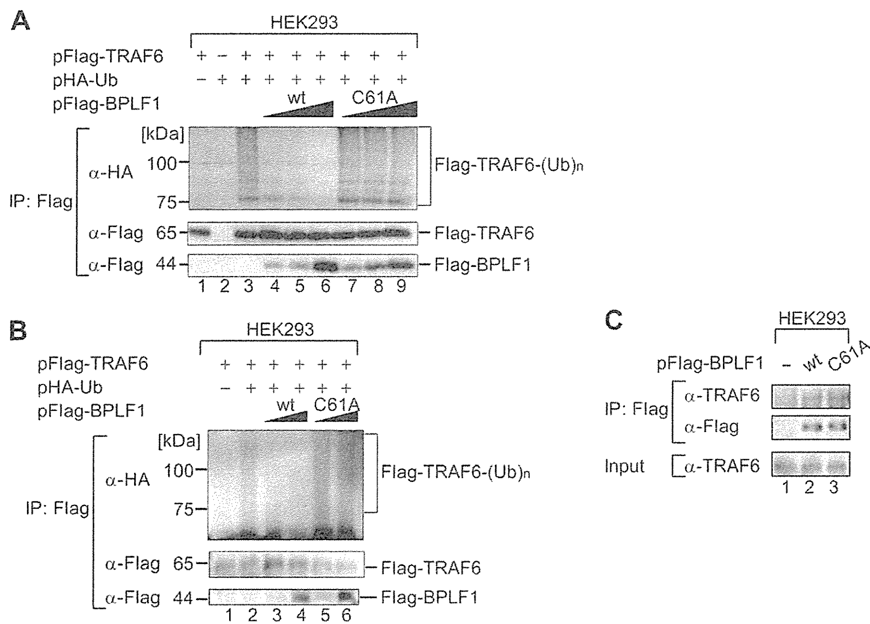

FIG 5 BPLF1 interacts with and inhibits ubiquitination of TRAF6. (A) HEK293 cells cultured in 6-well plates were cotransfected with hemagglutinin (HA)-tagged Ub (2 μg/well) and TRAF6 (3 μg/well) expression plasmids and increasing quantities (0.1, 0.2, or 0.5 μg/well) of the designated BPLF1 expression plasmid. Cell lysates were prepared at 24 hpi and immunoprecipitated (IP) with anti-Flag antibodies, and ubiquitin conjugation of the TRAF6 protein was verified by immunoblotting with anti-HA antibodies. Production of exogenously expressed tagged proteins was verified with the indicated antibodies. The experiment shown is a representative of three independent experiments. (B) The conditions were basically the same as described for panel A except that cells were lysed with the denaturing lysis buffer containing 2% SDS followed by a 10-min incubation at 95°C. The amount of transfected BPLF1 expression plasmid was 0.1 or 0.5 μg/well. The experiment shown is a representative of three independent experiments. (C) HEK293 cells cultured in 6-well plates were transfected with an empty plasmid or designated BPLF1 (0.5 μg/well) expression plasmids. Cell lysates were prepared at 24 hpi and immunoprecipitated with anti-Flag antibodies, followed by immunoblot analysis with anti-TRAF6 antibodies. Production of exogenously expressed BPLF1 proteins was verified with anti-Flag antibody. The experiment shown is a representative of two independent experiments.

biquitinating proteins such as BSLF1 and BXLF1 (36). We suggest that BPLF1 mainly deubiquitinates TRAF6 in the lytic phase of EBV replication, although BSLF1 and/or BXLF1 might also be involved in the deubiquitination of TRAF6.

Upon activation of the canonical NF-κB signaling pathway, IKK (IκB kinase) is activated by phosphorylation of β subunit (IKKβ), and then active IKK phosphorylates IκB, resulting in its proteasomal degradation (57). This liberates NF-κB, which translocates to the nucleus and binds to promoters of NF-κB-regulated genes. Overexpression of BPLF1 resulted in repression of IKKβ

