Effects of Coexpression of UGT1A9 on Enzymatic Activities of Human UGT1A Isoforms

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ABSTRACT:

We established stable HEK293 cell lines expressing double isoforms, UGT1A1 and UGT1A9, UGT1A4 and UGT1A9, or UGT1A6 and UGT1A9, as well as stable cell lines expressing each single isoform. To analyze the protein-protein interaction between the UGT1As, we investigated the thermal stability and resistance to detergent. UGT1A9 uniquely demonstrated thermal stability, which was enhanced in the presence of UDP-glucuronic acid (>90% of control), and resistance to detergent. Interestingly, UGT1A1, UGT1A4, and UGT1A6 acquired thermal stability and resistance to detergent by the coexpression of UGT1A9. An immunoprecipitation assay revealed that UGT1A6 and UGT1A9 interact in the double expression system. Using the single expression systems, it was confirmed that estradiol 3-O-glucuronide formation (decreased V_{max}) was enhanced in the presence of UDP-glucuronic acid (PK_{m} decreased) and that serotonin O-glucuronide formation was increased in the presence of UDP-glucuronic acid (V_{max} increased). By kinetic analyses, we found that the coexpressed UGT1A9 significantly affected the kinetics of estradiol 3-O-glucuronide formation (decreased V_{max}), imipramine N-glucuronide formation (increased K_{m} and V_{max}), and serotonin O-glucuronide formation (decreased V_{max}) catalyzed by UGT1A1, UGT1A4, and UGT1A6, respectively. On the other hand, the coexpressed UGT1A1 increased K_{m} and decreased the V_{max} of the propofol O-glucuronide formation catalyzed by UGT1A9. The coexpressed UGT1A4 and UGT1A6 also increased the V_{max} of the propofol O-glucuronide formation by UGT1A9. This is the first study showing that human UGT1A isoforms interact with other isoforms to change the enzymatic characteristics.

UDP-glucuronosyltransferases (UGTs) are a family of membrane-bound enzymes that catalyze the conjugation of endogenous and exogenous compounds with UDP-glucuronic acid (UDPGA) (Dutton, 1980). Human UGTs are classified into two subfamilies, UGT1A and UGT2B, on the basis of evolutionary divergence (Mackenzie et al., 2005). Characterization of genomic DNA clones encoding the UGT1 gene has shown that the UGT1 locus comprises multiple first exons that encode isoform-specific sequences and a single set of commonly used exons 2 to 5 that encodes the same sequence of all UGT1 isoforms (Bosma et al., 1992; Ritter et al., 1992). The C-terminal domain with very high homology is directly involved in UDPGA binding, whereas the N-terminal halves contain the aligyme binding site (Mackenzie, 1990). Among the UGT1A subfamily, UGT1A1, UGT1A4, UGT1A6, and UGT1A9 are expressed in human liver (Strassburg et al., 1999; Tukey and Strassburg, 2000).

UGTs have been reported to form homo- or hetero-oligomers as follows. Matern et al. (1982) suggested for the first time, on the basis of gel permeation chromatography, that UGTs existed as oligomers in rat liver microsomes. Radiation inactivation analyses revealed that UGT existed as oligomers composed of one to four subunits (Peters et al., 1984; Gschaidmeier and Bock, 1994). Moreover, cross-linking studies confirmed possible hetero-oligomerizations between UGT1As and UGT2B1 (Ikushiro et al., 1997). In addition, several experiments in recent years have verified the formation of homo- or hetero-oligomers (Ishii et al., 2001; Kurkela et al., 2004). However, the functional significance of the oligomerization is largely unknown. In the present study, we established stable HEK293 cell lines expressing simultaneously UGT1A1 and UGT1A9, UGT1A4 and UGT1A9, or UGT1A6 and UGT1A9 to investigate the effects of the heterodimerization on the enzymatic activities. Estradiol 3-O-glucuronide, imipramine N-glucuronide, serotonin O-glucuronide, and propofol O-glucuronide formations were determined as specific activities for UGT1A1, UGT1A4, UGT1A6, and UGT1A9, respectively. Using these marker activities, the effects of coexpression of other UGT1A isoforms on thermal stability, resistance to detergent, and the kinetic parameters were investigated.

