

Expression of β -catenin is regulated by PI-3 kinase and sodium butyrate in colorectal cancer cells

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Abstract. β -catenin has a dual function; it is implicated in intercellular junctions and transcriptional co-activation. Here we examined the regulation of the expression and localization of β -catenin in HT29 colorectal adenocarcinoma cells. Our results showed that inhibition of PI-3 kinase with wortmannin was accompanied by a considerably reduced expression of β -catenin. This effect was overcome by butyrate and occurred at the protein level, not at the level of mRNA. Moreover, NaBT significantly increased the phosphorylation of the ribosomal protein, S6, known to participate in the translational control of gene expression. This was accompanied by the increased phosphorylation of p70 S6K and MAPKs, the effector proteins that are upstream of protein S6 in the distinct signaling pathways. These facts indicate that different signaling pathways may be involved in the regulation of β -catenin synthesis. Modulation of β -catenin expression induced by NaBT appeared to occur at the level of protein translation, suggesting that NaBT may act as a translational regulator.

Introduction

β -catenin is a bifunctional protein that exists in two separate pools in the cell. β -catenin localized at the plasma membrane plays a role in adherens junctions. In the adhesion complex, the cadherin receptors associate with cytoplasmic proteins of the catenin family (β - or γ -catenin) and mediate an interaction with the cortical actin microfilament network via α -catenin (1). The cytosolic soluble pool of β -catenin is involved in transition of the Wnt signal from the plasma membrane to the

nucleus, where it acts as a co-activator of the Tcf transcription factor to stimulate the transcription of Wnt target genes, i.e. the cell cycle-promoting genes or genes necessary for invasive growth (2). Structurally, β -catenin is composed of 12 central Arm (armadillo) repeats, flanked by C- and N-terminal domains. The C-terminus contains the DNA-transactivating domain while the N-terminus contains several conserved serine and threonine residues. These, if phosphorylated, target cytosolic β -catenin for ubiquitination and rapid protein degradation. CK-1 and GSK-3 have been considered as the kinases responsible for the phosphorylation of β -catenin and they exist in a complex which also includes Axin and APC protein (3).

In unstimulated non-transformed cells, free β -catenin is unstable, labeled for ubiquitination and subsequent destruction (4). Destabilization of β -catenin in normal cells is mediated by a multi-protein complex of GSK-3, APC, PP2A, β -TrCP and Axin/Conductin (5). However, in colorectal cancer cells that express mutated APC, β -catenin destruction does not occur (6,7). Under these circumstances, free β -catenin accumulates in the cytoplasm and, eventually, functions as a transcriptional co-activator (4). Until recently, many reports suggested that the intranuclear translocation and transcriptional activity of β -catenin might participate in the initiation of mammalian tumors. Yet, Hagen *et al* (8) showed that β -catenin must first accumulate in the cytosol before being able to elicit its signaling activity in the nucleus. It appears that the nuclear localization of β -catenin occurs predominantly in the migrating invasive front of the colorectal carcinoma (9). Thus, β -catenin's transcriptional activity seems to be a marker of tumor de-differentiation and invasiveness rather than its initiation.

The integrity of adherens junctions and the stability of free β -catenin appear to be dynamically regulated by tyrosine phosphorylation. The existence of the adherens junctions can be disturbed, for example, by the *v-src* oncogene (10). β -catenin appears to be a direct substrate for protein-tyrosine phosphatases (PTPs), and PTPs were shown to inhibit cell migration (11). These data imply that β -catenin phosphorylated at tyrosine residues plays a critical role during invasive cell migration.

The problem of the stabilization of free β -catenin in developing tumors is yet to be elucidated. After dismantling the complex, soluble β -catenin has the ability to bind phosphatidylinositol-3 kinase (PI-3K), which might mediate the catenin stabilization. In this context, it is an important fact that a significant increase in PI-3 kinase activity occurs in

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Abbreviations: AP, alkaline phosphatase; NaBT, sodium butyrate; NPP, nitrophenyl phosphate; PI-3 kinase, phosphatidylinositol 3-kinase; PI, phosphoinositol; PIP, phosphoinositol phosphate

Key words: PI-3 kinase, sodium butyrate, ribosomal protein S6, colon, adenocarcinoma, control of translation

different tumors (12). Class I PI-3 kinases play key roles in cell proliferation, migration, apoptosis, gene expression and differentiation (13-15). Classical PI-3 kinases are considered as bifunctional lipid and serine kinases (16,17), whose specific product is phosphatidylinositol trisphosphate PI(3,4,5)P₃ (18). Many proteins have been defined as direct or indirect downstream targets for PI-3 kinase (17); the most explored effectors of PI-3 kinase signaling are PDK-1 and Akt (19). Our recent results suggested that PI-3 kinase may prevent differentiation of colorectal cancer cells and that it interacts with β -catenin in these cells (20; unpublished data). Therefore, we aimed to examine whether PI-3 kinase is involved in the regulation of the expression of β -catenin in colorectal adenocarcinoma cells.

There are many cell lines derived from human colorectal carcinomas, some of which represent a model for inducing transient differentiation. The HT29 cell line has been used frequently because these cells can differentiate only after stimulation with a differentiation agent. In our experiments, we used sodium butyrate (NaBT) to induce transient differentiation in HT29 cells. NaBT is a four-carbon short fatty acid salt produced *in vivo* in the colonic lumen by bacterial fermentation of a dietary fiber (21). This compound has significant physiological relevance to the integrity and function of the colonic epithelium (22). In cancer cell lines, NaBT exerts several antitumor effects, including inhibition of cell proliferation, stimulation of cell differentiation, and induction of apoptosis (23).

