Stimulation of the intestinal Cdx2 homeobox gene by butyrate in colon cancer cells

C Domon-Dell, Q Wang, S Kim, M Kedinger, B M Evers, J-N Freund

Background: The transcription factor encoded by the intestinal Cdx2 homeobox gene and treatment with sodium butyrate (NaB), a byproduct of fibre fermentation by colonic bacteria, exert similar effects on colon cancer cell lines as they both inhibit cell growth and stimulate cell differentiation and apoptosis.

Aim: To investigate whether NaB regulates expression of the Cdx2 gene in colon cancer cell lines.

Methods: Human adenocarcinoma cell lines Caco2 and HT29 were grown in the presence or absence of NaB. Cells were analysed for Cdx2 mRNA expression by reverse transcription-polymerase chain reaction, for protein expression by western blotting and electromobility shift assays, and for transcriptional activity of the Cdx2 promoter by transfection with luciferase reporter plasmids.

Results: In HT29 and Caco2 cells, NaB stimulated Cdx2 mRNA and protein expression as well as transcriptional activity of the Cdx2 promoter. Stimulation of the activity of the Cdx2 promoter by NaB was dose and time dependent. The Cdx2 promoter contains discrete regions that participate in or inversely blunt the stimulatory effect exerted by NaB. In addition, NaB stimulated the transcriptional activity of the Cdx2 promoter downregulated by oncogenic ras.

Conclusion: This study is the first report of an intestine specific transcription factor, Cdx2, stimulated by butyrate. Thus it provides a new mechanism whereby butyrate controls proliferation and differentiation of colon cancer cells.

ORIGINAL ARTICLE

Stimulation of the intestinal Cdx2 homeobox gene by butyrate in colon cancer cells

C Domon-Dell, Q Wang, S Kim, M Kedinger, B M Evers, J-N Freund

Stimulation of the intestinal Cdx2 homeobox gene by butyrate in colon cancer cells

C Domon-Dell, Q Wang, S Kim, M Kedinger, B M Evers, J-N Freund

Background: The transcription factor encoded by the intestinal Cdx2 homeobox gene and treatment with sodium butyrate (NaB), a byproduct of fibre fermentation by colonic bacteria, exert similar effects on colon cancer cell lines as they both inhibit cell growth and stimulate cell differentiation and apoptosis.

Aim: To investigate whether NaB regulates expression of the Cdx2 gene in colon cancer cell lines.

Methods: Human adenocarcinoma cell lines Caco2 and HT29 were grown in the presence or absence of NaB. Cells were analysed for Cdx2 mRNA expression by reverse transcription-polymerase chain reaction, for protein expression by western blotting and electromobility shift assays, and for transcriptional activity of the Cdx2 promoter by transfection with luciferase reporter plasmids.

Results: In HT29 and Caco2 cells, NaB stimulated Cdx2 mRNA and protein expression as well as transcriptional activity of the Cdx2 promoter. Stimulation of the activity of the Cdx2 promoter by NaB was dose and time dependent. The Cdx2 promoter contains discrete regions that participate in or inversely blunt the stimulatory effect exerted by NaB. In addition, NaB stimulated the transcriptional activity of the Cdx2 promoter downregulated by oncogenic ras.

Conclusion: This study is the first report of an intestine specific transcription factor, Cdx2, stimulated by butyrate. Thus it provides a new mechanism whereby butyrate controls proliferation and differentiation of colon cancer cells.

