Chromosome binding of Epstein-Barr virus EBNA1 protein is mediated by arginine residues within chromosome binding domains. International Congress on Oncogenic Herpesviruses and Associated Diseases. Philadelphia, USA. 2012 年 8 月.

47) Kawashima D, Tsurumi T. Involvement of Hsp90 in Epstein-Barr Virus Lytic Replication. The 11th International Congress of Hyperthermic Oncology (ICHO) & The 29th Japanese Congress of Thermal Medicine (JCTM). 京都. 2012 年 8 月.

48) 斉藤伸一、村田貴之、鶴見達也. Epstein-Barr virus deubiquitinase BPLF1 inhibits the canonical NF-kappaB pathway to promote viral lytic DNA replication. 第 71 回日本癌学会学術総会. 札幌. 2012 年 9 月.

49) 村田貴之、野田千恵子、神田輝、鶴見達也. Induction of Epstein-Barr virus Oncogene LMP1 by Transcription Factor AP-2 in Epithelial Cells. 第 71 回日本癌学会学術総会. 札幌. 2012 年 9 月.

50) 神田輝、村田貴之、鶴見達也. Mechamism of host chromosome binding of latently infected Epstein-Barr virus episomes. 第 71 回日本癌学会学術総会. 札幌. 2012 年 9 月.

51) 杉本温子、木村宏、鶴見達也. Epstein-Barr virus genome packaging factors converge at inner part of viral genome storeroom of the BMRF1 core within viral replication compartment. 第 71 回日本癌学会学術総会. 札幌. 2012 年 9 月.

52) 斉藤伸一、村田貴之、鶴見達也. Epstein-Barr virus 脱ユビキチン化酵素 BPLF1 は TRAF6 を介した NF-κB 経路の活性化を阻害することによってウイルス DNA 複製を促進する. 第 71 回日本ウイルス学会学術集会. 大阪. 2012 年 11 月.

53) 村田貴之、鶴見達也. EBV 再活性化におけるエピジェネティックヒストン修飾. 第 71 回日本ウイルス学会学術集会. 大阪. 2012 年 11 月.

54) 成田洋平、村田貴之、木村宏、鶴見達也. Pin1 は EB ウイルス複製に重要な因子である. 第 71 回日本ウイルス学会学術集会. 大阪. 2012 年 11 月.

55) 神田輝、鶴見達也. EBNA1 蛋白質の宿主染色体付着メカニズムの解析. 第 71 回日本ウイルス学会学術集会. 大阪. 2012 年 11 月.

56) 杉本温子、木村宏、鶴見達也. EBV capsid 形成・成熟・DNA packaging の場の解析. 第 71 回日本ウイルス学会学術集会. 大阪. 2012 年 11 月.

57) Tsurumi T. Epstein-Barr virus Replication Factory. The 11th Awaji International Forum on Infection and Immnity. Awaji, Japan 2012 年 9 月.

58) 鶴見達也 EBV 陽性細胞の増殖を制御するウイルス遺伝子の発現調節機構 感染と癌 -感染癌のエフェクター分子とその標的- 札幌 2012 年 9 月.

59) Kimura H. Chronic active Epstein-Barr

virus infection and related diseases in Japan. 11th Korean-Japanese Lymphoreticular Workshop 2012: Asian Hematopathology Symposium, Soul, Jan 29, 2012.1.

60) Kawada J, Ito Y, Torii Y, Kimura H, Iwata N, Remission of juvenile idiopathic arthritis with primary Epstein-Barr virus infection. International Congress on Oncogenic Herpesvirus and Asociated Disease, Philadelphia, USA, 2012.8.

61) Kawano Y, Iwata S, Kawada J, Kimura H, Ito Y, Plasma viral microRNA profiles reveal potential biomarkers for chronic active Epstein-Barr virus infection. ID Week 2012, San Diego, USA, 2012.10.

62) 木村　宏. ウイルス学の基礎よりみた臓器移植後の感染症. 第48回日本移植学会総会、教育セミナー. 名古屋　2012年9月.

63) 木村　宏. EBVと血液・腫瘍性疾患. 第74回日本血液学会学術集会　教育講演. 京都　2012年10月.

64) 中川光、岩田誠子、鎌倉真紀、五島典、木村宏. EBV関連悪性リンパ腫に対するヒストン脱アセチル化酵素阻害剤の効果. 第60回日本ウイルス学会学術集会、大阪市、2012年11月.

65) 河野好彦、岩田誠子、川田潤一、神谷泰子、鈴木道雄、鳥居ゆか、木村宏、伊藤嘉規. 慢性活動性EBウイルス感染用における EB ウイルス由来 miRNA の血漿中バイオマーカーとしての応用. 第60回日本ウイルス学会学術集会、大阪市、2012年11月.

66) 木村　宏. 難治性 EB ウイルス感染症～EBV-HLHとCAEBVの病態から治療まで～: CAEBV～アジア型と欧米型～. 第44回日本小児感染症学会学術集会　ワークショップ. 小倉 2012年11月.

H. 知的財産権の出願・登録状況

なし

Ⅱ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表雑誌名	巻号	ページ	出版年
Sato Y. and Tsurumi T.	Noise Cancellation: Viral Fine Tuning of the Cellular Environment for its Own Genome Replication.	***PLos Pathog.***	6	e1001158	2010
Nakayama S, Murata T, Yasui Y, Murayama K, Isomura H, Kanda T, Tsurumi T.	Tetrameric Ring Formation of EBV Polymerase Processivity Factor is Crucial for Viral Replication.	***J.Virol.***	84	12589-12598	2010
Murata T, Hotta N, Toyama S, Nakayama S, Chiba S, Isomura H, Ohshima T, Kanda T, Tsurumi T.	Transcriptional repression by sumoylation of Epstein-Barr virus BZLF1 protein correlates with association of histone deacetylase.	***J Biol Chem.***	285	23925-23935	2010
Sato Y, Shirata N, Murata T, Nakasu S, Kudoh A, Iwahori S, Nakayama S, Chiba S, Isomura H, Kanda T, Tsurumi T.	Transient increases in p53-responsible gene expression at early stages of Epstein-Barr virus productive replication.	***Cell Cycle***	9	807-814	2010
Iwata S, Wada K, Tobita S, Gotoh K, Ito Y, Demachi-Okamura A, Shimizu N, Nishiyama Y, Kimura H.	Quantitative Analysis of Epstein-Barr Virus (EBV)-Related GeneExpression in Patients with Chronic Active EBV Infection.	***J Gen Virol***	90	42-50	2010
Calatini S, Sereti I, Scheinberg P, Kimura H, Childs R, Cohen JI.	Detection of EBV genomes in plasmablasts/plasma cells and non-B cells in the blood of most patients with EBV lymphoproliferative disorders using Immuno-FISH.	***Blood***	116	4546-4559	2010

Gotoh K, Ito Y, Ohta R, Iwata S, Nishiyama Y, Nakamura T, Kaneko K, Kiuchi T, Ando H, Kimura H.	Immunologic and Virologic Analyses in Pediatric Liver Transplant Recipients with Chronic High Epstein-Barr Viral Loads.	***J Infect Dis***	202	461-469	2010
Ito Y, Takakura S, Ichiyama S, Ueda M, Ando Y, Matsuda K, Hidaka E, Nakatani A, ishioka J, Nobori T, Sasaki M, Kimura H.	Multicenter evaluation of prototype real-time PCR assays for Epstein-Barr virus and cytomegalovirus DNA in whole blood samples from transplant recipients" in its current form for publication in Microbiology and Immunology.	***Microbiol Immunol***	54	516-522	2010
Kanda T, Shibata S, Saito,S Murata T, Isomura H, Takada K, Tsurumi T.	Unexpected instability of family of repeats (FR) the critical cis-acting sequence required for EBV latent infection, in EBV-BAC systems.	***PLos One.***	11	E27758	2011
Noda C, Murata T, Kanda T, Yoshiyama H, Sugimoto A, Kawashima D, Saito S, Isomura H, Tsurumi T.	Identification and characterization of CCAAT enhancer-binding protein (C/EBP) as a transcriptional activator for Epstein-Barr virus oncogene latent protein 1.	***J Biol Chem.***	49	42524-42533	2011
Murata T, Noda C, Saito S, Kawashima D, Sugimoto A, Isomura H, Kanda T, Tsurumi T.	Involvement of Jun dimerization protein 2(JDP2) in the maintenance of Epstein-Barr virus latency.	***J Biol Chem.***	25	22007-22016	2011
Sugimoto A, Kanda T, Yamashita Y, Murata T, Saito S, Kawashima D, Isomura H, Nishiyama Y, Tsurumi T.	Spatiotemporally different DNA repair systems participate in Epstein-Barr virus genome maturation.	***J Virol.***	13	6127-6135	2011
Hoshino Y, Nishikawa K, Ito Y, Kuzushima K, Kimura H.	Kinetics of Epstein–Barr virus load and virus-specific CD8+ T cells in acute infectious mononucleosis.	***J Clin Virol***	50	244-246	2011

Iwata S, Yano S, Ito Y, Ushijima Y, Gotoh K, Kawada J, Fujiwara S, Sugimoto K, Isobe Y, Nishiyama Y, Kimura H.	Bortezomib induces apoptosis in T lymphoma cells and natural killer lymphoma cells independent of Epstein-Barr virus infection.	***Int J Cancer***	119	2263-2273	2011
Yamaguchi M, Kwong YL, Kim WS, Maeda Y, Hashimoto C, Suh C, Izutsu K, Ishida F, Isobe Y, Suzumiya J, Kodama T, Kimura H, Hyo R, Nakamura S, Oshimi K, Suzuki R.	Phase II study of SMILE chemotherapy for newly-diagnosed stage IV, relapsed or refractory extranodal NK/T-cell lymphoma, nasal type: the NK-cell Tumor Study Group (NKTSG) study.	***J Clin Oncol***	29	4410-4416	2011
Ito Y, Kawabe S, Kojima S, Nakamura F, Nishiyama Y, Kaneko K, Kiuchi K, Ando H, Kimura H.	Identification of Epstein-Barr virus-infected CD27+ memory B cells in patients after transplantation.	***J Gen Virol***	92	2590-2595	2011
Gotoh K, Ito Y, Maruo S, Takada K, Mizuno T, Teranishi M, Nakata S, Nakashima T, Iwata S, Goshima F, Nakamura S, Kimura H.	Replication of Epstein-Barr virus primary infection in human tonsil tissue explants.	***PLoS One***	6	E25490	2011
Nakamura M, Iwata S, Kimura H, TokuraY.	Elevated expression of activation-induced cytidine deaminase in T and NK cells from patients with chronic active Epstein-Barr virus infection.	***Eur J Dermatol***	21	780-782	2011

