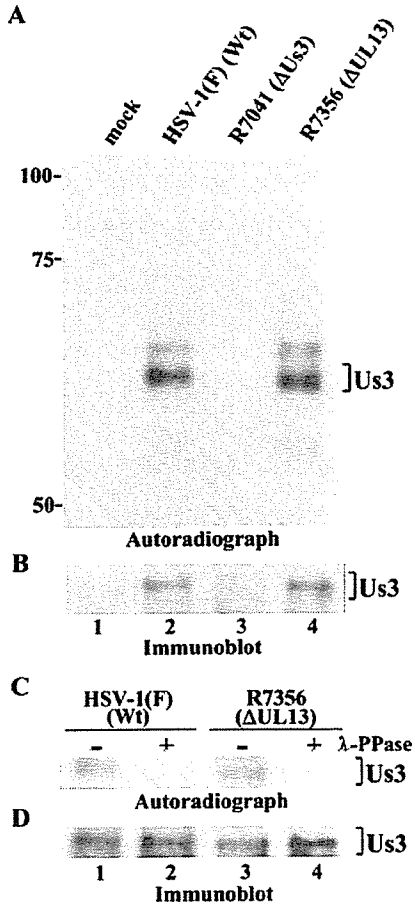

FIG. 3. Autoradiographic images of Us3 immunoprecipitates subjected to in vitro kinase assay. (A) Vero cells were mock infected (lane 1) or infected with HSV-1(F) (lane 2), R7041 (lane 3), or R7356 (lane 4); harvested at 12 h postinfection; and immunoprecipitated with antibody to Us3. The immunoprecipitates 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-Us3 antibody. (C) Immunoprecipitates prepared as in panel A were either mock treated (lanes 1 and 3) or treated with λ-PPase (lanes 2 and 4), separated on a denaturing gel, transferred to a nitrocellulose membrane, and analzyed by autoradiography. Wt, wild type. (D) Immunoblot of the nitrocellulose membrane in panel C using anti-Us3 antibody.

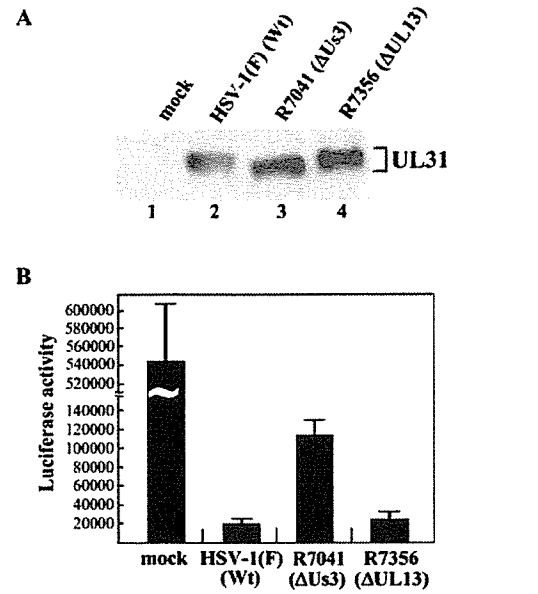

FIG. 4. (A) Immunoblot of electrophoretically separated lysates from Vero cells mock infected (lane 1) or infected with HSV-1(F) (lane 2), R7041 (lane 3), or R7356 (lane 4) at an MOI of 5. Infected cells were harvested at 12 h postinfection and immunoblotted with anti-UL31 antibody. (B) Caspase 3/7 activity of infected SK-N-SH cells after induction of apoptosis by osmotic shock. SK-N-SH cells were mock infected or infected with HSV-1(F), R7041, or R7356. At 12 h postinfection, the cells were exposed to sorbitol for 1 h, incubated for an additional 5 h, harvested, and assayed for caspase 3/7 activity using a Z-DEVD-aminoluciferin substrate. The values are the means and standard deviations for three independent experiments. Wt, wild type.

tion, solubilized, and immunoprecipitated with antibody to Us3. The immunoprecipitates were then used in kinase assays. To reduce the possibility that the anti-Us3 antibody might bring down contaminating kinase(s), the immunoprecipitates containing Us3 protein kinase were washed with high-salt buffer containing 1 M NaCl prior to in vitro kinase assays. As shown in Fig. 3A, Us3 protein in immunoprecipitates from HSV-1(F)- and R7356 (ΔUL13)-infected cells were labeled with [γ-^{32}P]ATP at similar levels, but no labeled protein bands at the apparent M_r corresponding to Us3 were detected in immunoprecipitates from R7041 (ΔUs3)-infected cells. The labeling of Us3 proteins was due to phosphorylation, as determined by studies showing that the labeling was eliminated by phosphatase treatment (Fig. 3C). The expression of each Us3 protein and identification of the Us3 radiolabeled band were verified by immunoblotting (Fig. 3B and D). These results indicate that Us3 proteins in lysates from cells infected with HSV-1(F) and R7356 (ΔUL13) have similar autophosphorylation activities.

We recently reported that Us3 directly phosphorylates UL31 in vitro and mediates posttranslational processing of UL31, which involves phosphorylation, in infected cells (25). To examine whether UL13 affects the posttranslational modification of UL31 in infected cells, in a second series of experiments, Vero cells mock infected or infected with HSV-1(F), R7041 (ΔUs3), or R7356 (ΔUL13) were harvested at 12 h postinfection, solubilized, electrophoretically separated in a denaturing gel, and subjected to immunoblotting with antibody to UL31. These data showed that UL31 protein produced in cells infected with R7041 (ΔUs3) migrated faster than that produced in cells infected with HSV-1(F) (Fig. 4A, lanes 2 and 3). In contrast, UL31 from cells infected with R7356 (ΔUL13) migrated as slowly as UL31 from cells infected with HSV-(F) (Fig. 4A, lanes 2 and 4). These results suggest that Us3 proteins expressed in cells infected with wild-type virus and UL13 deletion mutant virus induce similar posttranslational modifications of UL31.

