Cdx2 homeodomain protein regulates the expression of MOK, a member of the mitogen-activated protein kinase superfamily, in the intestinal epithelial cells☆

Toshihiro Uesaka*, Noriko Kageyama

Department of Cellular Biology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima 734-8553, Japan

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Abstract **Regulatory protein kinases are involved in various cellular processes such as proliferation, differentiation, and apoptosis. Using cDNA differential display, we identified MOK, a member of the mitogen-activated protein kinase superfamily, as one of the genes induced by a caudal-related homeobox transcription factor, Cdx2. Analysis of the 5′-flanking region of the MOK gene led to the identification of primary Cdx2 responsive element, and an electrophoretic mobility shift assay indicated that Cdx2 binds to that element. The interaction of Cdx2 with the MOK promoter region was further confirmed in vivo by chromatin immunoprecipitation assays. The expression of MOK mRNA and protein was limited to the crypt epithelial cells of the mouse intestine. We also determined the MOK activity associated with the growth arrest and induction of differentiation by sodium butyrate or Cdx2 expression in the human colon cancer cell line HT-29. Taken together, these data indicate that MOK is a direct target gene for Cdx2, and that MOK may be involved in growth arrest and differentiation in the intestinal epithelium.**

Keywords: Crypt; Differentiation; Target gene; IEC-6; ICK

1. Introduction

Cdx2 is a member of the caudal-related homeobox gene family due to its sequence homology to the *caudal* gene of *Drosophila melanogaster* [1]. This gene encodes a transcriptional factor that plays an important role in the development and homeostasis of the intestinal epithelium [2]. The Cdx2 gene is expressed in the epithelial cells of the intestine and colon, with undetectable levels in other tissues [1]. Cdx2 activates intestinal-specific gene expression and may direct normal intestinal epithelial development and differentiation [3,4].

In order to further understand Cdx2 function in determining the fate of epithelial cells in the intestine, we attempted to identify downstream target genes regulated by Cdx2. Differential display PCR approaches were used to characterize the effects on gene expression profiles following the overexpression of Cdx2 in IEC-6 cells, an undifferentiated intestinal cell line with very low levels of endogenous Cdx2 expression. One gene for MOK [5], a member of the Mitogen-activated protein (MAP) kinase superfamily, was found to be activated following Cdx2 overexpression. The MAP kinases are critical in signal transduction pathways involved in the regulation of cellular proliferation, differentiation, and apoptosis. MOK is similar (about 40% identity) to previously identified protein kinases, male germ cell-associated kinase (MAK; [6]), MAK-related kinase (MRK; [7]), and intestinal cell kinase (ICK; [8]), and possesses a Thr–Glu–Tyr (TEY) motif in the activation loop domain similar to those of the classical MAP kinases. However, MAP kinase-stimulating treatment of cells including serum addition, anisomycin treatment, and osmotic shock did not significantly activate MOK [5], suggesting that MOK may be indirectly involved in MAP kinase cascades, or possibly serves as a component of a different signal transduction pathway. To date, the physiological functions of this kinase remain largely unknown.

In the present study, a reporter gene assay, atomic force microscopy (AFM), an electrophoretic mobility shift assay (EMSA), and a chromatin immunoprecipitation assay (ChIP) were performed to determine whether Cdx2 interacts with the MOK promoter and upregulates MOK mRNA. Localization of the MOK gene and protein in the intestine was observed by RNA in situ hybridization and immunohistochemical staining. These findings may provide insight into important roles played by MOK in the differentiation of intestinal epithelial cells.

2. Materials and methods

2.1. Cell culture

The rat intestinal cell line, IEC-6, and the human colon cancer cell line, HT-29, were obtained from RIKEN Bioresource Center (Tsukuba, Japan) and from ATCC (Manassas, VA), respectively. IEC-6 cells were maintained under an atmosphere of 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum (FCS; P.A.A. Wiener Strasse, Linz, Austria), 0.1 units/ml insulin, 100 units/ml of penicillin, and 100 μg/ml streptomycin (Invitrogen, Grand Island, NY). HT-29 cells were grown in DMEM containing 10% FCS. Upon reaching 90% confluency, cells were

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* Corresponding author. Present address: Laboratory for Neuronal Differentiation and Regeneration, Center for Developmental Biology, Riken Kobe, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan.
E-mail address: uesaka-t@cdb.riken.jp (T. Uesaka).

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dissociated with 0.25% trypsin and 0.02% EDTA and replaced. Sodium butyrate (Wako, Kyoto, Japan) was used at a final concentration of 4 mM. The stably transfected cell line that expresses Cdx2 conditionally was established with the Tet-Off Gene Expression System (BD Biosciences Clontech, Palo Alto, CA, USA) as described previously [9].

2.2. RT-PCR

RT-PCR was performed with oligo-dT-primed cDNA. Gene-specific primers, Cdx2, glyceraldehyde-3-phosphate dehydrogenase (G3PDH), MOK, and ICK primers were designed as follows: Cdx2 forward, 5′-GGCGAAACCTTTGTGAATGG-3′; Cdx2 reverse, 5′-GTGAAATTCCTTCTCCAGCTCC-3′; G3PDH forward, 5′-ACCACAGTCCATGCCATCAC-3′; G3PDH reverse, 5′-TCCACCACCCTGTTGCTGTA-3′; rat MOK forward, 5′-CGGAGTGTCTATTCCAAGC-3′; rat MOK reverse, 5′-CATTCACACCAGGAAAGAG-3′; human MOK forward, 5′-GAGCTAATACGAGGGAGAAG-3′; human MOK reverse, 5′-TCAGTAGAGGTATTCCTGATCC-3′.

