

Modulation of UDP-Glucuronosyltransferase Function by Cytochrome P450: Evidence for the Alteration of UGT2B7-Catalyzed Glucuronidation of Morphine by CYP3A4

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ABSTRACT

Modulation of UDP-glucuronosyltransferase 2B7 (UGT2B7)-catalyzed morphine glucuronidation by cytochrome P450 (P450) was studied. The effects of P450 isozymes on the kinetic parameters of UGT2B7-catalyzed glucuronidation of the morphine 3-hydroxyl group were examined by simultaneous expression of UGT2B7 and either CYP3A4, -1A2, or -2C9 in COS-1 cells. Although coexpression of CYP3A4 with UGT2B7 had little effect on V_{\max} , the K_m was increased by about 9.8-fold compared with the UGT2B7 single expression system. The other P450 isozymes (CYP1A2 and CYP2C9) had some effects on K_m and V_{\max} values. Immunoprecipitation of UGT from solubilized human liver microsomes resulted in coprecipitation of CYP3A4 with UGT2B7. The protein-protein interaction between CYP3A4 and UGT2B7 was further confirmed by overlay

assay using glutathione S-transferase-CYP3A4 fusion protein. Addition of CYP3A4 to untreated COS microsomes expressing UGT2B7 had no or minor effects on morphine glucuronidation. In contrast, the formation of morphine-3-glucuronide by detergent-treated microsomes from COS-1 cells expressing UGT2B7 was reduced by CYP3A4, whereas the formation of the 6-glucuronide was enhanced. These results strongly suggest that 1) the glucuronidation activity of UGT2B7 toward morphine is specifically modulated by interaction with CYP3A4 in microsomal membranes and that 2) CYP3A4 alters UGT2B7 regioselectivity so that the ratio of morphine activation/detoxification is increased. This study provides the first evidence that P450 is not only involved in oxidation of drugs but also modulates the function of UGTs.

Drug-metabolizing enzymes that are present predominantly in the liver are involved in biotransformation of both endogenous and exogenous compounds to polar products to facilitate their elimination. These reactions are categorized into phase I and phase II reactions (Tukey and Strassburg, 2000; Nebert and Russell, 2002). The cytochrome P450 (P450) and the UDP-glucuronosyltransferase (UGT) families, the catalytic domains of which are localized in the cytosolic

and luminal sides of the endoplasmic reticulum (ER) membrane, respectively, are two major enzyme groups responsible for phase I and II reactions. Among the UGTs identified in humans, UGT2B7 is of particular interest because it is a major isoform involved in morphine glucuronidation (Coffman et al., 1997). We have reported previously that the catalytic properties of guinea pig morphine UGT (UGT2B21) are changed by hetero-oligomer formation with UGT2B22 (Ishii et al., 2001). Because other workers also demonstrated the presence of homo- and hetero-UGT oligomers (Ikushiro et al., 1997; Meech and Mackenzie, 1997; Ghosh et al., 2001; Kurkela et al., 2003), it is conceivable that the function of UGT may be modulated by oligomer formation. Furthermore, the formation of enzyme-enzyme complexes generally facilitates metabolite transfer between P450 enzymes (Alston et al., 1991) owing to the spatial proximity of the active sites.

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ABBREVIATIONS: P450, cytochrome P450; UGT, UDP-glucuronosyltransferase; ER, endoplasmic reticulum; BSA, bovine serum albumin; M-6-G, morphine-6-glucuronide; GST, glutathione S-transferase; PCR, polymerase chain reaction; CMV, cytomegalovirus; M-3-G, morphine-3-glucuronide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; CNX, calnexin.

Although P450-P450 and UGT-UGT interaction and their significance on the catalytic function have been reported as mentioned above, it has long been believed that the phase I and II reactions take place individually. UGT plays an important role in detoxifying potent carcinogenic metabolite(s) formed by P450 (Tukey and Strassburg, 2000). To minimize toxicity, it would be reasonable to expect that the reactive metabolite produced by P450 is directly transferred to the other enzymes participating in its subsequent metabolism (e.g., the UGTs) via protein-protein interactions. We have provided evidence supporting a P450-UGT interaction, by means of P450-immobilized affinity chromatography (Taura et al., 2000, 2004). UGT1A1, UGT1A6, and UGT2B7 may interact with CYP3A4 because these were coimmunoprecipitated with anti-CYP3A4 antibody (Fremont et al., 2005). However, the functional significance of the interaction between P450 and UGT has not yet been determined.

To validate the hypothesis of functional cooperation between P450 and UGT, we examined whether morphine glucuronidation by UGT2B7 is affected by P450 isozymes CYP3A4, CYP1A2, and CYP2C9, the most abundant P450s expressed in human liver (Evans and Relling, 1999). In this study, we have investigated the effect of the simultaneous expression of these P450 isozymes on kinetics of morphine glucuronidation catalyzed by UGT2B7. In addition, we examined the effect of CYP3A4 on morphine glucuronidation in permeabilized and nonpermeabilized microsomes from cells stably expressing UGT2B7. Our results strongly suggest that the catalytic function of UGT2B7 is modulated by the presence of CYP3A4 via enzyme-enzyme interactions.

