Protein−Protein Interactions between UDP-Glucuronosyltransferase Isozymes in Rat Hepatic Microsomes†

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ABSTRACT: The interactions between UDP-glucuronosyltransferase (UGT) isozymes, UGT1s and UGT2B1, in rat hepatic microsomes were investigated using an immunopurification technique with anti-peptide antibodies and a chemical cross-linking strategy. A 50 kDa protein coimmunopurified with UGT1s was identified as UGT2B1 by amino-terminal sequencing and immunodetection with anti-peptide antibody against UGT2B1. Evidence for direct interaction of UGT2B1 with UGT1s was obtained by the loss of UGT2B1 adsorption to immunoaffinity column in Gunn rat hepatic microsomes, which lack all UGT1 isozymes. When the microsomes were treated with the chemical cross-linking reagent 1,6-bis(maleimido)-hexane, a cross-linked product with an apparent molecular mass of 120−130 kDa was obtained that immunostained with antibodies against UGT1s and UGT2B1, indicating the formation of a heterodimer containing one of the UGT1 isozymes and UGT2B1. The effects of UGT complex formation on the stimulation of glucuronidation of testosterone and uptake of UDP-glucuronic acid (UDP-GlcUA) by UDP-*N*-acetylglucosamine (UDP-GlcNAc) were examined. Alkaline pH-induced dissociation of the complexes was associated with the loss of UDP-GlcNAc-dependent stimulation of glucuronidation, suggesting that two functional states of UGTs with different kinetic parameters correspond to the monomer and oligomer form of UGTs in the membranes. The UDP-GlcNAc-dependent stimulation of UDP-GlcUA uptake into the microsomal vesicles also was affected by the extent of complex formation. These results suggest that complex formation of the UGT isozymes affects the UDP-GlcNAc-dependent stimulation of glucuronidation via stimulation of UDP-GlcUA uptake.

UDP-Glucuronosyltransferases (UGTs,[1] EC 2.4.1.17) are a family of membrane-bound enzymes that catalyze the conjugation of endogenous and exogenous compounds with UDP-glucuronic acid (UDP-GlcUA) (Dutton, 1980; Burchell & Coughtrie, 1989). On the basis of sequence similarities of UGTs, the isozymes are divided into two families termed *UGT1* and *UGT2* (Burchell et al., 1991). The UGT1 family consists of several isozymes with variable amino-terminal domains and an identical carboxyl-terminal domain, which are produced by the *UGT1* gene complex (Iyanagi, 1991; Ritter et al., 1992; Emi et al., 1995, 1996; Ikushiro et al., 1995). The UGT1 isozymes catalyze the glucuronidation of bilirubin and phenol-like compounds. In contrast to the UGT1s, the UGT2 isozymes, which catalyze the glucuronidation of steroids, are encoded by separate distinct genes (Mackenzie et al., 1992). The carboxyl-terminal region of UGTs is highly conserved and involved in the binding of the common cosubstrate UDP-GlcUA (Mackenzie et al., 1990a). On the other hand, the variable amino-terminal halves of UGTs are involved in the binding of aglycons and are thought to determine the substrate specificity for glucuronidation.

UGT-dependent glucuronidation in the microsomal membranes is known to have complex regulatory properties. Glucuronidation activity is lower in intact microsomes as compared with those in membrane-disrupted microsomes, a phenomenon called "latency" (Zakim & Dannenberg, 1992; Jansen et al., 1992). From sequencing data, a common membrane topology for all UGTs is predicted, where UGTs are anchored in the endoplasmic reticulum (ER) membranes by a potential transmembrane region in the carboxyl-terminal domain with the bulk of the polypeptide chain located within the cisternal lumen of the ER (Iyanagi et al., 1986; Mackenzie, 1986, 1990a). Therefore, this predicted membrane topology necessitates translocation of UDP-GlcUA to the lumen or membranes of the ER, where the catalytic site of UGTs is located. According to this "compartmentation model", rate-limiting transport of UDP-GlcUA by a putative membrane transport protein(s) has been postulated as a reason for latency (Hauser et al., 1988; Bossuyt & Blanckaert, 1994; Radominska et al., 1994). Bossuyt et al. (1995) and Berg et al. (1995) reported that UDP-*N*-acetylglucosamine (UDP-GlcNAc) as an activator of glucuronidation trans-stimulates the uptake of UDP-GlcUA into microsomal vesicles via membrane transport systems. An alternative explanation for the latency in UGT activity is the "conformational model" proposed by Zakim and co-workers (Zakim & Dannenberg, 1992; Dannenberg et al., 1990; Kavecansky et al., 1992). On the basis of a kinetic study of UGTs in microsomes and in a reconstituted system, they have

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[1] Abbreviations: BMH, 1,6-bis(maleimido)hexane; CN-II, Crigler−Najjar syndrome type II; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulumn; GlcNAc, *N*-acetylglucosamine; GlcUA, glucuronic acid; NEM, *N*-ethylmaleimide; stilbenedisulfonate maleimide, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; UGT, UDP-glucuronosyltransferase.

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hypothesized that the UGTs in the membranes can exist in a different functional state with different activity. UGT activity in the membranes is functionally constrained by a suppressive conformation of UGT and the functional states may be affected by monomer−oligomer formation in UGTs. According to this model, UDP-GlcNAc acts as an allosteric activator with binding to UGTs (Vessey et al., 1973).