Takahashi E, Ohshima K, Kimura H, Hara K, Suzuki R, Kawa K, Eimoto T, Nakamura S.	Clinicopathological analysis of the age-related differences in patients with Epstein-Barr virus-associated extranasal NK/T-cell lymphoma with reference to the relationship with aggressive NK cell leukemia and chronic active Epstein-Barr virus infection-associated lymphoproliferative disorder.	***Histopathology***	59	660-671	2011
Murata T, Kondo Y, Sugimoto A, Kawashima D, Saito S, Isomura H, Kanda T, Tsurumi T.	Epigenetic histone modification of Epstein-Barr virus BZLF1 promoter during latency and reactivation in Raji cells.	***J Virol.***	86	4752-4761	2012
Kanda T, Ochi T, Fujiwara H, Yasukawa M, Okamoto S, Mineno J, Kuzushima K, Tsurumi T.	HLA-restricted presentation of WT1 tumor antigen in B-lymphoblastoid cell lines established using a maxi-EBV system.	***Cancer Gene Ther,***	19	566-571	2012
Iwata S, Saito T, Ito Y, Kamakura M, Gotoh K, Kawada J, Nishiyama Y, Kimura H	Antitumor activities of valproic acid on Epstein-Barr virus-associated T and natural killer lymphoma cells.	***Cancer Sci***	103	375-381	2012
Kimura H, Ito Y, Kawabe S, Gotoh K, Takahashi Y, Kojima S, Naoe T, Esaki S, Kikuta A, Sawada A, Kawa K, Ohshima K, Nakamura S.	Epstein-Barr virus (EBV)-associated T/NK lymphoproliferative diseases in non-immunocompromised hosts: prospective analysis of 108 cases.	***Blood***	119	673-86	2012
Kawabe S, Ito Y, Gotoh K, Kojima S, Matsumoto K, Kinoshita T, Iwata S, Nishiyama Y, Kimura H.	Application of flow cytometric in situ hybridization assay to Epstein–Barr virus-associated T/NK lymphoproliferative diseases.	***Cancer Sci***	103	1481-1488	2012

Ito Y, Kimura H, Maeda Y, Hashimoto C, Ishida F, Izutsu K, Sueoka E, Isobe Y, Takizawa J, Hasegawa Y, Kobayashi H, Okamura S, Kobayashi H, Yamaguchi M, Suzumiya J, Hyo R, Nakamura S, Kawa K, Oshimi K, Suzuki R.	Pretreatment EBV-DNA copy number is predictive of response and toxicities to SMILE chemotherapy for extranodal NK/T-cell lymphoma, nasal type.	***Clin Cancer Res***	18	4183-4190	2012
Narita Y, Murata T, Ryo A, Kawashima D, Sugimoto A, Kanda T, Kimura H, Tsurumi T.	Pin1 interacts with the epstein-barr virus DNA polymerase catalytic subunit and regulates viral DNA replication.	***J Virol.***	87(4)	2120-2127	2013
Sato Y, Tsurumi T.	Genome guardian p53 and viral infections.	***Rev Med Virol.***	Dec 17	Epub ahead of print	2012
Saito S, Murata T, Kanda T, Isomura H, Narita Y, Sugimoto A, Kawashima D, Tsurumi T.	Epstein-Barr Virus Deubiquitinase Down-regulates TRAF6-mediated NF-κB Signaling during Productive Replication.	***J Virol.***	Jan 30	Epub ahead of print	2013

Ⅲ. 研究成果の刊行物・別刷り

OPEN ACCESS Freely available online

PLoS PATHOGENS

Review

Noise Cancellation: Viral Fine Tuning of the Cellular Environment for Its Own Genome Replication

Yoshitaka Sato[1,2,3], Tatsuya Tsurumi[1,4]*

1 Division of Virology, Aichi Cancer Center Research Institute, Nagoya, Japan, **2** Department of Virology, Nagoya University Graduate School of Medicine, Nagoya, Japan, **3** Department of Cell Biology, G-COE, Kobe University School of Medicine, Kobe, Japan, **4** Department of Oncology, Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, Japan

Abstract: Productive replication of DNA viruses elicits host cell DNA damage responses, which cause both beneficial and detrimental effects on viral replication. In response to the viral productive replication, host cells attempt to attenuate the S-phase cyclin-dependent kinase (CDK) activities to inhibit viral replication. However, accumulating evidence regarding interactions between viral factors and cellular signaling molecules indicate that viruses utilize them and selectively block the downstream signaling pathways that lead to attenuation of the high S-phase CDK activities required for viral replication. In this review, we describe the sophisticated strategy of Epstein-Barr virus to cancel such "noisy" host defense signals in order to hijack the cellular environment.

Introduction

Cellular DNA damage responses initiate with activation and rapid recruitment of repair proteins to DNA damage sites [1,2]. Until the damage is repaired, cells are prevented from transitioning to the next stage of the cell cycle. The tumor suppressor p53 is phosphorylated by DNA damage–responsive kinases, resulting in stabilization of p53 and an increase in its protein level. This leads to activation of target gene transcription including p53 itself, which subsequently causes cell cycle arrest or apoptosis [3,4]. The replicated viral genomes of DNA viruses, including adenoviruses, the polyomavirus, and herpesviruses, are recognized by cellular DNA damage sensors, triggering activation of DNA damage responses [5,6,7,8,9]. Several lines of evidence revealed viral approaches to create an optimal environment for viral replication by manipulating the host defense systems. In this review, we describe the elegant strategies used by Epstein-Barr virus (EBV) to cancel "noisy" cellular signaling in order to manipulate the cellular environment for its own genome replication.

Life Cycle of the Epstein-Barr Virus

EBV, a human lymphotropic herpesvirus, infects more than 90% of world's population and is now known to contribute to a variety of human disorders, including infectious mononucleosis, nasopharyngeal carcinoma, Burkitt's lymphoma, and lymphoproliferative diseases occurring in immune-compromised individuals [10]. The lifecycle of EBV is quite distinctive, featuring two alternative infection cycles: "latent" and "lytic." Primary EBV infection targets resting B lymphocytes, inducing their continuous proliferation. In the resultant B lymphoblastoid cell lines that express a limited number of EBV gene products, the viral genomes are maintained as circular plasmids forming nucleosomal structures with histones [11], and there is no production of virus particles, this being called "latent" infection. In the latent state, viral DNA is replicated only once during S phase, just as host chromosomal DNA [11]. Only a small percentage of infected cells switch their states from the latent stage into the "lytic" cycle to produce progeny viruses. EBV DNA replication occurs at discrete sites in nuclei called "replication compartments," where all of the viral replication proteins are assembled [12]. During lytic replication, the circular genome becomes a ready template for amplification by the viral replication machinery, generating thousands of copies per cell. This reactivation is correlated with the emergence of human cancers [13,14]. The switch from latent to lytic replication is triggered by expression of the EBV BZLF1 gene product (also called Zta or ZEBRA) [15]. The BZLF1 protein is a lytic replication origin binding protein and acts to transactivate various viral promoters [16], leading to an ordered cascade of viral gene expression: activation of early genes followed by viral genome replication and late gene expression. Using the EBV system, the alteration in cellular conditions, from latent to virus-productive infection without overlapping signals triggered by virus entry, can be monitored [17,18].

Regulation of p53 during the Latent Phase of EBV Infection

In uninfected cells, p53 is hypophosphorylated [9] and its level is regulated by cellular E3 ubiquitin ligase MDM2 and cellular ubiquitin–specific protease USP7 (Figure 1A) [19]. The EBV latent protein, EBNA1, contributes to repression of p53-dependent DNA damage signaling by competition of the USP7 binding site with p53 (Figure 1A) [20] (Figure 1A). Furthermore, this interaction between EBNA1 and USP7 leads to the disruption of PML bodies, the nuclear structures important for p53 activation and DNA repair [21]. These findings suggest that EBNA1 expression protects cells from DNA damage–induced apoptosis by destabilizing p53 protein. This possible mechanism points to an important role of EBNA1 in cancer development by allowing

Citation: Sato Y, Tsurumi T (2010) Noise Cancellation: Viral Fine Tuning of the Cellular Environment for Its Own Genome Replication. PLoS Pathog 6(12): e1001158. doi:10.1371/journal.ppat.1001158

Editor: Marianne Manchester, University of California San Diego, United States of America

Published December 16, 2010

Funding: This work is supported by grants-in-aid for Scientific Research from the Ministry of Education, Science, Sports, Culture and Technology of Japan (no. 19041078, and 20390137 to TT). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: ttsurumi@aichi-cc.jp

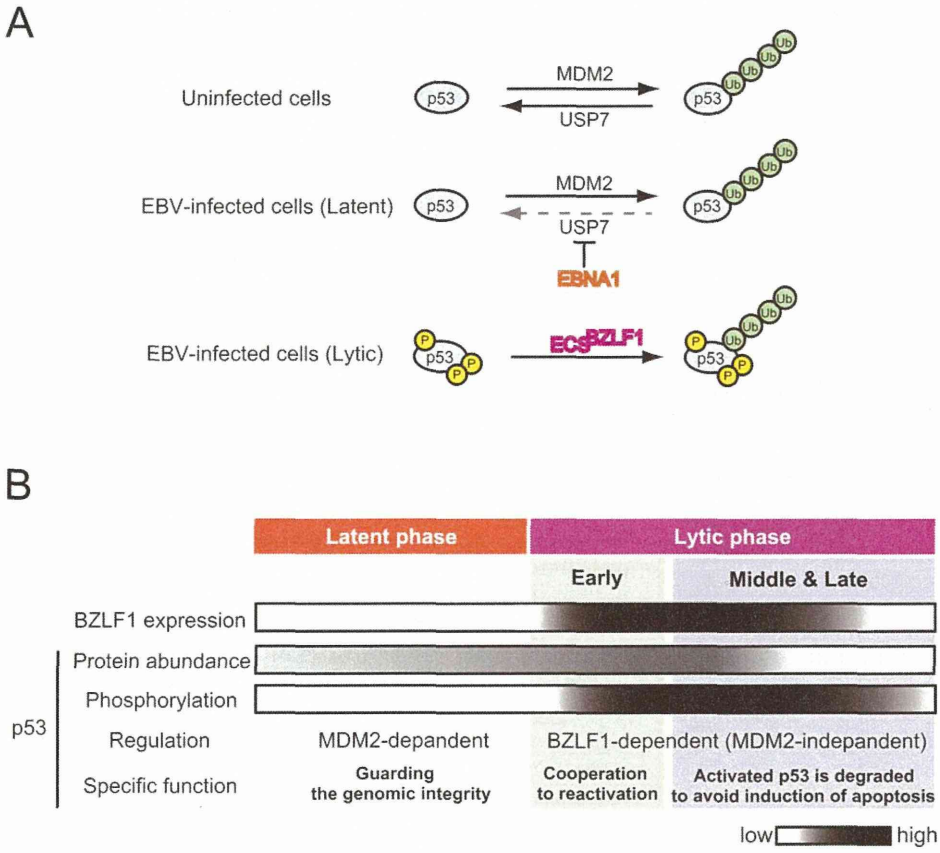

Figure 1. Stage-specific regulation of p53 during EBV infection. (A) The ubiquitination of p53 is regulated by both MDM2 E3 ligase and USP7 deubiquitinase in uninfected cells. During EBV latent infection, EBV latent EBNA1 protein inhibits USP7 and thereby drives the ubiquitination of p53. Phosphorylated p53 is ubiquitinated by BZLF1 protein-associated E3 ligase independently of MDM2 during lytic infection. (B) During the latent phase of EBV infection, p53 is quantitatively regulated by MDM2 ubiquitin ligase via the ubiquitin-proteasome pathway [36], serving as a guardian of genome stability. Expression of BZLF1 protein induces virus-productive (lytic) replication through the ordered cascades of viral gene expression, and concomitantly host DNA damage responses [9], leading to p53 phosphorylation and release of p53 from the MDM2-dependent regulation [36]. In the early stages of lytic infection, the inactive (hypophosphorylated) form of p53 cooperates with viral factors including BZLF1 protein to stimulate virus replication [26,27]. In the middle and late stages of infection, active (hyperphosphorylated) p53 is ubiquitinated by BZLF1 protein–associated ECS ubiquitin ligase complexes and is degraded in a proteasome-dependent manner to inhibit apoptosis [37].
doi:10.1371/journal.ppat.1001158.g001

uncontrolled cellular proliferation without inducing apoptosis in latent EBV-infected cells.

