Transcription Factor ZBP-89 Cooperates with Histone Acetyltransferase p300 during Butyrate Activation of p21<sub>waf1</sub> Transcription in Human Cells*

Received for publication, May 18, 2000, and in revised form, July 10, 2000
Published, JBC Papers in Press, July 17, 2000, DOI 10.1074/jbc.M004249200

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Inducible p53-independent regulation of the cyclin-dependent kinase inhibitor p21<sub>waf1</sub> transcription is mediated through proximal GC-rich sites. Prior studies have shown that Sp1, Sp3, and the histone acetylase co-activator p300 are components of the complexes binding to these sites. Although Sp1 and Sp3 collaborate with p300, a direct interaction between Sp1 and p300 does not occur. This study sought to determine whether ZBP-89 rather than Sp1 is the direct target of p300 during butyrate induction of p21<sub>waf1</sub>. ZBP-89 (BFCOL1, BERF-1, ZNF 148) is a Krüppel-type zinc finger transcription factor that binds to GC-rich elements and represses or activates known target genes. Adenoviral-mediated expression of ZBP-89 in HT-29 cells revealed that ZBP-89 potentiates butyrate induction of endogenous p21<sub>waf1</sub> gene expression. Further, cotransfection of a ZBP-89 expression vector with a 2.3-kilobase p21<sub>waf1</sub> reporter recapitulated the potentiation by butyrate. DNase I footprinting analysis of the human p21<sub>waf1</sub> promoter with recombinant ZBP-89 identified a binding site at −245 to −215. Electrophoretic mobility shift assays confirmed that both recombinant and endogenous ZBP-89 and Sp1 bind to this element. The potentiation was abolished in the presence of adenoviral protein E1A. Deletion of the N-terminal domain of ZBP-89 abolished the potentiation mediated by butyrate treatment. This same deletion mutant abolished the ZBP-89 interaction with p300. Cotransfection of p300 with ZBP-89 stimulated the p21<sub>waf1</sub> promoter in the absence of butyrate. p300 co-precipitated with ZBP-89 but not with Sp1, whereas ZBP-89 co-precipitated with Sp1. Together, these findings demonstrate that ZBP-89 also plays a critical role in butyrate activation of the p21<sub>waf1</sub> promoter and reveals preferential cooperation of this four-zinc finger transcription factor with p300.

The cyclin-dependent kinase inhibitor p21<sub>waf1</sub> controls cell cycle progression through binding to G1 cyclin/CDK complexes (1–3). DNA damage stimulates p21<sub>waf1</sub> transcription through p53-dependent mechanisms (4), whereas agents that regulate cellular differentiation may regulate p21<sub>waf1</sub> transcription through p53-independent mechanisms (5). Many of the studies reporting p53-independent regulation of p21<sub>waf1</sub> transcription demonstrate a requirement for GC-rich sites located within the first 100 bp of its promoter (6, 7). These sites have consistently been shown to bind members of the Sp family of transcription factors (8–13). Several studies have shown that Sp1 or Sp3 mediate activation of the p21<sub>waf1</sub> promoter by these extracellular regulators such as nerve growth factor and transforming growth factor-β; however, these same signals do not stimulate Sp1 binding or gene expression (10). The transcriptional co-activator p300 mediates growth arrest by catalyzing histone acetylation and subsequent chromatin rearrangements through its endogenous acetyltransferase enzyme activity (14). Taken together, these results raise the possibility that Sp1 transcriptional activity may be regulated by its association with a co-activator. As a result, the p300 co-activator was shown to co-precipitate in complexes with Sp1 (10). Moreover, activation of the p21<sub>waf1</sub> promoter by butyrate and nerve growth factor has been shown to require a functional collaboration between Sp1 and p300 (10, 15). Yet, p300 does not interact directly with Sp1 or Sp3 (15). Thus, although p300 and Sp1 are components of the complex activating p21<sub>waf1</sub>, the interaction is indirect raising the possibility that other factors are likely to participate in this transcriptional regulatory complex. Presumably, at least one or more of these other factors are capable of direct contact with p300.

ZBP-89 (BFCOL1, BERF1, ZNF 148) is a widely expressed four-zinc finger transcription factor that binds to GC-rich DNA elements in a variety of promoters involved in growth regulation, e.g. promotors for gastrin, T-cell α- and β-receptors, ornithine decarboxylase (ODC), enolase, type I procollagen, cyclin-dependent inhibitor p21<sub>waf1</sub>, vimentin, and stromelysin (16–23). In many instances, ZBP-89 appears to repress promoter activity by opposing the effect of Sp1, which also binds to the same or overlapping DNA element. Thus, competitive binding to the shared promoter elements may mediate transcriptional regulation by Sp1 and ZBP-89 (16, 19). ZBP-89 and Sp1 may also regulate transcription cooperatively because it has been shown that ZBP-89 directly binds Sp1 in co-precipitation assays (22).

