Bile Acids Up-regulate Death Receptor 5/TRAIL-receptor 2 Expression via a c-Jun N-terminal Kinase-dependent Pathway Involving Sp1*

Received for publication, August 26, 2003, and in revised form, September 22, 2003
Published, JBC Papers in Press, October 14, 2003, DOI 10.1074/jbc.M309476200

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Bile acids up-regulate death receptor 5 (DR5)/TRAIL-receptor 2 (TRAIL-R2) expression thereby sensitizing hepatocytes to TRAIL-mediated apoptosis. However, the precise mechanism by which bile acids enhance DR5/TRAIL-R2 expression is unknown. Although several bile acids enhanced DR5/TRAIL-R2 expression, deoxycholic acid (DCA) was the most potent. DCA stimulated JNK activation and the JNK inhibitor SP600125 blocked DCA-induced DR5/TRAIL-R2 mRNA and protein expression. Reporter gene analysis identified a 5′-flanking region containing two Sp1 binding sites within the DR5/TRAIL-R2 promoter as bile acid responsive. Sp1 binding to one of the two sites was enhanced by DCA treatment as evaluated by electrophoretic mobility shift assays and chromatin immunoprecipitation studies. JNK inhibition with SP600125 also blocked binding of Sp1 to the DR5/TRAIL-R2 promoter. Finally, point mutations of the Sp1 binding site attenuated promoter activity. In conclusion, Sp1 is a bile acid-responsive transcription factor that mediates DR5/TRAIL-R2 gene expression downstream of JNK.

In virtually all human liver diseases, hepatocytes undergo cell death by apoptosis (1, 2). This is also true for cholestatic liver diseases, pathophysiologic syndromes characterized by impaired hepatocellular secretion of bile acids into bile. In cholestasis, the intracellular accumulation of toxic bile acids within hepatocytes promotes cellular injury and the subsequent development of hepatic cirrhosis and liver failure (3). Numerous studies have shown that bile acids mediate their cytotoxicity by inducing hepatocellular apoptosis (4–6).

Bile acid-mediated apoptosis, in part, is likely regulated by their ability to markedly alter gene expression (7–11). These amphipathic molecules appear to alter gene transcription by one of two major pathways: (i) activation of nuclear hormone receptors and (ii) stimulation of mitogen-activated protein kinase (MAPK) pathways (5, 7, 12–18). Bile acids serve as ligands for the nuclear hormone receptors farnosid X receptor (FXR) (12–14), pregnane X receptor/steroid, and xenobiotic receptor (15, 16) and vitamin D receptor (17), which regulate genes involved in bile acid conjugation, transport proteins, intracellular binding proteins, and enzymes critical for xenobiotic biotransformation. However, these genes appear to serve in a cytoprotective capacity, and, indeed, an FXR agonist ameliorates cholestatic liver injury (19, 20). MAPK activation by specific bile acids, in contrast, can mediate cytotoxic signaling cascades (5, 7, 18, 21). At present three major groups of MAPKs are known: the classic p42/44 MAPK or extracellular receptor kinase 1 and 2 (ERK1 and 2), c-Jun N-terminal kinase (JNK1/2), and p38 MAPK. The p42/44 MAPKs are mainly involved in cell survival and proliferation pathways, and their inhibition potentiates bile acid-induced apoptosis in hepatocytes (5, 22).

Bile acid-triggered apoptosis involves death receptor activation. Consistent with their known effects on gene expression, bile acids stimulate transcription of the death receptor 5/tumor necrosis factor-related, apoptosis-inducing ligand-receptor 2 (DR5/TRAIL-R2) expression and aggregation, promoting a death receptor-dependent apoptosis (10). The importance of bile acid-mediated DR5/TRAIL-R2 induction has been amply demonstrated by in vitro and in vivo observations (10, 24). DR5/TRAIL-R2 is a highly inducible receptor whose expression is regulated by both p53-dependent and -independent pathways (10, 25–28). In previous studies examining bile acid-mediated DR5/TRAIL-R2 expression and cell death, a cell line with a defective p53 mutant was utilized, thereby, excluding a role for p53 in bile acid-DR5/TRAIL-R2 expression (10). An FXR agonist also failed to enhance expression of this death receptor. The observations make it likely that bile acid enhances DR5/TRAIL-R2 expression by a MAPK signaling pathway.

The overall objective of this study was to test the hypothesis that bile acid enhances DR5/TRAIL-R2 transcription by a MAPK-dependent process. To address this hypothesis, the following two questions were formulated: (i) Do MAPK inhibitors block muscular mobility shift assay; ERK, extracellular receptor kinase; FXR, farnosid X receptor; GC/DCA, glycochenodeoxycholic acid; GDCA, glycochenodeoxycholic acid; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor-κB; Sp1, specificity protein 1; Sp1 binding site 1; Sp1 binding site 2; TCDA, taurochenodeoxycholic acid; TDCa, taurodeoxycholic acid; DR5, death receptor 5; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TRAIL-R2, TRAIL receptor 2; UDCA, ursodeoxycholic acid; cons., consensus sequence; CMV, cytomegalovirus.

