SUMO Binding by the Epstein-Barr Virus Protein Kinase BGLF4 Is Crucial for BGLF4 Function

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An Epstein-Barr virus (EBV) protein microarray was used to screen for proteins binding noncovalently to the small ubiquitin-like modifier SUMO2. Among the 11 SUMO binding proteins identified was the conserved protein kinase BGLF4. The mutation of potential SUMO interaction motifs (SIMs) in BGLF4 identified N- and C-terminal SIMs. The mutation of both SIMs changed the intracellular localization of BGLF4 from nuclear to cytoplasmic, while BGLF4 mutated in the N-terminal SIM remained predominantly nuclear. The mutation of the C-terminal SIM yielded an intermediate phenotype with nuclear and cytoplasmic staining. The transfer of BGLF4 amino acids 342 to 359 to a nuclear green fluorescent protein (GFP)-tagged reporter protein led to the relocalization of the reporter to the cytoplasm. Thus, the C-terminal SIM lies adjacent to a nuclear export signal, and coordinated SUMO binding by the N- and C-terminal SIMs blocks export and allows the nuclear accumulation of BGLF4. The mutation of either SIM prevented SUMO binding in vitro. The ability of BGLF4 to abolish the SUMOylation of the EBV lytic cycle transactivator ZTA was dependent on both BGLF4 SUMO binding and BGLF4 kinase activity. The global profile of SUMOylated cell proteins was also suppressed by BGLF4 but not by the SIM or kinase-dead BGLF4 mutant. The effective BGLF4-mediated dispersion of promyelocytic leukemia (PML) bodies was dependent on SUMO binding. The SUMO binding function of BGLF4 was also required to induce the cellular DNA damage response and to enhance the production of extracellular virus during EBV lytic replication. Thus, SUMO binding by BGLF4 modulates BGLF4 function and affects the efficiency of lytic EBV replication.

Received 7 February 2012 Accepted 29 February 2012 Published ahead of print 7 March 2012

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doi:10.1128/JVI.00314-12
The BGLF4 phosphorylation of cellular elongation factor 1-δ (EF1-δ) may play a role in viral protein translation (54, 56). The phosphorylation of MCM4 by BGLF4 blocks host chromosomal DNA replication in the infected cells (60). The mimicry of cyclin-dependent kinase activity by BGLF4 results in the phosphorylation of both retinoblastoma protein (Rb) and p27 and leads to a prolonged pseudo S-phase environment suitable for efficient viral DNA replication (49, 61). An in vitro screen of human proteins phosphorylated by the herpesvirus conserved kinases identified more than 100 shared substrates with a statistical enrichment of proteins involved in the DDR (64). The BGLF4-dependent phosphorylation of several proteins in the DDR cascade, such as TIP60, ATM, and H2AX, is required for efficient viral replication (64, 94). In addition, BGLF4 phosphorylates the nuclearides analog drugs ganciclovir and acyclovir, and this activity may affect the drug-mediated inhibition of EBV lytic replication (40, 76). Finally, BGLF4 inhibits ZTA SUMOylation and enhances its transcriptional activity, although the underlying mechanism remains to be defined (45).

We identified SIMs in BGLF4 and found that several known BGLF4 functions wereSIM dependent. SUMO binding activity by BGLF4 was required for the BGLF4 suppression of ZTA SUMOylation, the suppression of global cellular SUMOylation, the efficient dispersion of promyelocytic leukemia (PML) bodies, the induction of the cellular DDR, and the facilitation of EBV lytic replication.

**MATERIALS AND METHODS**

**Antibodies.** The following antibodies were used: mouse anti-FLAG (M2)-horseradish peroxidase (HRP) (catalog no. A8592), rabbit anti-FLAG polyclonal (catalog no. F7425) and rabbit anti-β-actin polyclonal antibodies (catalog no. A5441) from Sigma-Aldrich; rabbit anti-TIP60 (phospho-Ser86) (catalog no. ab73207; Abcam); rat anti-hemagglutinin (HA) high-affinity antibody (catalog no. 11-867-431-001; Roche); anti-phospho-ATM (Ser1981) (catalog no. 5883; Cell Signaling); anti-phospho-H2AX (Ser139) (γ-H2AX) (catalog no. 9947; Cell Signaling); anti-phospho-KAP1 (Ser824) (catalog no. 4127; Cell Signaling); anti-H2AX (catalog no. 2595; Cell Signaling); mouse-anti-SUMO1/GMP-1 (catalog no. 33-2400; Invitrogen); and murine anti-ZTA antibody (catalog no. 11-007; Argene). Rabbit polyclonal anti-PML, directed against amino acid positions 484 to 498 of the human 90-kDa PML isomer, was described previously (2), as was mouse anti-BGLF4 antibody (99).

