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Interactions between human UGT1A1, UGT1A4, and UGT1A6 affect their enzymatic activities

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Abbreviations: UGT, UDP-glucuronosyltransferase; UDPGA, UDP-glucuronic acid.

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ABSTRACT

Protein-protein interactions between human UDP-glucuronosyltransferase (UGT) 1A1, UGT1A4, and UGT1A6 were investigated using double expression systems in HEK293 cells (UGT1A1/UGT1A4, UGT1A1/UGT1A6, and UGT1A4/UGT1A6). The substrates specific for UGT1A1 (estradiol and bilirubin), UGT1A4 (imipramine and trifluoperazine), and UGT1A6 (serotonin and diclofenac) were used to determine the effects of the coexpression of the other UGT1A isoforms on the enzymatic activity. The coexpression of UGT1A4 and UGT1A6 decreased the S_{50} and V_{max} values of UGT1A1-catalyzed estradiol 3-*O*-glucuronide formation, and increase the V_{max} value of UGT1A1-catalyzed bilirubin *O*-glucuronides formation. The coexpression of UGT1A1 decreased the V_{max} value of the UGT1A4-catalyzed imipramine *N*-glucuronide formation, but had no effect on the UGT1A4-catalyzed trifluoperazine *N*-glucuronide formation. The coexpression of UGT1A6 had no effect on the UGT1A4-catalyzed imipramine *N*-glucuronide formation, but increased the K_m and V_{max} of the UGT1A4-catalyzed trifluoperazine *N*-glucuronide formation. Both the coexpression of UGT1A1 and UGT1A4 increased the V_{max} values of the UGT1A6-catalyzed serotonin and diclofenac *O*-glucuronide formation. Thus, the effects of the coexpression of other UGT1A isoforms on the kinetics of specific activities were different depending on the UGT1A isoforms and substrates. Native PAGE analysis of the double expression systems showed multiple bands at approximately 110 kDa, indicating the existence of heterodimers as well as homodimers of UGTs. In conclusion, we found that human UGT1A1, UGT1A4, and UGT1A6 interact with each other, possibly by heterodimerization, and that their effects on the enzymatic activities are complex depending on the isoforms and substrates.

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INTRODUCTION

Human UDP-glucuronosyltransferases (UGTs; EC 2.4.1.17) are a family of membrane-bound enzymes of the endoplasmic reticulum. They catalyze the glucuronidation of various endogenous and exogenous compounds, converting them into more polar glucuronides (Tukey and Strassburg, 2000). The human genome encodes at least 19 different UGTs, and they are divided into three subfamilies, UGT1A, 2A, and 2B, according to the degree of nucleotide sequence identities and genomic organization (Mackenzie et al., 2005). UGT1A isoforms are encoded by a single gene that has multiple unique exon 1s and common exons 2 - 5. Five of the human UGT1A isoforms, UGT1A1, UGT1A3, UGT1A4, UGT1A6, and UGT1A9, are expressed in the liver and characterized by individual catalytic activity profiles (Tukey and Strassburg, 2000).

UGTs have been reported to form homo- and hetero-oligomers. Matern *et al*. (1982) proposed UGT homo-oligomerization for the first time based on gel permeation chromatography of rat liver microsomes. The homo-oligomer formation of rat UGT2B1 (Meech and Mackenzie, 1997), human UGT1A1 (Ghosh et al., 2001), and human UGT1A9 (Kurkela et al., 2003) was subsequently demonstrated by SDS-PAGE, two-hybrid analysis, and immobilized meta-chelating chromatography, respectively. In addition, radiation inactivation analysis revealed that dimers and tetramers of UGT have different catalytic roles (Peters et al., 1984; Gschaidmeier and Bock, 1994). Ishii *et al.* (2001, 2004) showed that the interaction between guinea pig UGT2B21 and UGT2B22 influenced UGT2B21-catalyzed morphine 3-glucuronide and 6-glucuronide formation in a heterologous expression system in COS-7 cells. It has also been reported that coexpression of human UGT1A4 affects the activity of human UGT1A9 (Kurkela et al., 2004). Unfortunately, a limitation of these studies was that the activities were evaluated by transient expression systems. The expression levels and the expression ratios of the two UGT isoforms differ in each transient transfection. To overcome the issue, we recently established stable cell lines expressing double human UGT isoforms, UGT1A1/UGT1A9, UGT1A4/UGT9, and UGT1A6/UGT1A9 in HEK293 cells (Fujiwara et

al., 2007). In that study, the interaction between UGT1A6 and UGT1A9 was suggested by the immunoprecipitation analysis, and the heterodimerization of UGT1A1, UGT1A4, and UGT1A6 with UGT1A9 was supported by thermal stability assay. Moreover, it was demonstrated that coexpression of UGT1A9 affected the kinetics of UGT1A1, UGT1A4, and UGT1A6 activities, and *vise versa*. The interactions between UGT1A1, UGT1A4, and UGT1A6 have remained to be elucidated. In the present extended study, we investigated the effects of the interactions between UGT1A1, UGT1A4, and UGT1A6 on their catalytic activities using the stable double expression systems. Furthermore, native PAGE analysis was carried out to determine the heterodimerization between these isoforms.

