Phosphorylation of p27Kip1 by Epstein-Barr Virus Protein Kinase Induces Its Degradation through SCF-Skp2 Ubiquitin Ligase Actions during Viral Lytic Replication*

Received for publication, March 10, 2009, and in revised form, April 30, 2009 Published, JBC Papers in Press, May 18, 2009, DOI 10.1074/jbc.M109.015123

Satoko Iwahori, Takayuki Murata, Ayumi Kudoh, Yoshitaka Sato, Sanae Nakayama, Hiroki Isomura, Teru Kanda, and Tatsuya Tsurumi

From the Division of Virology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan

Epstein-Barr virus (EBV) productive replication occurs in an S-phase-like cellular environment with high cyclin-dependent kinase (CDK) activity. The EBV protein kinase (PK), encoded by the viral BGLF4 gene, is a Ser/Thr kinase, which phosphorylates both viral and cellular proteins, modifying the cellular environment for efficient viral productive replication. Here, we provide evidence that the EBV PK phosphorylates the CDK inhibitor p27Kip1, resulting in ubiquitination and degradation in a proteasome-dependent manner during EBV productive replication. Experiments with BGLF4 knockdown by small interfering RNA and BGLF4 knock-out viruses clarified that EBV PK is involved in p27Kip1 degradation upon lytic replication. Transfection of the BGLF4 expression vector revealed that EBV PK alone could phosphorylate the Thr-187 residue of p27Kip1 and that the ubiquitination and degradation of p27Kip1 occurred in an SCF-Skp2 ubiquitin ligase-dependent manner. In vitro, EBV PK proved capable of phosphorylating p27Kip1 at Thr-187. Unlike cyclin E-CDK2 activity, the EBV PK activity was not inhibited by p27Kip1. Overall, EBV PK enhances p27Kip1 degradation effectively upon EBV productive replication, contributing to establishment of an S-phase-like cellular environment with high CDK activity.

The cyclin-dependent kinase (CDK)3 inhibitor p27Kip1 (kinase-inhibitory protein-1) belongs to the Cip/Kip family of proteins and plays a pivotal role in the regulation of cell cycle progression and cyclin-CDK complex activity (1, 2). It specifically inhibits the activity of CDK2 by binding to cyclin E-CDK2 and cyclin A-CDK2 complexes. Levels of p27Kip1 protein oscillate during the cell cycle, and one of the key mechanisms involved in their regulation is ubiquitin-dependent proteolysis (3). In S-phase, p27Kip1 is phosphorylated at Thr-187 by cyclin E-CDK2 and is then recognized and targeted for ubiquitination by SCF-Skp2 (S-phase kinase-associated protein 2), an F-box protein that functions as the receptor component of an SCF (Skp1/Cullin/F-box protein)-type ubiquitin ligase complex (4–8). Although p27Kip1-bound CDK2 is catalytically inactive in vitro, phosphorylation of p27Kip1 at Tyr-74 and Tyr-88 by Ab1 and Src family kinases can impair the inhibitory action of p27Kip1 for cyclin E-CDK2, p27Kip1 consequently becoming a substrate for cyclin E-CDK2 and degraded in the nucleus (9–11). Thus, phosphorylation of p27Kip1 at Tyr-74 and Tyr-88 residues is a prerequisite for phosphorylation of Thr-187 by cyclin E-CDK2. Alternatively, there exists another p27Kip1 degradation system in G1 phase, featuring phosphorylation at Ser-10 by kinase-interacting stathmin or the minibrain-related kinase in nuclei (12, 13), followed by nuclear export in a CRM1 (or exportin 1)-dependent manner (14, 15). Ubiquitin ligase KPC (Kip1 ubiquitylation-promoting complex) recognizes p27Kip1 in the cytoplasm, followed by proteasome-mediated degradation (16).

The Epstein-Barr virus (EBV) is a human lymphotropic herpesvirus with a linear double-stranded DNA, 172 kb in length (17). Primary EBV infection targets resting B lymphocytes, inducing their continuous proliferation. In resultant B lymphoblastoid cell lines, a limited number of viral proteins are usually expressed, and there is no production of virus particles, this being termed latent infection. Reactivation from latency can occur spontaneously or be induced artificially. A virus-productive lytic stage is initiated by the expression of the viral immediate early BZLF1 protein, a b-Zip transcriptional factor that binds to AP-1-like sequences present in the promoters of early lytic genes, followed by an ordered cascade of viral gene expression, viral DNA replication, and virion production (18). During lytic replication, the levels of cyclin E and cyclin A continue to be elevated, and cyclin E- and cyclin A-associated CDK activities increase, thereby causing accumulation of hyperphosphorylated forms of retinoblastoma (Rb) protein and an increase of the level of E2F-1 protein (19). Chemical CDK inhibitors, like purvalanol A and roscovitine, block viral lytic replication through prevention of viral immediate early and early gene expression (20). Thus, a cellular environment with high CDK activity is required for efficient viral replication.

