Detection of Epstein–Barr virus BGLF4 protein kinase in virus replication compartments and virus particles

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BGLF4 is the only serine/threonine protein kinase identified in Epstein–Barr virus (EBV); it is known to phosphorylate viral DNA polymerase processivity factor, EA-D (BMRF1), EBNA-LP, EBNA-2, cellular EF-1α and nucleoside analogue ganciclovir. However, the expression and biological functions of BGLF4 have not yet been clearly demonstrated in EBV-infected cells. To reveal authentic functions of BGLF4 protein within viral-replicating cells, a panel of specific monoclonal antibodies was generated and characterized. The major immunogenic regions of BGLF4 were mapped to aa 27–70 and 327–429. Using these antibodies, the expression kinetics and localization of BGLF4 were analysed in reactivated EBV-positive lymphoid and epithelial cells. BGLF4 was expressed as a phosphoprotein at the early lytic stage and was detected predominantly in the nucleus of EBV-positive cells, but small amounts of BGLF4 were observed in cytosolic and heavy membrane fractions at the late phase of virus replication. Additionally, it was demonstrated that BGLF4 co-localizes with viral DNA polymerase processivity factor, EA-D (BMRF1), in the virus replication compartment and that it is a virion component. Finally, possible functional domains at the N terminus of BGLF4 were analysed and it was found that aa 1–26 of BGLF4 are dispensable for EA-D phosphorylation, whereas deletion of aa 27–70 reduced kinase activity.

INTRODUCTION

Epstein–Barr virus (EBV) is a human gammaherpesvirus, which infects most of the world’s population. After primary infection, the virus becomes latent in B cells. Since elevated anti-EBV antibody titres are frequently detected in patients with EBV-associated malignancies, repeated reactivation of EBV in the host is believed to play a role in the disease development process (for a review see Kieff & Rickinson, 2001). Viral protein kinase is our special interest because of its possible ability to modify viral and cellular proteins to create an optimal cellular environment for virus replication.

Based on sequence alignment, EBV BGLF4 was identified as a UL kinase. Its homologues include UL13 of herpes simplex virus 1 (HSV-1), ORF47 of varicella-zoster virus, UL97 of human cytomegalovirus (HCMV), U69 of Human herpesvirus 6 and ORF36 of Human herpesvirus 8. The herpes protein kinases were found to have multiple biological functions for virus replication. For example, UL13-defective HSV-1 was severely impaired of the function of releasing VP22 upon infection of new cells, and the dissociation of major tegument proteins from virions was suggested to be mediated through virion kinase-associated phosphorylation (Morrison et al., 1998). In HCMV, UL97 was suggested to play roles in mature virion assembly (Wolf et al., 2001), efficient viral DNA replication (Wolf et al., 2001) and the nucleocapsid egress from the nucleus (Krosky et al., 2003; Marschall et al., 2005).

EBV BGLF4 was demonstrated to be a serine/threonine kinase (Chen et al., 2000a), and it was found to phosphorylate the viral DNA polymerase processivity factor, EA-D (BMRF1) (Chen et al., 2000a; Gershburg & Pagano, 2002), viral nuclear protein EBNA-LP (Kato et al., 2003), EBNA-2 (Yue et al., 2005) and cellular translation elongation factor, EF-1α (Kato et al., 2003; Kawaguchi et al., 2003). The C terminus of BGLF4 contains a non-classical nuclear localization signal and was demonstrated to be required for nuclear retention (Gershburg et al., 2004). Some potential functions of BGLF4 involving virus replication and infection were proposed. However, no clear evidence of BGLF4 in association with these functions was demonstrated within viral-replicating cells due to the lack of reagents.

The ability to phosphorylate nucleoside analogues such as ganciclovir (GCV) is another important characteristic shared by herpes UL kinases (Michel & Mertens, 2004).

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Deliberate induction of lytic EBV replication in tumour cells combined with GCV is one of the virally targeted therapies suggested for EBV-associated malignancies (Westphal et al., 1999; Israel & Kenney, 2003; Feng et al., 2004). The response of these cells to GCV was believed to be mediated by the kinase activities of EBV thymidine kinase (TK) and BGLF4 (Feng et al., 2004). Thus, it is important to study the protein expression characteristics and functional domains of BGLF4 for possible future application of kinase targeted clinical therapy.

