Carcinogenesis vol.24 no.7 pp.1247–1255, 2003
DOI: 10.1093/carcin/bgg088

Single amino acid mutations, but not common polymorphisms, decrease the activity of CYP1B1 against (−)benzo[*a*]pyrene-7R-*trans*-7,8-dihydrodiol

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Genetic differences that underlie inter-individual variation in the metabolism of common carcinogens are important potential sources of cancer susceptibility. Cytochrome P450 1B1 (CYP1B1), a central enzyme in the activation of the ubiquitous environmental carcinogen benzo[*a*]pyrene (B[*a*]P), has several genetic variants. This study investigated six rare mutations and four common polymorphisms for their effects on B[*a*]P metabolism. Five missense mutations associated with congenital glaucoma (Gly61Glu, Gly365Trp, Asp374Asn, Pro437Leu and Arg469Tryp) dramatically decreased the capacity of CYP1B1 to convert (−)benzo[*a*]pyrene-7R-*trans*-7,8-dihyrodiol (B[*a*]P-7,8-diol) to (±)benzo[*a*]pyrene-r-7,t-8-dihydrodiol-9,10-epoxides. These five mutations resulted in enzymes with 3–12% of normal activity when assayed *in vitro* using an *Saccharomyces cerevisiae* microsomal expression system. A 10 bp deletion mutation produced no detectable protein or activity. In contrast, proteins containing all possible combinations of four common single nucleotide polymorphisms (Arg48Gly, Ala199Ser, Val432Leu, Asn453Ser) had modest effects on B[*a*]P-7,8-diol metabolism. Michaelis–Menten analysis suggested that two alleles, Arg48, Ala119, Val432, Ser453 (RAVS) and Arg48, Ala119, Leu432, Ser453 (RALS), have K_M values 2-fold lower than Arg48, Ala119, Val432, Ser453 (RAVN): 1.4 ± 0.3 and 1.3 ± 0.4 μM, respectively, compared with 2.8 ± 0.8 μM ($P < 0.05$). However, these differences could not be confirmed with direct measurements of rate at low substrate concentration. There were no significant differences for either of two other kinetic parameters, k_{cat} or k_{cat}/K_M. Allele frequency analysis in three populations reveals the Ser453 variant is rare among those of Asian (<1%) and African ancestry (<4%), and more common in individuals of European ancestry (16%). Haplotypes containing the Ser453 variant were uncommon; only RALS was detectable in our small populations. The RALS allele occurred between 0.5% in Asians and 15% in Europeans. Our study demonstrates that rare, disease-associated mutations in CYP1B1 significantly decrease the enzyme's metabolism of B[*a*]P-7,8-diol; however, our results do not identify any major differences in this metabolism due to four common single amino acid polymorphisms.

Introduction

Benzo[*a*]pyrene (B[*a*]P) is a ubiquitous, carcinogenic polycyclic aromatic hydrocarbon (PAH) produced during combustion of organic materials. Significant human exposures occur with cigarette smoking and consuming certain foods, such as charbroiled meat (1). An individual's risk of cancer in tissues directly exposed to this carcinogen such as the skin, lung and colon could be modulated by inter-individual variation in the metabolism of B[*a*]P. Genetic differences, which change the activity of enzymes that activate or detoxify B[*a*]P would be expected to correlate with toxicity, and therefore with cancer risk, in the relevant organs. Recently, low activity in microsomal epoxide hydrolase (mEH), an enzyme responsible for detoxifying the epoxides produced by the oxidation of many PAH compounds, was found to double the relative risk of colon cancer compared with high activity mEH (2).

B[*a*]P has 12 carbon molecules available for oxidation reactions and oxidative metabolism produces a number of phenols, quinones and diones (for review see ref. 3). The most carcinogenic metabolites are the sterically hindered, but highly reactive diol-epoxides (4). These compounds are formed by two sequential oxidations. In the relevant reactions for our experiments, the first oxidation yields benzo[*a*]pyrene-7,8-epoxide, which is hydrolyzed by mEH predominantly to the enantiomer (−)benzo[*a*]pyrene-7R-*trans*-7,8-dihydrodiol (B[*a*]P-7,8-diol) (5); a second oxidation produces (±)B[*a*]P-r-7,t-8-dihydrodiol-c-9,10-epoxide (DE1) and (±)B[*a*]P-r-7,t-8-dihydrodiol-t-9,10-epoxide (DE2). The diol-epoxides are readily hydrolyzed *in vitro* into tetrol products, which can be separated by HPLC. DE2 is the more carcinogenic epoxide (4,6,7) and its production is highly favored over DE1 when B[*a*]P-7,8-diol is incubated with rat liver microsomes (5).

Both oxidation reactions can be catalyzed by several cytochrome P450s (8,9) including CYP1A1 and CYP1B1 (10), which are expressed in extra-hepatic tissues (11,12). Target-tissue expression of CYPs is a critical variable in metabolism, since the short half-lives of the ultimate carcinogens make these species likely to cause DNA damage primarily in the cells where they are formed. Therefore, inter-individual variation in the activity of CYP1B1 might be a cancer susceptibility factor in those organs where it is highly expressed (11,12).

Six common single nucleotide polymorphisms (SNPs) have been identified in *CYP1B1*, four of which change the amino acid sequence: Arg48Gly, Ala119Ser, Val32Leu and Asn453-Ser (Figure 1) (11,13,14). Primary congenital glaucoma, an

Abbreviations: B[*a*]P, benzo[*a*]pyrene; B[*a*]P-7,8-diol, (−)benzo[*a*]pyrene-7R-*trans*-7,8-dihydrodiol; CYP, cytochrome P450; DE1, (±)B[*a*]P-r-7,t-8-dihydrodiol-c-9,10-epoxide; DE2, (±)B[*a*]P-r-7,t-8-dihydrodiol-t-9,10-epoxide; EH, Estimated Haplotypes; mEH, microsomal epoxide hydrolase; RTTC, (±)benzo[*a*]pyrene-r-7,t-8,t-9,c-10-tetrahydrotetrol.

