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EphA2 Up-regulation induced by deoxycholic acid in human colon carcinoma cells, an involvement of extracellular signal-regulated kinase and p53-independence

Abstract *Purpose*: The EphA2 receptor protein tyrosine kinase gene has been shown to be over-expressed or functionally altered in a number of human tumors, including colon cancer, but little is known about the regulation of this new oncoprotein. In order to explore the mechanism of EphA2 up-regulation in cancer cells, we examined the change of expression of EphA2 gene induced by deoxycholic acid (DCA) and elucidated its possible pathways in human colon cancer cells. *Methods*: Western blot and RT-PCR were used to assess the protein expression and messenger RNA in several colon cancer cell lines, which harbor various p53 status. The inhibition study to interfere the MAPK pathway was performed by using various chemicals and by transfecting dominant negative mutant plasmids. *Results*: Up-regulation of EphA2 induced by DCA was observed in a dose- and time-dependent fashion both in mRNA and protein levels. This regulation is constant regardless of p53 status including wild, mutant or knocked out in the colon cell lines used. This induction was in part blocked by either erk1/2 inhibitors or dominant negative mutants erk1/2 plasmids. *Conclusions*: These results suggest that DCA induced up-regulation of EphA2 in colon cancer cells is due to activation of erk1/2 cascade, and is p53-independent. Taken together with the roles of EphA2 and DCA in tumorigenesis, which have been independently reported, our observation will provide a new mechanistic basis of DCA commitment in carcinogenesis.

Keywords Colon cancer · EphA2 · Deoxycholic acid · Extracellular signal-regulated kinase · p53

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Introduction

The Eph receptors constitute the largest subfamily of receptor tyrosine kinases-RTKs, with 14 related members (Cheng et al. 2002; Holder & Klein 1999; Wang et al. 2002). A growing body of evidence supports the importance of the concerted actions of ephrins and Eph molecules in tumor angiogenesis, morphogenesis (Cheng et al. 2002; Holder & Klein 1999) and oncogenic transformation (Zelinski et al. 2001). Over-expression of Eph receptors and elevated Eph activity has been found in a number of tumors, suggesting the role of Eph receptors in tumorigenesis (Walker-Daniels et al. 2003). Among them, the EphA2 gene has been reported as being frequently over-expressed in various human tumors, including breast, esophageal, prostate, and lung cancers (Walker-Daniels et al. 2003). Our recent unpublished observation using semi-quantitative RT-PCR also indicated that EphA2 was overexpressed in considerable number of all colon cancer cases. As far as we know, the mechanism of expression of the EphA2 oncogene remains unknown, except that it is negatively regulated by c-Cbl (Walker-Daniels et al. 2002; Wang et al. 2002), c-Myc and estrogen (Zelinski et al. 2002), and camptothecin(CPT)-induced up-regulation (Dohn et al. 2001). While screening for agents to influence EphA2 expression, we discovered that deoxycholic acid (DCA), a well-known constituent of bile acid and cancer promoter, up-regulates EphA2 expression.

Among the secondary bile acids, DCA has been particularly implicated in the promotion of colon tumorigenesis, which is supported by epidemiological (Martinez et al. 1998), mutagenic (Lechner et al. 2002), cell signaling (Yoon et al. 2002), and carcinogenesis studies (Qiao et al. 2001b). However, the molecular mechanisms of all these effects have not yet been fully evaluated.

In this paper, we report, for the first time, that the EphA2 tyrosine receptor kinase gene is upregulated

by DCA in several colon cancer cell lines and we further analyzed the possible pathways of this phenomenon.

Materials and methods

Chemicals and antibodies

The sodium salt of DCA and CPT were purchased from Sigma Chemical (St. Louis, Mo., USA). They were dissolved in water as a 100 mM of stock solution. A series of chemical inhibitors: PD98059 (to MEK1), U0126 (to MEK1/2), SB202190 (to p38 MAP kinase), and JNK inhibitor-1 (JNKI-1, to c-Jun-N-terminal kinase), were from Calbiochem (Darmstadt, Germany). These chemicals were stored as stock solutions in dimethyl sulfoxide. Anti-EphA2 (Clone D7) was purchased from Upstate Biotechnology (Upstate, N.Y., USA). Rabbit polyclonal antibodies against erk1/2, p38, and JNK total proteins or the corresponding phospho-specific antibodies which recognize activated phosphorylated MAPKs isoforms, were purchased from Cell Signaling Technology/New England Biolabs (Beverley, Mass., USA). Anti-p53 was from Novocastra Lab Ltd. (Newcastle, UK).

