Epstein-Barr Virus BZLF1 Gene Is Activated by Transforming Growth Factor-β through Cooperativity of Smads and c-Jun/c-Fos Proteins*

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Induction of Epstein-Barr virus (EBV) production in an EBV-positive cell is achieved by expression of the gene BZLF1 that switches the latent state into a lytic state. The expression of the BZLF1 gene is initiated from the promoter Zp, which is normally suppressed in EBV-transformed B cells. The BZLF1 gene can be induced for expression by activating agents, such as transforming growth factor-β (TGF-β) and 12-O-tetradecanoylphorbol-13-acetate. The 12-O-tetradecanoylphorbol-13-acetate-responsive element located in the Zp is the AP-1 motif. The TGF-β-responsive element, however, has not been determined. We demonstrated that the Smad4-binding element site, GTCTG, from −233 to −229, was located in the regulatory region of the Zp relative to the BZLF1 transcription initiation site and was physically associated with Smad4. This association was important for the TGF-β induction of Zp. We also showed from the results of co-transfection experiments and electrophoretic mobility shift assays that both the AP-1 motif and Smad4-binding element site appeared to be required for the TGF-β-induced activation of Zp. This effect was mediated through the cooperation of Smad3/Smad4 and c-Jun/c-Fos that formed a complex. TGF-β treatment of Rael cells induced production of infectious EBV particles that was capable of infecting EBV-negative CA46 cells and transforming normal cord blood B cells, in vitro. Those data support a mechanism that TGF-β induces the latent EBV in cells to enter the viral lytic cycle through regulation of key viral proteins by TGF-β signal transducers. Those findings also suggest a role of TGF-β in EBV-associated diseases.

The Epstein-Barr virus (EBV) is a herpes virus, which infects epithelial cells and B lymphocytes. During latency little or no viral replication takes place; instead, the EBV-infected B cell is immortalized through the expression of a small subset of viral genes, serving to establish and maintain cellular growth transformation. On the other hand, a switch in the genetic program can lead to the expression of viral replication-associated genes. It can be accomplished, in vitro, by treatment of latent B cells with various activating agents, including phorbol esters, butyrate, Ca2+ ionophores, and anti-immunoglobulin. These treatments trigger a variety of cellular signaling pathways resulting in the activation of cellular transcription factors activating transcription from the BZLF1 promoter Zp (1−5). The product of the BZLF1 gene is Zta, also known as EB1 and ZEBRA. Zta is a lytic switch transactivator, a protein that is a transcription factor of the b-Zip family, which exhibits homology to c-Fos and the CCAAT/enhancer binding protein (C/EBP) (3, 6, 7). The Zta protein activates the expression of EBV lytic genes necessary for lytic DNA replication (8, 9). Zta also binds to the lytic origin site of EBV replication, and this binding is a prerequisite for EBV DNA replication (10). Therefore, Zta expression is pivotal for activation of the EBV lytic cycle. Zta can also be translated from baculovirus mRNA that contains both BRLF1 and BZLF1. The mRNA is transcribed from Rp, located in a BamH1R fragment; however, translation of BZLF1 from the baculovirus mRNA requires Rta protein (11). Zta expression also increases the level of active endogenous transforming growth factor β1 (TGF-β1) and its related products. This contributes to the induction of EBV lytic cycle (12, 13), indicating an autocrine effect of TGF-β.

TGF-β is an important cytokine regulating cell growth, morphogenesis, cell differentiation, and apoptosis (14−16). A class of transcription factors, the Smad proteins, mediates the signaling of the TGF-β intracellular pathway (17). Among the Smad proteins, Smad2 and Smad3 are ligand-responsive. Smad4 (DPC4, named from a deletion in pancreatic carcinoma), is a tumor suppressor and functions as a co-mediator in the signaling pathways of the TGF-β superfamily. Responding to TGF-β binding (18, 19), TβRII, an active type II receptor kinase, phosphorylates and activates TβRI. The activated TβRI (20) is carboxyl-terminally phosphorylated. Smad2 and Smad3 transiently associate with TβRI/TβRII. These receptor-activated Smads form a heteromeric complex with Smad4 and then translocate into the nucleus where target genes are transcriptionally activated (21). The activation of TGF-β-induced transcription factors, the Smad proteins, mediates the signaling of the TGF-β intracellular pathway (17). Among the Smad proteins, Smad2 and Smad3 are ligand-responsive. Smad4 (DPC4, named from a deletion in pancreatic carcinoma), is a tumor suppressor and functions as a co-mediator in the signaling pathways of the TGF-β superfamily. Responding to TGF-β binding (18, 19), TβRII, an active type II receptor kinase, phosphorylates and activates TβRI. The activated TβRI (20) is carboxyl-terminally phosphorylated. Smad2 and Smad3 transiently associate with TβRI/TβRII. These receptor-activated Smads form a heteromeric complex with Smad4 and then translocate into the nucleus where target genes are transcriptionally activated (21). The activation of TGF-β-induced transcription factors, the Smad proteins, mediates the signaling of the TGF-β intracellular pathway (17). Among the Smad proteins, Smad2 and Smad3 are ligand-responsive. Smad4 (DPC4, named from a deletion in pancreatic carcinoma), is a tumor suppressor and functions as a co-mediator in the signaling pathways of the TGF-β superfamily. Responding to TGF-β binding (18, 19), TβRII, an active type II receptor kinase, phosphorylates and activates TβRI. The activated TβRI (20) is carboxyl-terminally phosphorylated. Smad2 and Smad3 transiently associate with TβRI/TβRII. These receptor-activated Smads form a heteromeric complex with Smad4 and then translocate into the nucleus where target genes are transcriptionally activated (21). The activation of TGF-β-induced transcription factors, the Smad proteins, mediates the signaling of the TGF-β intracellular pathway (17).
scription can be mediated through the binding element of Smad4 containing the CAGA box (22-24) or through the cooperation of Smad proteins and the AP-1 complex composed of c-Jun and c-Fos (25).

The BZLF1 gene promoter Zp is located between the coding sequence of BRLF1 (Rta) and BZLF1 (Zta). The previously identified responsive elements of Zp-221 (covering from −221 to +13 of Zp) contain four ZI elements, a ZII domain, and two juxtaposed elements ZIIIA and ZIIIB (4, 26). ZI domains can be regulated by Sp1 and Sp3. ZII domain contains a CREB/AP-1-like binding site, which is a key element for various inducing agents. ZIII domains are the major Zta auto-activation sites.

