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Cell Biology

$p21^{WAF1}$ is required for butyrate-mediated growth inhibition of human colon cancer cells

SONIA Y. ARCHER, SHUFEN MENG, AMY SHEI, AND RICHARD A. HODIN*

Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, 330 Brookline Avenue, Boston, MA 02215

Edited by Philip Leder, Harvard Medical School, Boston, MA, and approved April 10, 1998 (received for review November 13, 1997)

ABSTRACT **A diet high in fiber is associated with a decreased incidence and growth of colon cancers. Butyrate, a four-carbon short-chain fatty acid product of fiber fermentation within the colon, appears to mediate these salutary effects. We sought to determine the molecular mechanism by which butyrate mediates growth inhibition of colonic cancer cells and thereby to elucidate the molecular link between a high-fiber diet and the arrest of colon carcinogenesis. We show that concomitant with growth arrest, butyrate induces p21 mRNA expression in an immediate-early fashion, through transactivation of a promoter cis-element(s) located within 1.4 kb of the transcriptional start site, independent of p53 binding. Studies using the specific histone hyperacetylating agent, trichostatin A, and histone deacetylase 1 indicate that growth arrest and p21 induction occur through a mechanism involving histone hyperacetylation. We show the critical importance of p21 in butyrate-mediated growth arrest by first confirming that stable overexpression of the p21 gene is able to cause growth arrest in the human colon carcinoma cell line, HT-29. Furthermore, using p21-deleted HCT116 human colon carcinoma cells, we provide convincing evidence that p21 is required for growth arrest to occur in response to histone hyperacetylation, but not for serum starvation nor postconfluent growth. Thus, p21 appears to be a critical effector of butyrate-induced growth arrest in colonic cancer cells, and may be an important molecular link between a high-fiber diet and the prevention of colon carcinogenesis.**

Cancer of the colon is the third most common cancer in the United States and the second leading cause of death from cancer, affecting both men and women equally (1). Numerous epidemiological and experimental studies have identified an association between a high-fiber diet and a decreased incidence and growth of colon cancer (2). It appears that the key to these protective effects of dietary fiber lie in the production of butyrate by bacterial fiber fermentation within the colon. Butyrate, a four-carbon short-chain fatty acid, is a natural component of the colonic milieu and has been shown to inhibit the growth of colonic carcinoma cells, both *in vivo*, as in the prevention and decreased growth of chemically induced colonic cancers in the rat (3), and *in vitro* in many colorectal cancer cell lines such as HT-29 and LIM1215 (4, 5). Interestingly, the concentrations of butyrate that cause growth inhibition *in vitro* are similar to those measured within the mammalian colon (6).

Although the molecular mechanisms by which butyrate mediates its effects are not well understood, it is known to induce a variety of changes within the nucleus, including histone hyperacetylation and DNA methylation (7, 8). Of these changes, histone hyperacetylation has been the most extensively studied. Core histones, which package DNA into nucleosomes, can be acetylated on the ε-amino groups of specific lysine residues of the N-terminal tails. Acetylation and deacetylation are catalyzed by specific enzymes, histone acetyltransferase and deacetylase, respectively, with the net level of acetylation controlled by an equilibrium between these two enzymes. Butyrate causes histone hyperacetylation through a noncompetitive and reversible inhibition of histone deacetylase (9). Histone hyperacetylation generally has been associated with both a decrease in cell growth and the activation of specific genes (10, 11), effects similar to those of butyrate. Effects of butyrate attributable to histone hyperacetylation can be assessed by comparing the effects of butyrate to those of (R)-trichostatin A (TSA), a compound structurally unrelated to butyrate, which has been shown to be a very potent and specific inhibitor of histone deacetylase at very low concentrations (12).

In a variety of cell types, butyrate and specific histone hyperacetylating agents (such as TSA and trapoxin) have been shown to cause a G_1 cell cycle arrest (13–15), in addition to inducing differentiation characteristics. The cyclin-CDK inhibitor, p21, also is known to exert a G_1 cell cycle arrest in response to a variety of stimuli, e.g., by DNA damage, in a p53-dependent fashion (16), or during mammalian development and cellular differentiation, by p53-independent pathways (17, 18). Because p21, butyrate, and histone hyperacetylation all are associated with a G_1 cell cycle arrest, we sought to determine whether growth inhibition of human colonic cancer cells by butyrate was mediated by the p21 cell cycle inhibitor and whether this process involved histone hyperacetylation. Here, we present evidence that p21 expression is induced by butyrate through a process involving histone hyperacetylation, and that p21 is required for butyrate-mediated growth arrest in colon carcinoma cells.