The Important Role of p53 in the Early Stages of EBV Lytic Replication

We propose that, during the course of lytic replication, BZLF1 protein plays two distinct roles in the regulation of p53-mediated transactivation, which depend on the progression of lytic replication: the early stage and the middle to late stages (described below) (Figure 1B). Previous studies demonstrated that the EBV immediate-early protein BZLF1, which was either conditionally expressed [22,23] or overexpressed by a recombinant adenovirus [24,25], could induce G1 arrest in some cell lines. The BZLF1 protein causes the accumulation of both mRNA and protein of CDK inhibitor $p21^{Cip1/Waf1}$ [23], a well-known p53-target gene product. BZLF1 protein accelerates the rate of p53-DNA complex formation through physical interaction with p53 [26]. In the early phases of lytic replication, p53 is hypophosphorylated and therefore exhibits weak DNA binding ability to its recognition sequences [9]. The BZLF1 protein helps the hypophosphorylated p53 to bind to its recognition sequences, leading to the enhancement of p53-dependent transcription [26]. Levels of p53 and $p21^{Cip1/Waf1}$ are transiently elevated in the early stages of lytic replication, and then decline with the progression of lytic infection [26], probably reflecting the effects of BZLF1 expression.

Recently, we and other groups have shown that p53 is involved in reactivation of EBV [26,27]. Tsai and his colleagues have reported that induction of viral lytic proteins by a chemical inducer, sodium butylate, does not occur in p53-negative H1299A and Saos2A cells [27], although the ability of BZLF1 or BRLF1 protein to transactivate its downstream genes is not notably affected by the lack of p53 [28,29]. This implies that p53 might instead be required for a switch from the latent to the lytic cycle. Indeed, we found that overexpression of p53 in the early stages of lytic replication enhances subsequent viral genome replication [26].

In the case of human cytomegalovirus (HCMV), the level of p53 is elevated upon viral infection, but its downstream transcriptional targets remain inactivated [30,31]. It has been reported that cells infected with HCMV in the absence of p53 produce fewer infectious viral particles and cause delays in viral protein production and trafficking [30]. The HCMV genome has 21 potential p53-responsive sites [32]. HCMV gene expression is thought to be influenced by p53 molecules bound to HCMV genome at immediate-early and early stages of the infection, which

could explain the mechanism for reduced and delayed production of virions in p53-negative cells. Similarly, potential p53 recognition sequences are present on the EBV genome (T. Murata et al., unpublished results). Indeed, we have found that p53 is associated with EBV replication compartments [9]. Thus, in the early stages of EBV lytic infection, p53 could be recruited to the EBV genomic regions through its direct binding to the recognition sequences. The BZLF1-mediated enhancement of p53-DNA binding may therefore contribute to the expression of viral genes (Figure 1B).

Newly Synthesized Viral DNA Elicits Host DNA Damage Responses

Herpesviruses such as HSV, HCMV, and EBV modulate the cell cycle to promote a transition through G1-S phase and achieve the cellular environment with high S-phase CDK activities, called the S-phase-like condition, for virus-productive replication (reviewed in [18]). During the EBV lytic replication, the levels of cyclin E and cyclin A continue to be elevated, and cyclin E- and cyclin A-associated CDK activities actually increase [9]. Moreover, this elevation of S-phase CDK activities drives accumulation of the hyperphosphorylated form of retinoblastoma protein (Rb) and an increase in the level of E2F-1 transcription factor [9]. The observation that chemical CDK inhibitors, such as purvalanol A and roscovitine, block viral lytic replication through prevention of viral immediate-early and early gene expression [33] suggests that a cellular environment featuring high CDK activities is required for efficient viral replication. It is conceivable that expression of proteins involved in DNA metabolism may be promoted under S-phase conditions, when energy generation and other resources support viral replication [18]. However, cellular DNA synthesis is almost entirely blocked during the lytic phase of EBV DNA replication, despite S-phase-like cellular conditions with high CDK activities [17]. The EBV-encoding protein kinase (PK) BGLF4 phosphorylates MCM complex to inhibit its replicative helicase activity (Figure 2) [34]. Although the precise mechanism remains unclear, it might be one of the reasons for inhibition of chromosomal DNA replication and for the blockage of the cell-cycle progression from S to G2 phase.

The host cell DNA damage-sensing machinery recognizes the newly synthesized viral DNA in the lytic phase as "abnormal" DNA, activating ATM-dependent DNA damage signaling [9] (Figure 2). ATM phosphorylates histone H2AX (H2AX), which initiates the DNA damage response. The EBV BGLF4 PK might further amplify this response through phosphorylation of H2AX [35]. ATM phosphorylates p53 at Ser-15, which liberates p53 from MDM2-mediated degradation. The downstream kinases of ATM, Chk1, and Chk2 also phosphorylate p53 at various sites. Therefore, elicitation of DNA damage responses in general activates the transcriptional functions of p53.

Ubiquitin-Mediated Degradation of p53 in the Middle and Late Stages of Lytic Infection

Paradoxically, reactivation of EBV induces cellular DNA damage responses that causes phosphorylation of p53, which could lead to accumulation of p53 and subsequent activation of p53 downstream signaling (Figure 1), at the same time it establishes the S-phase-like cellular environment. At the middle to late stages of the lytic replication, the p53 target gene products are indeed maintained at low levels [9,17,26,36]. An explanation for this comes from the observation that p53 is degraded via the ubiquitin-proteasome pathway in the middle and late stages of lytic infection, allowing EBV to exploit cellular environments with high CDK activities for efficient viral replication (Figure 2).

A series of recent studies have shown that induction of the EBV lytic program leads to degradation of p53 via a ubiquitin-proteasome pathway independently of MDM2 [36]. The BZLF1 protein functions as an adaptor component of the ECS (Elongin B/C-Cul2/5-SOCS-box protein) ubiquitin ligase complex that targets both unphosphorylated and hyperphosphorylated p53 for degradation (Figure 2) [37]. The BZLF1 M3 mutant, which lacks the ability to bind to Cullin 2 and 5, degrades p53 very little in EBV-positive cells, and the yield of infectious viruses is poorer than in wild-type BZLF1-expressing cells [37]. The BZLF1 M3 mutant includes a mutation at residue E54, previously reported to prevent activation of the EBV lytic cycle, but the underlying mechanism was unknown [38]. These findings suggest that the deficiency of viral replication is due to the failure of p53 degradation.

Chk2 is known to mediate phosphorylation of p53 at Ser-366 and Ser-378 in response to genotoxic stresses [39]. Indeed, p53 is phosphorylated at least at Ser-15, Ser-20, Ser-366, and Ser-378 with progression of EBV lytic infection [37]. Intriguingly, C-terminal phosphorylation of p53 at both Ser-366 and Ser-378 enhances the association with BZLF1 protein and subsequent ubiquitination of p53 [37], possibly through the phosphorylation-induced conformational change of p53. These results suggest that DNA damage responses play a pivotal role in lytic infection.

In addition, inhibition of p53 degradation by the BZLF1 M3 mutant induces apoptotic cellular changes [37]. The maintenance of p53 at very low levels, therefore, is required not only for establishing S-phase-like conditions [9,17,36], but also for inhibiting apoptosis for efficient viral propagation. In fact, caspase activity is not induced during lytic infection [40]. Similarly, a body of evidence in the herpesvirus family shows that p53 is inactivated in lytic replication, although its molecular mechanism is controversial [41,42,43].

Thus, studies on the relationship between p53 alteration and viral DNA replication have demonstrated that BZLF1 enables hypophosphorylated p53 to transactivate the p53 target genes in the initial phase of lytic replication. In the middle and late stages, activated p53 is subjected to BZLF1-dependent degradation to maintain an S-phase-like environment for efficient viral propagation (Figure 1B).

Several groups have reported that BZLF1 is transiently expressed as an immediate-early gene following EBV primary infection of resting B lymphocytes, although early and late lytic gene expression is very low or undetectable [44,45,46]. A transient BZLF1 expression at the primary infection may contribute to establishing a latent infection, as speculated by Wen and colleagues [44]. This could be driven by degradation of p53, which blocks reprogramming of B lymphocyte proliferation. Interestingly, p53 serves as a negative regulator for reprogramming of somatic cells into induced pluripotent stem (iPS) cells [47,48,49,50,51]. Thus, it is possible that the degradation of p53 by BZLF1 protein-associated ECS ubiquitin ligases contributes to the efficient transformation of B lymphocytes.

Regulation of CDK Inhibitors during Lytic Replication

The large body of evidence implicating Cullin-based E3 ubiquitin ligase in the regulation of diverse cellular processes [52,53] provides us with new insights into their significance as potential targets of viruses manipulating the host cellular system. Post-translational modifications, especially phosphorylation and ubiquitination, play a crucial role in cell-cycle progression.