By determining Zp transcriptional regulation, in response to TGF-β, we discovered a potential SBE site ~12-bp proximal to the previously determined promoter region, −221 to +13. In order to substantiate that the SBE site is inducible, we examined a Zp reporter construct, Zp-236, which is 15 bp longer than the previously used construct. We discovered that the SBE site, GTGTCG, −233 to −229, is crucial to Zp activity. We also discovered that in response to TGF-β induction, the SBE site and the AP-1 motif act cooperatively. The activation is through the cooperation between Smad3/4, c-Jun, and c-Fos. Latent EBV harboring Rael cells treated with TGF-β induce production of infectious viruses, infecting EBV-negative CA46 cells and transforming normal cord blood B cells in vitro.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—The cell lines of EBV-positive type I Burkitt’s lymphoma (BL), Rael, and P3HR-1 and EBV-negative BL, CA46, and Ramos were maintained in a Roswell Park Memorial Institute (RPMI 1640) medium supplemented with 15% fetal bovine serum (FBS, v/v), 100 μg/ml penicillin, 100 μg/ml streptomycin, and 2 μM glutamine. The 293 cells (human kidney epithelial cell, ATCC CRL-1573) and F9 (mouse embryonal carcinoma cell, ATCC CRL-1720) were maintained in Dulbecco’s modified Eagle’s medium with 10% FBS, 100 μg/ml penicillin, and 100 μg/ml streptomycin. MDA-MB-468 (human breast cancer cell, ATCC HTB-132) was maintained in Leibovitz’s L-15 medium with 10% FBS.

**Plasmid Construction**—Various fragments of Zp containing the −236 to +13, corresponding to nt 103,182 to nt 103,430 of the EBV genome, were cloned into Smal-treated pGL2-Basic vector (Promega) to generate various Zp-containing reporter constructs. The Zp sequence was generated by PCR using B95-8 genomic DNA as a template and primers Zp-F (5′-TGGAGCGTTCTGCTATGAGTC-3′) and Zp-R (5′-CGCGGCTGCTGCGCAGGCG-3′), corresponding to nt 103,182 to nt 103,430 of B95-8 genome) and Zp-R. The reporter plasmids that contained mutated Zp with a mutation at the AP-1 site of pGEM3 (Promega). The c-Jun expression plasmid was constructed by inserting cDNA of c-Jun into pBluescript KS-vector. Both c-Jun and c-Fos expression plasmids were provided by Dr. S. L. Chen, National Defense Medical School. The dominant-negative Erk1/2 (KR), DN-p38, and DN-JNK expression plasmids were obtained from Dr. J. S. Yu, Chang-Gung University.

**DNA Transfection and Reporter Gene Assay**—Reporter plasmids were transfected into P3HR-1 and Rael, a BZLF1 gene induction in P3HR-1 cells. Upper panel, RT-PCR analysis of BZLF1 gene expression in response to TGF-β treatment. Total RNA was isolated from P3HR-1 cells treated with 4 ng/ml recombinant TGF-β for 1, 2, 4, 12, and 24 h. BZLF1 transcripts were amplified by using BZLF1-specific primers. The RT-PCR products were then subjected to Southern blot analysis. The percent increase of Zta expression is shown at the bottom. Expression of GAPDH gene is used as the control. Lower panel, Western blot analyses of Zta in response to TGF-β treatment. Cell extracts of untreated and TGF-β-treated P3HR-1 cells were assayed for Zta expression by Western blot analysis using anti-Zta antibody. Cell extract from TPA-treated B95-8 cells was used as the positive control for Zta expression. The percent increase of Zta expression is shown as described above. The percent increase of Zta expression is shown at the bottom.

The expression vectors pcDNA3-Smad3, Smad4, and DN Smad3 were described previously (17, 28). p3TP-Lux, a TGF-β-responsible reporter construct (29), was used as a positive control.

The sequences of these constructs were confirmed by Sanger’s dideoxy chain termination method (30) using T7 Sequenase version 2.0 DNA sequencing kit (Amersham Biosciences).

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To examine the effect of TGF-β, cells were transfected with plasmid DNA and treated with 4 ng/ml recombinant human TGF-β (R&D Systems) for 24 h. To test the effect of TPA treatment, cells were cultured in 5 ml of 0.4% FBS RPMI 1640 with 30 ng/ml TPA for 24 h. Plasmid DNA was purified by cesium chloride-ethidium bromide gradient centrifugation.

To examine the effect of sense or antisense oligonucleotides, P3HR-1 cells were co-transfected with Zp-236 and sense or antisense oligonucleotides. The sense Smad4 (5'-CAA ATG GAC AAT ATG TCT ATT ACG-3'), the antisense Smad 4 (5'-CGT AAT AGA CAT ATT GTG CAT TTG-3'), the sense c-Jun (5'-TCT ATT ACT GCA AAG ATG GAA ACG-3'), and the antisense c-Jun (5'-CAT ATT CTT TTC CTT GTG CAT AGT CAT AGA-3') were used. After 72 h, cells were then treated with 4 ng/ml TGF-β for another 12 h before harvesting.

Western Blot Analysis—Cells were washed three times with a phosphate-buffered saline, harvested, and lysed in a buffer containing 10 mM Tris-HCl, pH 7.1, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 1% (v/v) Triton X-100. Cells were disrupted by sonication, and the cell debris was removed by centrifugation at 10,000 × g for 30 min at 4 °C. The supernatant of the whole-cell lysate was then separated by 10% SDS-PAGE and then electrotransferred to the nitrocellulose membrane, followed by immunoblotting with antibodies. Anti-Zta monoclonal antibody was a gift from Dr. M. R. Chen and C. H. Tsai, National Taiwan University (31). Anti-tubulin antibody was purchased from Neo Markers. Anti-FLAG-Smad protein antibody (anti-FLAG antibody M2) was purchased from Sigma, and anti-Myc-Smad protein antibody (anti-Myc antibody) was purchased from CLONTECH. Anti-c-Jun antibody (KM-1) and anti-c-Fos antibody (6–2H-2F) were purchased from Santa Cruz Biotechnology. The blot was then subjected to the ECL system (Amersham Biosciences) for the detection of Zta and tubulin protein.

