0026-895X/97/030406-08$3.00/0

MOLECULAR PHARMACOLOGY, **51:**406–413 (1997).

Arginine 52 and Histidine 54 Located in a Conserved Amino-Terminal Hydrophobic Region (LX2-*R52*-G-*H54*-X3-V-L) Are Important Amino Acids for the Functional and Structural Integrity of the Human Liver UDP-Glucuronosyltransferase UGT1*6

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Received July 18, 1996; Accepted November 25, 1996

SUMMARY

The hepatic UDP-glucuronosyltransferase UGT1*6 is actively involved in the glucuronidation of short and planar phenols in humans. Based on the irreversible inhibition of the enzyme on chemical modification by 2,3-butanedione and diethyl pyrocarbonate, the roles of His54 and Arg52 were investigated by oligonucleotide site-directed mutagenesis. These amino acids belong to a consensus sequence LX2-*R52*-G-*H54*-X3-V-L located in a conserved hydrophobic region of the variable amino-terminal domain of UGT. Arg52 was replaced by alanine (mutant R52A), and His54 was replaced by alanine or glutamine (mutants H54A and H54Q). The immunological and catalytic properties of UGT1*6 and mutants were examined after stable expression in V79 cell lines. Immunoblots and immunoprecipitation studies revealed that the mutant and UGT1*6 proteins were expressed in the microsomal membranes in similar amounts. However, replacement of His54 by glutamine led to a complete loss of activity toward 4-methylumbelliferone, and the V_{max} value was decreased 4–5-fold in the mutants R52A and H54A compared with the wild-type enzyme. The dissociation constants that characterize the binding of 4-methylumbelliferone and UDP-glucuronic acid to UGT1*6 were not greatly affected by the mutations. Interestingly, H54Q was not recognized by specific antibodies to the amino-terminal portion of UGT1*6, thereby indicating that this amino acid was critical to antibody recognition. In contrast, the mutants R52A and H54A could not be differentiated from the wild-type protein by pH optimum or thermal denaturation. Furthermore, these mutants were still sensitive to irreversible inhibition by diethyl pyrocarbonate and 2,3-butanedione, with second-order inactivation constant values similar to those obtained for UGT1*6. Altogether, the strict conservation of His54 and Arg52 and the mutational analysis of these residues suggest that these amino acids in the hydrophobic amino-terminal consensus sequence LX2-*R52*-G-*H54*-X3-V-L are important for the function and the structure required for optimal catalytic efficiency of UGT1*6.

UGTs (E.C. 2.4.1.17) are a family of membrane-bound enzymes. They catalyze the conjugation of glucuronic acid to the nucleophilic functional group of structurally unrelated lipophilic substrates (1). More than 35 cDNAs have been isolated, which encode two families of proteins (2). The glucuronidation reaction results in the formation of a β-D-glucuronide with inversion of configuration of the α-glycosidic hydroxyl group, suggesting an SN_2 mechanism (3). These enzymes are centrally involved in the final metabolism of drugs, pollutants, and toxic substances (4). Furthermore, they metabolize and contribute to the regulation of the concentration of ligands of nuclear receptors (retinoic acid, steroid, and thyroid hormones) that are important for cell differentiation and growth (5).

This work was supported by the Région Lorraine, by the Institut Fédératif de Recherche "Protéines," by the Ministère de l'Education Nationale, and by National Institutes of Health Grant DK49715. This work is in partial fulfillment of the doctoral thesis of C.S. S.F.G. was the recipient of an INSERM-MRC senior research fellowship.

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ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; 4-MU, 4-methylumbelliferone; DMEM, Dulbecco's modified Eagle's minimum; SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

In terms of substrate specificity, liver microsomal UGTs from rat, human, and other mammalian species have been well characterized and documented (6). However, little is known about the peptide domains and amino acids that are located in the active site and involved in catalysis. This information is important to understand better and ultimately prevent the glucuronidation-mediated toxicity that is encountered with several compounds (e.g., arylamines, carboxylic acids) and to improve the efficacy of drugs that are glucuronidated.

The primary structure of many UGTs deduced from their nucleotide sequences suggests that the high specificity of the UGT isoforms for the common donor substrate UDP-glucuronic acid could be related to the highly conserved carboxyl-terminal domain of UGTs. On the other hand, the amino-terminal portion of the isoforms, encoded by one of the first exons of the gene coding for the members of family 1, is likely to specify the acceptor properties of each of the transferases (7). Functional expression of chimeric UGTs belonging to family 1 or 2 has confirmed this general organization (8).

UGT1*6, a human liver enzyme encoded by a gene belonging to the *UGT1* locus, glucuronidates planar and short phenols, including drugs (e.g., paracetamol, reduced naftazone) and carcinogens (9). Investigation of the active site topography has been performed mainly through the use of amino acid-specific reagents (10, 11). Chemical modification of recombinant UGT1*6 stably expressed in V79 cells established the key amino acid classes implicated in catalysis or in the vicinity of the active site. Recently, we showed that the histidine-acetylating reagent diethyl pyrocarbonate caused a rapid time- and concentration-dependent inactivation of UGT1*6. The pH dependence of this inactivation was in agreement with the involvement of a nonprotonated imidazole histidine residue, which may facilitate ionization of the nucleophilic group of the donor substrate (10). Furthermore, using carbodiimides, we showed that an aspartic or a glutamic acid residue could actively participate in the glucuronidation reaction through a general acid-base catalysis (11).

On the other hand, Zakim *et al*. (12) reported the susceptibility of a purified pig liver UGT (phenols) to the dicarbonyl reagent 2,3-butanedione, thus indicating the presence of a key arginine residue. Therefore, inhibition of the recombinant UGT1*6 by 2,3-butanedione was examined. Our results support the presence of an important arginine residue.

The sequence LX2-*R52*-G-*H54*-X3-V-L located in a conserved hydrophobic domain represents a unique consensus sequence in the variable amino-terminal portion of UGT proteins and other UDP-glycosyltransferases (Table 1). Based on the chemical reactivity of histidine and arginine residues toward irreversible inhibitors, oligonucleotide site-directed mutagenesis was used to examine the roles of His54 and Arg52 in the conjugation of phenolic substrates catalyzed by UGT1*6. For this purpose, we characterized the catalytic and immunological properties of wild-type and mutant enzymes after production in V79 cells. This system represents an efficient expression procedure and produces large quantities of active protein for structure-function studies (13).

