Epstein-Barr Virus Lytic Replication Elicits ATM Checkpoint Signal Transduction While Providing an S-phase-like Cellular Environment*

When exposed to genotoxic stress, eukaryotic cells demonstrate DNA damage response with arrest of cell-cycle progression, providing time for DNA repair. Induction of the Epstein-Barr virus (EBV) lytic program elicited a cellular DNA damage response, with activation of the ataxia telangiectasia-mutated (ATM) signal transduction pathway. Activation of the ATM-Rad3-related (ATR) replication checkpoint pathway, in contrast, was minimal. The DNA damage sensor Mre11-Rad50-Nbs1 (MRN) complex and phosphorylated ATM were recruited and retained in viral replication compartments, recognizing newly synthesized viral DNAs as abnormal DNA structures. Phosphorylated p53 also became concentrated in replication compartments and physically interacted with viral BZLF1 protein. Despite the activation of ATM checkpoint signaling, p53-downstream signaling was blocked, with rather high S-phase CDK activity associated with progression of lytic infection. Therefore, although host cells activate ATM checkpoint signaling with response to the lytic viral DNA synthesis, the virus can skillfully evade this host checkpoint security system and actively promote an S-phase-like environment advantageous for viral lytic replication.

Eukaryotic cells exhibit a variety of physiological responses, including cell cycle arrest, activation of DNA repair and apoptosis, upon DNA damage. Sets of checkpoint proteins that have been conserved with evolution are rapidly induced to prevent replication or segregation of damaged DNA before repair is completed. The related phosphatidylinositol 3-like kinases, ataxia telangiectasia-mutated (ATM) and ATM-Rad3-related (ATR), respond to a variety of abnormal DNA structures and initiate signaling cascades leading to a DNA damage checkpoint (1). ATM responds to the presence of DNA double-strand breaks (DSBs) induced by ionizing radiation (2). On the other hand, the ATR pathway can be stimulated by hydroxyurea, UV light, and base-damaging agents that interfere with the movement of replication forks (3). The ATR pathway also responds to DSBs, but more slowly than ATM (4).

A variety of checkpoint proteins have been identified as substrates for ATM and ATR kinases, including the checkpoint kinases Chk1 and Chk2, as well as γH2AX (5) and p53 (2, 6). ATM phosphorylates Chk2 at several sites including Thr-68, followed by Chk2 activation (6–9). Chk1 is phosphorylated at Ser-345 by ATR in response to UV and hydroxyurea, leading to a 3–5-fold increase in Chk1 activity (6, 7, 10). ATM is activated by intermolecular autophosphorylation on Ser-1981 (11). Both Mre11 and Nbs1 are also targets of ATM and possibly ATR (10, 12–14). The MRN complex consisting of Mre11, Rad50, and Nbs1 has been proposed to facilitate ATM activation (15–17) and recently demonstrated to function upstream of ATM activation as a damage sensor, in addition to acting as an effector of ATM signaling (15, 18).

ATM/ATR-initiated checkpoint signaling induces p53-dependent and p53-independent responses. The p53-dependent cell cycle checkpoint features (p21-mediated inactivation of Cd2/cyclin E) (19–21), while Chk2 inhibits Cd2/cyclin E activity by phosphorylation of Cd2 at Tyr-15, via down-regulation of CDC25A phosphatase (4), in a p53-independent fashion. Among Cd2-targets, the Rb protein is most important for cell cycle progression and the checkpoint pathways result in its hypophosphorylation, leading to G1 or G2/M cell cycle arrest.

The Epstein-Barr virus (EBV) is a human herpes virus that infects 90% of individuals. Primary EBV infection targets resting B lymphocytes, inducing their continuous proliferation. In the B lymphoblastoid cell lines (LCL) only limited numbers of viral genes are usually expressed and there is no production of virus particles, this being called latent infection. In the latent state, EBV maintains its 170 kbp genome as complete, multiple copies of plasmids that are synthesized only once in each S-phase of the cell cycle replication machinery, following the rules of chromosome replication (22).