Materials and Methods

Materials. UDP-glucuronic acid (trisodium salt) was purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). Morphine hydrochloride was obtained from Takeda Chemical Ind. Co., Ltd. (Osaka, Japan). Egg yolk L- α -phosphatidylcholine, saccharolactone, *n*-octylglucoside, and BSA, fraction V were purchased from Sigma-Aldrich (St. Louis, MO). Morphine-3-glucuronide (M-3-G) and morphine-6-glucuronide (M-6-G) were synthesized in our laboratory (Yoshimura et al., 1968). Recombinant CYP3A4 as a purified form was purchased from Panvera (Madison, WI). Geneticin solution (G418) was obtained from PAA laboratories GmbH (Linz, Austria). Pooled human liver microsomes (from 21 subjects) and microsomal preparations of individual organ donors were obtained, together with agreement for their use from donors, from BD Gentest (Woburn, MA) and stored at -80°C until use. Expression vector pGEX-6P-1 carrying glutathione *S*-transferase (GST; *Schistosoma japonicum*) cDNA and anti-GST polyclonal antibody were purchased from Amersham Biosciences (Uppsala, Sweden). All other reagents were of analytical grade.

Construction of Expression Plasmids for UGT2B7 and P450. The open reading frame of UGT2B7 (1.6 kilobase pairs) was amplified by PCR using isozyme-specific primers (5'-CAA GCA TTG CAT TGC ACC AGG -3', 5'-TCT TGC ATC ACA ATC TTT CTT GCT GG-3') and UGT2B7 cDNA (Jin et al., 1993) as a template. LA-*Taq*DNA-polymerase (TaKaRa, Japan) was used for the PCR reaction. The PCR product was then TA-cloned into the pTARGET vector (Promega, Madison, WI). The CYP1A2, CYP2C9, and CYP3A4 cDNAs were amplified by PCR using isozyme-specific primers (CYP1A2; 5'-CTC GAG GTT GGT ACA GAT GGC ATT GTC C-3' and 5'-TTC GAA GAC CGG AGT CTT ACC ACC ACA G-3'; CYP2C9; 5'-CTC GAG TTC TTA ACA GAG GAA TGA AGC-3' and 5'-CGT GAT CAT GCA GGT TCC GCG TAT GGA TTC TCT TG TG-3'; and CYP3A4; 5'-CGC GGT ACC TTT GCT GGG CTA TGT GCA-3' and 5'-GCG TCT AGA TCA GGC TCC ACT TAC GG-3'). The amplified

cDNA fragments of CYP1A2 and CYP2C9 were each subcloned into pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA) and KpnI/EcoRV sites of the pShuttle-CMV expression vector (Quantum Biotechnologies, Montreal, QC, Canada) to generate pShuttle-CMV-CYP1A2 and pShuttle-CMV-CYP2C9, respectively. The amplified cDNA fragment of CYP3A4 digested with KpnI/XbaI was inserted into KpnI/XbaI sites of the expression plasmid (pCMV4). The sequences of the expression plasmids were verified by DNA sequencing.

Transient Transfection of P450 Expression Plasmids. COS-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 0.45% D-glucose, 10 mM HEPES, 110 $\mu\text{g}/\text{ml}$ sodium pyruvate, 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin in a humidified incubator, in an atmosphere of 5% CO_2 , at 37°C . Two days before transfection, 1.5 to 2×10^6 COS-1 cells were seeded on a 100-mm culture dish. Transfection of the COS-1 cells that were approximately 70 to 80% confluent was carried out using a polyamine liposome reagent, Trans-IT LT1 (Mirus, Madison, WI), with a DNA/reagent ratio (20 μg of expression plasmid carrying P450 cDNA and 40 μl of the reagent) according to the manufacturer's recommendations. Cells were harvested 42–48 h after transfection, and microsomes were prepared. When P450s were simultaneously expressed with UGT2B7, transfections with expression plasmids for P450 were carried out using COS-1 cells stably expressing UGT2B7 (described below).

Stable Expression of UGT2B7. The transfection protocol described above was used to generate stable UGT2B7-transfectants of COS-1 cells, except that 20 μg of pTARGET-UGT2B7, a plasmid containing the neomycin-resistant gene, was used. After selection with G418, 4 clones of pTARGET-UGT2B7 transfectants were established. One of the clones that exhibited the highest UGT2B7 expression was used for further study.

Glucuronidation Assay. Glucuronidation of morphine was assayed according to Kuo et al. (1991). Unless otherwise stated, incubation mixtures (350 μl) consisted of 50 mM Tris-HCl buffer, pH 7.8, 10 mM MgCl_2 , 5 mM saccharolactone, 2 mM UDP-glucuronic acid, 0.75 to 1 mg of COS-1 cell microsomes expressing the recombinant UGT2B7 or 0.5 to 1 mg of protein of human liver microsomes, and 5 mM morphine. In some experiments, microsomes were treated before assay with a detergent, *n*-octylglucoside (0.5 mg detergent/mg of protein) on ice for 15 min. Sonicated L- α -phosphatidylcholine was included in all assays at a concentration of 0.5 mg/ml. Incubations were performed at 37°C for 4 h. Linearity of the time-dependent increase in M-3-G and M-6-G formation was confirmed up to 8 h of incubation. The reaction was terminated by addition of 0.117 ml of ice-cold 16.34% trichloroacetic acid, and the mixture was chilled in an ice bath for 30 min, followed by centrifugation at 9000 rpm for 8 min. The resulting supernatant was subjected to high-performance liquid chromatography and the formation of M-3-G and M-6-G was determined by a method described elsewhere (Oguri et al., 1996). The kinetic parameters with standard deviations were calculated using a nonlinear least square program MULTI (Yamaoka et al., 1981). In this procedure, the data were fitted to the Michaelis-Menten equation. Protein content was determined by the Bradford assay (Bradford, 1976) with bovine serum albumin as a standard.

