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Key motifs in EBV (Epstein–Barr virus)-encoded protein kinase for phosphorylation activity and nuclear localization

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Abstract

A sole EBV (Epstein–Barr virus)-encoded protein kinase (EBV-PK) (the *BGLF4* gene product) plays important roles in viral infection. Although a number of targets of this protein have been identified, the kinase itself remains largely unstudied with regard to its enzymology and structure. In the present study, site-directed mutagenesis has been employed to generate mutations targeting residues involved in nuclear localization of the EBV-PK, core residues in subdomain III of the protein kinase domain conserved in most protein kinases or residues in subdomain VIa conserved only within the HPK (herpesvirus-encoded protein kinase) group. Deletion of amino acids 389–391 resulted in exclusive cytoplasmic localization of the protein, indicating the involvement of this region in nuclear translocation of the EBV-PK. Mutations at the amino acids Glu¹¹³ (core component), Phe¹⁷⁵, Leu¹⁷⁸, Phe¹⁸⁴, Leu¹⁸⁵ and Asn¹⁸⁶ (conserved in HPKs) resulted in loss of EBV-PK autophosphorylation, protein substrate [EBV EA-D (early antigen diffused)] phosphorylation, and ability to facilitate ganciclovir phosphorylation. These results reiterate the unique features of this group of kinases and present an opportunity for designing more specific antiviral compounds.

Keywords

Epstein–Barr virus (EBV); ganciclovir; herpesvirus; phosphorylation; protein kinase

INTRODUCTION

EBV (Epstein–Barr virus) is a ubiquitous human gammaherpesvirus, which causes infectious mononucleosis and is closely associated with a number of human malignant diseases, including Burkitt's lymphoma, nasopharyngeal carcinoma, gastric carcinoma and lymphoproliferative disease in immunocompromised patients [1]. The EBV infection cycle consists of latent and lytic programmes. During latency, a limited number of genes are expressed and no progeny viruses are generated; conversely, most of the genes are expressed during the lytic programme, leading to *de novo* production of infectious virions [2]. The repertoire of viral genes expressed in the lytic programme includes a sole protein kinase

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AUTHOR CONTRIBUTION

Svetlana Gershburg designed experiments, analysed data and co-wrote the paper; Leann Murphy performed experiments and analysed data; Manfred Marschall designed experiments and analysed data; Edward Gershburg guided the experimental design and co-wrote the paper.

[EBV-PK (EBV protein kinase)] encoded by the *BGLF4* gene. EBV-PK belongs to a group of CHPKs (conserved herpesviral protein kinases) that are encoded by members of all three subfamilies of herpesviruses [3,4]; in human herpesviruses, these include the UL13 protein of HSV (herpes simplex virus) 1 and 2, pUL97 of HCMV (human cytomegalovirus), ORF47 protein of VZV (varicella zoster virus), U69 protein of human herpesviruses 6 and 7, and ORF36 protein of KSHV (Kaposi's sarcoma-associated herpesvirus). EBV-PK is a nuclear serine/threonine protein kinase that is expressed with early kinetics [5,6], exhibits certain functional similarities with HCMV (human cytomegalovirus) pUL97 (reviewed in [3,7]) and has been identified as a component of the virion tegument [8,9]. We demonstrated previously using an RNAi (RNA interference) approach that EBV-PK expression is crucial for production of infectious virions [10], which has been recently supported by a knockout approach [11,12]. The biological role of EBV-PK is thought to involve phosphorylation of lamin A/C, which in turn promotes disassembly/reorganization of the nuclear lamina required for an efficient nuclear egress [13,14].

An increasing number of EBV-PK targets is being identified, including: the products of lytic genes *BMRF1* [5,6,15], *BZLF1* [16,17] and *BGLF5* [11]; the products of latent genes *EBNA-LP* [18], *EBNA2* [19] and *EBNA1* [20]; and several cellular proteins {such as translation elongation factor 1 α [21], members of the interferon regulatory factor 3 signalling pathway [22,23], p27^{Kip1} [24], condensin and topoisomerase II [25], and MCM (minichromosome maintenance) 4 [26]}. Furthermore, additional targets of EBV-PK were recently identified by means of an EBV protein array [20], but have yet to be confirmed. In addition to its role in promoting nuclear egress, EBV-PK-mediated phosphorylation was found to: (i) reduce the ability of EBNA2 to transactivate LMP-1 expression [19]; (ii) recruit xeroderma pigmentosum C protein to enhance viral DNA replication [27]; (iii) induce premature chromosome condensation [25]; (iv) inhibit DNA helicase activity of the MCM4–MCM6–MCM7 complex [26]; and (v) facilitate phosphorylation of GCV (ganciclovir) [28,29]. Although studies to identify new targets of EBV-PK have intensified, research focusing on regulation of its activity has remained stagnant. To address this deficiency, we have generated a number of EBV-PK mutants targeting: (i) residues conserved among different groups of protein kinases (core residues); (ii) residues conserved only among CHPKs; and (iii) residues assumed to participate in nuclear translocation. Using this panel of mutants, we have mapped a region involved in nuclear localization of EBV-PK and identified a unique CHPK-conserved region crucial for its activity.

EXPERIMENTAL

Cell lines and transfection

HEK (human embryonic kidney)-293 (A.T.C.C. CRL-1573), and African green monkey kidney (Vero) (A.T.C.C. CCL-81) epithelial cells were maintained in DMEM (Dulbecco's modified essential medium) supplemented with 10% fetal bovine serum and antibiotic/antimycotic mixture (Gibco). All transfections were performed using TurboFect reagent (Fermentas) according to the manufacturer's protocol.

Plasmids, site-directed mutagenesis and transfections

The plasmids expressing FLAG-tagged wild-type EBV-PK and untagged EA-D (early antigen diffused) were as described previously [28,30]. The plasmids pHM829 and pHM840 expressing a β -gal (β -galactosidase)/GFP (green fluorescent protein) fusion protein were as described previously [31] and were generously provided by Thomas Stamminger (University of Erlangen-Nuremberg). The mutants were generated using the QuikChangeTM II Site-Directed Mutagenesis (Stratagene) protocol and the following sets of primers: Δ NLS-frw (5'-CAATTCTTGCAGTGGTGCAGAGCTACTTCTTCAAC-3') and Δ NLS-

rev (5'-GTTGAAGAAGTAGCTCTCGCACCACTGCAAGAATTG-3'), and ΔKKR-frw (5'-CAGTGGTGCCGGAGCCTTTTCAAGGAGAGCTACTTC-3') and ΔKKR-rev (5'-GAAGTAGCTCTCCTTGAAAAGGCTCCGGCACCACTG-3'). The plasmids pGFP/PK, pGFP/PK-(375–429) and pGFP/PK-(1–77) were generated by subcloning the wild-type EBV-PK, ΔNLS and ΔKKR mutants, and C-terminal (amino acids 375–429) and N-terminal (amino acids 1–77) regions into pEGFP-C2 (Clontech).