Studies by Hasegawa et al. (24) showed binding of ZBP-89 to...
a proximal Sp1 element from the mouse p21<sup>trans</sup> promoter. We hypothesized that ZBP-89 may be present in the p300/Sp1 activation complex and participate in the activation of p21<sup>trans</sup> transcription. p21<sup>trans</sup> is required for butyrate-mediated growth inhibition in HT-29 colorectal adenocarcinoma cells (25, 26). Thus the goals of this study were to examine the role of ZBP-89 in p21<sup>trans</sup> activation by butyrate. The results demonstrate that both ZBP-89 and Sp1 recognize the same p21<sup>trans</sup> regulatory sequences and that ZBP-89-dependent activation depends upon elevated histone acetyltransferase activity. We also found that Sp1, as reported (15), does not directly bind p300. Instead, we find that p300 cooperates directly with ZBP-89.

MATERIALS AND METHODS

Plasmid Constructs—The human p21<sup>trans</sup> reporter construct p21<sup>trans</sup>-Luc, which contains the human p21 promoter from −2386 to +16 ligated upstream of the luciferase cDNA, was a kind gift from Dr. W. El-Deiry (University of Pennsylvania). The pRcRSV/E1A-12S, pRcRSV/E1A-RG2, and pcDNA3/p300 expression vector, were gifts from Dr. Roland Kwok (University of Michigan). pBl9 E1A 12S ΔC′R is a gift from Dr. Tony Kouzarides (Wellcome/CRC Institute, Cambridge University). An ODC reporter construct was prepared by inserting the 2.3-kb XhoI-HindIII fragment from the p2-OS6.1 plasmid (a gift from M. A. Fojo) into pGL2 basic (Promega, Madison, WI) to generate pGL2-ODC. The resulting ODC promoter construct pODC2300-Luc contained 1.5 kb of 5' flanking sequence and 0.8 kb of downstream sequences including the first exon. The rat ZBP-89 construct subcloned into pBl9CMV (pcMV/rZBP-89) (Stratagene, La Jolla, CA) has been previously reported (16). A Tet-on inducible ZBP-89 expression vector (pBl-G/rZBP-89) was prepared by subcloning the full-length rat ZBP-89 cDNA as a blunt PCR fragment into the filled-in SalI site of the pBl-G vector (CLONTECH, Palo Alto, CA). Deletions of pcMV/rZBP-89 expression vectors were prepared using PCR-generated fragments containing 5' Nhel and 3' NotI sites followed by cohesive ligation into the pBl9CMV vector. The C-terminal deletion (ΔC-ter) containing residues 1 to 521 of rZBP-89 in pBl9CMV (Stratagene) has been previously reported (16). Deletion of the acidic domain (Δ2–112); basic domain 2 and C terminus (Δ301–794); the acidic domain and basic domain 1 (Δ2–153); the acidic domain and basic domain 2 (Δ2–112 and Δ301–794) and deletion of both amino and C-terminal domains leaving the zinc finger domain (Δ2–153 and Δ301–794) were all generated by PCR amplification and subsequent subcloning. Deletion of BD1 (Δ142–146 residues KKKKR) was generated using the QuickChange Mutation Kit (Stratagene) and the following primers: 5′-CAAGTGAGAGAGCCAGTAGACTTACT- CGAGAACAAAGCCTCCTGCAAAAAATC and 5′-GATTTTTGCCAGGATTGTTTCCGTAAAGTTACTCATCTCTCTTG-3′. The deletion was confirmed by DNA sequencing. A consensus Kozak sequence (ACCATG) and two tandem repeats of the Myc epitope tag were added to the N terminus. The Myc tag sequence was used: 5′-GACAAACCCCTCAGCAGAACCTGCGCATGCCGCGGAGAAAGAAC- CGAAAGGGTAC-3′ as the forward primer and 5′-GATTTTTGCCAGGATTGTTTCCGTAAAGTTACTCATCTCTCTCAGT-3′ as the reverse primer. The construct was confirmed by DNA sequencing and expression analysis. The expression was confirmed by anti-Myc antibody labeling.

ZBP-89 and p300 during Activation of p21<sup>trans</sup> Transcription

Construction of Recombinant Adenovirus—To construct a ZBP-89 expressing recombinant adenovirus, the 3.5-kb DNA fragment that contains the ZBP-89 cDNA, CMV promoter, and bovine growth hormone poly(A) signal sequence was excised from pCMV/Myc-ZBP-89 by NotI and PvuII and then blunt end-ligated into the EcolR site of the shuttle plasmid pAdMCSloxP (obtained from the University of Michigan Cancer Center Vector Core) (27). Recombinant replication-deficient adenovirus was produced by the Vector Core using the method of Aoki et al. (27). Briefly, the ZBP-89 adenoviral shuttle plasmid was recombined with the adenovirus type 5 cosmid, and then transfected into 293T cells. The resultant recombinant adenoviral particles were harvested from the cells and called Ad5-ZBP-89. The Ad5-ZBP-89 viral particles were purified by CsCl centrifugation and titrated.