Diet is an important determinant of the risk of developing colorectal cancers and dietary components are thought to influence gene expression in the gut. Although data reported in humans are still controversial, evidence has been provided that alimentary fibres exert a protective effect against colon carcinogenesis in rodents. The protective effect is linked to the capacity of fibres to be metabolised into short chain fatty acid byproducts such as butyrate. Direct administration of high doses of butyrate in the lumen of the colon increases cell proliferation at the crypt base and selectively decreases carcinogen induced crypt surface hyperplasia that is thought to represent an early event in neoplastic transformation. In colon cancer cell lines cultured in vitro, sodium butyrate (NaB) acts to oppose the malignant behaviour, inhibiting anchorage independent cell growth while stimulating cell differentiation and apoptosis. The cellular effects of NaB have been related (i) to stimulation of cell cycle inhibitors such as p16INK4a and p21WAF together with dephosphorylation of pRb and decline in cyclin D1, (ii) to decreased expression of the proto-oncogenes c-src and c-myc, (iii) to a rise in transforming growth factor β1 (TGF-β1) mRNA and modification of chemokine secretion, and (iv) to a reduced level of the apoptosis inhibitors Bcl-2 and Bc-X-L, together with upregulation of proapoptotic Bak and Bax, and caspase-3 mediated cleavage of poly-(ADP-ribose) polymerase.

The caudal related Cdx2 gene encodes an intestinal transcription factor of the homeoprotein family that is a key regulator of the development and homeostasis of the intestinal epithelium (for a review see Freund and colleagues and references therein). A number of studies performed on several intestinal cell lines have provided evidence that Cdx2 inhibits cell growth and stimulates overall cell differentiation. In combination with the related Cdx1 homeobox gene, Cdx2 also promotes cell apoptosis. Together, these results indicate that Cdx2 opposes the malignant behaviour of colon cancer cells. Apart from the effects reported on cell lines, a link has been proposed between colon cancers and Cdx2 status as human colorectal cancers and chemically induced colon tumours in the rat show reduced Cdx2 levels in relation to tumour grade. Homozygous Cdx2 deficiency leads to early embryonic lethality which fails to clearly delineate the role played by Cdx2 in the initiation and/or progression of colon cancer in vivo. However, hemizygous Cdx2+/- mice are viable and exhibit intestinal hamartomas and an altered rostrocaudal pattern of epithelial cell differentiation which suggests that altered Cdx2 expression participates in the broad changes associated with colon cancer.

The mechanisms of Cdx2 regulation are far from being elucidated. We have previously shown that this homeobox gene is downregulated by oncogenic Ras activation in colon adenocarcinoma cells. Cdx2 is also negatively targeted by the P13-kinase pathway that is antagonised by the dual specific phosphatase encoded by the PTEN tumour suppressor gene (unpublished results). On the basis of similar effects exerted by Cdx2 overexpression and by NaB treatment on intestinal cancer cells, we have investigated whether Cdx2 is regulated by NaB.

MATERIAL AND METHODS

Cells cultures

HT29 cells, the TC7 subclone of Caco2 cells, control and Ras activated Caco2-H and Caco2-T cell lines were cultured under standard conditions. Cells were plated at 1x10^5 cells/cm^2, and grown for 48 hours before addition of NaB or trichostatin A to the culture medium, as indicated below.

Abbreviations: NaB, sodium butyrate; TGF-β1, transforming growth factor β1; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; EMSA, electromobility shift assay.
Plasmids and transfections

The plasmid pCdx2-1Luc containing the murine Cdx2 promoter inserted into the pG3-baslic Luciferase reporter vector (Promega, Madison, WI, USA) has been previously described.29 It contains the promoter fragment −907/+117 with respect to the major Cdx2 transcription start site. Subclones extending respectively to positions −693, −551, −392, −338, and −171 were constructed by deletion with restriction enzymes and religation or by cloning polymerase chain reaction (PCR) subfragments in pGL3-basic. Point mutations were introduced into these plasmids using the GeneEditor in vitro Site-Directed Mutagenesis System, as recommended by the supplier (Promega). For transfection experiments, 1×10^6 cells/well were plated in triplicate 24 hours prior to addition of DNA. They were then transiently transfected with 0.4 µg of appropriate luciferase reporter plasmids using Exgen5000 following the protocol recommended by the manufacturer (Euromedex, Mundolsheim, France). The reporter pCMVβGal (0.04 µg/well) was cotransfected for normalization. After 9–48 hours of incubation, cells were harvested for luciferase and β-galactosidase measurements using the Dual Light Reporter Gene Assay System (Tropix, Bedford, Massachusetts, USA). At least three independent experiments were performed for each transfection condition.