Electrophoretic Mobility Shift Assay—The double-stranded oligonucleotide carrying the Z promoter (Zp) was used as the probe in electrophoretic mobility shift assay (EMSA). The oligonucleotide was labeled with T4 polynucleotide kinase and [γ-32P]ATP. The mixture of 293 cell nuclear extract and 15,000 cpm of γ-32P-oligonucleotide was incubated in a buffer containing 0.13 μg/μl poly(dI-dC), 4% glycerol, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, and 10 mM Tris-HCl, pH 7.5. For a competitive assay, a 200-fold excess of the individual cold

**Fig. 2.** Nucleotide sequence and schematic representation of Zp construct (Zp-236) and reporter plasmids containing various mutations. A, sequence analysis of −236 to +13 of Zp. Zp-F and Zp-R are the PCR primers used for amplification of Zp promoter fragment. The relative positions of SBE, SBE-like sequences, AP-1 motif, ZI domains, and ZII domains are as indicated in bold or underlined. +1 represents the transcription initiation site. B, the map of the Zp construct and its mutant constructs. m1 signifies mutation in the SBE site; m2 represents mutation in the SBE-like sequence, and mAP1 designates mutation in AP-1 motif. Construction of the deletion and site-specific mutants was carried out as described under “Experimental Procedures.”
The relative luciferase activity was the ratio of Zp-236 luciferase activity (fold increase) to that measured without TGF-β—specific 5’ primer (5’-TGGATCTGGGAAGGACTCATGAC-3’) and 3’ primer (5’-ATGCCAGTGAGCTCCCCGTACG-3’) were mixed with SYBR Green PCR Master Mix kit (Applied Biosystems). Fifty nmol of the Zta 5’ primer and Zta-3’ primer or glyceraldehyde-3-phosphate dehydrogenase (GAPDH)—specific 5’ primer (5’-TGGATCTGGGAAGGACTCATGAC-3’) and 3’ primer (5’-ATGCCAGTGAGCTCCCCGTACG-3’) were mixed with SYBR Green PCR Master Mix kit (Applied Biosystems, Inc.). The real time detection of emission intensity of SYBR Green bound to double-stranded DNA was analyzed by GeneAmp 5700 sequence detection system (Applied Biosystems, Inc.). All of the procedures were performed as per the manufacturer’s recommendations, and the quantitative PCRs were carried out in triplicate for each primer set. After PCR, background subtraction of the initial template concentration was followed by determination of the optimal threshold level (5% of the full scale) of fluorescent intensity using GeneAmp 5700 SDS software (Applied Biosystems). The level of mRNA for BZLF1 gene was expressed as a ratio to GAPDH mRNA value in each sample. Each PCR product was electrophoresed on 2% agarose gels to confirm the specific DNA band.

In Vitro EBV Infection and Immortalization Assays—To prepare the virus stock for infection of EBV-negative CA46 cells, the supernatant of Rael cells treated with or without 2 ng/ml TGF-β for 2 days was collected by centrifugation. The pellet went through three rounds of freezing and thawing before clearing by centrifugation. The cleared supernatant was filtered through a 0.45-μm pore size membrane, and the cleared supernatant was infected with the pellets resuspended in 3 ml of RPMI 1640—positive cell lines, Rael and P3HR-1. Cells treated with 4 ng/ml TGF-β1 or untreated was extracted using Trizol reagent (Invitrogen). Ten micrograms of total RNA were isolated from 500,000 cells and treated without or with 4 ng/ml TGF-β1 or 24 h. The total RNA was isolated by Trizol reagent as recommended by the manufacturer (Invitrogen). Oligo dT primer as a first strand cDNA synthesis by using a random hexamer primer (5’-ATGCACGGAGCTCCGCTACG-3’) and Taqman Reverse transcription kit (Applied Biosystems). Fifty nmol of the Zta 5’ primer and Zta-3’ primer or glyceraldehyde-3-phosphate dehydrogenase (GAPDH)—specific 5’ primer (5’-TGGATCTGGGAAGGACTCATGAC-3’) and 3’ primer (5’-ATGCCAGTGAGCTCCCCGTACG-3’) were mixed with SYBR Green PCR Master Mix kit (Applied Biosystems, Inc.). The real time detection of emission intensity of SYBR Green bound to double-stranded DNA was analyzed by GeneAmp 5700 sequence detection system (Applied Biosystems, Inc.). All of the procedures were performed as per the manufacturer’s recommendations, and the quantitative PCRs were carried out in triplicate for each primer set. After PCR, background subtraction of the initial concentration was followed by determination of the optimal threshold level (5% of the full scale) of fluorescent intensity using GeneAmp 5700 SDS software (Applied Biosystems). The level of mRNA for BZLF1 gene was expressed as a ratio to GAPDH mRNA value in each sample. Each PCR product was electrophoresed on 2% agarose gels to confirm the specific DNA band.

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To prepare the virus stock for the in vitro transformation assay, supernatants of Rael cells treated without or with various concentrations of TGF-β (0.25, 0.5, 1, 2, and 4 ng/ml) for 2 days were collected by centrifugation. Pellets were resuspended in the medium and filtered with a 0.45-μm pore size membrane. To transform the cells in vitro, each of 1 × 10^6 umbilical cord primary B cells derived from 10 healthy donors were placed in 20 ml of RPMI 1640 growth medium after ultracentrifugation. The infected cells were maintained in RPMI 1640, and the growing cells were scored 1½ months later.

RESULTS

TGF-β activates BZLF1 gene through its mediator Smad proteins—TGF-β treatment induces EBV to enter a lytic cycle through the action of the latent-to-lytic switching BZLF1 gene product (Zta) whose expression is activated (34). To examine how the BZLF1 gene was regulated, we first tested the time course of BZLF1 gene and Zta protein expression in two EBV-positive cell lines, Rael and P3HR-1. Cells treated with 4 ng/ml TGF-β and with or without 4 ng/ml TGF-β for 2 days were collected by centrifugation. The pellet went through three rounds of freezing and thawing before clearing by centrifugation. The cleared supernatant was filtered through a 0.45-μm pore size membrane, and the cleared supernatant was used to infect 2.5 × 10^6 EBV-negative CA46 cells. Forty eight h after infection, DNA of the infected CA46 cells was analyzed by PCR using EBV-specific primers, 30F and 30R, described previously (32). As a control, a similar preparation was obtained from a culture medium of Rael cells without TGF-β treatment.

