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Transcriptional Regulation of Human *UGT1A1* Gene Expression: Activated Glucocorticoid Receptor Enhances constitutive Androstane Receptor/ Pregnane X Receptor-Mediated UDP-Glucuronosyltransferase 1A1 Regulation with Glucocorticoid Receptor-Interacting Protein 1

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ABSTRACT

UDP-glucuronosyltransferase (UGT) 1A1 glucuronidates endogenous metabolites, such as bilirubin, and exogenous substances, and plays a critical role in their detoxification and excretion. In a previous article, we described the phenobarbital response activity to a 290-base pair (bp) distal enhancer sequence (−3499/−3210) of the human *UGT1A1* gene that is activated by the constitutive androstane receptor (CAR). Here, we show that dexamethasone at submicromolar concentrations enhances the pregnane X receptor (PXR) activator-mediated expression of the *UGT1A1* gene and protein in HepG2 cells. We investigated the molecular mechanism of UGT1A1 induction by glucocorticoids at submicromolar concentrations and PXR activators and the functional cross-talk between the glucocorticoid receptor (GR) and CAR/PXR. The glucocorticoid-response element (GRE) was characterized by cotransfection experiments, site-directed mutagenesis, and electrophoretic mobility shift assays. Analysis of the human *UGT1A1* promoter revealed GREs at −3404/−3389 and −3251/−3236 close to the CAR/PXR response element gtNR1 (−3382/−3367). Furthermore, in an in vitro reporter gene assay, dexamethasone effectively enhanced CAR/PXR-mediated transactivation of the 290-bp distal enhancer module in HepG2 cells and CV-1 cells in the presence of exogenously expressed GR and glucocorticoid receptor-interacting protein 1 (GRIP1). In glutathione *S*-transferase pull-down experiments, CAR and PXR interacted with GRIP1. Together, these results demonstrate a rational mechanistic basis for UGT1A1 induction by glucocorticoids and PXR activators, showing that activated GR enhances CAR/PXR-mediated UGT1A1 regulation with the transcriptional cofactor GRIP1 and that GR may be involved synergistically in the xenobiotic-responsive regulation of UGT1A1 by CAR/PXR.

UDP-glucuronosyltransferase UGT1A1 plays a critical role in the detoxification of potentially neurotoxic bilirubin by conjugating it with glucuronic acid for excretion in bile (Ostrow and Murphy, 1970), and conjugates drugs and other xenobiotics (King et al., 1996; Tukey and Strassburg, 2000). Reduced UGT1A1 activity causes unconjugated hyperbilirubinemia (Crigler-Najjar syndrome and Gilbert's syndrome). Phenobarbital is used as a therapeutic drug for patients with Crigler-Najjar type II syndrome because it increases the expression of bilirubin glucuronosyltransferase and reduces the

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ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; gtPBREM, phenobarbital-responsive enhancer module of UGT1A1; CAR, constitutive androstane receptor; bp, base pair; PXR, pregnane X receptor; PXRE, pregnane X receptor response element; AhR, aryl hydrocarbon receptor; GR, glucocorticoid receptor; GRE, glucocorticoid-response element; RXR, retinoid X receptor; AF, activation function; GRIP1, glucocorticoid receptor-interacting protein 1; kbp, kilobase pair; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; CITCO, 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime; PAGE, polyacrylamide gel electrophoresis; MMTV, mouse mammary tumor virus; pGL3-tk, pGL3-tk-firefly luciferase vector; tk, thymidine kinase promoter.

incidence of unconjugated hyperbilirubinemia (Mackenzie et al., 1997). These observations prompted us to find the enhancer module in the 5′-flanking region of the phenobarbital-inducible human *UGT1A1* gene. We identified the phenobarbital-responsive enhancer module at −3499/−3210 from the transcription start site of UGT1A1, gtPBREM, which is regulated by the nuclear receptor known as constitutive androstane receptor (CAR; NR1I3) in response to phenobarbital treatment (Sugatani et al., 2001). gtPBREM is a composite 290-bp element consisting of five nuclear receptor motifs: DR4 element, gtNR1 and DR3 element (Sugatani et al., 2001), pregnane X receptor (PXR; NR1I2) response element (PXRE) (Xie et al., 2003), and receptor-type transcription factor aryl hydrocarbon receptor (AhR) response element (xenobiotic response element) (Yueh et al., 2003). Xie et al. (2003) used an expression vector for constitutively active VP-PXR in HepG2 cells without ligands to identify the PXRE of the *UGT1A1* gene. It remains unknown whether the DR4, gtNR1, and DR3 elements play roles in PXR activator-induced transactivation. In addition, the induction of UGT1A1 by dexamethasone has been extensively documented both in vivo in liver (Emi et al., 1995) and in vitro in cultured rat hepatocytes (Jemnitz et al., 2002), although the molecular mechanism of this response has not been elucidated. Therefore, in this study, we investigated the molecular mechanism(s) of induction of human *UGT1A1* by glucocorticoids and PXR activators.

Most glucocorticoid actions are mediated by the glucocorticoid receptor (GR) (Hollenberg et al., 1985). Upon binding to glucocorticoids such as hydrocortisone and dexamethasone, GR is activated and translocated from the cytoplasm to the cell nucleus where it activates transcription by binding to the glucocorticoid-response element (GRE) in the promoter region of target genes (Becker et al., 1986; Htun et al., 1996). Schuetz et al. (2000) demonstrated that glucocorticoid receptor-deficient mice challenged with dexamethasone failed to induce CYP2B proteins, whereas CYP2B was readily induced in wild-type mice. These observations indicate that GR contributes to the inducible expression of CYP2B by glucocorticoids in mice. Moreover, dexamethasone has been reported to increase the expression of RXR, PXR, and CAR in cultured human hepatocytes, perhaps through a direct action on the GR, leading to a synergistic effect on the phenobarbital- and PXR activator-mediated induction of the *CYP2B6* and *CYP3A4* genes (Pascussi et al., 2000a,b). Therefore, in this study, we investigated whether glucocorticoids act as ligands for CAR, PXR, and RXR in UGT1A1 induction as they do in CYP2B6 and CYP3A4 induction, or whether glucocorticoids interact with the GR and in turn bind directly to the UGT1A1 promoter to induce transcriptional activation.

The molecular mechanism of steroid hormone receptor activation is believed to involve binding of a ligand to the receptor, altering its protein conformation to expose the activation function-2 (AF-2) in the hormone-binding domain, allowing interaction with coactivators such as steroid receptor coactivator 1 and glucocorticoid receptor-interacting protein 1 (GRIP1), and activating transcription (Hong et al., 1999; McKenna and O'Malley, 2002). Recent studies have demonstrated that CAR and PXR interact with steroid receptor coactivator 1 (Lehmann et al., 1998; Muangmoonchai et al., 2001), that GRIP1 mediates the nuclear translocation and activation of CAR (Min et al., 2002a), and that CAR antagonizes estrogen receptor-mediated transcriptional activity by interacting with the estrogen receptor and moreover, by limiting the amount of GRIP1 (Min et al., 2002b). In addition, the induction of a wide range of genes mediated by CAR and PXR seems to be regulated in a rather complex manner involving cross-talk between nuclear receptors and transcriptional cofactors (Pascussi et al., 2003) and to be influenced by other cellular signaling pathways in a similar way to modulation of the estrogen receptor transcriptional response by Rho GTPase (Su et al., 2002). Thus, it is possible that functional cross-talk between GR and CAR/PXR and/or the recruitment of coactivators and corepressors might influence transcriptional activation by the UGT1A1 290-bp distal enhancer module. Therefore, we examined whether CAR- or PXR-mediated transcriptional activity of the UGT1A1 290-bp distal enhancer module is altered by dexamethasone and whether cofactors such as GRIP1 influence cross-talk between GR and CAR, or GR and PXR.

Materials and Methods

Plasmids. Reporter plasmids containing subfragments of the 11-kbp flanking region (UA, UB, U2K, UC, UD, UE, UF, and the 1640-, 462-, 290-, and 190-bp subfragments of the U2K fragment)-pGL3-tk-firefly luciferase have been described previously (Sugatani et al., 2001, 2004). The UGT1A1 290-bp distal enhancer module (gtPBREM, −3499/−3210)-pGL3-tk-firefly luciferase reporter plasmid was prepared as described previously (Sugatani et al., 2001). Although we previously found a single nucleotide polymorphism changing T to G at nucleotide −3279 (Sugatani et al., 2002), we here used the wild-type 290-bp distal enhancer module having T at nucleotide −3279. Mutation of the DR4 element, gtNR1, and DR3 element (Sugatani et al., 2001), PXRE (Xie et al., 2003), and GRE half-sites (GRE1, GRE2, GRE3, and GRE4) in the 290-bp distal enhancer module was performed with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The primers used were 5′-TACACTAGTAA*G*G*C*G*C*-*C*CTCAATTCCAAGG-3′; 5′-AGAACAAACTTC*G*G*C*G*CC*TATATA-ACCTC-3′; 5′-TGGCCAAGGGTAGA*T*T*C*CAGTTTGAACAAAG-3′; and 5′-ACATTCTAAC*TT*TTCATAAAGGGTATTAGG-3′, 5′-ATGATTA-ACCAAAG*GG*C*G*TTCTAACGGTTC-3′, 5′-TGTTATCTCACCAG*GG*C-*G*AACTTCTGAGTT-3′, 5′-CAAAGCAATTTGAG*GG*C*G*TCAAAG-GAAGTT-3′, 5′-AAAGGAAGTTTGGG*TCG*C*G*GCAAGGGATCC-3′, and their complements for the DR4 element, gtNR1, DR3 element, PXRE, and GRE1, GRE2, GRE3, and GRE4, respectively. Underlined characters show the bases for the mutation. The fragment −6155/−5898 containing the putative GRE site −6015/−6001 was amplified using 5′-gtttcc*gctagc*CATCATTAAATTA-AGCCA-3′ and 5′-gtttaa*ctcgag*GGATAGGACCTCTGTTTG-3′ and cloned into the pGL3-tk-reporter plasmid at the NheI and XhoI sites. Bases in lowercase letters were added to allow digestion of the oligonucleotides with the restriction enzymes NheI and XhoI.