The EBV protein kinase (PK) encoded by the BGLF4 gene is a Ser/Thr protein kinase conserved throughout all subfamilies of Herpesviridae, targeting proline-directed Ser/Thr (21–23), expressed from an early stage of viral lytic replication and localized in viral replication compartments. EBV PK phosphorylates not only viral proteins, such as EBV BZLF1, BMRF1 (EA-D), EBNA-LP, EBNA2, and protein kinase itself, but also several
Degradation of p27^Kip1 Enhanced by EBV PK BGLF4

cellular proteins, including EF-1α, lamin A/C, MCM4, and MCM6 (22, 24–29). The phosphorylation sites targeted by the BGLF4 protein include those with CDK1 (Cdk2) and CDK2. Especially, phosphorylation of Thr-19 and Thr-110 residues on MCM4 results in loss of helicase activity of MCM4–6–7 complexes (26), providing an example of EBV PK-mediated protein phosphorylation causing a dramatic functional change. Knockdown of the EBV PK protein by small interfering RNA reduces the yield of infectious virus particles (30).

In this paper, we document evidence that, during EBV productive replication, EBV PK phosphorylates p27^Kip1 so that it is ubiquitinated by S6Kiep2 ubiquitin ligase and degraded in a proteasome-dependent manner. Unlike the cyclin E–CDK2 activity, the EBV PK activity is not inhibited by p27^Kip1. Overall, EBV possesses its own strategy to degrade p27^Kip1 upon onset of productive replication, contributing to provision of an S-phase-like cellular environment with high CDK activity.

EXPERIMENTAL PROCEDURES

Cells—HeLa and HEK293T cells were grown and maintained at 37 °C in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal calf serum. Tet-BZLF1/B95-8 cells, a marmoset B-cell line latently infected with EBV (31), was maintained in RPMI medium supplemented with 1 µg/ml puromycin, 250 µg/ml hygromycin B, and a tetracycline-free fetal calf serum. To induce lytic EBV replication, a tetracycline derivative, doxycycline, was added to the culture medium at a final concentration of 3 µg/ml. An EBV producer cell line, Akata (+) (32), was grown in RPMI medium supplemented with 10% fetal calf serum and mixed with polyclonal rabbit anti-human IgG (Dako) at a final concentration of 28.5 µg/ml in the culture medium to induce lytic replication. HEK293 cells latently infected with wild-type EBV-bacmid (293/EBV-WT), BGLF4-deficient EBV-bacmid (293/EBV-dbGLF4/NeoSt), and a revertant of BGLF4-deficient EBV-bacmid (293/EBV-dbGLF4/NeoSt/R) were established (33). 293/EBV-dbGLF4/NeoSt cells contain a bacmid genome in which the region between nucleotides 256 and 320 of the BGLF4 gene was replaced with the tandemly arranged neomycin resistance and streptomycin sensitivity (NeoSt') genes. 293/EBV-dbGLF4/NeoSt/R cells contain a bacmid genome in which the NeoSt' cassette of dbGLF4/NeoSt was replaced with a wild-type BGLF4 sequence.

Plasmids—pcDNA-FLAG/p27^Kip1, pcDNA-FLAG/p27^Kip1, S10A, pcDNA-FLAG/p27^Kip1-S178A, and pcDNA-FLAG/p27^Kip1-T187A were kind gifts from Dr. N. Ishida (34). pcDNA-HA-UB was prepared as described previously (35). pcDNA-BZLF1 was a gift from Dr. K. Kuzushima and Dr. R. Ohta. To prepare the expression vector for BGLF4, pcDNA-BZLF1 was a gift from Dr. K. Kuzushima and Dr. R. Ohta. To prepare the expression vector for BGLF4, pcDNA-FLAG/p27^Kip1−S10A, pcDNA-FLAG/p27^Kip1−S178A, and pcDNA-FLAG/p27^Kip1−T187A were kind gifts from Dr. N. Ishida (34). pcDNA-HA-UB was prepared as described previously (35). pcDNA-BZLF1 was a gift from Dr. K. Kuzushima and Dr. R. Ohta. To prepare the expression vector for BGLF4, pcDNA-BZLF1 was a gift from Dr. K. Kuzushima and Dr. R. Ohta. To prepare the expression vector for BGLF4, pcDNA-HA-UB was prepared as described previously (35). pcDNA-BZLF1 was a gift from Dr. K. Kuzushima and Dr. R. Ohta. To prepare the expression vector for BGLF4, pcDNA-BZLF1 was a gift from Dr. K. Kuzushima and Dr. R. Ohta. To prepare the expression vector for BGLF4, pcDNA-BZLF1 was a gift from Dr. K. Kuzushima and Dr. R. Ohta. To prepare the expression vector for BGLF4, pcDNA-BZLF1 was a gift from Dr. K. Kuzushima and Dr. R. Ohta. To prepare the expression vector for BGLF4, pcDNA-FLAG/p27^Kip1 were cotransfected into HEK293T cells (1.2 × 10^6) with 200 pmol of Skp2 siRNA or non-targeting/control siRNA were purchased from Santa Cruz Biotechnology. BGLF4-targeted siRNA, si-BGLF4 (5'-CCUCUAGUGAAGCGUCCGGAGAA-3'), and a control siRNA (5'-CCCGGUAUUUGCCGCCACUGAA-3') were purchased from Invitrogen, as described previously (22).