**Plasmids.** Plasmids that have been previously described are HA-BGLF4 (kinase dead [KD]), glutathione S-transferase (GST)-TIP60 (64, 108), ZTA and the ZTA mutant (mZTA; KF12/13AA) (86), and HA-SUMO2, GST-tagged SUMO1, SUMO2, and SUMO2 trimer expression vectors (109). Flag-tagged BGLF4 (mSIM-N), BGLF4 (mSIM-C), and BGLF4 (mSIM-NC) were generated using the QuickChange site-directed mutagenesis kit (Stratagene) and were expressed from the pSG5-Flag vector. Primers used were the following: mSIM-N (V139/40DR), 5′-GGCTTTTGGGGAAAGAGTGCAGTGCAAGATTTACTCTACCTTTCA GAC-3′ and 5′-GTCGTAAGAGATTAAATCTCAGTCACCT CCCAAAAGGCG-3′; mSIM-C first-round primers (V344/345ER), 5′-TGCTGAATTTGCACCGAAAAAGGAGGCTGACTCTGTC GCAG-3′ and 5′-CTGGACAGACCTCCTCCAGACGCGCTT GCTCGAACATTGAAA-3′; mSIM-C second-round primers (V349/ 350ER), 5′-CAGAGACGCCGTTAGGAGACGCGCATGTC TGGAACCTGAAC-3′ and 5′-GTCGAGGTTGACCATCCTGAGCCGGC TCTCCACAGGCCCTTGG-3′. The green fluorescent protein (GFP) reporter vector (GFP-NLS-PK; pGL22) for testing nuclear export sequences has been previously described (17). Two 57-mer synthetic oligonucleotides (forward, 5′-GATCCAAAGGTGGTGGTGGGAGGTG TTCGCAGATGTGGAACCTCTGAACTTAGAC-3′; reverse, 5′- GATCTGTCAAATCGTGTTGCACATCTGCGACACCTCACAG GCAGACCTTTTC-3′) encoding BGLF4 amino acids 342 to 359 plus linker sequences were annealed and ligated into the BamHI site of the GFP reporter vector to create a GFP-NLS-PK-NE3 vector (pGL659).

**Protein microarray SUMO binding assay.** SUMO2 monomer and trimer proteins were purified from bacteria as GST fusions and then cleaved from GST using acTEV protease (Invitrogen), which cleaves an engineered site in the polylinker of the Gateway vector (109). SUMO2 proteins were tagged with maleimide-Cy5 using a commercial kit (GE Healthcare) that chemically links Cy5 through maleimide to the single cysteine residue in SUMO2. EBV protein microarrays have been described previously (108). The EBV protein arrays were blocked for 3 h with 3% bovine serum albumin (BSA) in Superblock buffer (Pierce) and then incubated with Cy5-labeled SUMO2 at a final concentration of 40 nM in Superblock buffer at 4°C overnight. The chip was washed 3 times in TBST buffer (25 mM Tris-HCl [pH 7.4], 3 mM KCl, 150 mM NaCl, 0.05% Tween 20) for 5 min each time, rinsed with distilled water, and spun to dryness. The slides were then scanned with a GenePix 4000 scanner (Molecular Devices, CA), and the binding signals were acquired using GenePix software, EBVs proteins, where both duplicate spots were positive in three independent SUMO binding assays, were scored as positive.

**Cell lines and DNA transfection.** The Akata (EBV⁺)-vector-tetrascline (Tet)-, Akata (EBV⁺)-BGLF4 (WT)-Tet-, and Akata (EBV⁺)-BGLF4 (KD)-Tet-inducible cell lines were described previously (64, 108). The Akata (EBV⁺)-BGLF4 (mSIM-N)-tet cell line was constructed using previously described methods (108). Akata (EBV⁺)-BGLF4 cell lines were maintained in RPMI 1640 with 10% fetal bovine serum. The medium for Tet-inducible cell lines was supplemented with 1.0 μg/ml puromycin and 1.0 mg/ml G418. 293T, Vero, and U2OS cells were maintained in Dulbecco’s modified Eagle medium (DMEM) plus 10% fetal bovine serum.