MATERIALS AND METHODS

Cell Culture. HT-29 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). HCT116 (+/+), (+/−), and (−/−) cells were kindly provided by B. Vogelstein (Johns Hopkins University School of Medicine, Baltimore) (19). Experiments were performed on cells at 80% confluence, and the media were changed 24 hr before the start of each experiment. Cells were treated with various concentrations of sodium butyrate (NaBu) or TSA as indicated. Some cells were treated concomitantly with the protein synthesis inhibitor, cycloheximide (10 μg/ml). Some cells were also serum-starved or grown to 2 weeks postconfluence.

Northern Blot Analyses. Total RNA was extracted by using the guanidinium thiocyanate method (20). Northern blot analyses were performed by loading 20 μg RNA in each lane

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Abbreviations: TSA, trichostatin A; HDAC1, histone deacetylase 1; NaBu, sodium butyrate; CAT, chloramphenicol acetyltransferase.

*To whom reprint requests should be addressed at: Department of Surgery, Beth Israel Deaconess Medical Center, Room ST 932, 330 Brookline Avenue, Boston, MA 02215. e-mail: rhodin@bidmc.harvard.edu.

of an agarose/formaldehyde gel, separating by gel electrophoresis, transferring to nitrocellulose membranes, and baking for 2 hr at 80°C. ^{32}P-radiolabeled cDNA probes (random primer method, ref. 21) were made specific for p21, a 1.009-kb *Xho*I/*Eco*RI fragment derived from the human Cip1 cDNA (ATCC), and actin, a 1.0-kb *Pst*I fragment derived from the mouse β-actin cDNA (22). Conditions of hybridization were: 5× standard saline citrate (SSC)/50% (vol/vol) formamide/1% (wt/vol) SDS at 42°C. Washing conditions were: 2× SSC/0.1% SDS at 50°C.

^{3}H-thymidine Assay. Cells were seeded in six-well plates at equal density. Some cells were treated 48 hr later with NaBu or TSA and others were serum-starved or grown to 2 weeks postconfluence. Six hr before the assay, ^{3}H-thymidine was added to the media at a concentration of 1 μCi/ml. Cells were washed with PBS, treated with 5% trichloroacetic acid, and lysed with NaOH. Counts of cell lysates were measured by scintillation scanning and normalized for total protein content (Bradford assay).

DNA Transfer. Both transient and stable transfections were accomplished by using the $CaPO_4$/DNA coprecipitation technique (Stratagene). For transient transfection studies, to examine the effects of histone hyperacetylation on the p21 promoter, HT-29 cells were transfected with 10 μg of the murine p21CAT plasmid or empty vector (17), −/+ the histone deacetylase enzyme expression plasmid, pBJ5-HDAC1, or empty vector (23). Cells were then treated with ±5 mM NaBu or 0.3 μM TSA for 24 hr, then harvested, and total protein was isolated. Chloramphenicol acetyltransferase (CAT) assays were carried out with 100 μg of protein, using the Stratagene FlashCAT kit, and reaction products were separated by thin layer chromatography. Fluorimetric analyses of reaction products were performed and resulting numerical values were compared for each group. For control purposes, cells were transfected with 10 μg of pSVCAT or the thyroid hormone-responsive pTK14AACAT (24) −/+ HDAC1, and the TK14AACAT cells then treated with ±10 μM thyroid hormone (T3). Cotransfections with a pTKGH plasmid were used to control for transfection efficiency. For stable transfections, HT-29 cells were transfected with an expression plasmid encoding the neomycin resistance gene (pSV2-neo, ATCC) and/or the human p21 gene (pCMV-Cip1, ATCC). Pooled stable transfectants were selected based on growth in G418 media. Experiments were repeated with at least three different pools of transfectants to verify the results.

