Alternatively Spliced Products of the UGT1A Gene Interact with the Enzymatically Active Proteins to Inhibit Glucuronosyltransferase Activity In Vitro

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ABSTRACT:

UDP-glucuronosyltransferases (UGTs) are major mediators in conjugative metabolism. Current data suggest that UGTs, which are anchored in the endoplasmic reticulum membrane, can oligomerize with each other and/or with other metabolic enzymes, a process that may influence their enzymatic activities. We demonstrated previously that the UGT1A locus encodes previously unknown isoforms (denoted “i2”), by alternative usage of the terminal exon 5. Although i2 proteins lack transferase activity, we showed that knockdown of endogenous i2 levels enhanced cellular UGT1A-i1 activity. In this study, we explored the potential of multiple active UGT1A_i1 proteins (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, and UGT1A9) to interact with all spliced i2s by communoprecipitation. We further studied the functional consequences of coexpressing various combinations of spliced i1s and i2s from highly similar UGTs, namely UGT1A7, UGT1A8, and UGT1A9, based on expression profiles observed in human tissues. The i1 isoform of each UGT1A communoprecipitated its respective i2 homolog as well as all other i2s, indicating that they can form heteromeric complexes. Functional data further support the fact that i2 splice species alter glucuronidation activity of i1s independently of the identity of the i2, although the degree of inhibition varied, suggesting that this phenomenon may occur in tissues expressing such combinations of splice forms. These results provide biochemical evidence to support the inhibitory effect of i2s on multiple active UGT1As, probably through formation of inactive heteromeric assemblies of i1s and inactive i2s. The relative abundance of active/inactive oligomeric complexes may thus determine transferase activity.

Introduction

The human UDP-glucuronosyltransferases (UGTs) are conjugative enzymes responsible for the biotransformation/inactivation of various endogenous and xenogenous molecules. The human UGT1A gene exemplifies the alternative use of promoters, first exons, and terminal exons to diversify enzyme specificity and increase metabolic capacity (Ritter et al., 1992; Gong et al., 2001; Lévesque et al., 2007). The UGT1A locus is defined by 13 first exons, which are alternatively spliced to 3 other common exons and 2 alternative terminal exons 5. From the 27 possible mRNA isoforms, 9 produce functionally active polypeptides (UGT1A1, 1A3, 1A4, 1A5, 1A6, 1A7, 1A8, 1A9, and 1A10) called isoforms 1 (i1). Recent findings suggest that the alternative exon 5 (5b) might be used as terminal exon, leading to 9 alternatively spliced products termed isoforms 2 (i2) (Girard et al., 2007; Lévesque et al., 2007).

UGTs are endoplasmic reticulum (ER)-resident membrane proteins. The UGT1A_i2 splice variants lack the transmembrane domain encoded by exon 5a but contain an N-terminal ER-targeting signal peptide and complete binding sites for both the substrate and cosubstrate, glucuronic acid (UDP-glucuronic acid). Despite the fact that the UGT1A_i2 proteins are expressed in human tissues and reside in the ER, they lack the ability to transfer a glucuronic acid moiety to classic substrates of the UGT1A_i1 enzymes. Rather, they have a repressive effect on rates of formation of glucuronide products when coexpressed with their cognate UGT1A_i1 isoforms in HEK293 cells (e.g., UGT1A1_i1 coexpressed with UGT1A1_i2) (Girard et al., 2007; Lévesque et al., 2007; Bellemare et al., 2010). Because i1 and i2 proteins are in close proximity in the ER membrane (Girard et al., 2007; Lévesque et al., 2007; Bellemare et al., 2010), we hypothesized that these two isoform types may interact physically, thereby establishing a mechanism for the observed dominant-negative effect of i2s on the activities of i1 enzymes.
Previous work by others clearly support the fact that UGTs form oligomeric complexes (Finel and Kurkela, 2008; Bock and Köhle, 2009; Ishii et al., 2010). Homo-oligomerization of various UGTs has been demonstrated, such as that for rat UGT2B1 (Meech and Mackenzie, 1997), human UGT1A1 (Ghosh et al., 2001), and UGT1A9 (Kurkela et al., 2003). These interactions were further supported by a recent study using fluorescence resonance energy transfer technology that revealed the capacity of UGT1As to form homodimers in living cells (Oparaña and Tukey, 2007). Thereafter, the formation of various hetero-oligomers was described by means of several techniques. UGT1A1, UGT1A4, UGT1A6, and UGT1A9 have been found to form both homodimers and heterodimers (Fujiwara et al., 2007a; Nakajima et al., 2007). UGT1A1 was further demonstrated to interact with all members of the UGT1A family (Oparaña and Tukey, 2007) and more recently with its i2 isoform, UGT1A1_i2 (Lèvesque et al., 2007). Hetero-oligomerization had also been reported between members of the UGT1A and UGT2B families, thereby underscoring the breadth of diversity of interactions between the various isoforms (Ikushiro et al., 1997; Kurkela et al., 2007; Fujiwara et al., 2010). However, the potential for UGT1A i1 and i2 isoforms to form hetero-oligomers and mixed oligomers has not been addressed.