Northern blot analysis. For each sample, 10 μg of total RNA was separated on 1% formaldehyde-agarose gel and transferred to Hybond-N^+ (Amersham, Buckinghamshire, UK) by capillary action in 20× SSPE buffer (where 1× SSPE was 0.149 M NaCl, 0.001 M EDTA, and 0.01 M phosphate buffer, pH 7.7) for 12 h. Membranes were rinsed in 2× SSC (1× SSC is 0.149 M NaCl and 0.015 M sodium citrate, pH 7.0) and were briefly air-dried, and the RNA was cross-linked by UV irradiation. An anti-sense cRNA probe labeled with digoxigenin was generated from 161 bp of the PCR products (448–619) of MOK inserted into the pGEM-T Easy vector (Promega, Madison, WI) using a Dig RNA labeling kit (Roche Diagnostic, Indianapolis, IN) by means of in vitro transcription. After prehybridization, the membranes were hybridized overnight at 65 °C in a Rapid-hyb buffer (Amersham) containing the DIG-labeled probe and 0.1 mg/ml denatured salmon sperm DNA (Sigma, St. Louis, MO). Membranes were then washed twice with 2× SSC and 0.05% sodium dodecyl sulfate (SDS) at 58 °C for 20 min, as well as twice with 0.1× SSC and 0.1% SDS at 50 °C for 30 min. DIG detection was performed by using a DIG Luminescent Detection Kit, as described by the manufacturer (Roche Diagnotics).

2.3. Reporter assay

The 5′-flanking region of the human MOK gene between +183 and −677 was obtained from a human BAC clone 59L23 (Invitrogen). The DNA fragments of the MOK genomic clone were obtained by PCR, confirmed by sequence analysis, and subcloned into the multiple-cloning site of the promoterless vector pGL3 basic (Promega) to generate the reporter constructs pGL3/MOK 677 (−677/+183), pGL3/MOK 340 (−340/+183), pGL3/MOK 101 (−101/+183), and pGL3/MOK 77 (−77/+183). Preparation of mouse sucrase-isomaltase (SI) promoter-luciferase vector (pGL3/SI 130) was described previously [9]. The mutant reporter vectors, in which Cdx2-binding element from −84 to −88 (5′-**TTTAC**-3) is disrupted (5′-**TGCAC**-3), were constructed from pGL3/MOK 340 and pGL3/MOK 677 using a site-directed mutagenesis kit, QuickChange (Stratagene, La Jolla, CA). For the luciferase assay, HT-29 Tet-Off/Cdx2 cells plated 24 h prior to transfection at 2× 10^5 cells/well in 60 mm-well plates were co-transfected with 1 μg of the firefly luciferase reporter plasmid and 50 ng of the *Renilla* luciferase reporter plasmid pRL-TK (Promega) by using Effectene according to the manufacturer's instructions (Qiagen, Hilden, Germany). After 24 h of incubation, the cells were lysed in lysis buffer supplied by the manufacturer, followed by measurement of the firefly and *Renilla* luciferase activities on a luminometer TD-20/20 (Turner Designs, Sunnyvale, CA). The relative firefly luciferase activities were calculated by normalizing the transfection efficiency according to the *Renilla* luciferase activities. The experiments were performed in triplicate and similar results were obtained from at least three independent experiments.

A full-length, wild-type human Cdx2 and Cdx1 were amplified by RT-PCR from normal human colon total RNA. The Cdx2 and Cdx1 cDNA were inserted into the multiple cloning site of the expression vector pcDNA3.1 (Invitrogen). Transfection of cells at 60–80% confluency was performed with Effectene, pGL3/MOK 677, pRL-TK, and pcDNA3.1/Cdx2 or pcDNA3.1/Cdx1. At 24 h after transfection, cells were collected for luciferase assay.

2.4. Atomic force microscopy experiments

pGL3/MOK677 was digested to obtain the *Eco*RI fragment, which was subjected to 2% agarose gel electrophoresis. An 800 bp DNA band was then cut and purified using Wizard SV Gel and PCR Clean-Up System (Promega). The purified DNA sample was heated at 60 °C for 5 min and then rapidly cooled on ice. Purified Cdx2 protein and β-galactosidase was mixed with the DNA in 10 mM Tris–HCl buffer (pH 7.2) for 5 min at room temperature and diluted 50 times to final concentrations of 8 nM DNA and protein. Aliquots (10 μl) of the mixture were then dripped onto a mica substrate coated with spermine, rinsed with 1 ml of water, and blown with dry nitrogen gas. AFM analysis was carried out using a Nanoscope IIIa Multimode system (Digital Instruments Inc., Santa Barbara, CA, USA) in the tapping mode at room temperature in air. Silicon cantilevers of 125 μm in length with a spring constant of 42 N/m were purchased from NanoWorld (Neuchâtel, Switzerland). Typical resonant frequencies of these tips were 320 kHz. The 512 × 512 pixel images were collected at a rate of two scan lines per second. The contour lengths of the protein-DNA complex were determined using Image Gauge (FUJIFILM, Tokyo, Japan).

2.5. Electrophoretic mobility supershift assay

Nuclear extracts were obtained as described previously [10]. Cells were harvested and resuspended in buffer A (10 mM HEPES/KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), and Protease Inhibitor Cocktail; Sigma). The supernatant was removed by centrifugation at 600×*g* for 5 min and the nuclear pellet was resuspended in buffer B (20 mM HEPES/KOH, pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, and Protease Inhibitor Cocktail). The Cdx2 binding consensus region probe consisted of a double-stranded 30 bp oligonucleotide (5′-GATTTGCACAGT**ATTTACA**TGGAAGCGGAG-3′) from −101 to −72 of the human MOK gene. The sequence of the mutant oligonucleotide was 5′-GATTTGCACAGT**ATGCACA**TGGAAGCGGAG-3′. The probes were labeled with a DIG Oligonucleotide labeling kit (Roche Diagnostics) according to the manufacturer's instructions. The nuclear extract (2 μg) was incubated in the presence of 1 ng DIG-labeled probe with 0.5 μg poly dI–dC, 5 mM HEPES/KOH, pH 7.9; 0.25 mM KCl, 0.05 mM EDTA, 0.125 mM PMSF, 10% glycerol, and 100 μg/μl salmon testis DNA, for 15 min at room temperature. DNA–protein complexes were loaded on 6% polyacrylamide gel. For DIG detection, gels were transferred by electroblotting for 30 min at 40 mA to Hybond-N^+ membranes. DIG detection was performed as described by the manufacturer (Roche Diagnotics). Mouse monoclonal anti-Cdx2 antibody (BioGenex, San Ramon, CA) was used to detect Cdx2 protein.