Biochemical fractionation

HEK-293 cells were grown on 60-mm-diameter dishes and transfected as described above. At 24–36 h post-transfection, the cells were collected and washed with PBS. The fractionation was performed using the method of Nielsen et al. [32]. Briefly, the cells were resuspended in NIB (nuclear isolation buffer) [15 mM Tris/HCl, pH 7.4, 60 mM KCl, 15 mM MgCl₂, 15 mM NaCl, 1 mM CaCl₂, 1 mM PMSF and Halt protease inhibitor mixture (Pierce)] with 0.3% NP-40 (Nonidet P40) and incubated on ice for 10 min. The nuclei were pelleted by centrifugation at 2000 *g* for 5 min and the supernatants were transferred to a clean tube (cytosolic fraction). The pellets were washed in NIB with 0.6% NP-40 and pelleted again (nuclear wash I). The pellets were then resuspended in NIB/0.6% NP-40, treated with 30 units of micrococcal nuclease (Fermentas) for 8 min at room temperature (23°C), and centrifuged again to separate insoluble chromatin and soluble nuclear fraction (nuclear wash II). The final pellets were resuspended in 2 mM ice-cold EDTA to yield a soluble chromatin fraction. One-twentieth of each fraction was analysed by immunoblotting as described below.

Immunofluorescence staining

Vero cells were grown on 12-mm round coverslips inserted in a 12-well plate and transfected as described above. At 24–36 h posttransfection, the cells were fixed with 4% (w/v) paraformaldehyde and permeabilized with 0.5% Triton X-100 at room temperature. The fixed cells were incubated for 30 min with 5% (v/v) NGS (normal goat serum) and 3% BSA to block non-specific antibody binding. The blocking solution was then replaced with anti-FLAG M2 mouse mAb (monoclonal antibody) (Sigma) diluted 1:1000 in 2% NGS/PBS for 2 h at room temperature. The coverslips were washed with PBS and incubated with Alexa Fluor® 594-conjugated goat anti-mouse secondary antibodies used at 1:1000 in 2% NGS/PBS for 1 h. After additional washes, the nuclei were counterstained with Hoechst 33258 (1 µg/ml in water), washed in water, and mounted in FluorSave™ reagent (Calbiochem). The cells were imaged through an Olympus 60×/1.42 objective on Olympus IX71 fluorescence microscope and epifluorescence images were recorded at 12-bit depth with a Retiga EXi CCD (charge-coupled device) camera (QImaging).

Immunoblotting

HEK-293 cells were grown in 12-well plates and transfected as described above. At 24–36 h post-transfection, the cells were collected and washed with PBS and whole-cell lysates were prepared in WCL buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% NP-40, 1% SDS and 2 mM EDTA) supplemented with Halt protease inhibitor mixture. Cell debris was removed by centrifugation at 16000 *g* for 15 min. Then, 25–40 µg of the lysates were denatured in Laemmli loading buffer (5 min at 95°C), resolved by SDS/PAGE (10 and 15% gels), and transferred on to nitrocellulose membranes (Millipore). After incubation in blocking buffer [5% Difco skimmed milk (BD Biosciences) in TBST (Tris-buffered saline with 0.2% Tween 20)], the membranes were incubated with anti-FLAG M2 mouse mAb and anti-EA-D mAb (Bioworld Consulting Laboratories) diluted to 1:1000 in blocking buffer overnight. The membranes were washed in TBST and incubated for 1 h with IRDye-800CW-conjugated goat anti-mouse secondary antibody (Li-Cor Biosciences) diluted 1:20000 in blocking buffer. After additional washes, the proteins of interest were

visualized on an Odyssey® Infrared Imaging System (Li-Cor Biosciences). To verify the efficiency of the biochemical fractionation, membranes were reprobed with anti-histone H3 pAb (polyclonal antibody) (Sigma) and anti-HSP90 (heat-shock protein 90) mAb (StressMarq Biosciences) used at 1:4000 and 1:1000 dilutions respectively.

IP (immunoprecipitation)

HEK-293 cells were grown in 100-mm dishes and transfected as described above. At 24–36 h post-transfection, the cells were collected and washed with PBS, and whole-cell lysates were prepared in IP-150 buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP-40 and Halt protease inhibitor mixture). Cell debris was removed by centrifugation at 16000 *g* for 15 min, and the lysates were pre-cleared by incubation with normal mouse IgG and Protein A/G–agarose beads (Santa Cruz Biotechnology). The supernatants were then incubated with anti-FLAG M2–agarose beads overnight and washed extensively in IP-500 buffer (containing 0.5M NaCl), and the precipitated complexes were used in kinase assays.

Kinase assays

The immunocomplexes obtained after IP were washed twice in kinase buffer (75 mM Hepes, pH 7.3, 200 mM NaCl, 5 mM MgCl₂, 5mM MnCl₂, 1 mM dithiothreitol and 0.1% NP-40). The kinase assays were performed for 30 min at 37°C in the same buffer complemented with 50 μM ATP (Sigma) and 5 μCi of [γ -³²P] ATP (specific activity 4500 Ci/mmol, MP Biomedicals). The beads were washed three times with PBS and the proteins were denatured, resolved by SDS/PAGE (10% gels), and transferred on to nitrocellulose membranes as described above. The membranes were exposed to phosphor screens and analysed with a Cyclone PhosphorImager and OptiQuant software (PerkinElmer). Protein expression levels were verified by immunoblotting.

EBV-PK ‘in-cell’ activity assay

The assays were performed as described by Marschall et al. [33]. Briefly, HEK-293 cells were transfected in 96-well plates using Lipofectamine™ reagent (Invitrogen). At 24 h post-transfection, transfection media were replaced with serial dilutions of GCV in complete DMEM. At 7 days post-transfection, a change in the colour of the culture medium and cytotoxic effects were evaluated by direct microscopic observation and photometric quantification using an ELISA plate reader (O.D.₅₆₀).

