Homodimerization of Human Bilirubin-Uridine-diphosphoglucuronate Glucuronosyltransferase-1 (UGT1A1) and Its Functional Implications*

Siddhartha S. Ghosh‡§, Baljit S. Sappal‡§, Ganjam V. Kalpana¶, Sung W. Lee‡§, Jayanta Roy Chowdhury‡§, and Namita Roy Chowdhury‡§

From the Departments of § Medicine and ¶ Molecular Genetics and the § Marion Bessin Liver Research Center, Albert Einstein College of Medicine, Bronx, New York 10461

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Genetic lesions of bilirubin-uridine-diphosphoglucuronate glucuronosyltransferase-1 (UGT1A1) completely or partially abolish hepatic bilirubin glucuronidation, causing Crigler-Najjar syndrome type 1 or 2, respectively. Clinical observations indicate that some mutant forms of human UGT1A1 (hUGT1A1) may be dominant-negative, suggesting their interaction with the wild-type enzyme. To evaluate intermolecular interaction of hUGT1A1, Gunn rat fibroblasts were stably transduced with hUGT1A1 cDNA. Gel permeation chromatography of solubilized microsomes suggested dimerization of hUGT1A1 in solution. Nearest-neighbor cross-linking analysis indicated that, within microsomal membranes, hUGT1A1 dimerized more efficiently at pH 7.4 than at pH 9. Two-hybrid analysis in yeast and mammalian systems demonstrated positive interaction of hUGT1A1 with itself, but not with another UGT isoform, human UGT1A6, which differs only in the N-terminal domain. Dimerization was abolished by deletion of the membrane-embedded helix from the N-terminal domain of hUGT1A1, but not by substitution of several individual amino acid residues or partial deletion of the C-terminal domain. A C12T7 substitution abolished UGT1A1 activity, but not its dimerization. Coexpression of mutagenized and wild-type hUGT1A1 in COS-7 cells showed that the mutant form markedly suppressed the catalytic activity of wild-type hUGT1A1. Homodimerization of hUGT1A1 may explain the dominant-negative effect of some mutant forms of the enzyme.

Ur dine-diphosphoglucuronate glucuronosyltransferases (UGTs)³ constitute a family of enzymes that detoxify numerous endogenous and exogenous substances by catalyzing the transfer of the glucuronic acid moiety of uridine diphosphoglucuronate to the aglycone substrates (1). Based on structural homology, UGTs are classified into several families and subfamilies (1, 2). UGT1A1, which mediates bilirubin glucuronidation (3), belongs to a subfamily that is expressed from the UGT1A locus at human chromosome 2q37 (4). The four exons (exons 2–5) located at the 3′-end of this locus are used in mRNAs for several UGT1A isoforms and encode the identical carboxyl-terminal halves of these enzymes (5). Upstream of these exons is a series of at least 12 unique exons, only one of which is used in the mRNA for a given isoform (5). The unique region exon encodes the amino-terminal domain of these enzymes, which imparts their aglycone substrate specificity (6). UGTs are concentrated in the endoplasmic reticulum (ER) (7). Except for a 21–26-amino acid carboxyl-terminal cytoplasmic tail, the enzymes are located within the ER lumen, predicting the need for transport of the sugar donor substrate, UDP-glucuronic acid, from the cytosol to the catalytic site within the ER lumen.

UGT1A1 is encoded by exons 1A1 and 2–5. UGT1A1-mediated glucuronidation is essential for efficient biliary excretion of bilirubin. Genetic lesions within the coding region of UGT1A1 can abolish or markedly reduce UGT activity toward bilirubin, resulting in Crigler-Najjar syndrome type 1 or 2, respectively (8), both of which are characterized by unconjugated hyperbilirubinemia (9). The inheritance of Crigler-Najjar syndrome types 1 and 2 generally follows an autosomal recessive pattern (9) because, in most heterozygous carriers, the wild-type enzyme expressed from the structurally normal allele is sufficient to keep plasma bilirubin concentrations within normal limits. However, the heterozygous carriers of certain human UGT1A1 (hUGT1A1) mutations exhibit mild to moderate hyperbilirubinemia, suggesting an autosomal dominant pattern of inheritance (10). It has been postulated that, in these cases, mutant hUGT1A1 may act as a dominant-negative protein. An essential component of this hypothesis is intermolecular association of hUGT1A1 molecules.