Semiquantitative RT-PCR

RNA was extracted with TRI-Reagent (MRC, Cincinnati, Ohio, USA). Semiquantitative reverse transcription (RT)-PCR was performed as described previously20 for an increasing number of cycles (24–40 cycles), using the primers cdx2 b/c: dCCAGCGGCCGAGGCGGAAACCTGTT / dATTTGCTTCTTGCTTCTTTGTTCA. Results were standardized with primers PG193/194: dATGGTAAGCCTCTGGCG / dGTGTAATCTGCTCCACAGA corresponding to the mRNA of the 36B4 ribosomal protein. PCR fragments were run on 3% agarose gels and analysed using an Imaging Densitometer (Bio-Rad Laboratories, Hercules, California, USA).

Western blots and electromobility shift assays (EMSA)

Protein (50 µg) was incubated at 100°C for five minutes in Laemmli buffer containing 2% sodium dodecyl sulphate (SDS) and 100 mM DTT, separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 10% polyacrylamide gels and analysed using an Imaging Densitometer (Bio-Rad Laboratories, Hercules, California, USA).

RESULTS

NaB stimulates Cdx2 expression in colon cancer cells

Several studies, including our own, have shown that treatment with NaB at 1–2 mM promotes differentiation of intestinal cell lines. At higher doses (above 5 mM) NaB causes massive apoptosis, followed by increased proliferation of the remaining cells. Unless otherwise stated, we used NaB at 1 or 2 mM throughout the study. To investigate the effect of NaB on expression of the Cdx2 homeobox gene, human colon adenocarcinoma Caco2 cells were treated with NaB for 48 hours. Semiquantitative RT-PCR using Cdx2 specific primers, similar to western blot analysis with anti-Cdx2 antibody, indicated that mRNA and protein expression of Cdx2 were both stimulated in NaB treated Caco2 cells compared with control cells incubated with vehicle alone (fig 1A, B). In another human colon adenocarcinoma cell line, HT29, NaB treatment also increased levels of Cdx2 mRNA detected by RT-PCR (fig 1A). The intestinal specific sucrase-isomaltase gene is a typical target of the Cdx2 protein which binds the SIF-1 element in the proximal promoter.31 Nuclear proteins extracted from HT29 cells were used for EMSA with the double stranded SIF-1 oligonucleotide. Figure 1C (left panel) shows a higher amount of DNA-protein complex in NaB treated HT29 cells compared with controls. Preincubation of nuclear proteins with anti-Cdx2 antibody largely prevented the formation of the DNA-protein complex, confirming the identity of the Cdx2 protein in the complex (not shown). Taken together, these results provide evidence that Cdx2 expression is stimulated in human colon cancer cell lines by NaB treatment at a dose that promotes cell differentiation.

To investigate if NaB causes a general increase in DNA binding activity or if the effects are specific to Cdx2, EMSAs were performed with double stranded oligonucleotides for a series of other DNA binding factors. The SIF-3 and SIF-4 oligonucleotides correspond to cis-elements of the sucrase-isomaltase promoter that have been shown to bind, respectively, the homeoproteins of the HNF1 family and transcription repressor E4BP4.32,33 As shown in fig 1C, SIF-3 binding activity was reduced in NaB treated HT29 cells compared with controls whereas SIF-4 binding activity was unchanged. EMSA also revealed that the DNA binding activity of the AP-1 complex was not modified on cell treatment with NaB (fig 1C). Hence these data indicate that NaB has distinct effects on specific nuclear factors, including an increase in Cdx2 DNA binding activity.