Cell Culture and Transfections—The HT-29 human colorectal adenocarcinoma (HTB-38) and HeLa human cervix adenocarcinoma cell lines (CCL-2) were purchased from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal calf serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air at 37 °C. The 293/Tet-on cells were purchased from CLONTECH. Sodium butyrate and trichostatin A (TSA) were purchased from Sigma and used at final concentrations of 5 and 0.5 μM, respectively. The cells were plated in six-well plates and transiently transfected using FuGENE 6 (Roche Molecular Biochemicals). The p21<sup>trans</sup>-reporter plasmid p21<sup>trans</sup>-Luc and ODC reporter plasmid pCD2300-Luc were cotransfected with the pCMV/rZBP-89 expression vector (16). 48 h after transfection, the cells were harvested for luciferase, β-galactosidase, and protein assays. At least three transfections in triplicate were performed with each promoter construct. In experiments using butyrate or TSA, the luciferase activity was normalized to cell protein because these treatments stimulated the CMV promoter expressing β-galactosidase. However, in the absence of butyrate or TSA treatment, β-galactosidase was used to normalize transfections, and no significant differences in plasmid transfection efficiency were detected. Induction of the Tet-on promoter was accomplished with 2 μg/ml doxycycline.

Adenovirus Infection of HT-29 Cells—HT-29 cells were cultured in McCoy’s 5a medium, grown to 50% confluence, and then infected with the recombinant adenoviral particles (Ad5 vector or Ad5-ZBP-89) in F12 serum-free medium (Life Technologies, Inc.) at 5 × 10<sup>6</sup> viral particles/5 × 10<sup>6</sup> cells-100-mm plate (equivalent to 10 multiplicities of infection) for 6 h. The Ad5 vector, which contains the CMV promoter alone and the poly(A) sequence, was used as a control at the same multiplicity of infection. After infection, the viral particles were washed off, and fresh medium containing serum was added. 36 h later, the cells were treated with or without 5 mM sodium butyrate for another 12 h before they were processed for immunoblot analysis. 50 μg of whole cell extracts were separated on a 4–12% NuPAGE Bis-Tris gradient gel (Novex) and then transferred to polyvinylidene fluoride membrane for immunoblot analysis with designated antibodies. Enhanced chemiluminescence was used to detect the antigen-antibody complexes. The anti-FLAG M1 monoclonal antibody was purchased from Sigma, and the anti-actin (C-2) and anti-p21<sup>trans</sup> (F-5) monoclonal antibodies were purchased from Santa Cruz Biotechnology.

Ribonuclease Protection Assays—Riboprobes were generated from antisense templates for human ZBP-89 and human cyclophilin. The human ZBP-89 antisense template was constructed using the human ZBP-89 cDNA fragment (28), from +382 to +639 amplified with 5′-GATAGAGACAAAACAAACATCAGAGGGCTGAGATACG-3′ as the forward primer and 5′-GGTACTTCTGATGAAACGATGTCATG-3′ as the reverse primer. The PCR product generated was purified (QIAquick) and the pCR2.1 vector (Invitrogen) used to express the orientation. The template was linearized with AccI and used to transcribe a 290-nucleotide ZBP-89 antisense riboprobe that protected a 257-nucleotide fragment. The human pTRI-cyclophilin template (Ambion) generated a 165-nucleotide probe and a 103-nucleotide protected fragment. All riboprobes were used with MAXIscript In Vitro Transcription Kit (Ambion). Total RNA was isolated from butyrate-treated HT-29 cells using TRIZOL reagent (Life Technologies, Inc.) and then mRNA extracted using poly(A)Tract mRNA Isolation System (Promega). Total RNA was hybridized for 16 h with riboprobes at 45 °C in hybridization buffer (1× EDTA, 300 mM sodium acetate, pH 6.4, 100 mM sodium citrate, pH 6.4, 80% deionized formamide). After hybridization, the samples were digested at 37 °C for 30 min in an RNase A/T1 mixture containing 80 units/ml RNase A, 250 units/ml RNase T1 (Ambion) in digestion buffer (300 mM NaCl, 10 mM Tris, pH 7.4, 5× EDTA). The protected fragment was precipitated in isopropanol, dissolved in loading buffer (95% formamide, 0.025% xylene cyanol, 0.025% bromphenol blue, 0.5 mM EDTA, 0.025% SDS), and then resolved on a 6% polyacrylamide, 8 × urea gel. Protected fragments were quantified on a PhosphoImager and normalized to cyclophilin mRNA amounts. C.6.1 cells were infected with pBl-G/rZBP-89 using FuGENE6. The cells were treated with or without 2 μg/ml doxycycline to induce ZBP-89 production. The 293T cells were lysed in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 1% SDS, 1 mM dithiothreitol in phosphate-
buffered saline), and the protein concentration was determined. Whole cell extracts were precleared with rabbit preimmune serum and protein A/G-agarose (Santa Cruz) at 4 °C for 30 min. The supernatant was incubated with the primary antibody (5–10 μg) for 1 h at 4 °C followed by the addition of 20 μl of Protein A/G-Agarose for another 16 h. The pellets were collected and washed with RIPA buffer three times. The proteins were separated on a 4–12% NuPAGE Bis-Tris gel (Novex) and then transferred to polyvinylidene fluoride membrane for immunoblot analysis with designated antibodies. Enhanced chemiluminescence was used to detect the antigen-antibody complex. E1A, p300, CBP, and Sp1 antibodies were purchased from Santa Cruz Biotechnology.