RESULTS

Deletion of amino acids 389–391 results in cytoplasmic localization of the EBV-PK

The C-terminus of EBV-PK contains a stretch of basic amino acids (R³⁸⁶SLKKRFK³⁹³), which may target the protein to the nucleus based on its similarity with known NLSs (nuclear localization signals) (reviewed in [34]). Consistent with this possibility, we have demonstrated previously that deletion of 55 amino acids from the C-terminus of EBV-PK results in its cytoplasmic localization [35]. To determine whether amino acids 386–393 are necessary for the nuclear localization of the EBV-PK, the following mutants were generated: PKΔ386–393_{FLAG} in which eight amino acids containing five basic residues were deleted, and PKΔ389–391_{FLAG} in which only three amino acids that lie in the core of this stretch were deleted. The wild-type EBV-PK or the mutants were expressed ectopically in Vero cells and their subcellular distribution was visualized by immunostaining. In the majority of cells, wild-type EBV-PK localized exclusively in the nucleus (Figure 1) exhibiting a mostly diffuse nuclear pattern. In contrast, the mutant protein PKΔ386–393_{FLAG} localized in the cytoplasm in virtually all cells analysed, therefore suggesting that this region is involved in

nuclear localization of the EBV-PK. Likewise, the mutant protein PK Δ 389–391_{FLAG} demonstrated nearly identical cytoplasmic localization.

To validate further the results of immunofluorescence staining, we employed a biochemical fractionation approach, which allows separation of cytoplasmic, soluble nuclear and chromatin fractions from the cells expressing the wild-type EBV-PK or the mutants. HEK-293 cells were transfected with respective constructs and the cell lysates were fractionated (Figure 2A). We first tested the efficiency of the fractionation method by probing the resulting fractions for the cytoplasmic protein HSP90 α [36] and the chromatin-associated protein histone H3. The majority of HSP90 was detected in the cytoplasmic fraction (Figure 2B, lanes 1, 5 and 9), but residual amounts of HSP90 were detected in soluble nuclear fractions N₁ and N₂ (Figure 2B, lanes 2, 3, 6, 7, 10 and 11), perhaps due to the incomplete lysis of some cells or due to the fact that small amounts of the protein can localize in the nucleus. Histone H3 was detected only in the chromatin fraction (Figure 2B, lanes 4, 8 and 12). Wild-type EBV-PK co-fractionated with the chromatin fraction (Figure 2B, lane 4), which suggests that the protein is tightly bound to the chromatin. In contrast, both mutant proteins were mainly detected in the cytoplasmic fraction (Figure 2B, lanes 5 and 9 respectively) and low levels were also detected in the soluble nuclear fraction N₁ (Figure 2, lanes 6 and 10). Consistent with the data obtained by immunofluorescence staining (Figure 1), the results of biochemical fractionation indicated that the region between amino acids 386 and 393 is essential for nuclear localization of EBV-PK.

C-terminal amino acids 386–393 of EBV-PK are not sufficient for nuclear translocation

Classical NLSs [such as that of the SV40 (simian virus 40) large-T antigen] are able to act autonomously by translocating normally cytoplasmic proteins to the nucleus [37]. To examine whether the C-terminal amino acids 386–393 of the EBV-PK was sufficient to translocate a cytoplasmic protein to the nucleus, amino acids R³⁸⁶SLKKR³⁹³ or K³⁸⁹KR³⁹¹ of EBV-PK were introduced into plasmids that encode a chimaeric β -gal–GFP reporter protein and which were termed p β -gal/PK-(386–393)/GFP and p β -gal/PK-(389–391)/GFP (Figure 3). The plasmids were expressed ectopically in Vero cells, and the expression of the fusion proteins was monitored by fluorescence microscopy as shown in representative images in Figure 3. β -Gal is a large tetrameric protein lacking nuclear-targeting sequences and hence is known to localize strongly in the cytoplasm, as is evident for the control β -gal/GFP fusion protein (Figure 3). The autonomous NLS of SV40 large-T antigen cloned between the GFP and β -gal caused the fusion protein to translocate to the nucleus. However, neither R³⁸⁶SLKKR³⁹³ nor K³⁸⁹KR³⁹¹ were able to translocate the respective fusion proteins to the nucleus (Figure 3). Hence, amino acids 386–393 of EBV-PK are not sufficient for the nuclear translocation of an exogenous cytoplasmic protein.

We reasoned that the short region used in these assays may not contain all of the necessary components for nuclear localization and thus examined whether larger parts of the EBV-PK promoted nuclear translocation. To this end, additional GFP-fusion constructs were generated: pGFP/PK contains a wild-type EBV-PK fused with the C-terminus of the GFP; pGFP/PK-(375–429) contains 55 C-terminal amino acids fused with the C-terminus of the GFP; and pPK-(1–77)/GFP contains 77 N-terminal amino acids of the EBV-PK fused with the N-terminus of GFP. As in the previous assay, the constructs were expressed in Vero cells and GFP expression was monitored by fluorescence microscopy (Figure 4). Wild-type EBV-PK was able to target the GFP-fusion protein to the nucleus in the majority of cells analysed. In contrast, neither N- nor C-terminal parts of the EBV-PK were able to do so, and most cells analysed exhibited a mixed distribution identical with the distribution of the GFP expressed by itself (Figure 4). Taken together, these observations suggest that amino acids 386–393 and even larger parts of the EBV-PK (amino acids 375–429) are not sufficient for nuclear translocation and cannot operate autonomously.