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MATERIALS AND METHODS

Chemicals and reagents

UDP-glucuronic acid (UDPGA), alamethicin, estradiol, estradiol 3-*O*-glucuronide, and diclofenac sodium salt were purchased from Sigma-Aldrich (St. Louis, MO). Bilirubin, imipramine hydrochloride, serotonin, and trifluoperazine dihydrochloride salt were purchased from Wako Pure Chemicals Industries (Osaka, Japan). Rabbit anti-human UGT1A, anti-human UGT1A1, and anti-human UGT1A6 antibodies were obtained from BD Gentest (Woburn, MA). IRDye680-labeled goat anti-rabbit secondary antibody and Odyssey Blocking Buffer were from Li-COR Biosciences (Lincoln, NE). Perfect NT Gel M was purchased from DRC (Tokyo, Japan). All other chemicals and solvents were of the highest grade commercially available.

Stable expression of UGT1A isoforms in HEK293 cells

Expression vectors for UGT1A1, UGT1A4, and UGT1A6 were previously constructed (Fujiwara et al., 2007). HEK293 (American Type Culture Collection, Rockville, MD) cells were grown in Dulbecco's modified Eagle's medium containing 4.5 g/L glucose, 10 mM HEPES, and 10% fetal bovine serum with 5% CO_2 at 37°C. The cells in 6-well plates were transfected with 2 μg of UGT expression vector using Lipofectamine (Invitrogen, Carlsbad, CA). For the double expression systems, each expression vector was co-transfected at the ratio of 1:1. Stable transfectants were selected in medium containing 800 μg/mL of G418. The total cell homogenates were prepared as described previously (Fujiwara et al., 2007).

Immunoblot analysis

To determine the expression levels of UGT1A in the total cell homogenates, SDS-PAGE and immunoblot analysis were performed using anti-UGT1A, anti-UGT1A1, or anti-UGT1A6 antibodies and an Odyssey infrared imaging system (Li-COR Biosciences, Lincoln, NE). The total cell homogenates (1-10 μg of protein) were subjected to 10% SDS-PAGE and transferred to a PVDF membrane Immobilon-P (Millipore, Bedford, MA). The membrane was washed

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with phosphate buffered saline (PBS) two times and blocked with Odyssey Blocking Buffer (Li-COR Biosciences) for 1 h. The membrane was probed with the primary antibody diluted (1:500) with Odyssey Blocking Buffer containing 0.1% Tween-20 for 1 h. The membrane was washed with PBST (PBS containing 0.1% Tween-20) four times and incubated with IRDye680-labeled goat anti-rabbit secondary antibody diluted (1:5000) with PBST for 1 h. The expression level of UGT1A was defined based on a standard curve using the UGT1A1 single expression system (1 unit per 1 mg of cell homogenates), as described previously (Fujiwara et al., 2007).

Glucuronide formation

Estradiol 3-*O*-glucuronide formation, imipramine *N*-glucuronide formation, serotonin *O*-glucuronide formation, and trifluoperazine *N*-glucuronide formation were determined as described previously (Fujiwara et al., 2007). Diclofenac *O*-glucuronide formation and bilirubin *O*-glucuronides formation were determined according to the methods of King *et al.* (2001) and Jacobson *et al.* (1975), respectively. Bilirubin was freshly dissolved in 0.2 M NaOH, and analysis was performed under dim light. The sum of monoglucuronides and diglucuronides was evaluated. The quantification of estradiol 3-*O*-glucuronide was performed by comparing the HPLC peak height to that of the authentic standard. For the quantification of the imipramine *N*-, trifluoperazine *N*-, and serotonin *O*-glucuronides, the eluate containing each glucuronide was hydrolyzed with NaOH at 75°C for 30 min (Hawes, 1998). The hydrolyzed glucuronides were quantified using HPLC by comparing the peak heights to those of the external standard curve of the substrates. The activities of bilirubin *O*- and diclofenac *O*-glucuronide formation were determined by the decrease of the peak area of the parent compounds.

Kinetic parameters were estimated from the fitted curve using a computer program (KaleidaGraph, Synergy Software, Reading, PA) designed for non-linear regression analysis. The following equations were used:

Michaelis-Menten equation $V = V_{max} \cdot [S] / (K_m + [S])$

Hill equation $V = (V_{max} \cdot [S]^n) / (S_{50}^n + [S]^n)$

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where V is the velocity of the reaction at substrate concentration S, K_m is the Michaelis-Menten constant, V_{max} is the maximum velocity, S_{50} is the substrate concentration showing the half V_{max}, and n is the Hill coefficient.

