Cellular differentiation regulator BLIMP1 induces Epstein-Barr virus lytic reactivation in epithelial and B cells by activating transcription from both the R and Z promoters

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Abstract: EBV maintains a life-long latent infection within a subset of its host’s memory B cells, while lytic EBV replication takes place in plasma cells and differentiated epithelial cells. Therefore, cellular transcription factors such as BLIMP1 that are key mediators of differentiation likely contribute to the EBV latent-to-lytic switch. Previous reports showed that ectopic BLIMP1 expression induces reactivation in some EBV(+) B-cell lines and transcription from Zp, with all Z(+) cells in oral hairy leukoplakia being BLIMP1(+). Here, we examined BLIMP1’s role in inducing EBV lytic-gene expression in numerous EBV(+) epithelial and B-cell lines and activating transcription from Rp. BLIMP1 addition was sufficient to induce reactivation in latently infected epithelial cells derived from gastric cancers, nasopharyngeal carcinomas, and normal oral keratinocytes (NOK) as well as some, but not all B-cell lines. BLIMP1 strongly induced transcription from Rp as well as Zp, with there being three or more synergistically acting BLIMP1-responsive elements (BRE) within Rp. BLIMP1’s DNA-binding domain was required for reactivation, but BLIMP1 did not directly bind the nt -660 Rp BRE. siRNA knockdown of BLIMP1 inhibited TPA-induced lytic reactivation in NOK-Akata cells, cells that can be reactivated by R, but not Z. Thus, we conclude that BLIMP1 expression is both necessary and sufficient to induce EBV lytic replication in many (possibly all) EBV(+) epithelial-cell types, but in only a subset of EBV(+) B-cell types; it does so, at least in part, by strongly activating expression of both EBV immediately-early genes, BZLF1 and BRLF1.

Importance: This study is the first one to show that the cellular transcription factor BLIMP1, a key player in both epithelial and B-cell differentiation, induces reactivation of the oncogenic herpes virus, Epstein-Barr virus (EBV), out of latency into lytic replication in a variety of cancerous epithelial cell types as well as in some, but not all B-cell types that contain
this virus in a dormant state. The mechanism by which BLIMP1 does so involves strongly
turning on expression of both of the immediate-early genes of the virus, probably by directly
acting upon the promoters as part of protein complexes or indirectly by altering the expression or
activities of some cellular transcription factors and signaling pathways. The fact that EBV(+) cancers usually contain mostly undifferentiated cells may be due in part to these cells dying from
lytic EBV infection when they differentiate and express wild-type BLIMP1.

INTRODUCTION

Epstein-Barr virus (EBV) is a human γ-herpes virus that infects over 90% of the world’s
population. Like all herpes viruses, it has both latent and lytic phases to its life cycle. Initial
infection is generally mild and asymptomatic when the virus is acquired before adolescence. If
infected after adolescence, the host may develop infectious mononucleosis (IM) (1, 2). After the
primary lytic infection, EBV establishes a life-long latent infection in a subset of its host’s
memory B cells where its genome is maintained as an episome (3). Latent EBV infection has
been linked to several epithelial and B-cell malignancies, including Burkitt lymphoma (BL),
nasopharyngeal carcinoma (NPC), some gastric cancers, and post-transplant lymphoproliferative
disease (4–12).

EBV-positive [EBV(+)] cells can reactivate into lytic replication, leading to the
production and shedding of infectious virus. EBV lytic replication is initiated by activation of
transcription from one or both of its immediate-early (IE) promoters, Zp and Rp, leading to
production of its two IE proteins, Z (also called Zta, ZEBRA, EB1, the product of the BZLF1
gene) and R (also called Rta, EB2, the product of the BRLF1 gene), respectively (13). In most
EBV(+) cell lines, synthesis of Z is sufficient to initiate reactivation (14–17). R can frequently
initiate reactivation as well given R and Z are transcription factors that can usually activate each other’s promoters (18–24). However, R, not Z, is necessary in some cases such as telomerase-immortalized normal oral keratinocytes (NOK) that have been infected with the Akata strain of EBV (25). Together, R and Z induce expression of the viral early (E) genes, including BMRF1 which encodes the viral DNA polymerase-processivity factor [also known as early antigen diffuse (EAD)] (26–29). Thus, regulation of Zp and Rp serves as the gatekeeper to the EBV latent-to-lytic switch in a cell-type-dependent manner.

Zp has been intensively studied, with there being numerous well-mapped positive and negative regulatory domains [reviewed in (30–33)]. Unfortunately, much less is known to date about regulation of Rp [reviewed in (33); see Fig. 4A below]. Among Rp’s known cis-acting regulatory elements are binding sites for the cellular factors NF1, YY1, EGR-1, and ZEB1/2. Sp1/Sp3 binding sites have also been identified within Rp; they are necessary for both constitutive promoter activity and auto-activation by R (34). Rp is also activated by Z via three Z-responsive elements (ZRE) and further activated by Z and R in combination (35).

Spontaneous lytic reactivation of EBV is normally initiated during the differentiation of B cells into plasma cells (PC); it is also observed in the more differentiated layers of the oral epithelium, indicating that the environment of a differentiated cell plays a critical role in this process (36, 37). However, the identities of the several cellular factors that contribute to initiating the EBV latent-lytic switch during differentiation and the precise mechanisms by which they do so have yet to be determined. To elucidate the connection between cellular differentiation and lytic EBV reactivation, we have been investigating whether cellular factors known to play key roles in epithelial and/or B-cell differentiation can induce EBV reactivation. Previously, we (38) and others (39) showed that XBP-1 is one such factor, inducing lytic EBV
replication by activating transcription from Zp. Here, we examined the mechanisms of EBV reactivation by another such factor, B-lymphocyte-induced maturation protein-1 (BLIMP1, also known as PRDI-BF1 and PRDM1).

BLIMP1 is necessary for differentiation of B cells into plasma cells (40, 41). It is also a key player in epithelial cell differentiation (42, 43). The 789-amino acid BLIMP1 protein contains five zinc-finger domains, the first two of which are critical for the ability of the protein to bind directly to DNA via sequences resembling the BLIMP1 consensus site, 5’-(A/C)AG(T/C)GAAAG(T/C)(G/T)-3’ [see Fig. 5A below; (44, 45)]. BLIMP1 transcriptionally silences a myriad of genes during plasma cell differentiation, including those encoding the transcription factors PAX5, Oct-2, and ID3, as well as genes involved in cell cycle progression such as p53 and c-Myc (46–49). BLIMP1 represses its target genes through a variety of mechanisms, including recruiting Groucho family proteins, HDAC1/2, and histone methyltransferase G9a (50–54).

Given that the PAX5 and Oct-2 proteins inhibit Z activity (55, 56) and PAX5 represses synthesis of XBP-1 in lymphoid cells (57), one might expect that addition of BLIMP1 can induce EBV lytic-gene expression in some EBV(+) B-cell lines under some conditions; this hypothesis has been validated (58). Others reported that while expression of EBV Z protein was restricted to BLIMP1-positive epithelial cells in oral hairy leukoplakia (OHL) tissue samples, this relationship did not hold in IM tonsillar B cells, suggesting that BLIMP1 may be necessary for induction of EBV lytic-gene expression during differentiation of epithelial, but not B-cells (59).

Here, we show that expression of BLIMP1 is sufficient to induce EBV lytic-gene expression in EBV(+) epithelial cell lines derived from gastric cancers, NPCs, and NOK. We demonstrate that BLIMP1 is also necessary for differentiation-induced lytic reactivation of EBV.
in at least some epithelial cell types. We further show that BLIMP1 can induce transcriptional activation of Rp over 300-fold as well as Zp 20- to 50-fold in some epithelial cell lines and identify several regions within Rp that act synergistically to enable this high-level activation by BLIMP1. Thus, we conclude that BLIMP1 is a key player in EBV lytic reactivation in epithelial cells; it does so, at least in part, by inducing expression of the BRLF1 as well as BZLF1 gene.

