tion. Because there is a very limited number of anti-EBV drugs developed or being developed to date, including acyclic nucleoside analogs, such as acyclovir, and kinase inhibitors, such as maribavir (26, 27), a search for an effective molecular target has been needed. Pin1 may be a potential target for development of novel antiviral drugs.

MATERIALS AND METHODS

Cell culture and reagents. HEK293T and HEK293 EBV-bacterial artificial chromosome (BAC) cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum. B95-8 cells and an EBV-negative cell clone derived from Akata cells [Akata(−)] were cultured in RPMI medium supplemented with 10% fetal bovine serum. To induce lytic EBV replication, tetradecanoyl phorbol acetate (TPA), A23187, and sodium butyrate were added to the culture medium at final concentrations of 20 ng/ml, 1 μM, and 5 mM, respectively.

Antibodies. Rabbit anti-BZLF1, -BMRF1, -BALF2, and -BALF5 antibodies were as reported previously (28). An anti-EBV EA-D-p52/50 (BMRF1 gene product) protein-specific mouse monoclonal antibody, clone R3, was purchased from Chemicon Inc. Anti-Pin1 (H-123 and G-8) and anti-α/β-tubulin (2148) antibodies were purchased from Santa Cruz Biotechnology, Inc., and Cell Signaling, respectively. Horseradish peroxidase (HRP)-linked goat antibodies to rabbit IgG were from Amersham Biosciences.

shRNA and siRNA. Knockdown of Pin1 with short hairpin (shRNA) was carried out as described previously (29). As a control, we targeted the luciferase gene (designated shluc). Oligonucleotide sequences for the Pin1 shRNA (shPin1) were 5′-GATCCGCCGAGTGTACTACTTCAATTCAA GAGATTGAAGTAGTACACTCGGCTTTTTTAT-3′ (shPin1 for) and 5′-CGATAAAAAAGCCGAGTGTACTACTTCAATCTCTTGAATTGA AGTAGTACACTCGGCG-3′ (shPin1 rev), and the sequence for shluc was as noted previously (29). Duplexes of 21-nucleotide small interfering RNAs (siRNAs) were synthesized and annealed (Gene Design, Inc.). The sense and antisense sequences of the duplex were 5′-GCCAUUUGAAGA CGCCUCGdTdT-3′ and 5′-CGAGGCGUCUUCAAAUGGCdTdT-3′ for Pin1 and 5′-GCAGAGCUGGUUUAGUGAAdTdT-3′ and 5′-UUCA CUAAACCAGCUCUGCdTdT-3′ for the control siRNA.

Measurement of the viral genome by qRT-PCR. Cells were harvested at the time indicated in the figure legends and lysed with 200 μl of PCR lysis buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.001% Triton X-100, and 0.001% SDS). After treatment with 25 μg of proteinase K at 50°C for 2 h, samples were boiled at 95°C for 10 min. Quantitative real-time PCR (qRT-PCR) was performed in 10 μl of solution containing 1 μM each forward and reverse primer, 5 μl of FastStart Universal Probe Master (Rox) (containing 6-carboxy-X-rhodamine [ROX] dye; Roche Applied Science), 0.5 μl of eukaryotic 18S rRNA endogenous control (Applied Biosystems), and 1 μl of prepared sample DNA in PCR lysis buffer. The intensity of ROX dye was used to compensate for volume fluctuations among the tubes. PCR included 2 min at 50°C and 10 min at 95°C and then 40 cycles at 95°C for 15 s, followed by 1 min at 60°C. Immediately after the PCR, we carried out dissociation curve analysis and confirmed the specificity of each PCR product. A standard curve was constructed using serial dilutions of DNA and was used to quantify the amount of DNA. Primers and a probe for detection of the viral genome were designed using Primer Express (Applied Biosystems) within the BALF2-coding region. The sequences were as follows: 5′-GCCCGTCCG GTTGTCA-3′ (forward primer), 5′-AATATCTGGTTGTTGCCGTTG A-3′ (reverse primer), and 5′-FAM-CTGCCAGTGACCATCAACAAGT ACACGG-TAMRA-3′ (probe; where FAM is 6-carboxyfluorescein and TAMRA is tetramethyl rhodamine).

GST pulldown assays. For bacterial expression of glutathione *S*-transferase (GST)-tagged Pin1 (wild type [WT] or the W34A mutant), *Escherichia coli* strain DH5α was transformed with the pGEX expression vector for each protein (10). Expression of GST fusion proteins was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG; 0.5 mM), followed by incubation at 25°C for 4 h. GST pulldown assays were conducted as described previously (10). In brief, B95-8 or HEK293 cell proteins were lysed in GST lysis buffer at 4°C. After sonication and centrifugation (at 20,000 × *g* for 10 min at 4°C), the supernatant was preincubated with glutathione-Sepharose beads (GE Healthcare) for 30 min at 4°C. Afterwards, the supernatant was mixed with 50 μg of GST fusion protein and glutathione beads for 1 h at 4°C with rotation. The beads were then washed with GST lysis buffer five times and subjected to immunoblotting.

Transfection and IP. Cells were transfected with appropriate plasmids using Lipofectamine 2000 reagent (Invitrogen) or by electroporation using a Microporator (Disital Bio). The total amounts of plasmid DNAs were standardized by addition of an empty vector. For immunoprecipitation (IP), cells were solubilized in 200 μl of 0.5% Nonidet P-40 buffer (10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 0.5% Nonidet P-40, and protease and phosphatase inhibitor mixture). Cell extracts were then diluted with 800 μl of lysis buffer and precleared with protein G-Sepharose (GE Healthcare). Supernatants were then mixed with protein G-Sepharose and antibody and then incubated at 4°C for 4 h with rotation. Immunocomplexes were washed five times with the same buffer. Samples were subjected to SDS-PAGE, followed by immunoblotting with the antibodies indicated in the figures and figure legends. We used TrueBlot anti-rabbit IgG HRP-conjugated antibodies (eBioscience) as the secondary antibody to eliminate the immunoglobulin heavy chain/light chain-specific band.

BALF5 expression plasmid and mutagenesis. The expression vector for BALF5 was made by inserting the BALF5 open reading frame into the EcoRI/HindIII site of pcDNA3.1 (Invitrogen). Mutant vectors were generated by a PCR-based method using the following primers: TTCCATGT CTACGACATACTC (BALF5Δ1 For), ACACTTGGGAATGAGACGC (BALF5Δ1 Rev), AAGGTCACGCGCCGTTCCATT (BALF5Δ2 For), GA GGACTGCAAACTCCACGTC (BALF5Δ2 Rev), AGAAGAGCACAGGC TAGCC (BALF5Δ3 For), TTGTAGAATCCGGACAGGGG (BALF5Δ3 Rev), CTTCGAGTCATCTACGGGGAC (BALF5Δ4 For), GATGGAGAG GCAGGGAAAG (BALF5Δ4 Rev), GCCCCCTGCCGGGTCTCGG (BALF5-T178A For), and CCTGCGGTCGAAGGTGCTGG (BALF5-T178A Rev). The DNA sequence of each vector was confirmed by DNA sequencing using the following primers: GCATCGTCATCAAGCTACTG (BALF5-1), AGCTCGAGTACGACTGTGAG (BALF5-2), CACATCTAC AGCATCAACCC (BALF5-3), GATCCGCGTGTTCTCCTGC (BALF5-4), CCTTCTTGGCTAGTCTGTTG (BALF5-5), and TCCTGCCTGATG CTGATTAC (BALF5-6).

Genetic manipulation of EBV-BAC DNA. EBV-BAC DNA was provided by W. Hammerschmidt (30). Homologous recombination was undertaken in *E. coli* as described previously (28, 29, 31) with the following oligonucleotide primers: 5′-TGTGTGAACGTGTTTGGGCAGCAGGCC TACTTCTACGCCAGCGCGCCTCAGGGTCTGGACGGCCTGGTG ATGATGGCGGGATC-3′ (Neo/stFor), 5′-CGCGTGGCATCCACGTTG GCCTCAAAGATCCGACACCCGTGCTTGTCTTGCCACGTGTCAGA AGAACTCGTCAAGAAGG-3′ (Neo/stRev), 5′-AAGCCCTCTGGACTT CCATG-3′ (Transfer vector For), and 5′-CATTGTCCAGGACAAAGCG G-3′ (Transfer vector Rev). Electroporation was performed using a Gene Pulser III (Bio-Rad), and purification of EBV-BAC DNA was achieved with NucleoBond Bac100 (Macherey-Nagel, Germany).

RESULTS

Productive EBV DNA replication is strongly suppressed by knockdown of Pin1. To determine whether Pin1 might influence productive EBV replication, we first carried out knockdown experiments. In HEK293 EBV-BAC cells featuring EBV latent infection, Pin1 expression was suppressed by shRNA (shPin1) transduction (Fig. 1A). An shRNA against the luciferase gene served as the control. Cells were transfected with empty vector pcDNA3 or pBZLF1, an expression vector for BZLF1, the molecular switch from latent to lytic infection of EBV. After 24 h, levels of viral and cellular proteins were examined by immunoblotting (Fig. 1A). In the control HEK293 EBV-BAC shluc cells, BZLF1 transfection

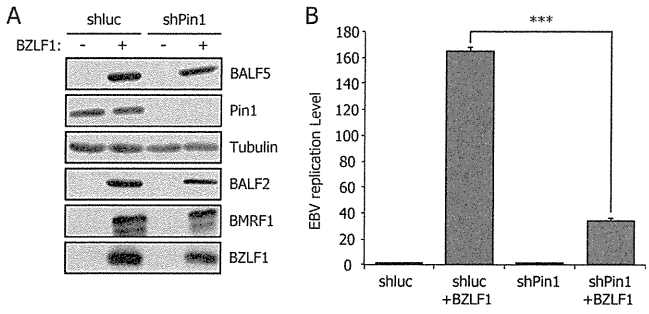

FIG 1 Knockdown of Pin1 decreases the level of EBV viral replication. (A) HEK293 EBV-BAC cells, transduced with control shRNA (shluc) or shRNA for Pin1 (shPin1), were transfected with 50 ng of BZLF1 expression vector or empty vector (pcDNA3). After 24 h, aliquots of cells were harvested and subjected to immunoblotting with the indicated antibodies. (B) Remaining cells transfected in panel A were subjected to qRT-PCR assays 60 h after transfection. The amount of EBV viral DNA was quantified and standardized with an 18S ribosome probe. Each bar represents the mean and standard deviation of three independent transfections and quantifications. ***, $P < 0.002$.

induced expression of viral genes such as BALF5, BALF2, and BMRF1, as expected, and knockdown of Pin1 (shPin1) had little effect on the expression levels.

We next checked the levels of viral DNA by qRT-PCR (Fig. 1B). The amount of synthesized EBV DNA was drastically and significantly reduced in Pin1-depleted cells upon induction compared to that in control cells, while levels of intrinsic, latent EBV genome copy numbers were comparable (Fig. 1B). Similar results were also obtained in lymphocytes (data not shown).

