BACULOVIRUS-DIRECTED EXPRESSION OF RABBIT UDP-GLUCURONOSYLTRANSFERASES IN SPODOPTERA FRUGIPERDA CELLS

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ABSTRACT:

The rabbit liver UDP-glucuronosyltransferase (UGT) cDNAs that encode the 4-hydroxybiphenyl UGT2B13 and 4-nitrophenol UGT1A6 have been cloned into baculovirus. Spodoptera frugiperda (SF-9) cells infected with the UGT recombinant baculovirus produced significant amounts of protein, which was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoblot analysis, and pulse chase with \(^{35}\)S-amino acids. The expression of the UGT proteins in SF-9 cells were detected at \(24\) hr postinfection, with maximal levels of protein seen at \(48\) hr. Immunoprecipitation of newly synthesized \(^{35}\)S-labeled proteins demonstrated that the maximal rate of protein synthesis in SF-9 cells infected with the UGT baculovirus occur at \(48\) hr postinfection, although the proteins are abundant in the cells for up to \(96\) hr. When compared with the expression levels of the same cDNAs through transient transfection into COS-1 cells, the insect-derived UGT proteins showed nearly \(50\)–\(100\)-fold greater protein accumulation. Although kinetic analysis demonstrated that turnover rate of the SF-9–expressed proteins were greater than their counterparts in COS-1 cells, \(K_m\) values for UGT1A6 and UGT2B13 in SF-9 and COS-1 cells were similar. Overall, SF-9 cells seem to serve as an efficient expression system for the production of the mammalian UGTs.

The UGTs, located in the endoplasmic reticulum of the cell are a “superfamily” of proteins that transfer glucuronic acid from UDPGA to virtually thousands of structurally different compounds, many of which come through our diet, our environment, or by drug administration. In addition, many exogenous compounds (such as steroids, hormones, and bile acids) are also substrates for these enzymes. In an organ such as the liver, the products of glucuronidation are made readily more water soluble, and by the compounds’ physical conversion into a more hydrophilic agent, the glucuronides are excreted into the bile or urine. By nature, most glucuronides are biologically inactive. Therefore, UGTs play a major role in the organisms’ ability to remove compounds that would potentially concentrate in the cell. Such a mechanism undermines the cells ability to detoxify itself from potentially harmful waste products that arise from the natural catabolic processes of the cell, in addition to the removal of agents that are acquired through environmental exposure.

The UGTs’ large substrate diversity results in part from the individual enzymes ability to conjugate structurally different agents, but also results from the suspected large number of unique enzymes. The identity of recombinant cDNA clones encoding these proteins has led to the implementation of techniques to characterize the substrate specificities of UGTs. Two primary methods have been adopted. The first is to introduce cDNA clones into monkey kidney COS cells through transient transfections (2, 3). Recombinant cDNA clones can be subcloned into expression vectors under the control of the large T-antigen promoter and adequate quantities of COS cells obtained that express the appropriate UGTs. Whereas there is detectable small phenolic UGT transferase activity that is resident to these cells, the very low levels of UGT transferase activity has made this cell line a valuable tool to examine substrate specificities. The second approach is to integrate the cDNAs in a stable fashion using cell lines such as fibroblast V79 cells (4). The significant advantage of this approach is the selection of a clonal cell line, with 100% of the cells expressing the recombinant protein of interest. Usually, a higher concentration of protein can be obtained from a clonal cell line than from cells that express the recombinant protein through transient transfection. A disadvantage, however, is the use of these techniques to obtain large quantities of cells and in turn greater amounts of active UGTs.

The use of baculovirus expression vectors and the insect SF-9 cells has served as a useful alternative for the generation of many different types of protein (5, 6). Baculovirus-expressed proteins that reside in the cytosol, as well as different membrane compartments in the cell, have been manufactured in a functional form in insect cells. The ability of these cells to target the different proteins to the appropriate cellular compartments demonstrates that adequate posttranslational processing of these proteins occurs, an important consideration when expressing the UGTs. Most, if not all of the UGTs are glycoproteins (7, 8), and all contain a hydrophobic leader sequence that is removed as the proteins intercalate into the membrane (9). In this study, we have examined the ability of baculovirus recombinants containing rabbit liver UGT2B13 and UGT1A6 cDNAs to express these proteins in SF-9 cells. We have also compared the levels of their expression in...
SF-9 cells with their expression in COS-1 cells, as conducted by transient transfection.

