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Biochemical and Biophysical Research Communications 315 (2004) 93–99
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Homeodomain protein CDX2 regulates COX-2 expression in colorectal cancer

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Received 19 December 2003

Abstract

CDX2 is an intestine-specific tumor suppressor gene encoding homeodomain-containing transcription factor, which is involved in a variety of developmental, proliferating, and differentiating processes. Moreover, the expression of CDX2 is reduced in a subset of primary colorectal cancers. In contrast, cyclooxygenase-2 (COX-2) is often up-regulated in human colorectal cancers. However, the molecular relationship between CDX2 down-regulation and COX-2 up-regulation is unknown. Here we show that CDX2 down-regulates COX-2 promoter activity by interacting with NF-κB. The ectopic expression of CDX2 was found to suppress PMA-induced COX-2 promoter activity in a dose-dependent manner. In addition, the treatment of colorectal cancer cells with PMA resulted in significant reduction in the level of endogenous CDX2 and a significant increase in the level of endogenous COX-2, in a dose-dependent manner. Furthermore, CDX2 was found to co-immunoprecipitate with the p65 subunit of NF-κB and to inhibit p65-induced NF-κB minimal promoter activity in colon cancer cells. These results suggest that reduced CDX2 expression may be involved in colorectal carcinogenesis by enhancing NF-κB-mediated inflammatory genes such as COX-2.

Keywords: CDX2; COX-2; Expression; Carcinogenesis; Colorectal cancer

The caudal-type homeobox gene, CDX2, encodes a transcription factor, which is expressed in the intestine, and seems to be critical during the development, differentiation, and for the maintenance of intestinal epithelial cells [1]. Previous studies suggest that CDX2 is a tumor-suppressor gene for the following reasons. First, heterozygous CDX2 knock-out mice give rise to a phenotype that includes multiple colonic polyps in the proximal colon, which have been reported to have features of hamartomas [2,3]. Second, the expression of CDX2 is reduced in a subset of primary colorectal cancers and was found to be attenuated in high grade colorectal cancers [4,5]. Third, CDX2 has been demonstrated to inhibit the proliferation of the undifferentiated intestinal cell line, IEC-6 cells, and human colon carcinoma cell line, HT-29 [6,7]. Finally, oncogenic *ras* is known to down-regulate CDX2 in human colon carcinoma Caco-2 and HT-29 cells [8].

COX-2 is essential enzyme in prostaglandin synthesis [9] and is induced by mitogens, cytokines, and growth factors [10,11]. COX-2 is usually absent or present only at low levels in normal colonic mucosa [12]. In contrast, COX-2 is often up-regulated in human sporadic colorectal cancers [13,14], and the expression of COX-2 mRNA is also markedly elevated in the tissues of human colorectal cancers as compared with the accompanying

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.01.020

normal mucosa [15]. Therefore, CDX2 and COX-2 may play important roles in colorectal cancers. However, the relationship between CDX2 and COX-2 expression remains elusive. We hypothesized that decreased CDX2 expression and increased COX-2 expression may be related to colorectal carcinogenesis.

In the present study, we investigated the molecular relationship between CDX2 down-regulation and COX-2 up-regulation.

Materials and methods

Cell culture and reagents. The human embryonic kidney (HEK) 293 cells, U87 cells, HCT-116, and SNU-C4 cells were obtained from the American Type Culture Collection (ATCC: Rockville, MD). The cells were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum. The cells were grown at 37 °C, 5% CO_2 in fully humidified air and subcultured twice weekly. Anti-COX-2 and anti-CDX2 antibodies were purchased from Cayman (Ann Arbor, MI) and Biogenex (San Ramon, CA), respectively. Other chemicals were purchased from Sigma (St. Louis, MO).

Western blot analysis. Cellular lysates were prepared by suspending 1×10^6 cells in 100 μl lysis buffer (137 mM NaCl, 15 mM EGTA, 1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM MOPS, and 2 μg/ml proteinase inhibitor E64, adjusted to pH 7.2). The cells were disrupted by sonication and extracted at 4 °C for 30 min. Fifty micrograms of cell lysate was electrophoresed on 10% SDS–polyacrylamide gels. The proteins were electro-transferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA). Detection of the specific proteins was carried out with an ECL kit following the manufacturer's instructions.