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TGF-β were subjected to the RT-PCR using sequence-specific primers for *BZLF1* gene. As illustrated in Fig. 1, Zta transcripts were significantly elevated 1 h after TGF-β treatment and were increased ~200% by 12 h. Zta protein was also easily detected by anti-Zta monoclonal antibody at 12 h after TGF-β treatment and increased to ~500% at 24 h in P3HR-1 cells. Similarly, in Rael cells, Zta transcripts were significantly elevated 2 h after TGF-β treatment and increased ~300% by 48 h. Zta protein was easily detected by anti-Zta monoclonal antibody at 12 h after TGF-β treatment and reached the peak level (~500%) at 48 h after TGF-β treatment (Fig. 1B). This level of activation remained as the treatment was prolonged. These results suggest that TGF-β induces *BZLF1* gene expression and Zta protein production.

The TGF-β signaling pathway involves the cooperation of the effector Smad proteins including Smad3 and Smad4 (16, 34). Among them, Smad4 recognizes and binds to a Smad4-binding element (SBE) or to a core “CAGA” sequence (or SBE-like sequences) within the regulatory region of the targeted genes (12, 24, 45). Sequence analysis of Zp-236 identifies a potential SBE site (~233 to ~229), two CAGA boxes (~166 to ~163 and ~59 to ~56), in addition to the previously reported AP-1 recognition motif (Fig. 2A). Therefore, to study the mechanism involved in the TGF-β-induced *BZLF1* gene activation, the Zp-236 was generated (Fig. 2B) and was used to examine the effect of TGF-β. P3HR-1 cells were transfected with Zp-236, and the transfected cells were treated with 0.1, 0.2, 0.5, 1, 2, 4, and 10 ng/ml TGF-β, respectively, for 24 h. Promoter strength, in terms of luciferase reporter gene activity, was measured. Activation of Zp was increasingly enhanced as a function of TGF-β concentration: from ~2-fold over the basal value with 0.1 ng/ml to ~3.5-fold with ~2 ng/ml (Fig. 3A), although the increment was relatively small. Zp promoter activity correlated with *BZLF1* gene transcription and Zta protein production as demonstrated in Fig. 1.

To examine the cooperation of the effector Smad proteins in Zp activation, Zp-236 with Smad3 or Smad4 or further with both expression vectors was co-transfected, respectively, into P3HR-1 cells, and the transfected cells were treated with 4 ng/ml TGF-β for 24 h. As illustrated in Fig. 3B, Zp-236 was activated 2–3-fold by the expression of the ectopically transferred Smad3 or Smad4 without TGF-β. In the presence of TGF-β, Zp activity of the control was enhanced ~2-fold, whereas it was stimulated ~6-fold over the basal level without TGF-β by Smad3 and ~4-fold by Smad4. When the cell contained both Smad3 and Smad4, Zp was activated ~6-fold without TGF-β; in contrast, in the presence of TGF-β, it was ~11-fold over the basal level without TGF-β. The addition of
dominant-negative Smad3 (DN Smad3) or dominant-negative Smad4 (DN Smad4), which inhibited the TGF-β-induced activation, eliminated the enhancement (Fig. 3B, data not shown for DN Smad4), confirming the activation observed above. As a positive control, p3TP-lux that would be induced by TGF-β induction was activated 4-fold with 4 ng/ml TGF-β (Fig. 3C).

Binding of Smad4 to the SBE Site and SBE-like Sequence—The effect of TGF-β on transcriptional activation of target genes is to form a Smad complex such as Smad3-Smad4 or Smad2-Smad3-Smad4, which enters the nucleus. As a result, Smad4 should recognize and bind to the SBE site (−233 to −229) and two SBE-like sites (−166 to −163 and −59 to −56) within Zp-236 (Fig. 2A). To test whether or not Smad4 indeed recognized these sites, 293 cells were infected with a FLAG-tagged Smad4 recombinant adenovirus, followed by TGF-β treatment. Nuclear extracts of the 293 cells were then prepared and used for EMSA. A DNA complex was formed using probe 1 (Fig. 4A and B). This complex was supershifted to a higher molecular weight complex detected by an anti-FLAG monoclonal antibody but not by an anti-ATF-2 antibody (a nonspecific antibody). This complex was also competed off with a 200-fold unlabeled probe 1 and with the CAGA motif (Smad4 consensus binding motif). The complex formation was not affected when mutated SBE sequences were added (Fig. 4B). Similar supershifts were obtained when probe 2 (−181 to −81) and probe 3 (−81 to +13), containing the two SBE-like sequences, respectively, were 5′ end-labeled with [γ-32P]dATP and used for EMSA (Fig. 4C and D). These results suggested that Smad4 indeed physically bound to the SBE site and the two SBE-like sequences located within Zp-236.

FIG. 5. MAP kinase signal pathway-independent induction of Zp activity by TGF-β. A, effect of MAP kinase inhibitor PD98059 on Zp-236 activity. P3HR-1 cells were co-transfected with Zp-236, and with Smad3- and Smad4-expressing vectors together were treated with TGF-β and PD98059 (10 or 20 μM), respectively or simultaneously, and relative Zp promoter activity was measured as described in Fig. 3. B, effect of dominant-negative mutants, Erk1/2(KR), of MAP kinase signaling pathway on Zp activity. P3HR-1 cells were co-transfected with Zp-236 and with either Erk1/2(KR)-expressing vectors, or the Smad3- and Smad4-expressing vectors, or both in the presence or absence of TGF-β, and the relative Zp promoter activity was measured. C, effect of dominant-negative JNK mutant on Zp activity. P3HR-1 cells were co-transfected with Zp-236, with JNK or dominant-negative (DN)-JNK-expressing vector, or the Smad3- and Smad4-expressing vectors, or both in the presence or absence of TGF-β, and the relative Zp promoter activity was measured. D, effect of dominant-negative mutant, DN-p38, of p38 MAP kinase signaling pathway on Zp activity. P3HR-1 cells were co-transfected with Zp-236 and with p38 or dominant-negative (DN)-p38-expressing vector, or the Smad3- and Smad4-expressing vectors, or both in the presence or absence of TGF-β, and the relative Zp promoter activity was measured. The fold increase marked at the top of each bar was determined from the luciferase value, set as 1, obtained in the cells transfected with only Zp-236. *signifies the presence of the agent. The value was presented as the average of at least six independent experiments. Vertical bars are the S.D.
TGF-β Signaling Mediators Induce EBV BZLF1 Gene Expression

**Fig. 6. Effect of TGF-β and TPA on Zp expression in EBV-positive P3HR-1 and EBV-negative Ramos cells.** A, Zp-236 and each of its mutant constructs shown in Fig. 1B were, respectively, transfected into EBV-positive P3HR-1 cells. These transfectants were then treated with TGF-β and/or TPA. The promoter activity, in terms of relative luciferase activity, in these cells was measured. The numbers shown at the top of each bar stands for “fold increase,” which was obtained by using the activity of untreated control for each promoter construct as 1. + indicates the presence of the factors. The value indicated is the average of at least six independent experiments. The S.D. is shown with vertical bars.