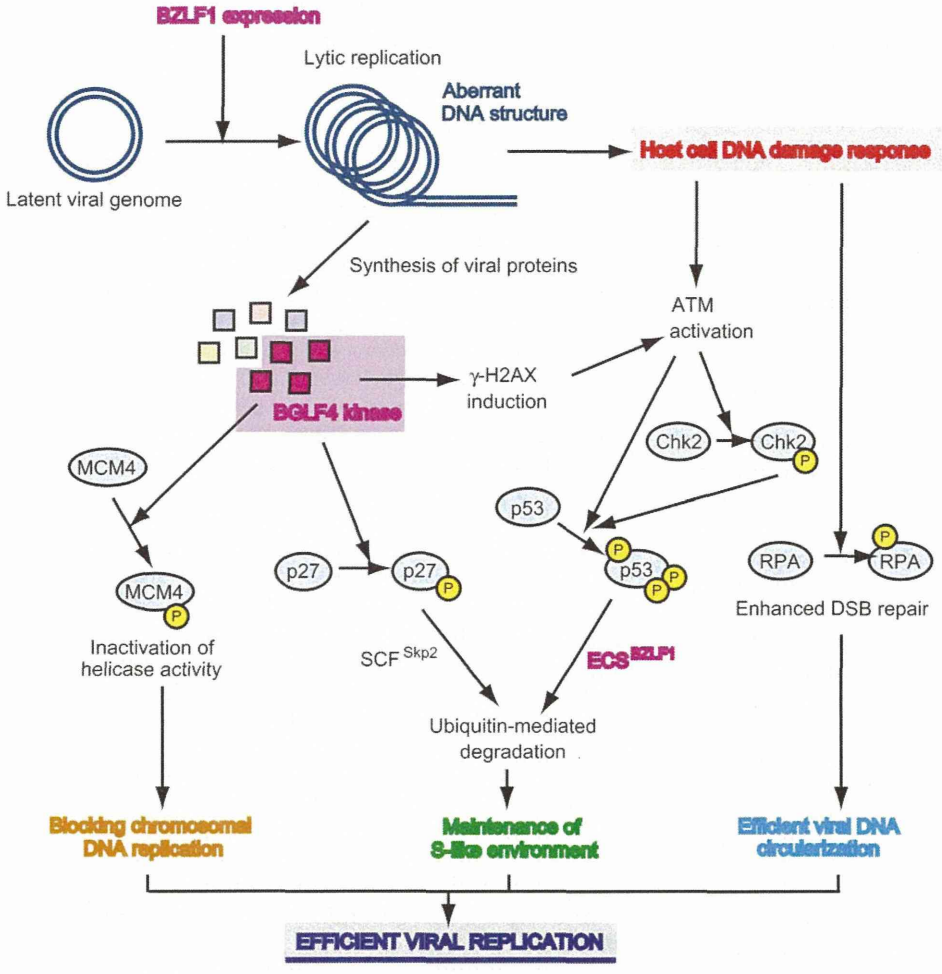

Figure 2. Viral strategy to manipulate the cellular environment for its own genome replication. Induction of lytic replication elicits ATM-dependent host cellular DNA damage responses, because newly synthesized viral DNA is sensed as "aberrant" [9]. The ATM signaling cascade, which is modified by BGLF4 kinase-mediated γ-H2AX induction [35], phosphorylates and activates downstream molecules including CHK2 and p53. However, phosphorylated p53, which can transactivate $p21^{Cip1/Waf1}$ CDK inhibitor, associates with high affinity to BZLF1 protein–formed ECS ubiquitin E3 ligase complex and then is ubiquitinated [37]. On the other hand, EBV protein kinase phosphorylates $p27^{Kip1}$ CDK inhibitor, thereby leading to phosphorylation-mediated ubiquitination by the SCF complex [65]. Since these ubiquitinated proteins are degraded in a proteasome-dependent manner, an S-phase-like environment with high CDK activity required for efficient viral replication is maintained during EBV lytic infection. In parallel with this, replicative helicase activity of the MCM complex is inactivated by BGLF4-mediated phosphorylation of MCM4, causing the inhibition of chromosomal DNA replication [34]. Phosphorylated RPA induced by the DNA damage response stimulates viral DNA replication through homologous recombinational repair [40]. Taken together, EBV manipulates various signaling cascades and thereby achieves efficient viral replication.
doi:10.1371/journal.ppat.1001158.g002

Phosphorylation controls the activity of proteins involved in G1-S and G2-M transitions. Ubiquitination and its mediated proteolysis are commonly facilitated to maintain threshold levels of cell-cycle regulators. Two distinct classes of E3 ubiquitin ligase regulate cell-cycle progression [52], possessing an adaptor protein to determine substrate specificity [54,55,56]. E3 ligase activity of the anaphase-promoting complex is required for the G2-M transition [57]. The SCF (Skp1-Cul1-F-box protein) family of E3 ligase promotes ubiquitination of phosphorylated substrates and typically targets the mediators of G1-S transition [58]. For instance, ubiquitin-mediated degradation of $p27^{Kip1}$ is regulated by the SCF^{Skp2} complex only when $p27^{Kip1}$ is phosphorylated at Thr-187 by the cyclin E-CDK2 complex, which induces S phase conditions [59,60,61].

The EBV lytic program promotes specific cell cycle–associated activity involved in progression from G1 to S phase, since virus-productive replication occurs under S-phase-like circumstances [18]. Similar to p53, CDK inhibitors are also regulated during lytic replication, contributing to establishment of an S-phase-like cellular environment with high-CDK activities [9,33]. γ-Herpesviruses possess their own strategies to degrade $p27^{Kip1}$. For example, Kaposi's sarcoma-associated herpesvirus (KSHV)-encoding cyclin (v-cyclin), a latent viral protein, forms a complex with CDK6 to phosphorylate Thr-187 on $p27^{Kip1}$, leading to down-regulation at the protein level [62,63]. Also, the viral cyclin encoded by murine herpesvirus 68 preferentially associates with CDK2 to phosphorylate Thr-187 on $p27^{Kip1}$ [64]. While EBV does not encode any v-cyclin homologue in its genome, our recent study revealed that the EBV protein kinase BGLF4 can phosphorylate the Thr-187 residue of $p27^{Kip1}$, resulting in its ubiquitination and degradation in an SCF^{Skp2} ubiquitin ligase-dependent manner [65] (Figure 2).

Manipulating the ubiquitin system by EBV involves two aspects of its regulation: attachment of ubiquitin to a substrate and removal from its substrate. As an EBV-encoding deubiquitinating enzyme, BPLF1 deubiquitinates and reduces activity of EBV

ribonucleotide reductase [66]. In this case, deubiquitination influences the function of the protein rather than targeting it for proteasomal degradation. A recent paper documented that BPLF1 also act as a deneddylase [67]. Neddylation, which is a conjugation of ubiquitin-like modifier NEDD8 to its substrate, is an important mechanism for regulating Cullin-based E3 ubiquitin ligase [68]. EBV BPLF1 binds to Cullins and attenuates the activity of the Cullin-RING ligases, resulting in accumulation of the licensing factor Cdt1 and induction of DNA re-replication. Inhibition of BPLF1 during the lytic infection prevents viral replication in the cells that carries a recombinant EBV [67]. These findings support the idea that manipulating ubiquitin system by virus promotes viral productive replication. Furthermore, two lytic proteins (BSLF1 and BXLF1) are found as deubiquitinases by a bioinformatic search on the EBV genome [69], although their functions in viral replication remain obscure. Further investigations are needed to determine the exact role of deubiquitination in the context of EBV lytic infection.

The level of another CDK inhibitor protein $p21^{Cip1/Waf1}$, of course, becomes low during lytic replication [36]. Although the detailed mechanisms remain unknown, one reason is that p53 is actively degraded during lytic infection and another is that the SCF^{Skp2} ubiquitin ligase complex directs $p21^{Cip1/Waf1}$ for degradation through S-phase CDK-mediated phosphoryration [70]. Recent study showed that KSHV-encoding microRNA, miR-K1 represses expression of $p21^{Cip1/Waf}$ in latent infection [71]. As an additional mechanism, an EBV-encoding miRNA that has yet to be discovered might regulate $p21^{Cip1/Waf1}$ for maintaining S-phase-like conditions.

On the other hand, maintaining low levels of CDK inhibitors results in accumulation of the hyperphosphorylated Rb protein due to high S-phase CDK activities and causes accumulation of active E2F-1 as lytic replication progresses [9]. E2F-1 in turn activates the transcription of many proteins involved in cellular DNA synthesis and cell-cycle progression [72], and probably transcription of the EBV DNA polymerase gene as well [73]. The available data suggest that E2F activity is required for lytic viral DNA replication. Alternatively, the EBV immediate-early transactivator BZLF1 and BRLF1 proteins are reported to increase the level of E2F-1 [74,75]. Furthermore, since activated ATM or Chk2 phosphorylates and activates E2F-1 in response to DNA damage [76,77], the DNA damage response induced by EBV lytic replication could activate E2F-1. To achieve effective viral lytic replication, EBV therefore possesses a variety of strategies to maintain the S-phase-like cellular environment.

Beneficial Aspects of DNA Damage Signaling on EBV DNA Replication

During EBV lytic replication, phosphorylated ATM and Mre11/Rad50/Nbs1 (MRN) complexes are targeted to replication compartments in nuclei. Simultaneously, homologous recombinational repair (HRR) factors such as replication protein A (RPA), Rad51, and Rad52, as well as MRN complex, are recruited and loaded onto the newly synthesized viral genome in replication compartments [40]. The 32 kDa subunit of RPA is extensively phosphorylated at sites in accordance with these events [40]. Hyperphosphorylation of RPA32 causes a change in RPA conformation, resulting in a switch from catalysis of DNA replication to participation in DNA repair. RNAi knockdown of RPA32 and Rad51 prevents viral DNA synthesis, suggesting that homologous recombination and/or repair of the viral DNA genome might occur, coupled with viral DNA replication to facilitate viral genome synthesis (Figure 2). Thus, the host DNA damage response induced by productive viral replication is essential for efficient EBV lytic genomic replication.

Conclusions

Replication of DNA viruses in host cells triggers a variety of cellular signaling cascades, including the DNA damage response. Recent studies indicate that such cellular responses to viral genomic replication paradoxically play a crucial role in EBV lytic replication by establishing cellular conditions appropriate for efficient viral replication. To achieve these conditions, EBV manipulates host ubiquitin-proteasome systems, and thereby cancels host antivirus signals. During lytic infection, the interaction between BZLF1 protein and ECS E3 ligase complexes leads to p53 degradation, and the SCF E3 complex recognizes and ubiquitinates phosphorylated $p27^{Kip1}$ through viral protein kinase. Therefore, by skipping the induction of checkpoint signaling and apoptosis, virus-producing cells stay in a persistent S-phase-like environment with high CDK activity.

Accession numbers

The Entrez Gene (http://www.ncbi.nlm.nih.gov/gene) accession numbers for genes and gene products discussed in this study are as follows: p53 (7157), $p21^{Cip1/Waf1}$ (1026), $p27^{Kip1}$ (1027), USP7 (7874), MDM2 (4193), E2F-1 (1869), ATM (472), Chk2 (11200), H2AX (3014), PARP (142), Skp2 (6502), Cdt1 (81620), ubiquitin (7314), NEDD8 (4738), Rb (5925), Cyclin E (898), Cyclin A (890), CDK2 (1017), CDK6 (1021), RPA32 (6118), Rad51 (5888), Rad52 (5893), KSHV v-cyclin (4961471), and EBV EBNA1 (3783709), BGLF4 (3783704) BPLF1 (3783726), BSLF1 (3783730), BXLF1 (3783741), and BZLF1 (3783744).

Acknowledgments

We thank Dr. Y. Nishiyama (Nagoya University) and Dr. S. Seino (Kobe University) for encouragement, and Dr. T. Igaki (Kobe University) for critical reading of the manuscript. We especially thank Dr. T. Murata (Aichi Cancer Center) for helpful comments on the manuscript and for sharing unpublished data.

