Bile acid regulates MUC2 transcription in colon cancer cells via positive EGFR/PKC/Ras/ERK/CREB, PI3K/Akt/IκB/NF-κB and p38/MSK1/CREB pathways and negative JNK/c-Jun/AP-1 pathway

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Abstract. MUC2 is a major secretory mucin normally expressed by goblet cells of the intestine, but is aberrantly expressed in colonic neoplasia. Bile acids have been implicated in colorectal carcinogenesis and, therefore, we sought to determine the effects of bile acids on MUC2 expression and regulation in colon cancer cells. Since deoxycholic acid (DCA), a secondary bile acid, has been reported to be a potent mucin secretagogue and tumor promoter, DCA-treated HM3 colon cancer cells were analyzed using promoter-reporter assays of the 5' flanking region of the MUC2 gene. Chemical inhibitors, mutant reporter constructs and EMSA showed that DCA upregulates MUC2 transcription via multiple pathways involving activation of EGFR/PKC/Ras/Raf-1/MEK1/ERK/CREB, PI3/Akt/IκB/NF-κB and p38/MSK1/CREB while DCA induced MUC2 transcription is inhibited by JNK/c-Jun/AP-1 pathway. These results provide new insight into the complex molecular mechanisms involved in the regulation of mucin gene by bile acids in colon cancer cells that may contribute to further elucidation of colorectal carcinogenesis.

Introduction

Gastrointestinal mucosal cell surface is covered by a thick layer of viscous mucus consisting mainly of secreted mucins (1,2). Mucins are heavily glycosylated high molecular weight glycoproteins which are broadly classified into two types; secretory or membrane associated (3-5). About 20 different mucin genes have been identified and many of these mucins are normally expressed in tissue- and cell type-specific manner (1,3,5,6). The intestinal mucus layer consists mainly of MUC2, a major intestinal mucin, synthesized and secreted by goblet cells, providing an effective barrier against pathogens, toxins, dietary components and endogenous and exogenous stimuli, such as digestive enzymes, bile acids and non-steroidal anti-inflammatory drugs (2,5-7). Quantitative and/or qualitative alterations in mucins have been reported in infection/inflammation and neoplasia (3-5). These changes occur due to altered regulation of expression of mucin core proteins and of glycosyltransferases that lead to altered glycosylation of mucin core proteins (3-5). Together these changes contribute to diverse biological properties of cancer cells and immune cells, such as cell-cell and cell-substratum interactions, differentiation and proliferation, invasion and metastasis and modulation of immune responses (3-5).

Bile acids are amphiphilic molecules that promote the processing of dietary fat absorption (8). Primary bile acids such as cholic acid and chenodeoxycholic acid are synthesized and conjugated by amino acids in the liver and are secreted in the bile into the intestinal lumen where they are hydrolyzed to secondary bile acids such as deoxycholic acid (DCA) and lithocholic acid by the action of bacterial enzymes in the colon (8). The secondary bile acids have been reported to be more toxic to colonic epithelial cells than the primary bile acids and also have been reported to be a tumor promoter in the animal models (9,10). Epidemiological studies also indicate that secondary bile acids may be implicated in colorectal carcinogenesis. For example, the high fat intake has been associated with increased fecal bile acids and a high incidence of colorectal cancer (11,12). Cholecystectomy which has been reported to be associated with higher incidence of colorectal cancer has been shown to result in higher levels of total fecal bile acids and a higher ratio of secondary to primary bile acids in the feces (13). Bile acids have also been reported to stimulate mucin secretion and expression in colon cancer cells (14-16). However, there is paucity of detailed data on the molecular mechanisms

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involved in the bile acid regulated expression of mucin in the cancerous colon cells.

In the present study, we sought to determine the effect of a secondary bile acid, DCA on MUC2 expression and on the molecular mechanisms involved in MUC2 regulation in colon cancer cells. Our study showed that DCA regulates MUC2 transcription in HM3 colon cancer cells via activation of CREB, NF-κB, Sp1, AP-1 mediated by either positive pathways involving EGFR/PKC/Ras/Raf1/MEK/ERK/CREB, PI3K/Akt/IκB/NF-κB and p38/MSK1/CREB or a negative pathway involving JNK/c-Jun/AP-1.

Materials and methods

Reagents. Forskolin, 8-bromo-3',5'-cAMP (8Br-cAMP) were obtained from Sigma. Dual-Luciferase™ Reporter Assay System was from Promega. Caffeic acid phenethyl ester (CAPE), calphostin C, bisindolylmaleimide 1, wortmannin, H89, SP600125, U0126, AG126, SB202190, BAY 11-7082, AG1478 and PD98059 were obtained from EMD Biosciences (La Jolla, CA). Antibodies against inhibitor of NF-κBα (IκBα) (no. 9242), Akt (no. 9272), phospho-Akt-Ser473 (no. 9271S), extracellular signal-regulated kinase (ERK)p44/42 (no. 9102), phospho-ERKp44/42 Thr202/Tyr204 (no. 9101), p38 (no. 9212), phospho-p38Thr180/Tyr204 (no. 9211), SAPK-JNK (no. 9252), phospho-SAPK-JNKThr183/Tyr185 (no. 9251), cAMP response element-binding protein (CREB) (sc-240X) and phospho-CREB (Ser133; no. 9191) were obtained from Cell Signaling Technology (Beverly, MA). Antibodies for p65 (sc-109X) and c-Rel (sc-70X), subunits of nuclear transcription factor-κB (NF-κB) and c-Jun (sc-45X), c-Fos (SC-253X), Fos B (SC-48X) and Fra 2 (SC-604X) subunit of activating protein-1 (AP-1) and specificity protein 1 (Sp1) (sc-059X) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). MUC2 antibody, Ccp58 was from Biomeda. Secondary antibodies were purchased from Zymed laboratories Inc. (South San Francisco, CA). Oligonucletides were synthesized by Operon, Alameda, CA.

Cell lines and plasmids. HM3 cells were maintained at 37˚C in 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) (high glucose) containing 10% heat-inactivated fetal calf serum, penicillin and streptomycin. The cell line HM3M2 is an HM3 cell line stably transfected with the MUC2 promoter region from -2864 to +19 subcloned into pGL2-Basic luciferase vector (Promega Corp., Madison, WI), along with pcDNA3 carrying the *neo* gene. This cell line was maintained in medium containing 600 μg/ml G418 Geneticin (Invitrogen).

Plasmids were prepared using the EndoFree Plasmid MAXI prep kit (Qiagen, Valencia, CA). MUC2 promoter-reporter deletion constructs containing regions -73/+19, -91/ +19, -171/ +19, -343/+19, -600/+19, -1308/+19, -1628/+19 and -2864/+19 were described previously (17). Dominant-negative form of NIK (K429-430A, subcloned into pRK) and IκBα (S32/36A) were described previously (18,19). Plasmid encoding dominant negative Akt was obtained from D. Stokoe, University of California, San Francisco. The expression vectors for Ha-Ras, and dominant-negative N17Ras were a kind gift from Dr G. Cooper (Boston University, MA). HMEK1 (K97R) was a kind gift from Dr A. Saltiel (Parke-Davis Pharmaceutical Research Division, MI). Dominant-negative SEK, dominant-negative p38 were gifts from Dr J.D. Li (U.S.C., CA) (20,21). KCREB was a kind gift from Dr R.H. Goodman (Oregon Health Science University, OR) (22). Nter-B-Raf was a kind gift from Dr R. Busca (INSERM, France) (23). Tam67 was a kind gift from Dr M.J. Birrer (National Cancer Institute, MA) (24).