Taken together, these experiments suggest that UL13-mediated phosphorylation of Us3 is not required for optimal Us3 protein kinase activity in infected cells. However, we cannot completely exclude the possibility that UL13 affects the Us3

protein kinase activity in vivo if cofactors are necessary for optimal Us3 protein kinase activity and/or that UL13 modulates the Us3 protein kinase activity against other Us3 substrates, except UL31, in vivo.

Level of caspase 3/7 activity in virus-infected SK-N-SH cells in which apoptosis was induced. To investigate whether UL13-mediated phosphorylation of Us3 affects Us3 regulation of apoptosis in infected cells, SK-N-SH cells were infected with HSV-1(F), R7041 (ΔUs3), or R7356 (ΔUL13), and at 12 h postinfection, apoptosis was induced by osmotic shock. The cells were then harvested and assayed for caspase 3/7 activity. As shown in Fig. 4B, caspase 3/7 activity induced by osmotic shock was significantly reduced (26.3-fold) in HSV-1(F)-infected cells (Fig. 4B). In R7041 (ΔUs3)-infected cells, there was less reduction of caspase 3/7 activity (4.7-fold), probably due to the lack of Us3 antiapoptotic activity. Similar results were reported previously (7). In R7356 (ΔUL13)-infected cells, caspase 3/7 activity was similar to that in HSV-1(F)-infected cells. The activity of Us3 to regulate apoptosis was not detected in SK-N-SH cells without induction of apoptosis, based on the observation that the level of the caspase 3/7 activity in SK-N-SH cells infected with R7041 (ΔUs3), without osmotic shock, was comparable to that in cells infected with wild-type virus (data not shown). These results suggest that the presence of UL13 does not affect caspase 3/7 activity in infected SK-N-SH cells.

UL13 is required for proper localization of UL34 and UL31 in infected cells. To investigate whether UL13-mediated phosphorylation of Us3 affects the role of Us3 in UL34 and UL31 localization, Vero cells were mock infected or infected with HSV-1(F), R7041 (ΔUs3), R7356 (ΔUL13), or R7356Rep (repair) at an MOI of 5; fixed at 12 or 15 h postinfection; and processed for indirect immunofluorescence assay with antibodies to UL34, UL31, and nucleoporin p62.

Previous studies reported that in HSV-1(F)-infected Vero and HEp-2 cells at 8 and 12 h postinfection, the UL34 and UL31 proteins colocalize at the nuclear envelope in a uniform distribution (61, 65). However, as shown in Fig. 5, the UL34 and UL31 distributions observed in the studies reported here differed from those results (61, 65), with both UL34 and UL31 showing nucleoplasmic localization in addition to nuclear-membrane localization (Fig. 5A, E, I, and M). The antibodies to UL34 and UL31 used in these studies were not able to detect any specific fluorescence in mock-infected cells (data not shown). In R7041 (ΔUs3)-infected cells, in agreement with previous reports (61, 65), UL34 and UL31 proteins were detected as punctate structures at the nuclear membrane (Fig. 5B, F, J, and N). However, although the previous studies found only UL34 and UL31 localized at the nuclear membrane in R7041 (ΔUs3)-infected cells, in the studies reported here, UL34 and UL31 were also detected as punctate structures in the nucleoplasm of R7041 (ΔUs3)-infected cells (Fig. 5B, F, J, and N). The nucleoplasmic staining of UL34 and UL31 in HSV-1(F)- and R7041 (ΔUs3)-infected cells did not appear to be specific to rabbit polyclonal antibodies generated in our laboratory. Thus, in HSV-1(F)- or R7041 (ΔUs3)-infected cells, the patterns of UL34 fluorescence detected by chicken polyclonal antibody to UL34, which was used in previously published studies (61, 62, 65), were almost identical to those of UL34 fluorescence detected by rabbit polyclonal antibody to UL34 generated in our laboratory (Fig. 6A, a, b, d, and e). As reported earlier (61), UL34 detected by chicken polyclonal antibody was clearly colocalized with UL31 detected by rabbit polyclonal antibody to UL31 in discrete punctate structures of R7041 (ΔUs3)-infected cells (Fig. 6B, b, f, and j). However, in the studies reported here, the punctate regions containing both UL34 and UL31 in R7041 (ΔUs3)-infected cells were detected not only at the nuclear membrane, but also in the nucleoplasm (Fig. 6B, b, f, and j).

In R7356 (ΔUL13)-infected cells, the UL34 protein was detected as punctate structures in the nucleus by rabbit polyclonal antibody to UL34 (Fig. 5C and G and 6A, c), as well as chicken polyclonal antibody to UL34 (Fig. 6A, f, and 6B, c). Similarly, R7356 (ΔUL13)-infected cells showed UL31 localization as nuclear punctate staining (Fig. 5K and O and 6B, g). Furthermore, UL34 and UL31 colocalized in the nuclear punctate structures of R7356 (ΔUL13)-infected cells (Fig. 6B, c, g, and k). These localization features of UL34 and UL31 in R7356 (ΔUL13)-infected cells seemed to be similar to those of the viral proteins in R7041 (ΔUs3)-infected cells (61). It should be noted, however, that the sizes of UL34 and UL31 stained speckles in R7356 (ΔUL13)-infected cells appeared to be larger than those in R7041 (ΔUs3)-infected cells, and the number of speckles in R7356 (ΔUL13)-infected cells was less than in R7041 (ΔUs3)-infected cells (Fig. 5B, C, F, G, J, K, N, and O and 6A, b, c, e, and f, and B, b, c, f, g, j, and k). Furthermore, it appeared that the effect of UL13 deletion on localization of UL31 was less than that on UL34 at 12 h postinfection. Thus, in most (approximately 80%) of the R7356 (ΔUL13)-infected cells, the UL34 protein appeared as punctate structures in the nucleus, but in the remainder (approximately 20%), UL34 staining was similar to that in HSV-1(F)-infected cells (Fig. 5C and G). In contrast, most (approximately 80%) R7356 (ΔUL13)-infected cells showed UL31 localization similar to that in HSV-1(F)-infected cells, and the remainder (approximately 20%) showed UL31 localization as nuclear punctate staining (Fig. 5K and O). At later times of infection (15 h postinfection), however, the UL31 protein appeared as punctate structures in the nuclei of most R7356 (ΔUL13)-infected cells, as observed with the UL34 protein (Fig. 6B, c to g).

