Bile Acid-induced Activation of Activator Protein-1 Requires Both Extracellular Signal-regulated Kinase and Protein Kinase C Signaling*

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Elevated concentrations of fecal bile acids are known to promote colon cancer and increasing evidence suggests that alterations in cellular signaling and gene expression may play an important role in this process. In this study, we examined the molecular mechanisms underlying bile acid-mediated gene regulation using GADD153 as our model gene. Promoter deletion analyses revealed that the activator protein-1 (AP-1) transcription factor was crucial for deoxycholic acid (DCA)-mediated GADD153 gene transcription. Electrophoretic mobility shift assays and transient transfection analyses demonstrated that both DNA binding and transactivation activities of AP-1 were induced by DCA in a dose-dependent manner. The AP-1 complex induced by DCA consisted of JunD, Fra-1, and c-Fos. Examination of the signaling pathways stimulated by DCA showed that extracellular signal-regulated kinases (ERKs) were required for AP-1 activation. Inhibition of ERK by the mitogen-activated protein kinase/ERK kinase inhibitor PD 98059 or by expression of a dominant negative mutant ERK suppressed AP-1 activation. Notably, the PKC inhibitor, calphostin C, also abolished DCA-induced AP-1 activation but did not affect DCA-mediated ERK activation, suggesting that ERK and PKC function in separate signaling pathways that cooperatively mediate DCA-induced AP-1 activation. Hence, bile acid-stimulated signaling appears to converge on the AP-1 protooncopogene.

Bile acids, amphiphilic derivatives of cholesterol, have been consistently associated with increased incidence of colon cancer. The primary bile acids, cholic and chenodeoxycholic acids, are synthesized in the liver and are excreted into the duodenum where they facilitate absorption of dietary lipids. Most of these bile acids are reabsorbed in the intestine; however, a small quantity remains unabsorbed and passes into the colon where it is converted to secondary bile acids, deoxycholic and lithocholic acids, by enteric bacteria. These secondary bile acids, particularly deoxycholic acid (DCA), have been linked to colon cancer. Comparison of populations with diverse eating habits have shown that consumption of a high fat/low fiber diet leads to elevated fecal concentrations of secondary bile acids, which is associated with an increased incidence of colon cancer (1). However, the most compelling data implicating bile acids in colon tumorigenesis come from animal studies where manipulation of fecal bile acid concentrations, in conjunction with carcinogen treatment, leads to increased tumor formation (2, 3). Importantly, bile acids by themselves cannot induce colon tumors, indicating that bile acid functions as tumor promoters rather than complete carcinogens. However, the mechanism of this tumor promoter activity is unknown.

Although there is some evidence that bile acids may induce DNA damage (4, 5), because of the inability of bile acids to initiate tumorigenesis by themselves, the general consensus is that bile acids are contributing to colonic carcinogenesis by disturbing the fine balance between proliferation, differentiation, and apoptosis in the colonic epithelial cells (6). Instead, it has been suggested that bile acids act by modifying intracellular signaling and gene expression, perhaps by altering the activity of protein kinase C (PKC). PKC is a group of important signaling molecules consisting of at least 11 isozymes. These PKC isotypes are subdivided into three groups: the conventional PKC members (cPKCs), the novel PKCs (nPKCs), and the atypical PKCs. PKC plays key roles in the regulation of cellular processes, including growth, differentiation, tumor promotion, and apoptosis (7). Studies have shown that bile acids activate PKC in both normal colonic epithelial cells and colon tumor cell lines (8, 9). Moreover, several genes, such as cholesterol 7α-hydroxylase, major histocompatibility complex class I, and cyclooxygenase-2, are regulated by bile acids through activation of PKC (10–12). Hence, activation of PKC and modification of signal transduction and gene expression appears to be an important aspect of the effect of bile acids in tumor promotion.

The purpose of this study was to examine the molecular mechanisms underlying bile acid-stimulated gene expression. Promoter deletion analyses were performed on the GADD153 (growth arrest- and DNA damage-inducible gene 153) promoter, which we previously showed, was inducible by DCA (13). We found that an activator protein-1 (AP-1)-binding site was crucial for activation of the GADD153 promoter by DCA. Further examination demonstrated that activation of both PKC and extracellular signal-regulated kinases (ERKs) were required for DCA-induced AP-1 activation. Importantly, these two kinases appear to regulate the activity of two distinct

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The abbreviations used are: DCA, deoxycholic acid; AP-1, activator protein-1; CA, cholic acid; CAT, chloramphenicol acetyltransferase; CDCA, chenodeoxycholic acid; C/EBP, CCAAT/enhancer-binding protein; cPKC, conventional PKC; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; nPKC, novel PKC; PKC, protein kinase C; UDCA, ursodeoxycholic acid; PCR, polymerase chain reaction; DMEM, Dulbecco’s modified Eagle’s medium.
ERK and PKC Mediate DCA-induced AP-1 Activation

