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Epstein-Barr Virus Protein Kinase BGLF4 Is a Virion Tegument Protein That Dissociates from Virions in a Phosphorylation-Dependent Process and Phosphorylates the Viral Immediate-Early Protein BZLF1

Risa Asai,[1,3] Ai Kato,[3] Kentaro Kato,[4]† Mikiko Kanamori-Koyama,[3,4]‡ Ken Sugimoto,[1,3] Takeshi Sairenji,[5] Yukihiro Nishiyama,[3] and Yasushi Kawaguchi[1,2,3,4]*

Division of Viral Infection, Department of Infectious Disease Control, International Research Center for Infectious Diseases, The Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo 108-8639[1]; PRESTO, Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012[2]; Department of Virology, Nagoya University Graduate School of Medicine, Showa-ku, Nagoya 466-8550[3]; Department of Cell Regulation, Medical Research Institute, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo 113-8510[4]; and Division of Biosignaling, Department of Biomedical Science, School of Life Science, Faculty of Medicine, Tottori University, Yonago 683-8503,[5] Japan

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Epstein-Barr virus (EBV) BGLF4 is a viral protein kinase that is expressed in the lytic phase of infection and is packaged in virions. We report here that BGLF4 is a tegument protein that dissociates from the virion in a phosphorylation-dependent process. We also present evidence that BGLF4 interacts with and phosphorylates BZLF1, a key viral regulator of lytic infection. These conclusions are based on the following observations. (i) In in vitro tegument release assays, a significant fraction of BGLF4 was released from virions in the presence of physiological NaCl concentrations. (ii) Addition of physiological concentrations of ATP and $MgCl_2$ to virions enhanced BGLF4 release, but phosphatase treatment of virions significantly reduced BGLF4 release. (iii) A recombinant protein containing a domain of BZLF1 was specifically phosphorylated by purified recombinant BGLF4 in vitro, and BGLF4 altered BZLF1 posttranslational modification in vivo. (iv) BZLF1 was specifically coimmunoprecipitated with BGLF4 in 12-*O*-tetradecanoylphorbol-13-acetate-treated B95-8 cells and in COS-1 cells transiently expressing both of these viral proteins. (v) BGLF4 and BZLF1 were colocalized in intranuclear globular structures, resembling the viral replication compartment, in Akata cells treated with anti-human immunoglobulin G. Our results suggest that BGLF4 functions not only in lytically infected cells by phosphorylating viral and cellular targets but also immediately after viral penetration like other herpesvirus tegument proteins.

Phosphorylation is one of the most common and effective modifications by which a cell or virus regulates protein activity. This modification is mediated by protein kinases that phosphorylate specific proteins, thereby regulating many cellular functions such as transcription, translation, cell cycle regulation, protein degradation, and apoptosis (5, 27). Herpesviruses encode protein kinases and, possibly, utilize them both to regulate their own replicative processes and to modify cellular processes by phosphorylation of specific viral and cellular proteins (16). In fact, viral protein kinases have been reported to play multiple roles in viral gene expression (33), apoptosis (25, 32), viral DNA synthesis (44), viral DNA encapsidation (44), and nucleocapsid nuclear egress (23, 34). Among the protein kinases encoded by herpesviruses, a subset exemplified by herpes simplex virus type 1 (HSV-1) UL13 is conserved in all *Herpesviridae* subfamilies (2, 37). The conservation of these viral protein kinases and their relatively high degree of amino acid sequence similarity suggests their importance in viral replication and pathogenesis.

Epstein-Barr virus (EBV) is a human gammaherpesvirus that is an etiologic agent of infectious mononucleosis and is associated with a variety of human malignancies, including endemic Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, gastric carcinoma, and lymphoproliferative diseases in immunosuppressed patients (20, 35). Once a person is infected with EBV, the virus persists for life due to its ability to establish a latent infection in B lymphocytes (35). Only a limited subset of viral genes is expressed in the latent state (35). Of these, those critical for EBV immortalization of primary human B cells in vitro are EBV nuclear antigens EBNA-1, EBNA-2, EBNA-3A, and EBNA-3C; EBNA leader protein (EBNA-LP); and EBV latent membrane protein LMP-1 (20). Latent virus in B cells occasionally switches from the latent stage to a virus-productive lytic stage, which results in amplification of viral DNA, release of infectious virions, and host cell death (20, 35). In lytic infection, viral gene expression

* Corresponding author. Mailing address: Division of Viral Infection, Department of Infectious Disease Control, International Research Center for Infectious Diseases, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. Phone: 81-3-6409-2070. Fax: 81-3-6409-2072. E-mail: ykawagu@ims.u-tokyo.ac.jp.

† Present address: Department of Veterinary Microbiology, Graduate School of Agricultural and Life Science, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan.

‡ Present address: Global Drug Discovery, Discovery Research Laboratories, Shionogi & Co. Ltd., 2-5-1, Settsu-shi, Mishima, Osaka 566-0022, Japan.

has been divided into three coordinately regulated, sequentially ordered phases designated immediate-early (IE), early (E), and late (L) (20). The switch to the lytic cycle is mainly controlled by one of the IE gene products, BZLF1, which functions as a transcriptional activator to initiate an ordered cascade of viral lytic gene expression (20).

The EBV UL13 homologue BGLF4 is a serine/threonine protein kinase and is the only protein kinase identified in the EBV genome (2, 3, 13, 14, 37). The BGLF4 gene is expressed in the lytic infection cycle E phase (9) and is detected mainly in nuclei of EBV-infected cells (8, 43). Since recombinant BGLF4 mutant viruses have not been constructed, the precise role(s) of this viral protein kinase in the EBV life cycle is largely unknown. However, identification of BGLF4 substrates has suggested biological roles for this protein kinase in EBV infection. Thus far, EBV BMRF1 (3, 9), the DNA polymerase processivity factor; BGLF4 itself (3, 13, 14); cellular translation elongation factor 1δ (EF-1δ) (13, 17); EBV EBNA-LP (14), a transcriptional coactivator of EBNA-2; and EBV EBNA-2 (47), a transcriptional regulator of viral and cellular genes, have been reported to be substrates for BGLF4. Among these substrates, the biological significance of BGLF4-mediated phosphorylation of BMRF1, EF-1δ, and BGLF4 itself remains unclear. In contrast, BGLF4-mediated phosphorylation of EBNA-LP and EBNA-2 has been shown to affect their transcriptional regulatory activity (14, 45, 47), suggesting that a function of BGLF4 in EBV-infected cells is to modify the activity of these target proteins via phosphorylation. It has also been reported that UL13 homologues, in general, tend to target the same sites in their substrates as cellular protein kinase cdc2 does (16). In agreement with this, BGLF4 phosphorylates EBNA-LP and EF-1δ at the same sites as cdc2 does (14, 17). These observations suggest that BGLF4 may function similarly to cdc2. However, more than 10 substrates have been reported for HSV-1 UL13 (16) and cdc2 is known to target a variety of cellular and EBV proteins (14, 21, 30, 46). These results suggest that there are many additional BGLF4 substrates which need to be identified for a further understanding of BGLF4 function.