Cell line, culture condition, and treatment

The human colon carcinoma cell lines HCT116 p53−/− and p53+/+ are kind gifts from Dr. Bert Vogelstein (John Hopkins University Cell Center, Baltimore, Md., USA) (Bunz et al. 1998). Other colon cancer cell lines DLD-1, SW620 were purchased from ATCC or RIKEN CELL BANK (Wako, Saitama, Japan). As recommended by the instructions from ATCC, HCT116 was maintained in McCoy'S 5A medium, DLD-1 was maintained in Dulbecco's modified Eagle's medium (DMEM), and SW620 was maintained in L-15 medium. All the media were supplemented with 10% fetal bovine serum (FBS), 2 mmol/l L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C. All the experiments for detection of protein, DNA, and mRNA were conducted with cells grown to 85–95% confluence. The p53 status in these cell lines was confirmed with PCR-SSCP (polymerase chain reaction-single stranded conformation polymorphism) and sequencing (Dobashi et al. 1994). HCT116 had the wild type p53, while the other two cell lines harbored mutant p53 in different sites: DLD-1 at Ser241Phe, SW620 at Arg273His, and Pro309Ser, respectively. Treatment of cells with chemical inhibitors was performed by pre-treatment with inhibitors for 30 min prior to addition of DCA unless otherwise specified. Cells treated with dimethyl sulfoxide were considered as the untreated controls.

Transient transfection

Dominant negative (DN) mutant erk2 plasmid: 19-II-pCR-bluntII-TOPO-erk2$^{T188A/Y190F}$ was a kind gift from Prof. Nishida E. (Kyoto University, Kyoto, Japan) and Prof. Matsuda M. (Osaka University, Japan). Another DN-erk2: pCMV5-erk2^{K52R} and pCEP4-MEK2 wild type plasmids were kind gifts from Prof. Cobb M.H. (Univ. of Texas Southwestern Medical Center, Dallas, Tex., USA). Transient transfection was conducted by cationic lipid-mediated DNA transfection using LipofectA-MINE™2000 (GIBCO Life Technology, Gaithersburg, Mass., USA) according to the protocol recommended by the manufacturer. The green fluorescent protein-harboring plasmid DNA, pEGFP-N_3, was co-transfected with target plasmid DNA (1:3) as the indicator. Thirty-six hours after transfection, cells were incubated with or without DCA for 12 h, and then the cell lysates were harvested.

Western blot analyses

Cells were treated with DCA for the indicated time periods. Collected cells were lysed in a lysis buffer containing 20 mM Tris-HCl (pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM sodium vanadate, 10 mM sodium fluoride, 10 μg/μl phenylmethylsulfonyl fluoride (Boehringer, Mannheim), 10 μg/μl aprotinin, and 10 μg/μl leupeptin). The whole cell extracts were quantitated by a Bio-Rad protein assay (Bio-Rad, Richmond, Calif., USA), an aliquot of 50 μg total cellular protein was subjected to SDS-PAGE in 8% gels and electrophoretically transferred to a nitrocellulose membrane. After blocking with 4% milk, the membrane was incubated with the first antibodies, washed with T-TBS (10 mM Tris, 0.15 M NaCl, 15 mM HCl and 0.05% Tween-20), incubated with the secondary antibody conjugated with alkaline phosphatase, and then developed for visualization.

### Extraction of total RNA and semi-quantitative RT-PCR analysis

Total RNA was extracted from colon cancer cells using Isogen RNA extract kits (Wako Pure Chemical Industries, Osaka, Japan) according to the methods recommended by the manufacturer. RNA was quantified spectrophotometrically.

Synthesis of the first strand of cDNA and PCR analysis were performed according to the method described previously (Li et al. 2002). All the PCR primer sets spanned different exons to exclude the artifact due to genomic DNA contamination. As an internal control, a set of primers for the 18SrRNA gene (5′-AT-GCTCTTAGCTGATGTCC-3′ and 5′-AACTACGACGTATCT-GATC-3′) was used. According to the genomic structure obtained from NCBI, a set of EphA2 primers to detect the coding region (5′-GCAACATCCTCGTCAACAGC-3′ and 5′-TGGCTTTCAT-CACCTCGTGG-3′) was used and a 260-nucleotide product was generated. Both genes were simultaneously and independently amplified in a 20-μl PCR reaction mixture at different cycle numbers to ensure that the assay was conducted in the linear range for each molecule tested. Following amplification, 10 μl aliquots were analyzed by electrophoretic separation on 2% agarose gels containing 0.5 μg/ml ethidium bromide. Under an electronic UV transilluminator, the gel was photographed using a digital image video camera (Fuji Photofilm, Japan). Quantitative image analysis of the PCR fragments on the gel was performed using the public domain NIH image 1.62 program. After obtaining the value of each band, the ratios of EphA2 :18S rRNA were calculated.