Materials and Methods

Chemicals and Reagents. UDPGA, alamethicin, estradiol, estradiol 3-O-glucuronide, 4-methylumbelliferone (4-MU), and 4-methylumbelliferone O-glucuronide were purchased from Sigma-Aldrich (St. Louis, MO). Imipramine hydrochloride, serotonin, trifluoperazine dihydrochloride, and G418 were pur-

ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; UDPGA, UDP-glucuronic acid; HPLC, high performance liquid chromatography; 4-MU, 4-methylumbelliferone; 7-HFC, 7-hydroxy-4-trifluoromethylcoumarin; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography.
The densities of the bands were determined using an ImageQuant (GE Healthcare Bio-Sciences, Japan). Propofol was kindly supplied by AstraZeneca (London, UK). Reconstituted from Wako Pure Chemicals (Osaka, Japan). 7-Hydroxy-4-trifluoromethylcoumarin (7-HFC) was obtained from Daishki Pure Chemicals (Tokyo, Japan). Propofol was kindly supplied by AstraZeneca (London, UK). Recombinant human UGT1A1, UGT1A4, UGT1A6, and UGT1A9 expressed in baculovirus-infected insect cells (Supersomes) and rabbit anti-human UGT1A1 polyclonal antibody were obtained from BD Gentest (Woburn, MA). Rabbit anti-human UGT1A6 peptide polyclonal antibody was prepared as described previously (Ishikuro et al., 2006). Primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). All other chemicals and solvents were of analytical grade or the highest grade commercially available.

Isolation of Human UGT1A CDNA and Construction of Expression Vectors. Human UGT1A1 (accession number NM000463), UGT1A4 (NM007120), UGT1A6 (NM001072), and UGT1A9 (NM021027) cDNAs were prepared by a reverse transcription-polymerase chain reaction technique using total RNA from human liver. The sequences of the primers are shown in Table 1. After an initial denaturation at 94°C for 5 min, amplification was initiated by the addition of UDPGA after a 3-min preincubation at 37°C. After incubation at 37°C for 30 min, the reaction was terminated by addition of 100 μM of cold acetonitrile. After removal of the protein by centrifugation at 15,000 rpm for 5 min, a 20-μl portion of the sample was subjected to HPLC.

Imipramine N-glucuronide formation was determined according to the method of Nakajima et al. (2002) with slight modifications. Briefly, a typical incubation mixture (100 μl of total volume) contained 50 mM Tris-HCl buffer, pH 7.4, 0.25 mM MgCl₂, 2 mM UDPGA, 25 μg/ml alamethicin, 0.25 mg/ml total cell homogenates and 2 to 100 μM imipramine. The reaction was initiated by the addition of UDPGA, following a 3-min preincubation at 37°C. After incubation at 37°C for 60 min, the reaction was terminated by addition of 100 μl of cold acetonitrile. After removal of the protein by centrifugation at 15,000 rpm for 5 min, a 20-μl portion of the sample was subjected to HPLC.

Serotonin O-glucuronide formation was determined according to the method of Krishnausamy et al. (2003) with slight modifications. Briefly, a typical incubation mixture (100 μl of total volume) contained 25 mM phosphate buffer (pH 7.5), 2.5 mM MgCl₂, 5 mM UDPGA, 25 μg/ml alamethicin, 0.25 mg/ml total cell homogenates, and 0.3 to 20 mM serotonin. The reaction was initiated by the addition of UDPGA after a 3-min preincubation at 37°C. After incubation at 37°C for 30 min, the reaction was terminated by addition of 100 μM of cold acetonitrile. After removal of the protein by centrifugation at 15,000 rpm for 5 min, a 20-μl portion of the sample was subjected to HPLC.

Propofol O-glucuronide formation was determined according to the method of Shimizu et al. (2003) with slight modifications. Briefly, a typical incubation mixture (200 μl of total volume) contained 50 mM potassium phosphate buffer (pH 7.4), 10 mM MgCl₂, 5 mM UDPGA, 25 μg/ml alamethicin, 0.25 mg/ml total cell homogenates, and 2 mM propofol. The reaction was initiated by addition of UDPGA after a 3-min preincubation at 37°C. After incubation at 37°C for 30 min, the reaction was terminated by addition of 200 μM of cold acetonitrile. After removal of the protein by centrifugation at 15,000 rpm for 5 min, a 20-μl portion of the sample was subjected to HPLC.