Several studies have emphasized that protein interactions between UGT isoenzymes often result in decreased enzyme activity or altered substrate specificity (Ikushiro et al., 1997; Meech and Mackenzie, 1997; Kurkela et al., 2004, 2007; Fujiwara et al., 2007a,b, 2010; Nakajima et al., 2007; Oparaña and Tukey, 2007). Functional implications of dimer formation have been studied in several ways; for example, the simultaneous expression of UGT2B7 and UGT1A1, UGT1A4, UGT1A6, and/or UGT1A9 results in enhanced glucuronide production (Kurkela et al., 2007; Fujiwara et al., 2010). The substrate specificity of a UGT also appears to be affected by the constituents of the heterocomplex, as suggested by Ishii et al. (2001). Taken together, these observations suggest that oligomerization between UGTs affects their enzymatic function although the extent of these interactions has never been quantified.

In this study, we examined the potential of protein-protein interactions between all splice forms of the UGT1A locus. After our finding that knockdown of endogenous i2 enhanced cellular UGT1A activity in colon cells (Bellemare et al., 2009), we explored the functional consequence of particular heterologous combinations between i1 and i2 proteins in vitro. The choices of isoforms that we tested, namely the highly similar UGT1A7, UGT1A8, and UGT1A9 often coexpressed in some tissues and sharing overlapping substrates, were based on our previous observations suggesting coexpression profiles of splice products in human drug-metabolizing tissues (Girard et al., 2007).

Materials and Methods

Materials. UDP-glucuronic acid was obtained from Sigma-Aldrich (St. Louis, MO), and blastcidin and Geneticin (G418) were purchased from Wisent Bioproduits (St-Bruno, QC, Canada). HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA). Protein assay reagents (Bradford, 1976) were obtained from Bio-Rad Laboratories (Hercules, CA), mycophenolate acid was from MP Biomedicals (Solon, OH), and SN-38 was prepared by hydrolysis of irinotecan-HCl (McKesson, ON, Canada) as described previously (Gagné et al., 2002; Bernard and Guillemette, 2004) and is reported as glucuronidation rates (picomoles per minute per milligram of protein) and normalized for UGT expression assessed by Western blotting. For Western blotting, 20 μg of membrane proteins at 2 mM UDP-glucuronic acid and were initiated by adding various concentrations of each individual substrate for determination of Km values. All enzymatic assays were incubated for 60 min at 37°C. Assays involving the glucuronidation of SN-38 and mycophenolic acid (MPA) were stopped with 100 and 200 μl of ice-cold methanol (1% HCl). Assays were then centrifuged at 14,000g for 10 min before analysis. Glucuronide formation was measured by high-performance liquid chromatography coupled with tandem mass spectrometry as described previously (Gagné et al., 2002; Bernard and Guillemette, 2004) and is reported as glucuronidation rates (picomoles per minute per milligram of protein) and normalized for UGT expression assessed by Western blotting. For Western blotting, 20 μg of microsomal proteins was resolved by 10% SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and probed with the anti-human UGT1A RC-71, as reported (Bellemare et al., 2010). A goat anti-rabbit IgG polyclonal antibody, horseradish peroxidase-conjugated (GE Healthcare, Baie d’Urfe, QC, Canada), was used as the secondary antibody, and the resulting immunocomplexes were visualized using the Western Lightning Plus-ECL chemiluminescence substrate (PerkinElmer, Woodbridge, ON, Canada).

Data Analysis and Statistics. Kinetic parameters were calculated using SigmaPlot 8.0 with Enzyme Kinetics 1.1 (SPSS, Chicago, IL). Eadie-Hofstee plots [velocity as a function of (velocity/substrate concentration)] and visual inspection of fitted functions (velocity as a function of substrate concentration) were used to select the best-fit enzyme kinetic model (Venkatakrishnan et al., 2001). Values are expressed as the mean ± S.D. of at least two experiments performed in triplicate. Differences in glucuronidation rates and kinetic parameters were evaluated for statistical significance by a paired Student’s t test (p < 0.05).