MATERIALS AND METHODS

Cell lines. The gastric carcinoma cell line, AGS [obtained from the American Type Culture Collection (ATCC)], and the EBV-infected clone derived from it, AGS-Akata (a gift from Lindsey Hutt-Fletcher), were maintained in F12 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals) and 100 U/ml penicillin and 100 μg/ml streptomycin (pen-strep; Life Technologies). The NPC cell line, HONE1 (a gift from Ron Glaser), the EBV-infected clone derived from it, HONE-Akata (a gift from Lawrence Young), the EBV-infected NPC line, CNE2-Akata (a gift from K. W. Lo via Diane Hayward), the naturally derived EBV-infected gastric carcinoma cell line, SNU-719 (60), and all EBV(+) B-cell lines were maintained in RPMI 1640 (Life Technologies) supplemented with 10% FBS and pen-strep. Though originally derived from NPCs, the HONE1 and CNE2 cell lines may have become contaminated with HeLa cells, resulting in somatic hybrids (61). NOK (a gift from Karl Munger via Paul Lambert) is a telomerase-immortalized normal oral epithelial keratinocyte cell line; NOK-Akata cells were derived from it by infection with the Akata strain of EBV (25). They were maintained in K-SFM supplemented with 0.2 ng/ml epidermal growth factor and 25 μg/ml bovine pituitary extract (Life Technologies). Akata EBV-infected epithelial cells were maintained in medium also supplemented with G418: 400 μg/ml for AGS-Akata and HONE-
Akata; and 50 µg/ml for NOK-Akata. The B-cell lines Sal and Oku (gifts from Alan Rickinson via David Vereide), MutuI (a gift from Alan Rickinson), and KemI and KemIII (gifts from Jeffrey Sample) are derived from EBV(+) BLs. Sal and Oku cells maintain a Wp-restricted latency (62, 63), MutuI and KemI cells maintain a type I latency, and KemIII cells maintain a type III latency. Raji cells were obtained from the ATCC. Human embryonic kidney 293T cells (obtained from the ATCC) were maintained in DMEM (Life Technologies) supplemented with 10% FBS and pen-strep. All cells were maintained at 37°C in a 5% CO₂ atmosphere.

**Plasmids.** Plasmid DNAs were purified using QIAGEN Plasmid Maxi Kits as described by the manufacturer. Plasmid pcDNA-FLAG-PRDI-BF1 (pcDNA3-BLIMP1) expresses an amino-terminal FLAG-tagged BLIMP1 (64). Plasmids pc-PRDI-BF1-ΔPR (pcDNA3-ΔPR), pc-PRDI-BF1-ΔPRO (pcDNA3-ΔPro), and pc-PRDI-BF1-ΔZn (pcDNA3-ΔZn) express the C-terminal FLAG-tagged BLIMP1 mutant variants described in reference (65); their structures are schematically shown in Figure 5A below. Plasmid pSG5-Z [a gift from Diane Hayward; (66)] expresses the EBV IE protein, Z. Plasmid pGL3-CIITAprom3 contains the firefly luciferase-coding region under the control of the promoter of the cellular CIITA gene which is known to be strongly repressed by BLIMP1 (65, 67). Plasmid pCpGL-Zp-668 (25) contains the nt -668 through +15 region of EBV Zp relative to the transcription initiation site cloned between the SpeI and BglII restriction sites of pCpGL, a CpG-free vector, driving expression of the firefly luciferase-coding region [a gift from Michael Rehli; (68)]. Plasmid pCpGL-Rp-673 is a luciferase reporter construct that contains the nt -673 to +38 region of Rp relative to the transcription initiation site cloned between the SpeI and BglII restriction sites of pCpGL.

All of the pCpGL-Rp mutant variants described here are derivatives of this “full-length” pCpGL-Rp-673 promoter construct. For 5’-deleted variants of Rp, the number in the plasmid
name indicates the endpoint of the deletion in nucleotides upstream of the transcription initiation site. To construct these pCpGL-Rp mutants, the appropriate PCR-amplified region of Rp was inserted between the SpeI and BgII restriction sites of pCpGL. The Rp 2-bp substitution mutants, mt1, 2, 3, 4, and 5, were produced by site-directed mutagenesis of pCpGL-Rp-673 according to the Strategene QuikChange Site-Directed Mutagenesis Kit protocol using the following primers:

- **mt1**: 5’-GACTAGTCAAGCTGACGGGACGTTAAAGCCACAAG-3’; mt2: 5’-
- **mt2**: 5’-CTAGTCAAGCTGACGGGACGTTAAAGCCACAAG-3’; mt3: 5’-
- **mt3**: 5’-CTAGTCAAGCTGACGGGACGTTAAAGCCACAAG-3’; mt4: 5’-
- **mt4**: 5’-GTCAAGCTGACCATGGGACGTTAAAGCCACAAGCTTG-3’; and mt5: 5’-
- **mt5**: 5’-GTCAAGCTGACCATGGGACGTTAAAGCCACAAGCTTG-3’.

Methylation of pCpGL-Zp-668, pCpGL-Rp-673, pCpGL-mt3, and pCpGL-Rp-655 was performed in vitro using M. SssI (NEB) according to the manufacturer’s instructions. Digestion with restriction enzyme HpaII (NEB), which cleaves its recognition sequence only when the DNA is unmethylated, was used to confirm methylation of the DNAs.

**Transient transfection and reporter gene assays.** For immunoblot analyses, HONE-Akata, AGS-Akata, NOK-Akata, and SNU-719 were transiently transfected with the indicated DNAs using Lipofectamine 2000 (Invitrogen) transfection reagent according to the manufacturer’s instructions. For luciferase assays, transient transfections of AGS, NOK, and 293T cells were carried out in 12-well plates using Lipofectamine 2000, with the medium changed 3 h after addition of the transfection reagent/DNA mixture to minimize cell death. Cells were harvested 48 h post transfection and processed with 5X Lysis Buffer (Promega) following the manufacturer’s instructions. Relative luciferase units were measured in a BD Monolight 3010 Luminometer (BD Biosciences) with Promega Luciferase Assay Reagent. Each condition was
performed in triplicate on two or more separate occasions with similar results. Extracts were also subjected to immunoblot analysis (not shown) to verify that similar BLIMP1 or Z protein levels were present in each sample following co-transfection with the indicated expression plasmids.

**Immunoblot analysis.** Cells were harvested in SUMO buffer and processed as previously described (69). Proteins in the whole-cell extracts were separated by electrophoresis at 100 V for 90 min in SDS 10% polyacrylamide gels and transferred to nitrocellulose membranes (ISC Biosystem). The membranes were blocked by pre-incubation for 1 h at 23°C with PBS containing 5% BSA, 0.1% Tween-20 solution when probing with BLIMP1 antibody and 5% milk, 0.1% Tween-20 solution when probing with all of the other antibodies. They were then incubated with the primary antibody in their respective blocking buffer overnight at 4°C.