This paper is available on line at http://www.jbc.org

*This work was supported by Grant DK41876 from the National Institutes of Health, and the Mayo Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: MAPK, mitogen-activated protein kinase; AP-1, activator protein-1; ATF, activating transcription factor; CDCA, chenodeoxycholic acid; ChIP, chromatin immunoprecipitation; CREB, cyclic AMP-responsive element binding protein; DCA, deoxycholic acid; DrR, death receptor; EMSA, electrophoretic mobility shift assay; ERK, extracellular receptor kinase; FXR, farnosid X receptor; GC/DCA, glycochenodeoxycholic acid; GDCA, glycochenodeoxycholic acid; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor-κB; Sp1, specificity protein 1; Sp1 binding site 1; Sp1 binding site 2; TCDA, taurochenodeoxycholic acid; TDCa, taurodeoxycholic acid; DR5, death receptor 5; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TRAIL-R2, TRAIL receptor 2; UDCA, ursodeoxycholic acid; cons., consensus sequence; CMV, cytomegalovirus.
bile acid-mediated DR5/TRAIL-R2 transcription? (ii) What is the transcription factor responsible for bile acid-associated DR5/TRAIL-R2 expression? The results indicate that DCA-mediated JNK1/2 activation contributes to DR5/TRAIL-R2 expression. The DCA-stimulated JNK1/2 pathway was found to be associated with the specificity protein 1 (Sp1) transcription factor. Thus, Sp1 appears to be a bile acid-responsive transcription factor that contributes to DR5/TRAIL-R2 gene expression downstream of JNK1/2.

**EXPERIMENTAL PROCEDURES**

**Materials**—Reagents were purchased from the following suppliers: DCA, GDCA, TDCA, CDCA, GCDCa, TCDCa, UDCA, and anti-FLAG M2 mouse monoclonal antibody (IgG1) were obtained from Sigma Chemicals Co. (St. Louis, MO); 4′,6-diamidino-2-phenylindole was from Molecular Probes Inc. (Eugene, OR); the JNK1/2 inhibitor Sp600125 was from BIO/MOL Research Laboratories (Plymouth Meeting, PA); the p42/44 MAPK inhibitor PD98059 and the p38 MAPK inhibitor SB203580 were from Calbiochem (San Diego, CA); horseradish peroxidase-conjugated anti-goat Igs and rabbit Igs were from BIOSOURCE (Camarillo, CA); protein G-Sepharose was from Zymed Laboratories, Inc. (San Francisco, CA).

**Cell Culture**—HuH-7 cells, a human hepatocellular carcinoma cell line stably transfected with the sodium-dependent taurocholate co-transporting polypeptide, were employed for this study (10, 29). Established clones (HuH-BAT for HuH-bile acid transporting) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100,000 units/liter), streptomycin (100 mg/liter), gentamicin (100 mg/liter), and G418 (1200 mg/liter).

**Immunoblot Analysis**—Immunoblot analyses were conducted as previously described in detail (10). The 12.5% SDS-PAGE, transferred to nitrocellulose, and blotted with appropriate primary antibodies at 4 °C. Polyvinylidene difluoride, and blotted with appropriate primary antibodies at dilution of 1:1,000. Horseradish peroxidase-conjugated secondary antibodies (BIO/SOURCE International, Camarillo, CA) were incubated at a dilution of 1:2,000 to 1:10,000. Bound antibody was visualized using a chemiluminescent substrate (ECL, Amersham Biosciences, Arlington Heights, IL) and exposed to Kodak X-Omat film.

**Quantitative Real-time PCR**—Total RNA was isolated from HuH-BAT cells using TRIzol reagent (Invitrogen, Carlsbad, CA), and cDNA was prepared using an random primer and murine leukemia virus reverse transcriptase as previously described in detail (10). The cDNA product was amplified by PCR with Taq DNA polymerase using standard protocols (10). PCR primers for human TRAIL-R2/DR5 were as follows: forward 5′-TGG AGT CTT GAT TGG-3′ and reverse 5′-GCA CCA CTG AGT CCA-3′.

**Chromatin Immunoprecipitation**—ChIP was performed using the Upstate Cell Signaling Solutions, Waltham, MA) according to the manufacturer's instruction. Briefly, cells were incubated in methanol (final concentration, 1%) at room temperature for 10 min, to obtain a cross-linking of DNA and DNA-bound proteins. After washing the cells twice with phosphate-buffered saline, cells were collected and lysed within 200 μl of the SDS lysis buffer provided by the kit. The cell extracts were sonicated to shear the DNA into 200- to 1,000-bp fragments, and centrifuged at 12,000 rpm for 10 min. The DNA concentration was estimated using the OD 260 nm. 100 μg of DNA were diluted with the ChIP dilution buffer obtained from the kit, to a final volume of 1,000 μl. 50 μl of each sample was used for non-immunoprecipitation control experiments (e.g., samples not treated with antisera).