Immunoblot Analysis—Cells were suspended in lysis buffer (20 mM Tris–HCl (pH 7.4), 0.5% Triton X-100, 300 mM NaCl, 1 mM EDTA, 0.1% SDS, 100 mM NaF, 2 mM Na3VO4, protease inhibitor mixture (Sigma)) and incubated on ice for 40 min followed by centrifugation. Equal amounts of proteins were separated by 15% (acrylamide (A)/bisacrylamide (B) 72:1) SDS-PAGE and transferred onto Immobilon transfer membranes (Millipore). Immunoreactivity was detected by Western blot analysis. For lytic induction of 293/EBV-WT, 293/EBV-dbGLF4, and HEK293T cells (1.2 × 10^6) were transfected with 2 µg of pcDNA-FLAG/BGLF4 or FLAG/kd BGLF4 using a microinjection system (Digital Bio). For detection of phosphorylation and degradation of p27^Kip1 in EBV-latently infected cells, 293/EBV-WT were transfected with pcDNA-FLAG/p27^Kip1, pcDNA-FLAG/p27^Kip1−S10A, pcDNA-FLAG/p27^Kip1−S178A, and pcDNA-FLAG/p27^Kip1−T187A were transfected into HEK293T cells (1.2 × 10^6) with 4 µg of pcDNA-FLAG/BGLF4 or FLAG/kd BGLF4 using Lipofectamine 2000 (Invitrogen). At 27 h post-transfection (hpt), the transfected cells were harvested and subjected to immunoblot analysis. For lytic induction of 293/EBV-WT, 293/EBV-dbGLF4/NeoSt, or 293/EBV-dbGLF4/NeoSt/R cells, the cells (1 × 10^6) were transfected with 2 µg of pcDNA-BZLF1 using a microinjection system. For siRNA transfection, 293/EBV-WT cells (1 × 10^6) and HEK293T cells (1.2 × 10^6) were transfected with 100 pmol of Skp2 siRNA or non-targeting/control siRNA using a microinjection system and Lipofectamine 2000, respectively. Tet-BZLF1/B95-8 cells (2 × 10^5) were transfected with 200 pmol of si-BGLF4 or control siRNA using a microinjection system. For overexpression of p27^Kip1 in EBV-latently infected cells, 293/EBV-WT cells (1 × 10^6) were transfected with 2 µg of pcDNA-BZLF1 and
Degradation of p27\textsuperscript{Kip1} Enhanced by EBV PK BGLF4

0.5 μg of pcDNA-FLAG/p27\textsuperscript{Kip1} using a microporator. Tet-BZLF1/B95-8 cells (1 \times 10^6) were transfected with 0.5 μg of pcDNA-FLAG/p27\textsuperscript{Kip1} using a microporator.

In Vitro Kinase Assay—In vitro kinase assays of BGLF4 were performed as described previously (26). Briefly, GST-tagged human p27\textsuperscript{Kip1} protein (400 ng; Abcam) was incubated with 20 ng of GST-fused wild-type BGLF4 or kinase-dead BGLF4 expressed in insect cells (26, 27) in a 50-μl reaction mixture containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 10 mM MgCl\textsubscript{2}, 1 mM ATP, and 0.2 mM Na\textsubscript{3}VO\textsubscript{4} at 37 °C for 60 min. The reactions were terminated by the addition of SDS gel loading buffer, and samples were subjected to immunoblot analysis with anti-p27\textsuperscript{Kip1}-phospho-Thr-187 and p27\textsuperscript{Kip1} antibodies. To assess inhibition by p27\textsuperscript{Kip1}, inhibition assays were performed with some modifications from the previous report (37). A 30-ng aliquot of GST-fused BGLF4 and 50 ng of cyclin E-CDK2 (Upstate Biotechnology) were preincubated with various amounts of GST-fused p27\textsuperscript{Kip1} (8, 40, 200, and 1000 ng) in the absence of ATP for 10 min at 30 °C. Phosphorylation reactions (40 μl) were conducted in 50 mM Tris-HCl (pH 7.4), 10 mM MgCl\textsubscript{2}, 1 mM dithiothreitol, 0.1 mM EGTA, 0.5 μg of histone H1, 100 μM ATP, and 5 μCi of [γ-\textsuperscript{32}P]ATP in each p27\textsuperscript{Kip1}/kinase mixture for 5 min at 30 °C. The reaction was terminated by the addition of SDS gel loading buffer, and the samples were separated by SDS-15% PAGE, followed by autoradiography.

Immuno precipitation—Immunoprecipitation was performed under denaturing conditions. HEK293T cells transfected with pcDNA-FLAG/p27\textsuperscript{Kip1}, pcDNA-WT or kd BGLF4, and pcDNA-HA-Ub were added with MG132 (10 μM) at 24 hpt, harvested at 40 hpt, and suspended in 50 μl of lysis buffer (1% SDS, 50 mM Tris-HCl (pH 8), 10 mM EDTA, 100 mM NaF, 2 mM Na\textsubscript{3}VO\textsubscript{4}, and protease inhibitor mixture (Sigma)) and boiled for 3 min to completely denature proteins and disrupt non-covalent interactions. Then samples were sonicated and centrifuged to remove cell debris. The lysate was diluted with 950 μl of dilution buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8), 167 mM NaCl, 100 mM NaF, 2 mM Na\textsubscript{3}VO\textsubscript{4}, and protease inhibitor mixture) and incubated with anti-FLAG M2 affinity resin for 3 h. The immune complex was washed four times with wash buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 0.1% Nonidet P-40, and 1 mM EDTA) and eluted with SDS gel loading buffer.