Results

Up-regulation of EphA2 protein induced by DCA in HCT116 cell line

To determine the effect of DCA on expression of EphA2 protein, passaged HCT116 colon cancer cells were grown for about 24 h to 95% confluence. Subsequently, DCA was added into the medium at different doses at the indicated periods. After the cells were harvested, their protein level of EphA2 was determined using Western blotting analyses. As shown in Fig. 1, EphA2 protein was constitutively expressed in HCT116 cells. The protein levels significantly increased when cells were exposed to 150 μM DCA for 12 h and 16 h, respectively. Furthermore, this increase of EphA2 by DCA was dose- and time-dependent (Fig. 1A and Fig. 1B). Meanwhile, as shown in Fig. 1B, the EphA2 protein level became steady when the cells had been growing for 16 h, but
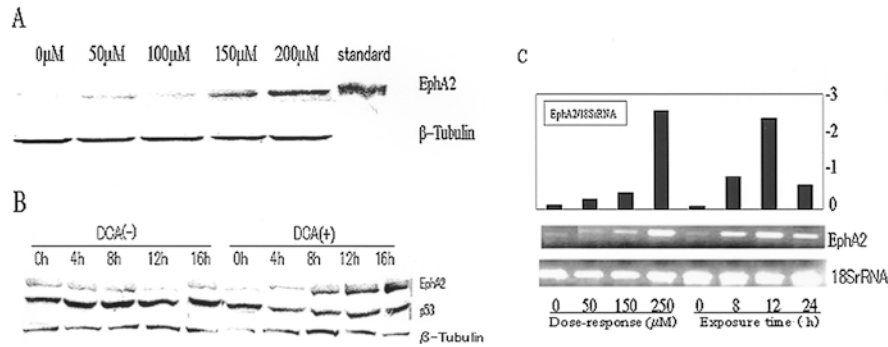
Fig. 1A–C DCA enhances EphA2 expression levels in human HCT116 colon cancer cell line. A HCT116 cells were cultured for 16 h in the presence and absence of DCA at various concentrations. Cell lysates were resolved by SDS-PAGE and immunoblot analysis performed for EphA2. β-Tubulin was performed as a control for protein loading, transfected wild type EphA2 protein was used as standard positive indicator; B HCT116 cells were treated with or without 150 μM of DCA for each indicated time period, and the cells were lysed. Immunoblot analysis was then performed for EphA2 and p53; C Dose response and time course effects of DCA on EphA2 mRNA expression in HCT116 cells. After cells were exposed to 0 μM, 50 μM, 150 μM, and 250 μM of DCA for 12 h (the **left four lanes**) or treated with 150 μM of DCA for 0 h, 8 h, 12 h, and 24 h (the **right four lanes**), respectively, total RNA was extracted and analyzed by RT-PCR. The normalized values indicate the relative abundance of EphA2 mRNA in untreated and DCA-treated HCT116 cells. Similar results were obtained in two or four independent experiments

when incubated with medium containing DCA, the EphA2 protein levels markedly increased. Therefore, the increase of EphA2 was not related to cell growth itself, but to the DCA-mediated effect.

The over-expression of EphA2 mRNA induced by DCA in HCT116 cancer cells

To determine whether an increase of EphA2 protein levels resulted from an increase in EphA2 transcript levels, the EphA2 mRNA level was evaluated with RT-PCR methods before and after DCA treatment. As shown in Fig. 1C, the expression of EphA2 mRNA in cells treated with DCA peaked at 12 h or at a 250 μM-dose exposure, with about a 24-fold or 28-fold greater increase than that in control cells. In a manner similar to the protein levels, the EphA2 mRNA levels also increased in a dose- and time-dependent manner (Fig. 1C). Taken together, these results demonstrated that DCA probably transcriptionally up-regulated the expression of EphA2 gene.