**Reporter Plasmid Construction**—The plasmid pGL2-DR5/TRAIL-R2-Full containing 1.6 kb upstream of the ATG site through intron 2 in human DR5/TRAIL-R2 genomic DNA has been described previously, and was a kind gift from El-Dayer and co-workers (University of Pennsylvania) (10, 26). The firefly luciferase-based reporter plasmids, pGL3-basic and pGL3-promoter, containing 195 bp of the SV40 minimal early promoter (pGL3-SV), were obtained from Promega. The 5′-flanking lesion (corresponding position at −1220/−2, numbers from the ATG site) and the downstream lesion containing exon 1 through intron 1 (nucleotide position at −1/416) were amplified by PCR using primers: forward 5′-GTC CAT CTT CGC GAA GCT TTC ACT CCT GAG CCA GTT-3′/reverse 5′-GCA CTC AGA TGT CCG GTA GGA GCT CCT TTG ATA G-3′ for −1220/−2, and −5′/AC CAT CTT CGC GAA GCT CAT GGA ACG GGC GAA GCA G-3′ and −5′/GCA CTC AGA TGT CCG GTA GGA GCT CCT TTG ATA G-3′ for −1/416 lesion. The PCR fragment was purified by 1% agarose gel electrophoresis and gel extraction and digested with Nhel and BglIII. The −1232/-2 fragment was subcloned into the pGL3-basic vector between Nhel and BglIII sites to make pGL3-5′-1223. The −1/416 fragment was subcloned into the pGL3-basic vector or pGL3-SV vector to make pGL3-Int1-416 and pGL3-SV-Int1-416, respectively. Deletion mutants of the pGL3-5′-1229 were generated by using an Erase-Base system (Promega) according to the manufacturer's instruction. Point mutations were introduced to the pGL3-5′-1220 by PCR-based site-directed mutagenesis technology using the primers: 5′-GGA TCT TGT TCG CAA AGT TCC GAA TGA TCC GGC C-3′ and 5′-GCC GTC ATT CCG CAG AAT TGC CAC ATC C-3′ for Sp1 binding site 1, and 5′-GAA AGT ACA GCC GCA AGA CCA ACG GGC TGT ACT TTG-3′ and 5′-CAG GAC TTC GAG TCG TAC CAC CTC AGC GGC TGT ACT TTC-3′ for Sp1 binding site 2. The sequences of all constructs were confirmed by nucleotide-sequence analysis.

**Reporter Gene Assay**—HuH-BAT cells cultured in 24-well plates were co-transfected with 5 ng of TR-Renilla-CMV and 250 ng of firefly luciferase-based reporter plasmids described above. Twelve hours after the transfection, cells were incubated with bile acids or media (control) for 8 h, and then cell lysates were prepared as previously described (10). Both firefly and Renilla luciferase activities were quantitated using the dual luciferase reporter assays system (Promega) according to the manufacturer's instructions, and firefly luciferase activity was normalized using Renilla luciferase activity.

**Intracellular Cholesterol Mobility Assay**—Nuclear protein extracts were prepared from the HuH-BAT cells using high salt extraction as described previously (30). The buffer composition of the final nuclear extract was 20 mM HEPES, pH 7.9, 20% glycerol, 140 mM NaCl, 16 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonil fluoride, and 1% concentration of the protase inhibitor mix (Roche Applied Science).

Single-stranded complimentary synthetic oligonucleotides were annealed and end-labeled with [γ-32P]ATP (3000 Ci/mmol) and T4 polynucleotide kinase. Six microliters of nuclear extract containing up to 6 μg of protein was incubated on ice in binding buffer (100 mM HEPES, 300 mM KCl, 20% Ficoll, 0.02% Nonidet P-40, 0.5 mg/ml bovine serum albumin, and 0.2 mg/ml gelatin) for 10 min at 4 °C. The gel filtration was initiated by addition of 20,000 cpm of labeled oligonucleotide probe and continued for 15 min at room temperature. Antibodies for supershifting or competing double-stranded oligonucleotides were added to the binding buffer 15 min prior to the addition of labeled oligonucleotides. Samples were loaded onto 4% or 5% polyacrylamide gel containing 0.5× Tris borate/EDTA. The gel was dried and exposed to BioMax MR films at −70 °C.

Double-stranded oligonucleotide probes and/or specific competitors used for EMSA were: AP-1 consensus sequence (AP-1 cons.); 5′-GCC TCG AGT ACG ACG GAA G-3′; CREB/ATF cons., 5′-AGA GAT AGC GAC GCA GGG C-3′; Sp1 cons., 5′-ATG TCT GAG TA ACC-3′; 5′-GCC GCC GAC-3′; Sp1 binding site 1; 5′-GCC TCG TGG CG CTG GCC GAC-3′; 5′- GCC GGC GTA-3′; and 5′-GAC TGG GCC GGC GAC-3′.

**Chromatin Immunoprecipitation—ChIP** was performed using the ChIP assay kit (Upstate Cell Signaling Solutions, Waltham, MA) according to the manufacturer's instruction. Briefly, cells were incubated in methanol (final concentration, 1%) at room temperature for 10 min, to obtain a cross-linking of DNA and DNA-bound proteins. After washing the cells twice with phosphate-buffered saline, cells were collected and lysed within 200 μl of the SDS lysis buffer provided by the kit. The cell extracts were sonicated to shear the DNA into 200- to 1,000-bp fragments, and centrifuged at 12,000 rpm for 10 min. The DNA concentration was estimated using the OD 260 nm. 100 μg of DNA were diluted with the ChIP dilution buffer obtained from the kit, to a final volume of 1,000 μl. 50 μl of each sample was used for non-immunoprecipitation control experiments (e.g., samples not treated with antisera).
experimental procedures.

RESULTS

Bile Acids Up-regulate DR5/TRAIL-R2 Expression—Initially, we screened several bile acids for their effects on DR5/TRAIL-R2 induction to ascertain which bile acids were the most potent in augmenting expression of this death receptor. Among the bile acids tested (100 \( \mu \)M), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), and the glycine-conjugated form of deoxycholic acid (GDCA) were the most effective in up-regulating DR5/TRAIL-R2 protein (Fig. 1A) and mRNA expression (Fig. 1B). Interestingly, the hydrophilic, cytoprotective bile acid ursodeoxycholic acid (UDCA) did not significantly enhance DR5/TRAIL-R2 expression. In contrast to their effects on DR5/TRAIL-R2 expression, bile acids altered neither DR4, DcR1, nor DcR2 protein expression. Thus, bile acid-mediated TRAIL-related receptor up-regulation is DR5/TRAIL-R2-specific. Because DCA was the most potent in inducing DR5/TRAIL-R2 expression, we choose this bile acid for the remainder of our studies.