Many observations have provided evidence for UGT complex formation. The tendency to form aggregates of purified UGT preparations has been reported. Purified chenodeoxycholic UGT isozyme appears to exist as a aggregate (Matern et al., 1982). Matsui and Nagai (1986) isolated a hybrid form of UGT isozymes, which consists of testosterone and androsterone UGTs. Another line of evidence for UGT complex is drawn from radiation inactivation analysis, a method to determine the molecular mass of membrane-bound enzymes *in situ* (Peters et al., 1984; Vessey & Kempner, 1989). From the radiation dose dependence of various glucuronidating activities, it has been suggested that the dimer and/or tetramer of UGTs is the active form for enzymatic activity. Bock et al. (Gschaidmeier & Bock, 1994; Bock et al., 1992) suggested that the unusually high induction factors for diglucuronide formation of polycyclic aromatic hydrocarbons is due to the interaction of multiple UGT subunits in the microsomes. Koiwai et al. (1996) reported the molecular analysis of the Crigler−Najjar syndrome type II (CN-II), which is characterized by unconjugated hyperbilirubinemia. They postulated that the disease in some cases of CN-II patients may be caused by a dominant mutation that results from formation of a tetrameric subunit structure of bilirubin−UGT. Thus, the interactions between UGTs may be responsible for the modulation of their glucuronidating activities including the problems of latency.

In this study, we have analyzed the protein−protein interactions between UGT isozymes, the UGT1s and UGT2B1, using an immunopurification procedures with anti-peptide antibodies and chemical cross-linking experiments. The complex formations between UGT isozymes in microsomal membranes could affect the UDP-GlcNAc-dependent stimulation of glucuronidation via the stimulation of UDP-GlcUA uptake into microsomal vesicles.

EXPERIMENTAL PROCEDURES

Preparation of Rat Hepatic Microsomes. Male Wistar rats and homozygous Gunn rats (5−6 weeks, 150−200 g) were obtained from Clea Inc. (Kyoto, Japan) and SLC Inc. (Shizuoka, Japan), respectively. Phenobarbital treatment of Gunn rats was performed as described previously (Emi et al., 1995). Hepatic microsomes were prepared as described previously (Ikushiro et al., 1995). Protein concentration of the microsomes was determined with BCA protein assays kits (Pierce Chemical, Co.) using bovine serum albumin as a standard.

Preparation of Anti-peptide Antibodies. UGT1-specific anti-peptide antibody, designated COM, was used for immunopurification and immunostaining (Ikushiro et al., 1995). To prepare UGT2B1-specific anti-peptide antibody, the region of carboxyl-terminal sequence (517−529, CRKTANMGKKKKE) in rat UGT2B1 deduced from cDNA sequence was selected (Mackenzie, 1986). The peptide was synthesized and monospecific anti-peptide antibody against UGT2B1, designated 2B1, was prepared as described previously (Ikushiro et al., 1995).

Immunopurification of UGT Isozymes Using Specific Antibody-Conjugated Sepharose-4B. For immunopurification procedures, COM- and 2B1-conjugated Sepharose-4B columns were prepared as previously described (Ikushiro et al., 1995). Rat hepatic microsomes (2 mg/mL) were solubilized with extraction buffer containing 20 mM Tris-HCl, pH 7.5, 200 mM KCl, 20% (v/v) glycerol, and 1% (w/v) Emulgen 913. For examination of pH effects on UGT complex formation, the pH used in extraction and column operations was maintained in a pH range from 6.5 to 9.0. The extracts (10 mg of proteins) were applied to a COM-conjugated Sepharose-4B (1.5 × 12 cm) and extensively washed with 0.5% (w/v) Emulgen 913 buffer. The adsorbed proteins were eluted by washing with 0.1 M glycine hydrochloride, pH 3.0, and then collected in 1 mL fractions. For elution by antigen peptide, a 0.4 mM peptide solution of COM-peptide was used. The immunopurified fractions were analyzed by immunostaining following SDS−PAGE and enzyme-linked immunosorbent assay (ELISA) with UGT-specific antibodies. For the analysis of immunostaining, the fractions were concentrated by the methods of Wessel and Flügge (1984).

To determine the amino-terminal amino acid sequence of the 50 kDa protein, the immunopurified fractions containing the 50 kDa protein were isolated following electroblot of SDS−PAGE. The band of 50 kDa proteins on the membrane was cut out and sequenced using an Applied Biosystems Model 473A protein sequencer.

Immunoblot and ELISA Analysis. The immunopurified fractions were subjected to SDS−PAGE (10% separation gel) and silver staining and/or immunostaining following the electroblot to nitrocellulose membranes. The membranes were blocked at 25 °C for 12 h in 50 mM Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl and 1.5% (w/v) BSA, followed by an incubation with diluted antibodies at 25 °C for 2 h. The membranes were washed and incubated with a 1:5000 dilution of goat anti-rabbit IgG−alkaline phosphatase. Immunodetection was developed by adding 1.7 mg/mL bromochloroindolyl phosphate and 0.7 mg/mL nitro blue tetrazolium. For ELISA, 10 μL/well of immunopurified fractions were incubated at 25 °C for 12 h in coating buffer containing 30 mM $NaHCO_3$, pH 10.4, and 1% (w/v) sodium cholate. Coated wells were blocked by incubation with 50 mM Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl and 1.5% (w/v) BSA at 25 °C for 4 h. The diluted antibodies were added to the wells at 25 °C for 2 h. The wells were washed four times with 10 mM potassium phosphate buffer, pH 7.5, containing 0.15 M NaCl and 0.05% (w/v) Tween 20. Goat anti-rabbit IgG−horseradish peroxidase was used in the UGT detection, following by color development with 0.75 mg/mL 2,2-azinobis(3-ethylbenzothiazoline)sulfonic acid in 10 mM PIPES, pH 6.5, and 0.001% (v/v) H_2O_2 detected at 405 nm.