Materials and Methods

The eukaryotic expression pSVL vector was purchased from Pharmacia (Piscataway, NJ). The baculovirus expression vector, pBlueBac, and linearized viral DNA were purchased from Invitrogen (San Diego, CA). Restriction endonucleases, modifying enzymes T4 DNA polymerase and Ligase, were purchased from New England Biolabs (Beverly, MA). Ni-NTAagarose filter was purchased from Millipore (Belford, MA). Medium and low melting point agarose was purchased from Fisher Scientific (Tustin, CA), and SeaPlaque low melting point agarose was purchased from FMC (Rockland, ME). [14C]UDPGA was purchased from New England Nuclear (Boston, MA). Dulbecco’s modified Eagle’s medium, chloroquine, DEAE-dextran, and fetal calf serum were purchased from Irvine Scientific (Santa Ana, CA). The insect cell line SF-9 was a generous gift from Dr. Max Summers (Texas A & M University). Grace’s and TMN-FH insect cell media, anti-goat IgG, and anti-sheep alkaline phosphatase-conjugated IgG, 3,3′-diaminobenzidine, 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium, were purchased from Sigma Chemical Co. (St. Louis, MO). Methionine-free media and trans-35S-labeled (145S)-methionine and (135S)-cysteine were purchased from ICN (Costa Mesa, CA).

cDNA Cloning. The 5′ noncoding region and all of the coding region of UGT2B13 (bases 1–1665) was removed from 2B13/pBluescript (10) by digestion with EcoRV, whereas a comparable region (bases 1–1708) encoded by the UGT1A6/pBluescript (8) was removed by digestion with HindIII. The UGT1A6 insert was treated with T4 DNA polymerase in the presence of dNTPs to generate blunt ends. The pBlueBac plasmid was linearized with restriction endonuclease Nhel and the 5′ overhangs filled in with T4 DNA polymerase I in the present of dNTPs. The blunt-ended UGT1A6 and UGT2B13 inserts were ligated with a molar excess of the linearized and blunt-ended pBlueBac vector, and transformed into E. coli strain DH10B. Recombinant clones containing the inserts were examined to ensure each was in the proper orientation by endonuclease restriction site mapping and DNA sequence analysis. The UGT2B13 and UGT1A6 inserts cloned into pSVL for expression in COS-1 cells has been previously described (8, 10).

Protein Expression in SF-9 Cells. SF-9 cells (2 × 10^6) were cotransfected with 3 μg of either 2B13pBluebac or UGT1A6pBluebac, 1 μg of linear AcMNPV DNA, and 20 μl of cationic liposome solution in 1 ml of Grace’s media with no supplements for 1 hr at room temperature. TNM-FH + 10% fetal calf serum medium was then added to the cells and incubated at 27°C, and a primary virus stock was collected at 48 hr posttransfection. To obtain pure recombinant UGT2B13 baculovirus, several rounds of plaque purification were performed. SF-9 cells in a 60 mm tissue culture dish were infected with a serial dilution of a primary stock virus. Virus solution was removed at 1 hr postinfection, and the cells were overlaid with TNM-FH + 10% fetal calf serum medium containing 1.5% low melting SeaPlaque agarose and 150 μg/ml isopropyl-β-D-thiogalactopyranoside. Cells were incubated at 27°C for 4–6 days, and isolated recombinant plaques (visualized as blue) were expanded to produce a stock of UGT virus. For protein expression, a multiplicity of infection of 10 plaque-forming units were used to infect SF-9 cells. Cells were collected at 48 hr postinfection and washed with 50 ml Tris-HCl (pH 7.6) containing 100 mM MgCl2. Cell extracts were made by homogenization of the cells using a Teflon homogenizer in 50 mM Tris-HCl (pH 7.6) and 10 mM MgCl2 buffer.

Protein Expression in COS-1 Cells. COS-1 cells (2 × 10^6/150 plate) were transfected with either 2B13pSVL (10) or UGT1A6pSVL (8) at 5 μg of DNA/ml of Dulbecco’s modified Eagle’s medium containing 0.25 mg/ml DEAE-dextran and 20 mM HEPEs (pH 7.5) for 1 hr at 37°C. Similar experiments have been described (8, 10). The transfection medium was removed and replaced with medium containing 100 μM chloroquine (11) for 5 hr at 37°C. Forty hours later, cells were rinsed and harvested with PBS. Cell extracts were made by homogenization in 50 mM Tris-HCl (pH 7.6) containing 10 mM MgCl2 buffer.

