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Coimmunoprecipitation of UDP-Glucuronosyltransferase Isoforms and Cytochrome P450 3A4

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ABSTRACT

Coimmunoprecipitation was used to investigate protein-protein interactions between several UDP-glucuronosyltransferase (UGT) isoforms and cytochrome P450 3A4. Solubilized human liver microsomes were incubated with specific antibodies to UGT2B7, UGT1A6, UGT1A1, and CYP3A4, and the immunoprecipitates were run on SDS-polyacrylamide gel electrophoresis. Western blots showed that UGT2B7, UGT1A6, UGT1A1, and CYP3A4 were successfully immunoprecipitated with the specific antibodies for each enzyme. Upon immunoprecipitating UGT2B7, the corresponding immunoblot showed that UGT1A6, UGT1A1, and CYP3A4 were immunoprecipitated. Similar studies found that different UGT isoforms or CYP3A4 immunoprecipitated along with the original immunoprecipitating enzyme. These data suggest that UGT isoforms may form complexes (dimers, tetramers, etc.) with each other in the endoplasmic reticulum and nuclear envelope. In addition, the UGT isoforms tested here may have interacted with CYP3A4 in the endoplasmic reticulum, suggesting that these enzymes may cooperate in the excretion of compounds in a multistep metabolic process.

The metabolism of endogenous and exogenous compounds is mediated by a number of drug metabolizing enzymes in the liver, including cytochromes P450 and UDP-glucuronosyltransferases (UGTs). P450s catalyze the hydrolysis, oxidation, and reduction reactions, whereas UGTs catalyze the addition of glucuronic acid to hydroxyl, carboxylic acid, sulfur, carbon, and nitrogen moieties. Each of these enzyme systems is localized within the endoplasmic reticulum (ER) and nuclear envelope of cells, where P450s are on the cytosolic side of the ER and UGTs are localized to the luminal side of the ER.

Compounds undergo metabolism through a variety of pathways, including phases I (e.g., P450s) and II (e.g., UGTs). The metabolites generated by P450s are often conjugated by phase II enzymes, including UGTs. The protein-protein interaction between phase I and II enzymes would permit the reactions to proceed more efficiently in the cell, but this concept has not been demonstrated in vitro. Research using P450s has demonstrated that coexpressed baculovirus P450 isoforms can alter one another's catalytic activity (Cawley et al., 1995; Tan et al., 1997; Yamazaki et al., 1997; Backes et al., 1998). Several investigators have reported that different UGT isoforms have protein-protein interactions; furthermore, Meech and Mackenzie (1997) suggested that the amino terminus is important in dimerization. Taura et al. (2000) showed that rat UGTs coeluted with rat CYP1A1 using a bovine serum albumin-conjugated Sepharose 4B column. This study investigates the immunoprecipitation of several human UGT isoforms and CYP3A4 in human liver microsomes.

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Materials and Methods

Human Liver Microsomes. Human liver was purchased from the International Institute for the Advancement of Medicine (Exton, PA). Liver microsomal preparations were prepared by standard differential centrifugation methods and stored in 0.25 M sucrose at −80°C until use as described by Baron and Tephly (1970).

Reagents. Polyclonal peptide antibodies, raised in rabbits, specific to UGT2B7, UGT1A1, and UGT1A6 were purchased from BD Gentest Corporation (Woburn, MA). Polyclonal peptide antibody specific to CYP3A4 was prepared as described previously by Wang and Lu (1997). Anti-ERp57 monoclonal antibody was purchased from Upstate Signaling Solutions (Lake Placid, NY). Horseradish peroxidase-conjugated goat anti-rabbit $F(ab)_2$ secondary antibody was purchased from ICN Biomedical (Costa Mesa, CA). All other reagents were of analytical grade.

Preparation of Protein A Beads. Protein A beads (0.2 g) (Sigma-Aldrich, St. Louis, MO) were added to 10 ml of coimmunoprecipitation buffer (CoB) (25 mM Tris HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% deoxycholate, 0.2% SDS, and 1% Triton X-100)

ABBREVIATIONS: P450, cytochrome P450; UGT, UDP-glucuronosyltransferase; PAGE, polyacrylamide gel electrophoresis; ER, endoplasmic reticulum; CoB, coimmunoprecipitation buffer.

