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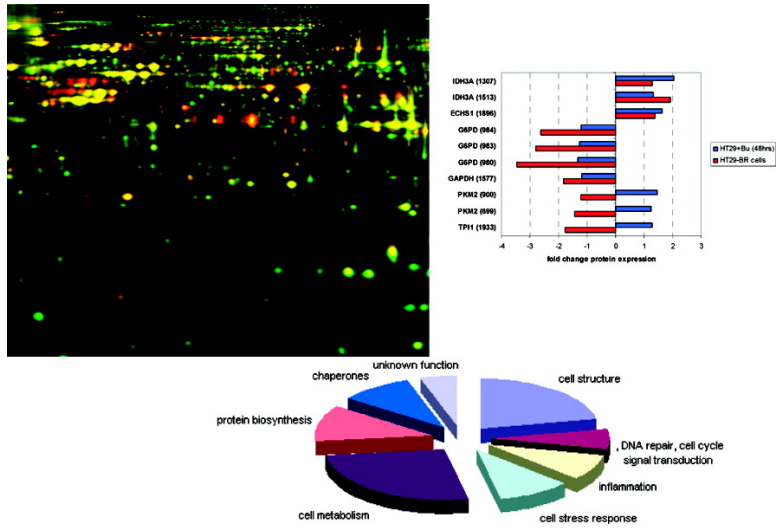

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Proteomic Analysis of Butyrate Effects and Loss of Butyrate Sensitivity in HT29 Colorectal Cancer Cells

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Butyrate, a fermentation product of the large bowel microflora, is potentially protective against the development of colorectal cancer. *In vitro*, butyrate has been shown to induce apoptosis and inhibit proliferation in numerous cancer cell lines, including colorectal cancer. Although these tumor suppressing properties of butyrate are well-documented in experimental systems, the mechanisms underlying the induction of these effects are not fully understood. Understanding these mechanisms in cancer cells, as well as the pathways involved in a cell's ability to overcome them and progress toward malignancy, is vital to determine therapeutic approaches for disease management. We have developed a colorectal cancer cell line (HT29-BR) that is less responsive to the apoptotic effects of butyrate through sustained exposure of HT29 cells to 5 mM butyrate and have used proteomics to investigate the mechanisms involved in the development of butyrate insensitivity. Proteomic analysis identified a number of cellular processes in HT29 and HT29-BR cells influenced by butyrate including remodeling of the actin cytoskeleton, inhibition of protein biosynthesis and dysregulation of the cell stress response. We describe novel roles for butyrate in the induction of its tumor suppressing effects and outline potential cellular pathways involved in the development of butyrate insensitivity in the HT29-BR cell population.

Keywords: Colorectal cancer • proteomics • butyrate • butyrate insensitive

Introduction

Colorectal cancer has emerged as one of the most prevalent types of cancer worldwide, and although genetic predisposition and chronic inflammatory conditions such as ulcerative colitis and Crohn's disease are recognized as risk factors for its development, epidemiological studies have drawn strong correlations between incidence and lifestyle factors such as body weight and diet.[1] Metabolism of dietary fiber by the gut microflora results in the production of short chain fatty acids (SCFA) such as acetate, propionate and butyrate.[2] Of the SCFAs, butyrate is believed to exert the greatest physiological effects on colonic epithelium. In addition to being the primary energy source for colonocytes, butyrate plays a significant role in maintaining normal physiological functions of the colonic mucosa where it regulates cell growth and differentiation and disruption of these processes may play a part in colorectal tumor growth and progression.[3,4]

In vitro studies involving exposure of a range of cancer cell lines, including those of colorectal origin, to physiological levels of butyrate have demonstrated effects such as inhibition of proliferation, induction of apoptosis and cell cycle arrest. These properties of butyrate have generated interest in the development of derivatives as a therapeutic for the treatment of various diseases,[5] including colorectal cancer. Numerous studies utilizing proteomics and gene microarrays have been conducted in an attempt to elucidate the pathways by which butyrate may exert its putative chemo-preventative actions.[4−10] While the majority of research efforts have focused on the mechanisms and potential pathways by which butyrate may suppress tumor growth, there is minimal information available describing how a subpopulation of tumorigenic cells is able to escape the apoptotic properties of butyrate and potentially progress toward malignancy. There is also evidence indicating that cancer cells, which are nonresponsive to the apoptotic effects of butyrate, display characteristics of chemo (or drug) resistance.[11] On the basis of these observations, butyrate resistant cell lines have been developed as *in vitro* models to study drug resistance, especially in the context of cancer therapy.[12,13] Investigations into the potential mechanisms involved in overcoming the physiological effects of butyrate and subsequent development of chemoresistance or malignancy is critical to identify novel therapeutic approaches for disease management.

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In an attempt to identify some of the underlying mechanisms associated with butyrate insensitivity and hence colorectal tumor progression, we have developed a cell line that is refractory to the apoptotic and differentiating effects of butyrate by chronic exposure of HT29 cells to physiological levels (5 mM) of butyrate. The HT29 colorectal cancer cell line is representative of an undifferentiated tumorigenic cell line and prolonged treatment of these cells with butyrate results in selection for a differentiated cell population characterized by irreversible morphological and phenotypic changes.[14,15] This differentiated cell population most likely represents a subpopulation of cells displaying a more aggressive malignant phenotype. Here, we describe a comprehensive comparative proteomic analysis between HT29 cells, HT29 cells exposed to butyrate for 48 h, and a butyrate insensitive HT29 cell line (HT29-BR). We outline some of the complex cellular responses the HT29-BR cell population adopts to circumvent the tumor suppressing effects of butyrate and also identify potential mechanisms associated with the apoptotic response in HT29 cells.