1,6-Bis(maleimido)hexane Cross-Linking Experiment. The cross-linking regent BMH (Pierce Chemical Co.) was dissolved in dimethyl sulfoxide in a 50 mM stock solution. The stock solution was added to 100 μL of microsomal protein solution containing 0.1 mg of proteins, 10 mM Tris-HCl, pH 7.5, 0.25 M sucrose, and 1 mM $MgCl_2$ to give a concentration in the assay of 0.1−2 mM BMH. For inhibition experiments by 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (stilbenedisulfonate maleimide, Molecular Probes Inc.), the microsomes were preincubated with 2 mM stilbenedisulfonate maleimide at 25 °C for 60 min before

the cross-linking reaction. The cross-linking reaction was carried out at 25 °C for 60 min. The reaction was quenched by adding 20 mM 2-mercaptoethanol. The protein samples were concentrated and dissolved with sample buffer. The samples (20 μg of proteins) were analyzed by SDS−PAGE in a gradient gel (4−10%) and immunostaining with antibodies COM and 2B1.

Testosterone Glucuronidation Assay. Glucuronidation of the 17β-hydroxyl group of testosterone was determined by the HPLC method of Matsui and Nagai (1980) with some modifications. The incubation medium containing the microsomal proteins (0.1 mg of proteins), 10 mM PIPES, pH 6.5−7.3, or 10 mM Tris-HCl, pH 7.5−8.8, 0.25 M sucrose, 1 mM $MgCl_2$, 0.1−4 mM UDP-GlcUA, and 1 mM testosterone in a total volume of 200 μL. Before assay incubation, the microsomes were preincubated at 37 °C for 60 min in the absence or presence of 1 mM UDP-GlcNAc. For the assay of the microsomal membrane permeabilized state, to the microsomes was added 0.05 mg of alamethicin, a pore-forming oligopeptide, (mg of protein)$^{-1}$ or 0.05% (w/v) Lubrol PX after the incubation with UDP-GlcNAc (Fulceri et al., 1994). The incubation was 5−20 min at 37 °C and was terminated by adding 100 μL of methanol. The reaction mixture was centrifuged at 15K rpm for 15 min and the resultant supernatant was analyzed under the following HPLC conditions. The glucuronides were chromatographed on Microsorb-MV C18 (4.6 × 150 mm, Rainin Inc.) using isocratic solvent containing 40% (v/v) methanol and 0.01 N HCl. The testosterone glucuronides were detected at 238 nm and quantified from the peak area of standard samples. The data and peak area determinations were made using an automatic system consisting of auto sampler (Model AS-8020, Tosoh Inc.), data processor system (MacIntegrator I, Rainin Inc.), and an HPLC system (Hitachi L-6200 Intelligent system, Hitachi Co.). Kinetic parameters for the glucuronidation were determined with a nonlinear-least-squares fitting program.

UDP-GlcUA Uptake Assay. Uptake of UDP-GlcUA into microsomal vesicles was determined using a rapid filtration assay (Hauser et al., 1988). Briefly, the microsomes (0.2 mg of proteins) were incubated within an assay mixture containing 10 mM PIPES, pH 6.5, or 10 mM Tris-HCl, pH 7.5 or 8.5, 0.25 M sucrose, 1 mM $MgCl_2$, and 2.5 μM UDP-GlcUA containing radiolabeled UDP-GlcUA [925 Bq of UDP-^{14}C(U)-GlcUA, ARC Inc.]. Before the uptake assay, the microsomes were preincubated for 60 min at 37 °C in the absence or presence of 1 mM UDP-GlcNAc. After assay incubation for 2 min at 37 °C, the reaction mixture were immediately filtered through nitrocellulose membranes (pore size 0.45 μm). The filter was washed with 0.4 mL of ice-cold buffer containin 3 mM UDP-GlcUA, followed by 5 mL of ice-cold buffer. Each filter was dissolved in 3 mL of Clearsol (Nacalai Tesq.) and the radioactivity was determined in a Beckman LS-6000TA scintillation counter.

RESULTS

Identification of 50 kDa Protein(s) Coimmunopurified with UGT1 Isozymes from Rat Hepatic Microsomes. We have prepared an anti-peptide antibody against carboxyl-terminal region of the UGT1 isozymes (Ikushiro et al., 1995). The antibody-conjugated Sepharose 4B columns were used to study the expression of UGT1 isozymes in rat hepatic microsomes. Figure 1A shows silver staining of immunopurified fractions using the UGT1-specific antibody- (COM-) conjugated column. Most solubilized UGT1 proteins from total microsomes (10 mg) completely adsorbed to the antibody column because of the absence of immunoreactive proteins in the flowthrough fractions. The main bands of the immunopurified proteins (fractions 54−61) migrated to the 53−57 kDa region (upper arrow in Figure 1A) and were identified as UGT1 isozymes (Ikushiro et al., 1995). In addition to the identified UGT1 proteins, fractions 51−57 contained other proteins, 50 kDa (lower arrow in Figure 1A) and 45 kDa, which were coeluted with UGT1s. Only the 50 kDa protein was coeluted by elution buffer containing COM-peptide as antigen, suggesting that the 50 kDa protein binds to COM directly or to the UGT1 isozymes indirectly (data not shown). The immunostaining and silver staining patterns of the fractions containing 50 kDa protein(s) (fractions 51−57) are shown in panels 1 and 2 of Figure 1B, respectively. The silver-stained band corresponding to the 50 kDa protein was found to not be immunostained with COM, which reacts with the common carboxyl-terminal region of UGT1 isozymes. This indicates that the 50 kDa protein does not belong to the family of UGT1 isozymes.

