Involvement of Jun Dimerization Protein 2 (JDP2) in the Maintenance of Epstein-Barr Virus Latency

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Reactivation of the Epstein-Barr virus from latency is dependent on expression of the BZLF1 viral immediate-early protein. The BZLF1 promoter (Zp) normally exhibits only low basal activity but is activated in response to chemical inducers such as TPA and calcium ionophore. We here found that Jun dimerization protein 2 (JDP2) plays a significant role in suppressing Zp activity. Reporter, EMSA and ChIP assays of a Zp mutant virus revealed JDP2 association with Zp at the ZII cis-element, a binding site for CREB/ATF/AP-1. Suppression of Zp activity by JDP2 correlated with HDAC3 association and reduced levels of histone acetylation. Although introduction of point mutations into the ZII element of the viral genome did not increase the level of BZLF1 production, silencing of endogenous JDP2 gene expression by RNA interference increased the levels of viral early gene products and viral DNA replication. These results indicate that JDP2 plays a role as a repressor of Zp and that its replacement by CREB/ATF/AP-1 at ZII is crucial role to triggering reactivation from latency to lytic replication.

The Epstein-Barr virus (EBV) is a human gamma-herpesvirus that predominantly establishes latent infection in B lymphocytes. Only a small percentage of infected cells switch from the latent stage into the lytic cycle to produce progeny viruses. Although the mechanism of EBV reactivation in vivo is not fully understood, it is known to be elicited by treatment of latently-infected B cells with chemical or biological reagents, such as 12-O-tetradecanoylphorbol-13-Acetate (TPA), calcium ionophore, sodium butyrate or anti-immunoglobulin, at least in cultured cells. Stimulation of the EBV lytic cascade by any of these leads to expression of two immediate-early genes, BZLF1 and BRLF1. The BZLF1 protein is a transcriptional activator that shares structural similarities to basic leucine zipper (b-Zip) family transcriptional factors and acts as an oriLyt binding protein essential for lytic viral DNA replication. BZLF1 expression alone can trigger the entire reactivation cascade (1-3).

Expression of the BZLF1 gene is tightly controlled at the transcriptional level. The BZLF1 promoter (Zp) normally exhibits low basal activity and is activated in response to TPA or the other reagents described above. The minimal sequence of Zp necessary for activation by the inducers is 233 bp in length (4). The region harbors at least three types of cis regulatory elements, referred to as ZI, ZII and ZIII. Four copies of the ZI element (ZIA-D) are distributed within the minimal Zp. The myocyte
enhancer factor 2D (MEF2D) binds to ZIA, ZIB and ZID (5), while Sp1 or Sp3 can bind to ZIA, ZIC and ZID (6). A single ZII element is located near TATA, sharing homology with binding sites for the cyclic AMP-response element-binding protein (CREB), activating transcription factor (ATF) and activator protein-1 (AP-1) family transcriptional factor such as Jun-B and Jun-D (7,8). Two copies of the ZIII element (ZIII-A, -B) bind to the BZLF1 protein. Previous studies have demonstrated that both ZI and ZII elements are necessary for the initial activation of the promoter by TPA/ionophore or anti-surface immunoglobulin IgG (2). Then, the expressed BZLF1 protein further activates Zp by binding to ZIIIA and B (9). BZLF1 also activates transcription of other viral immediate-early or early genes and enhances the lytic infection cycle of the virus.

The Jun dimerization protein 2 (JDP2) was initially identified as a binding partner of the AP-1 transcription factor, c-Jun (10). It appears ubiquitously expressed and is involved in a variety of biological phenomena, such as cell differentiation (11-14), apoptosis (15,16) and tumorigenesis (17-22). It can dimerize, through its b-Zip motif, with itself or other b-Zip proteins, such as c-Jun, JunB, JunD or ATF-2 (10,11,23), and function as a general repressor of, at least, AP-1, CRE and TRE-dependent transcription (10,23). It has been reported that JDP2 recruits histone deacetylase 3 (HDAC3) to the promoters of target genes and inhibits histone acetyltransferase activity, thereby suppressing transcriptional activity (14,24). Depending on the context and cell type, however, it can alternatively act as a transcriptional activator (25,26).

In the present study, we obtained evidence that JDP2 suppresses Zp mainly through interaction with the ZII cis-element. The lytic life cycle of the virus was found to be significantly enhanced by silencing of endogenous JDP2 expression. We also found JDP2 supported recruitment of HDAC3 to Zp. These results indicate that JDP2 plays critical roles in regulation of the latent-lytic switch in EBV infection.

**Experimental Procedures**

**Cell Culture and antibodies-** HEK293T, EBV-BAC 293 cells were maintained in Dulbecco’s modified Eagle medium (Gibco BRL) supplemented with 10% fetal bovine serum. HEK293 cells with BZLF1 knockout (BZLF1KO) EBV were prepared previously (27). B95-8 cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum. TPA and A23187 were added to induce lytic replication of EBV. Anti-human IgG (Dako Cytomation, A0423) was used for viral lytic induction in Akata cells, which were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum. Anti-JDP2 rabbit antiserum was a gift from Dr. A. Aronheim (The Rappaport Family Institute for Research in the Medical Sciences, Technion-Israel Institute of Technology, Israel). Anti-GAPDH, -HDAC3 and acetylated histone H3K9 antibodies were from Ambion, Abcam and Active Motif respectively. Both mouse and rabbit anti-Flag antibodies were from Sigma. Rabbit anti-BZLF1, -BMRF1, -BALF2 and -BALF5 antibodies were as reported previously (28). Horseradish peroxidase-linked goat antibodies to mouse or rabbit IgG were from Amersham Biosciences.