Results

Multiplicity of Protein-Protein Interactions between UGT1A Splice Products. We initially examined the potential of protein-protein interactions between isoforms i1 and i2 for each of the nine functional UGT1A isoforms. Coimmunoprecipitation (co-IP) experiments were conducted using total protein from cell lysates of double-transfected cell lines expressing transiently both splice forms. Immunopre-
coprecipitated with a given i1 were visualized by Western blotting with anti-V5. Myc-His with every i2 isoform, suggesting that there are broad interactions between isoforms was able to interact not only with its respective i2 but also with other i2 isoforms tagged with a V5-His epitope. A, coimmunoprecipitation assays. UGT1A isoforms 1 were subcloned and tagged with a Myc-His epitope, whereas isoforms 2 were tagged with a V5-His epitope. A, controls for co-IP experiments. Lanes 1 and 2, negative control co-IP experiments with anti-myc (1 mg); lane 1, expression of UGT1A1_i1-Myc-His alone; lane 2, expression of UGT1A1_i2-V5-His alone; lanes 3 and 4, expression (or no expression) of UGT1A1_i1-Myc-His + UGT1A1_i2-V5-His; lanes 5 and 6, positive control co-IP assays with anti-His (1 mg): lane 5, visualization of co-IP of UGT1A1_i2-V5-His (5 μg) was loaded onto the gel as a positive control for the Western blot. B, co-IP assays of various UGT1A_i1s-Myc-His + UGT1A_i2s-V5-His. co-IP assays were performed with anti-Myc (1 mg) to precipitate i1 isoforms and any interacting i2 isoform(s). The i2 species that coprecipitated with a given i1 were visualized by Western blotting with anti-V5.

Fig. 1. Evidence of interactions between multiple UGT1A spliced isoforms by coimmunoprecipitation assays. UGT1A isoforms 1 were subcloned and tagged with a Myc-His epitope, whereas isoforms 2 were tagged with a V5-His epitope. A, controls for co-IP experiments. Lanes 1 and 2, negative control co-IP experiments with anti-myc (1 mg); lane 1, expression of UGT1A1_i1-Myc-His alone; lane 2, expression of UGT1A1_i2-V5-His alone; lanes 3 and 4, expression (or no expression) of UGT1A1_i1-Myc-His + UGT1A1_i2-V5-His; lanes 5 and 6, positive control co-IP assays with anti-His (1 mg); lane 5, visualization of co-IP of UGT1A1_i2-V5-His (5 μg) was loaded onto the gel as a positive control for the Western blot. B, co-IP assays of various UGT1A_i1s-Myc-His + UGT1A_i2s-V5-His. co-IP assays were performed with anti-Myc (1 mg) to precipitate i1 isoforms and any interacting i2 isoform(s). The i2 species that coprecipitated with a given i1 were visualized by Western blotting with anti-V5.

Inactive i2s Inhibit i1-Mediated Glucuronidation Pathway. With knowledge of these expression profiles, we then tested the functional consequence of expressing specific i1 and i2 heterologous combinations using clonal cell lines overexpressing various combinations of UGT1A_i1s (tagged with the myc epitope) alone or in combination with i2 (tagged with the V5 epitope). Detailed kinetic analyses were performed by varying the amounts of substrate and/or cosubstrate. Three double stable HEK clones were used to address the functional consequence of expressing specific i1 and i2 heterologous combinations.

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TABLE 1

**Kinetic parameters of glucuronidation by UGT1A coexpressed splice isoforms**

<table>
<thead>
<tr>
<th>Substrates and HEK293 Cell Lanes</th>
<th>Applied $K_m$ (μM)</th>
<th>Relative $V_{max}$ (pmol/min·mg)</th>
<th>CL</th>
<th>%Inhibition vs. i1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SN-38</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1A7_i1</td>
<td>9.8 ± 1.5</td>
<td>5.0 ± 0.4</td>
<td>0.5 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>1A7_i1 + 1A8_i2</td>
<td>7.2 ± 1.9</td>
<td>3.1 ± 0.7*</td>
<td>0.5 ± 0.3</td>
<td>↓39</td>
</tr>
<tr>
<td><strong>MPA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1A8_i1</td>
<td>128 ± 40</td>
<td>1875 ± 205</td>
<td>14.7 ± 7.8</td>
<td></td>
</tr>
<tr>
<td>1A8_i1 + 1A7_i2</td>
<td>105 ± 7</td>
<td>1350 ± 225*</td>
<td>12.9 ± 2.3</td>
<td>↓28</td>
</tr>
<tr>
<td>1A8_i1 + 1A9_i2</td>
<td>89 ± 12</td>
<td>1223 ± 142*</td>
<td>13.7 ± 3.5</td>
<td>↓34</td>
</tr>
</tbody>
</table>

CL, clearance. *p < 0.05 compared with UGT1A_i1-alone. The substrate inhibition profile was observed for UGT1A7 and SN-38, whereas the Michaelis-Menten profile was fitted for UGT1A8 and MPA. Kinetic profiles remained unchanged in cell lines coexpressing i1 and i2 forms compared with those expressing i1 only.