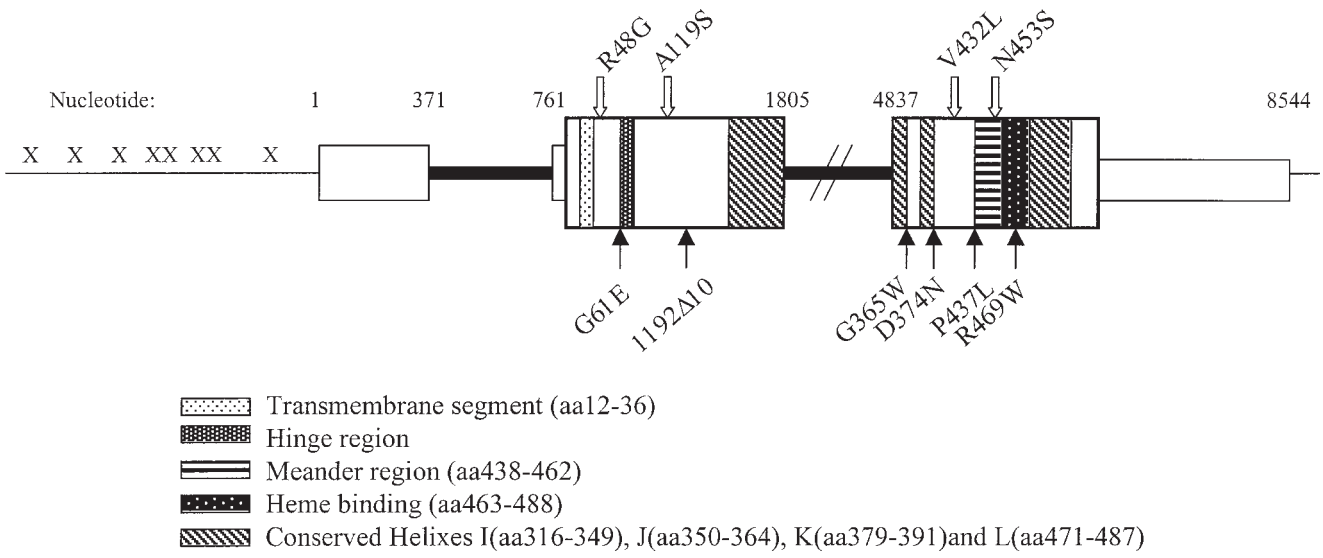

Fig. 1. Gene diagram (not to scale) showing the locations of the single common amino acid substitutions and mutations being studied in relation to regions conserved across the CYP superfamily (11,13,14). Mutations are indicated below with solid arrows, polymorphisms above with open arrows. Boxes are exons, smaller boxes are non-coding. Heavy lines are introns. XRE enhancer elements are identified as X.

autosomal recessive disease, has been mapped to *CYP1B1* and a variety of mutations segregate exclusively with disease (14–19). The mutant haplotypes are very rare and have only been observed in those families affected by congenital glaucoma. Among these are at least 15 missense mutations, found throughout the protein, generally representing non-conservative changes in highly conserved regions of the protein. These mutations are presumed to result in a major disruption of CYP1B1 activity. In contrast, the common polymorphisms are found in not well conserved areas of the protein and do not a priori suggest dramatic effects on function. However, several epidemiological studies have already examined one or more of the four *CYP1B1* common polymorphisms as disease risk factors with inconsistent results (20–25), and so an examination of the effects of these polymorphisms will help to clarify the literature. Since even small functional changes could have a large attributable risk if due to a common polymorphism or haplotype, we selected three non-diseased (control) populations to generate descriptive data on allele and haplotype frequencies for these loci.

We tested the *in vitro* activity of every possible haplotype generated by combinations of the four common polymorphisms in the oxidation of the proximate carcinogen B[*a*]P-7,8-diol. We also examined five missense substitutions that have been associated with congenital glaucoma: Gly61Glu, Gly365Trp, Asp374Asn, Pro437Leu and Arg469Trp, and a 10 bp deletion, 1192Δ10 that causes a premature stop (14–17).

Materials and methods

Yeast constructs of CYP1B1 polymorphisms

Starting with a cDNA clone described previously (11,26), a *Hin*dIII–*Sph*I 1.9 kb segment encoding unmodified, full-length CYP1B1 was subcloned into the pcDNAII vector (Invitrogen, Carlsbad, CA) for site-directed mutagenesis using the QuikChange kit (Stratagene, La Jolla, CA). Primers containing each of the single base pair changes and their reverse complements were purchased from Integrated DNA Technologies (Coralville, IA). Coding strand primers are as follows with base pair changes shown in bold and underlined): R48G, 5′-CGGAGGCGGCAGCTC**G**GGTCCGCGCCCCCC-3′; A119S, 5′-GCCGACCGGCCG**T**CCTTCGCCGCCGGC-3′; V432L, 5′-GTGAATCATGACCCA**C**TGAAGTGGCCTAACCCG-3′; N453S, 5′-GGCCTCATCA**G**CAAGGACCTGACCAGC-3′.

Similarly for the mutants, site-directed mutagenesis on the appropriate haplotype background was performed using the following primers (coding strand with mutations in bold and underlined, deletion indicated by…): G61E: 5′-GCGTGGCCACTGATCG**A**AAACGCGGCGGCGGTGGG-3′; 1192del10: 5′-CGGAGCACTGGAAGGTGC…CAGCCCACAGCATGATGCGCAAC-3′; G365W: 5′-GGATCAGGTCGTG**T**GGAGGGACCGTCTGCCTTGTATGGG-3′; D374N: 5′-CCGTCTGCCT**T**GTATGGGT**A**ACCAGCCCAACCTGCCC-3′; P437L: 5′-CCCACTGAAGTGGCCTAAC**C**T**T**GGAGAACTTTGATCCAGC-3′; R469W: 5′-CAGTGGGCAAAAGG**T**GGTGCATTGGCG-3′.

Constructs containing more than one polymorphic change were generated sequentially by additional rounds of site-directed mutagenesis with the appropriate primer pairs. All vectors were verified by DNA sequence analysis and then the *Hin*dIII–*Sph*I insert was subcloned into pYes2 (Invitrogen, Carlsbad, CA) followed by transformation into *S.cerevisiae* strain JL20 as described previously (26). Single colony stocks were grown in uracil deficient synthetic dextrose media (SD, –ura, 2% glucose, supplemented with leucine, histidine, methionine, tryptophan and adenine) and aliquots were frozen with 20% glycerol at −80°C and re-streaked to single colonies as necessary.

In order to distinguish the 16 polymorphic alleles with simple and meaningful shorthand, we used the single letter amino acid designations to give each allele a four-letter code corresponding to the amino acids found at each possible polymorphic site. For example, the initial cloned cDNA sequence (11), with amino acids Arg48, Ala119, Val432 and Asn453, is called RAVN, while the variant that differs at all four sites is called GSLS. Mutants associated with congenital glaucoma are designated by conventional single amino acid substitution and deletion nomenclature. Our wild-type sequence is defined as that of the initially reported cDNA sequence (11), accession number U03688, which is the RAVN variant, as this was the background for all of the mutants (except the 1192Δ10 construct, which is on the GSLN background) and was called wild-type in the original linkage analysis work (14–17).