RNA isolation and RT-PCR. Cells were seeded in six-well plates and then incubated for 48 h. The cells were then serum-starved for 17 h before treatment with DCA (200 μM). Cells were harvested using TriReagent (Molecular Research Center Inc., Cincinnati, OH) and RNA was isolated according to manufacturer's instructions. Total RNA (1.5 μg) was reverse transcribed using Superscript II (Invitrogen) primed with random hexamers, in a final volume of 25 μl. PCR amplification reactions were conducted in 10 μl reaction volume containing 0.2 mM dNTPs, 1X AmpliTaq buffer, 5% DMSO, 1 μl of reverse-transcribed reaction mixture, 0.3 μM of each primer, 0.25 unit of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA) and 0.6 μM of 18S rRNA primers (9:1 ratio) of 3'-blocked/unblocked (alternate) primers from 18S Internal Standards kit (Ambion, Austin, TX). Primers for MUC2 were 5'-TGC CTG GCC CTG TCT TTG-3' (forward) and 5'-CAG CTC CAG CAT GAG TGC-3' (reverse). The PCR reaction mixture was denatured at 94˚C for 5 min followed by 28 cycles at 94˚C for 30 sec, 60˚C for 30 sec and 72˚C for 60 sec. The final elongation step was extended for additional 6 min. PCR products were separated on ethidium bromide-stained gels, and bands were analyzed using NIH Image software.

Transient transfection and inhibitor assays. Cells were seeded in 24-well plates. After two days, they were co-transfected with 2 μg of MUC2-2.8 vector, 2 μg of specific expression vector and 0.1 μg of Renilla luciferase vector (pRL0, Promega) for internal, combined with 5 μl of Superfect (Qiagen). Total DNA amount was kept constant by supplementation with empty vector, pcDNA3. The transfected cells were incubated for 24 h and then were serum-starved and treated with DCA (200 μM) as before. The cells were harvested in 50 μl of Passive Lysis Buffer, and the luciferase activity of the supernatant was measured using Dual-Luciferase Reporter Assay System (Promega) using a Monolight 2010 Luminometer (Analytical Luminescent Laboratory). Firefly luciferase activities were normalized with respect to renilla luciferase activities. All transient transfections were carried in triplicate, with standard deviations indicated by error bars.

For inhibitor studies, HM3M2 cells were seeded in 24-well plates. After two days the cells were starved in 1.25% serum medium for 17 h, and then pretreated with inhibitors for 1 h before exposure to DCA (200 μM) for 4 h before preparation of lysates and assays. Cell viability was measured by trypan blue exclusion assay (25). Data are presented graphically as the average of triplicates with statistical significance determined by Student's t-test.

Western blotting. Cells were treated with or without DCA (200 μM) for various periods of time. Total cell lysates were

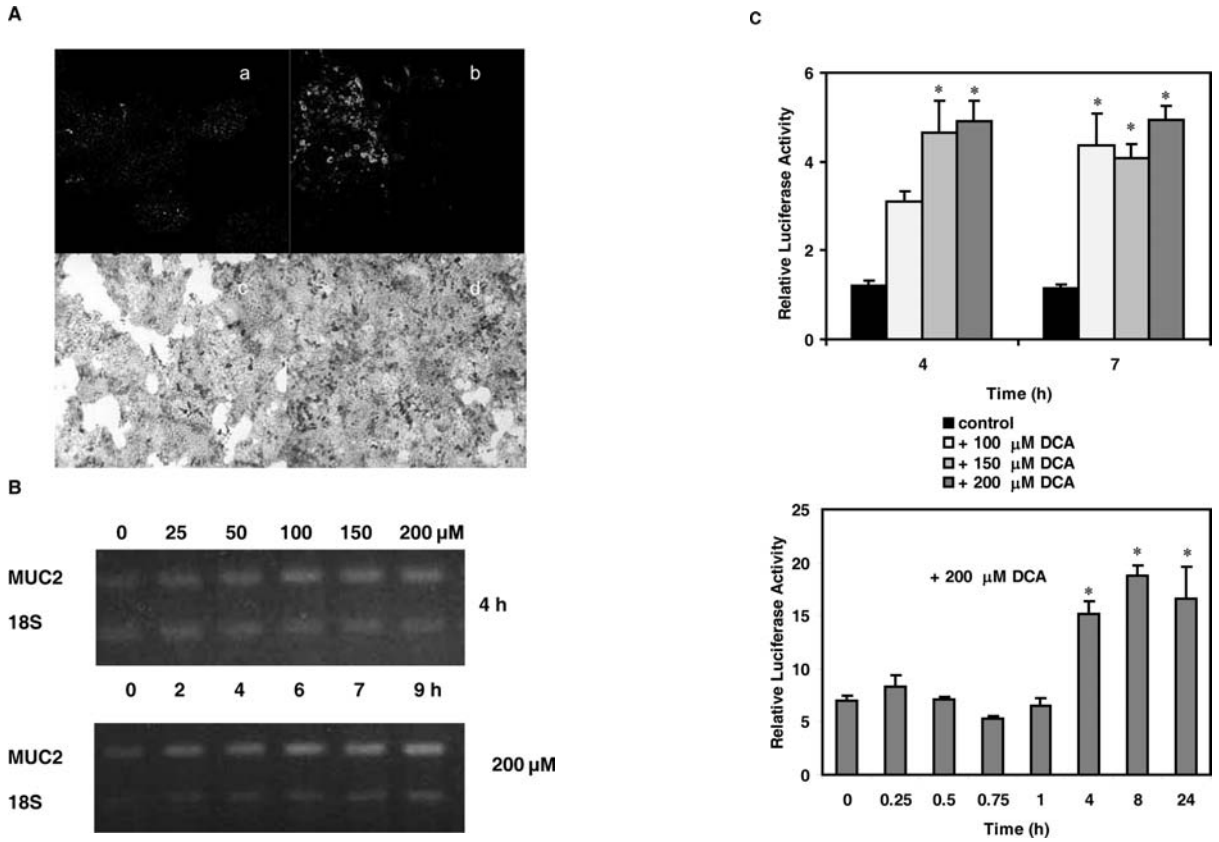

Figure 1. DCA upregulates MUC2 expression. (A) HM3 cells were treated with 200 μM DCA for 4 h. Newly synthesized MUC2 was detected by immunohistochemical staining (c, control; d, DCA treated) and confocal microscopy (a, control; b, DCA treated) using anti-MUC2 (Ccp) antibody. (B) RT-PCR analysis of mRNA from HM3 cells treated with DCA at various concentrations and for the indicated times. (C) HM3 cells were treated with DCA at indicated concentrations and for indicated times and then luciferase activity was measured and normalized with respect to untreated cells. *P<0.05.

prepared by lysing and scraping the cells off the culture plate with 10 mM Tris-HCl, pH 6.8, 0.4 mM EDTA, 2% SDS, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenymethyl-sulfonyl fluoride, 10 mM sodium fluoride, 0.4 mM sodium orthovanadate and 10 mM pyrophosphate. The protein concentration of the supernatant was determined by using the bicinchoninic acid based BCA Protein Assay Kit (Pierce Chemicals, Rockford, IL). Total cell lysate protein (20 μg/lane) was subjected to 10% SDS-polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes. Nitrocellulose blots were blocked with 5% non-fat milk in Tris-buffered saline (TBS; 10 mM Tris-HCl with 150 mM NaCl, pH 7.4), containing 0.1% Tween (TBST), and then probed overnight with indicated primary antibodies diluted 1:1000 in 5% bovine serum albumin in TBST. The membranes were subsequently washed three times for 5 min with TBST and probed with 1:2500 goat anti-rabbit IgG conjugated to horseradish peroxidase (Zymed Laboratories Inc.). After washing, bands were visualized by chemiluminescence using the Renaissance kit (NEN Life Science Products).

Electrophoretic mobility shift assay. Cells were treated with or without DCA (200 μM) and nuclear extracts were prepared according to Andrews and Faller (26), with protein concentration determined using the Bradford Assay method (Biorad, Hercules, CA). A double-stranded oligonucleotide probe corresponding to the human MUC2 promoter region from double-stranded oligonucleotide CREB probes used in this study were: from -2583 to -2554 (5'-TCA GGT GCC ATG TGA CGT CAG TGC TGC CT-3'); consensus CRE probe 5'-AGA GAT TGC TGA CGT CAG AGA GCT AG-3'; MUC2 Sp1 probe 5'-GCA GAT GCC ACA CCC ACC CTT GGC-3'; consensus Sp 1 probe 5'-GAT TCG ATG GGG CGG GGC GCG-3'; MUC2 NF-κB probe 5'-CGT CCT TGG GTT TCC CCA GGG CTC AGT GC-3'; consensus NF-κB probe 5'-GAG GTC CCA GCT TTG AGA GT-3'; MUC2 AP-1 probe 5'-CCT CCG ATA ACC TGA ATC AAT ATT T-3'; and consensus AP-1 probe 5'-CGC TTG ATG ACT CAG CCG GAA-3' were prepared by annealing forward and reverse-complementary deoxynucleotides end-labeled with [α-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Nuclear proteins were incubated with radiolabeled probes at 37˚C for 20 min in a solution containing 1 μg of poly (dI-dC) in 10 mM Tris-HCl at pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.05% NP-40 and 10% glycerol. For competition experiments, a 100-fold excess of unlabeled oligonucleotide was added. For supershift assays, antibodies specific for p65 or c-Rel, subunits of NF-κB, for CREB or pCREB of CREB, for c-Jun, c-Fos, Fra-2 or Fos-B, subunits of AP-1 and for Sp1 were added to the reaction during a 30-min. Samples were applied to 4% polyacrylamide gels in Tris-acetate-EDTA buffer at 140 volts for 3 h, the gel was dried and exposed to X-ray film.