Methods

Cell Culture. HT29 colorectal cancer cells (ATCC, Rockville, MD) were maintained in Dulbecco's Modified Eagle's Medium (DMEM)/F-12 Nutrient Mixture (Ham) Media with L-glutamine (1:1; 37 °C; 5% CO_2; Invitrogen) containing 5% fetal calf serum (FCS; Invitrogen) and 1% penicillin/streptomycin. Butyrate insensitive (HT29-BR) cells were selected by sustained exposure of HT29 cells to gradually increasing concentrations of sodium butyrate (0.5 mM increments) as described with the medium changed biweekly.[16] Cultures were initially exposed to 0.5 mM sodium butyrate and maintained at each concentration step for at least 2 passages until the butyrate concentration reached 5 mM. Surviving cells were maintained in medium containing 5 mM sodium butyrate. For all experimental work, cells were seeded at the appropriate densities and maintained in fresh media containing 3% FCS for 24 h prior to any treatment.

Apoptosis and Differentiation Assays. Apoptosis in HT29 and HT29-BR cells was measured 48 h following the addition of butyrate (concentration range 0−25 mM) to cells using the Apo-ONE homogeneous Caspase 3/7 Assay Kit (Promega) following manufacturer's protocols. Measurements were performed in triplicate and statistical analysis of the data was performed using Prism 4.0 Software (Graph Pad, San Diego, CA).

Cellular differentiation in HT29 and HT29-BR cells was measured using the Alkaline Phosphatase Detection Kit (Sigma Aldrich) according to manufacturer's specifications, except fluorescence was recorded at 355 nm/460 nm (Wallac Victor3 1420 multilabel counter, Perkin-Elmer). Measurements were performed in triplicate and statistical analysis of the data was performed using Prism 4.0 Software (Graph Pad, San Diego, CA).

Protein Isolation and 2D Electrophoresis. For proteomic analysis, cells were prepared at a concentration of approximately 6.5×10^6 cells/mL and an aliquot equivalent to 10×10^6 cells was dispensed into T175 flasks and maintained for 24 h prior to treatment. Cells were harvested after 48 h by trypsinization, and the pellets washed in PBS (3 times) to remove excess culture medium. Cells were lysed on ice (30 min) (150 mM NaCl; 1% IGEPAL; 1 mM $NaVO_4$; 10 mM NaF; 20 mM β-glycerophosphate; 50 mM Tris, protease inhibitors, pH 7.5) and centrifuged (10 000*g*, 15 min, 4 °C) to remove cell debris and the lysate was transferred to clean tubes. Lysates were obtained from untreated HT29 cells, and HT29 cells were treated with butyrate for 48 h, and HT29-BR cells.

Proteins were precipitated with cold $CH_3OH/CHCl_3$ (4:3, v/v). The protein pellet was isolated and washed with cold acetone (three times) to remove excess solvent prior to resuspension in 2DE rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, and 30 mM Tris). The protein concentration was determined using the Bradford protein assay (Sigma). Samples were aliquoted and stored at −80 °C until analysis. An aliquot of each protein sample (100 μg) was then labeled with either Cy3 or Cy5 dye (200 pmol) according to manufacturer's protocols (GE Healthcare). The internal standard was prepared by combining equal amounts (50 μg) of each respective sample and labeled with Cy2 dye (200 pmol) according to manufacturer's protocols.

Following labeling, the samples were combined and diluted in rehydration buffer (final volume 450 μL) containing 0.5% IPG buffer and trace amounts of bromophenol blue before passive rehydration into to 24 cm immobilized pH gradient strips (IPG 3−11 NL, GE Healthcare). First dimension isoelectric focusing was performed for approximately 70 kVh (hold at 300 V for 2 h, hold at 500 V for 2 h, hold at 1000 V for 2 h, ramp to 8000 V for 5 h, hold at 8000 V for 6 h) (IPGphor II system, GE Healthcare). Prior to second-dimension separation, cysteine side chains were reduced and alkylated by incubating focused strips in equilibration buffer (6 M urea, 50 mM Tris, pH 8.8, 30% glycerol, 2% SDS, and trace amounts of bromophenol blue) containing 1% DTT followed by 2.5% iodoacetamide (15 min, room temperature). Second-dimension separation (12.5% polyacrylamide gels) was performed at constant voltage (75 V) until the dye front had reached the bottom of the gel (DALT Twelve electrophoresis unit, GE Healthcare). Fluorescent gel images were obtained using the Ettan DIGE imager (GE Healthcare) according to manufacturer's specifications.