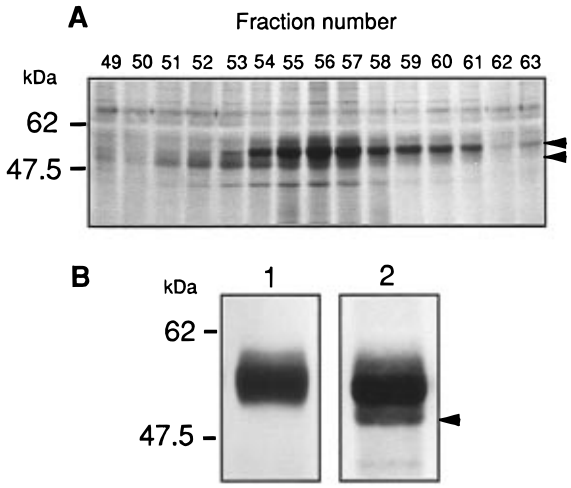

FIGURE 1: Coelution of a 50 kDa protein with UGT1s from UGT1-specific peptide antibody-conjugated Sepharose-4B column. (A) The immunopurified fractions eluted from UGT1-specific antibody-conjugated column were electrophoresed on an SDS−10% polyacrylamide gel and detected by silver staining. Upper and lower arrows point to UGT1 isozymes and 50 kDa protein, respectively. (B) The immunopurified fractions (fractions 51−57) were collected and concentrated. The sample was electrophoresed on an SDS−10% polyacrylamide gel and detected by immunostaining with COM (panel 1) and by silver staining (panel 2). Arrow points to the 50 kDa protein.

To identify the 50 kDa protein coeluted with UGT1 isozymes, the corresponding band on the SDS−polyacrylamide gel was transferred to membranes and sequenced (Table 1). The major amino-terminal amino acid sequence of 50 kDa protein was identical to that deduced from rat UGT2B1 cDNA, which is one of the isozymes of the UGT2 family (Mackenzie, 1986). Minor species having amino acid composition showed in parentheses in Table 1 were found to be other UGT2 isozymes, UGT2B3 and UGT2B6 (Mackenzie, 1987, 1990b).

To obtain further evidence that the major species of the 50 kDa protein is UGT2B1 isozyme, anti-peptide antibody against the carboxyl-terminal end of UGT2B1 (2B1) was prepared and used for immunoblot analysis of coeluted fractions with UGT1s. Figure 2A−C shows the elution patterns of immunopurified fractions immunostained with

Table 1: Comparison of Amino-Terminal Amino Acid Sequences between 50 kDa Protein(s) and UGT2 Isozymes

	Amino-terminal amino acid No.[a]									
	1	2	3	4	5	6	7	8	9	10
50kDa	G	K	V	L	V	W	P	T	E	Y
								(M)[b]		(F)
UGT2 Isozymes										
UGT2B1	G	K	V	L	V	W	P	T	E	Y
UGT2B2	G	K	V	L	V	W	P	M	D	F
UGT2B3	G	K	V	L	V	W	P	M	E	F
UGT2B6	G	K	V	L	V	W	P	M	E	F

[a] Amino-terminal amino acid sequences of UGT2 isozymes deduced from cDNA sequence are expressed as the putative mature form. [b] Residues in parentheses show the minor peak of sequence cycles.

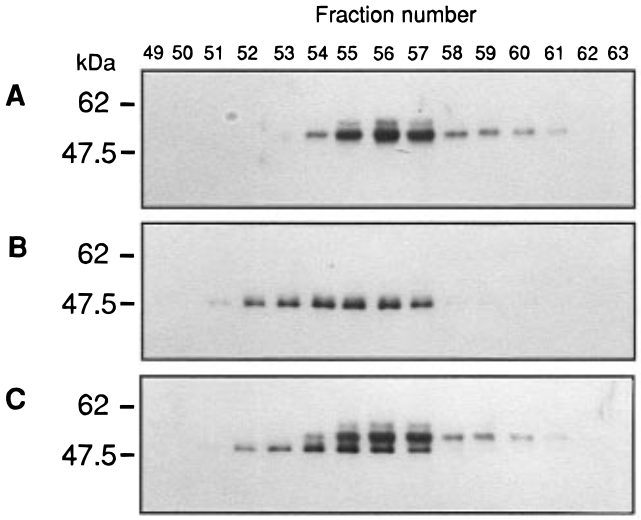

FIGURE 2: Identification of the 50 kDa protein coeluted with UGT1s as UGT2B1. The immunopurified fractions eluted from the COM-conjugated column corresponding to the fractions in Figure 1A were electrophoresed on an SDS−10% polyacrylamide gel and detected by immunostaining with (A) COM, (B) 2B1, and (C) COM plus 2B1.

COM, 2B1, and COM plus 2B1, respectively. The 2B1-immunostained bands between fractions 52 and 57 correspond to the coeluted 50 kDa protein. These results clearly establish that the 50 kDa protein coeluted with UGT1 isozymes is the UGT2B1 isozymes.

Direct Interaction of UGT2B1 with UGT1 Isozymes. In order to determine whether UGT2B1 bound directly or indirectly to the COM-conjugated column, the following experiments using Gunn rat hepatic microsomes were done. The Gunn rat is hyperbilirubinemic and has no UGT1 enzymatic activity because of the frameshift mutation in the common region of the *UGT1* gene complex (Emi et al., 1995; Ikushiro et al., 1995). Figure 3 shows the ELISA analysis of immunopurified fractions from normal Wistar (A) and Gunn (B) rat hepatic microsomes using the UGT2B1-specific antibody. Microsomes from phenobarbital-treated Gunn rats were used for immunopurification since the expression level of UGT2B1 was low in microsomes from untreated rats (data not shown). The immunoreactive peak corresponding to the coeluted UGT2B1 was found in the fractions between 53 and 60 (Figure 3A). In contrast to the normal rats, the immunoreactive peak of UGT2B1 was not found in Gunn rats (Figure 3B). The expression of UGT2B1 in Gunn rat microsomes was normal because of the presence of significant immunoreactive peak in flowthrough fractions (fractions 10−40). These results clearly show that UGT2B1 binds indirectly to the COM-conjugated column via UGT1 isozymes and establishes the interaction of UGT2B1 with the UGT1 isozymes.