Several previous observations have suggested the intermolecular association of some other UGT isoforms. Gel permeation chromatography of solubilized rat liver microsomal UGT isoforms with activity toward chenodeoxycholic acid (11) or phenols (12) suggested that they exist in a larger than monomeric size. Radiation inactivation analysis of rat liver microsomes also suggested that UGT1A1 may exist as dimers and tetramers (13). However, these studies did not differentiate between the homodimerization of UGT isoforms and interaction between one UGT isoform and other isoforms or non-UGT proteins. In fact, interaction between rat liver testosterone-UGT and androsterone-UGT isoforms has been reported (14). It has been proposed that heterodimerization of rat liver UGT2B1 with a UGT1A isoform may activate UDP-N-acetylgluco- coseamine-stimulated UDP-glucuronic acid transport into the ER lumen (15). The functional relevance of UGT dimerization was suggested by partial reconstitution of steroid-UGT activity.

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‡ To whom correspondence should be addressed: Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Tel: 718-430-2254; Fax: 718-430-8975; E-mail: chowdhur@aecom.yu.edu.

§ The abbreviations used are: UGTs, uridine-diphosphoglucuronate glucuronosyltransferases (prefix “h” indicates human); ER, endoplasmic reticulum; HPLC, high-pressure liquid chromatography; BMH, 1,6-bismaleimidohexane; DTBP, dimethyl 3,3′-dithiobiisopropionimidate; bp, base pair; ELISA, enzyme-linked immunosorbent assay; HIV, human immunodeficiency virus.
upon coexpression of two catalytically inactive mutant forms of rat liver UGT2B1 (16).

However, there is no direct evidence for homodimerization of human UGT1A1. Therefore, we have used a combination of gel permeation high-pressure liquid chromatography (HPLC), nearest-neighbor cross-linking analysis, and two-hybrid studies in yeast and mammalian systems to test the hypothesis that hUGT1A1 interacts with itself. We have also partially characterized the domain of hUGT1A1 that is required for such interaction.

**EXPERIMENTAL PROCEDURES**

**Stable Transduction of Gunn Rat Fibroblasts with hUGT1A1**—To express hUGT1A1 in a cell that does not express any other UGT isoform, we stably transduced skin fibroblasts from jaundiced Gunn rats, which lack the UGT1A subfamily of UGT isoforms, using a Moloney murine leukemia virus-based gene transfer system as previously described (17). The Gunn rat fibroblasts lack any other detectable UGT activity. These cells (termed GURF-hUGT1A1) express hUGT1A1 at a level comparable to that of normal human liver microsomes.

**SDS-Polyacrylamide Gel Electrophoresis under Reducing and Non-reducing Conditions**—To examine whether hUGT1A1 forms intermolecular disulfide bonds, GURF-hUGT1A1 cells were disrupted by sonication, and microsomal fractions were prepared as described (17). The microsomal proteins (25 μg/lane) were resolved by SDS-10% polyacrylamide gel electrophoresis with or without pretreatment with 100 mM dithiothreitol and with or without heating the sample with SDS. Immunoblot analysis was performed using a monoclonal antibody (WP1) against the human UGT1A group of proteins (18).

**Gel Permeation HPLC of Solubilized hUGT1A1**—Microsomes derived from GURF-hUGT1A1 cells were suspended in 50 mM HEPES (pH 7.8) containing 2% dithiothreitol. The sample was applied to a TSK-GEL PW1000 column (Toyo Soda, Tokyo), and the fractions (0.5 ml) were collected. Western blots were then performed using the WP1 antibody (1:4000 dilution) with minor modifications. Briefly, ELISA plates were coated overnight with the WP1 antibody (1:4000 dilution), blocked with phosphate-buffered saline containing 3% bovine serum albumin and 5% fetal calf serum, and then overlaid with lysates of the transfected cells. A rabbit antibody against a synthetic peptide corresponding to a unique region of hUGT1A1 (polycystic kidney disease 136–180) was applied at a 1:500 dilution. The detection system consisted of horseradish peroxidase-conjugated goat anti-rabbit IgG. Absorbance at 405 nm was determined using an ELISA plate reader.