As expected, in R7356Rep-infected cells with the UL13 deletion repaired, UL34 and UL31 localization was similar to that in HSV-1(F)-infected cells, confirming that the change in localization of UL34 and UL31 proteins in R7356 (ΔUL13)-infected cells was a result of the deletion of the UL13 open reading frame (Fig. 5D, H, L, and P and 6B, d, h, and l). Nucleoporin p62, a marker for the nuclear envelope, was evenly distributed in HSV-1(F)-, R7041 (ΔUs3)-, R7356 (ΔUL13)-, and R7356Rep-infected cells (Fig. 5Q, R, S, and T). These results indicate that UL13 plays a role in the proper localization of UL34 and UL31 in HSV-1-infected cells.

DISCUSSION

Cellular protein kinases are often regulated by phosphorylation cascades organized by other protein kinases (15, 77). The question we have investigated in the studies reported here is whether one HSV-encoded protein kinase can target another HSV-encoded protein kinase and what effect this might

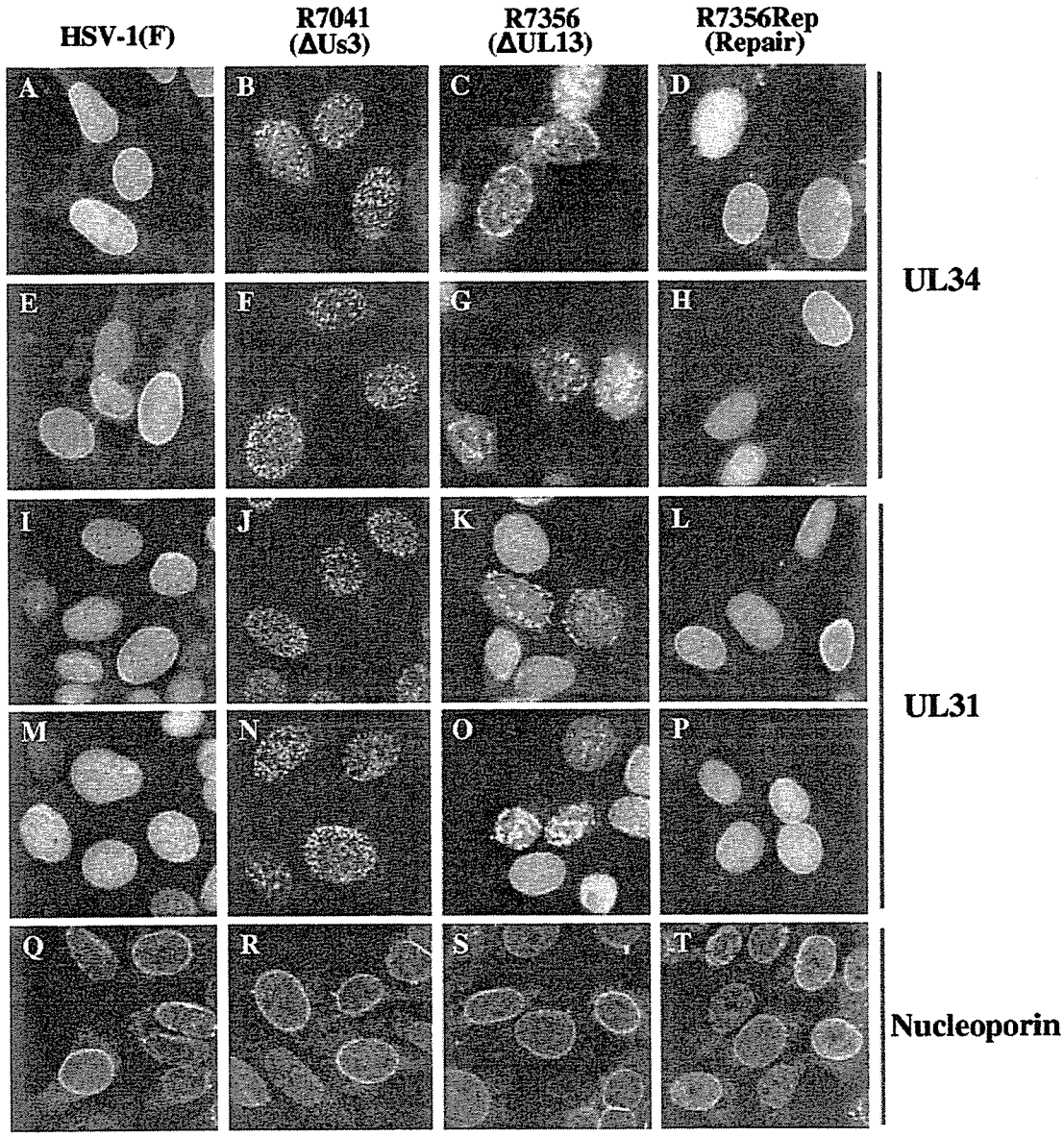

FIG. 5. Digital confocal microscope images showing localization of UL34, UL31, and nucleoporin p62 proteins in Vero cells infected with HSV-1(F) (A, E, I, M, and Q), R7041 (B, F, J, N, and R), R7356 (C, G, K, O, and S), and R7356Rep (D, H, L, P, and T). At 12 h postinfection, infected cells were fixed, permeabilized, and immunostained with rabbit polyclonal antibody to UL34 (A to H) detected with FITC-conjugated anti-rabbit IgG antibody, rabbit polyclonal antibody to UL31 (I to P) detected with Alexa Fluor 488-conjugated anti-rabbit IgG antibody, or mouse monoclonal antibody to nucleoporin p62 (Q to T) detected with Alexa Fluor 488-conjugated anti-mouse IgG antibody.

have on infected cells. The conclusions of these studies are as follows.