PLASMIDS AND REAGENTS—p5W1, a human GADD153 promoter-CAT reporter gene construct, was kindly provided by Dr. Nikkii J. Holbrook (14). pCMV-p41Ala21Ala26MAPK, a dominant negative mutant ERK2 expression construct, was a gift from Dr. Roger Davis (15). Phu 175-2A, a human GADD153 cDNA clone in pCD vector was generously provided by Dr. A. J. Fornace (14). pTZ-GAP contains a 0.75-kilobase human glyceroldehyde-3-phosphate dehydrogenase CDNA fragment (16). LipofectAMINE and enzymes including restriction enzymes, T4 DNA ligase, calf intestinal alkaline phosphatase, and Klenow fragment of DNA polymerase I were from Life Technologies, Inc. Cloned Pfu DNA polymerase was from Stratagene (La Jolla, CA). Acetyl-CoA and the sodium salts of cholic acid (CA), chenodeoxycholic acid (CDCA), DCA, and ursodeoxycholic acid (UDCA) were from Sigma. All of the bile acids were maintained as 100 mM stock solutions in water. Calphostin C, PD 90809 (2’-amino-3’-methoxyflavone), and SB 22190 (4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole) were from Calbiochem (La Jolla, CA) and were maintained as stock solution in dimethyl sulfoxide. All stock solutions were stored at −20 °C except for acetyl-CoA which was stored at room temperature.

Construction of GADD153 Promoter-CAT Reporter Gene Constructs—Serial and internal deletions of human GADD153 promoter region in p5W1 (−954 to +91) were performed using the polymerase chain reaction (PCR) method. For serial deletion, a common downstream primer and several upstream primers located at different positions on the GADD153 upstream region were designed for corresponding promoter-reporter gene constructs as follows: downstream primer, 5′-TACCAAGCTTCTTCTCAAGGTTCCACTCCACACTCTC-3′ (+91 to +72); upstream primers, pGC10 5′-TACGATCGATGATAGCCGTTGGGGCTACGGTGTACT-3′ (−302); (ii) pGC15.70 and pGC85.70, the DNA fragments deleted from pGC10 (310 to 295); (iii) pGC89.40 and pGC110.40, the DNA fragments deleted from pGC10 and p5W1, respectively; and (iv) pGC15.60, where only the putative C/EBP site at 248 containing both the putative C/EBP and AP-1-binding sites was deleted from pGC10 and p5W1, respectively. Amplified products were excised from plasmid DNA and inserted into pGEM containing a multiple cloning site of pBluescript II SK (Stratagene product), and cloned DNA polymerase. The following cycling program was used: 94 °C for 40 s, 50 °C for 1 min, 72 °C for 1 min. Five cycles were then performed after the 30-min incubation at room temperature. Samples were then cultured for another 12 h before harvest.

Stable transfection of HCT116 cells with pCMV-p41Ala21Ala26MAPK or the vector plasmid pCMV-BamHI-neo was performed by a calcium phosphate precipitation method (17). The stably transfected cells were selected in the presence of 500 μg/ml G418, and individual clones were subcultured.

CAT ASSAYS—CAT assays using 14C-chloramphenicol were performed essentially as described in Ref. 17. The spots of 14C-labeled chloramphenicol and acetylated chloramphenicol were visualized and quantitated by PhosphorImager (Molecular Dynamics). The percentage of conversion of chloramphenicol to the acetylated form was calculated by normalizing to protein content.

Isolation of Nuclear Protein, Gel Shift, and Supershift Assays—Control or treated HCT116 cells were homogenized with a Dounce homogenizer in 10 mM HEPES, pH 7.9 (at 4 °C), 1 mM MgCl2, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol. The nuclear extracts were then removed by microcentrifuge at 4 °C at 12,000 × g for 20 min.

