DNA despite replication of viral DNA (32). However, rather high S-phase CDK activity is associated with progression of lytic infection, indicating that the lytic replication occurs under S-phase like cellular conditions (31-33).

The EBV BGLF4 gene product is the only viral protein kinase (PK) which belongs to the group of herpesvirus-encoded Ser/Thr protein kinases, conserved in all herpesviruses (27). Expressed at an early stage after viral reactivation and localized in nuclei, the protein is reported to phosphorylate a number of viral and cellular proteins, including the EBV BMRF1 DNA Pol processivity factor (EA-D) (5), the EBV nuclear antigen leader protein (EBNA-LP) (26), EBNA2 (58), PK itself, and the cellular translation-elongation factor 1 delta (EF-1 δ) (26, 28). Most interestingly, EBV-PK phosphorylates EBNA-LP and EF-1 δ at the same sites as CDK1 (Cdc2) kinase. However, hitherto there has been no evidence that such phosphorylation influences functions of target proteins.

We report here that human MCM4, a subunit of the MCM complex, is phosphorylated in B95-8 and Akata cells when lytic replication is induced. The phosphorylation sites of Thr19 and Thr110 in MCM4 were identified as the same amino acid residues targeted by CDK2/CDK1 kinase, which inactivates DNA helicase activity of the MCM4-6-7 complex. Furthermore, expression of EBV-PK in HeLa cells phosphorylated the same sites on MCM4. In vitro, the sites of the MCM4 protein of the MCM4-6-7 complex was confirmed to be phosphorylated with EBV-PK with the same loss of enzyme activity as with CDK2/cyclin A. Although introducing mutations in the N-terminal six Ser and Thr residues of MCM4 reduced the inhibition by CDK2/cyclin A, EBV-PK inhibited the helicase activity of both wild type and mutant MCM4-6-7 hexamer. It was found that EBV-PK can phosphorylate MCM6 and other sites on

MCM4 in addition to the N-terminal residues. Thus, phosphorylation of MCM4-6-7 complex by redundant actions of CDK kinase and EBV-PK during the lytic replication might inhibit chromosomal DNA replication, including re-replication of chromosomal DNA and/or DNA fork progression, through inactivation of DNA unwinding by the MCM4-6-7 complex.

Materials and Methods

Cells

Tet-BZLF1/B95-8 cells, a marmoset B-cell line latently infected with EBV (32), and Tet-BZLF1/Akata cells, human EBV-positive Burkitt's lymphoma cells (31), were maintained in RPMI medium supplemented with 1 μg/ml of puromycine, 250 μg/ml of hygromycine B, and 10 % tetracycline-free fetal calf serum. To induce lytic EBV replication, a tetracycline derivative, doxycycline, was added to the culture medium at a final concentration of 2 μg/ml. HeLa cells were cultured in DMEM medium containing 10% FCS.

Protein preparation

Cells were harvested, washed with phosphate-buffer saline (PBS), and treated with lysis buffer (0.02 % sodium dodecyl sulfate [SDS], 0.5 % Triton X-100, 300 mM NaCl, 20 mM Tris-HCl[pH 7.6], 1 mM EDTA, 1 mM dithiothreitol) for 20 min on ice. After multiple protease inhibitors (Sigma; 25 μl/ml), 200 μM sodium vanadate and 20 mM sodium fluoride were added, samples were centrifuged at 18,000 g for 10 min at 4 ℃, and clarified cell extracts were assayed for protein concentration using a Bio-Rad kit.

Cellular fractionation

Tet-BZLF1/B95-8 cells (1.5×10^7) were harvested, washed with cold PBS, and then lysed for 10 min on ice with 1 ml of ice-cold modified cytoskeleton (mCSK)

buffer (10 mM Pipes pH6.8, 100 mM NaCl, 300mM Sucrose, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 1mM PMSF, 10μg/ml aprotinin,) containing 0.5 % Triton X-100, multiple protease inhibitors (Protease inhibitor mixture for mammalian cell extracts, Sigma; 25μl/ml), 0.1mM ATP, 200 mM sodium orthovanadate, and 20 mM NaF. The samples were then subjected to centrifugation (2000 x g, 3 min, 4 ℃) for separation into a Triton X-100-extractable fraction and a nuclear pellet. The latter was then washed once with mCSK buffer and resuspended in the same buffer containing 0.1 % Triton X-100, 1 mM ATP and 4 mM $MgCl_2$ at 1.5 x 10^7 nuclei/ml. To separate phosphorylated forms of MCM proteins, samples were applied for SDS-7.5 % (acrylamide, 72; bisacrylamide, 1) gel electrophoresis (SDS-PAGE).

Antibodies

Primary monoclonal antibodies to MCM6, the BMRF1 protein, and Cdk2 were purchased from Santa Cruz (MCM6 H-300), Chemicon (EBV BMRF1-R3) and BD Biosciences (CDK2 clone-55) respectively. Rabbit polyclonal antibodies to the BZLF protein and MCM7 proteins were prepared as described (13, 32). Anti-BGLF4 protein antibody was provided by Dr. Kawaguchi (Tokyo University)(25). Antisera against phosphothreonine at amino acids 19 or 110 of human MCM4 were also prepared as detailed earlier (21). MCM4 was detected either with a monoclonal antibody (kindly provided from Dr. Tamai, MBL. Co.) or with a commercially available polyclonal antibody (BD Biosciences). Highly cross-absorbed secondary reagents for dual-color detection (Alexa-488 and 594) were from Molecular Probes.