We have previously studied different aspects of the NaBT-stimulated differentiation of several colorectal adenocarcinoma cell lines, including HT29 (24-26). Yet, the mode and the degree of the response of these cells to butyrate treatment sometimes varied. Therefore, we have established several clones of the HT29 cell line in order to provide a better model for molecular and morphological studies of this type of cancer cells (unpublished data). Data acquired with these clones should contribute to better elucidation of the molecular principles of the differentiation process in adenocarcinoma cells.

Increased protein synthesis is necessary for tumor formation and progression. The translational control of gene expression, which is the major regulatory mechanism of protein biosynthesis, is mediated by signaling pathways targeting protein synthesis machinery. It occurs mainly at the level of initiation by reversible phosphorylation of several initiation factors and by phosphorylation of S6 protein, a component of the 40S subunit of eukaryotic ribosomes that is responsible for binding and decoding mRNA. Ribosomal subunits containing phosphorylated S6 protein are preferentially recruited into polysomes, rather than non-phosphorylated subunits (27).

In the present work, we examined the role of PI-3 kinase in the regulation of β -catenin expression and intracellular localization during proliferation and NaBT-stimulated differentiation in HT29 colorectal cancer cells. We found that inhibition of PI-3 kinase by wortmannin significantly reduced the level of β -catenin expression. This inhibition was overcome by NaBT. While neither wortmannin nor NaBT interfered with the transcription of mRNA for β -catenin, NaBT appeared to highly stimulate the phosphorylation of the ribosomal protein, S6, known to be involved in the control of mRNA

translation. These data suggest that NaBT may affect the expression of β -catenin at the level of translation.

Materials and methods

Materials. Tissue culture supplies were purchased from Life Technologies Inc. Sodium butyrate, wortmannin, phosphoinositol and Tri-Reagent were from Sigma. Silicagel/Kieselgur TLC plates were obtained from Merck. ECL detection reagent, G-Sepharose and [γ -³²P]-ATP were from Amersham-Biotechnologies. The NPP substrate was supplied by Fluka Chemie. The following primary antibodies were purchased: monoclonal anti-phospho-tyrosine antibody (PY99) and polyclonal rabbit anti-p70 S6K from Santa Cruz Biotechnologies; monoclonal anti- β -catenin and anti-phospho- β -catenin (Ser37) from Sigma; monoclonal anti-eIF-4E from Transduction Laboratories; polyclonal rabbit anti-S6 protein and anti-phospho-S6 protein (Ser235/236) and polyclonal anti-MAPK p42/44 and anti-phospho-MAPK p42/44 (Thr202/Tyr204) were from Cell Signaling Technology. HP-conjugated secondary antibodies against mouse and rabbit IgG were supplied by Bio-Rad. Anti mouse IgG₁ Alexa 488-conjugated secondary antibody was obtained from Molecular Probes (Eugene, OR). DAPI dye for staining nuclei was from Roche. All other chemicals were reagent grade and were obtained from commercial suppliers.

Cell culture. Human colorectal adenocarcinoma cells, HT29, or H8 and G9 clones were grown in H-MEM media supplemented with 10% fetal calf serum (FCS). For the 48-h experiments, confluent cells were subsequently washed and cultured in relevant media containing 2% serum with or without 5 mM NaBT. At appropriate time points, the cells were lysed and used for further experiments or fixed and used for immunocytochemistry.

For some experiments, the confluent cells were treated with DMSO or with 100 nM wortmannin in medium containing 2% serum and incubated for an additional 48 h. Some cells were first pre-treated with 100 nM wortmannin for 30 min, then 5 mM NaBT was added and the cells subsequently incubated for 48 h.

The cells were lysed at appropriate time points on ice for 30 min in basic buffer B [1% Triton X-100 (v/v); 100 mM HEPES (pH 7.9); 1 mM β -glycerolphosphate; 2 mM EGTA; 0.2 mM PMSF; 2 mM benzamidine; 2 mM Na₃VO₄; and protease inhibitors, leupeptin, pepstatin and antipain (0.4 μ g/ml each)] unless otherwise indicated. The cell extracts were cleared by centrifugation at 12,000 x g for 10 min at 4°C and supernatants were assayed for protein concentration using the Bradford method.

Cell fractionation. Cells were lysed with buffer C containing 10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT and 0.5 mM PMSF and subsequently passed 20 times through a 22 G needle and centrifuged at 560 x g to separate the cytoplasm from nuclei. This procedure was performed once more with buffer C supplemented with 0.1% Triton X-100. The pellet was then lysed with buffer N, consisting of 20 mM HEPES, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT and 0.5 mM PMSF, in order to

SPANDIDOS nuclear fraction. Both cytoplasmic and nuclear lysates were centrifuged at 15,300 x g for 15 min. The cytoplasmic pellet was further lysed with the lysis buffer C supplemented with 1% Triton X-100 for 30 min and centrifuged to obtain the membrane fraction.

Immunoblot analysis. For Western blotting, crude cell lysates (40 µg) or immunoprecipitated proteins (25) were resolved by SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes. The membranes were blocked with 5% (w/v) milk dissolved in PBS-0.1% Tween buffer and then the membranes were incubated with appropriate primary and secondary antibodies diluted with PBS-0.1% Tween buffer. For detection of bound antibodies, enhanced chemiluminescence (ECL) was utilized.