To gain further insight into BGLF4 within EBV-replicating cells, we successfully generated eight BGLF4-specific monoclonal antibodies (mAbs) to characterize BGLF4 protein through different approaches in this study. The major immunogenic regions of BGLF4 were mapped to aa 27–70 and 327–429. The expression of BGLF4 was then monitored through different approaches in this study. The major clonal antibodies (mAbs) to characterize BGLF4 protein within virus-replicating cells, we successfully generated eight BGLF4-specific monoclonal antibodies (mAbs) to characterize BGLF4 protein.

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**METHODS**

**Purification of bacterially expressed recombinant BGLF4.** To express His-BGLF4 protein, pSJC1 (Chen et al., 2000a) was transformed into BL21 (DE3), and then the bacteria were induced at exponential phase with IPTG and grown at 37 °C for 2 h. The resulting insoluble fraction containing BGLF4 was solubilized using the Protein Refolding kit (Novagen). The protein sample was then incubated with nickel nitrilotriacetic acid-agarose (Qiagen) at 4 °C with rotation overnight. The protein was eluted with a buffer [20 mM Tris/ HCl, pH 8.0, 10 % (v/v) glycerol] containing increasing concentrations of imidazole.

**Generation of BGLF4-specific mAbs.** Six-week-old BALB/c mice were immunized with 100 μg His-BGLF4 protein in complete Freund’s adjuvant subcutaneously, followed by three boosters with incomplete Freund’s adjuvant at 2 week intervals and a final tail vein booster 3 days before fusion. Hybridomas were established by fusing immunized splenocytes with mouse myeloma NS1 cells using polyethylene glycol and selected with HAT medium as described previously (Tsai et al., 1991).

**Plasmid construction.** For immunoeptide mapping, an EBNA-1 (408–446) tag recognized by mAb SC1 was applied to generate a panel of E1/BGLF4 constructs (Chen et al., 1999, 2000b). E1 fused wild-type (wt) and mutant BGLF4 coding regions were moved from pGEM2-derived plasmids into pYPW3, which is a modified pSG5 vector (Stratagene) containing HindIII–EcoRI–BglII sites within the multiple cloning region. E1/B(Δ1–26) (pYPW4), E1/B(Δ1–70) and E1/B(Δ1–108) were generated by moving the HindIII–EcoRI DNA fragment from pYPH8, pPHH4 and pPHH3 (Chen et al., 2000a) into pYPW3. DNA fragments encoding wt E1/BGLF4, E1/B(Δ175–204), E1/B(Δ213–230), E1/B(Δ294–429) and E1/B(Δ327–429) were also generated by removing the HindIII–EcoRI fragments from a pGEM2-based vector into pYPW3. DNA fragments encoding Δ175–204 and A213–230 were created by recombinant PCR (Chen et al., 2000a). For another set of plasmids expressing BGLF4 without the EBNA-1 tag, wt BGLF4 (pYPW17) was generated by cloning EcoRI–BamHI fragment of pYPW10 into pYPW3. For BGLF4 N-terminal deletion clones, Δ1–26, Δ1–70 and Δ1–108 were generated by PCR amplification using pHH8, pPHH4 and pPHH3 as templates with appropriate primer pairs. The amplified DNA fragments were digested with HindIII and PstI to replace the E1 tag and N-terminal coding region of parental plasmids. The kinase dead mutant (K102I) of BGLF4 was generated by recombinant PCR using mutagenesis primers 5′-CGGTCATTCTCTAGACTGTGTCGAC-3′ and 5′-ATAGGAAGAACGCTGACGATTCTTCT-3′, and appropriate external primers. The EA-D-expressing plasmid (pYPW26) was amplified using pBR322-BamM as template and cloned into BamHI and EcoRI sites of pSG5.