Native PAGE and immunoblot analysis

Native PAGE followed by immunoblotting was performed to detect homodimer and heterodimer formations of UGTs. The total cell homogenate was solubilized with PBS containing 1% Triton X-100 and 0.2% SDS on ice for 60 min and was centrifuged at 13,000 g for 30 min. To the 20-μL portion of supernatant, 1 μL of 60% glycerol containing 0.2% bromophenol blue was added. The sample including 0.02 to 0.03 unit of UGT1A was applied to Perfect NT Gel M (5-20% gradient). The electrophoresis was carried out at 10 mA for 5 h at 4° C in Tris-glycine electrophoresis buffer (25 mM Tris base, 192 mM glycine, and 0.1% SDS). The separated proteins were transferred to a PVDF membrane. UGT protein was detected by diaminobenzidine staining using rabbit anti-human UGT1A antibody, biotinylated anti-rabbit IgG, and a VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA) as described previously (Fujiwara et al., 2007).

Statistical analyses

Data are expressed as mean ± SD of three independent determinations. Statistical significance of the kinetic parameters was determined by analysis of variance (ANOVA) followed by Dunnett's test. A value of $P < 0.05$ was considered statistically significant.

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RESULTS

Establishment of stable expression systems of UGTs in HEK293 cells.

For each single (UGT1A1, UGT1A4, and UGT1A6) or double (UGT1A1/UGT1A4, UGT1A1/UGT1A6, and UGT1A4/UGT1A6) expression system, we isolated ten clones for each expression system. Immunoblot analysis revealed that the expression levels of the UGT1A isoforms varied between the clones (data not shown). The anti-UGT1A antibody recognized the UGT1A1, UGT1A4, and UGT1A6, whereas the anti-UGT1A1 and anti-UGT1A6 antibodies specifically recognized UGT1A1 and UGT1A6, respectively (Fig. 1). Each UGT1A isoform showed a single band at 50-55 kDa, possibly owing to the differences in the levels of glycosylation. For each single expression system of UGT1A1, UGT1A4, and UGT1A6, the clones with the highest protein levels were selected for subsequent analyses. Based on the expression level of 1.00 unit/mg protein in the UGT1A1 single expression system, as previously described (Fujiwara et al., 2007), the UGT1A4 and UGT1A6 levels in each single expression level were estimated as 0.56 and 0.88 unit/mg protein, respectively. Since the mobilities of UGT1A1 and UGT1A6 were similar, their expression levels in the UGT1A1/UGT1A6 double expression systems were determined using anti-UGT1A1 and UGT1A6 antibodies. Since UGT1A4 migrates more slowly than UGT1A1 and UGT1A6, the expression levels of the two isoforms in the UGT1A1/UGT1A4 and UGT1A4/UGT1A6 double expression systems were determined using anti-UGT1A antibodies. For subsequent experiments, clones in which the expression levels of the two isoforms were almost equal were selected. In the UGT1A1/UGT1A4 double expression system, the expression levels of UGT1A1 and UGT1A4 were 0.35 unit/mg protein and 0.46 unit/mg protein, respectively. In the UGT1A1/UGT1A6 double expression system, the expression levels of UGT1A1 and UGT1A6 were 0.26 unit/mg protein and 0.25 unit/mg protein, respectively. In the UGT1A4/UGT1A6 double expression system, the expression levels of UGT1A4 and UGT1A6 were 0.14 unit/mg protein and 0.15 unit/mg protein, respectively (Fig. 1). The small SD values of the protein levels revealed the reproducibility of the quantification of UGT1As.

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Kinetic analysis in double expression systems.

We confirmed that the estradiol 3-*O*- and bilirubin *O*-glucuronides formations were catalyzed by UGT1A1, but not by UGT1A4 and UGT1A6 (data not shown). The estradiol 3-*O*-glucuronide formation by the single expression system of UGT1A1 followed the Hill equation (Fig. 2A) yielding S_{50} of 8.7 μM, V_{max} of 975.0 pmol/min/unit, V_{max}/S_{50} of 112.1 μL/min/unit, and n of 1.7 (Table 1). The kinetic parameters were consistent with those of our previous study (Fujiwara et al., 2007). Coexpression of UGT1A4 decreased the V_{max} value to approximately one half resulting in a decreased V_{max}/S_{50} value (Table 1). In contrast, coexpression of UGT1A6 increased the V_{max}/S_{50} value with a decrease in the S_{50} value compared with those values in the single expression systems. The bilirubin *O*-glucuronides formation by the single expression system of UGT1A1 also followed the Hill equation (Fig. 2B), yielding S_{50} of 2.6 μM, V_{max} of 223.5 pmol/min/unit, V_{max}/S_{50} of 88.9 μL/min/unit, and n of 2.7 (Table 1). The S_{50} value was consistent with that of a previous study (Ciotti et al., 1998), although the V_{max} value could not be compared because we do not know the expression level of UGT1A1 in their expression system. Both the coexpression of UGT1A4 and UGT1A6 decreased the S_{50} value and increased the V_{max} value resulting in an increase of the V_{max}/S_{50} value. Thus, the effects of the coexpression of UGT1A4 or UGT1A6 on the UGT1A1 activities were different between the two substrates.