All expression vectors were sequenced by dye-terminator automated sequencing. The cDNA encoding the full-length human GRIP1 was amplified by polymerase chain reaction (PCR) with the primers 5′-gtttcc*ggatcc*ATGAGTGGGATGGGAGAAAAT-3′ and 5′-gtttaa*ctc-gag*TCAGCAATATTTCCGTGTTGTG-3′ and cloned into the BamHI and XhoI sites of pcDNA3 (Invitrogen, Carlsbad, CA) and pGEX4T3 (Amersham Biosciences Inc., Piscataway, NJ), respectively. Bases in lowercase letters were added to allow digestion of the oligonucleotides with the restriction enzymes BamHI and XhoI. The expression vectors pCR3-human CAR and pCR3-human PXR have been described previously (Sueyoshi et al., 1999; Sugatani et al., 2004). For the expression of GST-human GR, GST-human CAR, and GST-human PXR, BamHI/XhoI fragments containing human GR, human CAR, and human PXR cDNAs were inserted into pGEX4T3 digested

with the same enzymes. Human GRα expression vector was a generous gift from Dr. John Cidlowski (National Institute of Environmental Health Sciences, Research Triangle Park, NC). Vectors for the expression of 1) human CAR-ΔC8, 2) human PXR-ΔN38, and 3) human PXR-ΔC9 were constructed by inserting the corresponding cDNAs, which were amplified by PCR with the primers 1) 5′-gtttcc*ggatcc*ATGGCCAGTAGGGAAGATGAGCT-3′ and 5′-gttta*actcgag*TCACATCATGGCAGACAGG-3′; 2) 5′-gtttcc*ggatcc*ATGCAAATCTGCCGTGTATGTGGGGAC-3′ and 5′-gtttaa*ctcgag*TCAGCTACCTGTGATGCC-3′; and 3) 5′-gtttcc*ggatcc*ATGGAGGTGAGACCCAAAGAAAGC-3′ and 5′-gtttaa*ctcgag*TCACATGAGGGGCGTAGCAAAGGGGTG-3′, respectively, into the BamHI and XhoI sites of pCR3 and pGEX4T3, respectively. Bases in lowercase letters were added to allow digestion of the oligonucleotides with the restriction enzymes.

Cell Culture, Transfection, and Luciferase Assays. 1) Human liver-derived cells (HepG2 cell line; Cell Bank, RIKEN BioResource Center, Ibaraki, Japan) were seeded onto 24-well plates at 1×10^5 cells/ml in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, UT). Twenty-four hours later, they were transfected with UGT1A1-luciferase reporter constructs (0.2 μg) and expression vectors [pCR3-GR (0.2 μg), pCR3-CAR (0.2 μg), pCR3-CAR mutant (0.2 μg), pCR3-PXR (0.2 μg), or pCR3-PXR mutant (0.2 μg) and pRL-SV40 (Promega, Madison, WI) (0.2 μg) with or without pcDNA3-GRIP1 (0.6 μg)] using a calcium phosphate coprecipitation method (CellPhect transfection kit; Amersham Biosciences Inc.) unless otherwise stated. The medium (1 ml per well) was replaced after 12 h by Dulbecco's modified Eagle's medium supplemented with 10% dextran-coated charcoal-stripped fetal calf serum (Hyclone Laboratories). The cells were subsequently treated for 12, 24, and 48 h with dexamethasone (10^{-9}–10^{-5} M) (Sigma-Aldrich, St. Louis, MO), rifampicin (5×10^{-6} M) (Sigma-Aldrich), rifampicin (5×10^{-6} M) plus dexamethasone (10^{-7} M), clotrimazole (5×10^{-6} M) (Sigma-Aldrich), clotrimazole (5×10^{-6} M) plus dexamethasone (10^{-7} M) or hydrocortisone (10^{-7} M) (Sigma-Aldrich) as 400× concentrated stocks in dimethyl sulfoxide; controls received an equivalent volume of dimethyl sulfoxide. The total amount of DNA transfected was held constant in each transfection by using the corresponding empty vector. Transfected cells were washed once in phosphate-buffered saline and harvested in 1× passive lysis buffer, and luciferase activity was measured simultaneously with the dual-luciferase reporter assay system according to the manufacturer's instructions (Promega); the firefly luciferase values were normalized to the *R. reniformis* values for each sample. 2) Monkey kidney-derived cells (CV-1 cell line; Cell Bank, RIKEN BioResource Center) were transfected in suspension with FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) and plasmid DNAs [UGT1A1-luciferase reporter constructs (0.2 μg), expression vectors pCR3 (0.2 μg), pCR3-GR (0.2 μg), pCR3-CAR (0.2 μg), and/or pCR3-PXR (0.2 μg), and pSV-β-galactosidase control vector (Promega) (0.6 μg)] with or without pcDNA3-GRIP1 (0.6 μg) according to the manufacturer's instructions. The medium was replaced 24 h after transfection by either induction or control medium as described above. Luciferase and β-galactosidase assays were performed according to the specifications of the manufacturer (Promega).

mRNA Levels. Reverse transcription-PCR was used to confirm the differential expression of the genes. The following primers were synthesized and PCR amplification was conducted on cDNA from HepG2: UGT1A1, 5′-AACAAGGAGCTCATGGCCTCC-3′ and 5′-GTTCGCAAGATTCGATGGTCG-3′; PXR, 5′-GCAAGGGCTTTTTCAGGA-3′ and 5′-TCTTCCGCTTGATCAAGG-3′; CAR, 5′-ACATCAACACTTTCATGGTA-3′ and 5′-TCAGCTGCAGATCTCCTGGA-3′; and β-actin, 5′-GTGGGGCGCCCCAGGCACCA-3′ and 5′-CTCCTTAATGTCACGCACGATTTC-3′. The expected sizes of amplified cDNAs were 644, 204, and 540 bp for UGT1A1, PXR, CAR, and β-actin, respectively. PCR was performed in 50 μl on cDNA synthesized from 0.14, 0.05, 0.19, and 0.05 μg of total RNA with *Taq*DNA polymerase (Takara, Kyoto, Japan) for 40, 36, 40, and 20 cycles, respectively. The PCR cycle consisted of 94°C for 30 s, 59°C for 30 s, and 72°C for 1 min.

To measure the mRNA levels of UGT1A1, CAR, PXR, AhR, and GR in HepG2 cells treated with dexamethasone (10^{-8}–10^{-6} M), total RNA was prepared from HepG2 cells with TRIzol reagent. cDNA synthesized from 100 ng of total RNA was subjected to quantitative real-time PCR with an ABI GeneAmp 5700 (Applied Biosystems, Foster City, CA) as described previously (Sugatani et al., 2001, 2004) or by using Assays-on-Demand Gene Expression Products for human GR (Applied Biosystems). The mRNA levels were normalized against β-actin mRNA determined by predeveloped TaqMan assay reagents for human β-actin (Applied Biosystems).

Immunoblot Analysis. Microsomes of HepG2 cells cultured in three 75T flasks and treated with dexamethasone (10^{-7} M), rifampicin (5×10^{-6} M), dexamethasone (10^{-7} M) plus rifampicin (5×10^{-6} M), or vehicle (dimethyl sulfoxide) were prepared by differential centrifugation as described previously (Sugatani et al., 2004) and stored at −80°C. The microsomal protein concentration was determined with a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL) using bovine serum albumin as a standard. Microsomal proteins (20 μg) were resolved on a sodium dodecyl sulfate-12.5% polyacrylamide gel, electroblotted onto a polyvinylidene difluoride membrane (Millipore Corporation, Bedford, MA), and incubated with anti-human UGT1A1 antibody (BD Biosciences, Woburn, MA) or anti-human cytochrome P450 CYP3A4 antibody [generously given by Prof. Yasushi Yamazoe (Tohoku University, Tohoku, Japan)] (Miyata et al., 2004). After incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for the detection of UGT1A1 and CYP3A4, the resultant immunoproducts were visualized with an enhanced chemiluminescence system (Amersham Biosciences Inc.).

