AMINO ACID RESIDUE ILE211 IS ESSENTIAL FOR THE ENZYMATIC ACTIVITY OF HUMAN UDP-GLUCURONOSYLTRANSFERASE 1A10 (UGT1A10)

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ABSTRACT:

Conjugation of exogenous and endogenous compounds by uridine diphosphoglucuronosyltransferases (UGTs) is a pathway catalyzing the transfer of a glucuronic acid molecule from UDP glucuronic acid to lipophilic aglycones, which become more polar and more easily excretable in the bile or urine. UGTs are divided into two major families, UGT1 and UGT2. The isof orm UGT1A10, along with UGT1A7 and UGT1A8, is expressed exclusively in extrahepatic tissues, notably in the gastrointestinal tract. Here, we report the isolation of a mutant clone of the human UGT1A10, at position 211 of the protein, where a threonine residue replaces an isoleucine residue (allele Thr211). Because the isoleucine is conserved among many UGT1A isoforms, we proceeded to the analysis of the activity of the wild-type UGT1A10 (T211I) and compared it with that of the variant enzyme (I211T*). In vitro assays with microsomal extracts from stably expressing human embryonic kidney 293 (HEK293) cells showed that the mutant enzyme lost all detectable activity toward major substrates, which demonstrate that the residue isoleucine at position 211 is essential for UGT1A10 activity. Mutant UGT1A10 (I211T*) also lost all detectable activity toward mycophenolic acid. Genomic DNA from 103 unrelated individuals was sequenced for this mutation, and two heterozygous genotypes were detected for this mutation (frequency: 2 per 100 individuals). Because UGT1A10 appears to be expressed in all gastrointestinal tissues and is active toward a wide range of substrates, lack of activity of this isoform may have an impact on individual glucuronidation efficiency.

The glucuronidation of exogenous and endogenous compounds is catalyzed by a family of membrane-bound enzymes, the UDP-glucuronosyltransferases (UGTs), which are located in the lumen of the endoplasmic reticulum. This phase II mechanism of cellular detoxification corresponds to the transfer of a glucuronic acid group from uridine diphosphoglucuronic acid (UDPGA) to a variety of substrates, including exogenous and endogenous compounds (Mulder, 1992). The glucuronidated compounds are more polar, less toxic and more easily excretable from the body through the bile or urine. Hence, it is well accepted that glucuronidation generally terminates the biological and pharmacological activity of drugs and antibiotics (Mulder, 1992).

To date, over 60 different UGT enzymes have been isolated in various mammalian species (Mackenzie et al., 1997). UGT enzymes are classified into two major families, UGT1 and UGT2, on the basis of their amino acid sequence homology (Mackenzie et al., 1997). In humans, the UGT1 gene family is located on chromosome 2q37 (Harding et al., 1990; van Es et al., 1993; Clarke et al., 1997). UGT1A enzymes are all encoded by a complex gene consisting in 17 exons; different isoforms are generated by 13 alternative splicings of different exons 1 in combination with the four common exons 2 to 5 (Owens and Ritter, 1995; Gong et al., 2001). The physiological importance of UGT1A enzymes is demonstrated by mutations in UGT1A1 enzymes, which cause several diseases with varying degrees of hyperbilirubinemia (Crigler-Najjar syndrome types I and II, and Gilbert’s disease). These UGT1A1 mutations also provoke lowered or absent levels of bilirubin glucuronidation that lead to toxic levels of substrate accumulation in the body.

UGT1A10, along with UGT1A7, UGT1A8, and UGT1A9, is classified primarily as being active toward bulky phenols. Its activity on mycophenolic acid, simple phenols, anthraquinones/flavones, coumarins, secondary amines, heterocyclic amines, and estrogens has also been reported (Mojarrabi and Mackenzie, 1997; Cheng et al., 1999; Tukey and Strassburg, 2000). This isoform is expressed exclusively in extrahepatic tissues, namely in the esophagus, stomach, intestine, and colon (Mojarrabi and Mackenzie, 1997; Strassburg et al., 1997a, 1998a,b). It was also demonstrated that UGT1A10 conjugates 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, which has been involved in the etiology of colon cancer in humans (Nowell et al., 1999; Radominska-Pandya et al., 1999), and benz(a)pyrenes from tobacco (Elahi et al., 2003).

