Expression and Characterization of Recombinant Human UDP-glucuronosyltransferases (UGTs)

UGT1A9 IS MORE RESISTANT TO DETERGENT INHIBITION THAN THE OTHER UGTs AND WAS PURIFIED AS AN ACTIVE DIMERIC ENZYME*

Received for publication, June 20, 2002, and in revised form, September 24, 2002
Published, JBC Papers in Press, November 14, 2002, DOI 10.1074/jbc.M206136200

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Eight human liver UDP-glucuronosyltransferases (UGTs) were expressed in baculovirus-infected insect cells as fusion proteins carrying a short C-terminal extension that ends with 6 histidine residues (His tag). The activity of recombinant UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT2B4, UGT2B7, and UGT2B15 was almost fully inhibited by 0.2% Triton X-100. In the case of UGT1A9, however, glucuronidation of α-naphthol and scopoletin was resistant to such inhibition, whereas glucuronidation of entacapone and several other aglycones was sensitive. His-tagged UGT1A9 was purified by immobilized metal-chelating chromatography (IMAC). Purified UGT1A9 glucuronidated scopoletin at a high rate, whereas its glucuronidation activity toward entacapone was low and largely dependent on phospholipid addition. Recombinant UGT1A9 in which the His tag was replaced by hemagglutinin antigenic peptide (HA tag) was also prepared. Insect cells were co-infected with baculoviruses encoding both HA-tagged and His-tagged UGT1A9. Membranes from the co-infected cells, or a mixture of membranes from separately infected cells, were subjected to detergent extraction and IMAC, and the resulting fractions were analyzed for the presence of each type of UGT1A9 using tag-specific antibodies. In the case of separate infection, the HA-tagged UGT1A9 did not bind to the column. When co-infected with His-tagged UGT1A9, however, part of the HA-tagged enzyme was bound to the column and was eluted by imidazole concentration gradient together with the His-tagged UGT1A9, suggesting the formation of stable dimers that contain one His-tagged and one HA-tagged UGT1A9 monomers.

UDP-glucuronosyltransferases (UGTs)1 are a family of membrane-bound proteins of the endoplasmic reticulum that play an important role in the biotransformation of a large number of xenobiotics and endogenous compounds (for reviews see Refs. 1–5). The UGTs catalyze glucuronic acid transfer from UDP-glucuronic acid (UDP-GA) to acceptor compounds, aglycones, that are mostly lipophilic and rather small molecules. Glucuronidation renders the aglycone more water-soluble and facilitates its excretion from the body. Mutations that lead to low expression level or weak activity of UGT1A1 result in poor bilirubin glucuronidation and subsequent development of Crigler-Najjar or Gilbert’s Syndromes (6, 7). Mutations in UGT1A17 were suggested to increase the risk of colorectal cancer development (8), whereas UGT1A9 was shown to play an important role in the metabolism of certain carcinogenic amines (9). In addition to xenobiotics, UGTs of the 2B subfamily glucuronidate steroid hormones and bile salts, all of which may have important clinical implications. Hence, gaining deeper insight into the detoxification of carcinogens and metabolism of drugs and several endogenous compounds requires better understanding of the structure and function of UGTs.

The human genome contains many UGT genes (including some pseudogenes), most of which are grouped into two main subfamilies, UGT1A and UGT2B. The proteins are made of two large domains, a variable N-terminal half that is encoded by exon 1 of the respected genes, and a highly conserved C-terminal half that is encoded by exons 2–5. Due to exon sharing the C-terminal domain is identical in all the UGTs of the 1A subfamily. The C-terminal halves of the UGT2B enzymes are almost, but not fully, identical to each other, and they are also very similar to that of the UGT1A enzymes. Because all the UGTs employ UDP-GA as the sugar donor, the identity/similarity of the C-terminal half, as well as some experimental evidence, suggests that this domain harbors the UDP-GA-binding site (10, 11). The sugar acceptor specificity may thus be determined primarily by the structure of the N-terminal half (12). The aglycone specificity does vary among the individual UGTs, but large overlaps among them in this respect are well documented (e.g. Ref. 4 and references therein). Furthermore, each UGT can glucuronidate more than one substrate, a promiscuity that may be typical of detoxifying enzymes. Many UGTs are expressed in the liver, and in combination with partial overlapping in substrate specificity and substrate promiscuity, this means that the characterization of individual UGT activities in the native membrane is a very difficult task. The use of recombinant enzymes has thus become a common practice in the field, particularly when testing which UGT isoform glucuronidates a specific substrate of interest (e.g. Refs. 1, 4, 13–15).

The UGTs are bound to the endoplasmic reticulum membrane so that most of their mass is located on the luminal side.
of the membrane. A short trans-membrane segment is present close to the C-terminal of these 50–60-kDa proteins, and the last 20–22 amino acids are exposed on the cytoplasmic side of the membrane (2, 3). The single trans-membrane segment is not, however, the only way by which the enzymes bind to the membrane. Recombinant UGTs that carry a stop codon before the beginning of the trans-membrane segment were reported still to be bound to the membrane but to be catalytically inactive (16, 17). In contrast to the latter inactivation, however, truncation of UGT2B1 downstream from the trans-membrane segment did not abolish enzyme activity, indicating that the cytoplasmic “tail” is not essential for glucuronic acid transfer (16).