Pin1 interacts with EBV DNA polymerase BALF5. Since Pin1 was shown to contribute to EBV lytic replication (Fig. 1B), we next examined if certain EBV proteins could interact with Pin1 by GST pulldown assay. Whole-cell lysate from B95-8 cells treated with TPA-A23187-butyrate was incubated with purified GST-Pin1, GST-Pin1 W34A or GST alone expressed in bacteria. The W34A mutant of Pin1 served as a negative control because it cannot bind to the Ser/Thr-Pro motif of the substrates due to the mutation in its WW domain. We found that the viral DNA polymerase catalytic subunit, BALF5, was specifically and repeatedly coprecipitated with GST-Pin1 but not with GST alone or GST-Pin1 W34A

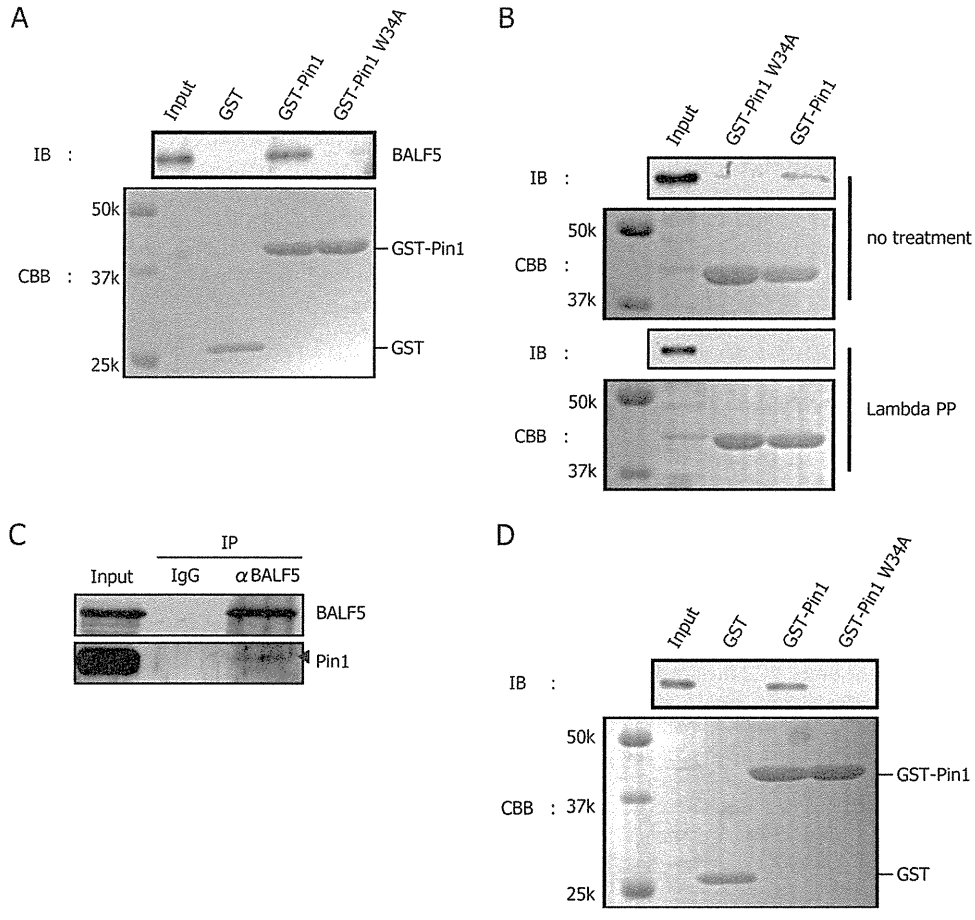

FIG 2 Pin1 interacts with EBV DNA polymerase BALF5. (A) GST-Pin1 binds to BALF5. Proteins from B95-8 cells, induced with TPA, A23187, and sodium butyrate for 24 h, were harvested and lysed in GST lysis buffer. GST pulldown assays were carried out using GST, GST-Pin1, or GST-Pin1 W34A. Pin1 W34A cannot bind with target proteins because of the mutation in its WW domain. (B) Phosphorylation-dependent association of Pin1. A GST pulldown assay was carried out as described for panel A except that the B95-8 cell lysate was incubated with lambda protein phosphatase (PP) (New England BioLabs) for 30 min at 30°C as indicated on the figure. (C) Immunoprecipitation assays confirmed the interaction. Cell proteins from lytic B95-8 lysate were subjected to immunoprecipitation using anti-BALF5 antibodies or normal IgG. The precipitates were then immunoblotted using anti-BALF5 or -Pin1 antibodies. α, anti. Arrowhead indicates size of Pin1. (D) Exogenously overexpressed BALF5 can bind to Pin1. Cell proteins from HEK293T cells transfected with BALF5 expression vector were subjected to GST pulldown assay. IB, immunoblotting; CBB, Coomassie brilliant blue. k, kilodaltons.

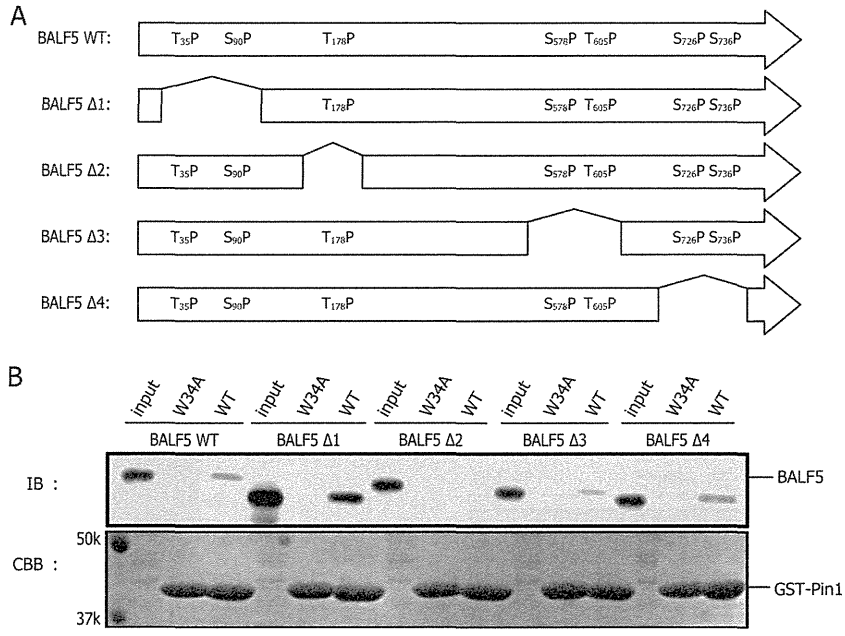

FIG 3 Pin1 interacts with BALF5 Thr178. (A) Scheme of BALF5 truncated mutants, featuring deletion of amino acids 34 to 93 (BALF5Δ1), 167 to 185 (BALF5Δ2), 578 to 607 (BALF5Δ3), and 704 to 748 (BALF5Δ4). Primers used for constructing these mutants are listed in Materials and Methods. (B) Cell proteins lysed from HEK293T cells, transfected with the WT BALF5 expression vector or its derivatives shown in panel A, were subjected to GST pulldown assay.

(Fig. 2A). Other than BALF5, viral factors, such as BZLF1, BMRF1, BBLF4, BBLF2/3, and BSLF1, were not copurified with Pin1 in our GST pulldown assays (data not shown).

To test if the interaction was phosphorylation dependent, an aliquot of the lysate was treated with lambda phosphatase while the rest was left untreated (Fig. 2B). Dephosphorylation by the phosphatase diminished the interaction (Fig. 2B), suggesting that the two proteins associate in a phosphorylation-dependent manner (Fig. 2B).

Immunoprecipitation assays were next conducted in order to confirm the association between endogenous Pin1 and BALF5 in B95-8 cells. Pin1 was coimmunoprecipitated with BALF5, as expected (Fig. 2C).

For the experiments shown in Fig. 2A to C, we collected viral proteins from EBV-positive B95-8 cells after induction of lytic replication and demonstrated that Pin1 interacts with BALF5 in a phosphorylation-dependent manner. Since EBV encodes a protein kinase, BGLF4, we then examined whether phosphorylation of BALF5 by the viral kinase BGLF4 was necessary for the Pin1-BALF5 association. To this end, EBV-negative HEK293T cells were transfected with a expression vector plasmid for BALF5, and at 24 h posttransfection, GST pulldown assays were carried out using the lysate (Fig. 2D). Because the interaction between BALF5 and Pin1 was reproduced in an overexpression system, the viral kinase is apparently not a prerequisite for the interaction, and cellular kinases, such as mitogen-activated protein kinases (MAPKs) or cyclin-dependent kinases (CDKs), may be sufficient to mediate the association (17).

Identification of the Ser/Thr-Pro motif in BALF5 required for association with Pin1. Since phospho-Ser/Thr-Pro is the binding motif for Pin1, we searched for its presence in BALF5 protein. As shown in Fig. 3A (top panel), BALF5 has seven Ser/Thr-Pro motifs. In order to map the domain in BALF5 important for the interaction, we generated a series of BALF5 truncation mutants (Fig. 3A). There are four Ser-Pro (Ser90, Ser578, Ser726, and Ser736) and three Thr-Pro (Thr35, Thr178, and Thr605) motifs in the EBV BALF5 protein. We prepared mutants designated BALF5Δ1, BALF5Δ2, BALF5Δ3, and BALF5Δ4, featuring deletion of amino acids 34 to 93, 167 to 185, 578 to 607, and 704 to 748, respectively. HEK293T cells were transfected with BALF5 wild type (WT) or its mutants, and at 24 h posttransfection whole-cell extracts were subjected to GST pulldown assays. Among the truncation mutants, BALF5Δ2 exhibited attenuated association with GST-Pin1, whereas other mutants showed comparable binding ability with the wild type (Fig. 3B). As the truncated BALF5Δ2 mutant lacks BALF5 Thr178-Pro, the motif is suggested to be a potential candidate Pin1 binding site. Pulldown of BALF5Δ1 with GST-Pin1 appeared strong, but we believe the result was just an artifact simply because the input level of the protein was also high.

Considering BALF5 T178 as the Pin1 binding motif, we next constructed an alanine substitution mutant, designated BALF5 T178A. BALF5 WT or BALF5 T178A was expressed in HEK293T cells, and then cell lysates were subjected to GST pulldown assays (Fig. 4A). Levels of copurified BALF5 T178A with GST-Pin1 were markedly lower than with the wild type, as expected (Fig. 4A). Furthermore, immunoprecipitation assays also confirmed that the T178A mutation diminished the BALF5 association with Pin1 (Fig. 4B). Therefore, we conclude that BALF5 Thr178-Pro is the Pin1 binding target.

BALF5 T178 is important for viral DNA polymerase activity. Our experiments indicated only one Pin1 binding site in the BALF5 amino acid sequence. To further extend and verify the finding, recombinant EBV with BALF5 deletion was prepared. As shown in Fig. 5A, part of the BALF5 sequence encompassing the Pin1 binding site (Thr178) was replaced with a marker cassette (for neomycin resistance and streptomycin sensitivity [Neo/st]).

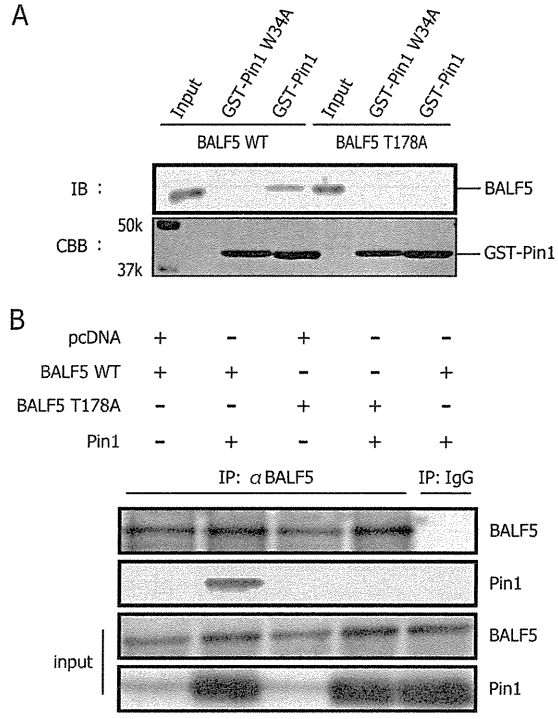

FIG 4 BALF5 T178A diminishes binding ability to Pin1. (A) Cell lysates from HEK293T cells, transfected with BALF5 wild type or T178A, were subjected to GST pulldown assays. (B) HEK293T cells were transfected with expression vectors for BALF5 wild type or T178A, with or without the expression vector for Pin1, as indicated. Cell proteins were lysed and subjected to immunoprecipitation using anti-BALF5 antibodies or normal IgG. The precipitates were then immunoblotted using anti-BALF5 or -Pin1 antibodies.

