TORC2, a Coactivator of cAMP-response Element-binding Protein, Promotes Epstein-Barr Virus Reactivation from Latency through Interaction with Viral BZLF1 Protein*5

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Reactivation of the Epstein-Barr virus from latency is dependent on expression of the viral BZLF1 protein. The BZLF1 promoter (Zp) normally exhibits only low basal activity but is activated in response to chemical inducers such as 12-O-tetradecanoylphorbol-13-acetate and calcium ionophore. We found here that Transducer of Regulated cAMP-response Element-binding Protein (CREB) (TORC2) enhances Zp activity 10-fold and more than 100-fold with co-expression of the BZLF1 protein. Mutational analysis of Zp revealed that the activation by TORC2 is dependent on ZII and ZIII cis elements, binding sites for CREB family transcriptional factors and the BZLF1 protein, respectively. Immunoprecipitation, chromatin immunoprecipitation, and reporter assay using Gal4-luc and Gal4BD-BZLF1 fusion protein indicate that TORC2 interacts with BZLF1, and that the complex is efficiently recruited onto Zp. These observations clearly indicate that TORC2 activates the promoter through interaction with the BZLF1 protein as well as CREB family transcriptional factors. Induction of the lytic replication resulted in the translocation of TORC2 from cytoplasm to viral replication compartments in nuclei, and furthermore, activation of Zp by TORC2 was augmented by calcium-regulated phosphatase, calcineurin. Silencing of endogenous TORC2 gene expression by RNA interference decreased the levels of the BZLF1 protein as well as CREB family transcriptional factors, binding sites for CREB family transcriptional factors, and 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 usually and is activated in response to TPA or the other reagents described above. The minimal sequence of Zp necessary for the 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 binds to ZIA, ZIB, and ZID (5), whereas 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) or the AP-1 family transcriptional factor (7, 8). Two copies of the ZIII element (ZIIIA, B) are bound by 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 IgG(2). Then, the expressed BZLF1 protein joins to further activate Zp by binding to the ZIIIA and B elements (9).

Epstein-Barr virus (EBV)3 is a human γ-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 and 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 some chemical or biological reagents, such as 12-O-tetradecanoylphorbol-13-acetate (TPA), calcium ionophore, sodium butyrate, or immunoglobulin (Ig). Stimulation of the EBV lytic cascade by any of those reagents leads to the expression of two presumed viral immediate-early genes, BZLF1 and BRLF1. The BZLF1 protein is a transcriptional activator that shares structural similarities to the basic leucine zipper (b-Zip) family transcriptional factors, and BZLF1 expression alone can trigger the entire reactivation cascade (1–3).

The abbreviations used are: EBV, Epstein-Barr virus; Zp, BZLF1 promoter; CREB, cyclic AMP-response element (CRE)-binding protein; TORC2, Transducer of Regulated CREB; TPA, 12-O-tetradecanoylphorbol-13-acetate; b-Zip, basic leucine zipper; CBP, CREB-binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IP, immunoprecipitation; IB, immunoblotting; ChiP, chromatin immunoprecipitation; siRNA, small interfering RNA; RT, reverse transcription; CMV, cytomegalovirus.

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TORC2 Promotes EBV Reactivation

Transducer of Regulated CREB (TORC) 1, 2, and 3 were identified from a lymphocyte cDNA library as a family of CREB co-activators that bind to CREB and enhance CRE-mediated transcription in an Ser-133 phosphorylation-independent manner (10, 11). It was reported recently that TORCs are activated by the calcium-regulated phosphatase, calcineurin (12, 13). Dephosphorylation of TORC by the phosphatase triggers release from 14-3-3 proteins and translocation from cytoplasm to nucleus. Interestingly, the activation of EBV Zp is blocked by calcineurin inhibitors, such as cyclosporin A or FK506 (14). Based on these studies, we hypothesized that TORCs might be involved in the transcriptional activation of Zp, leading to a switch from latent state to the lytic replication.

In the present study we show that TORC1, -2, and -3 can all enhance Zp, especially with co-expression of BZLF1. TORCs activate the promoter through interaction not only with CREB but also the BZLF1 protein. We also provide evidence that the activation of the promoter by TORC2 is up-regulated by calcium-regulated phosphatase, calcineurin. These results indicate involvement of TORCs in EBV reactivation from latency.