Immunoblot Analysis

Equal amounts of proteins were applied for SDS-10 % PAGE (acrylamide, 29.2; bisacrylamide, 0.8). To separate phosphorylated form of MCM proteins, gradient

SDS-PAGE (Daiichi Pure Chemicals: 2/15 PAG Mini), or SDS-7.5 % PAGE (acrylamide, 72; bisacrylamide, 1) were applied. The proteins were then processed as described previously (32). Detection of target proteins was with an enhanced chemiluminescence detection system (Amersham Biosciences).

Immunofluorescence analysis

Cells were treated with 0.5 % Triton X100-mCSK buffer for 10 min on ice, followed by fixation with 70 % methanol for 20 min on ice. The fixed cells were washed with PBS and blocked for 20 min in 10 % normal goat serum in PBS. Staining with primary antibodies (anti-MCM4, or anti-MCM7) was performed overnight at 4 °C in PBS/0.5 % goat serum. Staining with a mouse monoclonal antibody to BMRF1 was carried out for 1h at room temperature. Species-specific secondary antibodies were then applied for 1h at room temperature. Slides were mounted in Vectashield (Vector Labs) for analysis under fluorescence confocal microscope. Images were captured and processed using a Radiance 2000 Confocal System (Bio-Rad). All the primary antibodies were employed at 1:100 dilution, and the secondary antibodies at 1:500 dilution. All washes after antibody incubations were performed with 0.05 % Tween-20 in PBS at room temperature. When cells were stained singly for either antigen with inappropriate combinations of first and second antibodies, no fluorescence was observed. Also no immunofluorescence was observed with alternate filters.

Preparation of Anti-phospho specific antibodies

Antisera against phosphothreonine at amino acids 19 and 110 of human MCM4 were obtained by immunizing rabbits with a synthetic peptide of NH2-SRRGRA(pT)PAQTPRSEC-COOH (for aa 19) or NH2-CGTPRSGVRG(pT)PVRQRPDL-COOH (for aa 110), and purified as described

previously (21).

Purification of human MCM4-6-7 complexes.

Sf21 insect cells were co-infected with recombinant baculovirus expressing human MCM7 and virus expressing both human histidine-tagged MCM4 and -6 at an m.o.i. of 10 and harvested 3 days postinfection. MCM4-6-7 complexes containing histidine-tagged MCM4 were purified by nickel-nitrilotriacetic acid column chromatography and then applied for glycerol density gradient centrifugation (56) as described previously (21). Fractions containing the 600 kDa MCM4-6-7 hexamer determined by each specific antibody were collected and pooled.

Site-directed mutagenesis of the human MCM4 gene was conducted using a QuikChange site-directed mutagenesis kit (Stratagene). The oligonucleotide 5'-GATGCCAGGTCAGCTCCCTCTCAGAGACG-3' was used as primer to introduce a change from Ser to Ala at amino acid 32 and 5'-GGTGTTAGGGGCGCACCTGTGAGACAGAGG-3' was used for the change at amino acid 110. The oligonucleotide 5'-CATCACGGATCGGCCCCGGCGTCGGCCCCGAGCCGC-3' was used as the primer to change both Ser-3 and Thr-7 to Ala, 5'-CGTGGAAGGGCCGCCCCCGCCCAGACG-3' was used for the change at amino acid 19, and 5'-CCGATGCCAACCGCGCCTGGAGTGGAC-3' was used for the change at residue 54. The mutated MCM4 gene forms were cloned into a pAcUW31 vector (Pharmingen) in which the Mcm6 gene had been cloned (56). The entire MCM4 gene has been sequenced and all of the mutated sites were confirmed. Sf21 cells were co-infected with the recombinant baculovirus expressing proteins from mutated MCM4 and MCM6 and a virus expressing the MCM7 protein. The 600 kDa mutated MCM4a-6-7 hexamer was

purified by nickel column chromatography and then by glycerol gradient centrifugation (56).

In vitro kinase assays.

Human MCM4-6-7 or MCM4a-6-7 complexes (500 ng) were incubated with various amounts of EBV BGLF4 protein, kinase negative BGLF4K102I protein or human CDK2/cyclin A (purchased from Upstate.Inc.) in a 50 μl reaction mixture containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM ATP (or 100 μM ATP 2 μCi [gamma-^{32}P]ATP) and 0.2 mM sodium orthovanadate. The reaction mixtures were incubated for 1 h at 37 °C and the products were applied for electrophoresis on 7.5% polyacrylamide gels containing SDS and were detected with an Image guider (BAS2500, Fujifilm).

DNA helicase assays.