First, UL13 phosphorylates Us3 in vitro and in infected cells. Identification of the physiological substrate of a viral protein kinase requires demonstration that the substrate is specifically and directly phosphorylated by the kinase in vitro and that phosphorylation of the substrate in cells infected with a mutant virus lacking the protein kinase activity is altered. Although about 10 potential substrates of UL13 have been reported, only 3 (including gI/gE, ICP0, and EF-1δ) appear to fulfill the requirements to be natural UL13 substrates (4, 10, 20, 29, 32, 37, 44, 51, 57, 63). In the studies presented here, we have shown that a purified Us3 preparation was phosphorylated in vitro in the presence of purified recombinant UL13. The phosphorylation of Us3 was shown to be a direct effect of UL13 protein kinase activity and not of a contaminating kinase(s), because a kinase-negative mutant (GST-UL13K176M) was unable to phosphorylate Us3 in vitro. Furthermore, we found that Us3 phosphorylation was altered in cells infected with the UL13 deletion mutant virus. Thus, Us3 also fulfills the requirements to be a natural substrate of UL13 in infected cells.

Second, UL13 plays a role in the proper localization of UL34 and UL31 in infected cells. Previous studies have demonstrated that Us3 regulates the normal localization of the

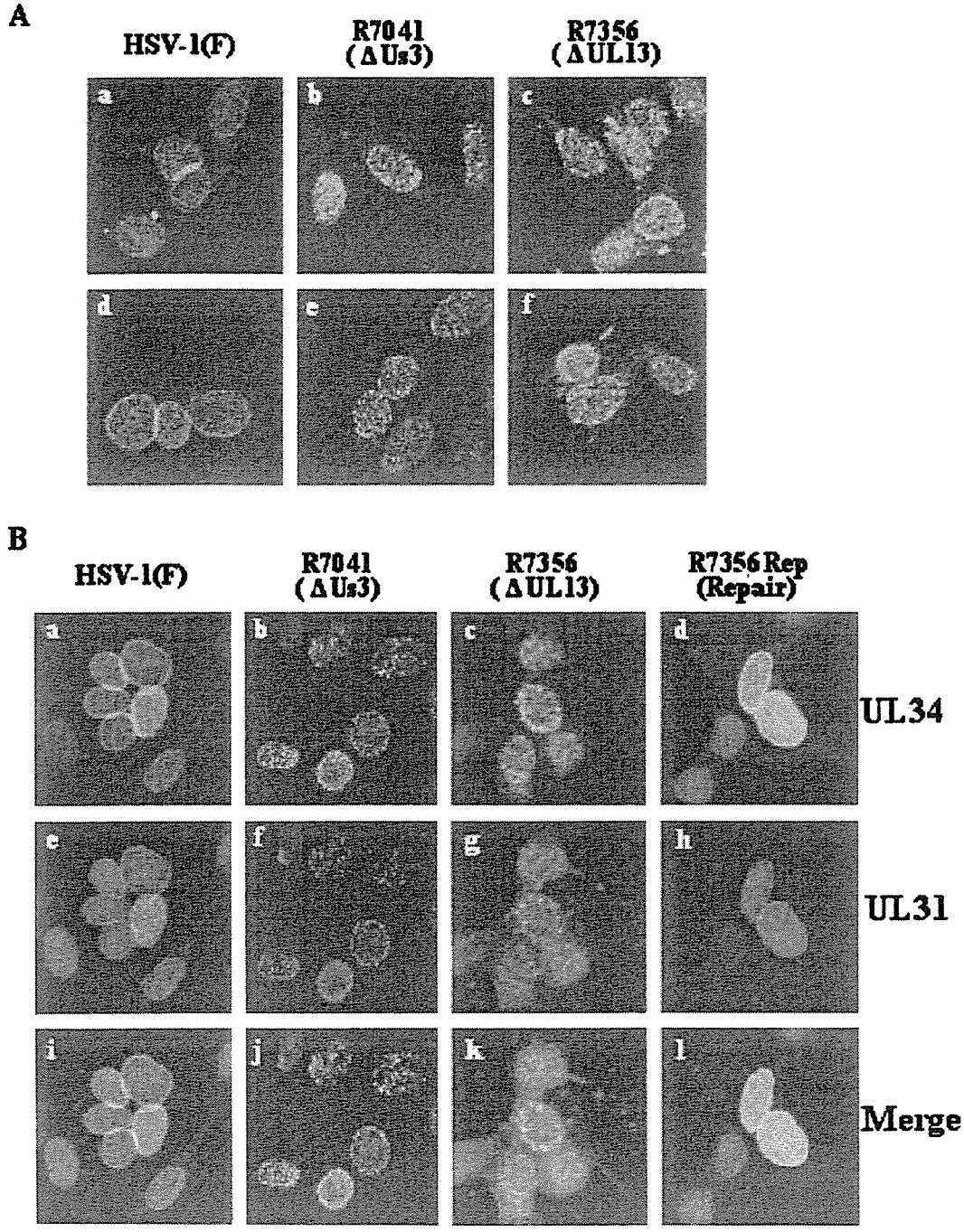

FIG. 6. (A) Digital confocal microscope images showing localization of UL34 in Vero cells infected with HSV-1(F) (a and d), R7041 (b and e), and R7356 (c and f). At 15 h postinfection, infected cells were fixed, permeabilized, and immunostained with rabbit polyclonal antibody to UL34 (a to c) detected with FITC-conjugated anti-rabbit IgG antibody or chicken polyclonal antibody to UL34 (d to f) detected with FITC-conjugated anti-chicken IgG antibody. (B) Digital confocal microscope images showing localization of UL34 and UL31 in Vero cells infected with HSV-1(F) (a, e, and i), R7041 (b, f, and j), R7356 (c, g, and k), and R7356Rep (d, h, and l). At 15 h postinfection, infected cells were fixed, permeabilized, and double labeled with a combination of chicken polyclonal antibody to UL34 (a to d) and rabbit polyclonal antibody to UL31 (e to h) and then detected with FITC-conjugated anti-chicken IgG antibody (green fluorescence) and Alexa-546-conjugated anti-rabbit IgG antibody (red fluorescence). Single-color images were captured separately and are shown in the upper (UL34) (a to d) and middle (UL31) (e to h) panels; the lower panels (i to l) represent simultaneous acquisitions of both colors. The yellow colors visualized in the merged images represent colocalization of UL34 and UL31.