References

1. Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S (2004) Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annu Rev Biochem 73: 39–85.
2. Rouse J, Jackson SP (2002) Interfaces between the detection, signaling, and repair of DNA damage. Science 297: 547–551.
3. Haffner R, Oren M (1995) Biochemical properties and biological effects of p53. Curr Opin Genet Dev 5: 84–90.
4. Ko LJ, Prives C (1996) p53: puzzle and paradigm. Genes Dev 10: 1054–1072.
5. Stracker TH, Carson CT, Weitzman MD (2002) Adenovirus oncoproteins inactivate the Mre11-Rad50-NBS1 DNA repair complex. Nature 418: 348–352.
6. Dahl J, You J, Benjamin TL (2005) Induction and utilization of an ATM signaling pathway by polyomavirus. J Virol 79: 13007–13017.
7. Shirata N, Kudoh A, Daikoku T, Tatsumi Y, Fujita M, et al. (2005) Activation of ataxia telangiectasia-mutated DNA damage checkpoint signal transduction elicited by herpes simplex virus infection. J Biol Chem 280: 30336–30341.
8. Gaspar M, Shenk T (2006) Human cytomegalovirus inhibits a DNA damage response by mislocalizing checkpoint proteins. Proc Natl Acad Sci U S A 103: 2821–2826.
9. Kudoh A, Fujita M, Zhang L, Shirata N, Daikoku T, et al. (2005) Epstein-Barr virus lytic replication elicits ATM checkpoint signal transduction while providing an S-phase-like cellular environment. J Biol Chem 280: 8156–8163.
10. Young LS, Rickinson AB (2004) Epstein-Barr virus: 40 years on. Nat Rev Cancer 4: 757–768.
11. Adams A (1987) Replication of latent Epstein-Barr virus genomes in Raji cells. J Virol 61: 1743–1746.

12. Daikoku T, Kudoh A, Fujita M, Sugaya Y, Isomura H, et al. (2005) Architecture of replication compartments formed during Epstein-Barr virus lytic replication. J Virol 79: 3409–3418.
13. Joab I, Nicolas JC, Schwaab G, de-The G, Clausse B, et al. (1991) Detection of anti-Epstein-Barr-virus transactivator (ZEBRA) antibodies in sera from patients with nasopharyngeal carcinoma. Int J Cancer 48: 647–649.
14. Feng WH, Cohen JI, Fischer S, Li L, Sneller M, et al. (2004) Reactivation of latent Epstein-Barr virus by methotrexate: a potential contributor to methotrexate-associated lymphomas. J Natl Cancer Inst 96: 1691–1702.
15. Hammerschmidt W, Sugden B (1988) Identification and characterization of oriLyt, a lytic origin of DNA replication of Epstein-Barr virus. Cell 55: 427–433.
16. Flemington EK, Goldfeld AE, Speck SH (1991) Efficient transcription of the Epstein-Barr virus immediate-early BZLF1 and BRLF1 genes requires protein synthesis. J Virol 65: 7073–7077.
17. Kudoh A, Fujita M, Kiyono T, Kuzushima K, Sugaya Y, et al. (2003) Reactivation of lytic replication from B cells latently infected with Epstein-Barr virus occurs with high S-phase cyclin-dependent kinase activity while inhibiting cellular DNA replication. J Virol 77: 851–861.
18. Tsurumi T, Fujita M, Kudoh A (2005) Latent and lytic Epstein-Barr virus replication strategies. Rev Med Virol 15: 3–15.
19. Li M, Chen D, Shiloh A, Luo J, Nikolaev AY, et al. (2002) Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. Nature 416: 648–653.
20. Saridakis V, Sheng Y, Sarkari F, Holowaty MN, Shire K, et al. (2005) Structure of the p53 binding domain of HAUSP/USP7 bound to Epstein-Barr nuclear antigen 1 implications for EBV-mediated immortalization. Mol Cell 18: 25–36.
21. Sivachandran N, Sarkari F, Frappier L (2008) Epstein-Barr nuclear antigen 1 contributes to nasopharyngeal carcinoma through disruption of PML nuclear bodies. PLoS Pathog 4: e1000170.
22. Cayrol C, Flemington E (1996) G0/G1 growth arrest mediated by a region encompassing the basic leucine zipper (bZIP) domain of the Epstein-Barr virus transactivator Zta. J Biol Chem 271: 31799–31802.
23. Cayrol C, Flemington EK (1996) The Epstein-Barr virus bZIP transcription factor Zta causes G0/G1 cell cycle arrest through induction of cyclin-dependent kinase inhibitors. EMBO J 15: 2748–2759.
24. Mauser A, Holley-Guthrie E, Simpson D, Kaufmann W, Kenney S (2002) The Epstein-Barr virus immediate-early protein BZLF1 induces both a G(2) and a mitotic block. J Virol 76: 10030–10037.
25. Mauser A, Saito S, Appella E, Anderson CW, Seaman WT, et al. (2002) The Epstein-Barr virus immediate-early protein BZLF1 regulates p53 function through multiple mechanisms. J Virol 76: 12503–12512.
26. Sato Y, Shirata N, Murata T, Nakasu S, Kudoh A, et al. (2010) Transient increases in p53-responsible gene expression at early stages of Epstein-Barr virus productive replication. Cell Cycle 9: 807–814.
27. Chang SS, Lo YC, Chua HH, Chiu HY, Tsai SC, et al. (2008) Critical role of p53 in histone deacetylase inhibitor-induced Epstein-Barr virus Zta expression. J Virol 82: 7745–7751.
28. Chevallier-Greco A, Manet E, Chavrier P, Mosnier C, Daillie J, et al. (1986) Both Epstein-Barr virus (EBV)-encoded trans-acting factors, EB1 and EB2, are required to activate transcription from an EBV early promoter. EMBO J 5: 3243–3249.
29. Hardwick JM, Lieberman PM, Hayward SD (1988) A new Epstein-Barr virus transactivator, R, induces expression of a cytoplasmic early antigen. J Virol 62: 2274–2284.
30. Casavant NC, Luo MH, Rosenke K, Winegardner T, Zurawska A, et al. (2006) Potential role for p53 in the permissive life cycle of human cytomegalovirus. J Virol 80: 8390–8401.
31. Jault FM, Jault JM, Ruchti F, Fortunato EA, Clark C, et al. (1995) Cytomegalovirus infection induces high levels of cyclins, phosphorylated Rb, and p53, leading to cell cycle arrest. J Virol 69: 6697–6704.
32. Rosenke K, Samuel MA, McDowell ET, Toerne MA, Fortunato EA (2006) An intact sequence-specific DNA-binding domain is required for human cytomegalovirus-mediated sequestration of p53 and may promote in vivo binding to the viral genome during infection. Virology 348: 19–34.
33. Kudoh A, Daikoku T, Sugaya Y, Isomura H, Fujita M, et al. (2004) Inhibition of S-phase cyclin-dependent kinase activity blocks expression of Epstein-Barr virus immediate-early and early genes, preventing viral lytic replication. J Virol 78: 104–115.
34. Kudoh A, Daikoku T, Ishimi Y, Kawaguchi Y, Shirata N, et al. (2006) Phosphorylation of MCM4 at sites inactivating DNA helicase activity of the MCM4-MCM6-MCM7 complex during Epstein-Barr virus productive replication. J Virol 80: 10064–10072.
35. Tarakanova VL, Leung-Pineda V, Hwang S, Yang CW, Matatall K, et al. (2007) Gamma-herpesvirus kinase actively initiates a DNA damage response by inducing phosphorylation of H2AX to foster viral replication. Cell Host Microbe 1: 275–286.
36. Sato Y, Shirata N, Kudoh A, Iwahori S, Nakayama S, et al. (2009) Expression of Epstein-Barr virus BZLF1 immediate-early protein induces p53 degradation independent of MDM2, leading to repression of p53-mediated transcription. Virology 388: 204–211.
37. Sato Y, Kamura T, Shirata N, Murata T, Kudoh A, et al. (2009) Degradation of Phosphorylated p53 by Viral Protein-ECS E3 Ligase Complex. PLoS Pathog 5: e1000530.
38. Deng Z, Chen CJ, Zerby D, Delecluse HJ, Lieberman PM (2001) Identification of acidic and aromatic residues in the Zta activation domain essential for Epstein-Barr virus reactivation. J Virol 75: 10334–10347.
39. Ou YH, Chung PH, Sun TP, Shieh SY (2005) p53 C-terminal phosphorylation by CHK1 and CHK2 participates in the regulation of DNA-damage-induced C-terminal acetylation. Mol Biol Cell 16: 1684–1695.
40. Kudoh A, Iwahori S, Sato Y, Nakayama S, Isomura H, et al. (2009) Homologous recombinational repair factors are recruited and loaded onto the viral DNA genome in Epstein-Barr virus replication compartments. J Virol 83: 6641–6651.
41. Fortunato EA, Spector DH (1998) p53 and RPA are sequestered in viral replication centers in the nuclei of cells infected with human cytomegalovirus. J Virol 72: 2033–2039.
42. Hsu CH, Chang MD, Tai KY, Yang YT, Wang PS, et al. (2004) HCMV IE2-mediated inhibition of HAT activity downregulates p53 function. EMBO J 23: 2269–2280.
43. Wilcock D, Lane DP (1991) Localization of p53, retinoblastoma and host replication proteins at sites of viral replication in herpes-infected cells. Nature 349: 429–431.
44. Wen W, Iwakiri D, Yamamoto K, Maruo S, Kanda T, et al. (2007) Epstein-Barr virus BZLF1 gene, a switch from latency to lytic infection, is expressed as an immediate-early gene after primary infection of B lymphocytes. J Virol 81: 1037–1042.
45. Halder S, Murakami M, Verma SC, Kumar P, Yi F, et al. (2009) Early events associated with infection of Epstein-Barr virus infection of primary B-cells. PLoS One 4: e7214.
46. Kalla M, Schmeinck A, Bergbauer M, Pich D, Hammerschmidt W (2010) AP-1 homolog BZLF1 of Epstein-Barr virus has two essential functions dependent on the epigenetic state of the viral genome. Proc Natl Acad Sci U S A 107: 850–855.
47. Hong H, Takahashi K, Ichisaka T, Aoi T, Kanagawa O, et al. (2009) Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. Nature 460: 1132–1135.
48. Li H, Collado M, Villasante A, Strati K, Ortega S, et al. (2009) The Ink4/Arf locus is a barrier for iPS cell reprogramming. Nature 460: 1136–1139.
49. Kawamura T, Suzuki J, Wang YV, Menendez S, Morera LB, et al. (2009) Linking the p53 tumour suppressor pathway to somatic cell reprogramming. Nature 460: 1140–1144.
50. Utikal J, Polo JM, Stadtfeld M, Maherali N, Kulalert W, et al. (2009) Immortalization eliminates a roadblock during cellular reprogramming into iPS cells. Nature 460: 1145–1148.
51. Marion RM, Strati K, Li H, Murga M, Blanco R, et al. (2009) A p53-mediated DNA damage response limits reprogramming to ensure iPS cell genomic integrity. Nature 460: 1149–1153.
52. Nakayama KI, Nakayama K (2006) Ubiquitin ligases: cell-cycle control and cancer. Nat Rev Cancer 6: 369–381.
53. Petroski MD, Deshaies RJ (2005) Function and regulation of cullin-RING ubiquitin ligases. Nat Rev Mol Cell Biol 6: 9–20.
54. Krafi C, Vodermaier HC, Maurer-Stroh S, Eisenhaber F, Peters JM (2005) The WD40 propeller domain of Cdh1 functions as a destruction box receptor for APC/C substrates. Mol Cell 18: 543–553.
55. Spruck C, Strohmaier H, Watson M, Smith AP, Ryan A, et al. (2001) A CDK-independent function of mammalian Cks1: targeting of SCF(Skp2) to the CDK inhibitor p27Kip1. Mol Cell 7: 639–650.
56. Hao B, Zheng N, Schulman BA, Wu G, Miller JJ, et al. (2005) Structural basis of the Cks1-dependent recognition of p27(Kip1) by the SCF(Skp2) ubiquitin ligase. Mol Cell 20: 9–19.
57. Harper JW, Burton JL, Solomon MJ (2002) The anaphase-promoting complex: it's not just for mitosis any more. Genes Dev 16: 2179–2206.
58. Skowyra D, Craig KL, Tyers M, Elledge SJ, Harper JW (1997) F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell 91: 209–219.
59. Carrano AC, Eytan E, Hershko A, Pagano M (1999) SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nat Cell Biol 1: 193–199.
60. Sutterluty H, Chatelain E, Marti A, Wirbelauer C, Senften M, et al. (1999) p45SKP2 promotes p27Kip1 degradation and induces S phase in quiescent cells. Nat Cell Biol 1: 207–214.
61. Tsvetkov LM, Yeh KH, Lee SJ, Sun H, Zhang H (1999) p27(Kip1) ubiquitination and degradation is regulated by the SCF(Skp2) complex through phosphorylated Thr187 in p27. Curr Biol 9: 661–664.
62. Mann DJ, Child ES, Swanton C, Laman H, Jones N (1999) Modulation of p27(Kip1) levels by the cyclin encoded by Kaposi's sarcoma-associated herpesvirus. EMBO J 18: 654–663.
63. Ellis M, Chew YP, Fallis L, Freddersdorf S, Boshoff C, et al. (1999) Degradation of p27(Kip) cdk inhibitor triggered by Kaposi's sarcoma virus cyclin-cdk6 complex. EMBO J 18: 644–653.
64. Yarmishyn A, Child ES, Elphick LM, Mann DJ (2008) Differential regulation of the cyclin-dependent kinase inhibitors p21(Cip1) and p27(Kip1) by phosphorylation directed by the cyclin encoded by Murine Herpesvirus 68. Exp Cell Res 314: 204–212.
65. Iwahori S, Murata T, Kudoh A, Sato Y, Nakayama S, et al. (2009) Phosphorylation of p27Kip1 by Epstein-Barr Virus Protein Kinase Induces Its