Questions about whether or not the UGTs exist as dimers, either homo- or heterodimers, and what the implications of such arrangements might be have often been raised (18–23). It was recently suggested that heterodimers including the guinea pig enzymes UGT2B21 and UGT2B22 might exhibit activity that is either weaker or even undetectable when either of the two participating UGTs is expressed alone (22). On the other hand, it was reported that UGT1A1 forms homodimers and not heterodimers and that dimers that contain one native and one mutant monomer are partly or completely inactive (23). Nevertheless, determination of the oligomeric state of human UGTs, either recombinant or in the native membrane, is not an easy task because of the absence of a good purification procedure that would yield sufficient amount of the pure and active enzyme. If an inactive enzyme turns out to be monomeric, it may be claimed that monomerization during the purification was the cause of activity loss; and if it is found to be oligomeric, then it may be argued that the enzyme aggregated during purification.

We have prepared a recombiant human liver UGTs that were expressed as fusion proteins with a C-terminal His tag. Among these UGTs, the activity of UGT1A9 is exceptional in its resistance to inhibition by high concentration of several detergents, at least when glucuronidation of some aglycones is concerned. In addition, our results indicate that the recombinant human UGT1A9 is a stable homodimeric enzyme.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—α-Naphthal, 4-methylumbelliferone, p-nitrophenol, saccharalactone, scopolentin, umbelliferone, UDP-GA, α-naphthyl-β-D-glucuronide, 4-methylumbelliferyl-β-D-glucuronide, and p-nitrophenyl-β-D-glucuronide were purchased from Sigma, and 7-hydroxycoumarin glucuronide was from UFC. Radiolabeled [3H]UDP-GA was from PerkinElmer Life Sciences. Entacapone was kindly provided by Orion Pharma (Espoo, Finland), and entacapone glucuronide was synthesized in our laboratory (24). Triton X-100 was purchased from BDH; dodecyl maltoside was from Anachemia, and phospholipids (phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol) were obtained from Avanti Polar Lipids (Alabaster, AL). The detergent tolerance test was performed at concentrations above the critical micellar concentrations depending on the detergent tested. The concentration of sonicated phospholipids in assays of the purified UGT1A9, when added, was 1 mg/ml. All the UGT activity assays were performed at 37 °C and the reaction time varied, within a linear range, from 15 to 60 min. The reactions were terminated, and the proteins were precipitated by an addition of cold perchloric acid to a final concentration of 365 mM and chilling in an ice bath for 10 min, followed by 5 min of centrifugation at 15,700 × g. Aliquots of the supernatants were analyzed using model 1100 high pressure liquid chromatography (Agilent) under the conditions described in Table I.

**Purification of UGT1A9**—All the steps were carried out on ice or at 4 °C. Membranes were thawed and suspended in extraction medium (25 mM Tris, pH 7.5, 500 mM NaCl, 1% Triton X-100) at about 1–2 mg of protein/ml. The suspension was mixed for 10 min and centrifuged at 41,000 × g for 1 h. The resultant supernatant was filtered through a 0.45-μm syringe filter to remove the remaining particles and loaded onto a nickel-charged His Hi-trap column attached to an AKTA prime machine (Amersham Biosciences) that has been pre-equilibrated with Buffer A (25 mM Tris, pH 7.5, 500 mM NaCl, 0.05% Triton X-100, 50 mM imidazole). After extensive washing the bound protein was eluted by imidazoline gradient, 50–400 mM, in the presence of NaCl and Triton X-100. The collected fractions were examined by SDS-PAGE and Western blotting, and selected fractions were pooled and concentrated using Centricon YM30 filter device (Millipore). Western Blotting—Proteins were separated by 12% SDS-PAGE (26) and transferred to PROTRAN 0.45-μm pore size nitrocellulose transfer membrane (Schleicher & Schuell) in the Mini PROTEAN 3 apparatus (Bio-Rad). His-tagged recombinant UGTs were detected using Tetra-His monoclonal antibodies (Qiagen) and HA-tagged UGT1A9 with the HA.11 monoclonal antibodies (Covance). The second antibody was horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology). The SuperSignal West Pico chemiluminescent peroxidase system (Pierce) was employed for visualization.

Protein concentrations were measured using the bicinchoninic acid (BCA) system (Pierce) and bovine serum albumin as a standard. N-terminal sequencing was performed on an Applied Biosystems 477A/120A automated sequencer, in the gas phase mode (27), following the procedure supplied by the manufacturer.
### Purification of Recombinant UGT1A9

Eight different human UGTs, 1A1, 1A3, 1A4, 1A6, 1A9, 2B4, 2B7, and 2B15 were cloned and expressed in baculovirus-infected insect cells as His-tagged proteins. The recombinant enzymes also contain an enterokinase cleavage site (Fig. 1), but due to the good specific activity of the expressed UGTs, and the usefulness of the His tag for immunodetection, enterokinase treatment was omitted. The expression of the different UGTs was aimed at getting a high yield of the active enzyme rather than a high protein production. To this end activity assays in the presence of suitable substrates were used in order to optimize infection with each virus stock and to develop the isolation protocol of the membranes. The outcome of these experiments indicated that in most cases there is an optimal value for the infection size and that the post-infection culturing should be carried out for about 48 h. During the experiments to improve sample preparation methods, we have observed that sonication reduces total activity and that such a treatment to disrupt insect cells that were cultured in suspension is unnecessary. Freezing, thawing, and mild osmotic shock render the cells permeable for both UDP-GA and the aglycone substrates. In line with these observations a total membrane fraction, rather than microsomes, was used for the subsequent activity assays.

