Changes in E2F Binding after Phenylbutyrate-induced Differentiation of Caco-2 Colon Cancer Cells

Qing Mei Wang, Rena Feinman, Fatah Kashanchi, Jean Marie Houghton, George P. Studzinski, and Lawrence E. Harrison

ABSTRACT
Differentiation agents use existing cellular systems to induce neoplastic cells to regain a normal phenotype and/or to cause growth arrest and therefore may offer novel chemotherapeutic approaches to treating solid tumors. In this study, we demonstrate in Caco-2 colon cancer cells that the differentiation agent phenylbutyrate (PB) causes a decrease in viable cells, an increase in cell differentiation, and a G1-S-phase block. The mechanism of this last effect is related to a PB-induced increase in p27Kip1, leading to a decrease in the activity of cyclin-dependent kinase 2 (CDK2), a positive regulator of the G1-S-phase cell cycle transition. Consistent with the decreased CDK2 kinase activity, we also observed a decrease in the phosphorylation state of the retinoblastoma protein after PB treatment. This was associated with increased binding and consequent inactivation of E2F, a transactivator of genes that regulate G1 to S phase cell cycle transition. These data suggest that the differentiation agent PB inhibits tumor growth by altering the availability of active E2F, with a subsequent G1-S-phase block. Additional studies should show whether PB is a clinically effective therapeutic agent against colorectal cancer.

INTRODUCTION
Each year, approximately 140,000 new cases of colorectal cancer are diagnosed in the United States, making it the third leading cause of cancer deaths (1). Cytotoxic chemotherapy has been used to treat patients with advanced colorectal cancer with limited success, often at the expense of severe side effects. Therefore, novel chemotherapeutic approaches are needed. An alternative to cytotoxic chemotherapy is differentiation therapy, which uses existing cellular systems to induce the cell to regain a normal phenotype and/or to cause growth arrest with subsequent apoptosis. Whereas it has been well documented that butyrate is an effective differentiation agent in vitro (2–4), clinical trials evaluating sodium butyrate as a therapeutic agent for treating malignancy have been disappointing. Based on the encouraging preclinical data, there has been an interest in developing derivatives of butyrate as clinically applicable differentiating agents. PB is a butyrate analogue closely related to the aromatic fatty acid phenylacetate. Whereas phenylacetate has recently been proposed as a differentiation agent and is undergoing active clinical trials for a variety of malignancies, its intensely unpleasant odor makes it a very impractical drug for clinical use. PB has been suggested as a more logical compound for clinical use because it is a nontoxic agent, can be taken in an oral form, and can safely achieve millimolar plasma concentrations in humans (5). Importantly, recent studies have suggested that PB is an active agent in treating cancer in both cell culture (6–8) and animal (9) models.

The purpose of this study was to investigate the growth-inhibitory and differentiating effects of PB in a human colon cancer cell model and to correlate these effects with PB-induced alterations in cell cycle control. We demonstrate that PB induces Caco-2 colon cancer cells to undergo growth arrest and cellular differentiation associated with a G1-S-phase cell cycle block. This G1-S-phase block appears to be regulated in part through a decrease in kinase activity of CDK2, which may be mediated through an increase in the expression of CDK inhibitor p27Kip1. This decrease in CDK2 activity relates to an observed hypophosphorylation of pRb after PB exposure. PB exposure was associated with increased pocket protein binding and subsequent inactivation of E2F, a transactivator of genes regulating G1 to S-phase cell cycle transition. These data suggest that the differentiation agent PB inhibits tumor growth by limiting the availability of active transcription factors that regulate cell cycle traverse.

MATERIALS AND METHODS
Materials. Unless otherwise indicated, all chemicals were obtained from Sigma (St. Louis, MO). All antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). PB was a kind gift from Dr. Hokan Cederberg (Triple Crown, Philadelphia, PA).