Like other herpesvirus UL13 homologues (16), BGLF4 is packaged in virions, probably in the tegument, a virion structural component located between the nucleocapsid and envelope (10, 43). Tegument proteins are the first to be exposed to the intracellular environment of a newly infected cell, and some play a role in establishing the conditions for efficient viral replication immediately after viral penetration. This role presumably requires dissociation of specific tegument proteins from the virion and their release as soluble proteins into the cytoplasm. It may be advantageous for the virus that virion-associated BGLF4, which can mimic cdc2, enters infected cells and expresses a cdc2-like activity to modulate the cellular environment, since cdc2 regulates a variety of cellular processes including transcription, translation, and structural changes in the nuclear envelope, cytoskeleton, and chromosomes (30, 31). However, although it has been reported that BGLF4 is detected in purified virions (10, 43), it is not known whether BGLF4 can dissociate from virions and, if so, what may be the mechanism of this dissociation.

We report here studies showing that BGLF4 is a tegument protein and dissociates from virions at physiological salt concentrations in a phosphorylation-dependent process. In addition, we identified BZLF1, the critical regulator of the EBV lytic cycle, as a novel substrate of BGLF4.

MATERIALS AND METHODS

Cells. Ramos is an EBV-negative Burkitt's lymphoma cell line. Akata is an EBV-positive Burkitt's lymphoma line. B95-8 is a marmoset cell line carrying infectious mononucleosis-derived EBV. These cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and antibiotics. A monkey kidney epithelial cell line, COS-1, was maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS and antibiotics. *Spodoptera frugiperda* Sf9 cells were maintained in Sf900IISFM medium (Invitrogen) supplemented with 10% FCS and antibiotics.

EBV lytic cycle induction. B95-8 cells were treated with 100 ng 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma) per ml. Akata cells were incubated in the presence of 100 μg anti-human immunoglobulin G (IgG; Cappel) per ml.

Plasmids. pGEX-BGLF4 was constructed by cloning the EcoRI-NotI fragment of pBS-BGLF4-stop (13) into pGEX4T-2 (Amersham-Biosciences) in frame with glutathione *S*-transferase (GST). pGEX-BcLF1-a, -b, and -c were constructed by amplifying the domain encoding BcLF1 codons 1 to 199, 467 to 659, and 656 to 857, respectively (Fig. 1), by PCR from EBV DNA isolated from B95-8 cells as described previously (13) and cloning the DNA fragments into pGEX4T-1. BS-Z (7), in which full-length BZLF1 cDNA is cloned into pBluescript II SK(−) (Stratagene), was kindly provided by E. Flemington (Dana-Farber Cancer Institute, Massachusetts). pcDNA3-BZLF1 was constructed by cloning the KpnI-XbaI fragment of BS-Z into pcDNA3 (Invitrogen). pMAL-BZLF1, pMAL-BZLF1d1, and pMAL-BZLF1d2 were constructed by amplifying the domain containing BZLF1 codons 1 to 245, 1 to 185, and 56 to 245, respectively (Fig. 1), by PCR from pcDNA3-BZLF1 and cloning the DNA fragments into pMAL-c (New England BioLabs) in frame with maltose binding protein (MBP). pMAL-BHRF1 (14) and pME-BGLF4(F) (13) were described previously.

Production and purification of MBP or GST fusion proteins expressed in *Escherichia coli*. GST fusion proteins (GST-BGLF4, GST-BcLF1-a, GST-BcLF1-b, and GST-BcLF1-c) were expressed in *E. coli* that had been transformed with pGEX-BGLF4, pGEX-BcLF1-a, pGEX-BcLF1-b, and pGEX-BcLF1-c, respectively, and purified as described previously (15). MBP fusion proteins (MBP-BZLF1, MBP-BZLF1d1, MBP-BZLF1d2, and MBP-BHRF1) were expressed in *E. coli* that had been transformed with pMAL-BZLF1, pMAL-BZLF1d1, pMAL-BZLF1d2, and pMAL-BHRF1, respectively, and purified as described previously (14).

Purification of GST fusion proteins from baculovirus-infected cells. GST-BGLF4 and GST-BGLF4K102I proteins were purified from Sf9 cells infected with Bac-GST-BGLF4 and Bac-GST-BGLF4K102I, respectively, as described previously (13, 17).

Antibodies. To generate rabbit polyclonal antibody to BGLF4 or BcLF1, two rabbits were immunized with purified GST-BGLF4 or a mixture of GST-BcLF1-a, GST-BcLF1-b, and GST-BcLF1-c by a standard protocol at MBL (Nagoya, Japan). A mouse monoclonal antibody to BZLF1 was described previously (40). A mouse monoclonal antibody to BMRF1 (mAb8186) was purchased from Chemicon (Temecula, CA).

Immunoblotting. Electrophoretically separated proteins were transferred to nitrocellulose sheets and blotted with antibodies as described previously (19).

Purification of virions. EBV virions were purified as described elsewhere (22, 41), with minor modifications. Briefly, B95-8 cells were treated with TPA and incubated for 7 days. Cell culture supernatants were then harvested by low-speed centrifugation and passed through 0.45-μm-pore-size filters. The EBV-containing supernatant (approximately 1.4 liters) was centrifuged for 2 h at 25,000 rpm in an SRP28S rotor (Hitachi Koki, Ibaragi, Japan). The pellet was resuspended in 0.5 ml TBSal (200 mM NaCl, 2.6 mM KCl, 10 mM Tris-HCl [pH 7.5], 20 mM $MgCl_2$, 1.8 mM $CaCl_2$), layered onto a 9-ml discontinuous sucrose gradient (30%, 40%, and 50%) in TBSal, and centrifuged for 2 h at 18,000 rpm in a P40ST rotor. Fractions (500 μl) were collected, pelleted by centrifugation for 2 h at 29,800 rpm in a PRS50 rotor, and analyzed by immunoblotting or immune complex kinase assays.