Alkaline phosphatase assay. Cells were lysed by sonication in a buffer consisting of 10% diethanolamine and 0.5 mM MgCl₂ (pH 9.8). The lysates were cleared by centrifugation at 15,000 x g for 4 min. Fifty µl lysates containing equal amounts of protein were mixed with 50 µl NPP substrate for the alkaline phosphatase dissolved in the lysis buffer (2.8 mg/ml) and incubated for 30 min at 37°C. The reaction was stopped with 50 µl 3 M NaOH. Absorbance (405 nm) values were measured using an ELISA reader and the enzyme activity was calculated using the molar extinction coefficient $\epsilon_{405} = 20.2 \mu\text{mol}^{-1}$ obtained from a p-nitrophenol calibration curve.

PI-3 kinase assay. Cells were extracted in basic buffer B, described above, that was supplemented with 40 mM β -glycerolphosphate and 5 mM DTT, and the assay of PI-3 kinase activity was performed essentially as described by Vojtechová *et al* (28). Briefly, protein extracts (2 mg) were immunoprecipitated overnight at 4°C with 2 µg of specific anti-pTyr antibody and 10 µl protein G-Sepharose. Phosphatidylinositol was prepared as a stock solution in chloroform. The solvent was evaporated *in vacuo* and the lipid was dispersed by sonication in 20 mM Tris-HCl pH 7.5, 1 mM EDTA and 5 mM DTT. The immunoprecipitates were pre-incubated with the lipid solution at 37°C for 10 min. The activity of PI-3 kinase was assayed in a reaction mixture containing 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 5 mM DTT, 10 mM MgCl₂, 100 µM ATP, 25 µg phosphatidylinositol and 1.5 µCi [γ -³²P]-ATP in 50 µl. The reaction was carried out for 10 min at 37°C and terminated with 200 µl 1 N HCl, 80 µl methanol and 80 µl chloroform. The mixture was then centrifuged, the upper phase was removed and the chloroform phase was washed twice with 200 µl mixture containing 1 N HCl-methanol (1:1). The chloroform extracts (20 µl) were spotted on an oxalate-treated Silicagel/Kieselgur TLC plate and chromatography was performed with n-propanol-2 M acetic acid (65:35) as the mobile phase. The developed plate was air-dried and autoradiographed.

Immunocytochemistry. Cells cultured on 4-chamber slides were washed with PBS and fixed for 20 min with 4% paraformaldehyde in PBS and subsequently permeabilized with methanol:acetone (50:50%, vol:vol) for 1 min. After additional washes with PBS, the cells were blocked with 4% BSA in

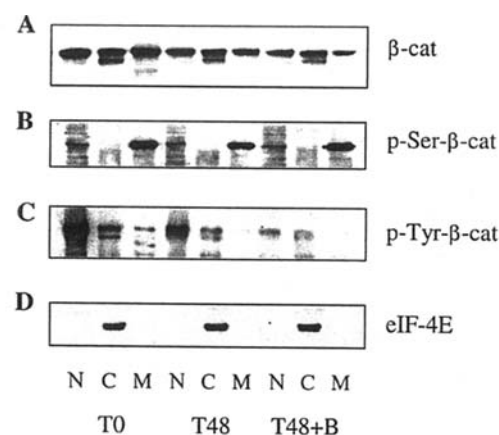


Figure 1. Examination of localization and phosphorylation of β -catenin in HT29 cells. Western blot analysis using anti- β -catenin antibody (A and C) and antibody against β -catenin phosphorylated at the Ser37 residue (B). In C, β -catenin was detected in the samples immunoprecipitated with the anti-phospho-tyrosine antibody. The analysis was performed with the nuclear (N), cytoplasmic (C) and membrane (M) fractions of HT29 cells untreated or treated with 5 mM NaBT for 48 h. Expression of the eIF-4E protein was used as a control of the cell fractionation (D). T0, cells lysed at their confluence; T48, cells cultured for 48 h in the media containing 2% serum; and T48+B, cells cultured in the same medium supplemented with 5 mM NaBT. For details of the fractionation procedure, see Materials and methods. Results are representative of three independent experiments.

0.2% Triton X-100 for 2 h RT. The cells were then incubated overnight at 4°C with primary monoclonal antibody, anti- β -catenin diluted with the blocking solution at a concentration 1:1,000. Then the cells were stained for 1 h in the dark with a mixture of secondary anti-IgG₁ antibody conjugated with Alexa 488 (1:1,000) and DAPI nuclear dye (1 µg/ml) diluted with 4% BSA in 0.2% Triton X-100. Fluorescent images were captured with a Nikon Eclipse E600W microscope using Lucia software (Laboratory Imaging, Ltd.).

Northern blot analysis. Total RNA was extracted from cells by Tri-Reagent and in amounts of 15 µg run on formaldehyde gels and blotted onto Hybond-N nylon membrane. After staining with methylene blue to determine the position of 18S and 28S rRNA markers, membranes were hybridized with ³²P-labelled probes under high stringency conditions. The probe for β -catenin was kindly provided by Professor W. Birchmeier (Berlin). As loading controls, S18 and S28 rRNA were used.

Statistical analysis. The data are presented as the mean of at least three independent experiments performed in duplicate \pm SD.