Gel shift assays were performed with 5 μg of nuclear protein extract and 10 ng of [α-32P]dATP end-labeled double strand 19-mer oligonucleotides containing the AP-1-binding site TGAACCTCA (12-O-tetradecanoylphorbol-13-acetate response element) and the flanking sequence in human GADD153 promoter (5′-TGGGATGACTGTTCAAC-3′ (sense)) and 5′-TGGGATGACTGTTCAAC (antisense) (14) in a 15–20 μl reaction mixture. Briefly, 5 μg of the nuclear protein extract was preincubated with 1 μg of poly(dI-dC) in the dialysis buffer at room temperature (20 °C) for 10 min, and then 10 ng of the 32P-labeled double strand probe was added and incubated at room temperature for 30 min. For supershift assays, 450 ng of rabbit polyclonal antibodies against various Fos and Jun proteins (Santa Cruz, CA) or rabbit polyclonal antibody specific for N-myc was added to the reaction after the 30-min incubation at room temperature. Samples were then incubated for an additional 60 min at room temperature. In the competition experiment, 1, 50-, or 100-fold excess unlabeled double strand oligonucleotides with the intact 12-O-tetradecanoylphorbol-13-acetate response element or mutated 12-O-tetradecanoylphorbol-13-acetate response element (5′-TGGGATGACTGTTCAAC-3′ (sense)) was added.
and 5'–GTGGTGGAGaCtGCGCAA-3' (antisense) were added to the reaction mixture along with the labeled probe. Samples were subjected to nondenaturing polyacrylamide gel electrophoresis in 4% gels. The gels were dried, and protein-DNA complexes were visualized by autoradiography.

Western Blot Analysis—Cells were lysed in 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate, and 10 mM sodium fluoride. 50 μg of the total cellular protein was subjected to SDS-polyacrylamide gel electrophoresis in 10% gels. Western blot analyses for expression and activation of MAPKs were performed using rabbit phospho-specific antibodies against ERK, p38, and JNK or the corresponding phospho-specific antibodies, which recognize activated phosphorylated MAPK isoforms (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (Santa Cruz, CA) and Luminol ECL reagents (Amersham Pharmacia Biotech) were used to detect proteins.

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RESULTS

Analyses of Bile Acid Response Elements in the Human GADD153 Promoter—GADD153 has been characterized as a stress response gene that is activated by a broad spectrum of genotoxic agents and physiological stresses (18). In our previous study, we found that the GADD153 promoter responded to DCA induction in a dose-dependent manner (13). Northern blot analysis also showed a dose-dependent significant induction of GADD153 mRNA by DCA in HeLa cells and human colon tumor cell lines, such as HCT116 cells (data not shown). The mRNA induction became to be evident by 2–4 h and increased along with further exposure of the cells to 200–500 μM DCA. These results indicate that GADD153 gene could be transcriptionally regulated by bile acids. These features make GADD153 an ideal model gene to explore the molecular mechanism and signal transduction underlying bile acid-mediated gene regulation.

To identify the bile acid response elements in the GADD153 promoter, serial and internal deletions of the promoter were constructed and linked to a CAT reporter gene (Fig. 1A). The reporter constructs were transiently transfected into HCT116 cells and either basal activity (Fig. 1B) or DCA-inducible activity quantified. Although serial deletion resulted in a progressive reduction in basal activity, DCA inducibility of the constructs remained relatively constant until the 190 base pairs region were −4-fold or less (Fig. 1C). This C/EBP-binding site was previously implicated by Sylvester et al. (19) as being an important regulatory element in the GADD153 promoter. To further elucidate the role of this element, the construct pGC15.70 was generated from pGC10, which lacked the nine base pairs of consensus C/E-binding sequence (5'–ATTGCATC-3'). Transient transfection experiments using this transfectant construct showed a dramatic increase in basal activity of the promoter, as well as a moderate increase in DCA inducibility. This suggested that the C/E-binding site might be a constitutive repressor of GADD153 promoter activity. None of the DNA sequence between −954 and −248, including the C/E-binding motif could confer DCA inducibility when linked to a basal promoter (Fig. 1C, pGC89.40 and pGC110.40). Hence, C/EIP appears to repress the GADD153 promoter. The effect of DCA on this transcription factor remains to be determined.

Other elements in the GADD153 promoter acted as positive regulators. In this regard we noted that deletion of 55 base pairs (−247 to −192) from pGC20 to generate pGC30 resulted in a marked decrease in DCA inducibility, dropping from −8-fold induction to −4-fold induction (Fig. 1C). Moreover, splicing this region onto pGC40, which did not respond to DCA, restored DCA inducibility (Fig. 1C, pGC211.40). Although there is a putative Es1-binding site located within 40 base pairs of the AP-1-binding site, it is unlikely that this element contributes to GADD153 activation because pGC30, which does not contain this element, exhibits the same degree of activation as pGC70 which does contain the Es1 element. However, the fact that both of these deleted promoters exhibit moderate DCA inducibility suggests that an additional positive regulatory element was located nearer to the transcription start site. There are several Sp1 consensus binding sites that may be responsible for this activity. This notion is based on the observation that pGC30 remains sensitive to stimulation by DCA and that both pGC15.70 and pGC85.70 exhibited similar moderate basal activity and similar DCA fold induction despite deletion of both the C/EBP- and AP-1-binding sites. We concluded that the major regulatory elements resided between −437 and −34 and that the important sequences likely included binding sites for the C/EBP and AP-1. Sp1 may also contribute to activation of the GADD153 promoter.