In addition to the UGT1A1 gene, the presence of polymorphic expression of other UGT1A members has also been demonstrated. Many allelic polymorphisms have been reported to date for the human UGT1A6 (Ito et al., 2002; Miners et al., 2002), UGT1A7 (Miners et al., 2002; Strassburg et al., 2002; Vogel et al., 2002), UGT1A8 (Huang et al., 2002), and UGT1A10 (Elahi et al., 2003; Jinno et al., 2003). Functional polymorphisms affecting UGT1A1, UGT1A3, and

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UGT1A6 activity in human gastric epithelium have also been observed (Strassburg et al., 1998b). These variations correlate with interindividual glucuronidation rates of exogenous compounds (Strassburg et al., 2000), thus suggesting that polymorphic expression of UGTs in the intestine could be responsible for differential conjugation of carcinogens such as benzo[a]pyrene, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, and acetylaminofluorene (Strassburg et al., 2000).

In the present study, we report a single mutation in the isoform UGT1A10 responsible for the loss of all detectable conjugation activity. We found that the isoleucine residue at position 211, which is conserved among human UGT1A, is crucial to this isoform since enzyme activity is completely lost when this residue is replaced by a threonine. This mutation is located in the putative portion responsible for aglycone binding. To further analyze this mutation, genomic DNA from 103 unrelated patients was sequenced, and we were able to detect two individuals who were heterozygous for this mutation (>1% polymorphism).

Materials and Methods

Materials. UDP-glucuronic acid and all aglycons were obtained from Sigma-Aldrich (St. Louis, MO) and Valeant Pharmaceuticals (Montreal, Quebec, Canada). Estrone was purchased from Steraloids, Inc. (Wilton, NH). [14C]-UDP-glucuronic acid (285 mCi/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Geneticin (G418) was purchased from Invitrogen (Carlsbad, CA). Bacterial S-HCI was purchased from ICN Pharmaceuticals Inc. and Exgen 500 was purchased from MBI Fermentas (Burlington, Ontario, Canada). Protein assay reagents were obtained from Bio-Rad (Hercules, CA). Restriction enzymes and other molecular biology reagents were obtained from Amersham Biosciences Inc. (Piscataway, NJ). Invitrogen, Stratagene (La Jolla, CA), and Roche Diagnostics (Indianapolis, IN). PfiTurbo DNA polymerase was purchased from Stratagene. Theramo Sequenase radiolabeled Terminator Cycle Sequencing Kit was obtained from USB (Cleveland, OH), and [3P]dTGTP, ddATP, ddCTP, and ddTTP (500 Ci/mmol) were purchased from Amersham Biosciences Inc. (Baie D’urfe, Quebec, Canada). HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA). QuickChange Site-Directed Mutagenesis Kit was obtained from Stratagene. Genomic DNA from 103 unrelated Caucasian patients was kindly provided by Drs. Jacques Simard and Vincent Raymond (Oncology and Molecular Endocrinology Research Center, Laval University, Medical Research Center (CHUL) and Laval University, Quebec, Canada).

Isolation of Human UGT1A10 Complementary DNA (cDNA). Isolation of the human UGT1A10 cDNA clones was achieved using a reverse transcriptase-polymerase chain reaction (PCR) technique as previously described (Beaulieu et al., 1996) with total RNA from human small intestine (BD Biosciences Clontech, Palo Alto, CA). The antisense primer used for the reverse transcriptase reaction was a 23-base pair oligo(dT). The PCR reaction was carried out by adding 100 pmol of the sense primer 5'-CAGTTCTCTCATCCTTGCGCCAGG-3', which is specific to exon 1 of human UGT1A10, and 100 pmol of the antisense primer 5'-AGAGCTCGATCTCAATGGGTCTTGTGAGG-3'. The identification of the PCR product was verified by direct sequencing (Ruehaim et al., 1991). The cDNA fragment of UGT1A10 was inserted in pBK-CMV vector via XhoI and PstI restriction sites.