Immunocytochemistry. HM3 and HM3M2 cells were seeded onto 1-cm glass coverslips in a 24-well plate. Cells were

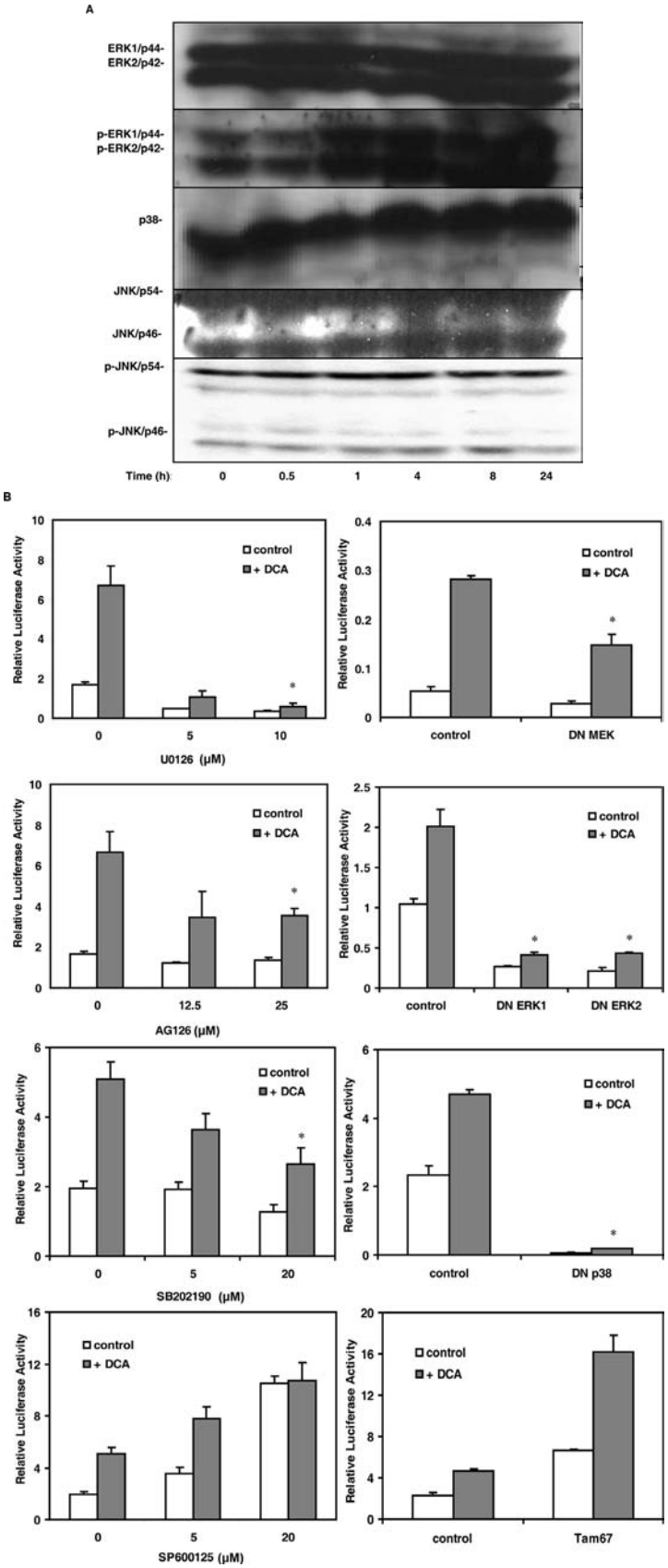

Figure 2. (A and B) Positive MEK/ERK and p38 pathways and negative JNK pathway mediate DCA-induced MUC2 promoter reporter activation. (A) DCA treatment activates ERK, p38 and JNK. HM3 cells were treated with 200 μM DCA for indicated time periods. Total cell lysates (20 μg/lane) were subjected to electrophoresis, and nitrocellulose blots were blotted with antibodies to phosphorylated and non-phosphorylated forms of ERK1/2, p38 and JNK(p54 and p46) as indicated. (B) One hour pretreatment with MEK1 inhibitor U0126, ERK1/2 inhibitor AG126 and p38 inhibitor SB20219 inhibited DCA-induced MUC2 promoter reporter activity. Dominant-negative forms of MEK, ERK/1/2 and p38 also inhibited DCA-induced MUC2 promoter activity. *P<0.05. By contrast, JNK inhibitor SP600125 did not inhibit DCA-induced MUC2 promoter activity but actually caused an increase in both basal and DCA-induced MUC2 promoter activity. c-Jun is a downstream target of JNK and co-transfection of dominant-negative c-Jun with MUC2 promoter (-2864/+19) reporter construct increased both basal and DCA-induced MUC2 promoter activity. *P<0.05.

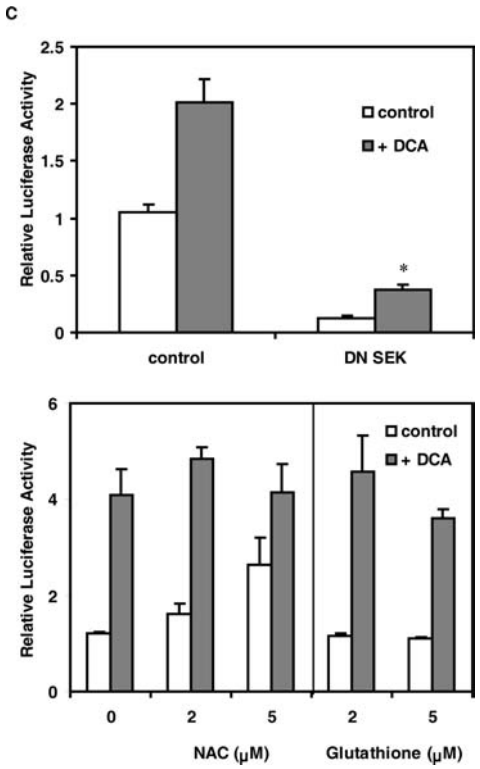

Figure 2. (C) Dominant-negative SEK which inhibits both MKK4/7 and MKK3/6 inhibited DCA-induced MUC2 promoter activity (*P<0.05) whereas antioxidants, N-acetyl-L-cysteine and glutathione showed no effect on DCA-induced MUC2 promoter activity.

serum-starved and treated with DCA according to several concentrations and times, then fixed for 10 min with 4:1 mixture of methanol acetic acid. Wells containing cells on coverslips were rinsed with methanol, and peroxidase activity quenched by 20 min treatment with 3% peroxide in 90% methanol. After rinsing with phosphate-buffered saline, fixed cells were blocked and stained using Zymed Histostain Plus Broad Spectrum Kit (Zymed) according to the manufacturer's instructions, and using a 1:300 dilution of Ccp58 antibody against MUC2 and a 1:200 dilution of caspase-3 antibody for apoptosis, with final detection using Aminoethyl Carbazole Substrate Kit (Zymed), to produce red staining. Nuclei were counterstained blue using hematoxylin solution (Zymed), and then coverslips were mounted onto slides with aqueous mounting medium.