For preparation of a substrate for DNA helicase activity, a 17-mer oligonucleotide (5'-GTTTTCCCAGTCACGAC-3') complementary to the M13mp18(+) strand was labeled at the 5'-end with polynucleotide kinase in the presence of [gamma-^{32}P]ATP and then annealed to M13 DNA. Approximately 50 fmol of the annealed oligomer was incubated at 37 °C for 1 h with MCM 4-6-7 proteins in 25 mM Hepes-NaOH, pH 7.5, 1 mM DTT, 4 mM ATP 10 mM $Mg(CH_3COO)_2$, and 0.1 mg of bovine serum albumin per ml. The reaction was terminated by adding SDS to a final concentration of 0.2 %, and an aliquot was electrophoresed on a 12 % acrylamide gel in Tris-borate-EDTA (TBE). The loaded oligomer in the gel was detected with an Image guider (BAS2500, Fujifilm) and quantitated.

To examine the effects of phosphorylation on DNA helicase activity of MCM4-6-7 complexes, EBV BGLF4 protein, kinase negative BGLF4K102I protein or

CDK2/cyclin A were added to the reaction mixture from the start of the DNA helicase assay, and the displaced oligonucleotide was analyzed as described above. Phosphorylation of MCM4 with EBV-PK or with CDK2/cyclin A reached plateau within 3 minutes incubation (data not shown).

Plasmids

The BGLF4 expression plasmid, pME-BGLF4(F), and the control plasmid, pME18S, were as described previously (25, 26, 28). In pME-BGLF4(F), the expression of the 3' Flag epitope-tagged BGLF4 is driven by the SR promoter. HeLa cells were transfected with these plasmid DNAs with Lipofectoamine 2000 (Invitrogen).

Purification of GST fused BGLF4 protein from baculovirus-infected cells.

Sf21 cells were infected with a baculovirus (either Bac-GST-BGLF4 or Bac-GST-BGLF4K102I) at an m.o.i. of 10 and harvested 3days thereafter. Cells were suspended in 10 ml of buffer A (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride and a Protease Inhibitor Mixture for Mammalian Cell Extracts (Sigma; 25 μl/ml) and then sonicated. After the insoluble materials were removed by centrifugation, the supernatants were reacted with 500 μl of a 50% slurry of glutathione-Sepharose beads (Amersham Pharmacia) for 2 h. The beads were then extensively washed with buffer A-2 (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride) and eluted in buffer A-2 containing 10 mM glutathione. The eluted proteins (GST-BGLF4, and GST-BGLF4K102I) were finally applied to Mono Q column chromatography and peak fractions were collected and pooled.

RESULTS

Effect of the EBV lytic replication on MCM loading onto chromatin.

It has been reported that infection of quiescent primary fibroblasts (G0 phase) with human cytomegalovirus (HCMV) induces expression of all pre-replicative factors like the ORC complex, CDC6, Cdt1, and the MCM complex, but that the virus prevents pre-replicative complex assembly by inhibiting MCM complex loading onto chromatin (4, 53). Therefore, we examined whether the MCM complexes are detached from chromatin after induction of EBV lytic replication in cycling cells latently infected with EBV. Tet-BZLF1/B95-8 cells were treated with doxycycline to induce lytic replication (32). We have previously demonstrated subcellular dynamics of EBNA1, viral replication proteins, histones, and human chromosomal DNA replication initiation proteins in lytic program-induced Tet-BZLF1/B95-8 cells using biochemical fractionation methods with a buffer containing non-ionic detergent Triton X-100 at relatively physiological salt condition (8, 9). The treatment extracts not only cytoplasmic but also nuclear proteins not tightly bound to nuclear structures (8, 9). Proteins remaining in extracted nuclei have been shown to indeed bind to chromatin or viral DNA/nuclear matrix material (12, 15). With these procedures the MCM4 and MCM7 proteins were extracted with the buffer, and most of the remainder were solubilized after DNase I treatment (8). Thus, the detergent resistant fractions of MCMs represent their chromatin binding. As shown in Fig.1A, all of the BZLF1 immediate-early protein became bound to chromatin or viral DNA/nuclear matrix material. In contrast, almost MCM4, 6, and 7 in the G2/M phase were sensitive to the detergent treatment, indicating release of the MCM complex from chromatin DNA. These results confirmed reproducibility of the fractionation procedure, consistent with

our previous observations (8, 9, 16).

In EBV latently infected cells about half of the MCM proteins proved resistant to the detergent treatment, indicating MCM loading onto chromatin DNA (Fig.1A. 0 h). Although two forms of MCM4 were detected, slower-migrated band represents hyper-phosphorylated form while faster-migrating band is hypo-phosphorylated. Levels of the slowly-migrating forms of MCM4, representing the hyperphosphorylated form as determined previously (16), increased with progression of lytic replication by an unknown mechanism, while level of CDC6 remained constant. The ratios of the chromatin-bound to unbound forms of MCM6, and MCM7 were essentially unchanged before and after induction. On the other hand, levels of the chromatin-unbound forms of MCM4 increased in some degree after induction. For example, most of MCM4 protein phosphorylated at Thr110 was detached from chromatin (Fig.1A), but about half of MCM4 phosphorylated at Thr19 became bound to chromatin (data not shown). Thus, the picture of regulation of chromatin binding of the MCM complex is likely to be very complex. Phosphorylation of some sites on MCM4 appears to trigger the dissociation of the MCM complex from chromatin. It was previously demonstrated that the EBV lytic replication arrests cell cycle and chromosomal DNA replication (32). Thus, the chromosomal DNA replication arrest during lytic infection might be partly due to dissociation of the MCM complex from chromatin. However, overall picture of MCM attachment did not dramatically change after induction of EBV lytic replication, suggesting that other mechanisms also operate at stages after loading MCM complex onto chromatin.