Immune complex kinase assays. Purified virions were pelleted from the virion-containing fraction as described above and lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40 [NP-40], 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) containing a protease inhibitor cocktail (Sigma). Supernatant fluids obtained after centrifugation of the lysate were precleared by incubation with protein A-Sepharose

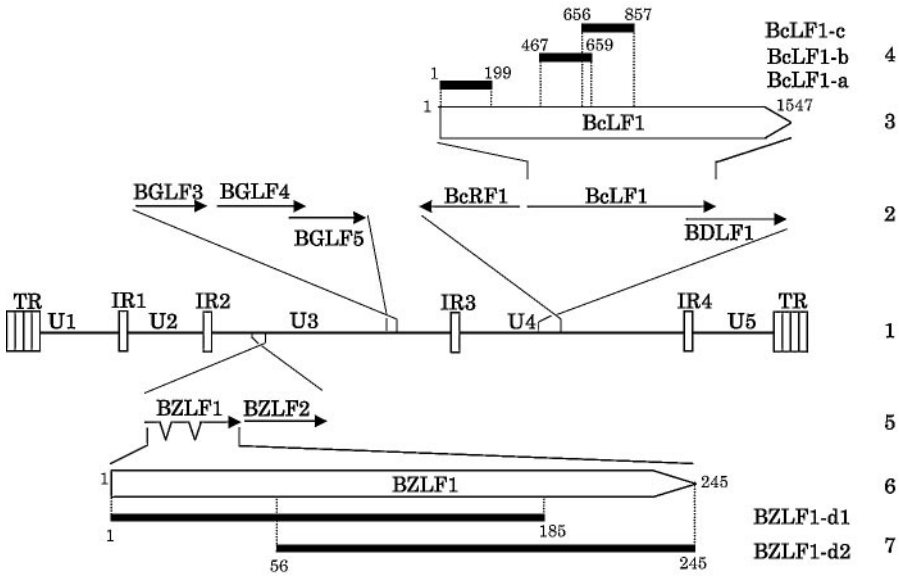

FIG. 1. Schematic diagram of the EBV genome and location of the genes studied in this report. Line 1: diagram of the EBV genome. The unique sequences are designated U1 to U5. The terminal and internal repeats flanking the unique sequences are shown as open rectangles with their designations above the rectangles. Line 2: expanded section of the domains encoding BGLF4 and BcLF1. The polarity and structure of the BGLF4 and BcLF1 coding regions are shown. Line 3: diagram of the 1,547 codons of BcLF1. Line 4: diagram of the BcLF1 peptides used to generate GST-BcLF1 fusion proteins. Line 5: expanded diagram of the domain encoding BZLF1. Line 6: diagram of the 245 codons of BZLF1. Line 7: diagram of the BZLF1 peptides used to generate MBP-BZLF1 fusion proteins.

beads (Amersham-Pharmacia) at 4°C for 30 min and then reacted with rabbit polyclonal antibody to BGLF4 at 4°C for 2 h. Additional protein A-Sepharose beads were added, and the reaction was continued for another 1.5 h. Immunoprecipitates were collected by a brief centrifugation; washed twice with high-salt buffer (1 M NaCl, 10 mM Tris-HCl [pH 8.0], 0.2% NP-40), once with low-salt buffer (0.1 M NaCl, 10 mM Tris-HCl [pH 8.0], 0.2% NP-40), six times with RIPA buffer, and twice with BGLF4 kinase buffer (50 mM Tris-HCl [pH 8.0], 200 mM NaCl, 50 mM $MgCl_2$, 0.1% NP-40, and 1 mM dithiothreitol); and analyzed by in vitro kinase assays. For these assays, BGLF4 kinase buffer containing 10 μM ATP and 10 μCi [γ-^{32}P]ATP was added to the protein A-Sepharose beads (15 μl) containing immunoprecipitated BGLF4 protein kinase, and the samples were reacted at 30°C for 30 min. After incubation, the samples were washed twice with TNE buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, and 1 mM EDTA) and analyzed by electrophoresis in denaturing gels with or without phosphatase treatment. After electrophoresis, the separated proteins were transferred from the gels to nitrocellulose membranes (Bio-Rad) and the membranes were exposed to X-ray film and then immunoblotted with anti-BGLF4 antibody.

Tegument release assay. The tegument release assay was performed essentially as described previously (29), with some modifications. Briefly, B95-8 cells were treated with TPA and incubated for 7 days. Cell culture supernatants obtained after low-speed centrifugation were passed through 0.45-μm-pore-size filters and centrifuged for 1 h at 25,000 rpm in an SRP28S rotor. Virion-containing pellets were resuspended in 0.2 ml lysis buffer A (20 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1% Triton X-100) containing 0, 0.15, or 1 M NaCl and incubated for 30 min on ice. The virion-containing pellets were also resuspended in 0.2 ml lysis buffer B (20 mM Tris-HCl [pH 7.5], 1% Triton X-100) containing 0.15 M NaCl for 1 h at 37°C in the presence or absence of 1 mM $MgCl_2$ and 1 mM ATP. The mixture was then layered onto 0.5 ml 35% sucrose in a microcentrifuge tube and centrifuged at 14,000 rpm for 30 min at 4°C. A 200-μl sample of the supernatant above the sucrose cushion interface was carefully removed and contained virion-dissociated soluble protein. After aspiration of the sucrose cushion, the pellet was removed and contained insoluble capsid-tegument structures. The supernatants and pellets were then analyzed by immunoblotting. In some experiments, 50 U calf intestinal phosphatase (CIP; New England BioLabs) was included in the incubation mixture.

In vitro kinase assays. MBP fusion proteins were captured on amylose beads (New England BioLabs) and used as substrates in in vitro kinase assays with 1 μg purified GST-BGLF4 and GST-BGLF4K102I, as described previously (14).

Transfection. COS-1 cells were transfected with appropriate expression vectors by the DEAE-dextran method described previously (18).

Two-dimensional electrophoretic analysis. Transfected COS-1 cells in 100-mm-diameter dishes were washed with phosphate-buffered saline, resuspended in 50 μl sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 20% glycerol), and lysed by brief sonication. After being boiled for 5 min, the samples were incubated on ice for a few minutes. The samples were then incubated without or with 50 U CIP for 2 h at 37°C, after which they were analyzed by two-dimensional electrophoresis. Two-dimensional electrophoresis was performed using an immobilized pH gradient (IPG) for first-dimension isoelectric focusing (IEF) (ZOOM IPGRunner System; Invitrogen) according to the manufacturer's instructions, with some modification. Briefly, a 20-μl sample prepared as described above was mixed with 120 μl sample rehydration buffer (8 M urea, 2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate [CHAPS], 0.5% ZOOM carrier ampholytes [Invitrogen], 20 mM dithiothreitol). IPG strips with a pH gradient of 6 to 10 (Invitrogen) were rehydrated in the sample solution (140 μl) for 2 h at room temperature, and IEF was performed at 200 V (20 min), 450 V (15 min), 750 V (15 min), and 2,000 V (30 min). IEF strips were then equilibrated for 15 min in equilibration buffer (Invitrogen). The equilibrated strips were overlaid onto a NuPAGE 4 to 12% Bis-Tris ZOOM gel (Invitrogen) and sealed with 0.5% agarose in NuPAGE MOPS (morpholinepropanesulfonic acid)-SDS running buffer (Invitrogen). Prestained molecular weight markers (Bio-Rad) were placed adjacent to the pH 10 end of the IPG strip. Second-dimension electrophoresis in SDS-polyacrylamide gels was performed at 145 V. Immunoblotting of electrophoretically separated proteins was performed as described above.

Coimmunoprecipitation. TPA-treated B95-8 cells or transfected COS-1 cells were lysed in NP-40 buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5% Nonidet P-40) containing a protease inhibitor cocktail (Sigma). Supernatant fluids obtained after centrifugation of the cell lysates were precleared by incubation with protein A-Sepharose beads (Amersham Biosciences) at 4°C for 30 min and then reacted with rabbit polyclonal antibody to BGLF4 at 4°C for 6 h. Protein A-Sepharose beads were then added, and the reaction continued for another 2 h. Immunoprecipitates were collected by a brief centrifugation, washed extensively with NP-40 buffer, and analyzed by immunoblotting with mouse monoclonal antibody to BZLF1.