2.6. Chromatin immunoprecipitation assay

HT-29 cells were transfected with Cdx2 expression plasmid containing a human cytomegalovirus promoter and Cdx2 open reading frame or vector alone. Chromatin immunoprecipitation assays were performed according to the manufacturer's recommendations (Upstate Biotech, Lake Placid, NY, USA). In brief, 24 h after transfection, cellular chromatin was cross-linked to the DNA by adding formaldehyde to a final concentration of 1%. Precipitated chromatin was incubated with the anti-Cdx2 antibody overnight at 4 °C. Then, the immune complexes were recovered by the addition of 60 μl of salmon sperm DNA/protein G-sepharose slurry, followed by incubation for 1 h at 4 °C. After washing the beads, the immune complexes were eluted by incubation for 15 min at 25 °C with 500 μl of elution buffer (1% SDS, 100 mM $NaHCO_3$, and 10 mM dithiothreitol (DTT)). To reverse the cross-linking of DNA, elutes were added to 50 μl of 5 M NaCl and incubated for 4 h at 65 °C, followed by treatment with proteinase K for 1 h at 45 °C. Quantitative PCR was carried out for 35 cycles using 5 μl of sample DNA solution and the PCR products were separated on 2% agarose gels in 1× Tris-acetate/EDTA.

2.7. In situ hybridization

Four- to six-week-old mice were sacrificed and the duodenum and ileum were surgically dissected. These tissues were fixed in Bouin's solution and processed through a standard embedding protocol. A series of 4-μm sections were cut for analysis. Hybridization was carried out in a hybridization solution containing 50% formaldehyde, 1× Denhardt's solution, 300 mM NaCl, and 30 mM sodium citrate kept at 50 °C overnight. Hybridized DIG-labeled cRNA was detected with an alkaline phosphatase-conjugated anti-DIG Fab fragment antibody (Roche Diagnotics), and the signals were visualized using 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphatase (Roche Diagnotics) as the chromogenic substrates.

2.8. Immunohistochemical staining

Following the manufacturer's instructions, we used the Vectastain Universal Quick kit (Vector Laboratories, Burlingame, CA) to detect primary antibodies on mouse tissue. Formalin-fixed, paraffin-embedded tissues were deparaffinized and hydrated. Antigen enhancement was performed by boiling the slides in a microwave oven for 6 min in 10 mM citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide in methanol for 20 min. After being washed and incubated with blocking reagent for 1 h, the slides were incubated with primary antibody for 1 h at room temperature. Mouse monoclonal anti-Cdx2 antibody (BioGenex) and rabbit polyclonal anti-MOK antibody (generously provided by Dr. Yoshihiko Miyata, Kyoto University) were used at 1:500 and 1:250 dilution, respectively. Sections were counterstained with hematoxylin and were then evaluated by light microscopy.

2.9. In vitro protein kinase assay

The open reading frame encoding for mouse MOK was inserted into a pENTR vector (Invitrogen) and then inserted into a pDEST 26 plasmid (Invitrogen) to obtain 6× His-tagged MOK. Plasmids were introduced into HT-29 cells by Effectene. The purification of 6× His-tagged protein was performed by anti-6× His and anti-MOK immunoprecipitation. The lysates were mixed with antibodies against 6× His-tag or MOK overnight. Protein G-Sepharose (Amersham) suspension (50%) was added to the mixture, which was further incubated at 4 °C for 90 min. The antibody-bound beads were washed thoroughly and were used for the in vitro protein kinase assay. Myelin basic protein (MBP), a substrate for phosphorylation, and PhosphoThreonine Antibody for the immunodetection of phosphorylated proteins were purchased from Upstate and Qiagen, respectively. To test the phosphorylation of MBP by MOK kinase, MBP was incubated with MOK at 30 °C for 60 min (50 mM Tris–HCl, pH 8.0, 100 mM NaCl, and 20 mM β-glycerophosphate). The final composition of the kinase assay mixture was 0.04 mM ATP. Reactions were stopped by the addition of SDS sample buffer to the samples, which were boiled for 5 min. The phosphorylation of the proteins was analyzed by Western blot analysis.

3. Statistics

All of the results are presented as the means ± S.E. Data were analyzed using the Mann–Whitney *U* test. Statistical values of $P < 0.05$ were considered significant. Statistical analysis was performed with SPSS software (SPSS, Chicago, IL).