**Plasmid Construction-** The expression vector for BZLF1 (pcDNABZLF1), b-Zip deletion form of BZLF1 (pcDNAdBZLF1), the reporter plasmid pZp-luc and its derivatives, pZpmZII-luc, pZpmZIII-luc and pZpmZII+III-luc, were constructed previously (29). An expression vector for Flag-tagged BZLF1 (pcDNAFlagBZLF1) was prepared by inserting the BZLF1 cDNA sequence into the EcoRI/XbaI site of pcDNAFlag (29). Flag-tagged expression vectors for
CREB (30) and c-Jun (31) were as reported previously. For pcDNAFlagXBP1(s), the cDNA sequence for XBP1(s) (32) was amplified using the following primers; 5’-CATGGGACTACAAGGACGACGATGACAG ATGGTGTGTTGGCACGGCC-3’ and 5’-CTTAGACACTAATCACTGGG-3’. Underlined nucleotides indicate the Flag epitope. The amplified DNA was phosphorylated by polynucleotide kinase and then inserted into the EcoRV site of the pcDNA3 vector. The pCMV-RL reporter plasmid was obtained commercially (Stratagene). pCMV-FlagJDP2 was made by inserting human JDP2 cDNA into the NotI site of pCMV_S-FLAG (RIKEN, RDB 5956). A deletion mutant at the b-Zip domain of JDP2 was generated by PCR using the following primers; 5’-CGCCCCACCTGCATCGTCC-3’ and 5’-TCGCTCCTCTTCCTCATCTAG-3’.

Transfection, Luciferase Assay and Immunoblotting- Plasmid DNA was transfected into HEK293T or EBV-BAC 293 cells using lipofectamine 2000 reagent (Invitrogen). The total amounts of plasmid DNAs were standardized by addition of an empty vector. Proteins were extracted from cells with the lysis buffer supplied in a Dual-Luciferase Reporter Assay System (Promega) kit and luciferase activities were measured using the kit. Akata cells were electronically transfected using a Microporator (Digital Bio). Protein samples were subjected to SDS-PAGE, followed by immunoblotting with the indicated antibodies as described previously (29).

Electromobility shift assay (EMSA)- Flag-tagged JDP2 proteins were produced using the TNT Quick Coupled Transcription/Translation System (Promega) according to the manufacturer’s instructions. The probe was prepared by 3’-end labeling using Klenow fragment (TOYOBO) and [32P]-dATP (Institute of Isotopes Co., Hungary). Unincorporated deoxynucleotide triphosphates were removed with Chromaspin-10 columns (Clontech). The in vitro translated protein and labeled DNA sequences were incubated in the EMSA binding buffer (20 mM Tris-HCl pH 7.6, 0.5 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 30 mM KCl, 3mM MgCl2, 0.5 mg/ml poly (dl-dC)) at room temperature for 30 min. The samples were then separated in a 4% non-denaturing polyacrylamide gel in 0.5x TBE buffer and the radioactivities were visualized by using BAS2500 system (Fuji Film). The sequences of oligonucleotide probes were as follows; ZII, 5’-CCCCAACCATGACATCACAGAG-3’ and 5’-TCCTCTCTGTGATGACATGTTCGTTTTTG-3’, ZIIIA, 5’-AATATGCACTGACGCGACAGG-3’ and 5’-ATGCGCTGCTGCTGATCATGTGTT-3’, ZIIIB, 5’-CACAGGCATGCTATGTG-3’ and 5’-GAGGTTACATAGCAATGTGCCGTGT-3’.

siRNA, Exogenous Expression and RTPCR- Duplexes of a 21-nucleotide small interfering RNA (siRNA) specific to JDP2 mRNA, including two nucleotides of deoxythymidine at the 3’ end, were synthesized and annealed (Gene Design, Inc). The sense and antisense sequences of the duplex were 5’-GUGAGCUAGAUGAGGAAGAdTdT-3’ and 5’-UCUUCCUAUCUAGCUCACdTdT-3’ (si-JDP2.1) and 5’-GCACAUACUCACCGAAUGUdTdT-3’ and 5’-ACAUUCGGUGAUGAGCAdTdT-3’ (si-JDP2.2), respectively. The control siRNA sequences were 5’-GCAGAGCUGGUUUAGUGAAdTdT-3’ and 5’-UUCACUAACCCAGCUUGCAdTdT-3’. Akata or B95-8 cells (1x10^5) were transfected with 50 pmol of the duplex RNA or with 300 ng of expression plasmid per well of 24-well plate, using a Microporator (Digital Bio). 24 or 48 h after transfection, TPA plus A23187, or anti-IgG was added to induce lytic replication and incubated for another 12 or 24 h. Cells were then harvested for quantification by Real-Time RT-PCR.
using One Step SYBR PrimeScript RT-PCR Kit II (TaKaRa). PCR was performed in 10 µl of solution containing 0.2 µM of primers, 0.2 µl ROX Dye, and the sample RNA in 1xOne Step SYBR RT-PCR buffer. Intensity of ROX Dye was used to compensate the volume fluctuations among the tubes. PCR included 5 min at 42 °C, 10 sec at 95 °C, and 40 cycles at 95 °C for 5 sec followed by 40 sec at 60 °C. Immediately after the RT-PCR reaction, we carried out dissociation curve analysis and confirmed the specificity of each PCR product. An arbitrary RNA was set to 1.0 and a standard curve was constructed using serial dilutions of RNA from the RNA set to 1.0. The amount of mRNA was quantitated based on the standard curve. Real-Time PCR with RNApolII primers was also performed to serve as an internal control for input RNA. Primers used for the Real-Time RT-PCR were as follows: for JDP2 mRNA, 5'- C T G T G G A G G A G C T G A A A T A C-3' and 5'-ATCTAGCTCACTTTTCACGG-3', for BZLF1 mRNA, 5'-AACAGCCAGAATCGCTGGAG-3' and 5'-GGCACATCTGCTTCAACAGG-3', for RNApolII 5'-GCACCACGTCCAATGACAT-3' and 5'-GTGCGGCTGCTTCCATAA-3'.