DNase I Footprinting Assays—Recombinant ZBP-89 protein was prepared as described previously (16). Protein concentration was determined by the method of Bradford (29). To footprint ZBP-89-binding sites from −556 to +6, the 560-bp fragment was PCR amplified from p21wt/2300-Luc with forward primer 5′-TTCTCCGGAGAGTGACCTAGT-3′ and reverse primer 5′-CTTCGGGACGTCTCACCAC-3′. The fragment was end labeled with T4 polynucleotide kinase then digested with BstEII to make the labeled antisense strand. To footprint the p21wt/2300-Luc promoter, p21wt/2300-Luc corresponding to −291 to +16 was digested with XbaI, end labeled with T4 polynucleotide kinase at +16, and digested with HindIII. The resulting probe was used to footprint the antisense strand. To footprint the sense strand, the same DNA fragment was restricted with SacI and HindIII, end-labeled with T4 polynucleotide kinase, and then digested with XhoI. Footprinting assays were performed with recombinant ZBP-89 protein as described above. Recombinant ZBP-89 was incubated first with probe on ice for 20 min in the binding buffer (25 mM Tris-HCl, pH 8.0, 50 mM KCl, 6.25 mM MgCl2, 0.5 mM EDTA, 10% glycerol, 0.5 mM dithiothreitol, 5% polyvinyl alcohol, 2 μg/ml poly(dI-dC)). DNase I digestion was then carried out for 1 min, and phenol/chloroform was extracted prior to resolving on 6% polyacrylamide, 8 M urea gel. The fragments were resolved at 200 V for 2–6 h at 4 °C. To perform supershift EMSAs, the protein was incubated first with each antibody for 30 min on ice before resolving on a 4% nondenaturing polyacrylamide gel containing 37% glycerol. The gel was fixed in 10% acetic acid, then transferred to polyvinylidene fluoride membrane. The membrane was blocked for 1 h at room temperature with 5% skim milk in TBST buffer for 1 h. The membrane was probed with anti-ZBP-89, p21, and actin. There was little effect of sodium butyrate. However, in the presence of butyrate, there was a 3-fold enhancement of elevated p21 expression, demonstrating that the butyrate effect on the ZBP-89 promoter was the result of direct binding to the p21 promoter element. This effect was confirmed by the use of a ZBP-89 expression vector (Fig. 2B).

RESULTS

ZBP-89 and p300 during Activation of p21 Transcription

DNA Affinity Precipitation—Quantitation of the changes in ZBP-89 and Sp1 binding to the p21wt promoter element was achieved by DNA affinity precipitation assays (DAPA) according to the method of Billon et al. (10). Briefly, oligonucleotides biotinylated at the 5′-termini and corresponding to the sense (−242 to −212 (5′-GGACTGGGGGAGGAGGAGGAGGAGGATGCCCTC-3′)) and antisense strands of the p21wt promoter element were annealed. The DAPA was performed by incubating 2 μg of biotinylated DNA probe with 300 μg of HT-29 whole cell extracts in binding buffer containing 20 mM HEPES, pH 7.9, 10% glycerol, 50 mM KCl, 0.2 mM EDTA, 1.5 mM MgCl2, 20 μM ZnCl2, 1 mM dithiothreitol, and 0.25% Triton X-100. The mixture was incubated on ice for 30 min prior to adding 100 μl of streptavidin-agarose (Novagen). 2 h later, the agarose beads were collected and rinsed with binding buffer three times. Protein was eluted from the DNA probe by adding Laemmli loading buffer and heating to 90 °C for 5 min. The eluted protein was resolved with a 12% polyacrylamide gel (Novex) and transferred to polyvinylidene fluoride membrane following the method noted for ZBP-89 or Sp1 protein levels. To analyze changes in ZBP-89 and Sp1 protein after butyrate treatment over 1 h, immunoblot analysis was performed on the same extracts used for the DAPA. Whole cell or nuclear extracts were prepared from HT-29 cells, separated on a 4–12% NuPAGE Bis-Tris gel (Novex), and transferred to polyvinylidene fluoride membrane. The membrane was blocked for 1 h in 5% UniBlock.
ZBP-89 and p300 during Activation of p21waf1 Transcription