Co-immunoprecipitation assay. Cells were transfected with pcDNA3.1-CDX2 (in some cases, together with pcDNA3-p65 expression vectors). Cell extracts were prepared by incubation in lysis buffer (50 mM Tris, pH 7.8, 1% Nonidet P-40, 150 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail Complete (Roche)). The cell extracts were incubated with anti-CDX antibody (or with anti-p65 antibody in the case of cells transfected with pcDNA3.1-CDX2 together with pcDNA3-p65) and the immunocomplexes were then precipitated by protein G–Sepharose (Pharmacia). Western blots were probed with anti-CDX antibody and anti-p65 antibody.

Reporter plasmids and luciferase assay. COX-2 promoter constructs were generously provided by Dr. H. Inoue (National Cardiovascular Center Research Institute, Japan). Briefly, the region from −1432 to +59 bp of COX-2 promoter was cloned into pGL2 [16]. NF-κB minimal reporter constructs including immunoglobulin (Ig)-κB-luciferase and interferon-β (IFN-β)-κB-luciferase were provided by Dr. Baltimore. These plasmids contain four copies of NF-κB-binding sites from Ig and three copies of NF-κB sites from IFN-β, respectively [17,18]. Cells were transfected using Lipofectamine reagent according to the manufacturer's instructions. To assess the luciferase reporter activity, cells were collected and disrupted by sonication in lysis buffer (25 mM Tris–phosphate, pH 7.8, 2 mM EDTA, 1% Triton X-100, and 10% glycerol). After centrifugation, aliquots of the supernatants were tested for luciferase activity using the luciferase assay system (Promega, Madison, WI) according to the manufacturer's instructions. Transfection efficiency was monitored by β-galactosidase activity using the pcDNA3-lacZ plasmid.

Immunohistochemistry. Cells were grown on glass coverslips overnight and treated with 10 nM, 50 nM PMA or the same volume of DMSO as control. After treatment, cells were fixed with cold methanol at −20 °C for 5 min and then washed three times with 0.1% Tween 20 in PBS and twice with PBS alone. The fixed cells were incubated with monoclonal anti-CDX2 antibody (1:100) and polyclonal anti-COX-2 antibody (1:300) in 1% BSA/PBS at room temperature for 2 h. Slides then were rinsed in PBS three times and incubated for 30 min with a biotinylated horse anti-mouse IgG secondary antibody (Vector) diluted 1:200 in PBS. A 1 h incubation with the avidin–biotin peroxidase complex (Vector) according to the manufacturer's instructions was followed by incubation with DAB (3,3′-diaminobenzidine-tetrachloride; Sigma, St. Louis, MO) chromogen development for 2–3 min.

Results

CDX2 down-regulates COX-2 expression

To assess the relationship between CDX2 function and COX-2 gene expression, COX-2 promoter activity was analyzed and performed on HEK 293 cells and U87 cells. HEK 293 cells or U87 cells were transfected with pcDNA3.1-CDX1 and pcDNA3.1-CDX2, and with luciferase reporter plasmid pGL2-COX-2 containing 1.4 kb of the COX-2 promoter. The result showed that the luciferase activity of the CDX2 transfected cells decreased by 2.5-fold as compared to the controls in both cell lines (Figs. 1A and B). Both CDX1 and CDX2 suppressed COX-2 promoter activity, but CDX1 transfected cells revealed less COX-2 promoter suppression effect than that of CDX2 transfected cells (Figs. 1A and B). Accumulating evidence suggests that the activation of COX-2 promoter in response to various stimuli could be regulated by protein kinases, including protein kinase C (PKC) [19]. PKC activator alone has been shown to induce COX-2 expression, thus, we examined whether CDX1 and 2 suppress PKC-mediated COX-2 induction. Treatment with a PKC activator, PMA, alone activated COX-2 promoter activity up to 8-fold in HEK 293 cells and U87 cells. Increased promoter activities by PMA were also inhibited by CDX1 and CDX2 cotransfection (Figs. 1C and D). To further examine the effect of CDX2 on the suppression of COX-2 expression, HEK 293 cells or U87 cells were cotransfected with various amounts of pcDNA3.1-CDX2 construct in the presence of pGL2-COX-2. As shown in Figs. 2A and B, basal and PMA-induced COX-2 promoter activities were diminished by CDX2 in a dose-dependent manner.