Viral DNA levels were assayed by dot-blot hybridization as described previously (28,33).

**Genetic manipulation of EBV-BAC DNA and cloning of HEK293 cells with EBV-BAC** - EBV-BAC DNA was provided by W. Hammerschmidt (34). Homologous recombination was carried out in *E. coli* as described previously (28).

To prepare an mZII mutant of EBV-BAC, a transfer DNA fragment for the first recombination was generated by PCR using PpsL-neo (Gene Bridges) as the template, with the following primers; 5'-AGCCACAGGCATTGCTAATGTACCTCATAGA CACACCTAAATTAGCAGCTCCCAAACCAGG CCTGGGTGATGATGGCGGGATC-3' and 5'-GAGTTACCTGTCTAACATCTCCCTCTTAAAGGC CAAGGCACCAGCCTCCTCTGTGATGTCATCA GAAGAACTCGTCAAGAAGG-3'. After the recombination, kanamycin-resistant colonies were selected and checked to make intermediate DNA. TheNeoSt+ cassette in the intermediate DNA was then replaced using the next transfer vector DNA, containing a mutation in the ZII cis-element of Zp. The transfer vector was made by PCR using pZpmZII-luc as the template with the following primers; 5'-ACCAGCTTATTTTAGACACTTC-3' and 5'-GTTTGGGTCCATCTTCCAG-3'. Streptomycin-resistant colonies were cloned and checked to make the EBV-BAC mZII mutant.

Likewise, a revertant of the mZII mutant (EBV-BAC mZII/R) was created by re-insertion of the NeoSt+ cassette and its rescue using a wild-type BZLF1 promoter sequence.

Electroporation of *E. Coli* was performed using Gene Pulser III (Bio-Rad) and purification of EBV-BAC DNA was achieved with NucleoBond Bac100 (Macherey-Nagel).

Recombination was confirmed with PCR products of the Zp region, electrophoresis of the BamHI-digested viral genome, and sequencing analysis.

EBV-BAC DNA was transfected into HEK293 cells using lipofectamine 2000 reagent (Invitrogen), which were then cultured on 10 cm dishes with 100-150 µg/ml of hygromycin B for 10-15 days for cloning of GFP-positive cell colonies as described previously (28). Briefly, for each recombinant virus, we picked up more than 10 hygromycin-resistant, GFP-positive cell colonies to obtain at least 3 typical clones exhibiting minimal spontaneous expression of viral lytic proteins and significant induction of these upon BZLF1 transfection.

**Chromatin IP (ChIP) assays**- ChIP assays were performed essentially as described (Upstate...
Biotechnology, Inc.) with formaldehyde cross-linked chromatin from 1x10^6 cells for each reaction. Cells were lysed, and chromatin was sonicated to obtain DNA fragments with an average length of 300 bp. Following centrifugation, the chromatin was diluted 10-fold with ChIP dilution buffer, and precleared with protein A agarose beads containing salmon sperm DNA (Upstate). Anti-Flag antibody or normal rabbit IgG was added to the sample and incubation proceeded overnight with rotation. Immune complexes were then collected by addition of protein A agarose beads and DNA was purified using a QIAquick PCR Purification Kit (Qiagen) after uncoupling of the cross-linking and proteinase K digestion. The recovered DNA was amplified by PCR using primers specific for Zp: 5'-TAGCCTCGAGGCCATGCATATTTCAACTGG-3', 5'-GCCAAGCTTCAAGGTGCAATGTTTAGTGAG-3', and for the EBNA1 open reading frame: 5'-GTCATCATCATCCGGGTCTC-3' and 5'-TTCGGGTTGGAACCTCCTTG-3'. The PCR products were then analyzed by agarose gel electrophoresis and visualized with ethidium bromide. The samples were also subjected to Real-Time PCR for quantification of DNA sequences using the same primers and SYBR Premix Ex Taq II (TaKaRa). Real-Time PCR was performed in 10 µl of solution containing 0.2 µM of primers, 0.2 µl ROX Dye, and the sample DNA in 1xOne Step SYBR RT-PCR buffer. Intensity of ROX Dye was used to compensate the volume fluctuations among the tubes. PCR included 10 sec at 95 °C and 40 cycles at 95 °C for 5 sec followed by 45 sec at 60 °C. Immediately after the PCR reaction, we carried out dissociation curve analysis and confirmed the specificity of each PCR product. An arbitrary DNA was set to 1.0 and a standard curve was constructed using serial dilutions of DNA from the DNA set to 1.0. The amount of DNA was quantitated based on the standard curve.

Immunoprecipitation Assay- For immunoprecipitation to detect the association between JDP2 and HDAC3, cells were lysed in NP-40 buffer (10 mM Tris-HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA, 0.2% NP-40, and protease inhibitor mixture). After sonication and centrifugation, lysates were precleared with protein G-Sepharose, then mixed with anti-Flag antibody and protein G-Sepharose for 4 h. The resin was washed 5 times with the same buffer and then samples were subjected to SDS-PAGE followed by immunoblotting with anti-JDP2 or Flag antibody.

Data analysis and statistics- Data are presented as mean±SD. Statistical analysis has been carried out using Student’s t-test and values are considered significantly different when p<0.05.