Next, the concentration-dependent response for DR5/TRAIL-R2 up-regulation by DCA was examined. DCA up-regulates DR5/TRAIL-R2 mRNA and protein level in a concentration-dependent manner with concentrations \( > 150 \mu \)M sufficient to induce maximum DR5/TRAIL-R2 expression (Fig. 1C and D). In contrast, DCA, at all concentrations tested, did not alter expression of the related DR4/TRAIL-R1 receptor. Thus, DCA selectively up-regulates DR5/TRAIL-R2 in a concentration-dependent manner.

Bile Acid-mediated DR5/TRAIL-R2 Induction Is JNK-dependent—Several mitogen-activated protein kinases (MAPKs) are activated by DCA (5, 18, 31) and can modulate gene expression. Therefore, we formulated the hypothesis that bile acid-activated MAPK activity was responsible for DR5/TRAIL-R2 gene induction by DCA. To test this hypothesis, several different kinase inhibitors, including the \( p42/44 \) MAPK inhibitor PD98059, the \( p38 \) MAPK inhibitor SB203580, and the JNK1/2 inhibitor SP600125 were screened for the effects on DR5/TRAIL-R2 protein expression. Among the inhibitors tested, the JNK1/2 inhibitor SP600125 completely abrogated DCA-mediated TRAIL-R2 up-regulation by DCA.

copy numbers. DCA, deoxycholic acid; GDCA, glycochenodeoxycholic acid; TDCDA, taurochenodeoxycholic acid; CDCA, chenodeoxycholic acid; GCDCA, glycochenodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; and UDCA, ursodeoxycholic acid.
with or without DCA (100 
HuH-BAT cells were incubated 
/H9262 gene transcription is regulated by the transcription factors p53 
and p38 MAPK inhibitors failed to block DR5/TRAIL-R2 induc-
tion (Fig. 2
A). Finally, we confirmed DCA-mediated JNK1/2 activation in HuH-BAT cells as demonstrated by an increase in phospho-JNK1/2 (Fig. 2C). These data provide a link between bile acid-induced signaling cascade and DR5/TRAIL-R2 gene induction.

**JNK and DR5 Induction**

**A**

| DCA | PD098059 | SB203580 | SP600125 |
|-----|----------|----------|----------|
| -   | +        | -        | +        |
| -   | -        | +        | -        |
| +   | -        | +        | -        |

**B**

![Graph showing DR5 mRNA expression](image)

**C**

![Graph showing phospho-JNK1/2 and total JNK1/2 expression](image)

**Fig. 2.** The JNK1/2 inhibitor SP600125 attenuates DCA-mediated DR5/TRAIL-R2 up-regulation. HuH-BAT cells were incubated with or without DCA (100 
µM) in the presence or absence of either the p42/44 MAPK inhibitor PD098059 (20 
µM), the p38 MAPK inhibitor SB203580 (20 
µM), or the JNK1/2 inhibitor SP600125 (20 
µM). Immunoblot analysis was performed to identify DR5/TRAIL-R2 protein levels (A) and JNK1/2 phosphorylation (C). Real-time PCR was performed to quantitate DR5/TRAIL-R2 mRNA levels (B). Data were expressed as mean ± S.D. from three individual experiments. p < 0.01 for DCA-treated group versus either Control, SP alone, or DCA plus SP (B).

induced DR5/TRAIL-R2 protein expression, whereas the p42/44 and p38 MAPK inhibitors failed to block DR5/TRAIL-R2 induction (Fig. 2A). The JNK1/2 inhibitor SP600125 also inhibited DCA-induced DR5/TRAIL-R2 mRNA induction as evaluated by real-time PCR (Fig. 2B). Finally, we confirmed DCA-mediated JNK1/2 activation in HuH-BAT cells as demonstrated by an increase in phospho-JNK1/2 (Fig. 2C). These data provide a link between bile acid-induced signaling cascade and DR5/TRAIL-R2 gene induction.

**JCA Enhances DR5/TRAIL-R2 Promoter Activity via the 5'-Flanking Region**—Current data indicate that DR5/TRAIL-R2 gene transcription is regulated by the transcription factors p53 (25, 26) and NF-κB (32, 33). These specific transcription factors bind to specific DNA sequences located within the DR5/TRAIL-R2 gene locus at the intronic promoter region (26, 32). In addition, the importance of the 5'-flanking promoter region of DR5/TRAIL-R2 gene in regulating its transcriptional activity has also been suggested by other studies (10, 34). Therefore, we first determined whether the 5'-flanking or the intronic promoter regions were responsible for bile acid-mediated DR5/TRAIL-R2 gene transcription. As shown in Fig. 3A, we compared different reporter constructs: pGL2-DR5/TRAIL-R2-full, which contains 1.6 kb upstream of the ATG site through intron 2 in the human DR5/TRAIL-R2 genomic locus; pGL3-5'-1220, which contains 1220 bp of the 5'-flanking region; pGL3-Int1+416, which contains exon 1 and part of intron 1, including both p53 and NF-κB binding sites; and pGL3-Int1-SV, which contains the same sequences of pGL3-Int1 upstream of SV40 minimal early promoter. DCA strongly enhanced the promoter activity of pGL3-5'-1220 containing the 5'-flanking region (12-fold elevation). In contrast, minimal activation by DCA was observed with the pGL2-DR5/TRAIL-R2-full, pGL3-Int1+416, or pGL3-Int1+416-SV constructs containing the intronic promoter region with the p53 and NF-κB binding sites. These results demonstrate that DCA enhancement of DR5/TRAIL-R2 promoter activity is dependent upon its 5'-flanking region. The fact that DCA-mediated DR5/TRAIL-R2 induction did not require the p53 and NF-κB binding regions is supported by additional information. First, p53 is mutated and not functional in this cell line. Second, although bile acids may activate NF-κB, adenovirus-mediated transduction with the I-κB super-repressor failed to attenuate DCA-mediated DR5/TRAIL-R2 induction (data not shown). This information further strengthens the relationship between bile acid-mediated DR5/TRAIL-R2 induction and its 5'-flanking region.