It may be asked whether or not the recombinant UGTs function like their counterparts in the native tissue, particularly when they are slightly modified. Nevertheless, the overlap in substrate specificity among many of the UGTs, and the expression of all the UGTs that are examined in this work in the liver, renders comparison of the kinetic properties between most recombinant and native UGTs very difficult. In the case of UGT1A9, however, it is feasible to do such a comparison because entacapone, a selective substrate for this enzyme, is poorly glucuronidated by the other liver UGTs (28). We have thus analyzed the rate of entacapone glucuronidation by human liver microsomes and the recombinant UGT1A9 that carries the C-terminal extension (Fig. 2). The similarity in specific activity between the two preparations is high, but this may partly be coincidental. Nevertheless, it is clear that the two different samples exhibit similar kinetics, including both the high affinity for entacapone as well as the substrate inhibition at high concentrations of the aglycone (Fig. 2). It can thus be concluded that the recombinant His-tagged UGT1A9 that was expressed in baculovirus-infected SF9 cells is a good model for the native enzyme.

The lack of a good method to purify individual UGTs in an active form was singled out as the major current hurdle in understanding their structure-function relationships (3). The recombinant UGTs described in this study carry His tags that should allow fast purification by immobilized metal affinity chromatography (IMAC) and are thus most suitable for the purification experiments. Being membrane proteins, purification of a UGT requires extraction by a detergent that does not inactivate the enzyme at concentrations above the critical micellar concentration (cmc) for the given detergent. We have tested the effects of 0.2% Triton X-100 and 0.1% dodecyl maltoside on the activity of recombinant UGTs. The effects of both detergents on the glucuronidation of α-naphthol and scopoletin by the different recombinant UGTs were very similar, and the Triton X-100 results are presented in Table II. In the case of both aglycones, UGT1A9 was the only enzyme that is not merely resistant to the detergent-induced inhibition but even stimulated by such treatments. Recombinant UGT1A4 was not tested in these experiments because it does not glucuronidate scopoletin or α-naphthol at measurable rates (not shown). The glucuronidation of 4-aminobiphenyl by recombinant UGT1A4

### TABLE I

| Substrate | Column | Mobile phase | Detection | Quantitation |
|-----------|--------|--------------|-----------|-------------|
| α-Naphthol | Symmetry C18, 150 × 3.9 mm | 50 mM phosphate, pH 3, MeOH, 50/50%, 1.0 ml/min | Fluorescence, 335/455 nm | Reference standard (Sigma) |
| Entacapone | Hypersil BDS-C18, 150 × 4.6 mm | 50 mM phosphate, pH 3, MeOH, 85/15%, 1.5 ml/min | Fluorescence, 316/382 nm | Reference standard (Sigma) |
| 4-Methyl-umbelliferone | Chromolith SpeedROD rp-18e, 50 m | 50 mM phosphate, pH 3, MeOH, 90/10%, flow | Fluorescence, 355/455 nm | Method utilizing labeled UDP-GA, as described by Kaivosaa et al. (31) |
| Scopoletin | Chromolith SpeedROD rp-18e, 50 m | 50 mM phosphate, pH 3, MeOH, 90/10%, flow | Absorbance, 310 nm | Reference standard (Luukkanen et al. 1999) |
| 4-Nitrophenol | Symmetry C18, 150 × 3.9 mm | 50 mM phosphate, pH 3, MeOH, 75/25%, 1.0 ml/min | Absorbance, 310 nm | Reference standard (Sigma) |

### RESULTS

Eight different human UGTs, 1A1, 1A3, 1A4, 1A6, 1A9, 2B4, 2B7, and 2B15 were cloned and expressed in baculovirus-infected insect cells as His-tagged proteins. The recombinant enzymes also contain an enterokinase cleavage site (Fig. 1), but due to the good specific activity of the expressed UGTs, and the usefulness of the His tag for immunodetection, enterokinase treatment was omitted. The expression of the different UGTs was aimed at getting a high yield of the active enzyme rather than a high protein production. To this end activity assays in the presence of suitable substrates were used in order to optimize infection with each virus stock and to develop the isolation protocol of the membranes. The outcome of these experiments indicated that in most cases there is an optimal value for the infection size and that the post-infection culturing should be carried out for about 48 h. During the experiments to improve sample preparation methods, we have observed that sonication reduces total activity and that such a treatment to disrupt insect cells that were cultured in suspension is unnecessary. Freezing, thawing, and mild osmotic shock render the cells permeable for both UDP-GA and the aglycone substrates. In line with these observations a total membrane fraction, rather than microsomes, was used for the subsequent activity assays.

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was, however, almost fully inhibited by Triton X-100.