Data Analysis. Relative changes in mRNA levels on Northern blots were assessed by laser densitometry of the autoradiograms and normalized for actin mRNA. Statistical analyses were carried out by using the Student's unpaired *t* test, $P < 0.05$, considered statistically significant.

RESULTS

Butyrate Induces p21 mRNA Expression. HT-29 human colon carcinoma cells undergo a G_1 cell cycle arrest in response to sodium butyrate (4). Because the cell cycle inhibitor, p21, is known to effect a G_1 arrest (25), we determined whether p21 levels increased in response to butyrate in HT-29 cells. As shown in Fig. 1*A*, p21 mRNA was induced by NaBu, with maximal effects occurring at the 5 mM concentration and remaining unchanged up to 20 mM. These concentrations of butyrate are similar to those normally present within the mammalian colon.

Fig. 1*B* shows that p21 mRNA induction by NaBu begins early (by 2 hr), and the levels remain elevated for up to 24 hr. Butyrate did not induce mRNA expression of two other cell cycle inhibitors, p16 and p27 (data not shown). Concomitant treatment of HT-29 cells with NaBu and the protein synthesis inhibitor, cycloheximide, revealed that p21 induction by NaBu is not blocked and, in fact, mRNA levels are slightly higher,

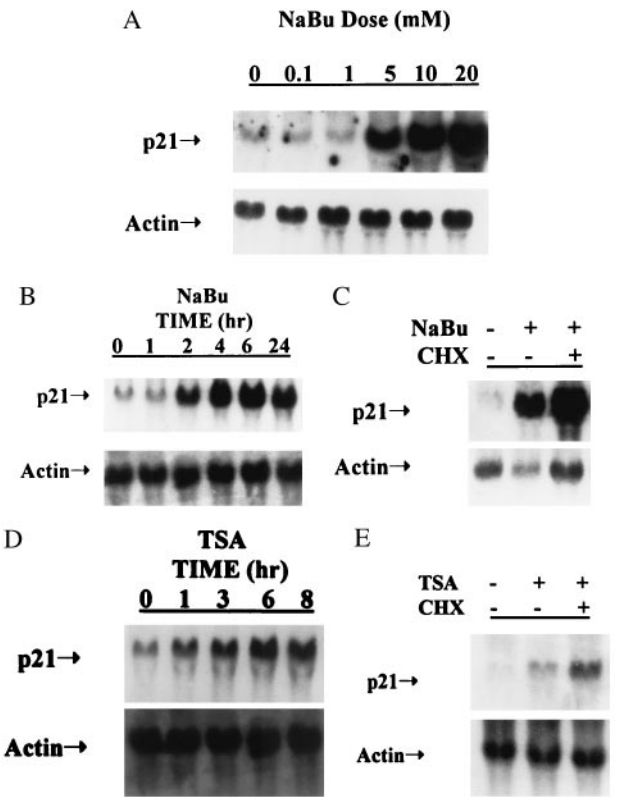

FIG. 1. NaBu and TSA induce p21 mRNA expression in HT-29 cells in an immediate-early fashion. (*A*) Dose response for p21 induction by NaBu. HT-29 cells were treated with various concentration of NaBu, 0–20 mM for 24 hr, and p21 mRNA expression was examined by Northern blot analyses. (*B*) Time course for induction of p21 by NaBu. Cells were treated with 5 mM NaBu for varying lengths of time, from 0 to 24 hr, and p21 induction was examined by Northern analysis. (*C*) p21 induction by NaBu is not blocked by protein synthesis inhibition. HT-29 cells were treated concomitantly with 5 mM NaBu and the protein synthesis inhibitor, cycloheximide (CHX, 10 μg/ml), for 48 hr. (*D*) Time course for p21 induction by TSA. Cells were treated with 0.3 μM TSA for varying lengths of time. (*E*) p21 induction by TSA is not blocked by protein synthesis inhibition. Cells were treated concomitantly with 0.3 μM TSA and 10 μg/ml CHX for 6 hr. Representative Northern blots ($n = 4$) are depicted in each figure. The actin control is shown in the lower panel. Twenty micrograms of total RNA was loaded in each lane, with equal loading verified by ethidium bromide staining.