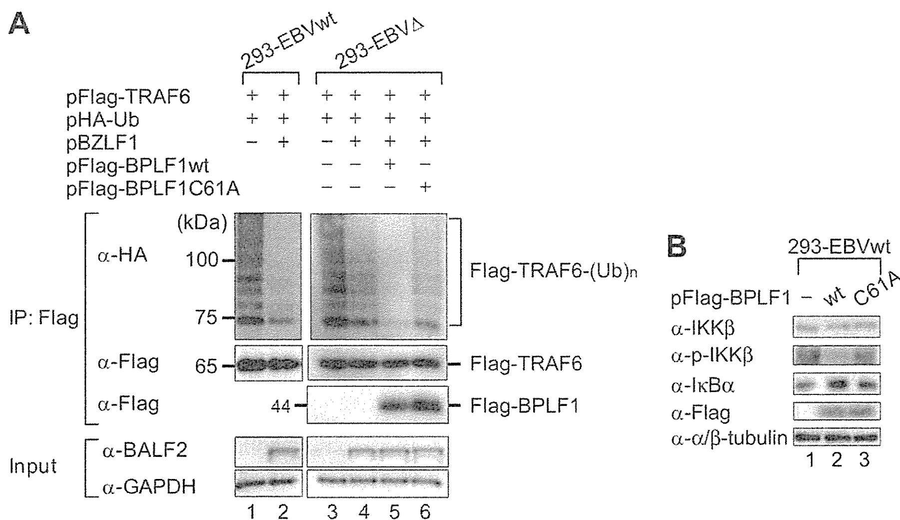

FIG 6 Endogenous BPLF1 deubiquitinates TRAF6. (A) 293-EBVwt and 293-EBVΔ cells cultured in 6-well plates were cotransfected with HA-tagged Ub (2 μg/well), TRAF6 (3 μg/well), and BZLF1 (1 μg/well) expression plasmids. Four hours after the initial transfection, the cells were further transfected with either wild-type or enzyme-dead BPLF1 expression plasmids (0.5 μg/well). Cell lysates were prepared at 24 h after initial transfection, and immunoprecipitation experiments were performed in the same fashion as described for Fig. 5A. The experiment shown is a representative of three independent experiments. (B) 293-EBVwt cells were transfected with empty plasmid or designated BPLF1 (0.5 μg/well) expression plasmid. Cell lysates were prepared at 24 hpi, and immunoblot analysis was performed using indicated antibodies. The experiment shown is a representative of two independent experiments.

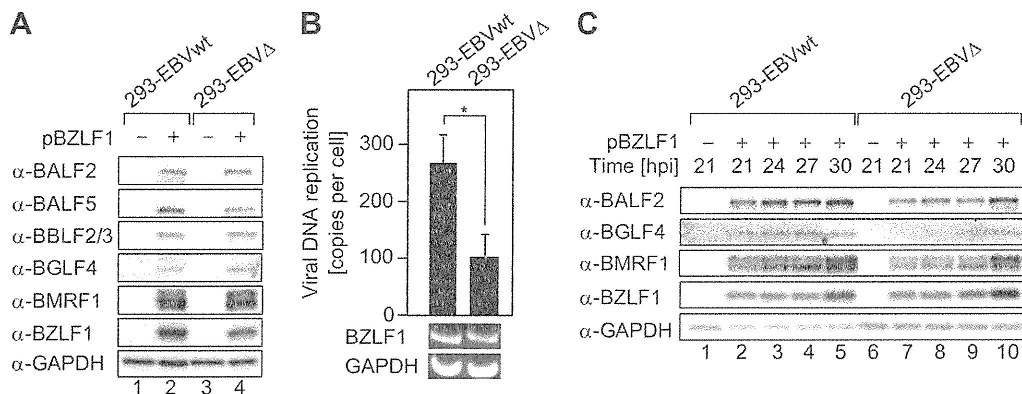

FIG 7 BPLF1 promotes EBV genome replication. (A) 293-EBVwt and 293-EBVΔ cells were transfected with pBZLF1 (1 μg) to induce lytic replication, harvested at 48 hpt, and washed with PBS (−), and then whole-cell lysates were extracted. Protein levels of viral early genes (BALF2, BALF5, BBLF2/3, BGLF4, and BMRF1) and the BZLF1 immediate-early gene were analyzed in 293-EBVwt and 293-EBVΔ cells by immunoblotting. GAPDH was used as an internal control. (B) At 48 h after pBZLF1 (1 μg) transfection, cells were washed with PBS (−), and total DNAs were extracted. qrt-PCR analysis was performed with BALF2- and GAPDH-specific primers. Intracellular viral DNA copy numbers were calculated as follows: BALF2 values were normalized to each GAPDH value, and the BALF2/GAPDH values were further compared to those for Namalwa cells, which maintain 2 EBV genomes per cell. RT-PCR data from one representative experiment are shown. Data are expressed as fold increase in comparison to untransfected cells and means ± SD of the results of 5 biological replicates. *, $P < 0.005$. (C) The threshold necessary amount (0.1 μg) of pBZLF1-transfected 293-EBVwt and 293-EBVΔ cells was cultured for the indicated periods. Protein levels of viral early genes (BALF2, BGLF4, BMRF1) were analyzed by immunoblotting. The experiment shown is representative of two independent experiments.

phosphorylation and accumulation of IκBα protein in 293-EBVwt cells in which the canonical NF-κB signaling is constitutively activated (Fig. 6B, lane 2). Collectively, the findings indicate that BPLF1 blocks ubiquitination of TRAF6, leading to inhibition of IκBα degradation to prevent NF-κB target gene expression.