**Protein expression and purification.** GST-tagged proteins were expressed and purified as described previously, with some modifications (65, 66). Briefly, Escherichia coli BL21 cells were transformed with the appropriate expression vectors (SUMO1, SUMO2, and SUMO2 trimer) and then cultured in LB medium at 37°C until the A600 reached 0.6. The bacteria were induced by adding 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 16°C for 12 to 16 h. Bacteria were harvested and then lysed by sonication. GST fusion proteins were purified by affinity chromatography using glutathione-Sepharose 4B (GE Healthcare Biosciences) according to the manufacturer’s instructions.

**GST affinity assay.** Cell lysate (500 μl) containing Flag-tagged wild-type (WT) or mutant BGLF4 was preclaved with 50 μl of 50% glutathione-Sepharose 4B at 4°C for 1 to 2 h. Twenty μl of GST, GST-SUMO1, GST-SUMO2, or GST-SUMO2 bound to glutathione-Sepharose 4B was added to the preclaved lysate, rotated on a vertical wheel at 4°C for 2 h, washed 4 times with lysis buffer, and boiled in 2X SDS sample buffer. GST-SUMO1), expressed 3 tandem copies of SUMO2 fused to GST (107). Proteins were separated on SDS-PAGE gels and detected by Western blotting using anti-Flag antibody.

**In vitro phosphorylation assay.** Flag-tagged WT, mSIM-N, and KD mutants of BGLF4 were transfected into 293T cells. Transfected cells were harvested 48 h posttransfection using radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% [vol/vol] NP-40, 1% [wt/vol] deoxycholate, 0.1% [wt/vol] SDS, 1 mM EDTA) containing protease inhibitors and phosphatase cocktail 1 and II (Sigma). Flag-tagged WT and mutants of BGLF4 were immunoprecipitated from the transfected 293T cells using anti-Flag-M2 agarose beads (catalog no. A2220; Sigma). The bound proteins were washed twice with lysis buffer and twice with kinase buffer (50 mM HEPES [pH 7.4], 10 mM MgCl₂, 10 mM MnCl₂, 300 mM KCl, and 0.5% NP-40). Immunocomplexes were then incubated with 1 μg purified recombinant GST-TIP60 in kinase buffer with 40 nM ATP for 30 min at 30°C. The reaction was terminated by adding 2X SDS sample buffer and heating at 90°C for 5 min. Proteins

May 2012 Volume 86 Number 5 jvi.asm.org 5413

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TABLE 1 SUMO binding proteins identified by proteomic screening

<table>
<thead>
<tr>
<th>Protein</th>
<th>Potential SIMs*</th>
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<tbody>
<tr>
<td>EBNA3C</td>
<td>203-LEIL,243-HEVIDL</td>
</tr>
<tr>
<td>EBNA3A</td>
<td>173-LEIL,206-VLIDL</td>
</tr>
<tr>
<td>EBNA3B</td>
<td>180-VQI,214-VQI,440-LSIKL</td>
</tr>
<tr>
<td>BGLF5</td>
<td>385-VNIV,419-VAVL</td>
</tr>
<tr>
<td>BGLF1</td>
<td>18-LVHL,183-LLEI,329-VSIL,342-LISV,368-VIPVV,452-LVAL</td>
</tr>
<tr>
<td>BBLF2/3</td>
<td>33-VVGIVPI,47-LIVV,217-VDLV,239-LKIL,644-VCLL</td>
</tr>
<tr>
<td>EBNA3A</td>
<td>37-LIEI,219-IIHL,867-VLDL</td>
</tr>
<tr>
<td>BZLF2</td>
<td>151-ALVVLLEL,149-LPVL</td>
</tr>
</tbody>
</table>

*The superscript numbers are the amino acid numbers.

then were separated on SDS-PAGE gels and checked by Western blotting using phospho-TIP60 (Ser86)-specific antibody.