Results

DCA increases MUC2 expression and transcription. Immunochemical analysis of DCA treated cells (HM3 and HM3M2 cell lines) with MUC2 antibody showed an increase in the number of positively-stained cells by both conventional microscopy and confocal microscopy (Fig. 1A). DCA-treatment of HM3 cells caused an increase in the level of MUC2 mRNA in dose- (200 μM) and time- (4 h) dependent manner as measured by RT-PCR (Fig. 1B).

MUC2 transcriptional activity, as measured by the MUC2 promoter-luciferase activity in DCA-treated HM3 cells was upregulated in a time- and dose-dependent manner up to 8 h of incubation at 200 μM DCA (Fig. 1C). The immunocytochemical study with a caspase antibody indicated no observable apoptosis and trypan blue dye exclusion study indicated good cell viability up to 200 μM DCA. However, at 300 μM or higher concentration of DCA treatment a significant apoptosis and cell death were observed in HM3 cells (data not shown).

Positive MEK/ERK and p38 signaling pathways and negative JNK pathway are involved in DCA-induced MUC2 transcriptional regulation. To investigate the involvement of downstream signaling pathways such as the MAP kinase pathways, we undertook Western blotting studies using antibodies against MAP kinase proteins (Fig. 2A). As shown in the figure, both ERK1/2 and p38 were phosphorylated in response to DCA treatment. SAPK/JNK, which typically occurs as multiple bands due to alternate splicing of long (p54) and short (p46) forms showed active phosphorylation at basal state and showed slight but definite increase in phosphorylation of both bands after DCA treatment (Fig. 2A).

To further investigate the involvement of MAP kinase, we examined MUC2 promoter activity in the presence of specific kinase inhibitor U0126, which inhibits MEK1 more potently than MEK2. It significantly blocked MUC2 induction by DCA in a dose-dependent manner (Fig. 2B). Consistent with this, transient transfection using expression vector-encoding dominant-negative form of MEK1 significantly inhibited the DCA-induced MUC2 promoter activity (Fig. 2B). AG126, an inhibitor of ERK tyrosine phosphorylation, inhibited DCA-induced MUC2 upregulation (Fig. 2B). Transient transfection of an expression vector-encoding dominant-negative ERK1 and dominant-negative ERK2 significantly inhibited the DCA-induced MUC2 promoter activity. Thus, DCA enhances MUC2 transcription via the MEK1/ERK pathway.

SB202190, a highly specific and negative p38 virtually abolished both basal and DCA-induced MUC2 transcription (Fig. 2B). SP600125, an inhibitor of JNK, increased both basal and DCA-induced MUC2 transcriptional activity (Fig. 2B). Consistent with this, cotransfection with dominant-negative c-Jun (Tam67) significantly increased both basal and DCA-induced MUC2 transcription (Fig. 2B).

Dominant-negative SEK, an MKK which acts upstream of both JNK and p38, inhibited MUC2 promoter-reporter activity (Fig. 2C). Thus, DCA-induced MUC2 transcription occur through two independent MAPK pathways, the MEK/ERK and p38 and negative pathway mediated by JNK. Since oxidative stress is one of the factors in the upstream of both p38 and JNK that activates both pathways, we examined the effect of antioxidants, N-acetyl-cysteine and glutathione, but neither agent had an effect on DCA-induced MUC2 transcription (Fig. 2C). This result suggests that other factors may be involved in activating p38 and JNK pathways.

Two upstream pathways, EGFR/PKC/Ras/Raf-1 and PI3K/Akt are involved in DCA-induced MUC2 upregulation. AG1478, a specific inhibitor of EGFR, inhibited the DCA-induced upregulation of MUC2 promoter activity in a dose-dependent manner (Fig. 3A) and dominant-negative EGFR inhibited the MUC2 induction by DCA (Fig. 3A). Calphostin C and

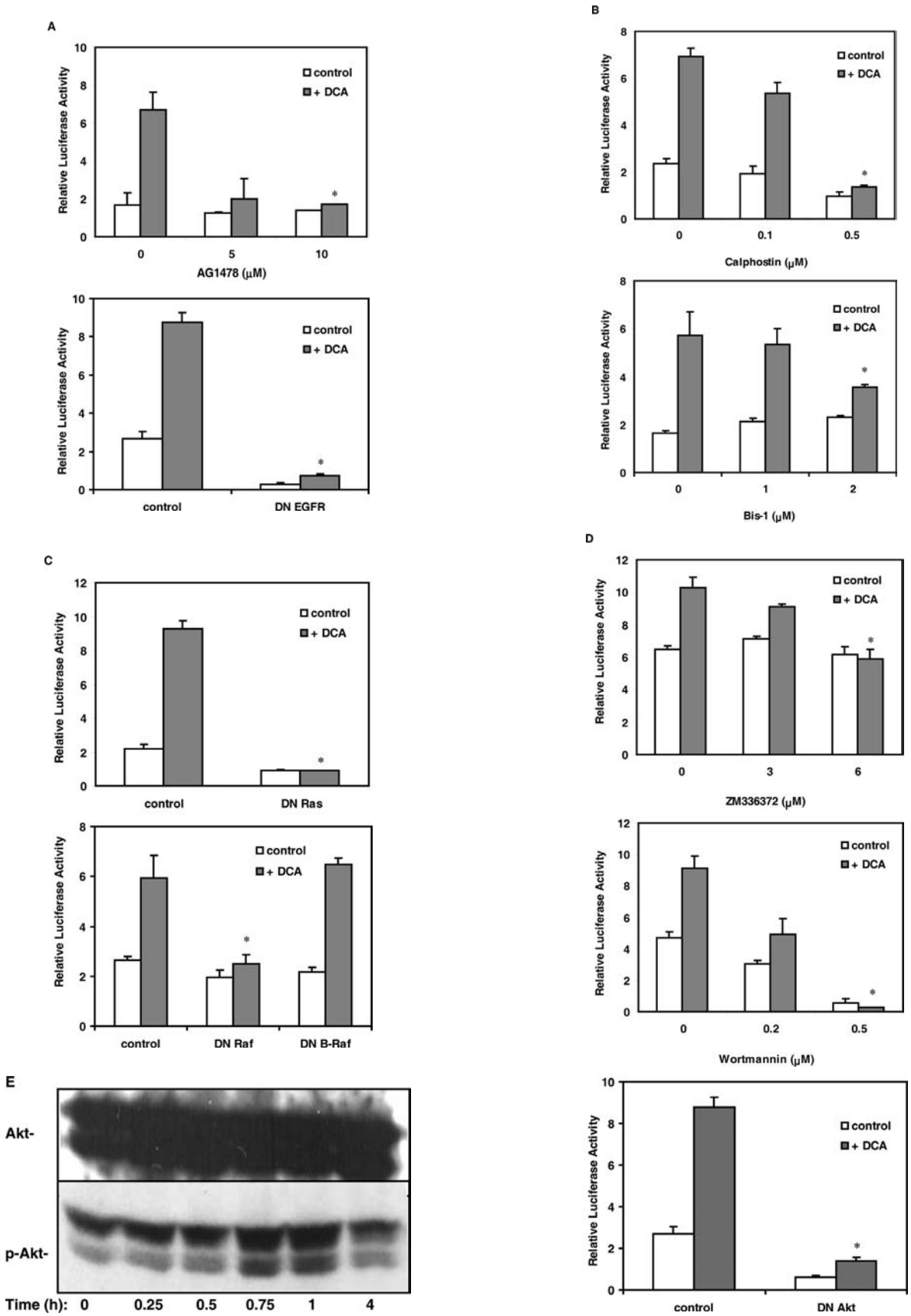

Figure 3. DCA upregulates MUC2 via pathways involving EGFR/Ras/Raf and EGFR/PI3K/Akt and PKC. (A) AG1478, an inhibitor of EGFR, inhibited DCA-induced MUC2 up-regulation after 1 h of treatment. Co-transfection with dominant-negative EGFR also inhibited DCA-induced MUC2 upregulation. *P<0.05. (B) One h pretreatment with calphostin and bis-1, PKC inhibitors, inhibited DCA-induced MUC2 upregulation. *P<0.05. (C) Dominant-negative Ras (N17 Ras) and Raf-1 (DN Raf) inhibited DCA-induced MUC2 upregulation but dominant-negative B-Raf did not. (D) ZM336372, an inhibitor of Raf-1 and wortmannin, an inhibitor of PI3K inhibited DCA-induced MUC2 upregulation. *P<0.05. (E) Western blotting of HM3 cell lysates after DCA treatment for indicated time periods probed with antibodies to phosphorylated and non-phosphorylated forms of Akt. When dominant-negative Akt was co-transfected with MUC2 promoter (-2864/+16) reporter construct, DCA-induced MUC2 upregulation was inhibited. *P<0.05.