EXPERIMENTAL PROCEDURES

Cell Culture and Antibodies—HEK293T, EBV-Bac-293, and GTC-4 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum. EBV-293 cells were prepared by transfection with EBV-Bac DNA (15) into HEK293 cells subcloned in our laboratory (16) followed by hygromycin selection. GTC-4 is a cell line established from an EBV-positive gastric cancer by Dr. M. Tajima (Teikyo University) (17). Akata, B95-8, and Tet-BZLF1/Akata cells were lysed in RPMI1640 as described previously (18, 19). To induce lytic EBV replication in Tet-BZLF1/Akata cells, doxycycline, was added to the culture medium at a final concentration of 2 μg/ml. The mouse anti-FLAG, hemaggulitin, -BZLF1, and -GAPDH antibodies were from Sigma, Roche Applied Science, Dako A/S, and Amboin, respectively. Rabbit anti-PCNA and -TORC2 antibodies were from Oncogene and Calbiochem, respectively, and rabbit anti-BMRF1 and -BALF5 antibodies have been reported previously (20). The anti-tubulin antibody was purchased from Cell Signaling. Horseradish peroxidase-linked goat antibodies to mouse or rabbit IgG were from Amersham Biosciences. Horseradish peroxidase (HPR)-linked goat antibody to rat IgG was obtained from Jackson ImmunoResearch, and TrueBlot HRP anti-mouse and rabbit IgG were from eBioscience.

Plasmid Construction—The pZp-luc reporter plasmid was constructed by inserting the minimal sequence of Zp (from -221 to +12) prepared by PCR into Xhol and HindIII sites of pGL4.10 (Promega). Primer sequences for the PCR were 5’-TAGGCTCCTGAGCCATGACTATTTCAAAGTAGG-3’ (forward), 5’-GCCAAGCTTCAAGGTGAATTTGATGAGG-3’ (reverse). Point mutations in the minimal Zp were introduced by PCR using following primers: mZII, 5’-TACGACGAGG-GTGTGGCC-3’ (forward), 5’-TGAATTCTGGTGGGAC-AGTGC-3’ (reverse); mZIII, 5’-GCACCGCTAATGCTACTATGAGG-3’ (forward), 5’-CTGTGAGTTCTCTGAGATTT-3’ (reverse). Expression plasmids for TORC3 and CREB1A have been reported elsewhere (21–23). TORC1 and TORC2 genes were amplified and cloned into EcoRI and Xhol sites of pCHA (22, 24) using the following primers: TORC1, 5’-AAAGAATTCATGGCGACTTGAACAACTCCGCGG-3’ (forward), 5’-AACTTGAGTCAAGGCGGTTCCATTGCGAGGT-3’ (reverse), TORC2, 5’-AAAGAATTCATTGTCCAGCTGGCTGGGAGGGAACGGG-3’ (forward), 5’-AACTTGAGTCAATTGGCTGGCGGTTCACTCGGAAA-3’ (reverse). The pCRE-Luc and pRL-TK reporter plasmids were obtained commercially (Stratagene). For the pcDNA3BZLF1 expression plasmid, the BZLF1 gene was cloned into pcDNA3 at BamHI and XhoI sites. The sequence in the b-Zip domain (amino acids 200–227) was deleted to generate pcDNA3BZLF1. To prepare the expression vector for Gal4-BZLF1 fusion protein, the BZLF1 sequence was recloned into EcoRI and XhoI sites of the pM vector (Clontech) after PCR using the primers 5’-CCGAGATCTGATGGAGACCCAAACTCGAC-3’ (forward) and 5’-CTTATATCTAGATGAAAATTAGAGATCAGTCG-3’ (reverse). The pGal4-luc reporter plasmid has been reported previously (21).

Transfection and Luciferase Assay—Plasmid DNA was transfected into HEK293T or EBV-293 cells using Lipofectamine 2000 reagent (Invitrogen). The total amounts of plasmid DNAs were standardized by the addition of an empty vector, pcDNA3. 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. The counts for firefly luciferase were normalized to those for renilla luciferase. GTC-4 and Akata cells were electrophoretically transfected using a MicroPorator (Digital Bio).

Immunoprecipitation (IP) and Immunoblotting (IB)—For IP, cells were lysed in 0.2% Nonidet P-40 buffer (10 mM Tris-HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, and protease and phosphatase inhibitor mixture). After centrifugation, lysates were precleared with protein G-Sepharose (Amersham Biosciences), and then incubated for 1 h. Immunocomplexes were recovered by incubating with G-Sepharose for 1 h, and the resin was washed 5 times with the same buffer. Samples were subjected to SDS-PAGE followed by IB with the indicated antibodies as described previously (24).