indicating that p21 is induced by butyrate as an immediate-early gene (Fig. 1*C*).

p21 mRNA Expression Is Induced by Histone Hyperacetylating Agents. Because butyrate is known to cause histone hyperacetylation, we compared its effects to those of another histone hyperacetylating agent, TSA, a potent and specific inhibitor of histone deacetylase. TSA, though a structurally distinct chemical, like butyrate causes growth arrest and differentiation of various cell types (26, 27). We found that TSA induces p21 mRNA expression, with maximal effects occurring at the 0.3 μM dose (data not shown), a concentration known to cause histone hyperacetylation (12). Similar to NaBu, the effects of TSA on p21 occur early, at 1 hr (Fig. 1*D*), and are not blocked by concomitant cycloheximide treatment (Fig. 1*E*), consistent with an immediate-early mechanism.

p21 Gene Transactivation Is Mediated by Histone Hyperacetylation, Independent of p53. Transient transfection assays then were used to further delineate the mechanism by which p21 is induced by NaBu and TSA. Plasmids containing the CAT reporter gene under the control of various portions of the mouse p21 promoter region (from 0 to 4.7 kb upstream of the transcriptional start site) were transiently transfected into

HT-29 cells, and the cells were treated with NaBu or TSA. As shown in Fig. 2*A*, both NaBu and TSA induce the p21 promoter constructs equally, from 1.4 to 4.7 kb, even in those plasmids lacking the two p53-binding sites, located at −2.85 and −1.95 upstream from the transcriptional start site. These results indicate that transactivation of the p21 gene by NaBu and TSA occurs via a cis-element(s) located within 1.4 kb of the transcriptional start site, in a p53-independent fashion.

The role of histone hyperacetylation in p21 induction was examined further by cotransfection of a plasmid encoding the recently cloned mammalian histone deacetylase enzyme 1, HDAC1. Overexpression of this HDAC1 expression plasmid by transfection into eukaryotic cells has been shown to cause an increase in histone deacetylase activity (23). We hypothesized that if p21 induction by butyrate were mediated by histone hyperacetylation, then overexpression of HDAC1 should block its induction by both NaBu and TSA. As shown in Fig. 2*B*, HDAC1 largely blocked induction of the p21 reporter constructs by both NaBu and TSA (74 and 70%, respectively). This effect was found to be dose-dependent, because increasing doses of HDAC1 resulted in progressively greater inhibition in p21 reporter gene activity (data not shown). For control purposes, HDAC1 was found to have no effects on the induction of a thyroid hormone-responsive reporter plasmid and only minimally altered the basal expression of the control plasmid, SVCAT. In addition, the HDAC1 empty vector had no effect on the p21 CAT reporter gene expression (data not shown). Thus, it appears that overexpression of histone deacetylase is specifically able to block induction of p21 by NaBu and TSA, consistent with a role for histone hyperacetylation in p21 induction by these agents.