Preparation of Microsomal Fractions and Glucuronidation Assay. HEK293 cells expressing wild-type human UGT1A10 or variant UGT1A10 were harvested, frozen twice in liquid nitrogen, and homogenized in phosphate buffer, pH 7.4, containing 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 2.5 μg/ml pepstatin, and 0.5 μg/ml leupeptin, using a Potter homogenizer (Glass-col, Terre Haute, IN) type homogenizer with a Teflon pestle at 4°C. The homogenates were centrifuged at 12,000g for 20 min to remove nuclei, unbroken cells, and mitochondria. The pellet was discarded and the supernatant was centrifuged at 105,000g for 60 min to obtain the microsomal pellet, which was resuspended in homogenization buffer at 10 mg of protein/ml and used for their stable expression in HEK293 cells.

Results

Expression of Wild-Type UGT1A10 and UGT1A10/I211T in HEK293 Cells. Eight positive UGT1A10 cDNA clones were obtained by the reverse transcriptase-PCR technique from commercially available human small intestine RNA. The cDNA clones were sequenced using specific UGT oligonucleotides. Using Xhol and PstI restriction sites, the wild-type and the mutant UGT1A10 cDNAs were inserted in the expression vector pcDNA3, and used for their stable expression in HEK293 cells.

Stable Expression of the Human Wild-Type and Mutant UGT1A10 Proteins. HEK293 cells were grown in Dulbecco’s modified Eagle’s medium containing 4.5 g/l glucose, 10 mM HEPES, 110 μg/ml sodium pyruvate, 100 IU of penicillin/ml, 100 μg/ml streptomycin, and 10% fetal bovine serum in a humidified incubator, with 5% CO2 at 37°C. Five micrograms of pcDNA3-UGT1A10 T211I and pcDNA3-UGT1A10 I211T were used to transfect HEK293 cells using Exgen 500 according to the manufacturer’s instructions (MBI Fermentas). Forty-eight hours post-transfection, stable transfectants were selected in media containing 10 μg/ml blasticidin S HCl. The clones demonstrating the highest activity for each enzyme were used for glucuronidation assays.

Western Blot Analysis. To ascertain the expression of the wild-type and mutant UGT1A10 proteins, 10 μg of microsomal protein from HEK293 cells stably expressing human wild-type UGT1A10 or mutant UGT1A10 were separated by 10% SDS-polyacrylamide gel electrophoresis. The gel was transferred onto a nitrocellulose membrane and probed with the antihuman UGT1A1 common carboxyl terminus region antisemur RC-71 (1:2000 dilution) (Barbier et al., 2001). To show the relative amount of proteins loaded in each lane of the Western blot, the same blot was subsequently probed with an anti-calnexin CT antibody (StressGen Biotechnologies, Victoria, British Columbia, Canada; 1:1000 dilution). A donkey antirabbit IgG antibody conjugated with horseradish peroxidase (Amersham Biosciences Inc.) was used as the second antibody, and the resulting immunocomplexes were visualized using an enhanced chemiluminescence kit (Renaissance Chemiluminescence Dupont, Markham, Quebec, Canada), following the manufacturer’s instructions, and exposed on Hyperfilm for 2 min (Eastman Kodak, Rochester, NY) for quantification by the PhosphorImager using Imagequant software (Amersham Biosciences Inc.).