RESULTS

JDP2 represses transcription from EBV Zp. Since JDP2 represses transcriptional activation of reporter constructs containing CRE or TPA-responsive elements (23), we hypothesized that JDP2 might regulate the BZLF1 transcription and thereby affect EBV reactivation. To investigate this possibility, we first carried out reporter gene assays. Co-transfection of the JDP2 expression vector clearly repressed reporter gene expression from pZp-luc, in the presence or absence of BZLF1 (data not shown). In this reporter assay, however, we co-transfected pCMV-RL, a renilla luciferase reporter driven by CMV IE promoter at the same time, and this reporter strongly responded negatively, regardless of BZLF1 (data not shown). This result pointed to a possibility that ectopic overexpression of JDP2 suppresses the CMV immediate-early promoter, as well as BZLF1 promoter and that this system is not suitable for analyses of specific effects of JDP2 on the Zp.
Therefore, we here adopted siRNA technology, using synthetic oligonucleotides that form duplex RNA encoding partial nucleotides from JDP2. As shown in Fig. 1A, cotransfection with BZLF1 expression vector (Z) caused increased Zp activity, due to its auto-activation ability. Treatment with an siRNA (si-JDP2.1) significantly elevated the transcriptional activity from wild-type BZLF1 promoter (pZp-luc(wt)), either in the presence or absence of BZLF1 (Fig. 1A). Another siRNA (si-JDP2.2) also resulted in increased Zp activity (Fig. 1A). Interestingly, silencing of endogenous JDP2 did not affect the CMV promoter as monitored by reporter assay (data not shown). On the other hand, when ZII motif in the Zp was mutated (pZpmZII-luc), significant response to JDP2 knockdown was not observed (Fig. 1A). Knockdown of JDP2 protein was checked by immunoblotting (Fig. 1B). Real-Time PCR analysis indicated that si-JDP2.1 and si-JDP2.2 reduced the JDP2 mRNA levels down to 23 and 43% of the control (data not shown). We also examined that levels of BZLF1 protein were relatively constant (Fig. 1B). In order to quantify the levels of JDP2 knockdown, protein samples transfected with si-JDP2.1 or si-JDP2.2 were compared with those from control cells after serial dilution (Fig. 1C). Either of the siRNAs reduced the JDP2 protein levels to approximately 30% or less in HEK293T cells. These results suggest that JDP2 inhibit Zp through the ZII cis-element, which is known to be bound by cellular transcription factors such as CREB/ATF/AP-1.

The site-directed mutation of the ZIII element, which prevents BZLF1 binding, failed to respond to ectopic expression of BZLF1 protein (Fig. 1D) as expected, but its promoter activity was increased by si-JDP2 treatment (Fig. 1D, pZpmZIII-luc). Although p values indicate that JDP2 knockdown did not always cause statistically significant augmentation of wild-type or mZIII promoter, these results imply that JDP2 decreases Zp activity and that the suppressive effect of JDP2 is mediated through ZII motif, but not through the ZIII of BZLF1 promoter.

**JDP2 binds to ZII motif in vitro.** Reporter assays suggested JDP2 association with the ZII element, but not with the ZIII elements. In order to confirm the binding of JDP2 to the ZII cis-element, EMISA was carried out in Fig. 2. Addition of Flag-tagged JDP2 produced a specific band of JDP2-ZII complex (Fig. 2 lane 2). Excess amounts of unlabeled ZII competitor clearly reduced the intensities of the shifted band (lane 3), whereas the cold ZIII A or ZIII B did not (lane 4 or 5, respectively). When JDP2 lacking b-Zip domain (FlagdJDP2, lane 8) was used instead of wild-type FlagJDP2 (lane 7), no obvious shift was detected (Fig. 2, right part). Supershift analysis with anti-Flag antibody demonstrated that the band actually contained Flag-tagged JDP2 protein (lane 9). Therefore, JDP2 binds to ZII, but not to ZIIIA or ZIIIB cis-element of Zp.

**Association of JDP2 with the BZLF1 promoter in vivo.** To further verify the reporter assays and EMISA results, we performed ChIP analysis using cells containing wild-type or BZLF1-knockout (BZLF1KO) EBV-BAC (27). We here used BZLF1KO mutant strain, in order to exclude the possibility that BZLF1 is involved in the association between JDP2 and the BZLF1 promoter. The BZLF1 promoter region was obviously co-precipitated with Flag-tagged JDP2 (Fig. S1A), either in wild-type or BZLF1KO. A primer set for the EBNA1 coding region was included as a negative control to prove that the signal for the Zp was specific. This experiment of BZLF1KO was repeated and quantified in Fig. 3. With wild-type JDP2, the Zp was significantly concentrated when compared to the level with dJDP2
Thus, the association proved to be intact, indicating that BZLF1 is not needed for JDP2 binding to the BZLF1 promoter sequence.

Since the above documented results indicated JDP2 acts to suppress the BZLF1 promoter by binding to the ZII cis-element, we generated recombinant EBV with a point mutation in the ZII element (Fig. 4A). To this end, we first replaced the ZII element with a marker cassette (NeoSt+), and then exchanged this with the mutated ZII sequence, to prepare EBV-BAC mZII. The mutated ZII sequence of the EBV was again swapped with a NeoSt+ cassette, followed by replacement with the wild-type sequence, to generate a revertant strain, EBV-BAC mZII/R. Sequencing analysis confirmed that the EBV-BAC mZII DNA had the same mutation as the pZpmZII-luc vector (Fig. 1), and that the EBV-BAC mZII/R had the same sequence as the wild-type virus, as intended. Integrity of the BAC DNA was checked by BamHI digestion followed by electrophoresis to confirm that the recombinant viruses did not carry obvious deletions or insertions (Fig. 4B). The BamHI-Z fragment was too short to be clearly detected.

Recombinant EBV-BAC DNA was introduced into HEK293 cells, followed by hygromycin selection, to establish cell lines in which multiple copies were maintained as an episome. More than 10 cell colonies from each recombinant virus were obtained and viral protein expression levels were examined. Fig. 4C shows immunoblotting data for typical cells. When the BZLF1 expression vector was transfected, the cells all responded and expressed viral genes, as expected. In the absence of BZLF1, however, levels of the viral genes in EBV-BAC mZII cells were noticeably lower than those in wild-type or revertant cells. Thus, introduction of point mutations into the ZII element of the viral genome did not increase BZLF1 production. We speculate that the suppressive JDP2 does bind to the ZII element in the latent phase but that its negative effect is counteracted by powerful stimulatory effects of other transcriptional factors, such as CREB/ATF/AP-1 family proteins. We here first demonstrated the significance of the ZII element in the context of the viral genome.