**Indirect immunofluorescence.** U2OS or Vero cells grown on 2-well slides were transfected using Lipofectamine 2000 (Invitrogen) to express the WT or the mSIM-N, mSIM-C, or mSIM-NC mutant of BGLF4. Two days after transfection, cells were fixed for 10 min in 1% paraformaldehyde, and slides were incubated in blocking buffer (2.5% normal goat serum, 0.3% Triton X-100, and 2% bovine serum albumen). Primary antibody (mouse anti-BGLF4 and rabbit anti-PML) was diluted 1:100 in blocking buffer and incubated with the cells for 2 h at room temperature. Slides were washed with phosphate-buffered saline (PBS) three times. Secondary antibody (fluorescein isothiocyanate [FITC] anti-mouse immunoglobulin or rhodamine anti-rabbit immunoglobulin [Jackson]) was used at a 1:500 dilution in PBS for 2 h at room temperature. Slides were rinsed with PBS three times and mounted in Vectashield containing DAPI (4',6-diamidino-2-phenylindole) (Vector Laboratories). Vero cells were similarly transfected with pGL22 and pGL659 vectors. Cells were fixed and images captured using a Nikon E800 Eclipse fluorescence microscope.

**Lytic induction of Akata (EBV+) cells and virus titration.** Akata (EBV+) cells were treated with 50 μg/ml of goat anti-human IgG (MP Biomedicals) for 48 h to induce the EBV lytic cycle. To measure EBV replication, virion-associated DNA present in the culture supernatant was determined by quantitative PCR (qPCR) analysis. The supernatant (180 μl) was treated with 4 μl RQ1 DNase (Promega) for 1 h at 37°C, and reactions were stopped by adding 20 μl of stop buffer and incubation at 65°C for 10 min; 12.5 μl proteinase K (20 mg/ml, Invitrogen) and 25 μl of stop buffer were then added to the reaction mixtures, which were incubated for 1 h at 65°C. DNA was purified by phenol-chloroform extraction followed by isopropanol-sodium acetate precipitation and then resuspended in 200 μl nuclease-free water. qPCR reaction mixes (20 μl) contained primers (100 nM), 2 μl DNA, and 10 μl SYBR green PCR master mix (Applied Biosystems). Cycle threshold (Ct) values were measured using a 7300 real-time PCR system (Applied Biosystems). Relative levels of virion DNA were normalized to supernatant virion DNA without lytic induction. The BALF5 primers used for quantitating EBV copy numbers were 5'-AGCTGTGTGGCTACTCTGTGTC-3' and 5'-CTTTG CGCGGGATCCTC-3'.

**RESULTS**

**Screening for EBV proteins that bind SUMO.** To systematically identify SUMO binding proteins, we employed a proteomic approach using Cys5-labeled SUMO2 and a previously described protein array displaying 60 EBV proteins expressed as GST fusions and purified from yeast (108). We identified 11 EBV proteins, including the known SUMO binding partner EBNA3C (84), that reproducibly scored as SUMO2 binding in three independent as-

say. At least two consensus SIM motifs ([V/I/L-X-V/I/L-X-V/I/L, or [V/I/L-X-V/I/L-X-V/I/L], where V is valine, I is isoleucine, L is leucine, and X is any amino acid), are present in each of these 11 proteins (Table 1). One of the newly identified SUMO binding proteins was the conserved protein kinase BGLF4. BGLF4 plays multiple roles in ensuring efficient EBV replication. We therefore focused on the interplay of BGLF4 and SUMO.

**Mapping the SIM motifs in BGLF4.** To validate the interaction between BGLF4 and SUMO2, we first performed an in vitro pulldown assay using GST-SUMO2, a GST-SUMO2 tandem trimer [GST-(SUMO2)3], and lyse from Flag-BGLF4-transfected cells. We found that both GST-SUMO2 and GST-(SUMO2)3, but not the GST control, bound BGLF4 (Fig. 1A, upper). In addition, we noted that the kinase-dead BGLF4 (KD) also bound GST-SUMO2 (Fig. 1B), indicating that the interaction between BGLF4 and SUMO2 is independent of BGLF4 kinase activity.

