Simian Virus 40 T/t Antigens and Lamin A/C Small Interfering RNA Rescue the Phenotype of an Epstein-Barr Virus Protein Kinase (BGLF4) Mutant

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Received 20 November 2009/Accepted 4 February 2010

The Epstein-Barr virus (EBV)-encoded viral protein kinase, EBV-PK (the BGLF4 gene product), is required for efficient nuclear viral egress in 293 cells. However, since EBV-PK phosphorylates a number of different viral and cellular proteins (including lamin A/C), the relative importance of each target during lytic viral replication remains unclear. We show here that an EBV PK mutant (PKmut; containing stop codons at residues 1 and 5 in EBV-PK) is highly defective for release of infectious virus from 293 cells but not 293T cells. Furthermore, the phenotype of the PKmut in 293 cells is substantially reversed by expression of the simian virus 40 (SV40) large (T) and small (t) T antigens. Efficient rescue requires the presence of both SV40 T/t proteins. We show that 293T cells have a much higher level of constitutive lamin A/C phosphorylation than do 293 cells over residues S22 and S392 that promote phosphorylation-dependent nuclear disassembly and that both large T and small t contribute to enhanced lamin A/C phosphorylation. Finally, we demonstrate that knockdown of lamin A/C expression using small interfering RNA also rescues the PKmut phenotype in 293 cells. These results suggest that essential roles of EBV-PK during lytic viral replication include the phosphorylation and dispersion of lamin A/C.

The Epstein-Barr virus (EBV) BGLF4 gene product, EBV-PK, is homologous to the cytomegalovirus (CMV) UL97 kinase and belongs to a conserved family of herpesviral protein kinases. EBV-PK is the only protein kinase encoded by the EBV genome (6) and is expressed as an early lytic viral protein during viral reactivation (11). EBV-PK is also packaged into the virion tegument and thus is delivered in an active form to cells immediately after virus penetration (3). In the context of the intact virus, several recent studies indicate that a major and essential role of EBV-PK is to promote viral egress from the nucleus during lytic infection (10, 13, 41). Although EBV-PK phosphorylates a number of different viral and cellular proteins in vitro and when overexpressed in cells, the relative importance of the potential EBV-PK cellular and viral targets in the context of the intact viral genome has not been well studied.

The nuclear EBV-PK protein is a serine/threonine kinase that shares many targets with cyclin-dependent cellular kinases. Indeed, half of the EBV-PK targets identified by a protein array in a recent study were also found to be in vitro substrates for the cellular kinase CDK1/cyclin B (63). Overexpression of EBV-PK in cells has been shown to result in phosphorylation of a number of different cellular proteins, including the cell cycle regulatory proteins p27 (19) and pRB (C. V. Kuny and R. F. Kalejta, unpublished data); nuclear lamin A/C (32); interferon regulatory factor 3 (IRF3) (54); cellular translational elongation factor 1α (24, 26); MCM4 (part of the MCM4-MCM6-MCM7 cellular replication origin binding complex) (29); and histone protein H2AX (52). The ability of EBV-PK to phosphorylate and inactivate two key cell cycle progression inhibitors, p27 and pRB, may provide a mechanism for inducing an S-phase-like environment in terminally differentiated nondividing cells, thus promoting the nucleotide synthesis required for lytic viral replication. Phosphorylation of nuclear lamin A/C by EBV-PK over serine residues 22, 390, and 392 promotes the disassembly of nuclear lamina (32), which could contribute to viral nuclear egress. EBV-PK phosphorylation of IRF3 inhibits its function and may thus decrease the innate immune response to the virus (54). Phosphorylation of MCM4 by EBV-PK inhibits its helicase activity and may play a role in blocking cellular DNA replication during lytic viral infection (29). Overexpression of EBV-PK in cells also leads to unscheduled chromosome condensation and stress fiber rearrangements independent of cellular DNA replication and cellular kinase CDK1/cyclin B activity (31), and activates a DNA damage response (by inducing H2AX phosphorylation) that may contribute to lytic viral replication (52).