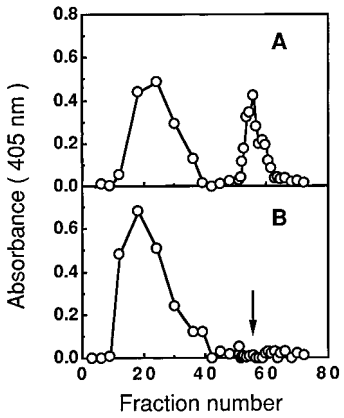

FIGURE 3: Adsorption of UGT2B1 to COM-conjugated column is lost when the sample is derived from Gunn rat hepatic microsomes lacking UGT1s. Hepatic microsomes from (A) normal or (B) Gunn rats were solubilized and applied to the COM-conjugated column as described in Experimental Procedures. The content of UGT2B1 in the each fraction was analyzed by ELISA with 2B1. Arrow in panel B shows the fraction correspond to elution peak fraction in panel A.

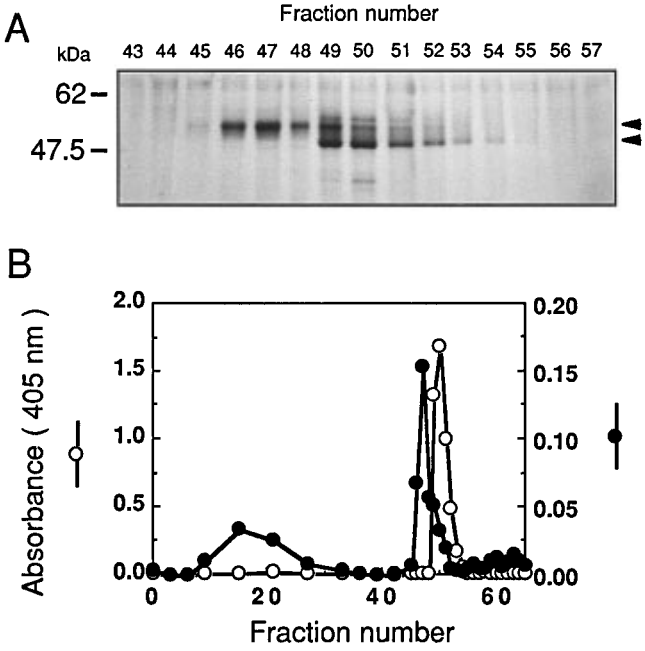

FIGURE 4: UGT1s can be coeluted with UGT2B1 from a UGT2B1-specific peptide antibody-conjugated Sepharose-4B column. (A) The immunopurified fractions eluted from the UGT2B1-specific antibody-conjugated column were electrophoresed on an SDS−10% polyacrylamide gel and detected by silver staining. Upper and lower arrows point to UGT1 isozymes and UGT2B1, respectively. (B) The same fractions were also analyzed by ELISA with 2B1 (○) or COM (●). Fractions 47 and 50 in panel A correspond to the peak fraction of UGT1s and UGT2B1 in panel B, respectively.

To confirm the protein−protein interactions between UGT isozymes, a 2B1 antibody-conjugated Sepharose-4B column was prepared. Figure 4A,B shows the silver staining and ELISA of immunopurified fractions using the 2B1-conjugated column, respectively. As expected, the UGT1 isozymes (fractions 45−51) were found to be coeluted with the UGT2B1 isozyme (fractions 49−53). These results clearly

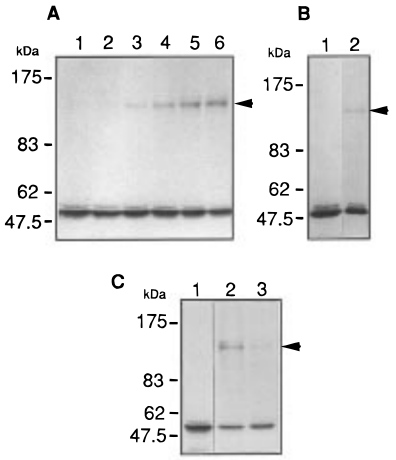

FIGURE 5: Cross-linking of a heterodimer formed between UGT1s and UGT2B1. Hepatic microsomes (20 μg of proteins) are incubated with various concentrations of BMH at 25 °C for 60 min and analyzed by immunostaining with (A, C) COM or (B) 2B1 following SDS−PAGE in a gradient gel (4−10%) as described in Experimental Procedures. Before cross-linking in lane 3 of panel C, the microsomes were treated with 2 mM stilbenedisulfonate maleimide at 25 °C for 60 min. BMH concentrations are as follows: (A) lane 1, 0 mM; lane 2, 0.1 mM; lane 3, 0.2 mM; lane, 4, 0.5 mM; lane 5, 1 mM; lane 6, 2 mM; (B) lane 1, 0 mM; lane 2, 2 mM: (C) lane 1, 0 mM; lanes 2 and 3, 1 mM. Arrows in each panel point to the cross-linked products of UGTs.

show the interaction between UGT1 isozymes and UGT2B1 isozyme to form a heterologous isozyme complex.

Cross-Linking of UGT Isozymes Using BMH. To verify the interactions between UGT isozymes and determine the number of UGT isozymes in the complex, we used the cross-linking reagent BMH, which is a homobifunctional cross-linker that reacts with sulfhydryl groups of proteins. Figure 5 shows immunostaining analysis of BMH-treated microsomes using COM (panels A and C) and 2B1 (panel B) antibodies. With increasing concentrations of BMH, the abundance of each cross-linked form increased while monomer forms decreased (Figure 5A,B). The apparent molecular mass of each cross-linked product on SDS−PAGE was about 120−130 kDa, which could be formed by a dimer of UGTs (50−60 kDa). The cross-linked products of the same apparent molecular mass were also formed with other cross-linking reagents, maleimidobenzoyl *N*-hydroxysuccinimide (MBS) and 1-ethyl-3-[3-(diethylamino)propyl]carbodiimide (EDC), but with very modest yields (data not shown). Pretreatment of the microsomes with membrane-impermeable stilbenedisulfonate maleimide inhibited the cross-linking to UGT by BMH (lane 3 in Figure 5C). This suggests that the cross-linked site of sulfhydryl group(s) in UGTs is located on the outside of microsomal membranes. Therefore, these results strongly suggest that one of the UGT1 isozymes interacts with UGT2B1 to form heteromeric dimers in microsomal membranes.