The primary antibodies were β-actin (Sigma-Aldrich A5441; 1:2,000), GAPDH (Genscript A00192; 1:5,000), α-tubulin (Sigma-Aldrich T5168; 1:5,000), BLIMP1 (Cell Signaling 9115S; 1:500), Involutrin (Sigma-Aldrich I9018; 1:3,000), Z (BZLF1; Santa Cruz sc-53904; 1:500), and EAD (BMRF1; Millipore MAB8186; 1:3,000). Additionally, we used a rabbit antibody specific to the EBV R protein (1:2,000) generated for us by Pierce Biotechnology from the peptide EDPDEETSQAVKALREMAD which corresponds to R amino acid residues 506-524, respectively. Membranes were washed and then incubated with the secondary antibody for 1 h at room temperature. Secondary antibodies were horseradish peroxidase (HRP)-conjugated mouse (ThermoScientific 31430; 1:10,000) or rabbit (GE Healthcare NA934; 1:10,000) antibody as appropriate. Blots were developed with Luminata Cresendo Western HRP Substrate (Millipore WBLUR0100). The band intensities were quantified with ImageJ software and internally normalized to GAPDH.
Infectious virus assay. CNE2-Akata cells (latently infected with a GFP-expressing EBV) were transfected in 22 mm-diameter wells with either (i) 100 ng pcDNA3-BLIMP1 plus 400 ng pcDNA3.1 or (ii) 500 ng pcDNA3.1 using Lipofectamine 2000. The medium was harvested four days later and passed through a 0.8-µm filter. Ten µl of this medium was added to 4 x 10^5 Raji cells in 15.4 mm-diameter wells. Twenty-four hours later, TPA (20 ng/ml) and sodium butyrate (3 mM) were added to the infected Raji cells and incubation was continued for another 24 h. The number of GFP (+) Raji cells, representing “green Raji units” (GRU), was determined by ultraviolet microscopy (70).

Chromatin immunoprecipitation (ChIP) assays. HONE-Akata cells were transfected in 10 cm-diameter dishes with 6 µg of pcDNA3.1 or pCDNA3-BLIMP1 as indicated. Two days later, the cells were cross-linked by incubation in fresh 1% paraformaldehyde for 10 min at room temperature. The cross-linking reaction was quenched by addition of glycine to 125 mM, and the cells were lysed by Dounce homogenization. The lysate was sonicated 3 times for 30 sec to yield DNA fragments approximately 500-bp in size. The DNA-protein complexes were immunoprecipitated by incubation at 4°C overnight with 2 µg anti-FLAG (Sigma; F1804) or mouse anti-IgG (Santa Cruz; sc-2025) as a control. Immunoprecipitated DNA-protein complexes were sequentially washed with low salt (0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), high salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), lithium chloride (0.5% sodium deoxycholate, 1 mM EDTA, 250 mM LiCl, 0.5% IGEPAL® CA-630 (Sigma; 18896), 10 mM Tris-HCl, pH8.1), and Tris-EDTA (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Protein-DNA cross-linking was reversed by incubation at 65°C overnight. DNA was purified using Qiagen Gel Extraction Kits.
PCR for 30 cycles was performed on the ChIPed DNAs to determine the relative presence of specific fragments using GoTaq® Flexi DNA Polymerase (Promega; M8291).

Primers were as follows: Rp +63 to -139: 5’-CTCTTACCTGCCTCTTTTG-3’ and 5’-

CTCTCTGCTGCCCAATAAGG-3’; c-Myc: 5’-CGCCTGCGATTTATATCTC-3’ and 5’-CGCCTACCACATTTTCTTTTG-3’

(55); Rp -512 to -770: 5’-

ATAAGGCTGCCAATAAGGTG-3’; and β-globin: 5’-AGGGCTGGGCAAAGTCGTA-3’

(42); Qp: 5’-GACCACCTGAGGAGTGTCCACACAG-3’ and 5’-

ACACCGTGCGAAAAGAAGCA-3’; and β-globin: 5’-AGGGCTGGGCAATAAGGTG-3’

(25). PCR products were electrophoresed at 100 V for 20 min in a 1% agarose gel in 1X Tris-borate-EDTA (TBE) buffer (pH 8.0) containing 0.5 µg/ml ethidium bromide and visualized by UV light.

Quantification of the ChIPed DNA was performed by quantitative PCR (qPCR) using iTaq™ Universal SYBR green supermix (Biorad; 172-5121). The DNAs were detected with an ABI Prism 7900 real-time PCR system (Applied Biosystems). Input samples were diluted to 5%, 1%, and 0.2% with dH₂O containing 100 µg/ml sheared salmon sperm DNA (Ambion; AM9680).

Primers were as follows: c-Myc: 5’- CGCCTGCGATTTATATCTC-3’ and 5’-

CGCCTACCACATTTTCTTTTG-3’ (42); Rp -40 to -173: 5’-

GCATGGGCGGGACAATCGCAATATAA-3’ and 5’-

GCGAGAGCTTTGGACACACAA-3’; Rp -708 to -859: 5’-

CAGACATCCAAATGACCACGTGAGG-3’ and 5’-GAGTCATCGAGGTGAGGATTTC-3’;

and β-globin: 5’-GGCAACCCTAAGGTGAAGGC-3’ and 5’-

GATGAGCCAGGGCCATCACTA-3’ (71). A standard curve was calculated from the threshold
cycle ($C_T$) of the input dilution series and used to calculate the relative amount of each specific DNA present in the ChIPed samples. All assays were performed in triplicate.

Electrophoretic mobility shift assays (EMSAs). HONE1 cells in 10 cm-diameter dishes were transfected with 6 μg pcDNA3-BLIMP1 or pcDNA3.1. Nuclear extracts were harvested 48 h post transfection and prepared as previously described (72). EMSAs were performed using these nuclear extracts essentially as previously described (73) with the addition of 0.5 μM ZnCl₂. As a control, the nuclear extracts were incubated with FLAG-specific antibody (Sigma; F1804) at 23°C for 20 min prior to addition of the double-stranded radiolabeled probe DNA and incubation for an additional 20 min at 23°C. Probe DNA sequences were as follows: CIITA p3: 5’-GTCCACAGTAAGGAAGTGAAATTAATTTCAGAG-3’ (64); Rp-673 to -633: 5’-CAAGCTGCACATGACCACGTAAAGCCACAAGCTTGTGGACC-3’; and Rp-642 to -601: 5’-CTTGTGGACCAACATGTCAGAGATTGACAGGACTACACTG-3’. These mixtures were electrophoresed in a non-denaturing 4% polyacrylamide gel in 0.5X TBE running buffer at 4°C for 2 h at 200 V. Gels were dried and exposed to a phosphor screen.

siRNA knockdown. NOK-Akata cells in a 12-well plate were transfected with 20 pmol of a universal scrambled control siRNA (SR30004; Origene) or a BLIMP1-targetting siRNA (SR300437B; Origene) using Lipofectamine RNAiMAX according to the manufacturer’s instructions. Three days later, the cells were treated with TPA (20 ng/ml) for 24 h immediately prior to harvesting and processing for immunoblot analysis.

Lentivirus infection of B cells. A BLIMP1-expressing lentivirus, CD713-BLIMP1, was constructed by insertion of the BLIMP1-coding sequences between the EcoRI and BamHI restriction sites of pCDH-MSCV-MCS-EF1-GFP-puro (System Biosciences; CD713B-1); the
former DNA was obtained by digestion of pCDNA3-BLIMP1 DNA with EcoRI and BamHI restriction enzymes (NEB) and gel purification.

Two lentiviruses expressing shRNAs against BLIMP1 mRNA that had been constructed in pLKO.1-TRC were originally purchased from Thermo Scientific; they can now be obtained from Sigma-Aldrich [product type: SHCLNG-NM_001198; TRC numbers: TRCN0000013612 (BLIMP1 #1) and TRCN0000013610 (BLIMP1 #2)]. Two non-targeting shRNAs, cntrl #1 [Addgene; #1864; (74)] and cntrl #2 (Sigma; SHC002), were used as controls.