**TGF-β-induced Smad Proteins Activate Zp Independent of the MAP Kinase Signaling Pathway**—A recent report (33) indicates that Zp may be activated by TGF-β indirectly by a mechanism that requires a MAP kinase signaling pathway. To distinguish the effect of TGF-β-mediated Smad proteins from MAP kinase signaling, Zp-236 activity was examined in the presence of TGF-β with or without a MEK/MAPK kinase inhibitor PD98059 (Calbiochem). As seen before, the basal level of Zp-236 activity generally increased ~3-fold in the presence of TGF-β (Fig. 5A) whether or not Smad proteins were present (from ~6-fold with ectopically expressed Smad3/4 in the absence of TGF-β to ~18-fold in the presence of TGF-β). However, the addition of 10 and 20 μM PD98059 showed little further enhancement whether or not the Smad proteins or TGF-β was present, indicating that Zp activity might not be activated by the MAP kinase signaling pathway as examined in P3HR-1 cells. When the test was carried out in cells transfected with two dominant-negative mutants of the MAP kinase signaling molecules, Erk1/2(KR), again, consistent with the result of Fig. 5A, only the effect of TGF-β and Smad proteins on Zp-236 activity was observed; the presence of Erk1/2(KR) showed little or no enhancement of Smad4 with or without TGF-β-mediated activation. In contrast, these dominant-negative mutants activated the Zp, implying that the Erk1/2 played an inhibitory role, rather than enhancement (Fig. 5B). Similar results were obtained from cells transfected with dominant-negative mutants of two other molecules, the DN-JNK and DN-p38 MAP kinases of the MAP kinase signaling pathways. As illustrated in Fig. 5, C and D, DN-JNK and DN-p38 did not inhibit Zp-236 activity in the presence or absence of TGF-β. Those results suggested that the TGF-β-mediated activity of Smad proteins to increase Zp activity was independent of the MAP kinase signaling pathways.

**Cooperative Activation of Zp by TGF-β and TPA**—To check the importance of the binding of Smad proteins to SBE or AP-1 sites on the Zp activity and its responsiveness to TGF-β, as well as on other TGF-β-responsive elements within Zp, a series of Zp reporter constructs (Fig. 2B) were prepared and examined. P3HR-1 cells were transfected with various Zp constructs, respectively, and then treated with TGF-β at 4 ng/ml for 24 h. The results are shown in Fig. 6A. The Zp-236m1 mutation in and the Zp-181 deletion of the SBE site resulted in more than 50% (open bars) and more than 60% (open bars) reduction of Zp activity, respectively, indicating that the SBE site was a positive regulatory element for Zp activity. The mutation at the AP-1 site (m1) or AP-1 alone or at both SBE site and AP-1 motif (double mutant) showed similarly reduced promoter activity of the wild type Zp-236, respectively. Treated with TGF-β, the promoter constructs containing an m1 mutation (Zp-236m1 and Zp-236m1mAP1) were less responsive. On the other hand, TPA failed to induce promoter activity in all AP-1 site mutants (diagonally crossed line bars). Furthermore, the presence of both TGF-β and TPA did not enhance the activity beyond what TGF-β had enhanced, suggesting the mutation at either site or both no longer activated the promoter cooperatively by the two agents. This was also true when shorter constructs (Zp-81 and Zp-81mAP1) were examined. Zp-81 and Zp-81mAP1, both lacking the ZI and ZIII domains, showed similarly low or only basal promoter activity. It is important to test if TGF-β acts on Zp directly in EBV-negative cells because TGF-β activates the endogenous Zta protein that might act on the Zp reporter. As shown in Fig. 6B, Zp-236 was activated moderately by TGF-β (~2-fold) and, in combination with TPA, was further increased to ~3-fold in Ramos cells. Mutations of the SBE (Zp-236m1), the AP-1 site (Zp-236m1mAP1), and both SBE and AP-1 sites (Zp-236m1mAP1) eliminated the TGF-β effect completely. Similar results were observed when other Zp constructs containing other deletions and mutations were used. Results suggested that Zp activity can be directly induced by TGF-β, and the SBE site and the AP-1 site are involved in and important for this induction.

**Cooperative Activation of Zp through Binding of Smad Proteins, c-Jun and c-Fos, to the SBE Site and the AP-1 Motif in Response to TGF-β Induction**—As described above, the SBE site and AP-1 motif were important for Zp activity in response to TGF-β treatment. The AP-1 motif is the key responsive element of Zp to the inducing agent TPA. The AP-1 complex, consisting of c-Jun and c-Fos, is involved in the TGF-β-induced transcription regulation in association with Smad proteins (35). To test whether Zp is activated through the cooperation of Smad proteins and the AP-1 complex in the presence of TGF-β,
P3HR-1 cells were first co-transfected with Zp-236 together with Smad3 and Smad4 expression vectors, respectively, or both, in the presence of either ectopically expressed c-Jun or c-Fos or both. These transfected cells were then treated with TGF-β. As shown in Fig. 7A, without TGF-β (open bars), c-Fos expression, or co-expression with Smad3 or Smad4 individually, increased Zp activity to only ~3–6-fold of base-line value (without any activating agent) when both Smad proteins were expressed. The c-Jun expression, or co-expression with Smad3 and Smad4 individually, increased Zp activity to ~4–8-fold when both Smad proteins were expressed. In the presence of TGF-β (solid bars), however, the activation was generally enhanced, and the enhancement was conspicuously higher when the Smad proteins were present; the activity was further increased to a maximum of 11- and 16-fold with the expression of both Smad proteins (the 4th c-Fos column and the 4th c-Jun + column). A stronger activation than the c-Fos cases was observed with c-Jun expression, but the differences between
transcripts by the quantitative RT-PCR method. As illustrated, BZLF1 was observed when Rael cells were used (data not shown). Nearly 18-fold when all four were present. Similar results were again, a substantial enhancement of the activity, reaching to 9-fold in the presence of either Smad protein, reaching to 20-fold as compared with the untreated in the presence of TGF-β. The result showed that various activating factors elevated the amount of BZLF1 transcript and Zta protein with or without TGF-β treatment. The level of activation at the transcriptional level (i.e., promoter activity and BZLF1 transcripts) is consistent with the elevated level of the Zta protein. Therefore, in the presence of TGF-β, Smad3, Smad4, c-Jun, and c-Fos of the AP-1, alone or in combination, all appeared to activate Zp to produce BZLF1 gene product, Zta protein.