Using the pGL3-5'-1220 constructs, we further observed the effects of various bile acids on the promoter activity (Fig. 3B). Consistent with their effects on DR5/TRAIL-R2 protein and mRNA expression, significant activation of luciferase activity was also observed with the unconjugated or glycine-conjugated forms of bile acids (DCA, GDCA, CDCA., and GCDCA) (Fig. 3B). Importantly, the JNK1/2 inhibitor SP600125 blocked DCA-induced DR5/TRAIL-R2 promoter activity (Fig. 3C). Thus, the bile acid DCA enhances DR5/TRAIL-R2 expression at a transcription level by a JNK1/2-dependent mechanism.

**DCA Enhances Sp1-DNA Binding to the DR5/TRAIL-R2 Promoter**—Next, we further evaluated the bile acid-responsive region in the DR5/TRAIL-R2 promoter by a luciferase reporter gene analysis using various deletion mutants of the pGL3-5'-1220 plasmid. Partial deletion of the DR5/TRAIL-R2 promoter 5'-flanking region upstream of −531 did not reduce DCA-mediated luciferase activity. In contrast, 5'-deletion at −240 significantly reduced luciferase activity (Fig. 4A), suggesting that the nucleotide region downstream of −531 contains bile acid response elements. By more detailed deletion analysis of this region, we observed that deletions of the −509/−437 and −243/−137 regions markedly reduced DR5/TRAIL-R2 promoter activity (Fig. 4B).

Next we performed an electrophoretic mobility shift assay using the probes containing the above base pair regions for the 5'-flanking region of the DR5/TRAIL-R2 open reading frame. Fig. 5A illustrates the location of potential transcription factor binding sites for the DR5/TRAIL-R2 promoter, including the −509/−437 and −243/−137 regions. The −509/−437 bp region contains two c-Myb binding sites, and the −243/−137 region contains two Sp1 binding sites as previously described (34). In addition, we noted that both the −509/−437 and −243/−137 regions contain TGACG sequences, which are the core binding elements for the JNK1/2-inducible transcription factors AP-1 and/or CREB/ATF (the computed score for these regions using the TRANSFAC MatInspector search were as follows: −449/−428: core similarity 1.000 and matrix similarity 0.831 for AP-1, core similarity 1.000 and matrix similarity 0.918 for CREB/ATF; −191/−170: core similarity 1.000 and matrix simi-
Figure 3. The DR5/TRAIL-R2 5'-flanking region is responsible for DCA-mediated promoter activation. A, the composition of the DR5/TRAIL-R2 gene locus and the reporter gene constructs used in these studies is schematically illustrated. HuH-BAT cells were transiently transfected with each plasmid as described under “Experimental Procedures.” Twenty-four hours after transfection, cells were further incubated in the presence or absence of DCA (100 μM) for an additional 12 h. Luciferase activity within the HuH-BAT cells was measured using a dual luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity. The 5'-flanking region of the DR5/TRAIL-R2 promoter (pGL3-5'-1220) contributed to DCA activation of luciferase activity. B, HuH-BAT cells transfected with the pGL3-5'-1220 plasmid were incubated with the indicated bile acids (100 μM) for 12 h. C, HuH-BAT cells transfected with the pGL3-5'-1220 plasmid were incubated with the indicated concentrations of DCA in the presence or absence of SP600125 (20 μM). A–C, all data were expressed as mean ± S.D. from at least three individual experiments.
FIG. 5. DCA enhances Sp1-DNA binding to specific DR5/TRAIL-R2 promoter sequences. A, potential Sp1 transcription binding sites located within the DR5/TRAIL-R2 promoter region −510/−411 and −235/−136 were used as probes. Nucleotide sequences for each oligonucleotide-DNA used as either an EMSA probe or in competition studies are shown. Sp1 cons., Sp1 consensus sequence; Sp1 BS1, Sp1 binding site 1; Sp1 BS2, Sp1 binding site 2; AP-1 cons., AP-1 consensus sequence; CREB/ATF cons., CREB/ATF consensus sequence. B, nuclear protein extracts were prepared from HuH-BAT cells incubated with or without DCA (100 μM) for 6 h, as described under "Experimental Procedure." EMSA was performed using [γ-32P]ATP-labeled double-stranded DNA probes (−510/−411 (left) and −235/−136 (right)). Lane 1, free probe; lanes 2 and 3, nuclear protein from control cells (3 and 6 μg within 15 μl of DNA/protein binding reaction); lanes 4 and 5, nuclear protein from DCA-treated cells (3 and 6 μg); lane 6, nuclear protein from DCA-treated cells (6 μg) plus unlabeled DNA competitor (100-fold of labeled probe). C, EMSA was performed using −235/−136 as a probe. Lanes 1 and 8, free probe; lanes 2 and 9, nuclear protein from control cells (6 μg); lanes 3–7 and 10–17, nuclear protein from DCA-treated cells (6 μg); lanes 4–6 and 11–16; either Sp1 cons., Sp1 BS1, or Sp1 BS2 unlabeled competitors (10-, 50-, and 100-fold) were added to the reaction; lanes 7 and 17, unlabeled DNA of −235/−136 (100-fold) was added in the reaction. D, supershift paradigms were performed using anti-Sp1 antisera. Lane 1, free probe; lane 2, nuclear protein from control cells (6 μg); lanes 3–7, nuclear protein from DCA-treated cells (6 μg); lanes 4–6, 0.5, 1.0, and 2.0 μg of anti-Sp1 antisera was added to the reaction; lane 7, 100-fold of unlabeled DNA competitor (−235/−136). E, lane 1, nuclear protein from control cells (6 μg); lanes 2–5, nuclear protein from DCA-treated cells (6 μg); lanes 3–5, either −235/−136, AP-1 cons., or CREB/ATF cons. unlabeled competitors (100-fold) were added in the reaction.
Sp1-related (Fig. 5, C and D). The formation of DNA-protein complexes C1, C2, and C3 was completely abolished by 10-, 50-, and 100-fold amounts of competitor oligonucleotide-DNA containing Sp1 consensus binding sequences (Sp1 cons., Fig. 5C, left). Consistently, incubation with another competitor Sp1 BS1 (containing Sp1 binding site 1 at −198/−189) also abrogated the formation of protein-DNA binding complexes (Fig. 5C, right). Interestingly, the Sp1 BS2 oligonucleotide (corresponding to the Sp1 binding site 2 at −152/−143 lesion) was less effective in these competition studies as compared with the Sp1 BS1 oligonucleotide. These results suggest that the bile acid-responsive DNA-protein binding is likely Sp1-related. Among the two Sp1 binding sites on the DR5/TRAIL-R2 promoter −235/−136 region, BS1 appears to be the only one which is bile acid-responsive.