The detergent effects in the inhibition experiments were rather rapid (Tables II and III, and data in the text below), because the detergents were added to the reaction mixture that already contained membranes, and neither preincubation nor centrifugation were performed before the initiation of the enzymatic reactions by substrate addition. The aglycone substrates selected for the initial experiments were \( \text{H}9251 \)-naphthol and scopoletin because they allow a parallel examination of the different UGTs. The results were somewhat surprising and similar for both the substrates, i.e. UGT1A9 is the only enzyme that is not severely inhibited by Triton X-100 (Table II). The observed detergent-induced stimulation of UGT1A9 activity (Table II) can probably be attributed to membrane disruption that makes previously sealed vesicles fully permeable to UDP-GA. Not every detergent, however, stimulates UGT1A9 activity. The glucuronidation activity of \( \text{H}9251 \)-naphthol by the membrane-bound UGT1A9 in the presence of 1% (w/v) CHAPS and sodium cholate was about 35% and less than 5%, respectively, of the control rate (no added detergent), and the glucuronidation of scopoletin was almost totally inhibited by 50 mM octyl glucoside.

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**Fig. 1.** Construction of plasmids for expression of recombinant UGTs as fusion proteins with a C-terminal His tag. The nucleotide sequence of the multicloning sites of the original pFastBac and its two derivatives that were prepared and employed in this study, pFBXHA and pFBXHC, are shown. Selected restriction enzyme sites are depicted above the sequences. The amino acid sequences of the C-terminal extensions, resulting from in frame ligation into the Sall sites, are shown below the nucleotide sequences.

**Fig. 2.** Kinetics of entacapone glucuronidation by membrane-bound recombinant UGT1A9 and human liver microsomes. The UDP-GA concentration in these assays was 1 mM, and the aglycone concentrations are shown on the abscissa. The glucuronidation rates at low entacapone concentrations are shown on a different scale in the inset.
The detergent was added to samples containing the respective membrane-bound UGT just before the onset of the reaction. The inhibition by detergent was done just before the onset of the reaction. The inhibition by detergent addition to samples containing membrane-bound UGT just before the onset of the reaction.

Table II
Effect of Triton X-100 (0.2%, w/v) on the activities of different recombinant UGTs

| UGT  | Aglycone* | Activity  | % remaining activity |
|------|-----------|-----------|----------------------|
|      | No detergent | + Triton X-100 |                     |
| 1A6  | α-Naphthol | 619.6     | 0.0                  |
| 1A6  | Scopoletin | 783.7     | 14.0                 |
| 1A7  | Scopoletin | 243.6     | 3.3                  |
| 1A8  | α-Naphthol | 2588.9    | 9.6                  |
| 1A9  | α-Naphthol | 876.5     | 0.4                  |
| 1A9  | Scopoletin | 137.0     | 181.3                |
| 1A9  | Scopoletin | 149.8     | 198.7                |
| 2B4  | α-Naphthol | 64.3      | 3.3                  |
| 2B7  | α-Naphthol | 405.3     | 4.9                  |
| 2B7  | Scopoletin | 123.0     | 0.5                  |
| 2B15 | α-Naphthol | 30.9      | 0.0                  |
| 2B15 | Scopoletin | 6.3       | 0.4                  |

* The aglycones and UDP-GA concentrations were 0.5 and 5 mM, respectively.

Activity was below detection limit and considered to be nil.

Table III
Effect of Triton X-100 (0.2%, w/v) on the glucuronidation of different aglycones by recombinant UGT1A9

Detergent addition to samples containing membrane-bound UGT1A9 was done just before the onset of the reaction. The inhibition by detergent addition, where indicated, was almost total.

The finding that glucuronidation of scopoletin and α-naphthol by UGT1A9 is not inhibited by Triton X-100 (Table II) was initially believed to suggest that the resistance to detergent inhibition arises solely from the protein structure. However, it soon turned out that this resistance is also dependent on the properties of the aglycone, because the glucuronidation of entacapone by recombinant UGT1A9 was almost fully inhibited by Triton X-100 at a concentration that did not inhibit the glucuronidation of scopoletin and α-naphthol (not shown). Following this observation we have tested the effect of 0.2% Triton X-100 on the glucuronidation of several other aglycones by the membrane-bound UGT1A9, and the results are summarized in Table III. With the exception of α-naphthol and scopoletin, we have thus far not found any aglycone for which the glucuronidation is resistant to detergent inhibition (Table III).

In order to find out whether the inhibition of the UGTs by Triton X-100 is reversible, we have incubated UGT1A6 in the presence of 0.2% Triton X-100, in 50 mM phosphate buffer at pH 7.4 and 37 °C for 30 min. Following this incubation the enzyme was diluted severalfold in a detergent-free buffer and was subjected to glucuronidation assay of α-naphthol. The results, presented in Fig. 3A as specific activity of the diluted UGT1A6, demonstrate that the detergent inhibition is, at least partly, reversible. The activity increased sharply once the concentration of the remaining Triton X-100 fell below its cmc of about 0.02%, even though the detergent to protein ratio was exactly the same in all the dilutions (Fig. 3). Recovery of the activity after such an incubation was, however, incomplete and reached about 30% of the activity of the control sample that was treated similarly but in the absence of Triton X-100. Another experiment was conducted with UGT1A9, in order to test the reversibility of the Triton X-100 inhibition on glucuronidation activity of entacapone. In addition, we wanted to test whether the high detergent concentration used in membrane extraction step of UGT1A9 purification (see below) induces irreversible changes in this enzyme. UGT1A9-containing membranes were thus incubated in the presence of 1% Triton X-100, albeit at 0 rather than 37 °C, and then diluted and assayed for glucuronidation rates of entacapone (Fig. 3B). The results clearly demonstrate that the high Triton-induced inhibition of the entacapone glucuronidation activity by UGT1A9 is reversible once the final detergent concentration drops below 0.02%. Furthermore, in contrast to the only partial recovery of activity observed for UGT1A6, the rate of entacapone glucuronidation by UGT1A9 at post-dilution detergent concentration of 0.01% (Fig. 3B) was as high as measured for the membrane-bound UGT1A9 that was never exposed to detergents.