Results

Localization and Tyr and Ser phosphorylation of β -catenin in HT29 cells. HT29 cells were analysed to ascertain whether butyrate has any effect on the localization and phosphorylation of β -catenin. The presence of total β -catenin or β -catenin phosphorylated either at Tyr residues or at Ser37 in these cells was examined by Western blotting in the membrane, cytoplasmic or nuclear fractions of HT29 cells treated or untreated with 5 mM NaBT.

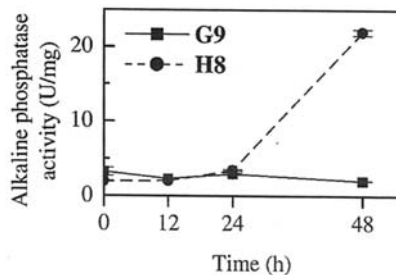


Figure 2. Effects of NaBT on the alkaline phosphatase activity of H8 and G9 clones of HT29 cells. Time course of alkaline phosphatase activity in H8 and G9 cells cultured for up to 48 h in media containing 2% serum supplemented with 5 mM NaBT. The activity of alkaline phosphatase was assayed in the cell lysates using NPP as a substrate as described in Materials and methods. Data are the means \pm SD of three individual experiments performed in triplicate.

As shown in Fig. 1, in HT29 cells, β -catenin was present in all three fractions. β -catenin phosphorylated at serine 37 was prevalently localized to the cell membrane (Fig. 1B) whereas β -catenin phosphorylated at tyrosines was detected mostly in the nucleus in HT29 cells (Fig. 1C). While tyrosine phosphorylation of β -catenin decreased significantly after 48-h treatment of the cells with 5 mM NaBT, β -catenin phosphorylation at serine 37 seemed stable (Fig. 1B). NaBT treatment appeared to have no effect either on the expression or intracellular localization of total β -catenin.

Activity of alkaline phosphatase in different clones of the HT29 cell line. Since, in previous experiments, we obtained variable results during analyses of human colorectal adenocarcinoma HT29 cells that differentiate only after stimulation with a differentiation agent (24,25), we have established several clones of this cell line differing in several morphological and molecular markers (unpublished data). For further experiments in this study, we used two of these clones, H8 and G9. Regarding alkaline phosphatase activity, which is one of the indicators of intestinal cell differentiation, clone H8 exerts a high sensitivity to NaBT and clone G9 exerts a very low NaBT sensitivity.

A comparison of the AP activities is shown in Fig. 2. In H8 cells, the activity of AP started to elevate 24 h after the addition of NaBT to the culture medium, while treatment of G9 cells with NaBT did not seem to change the activity of AP (Fig. 2).

PI-3 kinase activity in H8 and G9 clones. The *in vitro* activity of PI-3 kinase was determined in the clones, H8 and G9, incubated with or without 5 mM NaBT for 48 h. As shown in Fig. 3, PI-3 kinase activity was higher in untreated H8 cells than in G9 cells. The treatment of H8 cells with NaBT led to a strongly reduced PI-3 kinase activity (by 60%) while, in G9 cells, NaBT exerted no significant effect on PI-3 kinase activity (Fig. 3B).

Involvement of PI-3 kinase in the regulation of β -catenin expression in H8 cells. To ascertain whether the activity of PI-3 kinase has an effect on the expression of β -catenin in adenocarcinoma cells, we performed several different analyses of H8 cells cultured in the presence or absence of the selective

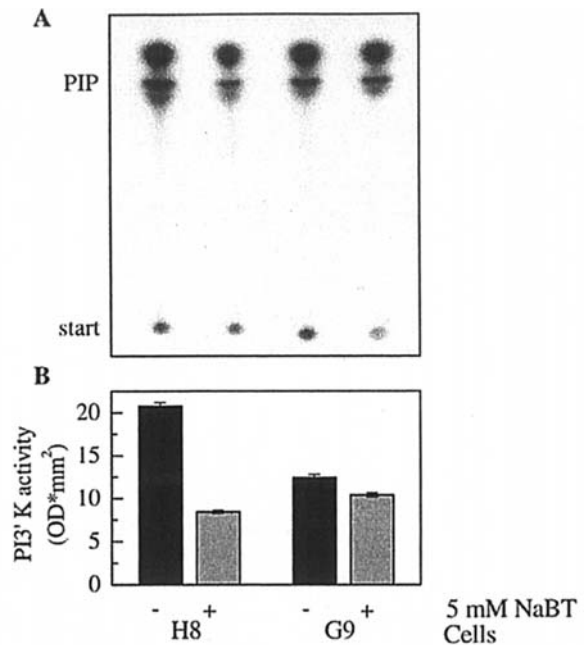


Figure 3. Examination of the PI-3 kinase activity in H8 and G9 clones. The cells were incubated for 48 h with or without 5 mM NaBT and the activity of PI-3 kinase was determined in the anti-phospho-tyrosine immunoprecipitates from these cells. (A) Chloroform extracts of the lipid products of the reaction were resolved by TLC as described in Materials and methods. The spots on the autoradiographs indicate generated phosphatidyl inositol phosphate (PIP). (B) Data obtained from densitometric analysis of the TLC autoradiograph. Black bars represent the cells cultured in the absence of NaBT and grey bars represent the cells cultured in the presence of 5 mM NaBT. The values represent means \pm SD of three independent experiments.

inhibitor of PI-3 kinase, wortmannin. For some experiments, the cells were pre-treated with 100 nM wortmannin and then treated with 5 mM NaBT.