To further determine if Sp1 binds to the region, a supershift analysis was performed using a Sp1-specific antisera (Fig. 5D). Indeed, the density of the C2 band dramatically decreased in the presence of anti-Sp1 antibody, and two additional bands (indicated as *1 and *2 in Fig. 5D) were observed. In contrast, the density of the other complexes (C1 and C3) was not altered by anti-Sp1 antisera. The additional band at the lower position (*2) appears to be a small molecular weight supershifted band, whereas the higher band (*1) likely represents the formation of larger molecular weight complexes because it increases in relationship to the anti-Sp1 antibody concentration. Taken together, DCA enhances C2 complex formation through the Sp1 binding site at −198/−189 (BS1).

Finally, as a control, we also tested the contribution of the known JNK1/2-regulated transcription factors, AP-1 and CREB/ATF, in the formation of these DNA-protein complexes. However, neither addition of the AP-1 nor the CREB/ATF consensus sequences (both 100-fold the amount of the probe) altered DNA-protein complex formation (Fig. 5E). Thus, neither the AP-1 nor the CREB/ATF transcription factor appears to bind to the −235/−136 region of the DR5/TRAIL-R2 promoter.

**DCA-mediated Sp1-DNA Binding Is JNK-dependent—** Because bile acid-mediated DR5/TRAIL-R2 induction is JNK1/2-dependent, we next tested if bile acid-mediated Sp1-DNA binding was JNK1/2-dependent. HuH-BAT cells were incubated with DCA in the presence or absence of SP600125, and the nuclear protein extracts from those cells were subjected to EMSA. JNK1/2 inhibition abrogated DCA-mediated augmentation of DNA-protein complexes C1, C2, and C3 on the −235/−136 DNA probe (Fig. 6A). The DNA-protein complex C2 was dramatically reduced by the anti-Sp1 supershifting antibody. Therefore, bile acid-mediated Sp1 binding to the DR5/TRAIL-R2 promoter is JNK1/2-dependent, likely by an indirect mechanism.

We next compared Sp1-DNA binding affinity between the Sp1 consensus sequence, Sp1 binding site 1 and Sp1 binding site 2 (Fig. 6B). Indeed, when the Sp1 consensus sequence was used as a probe (Fig. 6B, left), DCA dramatically enhanced Sp1-DNA binding as compared with control nuclear extract. More importantly, the JNK1/2 inhibitor SP600125 dramatically reduced formation of Sp1-DNA complex. The additional bands observed below the Sp1 bands were likely Sp3-DNA complexes. These results are consistent with the competition studies in that Sp1 BS1 sequence more effectively competed with the −235/−136 probe than the Sp1 BS2 sequences.

