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Human cytomegalovirus IE86 protein binds to cellular Mcm3 protein but does not inhibit its binding to the Epstein-Barr virus oriP in U373MG-p220.2 cells

Y.-J. SONG[1], M.F. STINSKI [2]

[1]Department of Life Science, Kyungwon University, Seongnam-Si, Kyeonggi-Do, 461-701, Korea; [2]Department of Microbiology, Carver College of Medicine, University of Iowa, Iowa City, IA 52242, USA

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Summary. – Human cytomegalovirus (HCMV) or its immediate-early IE86 protein alone induces cell cycle in quiescent primary human foreskin fibroblasts (HFFs), but blocks its progression at the G_1/S interphase and inhibits cellular DNA synthesis by a mechanism that is not clearly understood. It is assumed that, in this phenomenon, the binding of minichromosome maintenance (Mcm) proteins to replication origins is blocked. In this work, we analyzed the initiation of DNA replication in HCMV-permissive U373MG cells and used oriP of Epstein-Barr virus (EBV) as a simplified model of a cellular replication origin. Using U373MG cells we found that HCMV IE86 protein was bound to Mcm3, but did not inhibit the cellular DNA synthesis. Using U373MG-p220.2 cells carrying EBV oriP and expressing Epstein-Barr nuclear antigen 1 (EBNA1), we found that EBNA1 as well as Mcm3 were bound to oriP and that neither HCMV nor IE86 protein inhibited the binding of Mcm3 to oriP. Differences between the effects of HCMV on the cell cycle progression in HFFs and U373MG cells are discussed.

Keywords: cell cycle; Human cytomegalovirus; DNA replication

Introduction

HCMV (the subfamily *Betaherpesvirinae*) productively infects differentiated HFFs that are in the G_0/G_1 phase of the cell cycle with low amounts of biosynthetic enzymes for DNA precursor synthesis. Since HCMV is dependent on the host machinery for DNA replication, it induces HFFs to enter the cell cycle and progress into the S-phase to produce enzymes and nucleotides for DNA replication. Although precursors for DNA synthesis are highly activated in HCMV-infected HFFs cells, cellular DNA synthesis is inhibited, but viral DNA synthesis proceeds (Castillo and Kowalik, 2002; Kalejta and Shenk, 2002; Song and Stinski, 2002).

To initiate cellular DNA synthesis, pre-replication complexes (pre-RCs), which consist of the origin recognition complex (ORC1-6), cdt1, cdc6, and Mcm2-7 are formed at the origin of replication (Lei and Tye, 2001; Bell and Dutta, 2002). The ORCs bind to the replication origins throughout the cell cycle. During the G_1-phase of the cell cycle, cdt1 and cdc6 bind to the ORCs and recruit the Mcm proteins, which function as DNA helicases (Lei and Tye, 2001; Bell and Dutta, 2002). In HFFs, HCMV inhibits cellular DNA synthesis by preventing the binding of Mcm proteins to chromatin (Biswas *et al*., 2003; Wiebusch *et al*., 2003). Since the IE86 protein of HCMV inhibits cellular DNA synthesis and blocks cell cycle progression at the G_1/S phase in HFFs, it may be a viral factor that prevents the binding of Mcm proteins to the

E-mail: songyj@kyungwon.ac.kr; fax: +8231-7505389.

Abbreviations: Ad-IE86 = adenovirus vector expressing IE86 protein; Ad-GFP = adenovirus vector expressing green fluorescent protein; BrdU = bromo-deoxy uridine; cdc6 = cell division cycle; cdt1 = chromatin licensing and DNA replication factor 1; ChIP = chromatin immunoprecipitation; EBNA1 = Epstein-Barr nuclear antigen 1; EBV = Epstein-Barr virus; HA = hemagglutinin; HCMV = Human cytomegalovirus; HFF(s) = human foreskin fibroblast(s); IE86 protein = immediate early-86 protein; IE86HL = IE86 protein with histidine 446 and 452 converted to leucines; Mcm = minichromosome maintenance; ORC = origin recognition complex; pre-RC = pre-replication complex; Rb = retinoblastoma; WCEs = whole cell extracts

cellular origins of replication (Murphy *et al*., 2000; Wiebusch and Hagemeier, 2001; Noris *et al*., 2002).

EBV (the subfamily *Gammaherpesvirinae*) replicates as an episome in latently infected cells (Adams, 1987). During latency, a 1.7 kb region of the EBV chromosome, oriP, supports the replication and maintenance of the viral episome in the presence of EBNA1 (Yates *et al*., 1984, 1985; Chaudhuri *et al*., 2001; Hirai and Shirakata, 2001). To initiate the replication of the EBV episome, EBNA1 binds to a 120 bp component of oriP called the dyad symmetry (Gahn and Schildkraut, 1989; Harrison *et al*., 1994; Yates *et al*., 2000). Since EBNA1 lacks enzymatic activity (Middleton and Sugden, 1992), the EBV oriP utilizes cellular Mcm proteins to initiate replication (Chaudhuri *et al*., 2001; Hirai and Shirakata, 2001). Thus, EBV is a good model system for studying cellular DNA synthesis, because it replicates once per cell cycle using cellular proteins during the latent infection (Adams, 1987).

The aim of this study was (i) further analysis of the initiation of DNA replication in HCMV-permissive U373MG cells, namely the binding of IE86 to Mcm3 and the effect of IE86 on cellular DNA synthesis, and (ii) the use of EBV oriP as a simplified model of a cellular replication origin. For this purpose U373MG-p220.2 cells carrying EBV oriP and EBNA1 gene were generated and used for the testing of the binding of EBNA1 and Mcm3 to oriP and the effect of HCMV or IE86 on that binding.