Immunoblotting. Proteins separated by 7.5% or 9% SDS-PAGE were electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Immunochemical staining was performed with alkaline phosphatase-labeled secondary antibody. UGT2B7 was detected using goat anti-mouse low pI form UGT antibody (Mackenzie et al., 1984) or rabbit anti-UGT2B7 antibody obtained from BD Gentest. CYP3A4 was detected using WB-MAB-3A (BD Gentest) as primary antibody. CYP1A2 and CYP2C9 were detected using rat antibodies cross-reacting with human CYP1As (Nagata et al., 1985) and CYP2Cs (Baba et al., 1988) as primary antibodies, respectively. Rabbit anti-calnexin (CNX) antibody was purchased from StressGen Biotechnologies (Victoria, BC, Canada).

Immunoprecipitation. All procedures were carried out at 4°C . Solubilization of human liver microsomes with sodium cholate was

performed according to a method described elsewhere (Taura et al., 2000). Protein A-Sepharose CL4B (5 μ l; Amersham Biosciences) was suspended in 10 volumes of phosphate-buffered saline (PBS) containing 0.1% skim milk and gently mixed by rotator. The resin suspension was divided into two parts. One part was used for pretreatment of solubilized human liver microsomes and the other was reacted with anti-UGT IgG. As a control, nonimmune IgG was used instead of anti-UGT IgG. Solubilized human liver microsomes (100 μ g of protein) were pretreated as described above with the Protein A-Sepharose suspension for 30 min to remove nonspecific bindings and then mixed with goat anti-mouse low pI form UGT antibody (60 μ g IgG) bound to Protein A-Sepharose for 14 h. The anti-UGT antibody used in this procedure recognizes multiple forms of UGT with preference for the UGT2B subfamily (Mackenzie et al., 1984). Preliminary experiments showed the lower reactivity of the antibody to UGT1 isoforms. Control experiments were performed with Protein A-Sepharose treated with the same amount of nonimmune goat IgG. After low-speed centrifugation, the supernatant was removed, and the precipitates were washed three times for each of 5 min with 10 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol, 0.2% Emulgen 911, and 200 mM NaCl. The immunoprecipitates were subjected to SDS-PAGE/Western blot analysis for UGT2B7, CYP3A4, and CNX.

Construction of Plasmid for the Expression of GST-CYP3A4 Fusion Protein. The 5' end of the CYP3A4 cDNA in the pCMV4-CYP3A4 construct was modified by PCR using *Pfu* polymerase (Stratagene, La Jolla, CA) to remove hydrophobic membrane-spanning domain at the N terminus and introduce two NotI restriction sites at their ends. The forward primer (5'-ATA AGA ATG CCG CCG CAT ATG GGA ACC CAT TCA CAT GGA C-3') was designed to have a NotI site (underlined) upstream of the coding region of the CYP3A4 cDNA. The reverse primer (5'-ATA AGA ATG CCG CCG CAG AAG TCC TTA GGA AAA TTC AGG C-3') was also introduced a NotI site (underlined) downstream of the CYP3A4 termination codon. The PCR product obtained was purified, digested with NotI, purified again, and then ligated in frame to the GST cDNA cloned into the pGEX-6P-1 plasmid at the NotI site, giving rise to the expression of chimeric GST-CYP3A4 protein.

Heterologous Expression of GST-CYP3A4 Fusion Protein in *Escherichia coli*. A single colony of JM109 transformed with the plasmid carrying GST-CYP3A4 cDNA was grown overnight in 5 ml of Luria-Bertani broth containing 100 μ g/ml ampicillin and 0.2% glucose at 37°C. The overnight culture was added to 50 ml of 10-fold-diluted Luria-Bertani broth containing 100 μ g/ml ampicillin and 0.2% glucose, grown at 30°C with continuous shaking (190 stroke/min) until the absorbance at 600 nm was increased to be between 0.5 and 0.7. The bacteria were then treated with 0.1 mM freshly prepared isopropyl-1-thio- β -galactoside (concentration of the working solution, 0.1 M) and 0.5 mM δ -aminolevulinic acid, and the culture was continued for 4 h at 30°C with continuous shaking at 200 stroke/min. The cells were harvested by centrifugation at 10,000g for 20 min at 4°C, and the cells precipitated were washed once with ice-cold buffer A (100 mM Tris-HCl, pH 7.4, 500 mM sucrose, 0.5 mM EDTA, and 10 mM MgCl₂). The cells were resuspended in buffer A [100 mg of cells (wet weight)/ml] containing 0.2 mg/ml lysozyme, and incubated on ice for 30 min, followed by centrifugation at 4000g for 10 min at 4°C. The spheroplasts obtained were resuspended in ice-cold water (100 mg/ml) containing 2 mM dithiothreitol and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml leupeptin, and 1 μ g/ml pepstatin). The cells were lysed by adding *N*-laurylsarcosine in a final concentration of 1.5% and incubated for 15 min at 4°C. After incubation, the cells were moderately sonicated on ice for 30 s (power level 2, 50% duty cycle) in a Branson SONIFIER 250, and incubated for 15 min at 4°C in the presence of 1% Triton X-100. Triton X-100 used in above experiment was dissolved in buffer B consisting of 100 mM potassium/sodium phosphate, pH 7.4, 20% glycerol and 10 mM MgCl₂ at a concentration of 10%. After Triton-X-100 treatment, the cell solution was centrifuged at 12,000g

for 15 min at 4°C. The lysate containing GST-CYP3A4 fusion protein was collected and stored at -80°C until use. Approximately half amounts of the fusion protein expressed in *E. coli* were recovered from the cytoplasm and periplasmic fractions, and its specific content was shown to be 43 nmol/ μ g of protein by immunochemical assessment. The lysate containing GST that was not fused with CYP3A4 was prepared as above, following transfection of *E. coli* with pGEX-6P-1.