FIG. 2. ZBP-89 potentiates butyrate stimulation of p21waf1 gene expression in HT-29 cells. A, HT-29 cells were infected with viral particles containing Ad5 vector or Ad5-ZBP-89 at 10 multiplicities of infection for 6 h and then grown in complete Dulbecco’s modified Eagle’s medium for 36 h. The infected cells were incubated with or without 5 mM sodium butyrate for another 12 h before they were collected for immunoblot analysis. Lane 1, noninfected cells, without butyrate; lane 2, Ad5 vector infected cells, without butyrate; lane 3, Ad5-ZBP-89 infected cells, without butyrate; lane 4, Ad5 vector infected, with butyrate; lane 5, Ad5-ZBP-89 infected, with butyrate. The mouse anti-FLAG M1, anti-p21waf1, and anti-actin monoclonal antibodies were used at 1:1000 dilution, and the rabbit anti-ZBP-89 IgG was used at a 1:5000 dilution. B, Purified ZBP-89 or Sp1 bound to the 2.3-kb p21waf1 promoter (Fig. 3, A and B) was end labeled and incubated with HT-29 nuclear extracts. The percentage of activity relative to the untreated control is shown. Promoter activity of transformants in the absence (lane 1) and presence of butyrate (lane 2) is shown.}

ZBP-89 target genes (19, 24). Base-line regulation of the promoter (−T-fold) occurred with butyrate in the absence of constitutive exogenous ZBP-89 expression, suggesting a role for endogenous transcription factors, e.g. Sp1, Sp3, and ZBP-89 in butyrate-mediated induction. Taken together, these results suggest that ZBP-89 regulation of expression of p21waf1 transcription requires elevated histone acetyltransferase activity.

Identification of ZBP-89-binding Sites within the p21waf1 Promoter—To identify the preferred site of ZBP-89 binding, we performed footprinting analysis on the first 500 bp of the p21waf1 promoter (Fig. 3A). The results showed that a sequence at −245 to −215 was the major site footprinted. That sequence relative to the other Sp1 sites within the proximal p21waf1 promoter is shown (Fig. 3B). To study the complexes binding to the ZBP-89 footprint, a probe corresponding to −245 to −215 was end labeled and incubated with HT-29 nuclear extracts. Purified ZBP-89 or Sp1 bound to the −245 to −215 DNA element (Fig. 3C). Incubation with Sp1, Sp3, or ZBP-89 antibodies alone or in combination revealed the identity of the native complexes in nuclear extracts (Fig. 3D). Competition with WT versus mutant or known ZBP-89-binding sites demonstrated preference of the p21waf1 element for ZBP-89 binding (Fig. 3, C and D). Scanning mutations within the 30-bp footprinted element identified the ZBP-89 consensus site as GGGAGG or its converse CCCTCC (Fig. 4A). Both elements were also high affinity binding sites for Sp1. When that site was mutated within the context of the 2.3-kb p21waf1 promoter, the potentiation by ZBP-89 overexpression was blocked (Fig. 4B). Moreover, mutation of the downstream ZBP-89 consensus site CCCTCC had some effect on the enhancement. However, the double mutation of both sites resulted in the most significant decrease in the potentiation with the induction by butyrate suppressed below what was observed in the absence of ZBP-89 (Fig. 4B). Complete inhibition of the butyrate effect was not achieved perhaps because of intact downstream Sp1 sites that are known to mediate butyrate induction (5).

To determine whether an increase in ZBP-89 binding to the p21waf1 promoter occurred within the same time frame as activation of p21waf1 gene expression, both EMSAs and DAPA were performed. Antibody to Sp1 or ZBP-89 was used to supershift their respective antigens (Fig. 5A). Quantitation of the supershifted bands showed that there was no increase in ZBP-89 or Sp1 binding within 60 min of butyrate treatment. These results were confirmed by using DAPA (10) to detect DNA-protein interactions (Fig. 5B). Moreover, there was no significant increase in total ZBP-89 and Sp1 protein within the first 60 min of butyrate treatment (Fig. 5B). The lack of increased Sp1 binding to a p21waf1 element in response to nerve growth factor has been previously reported (10). Thus our results confirm other reports demonstrating no significant increase in complex binding to account for p21waf1 promoter induction.