UGT Assay. Reactions were conducted in 100 μl containing 50 mM Tris-HCl (pH 7.6), 10 mM MgCl2, 20 μM UDPGA, 0.04 μCi [14C]UDPGA (0.14 mCi/μg), 5 mM saffronin, 100 μg phosphatidylinositol, and 100 μg of COS-1 cell extract. For routine analysis of glucuronidation activities, substrates were added in methanol to a final concentration of 100 μM. For SF-9 cell extracts, the amount of protein used was 10–25 μg, unless otherwise stated. Reactions were incubated at 37°C for 1 hr and terminated with 200 μl ethanol. Proteins were removed by centrifugation in an Eppendorf microfuge for 5 min, and the supernatants were dried by speed vacuum centrifugation. Glucuronides were resuspended in 20 μl of methanol and spotted on a Whatman glass-bac TLC plate. TLC was conducted in a mixture of 35% n-butanol:35% acetic acid:20% water. The TLC plate was dried, sprayed with a thin layer of surface autoradiography enhancer, and exposed to X-ray film.

Kinetic Analysis. In the experiments performed to determine the apparent K_m and V_max values for substrates conjugated by expressed UGT1A6 and UGT2B13, the UDPGA concentration was 100 μM. UGT activities were measured over a range of 0.010–1 mM substrate and the apparent K_m and V_max values determined using linear regression analysis with Harvard Graphics to fit the data by Lineweaver-Burk analysis.

Western Blot Analysis. Proteins were separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membrane by electroblotting in 48 mM Tris-HCl (pH 8.3) containing 39 mM glycine, 0.037% SDS, and 20% methanol, with constant 25 V for 3 hr. Membranes were incubated for 2 hr in PBS and blocking buffer. Sheep-anti-rabbit UGT1A6 IgG (kindly provided by Dr. Thomas Tephly, University of Iowa) was added at a dilution of 1:2000 with blocking buffer, incubated for 2 hr, and washed free of the membrane by three washes in PBS/0.1% Tween-20. The membrane was then soaked in blocking buffer with a 1:4000 dilution of alkaline phosphate-conjugated goat-anti-sheep IgG for 2 hr, followed by additional washes with PBS containing 0.1% Tween-20. Phosphatase activities were detected by incubating the membrane in 100 mM Tris-HCl buffer at pH 8.0 containing 0.15 mg/ml 5-bromo-4-chloro-3-indolylphosphate, 0.30 mg/ml nitroblue tetrazolium, and 5 mM MgCl2.

35S Protein Labeling of COS-1 Cells. COS-1 cells (3 × 10^6) were transfected with 2B13pSVL or UGT1A6pSVL for 24 hr, washed 3 times with Met- and Cys-free medium and then incubated with Met- and Cys-free medium containing 100 μCi of trans-35S-label for 4 hr (8). Cells were then washed 3 times with ice-cold PBS and lysed on ice with 500 μl of RIPA buffer (pH 7.5). This method has been described previously (12). Cellulose DNA was broken by shearing through a 25 × 5 inch needle 8 times and removed by centrifugation at 4°C in an Eppendorf microfuge.

35S Protein Labeling of SF-9 Cells. The SF-9 (3 × 10^6) cells were infected with UGT virus for 24 hr and then washed 3 times with Met-free medium. Met-free medium containing 100 μCi of trans-35S-label was then added and incubated at 27°C for 12 hr. Cells were then washed 3 times with ice-cold PBS and lysed on ice with 500 μl of RIPA buffer. Cellulose DNA was broken by shearing through a 25 × 5 inch needle 8 times, and removed by centrifugation at 4°C in an Eppendorf microfuge.

Immunoprecipitation of the Expressed UGTs. To 50 μl of the cell lysate, 22 μg of sheep-anti-rabbit UGT1A6 IgG was added and incubated on ice for 4 hr. The lysate-antibody mixtures were then incubated at 4°C for 20 min, with 100 μl of a 10% suspension of Staphylococcus aureus added in PBS, 10% β-mercaptoethanol, and 3% SDS. S. aureus cells were collected by centrifugation in an Eppendorf microfuge and washed 4 times with RIPA buffer, once with 150 mM NaCl 50 mM Tris-HCl (pH 7.5) and once with 50 mM Tris-HCl (pH 6.8). S. aureus cells were resuspended in 50 μl of loading buffer (63 mM Tris-HCl, pH 6.8; containing 2% SDS, 5% glycerol, 5% β-mercaptoethanol, and 0.02% bromophenol blue), boiled for 5 min, and centrifuged for 5 min in an Eppendorf microfuge. Supernatants were then analyzed by SDS-PAGE on a 10% polyacrylamide gel. Gels were soaked in 1 M sodium salicylic acid for 30 min, dried, and exposed to X-ray film at ~70°C.