Degradation through SCFSkp2 Ubiquitin Ligase Actions during Viral Lytic Replication. J Biol Chem 284: 18923–18931.
66. Whitehurst CB, Ning S, Bentz GL, Dufour F, Gershburg E, et al. (2009) The Epstein-Barr virus (EBV) deubiquitinating enzyme BPLF1 reduces EBV ribonucleotide reductase activity. J Virol 83: 4345–4353.
67. Gastaldello S, Hildebrand S, Faridani O, Callegari S, Palmkvist M, et al. (2010) A deneddylase encoded by Epstein-Barr virus promotes viral DNA replication by regulating the activity of cullin-RING ligases. Nat Cell Biol 12: 351–361.
68. Rabut G, Peter M (2008) Function and regulation of protein neddylation. 'Protein modifications: beyond the usual suspects' review series. EMBO Rep 9: 969–976.
69. Sompallae R, Gastaldello S, Hildebrand S, Zinin N, Hassink G, et al. (2008) Epstein-barr virus encodes three bona fide ubiquitin-specific proteases. J Virol 82: 10477–10486.
70. Bornstein G, Bloom J, Sitry-Shevah D, Nakayama K, Pagano M, et al. (2003) Role of the SCFSkp2 ubiquitin ligase in the degradation of p21Cip1 in S phase. J Biol Chem 278: 25752–25757.
71. Gottwein E, Cullen BR (2010) A human herpesvirus microRNA inhibits p21 expression and attenuates p21-mediated cell cycle arrest. J Virol 84: 5229–5237.
72. Adams PD, Kaelin WG, Jr. (1995) Transcriptional control by E2F. Semin Cancer Biol 6: 99–108.
73. Liu C, Sista ND, Pagano JS (1996) Activation of the Epstein-Barr virus DNA polymerase promoter by the BRLF1 immediate-early protein is mediated through USF and E2F. J Virol 70: 2545–2555.
74. Mauser A, Holley-Guthrie E, Zanation A, Yarborough W, Kaufmann W, et al. (2002) The Epstein-Barr virus immediate-early protein BZLF1 induces expression of E2F-1 and other proteins involved in cell cycle progression in primary keratinocytes and gastric carcinoma cells. J Virol 76: 12543–12552.
75. Swenson JJ, Mauser AE, Kaufmann WK, Kenney SC (1999) The Epstein-Barr virus protein BRLF1 activates S phase entry through E2F1 induction. J Virol 73: 6540–6550.
76. Lin WC, Lin FT, Nevins JR (2001) Selective induction of E2F1 in response to DNA damage, mediated by ATM-dependent phosphorylation. Genes Dev 15: 1833–1844.
77. Stevens C, Smith L, La Thangue NB (2003) Chk2 activates E2F-1 in response to DNA damage. Nat Cell Biol 5: 401–409.

JOURNAL OF VIROLOGY, Dec. 2010, p. 12589–12598 Vol. 84, No. 24
0022-538X/10/$12.00 doi:10.1128/JVI.01394-10

Tetrameric Ring Formation of Epstein-Barr Virus Polymerase Processivity Factor Is Crucial for Viral Replication▽

Sanae Nakayama,[1] Takayuki Murata,[1] Yoshihiro Yasui,[2] Kazutaka Murayama,[3] Hiroki Isomura,[1] Teru Kanda,[1] and Tatsuya Tsurumi[1]*

Division of Virology, Aichi Cancer Center Research Institute, 1-1, Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan[1]; Division of Virology, Aichi Prefectural Institute of Public Health, Nagoya 462-8576, Japan[2]; and Division of Biomedical Measurements and Diagnostics, Graduate School of Biomedical Engineering, Tohoku University, Sendai 980-8575, Japan[3]

Received 5 July 2010/Accepted 24 September 2010

The Epstein-Barr virus BMRF1 DNA polymerase processivity factor, which is essential for viral genome replication, exists mainly as a C-shaped head-to-head homodimer but partly forms a ring-shaped tetramer through tail-to-tail association. Based on its molecular structure, several BMRF1 mutant viruses were constructed to examine their influence on viral replication. The R256E virus, which has a severely impaired capacity for DNA binding and polymerase processivity, failed to form replication compartments, resulting in interference of viral replication, while the C95E mutation, which impairs head-to-head contact *in vitro*, unexpectedly hardly affected the viral replication. Also, surprisingly, replication of the C206E virus, which is expected to have impairment of tail-to-tail contact, was severely restricted, although the mutant protein possesses the same *in vitro* biochemical activities as the wild type. Since the tail-to-tail contact surface is smaller than that of the head-to-head contact area, its contribution to ring formation might be essential for viral replication.

Epstein-Barr virus (EBV), a human gammaherpesvirus harboring a 172-kb double-stranded DNA (dsDNA) genome, is associated with several human cancers, including Burkitt's lymphoma and nasopharyngeal carcinoma (NPC) (14). EBV has two alternative life styles, latent and productive (lytic). Infection is primarily latent with no production of virus particles (14), but a switch to productive replication is triggered by expression of the BZLF1 gene product as a result of various stimuli (20). BZLF1 is a lytic replication origin binding protein which also transactivates various viral promoters (17), leading to an ordered cascade of viral gene expression. In the viral productive cycle, the EBV genome is amplified more than 100-fold by utilizing the viral replication machinery (15), which works at replication forks to synthesize leading and lagging strands of the concatemeric EBV genome (15).

The DNA polymerase processivity factor of EBV, BMRF1, associates with the polymerase catalytic subunit, BALF5, to enhance the polymerase processivity and exonuclease activities of the holoenzyme (51, 52), and it is the major early phosphoprotein expressed during EBV productive replication (7–9, 24–26, 29, 50). Judging from immunostaining data, together with the finding that almost all abundantly expressed BMRF1 proteins bind to double-stranded DNA (10), the factor not only acts at replication forks for polymerase processivity but also is widely distributed on newly synthesized EBV genomic DNA. Furthermore, it can transcriptionally activate the EBV BHLF1 promoter, one of two divergent early promoters located within the lytic origin of viral DNA replication, oriLyt (55), and enhance BZLF1-mediated transcriptional activation of the BALF2 promoter (39).

From our recent resolution of the crystal structure of C-terminally truncated BMRF1 protein (38), the molecular structure shares structural similarity with other processivity factors, such as herpes simplex virus type 1 (HSV-1) UL42, human cytomegalovirus (HCMV) UL44, and human proliferating cell nuclear antigen (PCNA). Most BMRF1 proteins form a C-shaped head-to-head homodimer, but some form ring-shaped tetramers through tail-to-tail association (Fig. 1). In general, processivity factors are associated with their cognate DNA polymerases on the template during replication. These proteins, which are also known as "sliding clamps," include PCNA from eukaryotes (19, 27) and archaebacteria (34), the β subunit of *Escherichia coli* DNA polymerase III (4), and gp45 from the T4 (35) and RB69 (47) bacteriophages. They assemble as toroidal, ring-shaped structures, forming a central channel to accommodate the template DNA. However, the herpesvirus polymerase processivity factors display different molecular assemblies. The HCMV UL44 forms a dimer in crystal structure as well as in solution. In contrast, the HSV-1 UL42 directly binds to DNA as a monomer (44). Electron microscopy observations have revealed that BMRF1 adopts a ring-shaped structure (32) which is almost twice as large as the previously reported PCNA ring structure.

In our previous study (38), several BMRF1 mutants were prepared: the C95E, H141F, and C206E mutations are predicted to affect the dimer interface, and the K19E, K29E, R87E, K99E, and R256E mutations are in the putative DNA binding region. Some were mapped on the molecular surface, as shown in Fig. 1. *In vitro* DNA binding assays suggested that basic amino acid residues (Lys^{19}, Lys^{29}, Arg^{87}, Lys^{99}, and

* Corresponding author: Mailing address: Division of Virology, Aichi Cancer Center Research Institute, 1-1, Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan. Phone and fax: 81-52-764-2979. E-mail: ttsurumi@aichi-cc.jp.

▽ Published ahead of print on 6 October 2010.