Sequence analysis revealed two potential SIM motifs in BGLF4,
one located at the N terminus (designated SIM-N) and another SIM at the C terminus (designated SIM-C) (Fig. 1C and Table 1). These motifs were mutated to generate: mSIM-N, mSIM-C, and mSIM-NC (in which SIM-N and SIM-C are mutated) (Fig. 1C). Each of these mutations disrupted the binding of BGLF4 to SUMO1 and to SUMO2, as evaluated by GST pulldown assays (Fig. 1D), confirming that these sequences represent functional SIMs.

**Effect of SUMO binding on BGLF4 subcellular localization.**
A previous study identified sequences that were necessary, but not sufficient, for BGLF4 nuclear localization (44). We investigated whether SUMO binding played any role in BGLF4 intracellular distribution. Immunofluorescence assays on transfected cells showed the expected nuclear localization for BGLF4 (Fig. 2A). The mSIM-N mutant had a predominantly nuclear localization with weak cytoplasmic staining (Fig. 2A). The mutation of the C-terminal SIM resulted in individual cells showing either nuclear or perinuclear plus cytoplasmic staining. The mutation of both N- and C-terminal SIMs led to BGLF4 adopting a cytoplasmic distribution (Fig. 2A). A second N- and C-terminal SIM mutant (mSIM-NC*) in which the C-terminal SIM mutation was restricted to the first two valine residues of the SIM then was generated. This BGLF4 SIM mutant also localized to the cytoplasm (Fig. 2A). The BGLF4 amino acid sequence immediately adjacent to the C-terminal SIM contains hydrophobic amino acids whose relative spacing is suggestive of a nuclear export motif. To test this possibility, sequences encoding BGLF4 amino acids 342 to 359 were transferred to a GFP-tagged nuclear protein, GFP-NLS-PK, that has previously been used to identify nuclear export signals (NES) in the EBV Mta protein (17). In transfected Vero cells, the GFP-NLS-PK protein had a strictly nuclear localization (Fig. 2B). The addition of BGLF4 amino acids 342 to 359 resulted in the relocalization of GFP-NLS-PK to the cytoplasm (Fig. 2B). Therefore, BGLF4 contains a functional nuclear export signal. The mutation of the adjacent C-terminal SIM is sufficient to cause a proportion of BGLF4 to display a cytoplasmic localization. However, the mutation of both N- and C-terminal SIMs is necessary for the complete abrogation of nuclear accumulation, suggesting that there is cooperation between the SIM motifs.

**BGLF4 inhibits ZTA and global cellular SUMOylation in a SIM-dependent manner.**
BGLF4 was previously shown to inhibit ZTA SUMOylation and regulate ZTA transcription activity (45). We asked whether SUMO binding by BGLF4 plays a role in the inhibition of ZTA SUMOylation. Since BGLF4 with the N-terminal SIM mutated lacked SUMO binding in vitro but still retained the wild-type BGLF4 nuclear localization phenotype, we selected this mutant for functional assays. ZTA or mZTA (KF12/13AA), HA-SUMO2, and WT or mSIM-N BGLF4 were transfected as indicated in Fig. 3A, and the presence of SUMOylated ZTA was examined by Western blotting. We found that ZTA was readily
SUMOylated in transfected cells with or without cotransfected HA-SUMO2 (Fig. 3A, lanes 1 and 2). ZTA SUMOylation was abolished by cotransfection with WT BGLF4 but not by mSIM-N BGLF4 (Fig. 3A, lanes 3 and 4). Covalent SUMO modification has been shown to occur on K12 of ZTA (45). The SUMO-ZTA bands were not detected when the ZTA mutant KF12/13AA was transfected (Fig. 3A, lanes 5 to 8). To ensure that the results were not influenced by the overexpression of transfected HA-SUMO2, we repeated the assay to examine ZTA modification by endogenous SUMO and similarly found that WT BGLF4 abolished ZTA SUMOylation, while the mSIM-N BGLF4 mutant had no effect on ZTA SUMOylation (Fig. 3B, lanes 3 and 4). To address whether the kinase activity of BGLF4 played a role in abolishing ZTA SUMOylation, ZTA was cotransfected with the kinase-dead BGLF4 mutant. Interestingly, we found that the elimination of BGLF4 kinase activity also abolished the ability of BGLF4 to interfere with ZTA SUMOylation (Fig. 3B, lanes 5 and 6). To ensure that the mutation of the N-terminal SIM did not inadvertently abolish BGLF4 kinase activity, an in vitro kinase assay was performed using WT, mSIM-N, and KD BGLF4 immunoprecipitated