Overlay Assay with GST-CYP3A4 Fusion Protein. The microsomes of insect cells transformed with baculovirus carrying UGT2B7 cDNA (Supersomes; BD Gentest) were used as the source of UGT2B7, and the microsomes are designated "baculosomes" herein. The baculosomes (2 mg of protein) were sedimented by ultracentrifugation at 100,000g for 15 min at 4°C. The precipitate was resuspended in 1 ml of PBS, pH 7.4, and resedimented at 100,000g for 15 min at 4°C. These steps were repeated five times, and washed baculosomes were treated with 1 ml of buffer B containing 1% *n*-octylglucoside for 30 min at 4°C. After ultracentrifugation at 100,000g for 1 h at 4°C, the supernatant was collected. Protein concentration was determined by Bradford assay (Bradford, 1976) with bovine serum albumin as a standard. Solubilized baculosomes were separated by SDS-PAGE (10% gel), and proteins in the gel were electrically transferred to a polyvinylidene difluoride membrane. Nonspecific binding of GST-CYP3A4 to the blotted membrane was blocked by immersion of the blot at 37°C for 2 h in 10% skim milk in binding buffer consisting of 10 mM potassium phosphate buffer, pH 7.4, 20% glycerol, 0.2% Emulgen 913, and 200 mM NaCl. After three 5-min washes with binding buffer, the membrane was incubated with either 50 μ g/ml GST-CYP3A4 fusion protein or GST in binding buffer containing 1% BSA at 4°C for 12 h. After three 5-min washes with binding buffer, the blot was incubated with 2000-fold-diluted goat anti-GST antibody at 4°C for 6 h. Location of anti-GST antibody on the membrane was visualized by as described under *Immunoblotting*.

Results

Kinetics of Morphine Glucuronidation in COS Cells Expressing UGT2B7/P450s and Human Liver Microsomes. To examine whether P450 isozymes can influence the function of UGT2B7, we determined the kinetic parameters for M-3-G formation catalyzed by UGT2B7 expressed in COS-1 cells in the presence and absence of coexpressed CYP3A4, CYP1A2, and CYP2C9. In some cases, the content of P450 and UGT2B7 in cells expressing both proteins was different from that in cells expressing each protein individually. (Fig. 1, A–D). In particular, it seems that cotransfection of CYP3A4 with UGT2B7 resulted in a lowered CYP3A4 content (Fig. 1B). The levels of UGT2B7 in P450/UGT2B7 simultaneous expression systems were 76% (CYP3A4), 90% (CYP1A2), and 103% (CYP2C9) that of the UGT2B7 single expression system, respectively. Thus, we normalized all results based on the UGT2B7 level in the single expression system. All microsomes from cells expressing UGT2B7 alone and UGT2B7/P450 exhibited apparent monophasic kinetics. When pooled human liver microsomes were used, kinetic plots clearly showed monophasic kinetics (data not shown), although M-3-G formation can be mediated by UGT isoforms other than UGT2B7 (Court et al., 2003). Likewise, M-3-G formation in hepatic microsomes from three individual donors, whose kinetic parameters are listed in Table 2, showed monophasic Michaelis-Menten kinetics (data not shown). The monophasic nature of M-3-G formation is likely to exist because morphine is a highly specific substrate for UGT2B7 and is poorly glucuronidated by other UGTs (Court et al.,

2003; Stone et al., 2003). These results were consistent with the reports by other workers (Hasselström and Säwe, 1993; Court et al., 2003; Soars et al., 2003). It is evident, therefore, that recombinant UGT2B7 expressed in COS cells exhibits the same mode of kinetics as that of the hepatic ER.

Recombinant UGT2B7 showed comparable velocities of M-3-G formation in UGT2B7-single and CYP3A4-UGT2B7 double expression systems (Tables 1 and 2). In contrast, simultaneous expression of CYP3A4 increased the apparent K_m value for M-3-G formation by 9.8-fold compared with UGT2B7 expressed in the absence of recombinant P450, resulting in a large reduction in morphine glucuronidation efficiency (V_{max}/K_m) (Table 1). Lower V_{max}/K_m and V_{max} values are seen in the CYP1A2 and CYP2C9 coexpression systems, respectively (Table 1). This observation suggests that CYP2C9 and CYP1A2 also exhibit some effects on UGT2B7-catalyzed morphine glucuronidation. These lines of evidence strongly suggest that UGT2B7 function is specifically suppressed by coexisting CYP3A4. Studies could not be conducted on the kinetics of M-6-G formation because of the low activity of expressed UGT2B7 toward the 6-OH group of morphine, especially at low substrate concentrations.

Coimmunoprecipitation of CYP3A4 and UGT2B7. To evaluate whether CYP3A4 and UGT2B7 proteins interact, we subjected human liver microsomes to immunoprecipita-

tion experiments. Human liver microsomes were solubilized with sodium cholate and treated with an antibody against UGTs. The resulting immunoprecipitates were analyzed by immunoblotting with antibodies specific for UGT2B7, CYP3A4, and CNX. As shown Fig. 2, the anti-UGT antibody efficiently precipitated CYP3A4 as well as UGT2B7. CYP3A4 was not recognized by the antibody used in the immunoprecipitation when Western blot analysis was performed with this immunoglobulin as primary antibody (data not shown). These data support the results of coimmunoprecipitation of UGT2B7 with anti-CYP3A4 antibody (Fremont et al., in press). To examine the possibility of nonspecific interactions between UGT and nonP450 proteins, we assessed whether CNX, an ER membrane protein that has the same topology as UGT (Oda et al., 2003), can interact with UGT. Immunoprecipitation followed by immunoblotting showed that CNX does not bind to UGT under our immunoprecipitation conditions. These observations support the possibility that the CYP3A4 and UGT2B7 proteins specifically associate and interact with each other.