Materials and Methods

Chemicals and reagents. 4-MU (free acid), 4-MU-β-D-glucuronide, diethyl pyrocarbonate, 2,3-butanedione, G-418 (geneticin), and donkey anti-goat alkaline phosphate-conjugated immunoglobulins were obtained from Sigma Chemical (L'Isle d'Abeau, St. Quentin Fallavier, France). L-[^{35}S]Methionine was from DuPont-New England Nuclear (Brussels, Belgium), and UDP-glucuronic acid was from Boehringer-Mannheim Biochemica (Mannheim, Germany). DMEM and Luria-Bertani medium were obtained from GIBCO BRL (Eragny, France). The restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Ozyme, Montigny le Bretonneux, France), and *Taq* polymerase was from Perkin-Elmer Cetus (St. Quentin, France). All other reagents were of the best quality available commercially.

Plasmid construction and mutagenesis. A full-length UGT1*6 cDNA isolated from the initial pKCRH2 expression vector (14) was subcloned into the pcDNAI (InVitrogen, Abingdon, UK) eukaryotic expression vector. Three mutants were constructed. His54 was replaced with alanine and glutamine, and Arg52 was replaced with alanine, forming the mutants H54A, H54Q, and R52A, respectively. For this purpose, site-directed mutagenesis of the UGT1*6 cDNA after subcloning into the *Hin*dIII site of the pGEM-3Z vector was performed using the "megaprimer" PCR-based method described by Sarkar and Sommer (15) with three PCR primers and two rounds of PCR. Primer A corresponds to the T7 promoter sequence of the pGEM-3Z plasmid, and primer B contains the sequence change for

TABLE 1

Alignment of the amino-terminal sequence of UDP-glycosyltransferases

The cDNA-derived amino acid sequences of different UGT isoforms, of a ceramide-galactosyltransferase (C-Gal-T), and of an ecdysteroid glucosyltransferase (E-Gluc-T) obtained from the SwissProt data bank were aligned using the PileUp program from the GCG package (Genetics Computer Group, Madison, WI). The number (n) indicates the position of the first amino acid of the alignment. Mutated residues are indicated in bold.

Enzyme	n																	
UGT1*6	46	Val	Glu	Val	Leu	Ser	Asp	**Arg**	Gly	**His**	Glu	Ile	Val	Val	Val	Pro	Glu	Val
UGT1*06	45	Val	Glu	His	Leu	Ser	Glu	**Arg**	Gly	**His**	Asp	Ile	Val	Leu	Val	Pro	Glu	Val
UGT1*1	47	Ile	Gln	Gln	Leu	Gln	Gln	**Arg**	Gly	**His**	Glu	Ile	Val	Val	Leu	Ala	Pro	Asp
UGT1*4	48	Leu	Arg	Glu	Leu	His	Ala	**Arg**	Gly	**His**	Gln	Ala	Val	Val	Leu	Trp	Pro	Glu
UGT1*0	45	Val	Arg	Glu	Leu	His	Ala	**Arg**	Gly	**His**	Asp	Ala	Val	Leu	Ala	Pro	Glu	Val
UGT2B8	43	Leu	Glu	Glu	Leu	Val	Gln	**Arg**	Gly	**His**	Gln	Val	Val	Leu	Thr	Ser	Ser	Ala
UGT2B4	43	Leu	Asp	Glu	Leu	Val	Gln	**Arg**	Gly	**His**	Glu	Val	Val	Leu	Ala	Ser	Ser	Ala
UGT2B7	43	Leu	Asp	Glu	Leu	Ile	Gln	**Arg**	Gly	**His**	Glu	Val	Val	Leu	Ala	Ser	Ser	Ala
UGT2B3	43	Leu	Asp	Glu	Leu	Val	Gln	**Arg**	Gly	**His**	Glu	Val	Val	Leu	Lys	Pro	Ser	Ala
UGT2B1	43	Leu	Asp	Glu	Leu	Val	Gln	**Arg**	Gly	**His**	Glu	Val	Val	Leu	Lys	Pro	Ser	Ala
UGT2B6	43	Leu	Asn	Glu	Leu	Ala	Gln	**Arg**	Gly	**His**	Glu	Val	Val	Leu	Val	Ser	Ser	Ala
Ugt2b5	43	Leu	Asp	Glu	Leu	Val	Gln	**Arg**	Gly	**His**	Glu	Val	Val	Leu	Lys	Pro	Ser	Ala
UGT2B2	43	Leu	Asp	Glu	Leu	Val	Gln	**Arg**	Gly	**His**	Glu	Val	Val	Leu	Arg	Pro	Ser	Ala
C-Gal-T	42	Ala	Ser	Ala	Leu	His	Glu	**Arg**	Gly	**His**	His	Thr	Val	Phe	Leu	Leu	Ser	Gly
E-Gluc-T	40	Ile	Glu	Ala	Leu	Ala	Glu	Lys	Cys	**His**	Asn	Val	Thr	Val	Val	Lys	Pro	Lys

each mutation: R52A, 3′-GAGTCACTGCGCCCAGTACTCTAAC-5′; H54A, 3′-CACTGGCCCCACGACTCTAACATCAC-5′; and R54Q, 3′-CACTGGCCCCAGTCCTCTAACATCAC-5′. In the first round of PCR, primers A and B were used in the presence of the template DNA to amplify fragment A–B. The amplified DNA was purified from an agarose gel and used as "megaprimer" in the second round of PCR in the presence of primer C (3′-GCTAGTAAGGATTGACG-5′) that matches a sequence upstream from the unique restriction site *Bst*XI of UGT1*6 cDNA. The segment A–C was then double-digested with *Bam*HI (located downstream from the T7 promoter in the pGEM-3Z polylinker) and *Bst*XI. The resulting fragment was exchanged with the nonmutated fragment in the initial pGEM-3Z/UGT1*6 vector. Finally, each of the mutated UGT1*6 cDNAs was subcloned into the *Hin*dIII/*Xba*I site of the pcDNAI expression vector. The plasmid DNA was isolated using Qiagen columns (Qiagen, Coger, Paris, France). Mutant clones were screened for *Taq* polymerase-introduced errors by dideoxy-sequencing.

Establishment and culture of V79 cells stably expressing wild-type and mutant UGT1*6. V79 cells (Chinese hamster lung fibroblasts) were cultured in DMEM supplemented with 10% Nuserum (Tebu, Le Perray en Yvelines, France), antibiotics, and antimycotics. Each of the recombinant wild-type and mutant plasmids was cotransfected into V79 cells at early passages seeded 24 hr before cotransfection at 0.5×10^6 cells/10-cm plate with the plasmid SFVneo as previously described (13). At 24 hr after transfection, cultures were split and placed in selection medium (G-418, 1 mg/ml). Thirty resistant colonies for each transfection were screened by Western blot analysis. The colonies expressing the highest level of protein were selected for further analysis. The stability of protein expression was checked over 10 passages.