bisindolylmaleimide 1, inhibitors of protein kinase C (PKC), inhibited the DCA-induced upregulation of MUC2 promoter activity (Fig. 3B). Dominant-negative Ras (Ras N17) inhibited the MUC2 induction by DCA (Fig. 3C) whereas dominant-negative Raf-1 (DN Raf1) but not dominant-negative B-Raf (N-B-Raf) inhibited DCA-induced MUC2 transcription (Fig. 3C). ZM336372, a specific inhibitor of Raf-1 significantly inhibited DCA-induced MUC2 promoter activity in a dose-dependent manner (Fig. 3D). Wortmannin, a specific inhibitors of phosphatidylinositol-3-OH kinase (PI3K), inhibited the DCA-induced up-regulation of MUC2 promoter activity (Fig. 3D). Western blotting of DCA-treated HM3 cell lysate showed that Akt was phosphorylated in response to DCA treatment (Fig. 3E). Dominant-negative Akt inhibited the MUC2 induction by DCA (Fig. 3E). Thus, upstream pathways mediated by EGFR/PKC/Ras/Raf-1 and EGFR/PI3K/Akt are involved in DCA-induced MUC2 upregulation.

DCA-induced MUC2 upregulation is not mediated by activation of cAMP/PKA. To determine whether DCA activates adenylate cyclase through G-protein-coupled receptor (GPCR) (15), we treated HM3M2 cells with the adenylate cyclase activator, forskolin, but no effect was observed on MUC2 promoter activity, indicating that cAMP is not involved (Fig. 4A). Likewise, treatment of HM3M2 cells with a stable cAMP analogue, 8Br-cAMP did not enhance the MUC2 promoter activity, suggesting that cAMP does not play a role in DCA-induced MUC2 transcription (Fig. 4A). H89, a protein kinase A (PKA) inhibitor, reduced DCA-induced MUC2 promoter-reporter activity in a dose-dependent manner, suggesting that PKA is responsible for DCA-induced MUC2 upregulation (Fig. 4B), but another PKA-specific inhibitor KT5720 did not inhibit DCA-induced MUC2 upregulation (Fig. 4B). Since H89 also inhibits ROCK and mitogen- and stress-activated kinase 1 (MSK1) we examined the effect of Y27632, an inhibitor of ROCK but no inhibition was observed (Fig. 4B). Therefore, by exclusion, MSK1 seems to be involved in MUC2 transcription. The observation that Src inhibitors, SU6656 and PP2 and its negative control PP3 had no effect on DCA-induced MUC2 promoter activity further support our conclusion that cAMP/PKA/Src pathway is not involved in DCA-induced MUC2 upregulation (Fig. 4B).

DCA upregulates MUC2 transcription via activation of transcription factors, CREB, NF-κB and Sp1. In order to identify the *cis* element(s) mediating the effects of DCA, a series of promoter-reporter deletion constructs were evaluated for their ability to respond to DCA. We identified three putative CRE elements located at base pairs, -2571 to -2563, -1022 to -1014 and -817 to -809 (Fig. 5A). Transient transfection assays carried out with truncated MUC2 promoter-reporter constructs in DCA-treated cells, showed significant differences in the activities of the construct -1308/+19 containing two CRE elements versus -600/+19, which contain no CRE element. However, we observed no significant differences between the activities of the constructs -2864/+19 and -1628/+19 which contain one CRE element (Fig. 5A). This indicated that the putative CRE element located at base pairs, -1022 to -1014 or -817 to -809 may play a dominant role in DCA-induced MUC2 transcription. We also observed significant differences between activities of the constructs -343/+19 and -228/+19, and between the constructs -91/+19 and -73/+19 (Fig. 5A), suggesting that additional elements other than CRE elements may be involved in DCA-induced MUC2 transcription.

We performed EMSA using oligonucleotides which were designed to include CRE *cis* elements (Fig. 5B). An oligonucleotide probe which included the base pairs, -2571 to -2563 CRE showed some binding to proteins in the nuclear extracts from HM3M2 cells. The intensity of the resulting bands was increased after 15-min treatment with DCA, and the binding further increased in a time-dependent fashion up to 4 h. This indicated that the CRE *cis* element which is located from -2571 to -2563 is important in DCA-induced MUC2 upregulation. The density of the band obtained using the -2571/-2563 sequence was decreased by the addition of unlabeled competitor oligonucleotide. This band was supershifted when nuclear protein was pre-incubated with antibody against CREB and c-Jun. We tested the effects of mutant expression vectors encoding dominant-negative CREB. Dominant-negative CREB (KCREB) inhibited DCA-induced MUC2 promoter-reporter activity (Fig. 5B).

A previous study identified a functional NF-κB site at base pairs, -1441 to -1452 of the MUC2 promoter (17). Since MUC2 promoter-reporter deletion construct studies showed that the construct -11628/+19, containing the functional NF-κB site was involved in DCA-induced MUC2 transcription, we examined the effect of chemical inhibitors of NF-κB on DCA-induced MUC2 transcription. We observed that NF-κB inhibitors, CAPE and parthenolide and a proteosome inhibitor, MG132 and an IKK inhibitor, BAY 11-7082 significantly inhibited DCA-induced MUC2 upregulation (Fig. 5C). EMSA and antibody supershift assays performed using an oligonucleotide probe (base pairs, -1458 to -1430 of MUC2 promoter) containing NF-κB binding-site, showed increased oligonucleotide binding to the nucleo-proteins of the DCA-treated HM3 cells and a slight decrease in the intensity of the band with pre-incubation with an anti-p65 antibody (Fig. 5D).

Western blot studies using antibodies against IκB kinase showed that IκBα is degraded in response to DCA treatment of the cells (Fig. 5D). Co-transfection with MUC2 promoter-reporter construct and the expression vector-encoding dominant-negative IκB or dominant-negative NIK inhibited MUC2 upregulation (Fig. 5D). EMSA and supershift assays performed using the oligonucleotide probe and anti-c-Jun antibody showed increased binding of the probe to the nuclear proteins of the DCA-treated cells and a slight reduction in the band intensity with the pretreatment of the cell lysate with anti-c-Jun antibody. Similar EMSA and supershift assays carried out using the oligonucleotide probe and anti-Sp1 antibody showed increased binding of the probe to the nuclear protein with DCA treatment of the cells and decreased intensity of the band with pretreatment of the nuclear protein with anti-Sp1 antibody (Fig. 5E). However, in the case of AP-1, we observed no supershift when nuclear protein was pretreated with antibodies against c-Jun, c-Fos, Fos B or Fra 2 (data not shown). These results indicate that activation of transcrition factors, CREB, NF-κB and Sp1 are involved in DCA-induced upregulation of MUC2 in colon cancer cells.