**Association of Smad Proteins and c-Jun/c-Fos Complex with Zp**—The above results indicated that the cooperative activity of Smad proteins and c-Jun and c-Fos might be manifested through the physical association of these factors. To confirm this, FLAG-Smad3 and FLAG-Smad4-expressing recombinant adenoviruses were introduced into 293 cells by infection, and the infected cells containing these plasmids were treated with TGF-β. Nuclear extracts of the treated 293 cells were prepared and incubated with a labeled probe covering the −236 to +13 region of Zp. The association was examined by EMSA. The results shown in Fig. 7D clearly indicated that when Smad proteins, c-Fos, or all of them were present, they formed a protein-DNA complex that was supershifted as detected by an anti-FLAG antibody (Fig. 7D, @Smads/DNA complex), an anti-c-Fos antibody (Fig. 7D, c-Fos/DNA complex), or both antibodies (Fig. 7D, #c-Fos/Smads/DNA complex). Incubation with a nonspecific antibody (anti-Sp1) did not detect (bind to) the supershifted complex. Furthermore, incubation of an anti-FLAG antibody with the nuclear extract derived from cells without infection of FLAG-Smad4-expressing recombinant virus (Fig. 7D, NE(−Flag-Smad4)) did not show a protein-DNA complex. The result indicated the physical association of the Smad proteins with c-Jun and/or c-Fos complex in response to TGF-β treatment.

**Cooperative Activation of Zp by Smad Proteins and c-Jun and c-Fos in EBV-negative Ramos Cells in Response to TGF-β**—The above Zp activation appeared to be the result of the direct action of Smads 3 and/or 4 rather than due to the endogenous Zta protein. This was supported by the results presented in Fig. 8A. In EBV-negative Ramos cells, Zp−236 was moderately activated (~2-fold, open bars) by ectopically expressed Smads 3 and 4 and c-Jun and c-Fos, respectively. TGF-β treatment further enhanced the activation ~3- to 4-fold. When all four were present, Zp was activated to ~4.5-fold in the absence of TGF-β and further to ~6-fold when TGF-β was present, demonstrating the additive effect of these two sets of factors. The TGF-β-inducible control reporter construct p3TP-Lux was activated by TGF-β in Ramos cells to ~10-fold (Fig. 8B).

**Inhibition of TGF-β-induced Zp Activation by Smad4 or c-Jun Antisense Oligonucleotides and in Smad4 or c-Jun/c-Fos Deficient Cells**—In order to test if TGF-β signaling is indeed a natural pathway for induction of Zp, cells were treated with antisense Smad4, antisense c-Jun, or both oligonucleotides individually for 72 h, followed by treatment with 4 ng/ml TGF-β for 12 h. The results are shown in Fig. 9A. The antisense Smad4 inhibited the Zp activity slightly in the absence TGF-β but inhibited ~65% activity (4.4-to 1.6-fold) in the presence of TGF-β.
Fig. 9. Inhibition of TGF-β-induced Zp activation by Smad4 or c-Jun antisense oligonucleotides and in Smad4 or c-Jun/c-Fos-deficient cells. A, effect of antisense Smad4 and antisense c-Jun on Zp activity in response to TGF-β. P3HR-1 cells were co-transfected with Zp-236 and the indicated sense or antisense oligonucleotides. 33 μg of each of the Smad4-specific oligonucleotides or 66 μg of each of the sense of TGF-β. Antisense c-Jun reduced the Zp activity to ~40% in the absence of TGF-β, and inhibited at least ~50% (from 2.7 to 1.3-fold) in the presence of TGF-β. The combination of antisense Smad4 and antisense c-Jun also showed ~50% inhibition in the absence of TGF-β and displayed ~70% inhibition (from 3- to 0.9-fold) in response to TGF-β treatment. As the controls, the sense Smad4 and sense c-Jun showed no inhibition of Zp activity. Accordingly, the protein levels of Smad4, c-Jun, and Zta were reduced in cells treated with antisense oligonucleotides as examined by Western blot analysis using protein-specific antibodies (Fig. 9A, inset). To confirm further if Zp activation by TGF-β requires Smad4 and c-Jun/c-Fos, Zp-236 and its mutants were transfected in Smad4-deficient cell line MDA-MB-468 and c-Jun/c-Fos-deficient cell line F9, respectively. As illustrated in Fig. 9B, ectopically expressed Smad3 and Smad4 activated Zp-236, and the activation was further enhanced in the presence of TGF-β, whereas Zp-236 alone showed a basal level of activity. In contrast, Smad3 and Smad4, with or without TGF-β treatment, did not activate Zp-236m1 (mutation in SBE site), Zp-236mAP1 (mutation in AP-1 site), or Zp-236m1AP1 (mutations in both SBE and AP-1 sites). Similar experiments were carried out using c-Jun/c-Fos-deficient F9 cells. As shown in Fig. 9C, ectopically expressed Smad3/c-Jun, c-Jun/c-Fos, or a combination of these proteins activated Zp-236 in the presence of TGF-β. However, Zp-236m1 was slightly activated by TGF-β only when c-Jun/c-Fos complex or a combination of Smad3/c-Jun and c-Jun/c-Fos was present, and Zp-236mAP1 was activated by TGF-β when Smad3/c-Jun was ectopically expressed. Furthermore, the double-site mutant Zp-236m1mAP1 was not activated by TGF-β in the presence or absence of these proteins. These results suggest that Smad3/c-Jun and c-Jun/c-Fos are required for Zp activation in response to TGF-β treatment.