Although effects of JDP2 on BZLF1 promoter activation were not observed in recombinant viruses, the ZII-mutant and its revertant viruses could still be used for binding assays. Fig. S1B shows ChIP results. JDP2 was recruited to the BZLF1 promoter in cells with the wild-type and revertant viruses, but this was impaired in cells with the mZII virus. Additionally, JDP2 with deletion in the b-Zip domain of the protein failed to target the BZLF1 promoter (Fig. S1B rightmost panels). Taken together, the results indicate that JDP2 is recruited to the BZLF1 promoter and binds to the ZII element through its b-Zip domain.

Since JDP2 and CREB, ATF, and AP-1 all bind to the ZII cis-element of BZLF1, we examined whether JDP2 could competitively block the binding of these b-Zip proteins (Fig. S2). HEK293 cells harboring wild-type EBV-BAC were transfected with siRNA against JDP2 or its control siRNA, together with or without the expression vector for Flag-tagged -BZLF1, -CREB and -c-Jun. We here used the viral b-Zip protein BZLF1 as a control, which associates with the ZIII element of the BZLF1 promoter (9). As expected, Flag-BZLF1 bound to Zp, and cotransfection of the JDP2 siRNA did not alter the binding efficiency to the promoter (Fig. S2 left panels). On the other hand, binding was lower with control siRNA, and si-JDP2 treatment significantly increased the association of Flag-tagged -CREB and -c-Jun with the promoter, compared with the si-control results (Fig. S2 left panels). Since a spliced form of X-box binding protein 1 (XBP1(s)) also reportedly targets the ZII element of Zp (35), we also
tested for association. We could observe XBP1(s) binding to the Zp to be increased by si-JDP2 treatment, although this was not obvious in control-si experiments.

The competitive nature of JDP2 with CREB was reconfirmed and quantified in Fig. 5. When JDP2 was knocked down, Flag-tagged CREB association with the Zp was significantly higher than that with the control siRNA (Fig. 5A). Immunoblotting data indicated the expression level of Flag-CREB was not so affected by si-JDP2 (Fig. 5C). These data indicate that JDP2 inhibits transcriptional activity of Zp by competitively occupying the ZII binding site.

Role of JDP2 in EBV reactivation from latency.
In order to examine the role of JDP2 in EBV reactivation from latency under physiological conditions, B95-8 cells, latently infected with EBV, were treated with the siRNAs against JDP2. As shown in Fig. 6B, 73 or 49% suppression of the levels of JDP2 mRNA resulted by si-JDP2.1 or si-JDP2.2. Although the knockdown of JDP2 mRNA might not be perfect, it did reduce JDP2 protein levels (Fig. S3). JDP2 knockdown, in the absence of chemical lytic induction, augmented BZLF1 levels 3.6 or 3.4-fold by si-JDP2.1 or si-JDP2.2, respectively (Fig. 6A), and the augmentation by si-JDP2.2, especially, was statistically significant. Treatment of TPA and A23187, a calcium ionophore, caused overall induction of BZLF1 gene, and si-JDP2.1 significantly induced BZLF1 mRNA levels (Fig. 6A). Similar increase was observed by si-JDP2.2, too (Fig. 6A).

We then examined whether silencing of JDP2 influence viral lytic replication another cell line (Fig. S4). Akata cells featuring latent infection were treated with the siRNA against JDP2. The levels of BZLF1 protein and early proteins, such as BMRF1, BALF5, and BALF2, were also enhanced, especially when treatment was combined with exposure to anti-IgG (Fig. S4).

In order to further confirm the role of JDP2, Effect of exogenous overexpression of JDP2 was monitored in B95-8 (Fig. 6C,D) or HEK293 (Fig. 6E,F) cells latently infected with EBV. Electroporation of Flag-tagged JDP2 expression vector (Flag/JDP2) or its deletion mutant (Flag/dJDP2) elicited JDP2 mRNA levels up to approximately 1,000-fold or even more (Fig. 6D,F). Expression of dJDP2 mutant served as a negative control, as it lacks its b-Zip domain, and thus be not functional. In B95-8 cells, BZLF1 mRNA levels were reduced significantly to about 50% by JDP2 overexpression with TPA and ionophore (Fig. 6C). Importantly, BZLF1 expression patterns in HEK293 EBV/BAC cells with wild-type or the revertant strain (mZII/R), responded in similar ways (Fig. 6E) to JDP2 overexpression, while the BZLF1 mRNA levels were not significantly affected by the overexpression in cells with mZII mutant virus (Fig. 6E). These experiments strongly suggest that JDP2 suppresses BZLF1 expression through ZII motif in the promoter upon reactivation from latency.

JDP2 mediates HDAC3 recruitment to the BZLF1 promoter. It has been reported that JDP2 recruits HDAC3 to the promoter of target genes and inhibits histone acetyltransferase activity, thereby suppressing transcriptional activity (14,24). As exhibited previously, exogenously expressed Flag-tagged JDP2 was able to associate with Zp (Fig. 7A, left part), whereas its b-Zip deletion mutant lost the ability (Fig. 7A, right part). Association of JDP2 with the BZLF1 promoter correlated with increased HDAC3 recruitment to the promoter (Fig. 7A). This HDAC3 recruitment by JDP2 correlated to reduced levels of histone H3 acetylation (Fig. 7B). To extend these results, endogenous JDP2 was knocked down by siRNA (Fig. 7C). Recruitment of endogenous HDAC3 to Zp was weak although appreciable in the si-control case, whereas treatment
with si-JDP2 decreased the HDAC3 interaction with Zp (Fig. 7C). The reduction in HDAC3 recruitment by si-JDP2 caused increment in histone H3 acetylation levels (Fig. 7D). Furthermore, JDP2 association with HDAC3 was confirmed by immunoprecipitation (Fig. 7E). These results suggest that HDAC3 recruitment to Zp, which coincides with lower levels of acetylated histone H3, is efficiently mediated by JDP2 protein.