Immunocytochemical analysis of β -catenin expressed in the H8 clone did not show any changes in its intracellular localization, which corresponds with the findings in the HT29 original cell line (Fig. 1A). However, the expression of β -catenin was significantly reduced upon treatment of H8 cells with wortmannin (Fig. 4C), indicating that the activity of PI-3 kinase may be responsible for changes in the expression of β -catenin in these cells. This observation was not a consequence of cell death, as documented by a sustained number of nuclei (Fig. 4C, blue DAPI-stained nuclei).

As shown in Fig. 4B, the treatment of H8 cells with 5 mM NaBT alone did not have any impact on the expression of β -catenin. Surprisingly, in comparison to the cells treated only with wortmannin (Fig. 4C), addition of 5 mM NaBT to the cell culture after 30-min pre-treatment with wortmannin resulted in an increased expression of β -catenin in H8 cells (Fig. 4D).

Effect of wortmannin and butyrate on mRNA or protein expression of β -catenin. Western blot analysis of the cells incubated with or without 100 nM wortmannin or with or without 5 mM NaBT (Fig. 5A) confirmed the immunocytochemical observations described above. The treatment of H8 cells with wortmannin decreased the amount of β -catenin by 27% compared to the amount of β -catenin in the cells treated with both wortmannin and butyrate (Fig. 5B).

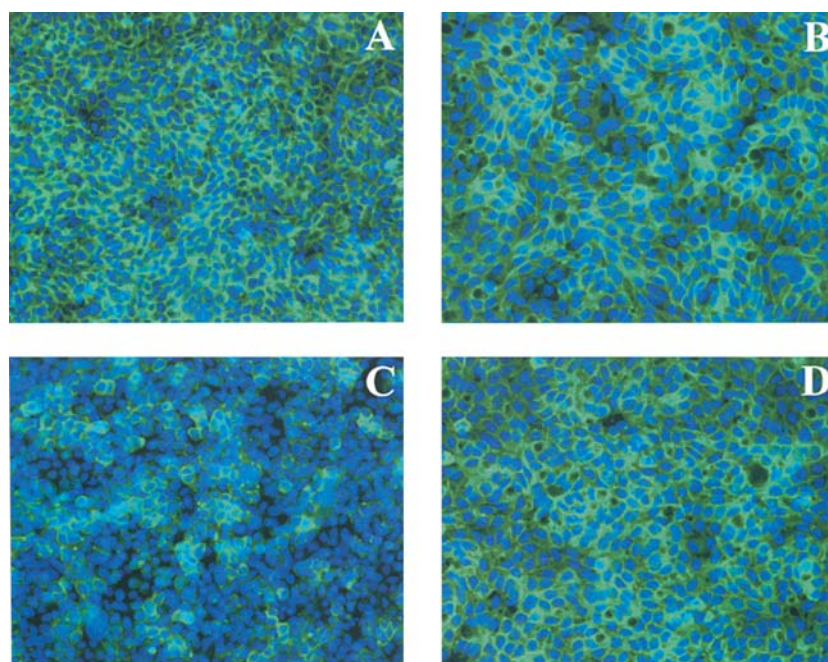


Figure 4. Immunocytochemical analysis of the effect of wortmannin and NaBT on the expression of β -catenin in H8 cells. The cells were treated with DMSO as a vehicle, or with 100 nM wortmannin for 30 min and subsequently incubated in the presence or absence of 5 mM NaBT for 48 h. Immunocytochemistry was performed with these cells using the anti- β -catenin antibody (green). The nuclei were visualized by DAPI staining (blue). (A) Cells treated with DMSO; (B) cells treated with 5 mM NaBT; (C) cells treated with wortmannin; (D) cells treated with both wortmannin and NaBT. A representative of three independent experiments is shown.

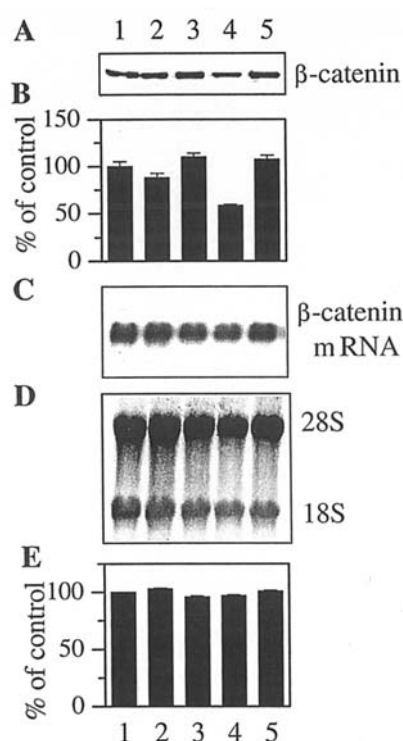


Figure 5. Analysis of β -catenin protein and mRNA expression in wortmannin and NaBT-treated H8 and G9 cells. (A) Western immunoblotting of β -catenin expression in H8 cells treated with DMSO as a vehicle (lane 2), with 100 nM wortmannin (lane 4) either treated together with 5 mM NaBT (lane 5) or incubated with NaBT alone (lane 3). (B) Histogram showing densitometric data obtained from the Western blot analysis of β -catenin. (C and D) Northern blot analysis of the expression of β -catenin mRNA in H8 cells (C) treated or untreated as in (A). As loading controls for C, 28S and 18S rRNAs are shown (D). Histogram (E) summarizes densitometric analysis of mRNA for β -catenin related to the amount of the relevant 28S rRNA.