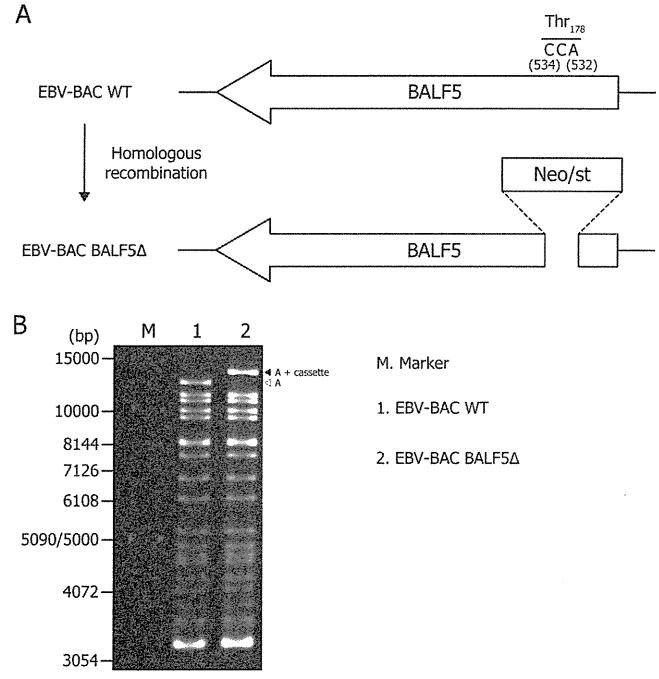

FIG 5 Scheme of EBV-BAC BALF5Δ construction. (A) Schematic arrangement of the recombination of the EBV genome using the neomycin-resistance and streptomycin-sensitivity genes (Neo/st) arranged in tandem. The sequence around the Pin1 binding site of BALF5 (Thr178) was replaced with the Neo/st cassette. (B) Electrophoresis of the recombinant viruses. The recombinant EBV genomes were digested with BamHI and separated in an agarose gel. A, the A fragment of BamHI-digested EBV-BAC.

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. The BamHI-digested A fragment of EBV-BAC BALF5Δ (Fig. 5B, filled arrowhead) migrated more slowly than that of the wild type (open arrow), as expected, since the Neo/st marker cassette was inserted into the fragment (Fig. 5B).

Recombinant EBV-BAC DNA was introduced into a virus-producing cell line, HEK293, followed by hygromycin selection, to establish cell lines in which the EBV-BAC genome was maintained as an episome. More than 10 cell colonies from each recombinant virus were obtained, and viral protein expression levels in the presence and absence of BZLF1 induction were examined (data not shown). As the result, we obtained BALF5 knockout EBV-BAC cells (EBV-BAC BALF5Δ) which exhibited a typical nature, i.e., viral lytic protein expression was restricted without BZLF1 and efficiently induced by BZLF1.

In the BALF5 knockout cell line, exogenous expression of BZLF1 led to induction of early genes, such as BALF2 and BMRF1, but failed to produce BALF5, in line with expectations (Fig. 6A, BZLF1). Because of the lack of BALF5, the DNA Pol catalytic subunit, the virus could not amplify viral DNA even after induction with BZLF1 (Fig. 6C, BZLF1). Next, in order to compare the efficiencies of complementation, HEK293 EBV-BAC BALF5Δ cells were transfected with either BALF5 WT or BALF5 T178A expression vectors in addition to BZLF1. Exogenous supply of BALF5 WT restored the replication and increased viral DNA levels by 14.6-fold (Fig. 6C, BZLF1+BALF5 WT). On the other hand, the BALF5 T178A mutant increased the viral DNA only 4.54-fold (Fig. 6C, BZLF1+BALF5 T178A), when the mutant BALF5 protein expression level was equivalent to that of the wild type (Fig. 6A and B). In addition, we measured viral particles produced from the cells (Fig. 6D). Culture supernatant from the cells were collected and cocultured with naive Akata(−) cells. Since the recombinant EBVs used here encode green fluorescent protein (GFP), Akata(−) cells infected with EBV become GFP positive. When wild-type BALF5 was transfected with BZLF1, 759 infectious particles were obtained per ml of supernatant on average. Although the DNA replication levels (Fig. 6C) and viral yield (Fig. 6D) were slightly weak, we assume that this experimental condition is still physiologically relevant. The T178A mutation of BALF5 caused a slight decrease in the viral yield (Fig. 6D). These results indicate that the T178 residue of BALF5 is needed for efficient lytic replication of the EBV genome and suggest that optimal activity of the DNA polymerase is mediated through the interaction with Pin1.

To further verify the conclusion, we lastly tested if knockdown of Pin1 could influence EBV replication under this condition, too. Transfection of siRNA to HEK293 EBV-BAC BALF5Δ cells caused a considerable decrease in Pin1 levels when levels of other markers like BALF5, BZLF1, and tubulin remained unchanged (Fig. 6E). Viral replication levels this time reached 17.8-fold with BZLF1 plus BALF5 (wild type) and control siRNA, but Pin1 knockdown resulted in only a 12.2-fold increase (Fig. 6F). Although the difference in the results shown in Fig. 6F was less remarkable than the difference shown in Fig. 1B, we speculate that this reduction level by siPin1 is convincing enough because knockdown of Pin1 here

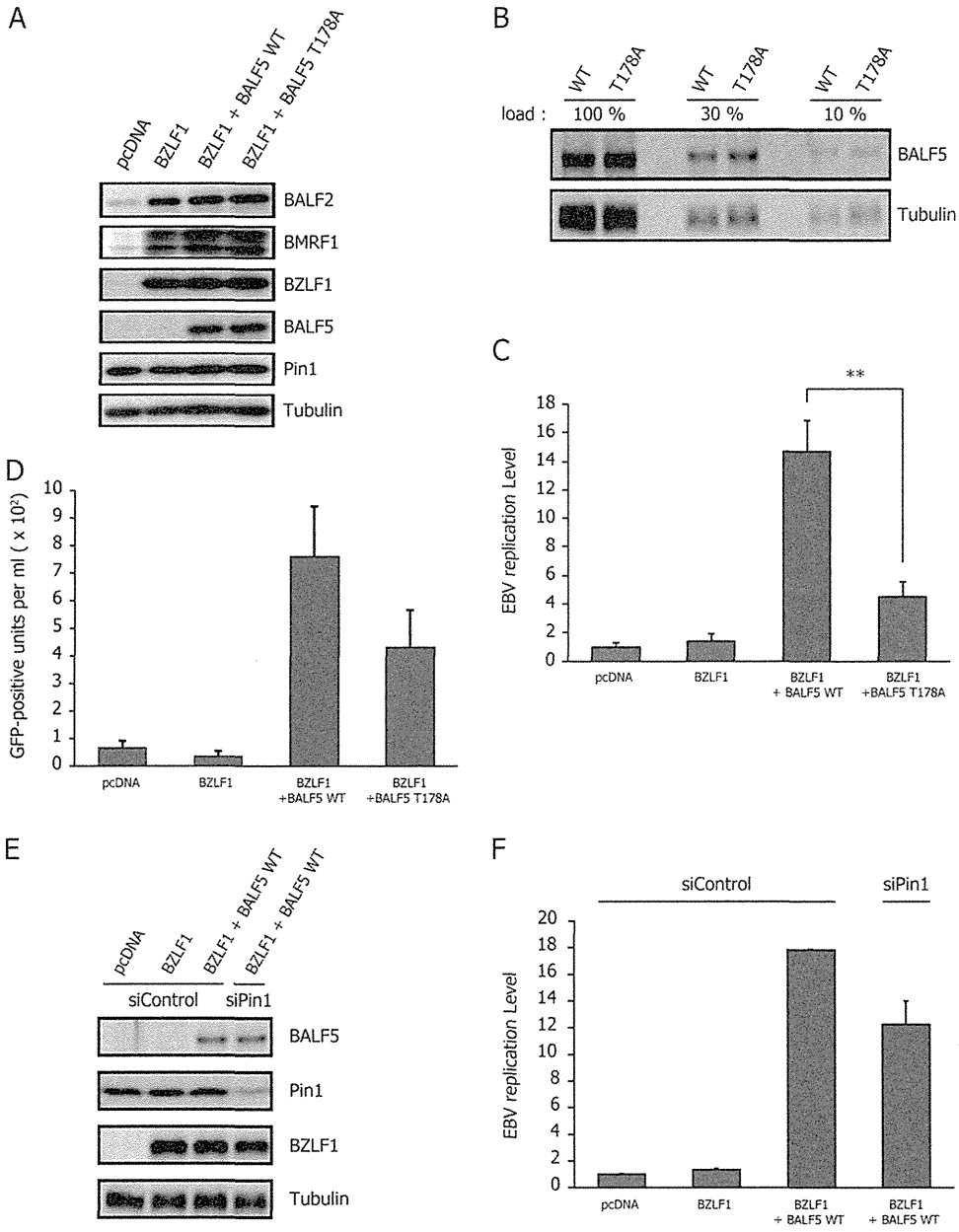

FIG 6 Significance of Pin1 binding to BALF5 Thr178 for viral replication. (A) HEK293 EBV-BAC BALF5Δ cells were transfected with 50 ng of BZLF1 and 10 ng of either BALF5 WT or BALF5 T178A expression vector using a Microporator (Digital Bio). Aliquots of cells were harvested at 24 h after transfection and subjected to immunoblotting with indicated antibodies. (B) The samples from the third and fourth lanes of the experiment shown in panel A were diluted and loaded as indicated, followed by immunoblotting with anti-BALF5 and -tubulin antibodies. The remaining cells were harvested at 36 h after transfection and subjected to qRT-PCR (C). Each bar represents the mean and standard deviation for the viral DNA level after normalization, calculated from three independent samples. **, $P < 0.005$. (D) Culture supernatants from HEK293 EBV-BAC BALF5Δ cells, transfected in the same fashion as described for panel C and followed by 3 days of incubation, were collected and used to infect naive Akata(−) cells. Viral load in the medium was determined by fluorescence-activated cell sorting analysis and is shown as the number of GFP-positive units per milliliter. (E and F) HEK293 EBV-BAC BALF5Δ cells were transfected with siRNA against Pin1 (siPin1) or a control siRNA (siControl). After 2 days, the cells were then transfected with expression vectors for BZLF1, BALF5 WT, BALF5 T178A, and/or the empty vector pcDNA, as indicated. Samples were harvested at 24 h after transfection of plasmids and subjected to immunoblotting with the indicated antibodies (E). The remaining cells were harvested at 36 h after transfection of plasmids for qRT-PCR (F).

(Fig. 6E) was not as complete as that in the experiment shown in Fig. 1A.

DISCUSSION

In this study, we obtained evidence for the first time of an interaction between the EBV lytic protein BALF5 and the cellular regulator Pin1. The results documented show clear involvement of the Pin1 protein in efficient EBV lytic replication. Initially, Pin1 was identified as a key regulator from knockdown experiments since silencing of Pin1 resulted in significant suppression of the viral replication level (Fig. 1).