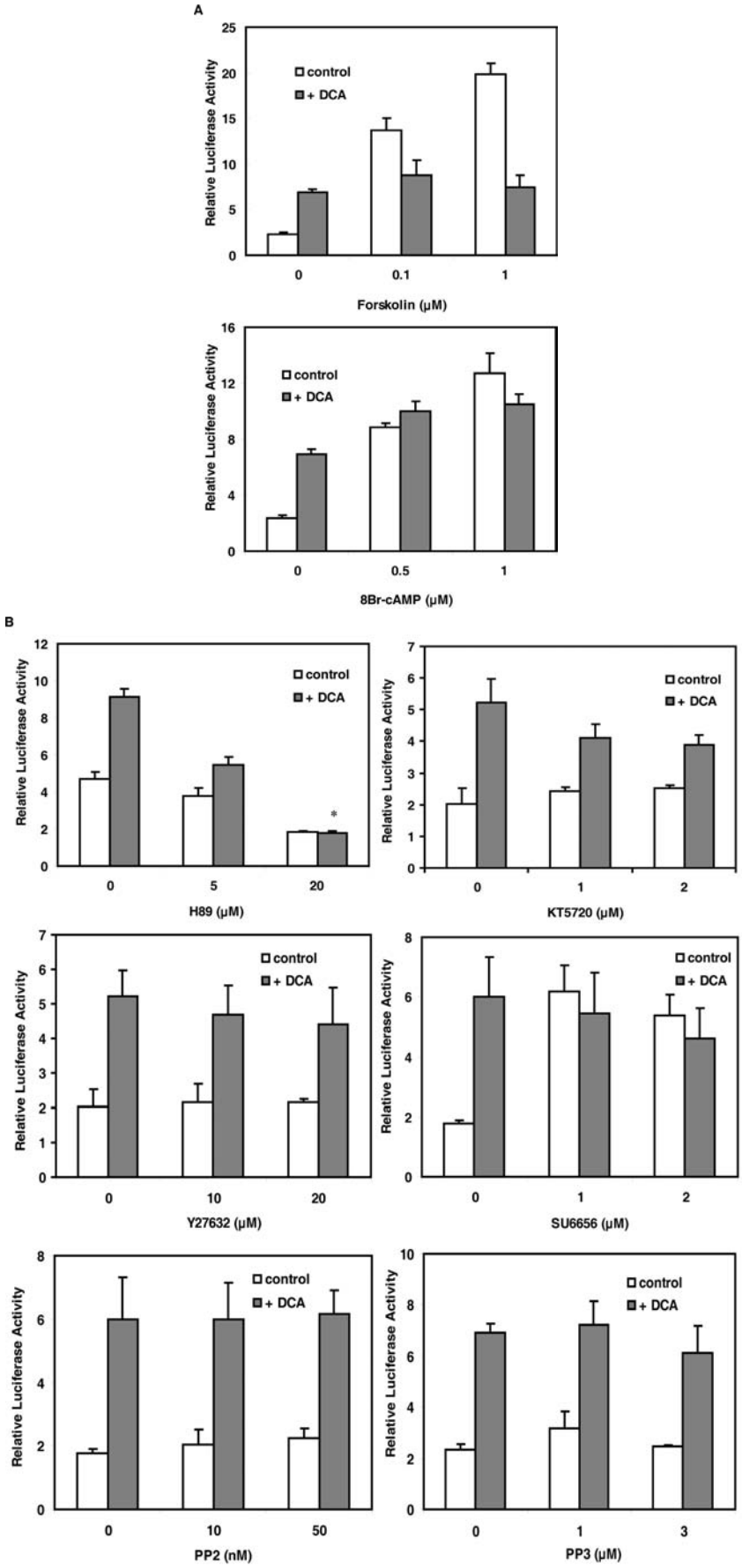

Figure 4. cAMP/PKA and Src pathways are not required or DCA-induced MUC2 upregulation. (A) One hour pretreatment of HM3M2 cells with either forskolin, an adenylate cyclase inhibitor, or 8BrcAMP, a stable cAMP agonist did not inhibit DCA-induced MUC2 upregulation. (B) One h pretreatment of HM3M2 cells with H89, a broad spectrum kinase inhibitor caused inhibition of DCA-induced MUC2 upregulation. *P<0.05. By contrast, 1 h pretreatment with KT5720, a PKA inhibitor, Y27632, a ROCK inhibitor, SU6656 and PP2, inhibitors of Src or PP3, a negative control of PP2 did not show any effect on DCA-induced MUC2 upregulation.

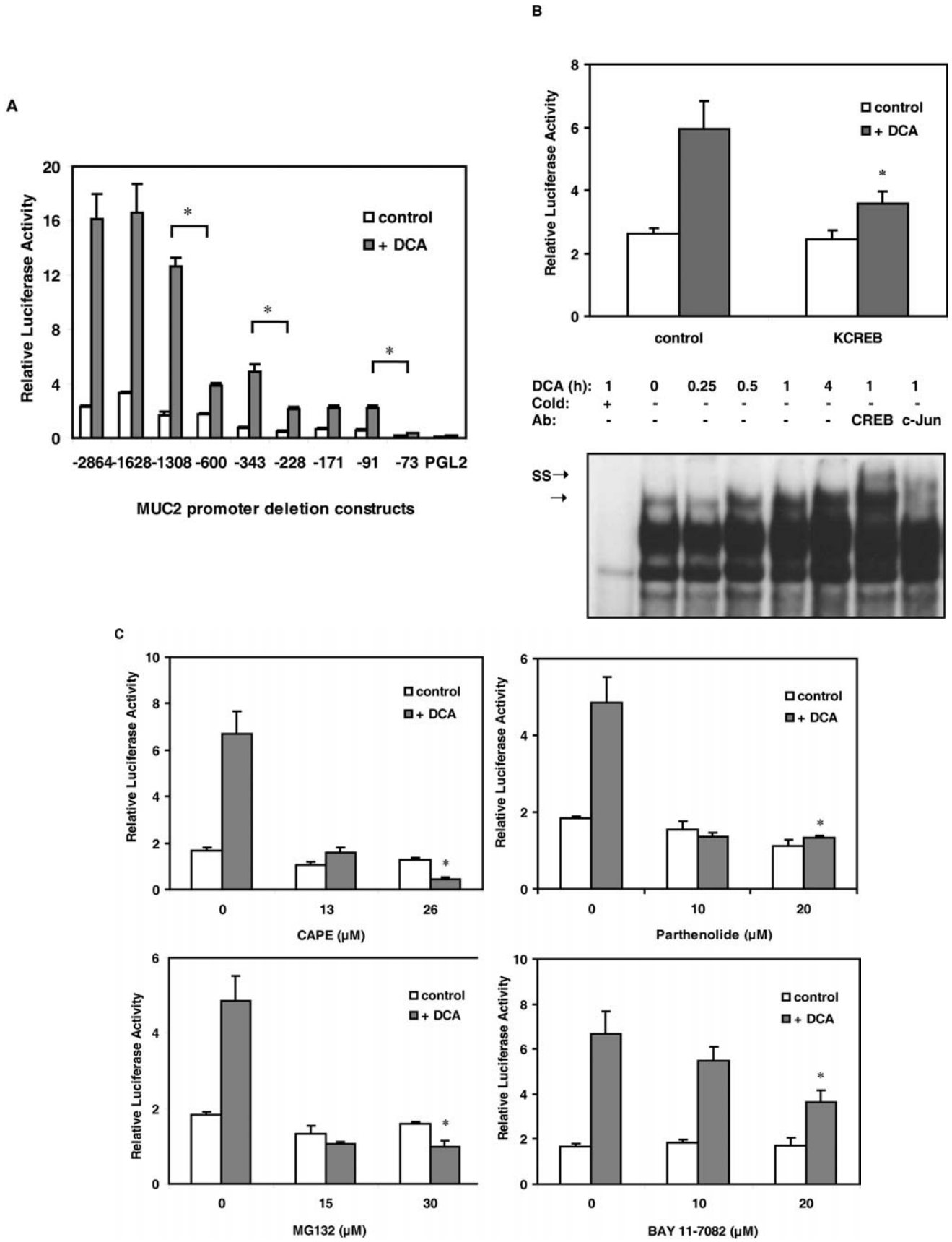

Figure 5. (A, B and C) CREB, NF-κB and Sp1 and AP-1 are involved in DCA-induced MUC2 regulation. (A) HM3 cells were transiently transfected with deletion constructs as indicated, and then treated with DCA for 4 h before harvesting for luciferase assays. (B) Co-transfection with MUC2 promoter (-2864/+19) reporter construct and dominant-negative CREB (KCREB) in HM3 cells inhibited DCA-induced MUC2 upregulation. *P<0.05. EMSA using double-stranded oligonucleotide probe corresponding to MUC2 CRE site (-2571/-2600 bases) was performed. 100-fold concentration of unlabeled (cold) oligonucleotides were added to demonstrate the specificity by competitively inhibiting the binding of labeled oligonucleotides. Antibodies used for supershift are indicated. Arrows indicate the shifted and the original bands. (C) One hour pretreatment with NF-κB inhibitors, CAPE, parthenolide, MG132 and BAY-11-7082 in HM3M2 cells inhibited DCA-induced MUC2 upregulation. *P<0.05.