To ascertain whether changes in β -catenin expression occurred at the level of transcription, Northern blot analysis of β -catenin mRNA isolated from the H8 cells non-treated and treated with wortmannin alone or with wortmannin and butyrate was performed. No significant changes in the amount of β -catenin mRNA upon these treatments were seen in these cells (Fig. 5C). The amount of each mRNA was related to the amount of the relevant loaded 28S rRNA (Fig. 5E). These data suggest that the reduced β -catenin expression, as a result of the inhibition of PI-3 kinase activity by wortmannin (Fig. 4C), may occur at the level of translation and not transcription in H8 cells.

Phosphorylation of ribosomal protein, S6; effect of NaBT. Since NaBT seemed to have an effect on β -catenin translation, we examined the state of phosphorylation of the ribosomal protein, S6, a component of the 40S subunit of eukaryotic ribosomes, which is one of the most important control mechanisms of protein translation. As shown in Fig. 6A and B, the treatment of both H8 and G9 cells with 5 mM NaBT highly stimulated the phosphorylation of the ribosomal protein, S6. In agreement with this finding, the phosphorylation of its major protein kinase, p70 S6K, was increased by butyrate treatment in both H8 and G9 cells (Fig. 6C). The state of activation of p70 S6K was analyzed by means of its migration on SDS-PAGE (increased phosphorylation and thus activation of p70 S6K is manifested by lower electrophoretic mobility on SDS-PAGE) (28). As shown in Fig. 6C, a highly phosphorylated form of p70 S6K, represented by the most slowly migrating protein band γ , was present only in H8 and G9 cells treated with butyrate. In addition, the phosphorylation of MAPKs (p42/44) that are upstream of another S6 kinase,

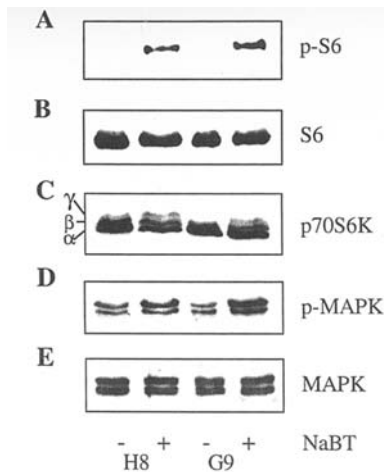


Figure 6. Effect of NaBT on the phosphorylation of S6, p70 S6K and MAPK in H8 cells. Western blot analysis of the phosphorylation level of ribosomal protein, S6, using the antibody against S6 protein phosphorylated at serine 235/236 (A), and of the total amount of S6 protein using the anti-S6 protein-specific antibody (B). The phosphorylation of protein kinase, p70 S6K, was detected using the anti-p70 S6K antibody. Symbols indicate different phosphorylated forms of the analyzed proteins (C). The phosphorylation of MAPK 1/2 was analyzed using the specific antibody against MAPKs p44/p42 phosphorylated at Thr202/Tyr204 (D). Results are representative of two independent experiments.

p90^{sk}, was also stimulated upon treatment of the cells with NaBT (Fig. 6D), suggesting that p90^{sk} may be involved in the phosphorylation of S6 protein induced by butyrate. Similar findings were observed in the original HT29 cell line (data not shown). These results indicate that butyrate may be involved in the translational control of β -catenin expression by regulating the phosphorylation of the ribosomal protein, S6.

Discussion

The objective of this study was to examine the regulation of the expression and intracellular localization of β -catenin in HT29 colorectal adenocarcinoma cells. Staal *et al.* (29) reported that, in order to transduce Wnt signaling, β -catenin in the nucleus must be dephosphorylated on its N-terminus where Ser37 and 41 residues reside. Our results are in accordance with these data, since β -catenin phosphorylated at serine 37 was found in HT29 cells, predominantly at the plasma membrane, and almost no Ser37-phosphorylation of β -catenin was found in the nucleus. Contrary to the tyrosine phosphorylation of β -catenin, its phosphorylation at serine 37 appeared to be NaBT-independent. We found that the overall tyrosine phosphorylation of β -catenin decreased considerably in HT29 cells after 48-h treatment with 5 mM NaBT. These results correspond with previous findings that NaBT down-regulates total cellular tyrosine kinase activity in colon cancer (30).

Recently, β -catenin has been revealed as one of the key binding partners for SHP-1 in human intestinal crypt cells and a substrate for tyrosine phosphatase, SHP-1, in these cells (31). In this context, it would be interesting to see whether NaBT stimulates the expression or activity of the tyrosine phosphatase, SHP-1, as a mechanism to control the dephosphorylation of β -catenin.