Titration of Virus Yields from 293/EBV Cells—293/EBV-WT, 293/EBV-dBGLF4/NeoSt, and 293/EBV-dBGLF4/NeoSt/R cells were transfected with the BZLF1 expression vector to induce lytic replication. Alternatively, 293/EBV-WT cells were transfected with BZLF1 expression plasmid together with non-targeting/control siRNA, Skp2 siRNA, or various amounts of pcDNA-FLAG/p27\textsuperscript{Kip1}. Cells and the culture supernatant were harvested at 60 hpt, freeze-thawed, and centrifuged. The supernatant from centrifugation was filtered and used as a virus stock. EBV-negative Akata(−) cells (38) were infected with the virus, and enhanced green fluorescent protein-positive cells were counted by fluorescence-activated cell sorting.

RESULTS

Degradation of p27\textsuperscript{Kip1} upon EBV Lytic Infection—EBV-lentently infected Tet-BZLF1/B95-8 cells, in which exogenous BZLF1 protein is conditionally expressed under the control of a tetracycline-regulated promoter (31), were treated with doxycycline to induce lytic replication. As shown in Fig. 1A, BZLF1 protein was detected by 10 h postinduction (hpi) and reached a plateau at 24 hpi. The early EBV proteins, BMRF1 (polymerase accessory protein) and BGLF4 (protein kinase), became strongly detectable at 24 hpi. Interestingly, the protein level of p27\textsuperscript{Kip1} decreased gradually from 24 hpi, and only 30%
Degradation of p27Kip1 Enhanced by EBV PK BGLF4

FIGURE 2. BGLF4 deficiency prevents the proteasome-dependent degradation of p27Kip1 upon EBV lytic replication. A, Tet-BZLF1/B95-8 cells were transfected with BGLF4-targeting siRNA (si-BGLF4) or non-targeting/control siRNA (si-control). At 17 hpi, cells were treated with doxycycline (DOX) and harvested at the indicated times. Equal amounts of whole cell lysates from each sample were subjected to immunoblot analysis with the indicated antibodies. B, HEK293 cells with WT, BGLF4-deficient (dBGLF4/NeoSt), and revertant EBV (dBGLF4/NeoSt/R)-bacmid genome were transfected with the BZLF1 expression vector (Z) or empty vector (Ev) and harvested at the indicated times. Equal amounts of whole cell lysates from each sample were subjected to immunoblot analysis with the indicated antibodies. C, HEK293 cells with wild-type (W), dBGLF4/NeoSt (Δ), and dBGLF4/NeoSt/R (R) EBV bacmid genome were transfected with the BZLF1 expression vector and harvested at 60 hpt. After freezing/thawing and centrifugation, the supernatants were co-cultured with Akata(−) cells. GFP-positive cells were counted by fluorescence-activated cell sorting. The results are the averages of three independent experiments and shown as values relative to the virus yield of dBGLF4/NeoSt (infectivity value of 1).

A proteasome inhibitor, MG132, restored the p27Kip1 protein level in doxycycline-treated Tet-BZLF1/B95-8 cells (Fig. 1B), suggesting that down-regulation of p27Kip1 upon EBV lytic infection is proteasome-dependent. Similarly, when Akata(+) cells, an EBV-positive B cell line derived from Burkitt’s lymphoma, were treated with polyclonal rabbit anti-human IgG to induce lytic replication, the p27Kip1 protein level became reduced remarkably upon lytic replication (Fig. 1C).

**Lack of EBV PK Prevents Proteasome-dependent Degradation of p27Kip1 upon EBV Lytic Replication**—There are two different pathways for the proteasome-dependent degradation of p27Kip1. During the G1 phase, p27Kip1 is phosphorylated at Ser-10, exported to the cytoplasm, and then ubiquitinated by KPC (14–16). On the other hand, p27Kip1 is phosphorylated at Thr-187 by cyclin E-CDK2 and ubiquitinated by the ubiquitin ligase SCFSkp2 in the nucleus, followed by degradation (4–8). Since EBV PK can phosphorylate CDK2 or CDK1 target sites (21, 26), there is a possibility that the EBV PK is involved in p27Kip1 phosphorylation and subsequent degradation. At first, we examined whether silencing of BGLF4 expression by siRNA strategy affects p27Kip1 levels after induction of lytic replication. Tet-BZLF1/B95-8 cells were transfected with the BZLF1 expression vector to induce lytic replication, the p27Kip1 protein level decreased (Fig. 2B), corresponding well with the data for Tet-BZLF1/B95-8 and Akata(+) cells (Fig. 1). In contrast, no reduction of p27Kip1 was observed in cells harboring the BGLF4 knock-out virus, whereas reduction was again observed in cells harboring revertant virus. Lytic replication appeared to be equally induced among the three viruses, since comparable amounts of BZLF1 protein were expressed. Lack of BGLF4 expression and change in phosphorylation states of BMRF1 protein in the cells with the knock-out virus were clearly observed, as expected. It was also confirmed that levels of viral DNA synthesis among those viruses are almost the same (data not shown), but the virus yield with the knock-out virus was significantly impaired as compared with wild-type and revertant virus cases (Fig. 2C). These results strongly suggest that EBV PK is involved in p27Kip1 degradation in the context of lytic replication.

**EBV PK Enhances Ubiquitination and Proteasome-dependent Degradation of p27Kip1**—We next investigated direct effects of BGLF4 expression on p27Kip1 protein levels in HEK293T and HeLa cells. Cells were transfected with empty vector or expres-
expression vectors for wild-type BGLF4 (WT BGLF4) or kinase-dead BGLF4 (kd BGLF4) containing a Lys-102 to Ile mutation (Fig. 3B, lanes 4, and 6). Thus, p27kip1 is subjected to degradation via cellular mechanisms, and the expression of EBV PK can further enhance proteasome-dependent degradation of p27kip1 in the absence of other viral factor(s).