4. Results

4.1. MOK expression induced by Cdx2

We previously demonstrated that the induction of Cdx2 expression in IEC-6 cells, an undifferentiated rat intestinal epithelial cell line, results in the expression of various genes [9]. To identify other genes that are induced by Cdx2, we used the cDNA differential display method to compare patterns of mRNA expression between IEC-6 cells that express Cdx2 and those that do not. For these experiments, we used the IEC-6 Tet-Off/Cdx2 cell line that we engineered to conditionally express Cdx2 when doxycycline (Dox) was removed from the culture medium [10]. Under one condition of differential display, PCR amplification was performed with the primers 5′-CATTATGCTGAGTGATATCTTTTTTTTTGA-3′ and 5′-ATTAACCCTCACTAAATGCTGGTGG-3′. A band was detected in the lane of IEC-6 Tet-Off/Cdx2 cells expressing Cdx2, but not in the lane of uninduced cells (Fig. 1A). Approximately 260-bp cDNA was reamplified, subcloned, sequenced, and identified as the 3′-untranslated region of the rat MOK mRNA. The differential expression of MOK was characterized further by RT-PCR and Northern blot analysis (Fig. 1B and C). IEC-6 Tet-Off/Cdx2 cells cultured in medium containing Dox expressed very low levels of MOK mRNA. By 24 h after the induction of Cdx2 expression, two transcripts were observed, which were approximately 2.0 and 4.0 kb, respectively. An additional larger band was reported in the heart, brain, kidney, and pancreas [5]. It is possible that the larger transcript may have resulted from alternative splicing or cross-hybridizing a different member of the MOK family. The Cdx2-induced MOK gene expression was also observed in

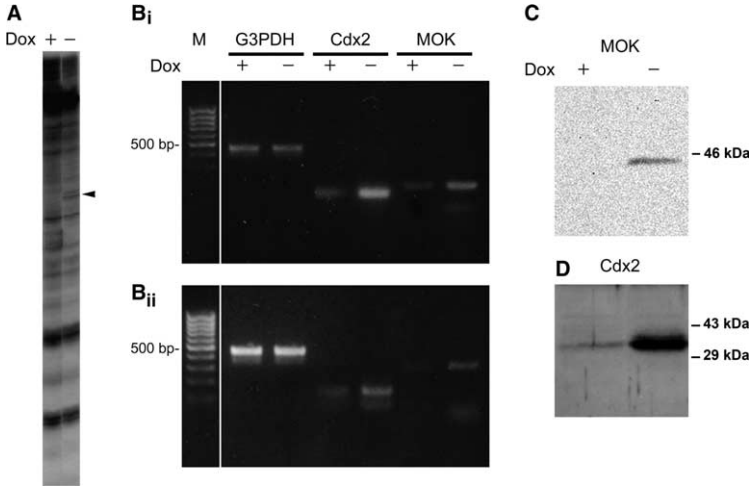

Fig. 1. Induction of MOK gene expression by Cdx2. (A) Differential display analysis of IEC-6 Tet-Off/Cdx2 mRNA. IEC-6 Tet-Off/Cdx2 cells were grown in the presence and absence of induction medium (Dox-free) and the RNA was isolated after 1 day. The arrow indicates differentially expressed cDNA, which was isolated for reamplification. (B) Effects of Cdx2 expression on MOK gene induction in the rat IEC-6 cells (i) and human HT-29 cells (ii). Total RNAs were isolated from IEC-6 Tet-Off/Cdx2 cells or HT-29 Tet-Off/Cdx2 cells treated with Dox (+) or vehicle alone (−) for 24 h and were analyzed by semi-quantitative RT-PCR. PCR was performed for 30 cycles for amplification. (C) MOK and Cdx2 proteins expression in IEC-6 Tet-Off/Cdx2 cells treated with (Dox+) or without (Dox−) for 24 h. Equal amounts of protein from cellular extracts were fractionated by SDS–10%PAGE. A polyclonal MOK antibody detects the roughly 46 kDa MOK protein in the Cdx2-induction medium (−) for 24 h but not in the basal solution (+).

human colorectal carcinoma cells, HT-29 Tet-Off/Cdx2 cells (Fig. 1D).

4.2. Identification of one Cdx2-responsive region in the MOK promoter

To determine whether or not the MOK gene is a potential target of Cdx2 transactivation, the ability of the human MOK promoter to drive transcription in HT-29 cells was tested using various lengths of the 5′-flanking region of the human MOK gene linked to the luciferase reporter gene (Fig. 2). Within the 677-bp 5′-flanking region of the MOK gene contained in the pGL3/MOK 677 plasmid, there were three Cdx2-binding element-like regions (TTTAC/T; [2,11]). The pGL3/MOK 340 and pGL3/MOK 101 plasmids contained only one Cdx2-binding element-like region. Fig. 3A shows the effects of Cdx2 expression on human MOK gene promoter activity. The basal promoter activities of those 5′-flanking regions of the human MOK gene were quite low. The minimal MOK promoter, containing −77 to +183, was not able to activate transcription. Removal of the sequence containing the Cdx2 binding elements between −677 and −340 did not change the Cdx2-induced activation of the promoter. The mutant (pGL3/MOK 340 Mut and pGL3/MOK 101 Mut), in which the Cdx2-binding element from −84 to −88 (5′-**TTTAC**-3) was disrupted (5′-**TGCAC**-3), revealed reduced Cdx2-induced promoter activity.

Another caudal-related homeobox gene, Cdx1, has the same homeodomain, which binds the same DNA sequence as Cdx2. We examined whether MOK was specific target of Cdx2 or was common downstream target of both Cdx2 and Cdx1. As shown in Fig. 3B, both of the Cdx2 and Cdx1 expression led to active transcription significantly by the minimal sucrase-isomaltase promoter containing one Cdx-binding motif. On the other hand, Cdx2 expression activates transcription by the MOK promoter significantly, whereas the Cdx1 expression exhibited quite a low transcription activity.