pH Effects on UGT Complex Formation. The effects of pH on the complex formation between UGT2B1 and UGT1s were examined using the COM-conjugated immunoaffinity column and ELISA analysis. The pH of solubilized microsomes and column elution buffers was changed in a pH range from 6.5 to 9.0. The elution patterns of immunopurified fractions containing UGT1s and UGT2B1 at pH 6.5, 7.5, and 8.5 are shown in Figure 6A. At pH 6.5, the elution patterns of UGT2B1 isozymes coeluted with UGT1s showed two peaks around fractions 54 and 57. The latter corresponded to the peak of UGT1s. Using pH 7.5 as a standard

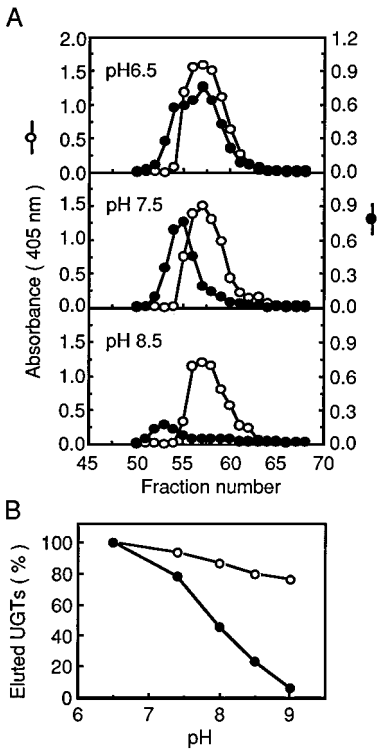

FIGURE 6: Loss of complex formation between UGT1s and UGT2B1 at alkaline pH. (A) The immunopurifications were performed at the following buffer pHs: pH 6.5 (upper panel), pH 7.5 (middle panel), and pH 8.5 (lower panel). The presence of UGT1s and UGT2B1 in the eluted fractions (fractions 50−68) were analyzed by ELISA with COM (○) and 2B1 (●). (B) The pH of solubilized microsomes and column elution buffers was changed in a pH range from 6.5 to 9.0. The amounts of eluted UGT (○, UGT1s; ●, UGT2B1) at each pH are expressed as ratio of peak area of eluted UGTs at pH 6.5.

buffer condition, the immunoreactive peak of UGT2B1 isozymes shifted to fractions 54−55. The coeluted UGT2B1 isozyme was significantly decreased at pH 8.5 compared to acid or neutral pH conditions. In contrast to the immunoreactive peaks of UGT2B1, the peaks of UGT1s were not so affected by pH change. As shown in Figure 6B, pH dependence of the complex formation confirms that the interaction of UGT2B1 with UGT1s is sensitive to pH change and the complex dissociates in alkaline pH conditions above pH 8.0.

pH Effects on UDP-GlcNAc-Dependent Stimulation of Testosterone Glucuronidation and UDP-GlcUA Uptake into Microsomal Vesicles. To examine the effect of UGT complex formation on the activity of glucuronidation in microsomes, glucuronidation of testosterone (which is mainly catalyzed by UGT2B1 in the microsomes) was measured under the same reaction conditions used in the pH study on complex formation. Figure 7A shows UDP-GlcUA concentration dependence of testosterone 17β-glucuronidating activity in the absence or presence of UDP-GlcNAc. The addition of UDP-GlcNAc to the microsomes changed the apparent V_{max} at pH 6.5 (3.3-fold) and pH 7.5 (2.0-fold) compared to each unstimulated condition. In contrast to acid or neutral pH conditions, the simulation effect of UDP-GlcNAc was not found at the alkaline condition of pH 8.5. Figure 7B shows the pH dependence of the specificity constant, V_{max}/K_m, values in a pH range from pH 6.5 to 8.8. The decrease of V_{max}/K_m value at alkaline pH is strongly correlated with loss of complex formation of UGT2B1 with UGT1s as shown in Figure 6B.

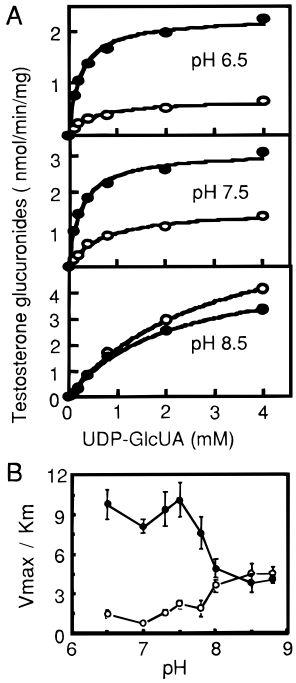

FIGURE 7: Alkaline pH induces the change of kinetic parameters of testosterone glucuronidation in the presence of UDP-GlcNAc. (A) The UDP-GlcUA concentration dependence on the initial rate of testosterone glucuronidation was measured at the following buffer pHs: pH 6.5 (upper panel), pH 7.5 (middle panel), and pH 8.5 (lower panel). The microsomes were preincubated at 37 °C for 60 min in the absence (○) or presence (●) of 1 mM UDP-GlcNAc. (B) pH dependence of specificity constants, V_{max}/K_m. Apparent kinetic parameters K_m (millimolar) and V_{max} (nanomoles per minute per milligram) were determined in a pH range from 6.5 to 8.8 as described under Experimental Procedures. Data are means for five separate determinations, and vertical bars denote the mean ± SD. The microsomes were preincubated at 37 °C for 60 min in the absence (○) or presence (●) of 1 mM UDP-GlcNAc.