293T cells in 10 cm-diameter dishes were co-transfected with (i) 4 µg of the indicated lentivirus vector, (ii) 1.4 µg pCMV-dR8.2 dvpr (Addgene; #8455), and (iii) 0.6 µg VSVG (Addgene; #12259). The medium containing the virus was harvested at 48 h and again at 72 h post transfection; both times, the virus was passed through a 0.8 µm pore-sized filter and shortly thereafter added to the B-cells being infected with it. KemI, Sal, and Oku cells were incubated with 1 µg/ml puromycin beginning at 72 h after infection to select for lentivirus-positive cells. MutuI and KemIII cells showed a robust initial infection and required no further selection for our short-term experiments.

RESULTS

BLIMP1 induces lytic replication in EBV(+) epithelial cells. BLIMP1 is an important factor in epithelial as well as B-cell differentiation. Whether BLIMP1 expression is sufficient to induce EBV lytic reactivation in epithelial cells has not been previously investigated. To answer this question, we examined the effect of addition of BLIMP1 to HONE-Akata, AGS-Akata, NOK-Akata, and SNU-719 cells on EBV lytic-gene expression. HONE-Akata and AGS-Akata cells were derived from a NPC and gastric carcinoma cell line, respectively, that had been
infected with the Akata strain of EBV. BLIMP1 expression in these cells induced high-level synthesis of the lytic viral proteins Z, R, and EAD (Fig. 1, lane 2 vs. lane 1 and lane 4 vs. lane 3, respectively).

NOK-Akata cells were derived from a telomerase-immortalized normal oral keratinocyte cell line that had been infected with the Akata strain of EBV (25). Given their origin, they represent a more physiologic model for EBV infection than do cells derived from tumors. Once again, addition of BLIMP1 strongly induced EBV lytic-gene expression (Fig. 1, lane 6 vs. lane 5). We also examined the effect of BLIMP1 addition to SNU-719, a cell line derived from a gastric carcinoma that maintains its original latent EBV. We observed strong induction of Z, R, and EAD synthesis (Fig. 1A, lane 8 vs. lane 7) here as well.

To determine whether BLIMP1-induced reactivation proceeded all the way to production of infectious virus, we harvested the culture medium from CNE2-Akata cells transfected 4 days earlier with pcDNA3-BLIMP1 or its empty vector as a control. CNE2-Akata is another EBV(+) epithelial cell line, this one derived from an NPC that had been latently infected with a GFP-marked Akata strain of EBV. After filtration to remove cells, the relative amount of infectious EBV present in the medium was determined by superinfection of Raji cells; the BLIMP1-expressing cells produced approximately 40-fold more virus than did the control ones (Fig. 1B).

Thus, five distinct EBV(+) epithelial cell lines derived from a variety of different, physiologically relevant cell types all similarly responded to BLIMP1 addition. Therefore, we conclude that BLIMP1 expression is, indeed, sufficient to induce EBV lytic reactivation in many (possibly all) EBV(+) epithelial cell lines.

**BLIMP1 stimulates EBV lytic reactivation in B cells in a cell-line-dependent manner.**

Given BLIMP1 addition was reported to induce EBV lytic-gene expression in Akata BL and
several LCLs (58), we next asked whether BLIMP1 does so in all EBV(+) B-cell lines. To answer this question, we infected a variety of EBV(+) BL-derived lines with a BLIMP1-expressing lentivirus, CD713-BLIMP1. We first examined two Wp-restricted latency lines, Sal and Oku, in which the EBV latency proteins EBNA1, -3A, -3B, -3C, and -LP are expressed. As expected, addition of BLIMP1 strongly induced Z, R, and EAD expression (Fig. 2, lane 2 vs. lane 1 and lane 6 vs. lane 5, respectively).

Next, we examined the effect of BLIMP1 addition in a type III latency line, KemIII, in which all nine EBV latency proteins are expressed. KemIII differs from the other cell lines examined here in that it constitutively expresses some BLIMP1 (Fig. 2, lane 9). Nevertheless, it was still somewhat responsive, although less so than Sal and Oku, with smaller increases in expression of Z, R, and EAD starting from a higher background level (Fig. 2, lane 10 vs. lane 9).

We also examined the effect of BLIMP1 addition in two type I latency cell lines, MutuI and KemI, in which EBNA1 is the only EBV protein expressed. Unexpectedly, addition of BLIMP1 was not sufficient to induce reactivation; rather, it led to decreased Z protein levels (Fig. 2, lane 14 vs. lane 13 and lane 18 vs. lane 17). Thus, we conclude that BLIMP1 addition induces reactivation in some, but not all EBV(+) B-cell lines.

TPA (12-O-tetradecanoyl-phorbol-13-acetate) is a well-known chemical inducer of EBV reactivation in some, but not all EBV(+) cell lines; it does so by activating the protein kinase C (PKC) pathway (75–77). The five B-cell lines examined here are among ones where incubation with TPA for 2 days has little, if any effect on EBV lytic-gene expression (Fig. 2, lane 3 vs. lane 1, lane 7 vs. lane 5, lane 11 vs. lane 9, lane 15 vs. lane 13, and lane 19 vs. lane 17). Nevertheless, when BLIMP1 addition was combined with TPA incubation, we observed strong synergistic reactivation in all five of these B-cell lines (Fig. 2, lanes 4, 8, 12, 16, and 20 vs. lanes 2 and 3,
lanes 6 and 7, lanes 10 and 11, lanes 14 and 15, and lanes 18 and 19, respectively). Thus, we conclude that the cellular environment can affect whether BLIMP1 expression is sufficient to induce EBV lytic reactivation in B cells.

BLIMP1 strongly activates both Zp and Rp. BLIMP1 was previously shown to induce transcription from Zp in a reporter assay (59); whether it affected Rp was not determined. Given R, but not Z, induces reactivation in NOK-Akata cells (25), we hypothesized that BLIMP1 also induces transcription from Rp. To test this possibility, we co-transfected EBV(-) AGS and NOK cells with a plasmid containing the firefly luciferase gene driven by the Z promoter (pCpGL-Zp-668), R promoter (pCpGL-Rp-673), or their empty vector, pCpGL, together with pcDNA3-BLIMP1 or its empty vector, pcDNA3.1. As expected, BLIMP1 activated transcription from Zp; however, it activated transcription from Rp even more, i.e., approximately 340-fold (Figs. 3A and 3B). Similar results were observed in EBV(-) HONE1 cells (data not shown).

DNA methylation status plays a major role in regulation of expression of EBV genes, with EBV genomes being highly methylated in EBV(+) cancers (25, 78–80). Therefore, we next asked whether BLIMP1 induces transcriptional activation from methylated as well as unmethylated Zp and Rp. To answer this question, we in vitro methylated versus mock-treated our pCpGL-Zp-668 and pCpGL-Rp-673 luciferase reporter DNAs prior to co-transfection into AGS cells along with the BLIMP1 expression plasmid. Methylation had no effect on BLIMP1-induced activation of transcription from Zp, while it reduced activation of transcription from Rp approximately 40%, with activation of Rp still several-fold higher than activation of Zp (Fig. 3C).

Furthermore, BLIMP1 induced EBV lytic-gene expression in cell lines regardless of whether the EBV genome was methylated (BL-derived lines; Fig. 2) or unmethylated [NOK-Akata; Fig. 1, lane 6; (25, 79, 80)].
Transcriptional activation is uncharacteristic of BLIMP1 given it usually functions as a repressor (46, 48, 50, 53, 81, 82). Thus, for a control, we examined likewise the effect of BLIMP1 addition on transcription from the promoter of the cellular gene CIITA. CIITA is a transcriptional co-activator that regulates MHC-II gene expression; its CIITAp3 promoter is known to be strongly repressed by BLIMP1 (65, 67, 83). 293T cells were used in this experiment because the CIITAp3 promoter exhibits high basal activity in these cells and, therefore, its repression by BLIMP1 can be readily observed. As expected, co-transfection of 293T cells with pcDNA3-BLIMP1 decreased expression from a CIITAp3-driven luciferase reporter by 90% (Fig. 3D).