To further determine if Sp1 binds to the endogenous DR5/TRAIL-R2 promoter, we performed chromatin immunoprecipitation experiments (Fig. 7, A and B). Indeed, Sp1-DR5/TRAIL-R2 promoter binding was dramatically enhanced by DCA treatment. The samples from DCA-treated HuH-BAT cells contained 9.50 ± 3.71 × 10^3 copies/μl of DR5/TRAIL-R2 promoter fragments, whereas 1.86 ± 1.30 × 10^3 copies/μl of the DNA fragments were co-precipitated from control cells. The number of co-precipitated DNA fragments were significantly lower in cells treated with the JNK1/2 inhibitor SP600125 plus DCA (0.74 ± 0.31 × 10^3 copies/μl and 0.52 ± 3.71 × 10^3 copies/μl, respectively). Although minimal nonspecific binding between DNA frag-
ments and the agarose beads was detected by the real-time PCR (<10^2 copies/μl), this was negligible by agarose gel electrophoresis. The Sp1 immunoprecipitation efficiency was equivalent in all immunoprecipitated samples as demonstrated under “Experimental Procedures.” One microliter of the immunoprecipitated samples and control samples not exposed to the antisera was subjected to real-time PCR to quantitate DR5/TRAIR2 sequences. The copy number of each sample was normalized to the DR5/TRAIR2 promoter copy number of samples not incubated with the antisera. Data were expressed as mean ± S.D. from three independent experiments. p < 0.01 for DCA-treated group versus either Control, SP alone, or DCA plus SP. B, PCR products from the ChIP experiments were analyzed by 1% agarose gel electrophoresis (top and middle panels). Immunoblot analysis was performed using anti-Sp1 antisera (bottom panel) to demonstrate equivalent immunoprecipitation efficiency. C, immunoblot analysis was performed after treatment of HuH-BAT with deoxycholate (100 μM) to analyze Sp1 cellular protein levels.

Fig. 6. JNK1/2 inhibition reduces Sp1-DNA binding activity. HuH-BAT cells were incubated with or without DCA (100 μM, 6 h) in the presence or absence of SP600125 (20 μM). A, EMSA was performed using the −235/+136 probe. Lane 1, free probe; lane 2, nuclear protein from control cells (6 μg); lanes 3, 6, and 7, nuclear protein from DCA-treated cells (6 μg); lane 4, nuclear protein from SP600125-treated cells (6 μg); lane 5, nuclear protein from SP600125 plus DCA-treated cells (6 μg); lanes 6 and 7, either anti-Sp1-supershifting antibody (1.0 μg) or Sp1 Cons., unlabeled competitor (100-fold) was added in the reaction. B, EMSA was performed using either Sp1 Cons., Sp1 BS1, or Sp1 BS2 oligonucleotide-DNA probes. Lane 1, nuclear protein from control cells (3 μg); lanes 2, 5, and 6, nuclear protein from DCA-treated cells (3 μg); lane 3, nuclear protein from SP600125 plus DCA-treated cells (3 μg); lanes 5 and 6, either anti-Sp1 supershifting antisera (1.0 μg) or Sp1 Cons., unlabeled competitor (100-fold) was added to the reaction.

Fig. 7. DCA promotes Sp1-DNA binding to the endogenous DR5/TRAIR2 promoter by a JNK1/2-dependent mechanism. HuH-BAT cells were incubated with or without DCA (100 μM, 6 h) in the presence or absence of SP600125 (20 μM). A, cells were fixed, lysed, sonicated, and subjected to Sp1 immunoprecipitation as described under “Experimental Procedures.” One microliter of the immunoprecipitated samples and control samples not exposed to the antisera was subjected to real-time PCR to quantitate DR5/TRAIR2 sequences. The copy number of each sample was normalized to the DR5/TRAIR2 promoter copy number of samples not incubated with the antisera. Data were expressed as mean ± S.D. from three independent experiments. p < 0.01 for DCA-treated group versus either Control, SP alone, or DCA plus SP. B, PCR products from the ChIP experiments were analyzed by 1% agarose gel electrophoresis (top and middle panels). Immunoblot analysis was performed using anti-Sp1 antisera (bottom panel) to demonstrate equivalent immunoprecipitation efficiency. C, immunoblot analysis was performed after treatment of HuH-BAT with deoxycholate (100 μM) to analyze Sp1 cellular protein levels.
Mutation of DR5/TRAIL-R2 Promoter at Sp1 Binding Sites Abrogates Transcriptional Activity—To further examine the function Sp1 binding in regulating DR5/TRAIL-R2 promoter activity, we introduced point mutations within the Sp1 binding sites of the DR5/TRAIL-R2 promoter. DCA-mediated DR5/TRAIL-R2 promoter activation was abrogated when the Sp1 BS1 was mutated (Fig. 8). In contrast, mutation of the Sp1 BS2 was less effective in attenuating luciferase activity. Thus, the Sp1 BS1 appears to be crucial for DCA-mediated DR5/TRAIL-R2 promoter activation. Taken together, DCA up-regulates DR5/TRAIL-R2 promoter activity by enhancing Sp1 binding to the DR5/TRAIL-R2 promoter at the Sp1 BS1.

DISCUSSION

The principal findings of this study relate to the molecular mechanisms by which bile acids up-regulate DR5/TRAIL-R2 expression. The results demonstrate that: (i) a JNK1/2 inhibitor SP600125 effectively suppresses DCA-mediated DR5/TRAIL-R2 mRNA and protein up-regulation; (ii) the transcription factor Sp1 binds to the 5’-flanking region of the DR5/TRAIL-R2 promoter/enhancer; (iii) DCA enhances Sp1-DNA binding activity to specific genomic sequences by a JNK1/2-dependent mechanism; and (iv) mutation of the Sp1 binding sequences abrogates DCA-mediated enhancement of DR5/TRAIL-R2 promoter activity. These results suggest bile acid-mediated cytotoxicity is, in part, mediated by a JNK1/2 signal transduction pathway resulting in Sp1 associated DR5/TRAIL-R2 up-regulation.