Microsomal preparations

Microsomal preparations were made from expanded single colonies as described previously (26). Small aliquots of microsomes in freezing buffer (10 mM Tris pH 7.5, 1 mM EDTA, 20% glycerol) were snap-frozen in a dry-ice:ethanol bath and stored at −80°C until needed. Total protein concentration was assayed using the Bradford reagent (Bio-Rad, Richmond, CA), which is less sensitive to the concentration of glycerol in the freezing buffer. Each sample was measured in triplicate at two different concentrations, and the measurements had <10% variation. Specific CYP1B1 protein content was estimated by quantitative western blot, in duplicate at two concentrations of total protein, using a previously described antibody (27). Standard curves were generated using CYP1B1 lymphoblastoid cell microsomes of known concentration determined spectrophotometrically (Gentest, Woburn, MA). Blots were scanned and optical density measured using MacBas (Fuji Films, Japan).

The variation in this method within a blot and between blots averaged 15% (CV; range 4–30%).

Cytochrome c reductase assay

To determine endogenous cytochrome P450 reductase activity, 25 μg of total protein from each microsomal preparation was diluted in 910 μl of 300 mM KPO_4, pH 7.7, to which 80 μl of 0.5 mM cytochrome *c* (in 10 mM KPO_4, pH 7.7) was added. A baseline scan at A_{550} was obtained for 3 min in a cuvette with 1 cm path length. Ten microliters of 10 mM NADPH was added and mixed quickly and A_{550} was again measured for 3 min. Cytochrome *c* reductase activity was calculated for the linear phase of the reaction as follows: nmole reduced/(μg × min) = ΔOD/(min × μg × 0.021) (28).

(−)-Benzo[a]pyrene-7R-trans-7,8-dihydrodiol oxidation assay

Reactions to measure B[*a*]P-7,8-diol metabolism were carried out as described (10). Briefly, an appropriate volume of microsomes to contain 5 pmol of CYP1B1 was resuspended in a final volume of 198 μl incubation buffer (100 mM $NaPO_4$, 5 mM $MgCl_2$, 1.4 mM NADPH final concentrations, pH 7.5). Each allele was assayed in triplicate at 0.0, 0.625, 1.25, 2.5, 5 and 10 μM B[*a*]P-7,8-diol. All reactions were carried out at low light levels in silanized glass test tubes. Reactions were pre-incubated at 37°C for 2 min, 2 μl of the appropriate 100× substrate stock in methanol was added; tubes were vortexed briefly and returned to 37°C for 15 min. Reactions were stopped by adding 1 vol ice-cold acetone and then extracted twice with 2 vol of ethyl acetate, dried under nitrogen and resuspended in an appropriate volume of HPLC grade methanol (150–300 μl) to maintain relatively consistent metabolite concentrations. These were stored at −20°C until use. We confirmed that the reaction conditions were in the linear range with respect to time and protein concentration (data not shown).

HPLC analysis was performed to separate the products as described with some modifications (10). Injections were performed either manually or using a Waters WISP712 autosampler. The 60 min run on a Bondclone C-18 column (Phenomenex, Torrance, CA) contained a 30 min isocratic phase of 50:50 methanol:H_2O followed by a 10 min gradient up to 100% methanol to elute the substrate, at constant flow rate of 1 ml/min. UV (Waters Tunable Absorbance Detector 486) and fluorescence (Ranin Dynamax FL-2, Varian, Walnut Creek, CA) signals were recorded and analyzed using Shimadzu Class-VP Chromatography software (Columbia, MD). Standard curves using authentic standards demonstrated that the quantification is linear between 2.5 and 25 pmol (data not shown). Standards at four concentrations were run every few weeks and slopes varied <10% (CV). Michaelis–Menten kinetics were fit to data using Prism software (GraphPad, San Diego, CA). Any experiment where the calculated K_M was lower than the lowest substrate concentration used was discarded as having insufficient data.

Genotype, allele and haplotype frequencies

Two novel PCR–RFLP assays were used to detect the four CYP1B1 polymorphisms: one assay for Arg48Gly and Ala119Ser, another for Val432Leu and Asn453Ser. For the Arg48Gly and Ala119Ser polymorphisms, the two PCR primers used were (forward) 5′-TCC CCA TCC CAA TCC AAG-3′ and (reverse) 5′-CGG CAG CCG AAA CAC AC-3′, which generate a 1223 bp fragment. The PCR contained 50 ng genomic DNA, H_2O, 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate, 5× DMSO and 0.28 U *Taq* DNA polymerase combined with an equal volume TaqStart™ antibody (Clontech Laboratories, Palo Alto, CA). The PCR cycling conditions used were 94°C for 4 min; 33 cycles of 94°C for 15 s, 61°C for 45 s, 72°C for 60 s; and 72°C for 4 min. PCR products were incubated for 3 h at 37°C with 2.5 U each *Ava*I, *Ngo*MIV and *Hin*fI. The Arg48 allele contains a polymorphic *Ava*I site; the Gly48 allele does not. The Ala119 allele contains a polymorphic *Ngo*MIV site; the Ser119 allele does not. *Hin*fI was added to improve fragment resolution.

For the Val432Leu and Asn453Ser polymorphisms, the two PCR primers used were (forward) 5′-GCC TGT CAC TAT TCC TCA TGC C-3′ and (reverse) 5′-GTG AGC CAG GAT GGA GAT GAA G-3′, which generate a 283 bp fragment. The PCR components were the same as previous, except 1.5 mM $MgCl_2$ was used. The PCR cycling conditions used were 94°C for 4 min; 33 cycles of 94°C for 15 s, 57°C for 30 s, 72°C for 30 s; and 72°C for 4 min. PCR products were incubated for 3 h at 37°C with 2.5 U each *Bsr*I and *Mwo*I. The Val432 allele contains a polymorphic *Bsr*I site; the Leu432 allele does not. The Asn453 allele contains a polymorphic *Mwo*I site; the Ser453 allele does not.

For both assays, PCR was performed on a GeneAmp® 9600 thermocycler (Applied Biosystems, Foster City, CA), and products were resolved on a 3.25% Metaphor® agarose gel (FMC BioProducts, Rockland, ME) stained with ethidium bromide and visualized using an Eagle Eye II® still video system (Stratagene, La Jolla, CA).