Electrophoretic Mobility Shift Assays. Human GR was synthesized in vitro with the TNT quick-coupled in vitro transcription translation system (Promega) according to the manufacturer's protocol. The following sets of complementary oligonucleotides were synthesized and annealed for electrophoretic mobility shift assays: UGT1A1 GRE2 5′ wt, 5′-gatcTGTTATCTCACCAGAACAAAC-3′ and 5′-gatcGTTTGTTCTGGTGAGATAACA-3′; UGT1A1 GRE2 5′ m1, 5′-gatcTGGGCTCCCACCAGAACAAAC-3′ and 5′-gatcGTTTGTTCTGGTGGGAGCCCA-3′; UGT1A1 GRE2 5′ m2, 5′-gatcTGTTATCTCACCAGGGCGAAC-3′ and 5′-gatcGTTCGCCCTGGTGAGATAACA-3′; UGT1A1 GRE2 3′, 5′-gatcACCAGAACAAACTTCTGAGTTTAT-3′ and 5′-gatcATAAACTCAGAAGTTTGTTCTGGT-3′; UGT1A1 GRE3 5′, 5′-gttggCAAAGCAATTTGAGAACATCA-3′ and 5′-gttggTGATGTTCTCAAATTGCTTTG-3′; UGT1A1 GRE3 3′, 5′-gttggTTGAGAACATCAAAGGAAGTT-3′ and 5′-gttggAACTTCCTTTGATGTTCTCAA-3′; UGT1A1 GRE4 5′ wt, 5′-gttggAAGGAAGTTTGGGGAACAGCA-3′ and 5′-gttggTGCTGTTCCCCAAACTTCCTT-3′; UGT1A1 GRE4 5′ m1, 5′-gttggAAGGCGCCTTGGGGAACAGCA-3′ and 5′-gttggTGCTGTTCCCCAAGGCGCCTT-3′; UGT1A1 GRE4 5′ m2, 5′-gttggAAGGAAGTTTGGGTCGCGGCA-3′ and 5′-gttggTGCCGCGACCCAAACTTCCTT-3′; UGT1A1 GRE4 3′, 5′-gttggTGGGGAACAGCAAGGGATCCA-3′ and 5′-gttggTGGATCCCTTGCTGTTCCCCA-3′; and MMTV GRE, 5′-gttggGTTACAAACTGTTCT-3′ and 5′-tggttAGAACAGTTTGTAAC-3′.

Bases in lowercase letters were added to allow labeling of the oligonucleotides with Klenow fragment and [α-^{32}P]dCTP. Binding assays were performed with human GR (1 μl) as described previously (Sugatani et al., 2001). For competition, a 100-fold concentration of unlabeled oligonucleotides was added before the radioactive probes.

GST-Dependent Protein-Protein Interaction Assays. GST fusion proteins were expressed in *Escherichia coli* BL21 [DE3(pLys)] and purified using glutathione-Sepharose (Amersham Biosciences Inc.) according to the manufacturer's protocols. ^{35}S-labeled proteins were synthesized by the TNT-coupled reticulocyte lysate system (Promega), and the labeled proteins were precleared by incubation with GST bound to glutathione-Sepharose for 30 min at 4°C. One microgram of GST or GST fusion protein bound to glutathione-Sepharose was incubated at 4°C for 2 h with 4 μl of precleared

reticulocyte lysate containing the ^{35}S-labeled proteins in 100 μl of binding buffer (25 mM HEPES-KOH buffer, pH 7.6, containing 0.15 M NaCl, 10% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mg/ml bovine serum albumin, 0.25% Nonidet P-40, and 0.2 mM phenylmethylsulfonyl fluoride) in the presence or absence of 1×10^{-7} M CITCO (BIOMOL Research Laboratories, Plymouth Meeting, PA), 5×10^{-6} M rifampicin, 1×10^{-7} M dexamethasone, or the vehicle (dimethyl sulfoxide) alone (0.5% volume). The resin was washed four times with 500 μl of the binding buffer. Thereafter, proteins were extracted from the resin with SDS-PAGE sample buffer, separated by SDS-PAGE, and the radioactive proteins were detected by autoradiography.

Results

Induction of UGT1A1 mRNA and Protein, and Enhancement of PXR Activator-Inducible Expression by Dexamethasone in HepG2 Cells. Treatment with dexamethasone at 10^{-7} M caused a slight but significant induction of UGT1A1 mRNA and protein in HepG2 cells (Figs. 1 and 2). Moreover, 10^{-7} M dexamethasone enhanced 5×10^{-6} M rifampicin- and 5×10^{-6} M clotrimazole-inducible expression of UGT1A1 mRNA in HepG2 cells to 1.8 ± 0.3- and 1.9 ± 0.3-fold (4.0 ± 0.9- and 6.1 ± 0.8-fold that of the vehicle-treated control, $n = 4$), respectively (Fig. 1B). As shown in Fig. 2, the rifampicin-inducible expression of UGT1A1 protein was synergistically enhanced by dexamethasone to a similar extent as that of CYP3As protein. Because the induction of *UGT1A1* is known to occur through three distinct mechanisms mediated by the nuclear receptors CAR, PXR, and AhR (Sugatani et al., 2001; Xie et al., 2003; Yueh et al., 2003), we tested the effects of dexamethasone on the expression of the *CAR*, *PXR*, and *AhR* genes, together with the *GR* gene. As shown in Fig. 1, dexamethasone at 10^{-7} and 10^{-6} M slightly induced PXR mRNA. But there was no significant change in AhR, CAR, and GR mRNA levels in HepG2 cells treated with 10^{-7} M dexamethasone, 5×10^{-6} M rifampicin, 10^{-7} M dexamethasone plus 5×10^{-6} M rifampicin, 5×10^{-6} M clotrimazole, and 10^{-7} M dexamethasone plus 5×10^{-6} M clotrimazole (data not shown).

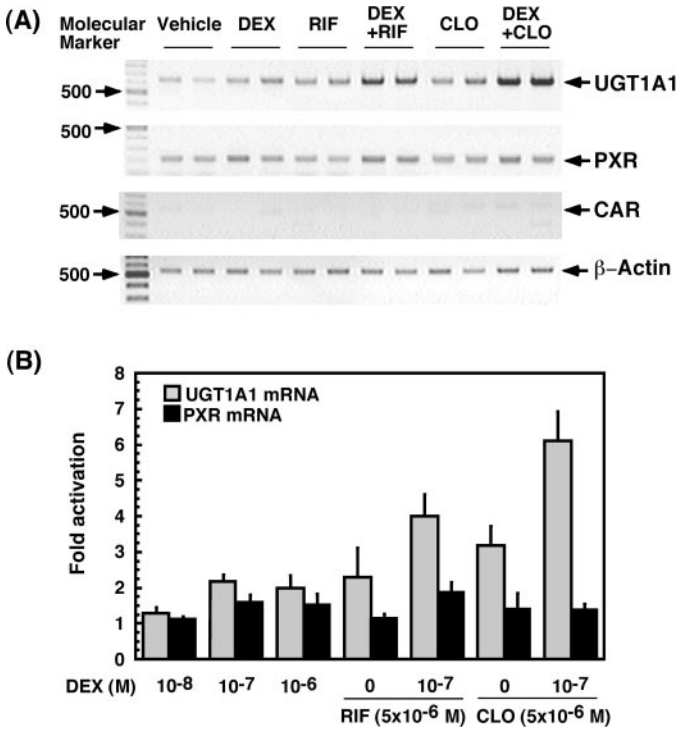

Fig. 1. Reverse transcription-PCR (A) and quantitative real-time PCR (B) for UGT1A1, PXR, and CAR. Total RNA was isolated from HepG2 cells treated for 24 h with the vehicle (dimethyl sulfoxide), dexamethasone (10^{-8}, 10^{-7}, and 10^{-6} M), rifampicin (5×10^{-6} M), dexamethasone (10^{-7} M) plus rifampicin (5×10^{-6} M), clotrimazole (5×10^{-6} M), or dexamethasone (10^{-7} M) plus clotrimazole (5×10^{-6} M). A, each gene product was amplified from HepG2 mRNA and separated on 1% agarose gel and stained by ethidium bromide. β-Actin mRNA was amplified as an internal standard. The left lane contains molecular size markers. B, relative mRNA levels are expressed by taking the control values obtained from the vehicle-treated cells as one. Data presented are the average ± S.D. of normalized mRNA abundance (four to six samples per treatment group). CLO, clotrimazole; DEX, dexamethasone; RIF, rifampicin.