Gel Image Analysis. Analysis of gel images was performed using the DeCyder software (v6.5, GE Healthcare) which allowed comparison of spot patterns and quantification of protein spot abundance changes across each of the different treatment groups. Each gel image was also manually examined to ensure gel spots were correctly matched across each treatment group. Only those proteins that were found to be regulated with fold change greater than ±1.5 between any two groups ($p < 0.05$, Student's t test) were selected for protein identification. Gels used for protein identification were stained with Coomassie blue and protein spots of interest were excised manually or using the Ettan Spot Handling Workstation (GE Healthcare).

Mass Spectrometry and Protein Identification. Selected protein spots were excised, destained (200 μL, CH_3CN/25 mM NH_4HCO_3, 1:1, v/v) and digested with trypsin (0.1 μg in 25 mM NH_4HCO_3; 37 °C, overnight; Promega, Madison, WI). Peptides were extracted with acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA) (1:1), dried under vacuum (SpeedVac), and resolubilized (0.1% TFA, 10 μL) prior to identification by mass spectrometry. Mass spectra were collected using an Ultraflex III MALDI TOF/TOF mass spectrometer (Bruker Daltonics) operated in the positive ion mode. Peptide mass maps and fragmentation spectra were collected using α-cyano-4-hydroxycinnamic acid as the matrix (Bruker Daltonics). Proteins were identified using the MASCOT search engine (MatrixScience, London, U.K.) and the search was performed against the IPI human protein database (v3.31). Database search parameters included carboxyamidomethylation of cysteine residues (fixed modification), methionine oxidation (variable modification) and mass

tolerance of ±50 ppm for peptide mass fingerprinting and ±0.5 Da for fragmentation spectra. Positive protein identifications were made based on Mascot scores indicating identity or extensive homology.

Western Blot Analysis. For validation of selected protein targets by Western blot analysis, HT29 and HT29-BR cells were seeded at the same density and maintained for 24 h prior to treatment. Untreated HT29 cells, HT29 cells treated with butyrate, and HT29-BR cells were harvested at 0 h (control) and 48 h following the addition of 5 mM butyrate. Cells were harvested as detailed above, and the cell lysate was stored at −80 °C until analysis. Lysate (equivalent to 15 μg of protein) was diluted into sample loading buffer and proteins were separated using 4−12% gradient Bis-tris mini-gels according to manufacturer's protocols (Invitrogen). Following electrophoresis, proteins were transferred onto Hybond-ECL membrane (GE Healthcare) and the membranes were blocked using skim milk powder in TBS-T prior to overnight incubation with mouse anti-pyruvate kinase (1:1000; AbCam, Cambridge, U.K.), goat anti-triosephosphate isomerase (1:50 000; Abnova, Taiwan), or rabbit anti-peroxiredoxin 1 (1:10 000; Upstate, NY). Rabbit anti-β-actin antibody (1:5000; AbCam, Cambridge, U.K.) was used as loading control. HRP-α-mouse IgG1 (1:10 000; Dako), HRP-α-goat IgG (1:5000; Dako) and HRP-α-rabbit IgG (1:5000; Dako) were used as secondary antibodies. Membranes were then visualized using enhanced chemiluminescence (ECL Plus, GE Healthcare) and quantitative analysis was performed using ImageQuant TL software 7.0 (GE Healthcare).

Results and Discussion

Apoptosis and Differentiation in the HT29 Butyrate Resistant Cell Line. A butyrate insensitive HT29 colorectal cancer cell line (HT29-BR) was generated by prolonged exposure of HT29 cells to butyrate and this unique cell population was significantly less responsive to the apoptotic and differentiating effects of butyrate (Figure 1). After 48 h, butyrate was found to induce apoptosis in both HT29 and HT29-BR cells at a concentration of 5 mM and greater ($p < 0.05$, Student's t test) as measured by caspase 3/7 activity (Figure 1A). Butyrate is known to induce differentiation of colorectal cancer cells *in vitro* and this is characterized by an increase in alkaline phosphatase activity.[17] In the HT29-BR cell line, a small but significant (Student's t test, $p < 0.005$) increase in alkaline phosphatase activity was also observed with butyrate treatment relative to untreated HT29 cells, but this increase was exceptionally minor in comparison to that seen in the HT29 cells exposed to butyrate for 48 h (Figure 1B). Kucerova et al.[17] also reported that alkaline phosphatase activity in colorectal cancer cells decreased with prolonged exposure to butyrate (>30 days) and that this decrease in enzyme activity correlated with decreased sensitivity to apoptosis. In this study, a similar correlation between alkaline phosphatase activity, apoptosis and duration of butyrate exposure was observed in the HT29 cells.

Proteomic Analysis of HT29 and HT29-BR Cell Lines. In total, 1347 protein spots were detected in all three treatment groups, with 139 and 323 differentially regulated protein spots observed when comparing whole cell lysates of HT29 and HT29 treated with 5 mM butyrate and for HT29 and HT29-BR cells, respectively (fold change > ±1.5, $p < 0.05$). An example of the master 2D DIGE gel image comparing HT29, HT29 cells treated with butyrate, and HT29-BR cells is shown in Supporting Information Figure 1. To determine regulation of protein expression in response to butyrate, our focus centered on those