Biosynthetic labeling of V79 cells and immunoprecipitation. Control and recombinant subconfluent cells were preincubated for 3 hr in methionine-free medium containing 5% (v/v) Nuserum in the presence or absence of tunicamycin (1 mM). Cells were then labeled for 6 hr with methionine-free medium containing 100 μCi of L-[^{35}S]methionine/plate with or without tunicamycin. After labeling, cells were chased in a complete medium supplemented with 0.5 mM unlabeled methionine for 12 hr, washed twice with ice-cold phosphate-buffered saline (GIBCO), and conserved as a pellet at -80o. Immunoprecipitation of the radiolabeled proteins was carried out as previously described (14). Pellets were resuspended in 50 mM lysis buffer [0.5% (w/v) sodium cholate, 1% (v/v) Triton X-100, 0.1% (w/w) SDS, 10 mM Tris·HCl, pH 7.5], and lysates were clarified by centrifugation for 10 min in a microcentrifuge. Supernatants were incubated for 1 hr with preimmune IgG in immunoprecipitation buffer [(0.5% (w/w) sodium cholate, 0.5% (v/v) Triton X-100, 150 mM KCl, 10 mM methionine, 50 mM Tris·HCl, pH 7.5] before the addition of Pansorbin cells (Calbiochem-Novabiochem). After centrifugation, the pellets were washed three times stringently in a solution containing 1 M KCl. After the final wash, the precipitated antigens were released by suspension of the cells in 50 μl of Laemmli's sample buffer and boiled for 5 min. The mixture was centrifuged, and the supernatant was analyzed by SDS-polyacrylamide gel electrophoresis on a 10% SDS-polyacrylamide gel according to the method of Laemmli (17). After soaking in Enhance solution (DuPont-New England Nuclear, Nottingham, UK), the gel underwent fluorographic analysis.

Membrane fraction preparation, enzyme activity, and protein analysis. Enriched membrane fractions were prepared as described previously (10). Briefly, pellets of V79 cells were homogenized in 0.25 M sucrose and 5 mM HEPES, pH 7.4, and subjected to three 5-sec sonications (Vibra Cell, Bioblock Scientific, Strasbourg, France). The homogenate was centrifuged at $12{,}000 \times g$ for 20 min, and the supernatant was centrifuged at $100{,}000 \times g$ for 60 min. The resulting pellet was suspended in the same buffer and represented the membrane-enriched fraction. The protein concentration was measured according to the method of Bradford with serum albumin as standard (16).

After SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels (17), the proteins were transferred onto Immobilon-P membranes (Millipore, Bedford, MA). Detection of recombinant proteins was carried out using antibodies raised against rat liver UGT (18), which recognize several UGT isoforms. Antibodies raised against a portion of the amino-terminal end of UGT1*6, which recognize only this isoform (19), were also used. Proteins from nontransfected V79 cells were used as positive controls. The intensity of the signals revealed on immunoblots was analyzed by densitometry (imaging densitometer model GS-670, BioRad, Hercules, CA). The intensity of the signal as a function of the amount of protein was found linear for a concentration range of 0–70 μg of protein loaded on the gel for both antibodies used.

UGT activity measurement and kinetic analysis. 4-MU was used as reporter substrate to measure the glucuronidation activity of UGT1*6 and of the mutants. Formation of 4-MU glucuronide was followed according to the method of Lilienblum *et al*. (20), with ethylacetate used instead of chloroform to extract unconjugated substrate. 4-MU (1 mM) was incubated with UDP-glucuronic acid (5 mM) in the presence of membrane fractions of V79 cells expressing the different enzymes. Fluorescence measurements of 4-MU-β-D-glucuronide, at excitation and emission wavelengths of 320 and 380 nm, respectively, were carried out on a Hitachi F2000 spectrofluorimeter (ScienceTec, Les Ulis, France) with authentic 4-MU-β-D-glucuronide (0–10 mmol) as standard.

The kinetic constants were determined from the glucuronidation rates measured as a function of 4-MU concentrations at different fixed concentrations of UDP-glucuronic acid, as described by Segel (21). Activities at V_{max} and the dissociation constants K and K' were determined (21) for an enzyme with a rapid-equilibrium, random-order kinetic mechanism.

The inhibition potency of 7,7,7-triphenylheptanoic acid (22) and several other inhibitors on the glucuronidation of 4-MU was estimated in terms of IC_{50}. These new inhibitors comprise a series of compounds containing a lipophilic *N*-acyl phenylaminoalcohol residue and a uridine moiety connected by a spacer that varies with each compound (23). The inhibitors were dissolved in dimethylsulfoxide and added to V79 membranes at a final concentration of 10–500 μM at 1 min before the addition of 4-MU. Controls without inhibitors represented 100% enzyme activity. IC_{50} values were determined using the linear portion of the semilogarithmic plots of the inhibition data.

Measurement of glucuronide formation in whole cells. Cells were grown to subconfluency in six-well tissue culture plates. At 24 hr before the experiment, 2 mM butyrate and 1.5 g/liter glucose were added to the DMEM. The culture medium was discarded, and plates were washed three times with Krebs-HEPES buffer (Krebs-Henseleit medium containing 6 g/liter glucose, 12.5 mM HEPES, pH 7.4). Incubations were carried out in 5 ml of the same buffer at 37o after start of the reaction by the addition of substrate [0.5 mM 4-MU dissolved in dimethylsulfoxide, 0.25% (v/v)]. Samples (200 μl) were taken from the extracellular medium at intervals of 0–8 hr. The formation of glucuronides was determined as described above.

Chemical modification by 2,3-butanedione or diethyl pyrocarbonate. Inactivation of UGT1*6 and the R52A mutant with 2,3-butanedione was performed at 25° in the dark with membrane fractions (1.8 mg/ml protein) in 125 mM Tris·HCl buffer, pH 7.4, and 20 mM $MgCl_2$. Inactivation was started by the addition of 2,3-butanedione freshly prepared in ice-cold absolute ethanol (20–60 mM). Aliquots were removed at times 0–30 min and quenched by a 50-fold dilution in buffer. 4-MU glucuronidation was determined as described above.