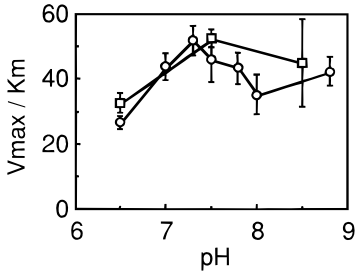

FIGURE 8: Alkaline pH-induced change of kinetic parameters of testosterone glucuronidation is lost in the permeabilized microsomes. For the UGT assay for membrane-permeabilized condition, the microsomes were added to alamethicin (○) or Lubrol PX (□). Apparent kinetic parameters K_m (millimolar) and V_{max} (nanomoles per minute per milligram) were determined in a pH range from 6.5 to 8.8 as described under Experimental Procedures. Data are means for five separate determinations, and vertical bars denote the mean ± SD.

Alamethicin is a pore-forming oligopeptide that makes the microsomes permit the free access of UDP-GlcUA to UGT isozymes in the lumen of microsomal vesicles. The addition of the reagent to microsomes is thus known to fully activate glucuronidation without the solubilization of UGT isozymes. As shown in Figure 8, the alamethicin treatment resulted in a increase of the V_{max} and V_{max}/K_m in a pH range from 6.5 to 8.8. In contrast to the intact microsomes (Figure 7B, ●), it should be noted that the decrease of V_{max}/K_m value in alkaline pH was not found in the permeabilized microsomes. The addition of a low concentration of Lubrol PX, a nonionic

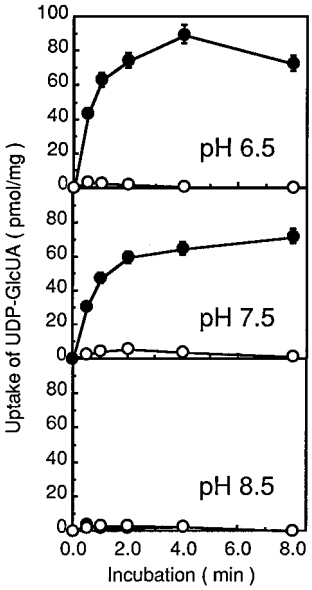

FIGURE 9: UDP-GlcNAc-dependent stimulation of UDP-GlcUA uptake into rat hepatic microsomes is lost at alkaline pH. The time course of UDP-GlcUA uptake into microsomal vesicles was observed at the following buffer pHs: pH 6.5 (upper panel), pH 7.5 (middle panel), and pH 8.5 (lower panel). After preincubation of microsomes with (●) or without (○) 1 mM UDP-GlcNAc, uptake of UDP-GlcUA was assayed by the rapid filtration method described in Experimental Procedures.

detergent, also caused the loss of the decrease of V_{max}/K_m value at alkaline pH (Figure 8, □). These results indicate that the alkaline pH influenced the kinetic parameter for the UDP-GlcNAc-dependent stimulation of glucuronidation only in intact membranes, which have poor permeability to UDP-GlcUA, but not in the membrane-permeabilized condition.

The effect of UGT complex formation on the uptake of UDP-GlcUA was also examined. Figure 9 shows the time course of UDP-GlcUA uptake into microsomal vesicles for the various pH conditions. Preincubation of the microsomes in the absence of the UDP-GlcNAc at 37 °C for 60 min resulted in the significant decrease of UDP-GlcUA uptake in all pH conditions described previously (Figure 9, ○) (Radominska et al., 1994). The stimulation effect of UDP-GlcNAc on UDP-GlcUA uptake was found only below pH 7.5 but not at pH 8.5. In all pH conditions, the microsomes remained intact and the UDP-GlcNAc uptakes in preincubation were weakly affected (data not shown). These data indicate that UDP-GlcNAc-dependent stimulation of UDP-GlcUA uptake is pH-dependent and is correlated with that of glucuronidation. These results suggest that UGT complex formation is involved in the UDP-GlcNAc-dependent stimulation of glucuronidation via the stimulation of UDP-GlcUA uptake into the microsomal membranes.

DISCUSSION

In the present study, we have provided direct evidence for protein−protein interactions between UGT1 isozymes and UGT2B1 in microsomal membranes using isozyme-specific anti-peptide antibodies and Gunn rat hepatic microsomes (Figure 3). Hepatic microsomes of Gunn rat lack all the UGT1 isozymes due to a mutation in the shared exon of the *UGT1* gene complex (Emi et al., 1995). The loss of the UGT2B1 adsorption in Gunn rat microsomes clearly indicates the direct interactions between the UGT1s and the UGT2B1. The major species of UGT1 isozymes is known

to be UGT1B1,[2] which catalyzes glucuronidation of bilirubin (Ikushiro et al., 1995; Clarke et al., 1992). The major population of the interacting isozymes is thus likely to be UGT2B1 and UGT1B1. From amino-terminal sequencing data of 50 kDa protein fractions (Table 1), minor species of coeluted proteins correspond to other UGT2 family members, UGT2B3 or UGT2B6, raising the possibility that interactions between UGT1s and other UGT2 isozymes can exist in the membranes. Furthermore, the immunopurification technique for the analysis of heterologous complexes does not exclude that UGTs can form a homologous complex between UGT1s or UGT2 isozymes.