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3 The abbreviations used are: PB, phenylbutyrate; CDK, cyclin-dependent kinase; pRb, retinoblastoma protein; ALP, alkaline phosphatase; EMSA, electrophoretic mobility shift assay; FUDR, fluorodeoxyuridine; PPAR, peroxisome proliferator-activated receptor; HDAC, histone deacetylase; Rb, retinoblastoma.
Cell Culture. Caco-2 cells were maintained in RPMI 1640 supplemented with 10% complement-inactivated bovine calf serum. Cells (3 × 10^5) were seeded and treated with PB for 0 (control), 24, and 48 h. Cells were harvested and counted by hemocytometer, and viability was determined by trypan blue (0.25%) exclusion.

ALP Activity. ALP activity was measured by a commercially available kit (Sigma). Briefly, total cellular lysates were prepared (in the absence of NaF and NaVO₄), and aliquots of 100 μg of protein were assayed for ALP activity by the addition of p-nitrophenyl phosphate substrate. After incubation at 37°C for 15 min, absorbance was read at 410 nm.

Flow Cytometry. For DNA content evaluation, 3 × 10^6 cells were fixed in 75% ethanol at −20°C for 24 h. After washing in ice-cold PBS, the cells were incubated with 0.5 ml of propidium iodide stain (10 μg/ml) in the presence of RNase (500 μg/ml) for 2 h. The DNA content was determined using the Epics Profile II Flow cytometer (Coulter, Hialeah, FL), and cell cycle distribution was analyzed by the Multicycle software package (Phoenix Flow Systems, San Diego, CA).

Protein Preparation, Immunoblotting, and Immunoprecipitation. Whole cell extracts were prepared by lysing cell pellets with a Dounce microtip homogenizer in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% NP40, 50 mM NaF, 0.2 mM NaVO₄, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 25 μg/ml aproatin, and 25 μg/ml pepstatin A). Cell debris was removed by centrifugation at 14,000 × g for 20 min at 4°C, and the supernatants were stored at −80°C.

Samples for immunoblotting were prepared by mixing aliquots of the protein extracts with 3× SDS sample buffer [150 mM Tris (pH 6.8), 30% glycerol, 3% SDS, bromphenol blue dye (1.5 μg/100 ml), and 100 mM DTT] and denatured by heating to 100°C for 4 min. Protein samples were then separated by SDS-PAGE, electrotransferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL), and incubated in 5% nonfat milk blocking buffer (Tris-buffered saline, 5% dry milk, and 0.05% Tween 20) for 1 h. The membrane was subjected to immunoblot analysis with the appropriate antibody, and proteins were visualized by the enhanced chemiluminescence method of detection (Amersham).

For immunoprecipitation, cells were lysed in lysis buffer, and cell debris was removed by centrifugation at 14,000 × g for 20 min at 4°C. Total protein (500 μg) was incubated with the appropriate antibody for 2 h at 4°C, followed by incubation with protein A/G-agarose beads for 1 h. The protein complexes were washed three times with immunoprecipitation buffer, released from the beads by boiling in 3× SDS sample buffer for 5 min, and separated by SDS-PAGE.

Kinase Reaction Assays. Total cellular lysates were prepared and immunoprecipitated as described above. The agarose beads were washed with immunoprecipitation buffer and subsequently washed with kinase reaction buffer (50 mM HEPES, 10 mM MgCl₂, 5 mM MnCl₂, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml aproatin, 10 μg/ml pepstatin A, 0.2 mM NaVO₄, 1 mM phenylmethylsulfonyl fluoride, and 50 mM NaF). The kinase reaction was carried out at 37°C for 30 min in 40 μl of kinase reaction buffer containing 10 μM ATP, 0.4 mM CTP [γ-32P]ATP (specific activity, 3000 Ci/mmol), and 2 μg of histone H1. The reaction was stopped by adding 3× SDS sample buffer, and the supernatant was separated on a 13% SDS-PAGE gel. The radioactivity of the 32P-labeled histone was detected by autoradiography.