CDX2 inhibits p65-induced NF-κB minimal promoter activity and co-immunoprecipitates with p65

To identify the *cis*-element responsible for the repression of COX-2 promoter activity by PMA treatment, we performed a luciferase reporter assay with serial deletion mutant reporters in the promoter region of COX-2. However, we failed to detect any *cis*-element (data not shown), which suggests that the transcriptional repression effect of CDX2 may occur in a

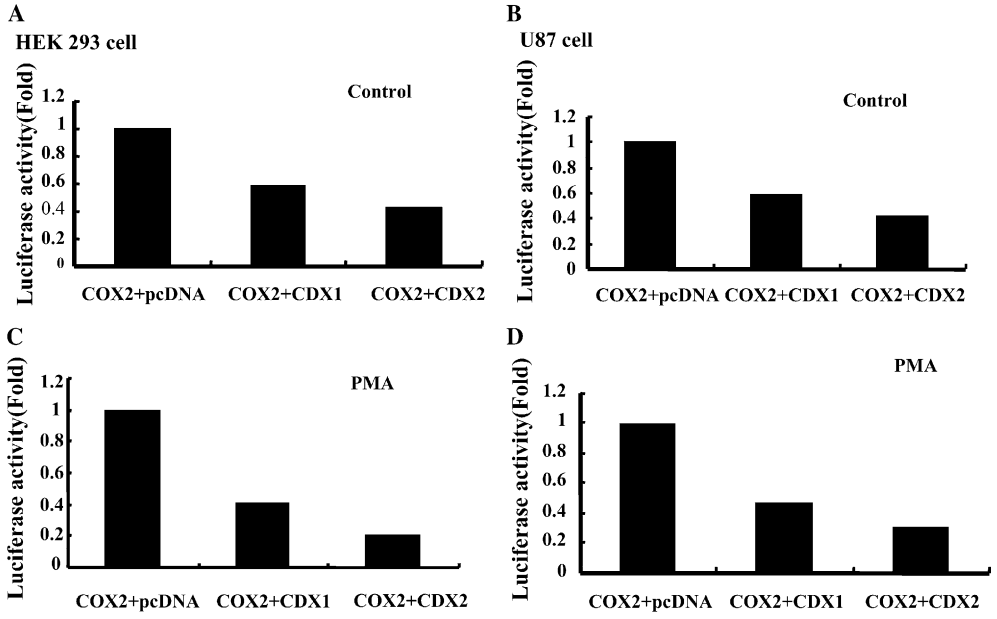

Fig. 1. Suppression of COX-2 expression by CDX2 in HEK 293 cells and U87 cells. HEK 293 cells (A) or U87 cells (B) were transfected with 1 μg of the reporter COX-2 plasmids and with 1 μg of pcDNA3.1, pcDNA3.1/CDX1, and pcDNA3.1/CDX2. HEK 293 cells (C) or U87 cells (D) were transfected and then treated with 50 nM PMA for 24 h. Cells were harvested and assayed for luciferase. Data are mean values obtained from three independent experiments.