The resistance of UGT1A9 to detergent-induced inhibition, at least when glucuronidation of scopoletin or α-naphthol is concerned, selected this isoform as an attractive candidate for purification. To this end the insect cell membranes that carry recombinant UGT1A9 were washed with high salt concentration in order to remove peripheral membrane proteins and also to remove EDTA prior to IMAC purification. The salt-washed membranes were solubilized using 1% Triton X-100, and the extracted UGT1A9 was purified by a single step of affinity chromatography in the presence of the detergent. SDS-PAGE analyses indicate that such single-step purification yields a highly purified enzyme (Fig. 4).

The activity of the purified enzyme, and its dependence on added phospholipids, was assayed in the presence of either scopoletin or entacapone (Fig. 5). Glucuronidation of scopoletin by the purified UGT1A9 was relatively high, and addition of phospholipids increased it further by about 20% (Fig. 5A). On the other hand, glucuronidation of entacapone by the purified enzyme in the absence of added phospholipids was very low, although measurable. Addition of sonicated phospholipids stimulated the glucuronidation of entacapone by about 400%, but it was still much lower than the glucuronidation of scopoletin by the same enzyme preparation (Fig. 5B). It may be noted here that the addition of sonicated phospholipids had no effect on the activity of the membrane-bound enzyme (not shown) and that membrane-bound UGT1A9 glucuronidates entacapone, at an optimal substrate concentration, much faster than it glucuronidates scopoletin (cf. data in Fig. 2 and Table II).

The mild effect of the added phospholipids on the glucuronidation rate of scopoletin by the purified UGT1A9 might
suggest that this activity does not require a membrane-like environment. In order to understand better this observation, we have examined the kinetics of scopoletin glucuronidation by both the purified UGT1A9, in the presence of added phospholipids, and membrane-bound enzyme (Fig. 6). The purification process increased the apparent $K_m$ of UGT1A9 for scopoletin about 10 times, from 61/11006 to 790/11006 M in the membrane-bound enzyme to 790/11006 M in the purified UGT1A9 (Fig. 6A). The apparent $K_m$ for UDP-GA was also increased, from 229/11006 to 704/11006 M (Fig. 6B), but in relative terms not as much as for the lipophilic aglycone, scopoletin. In any case, the high specific activity of the purified UGT1A9, about 500 times higher than the membrane-bound enzyme under the assay conditions used in these experiments (Fig. 6), indicates that it is a pure and active enzyme.

The UGTs are expressed as precursor proteins with an N-terminal signal sequence, even though the necessity of this peptide for directing the newly synthesized proteins to the endoplasmic reticulum was recently questioned (17). In any case, the availability of purified recombinant UGT1A9 (Fig. 4) allowed the determination of the exact cleavage point of the signal peptide by N-terminal protein sequencing. Purified UGT1A9 was directly subjected to 8 cycles of automated Edman degradation, and the resultant sequence was $\text{X KLLV-}$. Determination of the very first amino acid in this sequence, the $\text{X}$, was hampered by the presence of high background and noise in the first cycle. The N-terminal sequence (first 40 amino acids) of the precursor UGT1A9 protein is $\text{MACTGWTSPLPLCVCLLLTCGFAEAGKLLVVPMDG}$-$\text{SHWFT}$, and the underlined segment matches the sequence obtained from the purified recombinant enzyme. Hence, the signal peptide cleavage in UGT1A9 that was expressed in insect cells occurs between Ala-25 and Gly-26, exactly at the position predicted for eukaryotic cells by the SignalP program (29), available at www.cbs.dtu.dk/services/SignalP/.

Purification of an active UGT1A9 allows us to ask whether the UGTs are dimeric enzymes, as suggested previously (19, 22, 23). However, determination of the molecular weight of a purified membrane enzyme, e.g. by size exclusion chromatography, is often ambiguous due to the contribution of bound detergent micelle(s), the amount of which does not necessarily correlate with the number of monomers in the complex. In order to circumvent this obstacle, we have developed a new method to test whether the recombinant UGT1A9 is monomeric or oligomeric. The main element of the new system is preparation of a second version of the enzyme as a fusion protein that differs from the one used for the IMAC purification by the absence of the His tag and the presence of a new tag. The latter must fulfill two requirements, it should not mediate
protein binding to the column used for purification of the His-tagged enzyme, and it should allow reliable identification of the fusion protein, e.g. by immunodetection. The cells would then be co-transfected with baculoviruses (or equivalent gene delivery vehicles in other systems) encoding both the His-tagged and the differently tagged enzyme in order to allow formation of mixed oligomers in the membrane. The expressed recombinant proteins will be extracted and subjected to IMAC purification, and the collected fractions will be examined for the presence of either HA-tagged (see text and Experimental Procedures for further details). Equal volumes of undiluted samples from the indicated fractions were loaded on the two SDS-PAGES, except for the unbound sample that was diluted 10 times.