Results

Insects do not conduct glucuronidation (13), so the intestinal epithelium SF-9 may serve as an excellent recipient cell for the expression of UGT cDNAs. The approach that we have taken is to express rabbit liver UGT2B13 (10), a protein that catalyzes the glucuronidation of 4-hydroxybiphenyl, and UGT1A6 (8), which catalyzes the glucuronidation of small phenolic compounds such as 4-nitrophenol. The cDNAs encoding these transferases were subcloned into the baculovirus expression vector and transfected into SF-9 cells, with
linearized wild-type baculovirus. After several rounds of purification, the supernatant from the recombinant infected SF-9 cells was collected and used to infect an expanding monolayer of SF-9 cells. Parallel control experiments were performed with the wild-type baculovirus.

The first series of experiments were to examine the total cellular protein profiles by Coomasie Brilliant Blue staining from wild-type and recombinant UGT-infected SF-9 cells as displayed after resolution of the cellular proteins by SDS-PAGE. As shown in fig. 1A, wild-type infected cells displayed a predominant band at ~30 kDa, which represents the polyhedrin protein (14). When UGT2B13 recombinant infected cells were analyzed, the polyhedrin protein was no longer present, but a major protein of ~55 kDa was observed, which represents the predicted size of the encoded UGT2B13. A similar staining pattern was also observed with SF-9 cells transfected with the recombinant UGT1A6 virus (data not shown). To contrast the banding patterns observed in untransfected and transient transfected COS-1 cells, a parallel experiment is also shown. Using 50 times the protein concentration, it was not possible to distinguish clearly the expression of a unique protein in transient-transfected COS-1 cells by Coomasie Blue staining. When comparing the levels of expression of the UGTs in either SF-9 cells or COS-1 cells, it can be demonstrated by Western blot analysis (fig. 1B) that the levels of expression of UGTs in SF-9 cells is nearly 100-fold greater than that observed through transient transfection in COS-1 cells.

The temporal increase in UGT2B13 protein in SF-9 cells was shown to produce near-maximal levels of transferase by 48 hr (fig. 2A). In these experiments, ~10 µg of total cellular SF-9 protein was added to each lane. Also shown is the relative levels of UGT2B13 expression in comparison with a sample of purified rabbit liver UGT1A6. At 48 hr, the SF-9 cells are producing ~50 ng of UGT2B13/µg of total cellular protein, which is nearly 5% of the total cellular protein production of the cell. When synthesis of UGT2B13 is monitored by the incorporation of trans-35S-label into newly synthesized protein (fig. 2B), it can be demonstrated that maximal synthesis is complete by 48 hr, with a marked reduction in the rate of
and octylgallate as substrates for UGT2B13, the calculated 2-hydroxybiphenyl as substrates for UGT1A6 and 4-hydroxybiphenyl as characteristic of the expressed UGTs. Using 4-nitrophenol and values for glucuronide formation in SF-9 cells (table 1) were those calculated for synthesis in COS-1 cell extracts. Although UGTs are not frequently expressed in SF-9 cells, we felt it important to examine the $K_M$ values, because kinetic analysis can be interpreted as characteristic of the expressed UGTs. Using 4-nitrophenol and 2-hydroxybiphenyl as substrates for UGT1A6 and 4-hydroxybiphenyl and octylgallate as substrates for UGT2B13, the calculated $V_{max}$ values for glucuronide formation in SF-9 cells (table 1) were 5–10 times those calculated for synthesis in COS-1 cell extracts. However, the calculated $K_M$ values for each substrate as determined in COS-1 cells of SF-9 cell extracts were very similar, a result that suggests that the expressed protein in SF-9 cells maintains a conformational state similar to that of expressed protein in COS-1 cells.

**Discussion**

The recombinant baculovirus system was developed by Max Summers (5, 15) and Lois Miller (6) for the expression of heterologous proteins in insect cells. This expression system has been used for the production of a wide variety of proteins of differing molecular weights, ranging from 17 to >100 kDa (5), as well as soluble and membrane-associated proteins. A number of different proteins that play key roles in drug metabolism [such as cytochrome P450s (16, 17), NADPH-cytochrome P450 reductase (18), and cytochrome $b_5$ (19)] have also been successfully overexpressed in insect cells. An efficient means of producing significant quantities of catalytically active UGT can also be obtained through the expression of these glycoproteins in the insect SF-9 cells.