4.3. In vitro protein interaction with the Cdx2-responsive elements

An 800-bp DNA fragment of the MOK promoter (Fig. 4A) and Cdx2 protein were used for Nanoscope III AFM, which made Cdx2:DNA complexes images. Under the binding condition with β-galactosidase (100 kDa; as non-specific control), most of the DNA molecules appear with no protein attached (Fig. 4B, i). A typical image of Cdx2 interacting with MOK promoter DNA is shown in Fig. 4B, ii–iv. Most of the DNA–protein complexes showed one protein complex (ii and iii). Three protein complexes were not very often observed (iv). To further determine whether or not Cdx2 proteins in IEC-6 Tet-Off/Cdx2 cells bind to the caudal motif of the human MOK promoter gene, we incubated DIG-labeled DNA containing the caudal motif 1 and nuclear extracts from IEC-6 Tet-Off/Cdx2 cells in the absence or presence of Cdx2 antibody

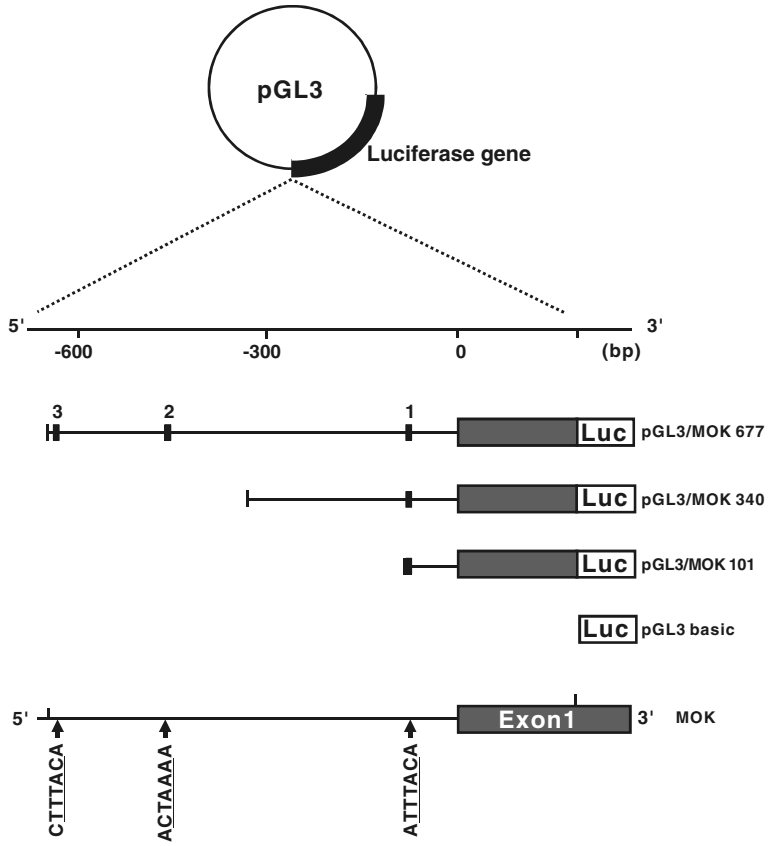

Fig. 2. Structure of the MOK promoter-luciferase fusion plasmids. The pGL3/MOK 677 (−677/+183), pGL3/MOK 340 (−340/+183), and pGL3/MOK 101 (−101/+183) plasmids contained 677-, 340-, and 101-bp fragments, respectively, of the human MOK gene promoter plus the first 183 bp of the exon 1 untranslated region, linked to a luciferase reporter. Cdx2-responsive element-like regions located in the MOK gene promoter region are shown (■). The plasmid pGL3-Basic is a negative control plasmid containing no MOK promoter.

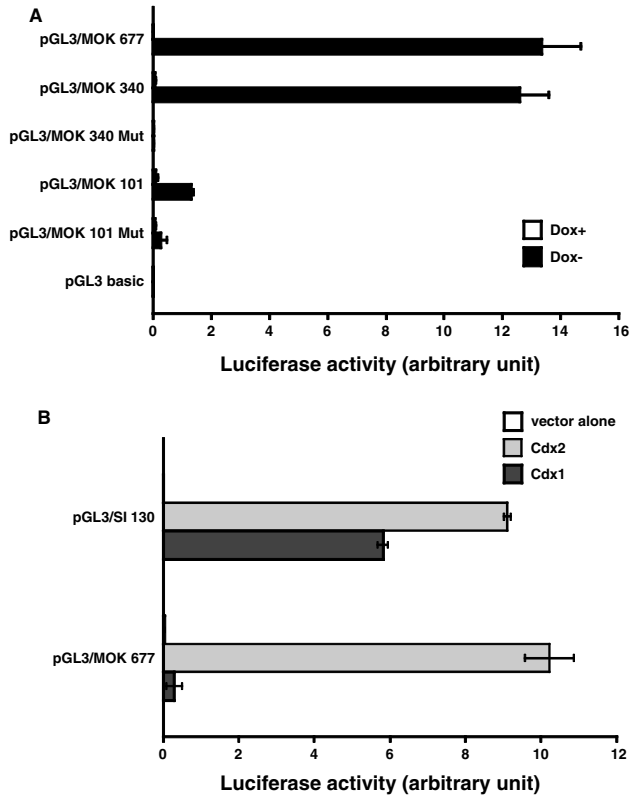

Fig. 3. Effects of Cdx2 expression on human MOK gene promoter activity. (A) Key Cdx2-responsible element for MOK transcription in HT-29 cells. Mutation was introduced to the Cdx2-responsive element-like sequences in the MOK gene promoter within the pGL3/MOK 340 and pGL3/MOK 101 plasmids, and the mutant reporter plasmids (pGL3/MOK 340 Mut and pGL3/MOK 101 Mut) were obtained. Luciferase activity was measured in HT-29 Tet-Off/Cdx2 cells co-transfected with the pRL-TK *Renilla* luciferase reporter and various luciferase reporters, as indicated in Fig. 2. The ratio of firefly luciferase activity to control *Renilla* luciferase activity is indicated (in arbitrary units), in response to the expression of Cdx2 (■) or no induction (□). All experiments are expressed as the means ± S.E. of triplicate cultures. (B) Luciferase assay of human MOK promoter (−677/+183) and minimal mouse SI promoter (−130/+45) in response to Cdx2 or Cdx1. MOK or SI promoter-luciferase constructs (pGL3/MOK 677 or pGL3/SI 130) were transfected into HT-29 cells with the pRL-TK and pcDNA3.1/Cdx2 or pcDNA3.1/Cdx1. Results are expressed as means ± S.E. of four independent experiments.