Chromatin IP (ChIP) Assay—ChIP assays were performed essentially as described (Upstate Biotechnology, Inc.) with formaldehyde cross-linked chromatin from 1 × 10⁶ cells for each reaction. Cells were lysed, and chromatin was sonicated to obtain DNA fragments with an average length of 300 bp. After 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 IgG or normal rabbit IgG were added to the sample and incubated overnight with rotation. The immune complexes were collected by the addition of the 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’-TAGGCTCCTGAGCCATGACTATTTCAAAGTAGG-3’ and 5’-GCCAAGCTTCAAGGTGAATTTGATGAGG-3’, and for the EBNA-1 open reading frame, 5’-GTCACATCATCATCCGGGTCTC-3’ and 5’-TTCGAGGTGGAAACCTCCTTG-3’. The PCR products were then analyzed...
by agarose gel electrophoresis and visualized with ethidium bromide staining.

**Immunofluorescence Assay**—For HEK293 cells, cells were fixed with 1% formaldehyde and permeabilized with 0.1% Nonidet P-40 in phosphate-buffered saline. The cells were washed and blocked in 1% bovine serum albumin in phosphate-buffered saline and then incubated with anti-FLAG antibody. Samples were then incubated with the secondary goat anti-mouse IgG antibody conjugated with Alexa Fluor 488. For Tet-BZLF1/B95-8, staining was carried out as described (25). Briefly, cells were washed with phosphate-buffered saline and lysed in ice-cold 0.5% Triton X-100-mCSK buffer (10 mM PIPES (pH 6.8), 100 mM NaCl, 300 mM sucrose, 1 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.5% Triton X-100, and protease inhibitors) and fixed with 70% ethanol. The cells were blocked and then incubated overnight with primary antibodies. The samples were then incubated for 2 h with the secondary goat anti-mouse and rabbit IgG antibodies conjugated with Alexa Fluor 488 and 594, respectively. After immunostaining, cells were then mounted and stained with 4′,6-diamidino-2-phenylindole (DAPI) using ProLong Gold antifade reagent with DAPI (Invitrogen). Image acquisition was performed with a Bio-Rad Radiance 2000 confocal laser-scanning microscope equipped with a PlainApo 100×1.4-numerical-aperture oil immersion objective lens.

**Small Interfering RNA (siRNA) and RT-PCR**—Duplexes of 21-nucleotide (siRNA) specific to TORC2 mRNA, including two nucleotides of deoxythymidine at the 3′ end, were synthesized and annealed (Sigma Genosys). The sense and antisense sequences of the duplex were 5′-CUGCGACUGGCAUCACAAdTdT-3′ and 5′-UUGUGUAUGCACACGAGdTdT-3′, respectively. GTC-4 or Akata cells (1 × 10⁵) were transfected with 50 pmol of the duplex RNA per well of a 24-well plate using a Microporator (Digital Bio). Twenty-four hours after transfection, TPA and A23187 or IgG were added and then incubated for another 24 h. Cells were then harvested for RT-PCR and IB. Primers used for the RT-PCR were as follows: for TORC2 mRNA, 5′-AAAGAATTCTACACAAAGGAGCTCTCATTATG-3′ and 5′-GCTTGTCCTGTTAAGTGCAG-3′; for GAPDH mRNA, 5′-GGGAAGGTGAAGGTCGGAGT-3′ and 5′-AAGACGCCAGTGGAACCTCCAC-3′.

**RESULTS**

**TORC Activates Transcription from EBV Zp**—To confirm that TORC proteins activate CRE-dependent transcription, luciferase assays using pCRE-luc or pRL-TK reporter vectors were performed (Fig. 1A). Expression of TORC1, -2, or -3
TORC2 Promotes EBV Reactivation

enhanced the CRE-mediated transcription by 147-, 79-, or 121-fold, respectively, whereas no obvious transcriptional stimulation from the herpes simplex virus thymidine kinase promoter was observed, demonstrating the specificity of TORCs for CRE-dependent transcription.

To test the effect of TORC proteins on Zp, a pZp-luc reporter plasmid containing the minimal sequence (4) sufficient for transcriptional activation by TPA/ionophore or IgG was used for the assay. It was found that TORC1, -2, and -3 alone enhanced transcription from Zp by 18-, 12-, and 26-fold, respectively (Fig. 1B). Expression of the BZLF1 protein elevated the transcription in the absence of TORC up to 3.9-fold. Co-expression of the BZLF1 protein and TORC1, -2, and -3 further enhanced pZp-luc activity, reaching 23-, 108-, and 58-fold, respectively.