A number of different viral targets of EBV-PK have also been identified, including the viral DNA polymerase processivity factor, BMRF1 (6, 12, 55); the latent viral proteins EBNA1 (63), EBNA2 (62), and EBNA-LP (25); and the lytic switch immediate-early protein, BZLF1 (3). Phosphorylation of EBNA2 and EBNA-LP by EBV-PK has been shown to decrease their transcriptional activation function (25, 60, 62), while phosphorylation of EBNA1 inhibits its ability to support the latent form of viral replication (63). EBV-PK phosphorylation of BMRF1 is not required for its replication function but appears to decrease its transcriptional function (58). Phosphorylation of BZLF1 by the EBV-PK is reported to inhibit its ability to activate its own promoter (4). In addition, EBV-PK...
may increase the expression level of two viral proteins important for nuclear egress (BFRF1 and BFLF2), although the mechanism for this effect is not clear (10, 13).

In the present study, we have examined the phenotype of a PK-mutant virus (PKmut; containing stop codons inserted at residues 1 and 5 in the EBV-PK open reading frame), in both 293 cells and 293T cells. In agreement with previously published findings (10, 13, 41), we find that the PKmut virus is severely impaired for release of infectious viral particles in 293 cells, although its DNA replication is not affected. Somewhat surprisingly, we find that wild-type (WT) and PKmut viruses release a similar amount of infectious virus in 293T cells. Furthermore, we show that the combination of large and small simian virus 40 (SV40) T antigens in 293 cells substantially rescues the phenotype of the PKmut virus and that both proteins contribute to this rescue. We demonstrate that 293T cells but not 293 cells have a high level of constitutive lamin A/C phosphorylation and that both SV40 T antigens contribute to this effect. Importantly, knockdown of lamin A/C using small interfering RNA (siRNA) also efficiently rescues the PKmut phenotype in 293 cells.

These results indicate that EBV-PK function during lytic EBV infection is cell line dependent and that SV40 virus encoded proteins (perhaps in conjunction with the adenovirus E1A/B proteins constitutively expressed in 293 cells) can at least partially substitute for EBV-PK during lytic EBV replication. Furthermore, our results suggest that the major rescue effect of the SV40 proteins in EBV PKmut-infected 293 cells may be mediated through enhanced nuclear egress via effects on lamin A/C.

MATERIALS AND METHODS

Construction of an EBV-PK mutant. EBV B95.8 bacmid p2089 (a gift from W. Hammerschmidt) has the complete genome of B95.8 strain EBV, plus inserted pcDNA3.1 was obtained from Invitrogen. The BZLF1 expression vector, to induce lytic replication. Three days later genomic DNA was isolated and an EBV terminus assay was performed essentially as described previously (37). A total of 4 × 10⁹ Raji cells were infected in 24-well plates with serial dilutions of virus supernatants. Raji cells were treated with phorbol-12-myristate-13-acetate (TPA; 20 ng/ml) and sodium butyrate (3 mM) 1 day after infection and scored for GFP expression by using a fluorescence microscope 2 days after infection. The number of green Raji cell units (GUR) per milliliter was used to calculate the concentration of infectious particles in virus stocks.

SV40 virus rescue experiments. For the SV40 rescue experiments, 293 PKmut cells were cotransfected with pCNA3-BZLF1, pSG5-R, and pG110 expression vectors in the presence or absence of vectors expressing both the large and the small T antigens (p219 LTag), expressing the large T antigen only [pRSV-t(t), which contains the SV40 nucleotides 4002 to 5171 (the t cDNA)] and expressing the small T antigen (p27 siRNA, or lamin A/C siRNA (Santa Cruz), or lamin A/C siRNA (Santa Cruz) using X-tremeGENE siRNA transfection reagent (Roche) according to the manufacturer's protocol. The next day, cells were transfected with 0.25 μg of pSG5-Z, 0.1 μg of pSG5-R, and 0.2 μg of pRSV-BALF4, in the presence of 20 pmol of control 2 siRNA, p27 siRNA, or lamin A/C siRNA using Lipofectamine 2000 (Invitrogen). Three days later, virus titer was determined by the Green Raji cell assay as previously described (18). A total of 4 × 10⁹ Raji cells were harvested in 24-well plates with serial dilutions of virus supernatants. Raji cells were transfected with plasmids for BZLF1, BRLF1, and gp110 in the presence or absence of a FLAG-tagged EBV-PK expression vector to induce lytic replication, and the supernatant from cells was used to infect Raji cells. The 293 or 293T cell clones that produced the highest virus titer were frozen at early passage and used for further study.