HSV-1 envelopment factors UL34 and UL31, showing that these viral proteins are localized abnormally in punctate structures at the nuclear membrane in cells infected with recombinant viruses lacking a functional Us3 protein (61, 62, 65). In the studies reported here, we have shown that the phenotype of the UL13 deletion mutant virus with respect to UL34 and UL31 localization is similar to that of the Us3 deletion mutant. Together with the observation that UL13 phosphorylates Us3 in infected cells, a reasonable hypothesis is that UL13-mediated phosphorylation of Us3 may regulate the ability of Us3 to determine the proper localization of the viral proteins UL34 and UL31. Although we have shown here that UL13-mediated phosphorylation of Us3 is not required for optimal Us3 protein kinase activity, such phosphorylation might alter some other Us3 activity, such as substrate specificity or subcellular localization. Further studies will be needed to clarify whether UL13-mediated phosphorylation of Us3 is required for regulation of UL34 and UL31 localization. Such studies will need to include identification of the Us3 site(s) for UL13-mediated phosphorylation, construction of a recombinant virus with a mutated phosphorylaton site(s) in Us3, and investigation of the phenotype of this mutant virus with respect to UL34 and UL31 localization.

Alternatively, UL13 may regulate UL34 and UL31 localization independently of Us3. We previously reported that HSV-1 UL13 and its counterparts in other herpesviruses, including human cytomegalovirus UL97 and EBV BGLF4, and cellular protein kinase cdc2 phosphorylate the same amino acid residues on target proteins (27–29). In addition, Advani et al. have shown that HSV-1 infection activates cdc2 and that UL13 is required for this activation (1). It is well known that cdc2 modifies nuclear membranes by direct phosphorylation of nuclear envelope proteins (5, 46). In particular, phosphorylation of nuclear lamina and the lamin B receptor by cdc2 results in disassembly of nuclear lamina during mitosis (11, 16, 22, 38, 47, 48, 53, 72). Interestingly, both the UL34 and UL31 proteins have been reported to interact in vitro with lamin A/C, a major component of nuclear lamina, and to be required for HSV-mediated modification of lamin A/C and chromatin (60, 67). Therefore, UL13 may act like a cdc2 kinase or may activate cdc2 kinase to phosphorylate nuclear envelope proteins for proper targeting of UL34 and UL31 proteins at the nuclear membrane.

The possibility that UL13 directly phosphorylates UL34 and/or UL31 to regulate their localization seems less likely, based on the following observations. First, we have shown here that, in cells infected with UL13 deletion mutant virus, post-translational processing of UL31, which is associated with phosphorylation (25), could not be differentiated from that in cells infected with wild-type virus. Second, Ryckman and Roller (65) reported that UL34 phosphorylation was completely abolished in infected cells when the Us3 kinase target sites in UL34 (threonine 195 and serine 198) (25, 58, 65) were mutated, indicating that UL34 is phosphorylated only at threonine 195 and serine 198. There are no reports that UL13 and Us3 target the same substrate phosphorylation site(s).

In the present study, we have demonstrated that UL34 and UL31 exhibited nucleoplasmic localization, in addition to nuclear membrane localization. Although several laboratories have investigated the localization of UL34 and UL31, it remains enigmatic. Thus, some laboratories clearly demonstrated that UL34 and UL31 were detected only at the nuclear membrane in HSV-1-infected cells and in cells cotransfected with a UL34- and UL31-expressing plasmid (61, 62, 65). In contrast, the others, including this laboratory, reported nucleoplasmic localization of UL34 and/or UL31, in addition to nuclear membrane localization, in cells infected with HSV-1 or HSV-2 and in cells transiently coexpressing UL31 and UL34 (8, 66, 67, 73, 74). Furthermore, the nucleoplasmic distribution of pseudorabies virus UL31 and UL34 homologues in infected cells has also been reported (19). At present, we do not know how to explain these discrepancies. Although it has been reported that UL34 is a membrane-anchored protein (58, 59, 66), UL34 could also function as a nucleoplasmic protein. There is also a possibility that the methods for fixation of cells, staining conditions, and equipment used in immunofluorescence assays affect the detection of the viral proteins. Further studies will be needed to clarify this subject.

In conclusion, we have provided data showing that Us3 is a physiological substrate of UL13 and that UL13 regulates the localization of the HSV envelopment factors UL34 and UL31. Although direct linkage between UL13-mediated phosphorylation of Us3 and the regulatory effects of Us3 on UL34 and UL31 localization remains to be elucidated, our observations raise the interesting possibility that UL13 is also involved in the nuclear egress pathway of HSV-1. In agreement with this possibility, it has been reported that the ability of mutant human cytomegalovirus UL97 to bud through the nuclear membrane is severely impaired (35).