The DCA-mediated EphA2 induction is p53 independent

To investigate the correlation of DCA-induced EphA2 up-regulation and p53 function, the levels of p53 protein were meanwhile detected in various p53 status-containing colon cancer cell lines before and after DCA

treatment. As shown in Fig. 2, in the cells harboring p53 mutation, DLD-1 cells (with p53^{Ser241Phe}) and SW620 cells (p53^{Arg273His} and p53^{Pro309Ser}), the EphA2 protein levels greatly increased when treated with 200 μM of DCA compared with the untreated cells. The increase of EphA2 was in a dose-dependent manner. Moreover, when DCA up-regulated the expression of EphA2, it seemed to degrade the level of p53 protein, whether the p53 is wild type or mutant type (HCT116 in Fig. 1B, 2 vs DLD-1 in Fig. 2). More interestingly, EphA2 was also induced by DCA in the HCT116 p53−/− cell line (Fig. 2, Fig. 3B). This indicates that the up-regulation of EphA2 induced by DCA was independent on the p53 function, unlike the case in CPT induced EphA2 up-regulation (Dohn et al. 2001).

The involvement of erk1/2 in the over-expression of EphA2 gene by DCA

DCA has been reported to activate erk1/2 and p38 MAPK, other than JNK in the HCT116 cell line (Qiao et al. 2001a). In order to elucidate the mechanism of the DCA-induced up-regulation of EphA2 in HCT116 colon cancer cells, activation of erk1/2 and expression of EphA2 were compared after treatment with DCA. As shown in Fig. 3A and Fig. 3B, the increase in phosphorylated erk1/2 was observed within 1 h. The quantity of phosphorylated erk1/2 continued to increase gradually over time and appeared to reach to the peak and stably remained at that level between 4 h and 8 h after DCA treatment. On the other hand, EphA2 appeared to reach the peak at 12 h after exposure, later than the case
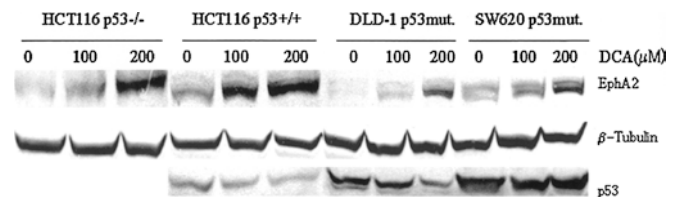
Fig. 2 p53 function and alteration of EphA2 induced by DCA. In four colon cancer cell lines, HCT116p53+/+ is the p53 wild type, HCT116p53−/− is p53 null type, while DLD-1 and SW620 are the p53 mutant types as shown in the materials and methods section. EphA2 protein and p53 protein were probed with western blot analysis before and after 100 μM and 200 μM of DCA treatment for 16 h. The blots represent two independent experiments
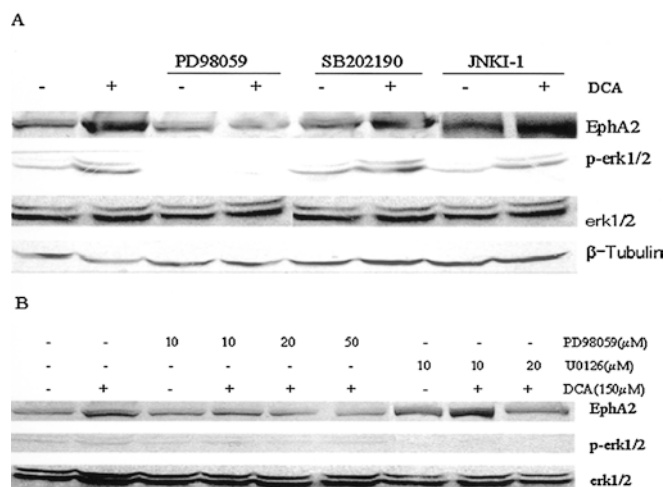
A