**Immunoprecipitation assay.** In vitro transcription/translation of pCP4 (E1/BGLF4) was performed with the TnT kit (Promega). For immunoprecipitation, 8 μl [35S]methionine-labelled in vitro translated E1/BGLF4 protein was incubated with 200 μl culture supernatant of individual hybridomas at 4 °C for 2 h. The immunocomplexes were pulled down with 50 μl 20% protein A–Sepharose and washed with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and complete protease cocktail (Boehringer)). The immunocomplexes were then displayed by SDS-PAGE and subjected to autoradiography.

**Cell culture and induction of viral lytic cycle.** Akata, Raji and P3HR1 are EBV-positive Burkitt’s lymphoma cell lines (Pulvertaft, 1964; Hinuma et al., 1967; Takada et al., 1991). NPC-TW01 is an NPC cell line lacking the EBV genome (Lin et al., 1993) and NA was derived from recombinant Akata EBV converted from NPC-TW01 (Chang et al., 1999). For lytic cycle induction, Raji, P3HR1 and NPC cells were treated with 40 ng 12-O-tetradecanoylphorbol-13-acetate (TPA) ml−1 and 3 mM sodium butyrate (Sigma). Akata cells were induced with goat anti-human immunoglobulin G (IgG) antibody (50 or 100 μg ml−1; ICN Pharmaceuticals).

**Transfection, protein extraction and phosphatase treatment.** Indicated plasmids were transfected into 293T cells with calcium phosphate-N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES) (Chen & Okayama, 1987), or into HeLa cells with Lipofectamine 2000 (Invitrogen). All the transfected or induced cells were resuspended in RIPA buffer. For phosphatase treatment, protein extract in RIPA buffer was in the presence or absence of 10 U alkaline phosphatase (calf intestinal, CIP; NEB) in NEB buffer 3 and incubated at 37 °C for 1 h.

**Western blot analysis.** Western blotting was performed similarly as described previously (Chen et al., 2000a). The primary antibodies used were Zta mAb 4F10 (Tsai et al., 1997), EA-D mAb 88A9 (Tsai et al., 1991), 125 kDa viral capsid antigen (VCA) mAb 343D12 (Tsai & Glaser, 1991), membrane antigen (MA) mAb 20ID6 (Tsai & Glaser, 1991), GAPDH Ab (Biodesign) and 32 kDa caspase-3-specific antibody (Imgenex).

**Subcellular fractionation.** The subcellular fractionation protocol was adapted from that described by Krajewski et al. (1993). Briefly, cells were covered or incubated with hypotonic buffer (5 mM Tris/ HCl, pH 7.4, 5 mM KCl, 1-5 mM MgCl2, 0.1 mM EDTA, 1 mM DTT and 1 mM PMSF) on ice for 1 h, harvested by scraping (for epithelial cells) and homogenized by passage through 27 gauge needles 15 times. Cell lysates were subjected to centrifugation at 500 g for 5 min at 4 °C. The resulting pellet was the nuclear fraction. The supernatant was then mixed with 3 vols LSB buffer (50 mM Tris/HCl, pH 7-5, 25 mM KCl, 5 mM MgCl2) and further centrifuged at 150000 g for 2 h at 4 °C. The resulting pellet contained...
heavy membrane (rough endoplasmic reticulum, mitochondria, peroxisomes, Golgi apparatus and lysosome) and light membrane (plasma membrane) fractions. The cytosolic fraction in the supernatant was further concentrated for gel analysis.

**Indirect immunofluorescence.** Akata cells were harvested at indicated time points post-induction (p.i.), applied onto glass slides and fixed in acetone at room temperature for 10 min. To detect BGLF4 in epithelial cells, HeLa cells, T9801 or NA cells, the cells were slide-cultured, harvested, air-dried and fixed with cold methanol at −20 °C for 20 min. To perform BGLF4 and EA-D double staining, rabbit anti-BGLF4 serum (1:10) and mouse anti-EA-D mAb 88 (Tsai et al., 1991) were used as primary antibodies. A published protocol (Liao et al., 2001) was followed for co-staining BrdU and BGLF4. FITC-conjugated anti-rabbit IgG (1:100; Jackson) and Rhodamin red-conjugated anti-mouse IgG antibodies (1:200; Jackson) were used as secondary antibodies. After PBS washing, cells were stained with 4.6-diamidino-2-phenylindole (DAPI, 1 μg ml⁻¹) at room temperature for 10 s, and mounted with medium (H1000; Vector) for confocal laser scanning (Leica) and fluorescence (Axioskop 40 FL; Zeiss) microscopy.