Transcription from the Cdx2 promoter is activated by NaB

The reporter luciferase plasmid pCdx2-1Luc containing the Cdx2 promoter fragment between positions −907 to +117 with respect to the major transcription start site was transfected into Caco2 or HT29 cells, and cells were then treated with 1 mM NaB or vehicle alone for 48 hours. Figure 2A shows that NaB stimulated the transcriptional activity of the Cdx2 promoter in both cell types by a factor of 4–5. No effect was observed using the empty luciferase vector pG3-baslic. Transcriptional stimulation of the Cdx2 promoter by NaB was dose dependent, and a faint effect was observed at concentrations as low as 0.1 mM NaB (fig 2B). We next treated pCdx2-1Luc transfected Caco2 cells with NaB at 0–5 mM, and luciferase activity was measured after NaB treatment for 9–33 hours (fig 2C). Maximal transcriptional activation was already observed nine hours after treatment, irrespective of the concentration of NaB. This activation was maintained throughout the time course of the experiment for NaB at 1 and 2 mM whereas stimulation was largely reduced after 24 hours and 33 hours of incubation with 5 mM NaB. No significant stimulation was observed with 10 mM NaB (not shown). Thus increased expression of the Cdx2 homeobox gene by NaB was

www.gutjnl.com
oncogenic Ras activation in Caco2 cells. In previous work, we showed that Cdx2 was downregulated by promoter downregulated by oncogenic Ras

NaB restores the transcriptional activity of the Cdx2 promoter

48 hours of treatment with 1 mM NaB or vehicle alone. The results are means of three independent experiments.

NaB restores the transcriptional activity of the Cdx2 promoter downregulated by oncogenic Ras

In previous work, we showed that Cdx2 was downregulated by oncogenic Ras activation in Caco2 cells. Thus we investigated whether NaB can abolish the negative effect of oncogenic Ras on the transcriptional activity of the Cdx2 promoter. For this purpose, we used the Caco2-T cell line stably transfected with the pH06T1 plasmid that encodes oncogenic Val12 Ha-Ras and the control Caco2-H cells transfected with the empty vector pH06. The reporter pCdx2-1 Luc plasmid was introduced into both cell lines, and luciferase activity was measured after 48 hours of treatment with 2 mM NaB or vehicle alone (fig 4). As shown earlier, basal activity of the Cdx2 promoter was threefold lower in Ras activated cells compared with controls. NaB stimulated by fourfold the Cdx2 promoter in control Caco2-H cells, in a similar manner to standard Caco2 cells shown above. In the Ras activated Caco2-T cells, NaB also resulted in fivefold stimulation of the transcriptional activity of pCdx2-1Luc which reached a level similar to that measured in control Caco2-H cells without NaB. Consistent with the

Figure 1  Stimulation of Cdx2 mRNA and protein expression by butyrate (NaB). Caco2 or HT29 cells were plated on culture dishes and treated for 48 hours with 1 mM NaB or vehicle alone. (A) RNA was used for reverse transcription-polymerase chain reaction analysis with the cdx2 b/c primers to detect Cdx2 mRNA and with PG193/194 primers to detect mRNA of the ribosomal protein 36B4. (B) Cellular proteins from Caco2 cells separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters were probed with anti-Cdx2 antibody and anti-β-actin antibody. (C) Nuclear cell extracts from HT29 cells were assayed for Cdx2 DNA binding activity using the labelled SIF-1 oligonucleotide (left panel), and for HNF-1, E4BP4, and AP-1 DNA binding activity with the oligonucleotides SIF-3, SIF-4, and AP-1, respectively. For competition experiments, 100-fold molar excess of unlabelled oligonucleotide was incubated with the nuclear extracts prior to addition of the labelled probe.