Immunofluorescence. Indirect immunofluorescence assays were performed as described previously (11), except that anti-mouse or anti-rabbit IgG conjugated to Alexa Fluor 546 was used as secondary antibody in addition to anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) and samples were examined with a Zeiss laser scanning microscope, LSM5.

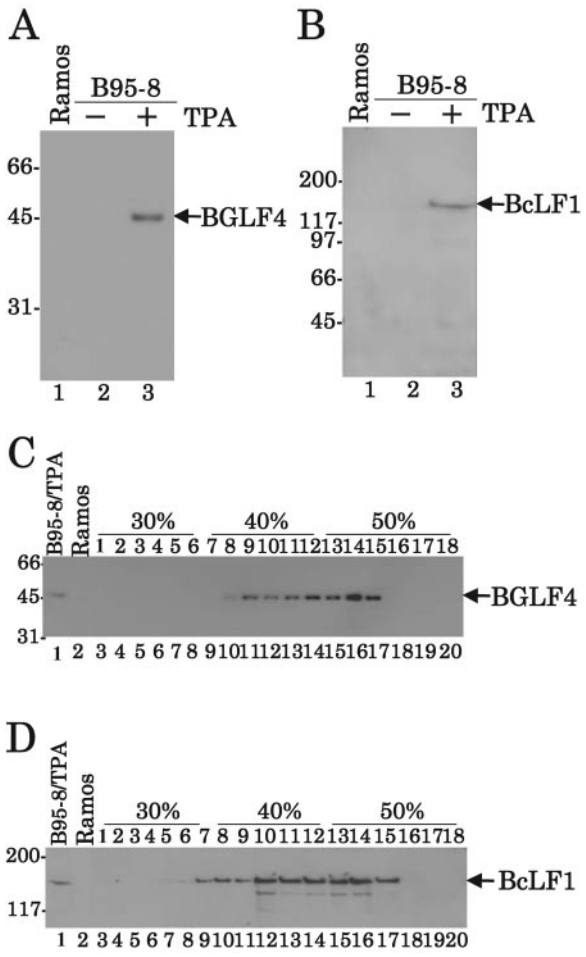

FIG. 2. Analysis of virion-associated BGLF4. (A and B) Immunoblots of electrophoretically separated lysates from Ramos (lane 1) and B95-8 cells without (lane 2) or with (lane 3) TPA treatment. Cell lysates were analyzed by immunoblotting with polyclonal antibody to BGLF4 (A) and BcLF1 (B). (C and D) Immunoblots of electrophoretically separated sucrose gradient fractions of BGLF4 (C) and BcLF1 (D). As described in Materials and Methods, virions harvested from the culture supernatants of TPA-treated B95-8 cells were separated in a discontinuous sucrose gradient. Fractions were collected, separated by electrophoresis, and immunoblotted with polyclonal antibody to BGLF4 (C) and BcLF1 (D). Lanes 1 and 2, whole-cell extracts of TPA-treated B95-8 and Ramos cells, respectively. Numbers at left are molecular masses in kilodaltons.

RESULTS

BGLF4 protein in virions. To facilitate the study of BGLF4 protein kinase and its role as a virion-associated protein, antibodies were raised against the BGLF4 protein and against the BcLF1 protein, which is a major capsid protein. As shown in Fig. 2A and B, antibodies to BGLF4 and BcLF1 reacted with specific bands with apparent M_rs of 43,000 and 150,000, respectively, in lysates from B95-8 cells in which lytic infection had been induced. Similar results for BGLF4 were reported by Gershburg et al. and Wang et al. (8, 43). No specific bands were detected using the antibodies to immunoblot lysates of EBV-negative Ramos cells. In uninduced B95-8 cells, the specific bands detected by the antibodies were seen only when the immunoblots were overexposed (data not shown).

To confirm that BGLF4 is a virion component, extracellular virions were harvested, pelleted, and separated in a discontinuous sucrose gradient. In this virion purification system, herpesvirus virions accumulate around the boundary between 40% and 50% sucrose (22, 41). Fractions were collected and analyzed by immunoblotting with anti-BGLF4 and anti-BcLF1 antibodies. As shown in Fig. 2C, BGLF4 was detected in fractions around the boundary between 40% and 50% (between fractions 8 and 15) and these fractions corresponded to those of the major capsid protein BcLF1 (Fig. 2D). These results confirm that BGLF4 protein is a component of EBV virions. In agreement with these results, Johannsen et al. and Wang et al. reported that BGLF4 proteins are detected in purified EBV virions (10, 43).

To test whether the BGLF4 protein in virions has kinase activity, sucrose gradient fraction 12 containing BGLF4, as described above, was immunoprecipitated with anti-BGLF4 antibody and the immunoprecipitate was used in kinase assays. The same fraction was prepared from the supernatant of EBV-negative Ramos cells and analyzed as a control. To reduce the possibility that the anti-BGLF4 antibody might bring down contaminating kinase(s), the immunoprecipitates containing BGLF4 protein were washed with high-salt buffer containing 1 M NaCl prior to the in vitro kinase assays. As shown in Fig. 3A, in these kinase assays, BGLF4 was labeled with [γ-^{32}P]ATP, but no labeled protein band at the apparent M_r corresponding to BGLF4 was detected in immunoprecipitates from Ramos cells. To confirm that BGLF4 labeling was due to phosphorylation, labeled BGLF4 was treated with phosphatase. As shown in Fig. 3C, BGLF4 protein labeling was eliminated by phosphatase treatment, indicating that BGLF4 was labeled with [γ-^{32}P]ATP by phosphorylation. The expression of each BGLF4 protein and identification of the BGLF4 radiolabeled band were verified by immunoblotting (Fig. 3B and D). These results indicate that BGLF4 proteins packaged in virions are likely to be enzymatically active. However, we cannot completely exclude the possibility that BGLF4 was phosphorylated by a cellular kinase strongly associated with BGLF4 that could not be removed by washing with 1 M NaCl and RIPA buffer.