Induction of AP-1 DNA Binding and Transactivation by DCA—Our GADD153 promoter deletion experiments showed that the AP-1-binding site at −245 (5'–TGACTC-3') functioned as a DCA-inducible positive regulatory element. To explore this further we investigated the effect of DCA on both DNA binding and transactivation activities of AP-1. HCT116 cells were treated with different concentrations of DCA for 4 h, nuclear protein extracts were prepared, and mobility shift assays were performed using 19-mer double-strand radiolabeled GADD153 promoter sequence containing the AP-1-binding site as a probe. An AP-1-DNA complex was found in untreated cells and significantly stimulated by DCA in a dose-dependent manner (Fig. 2A). Time course analysis (Fig. 2B) showed that the AP-1 DNA binding activity was stimulated as early as 2 h after treatment with 250 μM DCA and that the response reached a maximum by 4 h. Subsequently, the AP-1 DNA binding activity declined. Specificity of the protein/DNA interaction was confirmed by competition assays with the wild type and mutated probes (Fig. 2C). In the mutated probe, two base pairs of mutation were made within the consensus 12-O-tetradecanoylphorbol-13-acetate response element (20) as shown under “Materials and Methods.” 50- or 100-fold excess unlabeled wild type probe caused a significant reduction in DNA binding, whereas the same concentrations of mutated probe had little or no effect. The specificity of the AP-1 DNA binding was further confirmed by supershift assays using anti-Fos and anti-Jun antibodies as discussed later (see Fig. 4). These results indicated that AP-1 DNA binding activity specific to the consensus AP-1-binding site on the GADD153 promoter was significantly induced by DCA in a dose- and time-dependent manner.

To test whether DCA induction of AP-1 DNA binding activity correlates with functional activation of AP-1, pGC211.40, which contains the human GADD153 AP-1-binding sequence linked to the minimal GADD153 promoter driving a CAT reporter gene, was transiently transfected into HCT116 cells. The transfected cells were treated with increasing doses of DCA (Fig. 2D). CAT activity from pGC211.40 was stimulated in a dose-dependent manner, indicating that DCA not only stimulated AP-1 DNA binding activity but also induced AP-1 transactivation.
Different Bile Acids Exhibit Distinct Effects on AP-1 Activation

It has been suggested that different bile acids have different biological effects on cell growth (21), colonic carcinogenesis (22), cell transformation (23), and gene expression (24). For example, DCA and CDCA function as tumor promoters, which accelerate carcinogen-induced tumor formation and cell transformation, whereas UDCA acts as a chemopreventive agent, which suppresses cell proliferation and colon tumor development. CA has no discernible biological effect on cells. In previous studies, we found that different bile acids exhibited distinct biological effects on the HCT116 cell growth, i.e. DCA and CDCA inhibited cell growth by inducing apoptosis, whereas UDCA inhibited cell proliferation but did not induce apoptosis (21). We reasoned that different bile acids might have differing effects on AP-1 activity.

To examine the effects of different bile acids on AP-1 activation, we treated HCT116 cells with four different bile acids for 4 h at 250 μM. AP-1 DNA binding activity was examined by gel shift assays (Fig. 3A). Notably, DCA and CDCA caused a significant induction of AP-1 DNA binding activity, but UDCA and CA did not. The corresponding AP-1 transactivation induction assays were also conducted using pGC211.40 in HCT116 cells. The results showed that DCA and CDCA caused AP-1 transactivation but UDCA and CA did not (Fig. 3B). Hence the same bile acids that resulted in up-regulation of AP-1 DNA binding activity also caused AP-1 to become functionally activated.