Virus production assays. 293 or 293T WT and PKmut cells were transfected in six-well plates with BZLF1, BRLF1, and pG110 expression vectors to induce lytic replication. Virus supernatants were harvested, centrifuged, and filtered through a 0.8-μm-pore-size filter 3 days after transfection. Virus titer was determined by the Green Raji cell assay as previously described (18). A total of 4 × 10⁹ Raji cells were transfected in 24-well plates with serial dilutions of virus supernatants. Raji cells were treated with phorbol-12-myristate-13-acetate (TPA; 20 ng/ml) and sodium butyrate (3 mM) 1 day after infection and scored for GFP expression by using a fluorescence microscope 2 days after infection. The number of green Raji cell units (GUR) per milliliter was used to calculate the concentration of infectious particles in virus stocks.

pcDNA-BZLF1 expression vector contains BZLF1 cDNA sequences inserted into the pcDNA3 vector as previously described (39). pSG5-R contains genomic BRLF1 sequences downstream of the SV40 promoter in the pSG5 vector (a gift from S. Diane Hayward). The pRSV-BalF4 plasmid expresses the gpl10 glycoprotein and was a gift from H. J. Deleu (42). The C-terminal FLAG-tagged WT EBV-PK expression vector was described previously (11, 34) and was a gift from M. Marshall. An EBV-PK mutant (K102I) that changes lysine residue 102 into an isoleucine in the EBV-PK expression vector (M. Marshall) was a gift from Edward Gershberg. As previously described (55), this mutation inactivates the catalytic function of EBV-PK. The following SV40 vectors were used: pRSV-Bncod11440(T), which can produce large T antigen but not small T antigen (53); pRSV-t(t), which contains the SV40 nucleotides 4002 to 5171 (the t cDNA) and expresses small t antigen but not large T antigen (5); an SV40 WT p129/LTag expression vector that can express both SV40 large and small T antigens (pVUCR2, which expresses a mutant large T antigen that does not bind pRB, as well as a wild-type form of small t antigen (a gift from Charles Cole) (21). Plasmid Xhol1.9-Kb, containing unique DNA at the right terminus of EBV in the SP6 vector (45), was a gift from Nancy Raab-Traub and was used in the EBV terminus assay. pCQN-based expression plasmids for N-terminal hemagglutinin (HA)-tagged EBV-PK, CMV UL97, and HSV ORF36 sequences are described elsewhere (Kuny et al., unpublished).

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nus) with a random primer labeling system (GE Healthcare). A BamH1/BglII fragment derived from the pSG5-Z plasmid (which contains BZLF1 sequences) was also gel purified and labeled with random primer labeling system. After hybridization, membranes were washed with wash buffer (1% SDS, 20 mM Na2HPO4 [pH 7.2], 1 mM EDTA) three times (10 min for each wash), the membrane was exposed to film at −80°C overnight, and films were developed.