B
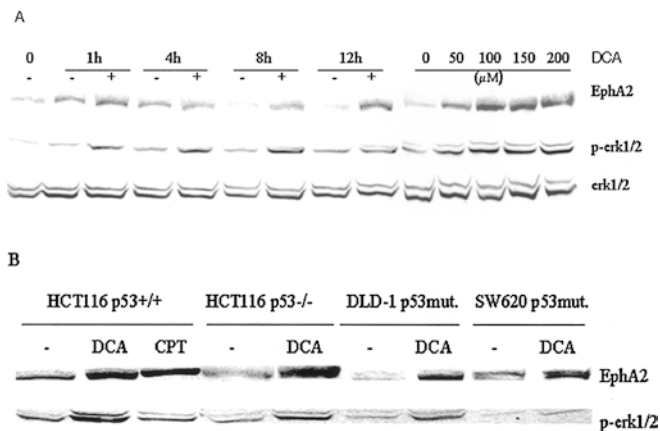
Fig. 3A,B Erk1/2 phosphorylation and EphA2 up-regulation. **A** With different DCA exposure periods and doses, EphA2 expression increased with the enhancement of erk1/2 phosphorylation levels; **B** In four human colon cancer cell lines which harbored different p53 statuses, EphA2 expression increased with enhanced erk1/2 phosphorylation regardless of the p53 status. CPT up-regulated EphA2, which was consistent with a previous report (Dohn et al. 2001), but did not activate erk1/2. Similar results were obtained in two independent experiments

A

B

Fig. 4A,B The effect of MAPK kinase inhibitors on the DCA-induced EphA2 expression. **A** HCT116 cells were pretreated with the following inhibitors for 30 min prior to DCA exposure. Cell lysates were separated by SDS-PAGE and immunoblot analysis performed for EphA2 and erk1/2; **B** Dose-dependent effects of PD98059 and U0126 on the inhibition of DCA-induced EphA2 expression were observed. Similar results could be repeated in two or three independent experiments
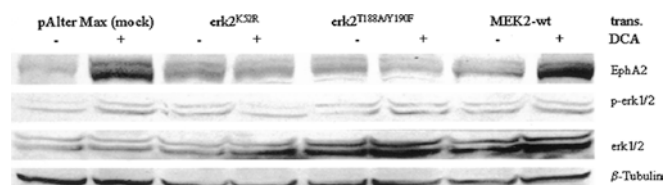
Fig. 5 Interfering effects of the dominant negative mutant erk2 constructs on the DCA-induced EphA2 expression. Thirty-six hours after transient transfection, cells were treated with DCA for 12 h, immunoblot analysis for the lysates was then performed for EphA2 and erk1/2. pAlterMax vector was used as a mock-transfected control. Similar results could be repeated in two independent experiments

with erk1/2 phosphorylation. This chronological relationship may suggest that the activation of erk1/2 MAPK might be an earlier event compared with the EphA2 up-regulation. Even in the HCT116 p53-null cell line, DCA activated erk1/2 and sequentially induced the EphA2 (as shown in Fig. 3B).

In order to confirm the significance of MAPK activation in the over-expression of the EphA2 gene, we investigated whether the inhibitors to MAPKs interfere with the induction of the EphA2 gene. PD98059 and U0126, specific inhibitors of MEK/ERK kinase, SB202190, a potent specific inhibitor of p38, and JNKI-1, an inhibitor of JNK, were employed to block DCA-induced erk1/2, p38 and JNK activation, respectively. After PD98059 or U0126 was added into the medium prior to the addition of DCA, the phosphorylation of erk1/2 markedly decreased, while there was no change in total erk1/2 levels. When the activation of erk1/2 was blocked, the expression of EphA2 was in part reduced in spite of the addition of DCA (Fig. 4A). Moreover, 50 μM of PD98059 and 20 μM of U0126 showed a higher inhibitory effect compared with 10 μM of the inhibitors, suggesting that the inhibitory effect shows a dose dependency (Fig. 4B). In contrast, SB202190 and JNKI-1 did not block the DCA-induced up-regulation of EphA2. These results indicated that among the 3 MAPK subfamilies, only erk1/2 takes part in DCA-induced EphA2 up-regulation.

Interference by the dominant negative mutant erk2 with DCA-induced EphA2 up-regulation

To further confirm the commitment of erk1/2 in the induction of EphA2, the dominant negative mutant erk1/2 was introduced into HCT116 cells to diminish the function of erk2 MAPK. After the cells were transfected with erk or MEK plasmid DNA, we monitored the stronger total erk1/2 levels, probably implying the successful transfection in this case. As shown in Fig. 5, after the cells were transfected with dominant negative mutant erk2^{K52R} or erk2$^{T188A/Y190F}$, DCA treatment did not increase the EphA2 expression effectively, while the wild type-MEK2 had no effect on the DCA-induced up-regulation of EphA2. Thus, erk1/2 MAPK is necessary for the up-regulation of EphA2.