Fig. 6. Kinetic analyses of membrane-bound and affinity-purified UGT1A9. The assays were carried out either with membrane-bound UGT1A9 at a protein concentration of 100 or 0.5 μg/ml purified UGT1A9 that was supplemented with 1 mg/ml sonicated phospholipids. The UDP-GA concentration was 2.5 mM (A), and the scopoletin concentration was 2 mM (B). Fitting to Michaelis-Menten equation was done using SigmaPlot (enzyme kinetics module 1.1, see text for kinetic values). Note that in both A and B the reaction velocities on the ordnates are pmol/mg protein/minute for purified UGT1A9 and pmol/mg protein/minute for the membrane-bound enzyme.

Fig. 7. Identification of both His-tagged and HA-tagged UGT1A9 in the peak fractions after IMAC purification of the enzyme from co-infected cells. Proteins from cells producing both His-tagged and HA-tagged recombinant UGT1A9 were extracted by 1% Triton X-100 and subjected to affinity purification by IMAC using a nickel-containing column. Fractions were analyzed by Western blots for the presence of either HA-tagged (A) or His-tagged (B) UGT1A9, using tag-specific monoclonal antibodies (see text and Experimental Procedures for further details). Equal volumes of undiluted samples from the indicated fractions were loaded on the two SDS-PAGES, except for the unbound sample that was diluted 10 times.

The co-infected cells were cultured, subjected to membrane isolation, Triton X-100 extraction, and affinity purification exactly as was done previously for His-tagged-only infections (e.g. the preparation shown in Fig. 4). Samples from different fractions of the chromatography were subjected, in parallel, to similar SDS-PAGES that were followed by Western blotting using monoclonal antibodies against either the HA tag or the His tag. The results (Fig. 7) show that only HA-tagged enzyme was present in the unbound fraction and that fractions 8–14 contained both His-tagged and HA-tagged UGT1A9 (Fig. 7). The enzyme in the latter fractions was eluted by imidazole concentration gradient, and its elution profile was practically identical to that of His-tagged-only enzyme in our regular purification experiments (not shown). Small amounts of HA-tagged UGT1A9 were also visible in some fractions that apparently lack His-tagged UGT1A9 (Fig. 7). In fraction 2 this could be assigned to traces of unbound enzyme (the tail of the large peak of unbound proteins), whereas in fractions 5 and 17 it is probably the beginning and the end of the major peak of purified UGT1A9. It may be noted in this respect that the HA tag is much more immunogenic than the His tag and that the anti-HA tag monoclonal antibodies used in this study appear to
be very efficient in detecting small amounts of the HA-tagged UGT1A9. The latter also means that no quantitative conclusion about the ratio between His/HA-mixed tagged dimers to His/His identically tagged dimers in fractions 8–14 can be drawn from these experiments (Fig. 7). Nevertheless, our results provide a new and independent indication that recombinant UGT1A9 forms oligomers, most probably dimers. These dimers should be considered homodimers and not heterodimers, regardless of the difference in the C-terminal tag between them, because both the monomers are UGT1A9.

In order to verify that the apparent dimerization (Fig. 7) is not caused by artifactual aggregation of the enzymes upon detergent extraction, we have carried out the following control experiment. Insect cells were infected with baculovirus encoding either His-tagged or HA-tagged UGT1A9, and the entire process of protein expression and membrane isolation and washing was done separately for each batch. The two membrane batches were later thawed and mixed together thoroughly, and in the subsequent steps of Triton X-100 extraction and IMAC purification they were treated as a single batch. The different fractions after the purification were analyzed by Western blotting as shown in Fig. 7, but the results were different. In the case of separate infection, HA-tagged UGT1A9 was only detected in the unbound fraction and not in the imidazole-eluted fractions. On the other hand, the distribution of the His-tagged enzyme among the different fractions of this control experiment was practically identical to the distribution of this type of recombinant UGT1A9 in the case of co-infected cells, i.e. as shown in Fig. 7B. It thus appears that the formation of HA/His tagged UGT1A9 dimers requires the expression of both types of enzyme in the same cell. Such mixed tag dimers are not induced by detergent solubilization or other conditions, e.g. high salt concentration, to which the enzyme is exposed during detergent extraction and IMAC purification.

**DISCUSSION**

Eight hepatic UGTs were cloned and expressed as active enzymes using baculovirus-infected insect cells. The main difference between the new recombinant UGTs and those that are commercially available is the presence of a C-terminal His tag (Fig. 1). The His tags enable easy purification of the UGTs by IMAC, regardless of whether or not they are active. In addition, the His tags allow the use of monoclonal antibodies against this peptide in order to follow expression, and it could provide a way to compare the amount of recombinant enzymes in different membrane preparations.

The addition of a short peptide to the C-terminal end of the UGTs might be expected to affect enzymatic activity, like several mutations in that region have done (16). However, a comparison of the glucuronidation activity of entacapone by the recombinant UGT1A9 with the native enzyme in human liver microsomes suggests that the former is a very good model for the latter UGT (Fig. 2). Extension of this conclusion to the other UGTs is currently hampered, however, by overlapping substrate specificities and co-expression in the same tissue. In any case, the positive results with UGT1A9 and the good activities of the other recombinant UGTs (Table II) suggest that the above conclusion may hold for the entire set of recombinant human UGTs.