We first speculated that knockdown of Pin1 might directly

influence viral lytic gene transcription because Pin1 reportedly regulates RNA polymerase II activity (32–34). Pin1 also impacts cellular signaling through factors such as Akt (35), c-Jun (36, 37), and p65/NF-κB (38). In fact, shPin1 might have slightly decreased EBV early gene expression (Fig. 1A) although we assume the levels are comparable.

Then, we found that the EBV DNA polymerase BALF5 interacted directly with Pin1, as demonstrated by GST pulldown (Fig. 2A), and that the interaction is dependent on phosphorylation of BALF5 Thr178 (Fig. 2B and 4). Results of immunoprecipitation assays also supported this conclusion (Fig. 2C and 4B). Although the association of Pin1 with BALF5 (Fig. 2 to 4) and its influence on viral DNA replication (Fig. 6) are clear, we cannot preclude the possibility that there may be other Pin1 substrates besides BALF5 that affect EBV genome amplification as Pin1 has a large number of substrates (12). Thus, the search for other Pin1 targets is still under way. In addition, it must also be noted that because there are a number of cellular target proteins that are up-/downregulated by Pin1, we cannot ignore the possibility that some of these cellular target proteins may cause even adverse effects on EBV replication. But as a whole, Pin1 clearly upregulates EBV lytic replication (Fig. 1), at least partly through the action of BALF5 (Fig. 6).

In this paper, we identified Pin1 interaction with the EBV DNA polymerase BALF5 enzyme at Thr178. The polymerase contains conserved domains, including polymerase catalytic and exonuclease domains at the C terminus, but the sequence around Thr178 is not conserved. We have no concrete idea of how Pin1 modulates BALF5 function, but it is possible that the N-terminal domain of BALF5, including Thr178, may somehow regulate its C-terminal functional domains by altering the structure of the protein in a subtle way. Further studies are required to gain an understanding of the molecular mechanism of how Pin1 regulates BALF5 enzymatic activity.

To our knowledge, EBV BALF5 is the only Pin1 target so far identified, not just in EBV but among all herpesvirus genes. Milbradt and others reported data suggesting that Pin1 may be involved in reorganization of nuclear lamin after phosphorylation by the human cytomegalovirus (HCMV)-encoded protein kinase UL97 (39). Elsewhere, Peloponese and others showed that Pin1 binds to the Tax protein of human T cell leukemia virus 1 and regulates Tax-induced NF-κB activation (40). Jeong and others demonstrated that Pin1 prolongs the Tax protein half-life by suppressing ubiquitination and proteasome-dependent degradation (41). Furthermore, Pin1 increased stability of the hepatitis B virus oncoprotein X and enhanced transactivation and cell proliferation (42). Hepatitis C virus replication is regulated by Pin1, probably through binding to NS5A/NS5B (43). Human immunodeficiency virus type 1 genome integration (44) and capsid uncoating (45) are also regulated by Pin1. Although exact roles of Pin1 in controlling herpesvirus replication remain elusive, it has already been proposed as an important modulator of viral proteins and a unique target for antiviral therapy (46).

A number of studies suggest that Pin1 has a role in tumorigenesis as it is overexpressed in a number of human cancers (47, 48). Since we determined that Pin1 is a positive regulator of EBV lytic replication, EBV-positive cancer tissue may be an efficient site for producing novel virus particles. While further studies are required to clarify the underlying mechanisms, Pin1 clearly warrants attention as a novel target for potential antiviral/cancer drug development.

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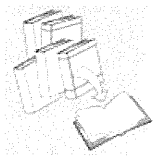
REVIEW



Genome guardian p53 and viral infections

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SUMMARY

Because virus infections elicit various cellular responses that inhibit viral replication and growth, viruses must intervene to attenuate antiviral measures in order to thrive. The genome guardian p53 plays a central part not only in DNA damage responses, inducing cell cycle arrest or apoptosis, but also in the innate host immune control of viral infections by orchestrating diverse signaling pathways originating from many different cellular receptors and sensors. Many viruses have acquired sophisticated mechanisms to regulate p53 functions by deploying subversive proteins and modulating its post-transcriptional status. In this review, we overview the mechanisms by which DNA and RNA viruses manage p53 signaling in favor of their continued survival. Copyright © 2012 John Wiley & Sons, Ltd.

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INTRODUCTION

Viruses intrinsically depend on their host cells during the course of infection. Cells infected by viruses utilize host surveillance mechanisms to ultimately block viral replication and dissemination. The coordinated genetic regulatory network in which a transcription factor p53 controls the expression of a set of diverse target genes is central to host defense. Actuary, p53-mediated apoptosis, which may be termed altruistic suicide, inhibits the further spread of infectious pathogens [1]. On the other hand, another important aspect of cellular responses is the immune system signaling elicited by infection with viruses, which usually leads to the production of type I IFN and inflammatory cytokines, resulting in elimination of the pathogens [2].

p53 is also activated in response to diverse cellular stresses such as DNA damage and oncogenic stress [3,4]. Induction of p53 triggers multiple cellular programs ranging from transient responses, such as DNA repair and cell cycle arrest, to terminal fates such as cell death and permanent cell cycle arrest, hence having central roles in tumor suppression and maintaining genomic integrity as a guardian of the genome [3,5,6].

Lane and Levine initially isolated p53 as a binding partner of SV40 LTag in 1979 [7,8]. Within a few years of its discovery, evidence of a cellular oncogene property appeared because the gene cloned from neoplastic cells could reproduce transformation [9]. Tumor-derived p53 mutants can promote cellular transformation through dominant-negative inactivation of endogenous wild-type p53, whereas wild-type p53 cannot [10]. Vogelstein and colleagues reported a common loss-of-heterozygosity at the p53 locus in human colorectal cancers [11], suggesting that p53 was actually a tumor suppressor gene rather than an oncogene. Indeed, p53 is mutated or lost in over 50% of human cancers [12], representing the most commonly mutated gene in human tumors.

In unstressed cells, p53 is kept at low levels by its negative regulator MDM2 (HDM2) through the ubiquitin-dependent proteasome pathway [13]. Upon DNA damage, p53 is phosphorylated to escape from proteasomal degradation [14], and then is stabilized and activated to function primarily as a transcription factor, consequently leading to cell

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Abbreviations used
Ad, adenovirus; DDRs, DNA damage responses; E6AP, E6-associated protein; HBX, hepatitis B virus X protein; HPV, human papillomaviruses; Inf., Influenza virus; KSHV, Kaposi sarcoma herpesvirus; LANA, latency-associated nuclear antigen; LTag, large T antigen; SV40, simian vacuolating virus 40; Vacc., vaccinia virus; VSV, Vesicular stomatitis virus.

cycle arrest or apoptosis through the p53-mediated gene expression cascades [4,15]. These cellular outcomes after stresses, including DNA damage, oncogene activation, hypoxia, nucleotide imbalance, and oxidative damage, are tightly linked to p53 dynamics mediated by both the levels and post-translational modifications of p53 [16,17]

Furthermore, p53 also contributes to immune responses that lead to eradication of pathogens such as viruses [18]. p53 directly transactivates the expression of several innate immunity-related genes such as IRF9, TRL3, ISG15, and MCP-1 [19–22], and interestingly, transcription of the p53 gene is induced by IFN-α/β signaling [1,23]. These findings suggest a positive feedback loop involving p53-mediated enhancement of IFN signals to boost antiviral immune responses. Viruses, in turn, have to evolve elaborate mechanisms to subvert IFN-mediated and p53-mediated host immune responses.

Viruses are grouped into two major categories: DNA and RNA viruses. Replication of viruses, especially RNA viruses, can induce type I IFNs, triggered by the production of dsRNA. On the other hand, DNA viruses activate DNA damage signaling, triggered by the production of viral DNA genomes. Viruses intervene at numerous stages in the pathways to attenuate the antiviral responses. Here, we review how viruses modulate p53 functions and its downstream signaling pathways during their propagation, the functional links between viral growth and post-translational status of p53, and the physiological importance of this interplay. The interplay between p53 and viruses is summarized in Figure 1.

DNA VIRUSES

Most DNA viruses replicate their genomes in nuclei and usually elicit DDRs, resulting in phosphorylation and stabilization of p53. Some exploit the DDR to facilitate their own genome replication, but in other cases, the DDR presents a block to viral replication, which must be overcome. Thus, DNA viruses employ a variety of strategies to inactivate or degrade p53 or sometimes to utilize p53 function for their proliferation. The prevention of p53 functions by virus, in turn, contributes to tumor progression in a certain tissue.

The high-risk HPV, which is associated with human cervical cancer, E6 protein can recruit the cellular E3 ubiquitin ligase E6AP, a prototype member of the homologous to the E6-associated protein carboxyl terminus (HECT) family, to a trimeric complex with p53 [24] that is degraded through the ubiquitin–proteasome pathway [25,26]. Degradation of p53 by the E6–E6AP complex reduces the net levels and then allows viral replication by inhibiting p53-mediated antiviral responses including DDR, apoptosis, and other stress signals.

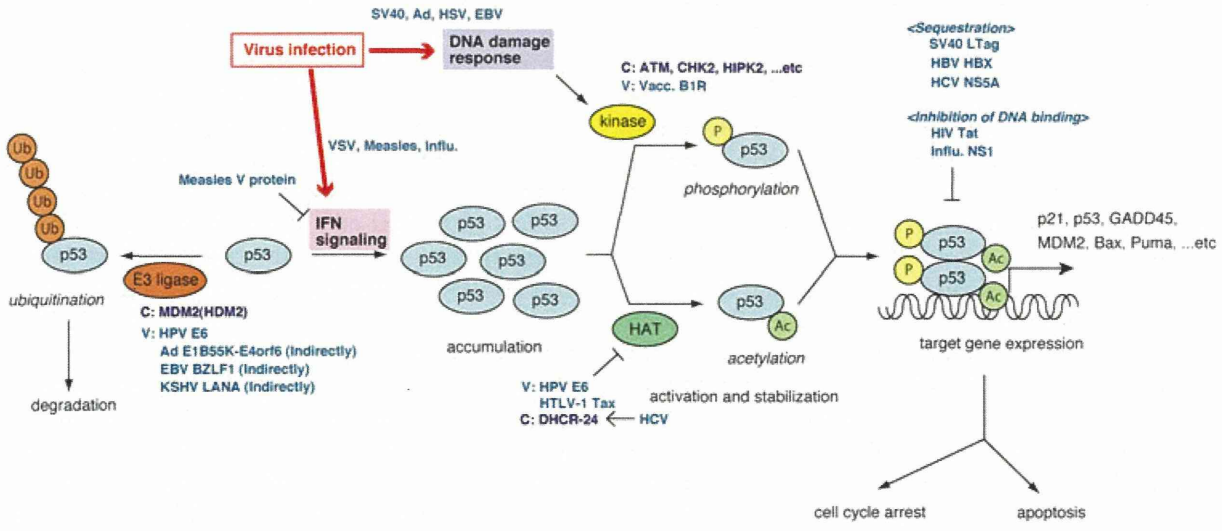

Figure 1. Intervention of viral proteins in p53-mediated antiviral responses. The viruses interfere with the p53 functions at several steps to permit a successful viral life cycle. Although infection with pathogens induces antiviral signaling pathways that stabilize and activate p53, viral factors manipulate these pathways by mimicking post-translational modifications of p53 and/or disrupting its downstream signals

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Many oncogenic DNA viruses including Ad, EBV, and KSHV also share the p53-inhibiting strategy that leads to p53 degradation through the ubiquitin–proteasome pathway. Ad E1B55K associates with E4orf6 to ubiquitinate p53 [27]. The EBV BZLF1 protein can function as an adaptor of the Elongin B/C-Cul2/5-SOCS-box protein (ECS) complex, which facilitates p53 degradation that has previously been phosphorylated in C-terminal region responses [28–30]. The KSHV-encoded LANA interacts with p53, resulting in inhibition of p53-mediated apoptosis and increased chromosome instability [31–33]. Thus, preventing p53 destruction by viral proteins might be a potent therapeutic target to combat virus-related carcinoma.