Levels of BZLF1 as well as TORC proteins were checked in Fig. 1C because we used the CMV promoter for BZLF1 expression, and TORC proteins might enhance the CMV promoter activity. Despite the fact that the CMV promoter has a CRE binding motif (26), levels of BZLF1 were comparable (Fig. 1C). A reporter assay also indicated that the CMV promoter was only marginally affected by TORC proteins (only 1.5–2.5-fold increase), at least under this condition, by TORC proteins (data not shown).

Analogous experiments were carried out in a B cell line as well (data not shown). In B cells, a single TORC2 expression caused a 9.9-fold enhancement, and with wild-type BZLF1, the activity reached to 48-fold (data not shown).

Taken together, although TORC alone can enhance transcription from the Zp, the BZLF1 protein is somehow able to further increase the transcription levels synergistically. Interestingly, although the activation of Zp by TORC2 alone was less potent than that by TORC1 or -3, co-expression of the BZLF1 protein dramatically enhanced the TORC2-mediated transcriptional activation. We also tested the effect of the BZLF1 deletion mutant dZ, which lacks the b-Zip domain, on the reporter gene as negative controls. The levels of luciferase activity were almost equal to those without the wild-type BZLF1 protein.

To further analyze the synergistic enforcement of Zp by TORCs, a pZpmZIII-luc plasmid was made, the ZII element being mutated as shown in Fig. 1B. This mutation disrupts the CRE/activation transcription factor motif and abrogates the induction from the promoter by TPA/ionophore or IgG (4). The basal luciferase activity from this reporter plasmid became very low (only 25% of wt pZp-luc), and the activity did not appreciably increase even with TORC proteins (Fig. 1B), showing that the Zp activation by TORCs in the absence of the BZLF1 protein is caused through the ZII domain containing the CRE/activation transcription factor motif. However, this reporter still responded to the BZLF1 expression, reaching the same levels of transcriptional activity as with wt pZp-luc because it still contained ZIII, the BZLF1 protein binding sites.

When TORC1, -2, or -3 were co-expressed with the BZLF1 protein, pZpmZIII-luc exhibited significant enhancement of the transcriptional activity to around 20–50-fold. Because TORCs could not enhance this mutant promoter activity without expression of the BZLF1 protein, this activity might be due to co-operation between TORC and the BZLF1 protein. It is also noteworthy that TORC2, with the BZLF1 protein, was the most efficient, suggesting again that TORC2 is particularly compatible with the BZLF1 protein regarding synergism of the transcription activation of Zp.

To extend this analysis, we constructed pZpmZIII-luc, in which ZIIIA and ZIIIB sites are mutated. This mutation almost completely disrupts the BZLF1 protein binding (27), thereby abolishing the response to the BZLF1 protein. As pZpmZIII-luc still bears the ZII element, the reaction to TORCs was almost comparable with wt pZp-luc in the absence of the BZLF1 protein. However, even in the presence of the BZLF1 and TORC proteins, the activity remained low.

Last, when both the ZII and ZIII elements were mutated, we could not observe any enhancement of the transcription, even with or without TORCs and/or the BZLF1 protein. Overall, these results imply that activation of transcription from Zp by TORCs is mainly mediated by ZII and ZIII elements but not via ZI or other elements.

TORCs Interact with the BZLF1 Protein and Function as Coactivators for the BZLF1-mediated Transcriptional Activation—Because the above results strongly suggest that TORC proteins co-operate with the BZLF1 protein to enhance the Zp promoter activity, we next analyzed protein-protein interactions by co-IP experiments. HEK293T cells were co-transfected with expression plasmids encoding the BZLF1 protein (+Z) and FLAG-tagged TORC1, -2, or -3. Complexes immunoprecipitated with anti-FLAG antibody were resolved by SDS-PAGE and analyzed by IB using anti-BZLF1 antibody. As shown in Fig. 2A, a 36-kDa band corresponding to the expected size of the BZLF1 protein was immunoprecipitated and identified as the BZLF1 protein (Fig. 2A). When we used the mutant BZLF1 protein lacking b-Zip sequence (+dZ), the interaction became less clear (Fig. 2A). Furthermore, because the N-terminal region of TORC proteins possesses a coiled-coil domain, a motif that has been implicated in protein-protein interactions (11), we tested if the region is involved in the interaction with the BZLF1 protein using the N-terminal deletion mutants of the TORC proteins (d1, d2, and d3). We found little or no association of the TORC mutants with the BZLF1 protein in the absence of the coiled-coil domain (Fig. 2A).