JunD, Fra-1, and c-Fos Are the Components of Bile Acid-induced AP-1 Complex in HCT116 Cells—The AP-1 protein is a dimer that consists of families of Jun and Fos proteins. There are three Jun proteins (c-Jun, JunD, and JunB) and four Fos proteins (c-Fos, FosB, Fra-1, and Fra-2) (20). It has been suggested that AP-1 complexes composed of different family members may be activated by different signaling pathways and may regulate different target genes (25). Therefore, it was of interest to identify the components of the AP-1 complex in bile acid-stimulated cells. For this purpose, supershift assays were performed with antibodies against various Fos and Jun proteins. DNA binding assays were conducted with nuclear extracts from HCT116 cells treated with 250 μM DCA. Antibodies against different Fos and Jun proteins were added to the reactions to test for the presence of different Fos and Jun proteins (Fig. 4). Antibodies against JunD, Fra-1, and c-Fos created clear supershift bands and caused reduction in AP-1 DNA binding (Fig. 4A). Simultaneous addition of the three antibodies caused a greater reduction in AP-1 DNA binding than any individual antibodies or combination of JunD with Fra-1 or c-Fos (Fig. 4B). The other antibodies did not create any supershifts and had no apparent effect on AP-1 DNA binding. These results indicated that JunD, Fra-1, and c-Fos were the components of the DCA-induced AP-1 complex. Furthermore, supershift assays using nuclear extracts from untreated cells and the cells treated with other bile acids, including UDCA, CDCA, and CA, showed the same Fos and Jun components as in DCA-treated cells. Because Fos proteins lack the ability to homodimerize, we suggest that two types of AP-1 complexes consisting of JunD and Fra-1 or JunD and c-Fos are involved in the DCA-induced AP-1 DNA binding. This is consistent with the observation that both c-Fos and Fra-1 form stable heterodimers with any of the Jun proteins, and these heterodimers have similar DNA binding activities and specificities (26).

DCA and CDCA Activate ERK and p38 MAPKs—Induction of AP-1 activity can be achieved by increasing expression of relevant Fos and Jun proteins and/or activating certain upstream phosphorylation pathways of AP-1. Western blot analyses for JunD, Fra-1, and c-Fos in HCT116 cells did not show changes in the protein level under stimulation of different bile acids (data not shown), suggesting that post-translational phosphorylation of pre-existing AP-1 proteins by upstream protein kinases could mediate bile acid-induced AP-1 activation.

MAPKs, including three structurally related subfamilies of the extracellular signal-regulated kinases (ERKs), the JNKs, and the p38 MAPKs (p38), are known to be key regulators of AP-1 proteins (26). To test whether bile acids could stimulate MAPK activity, HCT116 cells were treated with 250 μM bile acids for 4 h, and the phosphorylation status of ERK, p38, and

**Fig. 1.** Deletion analyses of human GADD153 promoter induction by DCA. **A,** a schematic of the serial and internal deletions of the human GADD153 promoter linked to a CAT reporter gene is depicted. Positions of important transcription factor-binding sites are marked. **B,** the relative basal activity of each CAT reporter gene constructs was determined after transient transfection into HCT116 cells compared with relative basal activity of each CAT reporter gene constructs was determined after transient transfection into HCT116 cells compared with basal activity of the intact promoter. **C,** similarly, DCA inducibility was assayed by transient transfection followed by incubation with medium containing 250 μM DCA for 12 h. The DCA inducibility was indicated by fold induction. Values are the means ± S.E. of at least three independent experiments.
JNK were examined by Western blot analyses using phospho-specific antibodies (Fig. 5). The results showed that both DCA and CDCA, which activated AP-1, significantly induced activation of ERK and p38, whereas UDCA and CA, which did not activate AP-1, had no apparent effects on the activity of these two MAPKs (Fig. 5, A and B). Notably, none of the bile acids affected the activity of JNK (Fig. 5C), suggesting that JNK is not stimulated by bile acids. Bile acid treatment did not alter the levels of cellular MAPK proteins (Fig. 5). We therefore conclude that ERK and p38 are activated by DCA and CDCA but not by UDCA and CA in a post-translational modification.

Inhibitor of ERK Abrogates DCA-induced AP-1 Activation—To investigate the role of ERK and p38 in DCA-induced AP-1 activation, PD 98059, a specific inhibitor of MAPK/ERK kinase (MEK), and SB 202190, a potent specific inhibitor of p38, were employed to block bile acid-induced ERK and p38 activation. First, the effects of the two inhibitors on DCA-induced ERK and p38 activation were determined. HCT116 cells were treated with 20 \( \mu \)M PD 98059 or 10 \( \mu \)M SB 202190 for 30 min prior to the addition of 250 \( \mu \)M DCA. 4 h after addition...
of DCA, crude cellular extracts were prepared, and Western blot analyses for activated phospho-ERK and phospho-p38 were performed. As shown in Fig. 6A, both basal and DCA-induced ERK activities were inhibited by PD 98059. No effect of PD 98059 was observed on DCA-induced p38 activation (Fig. 6B). As reported by previous studies (27), blocking of p38 activity by the p38 inhibitor SB 202190 stimulated DCA-induced ERK activation and p38 phosphorylation (Fig. 6A and B). Inhibition of p38 activity by SB 202190 was confirmed by testing the inhibitory effect of the inhibitor on DCA-induced phosphorylation of MAPK-activated protein kinase 2, a specific substrate of p38 (28) (data not shown). Furthermore, the effects of the two MAPK inhibitors on DCA-induced AP-1 activation were determined by testing AP-1 DNA binding activity. HCT116 cells were treated with DCA after pretreatment with the inhibitors, nuclear protein extracts were prepared, and AP-1 mobility shift assays were performed. The results showed that PD 98059 almost completely abolished the basal and DCA-induced AP-1 DNA binding activities in the cells (Fig. 6C). No inhibition of DCA-induced AP-1 DNA binding activity by SB 202190 was observed (Fig. 6D). These results suggest that ERK, but not p38, contributes to AP-1 activation in cells treated with DCA.