ZBP-89-dependent Transcriptional Activation of p21waf1 Requires the N-terminal Domain—To determine which domain of ZBP-89 was required for the butyrate-dependent activation, a series of ZBP-89 mutations were prepared (Fig. 6A). An immunoblot was performed to demonstrate that the constructs were expressed in nuclear extracts, and EMSAs were performed to demonstrate that the mutants could bind DNA (Fig. 6, B and C). Cotransfection of full-length ZBP-89 versus C-terminal and N-terminal truncated forms showed that the expected potentiation mediated by butyrate was significantly reduced with any combination of deletions that did not include the N-terminal acidic domain. There was less reduction of the enhanced activation with the C-terminal truncated form. Moreover, the zinc finger domain alone significantly reduced p21waf1 promoter induction below what was expected in the absence of the ZBP-89 expression vector (Fig. 6D). The N-terminal truncated forms did not mediate the enhanced butyrate induction yet were still capable of binding the p21waf1 promoter (Fig. 6C), probably preventing endogenous ZBP-89 from binding to the target promoter elements. The reduced potentiation reveals the contribution made by endogenous ZBP-89 to activation of the 2.3-kb p21waf1 promoter by butyrate. Hasegawa and co-workers (24) have shown that ZBP-89 binds to the third proximal GC-rich site of the mouse p21waf1 promoter that also binds Sp1. Thus the mutant ZBP-89 construct likely affects both the high affinity ZBP-89 site footprinted as well as lower affinity downstream sites. However, there are at least five other downstream Sp1 sites that do not appear to be affected by overexpression of the ZBP-89 ZF mutant. Thus as observed with the mutations of the upstream ZBP-89-binding sites (Fig. 4B), there still remained some residual activation of the p21waf1 promoter mediated presumably by Sp family members.

The Histone Acetyltransferase p300 Enhances ZBP-89 Acti-
To examine whether a more specific histone deacetylase inhibitor, TSA, recapitulated the butyrate effect, the HT-29 cells were transiently cotransfected with ZBP-89 and the p21<sup>waif<sup> reporter construct. The results show that the potentiation by ZBP-89 was similar with the two

FIG. 4. Mutation of p21<sup>waif<sup>-binding site for ZBP-89 abolishes the potentiation. A, scanning point mutations of the −245 to −215 DNA element were prepared as double-stranded oligonucleotides and used to compete (20- and 50-fold over labeled probe) then used to compete for recombinant ZBP-89 or purified Sp1 bound to the end labeled wild type (WT) p21<sup>waif<sup> element. B, transient cotransfections of HT-29 cells were performed with 0.2 μg p21<sup>waif<sup>/2300-Luc, p21<sup>waif<sup>/2300-Luc/M1, p21<sup>waif<sup>/2300-Luc/M6 or p21<sup>waif<sup>/2300-Luc/M1/6, and 0.2 μg of the pBKCMV/ZBP-89 or pBKCMV expression vectors. The transfected cells were treated with 5 mM butyrate for 20 h before performing enzyme and protein assays. All results represent the means ± S.E. of at least three independent experiments performed in triplicate. The mutations are indicated in bold type, and the fold induction is indicated.
complexes precipitated with the biotinylated probe corresponding to panel SS each specific DNA-protein complexes retarded, for an additional 20 min at room temperature. The blotted, and then incubated with ZBP-89 (shifted Sp1-DNA complexes. B, DAPA and immunoblot of HT-29 nuclear extracts treated for up to 60 min with butyrate. Lanes 1–4 contain complexes precipitated with the biotinylated probe corresponding to p21\textsuperscript{waf1} promoter sequences from –242 to –212. The HT-29 nuclear protein adherent to the probe was blotted then incubated with either ZBP-89 antibody (upper panel) or Sp1 antibody (lower panel). Lanes 5–8 contain nuclear extracts resolved by 4–12% NuPAGE Bis-Tris gel, blotted, and then incubated with ZBP-89 (upper panel) or Sp1 (lower panel) antibody.

![Fig. 5. Butyrate activation and protein complex binding to the –245 to –215 element of p21\textsuperscript{waf1}](image)