**FIG 3** SIM- and kinase activity-dependent inhibition of SUMOylation by BGLF4. (A) SIM-dependent inhibition of ZTA SUMOylation by BGLF4. Immunoblot analysis of cell lysates from 293T cells cotransfected as indicated. ZTA and SUMO-modified ZTA were detected using anti-ZTA or anti-HA antibodies. (B) SIM and kinase activity-dependent inhibition of ZTA SUMOylation by BGLF4. Upper) Immunoblot analysis of cell lysates from 293T cells cotransfected with ZTA and control vector, WT, mSIM-N, or kinase-dead (KD) BGLF4. SUMOylation by endogenous SUMO was detected using anti-ZTA antibody. (Lower) Expression of BGLF4 in the transfected cell extracts detected with anti-BGLF4 antibody. (C) The mSIM-N mutant of BGLF4 is phosphorylation competent. Western blot analysis of TIP60 substrate after in vitro phosphorylation reactions with the indicated combinations of GST-TIP60 and WT or mutant BGLF4. Phosphorylation was detected using pTIP60 S86-specific antibody. (D) Inhibition of cellular SUMOylation by BGLF4. Immunoblot analysis using anti-SUMO1 antibody of cell lysates from Akata (EBV+ cells) carrying control vector, WT, mSIM-N, or kinase-dead (KD) BGLF4. The cells were treated with doxycycline (DOX) for 48 h to induce BGLF4 expression. (Lower) Expression of BGLF4 and β-actin in the cell extracts detected with anti-BGLF4 and anti-β-actin antibodies, respectively. (E) Graphical representation of the relative density of SUMOylated substrates shown in panel D. The density of SUMOylated substrates was analyzed using the software TotalLab v2.0.1. The density in the vector control (lane 1) was set to 1.
from transfected cells. A known substrate of BGLF4 is the cell protein TIP60, which is phosphorylated at Ser86 (64). Using a pTIP60 S86-specific antibody, we demonstrated that both BGLF4 and mSIM-N BGLF4 phosphorylated TIP60 at Ser86, while the vector control and KD BGLF4 failed to phosphorylate TIP60 in vitro (Fig. 3C). Our results indicate that the SUMO binding and kinase activities of BGLF4 independently contribute to the inhibition of ZTA SUMOylation.

To address whether the BGLF4 inhibition of SUMOylation is confined to ZTA or is a more general phenomenon, we constructed an Akata (EBV/H11001) cell line that carries a Tet-inducible SIM mutant BGLF4 (mSIM-N) and examined global SUMOylation upon the doxycycline induction of BGLF4. As a comparison, we also tested three previously constructed cell lines expressing either the vector control, WT BGLF4, or KD BGLF4 (64). The results showed that WT BGLF4 globally suppressed SUMOylation events, while neither the mSIM-N nor KD BGLF4 protein had any significant effect on SUMOylation compared to the vector control (Fig. 3D and E). The SIM- and kinase-dependent BGLF4 suppression of ZTA SUMOylation thus extends to the modulation of cell protein SUMOylation.

**BGLF4 disrupts PML bodies in a SIM-dependent manner.**
The ability of BGLF4 to disrupt promyelocytic leukemia (PML) bodies has been controversial (61, 85). We reexamined this function of BGLF4 in transfected Vero cells. We observed that the number of PML bodies per cell was reduced upon the expression of WT BGLF4 compared to the level for the control cells (Fig. 4A). This is consistent with the results reported by Kuny et al. (61). The mSIM-N BGLF4 mutant also reduced the PML body number, although less efficiently than WT BGLF4 (Fig. 4A). As expected, there was no significant difference in the number of PML bodies in cells transfected with the cytoplasmic mSIM-NC* mutant (Fig. 4A). Quantitative analysis revealed that cells expressing WT BGLF4 had 2.5-fold fewer PML bodies than the control cells, while mSIM-N-transfected cells reduced the PML body number by 1.6-fold. Cells expressing mSIM-NC* retained the same number (99%) of PML bodies as control cells (Fig. 4B). The dispersal of PML bodies by BGLF4 is therefore another function of BGLF4 that is SIM dependent.