MCM proteins are localized outside of viral replication compartments in nuclei.

Tet-BZLF1/B95-8 cells were treated with doxycycline to induce lytic

replication (32), harvested and treated with a buffer containing the non-ionic detergent Triton X-100. This treatment is known to disrupt nuclear envelopes, but the nuclear lamina remains intact, thus maintaining nuclear structures (18). On confocal immunofluorescence analyses, the sites stained with anti BMRF1 replication protein specific antibodies completely coincided with foci of newly synthesized viral DNA as judged by 5-bromodeoxyuridine (BrdUrd) incorporation and FISH analyses (8, 33). Thus, BMRF1 protein-localized sites represent loci of viral DNA synthesis, termed viral replication compartments. As shown in Fig.1B, the detergent-resistant forms of MCM4 and MCM7 proteins were localized outside of these compartments in nuclei, indicating chromatin binding during lytic infection.

Induction of EBV lytic program elicits phosphorylation of Thr19 and Thr110 on MCM4.

Phosphorylation of Thr19 and Thr110 of MCM4 in human and mouse MCM4-6-7 complexes is associated with inhibition of the associated DNA helicase activity (20). In order to investigate whether chromosomal replication arrest induced by the EBV lytic replication is partly due to inactivation of the MCM complex, we examined the phosphorylation state of MCM4 on Thr19 and Thr110 in Tet-BZLF1/B95-8 cells after induction of lytic replication. Cells were harvested at the indicated times and applied for westernblotting analysis with specific antibodies (Fig.2). Detailed expression profiles of viral proteins after induction of lytic replication with doxycycline have been described previously in Tet-BZLF1/B95-8 cells (32), BZLF1 protein becoming detectable 4 h.p.i. (32) and reaching a plateau at 24 h p.i., as here shown in Fig. 2 (Tet-BZLF1/B95-8). The EBV-PK encoded by the BGLF4 gene also appeared at 6 h.p.i. (data not shown) with a plateau at 24 h.p.i. in lytic

replication-induced Tet-BZLF1/B95-8 cells. As shown in Fig.2, using phospho-specific antibodies raised against two sites (Thr-19 and Thr-110) on MCM4, it was established that induction of lytic replication in Tet-BZLF1/B95-8 and Tet-BZLF1/Akata cells caused a time dependent increase in the phosphorylation of Thr-19 and Thr-110 on MCM4 (Fig. 2), although their phosphorylation levels of MCM4 of B95-8 cells treated with doxycycline as a control were low and essentially constant (Fig.2). It has been shown that the amino-terminal region of MCM4 in an MCM4-6-7 complex is extensively phosphorylated with CDK2/cyclin A or CDK1/cyclin B (29) and that this phosphorylation inhibits DNA helicase activity of the complex in vitro and in vivo (20, 22). Thus, the finding that MCM4 is phosphorylated at Thr-19 and Thr-110 is suggestive of inactivation of DNA helicase activity of the MCM complexes during EBV lytic replication.

Expression of the EBV-PK in Hela cells leads to phosphorylation of Thr-19 and Thr-110 of MCM4.

It has been demonstrated that during lytic replication levels of cyclin E and cyclin A are elevated and eventually CDK2 activity rises and also that high CDK1/cyclin B activity is maintained (31-33). Thus, the MCM4 phosphorylation is conceivably due to high CDK2 or CDK1 activity. However, EBV also expresses viral protein kinase (PK) encoded by the BGLF4 gene, which is known to phosphorylate the same sites as CDK1 kinase (27). CDK2 kinase also sometimes phosphorylates the target sites of CDK1 kinase. Therefore, since there is a possibility that the EBV-PK is involved in MCM4 phosphorylation during lytic infection, we examined phosphorylation of MCM4 at Thr-19 and Thr-110 after BGLF4 expression plasmid, pME-BGLF4(F) (25) or the control vector, pME18S were transfected into HeLa cells. A

marked increase was observed in the level of phosphorylation at Thr-19 and Thr110 in cells expressing the BGLF4 protein (Fig. 3A), although levels of the slowly migrating forms of MCM4 with expression of EBV-PK were not so distinct. These observations clearly indicate that expression of EBV-PK results in phosphorylation of MCM4 including at least Thr-19 and Thr-110 sites in vivo.

Expression of the EBV-PK inhibits cell proliferation.

To determine whether expression of the EBV-PK inhibits cell proliferation, Hela cells were seeded at 6 x 10^5 per ml, and the cells were counted 0, 24, and 48h after BGLF4 expression plasmid, pME-BGLF4(F) or the control vector, pME18S were transfected into HeLa cells. (Fig. 3B). Growth of Hela cells slow down following transfection of pME-BGLF4(F). In contrast, when cells were transfected with the control vector, the cells continued to proliferate normally. Thus, it was clearly demonstrated that the expression of the BGLF4 protein inhibits the proliferation of Tet-BZLF1/B95-8 cells.