SV40 possesses tumorigenic properties in non-permissive cells. This transformation potential depends on the activity of LTag interacting with several cellular tumor suppressors, including p53 and pRb. Lilyestrom *et al.* reported the structure of p53 bound to LTag [34], featuring a circular Tag helicase domain hexamer with a p53 DNA-binding domain bound to the outside surface of each subunit, forming a pinwheel-like structure [34], suggesting that LTag subverts p53 function by preventing it from binding to DNA for appropriate regulation of p53.

On the other hand, HBX inhibits p53-mediated cellular processes by sequestration of p53 from the nucleus to the cytoplasm [35,36]. The EBV latent protein EBNA1 contributes to repress p53-dependent DDR by competing for the binding site of deubiquitinating enzyme USP7 with p53 [37]. The vaccinia virus-encoding Ser/Thr kinase B1R is able to directly hyperphosphorylate p53 in several residues including Thr 18 [38]. Interestingly, phosphorylation by B1R results in p53 degradation in an MDM2-dependent manner [38], illustrating the complexity of the structure of the p53 N-terminus region. Downregulation of p53 promotes viral DNA synthesis in cells infected with vaccinia virus [39] and also prevents p53-mediated responses, such as apoptosis [40]. Taken together, the complex between viral and cellular proteins suppresses p53 functions by distinct mechanisms that block p53 activity independently at various steps, suggesting that it is important for viruses to disrupt p53 activity in order to perform their efficient replication and dissemination.

However, it should be noted that some viruses require p53 for their replication. The cells infected with human cytomegalovirus (HCMV) in the absence of p53 produce fewer infectious viral particles, with delay in viral protein production and trafficking [41]. The HCMV genome has 21 potential p53 responsive sites [42]. The available data suggest that HCMV gene expression is influenced by p53 molecules bound to the HCMV genome at immediate-early and early stages of infection, which could explain the mechanism of reduced and delayed production of virions in p53-negative cells. Indeed, p53 has been demonstrated to be involved in regulation of viral UL94 protein expression [43]. Furthermore, in early stages of the EBV lytic infection, the inactive form of p53 cooperates with viral factors including BZLF1 protein to stimulate virus replication [44,45], although active p53 is ubiquitinated by BZLF1-ECS ubiquitin ligase complexes and degraded in a proteasome-dependent manner to inhibit apoptosis in the middle and late stages [30]. Therefore, virus has to well-organize p53 functions in both time-dependent and status-dependent manners for its efficient replication.

Some DNA viruses including HSV-1/2 and adenovirus induce the antiviral innate immune response that leads to type I IFN production [46]. Taniguchi and colleagues showed that IRF5 is critical for antiviral immunity by showing that *Irf5*$^{-/-}$ mice are highly vulnerable to HSV-1 infection, accompanied by a decrease in type I IFN induction in the sera [47]. The connections between the p53 family and IFN-mediated innate antiviral immunity have been established [1]. IFN signaling drives increased p53 mRNA and protein levels in order to evoke more robust p53 responses that trigger apoptosis of infected cells and restrict virus replication. This is also supported at the level of gene expression, as several gene targets of the IFN system are also subject to regulation by the p53 family [48,49]. Indeed, IRF5 is identified as a direct p53-target gene [50]. These findings indicate the crosstalk between p53 and the IFN pathway in the innate immunity.

RNA VIRUSES

Most RNA viruses undergo their entire replicative cycle in the cytoplasm except for two principal types, retroviruses and influenza viruses, both of which have an important replicative step in the nucleus. Infection with most RNA viruses induces antiviral responses mediated by IFN signaling.

VSV infection induces marked phosphorylation of mouse p53 at Ser 18 through ATM [1], and then,

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some p53-inducible genes are upregulated in wild-type but not IFN-α/β receptor 1-deficient mouse embryonic fibroblasts (MEFs), although phosphorylation of p53 is found in the latter. Thus, IFN does not activate p53 but contributes to enhancement of p53 responses by inducing the p53 gene [1]. Furthermore, the virus yield was found to be more than 30-fold higher in $p53^{-/-}$ than in wild-type MEFs, suggesting that p53 contributes to limiting virus replication. Thus, the p53 response to virus infection constitutes a critical aspect of antiviral protection and its replication. In the case of measles virus infection, the V protein binds to IFN signaling proteins, STAT1 and STAT2, allowing efficient evasion of the host IFN-induced antiviral immune response [51,52].

Furthermore, the HCV core, NS3, and NS5A proteins have been shown to associate with p53, modulating its functions without targeting p53 for degradation [53]. Knockdown of p53 actually enhances the HCV replication [54]. In addition, chronic HCV infection results in persistent liver inflammation and induces endoplasmic reticulum and oxidative stress, thought to contribute to hepatocarcinogenesis [55] due to increased risk of DNA damage and missegregation of chromosomes in proliferating cells. HCV causes expression of DHCR24 (also known as seladin-1), which catalyzes the reduction of sterol intermediates during cholesterol biosynthesis [56] in human hepatocytes, resulting in resistance to oxidative stress-induced apoptosis and suppressed p53 activity [57]. DHCR24 inhibits acetylation of p53 at Lys 373 and 382 in the nucleus without the modulation of phosporylated status of p53 [57]. Thus, expression of DHCR24 suppresses the p53 response to oxidative stress, consistent with the previous report that inactivation and mutation of p53 play a role in the development of hepatocellular carcinoma (HCC) [58]. Genetic inactivation of p53 is associated with late stage HCC [58] and HCV RNA levels are notably lower in cancerous tissues from HCV-positive HCC patients than in noncancerous tissues [59]. Thus, impairment of p53 function by HCV-induced overexpression of DHCR24 might play a crucial role in early stage disease progression, implying the relationship between p53 inhibition by virus and pathogenesis.

Retroviruses have a unique strategy for their propagation by which the viral genome is replicated to produce DNA from RNA genome templates by viral reverse transcriptase. The intermediate DNA is then transported to nuclei and incorporated into the host chromosomal genome by a virus-encoding integrase. This integration process elicits DDR [60,61]. Thus, retroviruses more directly affect events occurring in the nuclei of infected cells than other RNA viruses. The HTLV-1 Tax is crucial for viral replication and for initiating malignant transformation leading to development of adult T-cell leukemia [62]. Tax downregulates the p53 signaling through directly repress of p53 transcription [63,64]. However, the half-life of p53 protein is increased in the majority of Tax-transformed cells, suggesting functional inactivation [65]. Tax can activate expression of individual kinases as a transcriptional activator and then regulate both the phosphorylation status and transactivational functions of p53. This might be one of the mechanisms by which Tax can immortalize virus-carrying T-cells of HTLV-1-infected individuals. Thus, Tax inhibits p53 pathway by the control of p53 protein functions and by the decrease in p53 mRNA levels. Moreover, HIV-1 regulatory proteins Tat [66], Nef [67], Vpr [68], and Vif [69] modulate p53 for HIV-1 infection and replication. Although several distinct roles have been proposed for p53, the total effects of p53 on HIV-1 propagation remain controversial.

Infection with influenza virus induces apoptotic cell death in numerous cell types with an increase in p53 protein levels [70]. The nonstructural NS1 protein, which has multiple accessory functions including suppression of host immune and apoptotic responses [71], binds to p53 and suppresses p53-dependent transcription, leading to inhibition of p53-mediated apoptotic cell death [72] and presumably also to enhancement of viral replication. Indeed, the p53 pathway is overall downregulated by different subtypes of influenza A viruses [73]. In the case with H5N1 infection, a decrease in p53 mRNA expression is detected [73]. Intriguingly, in the human lung cell line, inhibiting p53 activity leads to elevated virus replication, potentially through the decrease in IFN signaling [74], suggesting that p53 is involved in the IFN-mediated antiviral response to influenza infection. Consistent with these findings, $p53^{-/-}$ mice show a more severe influenza A virus-induced disease compare with their wild-type counterparts [75]. Therefore, in addition to its established functions in tumor formation, p53 also serves as an antiviral factor that might be modulated to improve therapy and vaccines.

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CONCLUSIONS

Viral infection is tightly linked with host cell condition. Perhaps not surprisingly, given the central role of p53 attributed to various pathways in cells, changes in the activity of this protein by pathogens often alter the properties of cells such as cellular environment and cell fate in virus-infected cells.

A major conclusion of the work on cell proliferation and apoptosis is that loss of p53 functions may contribute to the initiation of virus-mediated cancer from these cells. The causative viruses of human cancer possess several distinct mechanisms to inactivate p53 functions and signaling by the alterations of post-transcriptional modification, localization, binding partner, turn over, and transcriptional activity. The activity of p53 is strictly controlled through a multistep process. Viruses have collectively acquired an impressive repertoire of molecules that target almost every aspect of the p53-mediated signaling pathway. An interesting aspect of these observations is that there are different ways of p53 inhibition within species, suggesting that virus obtained and adapted the mechanisms independently during its course of evolution. The connection between p53 and viral proteins is well established, but there are only a few demonstrations of the importance of these interactions in the control of biological processes related to p53 function. Purvis *et al.* recently demonstrate that p53 dynamics affects cell fate decision [17]. It would be interesting to investigate whether the interaction between p53 and viral factors influences the quality of signal in the cells. To clarify this, further studies are required.

Some studies have already provided evidence for the p53-mediated antiviral response. Infection of host cells with virus induces production of IFN-α/β and cytokines that concomitantly contributes to boost p53-mediated responses via accumulation of p53 protein. To counteract this, virus has to perturb the p53 functions. The antiviral effects by p53 are likely dependent on its ability to promote more rapid pro-inflammatory and antiviral gene expressions, strongly supporting the concept that enhancement of p53 functions as a host resistance factor against virus infection may be used as a host-targeted therapeutic strategy to develop antiviral therapies and vaccine adjuvants.

However, p53 is also necessary to construct a cellular environment for virus production before the onset of viral replication [41,42,45,69,76]. Taken together, the data indicate that, as a strategy for efficient virus survival and growth, it is important to maintain a delicate balance between activation and inhibition of p53 pathways.

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CONFLICTS OF INTEREST

The authors have no competing interest.

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Epstein-Barr Virus Deubiquitinase Downregulates TRAF6-Mediated NF-κB Signaling during Productive Replication

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Epstein-Barr virus (EBV), a human oncogenic herpesvirus that establishes a lifelong latent infection in the host, occasionally enters lytic infection to produce progeny viruses. The EBV oncogene latent membrane protein 1 (LMP1), which is expressed in both latent and lytic infection, constitutively activates the canonical NF-κB (p65) pathway. Such LMP1-mediated NF-κB activation is necessary for proliferation of latently infected cells and inhibition of viral lytic cycle progression. Actually, canonical NF-κB target gene expression was suppressed upon the onset of lytic infection. TRAF6, which is activated by conjugation of polyubiquitin chains, associates with LMP1 to mediate NF-κB signal transduction. We have found that EBV-encoded BPLF1 interacts with and deubiquitinates TRAF6 to inhibit NF-κB signaling during lytic infection. HEK293 cells with BPLF1-deficient recombinant EBV exhibited poor viral DNA replication compared with the wild type. Furthermore, exogenous expression of BPLF1 or p65 knockdown in cells restored DNA replication of BPLF1-deficient viruses, indicating that EBV BPLF1 deubiquitinates TRAF6 to inhibit NF-κB signal transduction, leading to promotion of viral lytic DNA replication.