A good purification method for any UGT, and particularly for the human enzymes, has been anticipated (e.g. Ref. 3). This work fulfills this expectation and also provides a possible explanation for the previous failures to purify these enzymes in active states. UGTs are membrane-bound enzymes, and they should be extracted from the membrane using a detergent prior to the separation from the other integral membrane proteins, e.g. by chromatography. It is well known that low concentration of a detergent may activate UGTs in liver microsomes (e.g. Ref. 30). In retrospect it seems that the optimal concentrations found for these activations resulted from a balance between making the membrane more permeable to UDP-GA on the one hand, and a minimal enzyme inhibition on the other hand. In any case, the optimal detergent concentrations in such assays were below the cmc and were thus insufficient for solubilization and subsequent purification.

One of the first steps in developing a new purification procedure for the recombinant UGTs was examining the effect of several detergents, at concentrations above their respective cmcs, on the activities of the different enzymes. Triton X-100 and dodecyl maltoside were examined first, the former because it is relatively inexpensive and has been used previously for UGT studies, and the latter because it is considered to be highly suitable for the purification of fragile membrane proteins. The initial results were somewhat surprising, and partly disappointing, because all the UGTs, with the exception of 1A9, were highly sensitive to both detergents (the effects of dodecyl maltoside were very similar to those of Triton X-100 that are summarized in Table II). It is not clear at this stage what makes the UGTs sensitive to these detergents. However, the partial or full recovery of activity by lowering the detergent concentration below its cmc without changing the detergent to protein ratio (Fig. 3) indicates that the effect of Triton X-100 is not an irreversible inactivation of the UGTs.

An interesting and unexpected finding was the dependence of the detergent inhibition on the type of aglycone substrate used (Table III). In order to gain a better understanding about this phenomenon, we have examined several aglycones that can be glucuronidated by membrane-bound UGT1A9, and more of them will be tested in the future. Thus far, however, we were not able to identify the factor(s) that determine whether or not the glucuronidation of a given substrate will be sensitive to inhibition by Triton X-100. In addition, it cannot be ruled out that there are aglycone substrates the glucuronidation of which by UGTs other than 1A9 may be resistant to detergent-induced inhibition. In line with this, it may be noted that purification of UGT1A9 would have never been attempted if umbelliferone or 4-methylumbelliferone were selected for the first detergent solubilization trials instead of α-naphthol and scopoletin (Tables II and III).

The rate of entacapone glucuronidation by the purified UGT1A9 was low, and it was stimulated significantly by the addition of sonicated phospholipid (Fig. 5B). On the other hand, glucuronidation of scopoletin by the purified enzyme was only slightly increased by such an addition (Fig. 5A). This substrate-dependent difference in response to phospholipid addition correlates well with the sensitivity of glucuronidation activities of the different aglycones by the membrane-bound UGT1A9 to inhibition by Triton X-100 (Table III). It may thus be suggested that the inhibitory effect of the detergent may be partly caused by delipidation of the protein. Nevertheless, the observed difference between the membrane-bound and purified UGT1A9 in the apparent K_m for scopoletin, and to a less extent also to UDP-GA, may indicate that complete reversal of the purification-induced changes in this enzyme cannot be achieve merely by phospholipid addition (Fig. 6). It remains to be studied if certain phospholipid head groups, fatty acid chains, or other components of biological membranes such as cholesterol could reconstitute the environment of the enzyme and fully restore the apparent kinetic parameters of the enzyme.

The question whether the UGTs are dimeric and, if so, are they homodimeric or heterodimeric have been examined and discussed in previous studies (e.g. Refs. 19, 22, and 23). One of the main reasons for the attempts to study the oligomeric state
of the recombinant UGT1A9 was the possibility that the mechanism of detergent-induced inhibition of most UGTs (Table II) is the monomerization of dimeric enzymes. Studying whether a given enzyme is dimeric requires the availability of an active preparation in order to avoid artifactual results, and this could not be guaranteed for the UGTs, because the activities of them are inhibited by the detergents we have tested so far. UGT1A9 was different in this respect, because its enzyme activity toward some substrates is sensitive to Triton X-100 inhibition, whereas toward other substrates it is not (Table III). We have used differently tagged recombinant UGTs to study dimerization of UGT1A9, and the results suggest that it forms dimers, if not larger oligomers, when expressed in baculovirus-infected insect cells (Fig. 7). The interaction between the two monomers in dimeric UGT1A9 appears to be tight and stable, and they are not easily dissociated from each other under the conditions used for extraction from the membrane and affinity purification.

In light of the present findings and previous reports (19, 22, 23), it may be suggested that dimer formation is indeed a property of many UGTs, and might be essential for UGTs activity. Nevertheless, detergent inhibition is not mediated by monomerization, at least as far as the entacapone glucuronidation activity of UGT1A9 is concerned.

Finally, the results and methods described in this paper open new avenues for detailed structural and functional studies of UGTs. In addition, this study raises new questions, particularly about the factors affecting the interactions of UGTs with detergents, and how this may lead to discrimination between certain aglycones. Extending the lines of research presented here and the development of new approaches to solve these questions are likely to significantly increase our understanding about this family of important enzymes.