Amino acid alignments of the CHPK kinase domains

Several previous reports have comprehensively compared sequence homology between the CHPKs using different alignment algorithms [7,38,39]. The authors of these studies concluded that overall sequence homology is very limited both within the group or when compared with cellular protein kinases. However, like most other kinases, CHPKs share a number of highly conserved key core components, which are involved in basic catalytic functions, such as ATP binding, transfer of γ -phosphate to substrate and/or release of ADP (reviewed in [40]). Of these, the following are known to perform catalytic roles: (i) an invariable glycine residue of the G-rich loop (GXG $\circ$ XXG) between β 1 and β 2 strands [Gly⁵² in PKA (protein kinase A)], which anchors the non-transferable phosphates of ATP; (ii) a basic residue (most often lysine) within the β 3 strand (Lys⁷² in PKA) that hydrogen bonds to the α and β oxygen atoms of bound ATP; (iii) an acidic residue (most often glutamate) in the C-helix (Glu⁹¹ in PKA) that hydrogen bonds to Lys⁷² and thus positions this basic residue; (iv) the DFG-motif aspartate residue (Asp¹⁸⁴ in PKA), which co-ordinates with ATP and the bound Mg²⁺; (v) the catalytic aspartate residue within the HXD-motif (Asp¹⁶⁶ in PKA); and (vi) an asparagine residue within the catalytic loop (Asn¹⁷¹ in PKA) that also co-ordinates with the ATP-bound Mg²⁺ (Figure 5). Since the reported alignments slightly differ in predictions of a few kinase core components, we used T-Coffee software [41,42] to generate additional alignments, two of which are presented in Figure 5: an alignment of kinase domains from seven CHPKs encoded by human herpesviruses of all three subfamilies (Figure 5A); and an alignment of CHPKs encoded by different gammaherpesviruses (Figure 5B). Both alignments identified the highly conserved elements mentioned above, but they also posed two interesting questions: (i) an invariable C-helix glutamate in subdomain III differs in different alignments, so that in the alignment of kinase domains (Figure 5A), the glutamate corresponding to EBV-PK Glu¹⁰⁹ was conserved, whereas in the alignment of gammaherpesviral CHPKs, Glu¹¹³ was conserved (Figure 5B); and (ii) a cluster of residues conserved in all CHPKs, but not in other known kinases [38] was identified. This cluster consists of mainly hydrophobic amino acids (Figure 5A) proximal to the catalytic aspartate residue in the HXD-motif (Asp¹⁹⁵ in EBV-PK). To address these questions, we employed site-directed mutagenesis to create point mutations corresponding to Glu¹⁰⁹, Glu¹¹³, Glu¹²⁸, Phe¹⁷⁵, Leu¹⁷⁸, Ala¹⁸¹, Phe¹⁸⁴, Leu¹⁷⁵, Asn¹⁸⁶ and Cys¹⁸⁹ in EBV-PK and tested their auto- and allo-phosphorylation activities.

Identification of the core glutamate in subdomain III of the EBV-PK

Since different alignment algorithms identified different conserved glutamate residues in subdomain III, we set to experimentally verify the activity of mutants in which the candidate glutamate residues were replaced with alanine residues (E109A, E113A and E128A respectively). The mutants were expressed in HEK-293 cells with or without EBV EA-D (*BMRF1* gene product), a known EBV-PK target [5,6,15], and their activity in IP/kinase assays (autophosphorylation) or their ability to hyperphosphorylate EA-D were assessed. Figure 6 demonstrates that the wild-type EBV-PK autophosphorylates (as visualized by incorporation of ³²P and autoradiography) and hyperphosphorylates EA-D (as evident from the retardation of its electrophoretic mobility [6]), whereas EBV-PK K102A mutant (in which the invariant Lys¹⁰² is mutated) was inactive in both assays (Figure 6, lanes 1 and 2). Both E109A and E128A mutants (Figure 6, lanes 3 and 5 respectively) exhibit levels of auto- and allo-phosphorylation activity comparable with that of the wild-type kinase (Figure 6, lane 1), suggesting that these residues are dispensable for the kinase activity. In contrast, E113A (Figure 6, lane 4) displayed minimal activity in both assays, suggesting that EBV-PK Glu¹¹³ is essential for the optimal kinase activity and is probably a homologue of Glu⁹¹ in PKA. Notably, the observation that E113A retained a certain level of activity correlates with its potential function, which is to position the invariant Lys¹⁰²; in the absence of this

glutamate residue, Lys¹⁰² becomes ‘disoriented’ and cannot interact efficiently with ATP, thus limiting, but not eliminating, the activity of the kinase.

Involvement of the CHPK conserved residues in EBV-PK activities

A number of hydrophobic residues in subdomain VIa are conserved in CHPKs (Figure 5A), but not in cellular protein kinases. In fact, this subdomain does not contain any conserved elements and is therefore thought to be less important for kinase activity [43]. High conservation of several residues in this region of the CHPKs led us to hypothesize that, in this group of enzymes, conserved residues in subdomain VIa are essential for their kinase activity. To determine the significance of these conserved residues, we replaced them with alanine (except for Ala¹⁸¹, in which the alanine residue was replaced with glycine), expressed the resulting constructs in HEK-293 cells, and tested their activity in autophosphorylation and in phosphorylation of the protein target, and their ability to promote the phosphorylation of GCV (Figure 7). The autophosphorylation was evaluated by the IP/kinase assay (Figure 7A), which showed that (i) L178A, F184A and N186A (Figure 7A, lanes 4, 6 and 8) had no detectable autophosphorylation activity [similar to the K102A mutant (Figure 7A, lane 2) described above], (ii) the autophosphorylation activity of F175A and L185A (Figure 7A, lanes 3 and 7) was significantly reduced in comparison with the wild-type EBV-PK (Figure 7A, lane 1), and (iii) the autophosphorylation activity of A181G (Figure 7A, lane 5) was comparable with that of the wild-type EBV-PK. The mutants were then assayed for their ability to hyperphosphorylate EBV EA-D (Figure 7B). The results of this assay corroborated the results obtained in IP/kinase assays as was evident by the lack of EA-D hyperphosphorylation for F175A, L178A, F184A and N186A mutants (Figure 7B, lanes 3, 4, 6 and 8). As in the previous assay, A181G exhibited wild-type levels of activity (Figure 7B, lane 5), and the activity of L185A nearly matched wild-type levels as well (Figure 7B, lane 7). From the results, we can conclude that Leu¹⁷⁸, Phe¹⁸⁴ and Asn¹⁸⁶, and, to a lesser extent, Phe¹⁷⁵ and Leu¹⁸⁵, are functionally crucial for both auto- and allo-phosphorylation, whereas Ala¹⁸¹ is not.

Since EBV-PK (similar to HCMV pUL97) is able to promote GCV phosphorylation when expressed in cell culture [35,44], we tested the ability of these mutants to phosphorylate GCV using a previously described ‘in-cell’ kinase activity assay [33]. The GCV is converted into its monophosphate (and eventually triphosphate) form more efficiently in the presence of active HCMV pUL97 or EBV-PK and this method is therefore quite useful in evaluating the activity of these enzymes. The A181G mutant again exhibited levels of activity comparable with the wild-type EBV-PK. The activities of the rest of the mutants was slightly higher than the background of the assay [activity of RFP (red fluorescent protein)] (Figure 7C). Expression levels of all proteins tested were equal as confirmed by immunoblotting. Hence the results of this assay are in agreement with the results observed with auto- and EA-D-phosphorylation experiments, which suggest that the CHPK-conserved residues (except for A181G) are functionally significant for all aspects of the EBV-PK (and potentially other CHPKs) activity.