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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

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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

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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

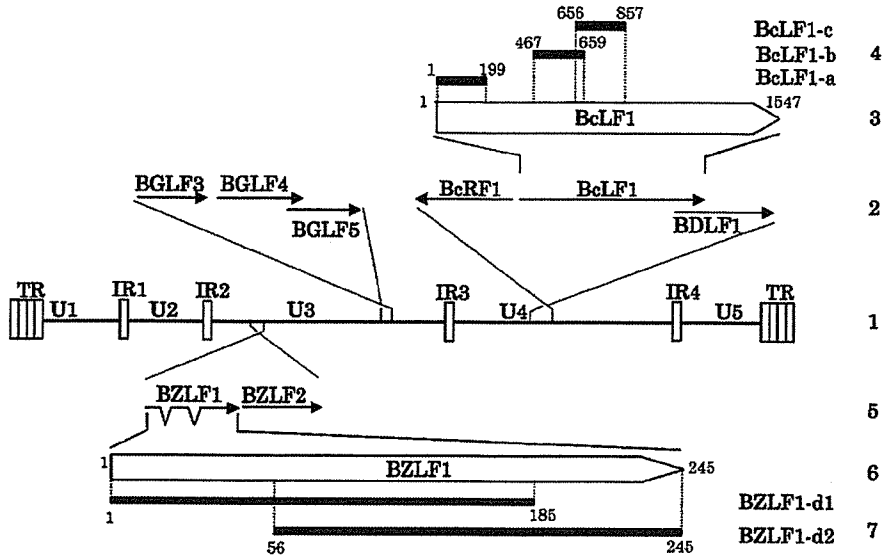

FIG. 1. Schematic diagram of the EBV genome and location of the genes studied in this report. Line 1: diagram of the EBV genome. The unique sequences are designated U1 to U5. The terminal and internal repeats flanking the unique sequences are shown as open rectangles with their designations above the 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.

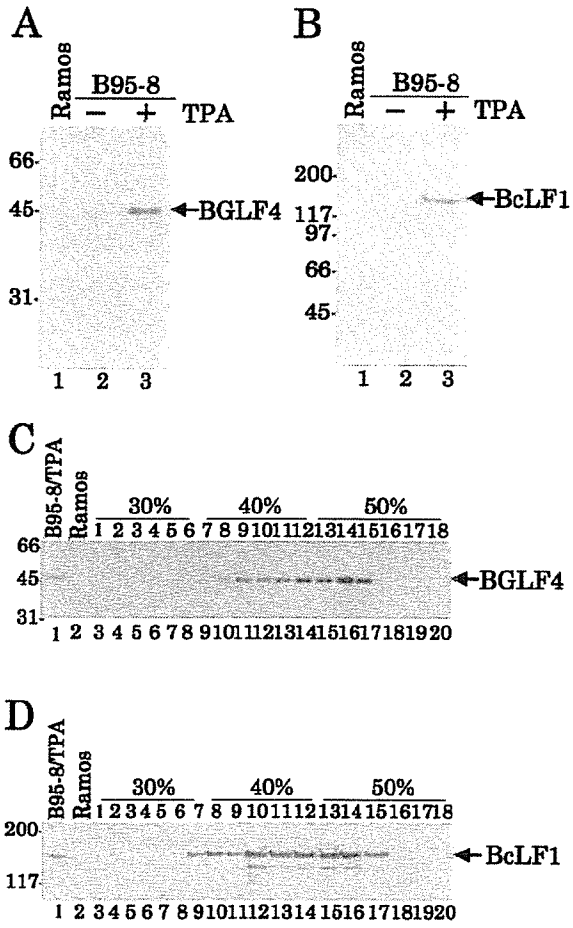

FIG. 2. Analysis of virion-associated BGLF4. (A and B) Immunoblots of electrophoretically separated lysates from Ramos (lane 1) and B95-8 cells without (lane 2) or with (lane 3) TPA treatment. Cell lysates were analyzed by immunoblotting with polyclonal antibody to BGLF4 (A) and BcLF1 (B). (C and D) 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

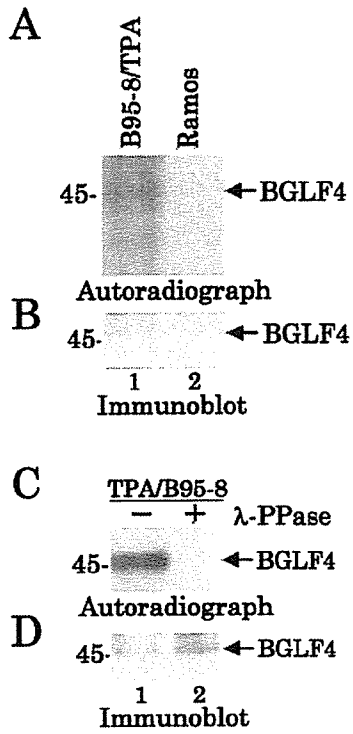

FIG. 3. Autoradiographs of BGLF4 immunoprecipitates after in vitro kinase assays and electrophoresis. (A) Purified virions from one of the virion-containing fractions described in Fig. 2C and D (lane 1) and from a corresponding fraction from the supernatant of Ramos cells (lane 2) were lysed and immunoprecipitated 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.

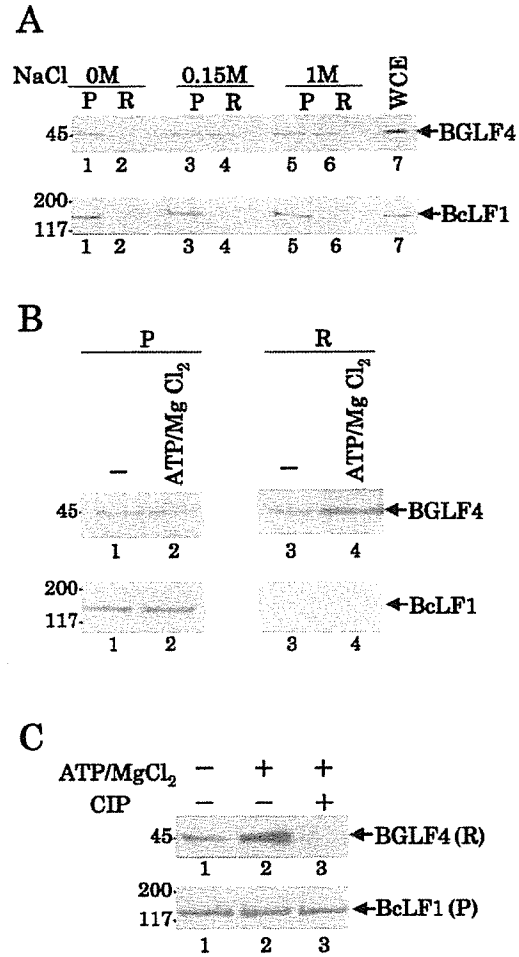

FIG. 4. Immunoblots of in vitro tegument release assays of BGLF4 and BcLF1. (A) EBV virions from the supernatant of TPA-treated B95-8 cells were treated with Triton X-100 in 0, 0.15, or 1 M NaCl. The samples were then separated by sucrose gradient centrifugation into pelleted (P) and released (R) fractions 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