Using standard methodologies of cloning into baculovirus, followed by several rounds of virus purification, SF-9 cells can be obtained that express a relatively large concentration of catalytically active UGT. Development of an efficient expression system is an important tool to examine catalytic activity, because many of the new UGT cDNAs that have been identified or may be identified in the future through recombinant DNA methodologies may have unknown catalytic functions (20). Whereas other means of protein expression—such as the introduction of cDNAs by transient transfection into COS cells or the stable expression of UGTs in fibroblasts—are also useful tools to examine catalytic activity, the use of SF-9 cells has several added advantages. The SF-9 cells cannot only be grown as monolayers, but also the cells can be grown as suspension cultures. As needed, this would allow for the production of significantly greater amounts of expressed protein. We have also noticed that because 5–10% of the cellular protein can be attributed to the production of recombinant UGTs, analysis of protein expression can be quickly monitored by visualization of the proteins after SDS-PAGE and protein staining of the gels. This simple technique could be of value in situations where the detection of the expressed UGTs cannot be monitored by Western blot analysis.

Quantitation of the expressed UGTs in SF-9 cells by Western blot analysis indicates that up to 5–10% of the total cellular protein is newly synthesized transferase. At present, we have no way of knowing if all of the expressed UGT is catalytically active. This could potentially be of importance, because several posttranslational modification steps are involved in protein processing and could significantly affect function, such as proteolytic cleavage of the leader sequence and targeting to the endoplasmic reticulum, as well as N-glycosylation of the proteins. However, because insect cells for the most part undergo posttranslational processing of recombinant proteins in a fashion similar to their own proteins (5), there is little reason

![Fig. 3. UGT assays with expressed UGT1A6 from SF-9 and COS-1 cells.](image)

**TABLE 1**

Kinetic analysis of UGTs expressed in SF-9 and COS-1 cells

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_M$ (μM)</th>
<th>$V_{max}$ (pmol/min/mg protein)</th>
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<tbody>
<tr>
<td>UGT1A6 (SF-9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-Nitrophenol</td>
<td>100</td>
<td>1250</td>
</tr>
<tr>
<td>2-OH-biphenyl</td>
<td>138</td>
<td>890</td>
</tr>
<tr>
<td>UGT1A6 (COS-1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-Nitrophenol</td>
<td>160</td>
<td>100</td>
</tr>
<tr>
<td>2-OH-biphenyl</td>
<td>140</td>
<td>85</td>
</tr>
<tr>
<td>UGT2B13 (SF-9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Octylgallate</td>
<td>225</td>
<td>850</td>
</tr>
<tr>
<td>4-OH-biphenyl</td>
<td>290</td>
<td>910</td>
</tr>
<tr>
<td>UGT2B13 (COS-1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Octylgallate</td>
<td>208</td>
<td>153</td>
</tr>
<tr>
<td>4-OH-biphenyl</td>
<td>333</td>
<td>200</td>
</tr>
</tbody>
</table>

UGT activities were performed with substrates ranging from 10 μM to 1 mM final concentrations, with a UDPGA concentration of 100 μM. For COS-1 cell extracts, 50 μg of protein was used. For SF-9 cell extracts, 10 μg of protein was used. Each assay was incubated for 1 hr. Activities at each substrate concentration were performed in duplicate. OH, hydroxy.
to believe that one of these processes would be rate-limiting in the production of catalytically active protein. Another concern regarding protein function is the cellular environment of the processed UGTs. For example, purification of the mammalian UGTs from tissues such as liver has demonstrated that the enzymes are highly dependent upon phospholipids for activity. When cellular extracts are made for analysis of catalytic function, phospholipid micelles are included as supplements in the enzyme reactions to ensure an adequate lipid environment for the enzyme. However, we have noticed little difference in enzyme activity if the supplemented phospholipids are not included. Because the lipid environment is important to providing an adequate environment for the delivery of the substrate to the active site of the enzyme. However, we have noticed little difference in enzyme activity if the supplemented phospholipids are not included. The lipid environment is important to providing an adequate environment for the delivery of the substrate to the active site of the transferase (21), the phospholipid composition of the SF-9 cells seems to provide a conducive environment for the mammalian UGTs. This is also supported by kinetic studies that demonstrate little difference in the $K_M$ values of the UGTs when expressed in either the SF-9 or COS-1 cell. Taken together, it would seem that the baculovirus expression of mammalian UGTs in SF-9 cells is an efficient means of producing significant quantities of these proteins.

References