**Immunoblot analysis.** 293 EBV WT or 293 PKmut cells were transfected with a BZLF1 expression vector, in the presence or absence of a cotransfected EBV-PK expression vector, to induce lytic replication. Cells were harvested 48 h posttransfection, washed once with cold 1× phosphate-buffered saline, and resuspended in a 1:3 mixture of SUMO buffer I (5% SDS, 0.15 M Tris-HCl [pH 6.8], 30% glycerol) and SUMO buffer II (25 mM Tris-HCl [pH 8.3], 50 mM NaCl, 0.5% NP-40, 0.5% deoxocholate, 0.1% SDS) and 1× complete protease inhibitors (Roche). The cells were briefly sonicated and centrifuged, and protein concentration of the supernatant was determined with the SUMO protein assay reagent (Bio-Rad). Equal amounts of protein were separated on a 10% SDS-polyacrylamide gel electrophoresis (PAGE) denaturing gel. The proteins were transferred onto a nitrocellulose membrane (Protran), blocked in 1× phosphate-buffered saline–5% milk–0.1% Tween 20 (PBST), and incubated with anti-Z (mouse; 1:250 [Argene]), anti-R (mouse; 1:250 [Argene]), anti-EBV diffuse early antigen (BMRF1; mouse; 1:250 [Vector Laboratories]), anti-EBV TK (rabbit; 1:2,000, a gift from Robert Gonnella), anti-BLF2 (mouse; 1:10; a gift from Robert Gonnella), anti-SM (rabbit; 1:1,000; a gift from Sankar Swaminathan), anti-BcLF1 (rabbit; 1:1,000; a gift from Lindsay Hurt-Fletcher), anti-p27 [Kip1] (mouse; 1:2,000 [BD Transduction Laboratories]), anti-BLF4 (C-ter; rabbit; 1:1,000 [ABGENT]), anti-EBV-induced latum A+C (rabbit polyclonal, phospho-lamin A/C 392–397; 1:2,000 [Abcam]), and (rabbit polyclonal phospho-lamin A/C S22; 1:1,000 [Cell Signaling], monoclonal anti-α-tubulin clone B5–1–2 (mouse; 1:2,000 [Sigma]), anti-SV40 Tag (mouse; 1:1,000 [Santa Cruz], anti-EBV VCA-gp125 (mouse; 1:1,000 [Chemicon), anti-HA-probe (rabbit; 1:500 [Santa Cruz]) and anti-β-actin (mouse; 1:5,000 [Sigma]) for 1 h. After primary antibody incubation, membranes were washed in PBST three times (5 min for each wash) and incubated in the horseradish peroxidase secondary antibody (Thermo Scientific) at a dilution of 1:10,000 (anti-rabbit) or 1:5,000 (anti-mouse) dilution. Membranes were then washed with PBST for three times (15 min for each wash) and visualized by ECL treatment (Pierce) and exposure to film.

**Lamin A/C fractionation studies.** 293 PKmut cells were transfected with 0.5 μg of BZLF1, in the presence or absence of EBV-PK, small t, or large T antigen expression vectors. After transfection, cells were changed to 1% fetal bovine serum medium. Cellular fractionation studies were performed 18 h after transfection as previously described (46) with some modifications. Briefly, cells were extracted in cytoskeleton buffer (CSK; 10 mM PIPES [pH 6.8], 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, and 1 mM EGTA, supplemented with 1 mM dithiothreitol, protease inhibitor cocktail, and 0.5% [vol/vol] Triton X-100). The suspension was incubated for 5 min on ice and centrifuged at 7,500 rpm for 5 min. The supernatant was kept as the “soluble” fraction. The pellet was washed with CSK buffer and resuspended with 1 mg of RNase free DNase I/ml in CSK buffer for 2 h at 37°C. Ammonium sulfate was added to a final concentration of 0.25 M. After 5 min at 4°C, samples were centrifuged again. The supernatant was kept as the “chromatin” fraction. The pellet was washed with 80 μl of 2 M NaCl in CSK buffer and then centrifuged. The remaining pellet was resuspended in 80 μl of 8 M urea in 10 mM Tris (pH 8) and was kept as the “nuclear matrix” fraction.

**RESULTS**

**Establishment of 293 or 293T cell clones stably infected with WT and PKmut EBV.** To create an EBV-PK mutant virus, stop codons were inserted in residues 1 and 5 in the EBV-PK open reading frame in the EBV bacterial artificial chromosome by site-directed mutagenesis as described previously and validated by sequencing and restriction enzyme analysis (51, 61). EBV WT or PKmut bacmids were then transfected into 293 and 293T cells, and stable GFP+ clones were obtained by using hygromycin selection. WT and PKmut cell clones (at least 30 clones for each construct in each cell type) were induced into the lytic form of viral replication by BZLF1/BRLF1/gp110 transfection (plus a cotransfected EBV-PK expression vector in the PKmut clones) and virus titers determined as described in Materials and Methods. Clones that produced high titers of virus were frozen at early passage and used for all further studies. Three different clones from each virus in both 293 and 293T cells were expanded and analyzed. Similar results were obtained with each mutant clone in a particular cell type.

To confirm that the PKmut virus does not express EBV-PK, WT or PKmut cells were transfected with a BZLF1 (Z) expression vector to induce lytic infection in the presence or absence of a cotransfected EBV-PK expression vector, and immunoblots performed to examine expression of EBV-PK, BZLF1, and β-actin. (B) 293 cells latently infected with WT or PKmut were transfected with a BZLF1 (Z) expression vector to induce lytic infection, in the presence or absence of a cotransfected EBV-PK expression vector, and immunoblotting was performed to examine the expression of BMRF1, BZLF1, and β-actin. The hypo- and hyperphosphorylated forms of BMRF1 are indicated.