DISCUSSION

Nuclear localization of the EBV-PK and CHPKs

All CHPKs have been characterized as nuclear proteins [7,25,35,45–47] except for HSV-1 UL13, for which the reports are conflicting [7,25]. HCMV pUL97 is probably the most studied member of the group with regard to intracellular localization [45,48]. The HCMV pUL97 sequence contains two NLS-like regions (identified on the basis of homology with several viral NLSs); however, neither of these regions was essential for nuclear translocation of the GFP-fusion protein. The existence of a non-conventional NLS located between amino

acids 48 and 110 of pUL97 has eventually been proposed, therefore suggesting that both identifiable NLSs are dispensable for the nuclear translocation [48]. Results of the present study imply that the NLS-like region at the C-terminus of EBV-PK is crucial for the nuclear localization of the protein (Figures 1 and 2), yet the same region or even the whole C-terminus (amino acids 375–429) were unable to act autonomously (Figures 3 and 4). In contrast, the wild-type EBV-PK efficiently translocated to the nucleus by itself (Figure 1) or as a fusion protein with GFP (Figure 4). Several conclusions can be drawn from these observations. First, nuclear translocation of the EBV-PK is an active transport (not a passive diffusion), which is evident from the nuclear localization of the GFP–protein kinase fusion protein, which exceeds the molecular mass threshold for passive transport (~50 kDa), and cytoplasmic localization of the PK Δ 386–393_{FLAG} and PK Δ 389–391_{FLAG} mutants. Secondly, amino acids 386–393 are essential, but not sufficient, for the nuclear localization. Thirdly, it is possible that some region (or regions) within the kinase domain contribute to the nuclear localization of EBV-PK; however, attempts to use a deletion approach inside the kinase domain will probably cause misfolding of the resulting protein, which, in turn, will distort its localization pattern.

Impairment of kinase activity by mutagenesis of conserved residues of the EBV-PK

Although over 1000 protein kinase structures are available, homology-based predictions of CHPK structures remain unreliable, suggesting that this group of kinases is sufficiently different from protein kinases found in other organisms. Furthermore, protein sequence homology even within the group remains sufficiently low (usually 15–20% [7]) and only increases when protein kinases from the same subfamily of herpesviruses are compared. Despite such a low homology, many key catalytic residues of the kinase fold are readily identifiable in CHPKs ([7,38] and Figure 5), albeit a few of them, such as an invariable glutamate residue in subdomain III or an APE (Ala-Pro-Glu) consensus motif in subdomain VIII, are harder, if not impossible, to identify. In the present study, we used protein sequence alignments as a guide to experimentally verify the importance of several conserved residues for the EBV-PK activity. We have considered three candidates for the subdomain III glutamate residue: Glu¹⁰⁹ (Figure 5A); Glu¹¹³ ([38] and Figure 5B); and Glu¹²⁸ [7], and found that only Glu¹¹³ was crucial for both protein substrate phosphorylation and autophosphorylation (Figure 6). In another set of experiments, we analysed the activity of EBV-PK mutants that targeted a group of amino acids in subdomain VIa uniquely conserved in CHPKs (Figure 5A) and found that most of them are critical for all EBV-PK activities tested (autophosphorylation, hyperphosphorylation of EA-D and GCV phosphorylation) (Figure 7). These results correlate with data reported for HCMV pUL97 mutant L446R (homologue of L185A), but contrast the data for HCMV pUL97 A442V (homologue of A181G) [49]. The activity of A442V was significantly reduced, whereas A181G was as active as a wild-type (Figure 7). The difference can be explained by the choice of the replacing residue: bulkier valine may have a stronger effect on activity than glycine. Overall, these results again reiterate the need for additional structural studies to gain a more complete understanding of the function of this region.

Studies of conserved herpesviral protein kinases illustrate several important points. First, from the evolutionary standpoint, this group demonstrates that divergence of herpesviruses resulted in significant sequence variation even for the proteins with presumably conserved activities, perhaps due to the adaptation to the specific host cell environment (i.e. neuronal and lymphoid persistence) or due to a gain of additional functions (such as phosphorylation of small metabolites in addition to protein substrates). On the other hand, even though CHPKs belong to a group of herpesvirus core (or ancestral) proteins [50–52], this divergence did not affect crucial key elements, which probably originated from a common herpesvirus ancestor including those components that are unique to this group of proteins.

Secondly, from the biochemical standpoint, this group of kinases represents a novel potentially diverse group, which will contribute to our general knowledge of protein kinase structure, substrate selectivity and evolution of the kinase fold. Finally, identification of the unique conserved region in CHPKs has an added practical significance: targeting this region may be an effective way to generate antiviral compounds with minimal off-target effects, unlike the majority of current compounds which target the ATP-binding pocket and are often not highly selective.

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Abbreviations used

β-gal	β-galactosidase
CHPK	conserved herpesviral protein kinase
DMEM	Dulbecco's modified Eagle's medium
EA-D	early antigen diffused
EBV	Epstein-Barr virus
EBV-PK	EBV protein kinase
GCV	ganciclovir
GFP	green fluorescent protein
HCMV	human cytomegalovirus
HEK	human embryonic kidney
HSP90	heat-shock protein 90
HSV	herpes simplex virus
IP	immunoprecipitation
mAb	monoclonal antibody
MCM	mini-chromosome maintenance
NGS	normal goat serum
NIB	nuclear isolation buffer
NLS	nuclear localization signal
PKA	protein kinase A
NP-40	Nonidet P40
SV40	simian virus 40
TBST	Tris-buffered saline with 0.2% Tween 20