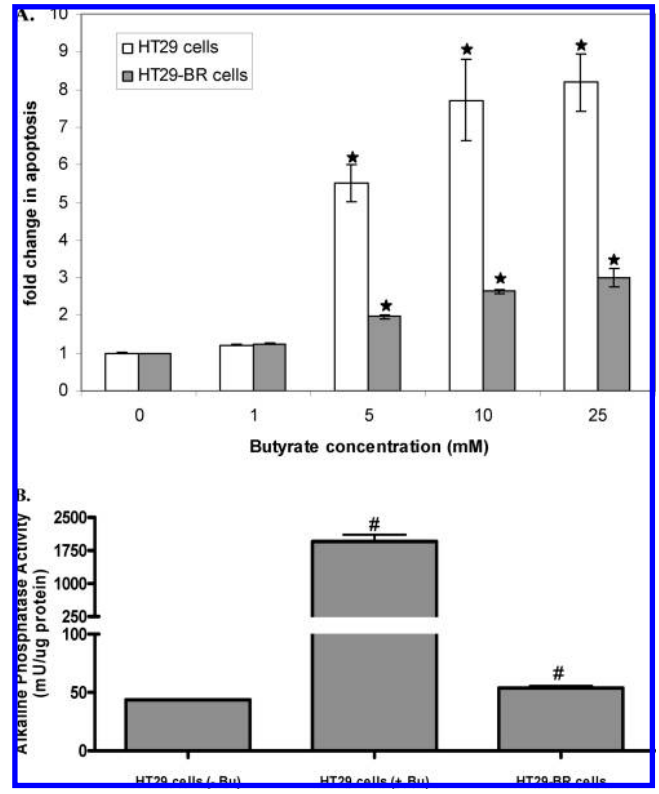

Figure 1. Apoptosis and differentiation in HT29 and HT29-BR cells following 48 h exposure to 5 mM butyrate. (A) Apoptosis in HT29 and HT29-BR cells as measured by caspase 3/7 activity. The apoptotic response was consistently lower in the HT29-BR cell line in comparison to the HT29 cells exposed to the same concentrations of butyrate, indicating that the BR cell line is less sensitive to the apoptotic effects of butyrate. A significant apoptotic response was observed in both HT29 and HT29-BR cells at concentrations of 5 mM and greater ($p < 0.02$, Student's t test). (B) Differentiation in HT29 and HT29-BR cells as measured by alkaline phosphatase activity. Comparison of alkaline phosphatase activity in HT29 cells, HT29 cells treated with 5 mM butyrate and HT29-BR cells after 48 h. Alkaline phosphatase activity was found to significantly increase (Student's t test, $p < 0.005$) when HT29 cells is treated with 5 mM butyrate and in the HT29-BR cell line. In comparison, the extent of differentiation in the HT29-BR cell line was less than that seen in the HT29 cells exposed to butyrate for 48 h. Symbols and abbreviations: asterisk (*) $p < 0.02$; pond symbol (#) $p < 0.005$. Student's t test compared to 0 mM control, Error bars represent standard error of the mean (SEM).

protein spots that were common between all 3 groups and 96 protein spots were found to be differentially regulated (One-way ANOVA, $p < 0.02$). Overall, 60 protein spots were identified representing 38 unique proteins, indicating that some of the protein spots most likely represent post-translationally modified forms, isoforms or variants of the parent protein (see Supporting Information Table 1). On the basis of GO annotations and KEGG pathway analysis, we were able to identify potential biological roles for these 60 proteins (see Supporting Information Figure 2) and determine cellular processes influenced by butyrate that either (a) supported its tumor suppressing effect in HT29 cells or (b) enabled a select population of cells to overcome these effects. These processes are summarized in Table 1. Butyrate was found to induce changes in at least 7 different cellular processes in HT29 and HT29-BR

Table 1. Cellular Processes Influenced by Butyrate

development of butyrate insensitivity	tumor suppression effects
↑ actin cytoskeletal remodelling	Pertubations in cellular redox homeostasis
↑ expression of oncogenic proteins	Mediation of growth factor signaling cascades
Cell cycle regulation	Inhibition of the glycolytic pathway
Activation of cell stress pathways	
↓ protein synthesis	

cells, indicating that butyrate influences multiple signaling pathways to affect this complex cellular response.

Cellular Processes Influenced during the Development of Butyrate Insensitivity. With the use of proteomics, we have identified a unique set of proteins that may act either individually or interact synergistically to enable the HT29-BR cell population to overcome the tumor suppressing effects of butyrate. Figure 2 compares the expression profiles of these proteins between the HT29-BR and HT29 cell line in response to butyrate and the expression of these proteins was found to alter significantly with prolonged butyrate exposure. Proteins known to interact with the actin cytoskeleton were identified (Figure 2A), as well as oncogenic proteins and proteins involved with cell cycle regulation (Figure 2B). Up-regulation of these proteins in cancer cells have been associated with the promotion of malignancy.[18−21] In addition, the expression of proteins linked to inhibition of apoptosis and drug resistance increased in the HT29-BR cell line (Figure 2C). Induced expression of these proteins may be a result of upstream activation of pathways which promote cell survival under conditions of cellular stress. For example, high levels of TG2 activity has been observed in tumor cells resistant to apoptosis and in drug resistant cell lines, and there is evidence to support its role in maintaining cell survival and promotion of tumor cell invasion.[22] It has also been reported recently that induction of HSP beta-1 expression occurs as a direct result of butyrate induced cell signaling via activation of G protein coupled receptors, in particular GPCR41 and 43, and that activation of these receptors may in turn mediate the cell stress response via the MAPK pathway.[23]