Expression of Dominant Negative Mutant ERK2 Inhibits DCA-mediated AP-1 Activation—To further explore the role of ERK in DCA-induced AP-1 activation, HCT116 cells were stably transfected with the dominant negative mutant ERK2 expression construct, pCMV-p41(Ala54Ala55)MAPK, in which mutation of ERK2 at the ATP-binding site to inhibit protein kinase activity was created by replacing Lys54 and Lys55 with Ala residues (15). It has been reported that constitutive expression of the mutant ERK could effectively interfere with the activation and function of ERK in the cells (15, 29). AP-1 DNA binding activity was investigated in the stable transfectants. In this study, two stably transfected clones were tested. Compared with parental HCT116 cells and the cells stably transfected with control plasmid pCMV-BamH-neo, DCA-mediated induction of phospho-ERK in both B6 and C6, two clones selected from stable transfection of HCT116 cells with the dominant negative mutant ERK2 expression construct, was significantly inhibited (Fig. 7A). Gel shift assays showed that both basal and DCA-induced AP-1 DNA binding activities in both B6 and C6 were significantly suppressed compared with parental HCT116 cells and vector control (Fig. 7B). These results combined with the observations using MAPK inhibitors (Fig. 6) indicated that activation of ERK was required for DCA-induced AP-1 activation.

Inhibition of ERK/AP-1 Signaling Alters Induction of GADD153 Transcription by DCA—To emphasize the role of ERK/AP-1 signaling in DCA-induced GADD153 transcription, the dominant negative mutant ERK2 transfectants, B6 and C6,
were treated with DCA, and GADD153 mRNA expression was determined by Northern blot analysis and compared with parental HCT116 cells and the vector control. As shown in Fig. 8A, DCA-induced GADD153 mRNA expression in B6 and C6 was significantly suppressed in comparison with vector control and parental HCT116 cells. However, at 8 h, both B6 and C6 exhibited dramatic induction of GADD153 mRNA upon DCA stimulation comparable with that in control cells. Given the observation that DCA induced a transient AP-1 DNA binding activity with a maximum by 4 h and declined afterward (Fig. 2B), this result suggests that ERK/AP-1 signaling is crucial for the early stage induction of GADD153 transcription by DCA. For comparison, these cells were also treated with the DNA damaging agent, etoposide (18), and GADD153 mRNA expression examined (Fig. 8B). Etoposide induced a transient increase in GADD153 mRNA level in both control cells, which was evident at 4 h but disappeared by 8 h. Importantly, disturbance of ERK/AP-1 signaling by constitutive expression of dominant negative mutant ERK2 in B6 and C6 did not inhibit etoposide induction of GADD153 transcription, suggesting that DNA damaging agents, at least etoposide, may activate GADD153 transcription through a mechanism independent of ERK/AP-1 signaling. Suppression of ERK/AP-1 signaling resulted in a sustained induction of GADD153 mRNA by etoposide in both B6 and C6 compared with control cells (Fig. 8B).

Hence, induction of GADD153 by DCA and by a genotoxic agent occurs with different kinetics.

**DCA-induced ERK Signaling Is Independent of PKC**—Bile acids activate PKC in *in vitro* and *in vivo* assays (8, 9, 30), and

![Fig. 6. Effects of PD 98059 and SB 202190 on DCA-induced ERK, p38, and AP-1 activation.](image)

HCT116 cells were treated with 250 μm DCA for 4 h after preincubation with 20 μM PD 98059 or 10 μM SB 202190 for 30 min. A and B, the total cellular protein extracts were prepared and Western blot analyses performed with 50 μg of protein using antibodies specific for total proteins or activated phosphorylated forms of ERK (A) and p38 (B), respectively. C and D, the nuclear protein extracts were prepared and gel shift assays for AP-1 DNA binding performed using radiolabeled GADD153 AP-1 probe.