Detection of UGT2B7-CYP3A4 Complex by Overlay Assay with GST-CYP3A4 Fusion Protein. We prepared GST-CYP3A4 fusion protein and carried out overlay assay with this probe to further confirm the formation of UGT2B7-CYP3A4 complex. The CYP3A4 in the fusion protein prepared in this study was designed to lack the N-terminal membrane-anchoring region (amino acid residues; 2nd–24th). The molecular mass of the fusion protein was expected to be approximately 80 kDa, and this was verified by SDS-PAGE and immunoblotting (data not shown). We carried out SDS-PAGE of baculosomes containing UGT2B7 and exam-

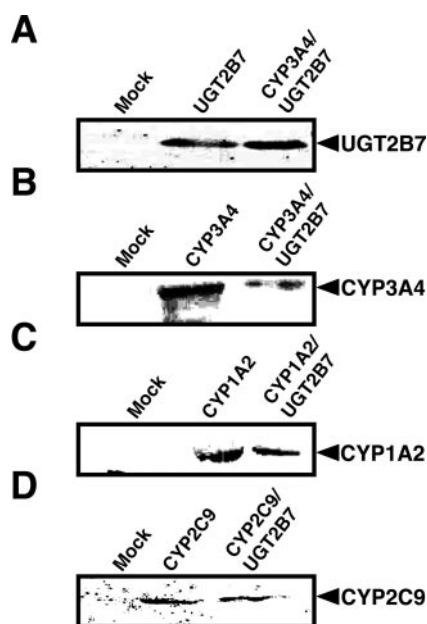


Fig. 1. Immunoblots of the expression of P450 isozymes and UGT2B7 in COS-1 cells. COS-1 cells were transfected with expression vector for UGT2B7 (pTARGET-UGT2B7). A G418-resistant clone stably expressing UGT2B7 was established. To obtain microsomes simultaneously expressing P450 and UGT2B7, this clone was transfected with expression vector carrying a P450 cDNA. For single P450 expression, intact COS-1 cells were also transfected with P450 expression vector. COS-1 cells transfected with the pTARGET vector without passenger DNA served as controls (Mock). The center and right lanes show samples of single and double transfect cells, the expressed protein(s) of which are indicated. Aliquots of 120 (A), 80 (B), and 100 (C and D) μ g of protein of microsomes from transfected COS cells were electrophoresed (SDS-PAGE). The proteins in the gel were electrically transferred to polyvinylidene difluoride membranes and blotted with anti-UGT(A), anti-CYP3A (B), anti-CYP1A (C), and anti-CYP2C (D), antibodies, respectively. The band intensity of UGT2B7 was quantified by using NIH Image software (version 1.61) and was confirmed to be linear up to 150 μ g of UGT2B7-expressing COS cell microsomal protein.

TABLE 1

Apparent kinetic parameters for morphine-3-glucuronide formation catalyzed by microsomes of COS-1 cells expressing UGT2B7 alone and in combination with P450s

UGT activity was determined with five to six substrate concentrations up to 20 mM in the presence of 2 mM UDP-glucuronic acid. Data were fit to the Michaelis-Menten equation. Numbers in parentheses represent the value normalized to UGT2B7 singly expressed in COS-1 cells on the basis of UGT2B7 protein expression levels. Results are the estimated value \pm S.D.

Cells	K_m mM	V_{max} pmol/min/mg of protein	V_{max}/K_m
UGT2B7	0.38 ± 0.12	8.49 ± 0.20 (8.5)	22.3 (22.3)
CYP3A4/UGT2B7	3.71 ± 0.68	7.23 ± 0.41 (9.47)	1.9 (2.55)
CYP1A2/UGT2B7	0.48 ± 0.14	5.22 ± 0.17 (5.77)	10.9 (12.0)
CYP2C9/UGT2B7	0.20 ± 0.12	4.43 ± 0.16 (4.3)	22.2 (21.5)

TABLE 2

Apparent kinetic parameters for morphine-3-glucuronide formation catalyzed by human liver microsomes

UGT activity was determined with 18 substrate concentrations up to 5 mM for pooled human liver microsomes (HLM) in the presence of 2 mM UDP-GlcA. For individual HLM, assays were performed with eight substrate concentrations up to 5 mM. Data were fit to the Michaelis-Menten equation. Numbers in parentheses represent the value normalized by the UGT2B7 content that was assumed by using baculovirus cell lysate containing recombinant UGT2B7 protein as the standard. Results are the estimated value \pm S.D.