DISCUSSION

In this report, we document evidence that JDP2 is able to suppress transcription from Zp, thereby inhibiting the entire life cycle in lytic infection. Our results indicate that JDP2 binds to the ZII cis-element of BZLF1 promoter, which has been reported to interact with b-Zip type transcriptional factors, such as CREB, ATF and AP-1. Despite the fact that JDP2 represses Zp via the ZII cis-element, introduction of a point mutation into the latter did not activate BZLF1 expression. Instead, the mutation even reduced the basal expression of BZLF1 (Fig. 4C). We speculate that effects of the potential activators such as CREB, ATF and AP-1 were more conspicuous than that of the negative regulator, JDP2 in the ZII element. Many reports have indicated a significance of the ZII domain for BZLF1 expression. For example, BZLF1 expression is highly leaky in AGS cells infected with EBV, at least partly due to abundant c-Jun and its binding to the ZII element (36). We are confident that JDP2 acts negatively on EBV reactivation, because knockdown of the gene substantially enhanced expression of viral immediate early, early genes and viral DNA replication (Fig. 6,S4).

In Fig. 6, huge amount (1,000-fold or even more of endogenous level of JDP2) of forced expression of JDP2 caused only 50% reduction in BZLF1 mRNA levels. One might argue that this reduction must have been more extensive. We speculate that the ZII motif of the promoter might already be occupied by endogenous b-Zip transcription factors, including JDP2, and thus exogenously supplied JDP2 could not replace effectively. Otherwise, JDP2 might be just one of several proteins that serve to restrict the BZLF1 expression, and the restriction by JDP2 could easily be achieved by the presence of relatively small amount of the protein.

Since JDP2 and viral BZLF1 are both b-Zip type transcriptional factors, we tested if they could interact with each other. Immunoprecipitation assays clearly demonstrated that JDP2 and BZLF1 associated through their b-Zip elements (data not shown), and we thus examined if the factors could act cooperatively on the BZLF1 promoter (Fig. 1). However, we observed no evidence of such cooperation, and the ZIII cis-element, the binding motif for BZLF1, did not appear to be involved in the suppression by JDP2 (Fig. 1).

Besides JDP2, several factors have been reported to suppress the BZLF1 promoter. SMUBP-2, a transcription factor that was first identified through its interaction with the immunoglobulin Smu region, represses Zp activation by TPA (37). ZEB proteins restrict BZLF1 expression by binding to the ZV element of the promoter (38-40). E-box binding proteins, E2-2 (41), and Yin Yang1 (YY-1) (42) have also been implicated. Suppression by JDP2 is distinct from that of these factors, since it relies on binding to th ZII element. Identification and detailed analysis of negative regulators of BZLF1 transcription should help to understand EBV reactivation mechanisms and provide a whole picture of the virus life cycle.

We found that the CMV promoter responded to JDP2 knockdown differently from the BZLF1 promoter, although both were similarly suppressed by JDP2 overexpression (data not shown). We speculate that the affinity between the repressor and those promoters might vary. Aronheim’s group recently demonstrated that
JDP2 activates transcription from TRE-dependent promoters through association with CHOP10 (26), suggesting that JDP2 can be a transcriptional activator, as well, depending on the context. One possible hypothesis is that the CMV promoter might be regulated both positively and negatively by JDP2, and JDP2 knockdown act in both ways, while exogenous overexpression causes emphatic effects on the negative side. The reasons remain to be determined, but it is clear that the JDP2 siRNA served, in this report, as the best tool to elucidate its biological roles under physiological conditions.

Knockdown of JDP2 by siRNA in EBV-positive Akata cells increased the levels of early gene expression and viral replication, when the viral lytic program was elicited by anti-IgG treatment (Fig. S4). Likewise, viral replication was augmented by such silencing in other cell lines, such as B95-8 and GTC-4 cells (data not shown), when treated with TPA and calcium ionophore. Interestingly, in EBV-positive HEK293 cells, knockdown of JDP2 still intensified viral lytic replication even when BZLF1 protein was exogenously supplied (data not shown). This result suggests that JDP2 can act at multiple steps in the lytic life cycle of the virus, and suppression at the BZLF1 promoter is not the only inhibitory point.

In conclusion, we here have provided evidence that JDP2 is involved in regulation of the latent-lytic switch in EBV infection. Elucidation of associated factors may contribute to development of anti-EBV agents in the future.
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**FOOTNOTES**

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**The abbreviations used are:** EBV, Epstein-Barr virus; Zp, BZLF1 promoter; JDP2, Jun dimerization protein 2; CREB, cyclic AMP-responsive element-binding protein; AP-1, activator protein-1; ATF, activating transcription factor; XBP1(s), spliced form of X-box binding protein 1; TPA, 12-O-tetradecanoylphorbol-13-Acetate; b-Zip, basic leucine zipper, CMV; cytomegalovirus; HDAC, histone deacetylase; TRE, TPA responsive element.
FIGURE LEGENDS