REFERENCES

1. Rickinson, AB.; Kieff, ED. Epstein–Barr virus. In: Knipe, DM.; Howley, PM., editors. *Fields Virology*. Philadelphia: Lippincott Williams & Wilkins; 2007. p. 2655–2700.
2. Kieff, E.; Rickinson, AB. Epstein–Barr virus and its replication. In: Knipe, DM.; Howley, PM., editors. *Fields Virology*. Philadelphia: Lippincott Williams & Wilkins; 2007. p. 2603–2654.
3. Gershburg E, Pagano JS. Conserved herpesvirus protein kinases. *Biochim. Biophys. Acta*. 2008; 1784:203–212. [PubMed: 17881303]
4. Kawaguchi Y, Kato K, Tanaka M, Kanamori M, Nishiyama Y, Yamanashi Y. Conserved protein kinases encoded by herpesviruses and cellular protein kinase cdc2 target the same phosphorylation site in eukaryotic elongation factor 1 β . *J. Virol*. 2003; 77:2359–2368. [PubMed: 12551973]
5. Chen MR, Chang SJ, Huang H, Chen JY. A protein kinase activity associated with Epstein–Barr virus BGLF4 phosphorylates the viral early antigen EA-D *in vitro*. *J. Virol*. 2000; 74:3093–3104. [PubMed: 10708424]
6. Gershburg E, Pagano JS. Phosphorylation of the Epstein–Barr virus (EBV) DNA polymerase processivity factor EA-D by the EBV-encoded protein kinase and effects of the L-riboside benzimidazole 1263W94. *J. Virol*. 2002; 76:998–1003. [PubMed: 11773375]
7. Romaker D, Schregel V, Maurer K, Auerochs S, Marzi A, Sticht H, Marschall M. Analysis of the structure–activity relationship of four herpesviral UL97 subfamily protein kinases reveals partial but not full functional conservation. *J. Med. Chem*. 2006; 49:7044–7053. [PubMed: 17125257]
8. Johannsen E, Luftig M, Chase MR, Weicksel S, Cahir-McFarland E, Illanes D, Sarracino D, Kieff E. Proteins of purified Epstein–Barr virus. *Proc. Natl. Acad. Sci. U. S. A*. 2004; 101:16286–16291. [PubMed: 15534216]
9. Wang JT, Yang PW, Lee CP, Han CH, Tsai CH, Chen MR. Detection of Epstein–Barr virus BGLF4 protein kinase in virus replication compartments and virus particles. *J. Gen. Virol*. 2005; 86:3215–3225. [PubMed: 16298966]
10. Gershburg E, Raffa S, Torrisi MR, Pagano JS. Epstein–Barr virus-encoded protein kinase (BGLF4) is involved in production of infectious virus. *J. Virol*. 2007; 81:5407–5412. [PubMed: 17360761]
11. Feederle R, Mehl-Lautscham AM, Bannert H, Delecluse HJ. The Epstein–Barr virus protein kinase BGLF4 and the exonuclease BGLF5 have opposite effects on the regulation of viral protein production. *J. Virol*. 2009; 83:10877–10891. [PubMed: 19710145]
12. Murata T, Isomura H, Yamashita Y, Toyama S, Sato Y, Nakayama S, Kudoh A, Iwahori S, Kanda T, Tsurumi T. Efficient production of infectious viruses requires enzymatic activity of Epstein–Barr virus protein kinase. *Virology*. 2009; 389:75–81. [PubMed: 19427010]
13. Lee CP, Huang YH, Lin SF, Chang Y, Chang YH, Takada K, Chen MR. Epstein–Barr virus BGLF4 kinase induces disassembly of the nuclear lamina to facilitate virion production. *J. Virol*. 2008; 82:11913–11926. [PubMed: 18815303]
14. Meng Q, Hagemeier SR, Kuny CV, Kalejta RF, Kenney SC. SV40 T/t antigens, and lamin A/C siRNA, rescue the phenotype of an Epstein–Barr Virus (EBV) protein kinase (BGLF4) mutant. *J. Virol*. 2010; 84:4524–4533. [PubMed: 20147387]
15. Yang PW, Chang SS, Tsai CH, Chao YH, Chen MR. Effect of phosphorylation on the transactivation activity of Epstein–Barr virus BMRF1, a major target of the viral BGLF4 kinase. *J. Gen. Virol*. 2008; 89:884–895. [PubMed: 18343828]
16. Asai R, Kato A, Kato K, Kanamori-Koyama M, Sugimoto K, Sairenji T, Nishiyama Y, Kawaguchi Y. 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. *J. Virol*. 2006; 80:5125–5134. [PubMed: 16698993]
17. Asai R, Kato A, Kawaguchi Y. Epstein–Barr virus protein kinase BGLF4 interacts with viral transactivator BZLF1 and regulates its transactivation activity. *J. Gen. Virol*. 2009; 90:1575–1581. [PubMed: 19321754]
18. Kato K, Yokoyama A, Tohya Y, Akashi H, Nishiyama Y, Kawaguchi Y. Identification of protein kinases responsible for phosphorylation of Epstein–Barr virus nuclear antigen leader protein at serine-35, which regulates its coactivator function. *J. Gen. Virol*. 2003; 84:3381–3392. [PubMed: 14645919]