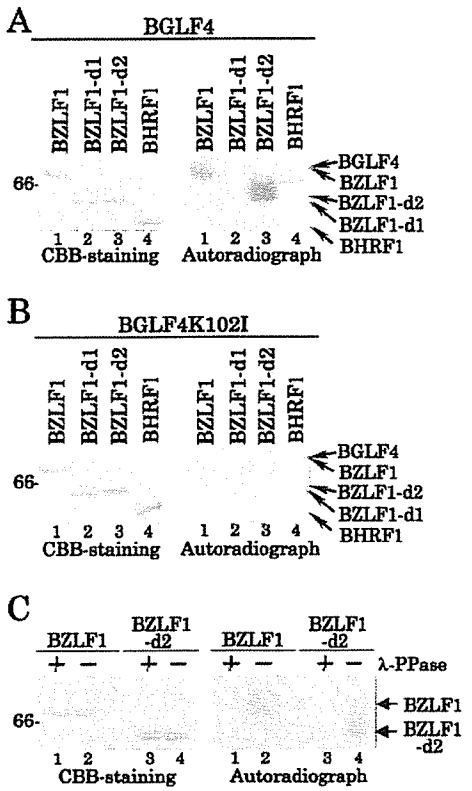

FIG. 5. Autoradiographs of in vitro BZLF1 phosphorylation. (A and B) Left panels: CBB-stained gels of phosphorylated BZLF1. Purified MBP-BZLF1 (lane 1), MBP-BZLF1d1 (lane 2), MBP-BZLF1d2 (lane 3), and MBP-BHRF1 (lane 4) were incubated in kinase buffer containing [γ-^{32}P]ATP and purified GST-BGLF4 (A) 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).

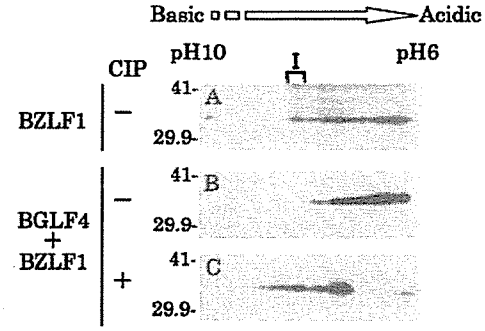

FIG. 6. Effect of BGLF4 expression on posttranslational modification of BZLF1 protein in COS-1 cells. COS-1 cells transiently expressing either BZLF1 alone (A) or both BZLF1 and BGLF4 (B and C) were solubilized, mock treated (A and B) or treated with CIP (C), separated by two-dimensional electrophoresis, 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 I 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-

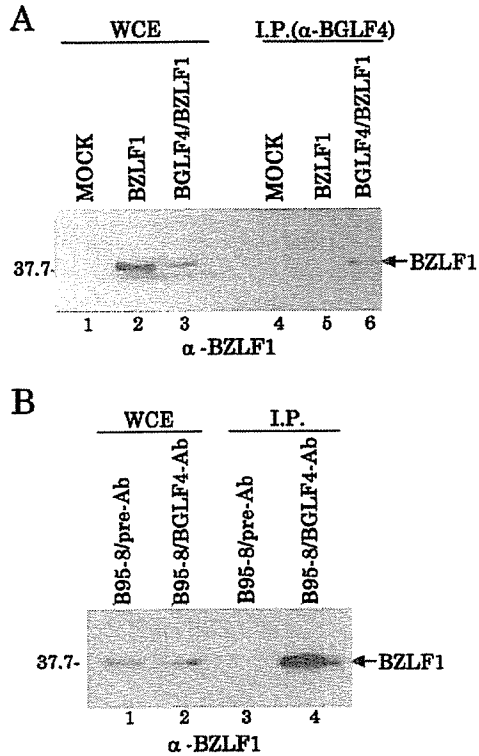

FIG. 7. Interaction of BGLF4 with BZLF1. (A) COS-1 cells mock transfected (lane 4) or transiently expressing BZLF1 alone (lane 5) or both BGLF4 and BZLF1 (lane 6) were solubilized and immunoprecipitated with antibody to BGLF4. The immunoprecipitates were analyzed by electrophoresis and immunoblotted 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.