It was confirmed that the imipramine and trifluoperazine *N*-glucuronide formations were catalyzed by UGT1A4 but not by UGT1A1 and UGT1A6 in accordance with previous reports (Nakajima et al., 2002; Uchaipichat et al., 2006). The imipramine *N*-glucuronide formation by the single expression system of UGT1A4 followed the Michaelis-Menten equation (Fig. 2C), yielding K_m of 1.1 mM, V_{max} of 134.9 pmol/min/unit, and V_{max}/K_m of 120.5 nL/min/unit (Table 2). The kinetic parameters were consistent with our previous study (Fujiwara et al., 2007). The coexpression of UGT1A1 decreased the V_{max} value of the imipramine *N*-glucuronide formation, although the coexpression of UGT1A6 did not affect the kinetics. The trifluoperazine *N*-glucuronide formation by the single expression system of UGT1A4 also followed the

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Michaelis-Menten equation (Fig. 2D) yielding K_m of 19.7 μM, V_{max} of 2.1 nmol/min/unit, and V_{max}/K_m of 108.1 μL/min/unit (Table 2). The K_m value was consistent with that of a previous study (Uchaipichat et al., 2006), although the V_{max} values could not be compared because we do not know the expression level of UGT1A4 in their expression system. The coexpression of UGT1A1 did not affect the trifluoperazine *N*-glucuronide formation, but the coexpression of UGT1A6 increased the K_m and V_{max} values. Thus, the effects of the coexpression of UGT1A1 or UGT1A6 on the UGT1A4 activities were different between two substrates.

As previously reported (Fujiwara et al., 2007), it was confirmed that serotonin *O*-glucuronide formation was catalyzed by UGT1A6, but not by UGT1A1 and UGT1A4. The serotonin *O*-glucuronide formation by the single expression system of UGT1A6 followed the Michaelis-Menten equation (Fig. 2E) yielding K_m of 4.6 mM, V_{max} of 1.5 nmol/min/unit, and V_{max}/K_m of 0.3 μL/min/unit (Table 3). The kinetic parameters were consistent with those of our previous study (Fujiwara et al., 2007). Both the coexpression of UGT1A1 and UGT1A4 increased the V_{max} value. Although it has been reported that diclofenac *O*-glucuronide formation is catalyzed by multiple human UGT isoforms (King et al., 2001; Kuehl et al., 2005), we confirmed that the activity was catalyzed by UGT1A6 but not by UGT1A1 and UGT1A4 (data not shown). The diclofenac *O*-glucuronide formation by the single expression system of UGT1A6 followed the Hill equation (Fig. 2F) yielding S_{50} of 69.8 μM, V_{max} of 28.4 pmol/min/unit, V_{max}/S_{50} of 0.4 μL/min/unit (Table 3). Both the coexpression of UGT1A1 and UGT1A4 increased the V_{max} value of the diclofenac *O*-glucuronide formation. Thus, the effects of the coexpression of UGT1A1 or UGT1A4 on the UGT1A6 activities were similar between two substrates.

The reproducibility of the kinetics of each glucuronide formation was confirmed using multiple preparations from other clones. Finally, it was confirmed that the mixing of the two single expression systems did not influence the kinetics of the enzymatic activities (data not shown). Thus, it is suggested that the co-expressed UGT isoforms functionally interacted with each other in the double expression systems.

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Formation of homodimers and heterodimers of human UGT1A.

Native-PAGE analysis was performed to investigate whether the UGT1As form homodimers and/or heterodimers. When the total cell homogenates of the UGT1A1, UGT1A4, and UGT1A6 single expression systems were separated, two obvious bands were observed at about 50-55 kDa and 110-125 kDa (Fig. 3). The lower and upper bands corresponded to monomers and dimers, respectively. The mobility of the upper bands corresponding to the UGT1A1 homodimer and UGT1A6 homodimer was higher than that corresponding to the UGT1A4 homodimer, although the mobility of the bands corresponding to UGT1A1 monomer and UGT1A6 monomer was lower than that corresponding to the UGT1A4 monomer. The intensities of the ~110 kDa band of the UGT1A4 and UGT1A6 homogenates were lower than that of UGT1A1, indicating the possibility that UGT1A4 and especially UGT1A6 unlikely form homodimers compared with UGT1A1.

Utilizing the differences in the mobility of the homodimers between the UGT1A isoforms, we extended the native-PAGE analysis for the double expression systems. Interestingly, three bands were detected at around 110 kDa with the UGT1A1/UGT1A4 double expression system. Among them, the upper and lower bands corresponded to the bands obtained with the single expression systems of UGT1A1 and UGT1A4, respectively. The middle band was unique to the double expression system of UGT1A1/UGT1A4 (Fig. 3, an open arrowhead). The mixture of the single expression systems of UGT1A1 and UGT1A4 showed only two upper and lower bands. The double expression system of UGT1A1/UGT1A6 showed a band at around 125 kDa with the same migration of the bands observed in the UGT1A1 and UGT1A6 single expression systems. The double expression system of UGT1A4/UGT1A6 showed two very faint bands at around 110 kDa and 125 kDa, which were similar to the mixture of the single expression systems of UGT1A4 and UGT1A6.