**Assay of UGT1A1 Activity**—Based on ELISA quantitation, equal amounts of hUGT1A1 from the various cell lines were assayed for UGT activity toward bilirubin in the presence of 80 μM bilirubin, 4.4 mM UDP-glucuronic acid, and diethyolphosphatidylcholine as described (3). In brief, after incubation at 37 °C for 4 h, the reaction was stopped by adding 0.4 mM glycine HCI (pH 1.8), and the pigments were extracted in chloroform/ethanol (1:1, v/v) (3). The solvents were evaporated under a stream of nitrogen. The pigments were dissolved in dimethyl sulfoxide, mixed with an equal volume of methanol, and analyzed by reverse-phase HPLC using a Waters μBondapak C18 column as described (25).

**Site-directed Mutagenesis**—Site-directed mutagenesis was done to introduce point mutations into hUGT1A1 cDNA. This was performed based on the method of Deng and Nickoloff (20) with some minor modifications of the protocols. Transformation mutants were cut out of GURF-hUGT1A1 cells were suspended in 50 mM Tris-HCl containing 0.25 mM sucrose and 5 mM MgCl2. The microsomal suspension (containing 100 μg of protein) was treated with DTBP at a final concentration of 2, 5, or 10 mM. The cross-linking reaction was carried out at 22 °C (optimum temperature) for 60 min. The reaction was stopped by adding 0.5 mM glycine (pH 7.0). SDS-4%–10% gradient polyacrylamide gel electrophoresis and immunoblot analysis was performed as described (19).

**Generation of a hUGT1A1 cDNA with an In-frame Deletion of Nucleotides 454–540**—To delete a segment of hUGT1A1 (amino acids 152–180) that spans a membrane-embedded helix, we generated an expression plasmid with in-frame deletion of nucleotides 454–540. A 453-base pair (bp) segment of the hUGT1A1 cDNA including the translation initiation codon (ATG) was amplified from plasmid pcDNA3.1/Zeo(+) + bUGT (21) using the following amplimers: sense, 5′-CCGGATCCGATGGCTGTGGGC-3′; anti-sense, 5′-GATCCGGCGCTTATGACCAGATG-3′. The 453-bp amplicon was digested with EcoRI to excise a 707-bp segment from the 5′-end of the hUGT1A1 coding region. The 619-bp fragment generated by ligation of the two polymerase chain reaction amplions was ligated to the EcoRI site of pcDNA3.1/Zeo(+). The resulting UGT1A1 cDNA with the deletion of nucleotides 454–540 was cloned in-frame at the BamHI and XhoI sites of the mammalian two-hybrid vectors.

**Transfection and Immunoblot Analysis—**COS-7 cells, grown in 100-mm dishes to 50–60% confluency, were transfected with pcDNA3.1/Zeo(+) containing normal or mutagenized hUGT1A1 sequences and a Zeocin® resistance gene using DEAE-dextran (Amer- Crobio Inc., Washington, D.C.) as described (21).

**Cross-linking Analysis—**To determine whether hUGT1A1 interacts with itself within the ER membrane, we performed “nearest-neighbor” analysis using two different cross-linkers. For generating intramolecular disulfide bonds, 10% homogenates of GURF-hUGT1A1 cells were prepared in 20 mM Tris-HCl (pH 8.0) containing 0.25 mM sucrose using a glass homogenizer by 30 up-and-down strokes of a motorized Teflon pestle. Microsomes were prepared as described (17) and suspended in 50 mM HEPES (pH 7.8) containing 1% dithiothreitol. The fractions (0.5 ml) were analyzed by immunoblotting using the WP1 monoclonal antibody. The immunoreactive bands were quantified by laser densitometry.