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To screen the substrates, 155 μM [14C]UDPGA, 1 mM unlabeled UDPGA, 200 μM aglycone, and 25 μg of proteins from microsomal preparations in 50 mM Tris-HCl (pH 7.55) were mixed with 10 mM MgCl2 and 100 μM phosphatidylcholine in a final volume of 100 μl. A preliminary time course, between 0 and 12 h, using 4-methylumbelliferone as a substrate for wild-type UGT1A10, was used to determine the linear portion of enzymatic activity. All subsequent assays were performed for 4 h at 37°C and were terminated by adding 100 μl of methanol. Samples were centrifuged at 13,500 rpm for 5 min to remove precipitated proteins. One hundred microliters of the aqueous phase were applied onto thin-layer chromatography plates (0.25-mm-thick silica gel; Whatman, Maidstone, UK) and chromatographed in a solvent of butanol, acetic acid, ammonium hydroxide, and water (7.5:1:8.0:15.6). The thin-layer chromatography plates were exposed for 24 h, and the extent of glucuronidation was assessed using a PhosphorImager (Amersham Biosciences Inc.). The glucuronidation activity is illustrated in picomoles per milligram of protein per minute.
and exhibited a cytidine base at position 632 instead of a thymidine, as previously reported for the UGT1A10 sequence (GenBank accession number U89508) (Strassburg et al., 1997b) (Table 1). Interestingly, other active members of the UGT1A subfamily, namely 1A7, 1A8, and 1A9, also possess a thymidine base at this position. The cytidine in place of a thymidine changes the amino acid residue at position 211 of UGT1A10 from isoleucine to threonine. By site-directed mutagenesis of mutant UGT1A10 to replace the cytidine by a thymidine, it was possible to obtain the wild-type UGT1A10 (T211I). Then, the two cDNAs were inserted in the expression vector pc-DNA3 to characterize the enzymatic properties in stably transfected HEK293 cells. Microsomal extracts were prepared for each enzyme, and their expression levels were quantified by Western blot analysis. Each of the cDNAs was translated in a full-length protein of 530 amino acids (60 kDa), and the variant I211T* had an additional band at 77 kDa (Fig. 1). HEK293 cells that were not transfected did not express a transcript that reacts with the antibody.

### Activity of Wild-Type UGT1A10 and UGT1A10/I211T.

In vitro glucuronidation assays were performed with the major substrates previously reported for UGT1A10 (Strassburg et al., 1997a, 1998a; Mojarrabi and Mackenzie, 1998; Cheng et al., 1999). Since UGT1A10 conjugates estrogens, flavonoids, coumarins, anthraquinones, phe-nolic compounds, and carcinogens, we tested estrone, 4-methyllumelic acid, belliferone, 7-hydroxyflavone, 1-naphthol, eugenol, and myco-phenolic acid. The wild-type UGT1A10 was active on all these substrates, whereas the mutant isoform UGT1A10/I211T showed no detectable activity on all substrates tested (Fig. 2).

### Identification of a Polymorphic UGT1A10 Allele.

To determine whether the variant isoform I211T* and wild-type T211I are transcribed from two separate genes or whether they originate from a single polymorphic gene, PCR analyses were performed using a pair of specific oligonucleotides that amplified the region encompassing the nucleotides at codon 632. The analyses were performed using genomic DNA samples from 103 unrelated Caucasian patients. Of the patients studied, two heterozygous genotypes for the mutation at position 211 were detected (Fig. 3). Thus, the allelic frequency of the wild-type allele was 99% (mutant allele frequency 1%). Under the Hardy-Weinberg equilibrium, the probability of obtaining the wild-type homozygous genotype was 98 per 100 individuals, 2 per 100 individuals for the heterozygous genotype, and a rate of 0.01 per 100 individuals for the mutant homozygous genotype (Table 2).