EBV-infected cell lines usually contain a small subpopulation of cells that have switched spontaneously from a latent stage of infection into the lytic cycle. The mechanism of switching is not fully understood, but one of the first detectable changes is expression of the BZLF1 immediate-early gene product. The BZLF1 protein, together with the other immediate-early protein, BRLF1 protein, transactivates viral promoters.

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(23) and leads to an ordered cascade of viral early and late gene expression. Early gene products include proteins involved in viral DNA replication and DNA metabolism. The lytic phase of EBV DNA replication is dependent on several viral replication proteins: BZLF1, an oriLyt-binding protein; BALF5, a DNA polymerase; BMRF1, a polymerase processivity factor; BBLF2, a single-stranded DNA-binding protein; and BBLF4, BSLF1, and BBLF2/3, predicted to be helicase-, primase-, and helicase primase-associated proteins, respectively (24). Viral lytic replication occurs in discrete sites in nuclei, called replication compartments in which viral replication proteins are assembled (25).

We have previously demonstrated that induction of the EBV lytic program results in inhibition of replication of cellular DNA as well as explosive replication of viral DNA (26). The levels of p53 and CDK inhibitors remain unaltered throughout the lytic infection, while the amounts of cyclin E/A and the hyperphosphorylated form of Rb increase as lytic infection progresses. The resultant S-phase-like cellular condition is found to be essential for the transcription of viral immediate-early and early genes probably attributed to transcription factors such as E2F-1 and Sp1 expressed during S phase (27).

It is of interest to determine whether host cells can monitor EBV lytic replication as DNA damage or abnormal DNA and, if so, how EBV blocks the checkpoint signaling to avoid G1 or G2/M cell cycle arrest and apoptosis. We have previously isolated EBV latently infected Tet-BZLF1/B95-8 cells in which exogenous BZLF1 protein is conditionally expressed under the control of a tetracycline-regulated promoter (26). Using this system, we show here for the first time that induction of EBV lytic replication elicits a cellular DNA damage response dependent on ATM. DNA damage sensor MRN complex and phosphorylated ATM are recruited to viral replication compartments, presumably recognizing newly synthesized viral DNAs as abnormal DNA structures. However, the ATM checkpoint signaling was blocked at downstream of p53. Therefore, although EBV lytic replication elicits ATM-dependent DNA damage response, the virus can skillfully block the host response and actively promote an S-phase-like environment advantageous for viral lytic replication.

**EXPERIMENTAL PROCEDURES**

**Cells**—Tet-BZLF1/B95-8 cells, a marmoset B-cell line latently infected with EBV (26), and Tet-BZLF1/Akata cells, human EBV-positive Burkitt’s lymphoma cells (27), were used. Cells were maintained in RPMI medium supplemented with 15% fetal bovine serum.

**Antibodies**—Primary antibodies were purchased from Cell Signaling (ATM-S1981, Chk1, Chk1-S345, Chk2-T68, p53/NBS1-S345, and p53-S15), Genetex (ATM-S1981, Chk1, Chk1-S345, Chk2-T68, p53/NBS1-S345, and p53-S15), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2),Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2),Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2),Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogene (p53, p21 and MDM2), Santa Cruz (Chk2, Chk2, Mre11, and Nbs1), Oncogen...
ATP containing 5 µCi of [γ-³²P]ATP, and kinase reactions were carried out for 60 min at 37 °C with 250 ng of histone H1 (Calbiochem) as substrate. Reactions were stopped by addition of 6 µl of 5× SDS loading buffer, and the products were resolved by 12% SDS-PAGE followed by autoradiography.

Quantification of Viral DNA Synthesis during Lytic Replication—Tet-BZLF1/B95-8 cells were incubated with 2 µg/ml of doxycycline and harvested at the indicated times. Total DNAs were purified from a total of 3.5 × 10⁶ cells and quantified. Dot-blot hybridization was performed, and quantification of the copy numbers of viral genome per cell were determined as described previously (26).