Trifluoperazine N-glucuronide formation was determined according to the method of Ghosal et al. (2004) with slight modifications. Briefly, a typical incubation mixture (200 μl of total volume) contained 50 mM Tris-HCl buffer, pH 7.4, 10 mM MgCl₂, 2.5 mM UDPGA, 25 μg/ml alamethicin, 0.25 mg/ml total cell homogenates, and 50 μM trifluoperazine. The reaction was initiated by addition of UDPGA after a 3-min preincubation at 37°C. After incubation at 37°C for 15 min, the reaction was terminated by addition of 100 μM of cold acetonitrile including 6% acetic acid. After removal of the protein by centrifugation at 15,000 rpm for 5 min, a 20-μl portion of the sample was subjected to HPLC.
including 6% acetic acid. After removal of the protein by centrifugation at 15,000 rpm for 5 min, a 20-μl portion of the sample was subjected to HPLC.

Quantification of estradiol 3-O-glucuronide or 4-MU O-glucuronide was performed by comparing the HPLC peak height to that of the authentic standard. For the quantification of the other glucuronides, the eluate from the HPLC column containing each glucuronide was collected, and a part of the eluate was hydrolyzed with NaOH at 75°C for 30 min (Hawes, 1998). The hydrolyzed glucuronides were quantified using HPLC by comparison of peak heights with those of the external standard curve of the substrates.

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:

\[ V = \frac{V_{\text{max}} \cdot [S]}{K_m + [S]} \]

Hill equation

\[ V = \frac{(V_{\text{max}} \cdot [S]^n)}{(S_{50}^n + [S]^n)} \]

Substrate inhibition equation

\[ V = \frac{V_{\text{max}} \cdot [S]}{(K_s + [S])} \]

where \( V \) is the velocity of the reaction, \( S \) is the substrate concentration, \( K_m \) is the Michaelis-Menten constant, \( V_{\text{max}} \) is the maximum velocity, \( S_{50} \) is the substrate concentration showing the half-\( V_{\text{max}} \), \( n \) is Hill coefficient, and \( K_s \) is the substrate inhibition constant. Data are expressed as the means ± S.D. of three independent determinations.

Effects of Heat Treatment and Detergent on Enzymatic Activities. To investigate the effect of heat treatment, the reaction mixtures containing the cell homogenate were treated at 37, 42, 47, 52, and 57°C for 15 min in the presence or absence of UDPGA or substrate. Immediately, the enzymatic activities were determined as described above with the incubation at 37°C for 15 min. To investigate the effect of the detergent, the incubation mixture was incubated with 0.02% (v/v) Triton X-100 for 15 min on ice. Immediately, the enzymatic activities were determined as described above with the incubation at 37°C for 15 min. To investigate the effect of the detergent, the incubation mixture was incubated with 0.02% (v/v) Triton X-100 for 15 min on ice. Immediately, the enzymatic activities were determined as described above with the incubation at 37°C for 15 min on ice. Immediately, the enzymatic activities were determined as described above with the incubation at 37°C for 15 min on ice. Immediately, the enzymatic activities were determined as described above with the incubation at 37°C for 15 min on ice. Immediately, the enzymatic activities were determined as described above with the incubation at 37°C for 15 min on ice. Immediately, the enzymatic activities were determined as described above with the incubation at 37°C for 15 min on ice.

Immunoprecipitation. Rabbit anti-UGT1A6 peptide antibody was conjugated with protein A-Sepharose CL-4B (GE Healthcare Bio-Sciences) using dimethylpimelimidate in PBS containing 0.2 M triethanolamine. The beads were washed with 50 mM ethanolamine in PBS for 5 min and 1 M glycine (pH 3.0) for 20 min. The beads were resuspended in lysis buffer (2 mM EDTA, 1% Triton X-100, 0.1% SDS in PBS). Cell homogenates were lysed with the lysis buffer at 4°C for 60 min. After centrifugation at 13,000g for 30 min at 4°C, the supernatants (0.2 mg) were incubated with the beads at 4°C for 12 h. The beads were washed three times with the lysis buffer. The bound proteins were eluted with 2 M guanidine hydrochloride for 2 h at room temperature. After guanidine hydrochloride was removed using a PAGEprep Advance Protein Clean-up and Enrichment kit (Pierce Biotechnology, Rockford, IL), the immunoprecipitates were subjected to immunoblot analysis.