(Fig. 4C). The probe alone migrated rapidly. A DNA–protein complex of slower mobility was detected. Cdx2-antibody recognized the nuclear protein bound to the Cdx2-region probe, resulting in a reduction in gel mobility. Unlabeled oligonucleotide provided in 100-fold excess could compete to the DIG-labeled probe, whereas an excess of mutant oligonucleotide was not able to compete for nuclear protein binding, which was recognized by Cdx2 antibody (resulting in a reduction of gel mobility).

4.4. Identification of proteins interacting in vivo with the Cdx2-responsive region

Since the EMSA performed on the Cdx2-responsive region in the MOK promoter provided clear evidence of the interaction of Cdx2 with that site in vitro, we further confirmed to assessed protein interactions with the responsive sequence in vivo using a chromatin immunoprecipitation (ChIP) assay. Protein-DNA complexes crosslinked by treatment with formaldehyde were immunoprecipitated with Cdx2 antibody. The DNA fragment of the 5′-flanking region of MOK could be amplified by PCR (Fig. 4D), as was the positive control (*Input*), whereas that immunoprecipitated without antibody was not amplified by PCR (*No antibody*). The specificity of the recovery of these promoter regions following ChIP with anti-Cdx2 antibody was verified by the fact that other irrelevant DNA fragments lacking Cdx2 binding sites (e.g., the G3PDH gene) were not recovered. These results indicate that Cdx2 binds directly to the MOK promoter.

4.5. MOK expression in the intestinal crypt

Small intestine tissue was subjected to RNA in situ hybridization with mouse MOK sense and antisense probes. These probes were generated to avoid potential cross-reactivity with other kinases. The hybridization of the antisense MOK probe was detected specifically in the crypt compartment of the small intestine (Fig. 5A), whereas the sense MOK probe was not detected (Fig. 5B). We also examined the expression of MOK protein in the mouse small intestine and stomach. In the intestine, Cdx2-positive epithelial cells were observed, except for in the lower portion of crypts containing Paneth cells (Fig. 5C). Similarly, MOK protein was detected in the intestinal epithelial cells (Fig. 5D), whereas Cdx2 and MOK expression was not detected in the epithelial cells of normal stomach tissue (Fig. 5E). Although the highly predominant immunostaining of MOK protein was observed within the lamina propria, this staining was seen in the control experiment with preimmune serum or biotin-streptavidin detection kit (data not shown). Therefore, the staining in the lamina propria seems to be non-specific.

4.6. MOK is activated in response to butyrate and Cdx2 expression

The phosphorylation of both Threonine and Tyrosine residues of the TEY sequence in the activation loop of MOK is necessary for the activation of MOK. However, upstream activators or extracellular stimuli were not readily apparent, suggesting that MOK may be involved indirectly in MAP kinase cascades. We examined the phosphorylation of MOK protein in response to butyrate treatment and to Cdx2 induction for 24 and 48 h. Butyrate, which is derived from the microbial metabolism of dietary fiber in the colon, has been reported to inhibit growth and induce differentiation and apoptosis in HT-29 cell lines [12,13]. Similarly, forced expression of the Cdx2 gene has been also reported to reduce proliferation and cause morphological changes in HT-29 cell lines [14]. The activation of transfected MOK by sodium butyrate or Cdx2 induction in the HT-29 cells is shown in Fig. 6A and B. In the HT-29 cells, the level of MOK phosphorylation and kinase activity (the phosphorylation of MBP, a target for phosphorylation by MOK) gradually increased after butyrate treatment or Cdx2 induction. On the other hand, Cdx2 expression in IEC-6 cells tended to stimulate cell proliferation instead of growth arrest [9,10]. In IEC-6 cell line, MOK phosphorylation did not increase significantly after Cdx2 induction (data not shown). Further investigation is necessary for understanding activation of MOK in the cell growth, differentiation or apoptosis of intestinal epithelial cells.

We tested whether the cell cycle arrest induced by Cdx2 expression is affected by transfection of the MOK dominant