Genomic DNA from individuals of Asian, African and European ancestry was genotyped for the four CYP1B1 polymorphisms. African- and European-American genomic DNA was extracted from whole blood samples obtained from a community-based population of healthy, unrelated African-American and European-American volunteers from Durham and Chapel Hill, North Carolina, recruited through newspaper advertisements. Asian genomic DNA was extracted from placental tissue obtained from unrelated, maternity patients with full-term, uncomplicated pregnancies at the Chang Gung Memorial Hospital in Taiwan and were provided by Dr L.L.Hsieh. All samples were collected under approved IRB protocols. 120 Asians, 111 African-Americans and 181 European-Americans were genotyped. We tested Hardy–Weinberg equilibrium within all three populations for all four loci. Because haplotype determination is complicated by incomplete genotype data, we only used individuals with complete data at all loci for that analysis: 98 Asians, 98 African-Americans and 143 European-Americans. While direct haplotype determination is possible for individuals heterozygous at most at one locus, estimation must be used for those heterozygous at two or more loci. We estimated haplotypes using the Estimated Haplotypes (EH) algorithm (29).

Results

Genotype, allele and haplotype frequencies

The public health importance of any inherited functional difference in CYP1B1 activity is dependent on both the magnitude of the difference and the allele frequency in the population. Therefore, we genotyped individuals of Asian, African and European ancestry in order to estimate allele and haplotype frequencies in these groups (Tables I and II). We found all loci were in Hardy–Weinberg equilibrium for each population. Allele frequencies differed widely among population groups. For example, the Gly48 allele occurred at a frequency of 0.19 in Asians, 0.5 in African-Americans and 0.33 in European-Americans, while the Leu432 allele occurred at frequencies of 0.91, 0.29 and 0.58, respectively. Asn453Ser was the most rare polymorphism among those of Asian and African descent (0.005 and 0.02, respectively) but occurred more commonly among those of European descent (0.16).

Table I. Genotype and allele frequencies of CYP1B1 polymorphisms

	Asian ancestry (n = 120)[a]				African ancestry (n = 111)				European ancestry (n = 181)			
	+/+[b]	+/−	−/−	VF[c]	+/+	+/−	−/−	VF	+/+	+/−	−/−	VF
Arg48Gly	67% (76)	29% (33)	4% (5)	0.19	25% (26)	48% (49)	26% (27)	0.50	44% (75)	46% (79)	9% (16)	0.3
Ala119Ser	67% (76)	29% (33)	4% (5)	0.19	29% (30)	47% (48)	24% (24)	0.47	44% (74)	46% (79)	10% (17)	0.3
Val432Leu	2% (2)	14% (14)	84% (87)	0.91	52% (55)	39% (41)	9% (10)	0.29	18% (28)	48% (73)	34% (52)	0.6
Asn453Ser	99% (102)	1% (1)	0% (0)	<0.01	96% (102)	4% (4)	0% ((0))	0.02	71% (111)	25% (39)	4% (6)	0.2

[a]Population sample sizes represent the total individuals genotyped. The numbers of readable genotypes are listed in the table.
[b]For each polymorphic site, wild-type is defined based on the originally reported CYP1B1 cDNA sequence with Arg48, Ala119, Val432 and Asn453 (11) and individuals are identified as homozygous wild type (+/+), heterozygous (+/−) or homozygous variant (−/−).
[c]Variant frequency (VF) of each allelic variant calculated for that ethnic group population sample shown.

Using the individuals for whom we had complete genotype information at each of the four polymorphic sites, we prepared estimates of haplotype frequency in the population samples (Table II). Applying the EH algorithm, RAVN, RALN, GSLN, GSVN and RALS haplotypes were predicted to occur among all three populations, although at different frequencies. The first three haplotypes were common among all groups. The RALN haplotype was estimated to account for 72.5% of alleles among Asians, but only 7 and 9% in African- and European-Americans, respectively. RAVN was the most common haplotype observed in the latter two populations (41 and 42%). Of note, among Asians and European-Americans the sequences at amino acid positions 48 and 119 were closely linked; the Arg48 occurred with Ala119 and Gly48 occurred with Ser119. The GAVN haplotype was uniquely observed among those of African descent, presumably a recombinant haplotype between RAVN and GSVN. The data indicate that all Ser453 alleles occur on Leu432 alleles, suggesting a more recent origin for the Ser453 polymorphism.

Table II. Estimated haplotype frequencies[a] (%) for CYP1B1

Allelic variant	Asian ancestry % (*n*)[b]	African ancestry % (*n*)	European ancestry % (*n*)
RAVN	8.0% (16)	41.0% (80)	42.0% (123)
RAVS	–[c]	–	–
RALN	72.5% (142)	7.0% (14)	9.0% (27)
RALS	0.5% (1)	2.0% (4)	15.0% (44)
GSVN	3.0% (6)	28.0% (55)	1.0% (3)
GSVS	–	–	–
GSLN	16.0% (31)	18.0% (35)	33.0% (97)
GSLS	–	–	–
RSVN	–	–	–
RSVS	–	–	–
RSLN	–	–	–
RSLS	–	–	–
GAVN	–	4.0% (4)	–
GAVS	–	–	–
GALN	–	–	–
GALS	–	–	–

[a]Haplotypes estimated using EH algorithm (29).
[b]Percent and number (*n*) for each population, see Table I.
[c]Dash (–) indicates haplotype not predicted from observed genotypes.

Microsome characterization

Specific protein concentrations in microsomes containing each of the 16 presumed normal alleles averaged 24 pmol/mg (range 15–32 pmol/mg) as determined by western blot (data not shown). This is consistent with earlier experience with the RAVN construct (26). Among the CYP1B1 mutants, all single amino acid substitutions resulted in expressed protein at levels similar to the normal alleles, except Gly365Trp, which was only found at 3 pmol/mg (data not shown). A 10 bp deletion construct that causes a frame-shift and premature stop did not produce any detectable protein. It is not known whether this is because the truncated protein is not expressed, is unstable, or has lost epitopes for the polyclonal antibody. Cytochrome *c* reductase activity levels averaged 134 ± 35 (SD) pmol/μg/min (range 86–220 pmol/μg/min; data not shown). The variance was $<15\%$ for measurements taken on a single day, and there was similar good agreement between repeated measures of an individual sample. There was no observable relationship between allele sequence and protein concentration or reductase level.