19. Yue W, Gershburg E, Pagano JS. Hyperphosphorylation of EBNA2 by Epstein–Barr virus protein kinase suppresses transactivation of the LMP1 promoter. *J. Virol.* 2005; 79:5880–5885. [PubMed: 15827205]
20. Zhu J, Liao G, Shan L, Zhang J, Chen MR, Hayward GS, Hayward SD, Desai P, Zhu H. Protein array identification of substrates of the Epstein–Barr virus protein kinase BGLF4. *J. Virol.* 2009; 83:5219–5231. [PubMed: 19244323]
21. Kato K, Kawaguchi Y, Tanaka M, Igarashi M, Yokoyama A, Matsuda G, Kanamori M, Nakajima K, Nishimura Y, Shimojima M, et al. Epstein–Barr virus-encoded protein kinase BGLF4 mediates hyperphosphorylation of cellular elongation factor 18 (EF-18): EF-18 is universally modified by conserved protein kinases of herpesviruses in mammalian cells. *J. Gen. Virol.* 2001; 82:1457–1463. [PubMed: 11369891]
22. Hwang S, Kim KS, Flano E, Wu TT, Tong LM, Park AN, Song MJ, Sanchez DJ, O’Connell RM, Cheng G, Sun R. Conserved herpesviral kinase promotes viral persistence by inhibiting the IRF-3-mediated type I interferon response. *Cell Host Microbe.* 2009; 5:166–178. [PubMed: 19218087]
23. Wang JT, Doong SL, Teng SC, Lee CP, Tsai CH, Chen MR. Epstein–Barr virus BGLF4 kinase suppresses the interferon regulatory factor 3 signaling pathway. *J. Virol.* 2009; 83:1856–1869. [PubMed: 19052084]
24. Iwahori S, Murata T, Kudoh A, Sato Y, Nakayama S, Isomura H, Kanda T, Tsurumi T. Phosphorylation of p27^{Kip1} by Epstein–Barr virus protein kinase induces its degradation through SCFSkp2 ubiquitin ligase actions during viral lytic replication. *J. Biol. Chem.* 2009; 284:18923–18931. [PubMed: 19451650]
25. Lee CP, Chen JY, Wang JT, Kimura K, Takemoto A, Lu CC, Chen MR. Epstein–Barr virus BGLF4 kinase induces premature chromosome condensation through activation of condensin and topoisomerase II. *J. Virol.* 2007; 81:5166–5180. [PubMed: 17360754]
26. Kudoh A, Daikoku T, Ishimi Y, Kawaguchi Y, Shirata N, Iwahori S, Isomura H, Tsurumi T. Phosphorylation of MCM4 at sites inactivating DNA helicase activity of the MCM4–MCM6–MCM7 complex during Epstein–Barr virus productive replication. *J. Virol.* 2006; 80:10064–10072. [PubMed: 17005684]
27. Lu CC, Chen YC, Wang JT, Yang PW, Chen MR. Xeroderma pigmentosum C is involved in Epstein–Barr virus DNA replication. *J. Gen. Virol.* 2007; 88:3234–3243. [PubMed: 18024891]
28. Marschall M, Stein-Gerlach M, Freitag M, Kupfer R, van den Bogaard M, Stamminger T. Direct targeting of human cytomegalovirus protein kinase pUL97 by kinase inhibitors is a novel principle for antiviral therapy. *J. Gen. Virol.* 2002; 83:1013–1023. [PubMed: 11961255]
29. Meng Q, Hagemeyer SR, Fingerroth JD, Gershburg E, Pagano JS, Kenney SC. The EBV-encoded protein kinase, EBV-PK, but not the thymidine kinase (EBV-TK), is required for ganciclovir and acyclovir inhibition of lytic viral production. *J. Virol.* 2010; 84:4534–4542. [PubMed: 20181711]
30. Kiehl A, Dorsky DI. Cooperation of EBV DNA polymerase and EA-D (BMRF1) *in vitro* and colocalization in nuclei of infected cells. *Virology.* 1991; 184:330–340. [PubMed: 1651595]
31. Sorg G, Stamminger T. Mapping of nuclear localization signals by simultaneous fusion to green fluorescent protein and to β -galactosidase. *BioTechniques.* 1999; 26:858–862. [PubMed: 10337476]
32. Nielsen AL, Ortiz JA, You J, Oulad-Abdelghani M, Khechumian R, Gansmuller A, Chambon P, Losson R. Interaction with members of the heterochromatin protein 1 (HP1) family and histone deacetylation are differentially involved in transcriptional silencing by members of the TIF1 family. *EMBO J.* 1999; 18:6385–6395. [PubMed: 10562550]
33. Marschall M, Stein-Gerlach M, Freitag M, Kupfer R, van den Bogaard M, Stamminger T. Inhibitors of human cytomegalovirus replication drastically reduce the activity of the viral protein kinase pUL97. *J. Gen. Virol.* 2001; 82:1439–1450. [PubMed: 11369889]
34. McLane LM, Corbett AH. Nuclear localization signals and human disease. *IUBMB Life.* 2009; 61:697–706. [PubMed: 19514019]
35. Gershburg E, Marschall M, Hong K, Pagano JS. Expression and localization of the Epstein–Barr virus-encoded protein kinase. *J. Virol.* 2004; 78:12140–12146. [PubMed: 15507600]
36. Nemoto T, Sato N, Iwanari H, Yamashita H, Takagi T. Domain structures and immunogenic regions of the 90-kDa heat-shock protein (HSP90): probing with a library of anti-HSP90

- monoclonal antibodies and limited proteolysis. *J. Biol. Chem.* 1997; 272:26179–26187. [PubMed: 9334185]
37. Kalderon D, Roberts BL, Richardson WD, Smith AE. A short amino acid sequence able to specify nuclear location. *Cell.* 1984; 39:499–509. [PubMed: 6096007]
38. Chee MS, Lawrence GL, Barrell BG. Alpha-, beta- and gammaherpesviruses encode a putative phosphotransferase. *J. Gen. Virol.* 1989; 70:1151–1160. [PubMed: 2543772]
39. Smith RF, Smith TF. Identification of new protein kinase-related genes in three herpesviruses, herpes simplex virus, varicella-zoster virus, and Epstein–Barr virus. *J. Virol.* 1989; 63:450–455. [PubMed: 2535748]
40. Kannan N, Neuwald AF. Did protein kinase regulatory mechanisms evolve through elaboration of a simple structural component? *J. Mol. Biol.* 2005; 351:956–972. [PubMed: 16051269]
41. Notredame C, Higgins DG, Heringa J. T-Coffee: a novel method for fast and accurate multiple sequence alignment. *J. Mol. Biol.* 2000; 302:205–217. [PubMed: 10964570]
42. Poirot O, O’Toole E, Notredame C. Tcoffee@igs: a web server for computing, evaluating and combining multiple sequence alignments. *Nucleic Acids Res.* 2003; 31:3503–3506. [PubMed: 12824354]
43. Hanks SK, Hunter T. Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J.* 1995; 9:576–596. [PubMed: 7768349]
44. Hagemeyer SR, Dickerson SJ, Meng Q, Yu X, Mertz JE, Kenney SC. Sumoylation of the Epstein–Barr virus BZLF1 protein inhibits its transcriptional activity and is regulated by the virus-encoded protein kinase. *J. Virol.* 2010; 84:4383–4394. [PubMed: 20181712]
45. Michel D, Pavic I, Zimmermann A, Haupt E, Wunderlich K, Heuschmid M, Mertens T. The UL97 gene product of human cytomegalovirus is an early-late protein with a nuclear localization but is not a nucleoside kinase. *J. Virol.* 1996; 70:6340–6346. [PubMed: 8709262]
46. Park J, Lee D, Seo T, Chung J, Choe J. Kaposi’s sarcoma-associated herpesvirus (human herpesvirus-8) open reading frame 36 protein is a serine protein kinase. *J. Gen. Virol.* 2000; 81:1067–1071. [PubMed: 10725434]
47. Thomas M, Rechter S, Milbradt J, Auerbach S, Muller R, Stamminger T, Marschall M. Cytomegaloviral protein kinase pUL97 interacts with the nuclear mRNA export factor pUL69 to modulate its intranuclear localization and activity. *J. Gen. Virol.* 2009; 90:567–578. [PubMed: 19218201]
48. Michel D, Schaarschmidt P, Wunderlich K, Heuschmid M, Simoncini L, Muhlberger D, Zimmermann A, Pavic I, Mertens T. Functional regions of the human cytomegalovirus protein pUL97 involved in nuclear localization and phosphorylation of ganciclovir and pUL97 itself. *J. Gen. Virol.* 1998; 79:2105–2112. [PubMed: 9747718]
49. Michel D, Kramer S, Hohn S, Schaarschmidt P, Wunderlich K, Mertens T. Amino acids of conserved kinase motifs of cytomegalovirus protein UL97 are essential for autophosphorylation. *J. Virol.* 1999; 73:8898–8901. [PubMed: 10482650]
50. Wang N, Baldi PF, Gaut BS. Phylogenetic analysis, genome evolution and the rate of gene gain in the Herpesviridae. *Mol. Phylogenet. Evol.* 2007; 43:1066–1075. [PubMed: 17196839]
51. Davison AJ. Evolution of the herpesviruses. *Vet. Microbiol.* 2002; 86:69–88. [PubMed: 11888691]
52. Davison A. Comments on the phylogenetics and evolution of herpesviruses and other large DNA viruses. *Virus Res.* 2002; 82:127–132. [PubMed: 11885939]