(Yamin et al., 1996) and incubated overnight at 4°C with constant agitation. The beads were centrifuged at 1500*g* for 5 min and washed three times in the CoB. Thereafter, the beads were incubated in Superblock (Pierce, Rockford, IL) for 60 min at 4°C, with constant agitation to diminish nonspecific binding. Superblock was removed by centrifuging at 1500 rpm for 5 min and washing the beads four times with 10 ml of CoB. The resultant pellet was resuspended in a 1:1 ratio (1-ml pellet = 1 ml of buffer) such that the final bead concentration was 0.2 μg/μl, and 0.2% sodium azide (Fisher Scientific, Fairlawn, NJ). Prepared beads were stored for no longer than 2 days.

Immunoprecipitation and Electrophoresis. CoB (100 μl) was added to 1.5-ml centrifuge tubes containing approximately 50 μg of human liver microsomal protein and sonicated for 20 s. Antibody-protein binding was facilitated by the addition of a 1:50 dilution of a specific antibody (UGT2B7, UGT1A6, UGT1A1, CYP3A4, or ERp57) and controls (preimmune serum and H_2O) to tubes containing CoB and human liver microsomes. The tubes were incubated for 3 h at 4°C with constant agitation. The prepared beads were added to the antibody-protein solution in a 1:1 ratio and incubated for 60 min at 4°C with constant agitation. The antibody-protein-bead solution was centrifuged in a 1.5-ml tube (Eppendorf, Mississauga, ON, Canada) at 2100*g* for 5 s. The supernatant was discarded, and the pellet was washed three times with 1 ml of CoB and finally with one volume of a low-salt buffer (25 mM Tris HCl, pH 8.0, 10 mM NaCl, 1 mM EDTA, 0.5% deoxycholate, 0.2% SDS, and 1% Triton X-100). The supernatant was discarded and the pellet was resuspended in phosphate-buffered saline (Invitrogen, Carlsbad, CA). To examine the resulting protein complex, 2 μg of the resuspended pellet was mixed with NuPAGE lithium dodecyl sulfate buffer (Novex, San Diego, CA) and boiled for 5 min at 90°C. The samples were centrifuged at 2100*g* for 5 s to pellet the beads, and the supernatant was electrophoresed on 8% Tris-glycine precast gels (Novex). The samples were then transferred to nitrocellulose membranes (Novex) using a semidry transfer cell (Bio-Rad, Hercules, CA).

Immunoblotting. The nitrocellulose membranes were washed twice for 5 min in Tris-buffered saline (100 mM Tris HCl, pH 7.5, and 0.9% NaCl) and twice for 5 min in Tris-buffered saline/Tween 20 (100 mM Tris HCl, pH 7.5, 0.9% NaCl, and 0.05% Tween 20). Finally, they were blocked with Superblock (Pierce) for 60 min at room temperature. For specific UGT, P450, or ERp57 protein detection, the nitrocellulose blots were incubated for 60 min with the primary antibody in a 1:1000 (v/v) dilution for UGT2B7, UGT1A6, and UGT1A1 and a 1:500 dilution for CYP3A4. Immunoblots were washed three times with Tris-buffered saline/Tween 20 followed by incubation with the secondary antibody $F(ab)_2$ anti-rabbit (MP Biomedicals, Irvine, CA) in a 1:20,000 (v/v) dilution at room temperature. (A control blot [$F(ab)_2$] was incubated in the absence of the primary antibody.) The blots were washed twice for 5 min each with Tris-buffered saline/Tween 20 followed by twice for 5 min each with Tris-buffered saline. The immunoblots were then subjected to chemiluminescence by the addition of Supersignal West Pico (Pierce), according to manufacturer's instructions, and exposed on Biomax MR film (Eastman Kodak, Rochester, NY).

Results and Discussion

Female human liver microsomes were solubilized and incubated with a specific anti-UGT antibody (1A1, 1A6, or 2B7), anti-CYP3A4 antibody, anti-ERp57 antibody, preimmune serum or water. The UGT antibodies used in this manuscript were generated to the amino terminus of the human UGT isoform (BD Gentest). Wang and Lu (1997) described previously the CYP3A4 inhibitory anti-peptide antibody. This antibody did not cross-react with CYP3A5 in Western blots and did not inhibit CYP1A2-, CYP2C9/10-, CYP2D6-, or CYP2E1-mediated metabolism. A monoclonal antibody to ERp57, a luminal protein of the ER and a member of the protein disulfide isomerase family, was used.