**Two-hybrid Analysis in the Yeast System**—In the yeast two-hybrid system, two physically functional domains are necessary, a DNA-binding domain and a transcription activation domain. A 2.2-kilobase pair fragment spanning the entire coding region of hUGT1A1 was amplified from plasmid pcDNA3.1/Zeo(+) + bUGT (21) using sense and antisense amplimers containing BamHI and XhoI linkers, respectively. The ampli-
con was digested with BamHI and XhoI and cloned in-frame into the BamHI and SalI sites of the “bait” vector pH21 so that hUGT1A1 is expressed as a fusion protein with the bacterial Lex DNA-binding domain. The nucleotide sequence of the insert was determined to confirm the fidelity of the amplification. The hUGT1A1 cDNA was also cloned in-frame into the BamHI and SalI sites of the “prey” vector Gal4-GADNOT so that hUGT1A1 is expressed as a fusion protein with the Gal4 transactivator protein (activation domain). The constructs (20 μg each) were cotransfected into yeast strain CTY. In this yeast strain, the LexA operator is engineered into the 5′-flanking region of the Escherichia coli β-galactosidase reporter gene (lacZ). The transformed cells were plated on selective dropout agar plates without histidine and leucine. After growing the yeast cells for 3–4 days at 30 °C, the colonies were blotted onto nitrocellulose membranes and stained for protein with the transcriptional activation domain of mouse NF-Xho domain of yeast Gal4. The test sequence (wild-type or mutagenized that hUGT1A1 is expressed as a fusion protein with the DNA-binding and nonreducing conditions, resulting in the transcription of LacZ and the appearance of blue colonies. HIV-1 integrase protein (IN) cloned into both the bait and prey vectors was used as a positive control. As a negative control, we used a bait construct expressing hUGT1A1 in combination with a prey construct without any fusion sequence or a bait construct without any fusion sequence in combination with a prey construct expressing hUGT1A1 (26).

Two-hybrid Analysis in the Mammalian System—In the mammalian two-hybrid assay system, the reporter plasmid pFR-Luc contains a synthetic promoter with five tandem repeats of the yeast Gal4 binding sites that control expression of the luciferase gene. This reporter gene expression occurs as a result of a reconstitution of a functional transcription factor by the association of two hybrid proteins. A 2.5-kilobase pair DNA segment containing the full-length hUGT1A1 cDNA was cloned in-frame into the BamHI and XhoI sites of the bait vector pCMV-BD so that hUGT1A1 is expressed as a fusion protein with the DNA-binding domain of yeast Gal4. The test sequence (wild-type or mutagenized hUGT1A1 or human UGT1A6) was cloned in-frame into the BamHI and XhoI sites of the pCMV-AD vector, where it is expressed as a fusion protein with the transcriptional activation domain of mouse NF-κB. The various coding sequences cloned into the prey vector are listed in Table I. These two constructs were cotransfected into COS-7 cells, cultured at 50% confluence, along with plasmid pFR-Luc. Positive interaction of the proteins expressed from the bait and prey vectors brings the NF-κB activation domain into proximity with the Gal4 DNA-binding domain, thereby activating the expression of luciferase. As a positive control, the coding regions of simian virus 40 large T antigen and p53 were cloned into the bait and prey vectors, respectively (27). Luciferase activity was assayed according to the manufacturer’s protocol (Stratagene). To determine whether the mutagenized forms of hUGT1A1 express stable proteins, the bait and prey plasmid constructs were transfected into COS-7 cells by the DEAE-dextran method (21), and the cell lysates were analyzed by immunoblotting using WP1.

Coexpression into COS Cells—To examine whether some mutant forms of hUGT1A1 can inhibit the function of wild-type hUGT1A1, we performed cotransfection experiments. A series of plasmids expressing wild-type hUGT1A1 and several mutant forms of hUGT1A1 were prepared. The plasmids were transfected into COS-7 cells singly (10 μg/75-cm² plate) or in pairs (5 μg each/75-cm² plate) using DEAE-dextran as the transfection vehicle (21). To confirm the expression of transfected plasmids, immunoblot experiments were performed using WP1. The expressed total hUGT1A1 proteins were quantified by sandwich ELISA as described above. UGT activity toward bilirubin was determined by a sensitive HPLC method as described above (25).

One mutagenized form of hUGT1A1 (C127Y) exhibited marked inhibition of the activity of the wild-type enzyme upon coexpression in COS-7 cells (see “Results”). To determine whether the wild-type and mutagenized forms were coexpressed, we extracted the total RNA from the lysate of the transfected COS-7 cells. A 646-bp segment (nucleotides 96–341) contains many UGT isoforms, which may potentially interact with each other. The sequence of the amplimer was determined by the dideoxy method (28).