Discussion

As a tumor promoter of human colon epithelial cells, DCA drastically alters the pattern of several gene expressions (Lechner et al. 2002; Yoon et al. 2002). Herein, we demonstrated that the EphA2 tyrosine

receptor kinase is one of the genes that undergo dramatic up-regulation following DCA treatment. The DCA-induced expression of EphA2 peaked at 16 h following DCA treatment. Further study demonstrated that DCA significantly increased the transcription level of EphA2. Meanwhile, we examined two ligands of EphA2, EphrinA1 and EphrinA4 by Western blot and RT-PCR methods and no up-regulation was found after treatment with DCA (data not shown). On the other hand, we did not find any over-expression of some other eph/ephrins induced by DCA, such as EphrinB2 and its EphB4 receptors (data not shown), although they were found to be over-expressed in colon cancer (Liu et al. 2002; Stephenson et al. 2001), Thus, DCA-induced upregulation is unique to EphA2 and it is not accompanied by concurrent overexpression of its ligands, this result supported the hypothesis: ligand binding is not necessary for EphA2 tyrosine kinase activity (Walker-Daniels et al. 2003). In addition to DCA, we tested three other bile acids, Urso-DCA, Cheno-DCA, and Taurocheno-DCA in our system. Among them, Urso-DCA also slightly induced the overexpression of EphA2 (data not shown).

In order to elucidate the mechanisms related to the DCA-induction of the EphA2 gene, we tested several pathways. Dohn et al. once reported that there was a p53 binding site within the EphA2 promoter and EphA2 may be up-regulated by members of the p53 family after activated by DNA damage agent (such as CPT, Dohn et al. 2001), although we found CPT did not activate erk1/2 afterwards (Fig. 3B). In addition to colon cancer HCT116p53+/+ and HCT116p53−/− cell lines, we further tested with mutant p53-harboring colon cancer cell lines: DLD-1, SW620. Interestingly, we found that DCA may induce over-expression of EphA2 regardless of the p53 status. Furthermore, when DCA up-regulated the EphA2, it seemed to promote the degradation of p53 probably by stimulating proteasome (Fig. 1B, Fig. 2). In our system, when the cell-permeable proteasome inhibitor, MG-132, was added to the DCA-harboring medium, the amount of EphA2 protein was reduced while p53 protein increased with dose-dependency (data not shown). Taken together, the DCA induced up-regulation of EphA2 gene occurs via a mechanism different from that induced by CPT, it is p53-independent (Dohn et al. 2001).

In colorectal cancer tissues and cells, bile acids may activate many signaling systems. An increasing amount of evidence suggest that bile acids may be a multiple stress-inducer (Crowley-Weber et al. 2002), and one of the best-documented pathways is the one involving protein kinase C (PKC) (Debruyne et al. 2001). Recently, many researchers reported that DCA might activate the Ras/Raf/ERK kinase pathway, then subsequently up-regulate some genes downstream (Yoon et al. 2002). In the present experiment, during the DCA activation of erk1/2-MAPK, we found activated-erk1/2 seemed to appear chronologically earlier than the induction of EphA2. When the specific inhibitors of erk1/2, PD98059 and U0126, was introduced into the mediums, the over-expression of EphA2 was blocked at least in part, but SB202190 and JNKI-1 did not exhibit any effect on EphA2 expression, suggesting that erk1/2, rather than the p38 and c-Jun-N-terminal kinase MAPK pathways, is probably involved in the EphA2 induction. Dominant negative erk2^{K52R} present a lower catalytic activity than that of wild type of erk2 (Robbins et al. 1993; Robinson et al. 1996). Another DN-erk2$^{T188A/Y190F}$ also disrupted the activity of erk2 phosphorylation (Adachi et al. 1999). Both of the dominant negative erk2 constructs blocked the induction of EphA2 by DCA (Fig. 5), suggesting that activation of extracellular signal-regulated kinase is required for the DCA induced up-regulation of the EphA2 gene.

In summary, these data demonstrate that DCA up-regulates EphA2, a new oncoprotein. Specifically, a novel mechanism was explored for how DCA induced increased expression of EphA2 in colon cancer cells. Our analyses further disclosed that erk1/2 MAPK was activated by DCA and played a role in inducing EphA2 expression in human colon cancer cells. Thus, our findings may imply a new perspective on the commitment of DCA and EphA2 in human colon carcinogenesis. Further studies are warranted to identify the transcriptional factors that may take part in the induction of EphA2.

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