**Purification of EBV particles and electron microscopy.** Culture supernatant of anti-IgG-induced Akata cells was harvested at 48 h p.i. for virus purification (Bortz et al., 2003). After depleting cell debris, virions were pelleted by centrifugation at 21,000 g for 90 min at 4 °C, resuspended in 1:5 ml low-salt TNE buffer (10 mM Tris/HCl, pH 7-4, 100 mM NaCl, 1 mM EDTA) and overlaid onto a 10 ml discontinuous sucrose-gradient (25-65%). After centrifugation at 25,000 g for 4.5 h at 4 °C, 15 of 0-9 ml fractions were collected from the top to the bottom of the tube. Seventy microlitres of each fraction was saved for EBV genome analysis and the rest of each fraction was centrifuged at 21,000 g for 90 min at 4 °C, resuspended in 50 μl low-salt TNE buffer, and subjected to protein concentration measurement and Western blot. For electron microscopy, purified EBV virions were spotted onto the Formvar-coated copper grids, negatively stained with saturated uranyl acetate for 1 min and observed on a Hitachi 7100 transmission electron microscope.

**Virion DNA extraction and PCR.** Each gradient fraction was incubated with 1 mg protease K ml⁻¹ in PCR lysis buffer (10 mM Tris/HCl, pH 8-0, 2-5 mM MgCl₂, 1% Tween 20, 1% NP-40) at 50 °C for 4 h and then inactivated at 72 °C for 10 min. To detect EBV genome, a 285 bp BamHI W fragment was amplified with primers 5'-CCCTGGTATAAAGTGTCCT-3' and 5'-AAGTCCACT- 

**RESULTS**

**Generation of BGLF4-specific mAbs**

In previous attempts to generate BGLF4-specific antibodies, weak activities of both rabbit and mouse sera were obtained when insoluble His-BGLF4 was used as antigen (Chen et al., 2000a). To improve immunogenicity, the insoluble fraction was solubilized with protein refolding buffer and purified through a nickel column. The His-BGLF4 protein eluted into the 250 mM imidazole fraction (Fig. 1a) was used to immunize animals for generating hybridomas. Among them, mAb 2616 showed the strongest signal in Western blotting, detecting E1/BGLF4 in transiently transfected 293T cells (Fig. 1b), whereas mAbs 2224 and 2260 reproducibly immunoprecipitated [³⁵S]methionine-labelled in vitro translated E1/BGLF4 with the highest efficiency (Fig. 1c).

**BGLF4 mAbs recognized epitopes within aa 27–70 and 327–429**

To map the major immunogenic epitopes of BGLF4, a panel of EBNA-1 tagged wt or truncated BGLF4 clones: E1/B(1–429), E1/B(A1–26), E1/B(A1–70), E1/B(A1–108), E1/B(A175–204), E1/B(A213–230), E1/B(A294–429) and E1/B(A327–429) were constructed and transfected into 293T cells (Fig. 2a). Expression was confirmed with EBNA-1 mAb 5C11 (Fig. 2b). The same lysates were then used to map the epitopes recognized by these eight BGLF4-specific mAbs (Fig. 2a). In summary, epitopes within aa 27–70 were recognized by mAbs 2224, 2130, 2034, 2444, 2527, 2260 and

![Fig. 1. Characterization of BGLF4 mAbs](http://vir.sgmjournals.org/3217.png)
2454 (Fig. 2c and data not shown), whereas mAb 2616 recognized an epitope within aa 327–429 of BGLF4 (Fig. 2d). As illustrated in Fig. 2(a), these two regions are outside the conserved kinase domains and were predicted to be immunogenic regions with high surface possibility according to Kolaskar & Tongaonkar (1990) and Hopp & Woods (1981) (data not shown).