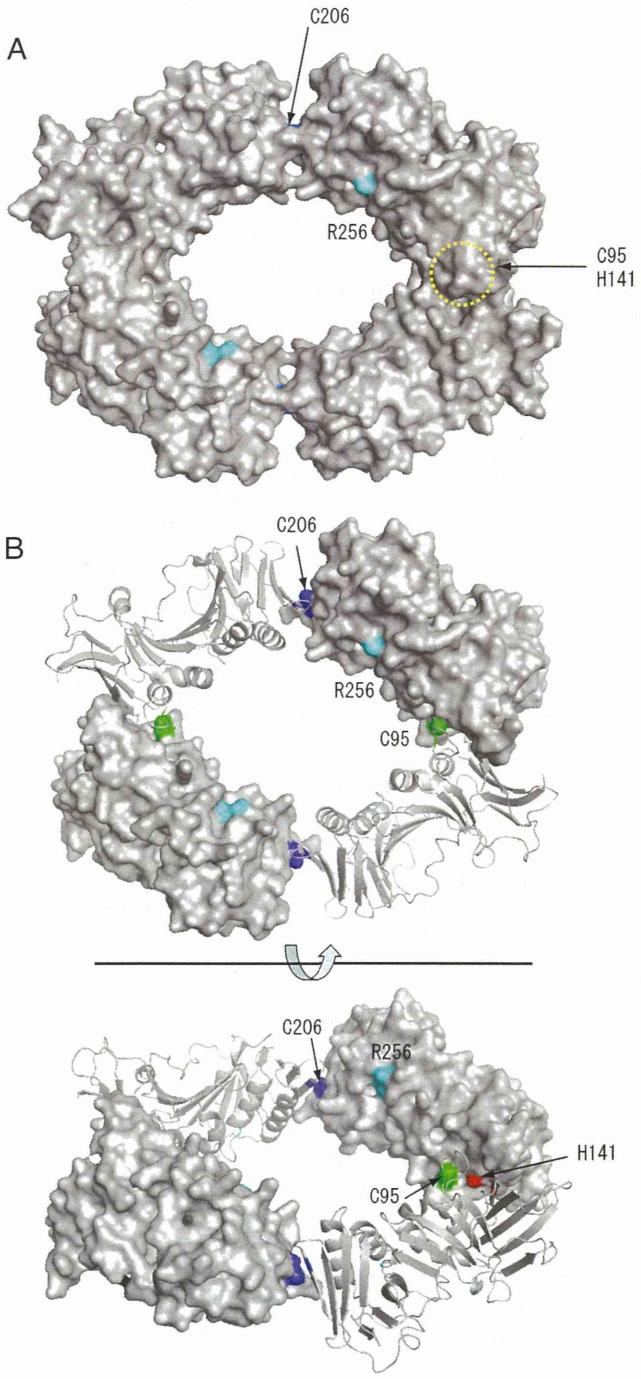

FIG. 1. Mutated amino acid residues of EBV BMRF1 (amino acids [aa] 1 to 314). (A) The ring-shaped crystal structure of a tetramer of C-terminally truncated BMRF1 protein (RCSB Protein Data Bank accession no. 2Z0L) is drawn as a surface model, in which the mutated amino acid residues are displayed in colors. (B) The mutated amino acid residues are displayed in colors in a gray surface model. The partner molecule forming a homodimer is drawn as a gray ribbon model. The lower panel provides a different view of the complex.

Arg^{256}) on the concave surface of the C-shaped head-to-head dimer play important roles in interactions with double-stranded DNA. A monomeric C95E mutant, which is impaired in head-to-head homodimerization, showed decreased DNA binding activity *in vitro*, suggesting that dimer formation enhances its DNA binding. The mutant, however, retained polymerase processivity activity (38) and could still enhance BZLF1-mediated transactivation of the BALF2 promoter (39). The C206E mutant, whose mutation should prevent the tail-to-tail contact, still formed a head-to-head dimer retaining DNA binding activity and polymerase processivity (38). Although the H141F mutant exhibited reduced polymerase processivity, it was still able to bind DNA and to dimerize. In contrast, DNA binding and processivity activities were dramatically inhibited by the R256E mutation.

For the present study, we constructed recombinant viruses with point mutation C95E, H141F, R256E, or C206E in BMRF1 and examined their effects on productive replication. Levels of viral DNA synthesis, processing of replication intermediates, and progeny virus production of the C95E and H141F viruses were comparable to those of wild-type and revertant viruses. On the other hand, the R256E and C206E mutant viruses exhibited severe reductions in viral DNA replication, maturation, and progeny virus production. These results indicate that the *in vitro* data do not necessarily reflect *in vivo* phenotypes and suggest that integrity of the tail-to-tail contact of BMRF1 is important for efficient viral productive replication.

MATERIALS AND METHODS

Cells. HEK293 cells were grown and maintained in Dulbecco modified Eagle medium (DMEM) (Sigma) supplemented with 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere containing 5% CO_2. Akata(−) cells were cultured in RPMI 1640 medium containing 10% FCS.

Plasmids. The BZLF1 protein expression vector (pCAG-Z) was constructed using an In-Fusion Advantage PCR cloning kit purchased from Clontech. A PCR-amplified fragment containing the complete BZLF1-coding region was inserted into the XhoI site of pCAGGS (42). Oligonucleotide primers used for PCR were as follows: CAGpZf, 5′-TTGGCAAAGAATTCCTCGAGATGATG GACCCAAACTCGAC-3′; and CAGpZr, 5′-TGAGGAGTGAATTCCTCGA GTTAGAAATTTAAGAGATCCT-3′. The BALF4 expression vector (pcDNA-BALF4) was kindly provided by W. Hammerschmidt (41). The C-terminal FLAG-tagged intact BMRF1 expression vector (pWT-f) and BMRF1(C95E) mutant (C95E-f) were constructed as described previously (39). All point mutants of FLAG-tagged BMRF1 expression plasmids were generated by site-directed mutagenesis using pWT-f as a template. Complementary oligonucleotide primers used for site-directed mutagenesis were as follows: 5′-AAGGTGT CCAAGAGCCACTTCACCTGCGCC-3′ for H141F-f; 5′-CTTAGCCTCTGC GAGATTCCGGCCGTTAGC-3′ for R256E-f; and 5′-CTGGGGAGGCCGAA CTCACCCTAGACTAC-3′ for C206E-f. The inserted DNA sequence of each vector was confirmed by DNA sequencing.

Genetic manipulation of EBV BAC DNA. Bacterial artificial chromosome BAC DNA of human EBV B95-8 (B95-8/F-BAC) was provided by W. Hammerschmidt (12). Homologous recombination was carried out in *E. coli* as described previously (21, 23). First, EBV BACΔM/neo DNA was produced by inserting a marker cassette containing neomycin resistance and streptomycin sensitivity genes and kanamycin selected as described previously (36, 37, 39). Revertant EBV BAC was prepared by second recombination using wild-type BMRF1 sequence DNA as a shuttle vector, followed by streptomycin selection, as detailed in our recent reports (36, 37, 39). Likewise, point mutations were introduced using mutant BMRF1 sequences as shuttle vectors. EBV BAC DNA was then transfected into HEK293 cells, followed by hygromycin selection. Cell clones were examined if the viral DNA demonstrated latent infection and if lytic replication could be induced upon BZLF1 expression (36, 37, 39).

Induction of lytic replication in HEK293 cells and transfection of BMRF1 plasmids. HEK293 cells in 12-well plates were transfected with 500 ng of pCAG-Z using a Microporator (Digital Bio) to induce a virus lytic cycle. To test whether impaired virus replication caused by BMRF1 knockout could be complemented by ectopic expression of BMRF1, FLAG-tagged, wild-type, or mutant BMRF1 plasmid was cotransfected with 200 ng of pCAG-Z into the EBV BACΔM/neo HEK293 cells.

TABLE 1. Summary of BMRF1 functions

BMRF1	Purified proteins[a]		Recombinant viruses			
	Mean (SD) DNA binding activity, %[b]	Processivity	Mean (SD) viral DNA synthesis[c]	Mean (SD) production of progeny[d]	Nuclear retention	Formation of replication compartments
Wild type	100	High	996.2 (54.91)	122.0 (5.71)	Strong	Yes
C95E	1.6 (0.18)	High	665.8 (14.65)	104.9 (10.60)	Moderate	Yes
H141F	96.3 (0.40)	Low	480.2 (11.30)	88.4 (19.80)	Strong	Yes
R256E	1.7 (0.07)	Low	199.1 (4.99)	5.8 (0.44)	Weak	Impaired
C206E	89.9 (4.32)	High	115.1 (9.68)	11.6 (1.62)	Strong	Impaired

[a] Data are from reference 38.
[b] Percentage of wild-type activity.
[c] Based on the data in Fig. 3 at 3 days after lytic induction.
[d] Progeny virus production levels ($\times 10^4$ GFP positive cells/ml) are based on the data in Fig. 4.

Antibodies. An anti-BMRF1-specific mouse monoclonal antibody (R3) was purchased from Chemicon International Inc., and an anti-FLAG-specific mouse monoclonal antibody (M2) and an anti-alpha-tubulin specific mouse monoclonal antibody were purchased from Sigma and Abcam, respectively. Affinity-purified anti-BALF5-, anti-BBLF2/3-, anti-BALF2-, anti-BZLF1-, and anti-BMRF1-specific polyclonal antibodies were prepared as described previously (10, 53, 54).

Quantification of viral DNA synthesis during lytic replication. Levels of viral DNA were determined by quantitative real-time PCR (22). Lytic replication-induced HEK293 cells (3×10^5 cells) were harvested and suspended in 200 μl of a lysis buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.001% Triton X-100, 0.001% SDS), followed by sonication and then incubation at 50°C overnight with 50 μg/ml of proteinase K (Sigma). Primers and a probe within the BALF2-coding region were designed using Primer Express (Applied Biosystems). The sequences were as follows: 5′-GCCCGTCCGGTTGTCA-3′ (forward primer), 5′-AATAT CTGGTTGTTGCCGTTGA-3′ (reverse primer), and 5′-6-carboxyfluorescein (FAM)-CTGCCAGTGACCATCAACAAGTACACGG-tetramethyl rhodamine (TAMRA)-3′ (probe). PCR was performed in 20 μl of aqueous solution containing 0.5 mM each primer, 0.2 mM labeled probe, Fast Start Universal Probe Master (Roche), and 1 μl of DNA mixture using the 7300 real-time PCR System (Applied Biosystems). PCR included 2 min at 50°C, 10 min at 95°C, and 40 cycles at 95°C for 15 s followed by 1 min at 60°C.

Viral yield assay. Titers of infectious viral particles of recombinant EBV were measured as follows (2). Culture supernatants were harvested at 3 days after lytic induction and then filtered through a 0.45-mm-pore-size filter (Millipore). EBV-negative Akata(−) cells (49) were infected with serially diluted mixtures of viruses. The infected cells were harvested at 3 days postinfection and fixed with 0.5% paraformaldehyde in phosphate-buffered saline (PBS). Since the recombinant EBV expresses green fluorescent protein (GFP), GFP-positive cells were counted using the FACSCalibur G5 system (Becton-Dickinson) according to the manufacturer's instructions.