RESULTS

SDS-Polyacrylamide Gel Electrophoresis under Reducing and Nonreducing Conditions—SDS-Polyacrylamide gel electrophoresis under reducing and nonreducing conditions, followed by immunoblot analysis. Microsomes from GURF-UGT cells stably expressing human UGT1A1 were subjected to SDS-10% polyacrylamide gel electrophoresis and immunoblot analysis as described under “Experimental Procedures.” Lane 1, electrophoresis after reduction with 100 mM dithiothreitol; lane 2, electrophoresis without treatment with a reducing agent.

FIG. 1. SDS-polyacrylamide gel electrophoresis under reducing and nonreducing conditions.
with each other. Therefore, to determine whether human UGT1A1 interacts with itself, we generated a stably transfected Gunn rat fibroblast cell line that expresses only one UGT isoform, human UGT1A1. To keep the enzyme in solution, gel permeation HPLC was performed in the presence of the non-ionic detergent \( n \)-octyl glucoside. The concentration of \( n \)-octyl glucoside was kept at 0.2%, which is below its critical micellar concentration of 0.6%, to avoid incorporation of the enzyme into large micellar particles. Detection of the enzyme in various fractions by immunoblot analysis using the human UGT1A subfamily-specific monoclonal antibody WP1 showed that a large fraction of hUGT1A1 eluted at a size class corresponding approximately to the dimeric form. The remainder exhibited a longer retention time, consistent with the monomeric size (Fig. 2, \( a-c \)). Both size classes were resolved to the monomeric size upon SDS-polyacrylamide gel chromatography (Fig. 2b).

Cross-linking Studies in Intact Microsomal Membranes—UGT1A1 activity toward bilirubin in the native microsomes of GURF-hUGT1A1 cells in the absence of detergent treatment was 630 pmol/µg of UGT1A1/h. In the presence of 0.17% \( n \)-octyl glucoside, hUGT1A1 in the microsomal vesicles was activated, resulting in an almost 5-fold enhancement of enzyme activity (3100 pmol/µg of UGT1A1/h). This indicated that our method of microsome preparation resulted in mostly sealed vesicles. Treatment of intact microsomal membranes of GURF-hUGT1A1 cells with the disulfide cross-linking agent BMH resulted in cross-linking of UGT1A1 molecules. At 2 mM cross-linker, more than half of the immunoreactive UGT1A1 migrated with an \( M_r \) of ~104,000, indicating dimerization of the
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The cross-linking reaction involved the cysteine residues located in the cytoplasmic carboxyl-terminal tail. To determine the effect of hydrogen ion concentration on the dimerization of UGT1A1, cross-linking experiments were also performed with the amino group cross-linker DTBP, which functions at both acid and alkaline pH values. Cross-linking efficiency was much higher at pH 7.4 (Fig. 3, lane 3) than at pH 9.0 (lanes 9–11).

Two-hybrid Analysis Using the Yeast System—The bait vector contained human UGT1A1 expressed as a fusion protein with the Lex DNA-binding protein. The prey vector contained human UGT1A1 expressed as a fusion protein with Gal4. For a positive control, IN (Fig. 4a, part 1) protein was expressed as fusion proteins in the bait and prey vectors, respectively. For a negative control, the bait and prey vectors were used without any fusion sequences (Fig. 4a, parts 3 and 4). Expression of the E. coli lacZ reporter gene clearly demonstrated interaction between UGT1A1 expressed in the bait and prey vectors (Fig. 4a, part 2).

Two-hybrid Analysis Using the Mammalian System—In subsequent experiments, to achieve greater sensitivity and to obtain quantitative information, we used the mammalian two-hybrid system. To determine the UGT1A1 domain responsible for the intramolecular interaction, we expressed wild-type hUGT1A1 in the bait vector as a fusion protein with the DNA-binding domain of the yeast protein Gal4. The prey vector consisted of a series of test constructs containing wild-type or mutagenized UGT1A1 expressed as fusion proteins with the transcriptional activation domain of mouse NF-κB. Expression of luciferase activity after cotransfection of the bait and prey vectors into COS-7 cells indicated a strong interaction between wild-type UGT1A1 molecules (Fig. 4b, first bar), whereas there was no detectable binding between UGT1A1 and UGT1A6 (second bar). Because the carboxyl-terminal domains of UGT1A1 and UGT1A6 are identical, this finding indicates that the amino-terminal domain of UGT1A1 is required for dimerization. This was consistent with the finding that UGT1A1 isozymes with partial truncation of the carboxyl terminus of UGT1A1 also dimerized with wild-type UGT1A1 (Fig. 4b, seventh and eighth bars). Several mutant forms of UGT1A1 with substitution of a single amino acid that abolished or reduced enzyme activity interacted efficiently with the wild-type protein, whereas other mutations markedly reduced the binding (Fig. 4b). When amino acids 152–180 (spanning the membrane-embedded helical region within the amino-terminal domain) were deleted from hUGT1A1, dimerization was abolished almost completely (Fig. 4b, ninth bar).