EMSA. EMSAs were performed as follows: a reaction mixture of binding buffer [50 mM KCl, 20 mM HEPES-KOH (pH 7.5), 10 mM MgCl₂, 10% glycerol, 0.5 mM DTT, and 1% NP40], 0.5 ng of 32P-labeled oligonucleotide probe, 2 μg of sonicated salmon sperm DNA, and 10 μg of nuclear protein was incubated at 25°C for 10 min, and the reaction products were separated on a 4% polyacrylamide gel in 0.25× TBE (22.5 mM Tris-borate and 0.5 mM EDTA). For antibody perturbation experiments, 2.5 μg of antibody were added 10 min before the addition of the oligonucleotide probe and incubated at 25°C. For oligonucleotide competition experiments, a 50-fold excess of unlabeled competitor oligonucleotide was added 10 min before the addition of the oligonucleotide probe and incubated at 25°C. The double-stranded DNA oligonucleotide for E2F is commercially available from Santa Cruz Biotechnology and contains the consensus binding site for E2F-1 (5′-ATTTAGTTTCCGC-CCCTTTCTCAA-3′).

RESULTS

PB Induces Differentiation, Growth Inhibition, and G1-S-phase Cell Cycle Block in Caco-2 Colon Cancer Cells. These experiments were designed to determine whether PB inhibits cell growth and induces differentiation in colon cancer cells. Caco-2 cells were treated with PB [0 (control), 1, 3, and 5 mM] for 48 h. In additional experiments, cells were treated with PB (3 mM) for 24, 48, and 72 h. The results of these experiments demonstrated that exposure of colon cancer cells to PB resulted in a significant growth inhibition and an increase in cellular differentiation (ALP activity; Fig. 1, A–D). ALP activity, a well-described measure of enterocyte differentiation (10), was determined in aliquots of equal protein from cell lysates in treated and untreated cells.

To determine whether this decrease in proliferation and increase in differentiation after PB treatment was related to an alteration in cell cycle traverse, cell cycle analysis of Caco-2 cells exposed to PB (3 mM for 48 h) or control cells (untreated) was performed by measuring DNA content. Exposure to PB resulted in a significant G1-S-phase block (Fig. 2), and this block was reversible after the removal of PB from growth medium (data not shown).

PB-induced Inhibition of the G1-S-phase Cell Cycle Checkpoint Is Associated with Decreased CDK2 Activity and Up-Regulation of p27kip1. Because the antiproliferative and differentiating effects of PB are associated with a G1-S-phase block, we next focused our studies on the major regulatory proteins responsible for G1-S-phase traversal. Treatment with PB (3 mM for 48 h) induced a modest decrease in the protein expression of the G1-S-phase CDK2, with no effect on the levels of CDK4 and CDK6 (Fig. 3A). However, when in vitro kinase activity of these CDKs was measured by 32P labeling of histone H1, CDK2 demonstrated decreased kinase activity (2.6-fold) after PB exposure (Fig. 3B). PB had no effect on the protein expression of cyclins D1 and E (Fig. 3C). Whereas PB had no effect on the protein level of p21WAF1, exposure to PB
resulted in a 5-fold increase in the CDK inhibitor p27\(^{kip1}\) (Fig. 3C).

PB-induced G\(_1\)-S-phase Block Is Associated with Hypophosphorylation of pRb and Decreased DNA Binding of E2F. Because the final common pathway for G\(_1\)-S-phase traverse is generally controlled by the pRb, Western blot analysis was performed on control and PB-treated cells for pRb and the Rb family pocket proteins p107 and p130. Exposure to PB (3 mM) resulted in hypophosphorylation of pRb, consistent with the G\(_1\)-S-phase block. In addition, the levels of p130 and p107 were significantly elevated (Fig. 4A). Protein levels of E2F-1 and E2F-4 before and after PB treatment were measured. Whereas the protein levels of E2F-1 were unaffected by PB, E2F-4 levels were significantly decreased after 48 h of exposure (Fig. 4B).