Expression of BGLF4 in reactivated EBV-positive lymphoid cells

To characterize BGLF4 within EBV-replicating cells, we detected BGLF4 in chemically induced Raji, P3HR1 and in anti-IgG-induced Akata cells. BGLF4 protein was observed with similar molecular masses in these three different cell lines (Fig. 3a). Since Raji cell harbours an EBV genome with a deletion at the coding region of the major DNA-binding protein and is unable to complete viral DNA replication, the detection of BGLF4 protein in Raji cells indicates its expression is independent of viral DNA replication (Fig. 3a). To examine whether BGLF4 is a phosphoprotein, total lysates from IgG-induced Akata and chemically induced Raji cells were treated with alkaline phosphatase (CIP). The molecular mass reduction after CIP treatment indicates that BGLF4 is phosphorylated in both cell lines (Fig. 3b). The expression kinetics of BGLF4 in correlation with other lytic viral proteins were then monitored in anti-IgG-induced Akata cells (Fig. 3c). The immediate early protein Zta became detectable at 4 h p.i. BGLF4 was visible at 6 h p.i. and gradually declined after 43 h p.i., showing an early protein expression pattern. Multiple species of EA-D resulting from different levels of phosphorylation were also detected as previously described (Tsurumi, 1993), and the hyperphosphorylation of EA-D correlated with the expression levels of BGLF4. The 125 kDa VCA was detectable at 12 h p.i. and showed maximal expression at 24 h p.i.

Subcellular localization of BGLF4 and EA-D was further analysed at 24 h p.i. As showed in Fig. 3(d), the majority of BGLF4 and EA-D was detected in the nuclear fraction but a small proportion of these proteins was detected in the cytosolic fraction and the membrane fraction, which contained light and heavy membranes. Apparently, hyperphosphorylated EA-D was mainly detected in the nucleus, suggesting that the nuclear function of EA-D might be regulated by phosphorylation.
Expression of BGLF4 protein in the EBV lytic cycle within epithelial cells

Since EBV-associated nasopharyngeal carcinoma (NPC) is a cancer of epithelial origin, and expression of BGLF4 in EBV-positive epithelial cells was never characterized, we analysed further the expression kinetics and subcellular localization of BGLF4 in reactivated NA cells. Overall, BGLF4 expression correlated with other viral proteins; similar to that observed in Akata cells, except for slower lytic cycle progression. As revealed in Fig. 4(a), both Zta and EA-D became detectable at 24 h p.i. and reached maximal expression levels at 60 h p.i., whereas structural protein 125 kDa VCA was detected at 48 h p.i. and gradually increased by 72 h p.i. A small amount of BGLF4 was detected at 24 h p.i. and reached maximal expression at 72 h p.i. Increasing hyperphosphorylated EA-D became obvious as virus replication progressed to 60 h p.i. and showed a slight reduction at 72 h p.i.

The subcellular localization of BGLF4 was then detected with indirect immunofluorescence. In transiently transfected HeLa cells, BGLF4 was mainly detected in the nucleus (Fig. 4b). In chemically induced NA, BGLF4 was detected as nuclear punctate structures in 3-4 % (5/194) of cells at 24 h p.i., and in 16-4 % (23/140) of cells at 48 h p.i., while weak cytoplasmic staining was also observed in 17-3 % (4/23) of positive cells at 48 h p.i. As virus replication progressed, 27-3 % (44/161) of cells were detected as being BGLF4-positive at 72 h p.i. Even though predominant nuclear staining was still observed, 25 % (11/44) of positive cells showed accompanying weak perinuclear cytoplasmic staining (Fig. 4c). Since other BGLF4 homologues were identified as virion-associated proteins, the cytoplasmic translocation of BGLF4 suggests it could be associated with other virion components at late virus replication (Wolf et al., 2001). Subcellular fractionation was performed to confirm the predominant nuclear localization of BGLF4, while increasing amounts of BGLF4 were also detected in the heavy membrane and cytoplasmic fraction between 48 and 60 h p.i. (Fig. 4d). The molecular mass of BGLF4 observed in cytosolic fractions was also slightly smaller than that detected in nuclear and heavy membrane fractions, as observed in Akata cells.