Figure 1. Amino acids 386–393 are involved in nuclear localization of the EBV-PK

The constructs EBV-PK_{FLAG}, PKΔ386–393_{FLAG} and PKΔ389–391_{FLAG} were expressed in Vero cells, fixed and immunostained with anti-FLAG antibodies (red). Nuclei were counterstained with Hoechst 33258 (blue). Images (at ×600 magnification) were recorded in separate channels and merged in Image-Pro Plus software (Media Cybernetics).

Figure 2. EBV-PK co-fractionates with chromatin

(**A**) Fractionation chart for the biochemical fractionation of EBV-PK-expressing cells (see the Experimental section for details). MNase, micrococcal nuclease; sup, supernatant. (**B**) The constructs EBV-PK_{FLAG}, PKΔ386-393_{FLAG} and PKΔ389-391_{FLAG} were expressed in HEK-293 cells and fractionated into cytosolic (C), soluble nuclear (N₁ and N₂), and chromatin (Ch) fractions. One-twentieth of each fraction was resolved by SDS/PAGE and protein expression was visualized by immunoblotting (IB) with antibodies specific to FLAG, histone H3 (chromatin) and HSP90 (cytosol).

Figure 3. Amino acids 386–393 of the EBV-PK do not translocate β-gal/GFP fusion protein to the nucleus

The DNA sequences encoding amino acids 386–393 or 389–391 of EBV-PK were cloned into pHM829 [31] ‘in-frame’ between the β-gal and GFP genes. The resulting constructs were transfected into Vero cells, and subcellular localization of the fusion protein (green) was monitored by fluorescence microscopy. Parental vector (expressing β-gal/GFP fusion protein) and pHM840 (carrying SV40 T-antigen NLS) (expressing β-gal/TAg-NLS/GFP fusion protein) served as markers for cytoplasmic and nuclear localization respectively. Nuclei were counterstained with Hoechst 33258 (blue). Images (at 600 × magnification)

were recorded in separate channels and merged in Image-Pro Plus software (Media Cybernetics).

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Figure 4. Intracellular localization of the EBV-PK/GFP fusion proteins

Full-length EBV-PK and its C- and N-termini (amino acids 375–429 and 1–77 respectively) were cloned into pEGFP vectors to yield respective GFP-fusion proteins. The resulting constructs were transfected into Vero cells and subcellular localization of the fusion protein (green) was monitored by fluorescence microscopy. Nuclei were counterstained with Hoechst 33258 (blue). Images (at $\times 600$ magnification) were recorded in separate channels and merged in Image-Pro Plus software (Media Cybernetics).

Figure 5. Protein sequence alignments of the conserved herpesviral protein kinases

Alignment of protein kinase domains of CHPKs from human herpesviruses (A) or gammaherpesviruses (B) were performed using the T-Coffee software [41,42], utilizing the program's default settings. Conserved residues are highlighted as follows: residues conserved in most protein kinases including CHPKs and candidates for subdomain III glutamate (Glu⁹¹ in PKA) are boxed with shading; residues conserved in CHPKs, but not in other protein kinases, are boxed without shading. The numbers refer to the EBV-PK sequence. The protein sequences used (with their Swiss-Prot accession numbers) are: HHV-1 UL13 (NP_044614), HHV-3 ORF47 (AAK19940), HHV-5 UL97 (Q68101), HHV-6A U69 (P24446), HHV-7 U69 (P52344), HHV-4 BGLF4 (P13288), HHV-8 ORF36 (YP_001129389), CalHV-3 ORF27 (AAK38235), CeHV-15 BGLF4 (AAK95452), AIHV-1 ORF36 (AAC58082), BoHV-4 ORF36 (AAK07955), CeHV-17 ORF36 (AAD21363), EHV-2 ORF36 (AAC13824), MHV-68 ORF36 (AAB66405) and HVS-2 ORF36 (CAA45659).

Figure 6. EBV-PK Glu¹¹³ is crucial for kinase activity

(A) The E109A, E113A, and E128A mutants of EBV-PK were co-expressed with EBV EA-D in HEK-293 cells. Whole-cell lysates were resolved by SDS/PAGE and the expression levels of EA-D and PK were visualized by immunoblotting (IB). Wild-type (lane 1) and the K102I mutant (lane 2) of the EBV-PK served as positive and negative controls for EA-D phosphorylation respectively. (B) The mutants were expressed in HEK-293 cells and immunoprecipitated with anti-FLAG antibodies from the whole-cell lysates. The autophosphorylation activity of the immunocomplexes was determined in kinase assays by incorporation of ³²P. Expression of the mutants was confirmed by immunoblotting (IB) with anti-FLAG antibodies.

Figure 7. Activity of the EBV-PK mutants carrying point mutations in residues of subdomain VIa conserved in CHPKs

(A) The F175A, L178A, A181G, F184A, L175A and N186A EBV-PK mutants were expressed in HEK-293 cells and immunoprecipitated from the whole-cell lysates with anti-FLAGM2–agarose beads. The autophosphorylation activity of the immunocomplexes was determined in kinase assays by incorporation of ³²P. Expression of the mutants was confirmed by immunoblotting (IB) with anti-FLAG antibodies. (B) The mutants were co-expressed with EBV EA-D in HEK-293 cells. Whole-cell lysates were resolved by SDS/PAGE and the EA-D and PK expression was visualized by immunoblotting (IB) with antibodies specific for each protein. Wild-type (lane 1) and K102I mutant (lane 2) of the

EBV-PK served as positive and negative controls for EA-D phosphorylation respectively. (C) The mutants were transfected into HEK-293 in triplicate and treated with GCV (concentration range 0–640 mM) the following day. At 7 days post-treatment, the cell viability was assessed by spectrophotometric measurements of conditioned media (D_{560}). The results are presented as percentage of kinase activity relative to the positive (HCMV pUL97) control. Equal expression of the mutants was confirmed by immunoblotting (IB) with anti-FLAG antibodies.