Cells	K_m mM	V_{max} pmol/min/mg of protein	V_{max}/K_m
Pooled HLM	1.18 ± 0.21	274 ± 24	232
Individual HLM A	1.30 ± 0.12	409 ± 14 (330)	315 (253)
Individual HLM B	0.76 ± 0.18	432 ± 32 (507)	568 (667)
Individual HLM C	1.17 ± 0.13	360 ± 14 (330)	308 (282)

ined whether exogenously added CYP3A4 can associate with the UGT on the PVDF membrane by detecting GST that was conjugated with CYP3A4. As shown in Fig. 3A, left, when the membrane was overlaid with neither GST nor GST-CYP3A4 fusion protein, anti-GST antibody used failed to detect any proteins on the membrane. A similar picture was observed when solubilized human liver microsomes were used (data not shown). On the other hand, when the membrane was overlaid with GST-tagged CYP3A4, a single band was detected (Fig. 3B; left). The apparent molecular size of this band corresponded to that of UGT2B7 in immunoblotting (Fig. 3A; right). Overlay with GST-CYP3A4 fusion protein on the membrane blotted with baculosomes not containing recombinant UGT2B7 exhibited no bands (Fig. 3B). In addition, the absence of signal in the lanes overlaid with nonfusion GST was also observed (Fig. 3B, right). These results strongly suggest that CYP3A4 specifically binds to UGT2B7.

Interactions between CYP3A4 and UGT2B7 in COS Cell Microsomes. The effects of the addition of purified CYP3A4 on morphine glucuronidation catalyzed by UGT2B7 in COS cell microsomes were examined. When untreated UGT2B7-expressing COS cell microsomes were used as the enzyme source, exogenous CYP3A4 caused no significant effect on the formation of M-3-G (Fig. 4A). However, the activity for M-6-G formation was slightly increased by CYP3A4 (Fig. 4B). The formation of M-3-G by UGT2B7 in *n*-octylglucoside-treated microsomes was comparable with that of the control (no treatment with detergent) (Fig. 4C). However, when the UGT2B7-expressing COS-1 cell microsomes were treated with *n*-octylglucoside, CYP3A4 significantly reduced activity to approximately half of that of the control (Fig. 4C). In contrast, the rates of M-6-G formation were significantly increased by CYP3A4 in a concentration-dependent manner (Fig. 4D). Thus, exogenous CYP3A4 produced marked effects on morphine glucuronidation only in UGT2B7-expressing microsomes that had been treated with detergent. This suggests that perturbation of the microsomal membrane is required to facilitate interaction between CYP3A4 and UGT2B7. The data described above also suggest that the regioselectivity of UGT2B7 is altered by the interaction with CYP3A4.

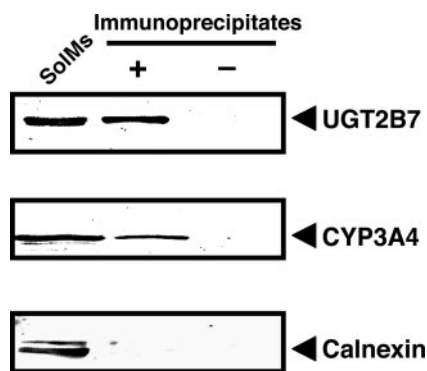


Fig. 2. Coimmunoprecipitation of UGT2B7 and CYP3A4 with goat anti-mouse UGT antibody. Sodium cholate solubilized human liver microsomes (100 μ g of protein) were subjected to immunoprecipitation with goat anti-mouse UGT antibody. Symbols (+) and (–) represent immunoprecipitates from anti-UGT antibody and nonimmune IgG, respectively. Immunoprecipitates obtained were subjected to Immunoblot analysis with primary antibodies against UGT2B7, CYP3A4, or CNX. SolMs represents solubilized human liver microsomes (10 μ g of protein). Details are described under *Materials and Methods*.

Discussion

The four complementary experimental approaches we have used strongly suggest functional interactions between P450 and UGT. To explore the significance of P450-UGT interactions on UGT function, morphine was used as a probe in this work because it is a substrate of UGT2B7 and does not require extensive metabolism by P450 before glucuronidation (Hasselström and Säwe, 1993). Morphine is an important and widely used opioid analgesic in clinical medicine, and its metabolism must be understood, given the influence of its metabolism on efficacy. Recombinant UGT2B7 expressed in COS cells showed some differences in the velocities of M-3-G formation in the absence and presence of coexpressed P450 (Table 1). It is interesting that coexpressed CYP3A4 greatly increased the K_m for M-3-G formation by UGT2B7 (Table 1). It is conceivable that this effect of CYP3A4 is because of changes in COS cell membrane properties by the overexpression of this P450. However, this possibility is unlikely because overexpression of other forms of P450 (CYP1A2 and -2C9) showed only minor effects on the kinetics of morphine glucuronidation. Although this study detected an interaction only between CYP3A4 and UGT2B7, the possibility that CYP1A2/2C9 might interact with UGTs distinct from UGT2B7 cannot be excluded. Ikushiro et al. (2004) reported