Because hypophosphorylated pRb binds (and inactivates) E2F, EMSAs were performed to assess E2F-DNA binding (Fig. 5). Caco-2 cells were treated in the presence or absence of PB (3 mM) for 48 h. Cells were harvested, nuclear extracts were prepared, and EMSA was performed using an oligonucleotide containing the consensus binding site for E2F. These experi-

Fig. 1 PB induces growth inhibition and differentiation in Caco-2 colon cancer cells. Treatment with PB resulted in a decrease in cell growth. Similarly, PB induced Caco-2 colon cancer cells to undergo differentiation, as measured by ALP activity. For all experiments, cells were initially seeded and, after 24 h, were exposed to 3 mM PB for 24, 48 and 72 h (A and C) or treated with PB (1, 3, and 5 mM) for an additional 48 h (B and D). Caco-2 cells treated with PB (○) demonstrated a significant decrease in cell proliferation as compared with the control cells (■). Cells grown for the entire 72 h in the absence of PB constitute the control cells for B and D. Experiments were performed in triplicate, and data are expressed as mean ± SE. * P < 0.05.

Fig. 2 PB induces a G\(_1\)-S-phase block in Caco-2 colon cancer cells. An example of the effect of PB on the cell cycle distribution of Caco-2 cells, as determined by DNA content, is shown. Cell cycle analysis of control and PB-treated (3 mM for 48 h) Caco-2 colon cancer cells shows that PB treatment resulted in a significant G\(_1\)-S-phase block (n = 3; P = 0.001).
ments demonstrated that the relative amount of free (active) E2F (Fig. 5A, complexes A and B) was decreased, and the amount of bound (inactive) E2F (Fig. 5A, complex C) was increased. Complexes A and B most likely represent different unbound forms of the E2F family (E2F1–5). Whereas there was an overall decrease in free E2F, PB exposure resulted in an increase in the intensity of complex A, which may represent a change in the relative amounts of E2F subtypes. Supershift experiments revealed that E2F-4 represents a significant component of E2F (Fig. 5A, complex D). Whereas the addition of Rb antibody did not demonstrate any evidence of a supershift, the addition of p130 (Fig. 5A) and p107 (Fig. 5B) resulted in a loss of intensity of complex C. The addition of an unrelated antibody (Fig. 5A, c-Rel) had no effect on protein-DNA binding, and the absence of nuclear extract (Fig. 5A, probe alone) in the reaction mixture did not produce any complexes. Unlabeled homologous competitor oligonucleotide (50× molar excess) competed out all three complexes almost completely (Fig. 5B).

DISCUSSION

Recent reports suggest that PB is an effective differentiation agent in a variety of cell types, including malignant gliomas, prostate cancer, leukemia, and melanoma and represents a promising compound for clinical use in treating cancer (7–9, 11). Although the biochemical basis of its antitumor activity is not well established, there is increasing evidence that changes in lipid metabolism and protein isoprenylation are important mech-

Fig. 3 The effect of PB on regulators of the G, S-phase traverse. Whereas PB modestly reduced the protein expression of CDK2, CDK4, and CDK6 (A), only CDK2 had decreased kinase activity after PB exposure (B). PB had no effect on the protein levels of the G, S-phase cyclins, cyclin D1 and E or p21CIP1. In contrast, PB exposure markedly increased the expression of the CDK inhibitor p27KIP1 (C). Equal protein loading was confirmed by Ponceau red staining (data not shown).