To examine whether TORC proteins have effects on BZLF1 protein-dependent transcriptional activity, we prepared an expression plasmid encoding a Gal4 DNA binding domain-BZLF1 fusion protein (Gal4-Z) and pGal4-luc, which contains five Gal4 binding sites and an SV40 minimal promoter. Expression of Gal4-BZLF1 fusion protein alone was able to activate pGal4-luc 3.6-fold (Fig. 2B). Because binding between the polypeptide of Gal4 DNA binding domain and the Gal4 binding sites in the promoter of the reporter construct is highly specific and exclusive, only the Gal4-BZLF1 fusion protein can be recruited onto the promoter, indicating that the increase reflects the net transcriptional activity of the BZLF1 protein but not of any other factors. Co-expression of Gal4-BZLF1 and TORC1, -2, and -3 proteins resulted in an 14-, 8.0-, and 9.9-fold increase, respectively, in the transcriptional activity as compared with Gal4-BZLF1 alone, whereas deletion mutants of the TORC proteins (d1, d2, and d3) failed to increase the levels.
TORC2 Promotes EBV Reactivation

To further analyze the behavior of TORC on the Zp promoter, we checked whether TORC was recruited onto the Zp in vivo by ChIP assays (Fig. 2C). We used TORC2 because it has been studied most extensively and was demonstrated to be the most crucial factor, at least in vivo (28–31) (also see Figs. 1, 4, and 5). EBV-293 cells harboring EBV Bac DNA were transfected with the expression plasmid of FLAG-tagged TORC2 with or without the BZLF1 expression vector, and then ChIP assays were performed using the anti-FLAG antibody and normal IgG as a negative control. With expression of the BZLF1 protein, FLAG-tagged TORC2 was recruited to the Zp of the EBV genome in cells (Fig. 2C, top panel, right). In contrast, TORC2 was hardly detected without BZLF1 on the promoter (Fig. 2C, top panel, left) unless the PCR cycle was increased (Fig. 2C, second panel). A primer set for the EBNA-1 coding region was included (Fig. 2C, third panel) as a negative control to prove that the signal for the Zp was specific. We also confirmed that the BZLF1 protein was also recruited to the Zp when both the BZLF1 protein and FLAG-tagged TORC2 were expressed (Fig. 2C, fourth panel).

In the second panel in Fig. 2C, TORC2 was shown to be recruited onto Zp without BZLF1. Speculating that TORC2 is on the promoter through the interaction with CREB, the association of CREB protein with the promoter was also tested in Fig. 2D. As expected, both CREB and TORC2 came onto Zp even without BZLF1 protein.

To further confirm the importance of the interaction between BZLF1 and TORC2, we also tested dBZLF1, which lacks the b-Zip motif, and dTORC2, which is devoid of coiled-coil motif (Fig. 2E). When wild-type BZLF1 and wild-type TORC2 were present, both were detected bound to Zp (Fig. 2E, right). Deletion of the coiled-coil domain in TORC2 caused significant loss of its binding (Fig. 2E, middle), and truncation of BZLF1 b-Zip also harmed its association with Zp (Fig. 2E, left).

Activation of TORC2 by Dephosphorylation and Nuclear Transport—It has been demonstrated that the phosphorylation state of TORC2 regulates its activity (12, 13). Under normal conditions, TORC2 is sequestered by cytoplasmic 14-3-3 proteins, which recognize phosphorylated proteins. In the pres-