Statistical Analyses. Statistical significances of the effects of the temperature and kinetic parameters were determined by analysis of variance followed by Dunnett’s test. Statistical significance of the effects of detergent was determined by unpaired Student’s t test. A value of \( P < 0.05 \) was considered statistically significant.

Results

Establishment of Single and Double Expression Systems of UGT1A in HEK293 Cells. To establish the stable cell lines expressing single isoforms of UGT1A1, UGT1A4, UGT1A6, or UGT1A9, we isolated five clones for each isoform. Immunoblot analysis revealed that the expression levels of UGT varied among the clones (data not shown). We selected the clones with the highest UGT1A protein levels for the subsequent analyses. As shown in Fig. 1A, the HEK293 single expression systems of each UGT1A isoform showed a single band at 50 to 55 kDa. In contrast, recombinant UGT1A4, UGT1A6, and UGT1A9 in baculovirus-infected insect cells (Supersomes) showed multiple bands, possibly owing to variability in the glycosylation (Malfatti and Felton, 2004). Based on the UGT1A1 level in HEK293 cells of 1.00 unit/mg protein, UGT1A4, UGT1A6, and UGT1A9 levels were estimated to be 0.94, 0.81, and 0.68 unit/mg protein, respectively (Fig. 1B). The minimal S.D. values of the protein levels (Table 2) revealed the reproducibility of quantification of UGT1As.

Because of the different mobilities, UGT1A1 and UGT1A9, UGT1A4 and UGT1A9, or UGT1A6 and UGT1A9 in each double expression system were separately detected in immunoblot analysis (Fig. 1C). The expression levels of UGT1As in the double expression systems ranged from 0.14 to 0.90 unit/mg protein (Table 2). Although the two expression vectors were transfected with equal content, the
Effects of Coexpression of UGT1A9 on Thermal Instability of UGT1A1, UGT1A4, and UGT1A6. We investigated the thermal instability of UGTs in the double expression systems. In the clone 19-3, estradiol 3-O-glucuronide formation catalyzed by UGT1A1 was restored by the coexpression of UGT1A9 (30% of control in clone 19-3 versus 1% of control in the UGT1A1 single expression system at 52°C (Fig. 4A). However, the restoration was weak in the absence of UDPGA (21% of control). Clones 19-1 and 19-2 showed similar profiles as the single expression system of UGT1A1 (data not shown). The imipramine N-glucuronide formation catalyzed by UGT1A4 was restored by the coexpression of UGT1A9 (Fig. 4B). In clone 49-3, the activity was restored to 61% of control at 57°C, whereas the activity of the single expression system was only 1% of control. However, the restoration was not observed in the absence of UDPGA. Clones 49-1 and 49-2 also showed the restoration, but the extents were lower than that in 49-3 (data not shown). In clone 69-3, serotonin O-glucuronide formation catalyzed by UGT1A6 was restored by the coexpression of UGT1A9 (58% of control in clone 69-3 versus 3% of control in the UGT1A6 single expression system at 57°C) (Fig. 4C). However, the restoration was weak in the absence of UDPGA (8% of control). Clones 69-1 and 69-2 also showed a profile similar to that for clone 69-3 (data not shown). On the other hand, coexpression of UGT1A1, UGT1A4, and UGT1A6 did not show any effect on the thermal stability of UGT1A9 measured by propofol O-glucuronide formation (data not shown).

Effects of Coexpression of UGT1A9 on the Inhibitory Effects of Triton X-100. The effects of Triton X-100 on the enzymatic activities of UGT1A in the single expression systems were investigated (Fig. 5). The activities of UGT1A1, UGT1A4, and UGT1A6 were abolished to <3% of the control activities by 0.02% Triton X-100, whereas the activity of UGT1A9 showed 66% of the control activity. Thus, UGT1A9 was resistant to the treatment with Triton X-100. We then examined the effects of Triton X-100 on the UGT1A activities in the double expression systems. Clones 19-3, 49-3, and 69-3 showed significantly higher residual activity compared with their single expression systems, suggesting that the coexpression of UGT1A9 could increase the resistance to Triton X-100. Clones 69-1 and 69-2 also showed the higher residual activities, although clones 19-1, 19-2, 49-1, and 49-2 showed residual activities similar to those of the single expression systems of UGT1A1 and UGT1A4 (data not shown). The differences in the three clones from each cotransfection were similar to those for the effects on thermal stability, depending on the relative expression ratio. The effects of Triton X-100 on UGT1A9 activity were not changed by the coexpression of UGT1A1, UGT1A4, or UGT1A6. These results suggested that the coexpression of UGT1A9 altered the properties of UGT1A1, UGT1A4, and UGT1A6.