BGLF4 protein dissociates from virions in a phosphorylation-dependent process. As described above, it has been recently reported that BGLF4 protein is a virion tegument or capsid-associated protein (10, 43). These studies, however, did not address the question of whether BGLF4 protein can dissociate from virions. To function in early postinfection events, BGLF4 would need to be released into the cytoplasm of newly infected cells. To study this question, the tegument release assay, which examines the effects of various conditions on the dissociation of herpesvirus tegument proteins from virions (29), was used. In the first series of experiments, equal aliquots of EBV virions were incubated on ice in lysis buffer A containing 0, 0.15, or 1 M NaCl. These samples were then fractionated into soluble and insoluble extracts by centrifugation through a sucrose cushion. The protein content of each fraction was analyzed by immunoblotting. As shown in Fig. 4A, BGLF4 was detected only in the insoluble fraction when virions were treated with lysis buffer A without NaCl. In contrast, treatment of virions with lysis buffer containing 0.15 M or 1 M NaCl

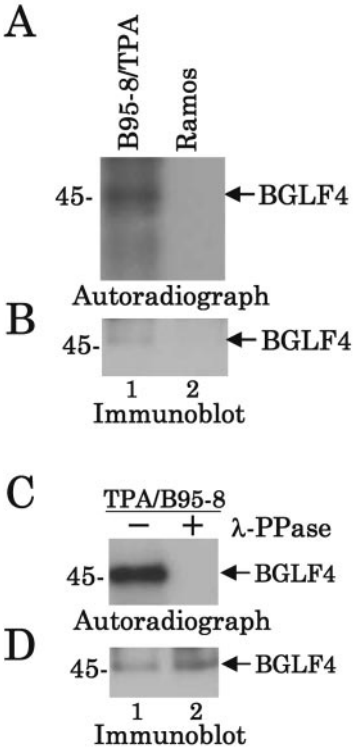

FIG. 3. Autoradiographs of BGLF4 immunoprecipitates after in vitro kinase assays and electrophoresis. (A) Purified virions from one of the virion-containing fractions described in Fig. 2C and D (lane 1) and from a corresponding fraction from the supernatant of Ramos cells (lane 2) were lysed and immunoprecipitated with antibody to BGLF4. The immunoprecipitates were incubated in kinase buffer containing [γ-^{32}P]ATP, separated on a denaturing gel, transferred to a nitrocellulose membrane, and analyzed by autoradiography. (B) Immunoblot of the nitrocellulose membrane in panel A using anti-BGLF4 antibody. (C) Immunoprecipitates prepared as in panel A either mock treated (lane 1) or treated with λ-PPase (lane 2), separated on a denaturing gel, transferred to a nitrocellulose membrane, and analyzed by autoradiography. (D) Immunoblot of the nitrocellulose membrane in panel C using anti-BGLF4 antibody. Numbers at left are molecular masses in kilodaltons.

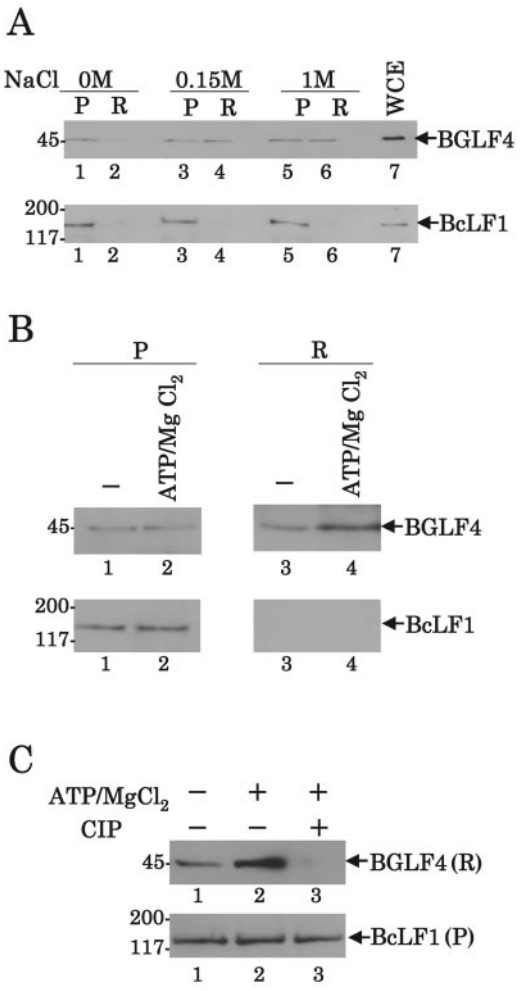

FIG. 4. Immunoblots of in vitro tegument release assays of BGLF4 and BcLF1. (A) EBV virions from the supernatant of TPA-treated B95-8 cells were treated with Triton X-100 in 0, 0.15, or 1 M NaCl. The samples were then separated by sucrose gradient centrifugation into pelleted (P) and released (R) fractions and analyzed by immunoblotting with antibody to BGLF4 (upper panel) or BcLF1 (lower panel). Lane 7 contains whole-cell extract (WCE). (B) EBV virions were treated with lysis buffer B in the absence or presence of 1 mM ATP and 1 mM $MgCl_2$ at 37°C for 1 h, fractionated as described in panel A, and analyzed by immunoblotting with antibody to BGLF4 (upper panels) or BcLF1 (lower panels). (C) EBV virions were treated with lysis buffer B with or without ATP and $MgCl_2$ in the absence or presence of CIP at 37°C for 1 h, fractionated as described in panel A, and analyzed by immunoblotting with antibody to BGLF4 or BcLF1. The fractions of released (R) BGLF4 (upper panel) and pelleted (P) BcLF1 (lower panel) are shown. Numbers at left are molecular masses in kilodaltons.

resulted in significant dissociation of BGLF4 from the virions. Interestingly, a portion of BGLF4 remained associated with virions even in 1 M NaCl. The capsid protein BcLF1 was found only in the insoluble fraction, as expected, eliminating the possibility that BGLF4 in the soluble fraction was due to degradation of virion structures during the in vitro tegument release assay. These results indicate that BGLF4 can be released from virions, but some BGLF4 remains tightly associated with the virions.

In the second series of experiments, EBV virions were incubated at 37°C for 1 h in lysis buffer B containing 0.15 M NaCl in the absence or presence of 1 mM ATP and 1 mM $MgCl_2$, fractionated into soluble and insoluble fractions as described above, and analyzed by immunoblotting. As shown in Fig. 4B, addition of ATP and $MgCl_2$ enhanced the release of BGLF4 from virions. However, CIP addition significantly reduced BGLF4 release (Fig. 4C). These results indicate that release of BGLF4 from virions is regulated by phosphorylation.

BGLF4 phosphorylates BZLF1 in vitro. Since BGLF4 is a protein kinase, identification of its specific substrate(s) is necessary to elucidate its function(s). For these studies, several EBV and cellular proteins fused to MBP were expressed in *E. coli* and purified. The MBP fusion proteins bound to amylose beads were reacted with purified GST-BGLF4 or GST-BGLF4K102I in in vitro kinase assays, and the reaction products were analyzed by electrophoresis in denaturing gels.