Next, to examine whether EBV PK promotes ubiquitination of p27kip1, expression vectors for FLAG-tagged p27kip1 and HA-tagged ubiquitin (Ub) were cotransfected into HEK293T cells with expression vectors for wild-type or kinase-dead BGLF4 in the presence of MG132 (Fig. 3B). Exogenously expressed p27kip1 proteins were immunoprecipitated with anti-FLAG antibodies and subjected to immunoblotting with anti-p27kip1 and HA antibodies. Successful precipitation of p27kip1 protein was confirmed in both WT BGLF4- and kd BGLF4-transfected cells (Fig. 3B, lanes 5 and 6). In contrast, HA-ubiquitinated p27kip1 was detectable in WT BGLF4-transfected cells but not in kd BGLF4-transfected cells (Fig. 3B, lanes 7 and 8). These observations indicate that kinase activity of BGLF4 protein enhances ubiquitination and proteasome-dependent degradation of p27kip1 in cells.

**EBV PK Phosphorylates p27kip1** on Thr-187 in Vivo and in Vitro—BGLF4 protein is a Ser/Thr-kinase that phosphorylates several cellular and viral substrates and often targets a proline-directed Ser/Thr (21–23). Human p27kip1 possesses three serine/threonine sites followed by proline residues (Ser-10, Ser-178, and Thr-187). To determine the phosphorylation site of p27kip1 targeted by EBV PK, we investigated the protein stability of p27kip1 with mutation in Ser-10, Ser-178, or Thr-187 (Fig. 4A). Wild-type BGLF4 (WT BGLF4) or kinase-dead BGLF4 (kd BGLF4) expression vectors were cotransfected into HEK293T cells with expression vectors for FLAG-tagged wild-type p27kip1 or FLAG-tagged mutant p27kip1 with an alanine mutation in either Ser-10, Ser-178, or Thr-187. The protein level of FLAG-tagged wild-type p27kip1 was significantly decreased when cotransfected with the WT BGLF4 expression vector. A specific antibody against phospho-Thr-187 on p27kip1 showed that wild-type p27kip1 could be phosphorylated at Thr-187 when cotransfected with the wild-type BGLF4 expression vector, whereas a specific antibody against phospho-Ser-10 showed that overexpressed p27kip1 was phosphorylated at Ser-10 on p27kip1 even in the absence of expression of BGLF4 protein. Phosphorylation of Ser-10 is known to be responsible for a shift in the electrophoretic mobility of p27kip1 (34). Consistent with this, the mobility of the p27kip1 Ser-10 to Ala mutant on SDS-PAGE was faster than that of wild-type p27kip1. These observations indicate overexpressed p27kip1 to be phosphorylated at Ser-10 on p27kip1 even without expression of the BGLF4 protein. Regarding the stability of p27kip1 protein, p27kip1 protein levels containing alanine mutations of Ser-10 or Ser-178 as well as wild-type p27kip1 were significantly decreased when WT BGLF4 protein was co-expressed. Thus, it was proved that Ser-10 and Ser-178 residues are not involved in degradation of p27kip1 enhanced by BGLF4 protein. In contrast, the protein level of the p27kip1 Thr-187 to Ala mutant was not so affected even when the WT BGLF4 protein was co-expressed. Thus, these observations strongly suggest that phosphorylation of the Thr-187 residue is mainly required for enhanced p27kip1 degradation. It should be noted that there is a possibility that other Ser/Thr residue(s) are phosphorylated by EBV PK, because the protein level of the p27kip1 Thr-187 to Ala mutant with expression of BGLF4 protein did not recover completely.

Next, we examined whether the BGLF4 protein can directly phosphorylate the Thr-187 residue on p27kip1 in vitro. As shown in Fig. 4B, immunoblot analysis using specific antibody against phospho-Thr-187 of p27kip1 demonstrated this to be the case.

We then tried to determine the phosphorylation state of Thr-187 on p27kip1 after induction of lytic replication. Unfortunately, phospho-Thr-187 of endogenous p27kip1 was not clearly detected (data not shown), probably due to rapid degradation of the phosphorylated p27kip1. Therefore, the phosphorylation states of overexpressed p27kip1 were examined in 293/EBV-WT cells before and after the induction of lytic...
Degradation of p27<sup>Kip1</sup> Enhanced by EBV PK BGLF4

replication. FLAG-tagged wild-type p27<sup>Kip1</sup> or FLAG-tagged p27<sup>Kip1</sup> Thr-187 to Ala mutant were expressed in 293/EBV-WT cells, which were induced to enter lytic replication by transfection of BZLF1 expression vector (Fig. 4C). The results revealed that the amounts of exogenously expressed p27<sup>Kip1</sup> did not change before and after the induction of lytic replication. Phosphorylation levels of Ser-10 were constant as well. In contrast, phosphorylation of Thr-187 on p27<sup>Kip1</sup> increased significantly after the induction of lytic replication. Similarly, the phosphorylation states of overexpressed p27<sup>Kip1</sup> were examined in Tet-BZLF1/B95-8 cells that were transfected with the expression vector of FLAG-tagged wild-type p27<sup>Kip1</sup> and then treated with doxycycline at 26 hpi (Fig. 4D). The expression levels of exogenous p27<sup>Kip1</sup> protein were constant until 48 hpi. Phosphorylation levels of Ser-10 on p27<sup>Kip1</sup> also proved constant until 48 hpi. In contrast, phosphorylation of Thr-187 on p27<sup>Kip1</sup> increased by 24 hpi, this correlating with the appearance of BGLF4 protein. Thus, it was demonstrated that p27<sup>Kip1</sup> is phosphorylated at Thr-187 upon EBV lytic replication.