Fig. 1. Knockdown of endogenous JDP2 enhances transcription from Zp. (A-C) HEK293T cells were transfected with duplexes of a 21-nucleotide siRNA against JDP2 (si-JDP2.1 or si-JDP2.2) or a control (si-control) siRNA, together with 10 ng pZp-luc or its derivative, pZpmZII-luc, and 1 ng of pCMV-RL with 10 ng of empty vector (C) or pcDNA-BZLF1 (Z). Luciferase assays (A) were carried out 36 h after the transfection, as described in the Experimental Procedures. Each bar illustrates the mean and SD of three independent transfections. The luciferase activities are shown as fold-activation of that for pZp-luc(wt), with the si-control and empty vector (leftmost bar). (B) Protein levels of BZLF1, JDP2 and GAPDH in the assay were examined by immunoblotting. (C) JDP2 levels in samples transfected with si-JDP2.1 or si-JDP2.2 in the assay were compared with 100, 30 or 10% of the control. (D) HEK293T cells were transfected with siRNA against JDP2 (si-JDP2.1) or a control (si-control) siRNA, together with 10 ng pZp-luc or its derivatives and 1 ng of pCMV-RL with 10 ng of empty vector (C), pcDNA-BZLF1 (Z) or pcDNA-dBZLF1 (dZ). pcDNA-dBZLF1 (dZ) expresses BZLF1 protein lacking b-Zip domain. Luciferase assays were carried out 36 h after the transfection, as described in the Experimental Procedures. (*) or ( **) indicates p<0.05 or p<0.02, respectively.

Fig. 2. JDP2 binds to ZII motif in the BZLF1 promoter in vitro. EMSA was carried out as described in the Experimental Procedures. Flag-tagged wild-type JDP2 protein (Flag-JDP2) or JDP2 protein lacking b-Zip domain (Flag-dJDP2, lane 8) was produced in vitro and incubated with 32P-labeled probe and excessive amounts of cold competitors (ZII (lane 3), ZIIIA (lane 4) and ZIIIB (lane 5)). Supershift analysis was performed using mouse anti-Flag monoclonal antibody (lane 9). The samples were then separated in a 4% polyacrylamide gel and analyzed with Fuji Image Analyzer BAS2500.

Fig. 3. Association of JDP2 with the BZLF1 promoter. HEK293 cells with BZLF1-knockout (BZLF1KO) EBV-BAC DNA were transfected with the Flag-JDP2 expression vector (FlagJDP2), or the Flag-JDP2 expression vector with a deletion in the b-Zip domain (Flag-dJDP2). After 24 h, ChIP experiments were carried out using crosslinked DNA-protein complexes from the cells, precipitated using normal IgG or anti-Flag antibody, followed by DNA extraction and PCR reactions to detect the Zp (A). A fragment for the EBNA1 open reading frame was also detected as a negative control.
Fig. 4. Construction of recombinant EBV featuring point mutation in the ZII cis-element of the BZLF1 promoter. (A) Schematic arrangement of the recombination of the EBV genome using the tandemly arranged neomycin-resistance and streptomycin-sensitivity genes (NeoSt+). The ZII element of the B95-8 genome was first replaced with the NeoSt+ cassette which was then replaced with a point mutated ZII sequence (asterisk) to construct EBV-BAC mZII. The mutated ZII sequence was replaced again with the NeoSt+ marker cassette and swapped with the wild-type ZII sequence to prepare the revertant clone, EBV-BAC mZII/R. (B) Electrophoresis of the recombinant viruses. The recombinant EBV genomes were digested with BamHI and separated in an agarose gel. (C) Expression of viral proteins from the recombinant viruses. The recombinant EBV-BAC DNAs were introduced into HEK293 cells, followed by hygromycin selection. Resultant cell clones were tested for protein viral expression. The indicated clones of cells were transfected with the BZLF1 expression vector (BZLF1) or its empty control vector (control vector). After 48 h, cell proteins were harvested, and immunoblotting was performed using anti-BZLF1, -BALF2, -BMRF1 and -GAPDH antibodies.

Fig. 5. Competitive binding of JDP2 with other b-Zip transcription factors. HEK293 cells with wild-type EBV-BAC DNA were transfected with the siRNA against JDP2 (si-JDP2.1) or the control (si-control), together with Flag-tagged expression vectors for CREB. After 36 h, ChIP experiments were carried out using crosslinked DNA-protein complexes from the cells, precipitated using normal IgG or anti-Flag antibody, followed by DNA extraction and PCR reactions to detect the Zp (A). A fragment for the EBNA1 open reading frame was also detected as a negative control (B). Levels of Flag-tagged CREB protein were examined by immunoblotting (C). (*) indicates \( p<0.05 \).

Fig. 6. JDP2 inhibits BZLF1 expression. (A,B) Knockdown of JDP2 enhances BZLF1 expression. B95-8 cells transfected with siRNA against JDP2 (si-JDP2.1 or si-JDP2.2) or a control (si-control) siRNA were cultured for 48 h, and then treated with TPA (20 ng/ml) and A23187 (1 mM) for an additional 12 h. Levels of BZLF1 (A) and JDP2 (B) were checked by Real-Time RT-PCR. (C,D) Exogenous overexpression of JDP2 decreases BZLF1 expression. B95-8 cells transfected with
pCMV-FlagJDP2, pCMV-FlagdJDP2, which lacks b-Zip motif of JDP2, or the empty vector were cultured for 24 h, and then treated with TPA (20 ng/ml) and A23187 (1 mM) for an additional 12 h. Levels of BZLF1 (C) and JDP2 (D) were checked by Real-Time RTPCR. (E,F) Exogenous overexpression of JDP2 decreases BZLF1 expression in HEK293 cells with recombinant EBV/BAC. Cells were transfected with pCMV-FlagJDP2, pCMV-FlagdJDP2, which lacks b-Zip motif of JDP2, or the empty vector. After 36 h, levels of BZLF1 (E) and JDP2 (F) mRNAs were quantified by Real-Time RTPCR. BZLF1 and JDP2 mRNA levels are normalized by RNApolIII mRNA levels and shown as fold-activation of that for control (leftmost bar). (*) or (**) indicates $p<0.05$ or $p<0.02$, respectively.