Coexpression of Wild-type hUGT1A1 and Catalytically Inactive Mutant Forms in COS-7 Cells—To examine whether some catalytically inactive mutant forms of UGT1A1 can inhibit the function of the wild-type enzyme, we performed cotransfection experiments. The wild-type hUGT1A1 cDNA was cotransfected with each of two mutagenized cDNAs that predict single amino acid substitutions (C127Y and C223Y). Both mutant forms lacked catalytic activity when expressed in COS-7 cells. The C127Y mutant retained the ability to dimerize with the wild-type enzyme as determined by the mammalian two-hybrid system, whereas dimerization was markedly reduced with the C223Y mutant. Expression of the wild-type and mutant forms of UGT1A1 was confirmed by immunoblot studies (Fig. 3).

![Fig. 4 Two-hybrid analysis](http://www.jbc.org/)

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**Table I**

| Bait    | Prey          | Intermolecular association |
|---------|---------------|---------------------------|
| hUGT1A1 | hUGT1A1       | +                         |
| hUGT1A1 | hUGT1A6       | –                         |
| hUGT1A1 | C127Y hUGT1A1 | +                         |
| hUGT1A1 | F171Y hUGT1A1 | +                         |
| hUGT1A1 | L175E hUGT1A1 | +                         |
| hUGT1A1 | C223Y hUGT1A1 | +                         |
| hUGT1A1 | L519X hUGT1A1 | +                         |
| hUGT1A1 | K530X hUGT1A1 | +                         |
| hUGT1A1 | hUGT1A1(-Δ152–180) | –                       |
a, and the expressed UGT1A1 was quantified by ELISA. Cotransfection of the wild-type UGT1A1 cDNA with the mutant UGT1A1 cDNA encoding the C223Y substitution resulted in a specific enzyme activity that was approximately half of the activity expressed by transfection of the wild-type UGT1A1 cDNA alone (Fig. 5b). This was the predicted outcome because, in all transfections, an equal amount of DNA was used, so in the cotransfection experiment, the amount of wild-type UGT1A1 cDNA used was half of that used when the wild-type cDNA was transfected alone. In contrast to these results, coexpression of a mutant UGT1A1 cDNA with a 381G→A transition (predicting the C127Y substitution) resulted in much lower activity (16%) than the predicted 50% specific UGT1A1 activity (Fig. 5b). The results were confirmed by repeated cotransfection experiments. To ascertain whether the mRNAs for both wild-type and C127Y mutant UGT1A1 were expressed upon cotransfection, we extracted the RNA from the transfected cells and amplified a 646-nucleotide segment containing the 381G→A mutation by reverse transcription-polymerase chain reaction. Nucleotide sequence determination of the amplicon indicated that the mRNA for both the wild-type and mutant forms had been expressed (Fig. 5c).

**FIG. 5.** Coexpression of wild-type hUGT1A1 and mutant hUGT1A1 (381G→A transition, predicting the C127Y substitution) in COS-7 cells. a, shown are the results from immunoblot analysis. Lane 1, GURF-hUGT1A1 cells (positive control); lane 2, wild-type hUGT1A1 (10 µg of cDNA); lane 3, mutant hUGT1A1 (381G→A; 10 µg of cDNA); lane 4, cotransfection of wild-type hUGT1A1 (5 µg of cDNA) and mutant UGT1A1 (381G→A; 5 µg of cDNA); lane 5, untransfected COS-7 cells. b, expression of the hUGT1A1 protein was determined by ELISA, and UGT1A1 activities were determined as described under “Experimental Procedures.” cDNAs were transfected as indicated. c, to ascertain whether both wild-type UGT1A1 and a mutant form (381G→A, predicting the C127Y substitution) were expressed upon cotransfection, a 646-nucleotide segment of the mRNA-spanning mutation site was amplified by reverse transcription-polymerase chain reaction, and the nucleotide sequence was determined by the dideoxy chain termination method.