**FIG. 1.** Creation of stable 293 and 293T cell lines latently infected with WT or PKmut viruses. (A) 293 cells latently infected with WT or PKmut EBV (PKmut) were transfected with a BZLF1 (Z) expression vector to induce lytic infection, in the presence or absence of a cotransfected EBV-PK expression vector, and immunoblots performed to examine expression of EBV-PK, BZLF1, and β-actin. (B) 293 cells latently infected with WT or PKmut were transfected with a BZLF1 (Z) expression vector to induce lytic infection, in the presence or absence of a cotransfected EBV-PK expression vector, and immunoblotting was performed to examine the expression of BMRF1, BZLF1, and β-actin. The hypo- and hyperphosphorylated forms of BMRF1 are indicated.
thymidine kinase (BXLF1), BFRF1, BFLF2, SM, and BcLF1) were found in cells infected with WT and PKmut viruses in both 293 cells and 293T cells (Fig. 2 and data not shown). In contrast to the results of a study that used siRNA to decrease EBV-PK expression (13), we did not find that loss of EBV-PK affects the expression level of the essential viral egress proteins, BFRF1 and BFLF2.

To determine whether loss of EBV-PK expression affects the amount of viral DNA replication in either 293 cells or 293T cells, we performed the “terminus assay.” In this Southern blot based assay with an EBV terminus probe, the fused form of the EBV termini is derived from both the latent episome and lytically replicated concatemer DNA, while the cleaved forms are derived exclusively from lytically replicated (linear) DNA. After BZLF1 transfection, the 293 cells infected with PKmut virus supported at least as much lytic viral replication as the cells infected with the WT virus (Fig. 3); similar results were obtained in the 293T cell clones (data not shown). These results indicate that the PKmut is not defective for viral DNA replication in 293 cells or 293T cells.

The PKmut virus is highly impaired for release of infectious viral particles in 293 cells, and this defect was not rescued by the HCMV or KSHV kinases. We next compared the amount of infectious virus released after lytic induction of 293 cells infected with the WT versus PKmut viruses using the green Raji cell assay to titer virus production as previously described (18). Similar to the results of two previous studies examining EBV-PK function in 293 cells (one which used siRNA to knock down EBV-PK expression [13] and one that examined the phenotypes of a PK mutant virus [41]), we found that 293 cells infected with the PKmut virus released only 1% as much infectious virus as the WT-virus-infected cells (Fig. 4). This defect in virus production was rescued when the PKmut cells were cotransfected with a WT EBV-PK expression vector but not with a kinase-dead EBV-PK expression vector (Fig. 4), indicating that the loss of virus production in PKmut-infected 293 cells is specifically due to the loss of EBV-PK activity. These results confirm that EBV-PK is required for efficient release of infectious viral particles in 293 cells.

We sought to determine whether the homologous protein kinases encoded by two other herpesviruses, human cytomegalovirus (HCMV) and Kaposi’s sarcoma herpesvirus (KSHV), can rescue the replication defect of PKmut in 293 cells. For this
study, we compared the viral rescue using three different viral kinase expression vectors in which an HA tag was fused to the amino terminus of the EBV, CMV (UL97), and KSHV (ORF36) kinases, respectively. Although the EBV-PK vector as expected rescued viral replication in 293 PKmut cells, neither the HCMV nor KSHV kinase vectors substantially rescued viral replication (Fig. 5A), although each kinase was expressed in the 293 PKmut cells (Fig. 5B). These results suggest that one or more essential EBV-PK targets are not shared by the other viral kinases or that one or more HCMV/KSHV PK substrates (not shared by the EBV-PK) act to inhibit EBV replication.

The PKmut virus is not impaired for viral release in 293T cells. We also compared the amount of infectious virus released by lytically induced 293T cells infected with the WT or PKmut virus. Surprisingly, the WT and PKmut viruses released a similar amount of infectious virus in 293T cells (Fig. 6A), which are derived from 293 cells and express the SV40 large T and small t antigens (30, 57). A similar result was observed in several different independently derived 293T PKmut and WT clones (data not shown).