A

**Antibody:**
- preimmune anti-ZBP-89
- anti-Sp1

**Time (min):**
- 0
- 10
- 30
- 60
- 0
- 10
- 30
- 60

B

**DAPA**

**Immunoblot**

**Time (min):**
- 0
- 10
- 30
- 60

**ZBP-89**

1
2
3
4
5
6
7
8

**Sp1**

1
2
3
4
5
6
7
8

**DISCUSSION**

Regulation of p21\textsuperscript{waf1} transcription by several extracellular signals is p53-independent and targets GC-rich sites within the proximal promoter (5). Sp1 and Sp3 almost invariably bind to these sites. Histone deacetylase inhibitors, e.g. butyrate or TSA, are examples of extracellular signals that induce growth arrest and cellular differentiation while stimulating p21\textsuperscript{waf1} gene expression (9, 25, 34). An increase in the level of histone acetylation and subsequent relaxation of chromatin at sites of active transcription is thought to be one mechanism by which butyrate activates gene expression (35). Site-specific transcription factors assist in the recruitment of co-activators, e.g. p300, to specific regulatory sites within targeted promoters (14). Although butyrate-dependent activation of p21\textsuperscript{waf1} transcription requires Sp1, the interaction between Sp1 and p300 is indirect raising the likelihood that other factors participate in the direct interaction with p300 (15). The novel result reported here is that ZBP-89 is one of those transcription factors interacting preferentially with p300. Both zinc finger transcription factors essentially recognize the same DNA-binding domain (16, 19) and interact with each other (22). Thus, a plausible model of p21\textsuperscript{waf1} promoter activation by butyrate is through direct association of ZBP-89 with the histone acetyltransferase co-activator p300 within the transcription regulatory complex that includes Sp1 (Fig. 9).

ZBP-89 is a four-zinc finger transcription factor that binds and represses or activates several target genes; yet, little is known about its regulation. Clues to its function have been deduced from observations implicating its role in cell growth (32, 36). In addition, all target genes that are repressed by ZBP-89 are themselves regulated by mitogens or developmental signals. To study its role in proliferation, the HT-29 colon cancer cell line was used because of its ability to undergo morphologic and biochemical differentiation with butyrate treatment. If the transcriptional repression mediated by ZBP-89 was related to its growth regulatory effects, then we predicted that extracellular mediators of growth arrest should stimulate ZBP-89 gene expression. Indeed, we found that butyrate stimulates ZBP-89 gene expression.

Focusing on a known target of ZBP-89 that mediates growth arrest, i.e. p21\textsuperscript{waf1}, we proceeded to define a mechanism by which ZBP-89 may inhibit cell growth. Two high affinity ZBP-89-binding sites were distal to the proximal GC-rich sites bound by Sp1. Mutating these sites effectively abolished enhanced p21\textsuperscript{waf1} activation mediated by butyrate, but complete suppression of the induction was not achieved. This was likely due to proximal Sp1 sites that are also recognized by ZBP-89 (24). Removing the N-terminal domain also abolished the potentialization, whereas overexpression of the zinc finger DNA-binding domain alone further reduced the induction of the promoter by butyrate perhaps because of its ability to displace endogenous ZBP-89 and weakly bound Sp1 from these proximal sites. The N-terminal domain was not only important for butyrate-mediated transactivation but was also required for enhancement by p300. Moreover, E1A adenoviral protein, which binds p300, inhibited the ZBP-89-mediated enhancement of p21\textsuperscript{waf1} induction by both butyrate and TSA. Consistent with the functional data, co-precipitation experiments revealed that p300 co-precipitated with ZBP-89 and E1A but not Sp1. However, E1A co-precipitated with both ZBP-89 and Sp1. It has been reported previously that Sp1 can co-immunopre-
cipitate with E1A (37). Our results also concur with those of Xiao et al. (15), who clearly demonstrated no direct interaction between p300 and Sp1. Moreover, co-precipitation of Sp1 with ZBP-89 documented in this report is consistent with the direct interaction between these two proteins as previously reported by Wieczorek et al. (22), implicating p300, ZBP-89, and Sp1 as components of a multi-factor regulatory complex.

The role of the ZBP-89 acidic domain is consistent with other Krüppel-type factors targeted by histone acetyltransferase co-activators. Passantino et al. (20) demonstrated an active regulatory domain within the N terminus of ZBP-89. We show here that ZBP-89-dependent activation as well as p300 potentiation requires an intact N-terminal domain. The first 70 amino acids of ZBP-89 are comprised of nearly 25% acidic residues (16). Thus, the N terminus of ZBP-89 like other Krüppel-like factors, e.g. gut-enriched Krüppel-like factor and erythroid Krüppel-like factor, is highly acidic and binds p300 (38, 39). In contrast, Sp1, which does not directly bind p300, contains a glutamine-

FIG. 6. Butyrate-dependent potentiation of the p21<sup>wafl</sup> promoter requires the N-terminal domain of ZBP-89. A, a series of deletion constructs were created to identify the domain of ZBP-89 required for p21<sup>wafl</sup> activation. B, the eight constructs shown in A were expressed in 293 cells and detected by immunoblot analysis using ZBP-89 antibody (upper panel) or anti-Myc tag antibody (lower panel). Lane 1, empty vector; lane 2, full-length ZBP-89; lane 3, ΔC-ter; lane 4, ΔBD2; lane 5, mutBD1; lane 6, ΔAD; lane 7, ΔAD and ΔBD1; lane 8, ΔAD & ΔBD2.