Proteomic analysis has also revealed that prolonged exposure of HT29 cells to butyrate had a negative impact on *de novo* protein biosynthesis. We describe a potentially novel role for butyrate as an inhibitor of the protein elongation phase as the expression of subunits comprising the eEF1 and eEF2 complexes have been altered and this aberration was found to be more pronounced in the HT29-BR cell population (Figure 2D). Of the eEF1B complex, two protein spots identified as eEF1γ were both up-regulated and eEF1δ was down-regulated. The eEF1δ subunit is responsible for mediating the GDP/GTP exchange on eEF1A, and reduced expression of this subunit would negatively regulate the rate of protein elongation and synthesis. The eEF1γ subunit is believed to facilitate the association of eEF1β/δ with eEF1A, thereby aiding the exchange of GDP for GTP on eEF1A and enabling eEF1A to bind and stabilize an appropriate amino acyl-tRNA for recruitment to the ribosome. Additionally, identification of multiple protein spots corresponding to eEF2 were identified, all of which were significantly up-regulated by butyrate. This indicates that eEF2 likely exists primarily in its inactive phosphorylated form, corresponding to a reduced rate of protein elongation and synthesis.

Although a link between protein synthesis and cancer progression is well-established, it is not known whether protein synthesis plays a significant role in the ability of HT29 cells to bypass the normal cellular apoptotic processes induced by butyrate. Butyrate has been reported to suppress proteasome activity via pathways involving histone deacetylase inhibition and activation of NF-κb.[24] More recently, it has been reported that simultaneous inhibition of both proteasome activity and histone deacetylase activity induces apoptosis in cancer cells, and that the induction of apoptosis was dependent on unperturbed protein synthesis.[25] It is possible that the disruption in protein biosynthesis observed in the HT29-BR cell population represents a pro-survival mechanism whereby this population of cells is able to overcome the apoptotic effects of butyrate.

The Tumor Suppressing Effects of Butyrate in HT29 Cells. *In vitro*, butyrate induces apoptosis and inhibits the proliferation of cancer cells and these properties have led to interest in the use of butyrate and butyrate analogues as potential chemopreventative agents. Even though the mechanisms of action of butyrate are unknown, this study identified a number of novel proteins that supports the tumor suppressing activity of butyrate. For instance, exposure of HT29 cells to butyrate reduced the expression of a number of proteins known to promote cell proliferation and growth, inhibit apoptosis and which are positively associated with cancer metastasis. Figure 3 compares the expression of these proteins in HT29 and HT29-BR cells in response to butyrate. Figure 3A shows the expression of proteins believed to play a role in stress-induced apoptosis and cellular proliferation. Included are 2 proteins involved in cellular redox homeostasis and which function to protect cells from oxidative stress-induced apoptosis, indicating that butyrate influences the ability of HT29 cells to scavenge potentially toxic reactive oxygen species. An overall decrease in the expression of glutathione transferase omega-1 was observed by 2D DIGE in response to butyrate. Peroxiredoxin-1, however, was paradoxically regulated. Perxoredoxin-1 is an antioxidant enzyme which functions to scavenge and sequester reactive oxygen species and whose expression is induced by oxidative stress. The expression of this protein was initially found to increase in HT29 cells exposed to butyrate for 48 h, and its expression was then significantly reduced in the HT29-BR cell line. Western blot analysis of this protein in HT29 cells, HT29 cells treated with butyrate for 48 h and HT29-BR cells verified the trend in protein expression obtained by 2D DIGE (Figure 4A). The functional significance of this protein in HT29 and HT29-BR cells in response to butyrate requires further investigation.

Proteins such as annexin A3, cortactin, hnRNP K, and valosin containing protein are known to mediate growth factor receptor signaling cascades to influence tumor growth.[26−30] Down-regulation of these proteins by butyrate most likely promotes the apoptotic response in HT29 cells. For example, overexpression of cortactin has been correlated with colorectal tumor growth and malignancy[31] and has been shown to provide a protective effect against apoptosis by mediating the EGFR signaling cascade.[32,33] Growth factor receptor signaling, including EGFR, regulates Src activity and this has been shown to promote colorectal tumorigenicity both *in vivo* and *in vitro*. Cortactin is a known substrate of Src and tyrosine phosphorylation of this protein is believed to promote remodeling of the actin cytoskeleton. Down-regulation of cortactin by butyrate may be a mechanism by which butyrate induces apoptosis in HT29 cells. Annexin A3 expression is regulated in