Transcriptional Activation of UGT1A1 Promoter by Dexamethasone. To investigate whether sequences upstream of the 5′-flanking region of *UGT1A1* may contribute to the activation process by dexamethasone at submicromolar concentrations, we tested various DNA fragments generated from a 11-kbp 5′-flanking region in *UGT1A1* and placed in front of the reporter luciferase gene for their enhancer activities in HepG2 cells in the presence of exogenously expressed CAR or PXR. The 290-bp fragment (−3499/−3210), which contains the binding domains for CAR and PXR (Sugatani et al., 2001; Xie et al., 2003), displayed the most prominent enhancement of CAR- and PXR-mediated transcriptional activation by 10^{-7} M dexamethasone (Fig. 3). To test the hypothesis that activated GR could influence CAR or PXR regulation of UGT1A1 gene expression, experiments were carried out in HepG2 cells transfected with the 290-bp reporter construct and expression plasmid of CAR or PXR in the absence or presence of exogenously expressed GR. The cells were cultured for 12, 24, and 48 h with or without 10^{-7} M dexamethasone or 10^{-7} M dexamethasone plus 5×10^{-6} M rifampicin, and luciferase activity was determined. In cells transfected with the GR expression plasmid, CAR- and PXR-mediated transcriptional activation of the 290-bp reporter construct was more prominently enhanced by dexamethasone at submicromolar concentrations, compared with cells in the absence of exogenously expressed GR (Fig. 4). GR enhancement of the CAR- and PXR-mediated transcriptional activation almost reached a plateau after 24 h (Fig. 4A) and peaked at 10^{-7} M dexamethasone (Fig. 4, B and C).

Moreover, to investigate whether the induction of UGT1A1 by glucocorticoids at submicromolar concentrations is caused directly by a distinct mechanism mediated by GR, we looked for a functional GRE in the *UGT1A1* gene. Computer analysis of the 11-kbp 5′-flanking region of the *UGT1A1* gene

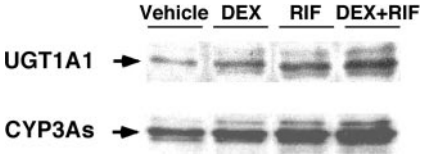

Fig. 2. Induction of UGT1A1 and CYP3As protein in HepG2 cells treated with dexamethasone, rifampicin, or rifampicin plus dexamethasone. The microsomal proteins (20 μg), which were prepared from cells treated with 10^{-7} M dexamethasone, 5×10^{-6} M rifampicin, 10^{-7} M dexamethasone plus 5×10^{-6} M rifampicin, or the vehicle (dimethyl sulfoxide) alone for 48 h were subjected to Western blot analysis using an anti-human UGT1A1 antibody or anti-human CYP3A4 antibody. Because the anti-CYP3A4 antibody used in this study could not distinguish the corresponding CYP3A subfamilies, we expressed the sum of immunoreactive proteins as CYP3As. DEX, dexamethasone; RIF, rifampicin.

using a MctInspector software program in conjunction with the TRANSFAC 4.0 database suggested the presence of numerous putative GREs, including putative GRE half-sites. To evaluate the role of these putative GREs, the various plasmids harboring UA, UB, gtPBREM (−3499/−3210), UD, UF, and −6155/−5898, which include putative GREs, were transfected into HepG2 cells with a plasmid expressing GR. The cells were then cultured for 24 h with or without 10^{-7} M dexamethasone or 10^{-7} M hydrocortisone, and luciferase activity was determined. In the cells transfected with GR expression plasmid, the luciferase activity of the −3499/−3210 construct was increased by 10^{-7} M dexamethasone and 10^{-7} M hydrocortisone [2.6 ± 0.2- and 1.7 ± 0.1-fold activation of the vehicle-treated control (n = 4 and 3), respectively], but the luciferase activity of the UA, UB, UD, UF, and −6155/−5898 constructs were not affected by 10^{-7} M dexamethasone (data not shown). Together, these results suggest a possible GRE located on the −3499/−3210 region of *UGT1A1*.

Identification of the UGT1A1-GRE by Direct Mutagenesis. To investigate whether GR can activate DR4, PXRE, gtNR1, or DR3 motifs in the UGT1A1 290-bp distal enhancer module, all four motifs were mutated by direct mutagenesis (Fig. 5A). The wild-type 290-bp distal enhancer module or the module mutated in all four motifs was transfected into HepG2 cells with the GR expression plasmid and then the cells were cultured with ligand (10^{-7} M dexamethasone). Even in the 290-bp distal enhancer module mutated in all four motifs, transcriptional activity was enhanced by 10^{-7} M dexamethasone to 1.9 ± 0.1-fold that of the vehicle-treated control ($p < 0.01$ versus vehicle, n = 4). In contrast, gtNR1 mutation resulted in a 100% decrease of both CAR-dependent enhancer activity and rifampicin-induced enhancer activity, whereas mutations of DR4, PXRE, and DR3 retained 56 to 76% in CAR-dependent enhancer activity and about 60% in PXR-dependent enhancer activity (Fig. 5B). Next, to evaluate the role of the four GRE half-sites in the UGT1A1 290-bp reporter gene, designated as GRE1, GRE2, GRE3, and GRE4, they were sequentially mutated by direct mutagenesis (Fig. 5A). Mutation of the GRE2 and GRE4 sites markedly repressed 10^{-7} M dexamethasone-induced transcriptional activation in HepG2 cells transfected with the GR expression plasmid (Fig. 5C). Moreover, also in HepG2 cells transfected with the expression plasmids for both CAR and GR, mutation of the GRE2 and GRE4 sites resulted in 89 ± 5 and 95 ± 12% decrease of the control transcriptional activation by 10^{-7} M dexamethasone (n = 4), respectively (data not shown). In contrast, the majority of transcriptional activity of GRE1-, GRE2-, GRE3-, and GRE4-mutated constructs was preserved in the presence of CAR and PXR (Fig. 5C). These results indicate that GRE2 and GRE4 contribute to transcriptional activation by GR.

GR Binds to GRE in the UGT1A1 290-bp Enhancer Module. To determine whether GR interacts directly with UGT1A1 GRE, and which GRE half-site participates in high-affinity GR-DNA binding, electrophoretic mobility shift assays were performed with in vitro-translated GR. As expected, GR bound a positive control probe containing GRE from a mouse mammary tumor virus (MMTV) (Fig. 6). The binding of GR to GRE2 3′, GRE3 5′, GRE3 3′, and GRE4 3′ probes was at trace level (Fig. 6). On the other hand, GR bound more strongly to GRE2 5′ and GRE4 5′ probes containing GRE2 and GRE4 half-sites, producing protein-DNA complexes consistent with those observed on binding of the dimeric GR-MMTV probe (Fig. 6). These bands were specifically eliminated by the addition of 100-fold molar excesses of unlabeled UGT1A1 GRE2 5′, UGT1A1 GRE4 5′, or MMTV GRE probe. Moreover, GR showed decreased binding to UGT1A1 GRE2 5′ and UGT1A1 GRE4 5′ probes mutated in

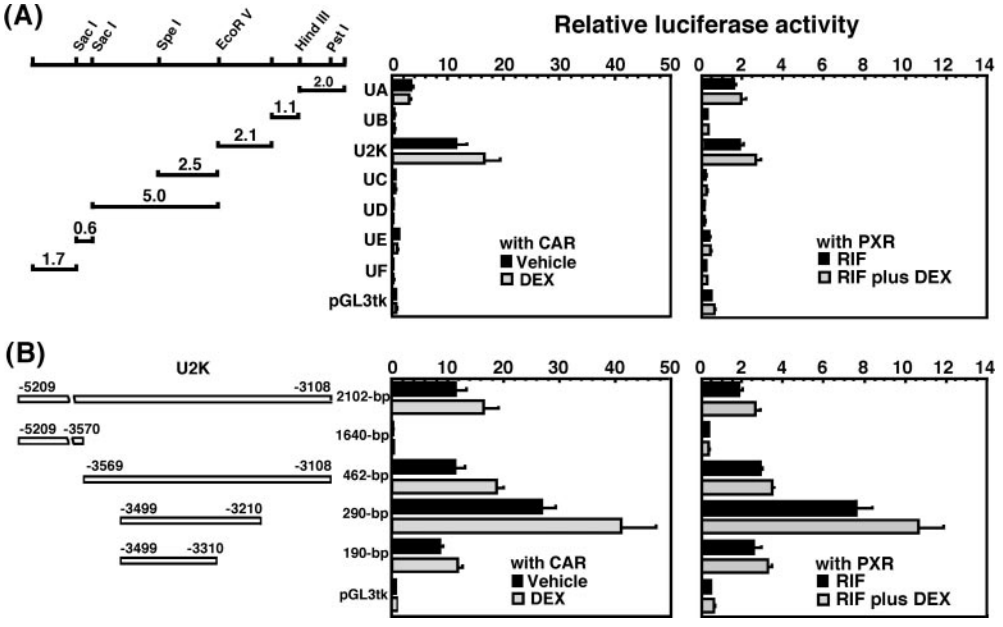

Fig. 3. Effect of dexamethasone on the transcriptional activity of various 5′-flanking fragments in HepG2 cells with exogenously expressed CAR or PXR. Various fragments were generated from the 11-kbp 5′-flanking DNA of the *UGT1A1* gene (A) and from the U2K DNA (B), constructed into the luciferase reporter gene plasmid (pGL3-tk) (0.2 μg), and cotransfected with pRL-SV40 (0.2 μg) and the expression vector for CAR or PXR (0.2 μg). The CAR-expressed cells were treated with the vehicle (dimethyl sulfoxide) alone or dexamethasone (10^{-7} M) for 24 h, and PXR-expressed cells were treated with rifampicin (5×10^{-6} M) alone or dexamethasone (10^{-7} M) plus rifampicin (5×10^{-6} M) for 24 h. The luciferase activity was determined as described under *Materials and Methods*. Relative luciferase activity, measured by pGL3-tk basic-reporter gene in vehicle-treated HepG2 cells, was calculated as 1. Data presented are the average of four independent experiments ± S.D. DEX, dexamethasone; RIF, rifampicin.

either the 5′ half-site (UGT1A1 GRE2 5′ m1 and UGT1A1 GRE4 5′ m1) or the 3′ half-site (UGT1A1 GRE2 5′ m2 and UGT1A1 GRE4 5′ m2) (Fig. 6). Together, these results suggest that the regions of −3404/−3389 and −3251/−3236, to which GR binds, are UGT1A1-GREs, although its affinity for these sites is lower than for MMTV GRE.