B

Blot

anti-ZBP-89

anti-myc

1 2 3 4 5 6 7 8 9 10

C

Construct: vector ΔAD ΔAD0BD1 ZfZ ΔAD0BD2

Δ-myc: + + + + + + + + + +

1 2 3 4 5 6 7 8 9 10

D

Relative Luciferase Activity

ZBP-89 Mutations

- NaBu + NaBu

Vector p8-9Δ C-ter ΔBD2 mBD1 ΔAD ΔAD ΔAD0BD1 ΔAD0BD2

- NaBu + NaBu

A

Relative Luciferase Activity

ZBP-89

- NaBu + NaBu

E1A

- + + + + + + + + + +

B

Relative Luciferase Activity

ZBP-89

- NaBu + NaBu

E1A

- + + + + + + + + + +

Fig. 7. Adenoviral protein E1A represses potentiation effect mediated by ZBP-89. A, HT-29 cells were co-transfected with pRSV E1A, ZBP-89, and the p21<sup>wafl</sup> reporter as indicated. Hatched bars are without butyrate treatment; solid bars are with butyrate treatment. Luciferase activity is expressed per protein and normalized to the untreated control from three independent experiments performed in triplicate. B, HT-29 cells were transfected as in A, and 0.3 mM trichostatin A was used instead of 5 mM butyrate.

The role of the ZBP-89 acidic domain is consistent with other Krüppel-type factors targeted by histone acetyltransferase co-activators. Passantino et al. (20) demonstrated an active regulatory domain within the N terminus of ZBP-89. We show here that ZBP-89-dependent activation as well as p300 potentiation requires an intact N-terminal domain. The first 70 amino acids of ZBP-89 are comprised of nearly 25% acidic residues (16). Thus, the N terminus of ZBP-89 like other Krüppel-like factors, e.g. gut-enriched Krüppel-like factor and erythroid Krüppel-like factor, is highly acidic and binds p300 (38, 39). In contrast, Sp1, which does not directly bind p300, contains a glutamine-
also forms a complex with at least one of these coactivators.

The co-precipitation studies show that ZBP-89 binds p300 but not CBP. Differences in co-activator targets have been reported (14). p300 does not acetylate CREB despite their tight association (42). Retinoic acid-dependent differentiation requires p300 but not CBP (43). p300-deficient and CBP-deficient mice exhibit different phenotypes (44). Mice null for p300 die in midgestation and exhibit defects in cell proliferation in addition to specific tissue defects (44). Recently, it has been demonstrated that CBP may regulate proliferation specifically in hematopoietic tissue (45). Point mutations in p300 have been found in gastric cancer (46). Collectively, these results suggest that p300 essentially behaves as a bifunctional co-activator mediating both growth-suppressing and -activating activities (14). The fact that CBP/p300 are both negative regulators of the cell cycle, mediate differentiation or apoptosis, and may be tumor suppressors (47) is consistent with the proposed role for ZBP-89 in growth inhibition. Although p53 binds p300 and DNA activation of p21<sub>wafl</sub> requires p53, HT-29 cells are p53-deficient (25). ZBP-89 appears to have a preference for p300 over CBP, which may reflect the direct involvement of p300 in growth suppression.

Through their ability to activate human proliferating cell nuclear antigen that interact with p53, CBP/p300 may sense DNA damage and activate DNA repair (47, 48). By this mechanism, the reported interaction of an 80-kDa GADD34-like (growth arrest DNA damage) protein with the mouse ZBP-89 homologue BFCOL1 (24) may have relevance because the GADD protein family senses DNA damage and arrests growth-independent of p53 status (49, 50). To our knowledge, association of a histone acetyltransferase gene product with GADD proteins has not been reported. Moreover, the role of GADD34 and ZBP-89 in p21<sub>wafl</sub> gene expression is not known (24).

In summary, these data support the aggregation of a large complex of factors binding to the p21<sub>wafl</sub> promoter that include ZBP-89, Sp family members and coactivators. Contact with p300 and subsequent transcriptional activation may depend upon the extracellular signal. In the case of butyrate, our data would support butyrate induction of p21<sub>wafl</sub> through promotion of a p300 and ZBP-89 interaction with the subsequent recruitment of Sp1 to the complex through its association with ZBP-89 (Fig. 9). Histone deacetylase 1 binds Sp1 in G<sub>s</sub>, raising the possibility that cofactors other than histone deacetylases may target this transcription factor in the resting state (51). Although not evaluated in this study, it is tempting to speculate on the possibility of butyrate inhibiting histone deacetylases associated with specific transcription factors on specific
promoters. In this way, the level of histone acetylation would rise like a rheostat to switch transcription on at specific promoter sites.

Acknowledgments—We thank Dr. W. El-Deiry (University of Pennsylvania), Drs. Kinzler and Vogelstein (Johns Hopkins University) for the p21 promoter constructs, and Dr. Roland Kwok (University of Michigan) and Dr. Tony Kouzarides (Cambridge University) for the E1A and p300 expression vectors.

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