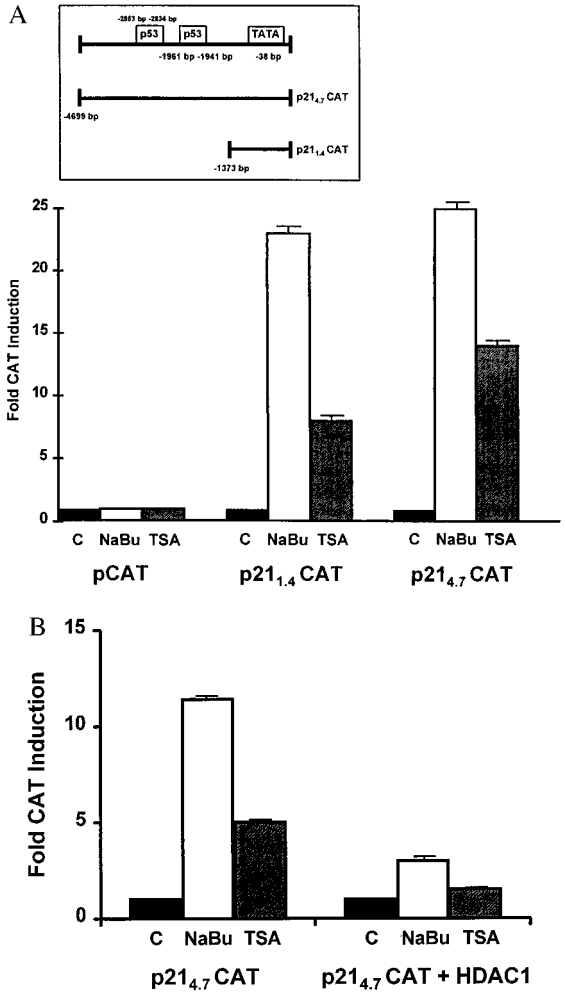

FIG. 2. Overexpression of histone deacetylase (HDAC1) blocks induction of the p21 promoter by NaBu and TSA. (*A*) NaBu and TSA induce p21 promoter activity. HT-29 cells were transiently transfected with CAT reporter plasmids under the control of various amounts of the mouse p21 promoter (0–4.7 kb upstream from the transcriptional start site). Transfectants were treated without or with 5 mM NaBu or 0.3 μM TSA, and total cellular protein was examined for CAT expression. Results are shown for the 0-kb (pCAT), 1.4-kb (p21$_{1.4}$CAT), and 4.7-kb (p21$_{4.7}$CAT) p21 promoter constructs and are expressed as fold CAT induction compared with the untreated negative control, arbitrarily taken as 1. (*B*) Cotransfection of HDAC1 blocks p21 promoter induction by NaBu and TSA. Transfections were performed with the p21$_{4.7}$ CAT −/+ HDAC1, and cells were treated without or with 5 mM NaBu or 0.3 μM TSA. The results are expressed as fold CAT induction compared with the untreated negative control (arbitrarily taken as 1). All results are shown as mean ± SEM ($n \geq 4$, in all cases).

Importance of p21 in Butyrate-Mediated Growth Inhibition. We next examined the importance of the p21 gene in the growth inhibition of colon cancer cells by NaBu and TSA. Fig. 3*A* shows that both NaBu and TSA markedly inhibit the growth of HT-29 cells as reflected by the dramatic decrease in ^{3}H-thymidine incorporation after 24 hr of treatment, supporting the link between histone hyperacetylation and decreased growth. HT-29 cells were then stably transfected with plasmids encoding the human p21 gene and/or the neomycin resistance

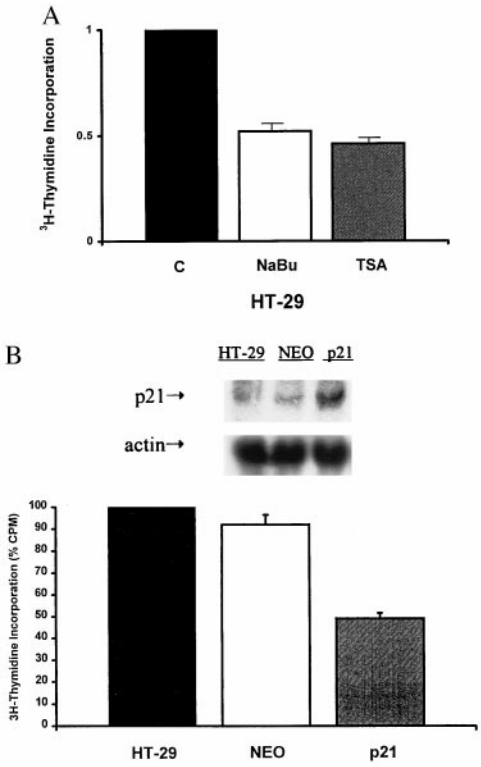

FIG. 3. NaBu, TSA, and p21 overexpression inhibit growth of HT-29 cells. (*A*) NaBu and TSA inhibit growth of HT-29 cells. Cells were plated at equal density in six-well plates. At 80% confluence, cells were treated without or with 5 mM NaBu or 0.3 μM TSA for 24 hr. ^{3}H-thymidine (1 μCi/ml) was added for the last 6 hr of treatment, and incorporation was measured by scintillation counting. Treatment results are expressed as percent change compared with untreated negative control, arbitrarily taken as 1. (*B*) p21 overexpression inhibits growth of HT-29 cells. Cells were stably transfected with an expression plasmid containing the human p21 gene, and overexpression of the gene in pooled stable transfectants compared with the parental and NEO controls ($n = 5$ each) was determined by Northern analyses (see *Inset*). ^{3}H-thymidine incorporation was measured in exponentially growing cells under basal conditions, and results are expressed as percent change compared with parental control, arbitrarily taken as 100%, and are shown as mean ± SEM ($n \geq 4$, in all cases).