B[a]P-7,8-diol metabolism

All 16 alleles with common polymorphisms produced the four expected tetrol products, (±)benzo[*a*]pyrene-r-7,t-8,c-9,c-10-tetrahydrotetrol (RTCC), (±)benzo[*a*]pyrene-r-7,t-8,c-9,t-10-tetrahydrotetrol (RTCT), (±)benzo[*a*]pyrene-r-7,t-8,t-9,c-10-tetrahydrotetrol (RTTC) and (±)benzo[*a*]pyrene-r-7,t-8,t-9,t-10-tetrahydrotetrol (RTTT), which were identified by co-migration with authentic standards (Figure 2). Vector

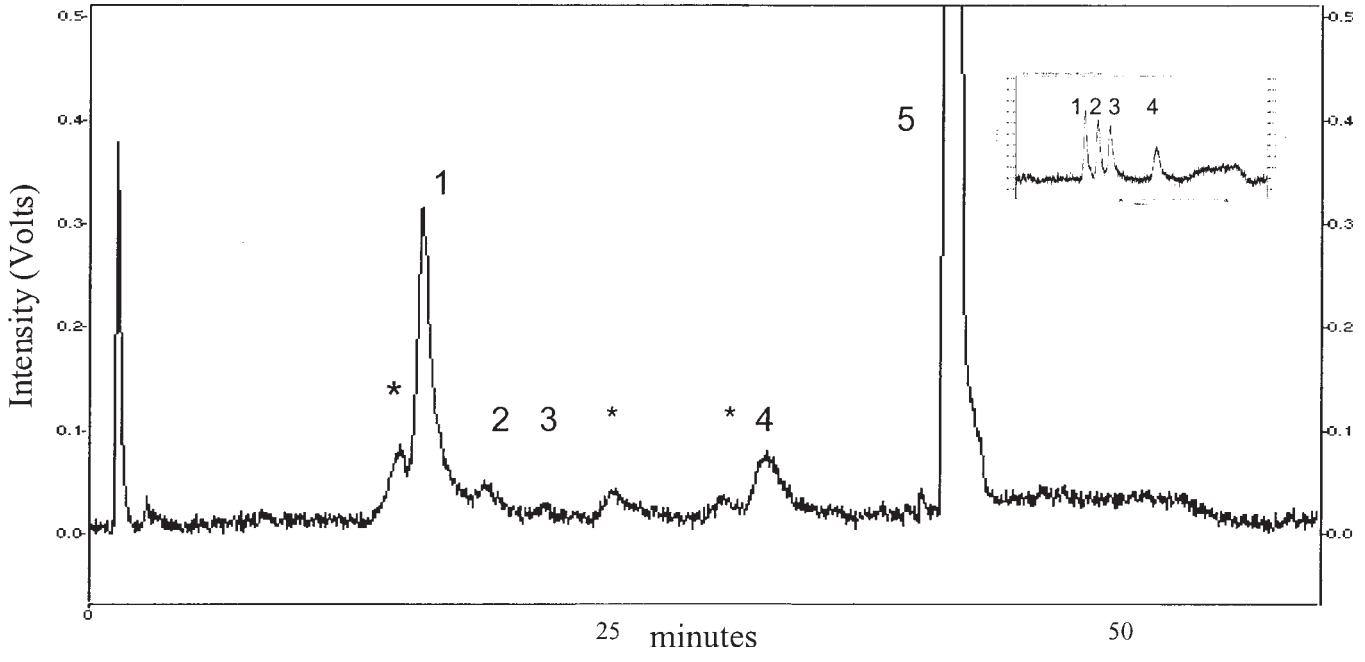

Fig. 2. Chromatogram of B[*a*]P-7,8-diol metabolites produced by CYP1B1. Separation of B[*a*]P-7,8,9,10-tetrols produced in the metabolism of 10 μM B[*a*]P-7,8-diol by CYP1B1. Tetrols are separated in 35 min by 50:50 methanol:water on a C18-reverse phase HPLC column, and detected by fluorescence with 340 excitation, 420 emission. Pure methanol is required to elute B[*a*]P-7,8-diol. Peaks were identified by co-migration with authentic standards (insert), which were used to generate standard curves for quantification of products. 1, RTTC; 2, RTCT; 3, RTTT; 4, RTCC; 5, B[*a*]P-7,8-diol; *, unknown.

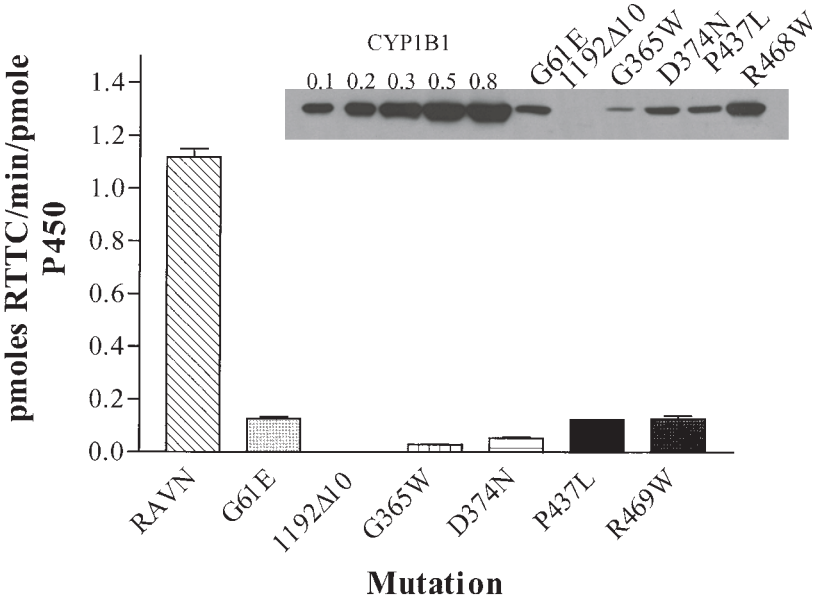

Fig. 3. Activity of mutant CYP1B1 enzymes associated with congenital glaucoma. Ten picomoles of each mutant enzyme or wild-type (RAVN) were assayed in triplicate with 10 μM B[*a*]P-7,8 diol for 15 min at 37°C. For 1192Δ10, where specific protein could not be quantified, 350 mg total protein was used, representing a similar volume of microsomes and maximal protein concentration. The average and standard deviation of the rate of production of RTTC for a triplicate experiment is shown for each enzyme. Activity of the missense mutations ranged from 3 to 12% compared with RAVN, while microsomes containing the truncated construct (1192Δ10) had no activity. Insert shows western blot using 15 μg total protein from microsomes containing each expressed mutant enzyme compared with a CYP1B1 standard curve of 0.1 to 0.8 pmol of enzyme.

control microsomes did not produce any metabolic products from B[*a*]P-7,8-diol (data not shown). Mutant constructs had such low activity that only the one or two most common tetrols were quantifiable. There was very little secondary metabolism observed at the substrate concentrations used, although CYP1B1 is able to further oxidize the tetrols, producing uncharacterized products that migrate between 4 and 10 min (data not shown). Three unidentified peaks were observed in samples with active CYP1B1, and probably represent triol products (5,10).