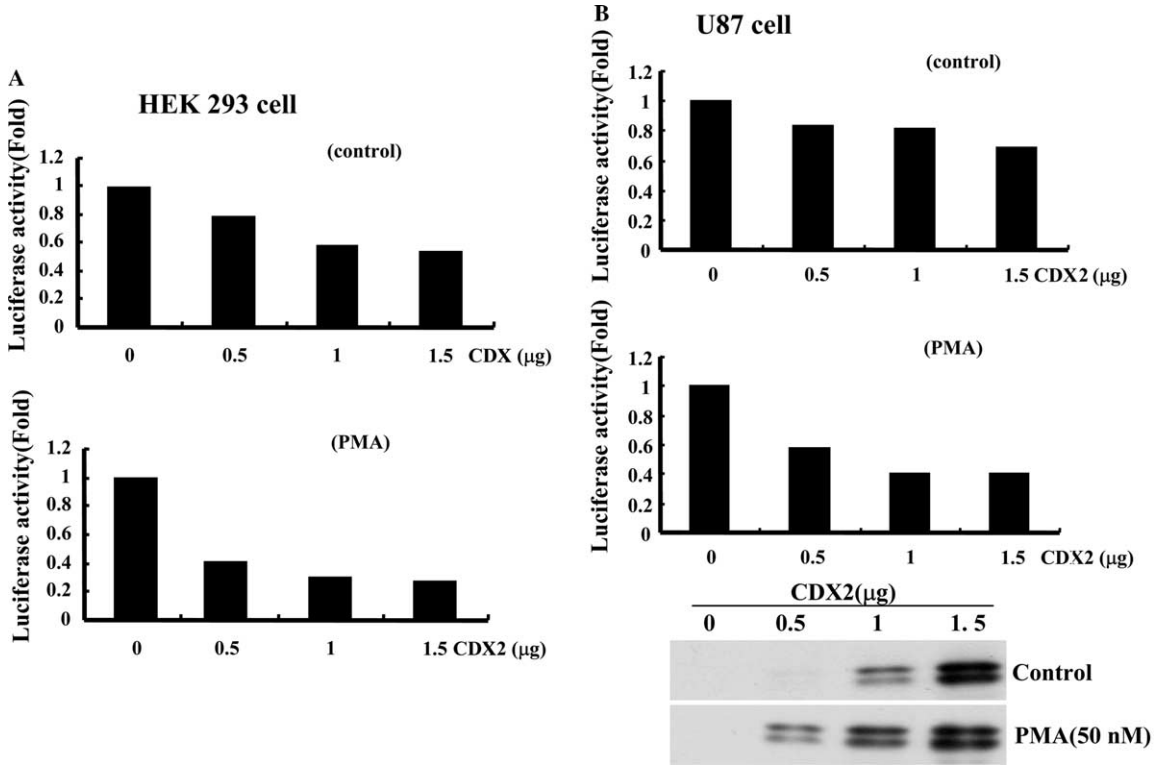

Fig. 2. Dose effect of CDX2 on COX-2 promoter activity. HEK 293 cells (A) or U87 cells (B) were transfected with 1 μg of the reporter COX-2 plasmids and with the indicated amount of pcDNA3.1/CDX2. Cells were incubated for 24 h in the presence or absence of 50 nM PMA, and then assayed for luciferase. Increasing CDX2 protein expression was monitored by Western blot analysis with anti-CDX2 antibody. Data are mean values obtained from three independent experiments.

DNA-binding-independent manner. As a previous report suggested that PMA-induced proinflammatory molecule production is mainly mediated by NF-κB in human intestinal epithelial cells [20], we reasoned that CDX2 may act as a non-DNA-binding co-repressor to inhibit the signal-induced NF-κB *trans*-activating capability. To test this possibility, we performed a luciferase reporter assay using two different NF-κB minimal promoters (IFN-β- and IgκB-) after PMA treatment in the presence or absence of CDX2 expression. The results showed that PMA-induced NF-κB reporter activity was almost completely abolished in the presence of CDX2 expression, in HEK 293 and HCT116 cell lines (Figs. 3A and B). Ectopic CDX2 expressions were found to have strong inhibitory effects on NF-κB reporter activity in a dose-dependent manner (Figs. 3A and B). We then investigated the effect of the p65 subunit of NF-κB on κB-dependent gene expression in the presence of CDX2 expression. As shown in Fig. 4A, CDX2 inhibited p65-induced luciferase gene expression in a dose-dependent manner. To understand the underlying molecular basis of this observed repression of p65-mediated activation, we investigated whether CDX2 and p65 physically interact with each other by using a co-immunoprecipitation assay. Immunoprecipitation with anti-p65 antibody (or anti-CDX2), followed by Western blot analysis, revealed that CDX2 interacts with p65 (Figs. 4B and C). These results indicate that CDX2 can form a protein–protein complex, directly or indirectly, with p65 and that their physical interaction may elicit transcriptional repression.