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DISCUSSION

Our recent study revealed that the coexpression of UGT1A9 affected the kinetics of UGT1A1, UGT1A4, and UGT1A6 enzymatic activities in HEK293 cells (Fujiwara et al., 2007). In the present extended study, we investigated the protein-protein interactions between UGT1A1, UGT1A4, and UGT1A6 using double expression systems of UGT1A1/UGT1A4, UGT1A1/UGT1A6, and UGT1A4/UGT1A6 in HEK293 cells. By the kinetic analyses for the enzymatic activities of UGT1A1 (estradiol and bilirubin), UGT1A4 (imipramine and trifluoperazine), and UGT1A6 (serotonin and diclofenac) using the double expression systems, puzzling results were obtained, that is, the coexpression of other isoforms variously affected the activities of the concerned isoform, depending on the substrates as well as isoforms. Although we previously (Fujiwara et al., 2007) investigated the effects of the coexpression of UGT1A9 on the UGT1A1, UGT1A4, and UGT1A6 activities with only a typical substrate (estradiol, imipramine, and serotonin, respectively), it is feasible that the effects might be different depending on the substrates. Thus, human UGT1A1, UGT1A4, UGT1A6, and UGT1A9, which are the main isoforms in human liver, complicatedly interact with each other affecting the enzymatic activity.

Previous studies have demonstrated inconsistencies in the K_m values of glucuronide formation between human liver microsomes and recombinant UGT. For example, the K_m value of the trifluoperazine *N*-glucuronide formation by human liver microsomes was about 2-fold higher than that by recombinant UGT1A4 (Uchaipichat et al., 2006). The K_m values of bilirubin and serotonin *O*-glucuronide formation by human liver microsomes were higher than that by recombinant UGT1A1 (Ciotti et al., 1998; Uchaipichat et al., 2006; Seppen et al., 1994; Soars et al., 2001; Krishnaswamy et al., 2003). In this study, we found that the coexpression of UGT1A6, and to a lesser extent UGT1A1, significantly increased the K_m value of UGT1A4-catalyzed trifluoperazine *N*-glucuronide formation up to 2.5-fold (Table 2). Thus, the interaction of UGT1A6 with UGT1A4 might be one of the factors for the different K_m values of trifluoperazine *N*-glucuronide formation between human liver microsomes and recombinant

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UGT. However, this theory cannot be applied to bilirubin and serotonin *O*-glucuronide formation from our results. It should be noted that not only UGT1A isoforms but also UGT2B isoforms are present in human liver microsomes. Moreover, recent studies have reported interactions between UGTs and other microsomal enzymes. Fremont *et al*. (2005) has demonstrated by coimmunoprecipitation assays that human UGT1A1, UGT1A6 and UGT2B7 interact with cytochrome P450 (CYP) 3A4. Takeda *et al*. (2005) has reported that morphine *O*-glucuronide formation catalyzed by UGT2B7 was affected by coexpressed CYP3A4. Therefore, although the present study focused on the potential effect of heterodimerization between UGT1A isoforms on the activities, further investigations are needed to clarify the effects of UGT2B and CYP enzymes on the UGT1A activities.

To detect homodimers or heterodimers of UGTs, several methods including cross-linking analysis (Ghosh et al., 2001; Ikushiro et al., 1997), two-hybrid assay (Ghosh et al., 2001), immunopurification (Ikushiro et al., 1997), immobilized meta-chelating chromatography (Kurkela et al., 2003), and fluorescence resonance energy transfer (FRET) analysis (Operana and Tukey, 2007) have been used. In our recent study (Fujiwara et al., 2007), heterodimerization was detected with immunoprecipitation and thermal stability assays. In the present study, we performed native-PAGE analyses to detect homodimerization and heterodimerization of UGTs, because accumulating data demonstrated the usefulness of this method to detect homo- and hetero-oligomeric complexes of protein (Garnier et al., 2001; Krause et al., 2004; Strohmeier et al., 2006). In all cases of single expression systems of UGT1A1, UGT1A4, and UGT1A6, bands corresponding to homodimers were observed (Fig. 3). According to the differences in the band intensities, it was considered that UGT1A4 and UGT1A6 have a lower capability to form homodimers than UGT1A1, although the solubilization condition might have differently affected the homodimerization of different isoforms. Although we believe that the bands correspond to UGT homodimers, a possibility cannot be excluded that they might be complex of UGT and unrelated protein. Nevertheless, the unique middle band by the double expression system of UGT1A1/UGT1A4 strongly suggested the heterodimerization of UGT1A1 and UGT1A4. This was supported by a result

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that the middle band as well as the upper band was also detected when the anti-UGT1A1 antibody was used (data not shown). Unfortunately, we could not judge whether UGT1A6 forms heterodimers with UGT1A1 or UGT1A4 by this method, but the kinetic analyses strongly suggested their interactions. Therefore, UGT1A1, UGT1A4, and UGT1A6 may form heterodimers as well as homodimers, although the extent varied between the UGT1A isoforms.