Thus, we conclude that BLIMP1 strongly induces transcription from both Zp and Rp; it does so regardless of whether these viral promoters are methylated.

**BLIMP1-induced activation of Rp involves several synergistically acting elements.**

To begin to understand how BLIMP1 expression induces high-level transcriptional activation of Rp, we asked what regions of Rp are needed for it to occur. Unfortunately, our current knowledge regarding how Rp is regulated is far from complete (Fig. 4A). Thus, we first looked for BLIMP1 consensus binding sites, 5’-(A/C)AG(T/C)GAAAG(T/C)(G/T)-3’, in Rp; none were identified. Next, we constructed a series of 5’-truncated variants of our full-length pCpGL-Rp-673 reporter and compared their ability to be activated by BLIMP1 in AGS cells. Over multiple replicates of this experiment, we observed that while Rp-664 was activated by BLIMP1 similarly to Rp-673, there was a 5- to 11-fold decrease in activation of Rp-655 (Fig. 4B). Further deletion up to -267 did not result in additional significant changes in activation by BLIMP1. However, deletion to -106 led to a further decrease of 4- to 6-fold, with BLIMP1-induced transcriptional activation of Rp-106 relative to its empty vector control still being a substantial 6- to 10-fold.
Thus, we conclude that Rp contains three or more elements through which BLIMP1 induces activation: one maps between nt -664 and -655, a second between nt -267 and -106, and a third between nt -106 and +38; these elements act synergistically, with each one contributing 5-fold or more for a total activation of over 300-fold.

**BLIMP1 activates Rp in part via a nt -660 region element.** To identify the exact bases involved in BLIMP1-induced activation via the nt -664 to -655 region of Rp, we constructed a series of variants of pCpGL-Rp-673 containing 2-bp substitution mutations throughout this region (Fig. 4C) and assayed them for activation by BLIMP1 as described above. All five Rp mutants exhibited some decrease in BLIMP1-induced activation (Fig. 4D). Activation of Rp-655 was almost as reduced as the deletion mutant Rp-655 which lacks this entire 10-bp region, while the basal transcriptional activities of these mutants were unaffected (Figs. 4D and 4E).

As an additional control to ensure that this 5-fold loss of Rp activation by BLIMP1 was not due to these mutations inadvertently impairing more generally the ability of Rp to function, we also tested the ability of Z protein to activate the two most impaired of these Rp mutants. Given Z strongly activates methylated Rp, but not unmethylated Rp (25, 78, 80), we *in vitro* methylated pCpGL-Rp-655 and full-length WT pCpGL-Rp-673 DNA prior to their co-transfection along with a Z expression plasmid; both of these Rp mutants were normally activated by Z (Fig. 4F). Therefore, we conclude that a sequence element that includes nt -660 and/or -659 is a significant contributor to high-level BLIMP1-induced activation of Rp.

We asked whether the sequence surrounding this nt -660 BLIMP1-responsive element (BRE) might also be present within the nt -267 to -106 or nt -106 to +38 BLIMP1-responsive regions of Rp; it was not. This finding indicates that BLIMP1 likely induces Rp through several mechanisms.
PR and Zn-finger domains of BLIMP1 are necessary for Rp activation. BLIMP1 contains five major domains: two acidic regions located at the N- and C-termini, a PRDI-BF1-RIZ1 (PR) domain, a proline-rich (Pro) domain, and a Zn-finger DNA-binding (Zn) domain. The Pro domain contributes to recruiting co-factors to BLIMP1 complexes, while the Zn-finger domain is required for its ability to directly bind DNA (51, 65). To try to gain further insights into the mechanism by which BLIMP1 induces EBV reactivation, we examined the activities of variants of BLIMP1 with deletions in these domains (depicted in Fig. 5A). First, we compared their abilities to induce EBV lytic-gene expression after transfection into HONE-Akata cells. While ΔPro induced expression of Z, R, and EAD to levels similar to those observed with wild-type BLIMP1, ΔPR and ΔZn failed to induce synthesis of any of these three EBV lytic proteins (Fig. 5B, lanes 2 and 4 vs. lanes 3 and 5). Therefore, we conclude that both the PR and Zn-finger domains are necessary for BLIMP1-induced reactivation of EBV in epithelial cells.

To determine whether the failure of these mutant proteins to induce reactivation was due, in part, to a defect in Rp activation, we co-transfected AGS cells with pCpGL-Rp-673 and these BLIMP1 expression plasmids. As expected, both ΔPR and ΔZn were also unable to induce high-level activation of Rp-673 (Fig. 5C). Thus, we conclude that the mechanism through which the PR and Zn-finger domains of BLIMP1 are necessary for induction of EBV lytic reactivation in epithelial cells likely involves, at least in part, their roles in inducing BRLF1 gene expression.

BLIMP1 associates with Rp in vivo. Given BLIMP1 requires its Zn-finger domain both to activate Rp and to induce EBV lytic reactivation, we hypothesized that BLIMP1 may bind Rp even though we failed to identify a BLIMP1 consensus sequence within Rp. To test this possibility, we performed ChIP assays for BLIMP1 binding to Rp. HONE-Akata cells were transfected with pcDNA3-BLIMP1 versus pcDNA3.1. Forty-eight hours later, the cells were
harvested and processed for ChIP. The ChIPed DNA was subjected to PCR analysis for the presence of Rp, c-Myc as a positive control, and EBV Qp and cellular β-globin as negative controls. Consistent with our hypothesis, BLIMP1 associated \textit{in vivo} with both the promoter proximal and distal regions of Rp that we showed above contain BREs (Figs. 6A and 6B).

To test whether BLIMP1 directly binds Rp DNA, we performed electrophoretic-mobility-shift assays (EMSAs) using a radio-labeled probe corresponding to the region of Rp encompassing our nt -660 BRE. As negative and positive controls, we used probes corresponding to a nearby region of Rp and the region of the CIITA p3 promoter known to bind BLIMP1 (64), respectively. While BLIMP1 bound well to the positive control, no binding above background was observed with the Rp probe encompassing the nt -660 BRE (Fig. 6C, lanes 7 and 8 vs. lanes 3 and 4). These data indicate that BLIMP1 does not activate Rp by directly binding to the nt -660 BRE; however, it may interact with this region of Rp indirectly as part of a protein complex.

\textbf{Requirement of BLIMP1 for EBV lytic-gene expression.} Incubation with TPA induces cellular differentiation in a variety of cell types, including epithelial and B cells (84–88). As expected, treatment of NOK-Akata cells with TPA induced them to differentiate as indicated by an increase in the level of the keratinocyte differentiation factor, involucrin, as well as activation of expression of endogenous BLIMP1 (Fig. 7A, lane 2 vs. lane 1). To determine if BLIMP1 was necessary for the induction of EBV reactivation during epithelial cell differentiation, we transfected NOK-Akata cells with a BLIMP1-targeting siRNA to inhibit BLIMP1 synthesis prior to adding TPA. Cells transfected in parallel with a control siRNA expressed a physiological level of BLIMP1 as a consequence of differentiation as well as Z, R, and EAD (Fig. 7B, lane 1). However, the levels of BLIMP1, Z, R, and EAD were reduced relative to the control by
approximately 90%, 90%, 50%, and 60%, respectively, in the cells that contained the BLIMP1-targeting siRNA (Fig. 7B, lane 2 vs. lane 1). Thus, we conclude that the physiological level of BLIMP1 that appears during differentiation of these epithelial cells is probably a necessary contributor to reactivation of EBV.