Inverse correlation between COX-2 and CDX2 expression in PMA treated colon cancer cells

To examine the in vivo significance of CDX2-mediated COX-2 expression in SNU-C4 cells, SNU-C4 cells were cultured in the presence or absence of PMA, and we examined CDX2 and COX-2 levels. The treatment of SNU-C4 colon cancer cells with PMA-induced COX-2 expression and a significant reduction of CDX2, in a dose-dependent manner (Fig. 5A). The immunocytochemical analysis showed the similar pattern as in the case of Western blot analysis (Fig. 5B), indicating inverse correlation between COX-2 and CDX2 expression in PMA treated colon cancer cells.

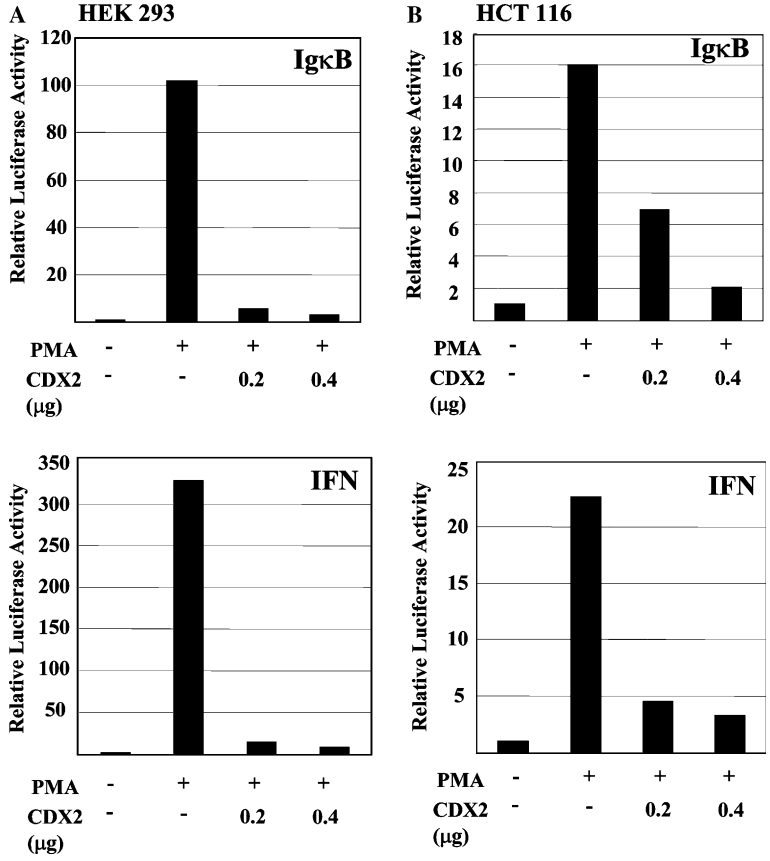

Fig. 3. The inhibition of PMA-induced NF-κB minimal reporter activity by the overexpression of CDX2 in HEK 293 (A) and HCT116 cells (B). IgκB-luciferase or IFN-κB-luciferase was co-transfected with the CDX2 construct in the presence of PMA treatment. Relative luciferase activity was measured as described in Materials and methods. Normalized luciferase activity in the absence of PMA treatment was taken arbitrarily as 1. Data are mean values obtained from at least three independent experiments.