### Discussion

Recent observations suggest that UGT1A10, which is expressed in the gastrointestinal tract, may be implicated in the inactivation of oral drugs and environmental pollutants. Thus, any alteration in UGT1A10 activity may influence the bioavailability of drugs or elimination of exogenous compounds. In this study, a cDNA for UGT1A10 was isolated from human small intestine RNA, which coded for a thre-noine residue instead of an isoleucine at position 211 of the protein. Interestingly, our data demonstrate that this amino acid substitution completely abolishes enzymatic activity. Thus, the isoleucine residue at position 211 is essential for the catalytic activity of the enzyme. Recent data reported by Jinno et al. (2003) indicated that a 50% reduction of UGT1A10 activity by the variant T202I could be obtained. To our knowledge, this is the first time that a complete inhibition by a mutant UGT1A10 is reported. In addition, by analyzing genomic DNA from 103 unrelated patients, we found two individuals carrying a heterozygous genotype for this mutation. The isoleucine residue, which is nonpolar and aliphatic, is conserved among 1A2, 1A7, 1A8, 1A9, 1A11, and 1A12 isoforms, notably in all UGT active on bulky phenols. Its replacement by a threonine, which is a small and neutral-polar molecule, suggests that the protein structure may differ from its original tridimensional form.

A large number of polymorphisms in the UGT1A gene locus have been reported, namely for UGT1A1, UGT1A6, UGT1A7, UGT1A8, and UGT1A10. For example, the glucuronidation of bilirubin by UGT1A1, which is an essential metabolic pathway in humans, is involved in inherited diseases of bilirubin metabolism (Ritter et al., 1991; 1992; Bosma et al., 1994; Monaghan et al., 1996; Beutler et al., 1998; Guillemette et al., 2000a). Three forms of inheritable unco-

### Table 1

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**Fig. 1.** Immunoblot analysis of HEK293 cells stably expressing wild-type and mutant human UGT1A10, using a specific UGT1A antibody.

Ten micrograms of microsomal proteins were isolated from stably transfected HEK293 cells. The wild-type (T211I) and mutant (I211T*) UGT1A10 proteins were separated on a 12% SDS-polyacrylamide gel electrophoresis gel and transferred onto a nitrocellulose filter and probed with the RC-71 antibody, which is specific to UGT1A proteins.
Gated hyperbilirubinemic diseases have been described: Crigler-Najjar syndrome type I, Crigler-Najjar syndrome type II, and Gilbert’s syndrome (Kadakol et al., 2000). These syndromes result from mutant UGT1A1 alleles or from UGT1A1 promoter polymorphisms and, to date, over 33 mutant UGT1A1 alleles have been identified (Tukey and Strassburg, 2000). The UGT1A6 has also been studied by site-directed mutagenesis, and it was shown that residues His54 and Arg52 are important for the function and the structure required for optimal catalytic efficiency of this UGT (Senay et al., 1997). More recently, six single-nucleotide polymorphisms were discovered in the first exon of the UGT1A7 gene, revealing a high structural heterogeneity at the UGT1A locus (Guillemette et al., 2000b). In the case of UGT1A8, which is expressed exclusively in extrahepatic tissues, two novel mutations were reported, creating three allelic variants of the wild-type UGT1A8*1 (A<sup>173</sup>C<sup>277</sup>): UGT1A8*1a (T<sup>255</sup>A>G), UGT1A8*2 (G<sup>173</sup>C<sup>277</sup>), and UGT1A8*3 (A<sup>173</sup>Y<sup>277</sup>). In comparison to UGT1A8*1a and UGT1A8*2, the isoform UGT1A8*3 (Y<sup>277</sup>) was completely inactive (Huang et al., 2002). Two mutations in UGT1A10 were reported by Jinno et al. (2003). The variants T202I and M59I were evaluated for their catalytic activity on 7-hydroxy-4-trifluoromethylcoumarin and estradiol. The activity of the variant M59I was similar to the wild type, whereas the activity of the T202I was reduced by 50%, which suggests that the lowered glucuronidation activity of this variant may affect the gastrointestinal glucuronidation of orally administrated chemicals and the enterohepatic circulation of biliary excreted metabolites. Detection of novel UGT1A10 polymorphisms and their association with orolaryngeal carcinoma risk were also recently reported by Elahi et al. (2003).