**FIG. 2.** Distribution of specific UGT1A7, UGT1A8, and UGT1A9 mRNA splice variants in selected human tissues. Specific primers located in exon 1 were used as sense primers with antisense primers specific for exon 5a (spliced forms i1) and 5b (spliced forms i2). The length of the amplification products for each UGT1A is indicated. The identity of all amplicons was confirmed by sequencing. One fifth of each reverse transcriptase-PCR was loaded on the gels. bp, base pairs.
splice species, whereas the degree of inhibition may vary depending of the specific i2, raising the possibility that this phenomenon may occur in tissues expressing particular combinations of i1 and i2 isoforms.

**Discussion**

In the present study, we used co-IP to demonstrate intermolecular interactions between active UGT1A_i1 and inactive UGT1A_i2 proteins. In addition, we provided biochemical evidence for the functional consequences of coexpressing various combinations of inactive i2 with active i1 proteins. These experiments support an inhibitory effect of individual i2s on various UGT1As and are consistent with formation of inactive heteromeric assemblies.

The heterodimerization of UGT1A proteins has been reported by measuring fluorescence resonance energy transfer in living cells, suggesting that all active UGT1A_i1s are able to physically interact (Operan˜a and Tukey, 2007). Our data suggest that i2 proteins are also capable of interacting with various active i1s. For instance, UGT1A1_i1 coimmunoprecipitated its corresponding UGT1A1_i2 homolog as well as all other i2s, including those of UGT1A3, 1A4, 1A6, 1A7, 1A8, 1A9, and 1A10. These results are consistent with previous observations supporting the strong potential of UGTs to oligomerize (Lêvesque et al., 2007; Finel and Kurkela, 2008; Bellemare et al., 2010). This approach was made possible by generating a library of UGT1A plasmids containing the Myc-His or V5-His epitope to evaluate interactions between different combinations of UGT1A_i1 and i2 isoforms. However, the quantitative evaluation of complexes was not measurable in that context. The amount of complexes is not a direct reflection of the intensity of the immunoprecipitates but rather may reflect variable levels of UGT expression in the protein preparations derived from transient transfections. Nevertheless, our data clearly demonstrate the broad diversity of heterooligomeric complexes between UGT1A i1 and i2 and raise the possibility that, if present in the ER membrane, any given i2 could potentially interact with any active i1. The suppression of endogenous i2 by RNA interference enhances glucuronidation activity in colon cancer cell lines, supporting the idea of a dominant-negative effect of splice products in cells (Bellemare et al., 2009). In addition, we recently reported that active UGT1As_i1 and inactive UGT1As_i2 isoforms are coproduced in the same tissue structures including liver, kidney, stomach, intestine, and colon (J. Bellemare, M. Rouleau, M. Harvey, I. Popa, G. Pelletier, B. Têtu, and C. Guillemette, submitted for publication) providing evidence for dominant-negative role of UGT1As_i2.

Similar to our observations, a large number of examples in the literature suggest that splice variants of various genes exert a dominant-negative effect through heterodimerization with their active counterpart (Stasiv et al., 2001; Kern et al., 2008). Moreover, several studies have indicated that UGTs can form hetero-oligomers, often with functional consequences (Ikushiro et al., 1997; Meech and Mackenzie, 1997; Kurkela et al., 2004, 2007; Fujiwara et al., 2007a; Nakajima et al., 2007; Operan˜a and Tukey, 2007). Indeed, some reports have suggested that the impact on UGT glucuronidation activity depends on which other monomer(s) is in the oligomer (Kurkela et al., 2004, 2007; Nakajima et al., 2007).

The influence of i2 on isoform 1-mediated activity seems to be a general mechanism, seemingly independent of the nature of the i2 species. However, the binding affinity of specific i2s toward any particular i1 may turn out to be an important factor in the selection of its partner in vivo. This observation is supported by the significant repression of UGT1A7_i1-mediated glucuronidation of SN-38 by UGT1A7_i2 described in Bellemare et al. (2010), compared with that for UGT1A8_i2 herein. Although both i2 isoforms are capable of interacting with UGT1A7_i1, the level of inhibition was greater in the
INTERACTION OF UGT1A SPliced FORMS INHIBIT EnZyme ACTIVITY

References