gene, with pooled transfectants selected by growth in G418 media. Overexpression of the p21 gene was verified by Northern blot analysis, which showed an approximate 5- to 10-fold increase in p21 mRNA expression over controls (see Fig. 3*B Inset*). On visual inspection, p21 stable transfectants grew slowly, and this was confirmed by a 50% decrease in ^{3}H-thymidine incorporation when compared with parental and neomycin controls (Fig. 3*B*). These results provide direct evidence that p21 overexpression results in growth inhibition in HT-29 cells, similar to that seen in other cell types (28).

p21 Is Required for Butyrate-Mediated Growth Inhibition. We next sought to determine whether the p21 gene was an absolute requirement for the growth inhibition by NaBu and TSA. For these studies, we used the parent human colon carcinoma, HCT116 p21 (+/+), along with HCT116 p21-deleted cells (−/−). These p21-deleted cells, created by homologous recombination techniques, have been used previously to demonstrate the importance of p21 in p53-mediated growth arrest in response to DNA damage (19).

First, we examined the induction of p21 by NaBu and TSA in the wild-type HCT116 +/+ cells. As in HT-29 cells, there was an early induction of p21 mRNA in the HCT116 +/+ cells, in response to both NaBu and TSA treatment (Fig. 4*A*). As expected, no mRNA band for p21 was observed in the HCT116 −/− cells (data not shown).

The importance of p21 induction in mediating the growth inhibition of the histone hyperacetylating agents then was assessed. As shown in Fig. 4*B*, both NaBu and TSA caused significant decreases in ^{3}H-thymidine incorporation in HCT116 +/+ cells (53 ± 9% and 46 ± 2.6%, respectively), similar to that seen in HT-29 cells (see Fig. 3*A*). The heterozygote HCT116 +/− cells also behaved similarly to the parental HCT116 +/+ cells in that they were growth-inhibited by NaBu (data not shown). In dramatic contrast, neither NaBu nor TSA inhibited growth in HCT116 −/− cells (Fig. 4*B*). Similar results were obtained in a different clone of HCT116 −/− cells (data not shown). To determine the specificity of this lack of response to the histone hyperacetylating agents, we exposed both HCT116 +/+ and HCT116 −/− cells to other conditions known to arrest cell growth, i.e., 24 hr of serum starvation or 2 weeks of postconfluent growth. As seen in Fig. 4*C*, the p21-deleted cells appropriately slowed their growth under these conditions, with an approximate 50% reduction in ^{3}H-thymidine, similar to the wild-type cells. Taken together, these data suggest that the p21 gene is critically important in the growth inhibition induced by the histone hyperacetylating agents, NaBu and TSA, whereas p21 is not required for growth arrest induced by either serum starvation or postconfluent growth.

DISCUSSION

Previous studies examining the protective effect of dietary fiber on colonic neoplasia have pointed to butyrate as the key mediator of these effects. This short-chain fatty acid is a product of fiber fermentation by resident anaerobic bacteria in the normal colon. *In vivo* studies using rat models of large bowel cancer indicate that high-fiber diets that are associated with high butyrate levels prevent or decrease the incidence of colon cancers. These findings have been corroborated by *in vitro* studies in which butyrate has been shown to slow the proliferation and promote the expression of phenotypic markers of differentiation in colon cancer cell lines. Indeed, of all the short-chain fatty acids studied, butyrate appears to have the most profound effects on growth inhibition and differentiation (5).