Figure 2  Stimulation of the transcriptional activity of the Cdx2 promoter by butyrate (NaB). (A) Caco2 or HT29 cells were transfected with the pCdx2-1 Luc reporter plasmid and pCMV-βGal and treated for 48 hours with 0.1–2 mM NaB or vehicle alone. (B) Caco2 cells were transfected with pCdx2-1 Luc and pCMV-βGal and treated for 48 hours with 0.1–2 mM NaB or vehicle alone. (C) pCdx2-1 Luc and pCMV-βGal transfected Caco2 cells were treated with 1, 2, or 5 mM NaB or vehicle alone for nine, 24, or 33 hours. In all experiments, luciferase activity was related to β-galactosidase and the results are expressed as fold induction compared with control cells treated with vehicle alone. The results are means of three independent experiments.

www.gutjnl.com
effects mediated by the TGF-β pathway. The TGF-β pathway is a signaling pathway that is involved in the regulation of gene expression via Smad family proteins. In this study, we observed that NaB, a histone deacetylase inhibitor, blunted the stimulatory effect of butyrate on the Cdx2 promoter.

**DISCUSSION**

Butyrate, a fermentation byproduct of dietary fiber, is known to act as a potent regulator of gene expression. Here, we investigated the effect of butyrate on the Cdx2 promoter.

**Figure 3**  Cdx2 promoter analysis for butyrate (NaB) stimulation. Caco2 cells were transfected with the pCdx2-1Luc reporter plasmid containing the promoter fragment between nucleotides −907 to +117 or with the shorter promoter fragments extending to positions −963, −551, −392, or −338. The arrow indicates the transcription start site. pCMV-βGal was cotransfected for normalisation. Cells were then treated for 48 hours with 1 mM NaB or vehicle alone. Luciferase activity was related to β-galactosidase and the results are expressed as fold induction compared with control cells treated with vehicle alone. The results are means of three independent experiments.

**Figure 4** Blunting effect of butyrate (NaB) on Cdx2 downregulation by oncogenic Ras. Control Caco2-H and Ras activated Caco2-T cells were transfected with the pCdx2-1Luc and pCMV-βGal plasmids and treated for 48 hours with 2 mM NaB or vehicle alone. Luciferase activity was related to β-galactosidase and the results are expressed as arbitrary units of luciferase activity. The results are means of three independent experiments.

results described above, the −693/+117 reporter plasmid was significantly less stimulated by NaB than the full length pCdx2-1Luc in both Caco2-H and Caco2-T cells whereas the −171/+117 reporter gave similar results as pCdx2-1Luc (not shown).

**ACKNOWLEDGEMENTS**

The authors are grateful to Dr C Gespach (INSERM U.482, Paris) for providing the Caco2-H and Caco2-T cell lines, and Dr M German (UCSF, CA) for the anti-Cdx2/3 antibody. We thank E Martin for skilled technical assistance. CD-D is a recipient of a fellowship of the Ligue Nationale contre le Cancer. This work was supported by INSERM and the Association pour la Recherche sur le Cancer.

**Authors’ affiliations**

C Domon-Dell, M Kedinger, J-N Freund, Institut National de la Santé et de la Recherche Médicale, Unité 381, Strasbourg, France

Q Wang, S Kim, B M Evers, Department of Surgery, University of Texas Medical Branch, Galveston, TX, USA
REFERENCES

Stimulation of the intestinal Cdx2 homeobox gene by butyrate in colon cancer cells

C Domon-Dell, Q Wang, S Kim, M Kedinger, B M Evers and J-N Freund

Gut 2002 50: 525-529
doi: 10.1136/gut.50.4.525

Updated information and services can be found at:
http://gut.bmj.com/content/50/4/525

These include:

References

This article cites 42 articles, 21 of which you can access for free at:
http://gut.bmj.com/content/50/4/525#BIBL

Email alerting service

Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Topic Collections

Articles on similar topics can be found in the following collections

Colon cancer (1547)

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/