To confirm that 293T cells infected with PKmut are actually missing EBV-PK function, we examined the level of BMRF1 hyperphosphorylation and endogenous EBV-PK expression. In contrast to the 293T EBV-WT cells, the 293T PKmut cells did not have the hyperphosphorylated form of BMRF1 or express endogenous EBV-PK, after BZLF1 transfection (Fig. 6B). These results indicate that the replication defect of the PKmut virus is cell line dependent and suggest the possibility that one or more SV40-encoded proteins can complement the functions of EBV-PK in 293 cells.

SV40 T/t antigen expression partially rescues the defect of the PKmut in 293 cells. If the SV40 small t and/or large T antigens contribute to the ability of PKmut to produce infectious virus in 293T cells, then they should be able to (at least partially) complement the defect of PKmut in 293 cells. To examine this, 293 PKmut cells were induced into lytic infection in the presence or absence of vectors that express only the SV40 large T or small t antigen. Although the expression level of the cotransfected EBV BALF4 (gp110), BZLF1 and BRLF1 proteins was similarly modestly increased in cells transfected with either large T antigen alone or both the large and small (T/t) antigens together (presumably reflecting the presence of the SV40 origin in the EBV vectors) (Fig. 7B), only the combination of both large and small T/t antigens could efficiently rescue EBV virus production (Fig. 7A). Expression of SV40 small t antigen alone was not sufficient to rescue the PKmut defect in 293 cells (Fig. 7A). These results suggest that both the SV40 large T and small t antigens...
contribute to the ability of PKmut to produce virus in 293T cells.

Since the SV40 large T antigen binds to and inactivates pRB (8, 16), and EBV-PK phosphorylates and inactivates RB (Kuny and Kalejta, unpublished), we also sought to determine whether the ability of large T antigen to inactivate pRB function contributes to T/t rescue of the PKmut phenotype. As shown in Fig. 7C and D, removal of the T antigen pRB binding domain (amino acids 107 to 112) considerably reduced the ability of the T/t combination to rescue PKmut virus production, although the expression level of the WT and mutant T antigens was similar. These results indicate that the ability of large T antigen to inactivate pRB function is partially responsible for the rescue effect of the T/t combination.

Knockdown of p27 also increases production of PKmut virus in 293 cells. Since EBV-PK was recently shown to phosphorylate p27 and lead to its degradation (19) and the SV40 small t antigen has likewise been reported to decrease p27 function (44), we examined the effect of p27 knockdown on viral production of the WT versus PKmut viruses in 293 cells (Fig. 8). Although treatment of 293 WT virus-infected cells with p27 siRNA only slightly increased virus production, it led to a 15-fold increase in virus production (versus control siRNA) in 293 cells infected with the PKmut virus (Fig. 8A). Treatment with p27 siRNA decreased p27 protein in both 293 WT and 293 PKmut cells (Fig. 8B). As previously reported, we found that overexpression of EBV-PK decreased the total p27 level in 293 PKmut cells (Fig. 8C). These results suggest that the ability of EBV-PK to inhibit p27 function in lytically infected cells also contributes to viral production.

Decreasing lamin A/C expression also enhances production of the PKmut virus in 293 cells. Phosphorylation of lamin A/C at residues S22 and S392 by the cellular CDK1/cyclin B kinase is required for the normal nuclear disassembly during mitosis (17, 43, 56). EBV-PK phosphorylates lamin A/C in vitro over the same residues, and overexpression of the EBV-PK in HeLa...
cells results in displacement of a GFP-lamin A/C protein from the nuclear envelope (32). However, whether EBV-PK phosphorylation of lamin A/C is required for viral nuclear egress in the context of the intact virus is unknown. To further examine this, we used siRNA to decrease lamin A/C expression in 293 PKmut cells or 293 WT cells and examined the effect on viral production. As shown in Fig. 9A, lamin A/C siRNA only slightly increased WT virus production, but increased PKmut production by 40-fold. Lamin A/C siRNA decreased lamin A/C expression in both WT and PKmut cells to a similar degree (Fig. 9B). These results suggest that the ability of EBV-PK to phosphorylate lamin A/C, and thus dislocate it from the nuclear envelope, plays a key role in promoting viral release.