Modification of UGT1*6 and both mutants by diethyl pyrocarbonate was carried out at 25° with membrane fractions adjusted to 1.3 mg of protein/ml with 50 mM sodium/potassium phosphate buffer, pH 6.0, and 5 mM $MgCl_2$ as previously described (10). Increasing concentrations of diethyl pyrocarbonate (0.1–0.5 mM) in absolute ethanol were used at various reaction times (0–80 sec). The pseudo-first-order rate constants of inactivation (k_i), the inactivation reaction

order (n), and the second-order inactivation constant k_i' were calculated as previously indicated (10, 11).

pH dependence and thermal denaturation. Optimal pH of UGT1*6 and mutants was determined over a pH range of 5.0–7.0 in 125 mM MES buffer containing 20 mM $MgCl_2$ and over a pH range of 7.0–9.1 with 125 mM Tris·HCl buffer and 20 mM $MgCl_2$.

Thermal inactivation plots were obtained by incubation of the enzyme at 50° for 1–35 min in Tris buffer. This temperature was previously shown to inactivate 50% of the initial enzyme activity after a 5-min exposure. The residual activity as a function of time was measured by the standard assay given above.

Results

Formation of 4-MU glucuronide from cultured V79 cells expressing UGT1*6 and mutants. We examined the rate of release of newly formed 4-MU glucuronide into the culture medium from V79 cell lines stably expressing UGT1*6 and the mutants H54A, H54Q, and R52A (Fig. 1). H54Q was unable to form the glucuronide, but the two other mutants were effective. However, the production of glucuronide by these two mutants was significantly lower than that for the wild-type.

Immunological studies. Microsomal fractions prepared from V79 cell homogenates containing either the wild-type enzyme or the UGT1*6 mutants were analyzed on 10% SDS-polyacrylamide gels. Fig. 2 shows that the expression level of the mutant proteins was similar to that of the wild-type, as judged from immunoblot analysis using nonspecific antibodies raised against rat liver UGT (Fig. 2B). In contrast, under the same conditions, when the immunoblot was probed with specific anti-amino-terminal UGT1*6 antibodies, no signal was seen for the H54Q mutant, whereas the wild-type UGT1*6 and the H54A and R52A mutants were readily detected (Fig. 2A). No signal was observed with nontransfected V79 cells (Fig. 2, A and B). To further verify that the absence of detectable signal for the H54Q mutant was not due to the absence of expressed protein, immunoprecipitation studies were performed on proteins radiolabeled with ^{35}S-methionine with nonspecific polyclonal anti-rat liver UGT IgG (18) (Fig. 3). By comparison with UGT1*6 and H54A, the H54Q mutant was labeled with a comparable intensity, thus indicating that the level of expression of this protein was not reduced.

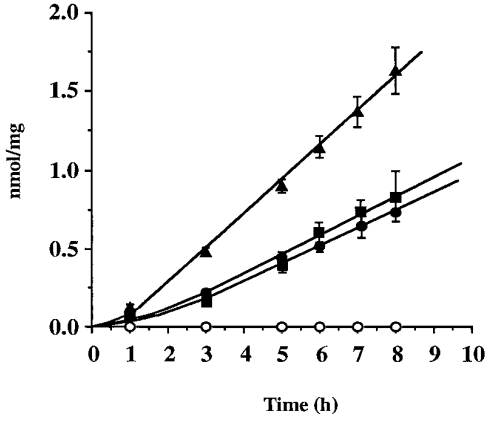

Fig. 1. Formation of 4-MU glucuronide from cultured V79 cells expressing UGT1*6 and mutants. V79 cells were incubated at 37° in Krebs-Heinseleit medium containing 6 g/liter glucose, 12.5 mM HEPES, pH 7.4, and 0.5 mM 4-MU. Results are expressed as nmol of glucuronide released into the culture medium/mg of cellular proteins. ▲, UGT1*6; ■, R52A; ●, H54A; ○, H54Q. Values are the mean ± standard deviation of three determinations of two independent experiments.

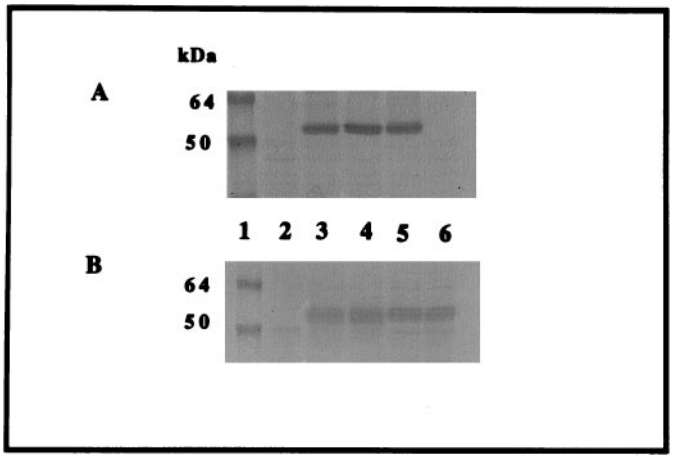

Fig. 2. Immunoblot analysis of UGT1*6 and H54A, H54Q, and R54A mutants expressed in V79 cells. Membrane-enriched fractions (50 μg) of nontransfected or recombinant cells were loaded onto 10% polyacrylamide gels. Immunoblots were probed with antibodies raised against the amino-terminal end of UGT1*6 (A) or antibodies developed against rat liver UGT (B), as described in Materials and Methods. *Lane 1*, molecular mass standards. *Lane 2*, membrane fraction of nontransfected V79 cells. *Lane 3*, UGT1*6. *Lane 4*, R52A. *Lane 5*, H54A. *Lane 6*, H54Q.

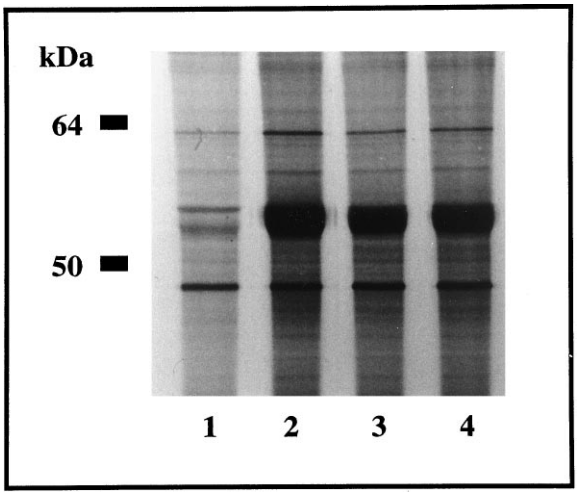

Fig. 3. ^{35}S-Methionine labeling and immunoprecipitation of UGT1*6 and H54A and H54Q mutants expressed in V79 cells. Recombinant proteins were radiolabeled as described in Materials and Methods before immunoprecipitation with nonspecific anti-UGT antibodies performed on cell lysates. *Lane 1*, nontransfected V79 cells. *Lane 2*, H54Q. *Lane 3*, UGT1*6. *Lane 4*, H54A.