Comunoprecipitation of UGT1A6-UGT1A9. To investigate the direct association between UGT1A9 and the other UGT1A isoforms, immunoprecipitation analysis was performed (Fig. 6). It was confirmed that the anti-human UGT1A6 antibody could immunoprecipitate UGT1A6 protein. When the cell homogenate from the double expression system of UGT1A6 and UGT1A9 was used, both UGT1A6 and UGT1A9 were immunoprecipitated. However, mixing of the single expression systems of UGT1A6 and UGT1A9 could not yield the immunoprecipitant of UGT1A9 (data not shown). The results suggested that UGT1A6 directly interacted with UGT1A9 in a
FIG. 2. Effect of heat treatment on the enzymatic activities of recombinant UGT1A1, UGT1A4, UGT1A6, and UGT1A9 in single expression systems. The reaction mixtures were treated at 37, 42, 47, 52, or 57°C for 15 min in the absence (○) or presence of the substrate (□) or UDPGA (△). Estradiol 3-O-glucuronide (A), imipramine N-glucuronide (B), serotonin O-glucuronide (C), propofol O-glucuronide (D), 4-MU O-glucuronide (E, G, and H), and trifluoperazine N-glucuronide (F) formations were measured at the substrate concentrations of 50 μM, 1 mM, 5 mM, 200 μM, 30 μM, and 50 μM, respectively. The residual activity was determined by comparing with the activity when preincubated at 37°C. Data are the means ± S.D. of three independent determinations. *, P < 0.05; **, P < 0.01, compared with the activities treated in the absence of both the substrate and UDPGA at each temperature.
common membrane. Unfortunately, the association between UGT1A1/UGT1A9 and UGT1A4/UGT1A9 could not be investigated, because antibodies for immunoprecipitation are not available.

Kinetic Analyses of Estradiol 3'-O-Glucuronide, Imipramine N'-Glucuronide, Serotonin O'-Glucuronide, and Propofol O'-Glucuronide Formations. It was confirmed that the estradiol 3'-O-gluc-
curonide, imipramine \(N\)-glucuronide, serotonin \(O\)-glucuronide, and propofol \(O\)-glucuronide formations were specifically detected by UGT1A1, UGT1A4, UGT1A6, and UGT1A9, respectively (data not shown). In addition, the reproducibility of the kinetics of each glucuronide formation by the single expression systems was confirmed using multiple preparations. The estradiol 3-\(O\)-glucuronide formation by the single expression system of UGT1A1 in HEK293 cells followed the Hill equation (Fig. 7A), yielding \(S_{50} = 8.5 \pm 0.2\ \mu M\), \(V_{\text{max}} = 983.7 \pm 21.2\ \text{pmol/min/unit}\), \(V_{\text{max}}/S_{50} = 115.7 \pm 1.2\ \mu l/\text{min/unit}\), and Hill coefficient, \(n = 1.8 \pm 0.1\) (Table 3). The kinetic parameters in the double expression systems of UGT1A1/UGT1A9 are shown in Table 3. The coexpression of UGT1A9 did not affect the \(S_{50}\) and Hill coefficient, but significantly decreased the \(V_{\text{max}}\), resulting in decreased \(V_{\text{max}}/S_{50}\) values. Clone 19-3 with the highest ratio of UGT1A9 to UGT1A1 showed the lowest \(V_{\text{max}}\) value (about one-sixth that of the single expression system).

Imipramine \(N\)-glucuronide formation by the single expression system of UGT1A4 in HEK293 cells showed substrate inhibition at substrate concentrations \(>1.5\ \mu M\), but the plot did not fit to the substrate inhibition kinetics (Fig. 7B). When the kinetics were analyzed by fitting to Michaelis-Menten kinetics with substrate concentrations up to \(1.5\ \mu M\), \(K_{m}\) and \(V_{\text{max}}\) were \(1.1 \pm 0.1\ \mu M\) and \(91.7 \pm 9.5\ \text{pmol/min/unit}\), respectively (Table 4). Coexpression of UGT1A9 significantly increased the \(K_{m}\) and \(V_{\text{max}}\) values, resulting in slightly increased \(V_{\text{max}}/K_{m}\). Clone 49-3 with the highest ratio of UGT1A9 to UGT1A4 showed 4-fold higher \(K_{m}\) and \(V_{\text{max}}\) than those of the single expression systems.
expression system (Table 4). In addition, the coexpression of UGT1A9 attenuated the substrate inhibition, shifting the substrate concentration and showing inhibition from 1.5 to 2.0 mM.