In conclusion, we found that human UGT1A1, UGT1A4, and UGT1A6 interact with each other, affecting the enzymatic activities. Their interactions would be complex depending on the isoforms and substrates. The data presented here suggest the difficulty of quantitative predictions of glucuronidation activities as well as the contribution of each UGT isoform to the activities in human liver microsomes.

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Footnote

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FIGURE LEGENDS

Figure 1 Immunoblot analyses of single or double expression systems of human UGT1As in HEK293 cells using rabbit anti-human UGT1A (upper panel), anti-human UGT1A1 (middle panel), and anti-human UGT1A6 (lower panel) antibodies. One to ten μg of total cell homogenates from the single expression systems were separated by electrophoresis using 10% SDS-PAGE. The expression levels of the UGT1A isoforms were defined based on the standard curve with the UGT1A1 single expression system (1 unit per 1 mg of cell homogenates). Columns are the mean ± SD of three independent determinations. M, mock transfection; ND, not detected.

Figure 2 Kinetic analyses of the formations of various glucuronides in single and double expression systems. (A) Estradiol 3-*O*-glucuronide formation catalyzed by UGT1A1 was determined with 0.25 mg/ml cell homogenates, 2 - 100 μM estradiol, and 2 mM UDPGA at 37°C with 60-min incubation. (B) Bilirubin *O*-glucuronides formation catalyzed by UGT1A1 was determined with 0.5 mg/ml cell homogenates, 1 - 10 μM (1A1) or 0.5 - 10 μM (1A1/1A4 and 1A1/1A6) bilirubin, and 2.5 mM UDPGA at 37°C with 30-min incubation. (C) Imipramine *N*-glucuronide formation by catalyzed UGT1A4 was determined with 0.25 mg/ml cell homogenates, 0.13 - 1.5 mM imipramine, and 5 mM UDPGA at 37°C with 60-min incubation. (D) Trifluoperazine *N*-glucuronide formation catalyzed by UGT1A4 was determined with 0.5 mg/ml cell homogenates, 2.5 - 100 μM trifluoperazine, and 2.5 mM UDPGA at 37°C with 30-min incubation. (E) Serotonin *O*-glucuronide formation catalyzed by UGT1A6 was determined with 0.25 mg/ml cell homogenates, 0.3 - 20 mM serotonin, and 5 mM UDPGA at 37°C with 30-min incubation. (F) Diclofenac *O*-glucuronide formation catalyzed by UGT1A6 was determined with 1.0 mg/ml cell homogenates, 25 - 250 μM diclofenac, and 2.5 mM UDPGA at 37°C with10-min incubation.

Figure 3 Native-PAGE analyses of UGT1A isoforms in single or double expression

systems. Total cell homogenates were solubilized with PBS containing 1% Triton X-100 and 0.2% SDS on ice for 60 min, and then were subjected to gradient gel (5-20%). After electrophoresis at 4°C, the proteins were transferred to a PVDF membrane, and immunoblotted with rabbit anti-human UGT1A antibody. The bands with closed and open arrowheads may indicate homodimerization and heterodimerization, respectively.

Table 1 Kinetic parameters for estradiol 3-*O*-glucuronide formation and bilirubin *O*-glucuronides formation by UGT1A1 in single and double expression systems

	Estradiol 3-*O*-glucuronide formation				Bilirubin *O*-glucuronides formation			
Clone	S_{50} (μM)	V_{max} (pmol/min/unit)	V_{max} / S_{50} (μL/min/unit)	n	S_{50} (μM)	V_{max} (pmol/min/unit)	V_{max} / S_{50} (μL/min/unit)	n
1A1	8.7 ± 0.3	975.0 ± 27.4	112.1 ± 1.8	1.7 ± 0.1	2.6 ± 0.4	223.5 ± 9.8	88.9 ± 17.0	2.7 ± 0.1
1A1/1A4	5.7 ± 0.1 **	498.4 ± 1.9 **	87.5 ± 1.8 **	1.9 ± 0.2	1.2 ± 0.1 **	256.3 ± 36.4	216.1 ± 46.2 *	2.6 ± 0.1
1A1/1A6	7.1 ± 0.1 **	917.3 ± 4.2 **	128.6 ± 1.0 **	1.9 ± 0.2	1.1 ± 0.2 **	303.7 ± 33.3 *	293.5 ± 71.0 **	3.0 ± 0.8

Data are mean ± SD of three independent determinations. * $P < 0.05$; ** $P < 0.01$ compared with single expression system.