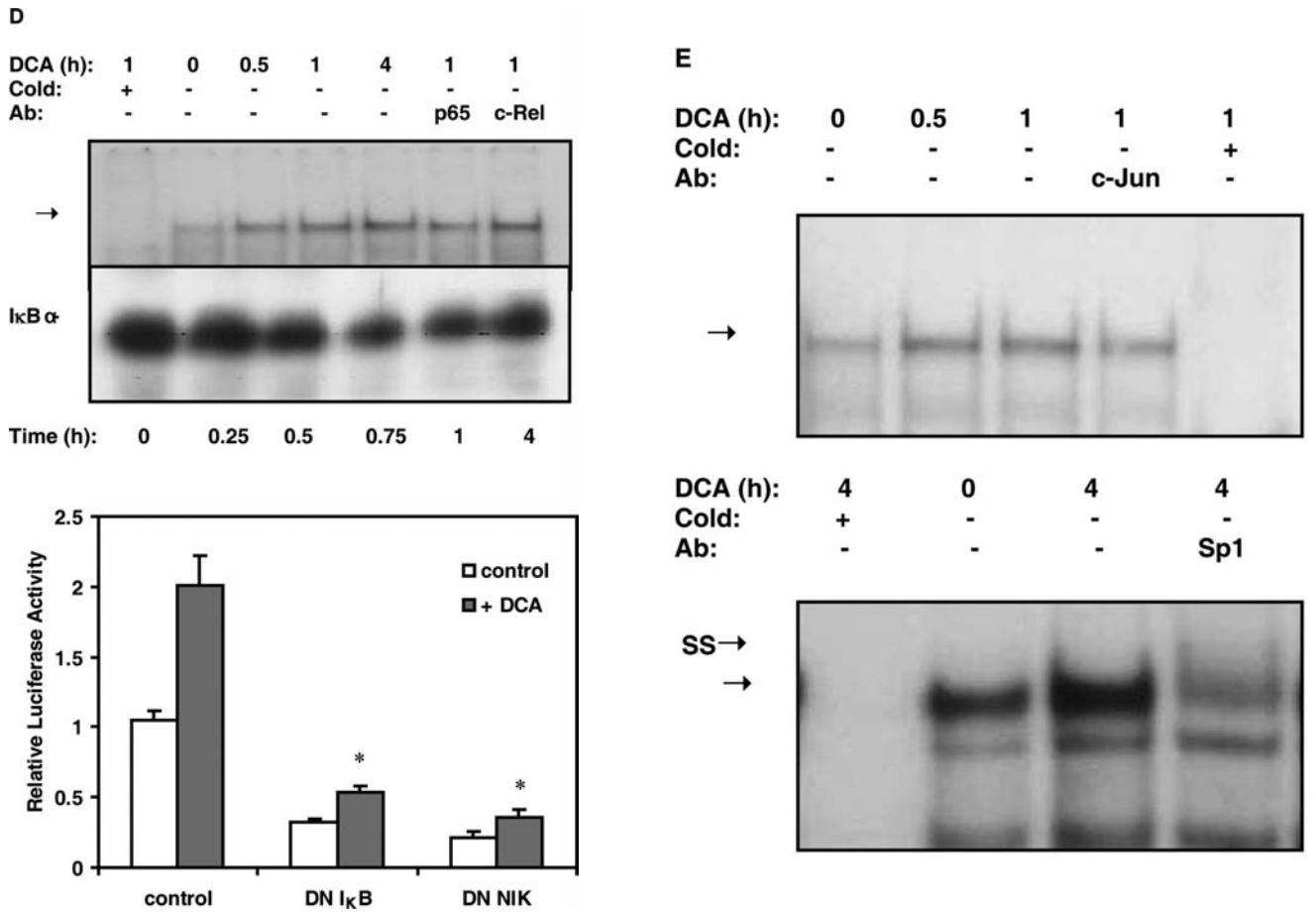

Figure 5. (D) EMSA using a double-stranded oligonucleotide probe corresponding to MUC2 NF-κB site (-1528/-140 bases) in the MUC2 promoter was performed. 100-fold concentration of unlabeled (cold) oligonucleotides were added to demonstrate the specificity. Antibodies used for supershifts are indicated. Arrows indicate the shifted and the original bands. (D) Western blot shows that IκBα is degraded in response to DCA treatment. Co-transfection with MUC2 reporter (-2864/+19) promoter construct and expression vectors encoding dominant-negative IκB or dominant-negative NIK in HM3 cells inhibited DCA-induced MUC2 upregulation. *P<0.05. (E) EMSA using double-stranded oligonucleotide probes corresponding to MUC2 AP-1 and Sp1 sites in the MUC2 promoter (described in the Materials and methods) was performed. No supershift was observed, but a slight decrease in band intensity was observed with c-Jun antibody but supershift was observed with Sp1 antibody.

Discussion

In colorectal carcinogenesis and inflammation, abnormal synthesis and secretion of mucin, one of the major functions of mature colonocytes have been reported (3,4). Secondary bile acids, such as DCA, which have been implicated in tissue injury and colorectal carcinogenesis have also been reported to cause altered expression of mucins such as MUC2, a major goblet cell mucin, in colon cancer cells (15,16). Bile acids have been reported to have diverse effects on the cells in the liver and gastrointestinal tract, such as oxidative stress, apoptosis and cell proliferation mediated by various cell signaling pathways (8,27).

We previously reported that the PKC/Ras/NF-κB pathway activated MUC2 synthesis in response to phorbol 12-myristate 12-acetate (PMA) (28) or TNF-α (20). In the present study we have shown that DCA regulates MUC2 transcriptional activity through several different pathways, some positive and some negative. Moreover, this is the first detailed analysis of signaling pathways utilized by bile acids in colonic epithelial cells as summarized in Fig. 6. Since diverse signaling pathways and cross-talk were involved in DCA regulation of MUC2 expression, we have divided our discussion into several sections dealing with specific pathways.

We first examined the possible involvement of activation of the mitogen-activated protein kinases (MAPKs), such as ERK1/2, p38 and c-Jun in DCA-induced MUC2 regulation. Significant inhibition of DCA-induced MUC2 transcription by dominant-negative MEK1 and MEK1 inhibitors (PD98059 and U0126) indicates the involvement of MEK1 in DCA-stimulated MUC2 transcription. MEK1 can activate both ERK1 and ERK2 and in our study, both dominant-negative ERK1 and dominant-negative ERK2 inhibited DCA-stimulated MUC2 transcription. An additional study, showing the inhibition of DCA-induced MUC2 upregulation by a specific inhibitor of ERK2 (AG126), further supports the involvement of MEK1/ERK1/2 in DCA-induced MUC2 transcription.

The MEK/ERK signaling pathway can exert proliferative or antiproliferative effects by targeting the downstream transcription factors such as, NF-κB, CREB, AP-1, Ets-1 and c-Myc (29). In the present study, we determined that the transcription factor, CREB is involved in DCA-induced MUC2 upregulation, because dominant-negative CREB inhibited DCA-induced MUC2 upregulation. Activation of

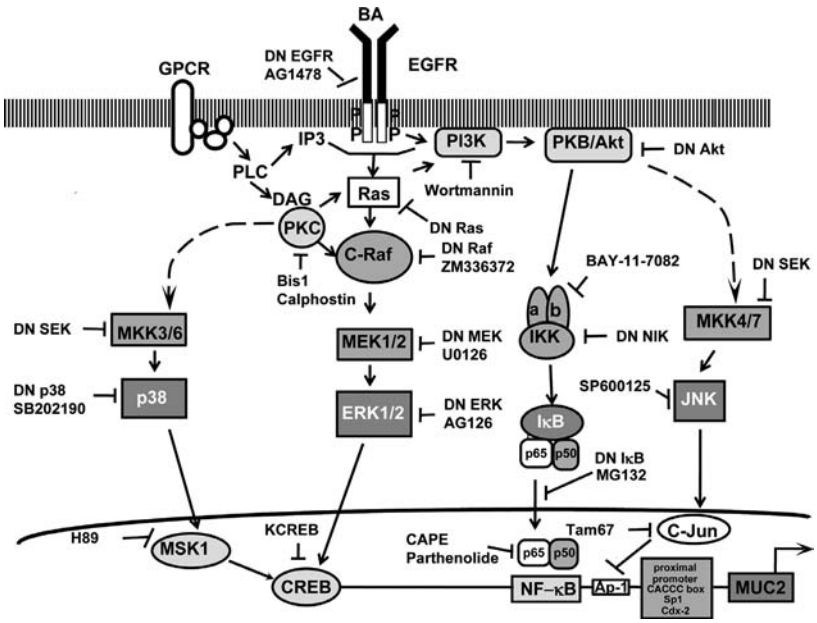

Figure 6. The pathways proposed to be involved in DCA mediated regulation of MUC2 transcription. Solid arrows indicate pathways demonstrated in this study, dotted arrows show possible but unproven pathways and blocked lines indicate inhibitory agents or pathways.