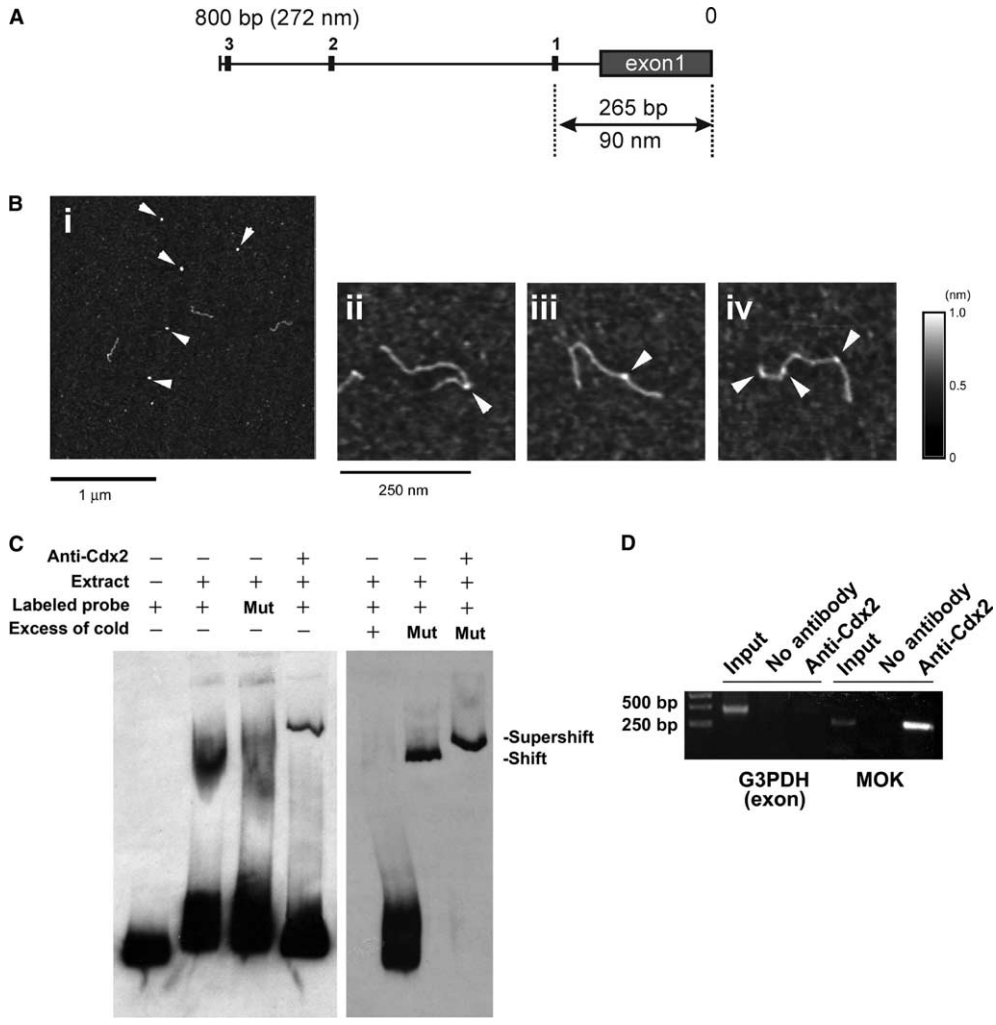

Fig. 4. Direct interaction of Cdx2 in the promoter region of the MOK gene. (A) Schematic picture of the MOK promoter showing three Cdx2-binding motifs. (B) AFM image of complexes of 800-bp DNA fragment of MOK promoter with β-galactosidase (i) Cdx2 protein (ii–iv; *arrowheads*). The images were acquired using a tapping mode AFM in air. The distinct light spot observed on the DNA molecules, which are marked with arrowheads, is bound Cdx2 protein. (C) Gel supershift assay with HT-29 Tet-Off/Cdx2 cell nuclear extracted, Cdx2 region probe, Cdx2-region mutant probe, and anti-Cdx2 antibody. DIG-labeled probes (wildtype or mutant) were incubated with nuclear extracts alone, or in the presence of excess of unlabeled oligonucleotide or Cdx2 antibody, and were separated on 6% non-denaturing polyacrylamide gel. DIG-labeled probes were incubated with nuclear extract Western blotting analysis of MOK and Cdx2 protein levels were added in Fig 1C. The early induction of the MOK protein was not observed (data not shown). Therefore, MOK gene induction was also observed 24 h after Cdx2 expression. Similar results were obtained from other two independent experiments. (D) Cdx2 binding to the MOK promoter region is shown by chromatin immunoprecipitation assay. The protein-DNA complexes were incubated with monoclonal antibodies against Cdx2 and were isolated by immunoprecipitation. PCR was performed for 35 cycles for the amplification of a 284-base MOK 5′-flanking region DNA fragment.

negative (MOK-K33A; deletion of the indispensable lysine residue for kinase activity). However, a remarkable effect was not observed (data not shown).

5. Discussion

To identify new molecules that may play important roles in affecting Cdx2 function in intestinal epithelial cells, we employed differential display PCR to identify genes induced following the overexpression of Cdx2 in a rat undifferentiated intestinal cell line, IEC-6, that has low levels of endogenous Cdx2 expression. The present study identified the MOK gene as a target for Cdx2. The rapid induction of MOK mRNA upon expression of Cdx2 in IEC-6 cells provided evidence that the regulation of the MOK gene is part of a cascade of events initiated by Cdx2 expression. Reporter gene assays allowed us to demonstrate the important role of a consensus Cdx2 DNA-binding element (−89 to −83) in the MOK promoter region in the transcription of MOK. Subsequent EMSA assays revealed that Cdx2 bound directly to the MOK promoter. The region −89 to −83 appears to play a role in MOK gene regulation by Cdx2. The ChIP assays performed here demonstrated that Cdx2 binds directly to the MOK promoter in vivo.

Immunohistochemical and in situ hybridization studies revealed that MOK localizes to intestinal crypt regions. The crypt compartment is associated with proliferation and it is from this compartment that cells ultimately differentiate into