BGLF4 co-localizes with EA-D to the viral DNA replication compartment

BGLF4 homologue HCMV UL97 was observed to modulate virus replication efficiency (Wolf et al., 2001). In addition, the viral DNA polymerase processivity factor, EA-D, was known to be phosphorylated by BGLF4. We thus examined
whether BGLF4 localizes at the virus replication compartment. As revealed by double immunostaining and confocal microscopy, BGLF4 co-localized with EA-D at specific compartments at early lytic phase in reactivated Akata cells (Fig. 5a). Similar to the expression pattern of EA-D, BGLF4 showed a speckled staining pattern at 6 h p.i., more homogeneous globular structures at 8 h p.i. and larger globular structures at 24 h p.i. These structures are very similar to viral DNA replication compartments. To demonstrate further that BGLF4 co-localizes with newly synthesized viral DNA, Akata cells were incubated with a thymidine analogue, BrdU, and detected with a specific antibody. As expected, co-localization of BGLF4 with BrdU staining was observed at 8 h p.i. (Fig. 5b). Since host DNA synthesis was blocked in virus-replicating cells, co-staining of BrdU and BGLF4 indicates that BGLF4 co-localizes to the viral DNA replication compartments. In the presence of herpesviral DNA polymerase inhibitor, PAA, which prevents viral DNA replication and the formation of mature virus replication compartment (Kiehl & Dorsky et al., 1991; Daikoku et al., 2005), both BGLF4 and EA-D showed a more diffuse staining pattern (Fig. 5c). These observations thus support the suggested role of BGLF4 in regulating viral DNA replication.

**BGLF4 protein kinase is a component of EBV viral particles**

To examine whether BGLF4 is a virion component, EBV particles were harvested from the supernatant of EBV-positive Akata cells and subjected to discontinuous
sucrose-gradient ultracentrifugation. Fractions were collected and analysed for the distribution of viral proteins (Fig. 6a and b). BGLF4 distributed similarly to viral structural proteins MA and VCA, and was most abundantly detected in fraction 8 in immunoblotting. The abundance of viral particles in fraction 8 was further demonstrated by PCR detection of the BamHI W fragment of viral DNA (Fig. 6c, d and e).
upper panel). Since no GAPDH DNA was amplified by PCR, the possibility of cellular DNA contamination was excluded (Fig. 6c, lower panel). To provide further evidence, fractions 6, 7, 8, 9 and 10 were examined under an electron microscope to confirm the virion peak at fraction 8 (Fig. 6d and e). As detected by immunoblotting, EA-D was also found as a virion component (Fig. 6b). In comparison with the hyperphosphorylated EA-D within Akata lysate, EA-D within viral particles appeared to be hypophosphorylated.

**N-terminal aa 1–26 of BGLF4 are dispensable for phosphorylation activity on EA-D**

In a recent study, a putative non-classical nuclear localization signal (NLS) (RSLKKRFK) at aa 386–393 was predicted to be responsible for nuclear localization of BGLF4, and nuclear localization of BGLF4 was found to be required for its ability to phosphorylate EA-D (Gershburg et al., 2004).

According to the alignment of amino acid sequences and our immunoepitope mapping, the N terminus of BGLF4 is less conserved in comparison to cellular kinases and is very possibly a surface regulatory domain. The conserved domain I, which is responsible for ATP binding, was present at around aa 80 of BGLF4 (Fig. 7a). Therefore, the contribution of N terminus to BGLF4 subcellular localization or kinase activity on co-transfected EA-D was then examined. wt BGLF4, Δ1–26, Δ1–70, Δ1–108 and K102I were co-expressed with EA-D in 293T cells to detect the phosphorylation ability of BGLF4 mutants on EA-D. Hyperphosphorylated EA-D of 58 kDa was observed in cells expressing wt BGLF4 or Δ1–26 but not other BGLF4 mutants (Fig. 7b), suggesting that residues 1–26 are dispensable for BGLF4 to phosphorylate EA-D, whereas aa 27–70 constitutes an important domain for BGLF4 kinase activity. Since EA-D appeared to locate mainly in the nucleus of transfected cells, we next examined whether BGLF4(Δ1–70) lost its nucleus localization ability.

