under ordinary conditions of human exposure.

In a separate experiment, CYP2E1 and CYP2A6 were compared for their ability to activate the low-molecular-weight nitrosamines *N*-nitrosodiethylamine and *N*-nitrosodimethylamine (*21*). Surprisingly, considering the structural similarities of these two nitrosamines, cells that express either P450 were sensitive to both chemicals but CYP2A6-expressing cells were considerably more sensitive to the toxicity and mutagenicity of *N*-nitrosodiethylamine and CYP2E1-expressing cells were more sensitive to *N*-nitrosodimethylamine.

Cells suitable for analyzing diverse classes of carcinogens and mutagens should simultaneously express multiple P450s. This has been accomplished to a degree by inserting four P450 cDNAs and epoxide hydratase cDNA

into a cell line. These cells, designated MCL-5, are responsive to small nitrosamines, polycyclic aromatic hydrocarbons, and aflatoxin B_1 (*32*) and are potential prototypes of cells that could be used to test unknown compounds for carcinogenicity and mutagenicity.

Interindividual Differences in P450 Expression and Cancer Susceptibility

Early studies in clinical pharmacology established that marked interindividual differences in P450 expression exist as a human polymorphism. The clearest example is the debrisoquine/sparteine drug oxidation genetic polymorphism, in which 75% of Caucasians possess two copies of a mutant *CYP2D6* gene and thus cannot metabolize debrisoquine and a growing number of other clinically used drugs (*33*). Almost all mutant genes can be diagnosed by polymerase chain reaction analysis of leukocyte DNA. Another drug oxidation polymorphism, associated with *S*-mephenytoin and involving a P450 in the CYP2C subfamily, exists, but it has not been elucidated at the molecular level.

Epidemiological evidence shows that CYP2D6 expression is associated with risk for smoking-associated nonadenocarcinoma of the lung (*34*). CYP2D6 is capable of metabolically activating the tobacco smoke-specific nitrosamine 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK) (*18*); however, several other P450s are also capable of activating this compound. Thus, the precise biochemical basis of the association between CYP2D6 and cancer is still unclear.

The question remains whether interindividual differences in levels of expression of other known carcinogen-activating P450s are associated with increased cancer risk. Marked differences exist in the levels of expression of CYP1A2 (*35–37*), CYP2A6 (*10*), CYP2B6 (*11*), CYP2E1 (*38*), and the CYP3A P450s (*23*), as detected by western immunoblot and northern blot analysis of protein and mRNA isolated from human liver specimens; there is also a large degree of variation in the expression of CYP1A1 mRNA in lung samples from smokers and nonsmokers. In order to establish whether expression of a P450 is associated with increased cancer risk, however, cohort and prospective epidemiological surveys must be conducted. These will be possible only when accurate, noninvasive assays can be provided for measuring P450 form-specific activities in humans. Several candidate compounds that can be used in this capacity have been proposed (*6*) but have yet to be used in cancer epidemiological studies.

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