The autograph of the gel from the reaction of MBP-BZLF1 with GST-BGLF4 showed a protein band labeled with

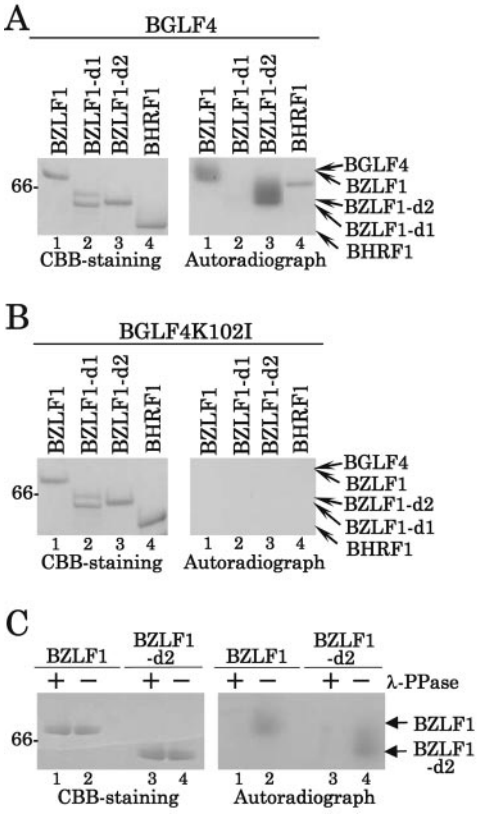

FIG. 5. Autoradiographs of in vitro BZLF1 phosphorylation. (A and B) Left panels: CBB-stained gels of phosphorylated BZLF1. Purified MBP-BZLF1 (lane 1), MBP-BZLF1d1 (lane 2), MBP-BZLF1d2 (lane 3), and MBP-BHRF1 (lane 4) were incubated in kinase buffer containing [γ-^{32}P]ATP and purified GST-BGLF4 (A) or GST-BGLF4K102I (B), separated on a denaturing gel, and stained with CBB. Right panels: autoradiographs of the gels in the left panels. (C) Left panel: purified MBP-BZLF1 (lanes 1 and 2) and MBP-BZLF1d2 (lanes 3 and 4) incubated in kinase buffer containing [γ-^{32}P]ATP and purified GST-BGLF4 were either mock treated (lanes 2 and 4) or treated with λ-PPase (lanes 1 and 3), separated on a denaturing gel, and stained with CBB. Right panel: autoradiograph of the gel in the left panel. Numbers at left are molecular masses in kilodaltons.

[γ-^{32}P]ATP with an M_r of 70,000 (Fig. 5A, right panel). Since GST-BGLF4 and MBP-BZLF1 show similar molecular masses, it was difficult to know whether the labeled band corresponded to labeled MBP-BZLF1 or GST-BGLF4. In in vitro kinase assays, protein kinase is sometimes pulled down by a specific substrate (12, 24). To resolve this problem, two deletion mutants of MBP-BZLF1 (MBP-BZLF1d1 and MBP-BZLF1d2) were constructed and tested in the in vitro kinase assays. MBP-BZLF1d1 and MBP-BZLF1d2 consist of MBP fused to peptides encoded by BZLF1 codons 1 to 185 and codons 56 to 245, respectively. As shown in Fig. 5A (right panel), MBP-BZLF1d2 was labeled with [γ-^{32}P]ATP in kinase assays using GST-BGLF4, while MBP-BZLF1d1 and MBP-BHRF1 were not. When the kinase-negative mutant GST-BGLF4K102I was used, none of the MBP fusion proteins were labeled (Fig. 5B, right panel). To confirm that MBP-BZLF1d2 labeling by GST-BGLF4 was due to phosphorylation, the labeled MBP-BZLF1d2 was treated with phosphatase. As shown in Fig. 5C (right panel), MBP-BZLF1d2 labeling by GST-BGLF4 was eliminated by phosphatase treatment, indicating that MBP-BZLF1d2 was labeled by phosphorylation. The presence of each MBP fusion protein and that of the radiolabeled MBP-BZLF1d2 band were verified by Coomassie brilliant blue (CBB) staining (Fig. 5A, B, and C, left panels).

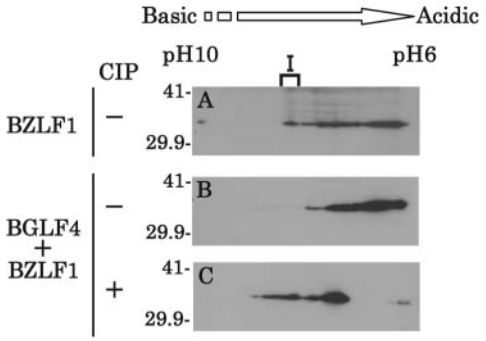

FIG. 6. Effect of BGLF4 expression on posttranslational modification of BZLF1 protein in COS-1 cells. COS-1 cells transiently expressing either BZLF1 alone (A) or both BZLF1 and BGLF4 (B and C) were solubilized, mock treated (A and B) or treated with CIP (C), separated by two-dimensional electrophoresis, and immunoblotted with mouse monoclonal antibody to BZLF1. Numbers at left are molecular masses in kilodaltons.

These results indicate that BGLF4 specifically and directly phosphorylates the BZLF1 peptide encoded by codons 186 to 245 in vitro.

BGLF4 alters posttranslational modification of BZLF1 in vivo. To test whether BGLF4 mediates posttranslational modification of BZLF1 in vivo, COS-1 cells were transfected with either BZLF1 expression vector (pcDNA-BZLF1) alone or both BZLF1 (pcDNA-BZLF1) and BGLF4 [pME-BGLF4(F)] expression vectors, solubilized, separated by two-dimensional electrophoresis, and immunoblotted with anti-BZLF1 antibody. As shown in Fig. 6A, when BZLF1 is expressed alone, it migrated as isoforms with different isoelectric points (pIs). This observation indicates that BZLF1 is posttranslationally modified by a cellular enzyme(s) in the absence of any other viral protein expression, as reported previously (1). When BZLF1 and BGLF4 were coexpressed, some BZLF1 isoforms in a gel region designated I, which were detected when BZLF1 was expressed by itself, were not detected (Fig. 6A and B). These results indicate that BGLF4 mediates posttranslational modification of BZLF1 in vivo. Furthermore, after phosphatase treatment of lysates of COS-1 cells expressing both BZLF1 and BGLF4, BZLF1 isoforms in region 1 were restored (Fig. 6C), strongly suggesting that the posttranslational modification of BZLF1 mediated by BGLF4 is due to phosphorylation.

BGLF4 interacts with BZLF1 in EBV-infected cells. To examine whether BGLF4 forms a stable complex with BZLF1, two series of experiments were performed.