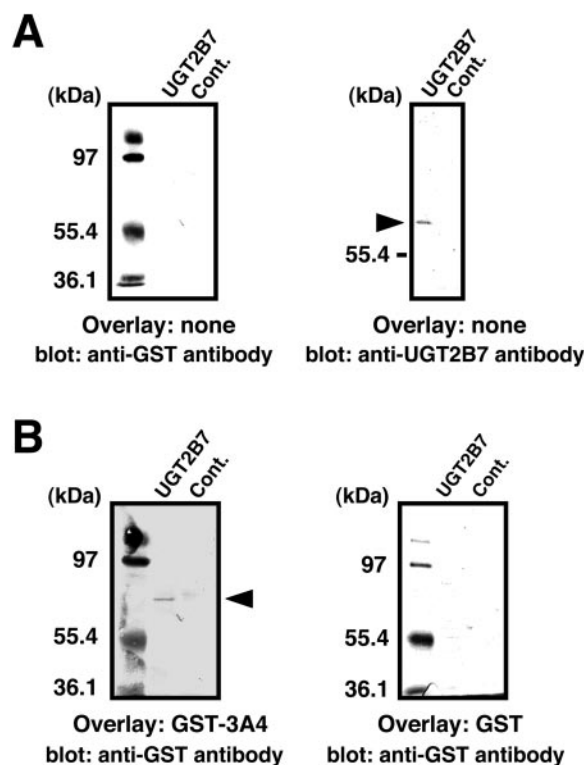


Fig. 3. Detection of interaction between CYP3A4 and UGT2B7 by overlay assay. Proteins in solubilized baculosomes (5 μ g) were separated by SDS-PAGE (10% gel), and transferred to polyvinylidene difluoride membrane. In the experiments shown in A, the membrane was treated either with anti-GST (left) or anti-UGT2B7 (right) antibody without pretreatment with GST/GST-CYP3A4. In the experiments shown in B, the membrane was overlaid either with GST-CYP3A4 fusion protein (left) or GST (right) and then treated with anti-GST antibody. The lanes labeled “UGT2B7” and “control” indicate baculosomes containing UGT2B7 and those lacking the UGT, respectively. The migration of molecular mass markers (kilodaltons) is indicated on the left of the figure. Arrowhead indicates UGT2B7 position.

recently that simultaneous expression of rat CYP1A1 and UGT1A6 in yeast does not alter the catalytic function of UGT1A6. Taking this and our present data into consideration, functional interactions between P450 and UGT probably occur in an isoform-specific manner. On the other hand, the absence of P450-UGT interactions in yeast may suggest the importance of the membrane environment for the interaction.

Biphasic kinetics of morphine-3-glucuronidation by human liver microsomes and/or recombinant UGT2B7 has been reported (Miners et al., 1988; Stone et al., 2003), although neither our experiments nor those of others observed the same mode of kinetics (Hasselström and Säwe, 1993; Court et al., 2003; Soars et al., 2003). The low- and high-affinity phases of biphasic kinetics were reported to show K_m values of 2.5 μ M and 798 μ M, respectively (Miners et al., 1988). If the biphasic nature of the kinetics is the case for UGT2B7-mediated glucuronidation, the CYP3A4-dependent alteration of UGT2B7 function observed in this work might affect only the low affinity-phase reaction.

Comparison of the morphine-glucuronidating activity of recombinant UGT2B7 with human liver microsomes revealed comparable K_m values for recombinant and hepatic microsomal UGT2B7, suggesting that the former is a good model for the latter UGT (Tables 1 and 2). The reason that the V_{max} for morphine glucuronidation varies between recombinant UGT2B7 and human hepatic microsomes seems to be attributable to the difference in the specific content of UGT2B7 in COS and hepatic microsomes. However, because recombinant and hepatic UGT2B7s exhibited comparable K_m values for M-3-G formation, these enzymes are expected to be

functionally equivalent. Recombinant CYP3A4 used in this study was confirmed to be functionally active using a specific substrate, 7-benzyloxy-4-(trifluoromethyl)coumarin. However, oxidation of morphine by P450 is not a cause of the increase in K_m exhibited by the CYP3A4-UGT2B7 double expression system, because the cofactor, NADPH, was not included in the incubation system. In vivo studies performed by other workers indicate that the major metabolites of morphine in human are the glucuronides and not oxidation products (Hasselström and Säwe, 1993). In addition, the K_m value for morphine oxidation mediated by P450 was reported to be more than 10 mM (Projean et al., 2003), which is greater than the K_m for UGT-mediated glucuronidation (1–4 mM). These data do not support the possibility that the binding of morphine to CYP3A4 is required for its interaction with UGT2B7.

When purified CYP3A4 was added to unperturbed COS cell microsomes containing UGT2B7, no change in the rates of formation of M-3-G was observed (Fig. 4A). However, when the microsomes were treated with a detergent, the activity of M-3-G formation was significantly reduced by exogenous CYP3A4 (Fig. 4C). This observation agrees with the data derived from experiments with the CYP3A4/UGT2B7 double expression system, in which CYP3A4 is shown to greatly reduce the catalytic efficiency of UGT2B7. It is assumed that the ratio of CYP3A4/UGT2B7 can be an important determinant for the modulation of UGT2B7 function by CYP3A4 for the following reason: minor increases (<2-fold) in the K_m for M-3-G formation were observed when purified CYP3A4 was added exogenously to the detergent-treated baculosomes in which recombinant UGT2B7 is expressed at far greater lev-