Table 2 Kinetic parameters for imipramine *N*-glucuronide formation and trifluoperazine *N*-glucuronide formation by UGT1A4 in single and double expression systems

	Imipramine *N*-glucuronide formation			Trifluoperazine *N*-glucuronide formation		
	K_m	V_{max}	V_{max} / K_m	K_m	V_{max}	V_{max} / K_m
Clone	(mM)	(pmol/min/unit)	(nL/min/unit)	(μM)	(nmol/min/unit)	(μL/min/unit)
1A4	1.1 ± 0.2	134.9 ± 1.6	120.5 ± 16.0	19.7 ± 0.6	2.1 ± 0.1	108.1 ± 5.7
1A1/1A4	1.4 ± 0.3	110.4 ± 3.2 **	78.3 ± 11.6 *	19.8 ± 2.9	2.0 ± 0.2	102.2 ± 14.0
1A4/1A6	1.4 ± 0.3	126.0 ± 10.8	92.4 ± 17.4	48.0 ± 15.2 *	5.1 ± 0.6 **	112.4 ± 31.4

Data are mean ± SD of three independent determinations. * $P < 0.05$; ** $P < 0.01$ compared with single expression system.

Table 3 Kinetic parameters for serotonin *O*-glucuronide formation and diclofenac *O*-glucuronide formation by UGT1A6 in single and double expression systems

	Serotonin *O*-glucuronide formation			Diclofenac *O*-glucuronide formation			
	K_m	V_{max}	V_{max} / K_m	S_{50}	V_{max}	V_{max} / S_{50}	n
Clone	(mM)	(nmol/min/unit)	(μL/min/unit)	(μM)	(pmol/min/unit)	(μL/min/unit)	
1A6	4.6 ± 0.4	1.5 ± 0.1	0.3 ± 0.0	69.8 ± 1.8	28.4 ± 3.0	0.4 ± 0.0	1.8 ± 0.3
1A1/1A6	4.4 ± 0.5	2.1 ± 0.1 **	0.5 ± 0.0	87.5 ± 20.8	88.0 ± 13.9 **	1.0 ± 0.1 **	1.8 ± 0.2
1A4/1A6	3.7 ± 0.6	6.1 ± 0.4 **	1.7 ± 0.1 **	112.9 ± 25.6	75.8 ± 13.6 **	0.7 ± 0.1 **	2.1 ± 0.3

Data are mean ± SD of three independent determinations. ** $P < 0.01$ compared with single expression system.

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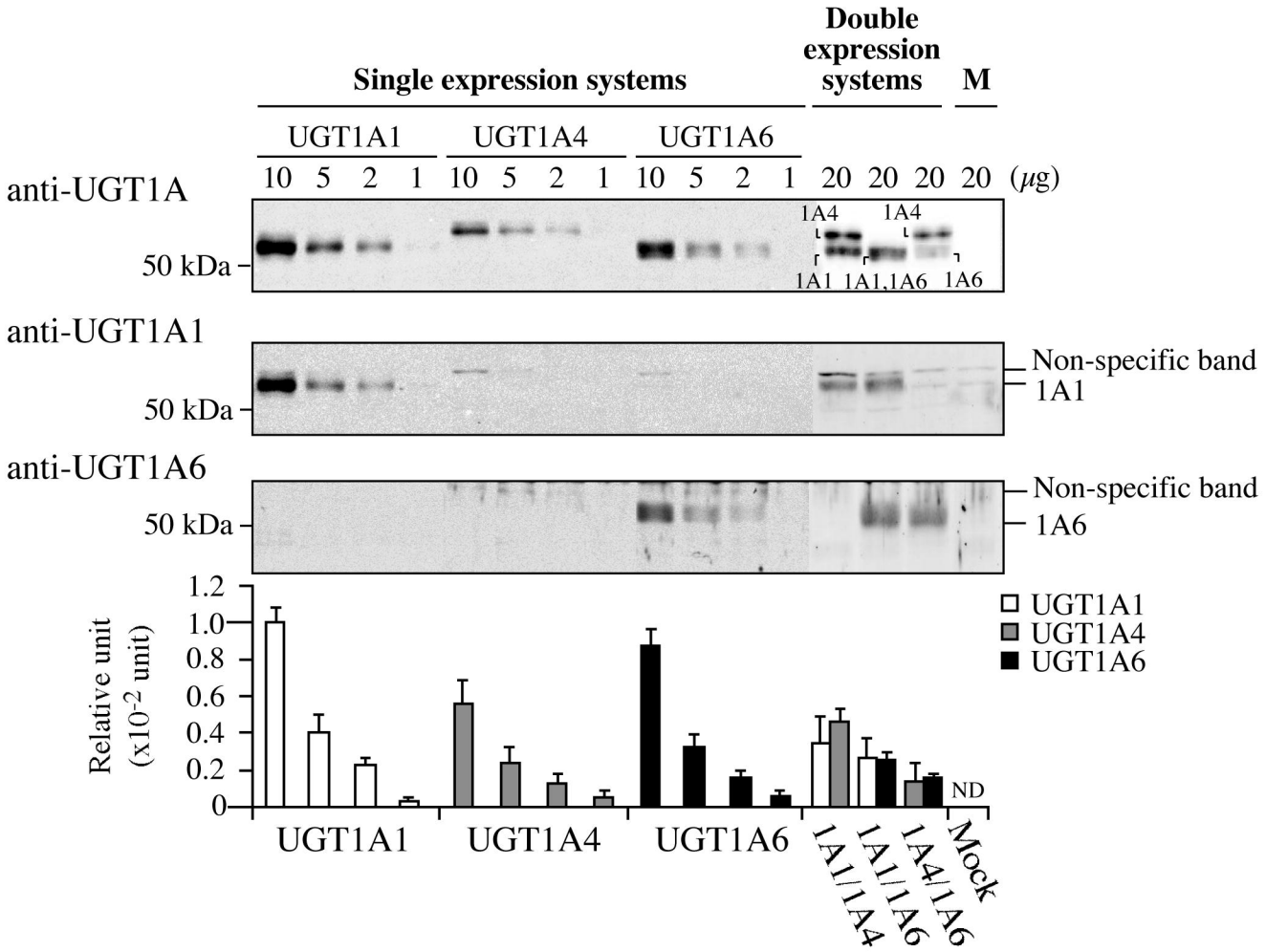