Enhancement of PXR- and CAR-Mediated Transactivation of UGT1A1 Gene by Activated GR and GRIP1. Because dexamethasone enhances the PXR activator-inducible expression of UGT1A1 mRNA and protein (Figs. 1 and 2), we were prompted to test whether activated GR influences PXR- or CAR-mediated transcriptional activation of the UGT1A1 290-bp reporter gene. Transient transfection assays in HepG2 cells and CV-1 cells were performed with exogenously expressed GR, PXR, and CAR. As shown in Figs. 7 and 8, 10^{-7} M dexamethasone elevated luciferase activity to about 2-fold that of the vehicle-treated control in HepG2 cells and CV-1 cells transfected with the GR expression plasmid. In the presence of exogenously expressed GR and CAR, 10^{-7} M dexamethasone enhanced CAR-mediated transcriptional activity to 2.2- and 2.8-fold that of the vehicle-treated control in HepG2 cells and CV-1 cells, respectively ($p < 0.001$) (Fig. 8).

In the presence of exogenously expressed GRIP1, GR, and PXR, not only dexamethasone (10^{-7} M)-induced transcriptional activation but also the enhancement of rifampicin (5×10^{-6} M)- and clotrimazole (5×10^{-6} M)-induced transcriptional activation by dexamethasone (10^{-7} M) was observed more effectively in HepG2 cells and CV-1 cells compared with those in the absence of exogenously expressed GRIP1 (Fig. 7). Likewise, in the presence of exogenously expressed GRIP1, GR, and CAR, the transcriptional activity was elevated more effectively by 10^{-7} M dexamethasone in HepG2 cells and CV-1 cells (Fig. 8). These results indicate that GRIP1 may contribute to the crosstalk by interacting with GR and PXR or with GR and CAR to form a functionally active complex in the transcriptional activation of the 290-bp distal enhancer module.

CAR and PXR Interact with GRIP1 in Vitro. To investigate whether CAR and PXR physically interact with GRIP1 in vitro, the binding of GST-GRIP1 to ^{35}S-labeled CAR, ^{35}S-labeled PXR, or ^{35}S-labeled GR, and of ^{35}S-labeled GRIP1 to

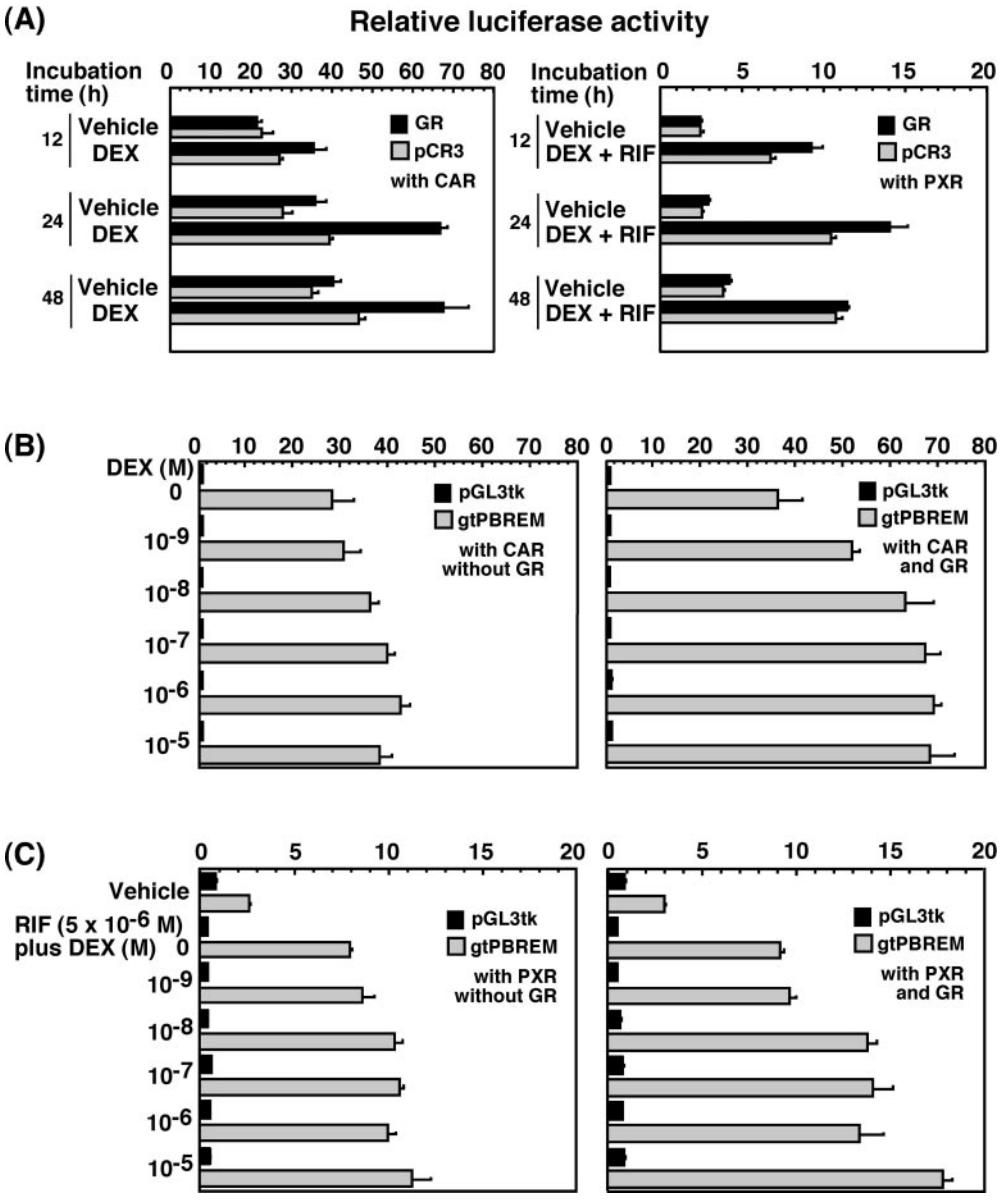

Fig. 4. Time (A)- and dose (B and C)-dependent transcriptional activation of UGT1A1 290-bp distal enhancer module by dexamethasone in HepG2 cells in the presence of exogenously expressed CAR or PXR. The UGT1A1 290-bp reporter gene or pGL3-tk basic reporter gene (0.2 μg) was cotransfected into HepG2 cells with pCR3-CAR (0.2 μg) and the control vector pCR3 (0.2 μg) or with both pCR3-CAR (0.2 μg) and pCR3-GR (0.2 μg) (A and B), or with pCR3-PXR (0.2 μg) and the control vector pCR3 (0.2 μg) or with both pCR3-PXR (0.2 μg) and pCR3-GR (0.2 μg) (A and C), together with pRL-SV40 (0.2 μg) as described under *Materials and Methods*. The transfected cells were incubated with 10^{-7} M dexamethasone or 10^{-7} M dexamethasone plus 5×10^{-6} M rifampicin for 12, 24, and 48 h (A) or with various concentrations of dexamethasone (B) or various concentrations of dexamethasone plus 5×10^{-6} M rifampicin (C) for 24 h, harvested, and assayed for luciferase activity. Relative luciferase activity, measured by pGL3-tk basic reporter gene in vehicle (dimethyl sulfoxide)-treated HepG2 cells, was calculated as 1. Data presented are the average of four independent experiments ± S.D. DEX, dexamethasone; RIF, rifampicin.

GST-CAR, GST-PXR, or GST-GR was analyzed by GST pull-down experiments. As shown in Fig. 9A, full-length CAR bound to GST-GRIP1 in the presence of the CAR agonist CITCO more strongly than in the absence of CITCO. In contrast, full-length PXR bound markedly to GST-GRIP1 in the presence of rifampicin at the same level as in the absence of rifampicin, whereas full-length GRIP1 bound only slightly to GST-PXR. In addition, the binding of full-length GR to GST-GRIP1 was not enhanced by treatment with 10^{-7} M dexamethasone (Fig. 9A). No binding of CAR, PXR, GR, or GRIP1 to GST was observed (Fig. 9). Whereas interaction of CAR with GST-GRIP1 was abolished by the deletion of eight amino acids (AF-2 domain) from the C terminus of CAR (CAR-ΔC8), the binding to GST-GRIP1 of PXR-ΔN38 and PXR-ΔC9, which were deleted 38 amino acids (AF-1 domain) from the N terminus of PXR and nine amino acids (AF-2 domain) from the C terminus of PXR, was decreased to 23.8 ± 9.2 and 9.1 ± 1.2% of that of wild-type PXR (n = 3), respectively (Fig. 9, B and C).