Both the EBV-PK and CDK2/cyclin A phosphorylate Thr-19 and Thr-110 sites on MCM4 of the MCM4-6-7 complex in vitro.

Next, we examined whether the EBV-PK can directly phosphorylate Thr-19 and Thr-110 sites on MCM4 of the MCM4-6-7 complex in vitro (Fig. 4). As shown in Fig.4A, the recombinant His-tagged MCM4-6-7 hexamer was purified through Ni-column chromatography and glycerol density gradient centrifugation from extracts of recombinant baculovirus-infected cells and used as a substrate in *in vitro* kinase assays (Fig. 4A). Also, wild-type (WT) and the kinase-inactive BGLF4 proteins were purified from the recombinant baculovirus-infected cells. Both the WT BGLF4 protein and CDK2/cyclin A complex could phosphorylate MCM4 of the MCM4-6-7 hexamer at

Thr-19 and Thr-110 sites in a dose dependent manner, whereas the kinase-inactive BGLF4K102I protein did not (Fig. 4B). The mobilities of the MCM4 phosphorylated by the BGLF4 protein and by CDK2/cyclin A differed, suggesting that EBV-PK might also phosphorylate many sites on MCM4 in addition to those by CDK2. Whatever the case, the data indicate that EBV-PK is able to phosphorylate at least Thr-19 and Thr-110 of MCM4 in MCM complexes directly.

DNA helicase activity of MCM4-6-7 hexamers is inhibited by EBV-PK as well as by CDK2/cyclin A.

To date, DNA helicase activity has been identified for the MCM4-6-7 complex exclusively. The effect of EBV-PK on MCM4-6-7 complex-associated DNA helicase activity was examined using a ^{32}P-labeled 17-mer annealed to M13ssDNA as the substrate (Fig.5). The purified MCM4-6-7 hexamer was able to effectively unwind the 17-mer primers from annealed M13 DNA substrates (Fig.5B) and this was inhibited by addition of CDK2/cyclin A, as reported previously (20). Similarly, EBV-PK inhibited the DNA helicase activity of the complex in a dose dependent manner, whereas kinase–negative GST-BGLF4K102I (up to 200 ng) did not (Fig 5B).

EBV-PK inhibits helicase activity of MCM4-6-7 hexamers containing mutations in amino N-terminal phosphorylation sites of MCM4 unlike CDK2/cyclin A.

To determine whether the suppression of MCM4-6-7 hexamer-associated helicase activity by EBV-PK is actually due to phosphorylation of specific sites on MCM4 or not, mutated MCM4-6-7 hexamers containing six amino acid mutations (to alanine) at amino N-terminal phosphorylation sites on MCM4 (3/7/19/32/54/110m), and therefore resistant to phosphorylation by CDK2/cyclin A, was examined for DNA helicase activity in the presence of EBV-PK. As shown in Fig.6A, the mutated MCM

was sensitive to the inhibition of DNA helicase activity by EBV-PK as well as the WT MCM complex. In contrast, the mutated MCM was resistant to the inhibition by CDK2/cyclin A (compare Fig.5B with Fig.6A). Thus, EBV-PK inhibited the MCM complex-associated helicase activity more powerfully than CDK2/cyclin A. Although phosphorylation of the N-terminal six amino acids in MCM4 is mainly responsible for inhibition of MCM4-6-7 associated DNA helicase, EBV-PK might further phosphorylate other sites of MCM4 or other subunits of the MCM complex, causing inhibition of the helicase activity.

EBV-PK phosphorylates MCM6 and other site(s) of MCM4 in addition to amino N-terminal six Ser/Thr residues.

We examined whether the EBV-PK can directly phosphorylate other site(s) besides the amino N-terminal six Ser/Thr residues on MCM4 and also other subunits of the MCM4-6-7 complex in vitro (Fig.6B). Human intact MCM4-6-7 and mutated MCM4a-6-7 hexamers containing six amino acid mutations (to alanine) at amino N-terminal phosphorylation sites on MCM4 (3/7/19/32/54/110m) were used as substrates for *in vitro* kinase assays. CDK2/cyclin A phosphorylated mainly MCM4 of the MCM4-6-7 hexamer but hardly the N-terminal mutated MCM4. Thus, CDK2/cyclin A phosphorylates the N-terminal Ser/Thr residues on MCM4 mainly. In contrast, EBV-PK could clearly phosphorylate the mutated MCM4 of the MCM4a-6-7 hexamers, indicating that EBV-PK phosphorylates other site(s) in addition to amino N-terminal phosphorylation sites on MCM4. Phosphorylation of MCM6 and 7 by CDK2/cyclin A was scarcely observed (Fig.6B). When intact MCM4-6-7 complex was reacted with EBV-PK, a broad band of MCM4 was observed in SDS-PAGE probably due to phosphorylation (Fig 6C). However, when the mutated complex was reacted with

EBV-PK, the mobility of the mutated MCM4 in SDS-PAGE was virtually unchanged, distinguishing the mutated MCM4 from MCM6 (Fig. 6C). As shown in Fig. 6B, the EBV-PK phosphorylated both MCM6 and the mutated MCM4. Collectively, EBV-PK inhibits the MCM complex-associated DNA helicase activity through phosphorylation of many sites including amino N-terminal six phosphorylation sites on MCM4 and also MCM6 of the MCM4-6-7 hexamer.