EBV PK Enhances p27<sup>Kip1</sup> Degradation Mediated by the ubiquitin ligase SCF<sub>Skp2</sub>—It was previously reported that Skp2 (S-phase kinase-associated protein 2), an F-box substrate recognition subunit of the SCF ubiquitin ligase complex, recognizes Thr-187-phosphorylated p27<sup>Kip1</sup> and promotes its ubiquitination and subsequent degradation (4, 5, 7, 8). We therefore examined whether SCF<sub>Skp2</sub> ubiquitin ligase is involved in BGLF4-mediated p27<sup>Kip1</sup> degradation. Non-targeting/control siRNA or Skp2-targeting siRNA were cotransfected into HEK293T cells with the FLAG-tagged p27<sup>Kip1</sup> expression vector and wild-type or kinase-dead BGLF4 expression vectors (Fig. 5A). Treatment with Skp2 siRNA reduced the Skp2 protein level by 54% compared with that of control siRNA and prevented the degradation of p27<sup>Kip1</sup> even when wild-type BGLF4 protein was expressed, strongly suggesting that Skp2 is involved in the EBV PK-mediated p27<sup>Kip1</sup> degradation.

**FIGURE 4.** The BGLF4 protein phosphorylates p27<sup>Kip1</sup> on Thr-187 in vivo and in vitro. **A,** HEK293T cells were transfected with expression vectors for FLAG-tagged WT BGLF4, kd BGLF4, or empty vector (−), together with expression vectors for FLAG-tagged WT p27<sup>Kip1</sup>, mutant p27<sup>Kip1</sup>-T187A, S10A, or S178A. Cells were harvested at 27 hpt, and whole cell lysates were prepared and subjected to immunoblot analysis with anti-GAPDH, p27<sup>Kip1</sup>-pT187, or p27<sup>Kip1</sup>-pS10 antibody or anti-FLAG antibody to detect exogenously expressed BGLF4 (FLAG-BGLF4) and p27<sup>Kip1</sup> (FLAG-p27). **B,** GST-tagged WT and kd BGLF4 proteins isolated from insect cells were incubated with p27<sup>Kip1</sup> in the presence of 1 mM ATP at 37 °C for 60 min. The samples were applied for immunoblot analysis with anti-p27<sup>Kip1</sup>-pT187 or p27<sup>Kip1</sup>-pT187 antibodies. **C,** expression vectors for FLAG-tagged WT p27<sup>Kip1</sup> or p27<sup>Kip1</sup>-T187A were cotransfected with the BZLF1 expression vector (Z) or empty vector (Ev) into 293/EBV-WT cells. Cells were harvested at 48 hpt, and equal amounts of whole cell lysates from each sample were subjected to immunoblot analysis with the indicated antibodies. **D,** FLAG-tagged WT p27<sup>Kip1</sup>-overexpressed Tet-BZLF1/B95-8 cells were cultured in the presence of doxycycline (DOX) and harvested at the indicated times. Equal amounts of whole cell lysates were subjected to immunoblot analysis with the indicated antibodies.

**FIGURE 5.** The BGLF4 protein enhances p27<sup>Kip1</sup> degradation mediated by the ubiquitin ligase, SCF<sub>Skp2</sub>. **A,** HEK293T cells transfected with the expression vector for FLAG-tagged p27<sup>Kip1</sup> were transfected with non-targeting/control siRNA (si-control) or Skp2 siRNA (si-Skp2) together with expression vectors for FLAG-tagged WT BGLF4, kd BGLF4, or empty vector (−). Cells were harvested at 32 hpt, and whole cell lysates were prepared and subjected to immunoblot analysis with anti-Skp2, GAPDH antibodies, and anti-FLAG antibody to detect exogenously expressed BGLF4 (FLAG-BGLF4) and p27<sup>Kip1</sup> (Flag-p27). **B,** 293/EBV-WT cells were subjected to transfection with the BZLF1 expression vector for induction of lytic EBV replication together with control siRNA or Skp2 siRNA and were harvested at 0, 24, and 48 hpi. Equal amounts of whole cell lysates were subjected to immunoblot analysis with the indicated antibodies.
We next examined the effect of Skp2 on the p27Kip1 degradation after induction of lytic replication in 293/EBV-WT cells by transfection of the BZLF1 expression vector in the presence of non-targeting/control siRNA or Skp2-targeting siRNA (Fig. 5B). When cells were treated with Skp2 siRNA, the protein level of Skp2 was reduced apparently by 24 hpt, whereas the protein level of p27Kip1 increased markedly (Fig. 5B). The amounts of exogenously expressed BZLF1 and subsequently expressed BMRF1 and BGLF4 proteins were almost the same in non-targeting/control siRNA- and Skp2 siRNA-treated cells. Thus, these observations strongly suggest that the SCF-Skp2 ubiquitin ligase is involved in the degradation of p27Kip1 during EBV lytic replication.