Cancers of the esophagus, stomach, bladder, liver, colon, lung, and pleura have been linked to chemical carcinogens inducing neoplastic transformations (Bock, 1991; Lai and Shields, 1999; Lutz, 1999), and it is believed that inactivation by UDP-glucuronosyltransferases may be important in this process by influencing the amount of available substrates (Lai and Shields, 1999). Tissue-specific and polymorphic regulation of UGT1A genes in the small intestine have been described and shown to represent molecular biological determinants that may contribute to interindividual differences in cancer susceptibility or extrahepatic drug metabolism in humans (Strassburg et al., 2000). Interestingly, down-regulation of UGT1A1 mRNA and microsomal activity has been observed in gastrointestinal tumors of the esophagus (Strassburg et al., 1999), stomach (Strassburg et al., 1998b), and liver and bile ducts (Strassburg et al., 1997a). It has also been observed that the UGT1A10 is expressed in gastric, esophageal, biliary, and colonic tissues (Strassburg et al., 2000). Thus, it is possible that compounds such as flavonoids, anthraquinones, coumarins, phenolic compounds, and benzo[a]pyrenes that are in part conjugated by UGT1A10 could be found in higher concentrations in individuals presenting the I<sub>211</sub>T* variant genotype of UGT1A10. For example, mycophenolate mofetil is the prodrug of mycophenolic acid, an immunosuppressive agent that is effective in the management of psoriasis and solid organ transplantation (Jones et al., 1998). Mycophenolic acid is conjugated by both UGT1A10 and UGT1A8 (Mojarrabi and Mackenzie, 1997; Cheng et al., 1999), and our data indicated that the variant I<sub>211</sub>T* had no activity toward mycophenolic acid. Although the frequency of the variant allele is low, a lack of UGT1A10 activity in these individuals could be partly responsible for adverse effects reported with the use of this compound.

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**Fig. 2.** Glucuronidation of eugenol, 1-naphthol, 4-methylumbelliferone (4-MU), 7-hydroxylflavone (7-OH-flavone), estrone, and mycophenolic acid (Myco.Ac) by wild type UGT1A10 (T<sub>211</sub>I) and mutant UGT1A10 (I<sub>211</sub>T<sup>*</sup>).

A, thin-layer chromatogram of glucuronidated compounds. B, glucuronidation formation expressed in pmol/mg of protein/min. Glucuronidation values represent the mean of duplicates of two experiments (±S.D.).

**Fig. 3.** Genomic PCR analysis of the UGT1A10 gene.

Genomic DNA sequence of the wild-type UGT1A10, heterozygous UGT1A10, and mutant UGT1A10. DNA sequence was performed on 103 unrelated Caucasian genomic samples.

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**TABLE 2**

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<th>Genotype</th>
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<td>Heterozygous</td>
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<td>Heterozygous</td>
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<td>Mutant homozygous</td>
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<td>Mutant homozygous</td>
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<td>Normal allele</td>
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<td>Normal allele</td>
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<td>Probabilities (under Hardy-Weinberg equilibrium)</td>
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<tr>
<td>Normal homozygous</td>
<td>98%</td>
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<tr>
<td>Mutant homozygous</td>
<td>0.01%</td>
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The sequencing of genomic DNA from 103 unrelated individuals indicates the relevance of 121IT* polymorphism. Frequencies of this mutation were in accordance with the Hardy-Weinberg equilibrium. Although the frequency is low, the possibility that this mutation may be found in some populations is present. Elahi et al. (2003) recently observed that the prevalence of the UGT1A10(240Met) variant was less than 0.01% in whites and blacks. Similarly, the prevalence of both the UGT1A10(139Lys) and UGT1A10(244Ile) variants was less than 0.01% in whites but it was significantly higher (0.04 and 0.05, respectively, P < 0.01) in blacks.

Although the frequency of the mutant allele was relatively low for UGT1A10, the importance of this mutation could possibly be extended to other UGT1A enzymes carrying a isoleucine at position 211 of the protein. Further studies are required to confirm this hypothesis.

References