**BGLF4 induction of the DDR and enhancement of viral lytic replication is SIM dependent.**
BGLF4 contributes to EBV lytic replication by inducing the DNA damage response (DDR) through TIP60 phosphorylation (Fig. 5A) (64). To examine the role of SUMO binding by BGLF4 in the induction of the DDR, we compared the phosphorylation status of several proteins in the DDR cascade, namely, TIP60, ATM, KAP1, and H2AX. After the doxycycline treatment of the Akata cells, the induction of WT BGLF4 led to the phosphorylation of each of these proteins (Fig. 5B). However, neither mSIM-N nor KD BGLF4 was capable of inducing the phosphorylation of TIP60, ATM, KAP1, or H2AX. The BGLF4 induction of the DDR is thus also SIM and kinase activity dependent.

**BGLF4 is required for efficient EBV replication.**
To determine whether the loss of SUMO binding by BGLF4 affects EBV lytic replication, we compared the extracellular virion DNA copy number in Akata B cells expressing WT, mSIM-N, or KD BGLF4. After a 6-h induction of these proteins, EBV lytic replication was activated via IgG cross-linking for 48 h, and then the supernatants were harvested to assay for extracellular virion DNA. As illustrated in Fig. 5C, the preinduction of WT BGLF4 led to the production of extracellular virus, while the preinduction of mSIM-N and KD BGLF4 had no effect above that of the vector control. Thus, eliminating the SUMO binding activity of BGLF4 affects the efficiency of lytic EBV replication to the same extent as the ablation of BGLF4 kinase activity.

**DISCUSSION**
High-throughput screening is a new tool for the investigation of pathogen-host interactions (9, 11, 32, 59, 87). We previously identified viral and host phosphorylation targets of the BGLF4

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**FIG 4** BGLF4 disperses PML bodies in a SIM-dependent manner. (A) Immunofluorescence assays performed on Vero cells transfected with WT or SIM BGLF4 mutants. Cells were stained with anti-BGLF4 (upper) and anti-PML (middle) antibodies. Nuclei were visualized with DAPI (bottom). Arrows denote BGLF4-expressing cells. (B) Histogram showing the average number of PML bodies per transfected cell. Data are presented as the means ± standard errors (n = 25). *, P < 0.001.
protein kinase using EBV and host protein microarrays (64, 108). In this study, we used a similar proteomic approach to identify 11 EBV proteins that bind to SUMO. We focused on the protein kinase BGLF4 and evaluated the importance of SUMO binding for BGLF4 function.

Viruses interact with the host SUMOylation pathway by modulating key components of the SUMO conjugation machinery and by encoding proteins that are themselves targets for covalent SUMOylation and noncovalent SUMO binding (8, 102, 103). EBV ZTA is SUMOylated by SUMO1/2/3, and the EBV protein kinase BGLF4 counteracts ZTA SUMOylation through an unknown mechanism (1, 45, 80). Here, we link BGLF4 SUMO binding and BGLF4 kinase activity to the BGLF4-mediated inhibition of SUMOylation. The phosphorylation-mediated regulation of SUMOylation has been described for a number of proteins. KRAB-associated protein 1 (KAP1), a corepressor for kruppel-associated box zinc finger proteins, recruits proteins involved in transcriptional repression and chromatin condensation. The phosphorylation and SUMOylation of KAP1 are mutually exclusive. The ATM-mediated phosphorylation of KAP1 Ser824 represses its SUMOylation, and conversely, SENP1-mediated de-SUMOylation of KAP1 enhances its phosphorylation (67, 68). The Kaposi’s sarcoma-associated herpesvirus (KSHV) protein kinase ORF36 also phosphorylates KAP1 and prevents KAP1 SUMOylation (50). The calcium/calmodulin-dependent protein kinase II (CaMKII)-dependent phosphorylation of liver X receptor (LXR) at Ser427 creates a docking site for SENP3 and promotes LXR de-SUMOylation (48). The loss of the SUMO modification of Elk-1 correlates with Elk-1 phosphorylation mediated by ERK pathway activation (104). The phosphorylation of IκBα inhibits its SUMO conjugation in vitro (24). The SUMOylation of ATF7 is abrogated by p38β2-mediated phosphorylation on Thr51 (12), and the SUMO modification of both c-Jun and p53 is downregulated by phosphorylation (79). ZTA has been shown to be phosphorylated at multiple sites (Thr14 and Ser 167/173/186/209) by cellular kinases and by the viral kinase BGLF4 (5, 26). It is plausible that BGLF4 binding to SUMOylated viral or host proteins enhances the phosphorylation of the targeted protein, and that this then either inhibits the continued SUMOylation of these targets or leads to the recruitment of SENPs and de-SUMOylation.