EBV PK Activity Is Not Inhibited by p27Kip1 and Phosphorylates p27Kip1 Efficiently—Although the p27Kip1-bound CDK2 is catalytically inactive in vitro, several groups have recently discovered that phosphorylation of p27Kip1 at Tyr-74 and Tyr-88 residues by Abl and Src family kinases is a prerequisite for p27Kip1 to become a substrate for cyclin E-CDK2 (9–11). To determine the difference between p27Kip1 phosphorylation by EBV PK and by cyclin E-CDK2, purified BGLF4 protein and cyclin E-CDK2 proteins were assayed for their ability to phosphorylate p27Kip1 in vitro (Fig. 6). BGLF4 protein and cyclin E-CDK2 were preincubated with various amounts of p27Kip1 in the absence of ATP, and then ATP and histone H1 as a substrate were added to the reaction. Cyclin E-CDK2 could phosphorylate histone H1 in vitro, but the kinase activity was inhibited by the addition of p27Kip1 in a dose-dependent manner. In contrast, BGLF4 protein phosphorylated histone H1 even at a concentration of p27Kip1 that completely inhibited cyclin E-CDK2 activity. With high concentrations of purified p27Kip1, cyclin E-CDK2 did not phosphorylate p27Kip1 at all. In contrast, BGLF4 protein still phosphorylated p27Kip1, correlating with the data in cells (Fig. 4A). Thus, unlike cyclin E-CDK2, EBV PK escapes from the inhibition of p27Kip1 and efficiently phosphorylates p27Kip1, thereby leading to p27Kip1 degradation.

Stable Retention of p27Kip1 Prevents Efficient Viral Lytic Replication—To examine whether inhibition of p27Kip1 degradation affects the virus yield, lytic replication was induced in 293/EBV-WT cells that were treated with Skp2-targeting siRNA (Fig. 7A). Treatment of Skp2 siRNA reduced the level of Skp2 while the protein level of p27Kip1 was increased, as shown in Fig. 5B. The virus yield from cells treated with Skp2 siRNA was 64%, compared with that with control siRNA at 60 hpt, taken as 100% (Fig. 7A), correlating with the impairment of virus production in cells with the BGLF4-deficient virus (Fig. 2C). Next, we investigated whether overexpression of p27Kip1 protein affects the virus yield by transfecting into 293/EBV-WT cells with the BZLF1 expression vector together with increasing amounts of p27Kip1 expression vector (Fig. 7B). Virus yields in 293/EBV-WT cells transfected with 1, 10, 50, and 100 ng of p27Kip1 expression vector were 86, 61, 59, and 47%, respectively, compared with that in the absence of p27Kip1 expression vector. Overall, p27Kip1 degradation by EBV PK should be required for efficient viral lytic replication.

DISCUSSION

EBV lytic replication occurs in an S-phase-like cellular environment with high CDK activity (20, 31). In this report, we document evidence that EBV PK phosphorylates Thr-187 on p27Kip1, a CDK inhibitor, so that it is ubiquitinated by SCF-Skp2 ubiquitin ligase and degraded to allow viral replication to proceed. The experimental data from siRNA-mediated EBV PK knockdown and with the BGLF4-knock-out virus thus clearly demonstrate that EBV PK is involved in p27Kip1 degradation in the context of viral lytic replication. Although cyclin E-CDK2 regulates the pathways of phosphorylation-dependent ubiquitination and degrada-
Degradation of p27Kip1 Enhanced by EBV PK BGLF4

Degradation of p27Kip1 during the cell cycle, the expression of EBV PK disrupts the pathways, leading to continuous degradation. To maintain a cellular environment with high CDK activity, which is advantageous for viral lytic replication, EBV might possess a variety of strategies to down-regulate CDK inhibitors like p27Kip1.

We reported previously that the levels of cyclin E and cyclin A continue to be elevated and cyclin E- and cyclin A-associated CDK activities increase as lytic replication progresses (19). This elevated activity might be achieved by not only increased levels of cyclin E and cyclin A but also p27Kip1 degradation. Moreover, we recently found that during the lytic infection, p53 was actively degraded, resulting in an undetectable level of another CDK inhibitor protein p21Cip1/Waf1 (39). Thus, EBV lytic replication possesses complex mechanisms to evade inhibition of CDK activity by p21Cip1/Waf1 and p27Kip1 to promote an S-phase-like cellular environment.

Knockdown of Skp2 expression by means of siRNA technology and p27Kip1-overexpression resulted in reduction of virus yields, corresponding with impaired virus production with the BGLF4-deficient virus (Figs. 2C and 7). This suggests that p27Kip1 degradation is required for efficient viral lytic replication and that it contributes to establishment of an S-phase-like cellular environment with high CDK activity for viral lytic replication. Actually, we earlier reported that purvalanol A and roscovitine, inhibitors of S-phase CDKs, blocked viral lytic replication when cells were treated at early stages of lytic infection (20). This observation supports the idea that cyclin A-CDK2 and cyclin E-CDK2 activities are somehow critical.

Virus production with the BGLF4-deficient virus (Fig. 2C) was here found to be severely impaired, whereas that of knockdown of Skp2 expression with siRNA and p27Kip1 overexpression was moderately reduced (Fig. 7). So far, it has been reported that BGLF4 phosphorylates a variety of viral and cellular proteins affecting cell growth, such as EF-1/H9253 (41) reported cell cycle profiles of asynchronous HeLa cells with or without expression of BGLF4 and showed that the cell population of G₁/S border increased ~10%, whereas the cell population of G₂/M border was slightly reduced with BGLF4 expression. Targets of BGLF4 are not only p27Kip1 found in this study but also several cellular proteins affecting cell growth, such as EF-1, lamin A/C, MCM complex, etc., as described in the Introduction. For example, phosphorylation of MCM4 by BGLF4 results in loss of helicase activity of MCM complex (26). Therefore, the cell cycle distribution in BGLF4-expressing cells could be caused not only by the enhanced degradation of p27Kip1 but through complex mechanisms.