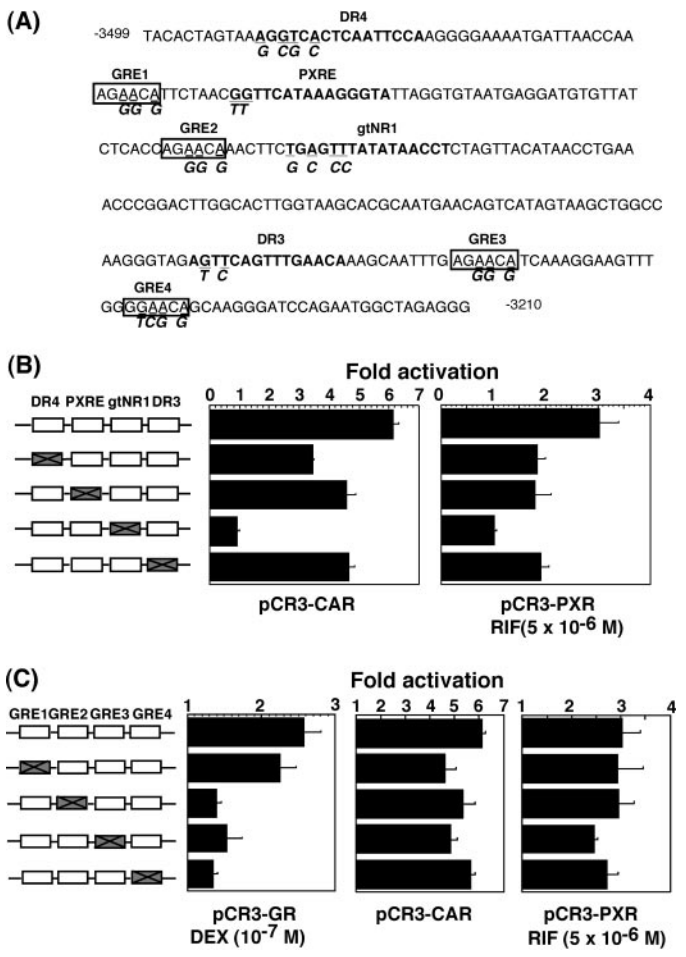

Fig. 5. Role of nuclear receptor binding elements and GRE half-sites in the transcriptional activation of the UGT1A1 290-bp distal enhancer module mediated by GR, CAR, and PXR. A, mutations of nuclear receptor binding elements and GRE half-sites, underlined, were introduced into the UGT1A1 290-bp distal enhancer module as described under *Materials and Methods*. Italicized characters show the bases for the mutation. B, each mutated DNA (indicated by the closed boxes with X) (0.2 μg) was cotransfected into HepG2 cells with pCR3-CAR or pCR3-PXR expression vector or the control vector pCR3 (0.2 μg), together with pRL-SV40 (0.2 μg) as described under *Materials and Methods*. C, GRE1-, GRE2-, GRE3-, or GRE4-mutated 290-bp reporter gene (0.2 μg) was cotransfected into HepG2 cells with the expression vector for GR, CAR, or PXR or the control vector pCR3 (0.2 μg), together with pRL-SV40 (0.2 μg) as described under *Materials and Methods*. The transfected cells were incubated for 24 h with vehicle (dimethyl sulfoxide) alone or dexamethasone (10^{-7} M) for GR-expressing cells, without ligand for CAR-expressing cells, or with the vehicle (dimethyl sulfoxide) alone or rifampicin (5×10^{-6} M) for PXR-expressing cells, harvested, and assayed for luciferase activity. Fold activation was calculated by dividing the activity with the inducer (dexamethasone or rifampicin) by that without the inducer for GR- and PXR-expressing cells, respectively, or by dividing the activity in the presence of CAR by that in the absence of CAR for CAR-expressing cells. Data presented are the average of four independent experiments ± S.D. DEX, dexamethasone; RIF, rifampicin.

Discussion

Reduced bilirubin glucuronosyltransferase (UGT1A1) activity is associated with the development of unconjugated hyperbilirubinemia (Crigler-Najjar syndrome and Gilbert's syndrome) and increased side effects of drug treatment, such as the predisposition of patients to SN-38-initiated toxicity (Tukey et al., 2002). UGT1A1 inducers can enhance glucuronosyltransferase activity against endogenous lipophilic compounds such as bilirubin and exogenous xenobiotics and dietary chemicals, facilitating their biotransformation to more water-soluble compounds. Glucocorticoids have been implicated in the regulation of specific genes, contributing to the modulation of many physiological actions such as metabolism, immune response, and behavior (Barnes, 1998; Malkoski and Dorin, 1999). Understanding the molecular mechanisms of the induction of this clinically important human UGT1A1, and of glucocorticoid action, may provide information for the prevention and treatment of unconjugated hyper-

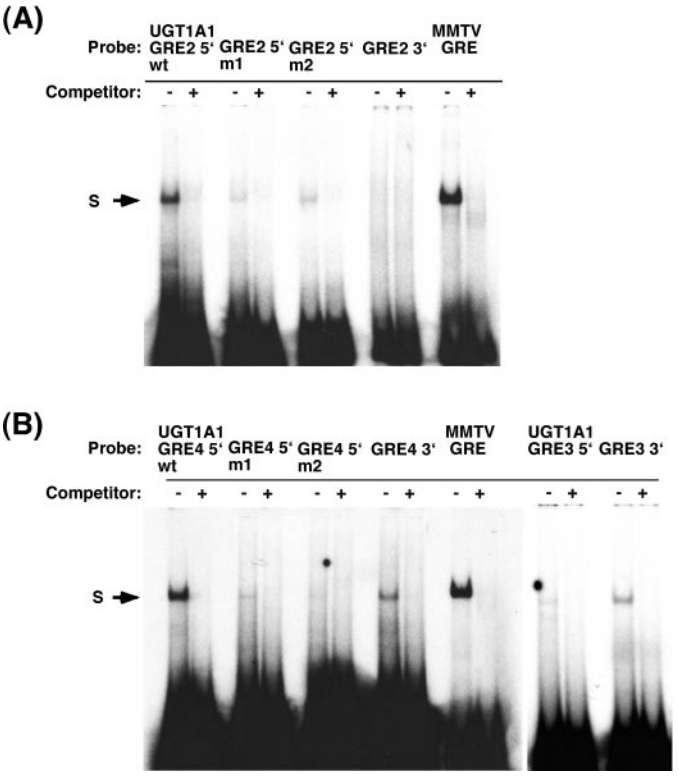

Fig. 6. DNA binding by GR. In vitro-transcribed and -translated GR was incubated with ^{32}P-labeled double-stranded GRE2 5′, GRE2 3′ (A), GRE3 5′, GRE3 3′, GRE4 5′, and GRE4 3′ (B) probes and electrophoresed in native polyacrylamide gels. MMTV GRE containing the palindromic consensus GRE from mouse mammary tumor virus was used as a control for binding to GR. Unlabeled competitor oligonucleotides were added at 100-fold molar excess.

bilirubinemia and the side effects of drug treatment, and moreover, may lead to the development of novel drugs targeting nuclear receptors and transcriptional cofactors to control the induction of drug-metabolizing enzymes and glucocorticoid action.

Xenobiotics that can induce human UGT1A1 include phenobarbital, rifampicin, clotrimazole (Sugatani et al., 2001, 2004), and dexamethasone (Figs. 1 and 2), and a similar spectrum of compounds can induce CYP2B6 (Sueyoshi et al., 1999), CYP2C9 (Gerbal-Chaloin et al., 2002), and CYP3A4 (Lehmann et al., 1998). Two nuclear orphan receptors, CAR and PXR, have a key role in phenobarbital- and rifampicin-mediated induction of the hepatic *CYP2B6*, *CYP2C9*, and *CYP3A4* genes (Pascussi et al., 2003). In our previous study (Sugatani et al., 2004), only a 290-bp fragment (−3499/−3210) in the 11-kbp 5′-flanking region of the *UGT1A1* gene displayed prominent PXR activation by rifampicin. Site-directed mutagenesis of gtNR1 abolished CAR- and PXR-mediated transcriptional activities in the reporter gene assay in HepG2 cells (Fig. 5B). Therefore, gtNR1 (−3382/−3367) in the 290-bp distal enhancer module (−3499/−3210) of the *UGT1A1* gene may play a central role in UGT1A1 induction by phenobarbital and rifampicin. On the other hand, even with the mutation of all four binding sites for CAR and PXR