RESULTS

Induction of the EBV Lytic Program Elicits a Cellular DNA Damage Response—Lytic replication was induced in Tet-BZLF1/B95-8 cells with doxycycline and cells were harvested at the indicated times. Detailed expression profiles of viral proteins after induction of lytic replication with doxycycline were described previously (26). BZLF1 protein became detectable 4 h post-induction (h.p.i.) (26) and reached a plateau at 48 h.p.i. (Fig. 1A). The other immediate-early protein, the BRLF1 protein also appeared at 6 h.p.i (26) with a plateau at 48 h.p.i. (Fig. 1A). Viral early gene products, the BALF2 single-stranded DNA-binding protein, the BBLF2/3 helicase-primase-associated protein, the BALF5 polymerase catalytic protein (data not shown), and the BMRF1 Pol accessory protein (data not shown) appeared after 12 h.p.i. and reached a plateau at 24 h.p.i.

The ATM kinase responds primarily to DSBs, and this pathway can act during all phases of the cell cycle. It has been recently proposed that ATM is usually present as an inactive multimer, and this is activated by autophosphorylation at Ser-1981 after DNA strand breaks or changes in the chromatin structure (11). As shown in Fig. 1B, immunoblotting of cell lysates revealed that the levels of the phosphorylated form of ATM at Ser-1981 increased upon induction, although the total levels of ATM remained constant throughout lytic infection. This was not the case with B95-8 cells treated with doxycycline.

In the presence of DSBs, activated ATM is known to phosphorylate Thr-68 on Chk2, which is required for its activation (9, 28). Immunoblotting with anti-Chk2 Thr-68-specific antibody showed phosphorylation of Chk2 at Thr-68 (Fig. 1B), this becoming detectable at 12 h.p.i., reaching a maximum by 24 h.p.i., and then decreasing by degrees. Phosphorylation of histone H2AX, the response of an ATM-controlled, but Chk2-independent branch of ATM signaling (29, 30), was also examined. As shown in Fig. 1B, significant increase was evident at 12 h post-induction. Next, we focused on phosphorylation of p53. Phosphorylation of p53 at Ser-15, a widely accepted target of ATM kinase activity, was conspicuous at 24 h.p.i. However, the EBV lytic replication program had no significant effect on expression levels of p53 protein throughout lytic infection, in agreement with our previous observation (26).

It should be noted that not only activated ATM but also ATR kinases phosphorylate Chk2 kinase at Thr-68 and up-regulate its activity (9, 28). Phosphorylation of histone H2AX or p53 at Ser-15 is also carried out by ATM/ATR kinases (31, 32). Therefore, EBV lytic replication could activate the ATM, ATR, or both kinases. The ATR kinase responds primarily to DNA replication stress during S phase (3). It can also respond to DSBs if within the S phase, but less efficiently than ATM. In contrast to Chk2, phosphorylation of Chk1 at Ser-345 is known to be carried out mainly by ATR kinase, leading to its activation (10). Therefore, we examined Chk1 phosphorylation at Ser-345 in lytic replication-induced B95-8 cells, but no significant phosphorylation was observed (Fig. 1B). Treatment of cells with hydroxyurea, a well-studied activator of the replication checkpoint, clearly induced Chk1 phosphorylation (Fig. 1B), showing the ATR/Chk1 pathway to be intact in the cells.

Thus, we conclude that EBV lytic replication elicits activation of ATM DNA damage checkpoint signaling rather than the ATR pathway that responds to replication stress.
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**Fig. 2.** Subcellular localization of ATM Ser-1981 after induction of lytic replication. *A,* architecture of viral replication compartments. Tet-BZLF1/B95-8 cells were cultured in the presence of 2 μg/ml doxycycline for 14 h and newly synthesized DNAs were labeled with 10 μM BrdUrd for 1 h prior to harvest. Cells were treated with 0.5% Triton X-100-mCSK buffer and were then fixed with methanol viral replication proteins (BMRF1 and BALF2 proteins), newly synthesized DNA, or viral DNA was visualized by immunofluorescence, BrdUrd staining, or FISH analyses, respectively. Right panels are merged images. *B,* sub-nuclear localization of phosphorylated ATM. Tet-BZLF1/B95-8 cells were cultured in the absence (panel *a*) or presence of 2 μg/ml of doxycycline and harvested at 14 h.p.i. (panels *b* and *d*) or 24 h.p.i. (panels *c* and *e*). Cells were treated with 0.5% Triton X-100-mCSK buffer. The nonionic-detergent-extracted cells were then fixed with methanol. Proteins (BALF2 protein and ATM phosphorylated at Ser-1981) and viral DNA was visualized by immunofluorescence and FISH analyses, respectively. Right panels are merged images.