Serotonin O-glucuronide formation by the single expression system of UGT1A6 fitted to the Michaelis-Menten kinetics (Fig. 7C) with $K_m = 4.8 \pm 0.2$ mM and $V_{max} = 1.9 \pm 0.1$ nmol/min/unit (Table 5).
increased the study, we established stable cell lines expressing double UGT isoforms. Therefore, in the present expression of UGT2B22 in COS-7 cells. Unfortunately, a limitation of curonide by guinea pig UGT2B21 was affected by the simultaneous expression of oligomers, but the effects of the oligomerizations on enzymatic acid to various lipophilic compounds. It has been reported that UGTs in the double expression systems. Coexpression of UGT1A9 significantly increased the $K_m$ and decreased the $V_{max}$ values, resulting in decreased $V_{max}/K_m$. The $V_{max}/K_m$ value of clone 69-3 with the highest ratio of UGT1A9 to UGT1A6 was 76% of that of the single expression system (Table 5).

Propofol O-glucuronide formation by the single expression system of UGT1A9 fitted to the substrate inhibition kinetics (Figs. 7, D–F) with $K_i = 59.8 ± 3.1$ μM, $V_{max} = 8.8 ± 0.4$ nmol/min/unit, and $K_i = 1.5 ± 0.1$ μM (Table 6). Coexpression of UGT1A1 significantly increased the $K_m$ value, decreased the $V_{max}$ value, and slightly increased the $K_i$ value. Clone 49-1, with the highest ratio of UGT1A1 to UGT1A9, showed a 3-fold higher $K_m$ value and 9-fold lower $V_{max}$ value than those of the single expression system, resulting in 25-fold lower $V_{max}/K_m$. Coexpression of UGT1A4 significantly decreased the $K_m$ value and increased the $V_{max}$ value, resulting in increased $V_{max}/K_m$. Clone 49-1, with the highest ratio of UGT1A4 to UGT1A9, showed a 5-fold higher $V_{max}/K_m$ than that of the single expression system. Coexpression of UGT1A6 significantly increased the $K_m$ and $V_{max}$ values, resulting in increased $V_{max}/K_m$. It was confirmed that mixing of each single expression system did not influence the kinetics of the enzymatic activities (data not shown). Thus, it is suggested that the coexpressed UGT isoforms functionally interacted with each other in the double expression systems.

**Discussion**

UGTs play a key role in detoxification by conjugating glucuronic acid to various lipophilic compounds. It has been reported that UGTs form oligomers, but the effects of the oligomerizations on enzymatic activities have not been completely investigated. There is only one report suggesting that UGT hetero-oligomerization results in a change of the substrate specificity (Ishii et al., 2001). The authors reported that the formation ratio of morphine 3-glucuronide/morphine 6-glucuronide by guinea pig UGT2B21 was affected by the simultaneous expression of UGT2B22 in COS-7 cells. Unfortunately, a limitation of the study was that the activity was evaluated by a transient expression system. The expression levels and the expression ratios of two UGT isoforms differ in each transient transfection. Therefore, in the present study, we established stable cell lines expressing double UGT isoforms with various expression ratios to examine the effects of simultaneous expression of UGT1A isoforms on the enzymatic activities.