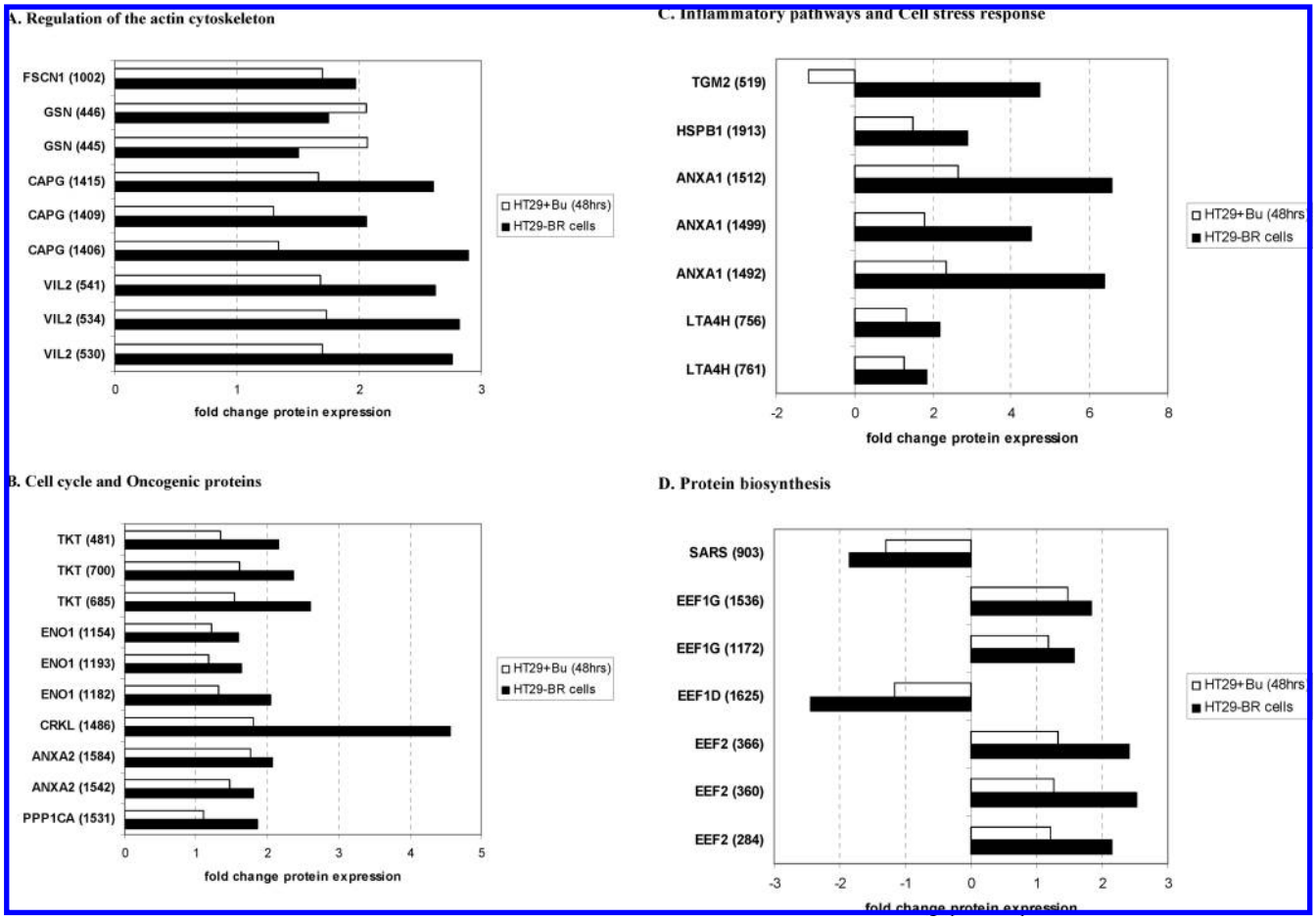

Figure 2. Expression profiles for proteins potentially contributing to survival of cells insensitive to the tumor suppressing effects of butyrate. Additional details for each protein can be found in Supporting Information Table 1. White bars represent fold change in protein expression in HT29 cells treated with 5 mM butyrate for 48 h. Black bars represent fold change in protein expression in HT29-BR cells treated with 5 mM butyrate for 48 h. Vertical axis, gene ID and corresponding master gel spot numbers; horizontal axis, fold change in protein expression. (A) Regulation of the actin cytoskeleton. These proteins, with the exception of gelsolin (GSN), are more highly expressed in the HT29-BR cell population, indicating that this cell line displays greater malignant potential. (B) Cell cycle and oncogenic proteins. Butyrate was found to increase the expression of oncogenic proteins such as Crk-like protein, α-enolase and transketolase. Proteins implicated in cell cycle regulation were also up-regulated in the HT29-BR cells. The expression of these proteins was found to increase in both the HT29 and HT29-BR cell lines. (C) Inflammatory pathways and cell stress response. Butyrate increased the expression of proteins involved in inflammatory pathways and in the cell stress response. A pronounced increase in the expression of these proteins was observed in the HT29-BR cells. (D) Protein biosynthesis. Butyrate inhibits protein biosynthesis in HT29 and HT29-BR cells, and more specifically, it inhibits the protein elongation phase. The expression of subunits comprising the eEF1 and eEF2 complexes has been altered and this aberration in the protein elongation process was found to be more pronounced in the butyrate resistant cell population.

various cancers, including colorectal, and is believed to influence tumor growth by activating the HIF-1 pathway and stimulating VEGF activity.[29,34] The expression of this protein initially increased in response to butyrate, but its expression was significantly reduced in the HT29-BR cell population (Figure 3A). The functional significance of this protein in response to butyrate requires further investigation, but down-regulation of annexin A3 in the HT29-BR cell population may support the tumor-suppressing properties of butyrate.