To ascertain whether lytic replication elicits ATM/Chk2 DNA damage checkpoint signaling in other EBV-latently infected B cells, we examined Akata cells, an EBV-positive B cell line derived from a Burkitt’s lymphoma. As shown in Fig. 1C, phosphorylation of ATM and Chk2 was again observed upon induction of lytic replication. Furthermore, as shown in Fig. 1D, it should be noted that expression of the BZLF1 protein alone in Hela cells did not phosphorylate NBS1 Ser-343, Chk2 at Thr-68, and p53 at Ser-15 as judged by Western blotting, suggesting that expression of the BZLF1 protein itself cannot elicit DNA damage response checkpoint signaling pathways.

Phosphorylated ATM Accumulates in Viral Replication Compartments After Induction of Lytic Replication—Many proteins involved in the DNA damage response accumulate in foci at sites of DSBs or abnormal DNA structures such as single-stranded DNA (33). It has previously been demonstrated that ATM protein becomes associated with chromatin upon ionizing radiation, using a biochemical fractionation procedure in which a portion of the ATM pool was found to be resistant to detergent extraction after treatment with agents that cause DSBs (18, 34). EBV lytic DNA replication occurs at discrete sites in nuclei, called replication compartments, where viral replication proteins cluster and viral DNAs are synthesized (25). Lytic replication-induced Tet-BZLF1/B95-8 cells were extracted with 0.5% Triton X-100-mCSK buffer to solubilize DNA-unbound forms of viral or cellular proteins (35). As shown in Fig. 2A, the BMRF1 and BALF2 viral replication proteins were colocalized in the nuclei after induction of lytic replication. The sites were completely coincided with the localized foci of newly synthesized viral DNA as judged by BrdUrd incorporation and FISH analyses (Fig. 2A, panels *b* and *c*). Thus, since the BALF2 or BMRF1 protein-localized sites represent loci of viral DNA synthesis, these were used as markers for viral replication compartments. We examined whether activated DNA damage responsive proteins accumulate in such foci after induction of lytic infection. As shown in Fig. 2B, in the lytic replication-induced cells, ATM phosphorylated at Ser-1981 was found to be resistant to detergent extraction and became colocalized with viral DNAs in the replication compartments, strongly suggesting that activated ATM recognizes and binds to newly synthesized viral DNA. In contrast, phosphorylated ATM was not observed in the latently infected cells (Fig. 2B, panel *a*).

The Nbs1 and Mre11 Proteins Constituting the MRN Complex Accumulate in Viral Replication Foci—The MRN complex consisting of Mre11, Rad50, and Nbs1 has been suggested to act as a damage sensor (14, 18), facilitating ATM activation (15). After ionizing radiation, relocalization of the MRN complex at sites of damage is readily observed in detergent-extracted cells (36) and independent of ATM and γH2AX (18, 31, 36). As shown in Fig. 3A, the levels of Mre11 and Nbs1 were constant throughout the lytic infection, unlike the case for adenovirus infection which degrades the MRN complex (18, 37). Activated ATM also phosphorylates Ser-343 on Nbs1 (12, 13). As shown in Fig. 3A, increase in levels of phosphorylated form of Nbs1 at Ser-343 was observed, this becoming detectable after 12 h.p.i. Next, we assessed the effect of induction of EBV lytic replication on the localization of Mre11 and Nbs1 by immunofluorescence after detergent extraction (Fig. 3B). No staining with Mre11-specific or Nbs1-specific antibodies was observed in the detergent-treated latently infected cells (Fig. 3B, panels *a* and *d*). As shown in Fig. 3B, panel *b*, ionizing radiation resulted in distinct staining of Nbs1 in the nuclei.
indicating that the MRN complex is activated and retained in the damaged sites. Upon induction of lytic replication, Mre11 and Nbs1 proteins became resistant to detergent treatment and colocalized predominantly in viral replication compartments represented by the BAF2 staining (Fig. 3B, panels c and e). Once the pools of endogenous Mre11 and Nbs1 protein were concentrated in this way, the associated fluorescence became resistant to extraction with a mild detergent-containing extraction buffer, indicating that the Mre11 and Nbs1 proteins became not only redistributed to, but also retained within the close vicinity of newly synthesized viral DNA. It is likely that the MRN complex recognizes newly synthesized viral genomic DNA in the replication compartments as abnormal DNA structures and binds to them.