One approach to understanding protein-protein interactions is to investigate the thermal stability of the proteins. It has been reported that protein shows increased thermal stability when it interacts with another protein (Ruvinov and Miles, 1994). Thus, we investigated the thermal stability of the double UGT1A expression system to analyze the protein-protein interactions and found three interesting results. First, we found that UDPGA could increase the thermal stability of UGTs. This phenomenon is reminiscent of MurG (UDP-N-acetylgalcosaminyltransferase from *Escherichia coli*) and GtfA (TDP-epi-vancomycinyltransferase from *Amycolatopsis orientalis*), which are in the glycosyltransferase-B superfamily as are UGTs. These enzymes have the C-terminal domains responsible for donor substrate binding and N-terminal domains responsible for aglycon binding. Second, UGT1A9 uniquely showed thermal stability compared with UGT1A1, UGT1A4, and UGT1A6. Conceivably, the binding of UDPGA may trigger the conformational change to a more stable form by making a closing between the N- and C-terminal domains. Therefore, in the absence of UDPGA, it was confirmed that UGT1A9, showing that there were protein-protein interactions with UGT1A9. However, the acquisition of thermal stability was weak in the absence of UDPGA. Thus, UDPGA may play roles in the conformational stabilization of UGTs not only for monomers or homodimers but also for heterodimers.

Because UGT in microsomes is latent, various types of detergents

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**TABLE 3**

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<td>1A1</td>
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<td>64.2 ± 3.4*</td>
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* $P < 0.01$ compared with the single expression system.

**TABLE 4**

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* $P < 0.01$ compared with the single expression system.

**TABLE 5**

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<tr>
<td></td>
<td>mM</td>
<td>pmol/min/unit</td>
<td>nmol/min/unit</td>
</tr>
<tr>
<td>1A6</td>
<td>4.8 ± 0.2</td>
<td>1.9 ± 0.1</td>
<td>391.6 ± 4.1</td>
</tr>
<tr>
<td>69-1</td>
<td>5.2 ± 0.2*</td>
<td>1.6 ± 0.1*</td>
<td>309.5 ± 8.3*</td>
</tr>
<tr>
<td>69-2</td>
<td>5.1 ± 0.2*</td>
<td>1.6 ± 0.1*</td>
<td>313.6 ± 11.8*</td>
</tr>
<tr>
<td>69-3</td>
<td>5.5 ± 0.2*</td>
<td>1.6 ± 0.1*</td>
<td>297.0 ± 1.1*</td>
</tr>
</tbody>
</table>

* $P < 0.01$ compared with the single expression system.

**TABLE 6**

<table>
<thead>
<tr>
<th>Clone</th>
<th>$K_m$</th>
<th>$V_{max}$</th>
<th>$V_{max}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/min/unit</td>
<td>μmol/min/unit</td>
<td>unit</td>
</tr>
<tr>
<td>1A9</td>
<td>59.8 ± 3.1</td>
<td>8.8 ± 0.4</td>
<td>147.3 ± 2.2</td>
</tr>
<tr>
<td>19-1</td>
<td>173.1 ± 36.6*</td>
<td>1.0 ± 0.1*</td>
<td>5.9 ± 0.6*</td>
</tr>
<tr>
<td>19-2</td>
<td>68.9 ± 11.2</td>
<td>6.7 ± 0.5*</td>
<td>98.7 ± 9.5*</td>
</tr>
<tr>
<td>19-3</td>
<td>59.8 ± 2.2</td>
<td>7.7 ± 0.3*</td>
<td>128.7 ± 3.6*</td>
</tr>
<tr>
<td>49-1</td>
<td>36.4 ± 4.6*</td>
<td>25.1 ± 0.6*</td>
<td>605.1 ± 60.3*</td>
</tr>
<tr>
<td>49-2</td>
<td>46.3 ± 10.7*</td>
<td>24.8 ± 0.4*</td>
<td>560.2 ± 57.5*</td>
</tr>
<tr>
<td>49-3</td>
<td>51.3 ± 10.5</td>
<td>14.8 ± 0.6*</td>
<td>397.2 ± 63.5*</td>
</tr>
<tr>
<td>69-1</td>
<td>81.2 ± 7.4*</td>
<td>25.4 ± 2.2*</td>
<td>313.4 ± 10.5*</td>
</tr>
<tr>
<td>69-2</td>
<td>70.3 ± 9.9*</td>
<td>26.4 ± 3.1*</td>
<td>377.0 ± 12.1*</td>
</tr>
<tr>
<td>69-3</td>
<td>90.4 ± 4.9*</td>
<td>24.7 ± 0.7*</td>
<td>273.8 ± 7.6*</td>
</tr>
</tbody>
</table>