Butyrate appears to suppress the production of energy by glycolysis and the pentose phosphate pathway with a more noticeable effect seen in the HT29-BR cell line (Figure 3B). The trends in protein expression for two proteins involved in glycolysis, triosephosphate dehydrogenase and pyruvate kinase were also confirmed by Western blot analysis (Figure 4B,C). Cancer cells are characterized by increased glycolytic activity due to greater requirements for energy to sustain rapid cell growth. Known as the Warburg effect, this is considered to be one of the fundamental changes associated with malignant transformation.[35] Although the molecular mechanisms leading to constitutive up-regulation of genes and proteins related to glycolysis in cancer cells is not fully understood, there is substantial evidence indicating that glycolysis and the related pentose phosphate pathway both play a major role in colorectal cancer development and may also contribute to the development of drug resistance in tumor cells.[36−38]

The role of butyrate in modifying cellular metabolism has not been investigated, although it may be possible that it indirectly targets upstream regulators of the glycolytic and the pentose phosphate pathways resulting in a general overall suppression of the Warburg effect. For instance, expression of the M2 isoform of pyruvate kinase (PKM2) in tumors is essential to the development of the Warburg effect, and regulation of this protein by tyrosine signaling pathways plays a key role in

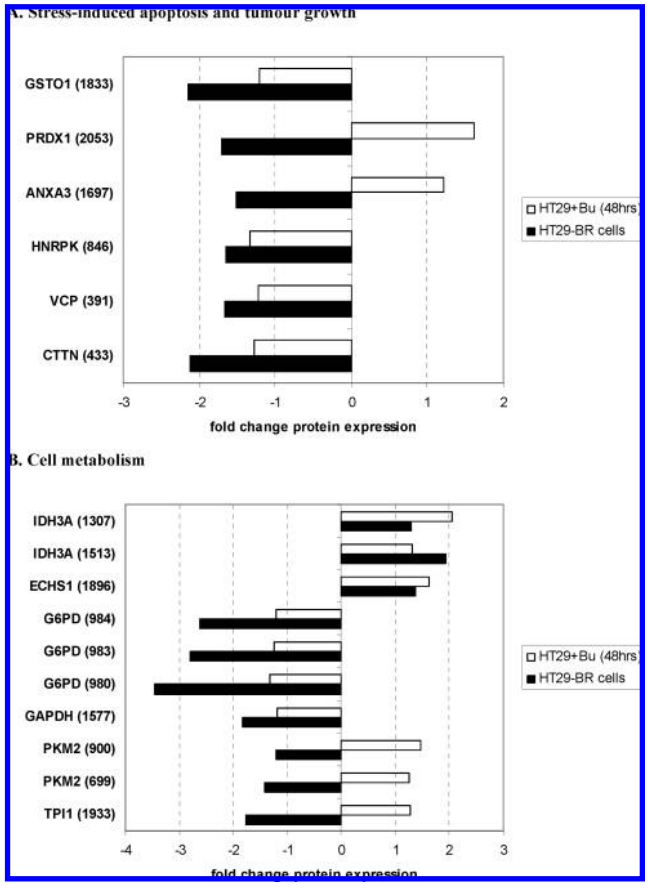

Figure 3. Expression profiles for proteins potentially contributing to the tumor suppressing effects and the apoptotic response observed in HT29 cells occurring as result of butyrate exposure. Additional details for each protein can be found in Supporting Information Table 1. White bars represent fold change in protein expression in HT29 cells treated with 5 mM butyrate for 48 h. Black bars represent fold change in protein expression in HT29-BR cells treated with 5 mM butyrate for 48 h. Vertical axis, gene ID and corresponding master gel spot numbers; horizontal axis, fold change in protein expression. (A) Stress-induced apoptosis and tumor growth. Butyrate was found to reduce the expression of proteins involved in cell proliferation and growth such as annexin A3, cortactin, hnRNP K and valosin containing protein. Two proteins, GST omega-1 and peroxiredoxin-1, are known to protect cells from stress-induced apoptosis. (B) Cell metabolism. The expression of the enzymes involved in cell metabolic pathways was altered by butyrate. In particular, glycolytic enzymes were found to decrease with prolonged butyrate exposure. In contrast, the expression of enzymes involved in alternate metabolic pathways (IDH3A and ECHS1) were up-regulated by butyrate.