DISCUSSION

EBV lytic infection induces hyperphosphorylation of Rb with elevated levels of E2F-1, cyclin E and cyclin A, and their associated CDK2 kinase activities, while cyclin B/CDK1 activity remains constant (32, 33). The present study provided evidence with phospho-specific antibodies that virus-mediated changes occur in the phosphorylation of MCM4 of the MCM complex, with involvement not only of CDK2/CDK1 but also of EBV-encoded protein kinase. These molecules are likely to act redundantly.

MCM4 exhibits different phosphorylation states that depend on the cell cycle phase and their association with chromatin (16). In the normal cell cycle, MCM4 is phosphorylated by CDK2/cyclin A and CDK1/cyclin B, primarily during S-phase and mitosis, respectively (17, 22). The phosphorylation of a mutant complex containing mouse MCM4 mutated at six sites (3/7/19/32/53109) showed almost no phosphorylation in this region of MCM4 (20). Thus, these three Ser-Pro (3,32, and 53) and three Thr-Pro (7,19, and 109) sites are required for phosphorylation of MCM4 in MCM4-6-7 complex by CDK2/cyclin A. The same sites in the amino-terminal region of MCM4 appeared to be required for the phosphorylation by CDK1/cyclin B in vitro (17), suggesting that both CDK2/cyclin A and CDK1/cyclin B have similar specificity of

substrate recognition. Furthermore, Komamura-Kohno et al. have recently reported that phosphorylation of MCM4 at Thr7, Thr19, Ser32, Ser88 and Thr110 in the M phase requires CDK1 (Cdc2), although they do not deny the possibility of the phosphorylation of these sites on MCM4 by CDK2 (29). Therefore, it must be noted that there is also a possibility of the involvement of CDK1/cyclin B in the MCM4 phosphorylation, since the CDK1/cyclin B activity remains essentially constant and high throughout lytic replication (33).

MCM proteins are present in cells as chromatin-bound as well as unbound forms, but at G2/M phase, almost all are heavily phosphorylated and present as the unbound form (16). Hyperphosphorylation of MCM complex prohibits inappropriate reloading of MCMs onto chromatin and thereby contributes suppression of re-replication. In heterohexameric Mcm2-7 complexes either the chromatin-bound or the unbound form may be formed (16). Although phosphorylation of MCM4 partly resulted in the dissociation of the MCM complex from chromatin as shown in Fig.1A, the preparation of the chromatin-bound form of MCM proteins was here found almost unchanged. It was suggested here that inhibition of the DNA unwinding function of the hexameric MCM complex on chromatin occurs during the EBV lytic replication as another role for the phosphorylation of MCM4.

In the case of HCMV, it has been reported that the virus prevents pre-replicative complex assembly by inhibiting MCM complex loading onto chromatin (4, 53) but quiescent primary fibroblasts (G0 phase) were used so that there was no expression of pre-replicative factors like the ORC complex, CDC6, Cdt1, and the MCM complex. However, both reactivation of EBV from latent infected B cells and infection of HCMV into cycling cells results in cell cycle arrest and blockage of chromosomal

DNA replication. Since levels of the chromatin-bound forms of MCM4, MCM6, and MCM7 were essentially unchanged before and after induction in our investigation, the chromosomal DNA replication arrest during EBV lytic infection might be cannot be explained only by dissociation of MCM complexes from chromatin. Overlap of multiple factors might result in the chromosomal DNA replication arrest.

Components of the replication fork include the trimeric, single-stranded DNA binding RPA complex and the DNA helicase. The identity of the replicative helicase which functions in the elongation step remains equivocal. Genetic and biochemical evidence supports a role for the MCM2-7 complex providing helicase activity in unwinding DNA at replication origins during initiation (37). The MCM2-7 proteins form a stable complex in vivo (14), although detectable helicase activity is only observed with the MCM4-6-7 subcomplex (19). Current models suggest that this subcomplex may represent the active helicase, while the remaining subunits may have an essential role in regulating its activity (38, 57). Moreover, a role for MCM complex has also been suggested during the elongation step and in budding yeast MCM4 appears to move away from replication origins after initiation of DNA synthesis (1, 34, 47). Although the MCM2-7 related protein MCM8 has been recently reported to possess ATP dependent helicase activity (38), MCM4-6-7 complex is still thought to be as a strong candidate for replicative helicase in the elongation step of DNA replication. Taking this into consideration, MCM4 phosphorylation during EBV productive replication might suppress re-firing of chromosomal DNA replication by blocking DNA unwinding activity and also prevent fork movement by blocking replicative DNA helicase activity. EBV might have adopted multiple redundant pathways to halt the cell cycle. Whatever the underlying mechanism, phosphorylation of MCM4 provides one

means to block chromosomal DNA replication in lytic infected cells.

ACKNOWLEDGEMENTS

We thank Y.Nishikawa for technical assistance. This work was supported by grants-in-aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports, Culture and Technology of Japan (17659138, 16017322 and 15390153 to T.T.) and partly Uehara Memorial Foundation.