Other γ-herpesviruses possess their own strategies to degrade p27Kip1. Kaposi’s sarcoma-associated herpesvirus-encoded cyclin (v-cyclin), a latent viral protein, forms a complex with CDK6 and phosphorylates Thr-187 on p27Kip1 and leads to down-regulation at the protein level (42, 43). Also, the viral cyclin encoded by murine herpesvirus 68 preferentially associates with CDK2, and the complex phosphorylates Thr-187 on p27Kip1 and leads to down-regulation (44). Although EBV does not encode any v-cyclin homologue in its genome, it is reported that latent protein EBNA3C expression prevents the accumulation of p27Kip1 (45). EBNA3C associates with the cyclin A-CDK2 complex and with the Skp2 subunit of SCF-Skp2, and the recruitment of SCF-Skp2 ubiquitin ligase to cyclin A-CDK2-p27Kip1 complex by EBNA3C results in ubiquitination and degradation of p27Kip1. Thus, with EBV latent infection, EBNA3C regulates p27Kip1 stability by manipulating Skp2 (46). On the other hand, EBV PK mainly appears to regulate the level of p27Kip1 in lytic replication.

Acknowledgments—We thank K. Kuzushima and R. Ohta (Aichi Cancer Center Research Center) for pcDNA-BZLF1; Y. Kawaguchi (University of Tokyo) for anti-BGLF4 antibody; N. Ishida (Tohoku University) for pcDNA-FLAG/p27Kip1, pcDNA-FLAG/p27Kip1-T187A, pcDNA-FLAG/p27Kip1-S10A, and pcDNA-FLAG/p27Kip1-S178A; and Y. Nishikawa (Aichi Cancer Center Research Institute) for technical assistance. Also, we are grateful to W. Hammerschmidt (German Research Center for Environment and Health) and H. J. Delecluse (German Cancer Research Center) for providing the EBV-Bac system.

REFERENCES

1. Sherr, C. J., and Roberts, J. M. (1999) Genes Dev. 9, 1149–1163
2. Sherr, C. J., and Roberts, J. M. (1999) Genes Dev. 13, 1501–1512
3. Pagano, M., Tam, S. W., Theodoras, A. M., Beer-Romerio, P., Del Sal, G., Chau, V., Yew, P. R., Draetta, G. F., and Rolfe, M. (1995) Science 269, 682–685
4. Bloom, J., and Pagano, M. (2003) Semin. Cancer Biol. 13, 41–47
5. Carrano, A. C., Eytan, E., Hershko, A., and Pagano, M. (1999) Nat. Cell Biol. 1, 193–199
6. Nakayama, K., Nagahama, H., Minamishima, Y. A., Matsumoto, M., Nakamichi, I., Kitagawa, K., Shirane, M., Tsunematsu, R., Tsukiyama, T., Ishida, N., Kitagawa, M., Nakayama, K., and Hatakeyama, S. (2000) EMBO J. 19, 2069–2081
7. Sutterlüty, H., Chatelain, E., Marti, A., Wirbelauer, C., Senften, M., Müller, U., and Krek, W. (1999) Nat. Cell Biol. 1, 207–214
8. Tsvetkov, L. M., Yeh, K. H., Lee, S. J., Sun, H., and Zhang, H. (2009) Curr. Biol. 19, 661–664
9. Chu, I., Sun, J., Arnaout, A., Kahn, H., Hanna, W., Narod, S., Sun, P., Tan, C. K., Hengst, L., and Slingerland, J. (2007) Cell 128, 281–294
10. Chu, I. M., Hengst, L., and Slingerland, J. M. (2008) Nat. Rev. Cancer 8, 253–267
11. Grümmler, M., Wang, Y., Mund, T., Cilensek, Z., Keidel, E. M., Waddell, M. B., Jäkel, H., Kullmann, M., Kriwacki, R. W., and Hengst, L. (2007) Cell 128, 269–280
12. Boehm, M., Yoshimoto, T., Crook, M. F., Nallamshetty, S., True, A., Nabel, G. J., and Nabel, E. G. (2002) EMBO J. 21, 3390–3401
13. Deng, X., Mercer, S. E., Shah, S., Ewton, D. Z., and Friedman, E. (2004) J. Biol. Chem. 279, 22498–22504
14. Rodier, G., Montagnoli, A., Di Marcotullio, L., Coulombe, P., Draetta, G. F., Pagano, M., and Meloche, S. (2001) EMBO J. 20, 6672–6682
15. Ishida, N., Hara, T., Kamura, T., Yoshida, M., Nakayama, K., and Nakayama, K. I. (2002) J. Biol. Chem. 277, 14355–14358
16. Kamura, T., Hara, T., Matsumoto, M., Ishida, N., Okumura, F., Hatakeyama, S., Yoshida, M., Nakayama, K., and Nakayama, K. I. (2004) Nat. Cell Biol. 6, 1229–1235
Degradation of p27Kip1 Enhanced by EBV PK BGLF4