Catalytic activities. To determine whether the His54 and Arg52 residues of UGT1*6 play a catalytic role, the influence of the mutations on the catalytic properties of UGT1*6 was investigated (Table 2). The kinetic constants of the mutants for glucuronidation of the standard phenolic substrate 4-MU were calculated and compared with those of the wild-type protein. The mutation of His54 and Arg52 strongly reduced the catalytic activity of UGT1*6. The most striking change was observed for the replacement of His54 with glutamine, which led to a complete loss of detectable enzyme activity. In addition, at pH 7.4, the V_{max} values of the substrate 4-MU or of the donor substrate UDP-glucuronic acid for the H54A and R52A mutants were reduced 4–5-fold

TABLE 2

Kinetic constants of mutant and wild-type UGT1*6 for the glucuronidation of 4-MU

Values are the average and standard deviation from bisubstrate kinetic analyses carried out in two independent preparations from cells of each kind of transfectant.

Enzyme	V_{max}	UDP-glucuronic acid		4-MU	
		K	*K'*	*K*	*K'*
	nmol/min/mg	*mM*			
UGT1*6	2.90 ± 1.4	0.11 ± 0.09	0.067 ± 0.01	0.22 ± 0.11	0.17 ± 0.08
H54A	0.70 ± 0.18	0.082 ± 0.016	0.15 ± 0.11	0.16 ± 0.07	0.23 ± 0.01
R52A	0.60 ± 0.20	0.13 ± 0.06	0.11 ± 0.006	0.22 ± 0.12	0.16 ± 0.06

compared with the wild-type enzyme (Table 2). These results indicated that His54 and Arg52 were important for the activity of UGT1*6. In contrast, the dissociation constant values of these mutants for the two substrates 4-MU and UDP-glucuronic acid were almost identical to those of the wild-type enzyme, indicating that the mutations did not provoke major changes in the affinity of the enzyme for the two substrates. The inhibition of glucuronidation of 4-MU by wild-type and mutated enzymes was compared using several representative molecules known to inhibit glucuronidation (competitive inhibitors, transition-state analog inhibitors) (Table 3). The IC_{50} values of inhibitors for the wild-type UGT1*6 were not significantly different from those obtained with H54A and R52A, confirming that H54A and R52A did not greatly alter the geometry of the active site.

Chemical modification. The glucuronidation rate of 4-MU was decreased as a function of time and concentration of 2,3-butanedione (Fig. 4, A and B). Plotted on a semilogarithmic scale, the residual activity as a function of time of inactivation showed a time- and dose-dependent inactivation of the enzyme by the reagent, which indicated that the inactivation followed pseudo-first-order kinetics (Fig. 4C). The reaction order (n) determined from plots of the logarithm of the apparent inactivation constants versus the logarithm of the concentration of inactivators was close to 1 for both UGT1*6 and the mutant R52A (Table 4).

To investigate the possible catalytic role of His54, the irreversible inhibition induced by the histidine-specific reagent diethyl pyrocarbonate was studied on the mutant H54A and compared with the wild-type UGT1*6. The results presented in Table 4 indicate that the inactivation profile was very similar, with correspondingly close second-order inactivation constants k_i' calculated from the slopes of the curves.

TABLE 3

IC_{50} values of transition-state analogs for wild-type and mutant UGT1*6

Inhibitor	IC_{50}		
	UGT1*6	H54A	R52A
		μM	
7,7,7-Triphenylheptanoic acid	46	31	46
PP 37	332	314	267
PP 50A	82	123	N.D.
PP 50B	127	145	80
PP 55A	260	230	314
PP 55B	168	213	170

PP 37, 5′-*O*-[[(2-decanoylamino-3-hydroxy-3-phenyl-propyloxycarbonyl)amino]sulfonyl]uridine; PP 50A and 50B, L- and D-, 5′-*O*[[(2-decanoylamino-3-phenyl-propyloxycarbonyl)amino]sulfonyl]-2′,3′-*O*-isopropylideneuridine; PP 55A and 55B, L- and D-5′-*O*-[[(2-decanoylamino-3-phenyl-propyloxycarbonyl)amino]sulfonyl]uridine. N.D., not determined.

Optimal pH and thermostability. Optimal pH for the glucuronidation of 4-MU by UGT1*6 was 7.4 when evaluated with Tris or MES buffer at a constant ionic strength. This value is in agreement with the involvement of a nonprotonated histidine residue with pK 6.1, as suggested previously (10). For the mutants H54A and R52A, the pH profiles were very similar to those observed for UGT1*6 (Fig. 5), with optimal pH 7.4, suggesting that an ionizable essential catalytic residue was apparently not modified by the mutations.

The thermostability of the mutants H54A and R52A was compared with that of the wild-type UGT1*6. For this purpose, membranes were incubated at 50° for various periods of time. The results (Fig. 6) show that the three enzymes were completely denatured after 35 min with no obvious differences between UGT1*6 and the mutants. The first-order inactivation constant k was 1.08 min^{-1} and similar for UGT1*6 and the mutants.

Discussion

The chemical modification of enzymes by amino acid-specific reagents is a powerful approach to investigation of the potential role of such amino acid residues in the structure of the protein or in the mechanism of the catalytic process. The mutagenesis of the conserved residues is also an efficient way to determine the key amino acids. The important role of UGT1*6 in the metabolism of drugs and carcinogens and its endogenous expression in human liver and kidney (19) prompted us to investigate the molecular basis of the glucuronidation reaction mediated by this isoform.

We recently reported the potent and irreversible inhibition of 4-MU glucuronidation on treatment of the recombinant human liver UGT1*6 with diethyl pyrocarbonate and several carbodiimides (10, 11). These studies indicated that both histidine and carboxylic acid (aspartic acid or glutamic acid) residues were implicated in a general acid-base catalysis for the glucuronidation of phenolic compounds. In addition, interactions of azidonucleotide photoaffinity probes with two peptide domains of UGT2B4 expressed in *Escherichia coli* suggested that a positively charged arginine residue located in the amino-terminal portion of the protein could be involved in the binding of UDP-glucuronic acid to the protein via an electrostatic bond with the carboxylate moiety of the cosubstrate (24). To further examine the potential role of arginine, we studied the inactivation induced by the dicarbonyl reagent 2,3-butanedione. Kinetic analysis of the irreversible inhibition strongly implicated a crucial arginine residue in the glucuronidation reaction. The presence of such an arginine has been suggested previously by Zakim *et al.* (12) in studies of a purified pig liver UGT that is active toward phenols.