FIGURE 2. TORCs associate with the BZLF1 protein to enhance transcription from Zp. A, TORCs coimmuno-precipitated with the BZLF1 protein. FLAG-tagged TORC1, -2, -3 or FLAG-tagged TORC with deletion at the coiled-coil domain of the protein (d1, d2, d3) expression vectors were co-transfected with wt BZLF1 (+Z) or BZLF1 without the b-Zip (−Z) expression vector. IP was carried out using anti-FLAG antibody and immuno-blotted with anti-BZLF1 antibody (top), then stripped and reprobed with anti-FLAG antibody (bottom). As a control, whole cell extracts (WCE) from the samples were also stained with anti-FLAG antibody (middle). B, BZLF1 protein-dependent transcription is enhanced by TORCs. HEK293T cells were transfected with 25 ng of a Gal4-luciferase reporter plasmid and 25 ng of the plasmid expressing the Gal4 DNA binding domain-BZLF1 fusion protein (+Gal4-Z) together with 100 ng of plasmids expressing TORC1, -2, -3 or the deletion mutant at the coiled-coil domain (d1, d2, d3). Luciferase assays were carried out as described under “Experimental Procedures.” The luciferase activity is shown as -fold activation of that with neither Gal4-Z nor TORC. Each bar represents the mean and S.D. of three independent transfections. Expression levels of FLAG-tagged TORC proteins and BZLF1 were also analyzed. C, ChIP assays were performed to evaluate the association of TORC2 with Zp. EBV-293 cells were transfected with a FLAG-tagged TORC2 expression plasmid with or without the BZLF1 expression plasmid. After fixation and sonication, protein-DNA complexes were immunoprecipitated with anti-FLAG (upper panels) or anti-BZLF1 (lower panels) antibody or normal IgG followed by uncoupling of the cross-linking and PCR reactions using primers for Zp or the coding region of EBNA-1 (ORF; open reading frame; ppt; precipitate). D, ChIP assays were carried out to show that both CREB and TORC2 were recruited to the Zp even without BZLF1. EBV-293 cells transfected with FLAG-tagged CREB and hemagglutinin-tagged TORC2 expression vectors were subjected to ChIP assay with normal IgG, anti-hemagglutinin (HA), or anti-FLAG antibody followed by PCR. E, ChIP assays showing the importance of b-Zip of BZLF1 and coiled-coil domain of TORC2 for their recruitment to the promoter. Wild-type or deletion mutant of BZLF1 and FLAG-tagged TORC2 were transacted in pairs as noted. Precipitations were done using the anti-BZLF1 or -FLAG antibody followed by detection.
cence of calcium signaling, TORC2 is dephosphorylated by calcineurin at phosphoserine 171, triggering disruption of the interaction with 14-3-3 and import into the nucleus where it can activate CRE-mediated transcription. We could confirm that the addition of A23187, a calcium ionophore, enhanced the translocation of FLAG-tagged TORC2 to nuclei as well as an alanine-substituted TORC2 mutant at Ser-171 (S171A) (Fig. 3A).

Using the construct, we examined the effect of the S171A mutation of TORC2 on the Zp of EBV (Fig. 3B). Wild-type TORC2 induced CRE-dependent reporter gene expression 59-fold and its S171A mutant 272-fold when compared with the luciferase activity without TORC. The result was quite similar to a previous report (12) indicating the reliability of this system. When both wt BZLF1 protein (+Z) and wild-type TORC2 were expressed, Zp was activated 150-fold as compared with the activity with neither wt BZLF1 protein nor TORC2. Co-expression of wt BZLF1 protein (+Z) and the S171A TORC2 exhibited 433-fold activation, whereas co-expression of the mutant BZLF1 protein lacking b-Zip sequence (+dZ) and the S171A TORC2 were without effect.

In Fig. 3C, coimmunoprecipitation assay not only confirmed the interaction between endogenous TORC2 and BZLF1 but also revealed that BZLF1 protein preferentially associates with faster-migrating species of TORC2, which are dephosphorylated forms of the protein (Fig. 3D) (12, 31).

In the EBV lytic replication, it was previously demonstrated that the BZLF1 protein localizes to replication compartments, the sites of viral genome replication and transcription, in the nuclei (3, 32). Immunofluorescence analysis showed that upon induction, TORC2 was recruited to the replication compartments and colocalized with BZLF1 protein in the nucleus in the lytic phase, whereas the protein was localized in the cytoplasm in the latent phase (Fig. 3E).

In addition, we confirmed that cyclosporin A, an inhibitor of calcineurin signaling pathway, clearly blocks the BZLF1 expression in B95-8 cells treated with TPA and calcium ionophore (supplemental Fig. S1). These results