Several viral proteins have been shown to bind SUMO via SIMs. For example, EBV EBNA3C upregulates EBNA2-mediated gene activation by SIM-dependent binding to a SUMOylated repressor (84). The SIM in human cytomegalovirus (HCMV) IE2 is required for efficient IE2 SUMOylation at K175/180 and for its binding to SUMOylated TATA-binding protein (TBP)-associated factor I2 (TAF12) (7, 58). The KSHV b-ZIP protein is a viral E3 SUMO ligase, and the ligase activity is dependent on the presence of a SIM (15). The herpesvirus protein kinases Us3, UL97, ORF36,
and BGLF4, encoded by herpes simplex virus type 2 (HSV-2) (52), HCMV (61, 82, 98), KSHV (61), and EBV (61), respectively, have a role in disrupting PML bodies. The kinase activity of Us3 and UL97 is required for this function. We now demonstrate that EBV BGLF4 requires SUMO binding for PML body disassembly. KSHV vIRF3/LANA2 and varicella-zoster virus (VZV) ORF61 also disrupt PML bodies in a SIM-dependent manner (74, 100).

BGLF4 localizes predominantly to the nucleus, although weak cytoplasmic and perinuclear staining also have been described (40, 99). We found that the mutation of both N- and C-terminal SIMs resulted in BGLF4 adopting a cytoplasmic distribution. We further demonstrated that the C-terminal SIM lies adjacent to a functional nuclear export signal. The data suggest that the accumulation of BGLF4 in the nucleus is dependent on the blocking of nuclear export through coordinate SUMO binding mediated by the two BGLF4 SIMs. Coordinated binding may increase the affinity of the SUMO interaction or may be required to mediate a change in BGLF4 protein conformation. The regulation of nucleocytoplasmic trafficking by covalent SUMOylation has been shown for a number of proteins (101), and several of these are covalently SUMOylated at sites in close proximity to nuclear export signals (25). SIMs have been associated with the regulation of the intranuclear localization of proteins, but we are not aware of previous reports implicating SUMO binding in nucleus-cytoplasmic distribution.

SUMOylation and SIM-dependent protein–protein interactions play an important role in the DDR and in double-strand break (DSB) repair (68, 90). SUMO, UBC9, and the SUMO E3 ligases PIAS1 and PIAS4 relocate to γ-H2AX foci after DNA damage (35, 78). The SUMOylation of TIP60 at lysines 430 and 451 is critical to the DDR and in double-strand break (DSB) repair (68, 90). SUMO, UBC9, and the SUMO E3 ligases PIAS1 and PIAS4 relocate to γ-H2AX foci after DNA damage (35, 78). The SUMOylation of TIP60 at lysines 430 and 451 is necessary for its p53-dependent DDR (20). The cellular DDR triggered by viral infection or lytic induction plays an important role in viral life cycle (63). The EBV infection of primary B cells elicits a robust DDR that restricts EBV-induced B cell transformation (81). Conversely, aspects of the DNA damage pathway are also required for efficient viral replication (63, 64). We demonstrate that the BGLF4-induced DDR requires the presence of a BGLF4 SIM. Therefore, BGLF4 appears to take advantage of the SUMO modification of DDR proteins to integrate into the complex formed at the site of DNA damage and to then facilitate the DDR. Overall, SUMO binding by BGLF4 was found to be as important as the kinase activity of BGLF4 for mediating functions that foster EBV replication.

ACKNOWLEDGMENTS

We thank Mei-Ru Chen for anti-BGLF4 antibody and Gary S. Hayward for anti-PML antibody. We also thank Ijee Uzoma and Eric Cox for assistance with the immunofluorescence assays.

This work was funded by NIH grant R37CA242245 to S.D.H.

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