The amount of β -catenin in butyrate-sensitive cells, H8, treated with wortmannin significantly decreased, indicating that inhibition of PI-3 kinase is responsible for the reduced expression of β -catenin. Despite the fact that the PI-3 kinase activity considerably declined in H8 cells treated with NaBT, when wortmannin pre-treatment was followed by the addition of 5 mM NaBT, the expression of β -catenin highly increased. This finding indicates that the expression of β -catenin is not dependent only on the activity of PI-3 kinase, since the decreased synthesis of β -catenin, evidently induced by the inhibition of PI-3 kinase activity, was overcome by the effect of sodium butyrate. Northern blot analysis performed with mRNA for β -catenin revealed that this effect occurred at the level of translation. This is also supported by the observation of the butyrate-stimulated phosphorylation of the ribosomal protein, S6. Thus, butyrate may be involved in the control of protein translation in colorectal cancer cells by regulating the phosphorylation of the ribosomal protein, S6. The phosphorylation of protein components of the translational apparatus provides the most important means of protein synthesis control. Ribosomal protein S6 is a component of the 40S subunit of eukaryotic ribosomes and a substrate for the p70 S6 kinase (p70 S6K), controlled by the mTOR signaling pathway (32). Also, protein kinase p90^{sk}, which is downstream of MAPKs in the Ras/MEK signaling pathway, catalyzes the phosphorylation of the ribosomal protein, S6 (33). Our findings may therefore indicate that at least two distinct signaling pathways that control mRNA translation through the regulation of S6 protein phosphorylation may be activated upon butyrate treatment in HT29 cells.

Sodium butyrate is known to upregulate the expression of some regulatory genes, such as p21 or p27, and some differentiation markers, such as cytokeratin, alkaline phosphatase and other brush border intestinal enzymes, as well as integrin β 1 and osteopontin (23). We have shown that β -catenin could be another target of NaBT in colorectal adenocarcinoma cells. So far, NaBT has been reported to alter only the transcription of a variety of genes by modulating histone acetylation or DNA methylation (34,35). Therefore, the possibility that NaBT may be responsible for the regulation of β -catenin expression at the level of translation would bring new insights into its molecular biological function.

It appears that the activities of either AP or PI-3 kinase of the G9 clone of HT29 cells were not altered by NaBT. Wortmannin did not cause any changes in β -catenin expression in the G9 clone, either (data not shown). On the contrary, NaBT stimulated the phosphorylation of the ribosomal protein, S6, as well as that of p70 S6K and MAPKs 1/2 in both H8 and G9 cells. The latter facts might indicate that the NaBT-induced regulation of protein translation is a general process in colon cancer cells.

In conclusion, our data indicate that in the regulation of β -catenin expression, the PI-3 kinase-dependent pathway may be involved, together with other, i.e. PI-3 kinase-independent, signal transduction pathway(s) stimulated by NaBT. The inhibition of PI-3 kinase, using wortmannin, caused a considerable decline in the expression of β -catenin. A very strongly reduced activity of PI-3 kinase was found in H8 cells treated with 5 mM NaBT. By contrast, NaBT alone did not change the level of expression of β -catenin. However, NaBT

SPANDIDOS PUBLICATIONS: the wortmannin-stimulated decrease of β -catenin in H8 cells treated with both agents. These facts suggest that NaBT could induce β -catenin expression via the transduction pathway independent of PI-3 kinase activity.

Another important finding of this study is that the prevention of the wortmannin-induced decrease of β -catenin expression by NaBT occurred at the protein level, not at the level of mRNA. Also, NaBT significantly increased the phosphorylation of the ribosomal protein, S6, playing an important role in the regulation of protein translation, and of its upstream effectors, p70 S6K and MAPKs 1/2. These results suggest that NaBT acts as a translational regulator, which may represent another biological function of sodium butyrate in colorectal adenocarcinoma cells.