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**Fig. 7.** Kinase activities of N-terminal deleted BGLF4 mutants on EA-D phosphorylation. (a) Schematic summary of BGLF4 deletion mutant. Domains I–XI are putative herpes viral protein kinase conserved domains identified by alignment. (b) Plasmids expressing EA-D and wt or mutant BGLF4 were co-transfected into 293T cells, harvested at 48 h post-transfection, lysed in RIPA buffer and analysed with immunoblotting. (c) BGLF4 proteins in HeLa cells were analysed in nuclear (N), heavy membrane (H) and cytosolic plus light membrane fractions (C) at 48 h post-transfection. BGLF4 was detected with mAb 2616. Poly (ADP-ribose) polymerases (PARP) and α-tubulin were detected as nuclear and cytoplasmic markers, respectively. NA cell lysates harvested at 24 h p.i. was loaded as a control. VC, Vector control.
Even though the proportion of all BGLF4 mutants within the cytosolic/membrane fraction increased by about 20–30% compared with wt, nuclear retention of wt BGLF4, Δ1–26, Δ1–70, Δ1–108 and K102I are still observed in transiently transfected HeLa cells (Fig. 7c). We thus concluded that the inability of BGLF4(Δ1–70) to phosphorylate EA-D was not because of the loss of nuclear retention.

**DISCUSSION**

EBV BGLF4 protein kinase was demonstrated to phosphorylate the viral DNA polymerase processivity factor, EA-D (Chen et al., 2000a; Gershburg & Pagano, 2002), and it was predicted to mimic the activity of cellular cdc2 (Kawaguchi et al., 2003). However, the biological function and behaviour of BGLF4 within EBV-replicating cells remained mostly elusive. With an intention to obtain a tool for monitoring BGLF4 protein and to identify its major immunogenic regions, a panel of mAbs against BGLF4 was generated. According to the sequence alignment of herpes UL and cellular protein kinases, only the very N-terminal 80 and C-terminal 100 aa of BGLF4 are unique regions and predicted to be immunogenic regions in animals. Indeed, fine mapping of the immunogenic epitopes of eight different hybridoma clones identified two regions within aa 27–70 and 327–429 of BGLF4. This result is accordant with the computer predicted surface possibilities of BGLF4 and suggests possible regulatory function of these two regions. mAbs obtained here will be useful reagents for studying the biochemical property and the structure–function relationship of BGLF4, or for detecting possible expression of BGLF4 within EBV-associated malignancies.

We detected BGLF4 expression in EBV-positive Raji, Akata, P3HR1 and NPC-derived NA at different time points post-induction. BGLF4 was expressed with early kinetics, it was detected predominantly in the nucleus of EBV-positive cells and increasing perinuclear cytoplasmic staining of BGLF4 was also observed between 48 and 72 h.p.i. in NA cells (Fig. 4c). Since the biological function of BGLF4 homologues was suggested to be involved in DNA replication and virion packaging, the nuclear punctate structures may indicate the association of BGLF4 with multiple protein complexes in the replication compartment (Fig. 5a and b). Similar translocation of UL97 from the nucleus to the cytoplasm was also observed at the late phase of CMV replication in HFF cells (Wolf et al., 1998). Additionally, UL97 was further demonstrated to be important for proper virus egress from the nucleus to the cytoplasm (Krosky et al., 2003). Therefore, the gradual change of subcellular localization correlates with the predicted function of BGLF4 in regulating viral DNA replication at the early phase and in the virion maturation process.

Detection of BGLF4 protein in virions hints further possible function of BGLF4 in the virion assembly, egress process or in the next round of infection. In HSV-1, UL13 was demonstrated to be required for nucleocapsid dissociation (Morrison et al., 1998). In the case of HCMV, UL97 is detected at 2 h post-infection, indicating that the kinase could be carried into the newly infected cells (van Zeijl et al., 1997). It would be interesting to examine whether BGLF4 kinase activity is important for the primary infection of EBV. Our observation also confirmed the report by Johannsen et al. (2004), showing with tandem mass spectrometry sequencing that BGLF4 was identified as a minor virion-associated protein.