We also investigated the effect of knocking down BLIMP1 in KemIII which constitutively express some BLIMP1 along with EBV lytic proteins (Fig. 2, lane 9). Being a B-cell line, these cells were infected with a lentivirus expressing a BLIMP1-targeting shRNA (BLIMP1 #1, BLIMP1 #2) or a non-targeting shRNA (cntrl #1, cntrl #2) for three days prior to infection with a BLIMP1-expressing lentivirus. Both BLIMP1-targeting shRNAs reduced not only the BLIMP1 level by greater than 80% relative to the control shRNAs, but also the levels of Z by greater than 90% and 70%, R by greater than 50% and 90%, and EAD by greater than 80% and nearly 50%, respectively (Fig. 7C, lanes 3 and 4 vs. lanes 1 and 2). Thus, we conclude that BLIMP1 is, in at least some cases, also necessary for EBV lytic-gene expression in B cells.

DISCUSSION

In this study, we examined the role of BLIMP1, an important regulator of both epithelial and plasma cell differentiation, in inducing EBV out of latency into lytic replication. We showed that BLIMP1 is sufficient to induce EBV lytic-gene expression in a variety of EBV(+) epithelial cells lines (Fig. 1) as well as in some, but not all EBV(+) B-cell lines (Fig. 2). We confirmed that BLIMP1 induces transcription from Zp, and discovered it also induced transcription from Rp, doing so to a very high level (Fig. 3). This latter transcriptional activation involved, in part, a cis-acting element that includes Rp nt -660 and/or -659 (Fig. 4) along with the PR and Zn-finger domains of BLIMP1 (Fig. 5). Knock-down of BLIMP1 in both epithelial and B cells led to...
reduction in expression of EBV lytic genes (Fig. 7). Combining these findings with ones reported previously (58, 59), we conclude that BLIMP1 is a key inducer of EBV lytic reactivation during both epithelial and B-cell differentiation, doing so by inducing expression of both of EBV's immediate-early genes, *BZLF1* and *BRLF1*; while not sufficient to induce EBV lytic-gene expression in some B-cell types, BLIMP1 expression appears to be sufficient in all of the epithelial cell types examined to date.

**BLIMP1-induced EBV reactivation.** A previous report showed that BLIMP1 can induce EBV lytic-gene expression in Akata BL and several LCLs (58). Here, we provided a much broader analysis of the range of EBV(+) cell lines in which BLIMP1 can induce EBV reactivation by looking at epithelial cell lines derived from three different tissue types and B-cell lines expressing three different types of EBV latency. While our findings confirmed that BLIMP1 addition was sufficient to induce EBV reactivation in some B-cell lines, we discovered others in which it failed to do so (Fig. 2). On the other hand, BLIMP1 induced reactivation in all five of the EBV(+) epithelial cell lines we examined (Fig. 1). Consistent with our findings, Buettner *et al.* (59) observed that all Z(+) cells within an OHL lesion are also BLIMP1(+), while only 1/3rd- to 2/3rd of Z(+) B cells from tonsils of IM patients contain detectable BLIMP1. Thus, we conclude that BLIMP1 expression leads to induction of EBV lytic-gene expression in many (possibly all) EBV(+) epithelial-cell types, but not all EBV(+) B-cell types.

Incubation with TPA greatly increased the responsiveness of EBV(+) B cells to BLIMP1, even in two type I latency cell lines, MutuI and KemI, that were otherwise unresponsive to it (Fig. 2). This finding reveals a synergy between BLIMP1 and changes in the cellular environment fostered by TPA. Incubation with TPA also led to higher levels of BLIMP1 in Sal, Oku, and KemIII cells. However, the synergy observed with TPA plus BLIMP1 was not solely
due to enhancement in BLIMP1 level since it was also observed in MutuI and KemI cells where TPA had the former effect without the latter one.

Incubation with TPA leads to activation of numerous cellular transcription factors through activation of the PKC pathway (76, 77) and stimulates cells to differentiate (84–88).

Given EBV is reactivated in one type I latency BL cell line, Akata, with BLIMP1 alone while two others, MutuI and KemI, require TPA along with BLIMP1, we hypothesize that there exists a balance of cellular transcription factors and pathways necessary for BLIMP1-induced reactivation that is already present (or absent) in Akata cells, with TPA modifying their levels and/or activities in MutuI and KemI cells and altering them somewhat in Sal, Oku, and KemIII cells. For example, one such factor may be the cellular transcription factor Ets-1; it can bind BLIMP1, inhibiting its activity (89, 90), while expression of Ets-1 is inhibited in B cells by incubation with TPA (91, 92).

**BLIMP1 vs. BLIMP1β.** Human BLIMP1 is expressed in two major isoforms, α and β. BLIMP1α is the full-length, fully functioning form described here as BLIMP1. BLIMP1β is a truncated isoform, arising from an alternative promoter, that differs from BLIMP1α by beginning with three unique amino acids, MEK, and continuing with BLIMP1 amino acids 102-789 (64). BLIMP1β is functionally impaired, exhibiting approximately half the repressive activity of BLIMP1 on natural target promoters (64). Infection of germinal center B cells with some strains of EBV leads to synthesis of BLIMP1β (93). We did not detect the presence of this isoform in any of our EBV(+) B-cell lines (Fig. 2); nor did we detect it in the Donor 4 LCL derived by infection of primary blood B cells with the B95.8 strain of EBV (data not shown). Therefore, we conclude that BLIMP1β is not expressed in many EBV(+) B-cell lines, and it need not contribute to BLIMP1-induced EBV lytic reactivation.
How does BLIMP1 induce EBV reactivation? Here, we showed that while both Zp and Rp are strongly activated by BLIMP1, Rp is activated even more than Zp in epithelial cells (Fig. 3). Thus, while BLIMP1 activation of Zp is probably the primary mechanism by which it induces EBV reactivation in B cells given EBV(+) B cells are much more responsive to Z than R, BLIMP1 activation of Rp may be the primary mechanism in epithelial cells given they are often more and sometimes exclusively responsive to R (15, 16, 20, 25).

To begin to understand the mechanism by which BLIMP1 induces high-level activation of Rp, we performed a deletional analysis of this promoter, identifying three distinct regions that functioned synergistically (Fig. 4). The most upstream BRE was precisely mapped to include nt -660 and/or -659. The only known transcription factor binding site sequence in this region is an E-box (5'-'CANNTG'-3') spanning nt -666 to -661. However, this E-box does not influence BLIMP1-induced Rp activation given our finding that pCpGL-Rp-664 was as strongly activated as pCpGL-Rp-673. The more promoter-proximal BREs have yet to be mapped precisely; with luck, the identities of the factors that recognize these BREs may become clear once they are.

We examined by both semi-quantitative and quantitative ChIP assays whether BLIMP1 activation of Rp might occur via it binding to these BREs, finding that BLIMP1 associates with Rp at or somewhere near (i) the nt -660 BRE and (ii) one or both of the promoter-proximal BREs (Fig. 6A and 6B). Given the limited resolution of these assays, ChIP-sequencing data would be needed to determine more precisely where BLIMP1 binds within Rp. ChIP assays also cannot be used to distinguish between directly binding to Rp versus indirectly binding via a protein complex. In the case of the nt -660 BRE, we addressed this later question by EMSAs, failing to observe BLIMP1 binding to a probe spanning Rp nt -674 to -633 (Fig. 6C). Thus, we conclude that BLIMP1 either directly binds the nt -660 BRE but too weakly or transiently to be detected.
by EMSAs, BLIMP1 directly binds Rp somewhat near but not at the -660 BRE, or BLIMP1 indirectly associates with Rp as part of a protein complex.