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**FIGURE 3. Dephosphorylation of TORC2 at Ser171 promotes nuclear localization and transcriptional activation for Zp.** A, immunocytochemical analysis showing the effects of ionophore (A23187) or the S171A mutation of TORC2 on its subcellular localization. Cells were stained with anti-FLAG antibody (green) and 4′,6-diamidino-2-phenylindole (DAPI; blue). wt, wild type. B, transient reporter assay results for the CRE-mediated promoter (left) and Zp (right). HEK293T cells were transfected with pCRE-luc or pZp-luc together with expression vectors for the wild-type or the S171A mutant of TORC2. For pZp-luc, plasmids with wt BZLF1 (+Z) or a mutant BZLF1 lacking the b-Zip domain (+dZ) were also transfected. Luciferase assays were carried out as described under "Experimental Procedures." The luciferase activity is shown as -fold activation of that without TORC2 for pCRE-luc (left part) and that with neither BZLF1 nor TORC2 for pZp-luc (right). Each bar represents the mean and S.D. of three independent transfections. C, dephosphorylated TORC2 interacts with BZLF1. Protein lysates from B95-8 cells treated with TPA and A23187 for 24 h were subjected to IP using normal IgG or anti-BZLF1 antibody followed by SDS-PAGE and IB with anti-TORC2 (upper panel) and -BZLF1 (lower panel) antibodies. WCE, whole cell extracts. D, phosphorylation of TORC2. Proteins from B95-8 cells treated with TPA and A23187 were lysed in calf intestine alkaline phosphatase buffer and incubated with or without calf intestine alkaline phosphatase for 1 h followed by SDS-PAGE and IB with anti-TORC2 antibody. E, localization of endogenous TORC2 (red) and BZLF1 protein (green) in Tet-BZLF1/B95-8 cells. Cells were mock-treated (middle panels) or treated with doxycycline and A23187 (top and bottom panels) and analyzed by immunofluorescence assay using confocal microscopy. 4′,6-Diamidino-2-phenylindole (blue) staining was also carried out. As a negative control, treatment with first antibodies was omitted for the bottom panels.
suggest a cooperative influence of the BZLF1 protein and dephosphorylated TORC2 in the presence of calcineurin signaling activation.

Role of TORC2 in EBV Reactivation from Latency—To examine the role of TORC2 in EBV reactivation from latency, GTC-4 cells, in which EBV is latently infected, were transfected with the TORC2 expression vector and incubated with or without TPA/A23187 (Fig. 4A). The cells expressed the BZLF1, BMRF1, and BALF5 proteins in response to TPA/A23187 treatment, and further exogenous expression of TORC2 increased the levels of the proteins. A similar result was obtained in Akata cells (Fig. 4B). Expression of S171A mutant of TORC2 appears to impact on BZLF1 levels significantly (data not shown).

We also tested EBV-293 cells, in which levels of exogenous gene expression are very efficient (Fig. 4C). Even in the absence of TPA/A23187, overexpression of TORC2 clearly enhanced BZLF1 protein levels. To examine the function of TORC2 under physiological conditions, we employed siRNA technology using a synthetic oligonucleotide that forms a duplex RNA encoding partial nucleotides from TORC2. As shown in Fig. 5A, treatment with siRNA against TORC2 reduced the level of TORC2 mRNA in HEK293T cells, whereas the level of GAPDH remained unchanged. TORC2 siRNA treatment also resulted in a decrease in the BZLF1-mediated transcription (Fig. 5B; si-TORC2) when compared with control siRNA treatment (+Z, si-Cont).

In addition, the effect of siRNA against TORC2 was also examined in GTC-4 and Akata cells, as shown in Fig. 5, C and D, respectively. Treatment with TORC2 siRNA suppressed the mRNA expression of TORC2, whereas the GAPDH gene was unaffected. The treatment also reduced the levels of viral lytic proteins including BZLF1.

To eliminate the possibility that the siRNA against TORC2 might elicit interferon signaling pathway, we analyzed interferon-β expression by RT-PCR (33) because activation of the signaling pathway provoked by double-stranded RNA causes the promoter activation. Treatment with TORC2 siRNA did not induce the levels of interferon-β (supplemental Fig. S2), indicating that interferon signaling is not activated by si-TORC2.
To deny the possibility that the TORC2 siRNA used in Fig. 5 might act through unknown off-target effects, we tested another TORC2 siRNA in supplemental Fig. S3. It also suppressed the expression level of the BZLF1 protein.

To test if not only TORC2 but also other TORCs might be involved in this process, all the members of TORC proteins were silenced simultaneously. In that case, however, reduction of the BZLF1 level was no stronger than that by si-TORC2 only (Fig. supplemental S4), suggesting the dominant role of TORC2 in this transcriptional activation. These results strongly suggest the importance of TORC2 in BZLF1 production and EBV reactivation from latency.

**DISCUSSION**

In this report we document evidence that TORC is able to enhance transcription from Zp and, more interestingly, that TORC interacts with the BZLF1 protein to activate the promoter very strongly. Fig. 6 shows our working model for Zp activation. Previous studies have demonstrated that both ZI and ZII elements are necessary for the initial activation (2, 34).

It has been reported that myocyte enhancer factor 2D, a transcription factor (MEF2D), is activated by calcineurin, a phosphatase that can be inhibited by CsA or FK506. TORC2 is able to associate with CREB or the BZLF1 protein and enhances Zp activity through binding to ZII and ZIII cis elements. PLC, phospholipase C; PKC, protein kinase C; DAG, diacylglycerol; CaM, calmodulin; BCR, B-cell receptor.