REFERENCES

1. **Aparicio, O. M., D. M. Weinstein, and S. P. Bell.** 1997. Components and dynamics of DNA replication complexes in S. cerevisiae: redistribution of MCM proteins and Cdc45p during S phase. Cell **91:**59-69.
2. **Bailis, J. M., and S. L. Forsburg.** 2004. MCM proteins: DNA damage, mutagenesis and repair. Curr Opin Genet Dev **14:**17-21.
3. **Bell, S. P., and A. Dutta.** 2002. DNA replication in eukaryotic cells. Annu Rev Biochem **71:**333-74.
4. **Biswas, N., V. Sanchez, and D. H. Spector.** 2003. Human cytomegalovirus infection leads to accumulation of geminin and inhibition of the licensing of cellular DNA replication. J Virol **77:**2369-76.
5. **Chen, M. R., S. J. Chang, H. Huang, and J. Y. Chen.** 2000. A protein kinase activity associated with Epstein-Barr virus BGLF4 phosphorylates the viral early antigen EA-D in vitro. J Virol **74:**3093-104.
6. **Correa-Bordes, J., and P. Nurse.** 1995. p25rum1 orders S phase and mitosis by acting as an inhibitor of the p34cdc2 mitotic kinase. Cell **83:**1001-9.
7. **Dahmann, C., J. F. Diffley, and K. A. Nasmyth.** 1995. S-phase-promoting cyclin-dependent kinases prevent re-replication by inhibiting the transition of replication origins to a pre-replicative state. Curr Biol **5:**1257-69.
8. **Daikoku, T., A. Kudoh, M. Fujita, Y. Sugaya, H. Isomura, N. Shirata, and T. Tsurumi.** 2005. Architecture of replication compartments formed during Epstein-Barr virus lytic replication. J Virol **79:**3409-18.
9. **Daikoku, T., A. Kudoh, M. Fujita, Y. Sugaya, H. Isomura, and T. Tsurumi.** 2004. In vivo dynamics of EBNA1-oriP interaction during latent and lytic replication of Epstein-Barr virus. J Biol Chem **279:**54817-25.
10. **Diffley, J. F.** 1996. Once and only once upon a time: specifying and regulating origins of DNA replication in eukaryotic cells. Genes Dev **10:**2819-30.
11. **Forsburg, S. L.** 2004. Eukaryotic MCM proteins: beyond replication initiation. Microbiol Mol Biol Rev **68:**109-31, table of contents.
12. **Fujita, M., Y. Ishimi, H. Nakamura, T. Kiyono, and T. Tsurumi.** 2002. Nuclear organization of DNA replication initiation proteins in Mammalian cells. J Biol Chem **277:**10354-61.
13. **Fujita, M., T. Kiyono, Y. Hayashi, and M. Ishibashi.** 1996. hCDC47, a human

member of the MCM family. Dissociation of the nucleus-bound form during S phase. J Biol Chem **271:**4349-54.

14. **Fujita, M., T. Kiyono, Y. Hayashi, and M. Ishibashi.** 1997. In vivo interaction of human MCM heterohexameric complexes with chromatin. Possible involvement of ATP. J Biol Chem **272:**10928-35.
15. **Fujita, M., C. Yamada, H. Goto, N. Yokoyama, K. Kuzushima, M. Inagaki, and T. Tsurumi.** 1999. Cell cycle regulation of human CDC6 protein. Intracellular localization, interaction with the human mcm complex, and CDC2 kinase-mediated hyperphosphorylation. J Biol Chem **274:**25927-32.
16. **Fujita, M., C. Yamada, T. Tsurumi, F. Hanaoka, K. Matsuzawa, and M. Inagaki.** 1998. Cell cycle- and chromatin binding state-dependent phosphorylation of human MCM heterohexameric complexes. A role for cdc2 kinase. J Biol Chem **273:**17095-101.
17. **Hendrickson, M., M. Madine, S. Dalton, and J. Gautier.** 1996. Phosphorylation of MCM4 by cdc2 protein kinase inhibits the activity of the minichromosome maintenance complex. Proc Natl Acad Sci U S A **93:**12223-8.
18. **Hozak, P., A. B. Hassan, D. A. Jackson, and P. R. Cook.** 1993. Visualization of replication factories attached to nucleoskeleton. Cell **73:**361-73.
19. **Ishimi, Y.** 1997. A DNA helicase activity is associated with an MCM4, -6, and -7 protein complex. J Biol Chem **272:**24508-13.
20. **Ishimi, Y., and Y. Komamura-Kohno.** 2001. Phosphorylation of Mcm4 at specific sites by cyclin-dependent kinase leads to loss of Mcm4,6,7 helicase activity. J Biol Chem **276:**34428-33.
21. **Ishimi, Y., Y. Komamura-Kohno, H. J. Kwon, K. Yamada, and M. Nakanishi.** 2003. Identification of MCM4 as a target of the DNA replication block checkpoint system. J Biol Chem **278:**24644-50.
22. **Ishimi, Y., Y. Komamura-Kohno, Z. You, A. Omori, and M. Kitagawa.** 2000. Inhibition of Mcm4,6,7 helicase activity by phosphorylation with cyclin A/Cdk2. J Biol Chem **275:**16235-41.
23. **Itzhaki, J. E., C. S. Gilbert, and A. C. Porter.** 1997. Construction by gene targeting in human cells of a "conditional' CDC2 mutant that rereplicates its DNA. Nat Genet **15:**258-65.
24. **Jares, P., and J. J. Blow.** 2000. Xenopus cdc7 function is dependent on licensing but not on XORC, XCdc6, or CDK activity and is required for XCdc45 loading. Genes Dev **14:**1528-40.
25. **Kato, K., Y. Kawaguchi, M. Tanaka, M. Igarashi, A. Yokoyama, G. Matsuda, M. Kanamori, K. Nakajima, Y. Nishimura, M. Shimojima, H. T. Phung, E. Takahashi, and K. Hirai.** 2001. Epstein-Barr virus-encoded protein kinase BGLF4 mediates hyperphosphorylation of cellular elongation factor 1delta (EF-1delta): EF-1delta is universally modified by conserved protein kinases of herpesviruses in mammalian cells. J Gen Virol **82:**1457-63.
26. **Kato, K., A. Yokoyama, Y. Tohya, H. Akashi, Y. Nishiyama, and Y. Kawaguchi.** 2003. Identification of protein kinases responsible for phosphorylation of Epstein-Barr virus nuclear antigen leader protein at serine-35, which regulates its coactivator function. J Gen Virol **84:**3381-92.
27. **Kawaguchi, Y., and K. Kato.** 2003. Protein kinases conserved in herpesviruses potentially share a function mimicking the cellular protein kinase cdc2. Rev