**SV40 small and large T antigens induce lamin A/C phosphorylation.** To further investigate the mechanism(s) by which the SV40 T/t antigens rescue the PKmut phenotype in 293 cells, we compared the level of lamin A/C phosphorylation in 293 cells versus 293T cells using phospho-specific antibodies. As shown in Fig. 10A, lamin A/C was found to be constitutively phosphorylated at both the S22 and S392 residues in 293T cells but not in 293 cells. The total amount of lamin A/C was similar in both cell lines. These results suggest that the ability of EBV-PK to phosphorylate lamin A/C is not required for viral release in PKmut-infected 293T cells because lamin A/C is already efficiently phosphorylated by cellular kinases. Although neither the large nor small SV40 T/t antigens have been reported to enhance the amount of phosphorylated lamin A/C in cells, small t antigen inhibits the activity of protein phosphatase 2A (2, 7) and thus could potentially increase lamin A/C phosphorylation by this mechanism. Large T antigen directly interacts with the lamin A/C proteins (27), although the functional effect of this interaction is unknown. To determine whether either large and/or small T antigens can induce lamin A/C phosphorylation, and/or enhance lamin A/C solubilization, in 293 cells, we transfected 293 PKmut cells with a BZLF1 vector in the presence or absence of an EBV-PK expression vector or vectors expressing the large or small SV40 T antigen. Cellular lysates were fractionated as described (46) to separate the nuclear matrix, chromatin, and soluble fractions, and the level of total and phosphorylated lamin A/C was examined in the soluble versus nuclear matrix fractions. 293 PKmut cells cotransfected with the BZLF1 and EBV-PK vectors clearly had an increased amount of phosphorylated lamin A/C in the soluble fraction compared to cells transfected with BZLF1 alone (Fig. 10B), a finding consistent with the previously described ability of EBV-PK to directly phosphorylate lamin A/C over residues that led to its disassociation from the nuclear envelope. Overexpression of the SV40 small t antigen and, to a lesser extent, the large T antigen also increased the amount of phosphorylated lamin A/C within the soluble fraction of the extracts. Equal amounts of tubulin were found in each soluble extract, confirming that separation of the soluble component in each condition was successful. The level of total lamin A/C in the nuclear matrix fraction was similar in all conditions (Fig. 10B). These results suggest that both the small and large forms of the SV40 T antigens contribute to the enhanced lamin A/C phosphorylation observed in 293T cells.

**DISCUSSION**

The EBV-encoded protein kinase, EBV-PK, phosphorylates cellular substrates that are common to a number of different herpesvirus protein kinases, including EF-16 (24), lamin A/C (32), and pRB (Kuny and Kalejta, unpublished). The pheno-
types of EBV mutants missing the viral PK gene (10, 41) are similar to that of HCMV UL97 mutants, since in both cases the mutant viruses are defective for nuclear egress. Nevertheless, it remains unclear which of the growing number of reported cellular and viral EBV-PK targets are actually important for promoting lytic replication in the context of the virus and whether the defects of EBV-PK mutants are cell type dependent. In the present study, we show that a mutant EBV virus containing inserted stop codons in the EBV-PK open reading frame is highly impaired for the release of infectious virus in 293 cells but is surprisingly not impaired in 293T cells. Our results suggest that both the large and small SV40 T antigens contribute to the rescue of the EBV PK mutant virus in 293T cells and that a critical factor in this rescue is the ability of SV40 proteins to enhance phosphorylation of lamin A/C over residues involved in the regulation of nuclear disassembly. Furthermore, we show that knockdown of lamin A/C expression by siRNA in 293 cells also rescues the PKmut phenotype.

A surprising and interesting aspect of our results was the unexpected discovery that the EBV-PK mutant is not impaired for viral production in 293T cells. The importance of SV40 large and small T antigens in this rescue was confirmed by our finding that coexpression of the small and large T antigens in 293 cells increased the titer of the EBV-PK mutant virus by 45-fold. Furthermore, we showed that both the small and large forms of the SV40 T antigens contribute to rescue of the EBV-PK mutant virus in 293 cells. Thus, rescue the PKmut phenotype in 293 cells by SV40 encoded proteins likely involves several different mechanisms.