Fig. 7. JDP2 mediates HDAC3 recruitment to the BZLF1 promoter. (A, B) HEK293 cells with wild-type EBV-BAC DNA were transfected with the Flag-JDP2 expression vector (FlagJDP2) or the expression vector with a deletion in the b-Zip domain (FlagdJDP2). After 24 h, ChIP experiments were carried out using crosslinked DNA-protein complexes from the cells, precipitated using normal IgG, anti-HDAC3, anti-Flag anti-histoneH3, or anti-acetylated histoneH3K9 antibody, followed by DNA extraction and PCR reactions to detect the Zp. A fragment for the EBNA1 open reading frame was also detected as a control. (C,D) HEK293 cells with wild-type EBV-BAC DNA were transfected with the siRNA against JDP2 (si-JDP2.1) or the control (si-control). After 48 h, ChIP experiments were carried out using normal IgG, anti-HDAC3 anti-histoneH3, or anti-acetylated histoneH3K9 antibody, followed by DNA extraction and PCR reactions to detect the Zp and a fragment for the EBNA1 open reading frame as a control. The samples in A-C were also subjected to Real-Time PCR analysis for quantification, and the relative values were indicated below the bands. (E) JDP2 associates with HDAC3. Proteins from HEK293T cells transfected with Flag-tagged JDP2 expression vector were subjected to immunoprecipitation using normal IgG or mouse anti-Flag monoclonal antibody followed by immunoblotting with rabbit anti-HDAC3 (upper panel) and rabbit anti-Flag antibodies (lower panel).
Fig 2

Cold competitor  →  ZII  →  ZIIA  →  ZIIB  →  Ab  →  —  →  anti-Flag

Protein  →  FlagJD2  →  FlagJD2  →  FlagJD2  →  FlagJD2

JDP2-ZII complex  →

Free probe  →

lanes 1 2 3 4 5 6 7 8 9

Probe:ZII
Fig3

A

\[ Z_p \]

\[
\begin{array}{c}
\text{Flag UDP2} \\
\text{Flag UDP2} \\
\text{Flag UDP2} \\
\text{Flag UDP2} \\
\text{Flag UDP2} \\
\text{Flag UDP2} \\
\text{Flag UDP2} \\
\text{Flag UDP2} \\
\end{array}
\]

\[
\begin{array}{c}
\text{0} \\
\text{0.5} \\
\text{1} \\
\text{1.5} \\
\end{array}
\]

% input

B

\[ EBNA1 ORF \]

\[
\begin{array}{c}
\text{Flag UDP2} \\
\text{Flag UDP2} \\
\text{Flag UDP2} \\
\text{Flag UDP2} \\
\text{Flag UDP2} \\
\text{Flag UDP2} \\
\text{Flag UDP2} \\
\text{Flag UDP2} \\
\end{array}
\]

\[
\begin{array}{c}
\text{0} \\
\text{0.5} \\
\text{1} \\
\text{1.5} \\
\end{array}
\]

% input

1/100 input normal anti-Flag IgG ppt ppt
Fig 5

A

Zp

% input

B

EBNA1ORF

% input

si-control  si-JDP2.1  si-control  si-JDP2.1  si-control  si-JDP2.1

1/100 input  normal IgG ppt  anti-Flag ppt

FlagCREB

C

IB: Flag  IB: FlagCREB  IB: GAPDH

Downloaded from http://www.jbc.org/ by guest on March 24, 2020
Fig7

A

|         | FlagJD2 | FlagdJD2 |
|---------|---------|----------|
| Zp      | 1/100 input | normal IgG | anti-Flag | anti-HDAC3 | anti-Flag |
|         | 1.00     | 0.11      | 0.02      | 0.02      | 0.02      |

EBNA1ORF

|         | 1/100 input | normal IgG | anti-Flag | anti-HDAC3 | anti-Flag |
|---------| 1.00       | 0.08      | 0.08      | 0.08      | 0.08      |


B

|         | FlagJD2 | FlagdJD2 |
|---------|---------|----------|
| Zp      | 1/100 input | normal IgG | anti-Flag | anti-H3K9Ac | anti-Flag |
|         | 1.00     | 4.11      | 0.49      | 0.02      | 0.02      |

EBNA1ORF

|         | 1/100 input | normal IgG | anti-Flag | anti-H3K9Ac | anti-Flag |
|---------| 1.00       | 0.00      | 0.00      | 0.00      | 0.00      |


C

|         | si-control | si-JDP2 |
|---------|------------|---------|
| Zp      | 1/100 input | normal IgG | anti-Flag | normal IgG | anti-Flag |
|         | 1.00 | 0.15 | 0.02 | 0.07 |

EBNA1ORF

|         | 1/100 input | normal IgG | anti-Flag | normal IgG | anti-Flag |
|---------| 1.00 | 0.01 | 0.58 | 0.07 |


D

|         | si-control | si-JDP2 |
|---------|------------|---------|
| Zp      | 1/100 input | normal IgG | anti-H3K9Ac | normal IgG | anti-H3K9Ac |
|         | 1.00 | 2.69 | 1.38 | 3.61 |

EBNA1ORF

|         | 1/100 input | normal IgG | anti-H3K9Ac | normal IgG | anti-H3K9Ac |
|---------| 1.00 | 0.01 | 0.38 | 0.01 |


E

|         | 1/20 input | IP: normal IgG | IP: anti-Flag |
|---------|------------|----------------|---------------|
| IB: HDAC3 |

IB: Flag
Involvement of Jun dimerization protein 2 (JDP2) in the maintenance of Epstein-Barr virus latency
Takayuki Murata, Chieko Noda, Shinichi Saito, Daisuke Kawashima, Atsuko Sugimoto, Hiroki Isomura, Teru Kanda, Kazunari K. Yokoyama and Tatsuya Tsurumi

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