Inhibition of p53 Downstream Target Gene Expression after Induction of EBV Lytic Infection—As described above, induction of EBV lytic replication elicited phosphorylation of ATM, Nbs1, γH2AX, Chk2, and p53. Phosphorylation of p53 at Ser-15 in response to DNA damage usually correlates with both accumulation of total p53 protein as well as with the ability of p53 to transactivate downstream target genes in wild-type cells. However, the level of p53 here proved to be constant throughout lytic replication (Fig. 1B). Therefore, we examined expression levels of p53-transcriptional targets such as p21cip1/waf1, a cyclin-dependent kinase inhibitory protein, and MDM2, a ubiquitin protein ligase, for p53 (Fig. 4A). When Tet-BZLF1/B95-8 cells were irradiated, the levels of p21cip1/waf1 and MDM2 were elevated remarkably in response. In contrast, the level of MDM2 ubiquitin ligase came down with progression of lytic replication, and the level of p21cip1/waf1 was low and almost unchanged throughout the lytic replication. These suggest that EBV lytic program possesses a defense system to prevent p53 downstream signaling. The levels of p53-independent CDK inhibitors such as p27kip1 and p16ink4a were also unchanged (data not shown). These observations suggest that p53 downstream signaling is blocked during EBV lytic infection despite the appearance of phosphorylated p53.

Next, the relative abundance of p53 was analyzed by Western blot in the presence of chemical proteasome inhibitor, MG132. As shown in Fig. 4B, the amounts of p53 in EBV latently infected cells were almost constant in the absence and presence of MG132. By contrast, MG132 stabilized p53, resulting in comparative accumulation of p53 in the lytic infection-induced cells. Thus, it was clearly demonstrated that p53 turn-
over is regulated by proteasome degradation after induction of the lytic infection. These results are intriguing because the degradation of p53 via proteasome appears to be one of the mechanisms that explain why the phosphorylated form of p53 at Ser-15 cannot transactivate its downstream factors such as p21 and MDM2. Since the level of MDM2 ubiquitin ligase came down with progression of lytic replication, degradation of p53 might be mediated by a direct interaction and recruitment of ubiquitin ligase activity other than MDM2.

Activation of S-phase-promoting CDK Activity throughout Lytic Infection—As shown in Fig. 4C, we observed that the levels of cyclin E and cyclin A. continued to be elevated, whereas the level of cyclin B was constant during the lytic replication, confirming our previous report (26). These data strongly suggest that S-phase-promoting CDK, namely cyclin A/E-Cdk2, is activated during lytic infection. In fact, as shown in Fig. 4D, cyclin E- and cyclin A-associate CDK activity increased as lytic replication progressed, whereas cyclin B-associate kinase activity was unchanged and rather down-regulated at 48 h.p.i. Slow migrating hyperphosphorylated Rb proteins accumulated with progression of lytic infection (Fig. 4C), probably because of elevated cyclin E- and A-associate CDK activity. Also, the levels of E2F-1 increased with progression of lytic infection (Fig. 4C). Hyperphosphorylation of Rb may result in release of an active form of E2F-1, which binds to its own binding site on E2F-1 promoters and enhances expression (39). Thus, these observations clearly indicate that EBV lytic replication occurs in an S phase-like cellular environment regardless of elicitation of the ATM DNA damage response.

p53 Physically Interacts with EBV BZLF1 Protein in Vivo and Is Recruited to Viral Replication Compartments—Zhang et al. (40) has previously reported that the BZLF1 protein inhibits transactivation activity of p53, likely through the direct interaction demonstrated using an adenovirus overexpression system. To confirm actual protein-protein interaction in vivo and resolve the paradox between induction of ATM DNA damage response and inhibition of p53 downstream events, immunoprecipitation analyses with an anti-BZLF1 protein-specific antibody were performed with lytic replication-induced B95-8 cell extracts. As shown in Fig. 5A, the antibody immunoprecipitated phosphorylated p53. Thus, the physical interaction between the BZLF1 protein and the phosphorylated form of p53 was confirmed not only in the overexpression system (40) but also in the lytic phase-induced B95-8 cells.