BPLF1 promotes EBV genome replication. Regarding the effects of BPLF1 DUB activity on EBV lytic DNA replication, immunoblotting revealed that the protein levels of viral early genes (BALF2, BBLF2/3, BGLF4, and BMRF1) were not affected by disrupting BPLF1 expression at 48 hpi, although the level of BALF5 DNA polymerase was to some extent lower (Fig. 7A). However, qrt-PCR using EBV genome DNA-specific primers revealed that viral DNA synthesis in 293-EBVΔ cells at 48 hpi was significantly impaired, comparing with the 293-EBVwt case (Fig. 7B), suggesting that BPLF1 promotes viral genome replication. In addition, when lytic replication was induced with the smaller amount of pBZLF1 (0.1 μg), lytic gene expression in 293-EBVΔ appeared to be lower than in 293-EBVwt at 21, 24, and 27 hpi, although after 30 hpi, comparable levels of expression were observed, suggesting that BPLF1 affects early gene expression under conditions of lower levels of BZLF1 expression (Fig. 7C).

Since ectopically expressed BPLF1 is sufficient for at least inactivation of cellular NF-κB activity (Fig. 1 and 3) and deubiquitination of TRAF6 (Fig. 5 and 6), we tested whether ectopic expression of the BPLF1 DUB domain in 293-EBVΔ cells might promote viral genome replication. The expression of pBPLF1wt in 293-EBVΔ distinctly restored viral DNA synthesis, while expression of the BPLF1 mutant did not (Fig. 8A).

Knockdown of p65 promotes viral DNA replication. We further examined whether inhibition of NF-κB signaling actually promotes viral DNA replication in 293-EBVΔ cells. Cotransfection of p65-targeting siRNA together with pBZLF1 resulted in increased numbers of copies of the EBV genome that are comparable to the level seen with the EBV genome in lytic replication-induced 293 WT cells, while cotransfection of control siRNA (DsRed) had no significant effect (Fig. 8B). Taking our results together, BPLF1 appears to promote lytic viral genome replication, at least partly by blocking the canonical NF-κB signaling via DUB activity.

DISCUSSION

Our present study clearly demonstrated that the DUB activity of BPLF1 is involved in the downregulation of NF-κB signaling in

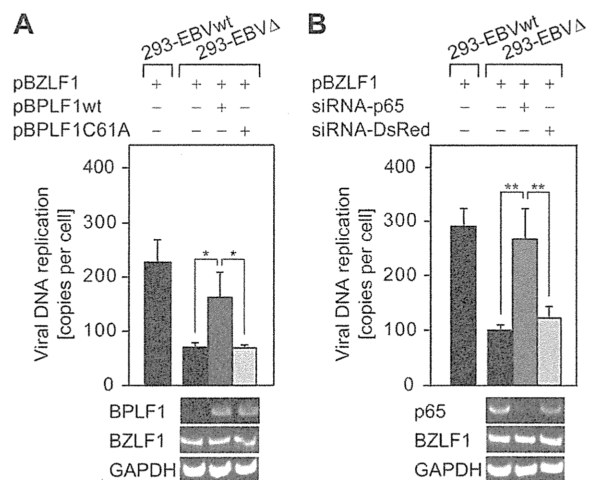

FIG 8 Exogenous expression of BPLF1 deubiquitinase or p65 knockdown restores viral DNA replication of the BPLF1-deficient virus. (A) The BZLF1 expression plasmid (0.5 μg/well) was transfected into 293EBVΔ cells using an electroporator, and 4 h after the initial transfection, the cells were further transfected with wild-type or enzyme-dead BPLF1 expression plasmids (0.5 μg/well) using Lipofectamine 2000. At 48 h after the initial transfection, cells were washed with PBS (−), and total DNA was extracted. qrt-PCR analysis was performed with the same method as described for Fig. 7B. cDNAs were prepared from the mRNAs extracted in parallel with the total DNAs. RT-PCR data from one representative experiment are shown below the graph. (B) p65-targeted or control siRNA (0.2 μg/well) was cotransfected with the BZLF1 expression plasmid (0.2 μg/well) into 293-EBVΔ cells using an electroporator and cultured for 48 h and processed similarly to the method described for panel A. Data are expressed as fold increase in comparison to untransfected cells and means ± SD of the results of 3 biological replicates. **, $P < 0.001$; *, $P < 0.01$.