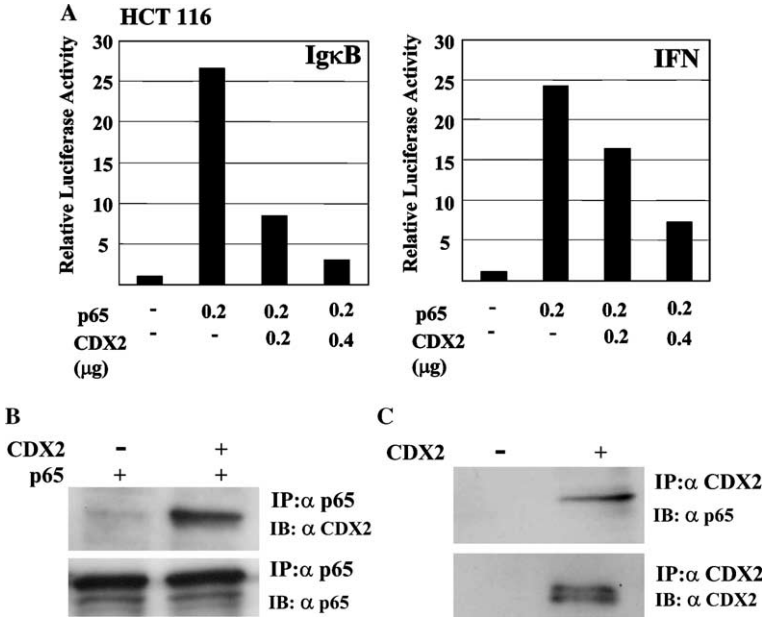

Fig. 4. (A) The inhibition of p65-induced NF-κB minimal reporter activity by the overexpression of CDX2 in HCT116 cells. Relative luciferase activity measurements were performed and normalized luciferase activity in the absence of p65 co-transfection was taken arbitrarily as 1. Data are mean values obtained from at least three independent experiments. (B) Immunoprecipitation of CDX2 by anti-p65 antibody. Caco-2 cells were transfected with p65 together with CDX2. Cells transfected with p65 alone were used as a control. Cell lysates were immunoprecipitated with anti-p65 antibody and subsequently subjected to Western blot analysis. Blots were probed with anti-CDX2 antibody and anti-p65 antibody. (C) Immunoprecipitation of endogenous p65 by anti-CDX2 antibody. Caco-2 cells were transfected with CDX2 and mock-transfected cells were used as a control. Cell lysates were immunoprecipitated by anti-CDX2 antibody and subsequently subjected to Western blot analysis. Blots were probed with anti-CDX2 antibody and anti-p65 antibody.

Discussion

CDX2, the intestinal-specific homeobox gene, is known to play crucial roles in the regulation of cell proliferation and differentiation in the intestine [1]. CDX2 seems to have a complex role in tumorigenesis in the gastrointestinal tract. Mice heterozygous for a germline, inactivating mutation of the *CDX2* gene develop multiple polyps in the small intestine and colon, with prominent involvement of the proximal colon [2,3]. Although studies on colorectal carcinomas arising in humans have not offered definitive proof of the causal role for CDX2 inactivation in the cancer process, it is apparent that the loss of CDX2 expression occurs in a subset of primary colorectal carcinomas, particularly large cell minimally differentiated carcinomas [21]. In addition, several recent studies on CDX2 have focused attention on the potential critical role of aberrant CDX2 expression in the development of intestinal metaplasia and some gastric carcinomas [22–24].

Cyclooxygenases are rate-limiting enzymes in the synthesis of prostaglandins. Two isoforms, COX-1 and COX-2, are involved in the process. COX-1 is the constitutive isoform and is expressed in various types of tissues, whereas COX-2 is an inducible enzyme [25]. The overexpression of COX-2 has been reported in colorectal carcinomas [15]. Studies in animal models indicate that COX-2 plays an important role in cell adhesion, apoptosis, and angiogenesis [26,27]. However, the molecular relationship between CDX2 and COX-2 in colorectal cancers is poorly defined. We hypothesized the inverse correlation between CDX2 and COX-2 expressions in colorectal carcinogenesis. To explore this hypothesis, we used a CDX2 promoter assay, a luciferase reporter assay using serial deletion mutant reporters, and immunohistochemistry. In this paper, we report that the homeodomain protein CDX2 interacts with the transcription factor NF-κB in the COX-2 promoter, and that the overexpression of CDX2 protein suppresses COX-2 transcription. This provides a first direct link between CDX2 and COX-2 expression. The serial deletion mutant reporter assay in the promoter region of COX-2 suggests that the transcriptional repressive effect of CDX2 may be DNA-binding-independent (data not shown). The COX-2 promoter region from −840 to +123 bp includes the binding sites for many transcriptional factors such as NF-κB, C/EBP, CRE/ATF, E-box, STAT3, and NF-IL-6 [16,28,29]. Recently, it was reported that COX-2 expression can be regulated through different signaling pathways, and that the particular signaling pathway involved is dependent on the type of stimulus [30–32]. We have shown in the present experiments that ectopic CDX2 expression suppresses COX-2 gene expression by inhibiting NF-κB activity. This inhibition of NF-κB activity was