In the first series of experiments, COS-1 cells transfected with BGLF4 and BZLF1 expression vectors were lysed and immunoprecipitated with anti-BGLF4 antibody. The immunoprecipitates were analyzed by electrophoresis and immunoblotted with anti-BZLF1 antibody. As shown in Fig. 7A, when BZLF1 and BGLF4 were coexpressed in COS-1 cells, anti-

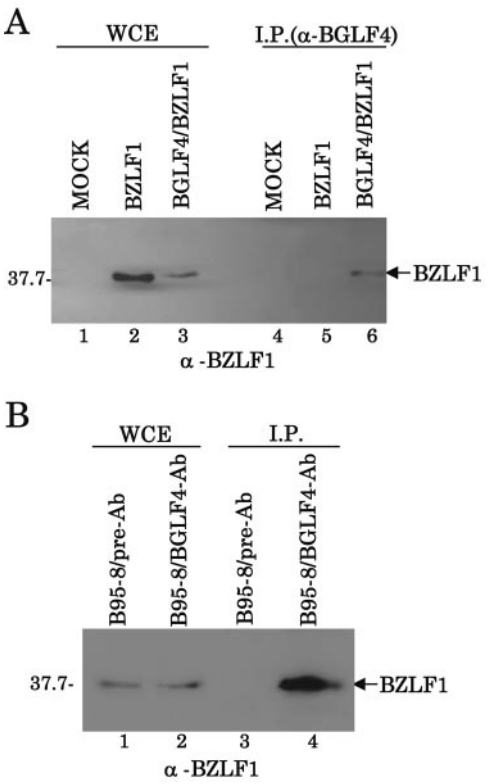

FIG. 7. Interaction of BGLF4 with BZLF1. (A) COS-1 cells mock transfected (lane 4) or transiently expressing BZLF1 alone (lane 5) or both BGLF4 and BZLF1 (lane 6) were solubilized and immunoprecipitated with antibody to BGLF4. The immunoprecipitates were analyzed by electrophoresis and immunoblotted with antibody to BZLF1. One-sixtieth of the COS-1 whole-cell extract (WCE) used in the reaction mixtures for lanes 4, 5, and 6 was loaded in lanes 1, 2, and 3, respectively. (B) TPA-treated B95-8 cells were solubilized and immunoprecipitated with anti-BGLF4 antibody (lane 4) or preimmune serum (lane 3). The immunoprecipitates were analyzed by electrophoresis and immunoblotted with mouse monoclonal antibody to BZLF1. One-sixtieth of the B95-8 whole-cell extract (WCE) used in the reaction mixtures for lanes 3 and 4 was loaded in lanes 1 and 2, respectively. Numbers at left are molecular masses in kilodaltons.

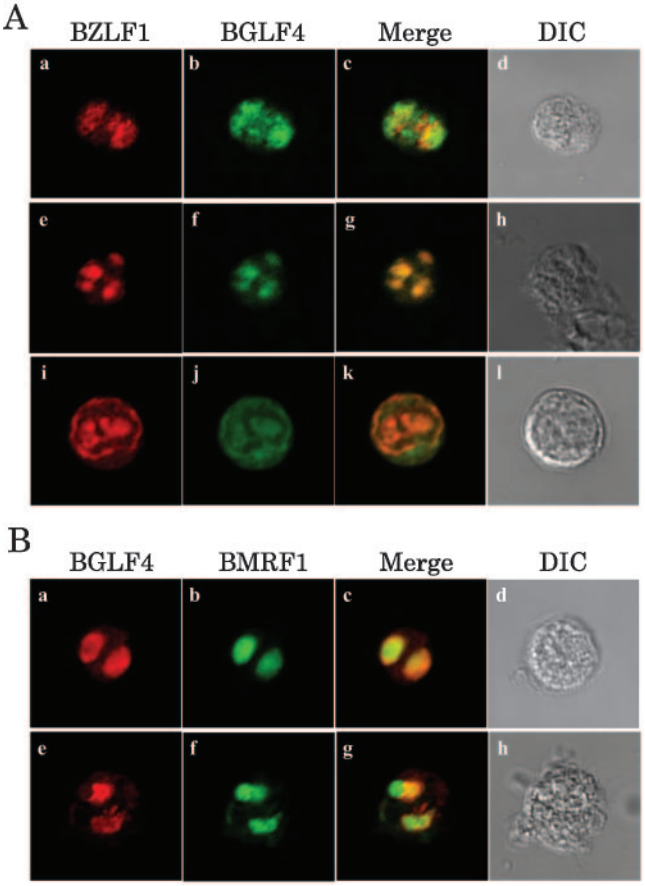

FIG. 8. Immunofluorescent localization of BGLF4, BZLF1, and BMRF1 in EBV-infected cells. (A) Digital confocal microscope images showing localization of BGLF4 and BZLF1 in Akata cells treated with anti-human IgG for 9 h. The cells were then fixed, permeabilized, and double labeled with a combination of mouse monoclonal antibody to BZLF1 (a, e, and i) and rabbit polyclonal antibody to BGLF4 (b, f, and j), which were detected with Alexa 546-conjugated anti-mouse IgG antibody (red fluorescence) and FITC-conjugated anti-rabbit IgG antibody (green fluorescence), respectively. Single-color images were captured separately and are shown in panels a, b, e, f, i, and j. Panels c, g, and k and panels d, h, and l show simultaneous acquisitions of both colors and differential interference contrast (DIC), respectively. (B) Digital confocal microscope images showing localization of BGLF4 and BMRF1 in Akata cells treated with anti-human IgG for 9 h. The cells were then processed as described in panel A and double labeled with a combination of rabbit polyclonal antibody to BGLF4 (a and e) and mouse monoclonal antibody to BMRF1 (b and f), which were detected with Alexa 546-conjugated anti-mouse IgG antibody (red fluorescence) and FITC-conjugated anti-rabbit IgG antibody (green fluorescence), respectively. Single-color images were captured separately and are shown in panels a, b, e, and f. Panels c and g and panels d and h show simultaneous acquisitions of both colors and differential interference contrast, respectively.

BGLF4 antibody coprecipitated BZLF1 and BGLF4 (lane 6). In contrast, when BZLF1 was expressed by itself, BZLF1 was not coprecipitated by the antibody (lane 5). These results indicate that BGLF4 interacts with BZLF1 in vivo.

In the second series of experiments, TPA-treated B95-8 cells were used for coimmunoprecipitation studies as described above. As shown in Fig. 7B, anti-BGLF4 antibody coprecipitated BZLF1 and BGLF4 but preimmune serum did not, indicating that BGLF4 forms a complex with BZLF1 in EBV-infected cells.

BGLF4 is colocalized with BZLF1 in replication compartment-like structures of EBV-infected cells. To investigate subcellular localization of BGLF4 and BZLF1 in EBV-infected cells, Akata cells in which EBV lytic infection had been induced were treated with anti-human IgG for 9 h and then fixed and processed for indirect immunofluorescence assays with antibodies to BGLF4, BZLF1, and BMRF1. As shown in Fig. 8A, BGLF4 accumulated in intranuclear globular structures and colocalized with BZLF1. It has been previously reported that BZLF1 is localized in replication compartments in cells lytically infected with EBV (26, 39). Since the globular structures containing both BZLF1 and BGLF4 observed in these studies (Fig. 8A) resembled the replication compartments reported previously (26, 39), we also examined whether BGLF4 is localized in these compartments in EBV-infected cells by double labeling experiments with anti-BGLF4 and anti-BMRF1 antibodies, since the BMRF1 is a marker of replica-

tion compartments (42). These studies showed that BGLF4 colocalized with BMRF1 in globular structures in induced Akata cells (Fig. 8B). The colocalization of BGLF4 and BMRF1 has also been reported recently by Wang et al. (43). Taken together, these results indicate that BGLF4 is colocalized with BZLF1 in replication compartment-like structures in cells lytically infected with EBV.