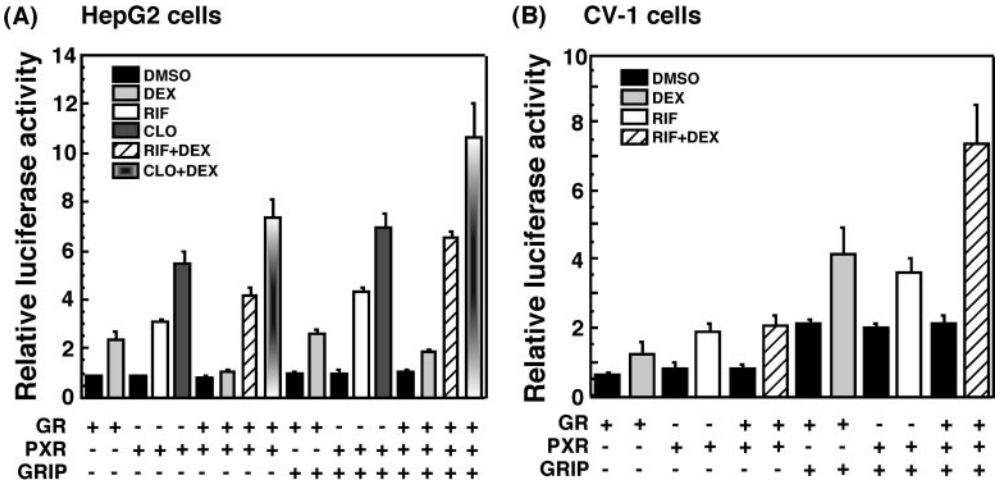

Fig. 7. Dexamethasone enhances PXR activator-induced transcriptional activity of the UGT1A1 290-bp promoter through the GR. HepG2 cells (A) and CV-1 cells (B) were transfected with pCR3-GR (0.2 μg), pCR3-PXR (0.2 μg), and/or pcDNA3-GRIP1 (0.6 μg), together with pRL-SV40 (0.2 μg, HepG2 cells) or pSV-β-galactosidase (0.6 μg, CV-1 cells) control vector, and the UGT1A1 290-bp reporter gene (0.2 μg). The transfected cells were treated with the vehicle (dimethyl sulfoxide), dexamethasone (10^{-7} M), rifampicin (5×10^{-6} M), dexamethasone (10^{-7} M) plus rifampicin (5×10^{-6} M), clotrimazole (5×10^{-6} M), or dexamethasone (10^{-7} M) plus clotrimazole (5×10^{-6} M) for 24 h, harvested, and assayed for luciferase activity. The values for firefly luciferase were normalized by dividing by the *R. reniformis* luciferase values (A) or by the β-galactosidase values (B). Relative luciferase activity level, measured in cells transfected with control pCR3 (0.4 μg) and pcDNA (0.6 μg) plasmids and the 290-bp reporter plasmid (0.2 μg) and treated with the vehicle, is expressed as 1. Data presented are the average of four independent experiments ± S.D. CLO, clotrimazole; DEX, dexamethasone; RIF, rifampicin.

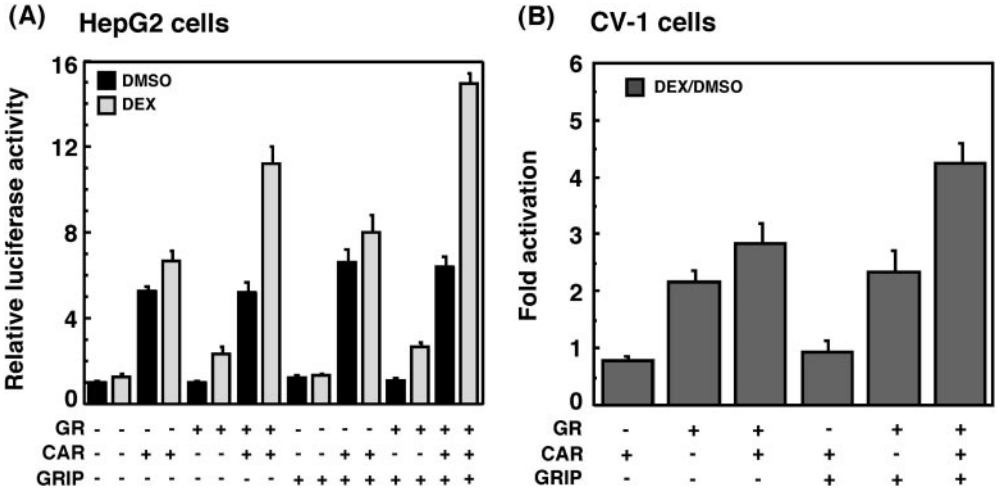

Fig. 8. Dexamethasone enhances CAR-mediated transcriptional activity of the UGT1A1 290-bp promoter through the GR. HepG2 cells (A) and CV-1 cells (B) were transfected with expression constructs for pCR3-GR (0.2 μg), pCR3-CAR (0.2 μg), and/or pcDNA3-GRIP1 (0.6 μg), together with pRL-SV40 (0.2 μg, HepG2 cells) or pSV-β-galactosidase (0.6 μg, CV-1 cells) control vector, and the UGT1A1 290-bp reporter gene (0.2 μg). The transfected cells were treated with the vehicle (dimethyl sulfoxide) alone or dexamethasone (10^{-7} M) for 24 h (HepG2 cells) or 48 h (CV-1 cells), harvested, and assayed for luciferase activity. The values for firefly luciferase were normalized by dividing by the *R. reniformis* luciferase values (A) or by the β-galactosidase values (B). A, relative luciferase activity level, measured in cells transfected with control pCR3 (0.4 μg) and pcDNA3 (0.6 μg) plasmids, and the 290-bp reporter plasmid (0.2 μg) and treated with the vehicle, is expressed as 1. B, fold activation was calculated by dividing the activity with dexamethasone by that without dexamethasone. Data presented are the average of four independent experiments ± S.D. DEX, dexamethasone.

(DR4, PXRE, gtNR1, and DR3 sites) in the 290-bp distal enhancer module, transcriptional activity in response to dexamethasone was preserved. Moreover, we demonstrated that dexamethasone at 10^{-7} M maximally elevates the level of UGT1A1 mRNA but does not affect those of PXR and CAR mRNAs (Fig. 1). In addition, 10^{-7} M dexamethasone synergistically enhanced the PXR activator-induced expression of *UGT1A1* gene in HepG2 cells (Fig. 1) and CAR- and PXR-mediated activation of UGT1A1 reporter gene was greatly enhanced by activated GR (Fig. 4). These results demonstrate that induction by dexamethasone of the human *UGT1A1* gene does not occur via an indirect pathway through the activation of CAR and PXR.

Mutations of the UGT1A1 290-bp distal enhancer module GRE half-sites GRE2 and GRE4 significantly decreased GR-mediated transcriptional activity of the UGT1A1 reporter gene by 10^{-7} M dexamethasone (Fig. 5C). In vitro-translated GR bound to probes containing UGT1A1 GRE2 and GRE4 (Fig. 6). Moreover, the binding of GR to UGT1A1 GRE2 5′ and UGT1A1 GRE4 5′ probes mutated in either the 5′ half-site or the 3′ half-site was markedly decreased (Fig. 6). Therefore, we identified the −3404/−3389 and −3251/−3236 elements in the regulatory region of the *UGT1A1* gene as GREs (Figs. 5 and 6). It is interesting that these elements are located in the 290-bp distal enhancer module that directs CAR/PXR-mediated transactivation of the *UGT1A1* gene. The UGT1A1 GREs have imperfect palindromic sequences composed of two half-sites separated by four nucleotides. The lower affinity of GR for UGT1A1 GREs in an electrophoretic mobility shift assay compared with the affinity for MMTV GRE (Fig. 6) may result from the change from consensus GRE (AGAACAxxxTGTTCT). These results show that like *CYP2C9* (Gerbal-Chaloin et al., 2002) and *CYP2C19* (Chen et al., 2003), *UGT1A1* is a glucocorticoid-responsive gene activated by direct binding of GR to GRE(s) in the regulatory region. This report is the first to describe the molecular mechanisms controlling *UGT1A1* expression in response to glucocorticoids.