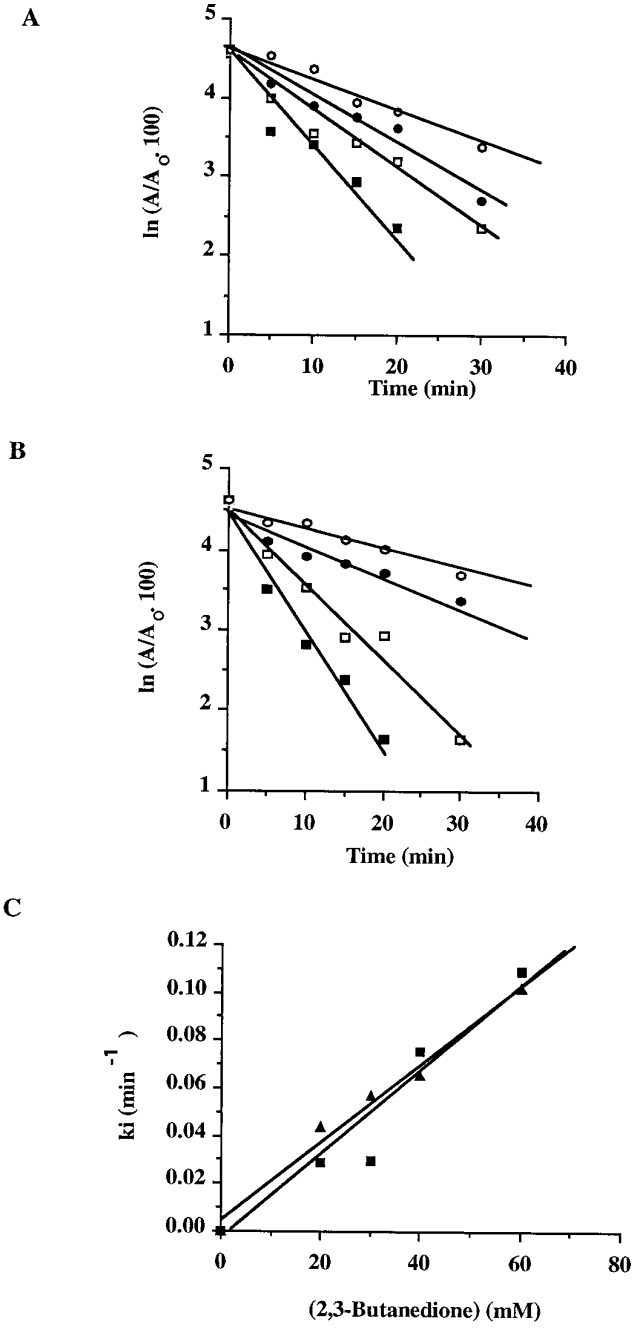

Fig. 4. Inactivation of the human liver recombinant UGT1*6 (A) and R52A mutant (B) by 2,3-butanedione. Inactivation was carried out by incubation of various concentrations of 2,3-butanedione (○, 20 mM; ●, 30 mM; ▲, 40 mM; ■, 60 mM) with the membrane proteins in 125 mM Tris·HCl buffer, pH 7.4, and 20 mM $MgCl_2$ in the dark. Inactivation was quenched at the indicated times, and the residual activity A/A_0 was determined. C, Plot of the pseudo-first-order inactivation rate constants as a function of 2,3-butanedione concentration (▲, UGT1*6; ■, R52A).

TABLE 4

Kinetic constants of the irreversible inactivation of the human wild-type and mutant UGT1*6 by 2,3-butanedione and diethyl pyrocarbonate

The enzymes were inactivated by 2,3-butanedione, as indicated in the legend of Fig. 4, or by diethyl pyrocarbonate, as previously reported (10). The second-order inactivation rate constant (k_i') for 2,3-butanedione or for diethyl pyrocarbonate was calculated from the slope of the plot of the pseudo-first-order inactivation rate constants (k_i) as a function of inhibitor concentration. The inactivation reaction order (n) with respect to inhibitor concentration [I] was calculated using the equation $\log k_i = n \cdot \log[I] + \log k_i'$.

Enzyme	2,3-Butanedione		Diethyl pyrocarbonate	
	k_i'	n	k_i'	n
	$M^{-1} \cdot min^{-1}$		$M^{-1} \cdot min^{-1}$	
UGT1*6	1.87	0.97	2645	1.3
H54A	N.D.	N.D.	2136	0.86
R52A	1.64	1.10	N.D.	N.D.

N.D., not determined (not applicable).

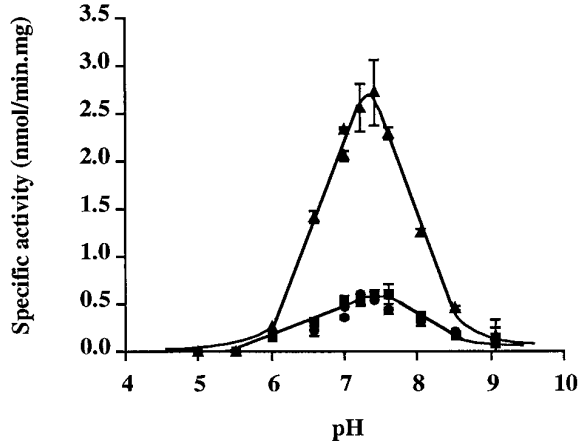

Fig. 5. pH dependence of the glucuronidation of 4-MU by UGT1*6 (▲) and the mutants H54A (●) and R52A (■). The activity was measured with 125 mM MES buffer and 20 mM $MgCl_2$ for pH 5.0–7.0 and with 125 mM Tris·HCl buffer and 20 mM $MgCl_2$ for pH 7.0–9.1. Values are mean ± standard deviation of three determinations of two independent experiments.

Histidine is a versatile amino acid that can interact with other residues through mechanisms mediated by hydrophobicity, electric charge, and hydrogen bonding. To investigate further the possible role played by a histidine residue and to identify its location within the primary sequence of UGT1*6, site-directed mutagenesis was performed. The choice of the candidate amino acid was based on sequence comparison of UGTs, which revealed the presence of a hydrophobic consensus sequence LX2-*R52*-G-*H54*-X3-V-L in the amino-terminal part of the proteins. His54 and Arg52 were mutated, and the corresponding cDNAs were stably expressed in V79 cells through use of a technique similar to that described by Fournel-Gigleux *et al.* (13). This approach has been found to be very efficient and to provide large amounts of active proteins.