The present data indicate that butyrate achieves its growth inhibition in colonic cancer cells by transcriptional upregulation of the cell cycle inhibitor, p21. The mechanisms that regulate p21 expression fall into two general categories: those that are either dependent or independent of the tumor suppressor, p53. p53 appears to be important in p21 activation in response to signals of cellular stress, e.g., DNA damage by γ-irradiation or nucleotide pool depletion (16, 29). p53 mediates induction of the p21 gene by transcriptional activation via cis-elements located 1.95 and 2.85 kb upstream from the transcriptional start site in the mouse and −2.4 kb in the human p21 gene (18, 28). p53-independent mechanisms of p21 induction and growth arrest have been less extensively studied but appear to operate under conditions of normal mammalian development as well as during differentiation of a variety of cell lines in culture. Emerging data have identified some

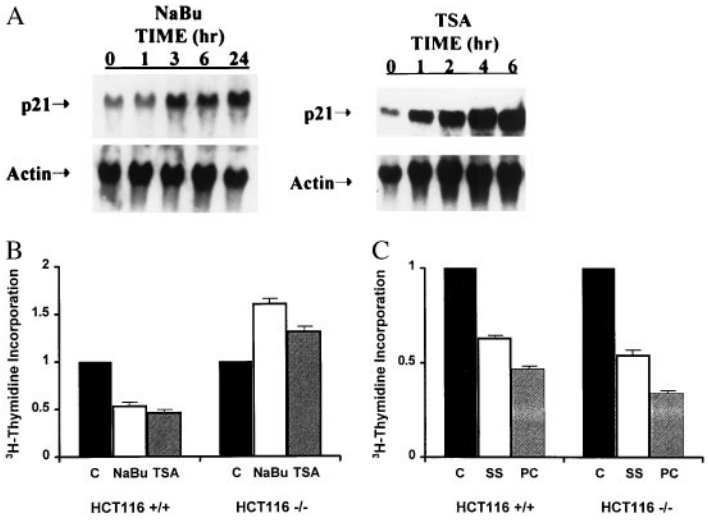

FIG. 4. p21 is required for NaBu-induced growth inhibition of colon cancer cells. (*A*) p21 mRNA is induced early in HCT116 +/+ cells. Cells were treated at 80% confluence with 1 mM NaBu or 0.15 μM TSA (higher concentrations of either agent led to cell death), and p21 mRNA expression was examined by Northern analysis. (*B*) p21 deletion prevents growth inhibition by butyrate and TSA. HCT116 +/+ and −/− cells were treated with 1 mM NaBu or 0.15 μM TSA for 24 hr, and 1 μCi/ml ^{3}H-thymidine was added for the last 6 hr of treatment. Incorporation was measured by scintillation counting. (*C*) HCT116 −/− cells are growth-inhibited by serum starvation and postconfluent growth. HCT116 +/+ and −/− cells were serum-starved for 24 hr or grown to 2 weeks postconfluence, and ^{3}H-thymidine incorporation was measured. Cells grown in standard McCoy's 5A medium with 10% fetal bovine serum and that were preconfluent were used as controls. Results are expressed as percent change compared with control groups (arbitrarily taken as 1) and are shown as mean ± SEM ($n \geq 4$, in all cases).

p53-independent transcriptional activators of p21 and include MyoD (during myocyte differentiation, ref. 30), STAT 1 (by interferon γ treatment, ref. 31), and, most recently, BRCA1 (32). Our studies show that p21 induction occurs in an immediate-early fashion, independent of p53, and via a cis-element(s) located within 1.4 kb of the transcriptional start site. Additional studies will be needed to identify the specific transcription factors that mediate p21 induction.