Mixing Studies Using Intact or Detergent-treated Preparations—When unperturbed homogenates of COS cells transfected with wild-type UGT1A1 or the C127Y mutant were mixed, there was no inhibition of the activity of the wild-type enzyme (Fig. 6). However, when the cell homogenates were first treated with 1% n-octyl glucoside to release the expressed UGT1A1 by solubilizing the microsomal vesicles, mixing of the two homogenates resulted in reduction of the activity of the wild-type UGT1A1 to approximately one-third (Fig. 6). This suggests that UGT1A1 molecules that are compartmentalized in different microsomal vesicles cannot undergo intermolecular association, but such association occurs upon dissolution of the membranes.

**DISCUSSION**

We have used three strategies to examine the interaction of human UGT1A1 with itself. These approaches provide different but complementary information and, considered together, have provided evidence that human UGT1A1 forms homodimers. Gel permeation HPLC of solubilized microsomal fractions of GURF-hUGT1A1 cells showed that, in the native form, the enzyme exists in two size classes, one corresponding
to the dimeric form and the other to the monomeric form. As the GURF-hUGT1A1 cells do not contain any other detectable UGT activity, the findings suggest that human UGT1A1 dimerizes with itself, although association with non-UGT proteins cannot be excluded in this experiment. We used concentrations of n-octyl glucoside that are much lower than its critical micellar concentration to obviate the formation of large micellar aggregates. The higher molecular size species of UGT1A1 resolved to monomeric size upon SDS-polyacrylamide gel electrophoresis, indicating that the interaction of the molecules does not involve disulfide bonding or other types of covalent bonding. We have observed that gel permeation HPLC of solubilized human liver microsomes also results in the elution of UGT1A1 in two size classes. However, as human liver microsomes contain numerous UGT isoforms, the latter finding could have resulted from interaction with other UGT isoforms. In fact, interaction between rat liver UGT1A1 and UGT2B1 has been reported (15). We have observed that gel permeation HPLC of solubilized human liver microsomes also results in the elution of UGT1A1 in two size classes. However, as human liver microsomes contain numerous UGT isoforms, the latter finding could have resulted from interaction with other UGT isoforms. In fact, interaction between rat liver UGT1A1 and UGT2B1 has been reported (15).

To determine whether the intermolecular binding of human UGT1A1 occurs within intact microsomal membranes, we performed cross-linking analysis. Nearest-neighbor cross-linking analysis with the disulfide cross-linking agent BMH showed that a large proportion of the human UGT1A1 molecules exist in a size class consistent with its dimer. This type of chemical disulfide bonding occurs via cysteine residues. Human UGT1A1 contains 11 cysteine residues, one of which is within the signal peptide that is not present in the mature enzyme. The primary structure of human UGT1A1 predicts that most of the molecule lies within the ER lumen, with only a 26-amino acid segment at the carboxyl terminus remaining in the cytoplasm. The cytoplasmic tail contains 3 cysteine residues. Dimerization of UGT1A1 by BMH treatment was blocked by the membrane-impermeable inhibitor stilbenedisulfonate maleimide. UGT1A1 activity exhibited marked latency in our microsomal preparation, and the latency was abolished by detergent treatment, indicating that the microsomal vesicles were mostly sealed. Therefore, inhibition of dimer formation by this membrane-impermeable agent indicates that the chemical cross-linking occurs through one or more of these residues. It should be noted, however, that in the predicted natural dimerization of the protein within the ER membrane, disulfide bond formation is not significant since, even without treatment with a reducing agent, human UGT1A1 is mostly resolved to its monomeric size during SDS-polyacrylamide gel electrophoresis of microsomal proteins. Previous studies have shown that heterodimer formation between rat liver UGT1A1 and UGT2B1 is disrupted at pH 9.0 (15). To evaluate the effect of pH on the dimerization of human UGT1A1, we used a different cross-linker (DTBP) with a wider pH range of effectiveness because BMH is not an effective cross-linker at alkaline pH. This experiment showed that the homodimerization of UGT1A1 is also disrupted at pH 9.0.