Western blots of the immunoprecipitates illustrated that UGT2B7 (Fig. 1A), UGT1A6 (Fig. 1B), UGT1A1 (Fig. 1C), and CYP3A4 (Fig. 3D) were all immunoprecipitated effectively with their specific antibodies. ERp57, preimmune serum, and water (negative controls) showed no bands between 40 and 100 kDa (data not shown). Two nonspecific bands were seen at approximately 190 and 30 kDa in the immunoprecipitate lanes; the 190-kDa band is probably the anti-UGT, anti-CYP3A4, or anti-ERp57 antibody (data not shown) because it was not in the preimmune serum or water lanes. Incubation of immunoblots with the secondary antibody also showed only these two bands. The lower 30-kDa band was seen in all lanes and may represent some other protein in the liver microsomes that bound to the protein A beads.

Western blots of UGT1A1-immunoprecipitates showed that UGT2B7 (Fig. 1A) and UGT1A6 (Fig. 1B) coprecipitated with UGT1A1. Immunoblots of UGT1A6-immunoprecipitates showed that UGT2B7 (Fig. 1A) and UGT1A1 (Fig. 1C) coprecipitated with UGT1A6. Likewise, UGT2B7 immunoprecipitates demonstrated that UGT1A6 (Fig. 1B) and UGT1A1 (Fig. 1C) were coprecipitated with UGT2B7. To our knowledge, this is the first demonstration of a protein-protein interaction between different UGT isoforms in human liver microsomes, suggesting that different UGT isoforms may participate in a UGT complex. Ikushiro et al. (1997) noted that rat UGT1 isoenzymes coeluted with UGT2B1 on an anti-UGT1 antibody conjugated column. Furthermore, heterodimers (120- to 130-kDa complexes) were formed between UGT isoforms when using 1,6-bis(maleimido)hexane, a cross-linker of protein sulfhydryl groups (Ikushiro et al., 1997). UGT2B1 was also shown to dimerize at the amino terminus by Meech and Mackenzie (1997). Using a fusion protein containing the amino-terminal domain of UGT2B1 and coexpressing it with catalytically active UGT2B1 in COS7, Meech and Mackenzie (1997) demonstrated that the

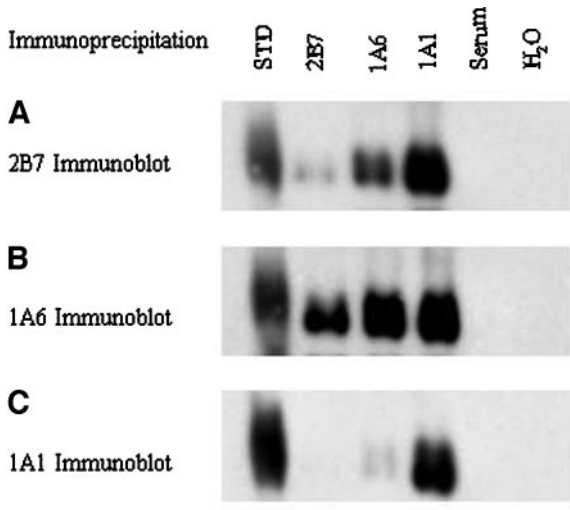

Fig. 1. UGT immunoprecipitation and immunoblots. Identification of the interaction between various UGT isoforms demonstrated by immunoprecipitation of UGT2B7, UGT1A6, UGT1A1, serum, and H_2O followed by the immunoblot with a UGT2B7 antibody (A), UGT1A6 antibody (B), and UGT1A1 antibody (C). Standard in lane 1 (STD) is glutamate dehydrogenase (56 kDa). Procedures for immunoprecipitations and Western blots are described under *Materials and Methods*.

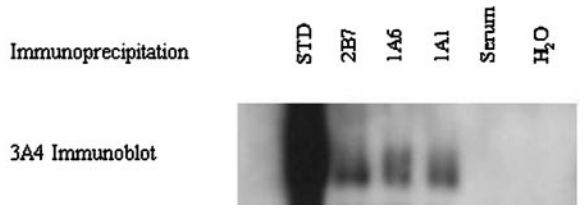

Fig. 2. UGT immunoprecipitation and CYP3A4 immunoblot. Demonstration of the interaction between various UGT isoforms and CYP3A4 by immunoprecipitating UGT2B7, UGT1A6, and UGT1A1 followed by an immunoblot with a specific CYP3A4 antibody. Serum or H_2O controls did not show a 50-kDa CYP3A4 band. Standard in lane 1 (STD) is glutamate dehydrogenase (56 kDa). Procedures for immunoprecipitations and Western blots are described under *Materials and Methods*.