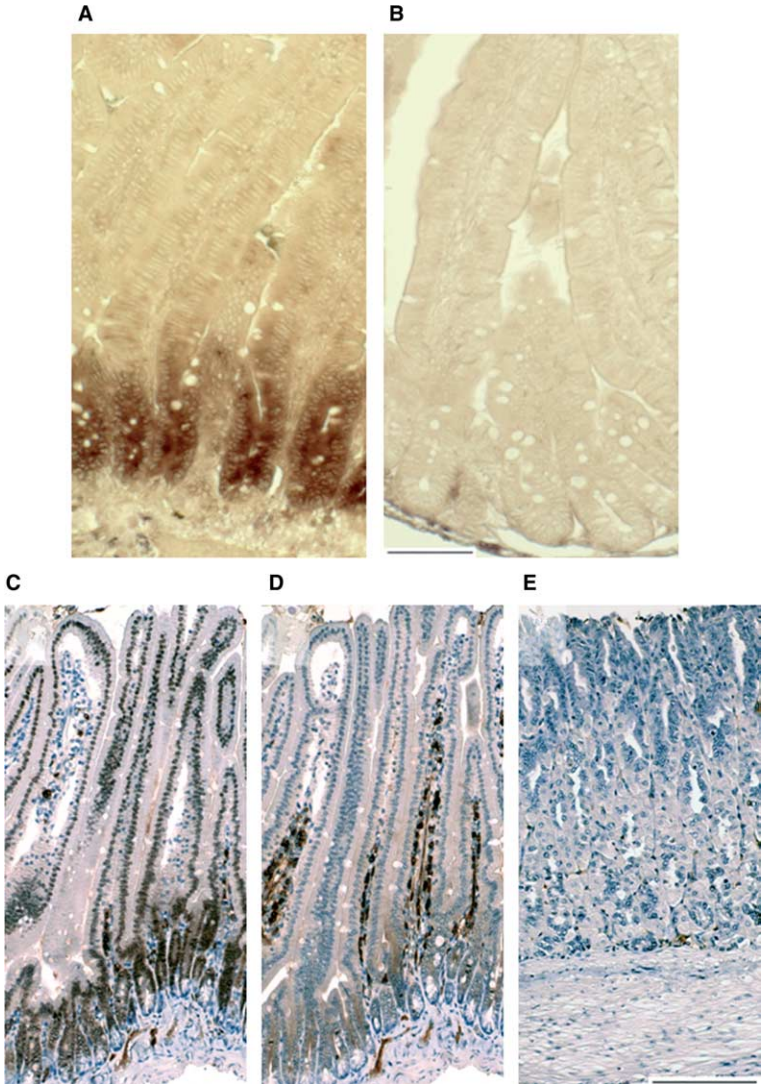

Fig. 5. Mouse MOK localizes to intestinal crypt regions by RNA in situ hybridization. An antisense (A) and sense (B) probe for the mouse MOK fragment was used (see Section 2). No significant signal was detected with the sense probe that served as a control. The scale bar is 50 μm. Immunohistochemical analyses of Cdx2 (C) and MOK protein expression (D) in the mouse intestine and stomach tissue (E). Immunohistochemistry was performed on formalin-fixed and paraffin-embedded tissue using mouse monoclonal anti-Cdx2 and rabbit polyclonal anti-MOK antibodies. Brown cells show positive immunostaining. Nuclear staining for Cdx2 was present in the normal intestinal epithelial cells. MOK expression was detected primarily in the crypt of intestine. Cdx2 and MOK expression was not detected in the normal stomach epithelial cells. The scale bar is 50 μm.

different lineages. Similarly, localization of related kinases may bring a clue about those functions. MAK is expressed in testicular germ cells at stages around meiotic cell division [15] and MRK, by virtue of expression in cardiac myocytes, has been implicated in development [7]. In HT-29 cells, which closely resemble intestinal crypt cells [16], butyrate addition resulted in terminal differentiation and a decrease in extracellular signal-regulated protein kinases (ERKs) phosphorylation [17–19]. The present study revealed that butyrate treatment caused phosphorylation of MOK and an increase in its kinase activity in HT-29 cells. The activation of MOK activity was also induced by the expression of Cdx2, which leads to the arrest of proliferation and then induces differentiation [4]. MOK may play a role in the processes of growth arrest and differentiation in upper crypt and lower villus cells. The upstream modulators and the physiological substrates of MOK remain to be clarified.

MOK shares about 40% homology with ICK, which is expressed in the intestinal crypt [8]. The 5′-flanking region of the human ICK gene contains several Cdx2-binding consensus sites, suggesting that its expression may also be, at least in part, regulated by Cdx2. The semi-quantitative RT-PCR analysis performed here revealed that the expression of the ICK gene was also induced by Cdx2 expression (data not shown). It is thus increasingly clear that the same pattern of MAP kinase signaling is used by more than one pathway employing the sequential activation of distinct isoforms at each level of the cascade. It will be interesting to identify the upstream kinase and the physiological substrates of MOK and ICK in the intestinal epithelium.

In conclusion, we demonstrated that Cdx2 protein directly binds to the MOK promoter and enhances MOK gene expression. MOK localizes to the intestinal crypt epithelial cells and might be activated by differentiation process. MOK kinase

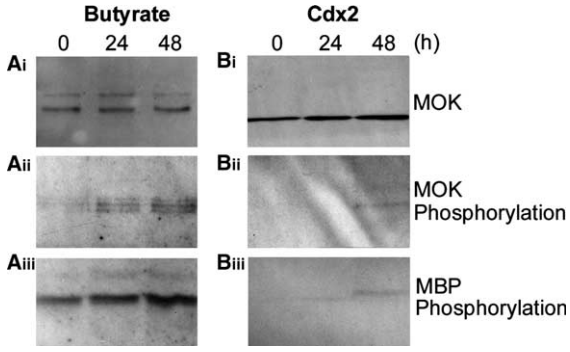

Fig. 6. Activation of MOK by butyrate treatment (A) or Cdx2 induction in HT-29 cells (B). Anti-MOK immunoprecipitates were prepared from MOK-transfected HT-29 cells treated with 4 mM sodium butyrate or in the presence of the medium for the induction of Cdx2 expression. (i) The same immunoprecipitates of MOK were probed by Western blotting with anti-MOK antibody. (ii) The phosphorylation of MOK was detected by Western blot analysis with PhosphoThreonine Antibody. (iii) The MBP phosphorylation activities by MOK were investigated by Western blot analysis with PhosphoThreonine Antibody. Protein concentration was determined by a bicinchoninic acid protein assay. Similar results were obtained from other two independent experiments.

in the intestinal epithelium must be important to understand the molecular mechanisms that underlie regulation of proliferation and differentiation.

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