By use of the chemical cross-linking reagent BMH, heteromeric dimer formation between one of the UGT1 isozymes and UGT2B1 was found in the microsomal membranes (Figure 5). The BMH has a sulfhydryl-reactive bismaleimide containing 16.1 Å of spacer and has been used to cross-link oligomeric proteins such as G_o protein subunits (Yi et al., 1991). The cross-linked products of UGTs by BMH was specific and the yield was higher than with other cross-linking reagents. It is thus expected that there are sulfhydryl group(s) available for cross-linking in the UGTs. The membrane-impermeable reagent stilbenedisulfonate maleimide has been used to determine the membrane topology of P-glycoprotein (Loo & Clarke, 1995). Pretreatment of the microsomes with stilbenedisulfonate maleimide resulted in the inhibition of UGT cross-linking by BMH (Figure 5C), suggesting that the location of the cross-linking site is on the cytosolic side of microsomal membranes. Indeed, there are some cysteine residues in the carboxyl-terminal region of UGT isozymes (for examples, Cys 511, 515, and 518 in UGT1B1 and Cys 513, 516, and 517 in UGT2B1) that are located on the cytosolic side of the potential transmembrane region (Mackenzie, 1990). Yamashita et al. (1995) reported the presence of one or more reactive sulfhydryl groups in UGT isozymes that are involved in the autoacylation of UGT. The interaction site of UGT dimers is likely located within the carboxyl-terminal region of UGTs containing reactive sulfhydryl group(s) on the cytosolic side of the microsomal membranes.

The interactions between UGT isozymes led us to consider what function in glucuronidation the complex formation might play. Evidence for protein−protein interactions between isozymes in the membranes indicated that complex formation could occur and may be involved in the modulation of their functions. Alston et al. (1991) showed complex formation among cytochrome P-450s to occur in rat hepatic microsomal membranes using a photoreactive cross-linking reagent and the interaction to be responsible for the modulation of their activity. Bovine adrenocortical mitochondrial P-450s, P-450(scc) and P-450(11β), interact with each other to modulate the steroidogenic activity via changing the successive hydroxylation (Ikushiro et al., 1992; Kominami et al. 1994). As shown in Figures 6 and 7, the values of V_{max}/K_m for the glucuronidating activity on testosterone in the absence of the UDP-GlcNAc, which is catalyzed by UGT2B1 in the microsomes, were not much affected by the extent of complex formation of the UGT2B1 with UGT1s. An interesting finding was that the loss of UDP-GlcNAc-dependent stimulation of glucuronidation at alkaline pH was accompanied with the decrease in complex formation. In the kinetic data for glucuronidation, the sharp drop of V_{max}/K_m values above pH 7.5 demonstrates the interrelation between the change of the functional states in UGT2B1 and the change of strength of the interaction of UGT2B1 with UGT1s. Dannenberg et al. (1990) and Kavecansky et al. (1992) reported that treatment of microsomes at high pressure induces the activation of glucuronidation and the loss of the UDP-GlcNAc-dependent stimulation. They interpreted the results to mean that the treatment at high pressure perturbs the interactions between UGTs to dissociate the complexes and changes the functional states of UGTs. It is likely that the pH-induced changes of activity in our results are consistent with the pressure-induced changes. It may thus be reasonably assumed that the UGTs in the membranes exist in two functional states at different pH conditions. At pH 7.5 or below, one corresponds to the oligomer form of UGTs, which is unactivated and competent to be stimulated by UDP-GlcNAc. At alkaline pH, the other state corresponds to the monomer form, which is already activated and is insensitive to UDP-GlcNAc-dependent stimulation. The effects of pH on the UDP-GlcNAc-dependent stimulation of glucuronidation may be considered due to the change of organization of the UGTs from the oligomer to the monomer form that results from dissociation of the complex in the membranes. In contrast to the intact membranes, the V_{max}/K_m values in membrane-permeabilized states were not changed by the pH (Figure 8), suggesting that UDP-GlcNAc-dependent stimulation induced by oligomer formation in UGTs is linked to the membrane structure, in which the permeability of UDP-GlcUA to microsomal membrane is limited.

Bossuyt et al. (1995) and Berg et al. (1995) reported that UDP-GlcNAc acts as a trans-stimulator in the uptake of UDP-GlcUA into the microsomal vesicles via a putative transport system(s). Based on the pH dependence of the uptake of UDP-GlcUA into the microsomal vesicles (Figure 9), UGT complex formation is associated with the UDP-GlcUA uptake process stimulated by UDP-GlcNAc. These results lead us to consider the possibility that the dimer and/or higher oligomer forms of UGTs can act as channels for UDP-GlcUA access from the cytosolic side to the UGT active site in cisternal lumen or within membranes. It is predicted that the channel formed from the oligomer of the UGTs is composed of the hydrophobic α-helical transmembrane region and some reactive sulfhydryl group(s) in the carboxyl-terminal end of UGT. Treatment of microsomes by *N*-ethylmaleimide (NEM) inhibits the UDP-GlcNAc-dependent stimulation of the glucuronidation and the UDP-GlcUA uptake (Burchell et al., 1983; Bossuyt & Blanckaert, 1994). NEM modifications of UGTs is inhibited by the presence of UDP-GlcUA, suggesting the involvement of sulfhydryl groups in the binding of UDP-GlcUA.[3] In the functional oligomer of UGTs, the carboxyl-terminal sulfhydryl groups located in the interaction sites may thus be involved in the uptake of UDP-GlcUA. The preloaded UDP-GlcNAc into the microsomes may act as antitransport substrate to stimulate the uptake of UDP-GlcUA into the catalytic site of glucuronidation via the channel, resulting in the UDP-GlcNAc-dependent stimulation of glucuronidation.

In conclusion, we have demonstrated direct interactions between the UGT isozymes, UGT1s and UGT2B1, in the microsomal membranes. The pH-induced change of the

[2] According to a recommended nomenclature system proposed by Burchell et al. (1991), UGT1B1 is defined as UGT1*01.

[3] S. Ikushiro et al., unpublished results.

interaction between UGTs corresponds to the change of functional states of the UGTs in the membranes. Our present data can partly support the conformational model for latency of UGT activity, in which UGTs exist in the different states corresponding to monomer and oligomer forms. Further experiments with purified UGT or cDNA-expressed microsomal systems must be performed to confirm our "oligomer model" for latency and to solve the physiological roles of UGT complex formation.

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