A recent report showed that the phenotype of a HCMV UL97 mutant can be partially rescued by expression of the human papillomavirus E7 protein and that this rescue is largely due to the ability of E7 to inactivate pRB function (23). However, the major effect of the E7 protein in regard to the rescue of the UL97-null HCMV virus occurred at the level of viral DNA replication, and this rescue was much more impressive in nondividing versus dividing cells (23). In contrast, the major effect of the SV40 T/t proteins in rescuing the phenotype of the EBV-PK mutant in 293 cells likely occurs at the level of viral nuclear egress, since the viral DNA replication of the mutant EBV virus is not impaired in this cell type. The lack of a viral replication defect of the EBV-PK mutant in 293 cells may be due to their constitutive expression of adenoviral proteins (E1A/E1B) that inhibit pRB function. Nevertheless, our finding that a large SV40 T antigen mutant that is impaired for inhibition of pRB function was also impaired for rescue of the PKmut virus in 293 cells suggests that pRB may not be totally inactivated by adenovirus proteins in 293 cells. Alternatively, the same region of SV40 large T antigen may also be required for some other function that contributes to PKmut rescue. In addition, we also found that knockdown of the cyclin-dependent kinase (CDK) inhibitor p27 (using siRNA) enhances the replication of the PKmut virus much more dramatically than it affects replication of the WT virus. This finding, combined with the recent report showing that EBV-PK phosphorylates and degrades p27 (19), suggests that the previously described ability of the SV40 small T antigen to inhibit p27 function (44) may be important for its ability to rescue the EBV PK mutant in conjunction with the large SV40 T antigen.

Another unexpected finding was our discovery that knock-
Furthermore, cells missing lamin A/C are phenotypically similar to cells missing pRB (20). Hence, the efficient rescue of the PKmut phenotype by lamin A/C siRNA in 293 cells likely reflects not only the disruption of the normal nuclear membrane structure, but the inhibition of pRB/p107 function (20).

Although EBV-PK was reported to partially complement the replication defect of an HCMV mutant with UL97 deleted (47), we show here that the reverse is not true, since our EBV-PK mutant was not effectively rescued by either the HCMV or KSHV kinases in 293 cells. This result suggests that EBV-PK recognizes one or more specific and essential substrates not recognized by the HCMV or KSHV kinases. It is clear that whereas some of the targets of the EBV, HCMV, and KSHV kinases are shared in common, other targets are unique. For example, we found that while the EBV kinase induces hyperphosphorylation of the EBV BMRF1 protein, neither the HCMV nor the KSHV kinases can induce this hyperphosphorylation (data not shown). In addition, EBV-PK induces nuclear disassembly more efficiently than either the UL97 or the ORF36 kinases (32), and EBV-PK phosphorylates more sites on lamin A/C than does UL97 (32). Alternately, EBV-PK may have a role in EBV replication that is independent of its kinase function and not shared by the other viral kinases. It is also possible that certain phosphorylated cellular targets of the HCMV and KSHV kinases are actually detrimental to EBV lytic replication.

Finally, the discovery of a cell line that efficiently supports the lytic form of EBV-PK mutant virus replication has provided us with a mechanism to ask what role, if any, EBV-PK plays in conferring antiviral susceptibility of EBV to the nucleoside analogues, acyclovir and ganciclovir. Although it is known that the HCMV UL97 kinase is required for HCMV replication, little is known about the role of EBV-PK mutant on viral susceptibility to ganciclovir and that the UL97 kinase can disrupt ganciclovir activity associated with the nuclear lamina (32). Alter- 

ACKNOWLEDGMENTS

We thank our collaborators Xiaoming Yu and Janet Mertz at the University of Wisconsin-Madison for SV40 expression vectors and help in constructing the EBV-PK mutant. We also thank Henri-Jacques Delecluse and Jim Pipas for providing valuable reagents.

This study was supported by grants R01-CA58853, R01-CA66519, R01-H0064851, P01-CA022443, and P01-CA019014 to S.C.K.; training grant T32-AI078985 to C.V.K. and S.R.H.; and R01-AI074984 to R.F.K. R.F.K. is a Burroughs Wellcome Fund Investigator in the Pathogenesis of Infectious Diseases.

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