Epstein-Barr virus (EBV), a human lymphotropic gammaherpesvirus with a linear double-stranded DNA, 172 kb in length (1), infects resting B lymphocytes, inducing their continuous proliferation without production of virus particles, this being termed latent infection. In the latent phase, a limited number of viral genes are expressed, and the expression pattern of viral latent genes varies depending on the tissue origin and the state of the cells/tumors. Productive (lytic) infection, which occurs spontaneously or can be induced artificially, is triggered by BZLF1 immediate-early protein and characterized by the expression of a number of lytic genes, leading to virus production. The EBV genome is thereby amplified several-hundred-fold by viral replication machinery.

In lymphocytes that are latently infected with EBV, latent membrane protein 1 (LMP1) is expressed to promote survival and proliferation of the cells. LMP1 is uniformly expressed in latency III EBV infection of human B lymphoblastoid cell lines (LCLs), and also in latent II EBV infection in Hodgkin's disease B lymphocytes and in nasopharyngeal carcinoma (NPC) epithelial cells (2). It is a transmembrane protein consisting of a short cytoplasmic N-terminal domain, six transmembrane domains, and a long cytoplasmic C-terminal domain (3, 4). Two subdomains within the C-terminal domain, C-terminal activating region 1 (CTAR1) and CTAR2, associate with tumor necrosis factor receptor-associated factors (TRAFs) which are critical for LMP1 signaling (3, 5, 6). LMP1 is a functional mimic of the tumor necrosis factor receptor superfamily member CD40, an activating receptor constitutively expressed on B cells, macrophages, and dendritic cells (7, 8). As a result, LMP1 causes constitutive activation of cellular signaling, with upregulation of factors such as NF-κB, mitogen-activated protein kinase (MAPK), JAK/STAT, and Akt (9–13). Of several transcriptional activators targeted by LMP1, NF-κB is most important for LMP1-stimulated gene expression (14–18).

The canonical NF-κB, consisting of p65/RelA and p50, plays an important role in regulation of a variety of genes involved in host immune responses and in different features of carcinogenesis, including proliferation, enhanced survival, inflammation, and angiogenesis (19). NF-κB is usually under tight regulation, being kept inactive in the cytoplasm by certain mechanisms, including binding of inhibitors of kappa B (IκBs). A series of NF-κB-activating stimuli converge on the activation of IκB kinase (IKK) complexes composed of a IKKγ regulatory subunit or the NF-κB essential modulator (NEMO), and two kinases, IKKα and IKKβ. The IKK complexes phosphorylate and promote proteasomal degradation of IκB, resulting in release of NF-κB from the inhibitor complex. It was recently demonstrated that TRAF6 associates with the CTAR1 subdomain of LMP1 and is critical for LMP1-mediated activation of NF-κB signaling (5, 20). TRAF6 activates IKK in a K63-ubiquitin (Ub) chain-dependent manner. Ub chains conjugated to signaling molecules during activation of the NF-κB pathway can be inactivated by cellular deubiquitination enzymes (DUBs) such as A20, CYLD, and DUBA (21–23), suggesting that ubiquitin modification enzymes and DUBs play critical roles in the NF-κB response, leading to modulation of immune responses.

High levels of NF-κB protect the cell from the cytopathic effects by viral protein synthesis and promote the establishment of a latent infection. In contrast, EBV lytic reactivation requires downregulation of NF-κB because basal or LMP1-stimulated NF-κB activity suppresses the expression and function of lytic transactivator BZLF1 (also known as ZEBRA and EB1), resulting in inhibition of lytic cycle induction (24, 25). However, LMP1 is para-

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doxically expressed during the lytic cycle in EBV-positive B cells (26).

EBV-encoded BPLF1 protein is a lytic gene product with DUB activity. Whitehurst et al. (2009) showed that its N-terminal fragment deubiquitinates viral ribonucleotide reductase (RR), resulting in downregulation of viral RR activity (27). Also, Gastaldello et al. (2010) showed that a 325-amino-acid (aa)-length N-terminal fragment of BPLF1 cleaves ubiquitin and NEDD8 conjugates and promotes EBV replication (28). More recently, Whitehurst et al. reported that BPLF1 deubiquitinates the cellular DNA polymerase processivity factor PCNA and attenuates Polη to DNA damage sites (29). In this study, we demonstrated that BPLF1 interacts, directly or indirectly, with and deubiquitinates TRAF6 to block cellular NF-κB signal responses during lytic replication. Cells harboring BPLF1-deficient EBV exhibited poor viral lytic DNA replication, and exogenous expression of BPLF1 restored it. Thus, DUB activity of BPLF1 is required for efficient viral genome replication.

MATERIALS AND METHODS

Cells. AGS cells transduced with CR2/CD21, the receptor for the EBV expression vector, and infected with enhanced green fluorescent protein (EGFP)-EBV (30) (AGS-EBV cells) were established previously (31) and maintained at 37°C in RPMI 1640 supplemented with 10% fetal calf serum and 150 μg/ml hygromycin B. B95-8 and Namalwa cells were maintained at 37°C in RPMI 1640 supplemented with 10% fetal calf serum. HEK293 cells and derivatives were grown and maintained at 37°C in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum. HEK293 cells latently infected with recombinant EBV-bacmid (293-EBV) were maintained as previously reported (32).

BAC mutagenesis and transfection. Wild-type (WT) EBV-bacmid (EBV-WT) has been described previously (33). The region between nucleotides (nt) 1 and 975 of the BPLF1 open reading frame (ORF) was replaced with tandemly arranged neomycin resistance and streptomycin sensitivity ($NeoSt^+$) genes using homologous recombination to construct a BPLF1-deficient EBV-bacmid (EBV-dBPLF1/NeoSt) (34). DNA fragments for recombination were generated by PCR with the following primers: 5′-GCG TAA GAC CCC GGA CCA GAA GGG GGG CGA CAA GGC GTC CTC CCC GCC CCA CCG CCG AAG GGC CTG GTG ATG ATG GCG GGA TC-3′ (forward) and 5′-GGG CCG CAG AGG CCG GGG CCG CAG AGG CCG GAG ACG ACG GCG GGG AGT TGG TCT TTG CAG TCA GAA GAA CTC GTC AAG AAG G-3′ (reverse). Electroporation of *Escherichia coli* was performed using Gene Pulser III (Bio-Rad). DNAs of EBV-WT and EBV-ΔBPLF1/NeoSt were purified using NucleoBond Bac 100 (Macherey-Nagel, Germany) and transfected into HEK293 cells using Lipofectamine 2000 reagent (Invitrogen) to establish HEK293 cells latently infected with either EBV-WT (293-EBVwt) or EBV-dBPLF1/NeoSt (293-EBVΔ).

Plasmids. pcDNA-Flag/TRAF6 (pFlag-TRAF6) was a kind gift from E. Harhaj (University of Miami), and pcDNA-BZLF1 (pBZLF1) was generously donated by K. Kuzushima and R. Ohta (Aichi Cancer Center Research Institute). pcDNA-HA-Ub (pHA-Ub) was prepared as described previously (35). To prepare the expression vector for the Flag-tagged N-terminal fragment of BPLF1, pFlag-BPLF1, a portion of the BPLF1 ORF sequence (nt 1 to 975, which is sufficient for deubiquitinase activity), was cloned into EcoRI and XhoI sites of pcDNA3 with a Flag tag (28). Primers used for BPLF1 amplification were as follows: 5′-GAC GAC GAT GAC AAG GAA TTC ATG AGT AAC GGC GAC TGG GGG-3′ (forward) and 5′-AGA TGC ATG CTC GAG TCA AGG ACT ATA CCT GGC GGC AGG GAA TGA GTC-3′ (reverse). A BPLF1 point mutation (C61A, which is a catalytically inactive mutation) was introduced to make pFlag-BPLF1C61A by PCR using the following primers: 5′-ACT GCG TCC TCT ACC TGG TCA AGA G-3′ (forward) and 5′-TGC TGA CTG CCT GGA TGC CG-3′ (reverse) (36).

Antibodies and reagents. Primary antibodies were purchased from Cell Signaling Technology (IKKβ, phosphorylated-IKKα/β, IκBα, α/β-tubulin, TRAF6), Chemicon (EBV BMRF1-R3, GAPDH [glyceraldehyde 3-phosphate dehydrogenase]), Roche Applied Science (hemagglutinin [HA]-3F10), and Sigma (Flag-M2). The antibodies to BZLF1, BALF2, BALF5, BGLF4, BBLF2 and -3 (BBLF2/3), and LMP1 have been described previously (37–42). Human p65-targeted small interfering (siRNA) was purchased from Santa Cruz. Control siRNA sequence (siRNA-DsRed) was 5′-GCA GAG CUG GUU UAG UGA AdT dT-3′ and 5′-UUC ACU AAA CCA GCU CUG CdT dT-3′, where dT means deoxythymidine.

Transfection and luciferase assays. Plasmid DNA was transfected into HEK293, 293-EBVwt, or 293-EBVΔ cells using a MP-100 microporator (Digital Bio). The total amounts of plasmid DNA 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 kit (Promega), and luciferase activities were measured using the kit. The counts of firefly luciferase were normalized to those of Renilla luciferase. The protein samples were then subjected to SDS-PAGE followed by immunoblotting.

Immunoprecipitation. To detect ubiquitinated forms of TRAF6 or physical interaction between BPLF1 and TRAF6, HEK293, 293-EBVwt, or 293-EBVΔ, cells transfected with expression plasmids were lysed 24 h posttransfection (hpt) in 100 μl of TX-100mCSK buffer (10 mM PIPES [pH 6.8], 100 mM NaCl, 300 mM sucrose, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 0.1% Triton X-100, and protease inhibitor mixture [Roche]). Cell lysates were then diluted with the same buffer. Immunoprecipitation under stringent conditions was carried out as described previously (43). In brief, denaturing lysis buffer (50 mM Tris-HCl [pH 7.5], 2% SDS) was used in place of TX-100mCSK buffer, and the lysate was subsequently incubated at 95°C for 10 min followed by dilution with the dilution buffer (950 mM Tris-HCl [pH 7.5], 2% bovine serum albumin [BSA]). Diluted cell lysates were precleared with protein G-Sepharose (Amersham Biosciences). Supernatants were then mixed with anti-Flag antibodies and incubated overnight at 4°C. Immunocomplexes were recovered by incubating protein G-Sepharose for 1 h, and the resin was washed five times with the same buffer. The immunoprecipitates were then subjected to SDS-PAGE followed by immunoblotting.