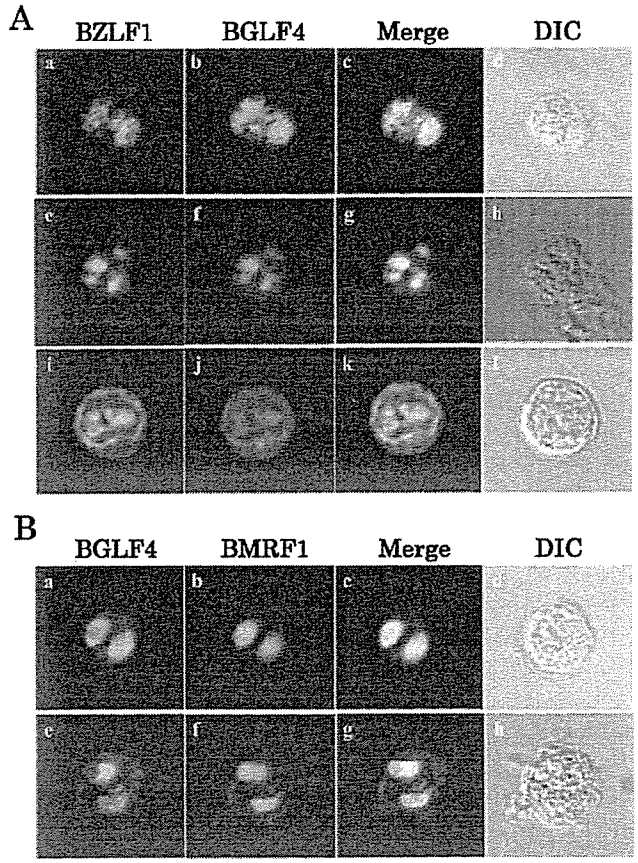

FIG. 8. Immunofluorescent localization of BGLF4, BZLF1, and BMRF1 in EBV-infected cells. (A) Digital confocal microscope images showing localization of BGLF4 and BZLF1 in Akata cells treated with anti-human IgG for 9 h. The cells were then fixed, permeabilized, and double labeled with a combination of 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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Phosphorylation of MCM4 at Sites Inactivating DNA Helicase Activity of the MCM4-6-7 Complex during Epstein-Barr Virus Productive Replication

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ABSTRACT

Induction of Epstein-Barr virus (EBV) lytic replication blocks chromosomal DNA replication notwithstanding an S-phase like cellular environment with high CDK activity. We report here that the phosphorylated form of MCM4, a subunit of the MCM complex essential for chromosomal DNA replication, increases with progression of lytic replication, Thr-19 and Thr-110 being CDK2/CDK1 targets whose phosphorylation inactivates MCM4-6-7 complex-associated DNA helicase. Expression of EBV-encoded protein kinase (EBV-PK) in HeLa cells caused phosphorylation of these sites on MCM4, leading to cell growth arrest. In vitro, the sites of MCM4 of the MCM4-6-7 hexamer was confirmed to be phosphorylated with EBV-PK with the same loss of helicase activity as with CDK2/cyclin A. Introducing mutations in the N-terminal six Ser and Thr residues of MCM4 reduced the inhibition by CDK2/cyclin A, while EBV-PK inhibited the helicase activity of both wild type and mutant MCM4-6-7 hexamer, probably due to that EBV-PK can further phosphorylate MCM6 and other site(s) of MCM4 in addition to the N-terminal residues. Therefore, phosphorylation of the MCM complex by redundant actions of CDK and EBV-PK during lytic replication might provide one mechanism to block chromosomal DNA replication in the infected cells, through inactivation of DNA unwinding by the MCM4-6-7 complex.

INTRODUCTION

A number of replication initiation sites that are present in the genome of eukaryotic cells are utilized in a temporal order during the DNA synthesis (S) phase of the cell cycle. Reinitiation of DNA replication is prevented, and only a single round of DNA replication is performed in a cell cycle. This regulation of DNA replication by the so-called replication licensing system is regulated by the loading of the minichromosome maintenance (MCM) complexes on chromatin DNA and their phosphorylation (37, 50, 51).

During the G1 phase of the cell cycle, replication origins in DNA are licensed by the assembly of pre-replicative complexes (pre-RC) comprising the origin recognition complex (ORC), Cdc6, Cdt1 and the MCM complex (47, 51). The ORC binds to origins of DNA replication and remains bound during most of the cell cycle (30, 40, 48). Cdc6 and Cdt1 then bind to the complex and facilitate the loading of MCM2-7 complex. Cdt1 itself is regulated by geminin, which blocks the binding of the MCM complex to the pre-RC (39, 46, 54). Activation of the pre-RC occurs at the G1/S boundary after licensing and is mediated by the action of S-phase cyclin-dependent kinases (CDKs), primarily cyclin A/CDK2, cyclin E/CDK2, and Cdc7/Dbf4 (3, 10, 42, 45), which trigger a chain of reactions that lead to the binding of Cdc45 to the origin and phosphorylation of Cdc6 and the MCM complex. As a result, the DNA duplex unwinds, facilitating loading of the DNA polymerase machinery (24, 41, 52, 59). The phosphorylation of key components of this process by the CDKs leads to initiation of replication and at the same time helps to prevent re-replication during the S, and G2/M phases of the cell cycle (6, 7, 23, 55).

All of the members of the MCM protein family contain highly conserved

DNA-dependent ATPase motifs in the central domain (3, 44) and form several stable sub-assemblies including MCM2-3-4-5-6-7, MCM2-4-6-7, MCM4-6-7 and MCM3-5 complexes (35, 49, 56). DNA helicase activity has been identified in the MCM4-6-7 complexes of human, mouse and fission yeast (19, 35, 36, 56), while MCM2 and MCM3-5 are known to inhibit this by converting the double trimer structure into a heterotetramer or a heteropentamer (43, 56). MCM4-6-7 proteins form trimers or hexamers to function as DNA helicases in vitro (37). Such DNA helicase activity is not processive under standard conditions of DNA helicase assay. During S phase, MCM proteins are released from origins of replication after initiation of DNA replication and move with replication forks where they are thought to function as DNA helicases. The mechanisms ensuring replication of DNA only once per cycle involve release of MCM proteins from chromatin after firing of the origins of replication and prevention of reloading (2, 11). Moreover, the phosphorylation of MCM4 with CDK2/cyclin A is associated with inactivation of the DNA helicase (unwinding) activity of the MCM4-6-7 complex (22) and the sites critical for phosphorylation have been determined (20, 22).

Primary infection with the Epstein-Barr virus (EBV), a human herpesvirus that infects 90% of individuals, targets resting B lymphocytes and induces their continuous proliferation. In B lymphoblastoid cell lines there is no production of virus particles, this being termed latent infection, but EBV-infected cell lines sometimes switch from a latent stage of infection into a virus-productive lytic stage, featuring an ordered cascade of viral early and late gene expression. The lytic phase of EBV DNA replication is dependent on seven viral replication proteins and occurs in discrete sites in nuclei, called replication compartments (8). We have previously demonstrated that induction of the EBV lytic program results in inhibition of replication of chromosomal