In this study, EA-D phosphorylation patterns within virus-replicating cells and virions were also examined. Extensive phosphorylation of EA-D was observed correlating with the expression amounts of BGLF4 in both lymphoid and epithelial cells. However, it remains to be determined whether the phosphorylation was induced by BGLF4 directly or through a secondary signalling effect. Based on the finding that transient expression of its homologue, KHSV ORF36, activated the c-Jun N-terminal kinase (JNK) signalling pathway in transiently transfected cells (Hama et al., 2004), we examined the phosphorylation status of JNK in BGLF4-expressing HeLa cells, and did not observe enhanced phosphorylation of JNK (data not shown). Another interesting observation is the hypophosphorylation status of EA-D within viral particle (Fig. 6b). The phosphorylation changes of EA-D found in this study is very similar to that of HSV-1 VP22, which is also phosphorylated by UL13 and other cellular kinases (Elliott et al., 1996). VP22 was found to be hyperphosphorylated during virus replication and hyperphosphorylated in the virion. EA-D is the viral DNA polymerase processivity factor and known to transactivate BHLF1 promoter (Zhang et al., 1997; Holley-Guthrie et al., 2005). Using an in-cell biochemical fractionation protocol, EA-D was not only associated with DNA polymerase complexes at the viral DNA replication fork but also distributed over newly replicated viral DNA (Daikoku et al., 2005). We suggest that the hypophosphorylated EA-D within the viral particles may provide protection for the viral genome, but how phosphorylation affects the translocation or function of EA-D will need further investigation. Whether a common mechanism is involved in regulating viral protein targeting through a viral kinase-dependent pathway will be an interesting issue to study in the future.

As detected with BrdU incorporation and immunofluorescence staining, BGLF4 co-localized with EA-D to the virus replication compartment, this suggests possible modulator function of BGLF4 in the DNA replication complexes. In UL97 mutant HCMV, viral DNA accumulation exhibits a defect at early replication of four- to sixfold compared with wt virus (Wolf et al., 2001). We suggest that BGLF4 may modulate lytic replication of EBV DNA or transcriptional activity of viral promoters. Similarly in HSV-1, reduced phosphorylation of both ICP22 and ICP0 was observed in an UL13 mutant, correlating with reduced production of a subset of viral mRNA (Purves et al., 1993; Purves & Roizman, 1992).
In deletion and mutation analysis, increased cytosolic/membrane retention of BGLF4 was observed in kinase dead or mutants without aa 1–70. It is not clear why a doublet was detected in cells expressing Δ1–26, and the distribution of these two bands seemed to be slightly different in heavy membrane and cytosolic fractions (Fig. 7d). Size differences in the cytosolic fraction and nuclear fraction were not observed in Δ1–70 and Δ1–108 (Fig. 7c), suggesting possible modification at the very N-terminal region of BGLF4. The N-terminal 26 aa of BGLF4 (MDVNMAELPTNSSSSSG-ELSVSPEP) contain seven serines and one threonine (bold), including predicted phosphorylation sites for CK1, CK2 and GSK3 (underlined). In our previous study, this region was demonstrated to be the major autophosphorylation site of BGLF4 in an in vitro kinase assay (Chen et al., 2000a). How the N terminus may contribute to the cytoplasmic retention of BGLF4 is unclear. Recently, a novel class of serine/threonine-rich NLS was identified within mineralocorticoid receptor at aa 592–602 (LRVSTGSSSSPSK), in addition to two basic NLSs (Walther et al., 2005). Further experiments are required to characterize whether the N-terminal serine/threonine-rich region of BGLF4 is also a serine/threonine NLS, which may act in concert with the C-terminal NLS to direct nuclear translocation, or whether other modifications at this region is responsible for nuclear retention of BGLF4.

Based on the observation of BGLF4 expression and subcellular localization at different lytic time points within EBV-replicating cells and in virion, we suggest that BGLF4 may play multiple roles at different stages of virus replication. The recently identified non-classical NLS at the C-terminus of BGLF4 (Gershburg et al., 2004) and our findings here indicate that complex mechanisms may be involved in regulating BGLF4 subcellular localization.

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Expression of EBV kinase BGLF4

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