Of the two epoxides formed by oxidation of B[*a*]P-7,8-diol, the *trans*-diol-epoxide (DE2, yielding RTTC and RTTT) is thought to be more reactive with DNA than the *cis*-diol-epoxide (DE1, yielding RTCC and RTCT). Therefore, we first examined whether any of the polymorphisms change the reaction stereospecificity by calculating the ratio of *trans*- and *cis*-epoxide formation at high substrate concentration. The ratio of DE2:DE1 ranged from 1.5 to 2.4, and none were significantly different from RAVN (Kruskal–Wallis, Dunn's post test for multiple comparisons, data not shown). This is in good agreement with a previously reported ratio of 2.4 for RAVN (10). All further analysis therefore compared enzymes by the production of the highly favored tetrol, RTTC, derived from the biologically significant DE2.

Five missense mutations and one 10 bp deletion associated previously with congenital glaucoma (14,16) were assayed for their ability to metabolize B[*a*]P-7,8-diol at a saturating substrate concentration of 10 μM. Each reaction included 10 pmol enzyme. Since the deletion construct could not be detected on a western blot, we used 350 μg of total protein, twice the average total protein in other experiments. All mutant alleles except the deletion construct had low levels of metabolic activity, ranging from 0.03–0.13 pmol RTTC/min/pmol P450, which represents 3–12% of normal activity for the RAVN clone (Figure 3). The deletion mutant generated no detectable product.

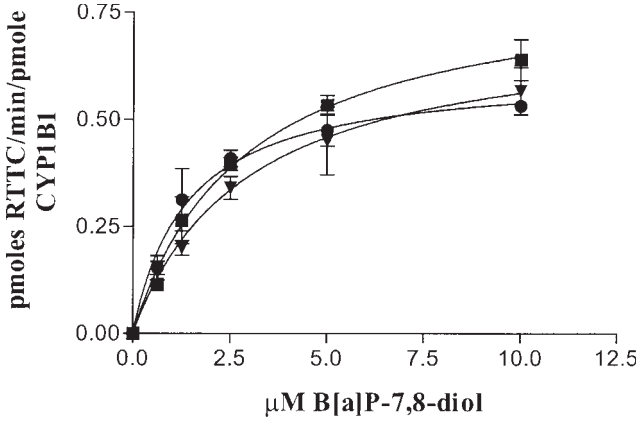

Fig. 4. Michalis–Menten curves for three common haplotypes. Representative curves for each haplotype show similar kinetcs. Each allele was assayed in triplicate at 0.0, 0.625, 1.25, 2.5, 5 and 10 μM B[*a*]P-7,8-diol, with points representing mean and standard deviation: RAVN (square), RALN (circle), GSVN (inverted triangle).

All enzymes examined followed Michaelis–Menten kinetics, as shown by representative curves for three common haplotypes (Figure 4). Initial estimates of kinetic parameters for each of the 16 presumed normal alleles indicated a potential 6-fold range of K_M values, from 0.76 to 4.67 μM, a 3-fold range in k_{cat}, from 0.44 to 1.35 pmol RTTC/min/pmol CYP1B1, and a 5-fold range in k_{cat}/K_M, ranging between 0.24 and 1.25 (Table III). Both Michaelis–Menten curve fitting and Eadie–Hofstee plots yielded similar estimates of the kinetic parameters (data not shown). There was no pattern of any consistent effect on the various parameters with a particular polymorphism. We therefore chose to repeat measurements for those haplotypes with the highest and lowest values for each constant in order to be able to assess the differences statistically: RAVN and RSVN had the highest and lowest K_M values, respectively, while RAVS and RALS represent

Table III. Estimate of kinetic parameters for 16 CYP1B1 alleles

Allelic variant[a]	K_M (μM)[b]	k_{cat} (min^{-1})[b]	k_{cat}/K_M[c]
RAVN	4.67 ± 1.13	1.14 ± 0.13	0.24
RAVS	1.08 ± 0.26	1.35 ± 0.08	1.25
RALN	1.84 ± 0.25	1.02 ± 0.04	0.55
RALS	0.76 ± 0.20	0.44 ± 0.03	0.58
GSVN	2.90 ± 0.52	0.72 ± 0.05	0.26
GSVS	2.56 ± 0.43	1.00 ± 0.06	0.39
GSLN	1.71 ± 0.49	0.64 ± 0.06	0.37
GSLS	2.08 ± 0.75	0.84 ± 0.12	0.40
RSVN	0.76 ± 0.20	0.94 ± 0.05	1.23
RSVS	1.04 ± 0.31	0.66 ± 0.05	0.64
RSLN	1.73 ± 0.24	0.69 ± 0.03	0.40
RSLS	1.94 ± 0.40	0.78 ± 0.06	0.40
GAVN	0.86 ± 0.25	0.68 ± 0.04	0.79
GAVS	1.25 ± 0.27	0.53 ± 0.03	0.42
GALN	1.29 ± 0.32	0.77 ± 0.06	0.59
GALS	1.32 ± 0.32	0.56 ± 0.04	0.42

[a]Microsomes were pooled from at least two preparations for each allele.
[b]Parameters reported are the best estimate and the standard deviation of that estimate from a single experiment at five concentrations performed in triplicate using pooled microsomes.
[c]The ratio of best fit estimates of each parameter, as shown in columns 2 and 3.

Table IV. Replicated kinetic parameters for six CYP1B1 alleles

Allelic variant[a]	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M
RAVN	2.79 ± 0.80	0.85 ± 0.17	0.33 ± 0.12
RAVS	1.38 ± 0.25[b]	0.88 ± 0.31	0.68 ± 0.35
RSVN	1.71 ± 0.61	0.72 ± 0.24	0.50 ± 0.36
RALS	1.32 ± 0.41[c]	0.48 ± 0.16	0.40 ± 0.18
RALN	1.79 ± 0.04	0.76 ± 0.40	0.42 ± 0.22
GSVN	2.79 ± 0.75	0.63 ± 0.10	0.24 ± 0.06

[a]Each allelic variant was assayed in five to nine experiments, using two to three independent microsomal preparations.
[b]$P < 0.05$, [c]$P < 0.01$ compared with RAVN, by Kruskal–Wallis and Dunn's post test for multiple comparisons.

the highest and lowest k_{cat} values. This set included the alleles, RAVN and RAVS, representing the extremes of k_{cat}/K_M. We also included in this analysis two of the most common haplotypes, RALN and GSVN.