In some cases, BLIMP1’s proline-rich domain is necessary for transcriptional repression via recruitment of Groucho family proteins and HDACs (51–53). In other cases, repression of a target promoter is independent of the proline-rich domain, but partially dependent upon BLIMP1’s PR domain (65). Here, we mapped the domains of BLIMP1 critical for activation of Rp and induction of EBV lytic-gene expression; one of them was the PR domain (Fig. 5). How BLIMP1’s PR domain contributes to repression of target genes such as CIITA (65) has yet to be determined; the homologous region of the RIZ1 protein is known to serve as a protein-binding interface, allowing for the formation of multi-protein complexes (94). Taken together with our EMSA and ChIP findings (Fig. 6), these data indicate that BLIMP1 may induce Rp activation in part as a component of a protein complex that binds the nt -660 BRE as an activator (Fig. 8A).

The zinc-finger DNA-binding domain of BLIMP1 was also found to be essential for induction of EBV lytic-gene expression and Rp activation (Fig. 5). Given BLIMP1 only requires its two N-terminal-most zinc-fingers for DNA-binding function (44), we also examined a BLIMP1 mutant variant lacking only these two zinc-fingers; it was similarly unable to induce EBV lytic-gene expression and Rp activation (data not shown). Thus, the mechanism of EBV reactivation probably involves BLIMP1 binding to both DNA and some transcriptional co-regulators.

Although BLIMP1 has been reported to act as a transcriptional repressor, many transcription factors have been shown to act as either activators or repressors depending upon the promoter context and cellular environment. Given our ChIP data, we hypothesize that BLIMP1 may activate Rp by indirectly binding to it as one component of multi-protein transcription
activating complexes (Fig. 8A). Alternatively, BLIMP1 may act indirectly by repressing synthesis of a protein(s) that inhibits the activity or level of another protein(s) that post-translationally modifies one or more transcriptional regulators of Rp, altering the latter’s activities (Fig. 8B). For example, BLIMP1 may inhibit the synthesis of some phosphatases or proteases that target kinases that phosphorylate some co-activators of Rp into their functionally active forms. Another possibility is BLIMP1 may repress synthesis of a factor(s) that, in turn, represses synthesis of an activator(s) of Rp (Fig. 8C). An example of the latter in B cells might be BLIMP1 repressing expression of PAX5 (48), thereby relieving repression of XBP-1 by PAX5 (57), thus enabling XBP-1 to activate both Rp and Zp (38, 39). These models are not mutually exclusive. Given several BREs exist on Rp, they could potentially function via different mechanisms, increasing the possible ways by which Rp can be activated under various growth conditions and cell types.

**Requirement for BLIMP1.** Lastly, we showed in both epithelial and B cells that knock down of BLIMP1 led to a reduction in EBV lytic-gene expression (Fig. 7). Thus, BLIMP1 is probably a key contributor to induction of EBV lytic reactivation during cellular differentiation given BLIMP1 expression is activated during the normal physiological process of differentiation in epithelial and B cells (Fig. 9). Combining all of our findings together with those of Buettner et al. (59), we conclude that this activation of BLIMP1 expression may well be necessary as well as sufficient in epithelial cells, but not in B cells, for induction of EBV reactivation.

**Conclusion.** BLIMP1, a key player in both epithelial and B-cell differentiation, also plays a major role in the induction of EBV lytic-gene expression during differentiation of epithelial and B cells. BLIMP1 is sufficient and, at least in some cases, necessary for EBV lytic reactivation in epithelial cells; it does so via inducing expression of both of EBV’s immediate-
early genes, *BZLF1* and *BRLF1*. The fact that EBV(+) cancers usually contain mostly undifferentiated cells may be due in part to these cells dying from lytic EBV infection when they differentiate and express wild-type BLIMP1.

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FIG 1  Immunoblots showing BLIMP1 induction of lytic reactivation in EBV-positive epithelial cell lines. (A) EBV(+) HONE-Akata, AGS-Akata, and NOK-Akata in 35 mm-diameter wells were transiently transfected as follows: HONE-Akata, (i) 300 ng pcDNA3-BLIMP1 or pcDNA3.1 plus (ii) 700 ng SG5 empty control vector; and AGS-Akata and NOK-Akata, 1 μg pcDNA3.1 or 750 ng pcDNA3-BLIMP1 plus 250 ng pcDNA3.1. GAPDH served as a loading control. SNU-719 cells in 22 mm-diameter wells were transiently transfected with 500 ng pcDNA3.1 or 100 ng pcDNA3-BLIMP1 plus 400 ng pcDNA3.1. Whole-cell extracts were prepared 48 h post transfection. The control samples were processed concurrently with their corresponding BLIMP1-containing ones, with irrelevant intervening lanes omitted where indicated. β-actin served as a loading control. (B) Raji assay showing addition of BLIMP1 to CNE2-Akata cells induces the entire lytic cycle of infection, leading to greatly enhanced production of infectious virus within 4 days.

FIG 2  Immunoblots showing BLIMP1 induction of lytic reactivation in EBV-positive B cells. EBV(+) BL cells were infected with the lentivirus CD713-BLIMP1 or CD713, its empty vector, as a control. CD713-positive KemI, Sal, and Oku cells were selected by incubation with puromycin (1 μg/ml) for 2 weeks, and then incubated with TPA (20 ng/ml) or DMSO (1 μl/ml) as a control for 48 h immediately prior to preparation of whole-cell extracts. Given high-level lentivirus infection, MutuI and KemIII cells were simply incubated for 4 days post infection without puromycin selection prior to incubation with TPA or DMSO for 48 h and harvesting. GAPDH served as a loading control.
FIG 3  Luciferase reporter assays showing BLIMP1 strongly induces transcriptional
activation from both Zp and Rp. (A) EBV(-) AGS cells in 22 mm-diameter wells were co-
transfected with (i) 50 ng pcDNA3-BLIMP1 or pcDNA3.1, and (ii) 450 ng of the CpG-free
luciferase reporter (pCpGL) containing no promoter (cntrl), Zp-668, or Rp-673. Fold activation
is presented on a log_{10} scale normalized to each promoter’s activity in the absence of BLIMP1.
(B) EBV(-) NOK cells in 22 mm-diameter wells were co-transfected with (i) 200 ng pcDNA3-
BLIMP1 or pcDNA3.1, and (ii) 300 ng of pCpGL-Zp-668, pCpGL-Rp-673, or pCpGL. Fold
activation is presented on a log_{10} scale. (C) BLIMP1-induced transcriptional activation from
methylated Zp and Rp DNA relative to activation from the unmethylated versions of these same
dNAs. AGS cells in 22 mm-diameter wells were co-transfected with (i) 50 ng of mock-treated or
methylated pCpGL-Zp-668 or pCpGL-Rp-673, (ii) 50 ng pcDNA3-BLIMP1 or pcDNA3.1, and
(iii) 400 ng pSG5. (D) 293T cells in 22 mm-diameter wells were co-transfected with (i) 10 ng
pcDNA-BLIMP1 or pcDNA3.1, and (ii) 490 ng pGL3-CIITAp3 or its empty vector, pGL3-Basic.
Cells were harvested 48 h post transfection. Data were normalized to each promoter’s activity
when co-transfected with pcDNA3.1. They are representative means of assays performed in
triple on two or more occasions. Error bars indicate standard deviations within one
representative set of assays performed in triplicate.