There are three genes for JNK, each with specific functions. JNK1 and -2 are ubiquitously expressed, whereas JNK3 is neuronal specific (35, 36). Bile acid activation of JNK1/2 has previously been implicated in gene regulation. For example, taurolithocholate has been shown to suppress cholesterol 7α-hydroxylase by a JNK1/2-dependent process (37, 38). JNK1/2 has also been implicated in the regulation of the death ligands Fas ligand and tumor necrosis factor-α (39–42). The present study suggests JNK1/2 can up-regulate death receptors, namely DR5/TRAIL-R2. Whether JNK1 or -2 mediates bile acid-induced DR5/TRAIL-R2 expression is unclear and will require further examination. However, given the recent observation that JNK1 is cytotoxic in the liver (35, 43), toxic bile acids may up-regulate DR5/TRAIL-R2 potentially by a JNK1-mediated signaling cascade.

Several bile acid response elements have been identified in a variety of genes (44). In the current study, we found that the DR5/TRAIL-R2 promoter also contains bile acid response elements. By reporter gene analysis we initially identified two regions of the DR5/TRAIL-R2 promoter, −235/−136 and −510/−411, that appeared to be important in the regulation of DR5/TRAIL promoter activity by DCA. However, when used as probes in electrophoretic mobility gel shift assays, only probes from the −235/−136 region displayed evidence for bile acid-mediated DNA-protein complex formation. Although probes from the −510/−411 region displayed protein-DNA complexes, the complex formation was not enhanced following bile acid treatment. The mechanism by which this region potentially contributes to DR5/TRAIL-R2 promoter activity therefore remains unknown and is a subject for future investigations.

We further examined the region −235/−136. This 5’-flanking sequence contains two putative Sp1 binding sites. The first putative binding site bound Sp1 more avidly than the second site, a finding consistent with their nucleotide sequences. The first binding region contains the complete Sp1 consensus binding sequence, whereas one guanine is replaced with a cytosine in the second sequence. The first sequence was also the only site responsive to DCA in a reporter gene assay. Consistently, reporter gene analysis revealed that mutations in the first site completely abrogated DR5/TRAIL-R2 promoter activity in response to the bile acid. Taken together, bile acids appear to activate DR5/TRAIL-R2 gene transcription by facilitating Sp1-DR5/TRAIL-R2 promoter binding at the first Sp1 binding site in the −235/−136 5’-flanking region of this death receptor gene.

Sp1 is the first identified member of Sp/Krüppel-like family proteins that binds to G-rich elements such as GC-box (GGGCGGGG) and GT-box (GGTGTGGGG) (45). Although Sp1 was initially identified as a basal transcription factor supporting transcription of so-called housekeeping genes (46), it is now well established that Sp1 is involved in inducible gene expression (45, 47–49). Sp1 participation in inducible gene expression occurs by post-translational modifications of this transcription factor (45, 46, 50, 51). Currently, several protein kinases, including MAPKs, have been implicated to modulate Sp1 transcription factor activity (45, 52). In our present study, DCA enhanced Sp1-DNA binding activity, and a JNK1/2 inhibitor SP600125 blocked DCA-mediated Sp1 activation. The results indicate that Sp1 is a bile acid-regulated transcription factor, and JNK1/2 is an intermediary kinase in this relationship. These data provide insight into the p53-independent mechanisms regulating DR5/TRAIL-R2 expression. Given the potential importance of this death receptor in cancer therapy (53), these data maybe important in developing strategies to enhance DR5/TRAIL-R2 expression in other cell types.

The precise mechanism by which JNK1/2 regulates Sp1 function was not elucidated in these studies. Because Sp1 contains Ser/Thr-rich regions and is a known phosphoprotein (45), it is possible that JNK1/2 may directly phosphorylate Sp1, thereby, altering its DNA binding activity. On the other hand, Sp1-DNA binding activity may be regulated by interaction with other co-activating proteins. For instance, binding between Sp1 and Egr-1 is regulated by Egr-1 phosphorylation, and Egr-1 phosphorylation results in an increase in Sp1-DNA binding (54). Other studies have also implicated yet to be identified co-interacting proteins in the regulation of Sp1 transcriptional activity (45, 55, 56). AP-1, a classic downstream target of JNK1/2 (35), has also been implicated in a cooperative role with Sp1 in gene induction (57–60). Indeed, in DCA-treated HuH-BAT cells, AP-1 activation was observed by EMSA (not shown). Thus, it is likely that AP-1 contributes to DCA-mediated DR5/
TRAIL-R2 induction by co-operating with Sp1. In summary, these data suggest bile acids sensitize HuH-7 cells to TRAIL-mediated apoptosis by up-regulating DR5/TRAIL-R2 expression via a JNK1/2/Sp1-dependent cascade. Given the number of genes regulated by bile acids, this paradigm likely pertains to many other bile acid-regulated genes (44). The results are also germane to cholestatic liver injury and cancer therapy (2). In cholestasis, elevated hepatic bile acid concentrations increase DR5/TRAIL-R2 expression and sensitize the liver to TRAIL cytotoxicity (24). Because JNK1/2 and cancer therapy (2). In cholestasis, elevated hepatic bile

Acknowledgment—The secretarial assistance of Beverly Colbourn is gratefully acknowledged.

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