Fig. 4 The effect of PB on the Rb-like pocket proteins and E2F. A, exposure to PB (3 mM) resulted in a significantly increased proportion of the hypophosphorylated form of Rb by 24 h. After 48 h, the majority of Rb was hypophosphorylated. PB also increased the expression of p130 and p107. B, whereas PB had little effect on E2F-1 protein levels, E2F-4 levels were decreased after 48 h of exposure to PB.
PB has been shown to be an inhibitor of HDAC (11). This is the first report that explores the mechanism of PB-induced cell differentiation and G1-S-phase cell cycle arrest. This study demonstrates that exposure of Caco-2 colon cancer cells to PB induces growth inhibition and cellular differentiation associated with a G1-S-phase block. This G1-S-phase block appears to be regulated in part through a decrease in kinase activity of CDK2, which results from an increase in the CDK inhibitor p27Kip1. The decrease in CDK2 activity relates to an observed hypophosphorylation of pRb. In addition, PB treatment also resulted in an increase in the expression of p130 and p107, which was associated with increased binding and subsequent inactivation of E2F, a transactivator of genes regulating cell cycle transition (Fig. 6).

Similar phenotypic changes after PB exposure have also been reported in other tumor types, including prostate cancer (9), melanoma (6), and leukemia (13). In a study of primary cultures of bone marrow samples from patients with myeloid neoplasm, Gore et al. (7) demonstrated that PB inhibited the proliferation of primary acute myeloid leukemia cells in culture. In addition, they also reported an increase in apoptosis as well as in the expression of the monocytic marker CD14 in response to PB.

In addition to growth inhibition and differentiation, PB exposure has been associated with a block in the G1-S-phase traverse (13, 14). Huang and Waxman (15), evaluating PB as an adjuvant to FUDR in two subclones of HT29 cells, demonstrated that the combination of FUDR and PB resulted in enhanced growth inhibition, increased differentiation, and decreased clonogenicity. Importantly, they also observed a G1-S-phase block and up-regulation of p21WAF1 after combining PB and FUDR. In addition, DiGiuseppe et al. (13) reported that PB induced a G1 arrest associated with an increase in apoptosis and

Fig. 5  The effect of PB on the E2F-DNA binding complexes. Caco-2 cells were treated in the presence or absence of PB (3 mM) for 48 h. The cells were harvested, nuclear extracts were prepared, and EMSA was performed using an oligonucleotide containing the consensus binding site for E2F. These experiments demonstrated that the relative amount of free (active) E2F (A, complex A and B) was decreased, and the amount of bound (inactive) E2F (A, complex C) was increased. Whereas there was an overall decrease in free E2F, PB exposure resulted in an increase in the intensity of complex A, which may represent a change in the relative amounts of E2F subtypes. Supershift experiments revealed that E2F-4 represents a significant percentage of E2F (A, complex D), whereas the addition of Rb antibody did not demonstrate any evidence of a supershift, the addition of p130 (A) and p107 (B) resulted in a loss of intensity of complex C. The addition of c-Rel antibody (negative control) had no effect on protein-DNA binding (A). Cold homologous competitor oligonucleotide (50× molar excess) nearly competed out all three complexes (B).
differentiation in ML-1 cells, a myeloid leukemia cell line. They also reported that PB induced p21\textsuperscript{waf1} expression and pRb hypophosphorylation. Similar to these findings, we also noted a G\textsubscript{1}-S-phase block and pRb hypophosphorylation in Caco-2 and HT29 (data not shown) colon cancer cell lines. Although we did not note any change in p21\textsuperscript{waf1} expression in our system, we did demonstrate an increase in p27\textsuperscript{Kip1} protein levels. Increases in both p21\textsuperscript{Waf1} and p27\textsuperscript{Kip1} associated with pRb hypophosphorylation have also been reported in other models of differentiation (16, 17).