Med Virol **13:**331-40.
28. **Kawaguchi, Y., K. Kato, M. Tanaka, M. Kanamori, Y. Nishiyama, and Y. Yamanashi.** 2003. Conserved protein kinases encoded by herpesviruses and cellular protein kinase cdc2 target the same phosphorylation site in eukaryotic elongation factor 1delta. J Virol **77:**2359-68.
29. **Komamura-Kohno, Y., K. Karasawa-Shimizu, T. Saitoh, M. Sato, F. Hanaoka, S. Tanaka, and Y. Ishimi.** 2006. Site-specific phosphorylation of MCM4 during the cell cycle in mammalian cells. Febs J **273:**1224-39.
30. **Kreitz, S., M. Ritzi, M. Baack, and R. Knippers.** 2001. The human origin recognition complex protein 1 dissociates from chromatin during S phase in HeLa cells. J Biol Chem **276:**6337-42.
31. **Kudoh, A., T. Daikoku, Y. Sugaya, H. Isomura, M. Fujita, T. Kiyono, Y. Nishiyama, and T. Tsurumi.** 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:**104-15.
32. **Kudoh, A., M. Fujita, T. Kiyono, K. Kuzushima, Y. Sugaya, S. Izuta, Y. Nishiyama, and T. Tsurumi.** 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 77:851-61.
33. **Kudoh, A., M. Fujita, L. Zhang, N. Shirata, T. Daikoku, Y. Sugaya, H. Isomura, Y. Nishiyama, and T. Tsurumi.** 2005. Epstein-Barr virus lytic replication elicits ATM checkpoint signal transduction while providing an S-phase-like cellular environment. J Biol Chem **280:**8156-63.
34. **Labib, K., J. A. Tercero, and J. F. Diffley.** 2000. Uninterrupted MCM2-7 function required for DNA replication fork progression. Science **288:**1643-7.
35. **Lee, J. K., and J. Hurwitz.** 2000. Isolation and characterization of various complexes of the minichromosome maintenance proteins of Schizosaccharomyces pombe. J Biol Chem **275:**18871-8.
36. **Lee, J. K., and J. Hurwitz.** 2001. Processive DNA helicase activity of the minichromosome maintenance proteins 4, 6, and 7 complex requires forked DNA structures. Proc Natl Acad Sci U S A **98:**54-9.
37. **Lei, M., and B. K. Tye.** 2001. Initiating DNA synthesis: from recruiting to activating the MCM complex. J Cell Sci **114:**1447-54.
38. **Maiorano, D., O. Cuvier, E. Danis, and M. Mechali.** 2005. MCM8 is an MCM2-7-related protein that functions as a DNA helicase during replication elongation and not initiation. Cell **120:**315-28.
39. **McGarry, T. J., and M. W. Kirschner.** 1998. Geminin, an inhibitor of DNA replication, is degraded during mitosis. Cell **93:**1043-53.
40. **Mendez, J., X. H. Zou-Yang, S. Y. Kim, M. Hidaka, W. P. Tansey, and B. Stillman.** 2002. Human origin recognition complex large subunit is degraded by ubiquitin-mediated proteolysis after initiation of DNA replication. Mol Cell **9:**481-91.
41. **Mimura, S., T. Masuda, T. Matsui, and H. Takisawa.** 2000. Central role for cdc45 in establishing an initiation complex of DNA replication in Xenopus egg extracts. Genes Cells **5:**439-52.
42. **Nurse, P.** 1994. Ordering S phase and M phase in the cell cycle. Cell **79:**547-50.