CREB may be mediated, either by direct phosphorylation of CREB or by enhancing translocation of p-CREB by activation of transcriptional coactivators, CBP/p300 (29,30).

With regard to p38 and JNK, significant inhibition by both dominant-negative p38 and chemical inhibitors of p38 indicated the involvement of this kinase in DCA-stimulated MUC2 transcription. However, specific JNK inhibitor (SP600125) enhanced MUC2 promoter-reporter activity, suggesting negative involvement of JNK in MUC2 transcription. Upstream of p38 and JNK, MKK3/6 or MKK4/7 are known to play a significant role (31). Among these, we focused on MKK4 (SEK) because it activates both p38 and JNK (31,32). The observation that dominant-negative SEK inhibited DCA-induced MUC2 activation suggests that the positive effects of p38 activation effectively counterbalance the apparent negative effects of JNK. Negative cross-talk between these two MAP kinase has been reported. For example, TNF-α-induced VCAM-1 expression is oppositely regulated by p38 and JNK in HTB-94 cells (33). Moreover, the p38 pathway was reported to inhibit JNK in NIH3T3 cells (34). AP-1 is a homo- or hetero-dimeric transcription factor composed of members of the Jun and Fos family of DNA-binding proteins (35,36). JNK is known to play an important role in AP-1 activity through phosphorylation of c-Jun (36,37). Our result showing negative regulation of DCA-induced MUC2 transcription by dominant-negative c-Jun indicates that JNK inhibits DCA-induced MUC2 upregulation via activation of c-Jun.

Bile acids have been reported to alter mitochondrial bioenergetics that result in the increased formation of reactive oxygen species causing oxidative stress in the cells (38,39). However, oxidative stress-mediated activation of stress-related MAPKs, p38 and JNK (40) appears not to be involved, since antioxidants (glutathione and N-acetyl-L-cysteine) did not inhibit DCA-induced MUC2 transcription, indicating that the upstream pathways other than oxidative stress is involved. Reactive oxygen species have also been reported to inhibit protein tyrosine phosphatases as these enzymes have a sensitive cysteine residue within their active site (8,41). Indeed, cell extracts prepared from hepatocytes treated with DCA had a decreased ability to dephosphorate the EGFR. Because protein phosphatase generally have much higher turnover numbers compared with protein kinases (42), this might account for the increased 'steady-state' phosphoration of EGFR observed in cells treated with DCA. The three best-characterized signaling pathways induced through EGFR are Ras-mitogen-activated protein kinase (Ras-MAPK) (43), phosphatidylinositol 3' kinase-protein kinase B (PI3K-PKB/Akt) and phosphatase C-protein kinase C (PLC-PKC) pathways (44-46). In our study, dominant-negative EGFR and several inhibitors of EGFR inhibited DCA-induced MUC2 transcription, indicating the involvement of EGFR pathways.

We previously reported that PMA activates MUC2 through the Ras pathway (28). In the present study, downstream of EGFR, dominant-negative Ras inhibited DCA-induced MUC2 transcription, indicating that DCA-induced MUC2 transcription is Ras-dependent. Both Raf-1 and B-Raf, are serine/threonine kinases that function downstream of Ras, activating MEK1/2 and ERK1/2 (29). We observed that dominant-negative form of Raf-1 inhibited DCA-induced MUC2 transcription while that of B-raf did not, indicating that Raf-1 is involved in DCA-induced MUC2 upregulation. The involvement of PI3K/Akt in DCA-induced MUC2 upregulation was demonstrated by the inhibition by an inhibitor of PI3K (Wortmannin) and by transient transfection with dominant-negative Akt and Western blotting using antibody to Akt.

We concluded that cAMP was not involved in DCA-induced MUC2 promoter activation, since neither, adenylate cyclase activator, forskolin nor cAMP agonist, 8Br cAMP induced MUC2 transcription. A broad spectrum inhibitor of

PKA, H89 significantly inhibited DCA-induced MUC2 promoter activity but H89 is also an inhibitor of MSK1 and ROCK. As specific inhibitors of ROCK and PKA did not inhibit DCA-induced MUC2 transcription, MSK1 which acts downstream of p38 seems to be involved in DCA-induced MUC2 transcription. PKC, a key enzyme involved in the signaling mediated by G-protein coupled receptors (GPCR) coupled to phosphoinositide-specific phospholipase C (PI-PLC) has been reported to be activated by the secondary bile acids such as DCA but not by the primary bile acids (47,48). The inhibitor studies (Bis 1 and calphostin) indicate the involvement of PKC in DCA-induced MUC2 upregulation. PKC activation by DCA may be mediated either by the activation of EGFR or GPCR or both. With respect to the downstream targets of PKC, p38 has been reported to activate CREB through MSK1 (49). Thus, our study indicates that PKC/p38/MSK1/CREB pathway may be involved in DCA-induced MUC2 up-regulation. An additional possibility is that because p38 inhibits JNK, the relief of negative regulation by JNK may result in the up-regulation of MUC2 transcription by p38. The negative regulation of MUC2 by JNK could also explain why DCA activates MUC2 transcription less effectively than PMA, which does not simultaneously activate JNK (28).

Transcription factor NF-κB plays a crucial role in the regulation of many genes involved in inflammation and cancer and can be activated by a variety of stimuli, including cytokines, viral proteins and stress inducers (50,51). NF-κB has also been reported to be activated by DCA in esophageal cells (52). A functional NF-κB site was previously identified at base pairs -1441 to -1452 of the MUC2 promoter (17). The transcriptional competence of NF-κB at its putative *cis* element was demonstrated using promoter-reporter constructs containing this region of the MUC2 promoter (-1528/-1430) cloned upstream of the minimal thymidylate kinase promoter. The involvement of NF-κB was further demonstrated by chemical inhibitors, CAPE, parthenolide and MG132. Phosphorylation of IκB by IKK triggers the degradation of IκB through the ubiquitin system, resulting in the nuclear translocation of free NF-κB to activate transcription (53). The Western blot showing rapid degradation and resynthesis of IκB and inhibition of MUC2 transcription by co-transfection with dominant-negative IκBα in HM3 cells treated with DCA indicate the involvement of IκB in DCA-induced MUC2 upregulation. An inhibitor of IKK (Bay-11-7082) and dominant-negative NIK also caused inhibition of DCA-induced MUC2 transcription indicating the involvement of classic IKK/IκB/NF-κB pathway. In addition, EMSA studies together with the data obtained with dominant-negative c-Jun indicated that transcription factors, AP-1 and Sp1 are also involved in DCA-induced MUC2 regulation.

This is the first comprehensive analysis of DCA-induced regulation of MUC2 transcription in colon cancer cells, showing that DCA induces MUC2 transcription by activation of ERK and p38 MAP kinases, while JNK activation leads to inhibition of DCA-induced MUC2 upregulation. Downstream of these kinases, several transcription factors are involved in DCA-induced MUC2 regulation, such as CREB, NF-κB, Sp1 and AP-1. Thus, the present study indicates that DCA induces MUC2 upregulation in colon cancer cells by activation of at least three pathways, EGFR/PKC/Ras/Raf-1/MEK1/ERK/CREB, p38/MSK1/CREB and EGFR or GPCR/PI3K/Akt/IκB/NF-κB. By contrast, JNK/c-Jun/AP-1 pathway inhibits DCA-induced MUC2 transcription. The molecular mechanisms involved in the cross-talk among these pathways remain to be elucidated.

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