Glucocorticoids exert their effects via direct or indirect regulation of the transactivation of target genes by GR. Recent studies have demonstrated that the molecular mechanism of induction by dexamethasone of the human *CYP3A4* gene is not based on direct interaction of GR with the human *CYP3A4* promoter, but on indirect pathways through increased PXR and RXR levels (Pascussi et al., 2000a) and through PXR-independent hepatocyte nuclear factor 3 and/or CCAAT/enhancer-binding protein α activation (El-Sankary et al., 2002). Gerbal-Chaloin et al. (2002) demonstrated that

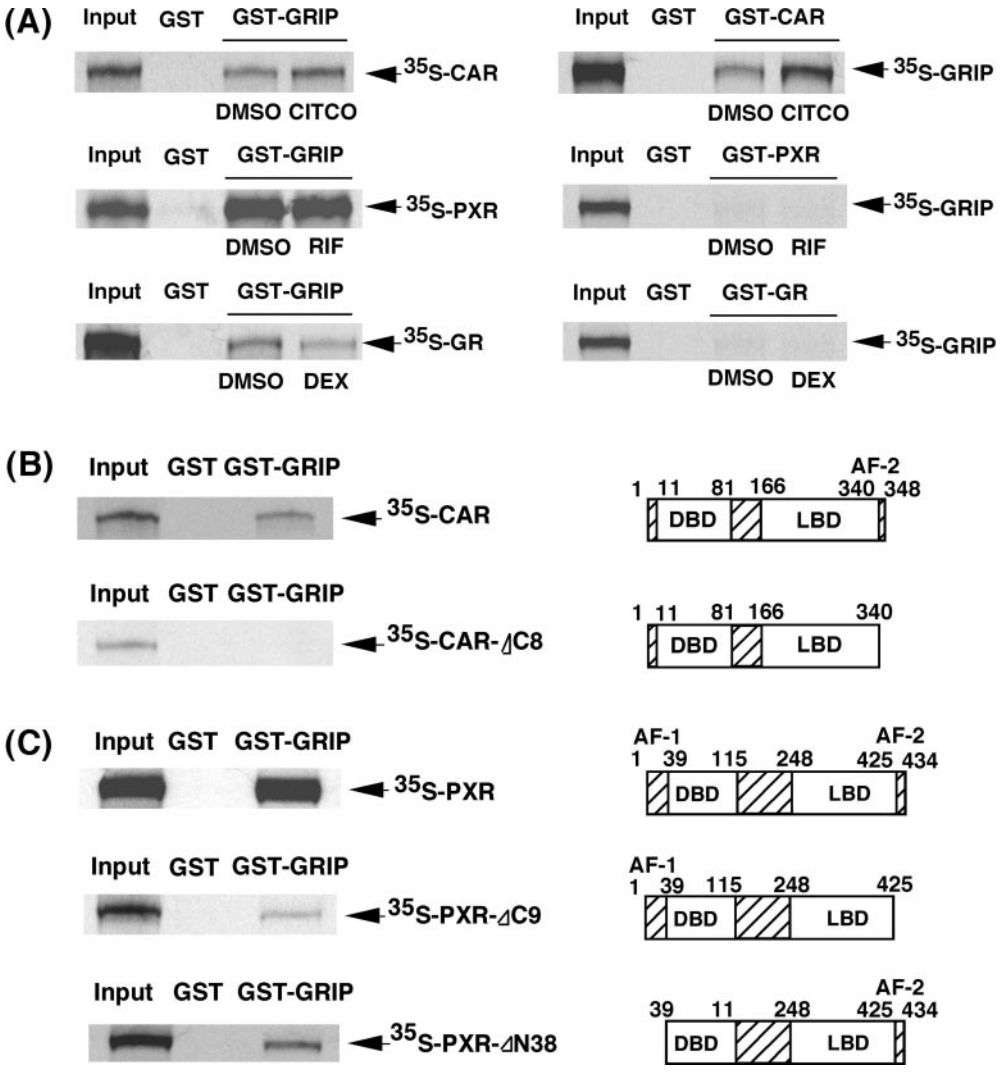

Fig. 9. Interaction of GRIP1 with CAR, PXR, and GR (A), CAR and its mutant (B), and PXR and its mutants (C) assessed by GST pull-down assays. Recombinant GST-GRIP1 or GST alone was produced in bacteria and immobilized on glutathione-Sepharose beads. Full-length GRIP1, CAR, PXR, or GR was produced by in vitro translation and labeled with [^{35}S]methionine. A, GST-fusion protein (GST-GRIP1, GST-CAR, GST-PXR, or GST-GR) or GST as a negative control for pull-down assays were incubated with the ^{35}S-labeled protein (CAR, PXR, GR, or GRIP1) in the presence or absence of ligands as indicated. B and C, CAR-ΔC8 and PXR-ΔC9 were generated by the deletion of eight and nine amino acids from the C terminus, respectively, and PXR-ΔN38 was generated by the deletion of 38 amino acids from the N terminus. ^{35}S-labeled in vitro-translated CAR and its mutant (B) and PXR and its mutants (C) were incubated with purified GST-GRIP1 fusion protein or GST as a negative control for pull-down assays. After washing, the labeled proteins bound to the GST-fusion proteins were analyzed by SDS-PAGE and visualized by autoradiography. The input lane contains 10% of the amount of the labeled protein used in each pull-down reaction. Signal intensities were determined with a Fujix BAS-2000 Bioimaging Analyzer. DEX, dexamethasone; DMSO, dimethyl sulfoxide; RIF, rifampicin.

a functional GRE (−1684/−1648) resides close to a CAR/PXR response element (−1818/−1803) in the regulatory region of human *CYP2C9*, and Chen et al. (2003) identified a functional GRE (−1676/−1662) separated by 126 bases from a CAR/PXR response element (−1891/−1876) of human *CYP2C19*. We demonstrated here that functional GREs (−3404/−3389 and −3251/−3236) also reside close to CAR/PXR response elements in the 290-bp regulatory region (−3499/−3210) of human *UGT1A1* (Sugatani et al., 2001; Xie et al., 2003). Stoltz et al. (1998) reported that the *CYP2B2* phenobarbital response unit (−2317/−2155) contains a putative glucocorticoid response element essential for conferring maximal phenobarbital responsiveness. There may be functional cross-talk between GR and CAR/PXR that is mediated by glucocorticoids at physiological (submicromolar) concentrations and by CAR/PXR activators.

In this study, we demonstrated that GR activity can be modulated by the action of other nuclear receptors, CAR and PXR, and by the transcriptional coactivator GRIP1, and vice versa. Synergistic transactivation by CAR/PXR and activated GR occurred in the presence of sufficient amounts of GRIP1 (Figs. 7 and 8). However, the mode of CAR interaction with GRIP1 may differ from that of PXR, because PXR is a ligand-dependent nuclear receptor, with rifampicin as one of its ligands, and CAR is a ligand-independent receptor. Phenobarbital, which induces *CYP2B* genes through a CAR-dependent mechanism, is believed to cause translocation of CAR to the nucleus. The interaction of GRIP1 with CAR was enhanced by CITCO, which induces nuclear translocation of CAR (Maglich et al., 2003), whereas the interaction with PXR and GR was not affected by agonists such as rifampicin and dexamethasone (Fig. 9A). PXR is structurally characterized by three distinct domains: an N-terminal transcriptional activation domain (AF-1), a DNA-binding domain, and a C-terminal ligand-binding domain, whereas CAR contains the DNA-binding domain and LBD domains but no AF-1 domain. Consistent with previous reports that the binding of CAR to GRIP1 is based on the AF-2 domain within the ligand-binding domain of CAR (Min et al., 2002a,b), the deletion of eight amino acids from the C terminus of CAR, the AF-2 domain, abolished the interaction with GRIP1 (Fig. 9). In contrast, the deletion of nine amino acids from the C terminus of PXR, the AF-2 domain, decreased the interaction with GRIP1 to 9.1% that of the wild type (Fig. 9). In addition, the deletion of 38 amino acids from the N terminus of PXR, that is, the AF-1 domain, also decreased the interaction with GRIP1 to 23.8% that of the wild type (Fig. 9). These results show that GRIP1 can interact with the AF-1 domain of PXR, but the binding of GRIP1 to the AF-2 domain seems stronger than that to the AF-1 domain. The difference of ligand responsiveness for GRIP1 binding may result from the differences in the mode of GRIP1 binding between CAR and PXR.

A significant question is whether induction of UGT1A1 expression by glucocorticoids is associated with pathophysiological actions. Human bilirubin UDP-glucuronosyltransferase activity in livers 1 to 10 days after birth is low at less than 10% of that of adults (Coughtrie et al., 1988). Recent study suggests that a functional deficit of CAR activity in neonatal human liver may account for low levels of bilirubin UDP-glucuronosyltransferase activity, which is associated with physiological neonatal jaundice, together with an increased rate of erythrocyte turnover (Huang et al., 2003). Braun et al. (1998) have reported that transcriptional induction of UGT1A1 was observed in diabetic rats and starved rats. Because the normal/metabolic alterations observed in diabetes and starvation are considered comparable with the postnatal metabolic state, Braun et al. (1998) have further suggested that the sudden interruption of maternal glucose supply could signal enhanced expression of UGT1A1 in newborn infants. However, it was previously shown that short-term starvation (72-h fasting) induces significant increases in the 24-h mean cortisol concentration in healthy men (Chan et al., 2003). Thus, the results described in the present study suggest that glucocorticoids could signal enhanced expression of UGT1A1 in response to increased demand in newborn infants to defend or suppress icterus neonatorum, via GR activation or GR enhancement of PXR- or decreased-neonatal CAR-mediated UGT1A1 regulation, or may maintain the plasma/serum bilirubin level within physiological range.

In conclusion, inducers of UGT1A1 such as dexamethasone and hydrocortisone at submicromolar concentrations and rifampicin act via the GR and the PXR, respectively. The present study shows that GRIP1 modulates CAR/PXR-mediated transactivation of the UGT1A1 gene by affecting a functional cross-talk between GR and CAR/PXR and enhancing the dexamethasone action. We are in progress to study whether the functional cross-talk of GR with other nuclear receptors is associated with physiological action.

Acknowledgments

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