Fig. 2.

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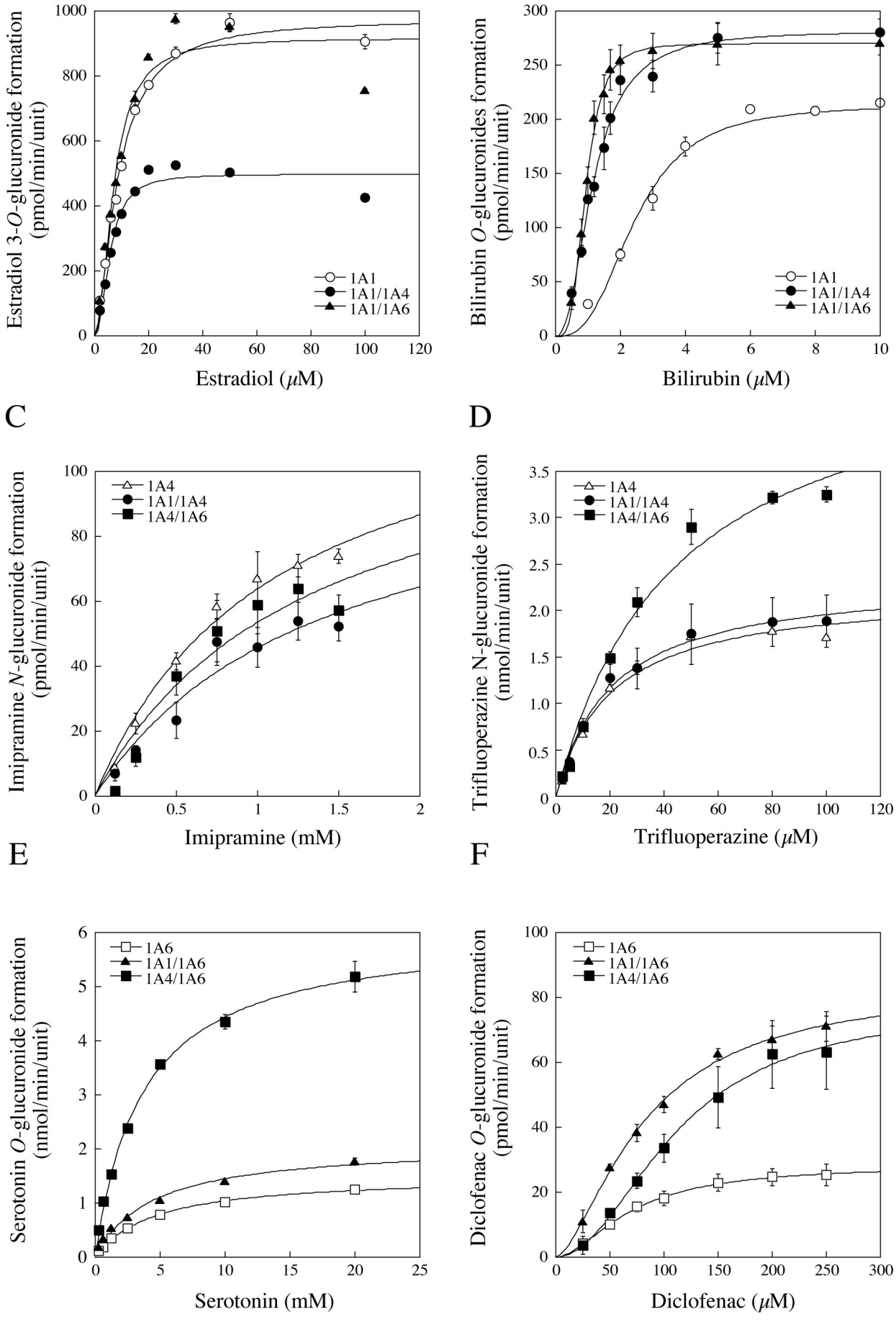

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Fig. 3.