Immunoblotting. Cells were suspended in 1× sample buffer (65 mM Tris-HCl [pH 6.8], 3% SDS, 10% glycerol, 2% 2-mercaptoethanol) and then sonicated. The debris was removed by centrifugation, and the supernatants were applied for SDS-PAGE and immunoblotting, carried out as described previously (37).

qrt-PCR and PCR analysis. Lytic replication-induced 293-EBV-WT or 293-EBVΔ cells (1×10^6 cells) were harvested, and total cellular RNA was purified using TriPure isolation reagent (Roche) followed by conversion to cDNA using a SuperScript III first-strand synthesis system (Invitrogen). Quantitative real-time PCR (qrt-PCR) was performed with SYBR Premix *Ex Taq* II Tli RNaseH Plus (TaKaRa Bio), an ABI Prism 7300 machine (Applied Biosystems), and 3-step cycling conditions (95°C for 30 s, followed by 50 cycles of 95°C for 5 s, 55°C for 30 s, and 72°C for 1 min). Dissociation curves were recorded after each run. Cycle threshold (C_T) values were determined by automated threshold analysis with ABI Prism version 1.0 software. qrt-PCR assays were performed in triplicate. The value for an arbitrary RNA in the isolated RNAs was set to 1.0, and a standard curve was constructed using serial dilutions of cDNA from the RNA set to 1.0. A constant amount of RNAs was quantitated based on the standard curve. qrt-PCR with GAPDH primers was also performed to serve as an internal control for input RNA. Primer sequences used were as follows: for interleukin-8 (IL-8), 5′-CAA ACC TTT CCA CCC CAA AT-3′ (forward) and 5′-CTC TGC ACC CAG TTT TCC TT-3′ (reverse); for intercellular adhesion molecule 1 (ICAM-1), 5′-CAA CCG GAA GGT GTA TGA AC-3′ (forward) and 5′-CAG CGT AGG GTA AGG TTC-3′ (reverse); for AGT, 5′-GGA TGA GAG AGA GCC CAC AG-3′ (forward) and 5′-CTC ACT CCA TGC AGC ACA CT-3′ (reverse); for CCL2, 5′-CAT TGT GGC CAA GGA GAT CTG-3′ (forward) and 5′-CTT CGG

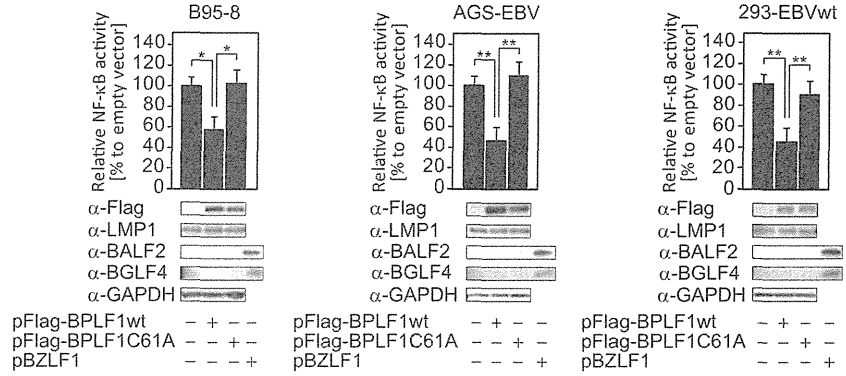

FIG 1 Ectopic expression of BPLF1 decreases NF-κB-dependent promoter activity in cells latently infected with EBV. Latently infected B95-8, AGS-EBV, and 293-EBVwt cells were transfected with the NF-κB-Fluc reporter plasmid (0.2 μg/well), along with the pCMV-Rluc plasmid (0.02 μg/well) and either pBPLF1wt or pBPLF1C61A (0.1 μg/well), in 24-well plates. Luciferase assays were performed at 24 hpt. Firefly luciferase activity was normalized to Renilla reniformis luciferase, and the value obtained by transfecting an empty-vector control was set to 100%. Data are shown as means ± SD of the results of 3 biological replicates. **, $P < 0.001$; *, $P < 0.005$. Sample lysates were subsequently subjected to immunoblotting with the specific antibodies indicated, and representative results are presented below the graph. In addition, sample lysates of cells transfected with BZLF1 were also included as controls for lytic replication.

AGT TTG GGT TTG CTT-3′ (reverse); and for GAPDH, 5′-GGG AAG GTG AAG GTC GGA GT-3′ (forward) and 5′-AAG ACG CCA GTG GAC TCC AC-3′ (reverse). Quantification of viral DNA synthesis during lytic replication was essentially conducted as described previously (44).

PCR analysis was performed with GoTaq Green Master Mix (Promega) and a Veriti thermal cycler (Applied Biosystems), and the PCR conditions used were 94°C for 30 s, 35 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 1 min. Primer sequences used in reverse transcription-PCR (RT-PCR) analysis were as follows: for BPLF1, 5′-GGA CCA TGG ATG TGA ATG C-3′ (forward) and 5′-GAG TCG GAT GTG AAA GAT CG-3′ (reverse); for BZLF1, 5′-AAC AGC CAG AAT CGC TGG AG-3′ (forward) and 5′-GGC ACA TCT GCT TCA ACA GG-3′ (reverse); and for GAPDH, 5′-TGC ACC ACC AAC TGC TAG C-3′ (forward) and 5′-GGC ATG GAC TGT GGT CAT GAG-3′ (reverse) (45).

Statistical analysis. Results are expressed as means ± standard deviations (SD). Values were compared between groups using analysis of variance (ANOVA) and Fisher's protected-least-significance-difference test. Results were considered statistically significant at a P of <0.05.

RESULTS

Ectopic expression of BPLF1 decreases NF-κB-responsive promoter activity in latently EBV-infected cells. Ubiquitination is involved in multiple steps of the NF-κB signaling pathway. Therefore, we first tested whether the EBV-encoded deubiquitinating enzyme BPLF1 inhibits NF-κB-dependent promoter activity in latently EBV-infected cells expressing LMP1. B95-8, AGS-EBV, and 293-EBVwt cells were transfected with reporter plasmids (pNF-κB-Fluc and pCMV-Rluc) and pBPLF1wt or pBPLF1C61A expression vectors, and luciferase assays were performed. Ectopic expression of the 325-aa-length N-terminal domain of BPLF1 exhibiting DUB activity decreased NF-κB-dependent promoter activity in these cells (Fig. 1). However, the EBV BPLF1C61A mutant, a mutant that is enzymatically defective due to the mutation of cysteine 61 to alanine (36), showed no significant inhibition (Fig. 1). The result suggests that DUB activity is essential for BPLF1 to suppress LMP1-induced NF-κB-dependent promoter activity.

In addition, we tested if overexpression of BPLF1 alone could induce EBV lytic cycle, because BPLF1 reduced NF-κB activity (Fig. 1) and because it was previously reported that inhibition of NF-κB by specific inhibitors causes spontaneous lytic gene expression in EBV-positive cells (46–49). While expression of immediate-early BZLF1 enhanced expression of early genes, including BALF2 and BGLF4, ectopic expression of BPLF1, either wild type or C61A, did not induce expression of the lytic genes (Fig. 1). Therefore, it is likely that, whereas BPLF1 inhibits NF-κB signaling, its expression alone is not sufficient for induction of EBV lytic replication.

Construction of BPLF1-deficient recombinant virus. We then constructed a BPLF1-deficient recombinant virus to determine the effect of BPLF1 on the NF-κB signaling pathway in EBV lytic replication. A marker cassette was inserted into the BPLF1 gene (nt 1 to 975, encoding its catalytic domain) of EBV-WT to construct dBPLF1/NeoSt, and, as a result, nt 181 to 183 encoding the C61 residue, crucial for deubiquitinase activity, were disrupted (Fig. 2A). The DNA of recombinant EBV bacmid was analyzed by digestion with BamHI or EcoRI (Fig. 2B and C) and PCR (Fig. 2D). Restriction enzyme digestion of wild-type and recombinant bacterial artificial chromosome (BAC) DNAs verified that no large deletions or rearrangements of the EBV genome occurred during recombination and that the BamHI-P and EcoRI-H fragments were of the expected sizes in the wild type (Fig. 2B and C, open arrowheads) and were increased in size by the insertion of a NesSt cassette into the deletion mutant (Fig. 2B and C, closed arrowheads). PCR analysis performed with BPLF1-specific primers amplified an DNA fragment of the expected size in the case of EBV-WT DNA, but not in the case of dBPLF1/NeoSt DNA (Fig. 2D). DNAs of the wild type and dBPLF1/NeoSt were introduced into HEK293 cells, and hygromycin-resistant cell colonies were cloned for further analysis. HEK293 cells containing EBV-WT and dBPLF1/NeoSt DNAs were designated 293-EBVwt and 293-EBVΔ, respectively. For RT-PCR, total RNAs were prepared from the HEK293 cells containing the wild-type or the recombinant EBV genome at 48 hpt with the pBZLF1. While comparable amounts of BZLF1 and GAPDH mRNA were detected in the two cell lines, as expected, BPLF1 mRNA was detected only in 293-EBVwt (Fig. 2E). The 293-EBVwt and 293-EBVΔ cells maintain about 1.9 and 2.1 copies of EBV-BAC DNA, respectively (Fig. 2F). Western blotting with anti-LMP1 antibody verified that 293-EBVwt and 293-EBVΔ cells express similar levels of LMP1

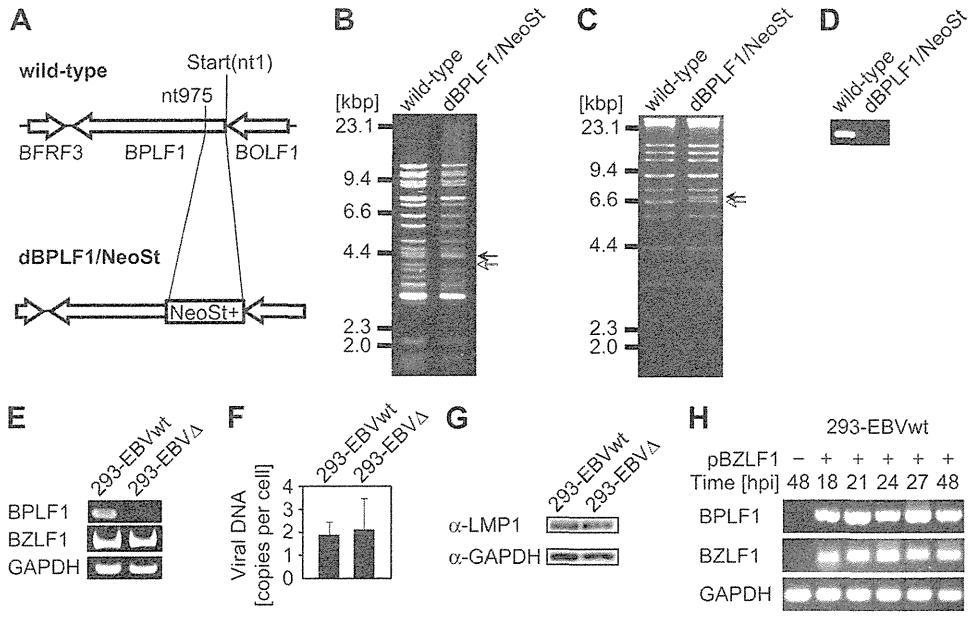

FIG 2 Recombinant EBV-BAC genome structures. (A) Schematic arrangement of recombination of the EBV genome using the neomycin resistance and streptomycin sensitivity genes. The region between nucleotides 1 and 975 of the BPLF1 ORF was replaced with tandemly arranged neomycin resistance and streptomycin sensitivity (NeoSt+) genes to make dBPLF1/NeoSt. (B and C) Electrophoresis of wild-type and recombinant EBV-BAC DNAs. EBV-BAC DNAs were digested with BamHI (B) or EcoRI (C) and separated in a 0.8% agarose gel. The sizes of BamHI-P fragment and a corresponding EcoRI fragment of the EBV-BAC DNAs (open arrows) were shifted by integration of the marker cassettes (closed arrows). Sizes (kbp) for molecular mass markers are indicated at the left side of the panels. (D) PCR analysis of the wild-type and the recombinant BAC DNAs with BPLF1 ORF-specific primers. The PCR product was detected by 1.5% agarose gel electrophoresis. (E) RT-PCR analysis of BPLF1 expressed in pBZLF1-transfected 293-EBVwt and 293-EBVΔ cells. Total RNAs were extracted at 48 hpi, and cDNAs were synthesized as described in Materials and Methods. PCR was performed on cDNA templates with specific primers. BZLF1 was used as an induction marker and GAPDH as an internal control. (F) Total DNAs prepared from 293-EBVwt and 293-EBVΔ cells were applied to qrt-PCR using BALF2-specific primers to quantify intracellular EBV-BAC DNA copies. The values were normalized to that of Namalwa cells, which maintain 2 EBV genomes per cell. (G) Western blotting using anti-LMP1 antibody was performed using whole-cell lysate prepared from 293-EBVwt and 293-EBVΔ cells to confirm that comparable amounts of the latent protein are expressed in both cells. (H) 293-EBVwt cells transfected with 1 μg of pBZLF1 were cultured for indicated periods, and expression levels of BPLF1 mRNA were measured by RT-PCR. Three biological replicates were carried out for the time-course analysis. Data from one representative experiment are shown.