![Fig. 7. Expression of dominant negative mutant ERK2 inhibits DCA-induced ERK and AP-1 activation.](image)

The HCT116 cells stably transfected with the dominant negative mutant construct, pCMV-p41(Ala44Ala55)MAPK, or the control plasmid, pCMV-BamH-neo, and the parental HCT116 cells were treated with 250 μM DCA for different time periods as indicated. B6 and C6 are two clones selected from stable transfection of HCT116 cells with pCMV-p41(Ala44Ala55)MAPK. A, the total cellular protein extracts were prepared, and Western blot analyses were performed with 50 μg of protein using the antibodies specific for activated phosphorylated ERK. B, the nuclear protein extracts were prepared, and gel shift assays for AP-1 DNA binding were performed using radiolabeled GADD153 AP-1 probe.

![Fig. 8. Comparison of GADD153 induction by DCA and etoposide.](image)

HCT116 cells and the cells stably transfected with the dominant negative mutant ERK2 expression construct, pCMV-p41(Ala44Ala55)MAPK, or vector plasmid pCMV-BamH-neo were treated with 250 μM DCA (A) or 100 μM etoposide (B) for different time periods as indicated. B6 and C6 are two clones selected from stable transfection of HCT116 cells with pCMV-p41(Ala44Ala55)MAPK. Total RNA was isolated from the treated and untreated cells, and Northern blot analysis was performed with 10 μg of RNA using radiolabeled GADD153 cDNA probe. The RNA blot was reprobed with radiolabeled glyceraldehyde-3-phosphate dehydrogenase cDNA fragment to confirm equal loading and transfer of RNA among different lanes.
ERK and PKC Mediate DCA-induced AP-1 Activation

PKC activity is required for induction of gene expression (10, 11, 12) and apoptosis in HCT116 cells (21). Previous studies have shown that the cPKC and nPKC members, but not the atypical PKC isozymes, can activate Raf/MEK/ERK signaling cascade (31, 32). To test whether PKC acts through the ERK pathway during DCA-induced activation of AP-1, HCT116 cells were pretreated with calphostin C, and ERK and AP-1 activities examined after DCA treatment. Calphostin C specifically inhibits cPKC and nPKC by competing for the binding site of diacylglycerol and phorbol esters (33). As shown in Fig. 9, calphostin C had no effect on activation of ERK in response to DCA. Nevertheless, calphostin C significantly suppressed DCA-induced AP-1 activation (Fig. 9B). These results suggest that ERK activation by DCA is in a PKC-independent mechanism and that both PKC and ERK signaling are essential for DCA-induced activation of AP-1.

**DISCUSSION**

Fecal bile acids are promoters of colon cancer. Increasing evidence suggests that alterations in cellular signaling and gene expression play an important role in this process. In this study, we examined the signaling mechanisms underlying bile acid-induced gene expression using GADD153 as our model gene. We demonstrated that DCA may stimulate multiple transcription factors that contribute to the activation of GADD153 gene. AP-1 activation was crucial for DCA-induced GADD153 gene expression. Examination of signaling pathways upstream of AP-1 demonstrated that ERK and p38 MAPKs were activated by tumor promoting bile acids and that ERK and PKC cooperatively mediated DCA-induced AP-1 activation through two distinct signaling mechanisms.

Activation of AP-1 by the tumor promoting bile acids, DCA and CDCA, but not by either UDCA or CA is likely to have important implications for colon tumorigenesis. AP-1 is a known protooncogene, and several lines of evidence support a role for AP-1 in cell proliferation and tumor promotion. Its activity is enhanced when cells are stimulated by tumor promoters (20). Elevated levels of Fos and Jun proteins are observed in transformed rapidly growing cells (20). In addition, studies in mouse skin have shown AP-1 as a critical component in epidermal tumor development (34). Moreover, blocking tumor promoter-induced AP-1 activity can inhibit neoplastic transformation (35). Here we demonstrated that the DCA-induced AP-1 complex consisted of JunD, Fra-1, and c-Fos (Fig. 4). Recent evidence indicates that these three proteins are involved in tumorigenesis (36–38). It has been reported that the tumor promoting bile acids stimulate proliferation at subtoxic concentrations in colonic epithelial cells (9, 39). Therefore, bile acid-induced AP-1 activation may be an important etiological factor in human colon tumorigenesis.