PFGE. Samples for pulsed-field gel electrophoresis (PFGE) were prepared from HEK293 cells with latently infected EBV genomes as follows. Cells (1.5×10^5) were detached from 24-well culture plates by incubation in PBS containing 1 mM EDTA. After centrifugation at $800 \times g$, the pellet was resuspended in 40 μl of PBS containing 87.5 mM EDTA and 0.5% of low-melting-point melted agarose (Nippon Gene). After gelling, agarose plugs containing cells were incubated at 37°C for 2 overnight periods in 20 volumes of lysis buffer (10 mM Tris-HCl [pH 8.0], 100 mM EDTA, 1% *N*-lauroylsarcosine sodium salt, 0.1 mg/ml proteinase K [Sigma]) and washed five times in Tris-EDTA (TE) buffer.

PFGE was performed using the Chromosomal DNA Electrophoresis System (Bio Craft) for 24 h at 150 V and 13°C in 0.5× Tris-borate-EDTA (TBE) buffer, using a pulse time of 50 s. The Lambda Ladder PFG marker (NEB) was used as a size marker. After the PFGE was run, the gel was stained with ethidium bromide and photographed. DNA separated by PFGE was transferred to a Hybond-N positively charged membrane. For the probes, DNA fragments within the W repeat region were amplified by PCR using primer pair 5′-TACCAGAG GGGGCCAAGAA-3′ and 5′-AGGAGAGGCAGGGCCTGAA-3′. Labeling of probes and hybridization were performed using the DIG High Prime DNA labeling system (Roche). The images were processed with LumiVision PRO 400EX.

Immunofluorescence analysis. Immunostaining of HEK293 cells was achieved as described previously (11). Cells were harvested 24 h after lytic induction and lysed for 10 min on ice with ice-cold 0.5% Triton X-100–mCSK buffer [10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) (pH 6.8), 300 mM sucrose, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100] containing multiple protease inhibitors (Roche), 0.2 mM Na_3VO_4, 20 mM NaF, and 150 mM NaCl. After centrifugation ($2,000 \times g$, 3 min, 4°C), the extracted nuclear pellets were fixed with 70% methanol. The fixed cells were immunostained with anti-BMRF1 monoclonal and anti-BALF2 polyclonal antibodies, followed by secondary goat anti-mouse or rabbit IgG antibodies conjugated with Alexa Fluor 594 or 488. Slides were mounted with ProLong Gold antifade reagent with DAPI (4′,6′-diamidino-2-phenylindole) (Invitrogen) for analysis under a fluorescence confocal microscope (Zeiss). Images were captured and processed using the Zeiss LSM Image Browser (Zeiss).

Biochemical cellular fractionation. Fractionation of HEK293 cells was performed as described previously (11). Cells were harvested at 3 days after lytic induction and lysed for 10 min on ice with ice-cold 0.5% Triton X-100–mCSK buffer containing multiple protease inhibitors (Roche), 0.2 mM Na_3VO_4, 20 mM NaF, and one of several concentrations of NaCl (50, 100, or 150 mM). The samples were then subjected to centrifugation ($2,000 \times g$, 3 min, 4°C) to obtain Triton X-100-extractable supernatants and extracted nuclear pellets. Each sample was adjusted to the same volume by adding SDS sample buffer and boiled, and aliquots corresponding to 1.8×10^4 cells per lane were applied for SDS-PAGE. The intensity of fluorescence was measured with a Lumi Vision Analyzer 2.0 (AISIN).

RESULTS

Construction of recombinant viruses. Based on our crystal structure imaging of the EBV BMRF1 (38), we previously analyzed the significance of panels of point mutations affecting biochemical properties of the protein with regard to dimer formation (38), DNA binding ability (38), transcriptional activation (39), and polymerase processivity *in vitro* (38). We here chose four representative mutations (Table 1) out of those characterized in our previous study and generated recombinant viruses expressing mutated BMRF1 proteins to examine the significance of those mutations in the context of viral infection. Figure 1 illustrates a surface-and-ribbon model of tetrameric BMRF1. The mutated amino acid residues are displayed in colors. Cys^{95} is located at the interface of head-to-head contact for the homodimer formation and appears to be involved in disulfide bond formation under oxidative conditions. C95E substitution would therefore impair dimer formation *in vitro*. Although the resultant monomer BMRF1 exhibits reduced DNA binding activity *in vitro*, it still can act as a polymerase processivity factor (38). His^{141} also lies at the interface of head-to-head contact for homodimer formation, although exchange of the residue (H141F) has no effect on dimer formation but rather diminishes the processivity function (38). While Cys^{206} is at the tail-to-tail interface, replacement of the residue (C206E) had no obvious effect on DNA binding and

processivity *in vitro* (38). Arg^{256} is situated at the DNA binding domain, and the R256E mutation abrogates DNA binding and processivity *in vitro* (38).

For construction of recombinant viruses harboring each point-mutated BMRF1 gene, we first constructed a BMRF1-deficient insertion mutant virus, ΔM/neo, as shown in Fig. 2A. The marker cassette containing the neomycin resistance and streptomycin sensitivity genes was inserted at nucleotides (nt) 80001 to 80876 to block the expression of BMRF1 proteins completely. The cassette was then replaced with a wild-type BMRF1 sequence or each point-mutated BMRF1 sequence to prepare revertants or viruses carrying the intended mutations, respectively (Fig. 2A).

The recombinant EBV genomes were analyzed by BamHI digestion, followed by agarose gel electrophoresis (Fig. 2B). The BamHI-M fragment is present in wild-type and mutated viruses, but the corresponding band of ΔM/neo virus migrated slowly in the gel at about 1.8 kb, which is the size of the cassette.

The recombinant EBV DNAs were then introduced into HEK293 cells, and hygromycin-resistant cell colonies were screened for further analysis. The colonies formed by the wild type and each recombinant virus were comparable in size and numbers. We calculated the copy number of recombinant EBV DNA in each infected cell using quantitative real-time PCR. The average values from three independent experiments were as follows: wild type, 3.1 copies/cell; C95E, 5.3 copies/cell; H141F, 3.8 copies/cell; R256E, 5.4 copies/cell; C206E, 3.0 copies/cell; revertant, 4.8 copies/cell; and ΔM/neo, 3.0 copies/cell. Exogenous expression of BZLF1 induced adequate expression of early proteins, BALF2 single-stranded DNA (ssDNA) binding protein, BALF5 polymerase catalytic subunit, and BBLF2/3 helicase-primase accessory protein, as well as BMRF1 (Fig. 2C), although ΔM/neo did not produce BMRF1 at all, as expected (Fig. 2C).

Viral DNA synthesis in recombinant viruses. When levels of viral DNA synthesis in HEK293 cells with the wild-type or recombinant viruses were examined using quantitative real-time PCR (Fig. 3), the amplification of viral DNA was found to reach nearly 1,000-fold at 3 days postinduction in cells harboring the wild-type genome, whereas it was completely blocked in cells with the BMRF1 knockout ΔM/neo, as reported previously (40). Viral DNA replication of the C95E mutant, which was defective in head-to-head dimerization, was at almost the same level as that of the revertant. Although the H141F mutant BMRF1 exhibited rather impaired polymerase processivity *in vitro* (38), the mutation caused only a mild reduction of viral DNA replication in the context of infection. In contrast, although the polymerase processivity of the C206E mutant *in vitro* was not impaired at all (38), viral DNA replication was severely impaired in the context of infection. These results suggest that *in vitro* processivity data do not accurately reflect the actual function in cells. In lytic replication-induced cells, other viral replication factors such as BALF2 single-stranded DNA binding protein and BBLF4/BSLF1/BBLF2-3 helicase-primase proteins might cooperate and compensate for the reduced processivity activity of the BMRF1, especially since BALF2 can enhance the processivity of the BALF5 polymerase catalytic subunit in a manner different from that of BMRF1 (53). On the other hand, replication of the R256E mutant,

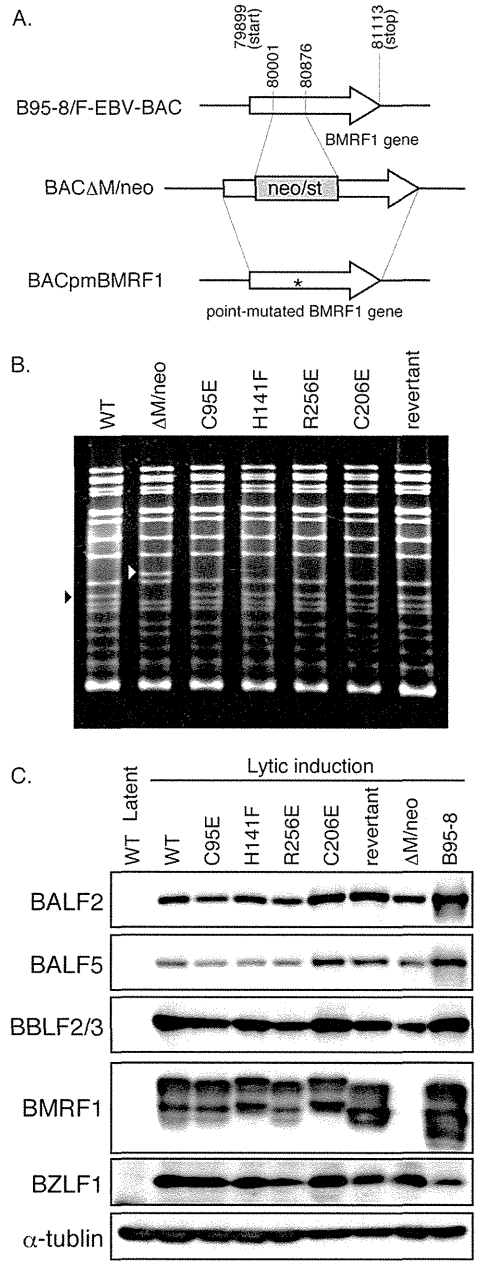

FIG. 2. Recombinant EBV BAC genome structures. (A) Schematic arrangement of the recombination of the EBV genome using neomycin resistance and streptomycin sensitivity genes (neo/st). The region between nucleotides 80001 and 80876 of the EBV gene (GenBank accession no. V01555) was replaced with the neo/st genes to make BACΔM/neo. The BMRF1 gene, including this neo/st cassette, was replaced with each point-mutated BMRF1 and wild-type BMRF1 sequence to construct BACpmBMRF1 and the revertant virus, respectively. The asterisk indicates the point-mutated site. (B) Electrophoresis of the recombinant viruses. EBV BAC DNAs were digested with BamHI and separated in a 1.0% agarose gel. The black arrowhead indicates the BamHI-M fragment of the virus, and the white arrowhead indicates the size of BamHI-M fragments plus the marker cassettes. (C) Expression levels of BALF2, BALF5, BBLF2/3, BMRF1, and BZLF1 proteins determined by immunoblot analysis with specific polyclonal antibodies. Lysate from HEK293 cells infected with recombinant viruses was harvested at 48 h posttransfection with BZLF1 expression plasmids. Lysate from B95-8 cells treated with tetradecanoyl phorbol acetate(TPA), A23187, and sodium butyrate for 48 h was included as a positive control.