TGF-β Induction of EBV Production in Type 1 Rael Cells—The EBV particles produced in lytic cycle switched from a latent stage is infectious and capable of infecting EBV-negative B lymphocytes. To test whether the infectious EBV particles were produced after TGF-β treatment, Rael cells were treated with TGF-β for 48 h and centrifuged at low speed. The supernatant was filtered through 0.45-μm filter paper, and the filtrate was concentrated by ultracentrifugation. Samples prepared from Rael cells without TGF-β treatment were used as control. Pellets, marked as prep1 and prep2 in Fig. 10A, were resuspended in an RPMI 1640 medium and added to infect EBV-negative CA46 cells. Forty eight h after infection, the CA46 cells were harvested, and the cellular DNA, marked as prep3 and prep4, was extracted for detection of EBV genome.

c-Jun-specific oligonucleotides were used. After 72 h, cells were then treated with 4 ng/ml TGF-β for 12 h before harvested. The relative Zp promoter activity was measured as described in Fig. 3. B, Zp activity in Smad4-deficient MDA-MB-468 cells. Smad4-deficient MDA-MB-468 cells were co-transfected with Zp-236, Zp-236m1, Zp-236mAP1, and Zp-236m1AP1 and with the Smad3- and Smad4-expressing vectors in the presence or absence of TGF-β (4 ng/ml), and the relative Zp promoter activity was measured. The fold increase marked at the top of each bar was determined from the luciferase value, set as 1, obtained in the cells transfected with only the indicated Zp reporter construct. C, Zp activity in c-Jun/c-Fos-deficient F9 cells. The c-Jun/c-Fos-deficient F9 cells were co-transfected with Zp-236, Zp-236m1, Zp-236mAP1, and Zp-236m1AP1 and with either the Smad3- and Smad4-expressing vectors, or c-Jun/c-Fos-expressing vectors, or both in the presence or absence of TGF-β (4 ng/ml), and the relative Zp promoter activity was measured. The fold increase marked at the top of each bar was determined from the luciferase value, set as 1, obtained in the cells transfected with only the indicated Zp reporter construct. The value was presented as the average of at least five independent experiments. Vertical bars are the S.D. Inset, Western blot analysis of Zta, Smad4, c-Jun, and tubulin proteins from cells treated with sense-Smad4 and sense-c-Jun oligonucleotides (s) or their antisense (as) oligonucleotides.
EBV production induced by TGF-β

Rael cells

- TGF-β

| M | prep1 | prep2 | prep3 | prep4 | Rael DNA |
|---|-------|-------|-------|-------|----------|
| 400 |       |       |       |       |          |
| 300 |       |       |       |       |          |
| 200 |       |       |       |       |          |
| 100 |       |       |       |       |          |

CA-46 cells (prep3) CA-46 cells (prep4)

![Figure 10](Image)

**Fig. 10. In vitro infection assay.** A, production of infectious EBV virus induced by TGF-β treatment. The chart shows the procedure for induction of infectious EBV by TGF-β. Rael cells were treated with 2 ng/ml TGF-β for 48 h. Virus stock was prepared as described under “Experimental Procedures.” B, infection of EBV-negative CA46 cells with prep3 and prep4 described in A. The PCR products using the prep4 as the DNA template showed a 286-bp DNA fragment, indicating the success of infection of EBV-negative CA46 cells. The prep2, which was used for infection, was also positive for EBV. Total cellular DNA of Rael cells was used as a positive control for PCR.

Viruses preparations from Rael cells treated with various concentrations of TGF-β (0.25, 0.5, 1, 2, and 4 ng/ml) were used to infect primary B cells isolated from cord blood samples of three individuals. One and half months after infection of \(1 \times 10^7\) primary B cells, three lymphoblastoid cell lines were established from cells by prep2 obtained from cells treated with 2 and 4 ng/ml TGF-β but not by prep1 from the untreated cells or from cells treated with lower concentrations of the cytokine (Table I). The presence of EBV DNA in newly established lymphoblastoid cell lines was confirmed by PCR using EBV-specific primers (data not shown), as described under “Experimental Procedures.” This result strongly supported the finding that infectious EBV was produced from the B cells in a latent stage after TGF-β treatment.

**Discussion**

The EBV lytic cycle can be induced in the latently infected cells by TGF-β (13); however, the mechanism is not fully understood. By examining the regulation of the EBV lytic promoter Zp, which directs the transcription of the lytic cycle activating BZLF1 gene, we identified the elements, the SBE and the AP-1 sites, that were critical for Zp activity. The activation was achieved by the complex of Smad proteins associated with the c-Jun/c-Fos of the AP-1 complex. Once TGF-β triggered Zp activation, the EBV entered the lytic cycle, producing infectious EBV, which, in turn, infected and transformed cord blood B lymphocytes.

Our data from the analysis of the Zp-236 construct, which contained a 15-bp sequence proximal to −221, were consistent with the previous report by showing enhanced promoter activity after the TPA treatment. More significantly, we showed that the extra 15-bp region included an SBE site, associated with Smad4, an effector protein in TGF-β signaling. The SBE site sequence located at −233 to −229 was complementary to 5′-CAGAC-3′, a preferred Smad4-binding site (22). This SBE functioning as a transcription enhancer for Zp activity and was required for the cooperative activation of TGF-β and TPA (Figs. 6–9). TGF-β treatment of the latently infected P3HR-1 cells and Rael cells expressed elevated levels of BZLF1 transcripts and Zta protein (Fig. 1 and Fig. 7, B and C). These results are consistent with the previous report (33) as examined in Raji cells, except the induction kinetics of TGF-β was different. In their study, the BZLF1 transcripts were induced −2 h after TGF-β treatment, and Zta protein was expressed −4 h after the treatment. However, in Rael and P3HR-1 cells (this study), the Zta transcripts were significantly elevated 1–2 h after TGF-β treatment and were increased −200% by 12 h, but Zta protein was detected at 12 h after TGF-β treatment (Fig. 1).

Zp (−221 to +13) has been shown to contain several cis elements that are responsive to viral lytic cycle-inducing agents. The cellular transcription factors Sp1 and Sp3 and also the myocyte enhancer factor 2D (MEF2D) bind to the four ZI domains, which share homology to a protein-binding domain in the promoter region of the BRLF1 gene (36, 37). The binding affinity of Sp1 and Sp3 to the ZI domain is relatively low, which may contribute to the low level basal activity of the BZLF1 promoter (36). The ZII domains (ZIIA and ZIIB) are major Zta auto-activation sites. The ZII domain contains a consensus CREB/AP-1 motif (26) recognized by c-Jun and c-Fos, which plays a more critical role in the induction of Zta expression in...
were also observed in Rael cells. This discrepancy might be due to the different cellular backgrounds between the cell lines examined in this study and those in the other study (33). Results from this study suggest that the effect of TGF-β signaling pathway on Zp was more prominent than that by MAP kinase signaling pathways under the experimental conditions.