Average values for at least three independent experiments with at least two independent microsomal preparations are shown in Table IV. In this analysis, both RALS and RAVS appeared to have K_M values that are significantly lower than RAVN, which has the highest, when analyzed using Kruskal–Wallis and Dunn's post-test for multiple comparisons. K_M values were found to be 1.38 ± 0.25, 1.32 ± 0.41 and 2.79 ± 0.8 for RAVS, RALS and RAVN, respectively. There are no significant differences in either k_{cat} or catalytic efficiency (k_{cat}/K_M). This suggests that these enzymes all perform with similar efficiency when the concentration of substrate is very small compared with K_M.

In order to test the reliability of the differences predicted by estimating the kinetic parameters, we compared the reaction rates for RAVS and RALS with RAVN at one of two substrate concentrations. Varying reaction time provides a robust measure of rate with which to compare enzymes. For enzymes with the same k_{cat}, a 2-fold lower K_M means the higher affinity enzyme will generate product at twice the rate at a substrate concentration equal to the lower K_M; so the slope of the curve product versus time for RAVS should be approximately twice that of RAVN at 1 μM B[*a*]P, as would the slope for RALS if the apparent 2-fold difference in k_{cat} is truly significant. If RALS has a 2-fold lower k_{cat} as well, the difference in rate at low concentration will be much less apparent, but at saturating concentrations, the 2-fold difference in k_{cat} would be clearly observed. The rates of RTTC production by each of the Ser453 alleles were not different when compared with RAVN at either 1 or 10 μM B[*a*]P-7,8-diol (Figure 5), meaning we were unable to confirm that RALS and RAVS have 2-fold lower K_M compared with RAVN. RALS showed a trend towards a lower k_{cat} compared with RAVN (Figure 5D), but much smaller than the 2-fold difference suggested in the earlier experiments, and this difference could not be established with statistical significance.

Discussion

Several presumed low activity alleles of *CYP1B1* have been identified as co-segregating with one form of primary congenital glaucoma in family studies of the inherited disease. We have demonstrated that six of these mutations severely reduce enzyme function. Similar results have been reported for two of the mutations, G61E and R469W, in the metabolism of steroids (30). Yeast microsomes containing enzymes with any of five missense mutations exhibit between 3 and 12% of normal RAVN activity for the activation of B[*a*]P-7,8-diol, while a deletion mutation shows no activity and cannot be detected on western blot by a polyclonal antibody, suggesting that the truncated protein is unstable. Our results suggest an explanation for the observation, made in a study of congenital glaucoma among the Amish, that individuals homozygous for a substitution mutation had a less severe phenotype than those homozygous for a truncating mutation (19).

In order to test individual or interactive effects of four commonly occurring polymorphisms (Arg48Gly, Ala119Ser, Val432Leu and Asn453Ser), we systematically created all 16 possible recombinant alleles and expressed them in yeast. In contrast to the glaucoma-associated mutations, these polymorphisms have modest effects on CYP1B1 kinetics. There were no differences that could be demonstrated in either k_{cat} or catalytic efficiency (k_{cat}/K_M) for the activation of B[*a*]P-7,8-diol. Repeated kinetic measurements revealed a 2-fold lower K_M in two haplotypes, RAVS and RALS, compared with the wild-type RAVN for this substrate. This difference, K_M = 1.3 μM versus K_M = 2.8 μM, is a small but statistically significant difference ($P < 0.05$). However, comparing metabolic rates at single substrate concentrations, the alleles did not appear to differ.

The biological relevance of the kinetic differences observed needs to be evaluated in three contexts: (i) typical exposure levels to the carcinogen in the general population; (ii) the range of likely enzyme induction; and (iii) the frequency of the higher risk alleles in the population. Dietary exposures to carcinogens such as B[*a*]P are in the nanograms/day range, which means that in non-smokers, cellular concentrations are likely to be quite low compared with K_M. As at low substrate concentrations kinetics are determined by the catalytic efficiency (k_{cat}/K_M), which is the first order rate constant for the reaction when substrate is much lower than K_M, our results suggest that there will not be differences in susceptibility correlated with the genotypes investigated for non-smokers. In smokers, elevated exposures have been shown to lead to