Next, we examined the localization of p53 using a specific antibody to p53 or phospho-Ser-15 p53. In latently infected cells p53 was sensitive to detergent treatment and no staining with a p53-specific antibody was observed (Fig. 5B, panels a and c). In contrast, the p53-associate fluorescence became resistant to extraction by a mild detergent-containing buffer after induction of lytic replication. Thus, p53 became retained in the nuclei and colocalized with the BZLF1 protein in the replication compartments. Also, p53 phosphorylated at Ser-15 was recruited to viral replication compartments. Tet-BZLF1/B95-8 cells were untreated or treated with 2 μg/ml doxycycline and harvested at 48 h.p.i. Clarified lysates were prepared and subjected to immunoprecipitation (IP) analysis with anti-BZLF1 IgG or control rabbit IgG beads. Aliquots of the immunoprecipitated proteins and lysates (Input) were analyzed by Western blotting with anti-p53 monoclonal IgG, anti-p53Ser-15 polyclonal IgG, or anti-BZLF1 polyclonal IgG. B, colocalization of p53 with the BZLF1 protein in viral replication compartments. Tet-BZLF1/B95-8 cells were cultured in the absence (panels a and c) or presence (panels b and d) of 2 μg of doxycycline/ml and harvested at 24 h.p.i. Cells were treated with 9.5% Triton X-100-mCSK buffer, fixed with methanol, and coimmunostained with the indicated antibodies. Panels a and b show images of BZLF1 protein and p53. Panels c and d show images of BMRF1 protein and p53 phosphorylated at Ser-15. Right panels are merged images.

Inhibition of ATM-dependent DNA Damage Response Induced by EBV Lytic Replication Does Not Affect Viral Lytic Replication—Phosphorylation states of ATM DNA damage responsive proteins and expression levels of viral lytic proteins during lytic infection with EBV were examined in the presence or absence of caffeine, a dose that inhibits the kinase activity of both ATM and ATR in vitro (41). Although caffeine treatment of lytic program-induced Tet-BZLF1/B95-8 cells abrogated phosphorylation of target proteins of ATM kinase activity such as Chk2 at Thr-68, ATM at Ser-1981, and Nbs1 at Ser-343, the expression levels of viral replication proteins were not affected at all (Fig. 6A). These data suggest that a caffeine-sensitive kinase is involved in the checkpoint signaling evoked during the EBV lytic infection, while ATM signaling is not absolutely required for lytic replication. Moreover, in order to clarify the effects of caffeine on EBV genome synthesis, Tet-BZLF1/B95-8 cells were treated with doxycycline in the presence or absence of the compound. Total DNA was extracted from the cells, and EBV DNA-specific signals were quantitated. As shown in Fig. 6B, the copy number of the viral DNA was amplified up to more than 1500 copies per cell after 48 h.p.i. in the absence of caffeine. Inhibition by caffeine of ATM-dependent checkpoint activation induced by EBV lytic replication did not affect viral lytic replication at all. It is likely that since ATM DNA damage signaling induced by the lytic replication is blocked at least at the level of p53 by the BZLF1 protein, inhibition of ATM/ATR.
kinase activity by caffeine might have almost no effect on viral lytic replication. We conclude that ATM signaling is not absolutely required for lytic replication.

DISCUSSION

It has been clearly demonstrated here that EBV lytic replication indeed induced the ATM DNA damage response, which was blocked through the interaction between the BZLF1 protein and p53. Also, activated Chk2 is known to inhibit Cdk2/cyclin E or A activity by phosphorylation of Cdk2 at Tyr-15, via down-regulation of CDC25A phosphatase (4), in a p53-independent fashion. Although Chk2 was phosphorylated by ATM as shown in Fig. 1, cyclin A- and E-associated CDK2 activity was increased with the progression of lytic replication. It has been very recently reported that EBNA3a, one of viral proteins expressed during latent infection, interacts with Chk2 to disrupt G2/M checkpoint (42), suggesting inhibition of p53-independent DNA damage signaling pathway. Since the G1- or G2/M-arrested state or apoptosis following DNA damage checkpoint signaling would be unsuitable for viral lytic replication, EBV would create the cell to become an S-phase-like condition.