the context of viral lytic replication. The immediate-early BZLF1 protein, a key initiator of EBV lytic replication, is known to interact with the NF-κB family member p65/RelA to inhibit its transcriptional activity, and p65/RelA in turn inhibits the transcriptional activity of BZLF1 (58). Also, Brown et al. reported that overexpression of p65 inhibits EBV lytic replication, and they predicted that cells expressing a high level of active NF-κB would hardly enter the lytic life cycle (24). It was also reported that NF-κB activation inhibits lytic cycle induction (25). Furthermore, in some cell lines such as B95-8 and HH514 (59), lyLMP1, an amino-terminally truncated and late-lytic-cycle-associated form of LMP1, is expressed to function as a dominant-negative regulator of NF-κB signaling by LMP1 (59, 60). Thus, EBV appears to utilize various strategies to downregulate NF-κB activity during lytic replication, highlighting the biological relevance of NF-κB inhibition by BPLF1. Our results, together with those of previous reports (24, 25), strongly support the idea that BPLF1 is necessary to establish cellular circumstances with decreased NF-κB activity for the lytic life cycle to proceed.

It has been reported that upregulation of NF-κB promotes host cell survival but inhibits the initiation of lytic replication in EBV-infected cells (61). There are several reports demonstrating that NF-κB inhibitors cause spontaneous apoptosis and lytic gene expression in EBV-positive B-lymphocytes and in nasopharyngeal and Burkitt's lymphoma cells (46–49). We showed here that expression of the N-terminal 325-aa region of BPLF1 carrying DUB activity was sufficient to suppress NF-κB activity in latently EBV-infected cells. However, expression of BPLF1 itself did not induce early lytic gene expression in B95-8, AGS-EBV, and 293-EBVwt cells (Fig. 1). Also, no death was observed in cells transfected with pFlag-BPLF1. Our results indicate that BPLF1 downregulates NF-κB signaling in a DUB activity-dependent manner, but it likely causes neither spontaneous apoptosis nor lytic gene expression in latently infected cells, at least under our conditions.

The known deubiquitinases of other viruses, including PLP2 of murine hepatitis virus A59 (62), ORF64 of Kaposi's sarcoma-associated herpesvirus (63), and L^{pro} of foot-and-mouth disease virus (64), were reported to provide an opportunity for effective virus invasion into a new host by downregulating beta interferon (IFN-β) activity. Downregulation of IFN-β activity may result in suppression of numerous IFN-stimulated genes, including important antiviral molecules such as PKR, MX1, OAS1, ISG15, and TRIM5 (65). Meanwhile, whether downregulation of canonical NF-κB target genes such as AGT, CCL-2, and ICAM-1 is physiologically advantageous to EBV lytic replication has yet to be elucidated. The AGT gene encodes angiotensinogen, a precursor of angiotensin II, which conversely activates such transcription factors, including the canonical NF-κB (66). Monocyte chemoattractant protein-1 (MCP-1; also known as CCL2) has been shown to mediate recruitment of monocytes to inflamed sites (67–69). Intercellular adhesion molecule 1 (ICAM-1) is a glycosylated, integral membrane protein that plays an important role in inflammatory responses by promoting cell-cell interactions (70). It also serves as a counterreceptor for lymphocyte function-associated antigen 1, which is found on all types of leukocytes and has been implicated in migration of leukocytes to sites of inflammation (71–73). A variety of viral proteins are expressed in lytic replication-induced cells, and they should be targeted by the host immune system. EBV may downregulate the expression of molecules that otherwise recruit monocytes and leukocytes to the infected cells, which would be disadvantageous to EBV productive replication.

The BPLF1 protein is conserved among members of the herpesvirus family and has been classified as a potential tegument protein by theoretical computer analysis using the Swiss-Prot database (74). Many details concerning functional domains have been elucidated, primarily through studies on BPLF1 homologs such as herpes simplex virus 1 (HSV-1) pUL36 and pseudorabies virus (PrV) pUL36. The results of these studies collectively suggest that the C-terminal part of the protein containing multiple binding sites for the capsid protein is critical for the virus production. In cells infected with HSV-1 lacking UL36 or a mutant encoding only the first 361 aa of pUL36, the capsids reach the cytosol, but there is no secondary envelopment, no cell egress, and no plaque formation (75, 76). Also, the PrV pUL36 is essential for production of virus particles (77, 78). Whitehurst et al., however, have previously reported that knockdown of BPLF1 expression with short hairpin RNA resulted in decreased viral particle production but that it did not completely inhibit the production (27). We also confirmed that induction of lytic replication in cells harboring the BPLF1-deleted EBV genome produced infectious viruses, although the yield of BPLF1-deleted EBV was 0.65-fold lower than that of wild-type virus.