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References

- Stockinger A, Eger A, Wolf J, Beug H and Foisner R: E-cadherin regulates cell growth by modulating proliferation-dependent beta-catenin transcriptional activity. *J Cell Biol* 154: 1185-1196, 2001.
- Moon RT, Kohn AD, De Ferrari GV and Kaykas A: Wnt and β -catenin signalling: diseases and therapies. *Nat Rev Genet* 5: 689-699, 2004.
- Polakis P: Wnt signaling and cancer. *Genes Dev* 14: 1837-1851, 2000.
- Gavert N, Conacci-Sorrell M, Gast D, Schneider A, Altevogt P, Brabletz T and Ben-Ze'ev A: L1, a novel target of β -catenin signaling, transforms cells and is expressed at the invasive front of colon cancers. *J Cell Biol* 168: 633-642, 2005.
- Malbon CC, Wang H and Moon RT: Wnt signaling and heterotrimeric G-proteins: strange bedfellows or a classic romance? *Biochem Biophys Res Commun* 287: 589-593, 2001.
- Murray NR, Davidson LA, Chapkin RS, Gustafson WC, Schattner DG and Fields AP: Overexpression of protein kinase C beta II induces colonic hyperproliferation and increased sensitivity to colon carcinogenesis. *J Cell Biol* 145: 699-711, 1999.
- Ilyas M, Tomlinson IPM, Rowan A, Pignatelli M and Bodmer WF: Beta-catenin mutations in cell lines established from human colorectal cancers. *Proc Natl Acad Sci USA* 94: 10330-10334, 1997.
- Hagen T, Sethi JK, Foxwell N and Vidal-Puig A: Signalling activity of β -catenin targeted to different subcellular compartments. *Biochem J* 379: 471-477, 2004.
- Brabletz T, Jung A, Rey S, Porzner M, Hlubek F, Kunz-Schughart LA, Knuechel R and Kirchner T: Variable β -catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. *Proc Natl Acad Sci USA* 98: 10356-10361, 2001.
- Gómez S, Del Mont Llosas M, Verdú J, Roura S, Lloreta J, Fabre M and García de Herreros A: Independent regulation of adherens and tight junctions by tyrosine phosphorylation in Caco-2 cells. *Biochim Biophys Acta* 1452: 121-132, 1999.
- Müller T, Choidas A, Reichmann E and Ullrich A: Phosphorylation and free pool of beta-catenin are regulated by tyrosine kinases and tyrosine phosphatases during epithelial cell migration. *J Biol Chem* 274: 10173-10183, 1999.
- Benistant C, Chapuis H and Roche S: Specific function for phosphatidylinositol 3-kinase alpha (p85alpha-p110alpha) in cell survival and for phosphatidylinositol 3-kinase beta (p85alpha-p110beta) in *de novo* DNA synthesis of human colon carcinoma cells. *Oncogene* 19: 5083-5090, 2000.
- Krasilnikov MA: Phosphatidylinositol-3 kinase dependent pathways: the role in control of the cell growth, survival, and malignant transformation. *Biochemistry* 65: 59-67, 2000.
- Nelson CM and Chen CS: Cell-cell signaling by direct contact increases cell proliferation via a PI3K-dependent signal. *FEBS Lett* 514: 238-242, 2002.
- Turecková J, Wilson EM, Cappalunga JL and Rotwein P: Insulin-like growth factor-mediated muscle differentiation: collaboration between phosphatidylinositol 3-kinase-Akt-signaling pathways and myogenin. *J Biol Chem* 276: 39264-39270, 2001.
- Bondeva T, Pirola L, Bulgarelli-Leva G, Rubio I, Wetzker R and Wymann MP: Bifurcation of lipid and protein kinase signals of PI3Kgamma to the protein kinases PKB and MAPK. *Science* 282: 293-296, 1998.
- Cantrell DA: Phosphoinositide 3-kinase signalling pathways. *J Cell Sci* 114: 1439-1445, 2001.
- Vanhaesebroeck B, Leevers SJ, Panayotou G and Waterfield MD: Phosphoinositide 3-kinases: a conserved family of signal transducers. *Trends Biochem Sci* 22: 267-272, 1997.
- Toker A and Newton AC: Cellular signaling: pivoting around PDK-1. *Cell* 103: 185-188, 2000.
- Turecková J, Vojtechová M, Kucerová D, Velek J and Tuháčková Z: Sodium butyrate-mediated differentiation of colorectal cancer cells: regulation of PKC β II by PI 3-kinase. *Int J Mol Med* 15: 329-335, 2005.
- Fleming SE and Arce DS: Volatile fatty acids: their production, absorption, utilization, and roles in human health. *Clin Gastroenterol* 15: 787-814, 1986.
- Soergel KH: Colonic fermentation: metabolic and clinical implications. *Clin Investig* 72: 742-748, 1994.
- Emanuele S, D'Anneo A, Bellavia G, Vassallo B, Lauricella M, De Blasio A, Vento R and Tesoriere G: Sodium butyrate induces apoptosis in human hepatoma cells by a mitochondria/caspase pathway, associated with degradation of β -catenin, pRb and Bcl-X_L. *Eur J Cancer* 40: 1441-1452, 2004.
- Kucerová D, Sloncová E, Tuháčková Z, Uhlířová M, Kos M and Sovová V: Changes of E-cadherin and β -catenin levels during induced differentiation of colorectal carcinoma cells. *Int J Mol Med* 4: 541-544, 1999.
- Kucerová D, Sloncová E, Tuháčková Z, Vojtechová M and Sovová V: Expression and interaction of different catenins in colorectal carcinoma cells. *Int J Mol Med* 8: 695-698, 2001.
- Stokrová J, Sovová V, Sloncová E, Kucerová D, Tuháčková Z and Korb J: Response of HT115, a highly invasive human colorectal adenocarcinoma cell line, to sodium butyrate treatment and glucose deprivation. *Int J Oncol* 26: 793-799, 2005.
- Gingras A-C, Raught B and Sonenberg N: Regulation of translation initiation by FRAP/mTOR. *Genes Dev* 15: 807-826, 2001.
- Vojtechová M, Sloncová E, Kucerová D, Jiricka J, Sovová V and Tuháčková Z: Initiation factor eIF2B not p70 S6 kinase is involved in the activation of the PI-3K signalling pathway induced by the v-src oncogene. *FEBS Lett* 543: 81-86, 2003.
- Staal FJT, van Noort M, Strous GJ and Clevers HC: Wnt signals are transmitted through N-terminally dephosphorylated β -catenin. *EMBO Rep* 3: 63-68, 2002.
- Keri G, Balogh A, Teplan I and Csuka O: Comparison of the tyrosine kinase activity with the proliferation rate in human colon solid tumors and tumor cell lines. *Tumour Biol* 9: 315-322, 1988.
- Duchesne C, Charland S, Asselin C, Nahmias C and Rivard N: Negative regulation of β -catenin signaling by tyrosine phosphatase SHP-1 in intestinal epithelial cells. *J Biol Chem* 278: 14274-14283, 2003.
- Hay N and Sonenberg N: Upstream and downstream of mTOR. *Genes Dev* 18: 1926-1945, 2004.
- Roux PP, Richards SA and Blenis J: Phosphorylation of p90 ribosomal S6 kinase (RSK) regulates extracellular signal-regulated kinase docking and RSK activity. *Mol Cell Biol* 23: 4796-4804, 2003.
- Parker MI, De Haan JB and Gevers W: DNA hypermethylation in sodium butyrate-treated WI-38 fibroblasts. *J Biol Chem* 261: 2786-2790, 1986.
- Turner BM: Histone acetylation and control of gene expression. *J Cell Sci* 99: 13-20, 1991.