Butyrate is known to induce general histone acetylation, but more specifically, hyperacetylation of the H3 and H4 species, through a noncompetitive inhibition of the histone deacetylase enzyme. This action likely occurs *in vivo*, because in rats fed a high-fiber diet, high butyrate levels were correlated with histone hyperacetylation in colonic epithelial cells (33). Histone hyperacetylation has been generally associated with areas of increased transcriptional activity within the genome. Hyperacetylation of histones neutralizes their positive charge, disrupting their ionic interaction with DNA, and thereby allowing transcription factors to access and activate specific genes (34). Recent studies have identified a number of transcriptional activators and coactivators possessing histone acetyltransferase activity, such as Gcn5p and CBP/300, as well a subunit of the basal transcriptional machinery, $TAF_{II}250$ (35–37). Conversely, several transcriptional repressors have been associated with histone deacetylase activity, e.g., Mad and members of the nuclear receptor superfamily (38–40). The recent cloning and molecular characterization of human HDAC1 has made it possible to further analyze the role of histone hyperacetylation in gene activity. Thus, it appears that specific genes are targeted for activation or repression by histone-acetylating or histone-deacetylating activity of specific transcription factors or cofactors. Little is known, however, regarding which specific genes are regulated by the process of histone hyperacetylation, though it has been suggested as an important regulatory mechanism for some yeast genes and the human endothelial tissue-type plasminogen activator gene (41, 42). Our data suggest that histone hyperacetylation is at least partly responsible for the induction of p21, because both butyrate and the specific inhibitor of histone deacetylase, TSA, resulted in a similar induction of p21. This model is supported by the finding that p21 mRNA induction does not require new protein synthesis (suggesting modification of an already existent protein that directly mediates changes in p21 gene acetylation state) and that p21 promoter induction by NaBu and TSA is specifically inhibited by overexpression of the HDAC1. Although it is not known exactly how HDAC1 works, the current model is that HDAC1 is targeted to specific genomic regions by association with specific transcription factors, thus contributing to repression of these genes (39, 43). It is likely, therefore, that butyrate and TSA are working to inhibit HDAC1 at the level of the p21 gene, leading to changes in chromatin that allow transcriptional activation of this gene. It is possible that other mechanism(s) also may be playing a role in p21 induction because HDAC1 was unable to completely block p21 reporter gene transactivation. Because butyrate is also known to inhibit the deacetylation of nonhistone proteins such as high-mobility group proteins (44), it is possible that other acetylated proteins may be involved in p21 gene regulation. Alternatively, other biochemical effects of butyrate may be important, e.g., DNA methylation.

Our studies suggest that the cyclin-CDK inhibitors p16 and p27 may not be important in butyrate-mediated growth arrest because they were not induced by butyrate. Additionally, our studies indicate that p21 is not required for two other modes of growth arrest, i.e., serum starvation and postconfluence growth. Other cell cycle inhibitors may be important under these conditions. For example, p27 has been shown to mediate inhibitory responses under postconfluent conditions in Mv1 Lu Mink epithelial cells (45) and cellular quiescence resulting from withdrawal of mitogens in T cell clones (46). It is important to note that though butyrate is known to cause apoptosis in colon cancer cells, it does not appear that apoptosis is the explanation for the growth inhibition by butyrate in these studies. Hague *et al.* (47) have shown that there is minimal apoptosis in control and butyrate-treated colon cancer cells that were adherent, but extensive in those that were floating. Our ^{3}H-thymidine incorporation assays were performed on adherent cells, and the cell counts were normalized for total cellular protein content. Also, few cells were observed to be floating in the media at 24 hr of butyrate (and TSA) treatment. In addition, Barnard and Warwick (4) showed that the growth-inhibitory effect of butyrate in HT-29 cells was reversible upon its removal and that the cells were able to reenter the cell cycle and proliferate. This suggests that the major growth inhibitory effects of butyrate under these conditions (similar to those in our studies) are not related to apoptosis.

The present studies show that p21 is an absolute requirement for growth arrest by butyrate. Other investigators have shown a similar role for p21 in the growth arrest response to other stimuli, e.g., γ-irradiation and, most recently, BRCA1 (21, 32). Growth of the HCT116 $-/-$ cells was not inhibited by butyrate, and in fact, both butyrate and TSA treatment appeared to potentiate growth. The reason for the observed increase in HCT116 $-/-$ cell growth in response to butyrate is not clear. It is interesting, however, to note that these cells have been shown to display S/M uncoupling (several rounds of S phase cycles despite a G_2 block) in response to certain chemotherapeutic agents (48). Nevertheless, our results clearly indicate the vital role that p21 plays in butyrate-induced growth arrest in colon carcinoma cells, though other downstream gene products may cooperate in mediating this effect.

The results of these studies are of potential clinical interest, because agents that increase histone hyperacetylation and p21 expression within colon cancer cells may be incorporated into strategies directed toward the prevention and treatment of colon cancer.

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