(Fig. 2G). Thus, induction of lytic replication could be started from the similar genome copy and similar latency backgrounds. Expression of BPLF1 mRNA was detected at least by 18 h postinduction (hpi) and continued at least until 48 hpi (Fig. 2H).

DUB activity is essential for BPLF1 to block activation of the NF-κB pathway in EBV lytic replication. We then examined whether EBV BPLF1 is involved in regulation of NF-κB signaling in EBV lytic replication. 293-EBVwt or 293-EBVΔ cells were transfected with pBZLF1 and reporter plasmids (pNF-κB-Fluc and pCMV-Rluc). The intrinsic NF-κB reporter activity (normalized with RLuc expression driven by a cytomegalovirus [CMV] promoter) in 293-EBVΔ cells was almost the same as that in 293-EBVwt cells. Transfection of pBZLF1 resulted in downregulation of NF-κB activity in 293-EBVwt compared with cells transfected with a control vector (Fig. 3A, lanes 1 and 2), consistent with a previous report that viral lytic reactivation requires downregulation of NF-κB (50). In contrast, transfection of pBZLF1 into 293-EBVΔ did not decrease NF-κB activity (Fig. 3A, lanes 3 and 4). We then examined the effect of the BPLF1 expression on NF-κB-dependent promoter activity in 293-EBVΔ. Cotransfection of pBPLF1wt together with pBZLF1 into 293-EBVΔ decreased the NF-κB promoter activity to a level comparable to that seen with pBZLF1-transfected 293-EBVwt (Fig. 3A, lane 5), while transfection of enzyme-dead mutant pBPLF1C61A did not (Fig. 3A, lane 6). In the parental EBV-negative HEK293 cells, transfection of BZLF1 did not significantly affect NF-κB activity under our assay conditions (Fig. 3B, lanes 1 and 2). Up- or downregulation of NF-κB activity observed in 293-EBVwt and 293-EBVΔ would be dependent on BZLF1-induced lytic gene expression (Fig. 3A, lanes 2 and 4). These data suggest that EBV BPLF1 is required to downregulate NF-κB-dependent promoter activity during EBV lytic replication, too, and that DUB activity is critical for BPLF1 to antagonize NF-κB functions.

Moreover, since exogenous expression of BPLF1 could attenuate the NF-κB activity even in parental HEK293, which is devoid of EBV (Fig. 3B, lane 3), we speculate that suppression of NF-κB activity by BPLF1 is not specific to LMP1 or to EBV.

BPLF1 suppresses canonical NF-κB-regulated genes during the EBV lytic life cycle. Expression of canonical NF-κB-regulated genes, including AGT, CCL2 (monocyte chemoattractant protein-1 [MCP-1]), ICAM-1, and IL-8 (51–54), was conducted to confirm that BPLF1 actually inhibits NF-κB target gene expression during EBV lytic replication (Fig. 4). Total RNA was extracted, reverse transcribed into cDNA, and analyzed by qrt-PCR. Induction of EBV lytic replication in 293-EBVwt cells resulted in downregulation of a series of NF-κB-regulated genes, such as AGT (0.034-fold), CCL2 (0.104-fold), and ICAM-1 (0.528-fold) (Fig. 4A, top panel). Despite IL-8 expression being upregulated by the canonical NF-κB (55), IL-8 expression remained unchanged. It was earlier reported that BZLF1 induces IL-8 expression at both

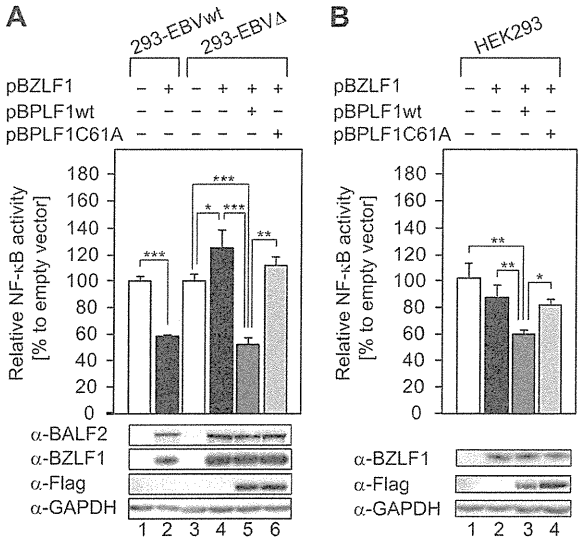

FIG 3 DUB activity is essential for BPLF1 to block activation of the NF-κB pathway during EBV lytic replication. Wild-type and recombinant BPLF1 expression vectors (0.1 μg each) were cotransfected into 293-EBVwt and 293-EBVΔ cells along with the pBZLF1 plasmid (1 μg), the pNF-κB-Fluc reporter plasmid (0.2 μg), and pCMV-Rluc (0.02 μg) using an MP-100 electroporator. An empty vector (pcDNA3) was used as a control. Cell extracts were collected at 24 hpt and analyzed for firefly and Renilla luciferase expression. Firefly luciferase activity was normalized to the Renilla reniformis luciferase, and the values obtained by transfecting the empty-plasmid control into 293-EBVwt or 293-EBVΔ were set to 100%. Sample lysates were subsequently subjected to immunoblotting with specific antibodies, and a representative result is presented below the graph. BZLF1 (immediate-early) and BALF2 (early) were used as induction markers. Data are shown as means ± SD of the results of 5 biological replicates. ***, $P < 0.001$; **, $P < 0.005$; *, $P < 0.01$.

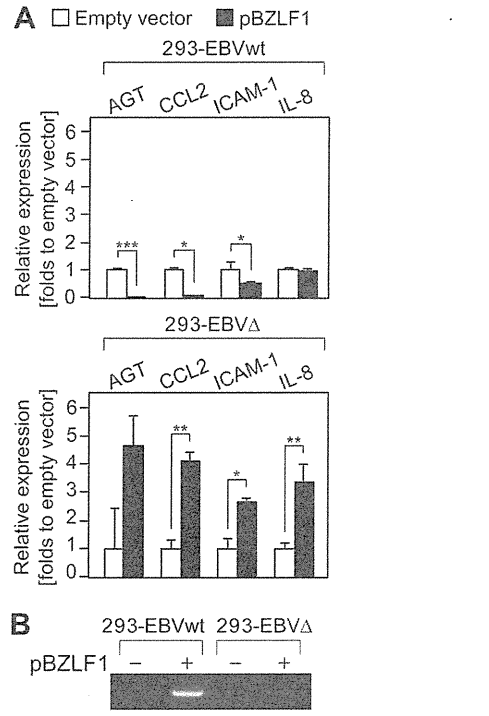

FIG 4 BPLF1 suppresses expression of NF-κB-regulated genes during the EBV lytic life cycle. (A) 293-EBVwt and 293-EBVΔ cells were transfected with control or BZLF1 expression plasmids. At 24 hpi, cells were subjected to qrt-PCR to measure the mRNA levels of NF-κB-dependent genes. Values were normalized to GAPDH mRNA, and the value obtained by transfecting an empty-plasmid control into 293-EBVwt or 293-EBVΔ was set to 1. Data are shown as means ± SD of the results of 3 biological replicates. ***, $P < 0.001$; **, $P < 0.005$; *, $P < 0.05$. (B) RT-PCR was carried out in order to detect BPLF1 mRNA in the same samples described for panel A, followed by an agarose electrophoresis.

the protein and mRNA levels by directly binding to BZLF1-responsive elements in the IL-8 promoter (56), suggesting that the level of IL-8 expression in 293-EBVwt was compensated by BZLF1 during lytic replication. In contrast, the expression of AGT, CCL2, ICAM-1, and IL-8 was markedly elevated (4.7-fold, 4.1-fold, 2.7-fold, and 3.4-fold, respectively) in 293-EBVΔ (Fig. 4A, bottom panel). In addition, we confirmed, by RT-PCR, that BPLF1 was induced by BZLF1 in the wild type and that no BPLF1 signal was obtained in the knockout virus (Fig. 4B). Thus, BPLF1 appears to prevent canonical NF-κB-regulated gene expression in EBV lytic replication.

Inhibition of NF-κB signaling by BPLF1 correlates with TRAF6 deubiquitination and increased IκBα. Ubiquitination or deubiquitination of key signaling molecules is an important regulatory mechanism in NF-κB signaling. It is known that TRAF6 is an especially critical host factor for LMP1-mediated B cell activation, and its ubiquitination activates NF-κB signaling in latently infected cells (20). Therefore, we set out to examine whether BPLF1 could target TRAF6 to suppress NF-κB signaling. Ubiquitination assays performed by means of a heterologous expression system with 293 cells demonstrated that overexpressed TRAF6 became polyubiquitinated (Fig. 5A, lane 3). The assays also revealed that ubiquitinated TRAF6 was deubiquitinated by a coexpressed wild-type BPLF1 in a dose-dependent manner (Fig. 5A, lanes 4 to 6), but not by the enzymatically defective BPLF1C61A mutant (Fig. 5A, lanes 7 to 9). Since there is a possibility that TRAF6 may interact with other protein(s) that may also be polyubiquitinated, we then performed immunoprecipitation under stringent conditions to avoid that possibility (Fig. 5B). Similar results were obtained under the stringent conditions, supporting the result shown in Fig. 5A.

A coimmunoprecipitation assay revealed that endogenously expressed TRAF6 protein was coprecipitated with Flag-tagged BPLF1 protein (Fig. 5C). Similar amounts of TRAF6 were also coprecipitated with enzymatically defective BPLF1. The result indicates that BPLF1 interacts with TRAF6, directly or indirectly, independently of its catalytic activity.

We further investigated whether the ability of BPLF1 to antagonize NF-κB is associated with deubiquitination of TRAF6 in EBV lytic replication. Ubiquitination states of TRAF6 in 293-EBVwt and 293-EBVΔ cells were compared when they were induced to perform lytic replication. When 293-EBVwt cells were transfected with pBZLF1, the TRAF6 polyubiquitination was markedly inhibited (Fig. 6A, lanes 1 and 2). In contrast, when 293-EBVΔ cells were transfected with pBZLF1, reduction of the TRAF6 ubiquitination state was much less significant compared to the case of 293 WT cells (Fig. 6A, compare lane 2 with lane 4). Furthermore, coexpression of wild-type BPLF1 in 293-EBVΔ diminished ubiquitination of TRAF6 (Fig. 6A, lane 5), while coexpression of the BPLF1C61A mutant did not show such an effect (Fig. 6A, lane 6). Partial reduction in TRAF6 ubiquitination in lytic replication-induced 293-EBVΔ cells might be due to other EBV-encoded deu-