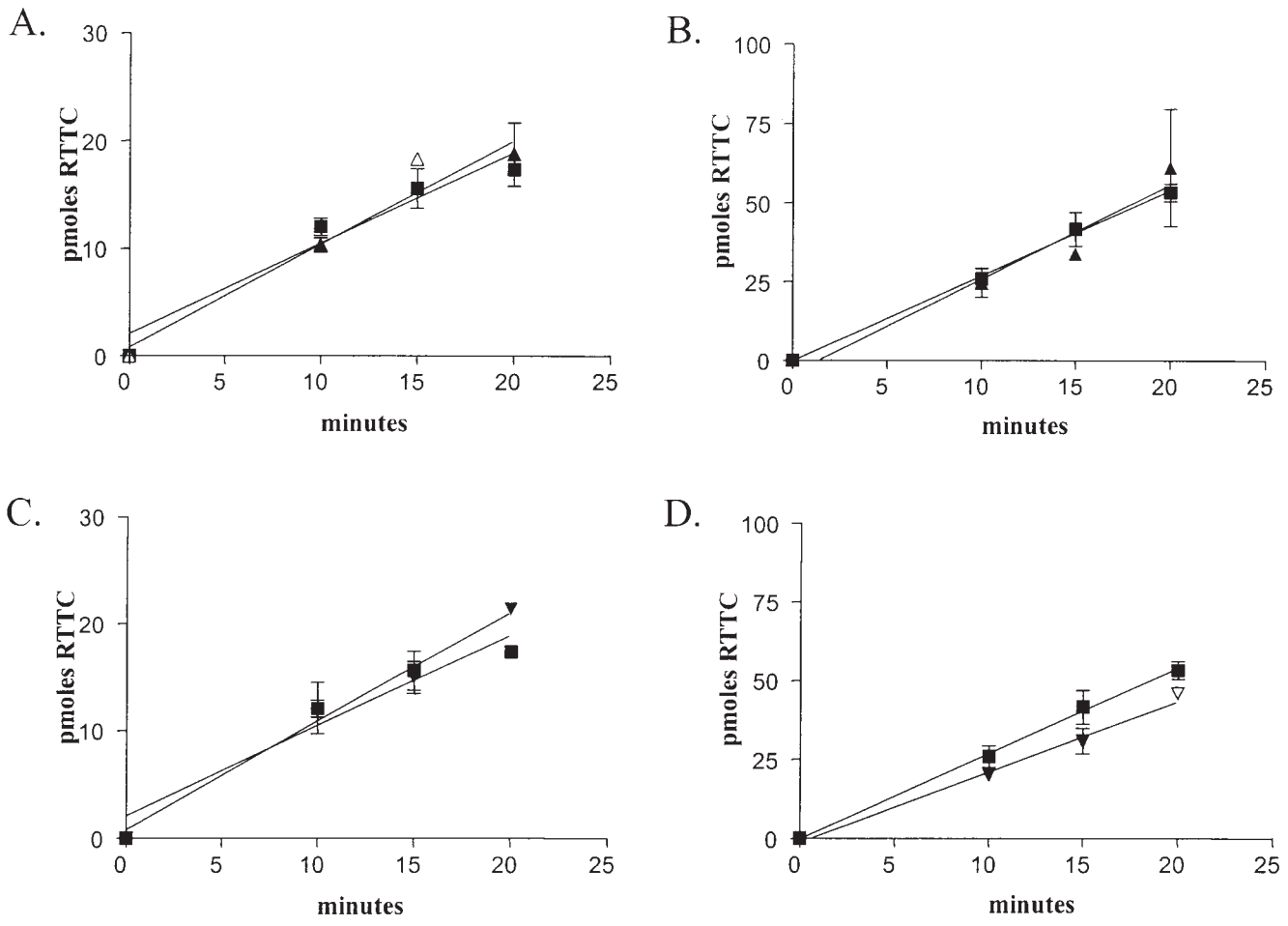

Fig. 5. Rate determinations for RAVS, RALS and RAVN. Rates at low and high substrate concentration for RAVS (triangles, **A** and **B**) or RALS (inverted triangles, **C** and **D**) compared with wild-type RAVN (squares). Experiments were performed in duplicate at 0, 10, 15 and 20 min with B[*a*]P-7,8-diol concentrations of 1 μM (A and C), where a difference in slope would reflect a difference in K_M, or 10 μM (B and D), where a difference in slope would reflect a difference in k_{cat}. None of the slopes were significantly different from wild-type. Symbols specify mean, error bars indicate range where range is greater than the size of the symbol; open symbols indicate only a single data point was available.

enzyme induction, particularly in the lung (31). This induction leads to mRNA expression levels that vary by 40-fold, which, if the protein is mostly active, would create a greater variation in total enzyme activity than that due to the small, observed differences in K_M.

Small differences in individual risk due to variation in metabolism may have a large impact on population-based risk estimates if the risk factor is at high frequency in the population. Allele frequency estimates in the population groups examined in this study are consistent with those reported in the literature (20,22,23,32). The RALN, RAVN, GSLN and GSVN haplotypes (all functional) together account for the majority of alleles across all population groups tested. Alleles containing the Ser453 variant (RAVS and RALS, the possible high activity alleles) are uncommon, with RAVS being undetectable in our small populations. The RALS allele occurs between 0.5% in Asians and 15% in European-Americans. It would appear that risk associated with this allele would be most easily detected in those of European descent. One study has looked for an increased risk of breast cancer due to the Ser453 variant among 328 Caucasians and found no association (23). Statistical power calculations ($\alpha < 0.05$ and $\beta > 80$) suggest that a study of over 600 individuals would be needed in order to detect a 2-fold increase in risk among homozygous RALS individuals with reasonable probability.

The locations of the rare mutations examined within CYP1B1 are consistent with our results that the mutations associated with congenital glaucoma have a large impact on function. Those point mutations associated with congenital glaucoma are spread throughout the protein but are generally well-conserved residues (14). Gly61Glu is in the hinge region and Arg469Trp is in the heme-binding region, both highly conserved segments of the protein, although the latter amino acid is not conserved outside of the CYP1 family. Pro437Leu is situated on the boundary of the so-called meander region and is highly conserved, while Gly365Trp is on the boundary of helix J and is conserved across several human CYP families and within the CYP1 family across species. Only Asp374Asn is in a non-conserved region between helixes J and K. However, this substitution exchanges an acidic residue with a neutral one, and could have important consequences for proper protein folding, which could in turn disrupt the active site or substrate binding. In contrast, the common SNPs examined here are well outside conserved regions with the exception of Asn453Ser, which is found within the meander region.

Although Asn453Ser does not represent a conserved residue in the CYP1 family broadly, it is conserved in the *CYP1B1* sequence of human, mouse and rat.

Only one other group has compared four *CYP1B1* alleles for the metabolism of B[*a*]P-7,8-diol, and they also do not report differences, although the experiment is done in *Escherichia coli* and much higher overall K_M values were obtained than reported here (33). This discrepancy in K_M measurements between *E.coli* and yeast experiments is consistent with that found for estradiol metabolism, and may be due to inadequate association between CYP1B1 and reductase in the *E.coli* plasma membranes (26,34–36). A second paper that compared alleles in the metabolism of B[*a*]P cannot be evaluated as the assay was not linear with time for the interval used, so kinetic parameters cannot be accurately derived (37).

CYP1B1 can metabolize a broad range of substrates, many of which are carcinogenic (12). CYP1B1 is also the most catalytically efficient 17β-estradiol 4-hydroxylase described to date (26). This metabolite is believed to be important in estrogen carcinogenesis (38). A thorough study of estradiol metabolism in yeast needs to be undertaken, as it is not known whether kinetic analysis can be generalized across substrates. Groups investigating estradiol metabolism so far in *E.coli* have not found differences in k_{cat}, and have divergent findings for K_M. Reports include no differences, a higher K_M value for RAVN compared with other alleles, and higher K_M values for RALN and RALS compared with others (33,34,36,37).

Logical pursuit of genetic variation as a risk factor for disease rests upon the biological plausibility that the variation could affect the disease process. For a metabolic enzyme hypothesized to affect cancer risk, the critical variation is in the contribution it makes to the availability of an ultimate carcinogen. Here we have taken a rigorous biochemical approach to investigate nine single amino acid substitutions and one deletion in CYP1B1, encompassing both disease-associated mutations and all possible haplotypes from four common polymorphisms, in order to better understand the range of normal and abnormal function of this enzyme in the production of B[*a*]P diol epoxides. We have begun the investigation of amino acid substitutions with B[*a*]P-7,8-diol because it is a well characterized and ubiquitous carcinogen. CYP1B1, like most other CYP enzymes, is able to activate a wide range of substrates, and a complete characterization of how genetic polymorphisms might affect cancer risk will require a broader investigation into the metabolism of other substrates. As we build an understanding of how gene–environment interactions can be generalized and predicted, a systematic approach to examining the functional consequences of polymorphisms could ultimately allow us to predict what types of genetic changes in a family of enzymes would have important effects on particular substrates.

Acknowledgements

Thank you to Dr Randy Bryant for his advice and feedback. This research was supported by the National Institutes of Health grants ES08148 and ES03819.

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Received January 20, 2003; revised April 23, 2003; accepted May 4, 2003