ATM and the MRN complex function in a common pathway (13), and the MRN complex can function to activate ATM as a damage sensor, in addition to acting as an effector of ATM signaling (18). Clustering of ATM, Mre11, and Nbs1 proteins to the EBV replication compartments from early stages of lytic infection strongly suggests that these damage sensors recognize newly synthesized viral DNAs as abnormal DNA structures. Although ATM/ATR kinase phosphorylates histone H2AX and p53 at Ser-15, ATR kinase activity predominantly phosphorylates Chk1 at Ser-345 leading to increased Chk1 activity (6, 7, 10). Surprisingly, we could not detect any phosphorylation of Ser-345 on Chk1 (Fig. 1B). Further, recruitment of ATR to EBV replication compartments as judged by confocal immunostaining analyses was not observed (data not shown). The ATR kinase responds primarily to chromosomal DNA replication stress during S phase (3). Taken together, our results indicate that EBV lytic replication preferentially activates the ATM DNA damage response. At 24 h after lytic induction, some cells exhibited BrdUrd staining throughout nuclei. We could not observe any viral replication compartments stained with specific antibodies to the BMRF1 or the BALF2 lytic proteins in such cells, suggesting that EBV lytic replication might not occur in S-phase cells in which chromosomal DNA replication has already started. If EBV lytic replication had arrested fork movements of chromosomal DNA replication, ATR DNA damage checkpoint could be activated. Further experiments are needed to clarify this point.

The expression level of E2F-1 is elevated as lytic replication progresses. Although the precise mechanism remains to be determined, maintaining low levels of CDK inhibitors during lytic infection might result in accumulation of the hyperphosphorylated form of Rb protein by cyclin E and A associated kinase activity. Phosphorylation of the Rb protein leads to release from its inhibitory effects on E2F-1 as transcription factor (43), thereby deregulating E2F-1 in favor of an S-phase environment. E2F-1 can transactivate not only the cyclin E and cyclin A genes but also its own expression. Alternatively, it has been very recently reported that the BZLF1 protein by itself activates E2F-1, cyclin E, and Cdc25A involved in cell cycle progression in telomerase-immortalized human keratinocytes and primary tonsil keratinocytes (44) and further that the other immediate-early transactivator, BRLF1 protein, can in-
duce contact-inhibited, quiescent human fibroblasts to enter the S phase with dramatic increase in the level of E2F-1 (45). Also, activated ATM or Chk2 phosphorylate and activate E2F-1 in response to DNA damage (46, 47) and thus the fact that inhibition of ATM kinase activity by caffeine treatment reduced the level of E2F-1 (data not shown) is in line with the literature.

Infection with an E4-deleted adenovirus results in synthesis of end-joined large viral genomes that are recognized as abnor-
mal DNA structures by the MRN complex, leading to activation of cell cycle checkpoints. As a result, ATM damage response signaling is activated and Chk1, Chk2, 53BP1, p53, Nbs1 and ATM are phosphorylated (18, 37). Similarly, in the case of EBV, intermediate replication products in lytic phase are thought to be large head-to-tail concatemers with branched structures that might be generated by homologous recombination coupled with repetition events (48, 49). In homologous recombination repair the MRN complex, most likely with help of other nuclease, might resect the DNA to provide ssDNA overhangs necessary for DNA pairing and strand exchange, since the MRN complex is known to possess exonuclease activity. The Rad50 subunit of MRN has ATPase activity that is believed to facilitate DNA unwinding. Thus, such higher order intermediate viral genome structures might be recognized and made by the MRN complex. We speculate that this is the case and that the MRN complex then modifies the DNA to create a platform for ATM and other signaling factors (14). When viral lytic DNA replication is blocked by the addition of phosphonoacetic acid (PAA), a herpes virus DNA polymerase specific inhibitor, viral pr...
Epstein-Barr Virus Lytic Replication Elicits ATM Checkpoint Signal Transduction While Providing an S-phase-like Cellular Environment

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