Zp was activated by TGF-β or by ectopically expressed Smad3 and Smad4 in the absence of EBV as tested in the EBV-negative Ramos cells. This result also support the notion that Zp activity is directly induced by TGF-β. Furthermore, mutations of the EBV site (Zp-236m1), the AP-1 site (Zp-236mAP1), or both (Zp-236m1mAP1) caused their loss of responsiveness to TGF-β (Fig. 6B). Notably, the fold activation was lower in EBV-negative cells than previously detected in EBV-positive cells. This might be partially due to the autoactivation of Zp by its gene product Zta through the ZIII domains, which is absent in EBV-negative cells.

Like other inducing agents, TGF-β can induce the full cycle of viral lytic replication in at least two BL cell lines (Rael and P3HR-1) harboring the latent EBV. Our data also showed that the interaction of two sets of transcription factors, the c-Jun/c-Fos of the AP-1 complex and Smad proteins, activate a viral promoter in response to TGF-β treatment. Taken together, the present results and the results of others, a model demonstrating control of the EBV reactivation and production by viral and cellular factors in response to TGF-β is shown in Fig. 11. Expression of the EBV latent gene EBNA1 is initiated from a latent promoter Qp in BL cells (48). Upon TGF-β induction, Qp is down-regulated by TGFβ and Smad4 cooperatively via the SBE sites within Qp (27). On the other hand, upon stimulation by TGF-β, Zp is activated by interaction of Smad3/Smad4 with c-Jun and c-Fos, resulting in production of Zta protein (this study). Zta, in turn, down-regulates Qp via the signal transducers and activators of transcription-binding site near the transcription initiation site and induces expression of endogenous TGF-β (12, 49). Thus, the autocrine/paracrine function of TGF-β is generated by up-regulation of Zta, which then activates the transcription of TGF-β, thereby forming a positive feedback loop to initiate the EBV lytic cycle. These cells will eventually undergo a full cycle of viral replication, followed by release of infectious viruses. Rael cells, after 48 h of induction by TGF-β, were able to release infectious EBV particles, which in turn transformed cord blood B cells in vitro (Table I). Thus, the control of promoter activities of EBV key genes by a cytokine such as TGF-β may raise the potential for a therapeutic attack on EBV-associated B cell lymphoma.

In summary, in addition to EBNA1 gene promoter Qp, the EBV BZLF1 gene promoter Zp was also a direct target for the TGF-β signaling pathway. This regulation was mediated through a cooperation of Smad proteins and the c-Jun/c-Fos of the AP-1 complex. The biological and pathological significance of TGF-β stimulation in EBV-positive BL cells is still under investigation.

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| Primary B cells | The concentrated culture medium (ng/ml) | TPA + n-butyrate |
|----------------|----------------------------------------|-----------------|
| A              | 0 0.25 0.5 1 2 4                       | +               |
| B              | − − 0.25 0.5 1 2 4                     | +              |
| C              | − 0.25 − 0.5 1 2 4                     | ND* |

* A, B, and C represent the primary B cells isolated from cord blood samples of three individuals.
** The concentrated culture medium is prepared as described under “Experimental Procedures.”
** +, lymphoblastic cell line (LCL) was established after infection of the primary B cells obtained from the cord blood samples with the concentrated culture medium of TGF-β-treated Rael cells. –, no LCL was established.
* ND, not done.

response to TPA, anti-surface immunoglobulin antibodies, and Ca2+ ionophores (38–40). We presented data to show further that the AP-1 site of the ZII domain is also important for TGF-β-induced Zp activation. TGF-β-mediated transcription activation may be involved in the recruitment of other transcription activators to the Smads, i.e. c-Jun and c-Fos, which can function independently from Smads and receive inputs from other signaling pathways. In this context, the Smad proteins may not initiate unique transcription programs; however, they may function as co-modulators to regulate pre-existing gene expression patterns. CREB-binding protein and its closely related p300 can act as co-activators to Smad2 or Smad3 through a physical interaction with TGF-β-activated Smads (41–44). The cooperation between Smad3/Smad4 and c-Jun/c-Fos can mediate TGF-β-induced transcription through the TGF-β-induced association of c-Jun and Smad3 as well as physical interaction of Smad3 and c-Fos (see Refs. 25, 35, and this study). Therefore, it is likely that Zp is activated through an induced complex consisting of Smads/c-Jun/c-Fos/CBP/p300. CBP and p300 have histone acetyltransferase activity (45, 46). The acetylation state of core histones plays a critical role in transcription, suggesting that the recruitment of histone acetyltransferase activity by promoter-bound factors is a general feature of transcription activation. In this study, Smad proteins and c-Jun/c-Fos cooperatively activated Zp, indicating the potential involvement of histone acetyltransferase activity. The SBE site and AP-1 motif may interact in the presence of TGF-β. Therefore, both sites may function as response elements to the TGF-β treatment.

In addition to SBE site (~233 to ~229), two other SBE-like sequences appeared also to physically associate with Smad4. In EBV-positive cells, Zp-181m2 (mutation of ~166 to ~163 region) showed lower activity but remained responsive to TGF-β treatment. However, in EBV-negative Ramos cells, the mutants no longer responded to TGF-β treatment. These results suggested that, besides TGF-β, other signaling pathways in EBV-positive cells might activate Zp with m2 mutation.

A recent report (33) showed that the BZLF1 gene might be activated by an indirect mechanism that requires a MAP kinase signaling pathway for activation. However, our results suggested a different mechanism for the activation of Zp. We found that the MAP kinase pathway inhibitor PD98059 did not, in fact, repress slightly activated Zp at a concentration as low as 10 μM (Fig. 5A). Zp contains the ZII domain and the AP-1 motif, which can be regulated by the MAP kinase signaling (47). However, this does not seem to be the case for Zp regulation by TGF-β because Zp was activated in the presence of the dominant-negative mediators of MAP kinase signaling pathways as follows: dominant-negative Erk1 (KR) and Erk2 (KR) mutants (Fig. 5B), dominant-negative DN-IKK mutant (Fig. 5C), and dominant-negative DN-p38 (Fig. 5D). Similar results
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