FIG 4  High-level BLIMP1-induced transcriptional activation of Rp involves three or more
elements that act synergistically, including one requiring nt -660 and/or -659. (A) Schematic
indicating factors known to be involved in regulating Rp expression and the cis-acting elements
through which they act. Not drawn to scale. (B) Luciferase reporter assays performed with
deleted variants of Rp. AGS cells in 22 mm-diameter wells were co-transfected with (i) 50 ng
pcDNA3-BLIMP1 or pcDNA3.1, and (ii) 450 ng of a variant of pCpGL-Rp-673 containing the indicated 5′-deletion end point of Rp. Data were normalized both to each promoter’s activity when co-transfected with pcDNA3.1 and to the relative activity observed with Rp-673. (C) Sequences of the Rp nt -673 to -650 region of the wild-type Rp-673 and 2-bp substitution mutant variants of it studied here, with the mutations indicated in bold font. (D) Luciferase reporter assays performed with the 2-bp substitution mutants shown in panel C. AGS cells in 22 mm-diameter wells were co-transfected with (i) 50 ng pcDNA3-BLIMP1 or pcDNA3.1, and (ii) 450 ng pCpGL-Rp-673, pCpGL-Rp-655, or one of the 2-bp mutant variants of pCpGL-Rp-673. Data were normalized both to each promoter’s activity when co-transfected with pcDNA3.1 and to the activity of the WT Rp-673. (E) Basal transcriptional activity of some of the Rp mutants. The data were obtained from the luciferase assays shown in panel D performed with pcDNA3.1, with normalization to the level observed with Rp-673. (F) Luciferase reporter assays showing the Rp mutants are not defective in activation by Z. AGS cells in 22 mm-diameter wells were co-transfected with (i) 50 ng mock-treated or methylated pCpGL-Rp-673, pCpGL-Rp-mt3, or pCpGL-Rp-655, and (ii) 5 ng pSG5-Z plus 445 ng pSG5 or 450 ng pSG5. Data were normalized to each promoter’s activity in the absence of pSG5-Z. All data are representative means of assays performed in triplicate on two or more occasions; error bars indicate standard deviations within one representative set of assays performed in triplicate.

**FIG 5** PR and zinc-finger domains of BLIMP1 are critical for inducing reactivation. (A) Schematic showing structures of BLIMP1 wild-type and variants deleted in the PRDI-BF1-RIZ1 (PR), Proline-rich (Pro), and Zn-finger DNA-binding (Zn) domains. Not drawn to scale. (B) Immunoblot showing relative levels of some EBV lytic proteins following expression of WT
BLIMP1 and the BLIMP1 variants depicted in panel A. HONE-Akata cells in 35 mm-diameter wells were transfected with (i) 300 ng of the indicated pcDNA3-BLIMP1 variant or pcDNA3.1, and (ii) 700 ng pSG5. Whole-cell extracts were prepared 48 h post transfection. All samples were present in the same immunoblots, with irrelevant lanes omitted. GAPDH served as a loading control. (C) Luciferase assays showing relative abilities of the variant BLIMP1 proteins to activate Rp. AGS cells in 22 mm-diameter wells were co-transfected with (i) 450 ng pCpGL-Rp-673, and (ii) 50 ng of the indicated pcDNA3-BLIMP1 variant depicted in panel A or pcDNA3.1. Data were normalized to WT BLIMP1-induced activation of Rp-673. They are representative means of assays performed in triplicate on two separate occasions. Error bars indicate standard deviations within one representative set of assays performed in triplicate.

FIG 6  BLIMP1 associates with Rp in vivo, but does not directly bind the nt -660 BRE in vitro. (A) Semi-quantitative ChIP assay for BLIMP1 association with Rp. HONE-Akata cells in 10 cm-diameter dishes were transfected with 6 µg pcDNA3-BLIMP1 (encoding FLAG-tagged BLIMP1) or pcDNA3.1. Cells were processed for ChIP 48 h later and immunoprecipitated with a FLAG-specific or mouse IgG control antibody. ChIPed DNA was subjected to PCR analysis with the indicated primers specific to Rp, c-Myc as a positive control, and EBV Qp and cellular β-globin as negative controls. (B) Quantitative PCR analysis of the ChIPed DNA from the experiment described in panel A. The ChIPed DNA was subjected to qPCR with primers specific to Rp, cellular β-globin, and c-Myc. (C) EMSA to look for direct binding of BLIMP1 to the Rp nt -660 BRE in vitro. HONE1 cells in 10 cm-diameter dishes were transfected with 6 µg pcDNA3-BLIMP1 or pcDNA3.1. Nuclear extracts were prepared 48 h post transfection and incubated with radio-labeled probes corresponding to the indicated regions of Rp or the cellular
promoter, CIITA<sub>p3</sub>, as a positive control. A FLAG-specific antibody was used to supershift FLAG-BLIMP1:DNA complexes.

**FIG 7** Requirement of BLIMP1 for EBV lytic-gene expression in NOK-Akata and KemIII cells. (A) Immunoblot showing effect of TPA treatment on the expression of markers of differentiation in NOK-Akata cells. NOK-Akata cells in 22 mm-diameter wells were treated with TPA (20 ng/ml) or DMSO (1 µl/ml) as a control for 48 h immediately prior to preparation of whole-cell extracts. The samples were present on the same blots, with an irrelevant intervening lane omitted. α-tubulin served as a loading control. (B) Immunoblot showing effect of BLIMP1 knockdown in NOK-Akata cells. NOK-Akata cells in 22 mm-diameter wells were transfected with 20 pmol of either a universal scrambled control or a BLIMP1-targeting siRNA. Three days later, the cells were incubated with TPA (20 ng/ml) for an additional 24 h prior to preparation of whole-cell extracts. (C) Immunoblot showing effect of BLIMP1 knockdown in KemIII cells. KemIII cells were infected with a lentivirus expressing the indicated shRNAs. Three days later, the cells were infected with the BLIMP1-expressing lentivirus, CD713-BLIMP1, and incubated for an addition 48 h prior to preparation of whole-cell extracts. GAPDH served as a loading control in panels B and C.

**FIG 8** Models for BLIMP1-mediated transcriptional activation of Rp. (A) BLIMP1 may act by indirectly binding to Rp as part of a multi-protein complex(es) that activates transcription from Rp. (B) BLIMP1 may act indirectly through repression of synthesis of a protein(s) (Q) that inhibits the activity or level of a protein(s) (P) that post-translationally modifies transcriptional regulators of Rp (X, Y, Z), altering the latter’s activities. (C) BLIMP1 may act indirectly through
repressing synthesis of a repressor(s) (Rep) of factors (X, Y, Z) that activate transcription from Rp. Each BRE on Rp could potentially function via one or more of these non-mutually exclusive mechanisms depending upon the cell type and culture conditions.

FIG 9  Model for BLIMP1 induction of EBV reactivation during cellular differentiation. As epithelial and B cells differentiate, expression of the gene encoding BLIMP1 is activated, leading to synthesis of BLIMP1 and, consequently, the induction of lytic EBV infection with production of infectious virus in EBV(+) epithelial and some B cell types.
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<td><img src="%CE%94Pro.png" alt="Image" /></td>
<td><img src="%CE%94Zn.png" alt="Image" /></td>
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<td><img src="%CE%94Zn.png" alt="Image" /></td>
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</table>

C. **Fold Rp-673 activation (rel. to WT BLIMP1):**

<table>
<thead>
<tr>
<th>BLIMP1</th>
<th>WT</th>
<th>ΔPR</th>
<th>ΔPro</th>
<th>ΔZn</th>
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<td><img src="%CE%94Pro.png" alt="Image" /></td>
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</tr>
</tbody>
</table>
Lytic Replication

B cells

Latency

BLIMP-1

Differentiation

Epithelial cells