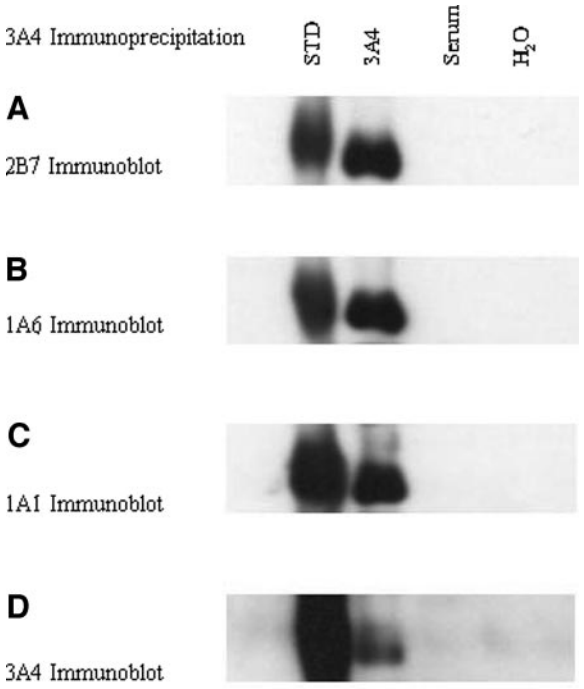

Fig. 3. CYP3A4 immunoprecipitation and UGT immunoblot. Further evidence suggesting that UGT isoforms are interacting with CYP3A4 in the endoplasmic reticulum and the nuclear envelopes of cells through the immunoprecipitation of CYP3A4. Immunoprecipitation was followed by an immunoblot with a UGT2B7 antibody (A), a UGT1A6 antibody (B), a UGT1A1 antibody (C), and a CYP3A4 antibody (D). Bands of ~52–56 kDa did not show in any of the preimmune serum or H_2O controls. Standard in lane 1 (STD) is glutamate dehydrogenase (56 kDa). Procedures for immunoprecipitations and Western blots are described under *Materials and Methods*.

catalytic activity of UGT2B1 was compromised. They also coexpressed two forms of UGT2B1 that were catalytically inactive when expressed alone, but UGT activity was restored when the two forms were coexpressed.

A Western blot of the UGT immunoprecipitates was also investigated with a specific polyclonal human CYP3A4 antibody. It was interesting that CYP3A4 coimmunoprecipitated with all of the UGT isoforms tested (Fig. 2). Immunoprecipitating CYP3A4 (Fig. 3, A–D) and immunoblotting for the specific UGT isoforms, the Western blot shows that UGT1A1, UGT1A6, and UGT2B7 were immunoprecipitated. Taura et al. (2000) showed that rat CYP1A1 coeluted with microsomal epoxide hydrolase and UGTs using an anti-CYP1A1 Sepharose column. As early as 1972, Oesch and Daly reported P450 and microsomal epoxide hydrolase to interact in the ER membrane in crude purification procedures. Until now, human UGTs have not been shown to interact with other enzymes involved in drug metabolism, and these data strengthen several other UGT-UGT interaction studies (Meech and Mackenzie, 1997; Taura et al., 2000; Ishii et al., 2001). Although the nonspecific binding of the antibody cannot be ruled out for the immunoprecipitation of these proteins, it is highly unlikely because the UGT antibodies do not cross-react (in this laboratory) using Western blot techniques, and the CYP3A4 antibody generated by Wang and Lu (1997) was shown to be specific for that P450 isoform. Furthermore, ERp57, an ER protein, did not immunoprecipitate any of the metabolic enzymes tested here but did immunoprecipitate its own protein (data not shown). Further studies are warranted on the interactions between other P450 isoforms and UGTs. Although this work does not support a direct P450-UGT interaction, it does strengthen the claim that different drug-metabolizing enzymes may cohabit within the ER membrane to more efficiently metabolize and ultimately eliminate compounds from the body (Taura et al., 2000).

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