His54 and Arg52 were replaced by alanine, which presents a nonreactive methyl group in its side chain. These changes test the effect of size and the lipophilicity of this moiety on the structure and activity of UGT1*6. To examine the possible role played by nitrogen atoms associated to an imidazole ring as well as the bulkiness of the side chain, His54 was mutated into glutamine.

Western blots and immunoprecipitation studies using an antibody raised against the full-length sequence of a rat liver UGT that cross-reacts with human UGT1*6 showed that all mutants were expressed at levels similar to those of the wild-type protein. Thus, the decrease in V_{max} values found for the mutants was not due to differences in the expression level of the recombinant UGT proteins. Indeed, glucuronidation of 4-MU was strongly impaired in the H54A and R52A

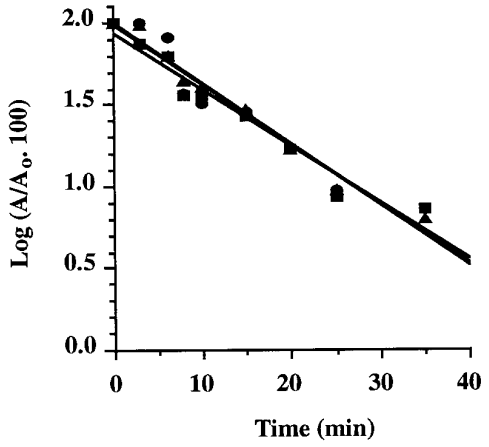

Fig. 6. Thermal denaturation of UGT1*6 and H54A and R52A mutants. The proteins were incubated at 50° for 1–35 min in 125 mM Tris·HCl buffer, pH 7.4, and 20 mM $MgCl_2$, and the glucuronidation of 4-MU was monitored as described in Materials and Methods. ▲, UGT1*6; ●, H54A; ■, R52A.

mutants and was even totally abolished in H54Q. A detailed analysis of the kinetics of the 4-MU glucuronidation indicated that the V_{max} values were markedly affected by the mutation, but the dissociation constants of the different enzyme/substrate complexes were essentially unchanged. These results indicated that the mutated amino acids are not directly involved in the binding of the substrates but are probably necessary for maintenance of a proper conformation compatible with normal activity.

The H54Q mutant was totally inactive and was not recognized by a specific antibody raised against the amino-terminal end of UGT1*6. The amino-terminal half of UGTs, which has a primary sequence that differs among isoforms, is believed to interact with the aglycone. It is likely that the loss of activity was a consequence of a dramatic change in conformation of an important part of the protein after substitution of the histidine residue by glutamine. A similar situation has been recently reported with the human cytochrome P450 3A (25), for which a valine residue was found to be critical for antibody recognition. When compared with the other mutant in which histidine was replaced by alanine, the larger side chain with electronegative atoms of glutamine could induce a significant modification of the UGT structure, causing the loss of both glucuronidation activity and epitope folding and recognition, whereas the methyl group of alanine could fit within a protein that retained some activity, although the activity was reduced. Indeed, replacement of histidine or arginine by alanine did not greatly affect the catalytic properties of UGT1*6. The mutants exhibited a similar optimal pH and were also similarly susceptible to heat denaturation and inhibition. A powerful competitive inhibitor of bilirubin UGT (22), 7,7,7-triphenylheptanoic acid, inhibited both UGT1*6 and mutants to similar extents. The same situation occurred with the transition-state analog inhibitors of UGT1*6 (23), which are believed to interact at both the 4-MU and UDP-glucuronic acid binding sites. These inhibitors also failed to discriminate between the mutants and UGT1*6. These results indicate that the peptide domains that interact with both 4-MU and UDP-glucuronic acid were not affected by the mutations. Based on these results and on the assumption that the essential histidine residue is not mainly in the nonionized form (pK_a 6.1) at optimal pH (7.4), it is likely that the main contribution of His54 to the protein structure is related to the bulkiness and the geometry of the imidazole side chain rather than to charge and hydrogen bonding. Similarly, ionization of the Arg52 residue was not a prerequisite for enzyme activity because its replacement by alanine only decreased the glucuronidation rate, without affecting the dissociation constants of the various enzyme/substrate complexes.

The susceptibility of the mutants H54A and R52A to inactivation by diethyl pyrocarbonate and 2,3-butanedione, respectively, with second-order inactivation constants k_i' in the same range as those determined for UGT1*6, suggests that both histidine and arginine residues are indeed implicated in the glucuronidation reaction but are located at different positions within the protein structure. Nevertheless, the results of the current study clearly show the importance of His54 and Arg52 for the functional and structural integrity of UGT1*6. These two conserved residues are essential to maintenance of the tertiary structure of the active site of UGT1*6. Arg52 and His54 are positioned at the beginning of the amino-terminal end, which is believed to contain a peptide domain that interacts with the aglycone and confers protein specificity. It is likely therefore that any change within this important region could affect the glucuronidation activity of the enzyme.

A complementary strategy to identify the critical amino acid residues has been developed for the isoforms of bilirubin UGT that are encoded by the same *UGT1* gene locus as UGT1*6. Mutations that lead to the complete absence of activity have been detected on the corresponding genes that cause severe disease in humans (Crigler-Najjar type I and type II syndromes) as well as in the Gunn rat. The defects were heterogeneous and affect all five exons of the gene. Ciotti *et al*. (26) reported, in a Crigler-Najjar type I patient, that the substitution of Arg92 for glycine abolished a conserved, structurally important di-glycine. On the other hand, Crigler-Najjar syndrome type II was associated with several point mutations resulting in the change of glycine to arginine and of tyrosine to aspartic acid at amino acid positions 71 and 486 of the UGT1A protein and of leucine to proline and of tyrosine to aspartic acid at amino acid positions 132 and 487, respectively (27). Compared with the results obtained with UGT1*6, these observations indicate the importance of other amino acids located in different positions along the UGT sequence.

In conclusion, we demonstrated that the strictly conserved, hydrophobic amino-terminal sequence contains Arg52 and His54 residues that are critical for the function and structural integrity of the human liver UGT1*6, although they are not directly involved in catalysis or substrate binding. Work is in progress to find other important residues in the conserved peptide domains of this protein.

Acknowledgments

We thank H. Leh for his help in the creation of the R52A mutant. The technical assistance of K. Lozach is gratefully acknowledged.