* $P < 0.01$ compared with the single expression system.
increase the enzymatic activities by improving the permeability of substrates or UDPGA. However, high concentrations of detergents over the optimal condition conversely decrease the activity due to progressive disruption of the membrane organization of the UGT (Luuukkanen et al., 1997). Kurkela et al. (2003) have reported that UGT1A9 was uniquely tolerable to the inhibitory effects of detergent. In the present study, we found that UGT1A1, UGT1A4, and UGT1A6 acquired tolerance to detergent by the coexpression of UGT1A9. The phenomenon may result from protein-protein interactions. Although the mechanism determining the tolerance to detergent remains to be clarified, particular properties of UGT1A9 per se or interaction with membrane components such as phospholipids might be relevant to the stability against heat and detergent. Finally, the immunoprecipitation assay proved an association between UGT1A6 and UGT1A9. These results strongly demonstrated the existence of protein-protein interactions between UGT1A1/UGT1A9, UGT1A4/UGT1A9, and UGT1A6/UGT1A9 in the double expression systems.

To examine the effects of the interactions on the enzymatic activities of the UGTs, single and double expression systems were used for kinetic analyses. We found that coexpression of UGT1A9 significantly affected the kinetics of the activities by UGT1A1, UGT1A4, and UGT1A6 and vice versa. The decrease in the $V_{\max}$ value of estradiol 3-O-glucuronide formation by UGT1A1 was 19-1 < 19-2 < 19-3. The increase in $K_m$ and $V_{\max}$ values in imipramine N-glucuronide formation was 49-1 < 49-2 < 49-3. Thus, the effects of UGT1A9 depended on the expression ratio of UGT1A9 toward UGT1A1 or UGT1A4 (Table 2). However, the effects of UGT1A9 on serotonin O-glucuronide formation by UGT1A6 were approximately equal within three clones, because the expression ratios UGT1A6/UGT1A9 were almost the same (Table 5). The expression ratio-dependent effects were also observed in propofol O-glucuronide formation by UGT1A9 (Table 6). These results suggested that the effects of coexpression of other UGT1A isoform on the kinetics were dependent on the expression ratios of each UGT1A isoform.

Extending the results obtained in the present study, comparison of $K_m$ or $S_{\max}$ between recombinant systems and human liver microsomes would be useful to understand the enzymatic characteristics, but the $V_{\max}$ values cannot be compared, because the lack of specific antibodies prevents determination of the expression levels of each UGT1A isoform in human liver microsomes. The present study demonstrated that the $K_m$ value of propofol O-glucuronide formation by UGT1A9 was significantly increased from 59.8 to 173.1 $\mu$M by the coexpression of UGT1A1 or UGT1A6 (Table 6). The $K_m$ values for recombinant UGT1A9 expressed in insect cell and human liver microsomes were reported to be 37 $\mu$M (Soars et al., 2003) and 190 to 213 $\mu$M (Shimizu et al., 2003; Soars et al., 2003), respectively. The higher $K_m$ values in human liver microsomes might result from protein-protein interactions between UGT1A9 and UGT1A1 or UGT1A6. Imipramine N-glucuronide formation showed substrate inhibition at concentrations >1.5 $\mu$M in the single expression system of UGT1A4 but at >2.0 $\mu$M by the coexpression of UGT1A9 (Fig. 7B). Interestingly, the kinetics in human liver microsomes also showed substrate inhibition at >2.0 to 2.5 $\mu$M (Nakajima et al., 2002; Qian and Zeng, 2006). Thus, the change of the substrate concentration showing inhibition might also result from protein-protein interactions between UGT1A4 and UGT1A9. Although the $K_m$ values for estradiol 3-O-glucuronide and serotonin O-glucuronide formations were not changed by the coexpression of UGT1A9, these values were close to that in human liver microsomes (Fisher et al., 2000; Krishnaswamy et al., 2003; Lepine et al., 2004). The $V_{\max}$ values were decreased or increased by the coexpression of other isoforms. Therefore, to predict the glucuronide formations in human liver microsomes, it might be necessary to understand these UGT-UGT interactions.

In conclusion, we found that UGT1A1, UGT1A4, and UGT1A6 interact with UGT1A9, acquiring the enzymatic characteristics of the other isoform, resulting in a change of the kinetics of the enzymatic activities. Extending the lines of research presented here to the interaction between UGT1A1 and UGT2B may also increase our understanding of UGT activities in human liver microsomes.

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References


Qian MR and Zeng S (2006) Biosynthesis of imipramine glucuronide and characterization of...

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