**FIGURE 6. Proposed model for EBV Zp activation.** TORC2, as well as myocyte enhancer factor 2D (MEF2D), is activated by calcineurin, a phosphatase that can be inhibited by CsA or FK506. TORC2 is able to associate with CREB or the BZLF1 protein and enhances Zp activity through binding to ZII and ZIII cis elements. PLC, phospholipase C; PKC, protein kinase C; DAG, diacylglycerol; CaM, calmodulin; BCR, B-cell receptor.

Also potentiates the promoter activity by binding to the ZIII element through the BZLF1 protein. Calcineurin, a serine/threonine-phosphatase sensitive to cyclosporin A is responsible for the dephosphorylation and the activation of TORC. In turn, cyclosporin A and FK506 are very effective for suppressing EBV.

A number of cellular proteins have been reported to interact with the BZLF1 protein, including p53 (35, 36), C/EBPα (37), NF-κB (38), basic transcriptional machinery TFII components (39), and CREB-binding protein (CBP) (40, 41). Among these, CBP has histone acetyltransferase activity and cooperates with the BZLF1 protein to transactivate BZLF1-dependent transcription, inducing the viral lytic cycle. Mutation analysis revealed that at least the homodimerization domain (b-Zip) of the BZLF1 protein is required for its interaction with CBP, but other parts of the protein also must be involved in the association (40, 41). The BZLF1 protein also interacts with TFII components mainly through the transcriptional domain and stabilizes the association of initiation complexes on DNA. Stable assembly of general transcriptional machinery might promote transcription from BZLF1-responsive promoters. Interestingly, TORC enhances the interaction of CREB with the TAF1130 component of TFIIID (11), and at least TORC2 mediates target gene activation by associating with CBP/p300 and increasing its recruitment to CREB-responsive promoters (31). From these studies and our own results, the BZLF1 protein may not only directly recruit CBP/p300 and basic transcriptional machinery but also be able to recruit them through TORC2.

Besides EBV, transcription from human T-cell leukemia virus type 1 long terminal repeats is also affected by TORC proteins (21, 42). TORC activates long terminal repeats through interaction with the viral transcriptional factor Tax as well as CREB. So this mode of the action is quite parallel to the situation with EBV. Because both human T-cell leukemia virus type 1 and EBV are lymphotropic viruses, there is a possibility that other lymphotropic viruses such as the human immunodeficiency virus might also be controlled by TORC proteins.

Curiously, although CMV immediate-early promoter has a CREB binding motif (26), we here observe that transcription from the promoter is less affected by TORC proteins (Fig. 1C) when compared with the BZLF1 promoter, an EBV immediate-early gene. Others also have used expression vectors driven by the CMV immediate-early promoter and shown that the promoter activity is relatively unaffected (22, 42). It is speculated that this might be because the activation by TORC is dependent on the promoter context (11, 43). Because immediate-early genes of herpesviruses are crucial for lytic infection, distinct dependence of the promoters on TORC proteins may reflect differences in the characters of those herpesviruses.

Although TORC proteins could enhance Zp 100-fold in reporter assays, overexpression or ablation of TORC2 had only a relatively small impact on BZLF1 production under physiological conditions. It is likely that transcriptional suppressors of the promoter such as YY1 (44) might inhibit transcription. Another intriguing possibility is that there might be epigenetic regulation such as DNA methylation or histone deacetylation. Interestingly, Gruffat et al. (45) reported that myocyte enhancer factor 2 family protein, a crucial transactivator for the
Zp, recruits class II histone deacetylases to suppress transcription from Zp. They argued that the switch from latency to the productive cycle is dependent at least in part on the post-translational modification of myocyte enhancer factor 2 and local acetylation state of histones around the Zp. Likewise, the transcriptional co-activator TORC2 can associate with BCL-3, which recruits histone deacetylases to inhibit transcription (22). These results and the cited reports suggest that the molecular mechanism regulating EBV reactivation from latency is not quite as simple as expected, and further clarification of the mechanism regulating EBV reactivation is necessary. Elucidation of associating factors and chromosomal environment of the Zp proximity may contribute to the development of anti-EBV compounds.

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TORC2 Promotes EBV Reactivation

MARCH 20, 2009 • VOLUME 284 • NUMBER 12 JOURNAL OF BIOLOGICAL CHEMISTRY 8041