References

1. Dutton, G. J. *Glucuronic Acid Free and Combined* (G. J. Dutton, ed.). Academic Press, New York/London (1966).

2. Burchell, B., D. W. Nebert, D. R. Nelson, K. W. Bock, T. Iyanagi, P. L. M. Jansen, D. Lancet, G. J. Mulder, J. Roy Chowdhury, G. Siest, T. Tephly, and P. I. Mackenzie. The UDP-glucuronosyltransferase gene superfamily: suggested nomenclature based on evolutionary divergence. *DNA Cell Biol.* **10:**487–494 (1991).
3. Magdalou, J., Y. Hochman, and D. Zakim. Factors modulating the catalytic specificity of a pure form of UDP-glucuronosyltransferase. *J. Biol. Chem.* **257:**13624–13629 (1982).
4. Burchell, B., and M. W. H. Coughtrie. UDP-glucuronosyltransferases. *Pharmacol. Ther.* **43:**261–289 (1989).
5. Nebert, D. W. Proposed role of drug-metabolizing enzymes: regulation of steady state levels of the ligands that effect growth, homeostasis, differentiation, and neuroendocrine functions. *Mol. Endocrinol.* **5:**1203–1214 (1991).
6. Ebner, T., and B. Burchell. Substrate specificities of two stably expressed human liver UDP-glucuronosyltransferase. *Drug Metab. Dispos.* **21:**50–55 (1993).
7. Ritter, J. K., F. Chen, Y. Y. Sheen, H. M. Tran, S. Kimura, M. T. Yeatman, and I. S. Owens. Two human liver cDNAs encode UDP-glucuronosyltransferases with 2 log differences in activity toward parallel substrates including hyodeoxycholic acid and certain estrogen derivatives. *J. Biol. Chem.* **267:**3257–3261 (1992).
8. Mackenzie, P. I. Expression of chimeric cDNAs in cell culture defines a region of UDP-glucuronosyltransferase involved in substrates selection. *J. Biol. Chem.* **265:**3432–3435 (1990).
9. Bock, K. W. Roles of UDP-glucuronosyltransferases in chemical carcinogenesis. *Crit. Rev. Biochem. Mol. Biol.* **26:**129–150 (1991).
10. Battaglia, E., M. Pritchard, M. Ouzzine, S. Fournel-Gigleux, A. Radominska, G. Siest, and J. Magdalou. Chemical modification of human UDP-glucuronosyltransferase UGT1*6 by diethyl pyrocarbonate: possible involvement of a histidine residue in the catalytic process. *Arch. Biochem. Biophys.* **309:**266–272 (1994).
11. Battaglia, E., C. Senay, S. Fournel-Gigleux, R. Herber, G. Siest, and J. Magdalou. The chemical modification of human UDP-glucuronosyltransferase UGT1*6 reveals the involvement of carboxyl group in catalysis. *FEBS Lett.* **346:**146–150 (1994).
12. Zakim, D., Y. Hochman, and W. C. Keney. Evidence for an active site arginine in UDP-glucuronosyltransferase. *J. Biol. Chem.* **263:**5164–5169 (1983).
13. Fournel-Gigleux, S., L. Sutherland, N. Sabolovic, B. Burchell, J. Magdalou, and G. Siest. Stable expression of two human liver UDP-glucuronosyltransferase cDNA in V79 cell culture. *Mol. Pharmacol.* **39:** 177–183 (1991).
14. Harding, D., S. Fournel-Gigleux, M. R. Jackson, and B. Burchell. Cloning and substrate specificity of human phenol UDP-glucuronosyltransferase expressed in COS-7 cells. *Proc. Natl. Acad. Sci. USA* **85:**8381–8385 (1988).
15. Sarkar, G., and S. S. Sommer. The megaprimer method of site-directed mutagenesis. *Biotechniques* **8:**404–407 (1990).
16. Bradford, M. M. Rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:**248–254 (1976).
17. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227:**680–685 (1970).
18. Coughtrie, M., B. Burchell, J. E. A. Leakey, and R. Hume. The inadequacy of perinatal glucuronidation: immunoblot analysis of the developmental expression of individual UDP-glucuronosyltransferase isoenzymes in rat and human liver microsomes. *Mol. Pharmacol.* **34:**729–735 (1988).
19. Ouzzine, M., T. Pillot, S. Fournel-Gigleux, J. Magdalou, B. Burchell, and G. Siest. Expression and role of the human liver UDP-glucuronosyltransferase UGT1*6 analysed by specific antibodies raised against a hybrid protein produced in *Escherichia coli*. *Arch. Biochem. Biophys.* **310:**196–204 (1994).
20. Lilienblum, W., A. K. Walli, and K. W. Bock. Differential induction of rat liver microsomal UDP-glucuronosyltransferase activities by various inducing agents. *Biochem. Pharmacol.* **31:**907–913 (1982).
21. Segel, I. H. *Enzyme Kinetics*. John Wiley & Sons, New York, 274–284 (1975).
22. Fournel-Gigleux, S., S. R. P Shepherd, M. C. Carré, B. Burchell, G. Siest, and P. Caubère. Novel inhibitors and substrates of bilirubin: UDP-glucuronosyltransferase arylalkylcarboxylic acids. *Eur. J. Biochem.* **183:** 653–659 (1989).
23. Battaglia, E., A. Elas, R. R. Draket, P. Paul, S. Treat, J. Magdalou, S. Fournel-Gigleux, T. Pillot, M. Ouzzine, G. Siest, G. Vergoten, R. Lester, and A. Radominska. Characterization of a new class of inhibitors of a recombinant human liver UDP-glucuronosyltransferase, UGT1*6. *Biochim. Biophys. Acta* **1243:**9–14 (1995).
24. Pillot, T., M. Ouzzine, S. Fournel-Gigleux, C. Lafaurie, D. Tebbi, S. Treat, A. Radominska, R. Lester, G. Siest, and J. Magdalou. Determination of the human liver UDP-glucuronosyltransferase 2B4 domains involved in the binding of UDP-glucuronic acid using photoaffinity labeling of fusion proteins. *Biochem. Biophys. Res. Commun.* **197:**785–791 (1993).
25. Leeder, J. S., A. Gaedigk, X. Lu, and V. A. Cook. Epitope mapping studies with human anti-cytochrome P450 3A antibodies. *Mol. Pharmacol.* **49:** 234–243 (1996).
26. Ciotti, M., M. T. Yeatman, R. J. Sokol, and I. S. Owens. Altered coding for a strictly conserved di-glycine in the major bilirubin UDP-glucuronosyltransferase of a Crigler-Najjar type I patient. *J. Biol. Chem.* **270:**3283–3291 (1995).
27. Aono, S., Y. Yamada., H. Keino., N. Hanada, T. Nakagawa, Y. Sasaoka, T. Yazawa, H. Sato, and O. Koiwai. Identification of defect in the genes for bilirubin UDP-glucuronosyltransferase with Crigler-Najjar syndrome type II. *Biochem. Biophys. Res. Commun.* **197:**1239–1244 (1993).

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