The current dogma is that the central event for the G\textsubscript{1}-S-phase transition is the phosphorylation of the tumor suppressor pRb and the other Rb-like “pocket” proteins, p130 and p107. These proteins are believed to control the entry into S phase by interacting with a member of the transcription factor E2F family (E2F1–5), a family whose members are active when they form heterodimeric complexes with one of the E2F-related transcription factors (DP-1, DP-2, or DP-3). Within the E2F family, E2F-1, E2F-2, and E2F-3 bind with high affinity to pRb, whereas E2F-4 and E2F-5 preferentially bind to p107 and p130 (18). On phosphorylation, the pocket proteins free E2F, which is capable of activating genes necessary for S-phase initiation (10). This study demonstrates that binding of the transcription factor E2F to its DNA binding site is modulated during PB-induced Caco-2 cell differentiation. Whereas the majority of E2F in the untreated Caco-2 cells was in its free form (Fig. 5, complex A and B), after PB-induced differentiation, the major proportion of E2F was bound (inactive). This decrease in free E2F has been described in other cell models of differentiation (19–21), and the present findings further support the concept that modulation of E2F-DNA binding appears to be an important event during cellular differentiation.

The loss of free E2F after PB-induced differentiation may involve both an increase in binding to the pocket proteins and an
overall decrease in the expression of E2F-4. This change in E2F-4 levels may contribute to the observed change in the relative intensities of complexes A and B (Fig. 5). In addition to pRb hypophosphorylation, the increase in overall p130 and p107 levels after PB treatment may also contribute to the decrease in free E2F. The role of the p130-E2F-4 complex in cellular differentiation has been noted in various cell models (16, 18, 20, 22, 23). Whereas these studies report increased protein levels of p130 after cells differentiate and/or become quiescent, it has been suggested that p107 shows an opposite pattern of expression during cell growth and differentiation (18, 24). Kiess et al. (22) noted that during the differentiation of skeletal myoblasts, p107 levels were reduced, and p130 protein levels increased 8-fold. In contrast, we demonstrated that both p130 and p107 protein levels increased after PB-induced differentiation. An increase in p107 protein levels has also been reported after hexamethylene bisacetamide-induced erythroid differentiation (19). The increase in p107 in our model may contribute to the observed decrease of free E2F, which is supported by the observation that in transient transfection experiments, overexpression of p107 represses E2F-mediated transcription, with growth arrest in the G₁ phase of the cell cycle (25, 26).

Although the data presented support the conclusion that PB induces growth arrest through increased levels of p27Kip1 with subsequent pRb hypophosphorylation, additional effects of PB may also contribute to the observed G₁-S-phase block. PB has been reported to activate PPAR-α and PPAR-γ. Pinea et al. (27) demonstrated that PB exposure induced PPAR activation as evidenced by increased expression of chloramphenicol acetyltransferase linked to a PPAR response element from both rat acyl-CoA oxidase and rabbit CYP4A6 genes. Related to our findings, Altiko et al. (28) demonstrated that activated PPAR-γ results in decreased E2F-DNA binding. However, it shows that this decreased E2F binding was independent of pRb phosphorylation. An additional effect of PB on cell cycle traverse may occur as a result of its inhibitory effect on HDAC1 activity (11, 29). HDAC1 participates in the transcription of genes regulating the G₁-S-phase traverse and may also be involved in regulating PPAR activity (30, 31).

In summary, we demonstrate that PB causes a decrease in proliferation, an increase in differentiation, and a G₁-S-phase block in Caco-2 colon cancer cells. The mechanism of this effect is related to a decrease in the activity of CDK 2, which may be modulated by an increase in the expression of the CDK inhibitor p27Kip1. Consistent with the decreased CDK2 kinase activity, we also observed decreased phosphorylation of pRb and an increase in p130 and p107 after PB treatment. This was associated with increased binding and subsequent inactivation of E2F. These data suggest that the differentiation agent PB inhibits tumor growth by limiting the availability of active E2F, with a subsequent G₁-S-phase block. Importantly, PB may be a clinically effective therapeutic agent for treating colorectal cancer.

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