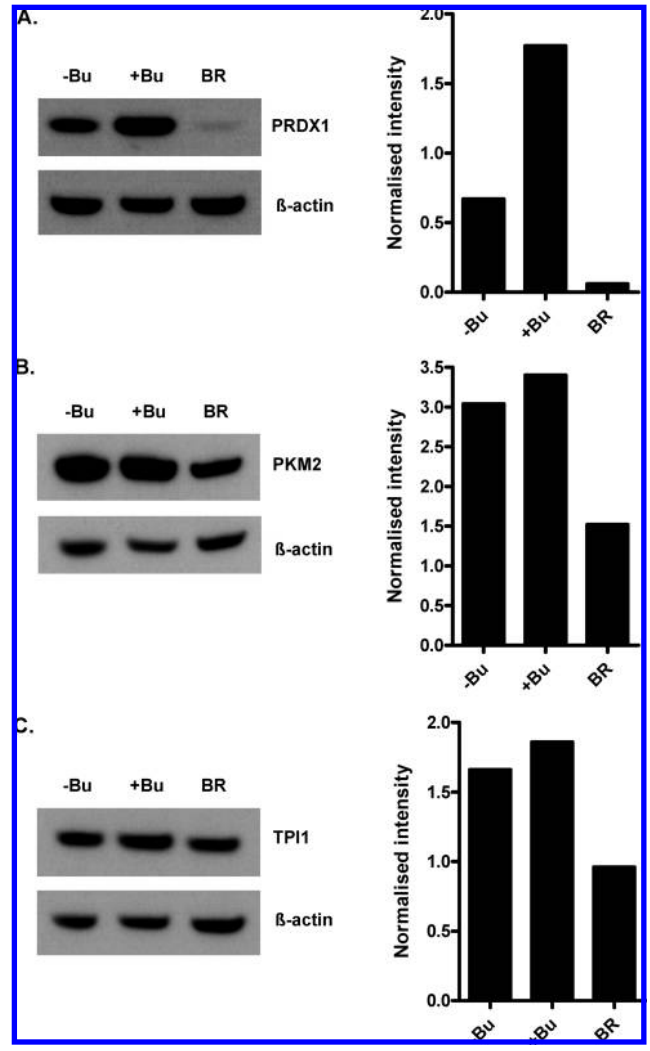

Figure 4. Representative Western blot analysis for selected differentially expressed proteins identified by proteomic analysis in HT29 cells, HT29 cells treated with butyrate for 48 h, and HT29-BR cell lysates. Densitometry results were normalized using β-actin as a loading control. (A) PRDX1 (peroxiredoxin 1): proteomic and Western blot analysis indicated that the expression of this protein increased in HT29 cells treated with butyrate for 48 h, and then subsequently decreased in HT29-BR cells. (B) PKM2 (pyruvate kinase): proteomic and Western blot analysis indicated that the expression of this protein was found to increase slightly in HT29 cells treated with butyrate (48 h). Conversely, expression of this protein decreased in the HT29-BR cell population. (C) TPI1 (triosephosphate isomerase): proteomic analysis indicated that the trend in expression of triosephosphate isomerase was similar to that of pyruvate kinase and this trend was confirmed by Western blot analysis. Expression of this protein was found to be slightly up-regulated in response to 48 h butyrate treatment, and a decrease in expression was observed in HT29-BR cells. Abbreviations: -Bu, untreated HT29 cells; +Bu, HT29 cells treated with butyrate for 48 h; BR, HT29-BR cells.

promoting proliferation of cancer cells.[39,40] The expression of this protein was significantly down-regulated with prolonged butyrate exposure (Figures 3B and 4B). It has also been recently demonstrated that HIF-1α can directly stimulate the glycolytic process during carcinogenesis[41] and butyrate may adversely influence these pathways to switch off the metabolic processes characteristic of tumor cell survival.

Conclusions

We have used proteomics to identify proteins potentially involved with (a) the tumor-suppressing effects of butyrate observed in HT29 colon cancer cell lines upon short-term exposure to this short chain fatty acid and (b) to support the phenotypic changes occurring when HT29 cells are rendered less sensitive to these effects as a result of prolonged butyrate exposure. This study has revealed a number of proteins in the HT29-BR cell population that act synergistically to develop a butyrate insensitive phenotype. This includes up-regulation of proteins which have been associated with tumor progression and malignancy, including proteins such as α-enolase, tran-

sketolase, cell cycle proteins and actin associated proteins. It is possible that reduced sensitivity to the tumor suppressing effects of butyrate and development of an altered phenotype in this cell population occur via GPCR activation of the MAPK/ERK signaling pathway and subsequent up-regulation of proteins such as heat shock beta-1. Additional investigations into these downstream effects of butyrate in the HT29-BR cell population may provide valuable insight into colorectal tumor progression and the ability of a subpopulation of cells to evade current chemotherapeutic strategies.

The tumor suppressing effect of butyrate in HT29 cells is supported by down-regulation of a number of potential proto-oncogenic proteins such as Crk-like protein. Further support of the tumor suppressing activity of butyrate is its ability to alter the metabolic profile of HT29 cells; that is, proteins identified as a result of exposure of HT29 cells to butyrate may reflect suppression of the Warburg effect. In addition, proteomic analysis has provided some evidence to indicate that the expression of proteins, such as cortactin and valosin containing protein, regulated by butyrate exposure, inhibits signaling cascades such as EGFR, Src and NFκB which are known to promote colorectal cancer progression. Further functional studies are required to determine the role of butyrate in mediating these signaling cascades to induce apoptosis in colorectal cancer cells.

Abbreviations: SCFA, short chain fatty acid; Bu, butyrate; HT29-BR, butyrate resistant HT29 colorectal cancer cells.

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Note Added after ASAP Publication. The manuscript was originally published on the Web on February 5, 2009, missing an author name. The version published on the Web February 10, 2009 is correct.

Supporting Information Available: Supplementary Figure 1, representative 2D DIGE image and protein spots determined to be differentially expressed; Supplementary Figure 2, functional classification of proteins identified by mass spectrometry based on GO annotations; Supplementary Table 1, proteins identified by mass spectrometry as being differentially expressed. This material is available free of charge via the Internet at http://pubs.acs.org.

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