In light of the potential role of AP-1 activation in colon tumorigenesis, it was of interest to examine the signaling pathways of bile acid-induced AP-1 activation. It has been suggested that AP-1 activity could be regulated at multiple levels, including transcriptional regulation altering the protein levels of Fos and Jun and post-translational regulation modulating the DNA-binding affinity, transcriptional activity, and stability of AP-1 proteins (25). In this study, we observed that JunD, Fra-1, and c-Fos proteins were equally abundant before and after bile acid treatment in HCT116 cells (data not shown). These results indicated that bile acids activated pre-existing AP-1 proteins through post-translational modification. At present, MAPKs, including ERK, JNK, and p38, are the most important protein kinases that mediate the post-translational modification of AP-1 proteins (26). Western blot analyses with phospho-specific antibodies showed that ERK and p38 were activated by DCA and CDCA but not by UDCA and CA (Fig. 5). This observation was correlated well with AP-1 activation (Fig. 3). Inhibition of ERK activation, but not p38, resulted in suppression of DCA-induced AP-1 activation (Figs. 6 and 7), indicating that ERK is necessary for DCA-induced AP-1 activation. The DCA-induced AP-1 complex consisted of JunD, Fra-1, and c-Fos (Fig. 4). It has been reported that ERK can activate c-Fos through p90 ribosomal S6 kinase (p90Rsk) (40). Because p90Rsk inhibits glycogen-synthase kinase 3 (41) and glycogen-synthase kinase 3 negatively regulates c-Jun and JunD (42, 43), ERK may mediate bile acid-induced AP-1 activation by modulating both c-Fos and JunD. The involvement of additional kinases could result in amplification of bile acid-stimulated signaling downstream of ERK.

MAPKs are activated in a three-kinase module involving MAPK, MAPK kinase, and MAPK kinase kinase (44). MEK directly activates ERK by dual phosphorylation at conserved threonine and tyrosine residues. Several MAPK kinase kinases that activate MEK have been identified, including Raf, Mos, MEK kinases 1, 2, and 3, and TPI-2, of which the Raf protein acts as prominent mediator of extracellular stimulus-induced ERK activation (44). Raf mediates extracellular stimulus-induced ERK activation in a Ras- or PKC-dependent mechanism (45). Diverse studies have shown that the cPKC and nPKC members, but not the atypical PKC isozymes, can activate Raf by direct phosphorylation (31, 32). PKC is widely reported to activate MEK by direct phosphorylation at conserved residues (8, 9, 30). Our previous studies showed that calphostin C, a highly specific inhibitor of cPKC and nPKC (33), inhibited DCA-induced apoptosis in HCT116 cells, suggesting that cPKC and nPKC play key roles in DCA-mediated cellular alterations (21). Thus, we reasoned that PKC could act upstream of bile acid-induced ERK activation. Surprisingly, however, inhibition of PKC by calphostin C did not affect DCA-induced ERK activation (Fig. 9), suggesting
that DCA-mediated ERK activation was PKC-independent. Given the well-documented link between Ras and the Raf/MEK/ERK signaling cascade (45), it may be that bile acids stimulate the Ras/Raf/MEK/ERK signal cascade leading to AP-1 activation and that this plays an important role in tumor promotion by DCA. Oncogenic activation of Ras has been shown to be an early event in multistage tumor progression such as colon cancer (45). Inhibition of Ras in colon cancer cell lines causes the cells to revert to a more controlled state of growth, indicating a requirement of Ras in malignant transformation of colorectal epithelial cells (46). A direct association between Ras and AP-1 was established by the observation that cellular transformation and malignancy induced by Ras required c-Jun (47). In addition, ERK was required for Ha-Ras-mediated AP-1 activation (48). Combined with the proliferative or oncogenic properties of ERK and AP-1, it appears that activation of the Ras/Raf/MEK/ERK/AP-1 signaling cascade plays an important role in tumor promotion by DCA. This notion is strengthened by the correlation between tumor promoting activity of bile acids and their capacity to activate ERK and AP-1 (Figs. 3 and 4).

Importantly, inhibition of PKC by calphostin C could also suppress DCA-induced AP-1 activation without affecting DCA-signaling mediated ERK activation (Fig. 9). Hence, at least two separate signaling pathways, which are ERK- and PKC-dependent, respectively, are involved in bile acid-mediated AP-1 activation. It has been reported that two types of phosphorylation sites with positive and negative effects, respectively, were involved in the post-translational regulation of Jun proteins (43). Furthermore, activation of PKC decreases phosphorylation of c-Jun at sites that negatively regulate its DNA-binding activity (49). Thus, it may be that activation of the ERK signaling pathway contributes to the phosphorylation of AP-1 proteins at the sites that positively regulate AP-1 activity, whereas stimulation of the PKC signaling pathway inhibits phosphorylation of the AP-1 proteins at sites that negatively regulate AP-1 activity. Both events are essential for bile acid-induced AP-1 activation.

In conclusion, we showed that two distinct signaling pathways, ERK and PKC, cooperatively mediated DCA-induced AP-1 activation. Importantly, we showed, for the first time, that the distinct biological activities of DCA, a tumor promoter, and UDCA, a chemopreventive agent, can be attributed at least in part to the capacity to activate ERK and PKC/AP-1 signaling pathway. These results may provide an important insight into the molecular mechanisms by which bile acids regulate gene expression and exert their biological effects.