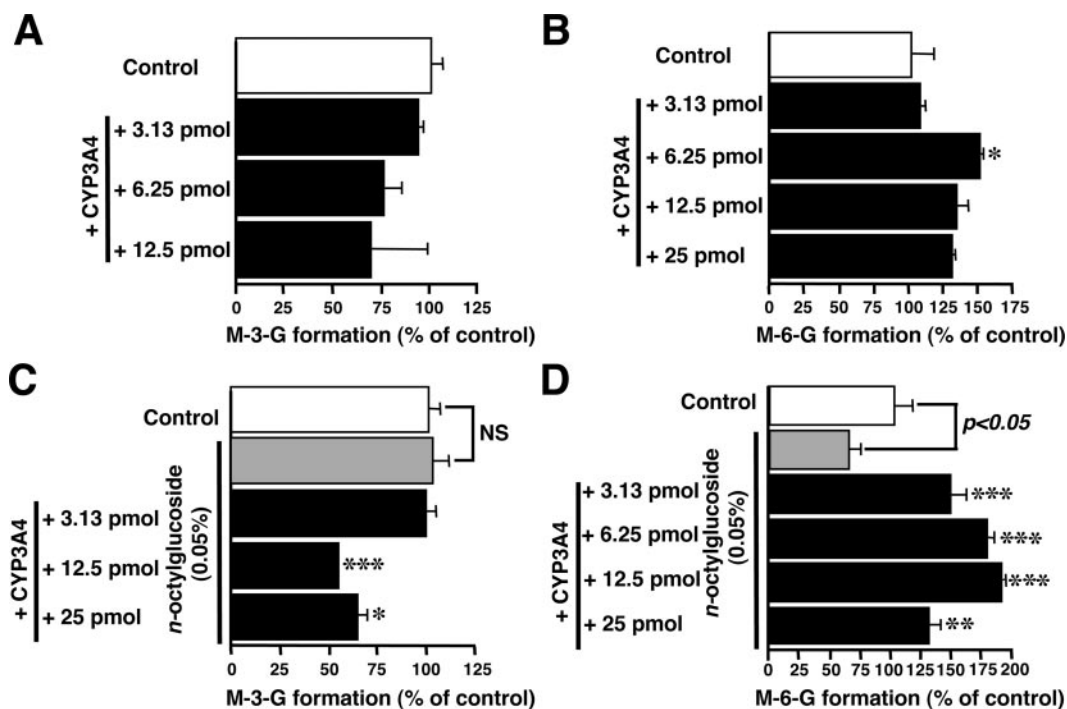


Fig. 4. The effect of purified CYP3A4 on UGT2B7-catalyzed morphine glucuronidation in the presence and absence of pretreatment of cellular microsomes with detergent. Microsomes prepared from UGT2B7 transfected cells were pretreated (C and D) or not pretreated (A and B) with *n*-octylglucoside as described under *Materials and Methods*. These microsomes were used for the assay of UGT2B7 activity toward the 3-hydroxyl (A and C) or 6-hydroxyl group (B and D) of morphine in the presence and absence of exogenous CYP3A4. The values are given as a percentage of the M-3-G or M-6-G formation activity in COS-1 cell microsomes expressing UGT2B7 alone (Control). Each bar represents the mean \pm S.D. of three determinations. In A and B, significant difference from control group (*, $p < 0.01$) is indicated. In C and D, significant difference from *n*-octylglucoside treated control group (**, $p < 0.005$; ***, $p < 0.001$) is indicated. NS, not significant. Statistical significance was calculated by Scheffé's F test.

els (>100-fold) than in COS-1 cells transfected with UGT2B7-carrying vector (data not shown). These phenomena were consistent with the results presented in Fig. 4. The data also suggest that productive interactions between UGT2B7 and CYP3A4 require cointegration into the microsomal membrane, as in the coexpression system or perturbation of the membrane environment (i.e., with detergent) to facilitate the interaction. Furthermore, because the purified recombinant CYP3A4 used in these experiments lacks the N-terminal membrane anchor region, this region would not be involved in the interaction between CYP3A4 and UGT2B7. In agreement with this, overlay experiment in this study showed that CYP3A4 lacking its N terminus binds to UGT2B7. The P450 molecule, except for its N terminus, was thought to be exposed on the cytoplasmic side of the ER (Sato et al., 1990; Edwards et al., 1991). However, accumulating evidence suggests that some domains of the P450 molecule are integrated into the ER membrane to permit full catalytic activity (Ahn et al., 1998). These domains may be involved in interaction with the UGT2B7 protein. The data from the P450/UGT2B7 double expression and immunoprecipitation experiments demonstrate that UGT2B7 favorably associates with CYP3A4 but not other ER proteins including other P450s and CNX. In addition, we examined the effect of BSA as a drug carrier protein on morphine UGT activities of COS-1 cells containing UGT2B7 and human liver. The results showed that BSA exhibits no marked effect on M-3-G formation in either preparation, indicating the absence of BSA-facilitated metabolism or enzyme inhibition on M-3-G formation (data not shown). This phenomena was consistent with the observation that the majority (>80%) of morphine in the body exists as the free form (Wu et al., 1997) and would not require any carrier protein(s) for biotransformation.

It has long been known that there are marked interindividual differences in the pharmacological effects of morphine. Genetic variations of the opioid μ receptor reported thus far have not successfully explained these differences (van Lingen et al., 2002). They may be partly explained by genetic polymorphisms in the coding and regulatory regions of the *UGT2B7* gene. However, morphine glucuronide-to-morphine plasma ratios are unaffected by two major alleles *UGT2B7*1* (His268) and *UGT2B7*2* (Tyr268) in cancer patients on long-term morphine therapy (Holthe et al., 2002). In agreement with this, no effect of *UGT2B7*1* and *UGT2B7*2* genotype on human liver microsomal glucuronidation or UGT2B7 protein content and stability was observed (Court et al., 2003; Barbier et al., 2000). However, it should be noted that mutation of the *UGT2B7* gene promoter reduces transcription, and this may contribute to the different sensitivity toward morphine in rare cases (Duguay et al., 2004). We propose another cause of this variability. Our data demonstrate that higher expression of CYP3A4 increases morphine action not only by suppressing detoxication (M-3-G formation) but also by increasing activation (M-6-G formation). Because the expression levels of CYP3A4 vary among persons (Thummel and Wilkinson, 1998), it seems reasonable to suppose that CYP3A4 content in hepatic microsomes is one of the determinants of morphine clearance in vivo. To our knowledge, this is the first report to demonstrate a functional interaction between P450 and UGT. This study supports the novel concept that P450 enzymes can affect glucuronidation capacity by direct protein-protein interactions with UGT.

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