Since Brown et al. reported that the canonical NF-κB inhibits activation of the early lytic BHLF1 promoter harboring BZLF1- and BRLF1-responsive elements (24), we speculate that NF-κB-mediated inhibition of early lytic genes is released by BPLF1 at the beginning of lytic replication. They also indicated that inhibition of lytic promoters by NF-κB is reversible: overexpression of BZLF1 and BRLF1 restored lytic promoter activation. In accordance with the literature cited above, early lytic protein expression in 293-EBVΔ appeared to be lower than that in 293-EBVwt when the lytic replication was induced with a smaller amount of pBZLF1 (Fig. 7C). The decreased levels of lytic proteins were observed until 27 hpi, but comparable levels were observed at 30 hpi. Our results suggest that the impairment of viral DNA synthesis in 293-EBVΔ cells could be partly due to the decreased expression of early genes at around 27 hpi, although the impairment was observed even with the higher BZLF1 expression. A study on human CMV (HCMV) UL48, the counterpart of BPLF1, demonstrated that the mutant virus, which has full-length but catalytically inactive UL48(C24S), replicated more slowly than the wild type and with lower yields of extracellular virus (79). Our results, together with those of the study on UL48, suggest that the loss of viral DUB activity was partly attributable to the decreased and delayed expression of early genes. Interestingly, the growth kinetics of the UL48(C24S) mutant virus were similar to those of the wild type at a multiplicity of infection (MOI) of 3, whereas the mutant virus infection produced about 10-fold-fewer progeny virions than did wild-type virus at an MOI of 0.1. In addition, slightly reduced levels of viral immediate-early, early, and late proteins were observed in Western blot analysis in the mutant virus compared to the wild type at a low MOI.

It is reported that EBV BILF1 and BLLF3 proteins expressed in later stages of lytic replication again upregulate NF-κB signaling (80, 81). EBV G-protein-coupled receptor (EBV BILF1) also appeared to activate the NF-κB pathway in COS-7 and Burkitt's lymphoma cells (82). Furthermore, activation of NF-κB by the EBV dUTPase (EBV BLLF3) through TLR-2 has been previously described (80). These proteins might protect the host cell from

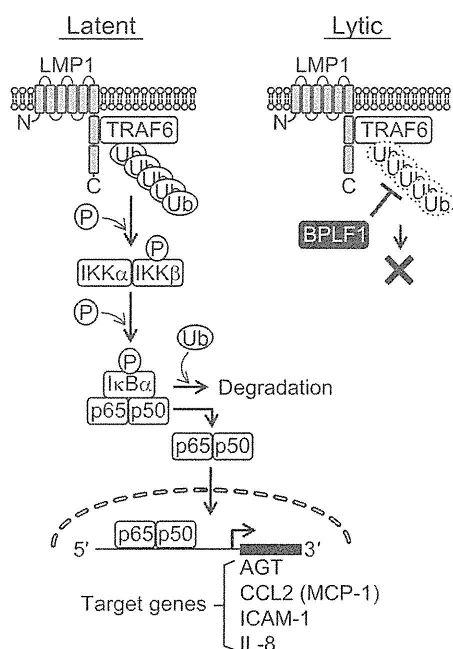

FIG 9 A schematic model demonstrating the inhibition of NF-κB signaling by BPLF1 in the EBV life cycle. In EBV latent infection, NF-κB is activated by viral LMP1 protein; TRAF6 associates with LMP1 and is constitutively polyubiquitinated. Activation of NF-κB confers cell survival (83) and inhibition of spontaneous lytic replication as well (24). Changes in the host cell microenvironment or other unknown triggers can downregulate the NF-κB activity and disrupt the balance between the latent cycle and the lytic cycle of EBV (61). Once lytic replication is induced, BPLF1 then deubiquitinates and inactivates TRAF6 to further block NF-κB signaling, promoting efficient viral genome replication.

death caused by cytopathic effects of viral infection in later phases of lytic replication. We propose that BPLF1 is an accelerating agent of the latent-to-lytic switch that antagonizes NF-κB function at the earlier phase of lytic replication (Fig. 9). BPLF1 may reduce the biological threshold of NF-κB activity required for switching from the latent to the lytic life cycle of EBV.

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