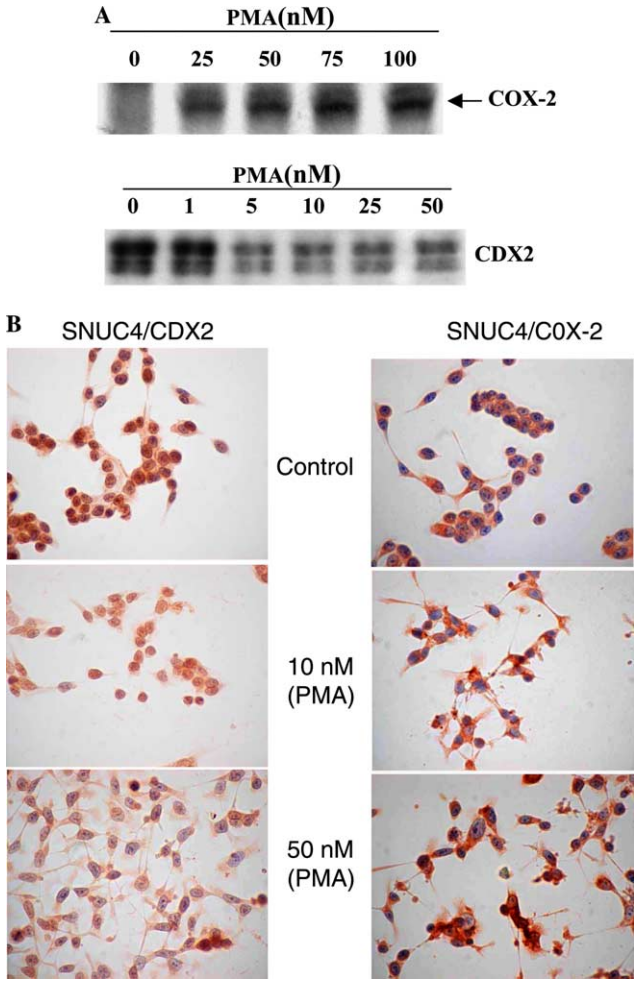

Fig. 5. Inverse relation between COX-2 and CDX2 expression in PMA treated colon cancer cells. (A) SNU-C4 cells were treated with the indicated concentrations of PMA for 24 h. The cells were lysed and the lysates were analyzed by immunoblotting using anti-CDX2 and COX-2 antibodies. The results presented are representative of three independent experiments. (B) SNU-C4 cells were treated with the indicated concentrations of PMA for 24 h. The cells were then stained with anti-CDX2 and COX-2 antibodies.

mediated by the CDX2 protein complex, either directly or indirectly with p65 NF-κB.

In conclusion, these results clearly show significant roles of CDX2 in colorectal cancer. Reduced CDX2 expression may be involved in colorectal carcinogenesis by enhancing NF-κB-mediated inflammatory genes such as COX-2.

Acknowledgments

This work was supported by Grant No. R13-2002-028-01002-0 from the Basic Research Program of the Korea Science and Engineering Foundation (MRC), and research-promoting grant from the Keimyung University Dongsan Medical Center in 2002. W.J.L. received support from Center for Cell signaling Research and a research grant from Ewha Womans University.

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