Yeast and mammalian two-hybrid systems are powerful tools for the study of protein-protein interaction. Both systems demonstrated positive interaction between human UGT1A1 molecules. To identify the regions of UGT1A1 required for dimer formation, we utilized the mammalian two-hybrid system. Switching the amino-terminal domain of UGT1A1 to the amino-terminal domain of UGT1A6 completely abolished the interaction, indicating that the amino-terminal domain is important in the selection of UGT isoforms with which UGT1A1 can dimerize. However, this does not preclude its potential interaction with some other UGT isoforms, such as that reported between rat UGT1A1 and UGT2B1 (15). Substitution of several individual amino acids within the N-terminal domain resulted in the loss or severe reduction of the catalytic activity of UGT1A1, but did not affect homodimerization of the molecule (e.g. C127Y and F171Y). Other substitutions (e.g. L175E and C223Y) significantly reduced the interaction. A membrane-embedded helix has been identified recently within the amino-terminal domain of UGT1A1 by computer analysis (28). This region has a significant degree of homology to the corresponding site in UGT1A6, but differs in some specific amino acid residues. We found that deletion of these amino acids abolished the positive interaction with wild-type UGT1A1, although immunoblot analysis showed that the protein containing the deletion was expressed after transfection of the prey plasmid in the mammalian two-hybrid system.

The intermolecular binding of UGT1A1 may be functionally significant for several reasons. Interaction of two inactive forms of rat liver UGT2B1 carrying two different mutations was reported to result in complementation and reconstitution of the enzyme activity toward testosterone upon coexpression in COS-7 cells (16). We coexpressed several pairs of mutant forms of UGT1A1 carrying two different mutations in COS-7 cells, but did not find reconstitution of the enzyme activity toward bilirubin (data not shown). On the other hand, it has been proposed that intermolecular binding between some mutant forms of hUGT1A1 and the wild-type enzyme may result in inhibition of the catalytic activity of wild-type hUGT1A1. We examined this possibility by coexpressing wild-type hUGT1A1 with a catalytically inactive form of hUGT1A1 containing a C127Y mutation in COS-7 cells. This mutation does not interfere with positive interaction with wild-type hUGT1A1. Expression of both forms of UGT1A1 was shown by performing reverse transcription-polymerase chain reaction on the relevant region, followed by nucleotide sequence analysis of the amplicons. The significant inhibition of wild-type hUGT1A1 by catalytically inactive mutant hUGT1A1 provided evidence that some mutant forms of hUGT1A1 can function as dominant-
negative proteins. In contrast, coexpression of mutant hUGT1A1 with a C223Y substitution, which does not strongly interact with wild-type UGT1A1, did not result in inhibition of enzyme activity. This observation may provide a mechanistic explanation for the finding of unconjugated hyperbilirubinemia in heterozygous carriers of certain single nucleotide transitions within the coding region of human UGT1A1 (10). Interestingly, mixing unperturbed homogenates of COS cells expressing wild-type and C127Y mutant UGT1A1 did not result in inhibition of enzyme activity. This observation may provide a mechanistic explanation for the finding of unconjugated hyperbilirubinemia in heterozygous carriers of certain single nucleotide transitions within the coding region of human UGT1A1 (10). Interestingly, mixing unperturbed homogenates of COS cells expressing wild-type and C127Y mutant UGT1A1 did not result in inhibition of the catalytic activity of the wild-type enzyme, but such inhibition was seen when the homogenates were treated with a detergent. Considered together with the results of the cotransfection experiments, these findings indicate that UGT1A1 molecules coexpressed in the same endoplasmic reticulum membrane exhibit intermolecular association, but such association does not occur between molecules that are segregated in different microsomal vesicles.

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Homodimerization of Human Bilirubin-Uridine-diphosphoglucuronate Glucuronosyltransferase-1 (UGT1A1) and Its Functional Implications
Siddhartha S. Ghosh, Baljit S. Sappal, Ganjam V. Kalpana, Sung W. Lee, Jayanta Roy Chowdhury and Namita Roy Chowdhury

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