Acknowledgments—We thank Jaana Tolmunen, Juanita Korsström, and Sanna Sistonen for technical assistance.

REFERENCES
1. Burchell, B., Briolay, C. H., and Rance, D. (1995) Life Sci. 57, 1819–1831
2. Meech, R., and Mackenzie, P. I. (1997) Clin. Exp. Pharmacol. Physiol. 24, 907–915
3. Radominska-Pandya, A., Czernek, P. J., Little, J. M., Battaglia, E., and Mackenzie, P. I. (1999) Drug Metab. Rev. 31, 817–899
4. Tukey, R. H., and Strassburg, C. P. (2000) Annu. Rev. Pharmacol. Toxicol. 40, 581–616
5. King, C. D., Ries, G. R., Green, M. D., and Tephly, T. R. (2000) Curr. Drug Metab. 1, 143–161
6. Kadakol, A., Ghosh, S. S., Sappal, B. S., Sharma, G., Chowdhury, J. R., and Chowdhury, N. R. (2000) Hum. Mutat. 16, 297–306
7. Burdell, B., Sears, M., Mochini, G., Cassidy, A., Smith, D., and Ethell, B. (2000) Toxicol. Lett. 113, 333–340
8. Strassburg, C. P., Vogel, A., Kneip, S., Tukey, R. H., and Manns, M. P. (2002) Gut 50, 851–856
9. Yueh, M.-F., Nguyen, N., Famourzadeh, M., Strassburg, C. P., Oda, Y., Guengerich, F. P., and Tukey, R. H. (2001) Carcinogenesis 22, 943–950
10. Pilot, T., Ouzinne, M., Fournel-Gigleux, S., Lafaurie, C., Tebbi, D., Treat, S., Radominka, A., Lester, R., Siest, G., and Magdalou, J. (1993) Biochem. Biophys. Res. Commun. 197, 785–791
11. Battaglia, E., Terrier, N., Mizeraacka, M., Senay, C., Magdalou, J., Fournel-Gigleux, S., and Radominska-Pandya, A. (1998) Drug Metab. Dispos. 26, 812–817
12. Mackenzie, P. I. (1999) J. Biol. Chem. 265, 3432–3435
13. Nguyen, N., and Tukey, R. H. (1997) Drug Metab. Dispos. 25, 745–749
14. Guengerich, F. P., Parikh, A., Johnson, E. F., Richardson, T. H., von Wachenfeldt, C., Cosme, J., Jung, F., Strassburg, C. P., Manns, M. P., Tukey, R. H., Pritchard, M., Fournel-Gigleux, S., and Burchell, B. (1997) Drug Metab. Dispos. 25, 1234–1241
15. Ethell, B. T., Beaumont, K., Rance, D. J., and Burchell, B. (2001) Drug Metab. Dispos. 29, 48–53
16. Meech, R., and Mackenzie, P. I. (1998) Arch. Biochem. Biophys. 356, 77–85
17. Ouzinne, M., Magdalou, J., Burchell, B., and Fournel-Gigleux, S. (1999) J. Biol. Chem. 274, 31401–31409
18. Kowai, O., Aono, S., Adachi, Y., Kamisako, T., Yasui, Y., Nishizawa, M., and Sato, H. (1996) Hum. Mol. Genet. 5, 645–647
19. Meech, R., and Mackenzie, P. I. (1997) J. Biol. Chem. 272, 26913–26917
20. Ikushiro, S.-I., Emi, Y., and Iyanagi, T. (1997) Biochemistry 36, 7154–7161
21. Guernaud, P., and Paris, A. (1998) Gen. Pharmacol. 31, 685–688
22. Ishii, Y., Miyoshi, A., Watanabe, R., Tsuruda, K., Teuda, M., Yamaguchi-Nagamatsu, Y., Yoshisue, K., Tanaka, M., Maji, D., Ogisiya, S., and Oguri, K. (2001) Mol. Pharmacol. 60, 1040–1048
23. Ghosh, S. S., Sappal, B. S., Kalpana, G. V., Lee, S. W., Chowdhury, J. R., and Chowdhury, N. R. (2001) J. Biol. Chem. 276, 42108–42115
24. Lukkkonen, L., Kilpelainen, I., Kangas, H., Ottola, P., Elovaara, E., and Taskinen, J. (1999) Bioconjugate Chem. 10, 150–154
25. Forsman, T., Lautala, P., Lundstrom, K., Monastyrskaya, K., Ouzinne, M., Burchell, B., Taskinen, J., and Ulmanen, I. (2000) Life Sci. 67, 2473–2484
26. Laemmli, U. K. (1970) Nature 227, 680–685
27. Baumann, M. (1990) Anal. Biochem. 190, 198–208
28. Lautala, P., Ethell, B. T., Taskinen, J., and Burchell, B. (2000) Drug Metab. Dispos. 28, 1385–1389
29. Nielsen, H., Engolbrecht, J., Brunak, S., and von Heijne, G. (1997) Protein Eng. 10, 1–6
30. Lukkkonen, L., Elovaara, E., Lautala, P., Taskinen, J., and Vainio, H. (1997) Pharmacol. Toxicol. 80, 152–158
31. Kaivosaari, S., Salonen, J. S., Mortensen, J., and Taskinen, J. (2001) Anal. Biochem. 292, 178–187