DISCUSSION

The major findings in this report relate to two of the less well understood aspects of EBV-encoded protein kinase BGLF4. The first of these is the action of BGLF4, as a virion tegument protein, immediately after viral penetration. The second is the identification of the substrate(s) of the BGLF4 protein kinase.

After herpesvirus penetration, tegument proteins enter the cell with the capsid. Some tegument proteins are released into the cytoplasm and function in early postinfection to modulate the cellular environment. Tegument proteins with such activities have been studied in detail for some herpesviruses. For example, upon release from the virion, HSV-1 tegument protein UL48 (VP16) interacts with cellular proteins HCF and Oct-1 and the complex promotes transcription of viral IE genes by binding to cognate responsive elements in IE gene promoters in the nucleus (36). Another HSV-1 tegument protein, UL41, plays a role in the shutoff of host protein synthesis by degrading cellular mRNAs in the cytoplasm, producing preferential translation of viral mRNAs by host cell ribosomes (36). Thus, the functions of tegument proteins in early postinfection have been well established in HSV-1 and some other herpesviruses (4, 28, 36). In contrast, there is a lack of information on the function(s) of EBV tegument proteins. To function in early postinfection, virion tegument proteins must dissociate from the virions and be released into the cytoplasm as soluble proteins. The BGLF4 protein fulfills this requirement, based on the observations reported here that a significant fraction of BGLF4 can dissociate from virions and be solubilized at physiological salt concentrations in in vitro tegument release assays. This property of BGLF4 is different from that of HSV-1-encoded protein kinase UL13. In similar tegument release assays, HSV-1 UL13 was barely detectable in the soluble fraction, while other HSV virion tegument proteins (e.g., UL47 [VP13/14], UL48 [VP16], and UL49 [VP22]) were efficiently released from virions under the same conditions, suggesting that most HSV-1 UL13 is tightly virion associated (29).

In the present study, we showed that phosphorylation of BGLF4 mediates its dissociation from virions in vitro. This conclusion was supported by tegument release assays showing that physiological concentrations of ATP and $MgCl_2$, which activate protein kinase-mediated phosphorylation, enhanced BGLF4 release from virions and phosphatase treatment of virions severely inhibited BGLF4 release. Consistent with these conclusions, it has been reported that dissociation of major tegument proteins from alphaherpesvirus virions, including HSV-1 and equine herpesvirus 1, was promoted by phosphorylation (29), implying that phosphorylation of virion-associated proteins is a general mechanism for regulating the dissociation of herpesvirus tegument structures. At present, the source of the virion-associated protein kinase activity responsible for the release of BGLF4 is not known. Since it has been reported that the HSV-1 virion-associated protein kinase UL13 mediates release of the major HSV-1 tegument protein UL49 (29), a reasonable hypothesis is that autophosphorylation of BGLF4 is involved in its dissociation from virions. However, it remains possible that a significant amount of a contaminating cell-derived kinase was present in the purified virion preparations in the studies reported here.

At present, the target proteins of virion-associated BGLF4 are not known. EBNA-LP is one potential target, since it is the first gene product expressed, together with EBNA-2, after EBV infection of B cells. We previously mapped the major phosphorylation site of EBNA-LP by cellular kinase(s) to serine 35 (Ser-35) and showed that amino acid substitutions at Ser-35 significantly reduced the ability of EBNA-LP to induce LMP-1 expression concurrently with EBNA-2 in B cells (45). We have also shown that both cellular cdc2 and virally encoded BGLF4 protein kinases phosphorylate EBNA-LP Ser-35 both in vitro and in vivo (14). These results indicate that the EBNA-LP coactivator function can be regulated by phosphorylation at Ser-35 mediated by cdc2 and BGLF4. Since EBNA-LP is expressed first after EBV infection of B cells, BGLF4 proteins brought into infected cells by the virions may phosphorylate the nascent EBNA-LP. There may be an advantage for the virus in bringing a virion-associated protein kinase into infected cells to phosphorylate EBNA-LP Ser-35, thereby expressing the EBNA-LP coactivator function independent of the intracellular conditions of the host cells, since EBV can infect resting B cells in which cdc2 activity, which would mediate EBNA-LP phosphorylation, is down-regulated (38). Yue et al. also recently reported that BGLF4 phosphorylates EBNA-2 in vitro and in vivo and that this phosphorylation affects EBNA-2 function (47). Virion-associated BGLF4 might target these first viral gene products that are expressed in early postinfection.

The studies reported here showed that the viral immediate-early protein BZLF1 binds to and is phosphorylated by BGLF4. Identification of the physiological substrate of a viral protein kinase requires demonstration that phosphorylation of the substrate in cells infected with a mutant virus lacking the protein kinase activity is altered and that the substrate is specifically and directly phosphorylated by the kinase in vitro since such in vivo analyses cannot eliminate the possibility that the protein kinase activates or induces another kinase(s) that phosphorylates the target substrate (12, 24). In the studies reported here, we have shown that purified BZLF1 was phosphorylated in vitro in the presence of purified recombinant BGLF4. BZLF1 phosphorylation was shown to be due to BGLF4 protein kinase activity and not to contaminating kinase(s) from the purification procedure, because a kinase-negative mutant (GST-BGLF4K102I) was unable to phosphorylate BZLF1 in vitro. Since recombinant BGLF4 mutant viruses are not available at present, it is not known whether BGLF4 mediates BZLF1 phosphorylation in EBV-infected cells. However, our data show that BGLF4 mediates posttranslational modification of BZLF4 in vivo, which is sensitive to phosphatase treatment. Furthermore, we showed that BGLF4 forms a complex with and colocalizes with BZLF1 in cells lytically infected with EBV. Taken together, it is likely that BZLF1 is a physiological substrate of BGLF4 in EBV-infected cells.

At present, we can only speculate about the functional sig-

nificance of the BGLF4-BZLF1 interaction. BZLF1 is thought to belong to the family of basic zipper (bZIP) transcription factors, including c-jun and c-*fos*, and to be a master switch between the EBV latent and lytic cycles (20). In addition, BZLF1 has been reported to play an essential role in EBV DNA replication in lytic infection by binding to the lytic origin of viral DNA replication (*oriLyt*) (6, 42). In the present study, we showed that BGLF4 colocalizes with BMRF1 in discrete nuclear structures, suggesting that BGLF4 is localized in the replication compartment where viral DNA replication takes place (42). Since BGLF4 has also been reported to phosphorylate the DNA polymerase processivity factor BMRF1 (3, 9), which is required for viral lytic-phase DNA replication (6), a possible BGLF4 function may be to regulate viral DNA replication by phosphorylating the essential EBV core DNA replication gene products BMRF1 and BZLF1. Further studies of identification of the BGLF4-mediated phosphorylation site(s) in BZLF1 and investigation of the phenotype of BZLF1 mutants, in which the phosphorylation site(s) has been mutated, will be needed and are in progress in this laboratory.

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