Materials and Methods

Cells, virus, adenovirus vectors, and plasmids. Maintenance and propagation of primary HFFs, U373MG – human glioblastoma-astrocytoma cells and HCMV Towne strain has been described previously (Stinski, 1977). For serum starvation, the cells were washed twice with serum-free medium and grown in a low serum (0.1%) medium for 48 hrs. Replication-defective E1a$^-$, E1b$^-$, and E3$^-$ adenovirus vectors expressing either the green fluorescent protein (Ad-GFP), or the IE86 protein (Ad-IE86) were grown in 293 cells as described previously (Murphy *et al*., 2000). pDEST. SG5-HA-IE86 was a gift from Dr. Jin Hyun Ahn (Sungkyunkwan University, Korea). pDEST SG5-HA-IE86HL was developed using the Quikchange mutagenesis kit (Stratagene).

Generation of U373MG-p220.2 cells. To generate cell lines carrying an autonomously replicating EBV plasmid, U373MG cells were transfected with plasmid p220.2 containing the EBV oriP, the EBNA1 gene and the hygromycin B resistance gene (a gift from Bill Sugden, University of Wisconsin) (Yates *et al*., 1985; Mecsas and Sugden, 1987) by the calcium precipitation method (Graham and van der Eb, 1973). After 24 hrs, hygromycin B resistant U373MG cells were selected by the addition of 100 μg/ml of hygromycin B. After 2–3 weeks, hygromycin B resistant colonies were picked up and sub-cultured.

Immunoprecipitation and Western blot analysis. 1 x 10^7 cells were lysed in buffer containing 20 mmol/l Tris-HCl (pH 7.5), 100 mmol/l NaCl, 0.5 mmol/l EDTA, 1% NP-40, 1 mmol/l PMSF, and protease inhibitor cocktail (Roche). Whole cell extracts (WCEs) were pre-cleared with protein A/G agarose beads (Santa Cruz Biotechnology) and incubated at 4°C overnight with hemagglutinin (HA)-antibody conjugated agarose beads (HA-probe F-7, Santa Cruz Biotechnology). After washing three times with lysis buffer, protein complexes were resuspended with 2x SDS-sample buffer and subjected to western blot analysis with either α-HA or α-Mcm3 antibody (Santa Cruz Biotechnology).

Agarose gel electrophoresis. Gel electrophoresis was performed according to Gardella *et al.* (1984). After electrophoresis, cellular DNAs were analyzed by Southern blot hybridization (Chen and Stinski, 2000).

Chromatin immunoprecipitation (ChIP) and PCR for oriP. ChIP assay was performed according to the manufacturer's instructions (Upstate) with some modifications. 4 x 10^6 cells were fixed with 1% formaldehyde in PBS and harvested (Matsushime *et al*., 1994). Cross-linked chromatin was sonicated to shear DNA using a Virsonic cell disrupter 475 (Virtis). WCEs were diluted 10x in ChIP dilution buffer (16.7 mmol/l Tris-HCl, pH 8.1 containing 0.01% SDS, 1.1% Triton X-100, 1.2 mmol/l EDTA, and 167 mmol/l NaCl) (Matsushime *et al*., 1994) and pre-cleared with non-immune serum and 80 μl of 50% slurry of salmon sperm DNA/protein A agarose at 4°C for 30 mins. Pre-cleared supernatants were incubated with either Mcm3 (a gift from H. Kimura, Mitsubishi Kasei Institute of Life Sciences, Tokyo, Japan) or EBNA1 (a gift from Bill Sugden, University of Wisconsin) antibody or with non-immune serum followed by 60 μl of 50% slurry of salmon sperm DNA/protein A agarose. Immunoprecipitates were washed and eluted by adding 200 μl of elution buffer (0.1 mol/l $NaHCO_3$ containing 1% SDS). Protein-DNA cross-links were reversed by incubating at 65°C for 6 hrs and immunoprecipitated DNA was purified using QIAquick PCR purification kit (QIAGEN). PCR was performed using 1/30th of the purified DNA for 25 cycles for 30 secs at 94°C, 30 secs at 55°C, and 1 min at 68°C to detect the EBV oriP. The primers used to amplify a dyad symmetry of EBV oriP was described (Chaudhuri *et al*., 2001).

Bromodeoxy uridine (BrdU) labeling and immunofluorescence assay. Serum starved U373MG cells were infected with 10 PFU/cell of either Ad-IE86 or Ad-GFP, plated at low density on glass coverslips and grown in high serum medium with 10 μmol/l BrdU (Sigma) to allow re-entry into the cell cycle. After 18 hrs incubation at 37°C, the cells were fixed as described (Korgaonkar *et al*., 2002) and stained with rabbit α-IE86 antibody 6655 (a gift from Jay Nelson, Oregon Health Science University) followed by a FITC-conjugated anti-rabbit immunoglobulin (Santa Cruz Biotechnology). The cells were stained with α-BrdU monoclonal antibody (Amersham Biosciences) and BrdU incorporation was detected with a Texas Red-conjugated anti-mouse immunoglobulin (Santa Cruz Biotechnology). The nuclei were stained with DAPI (Sigma). After staining, the cells were visualized with an Olympus BX-51 light microscope.

Results

IE86 binds to Mcm3 in U373MG cells

In a yeast two hybrid assays, wild type IE86 protein bound strongly to the Mcm3 (data not shown). IE86HL,

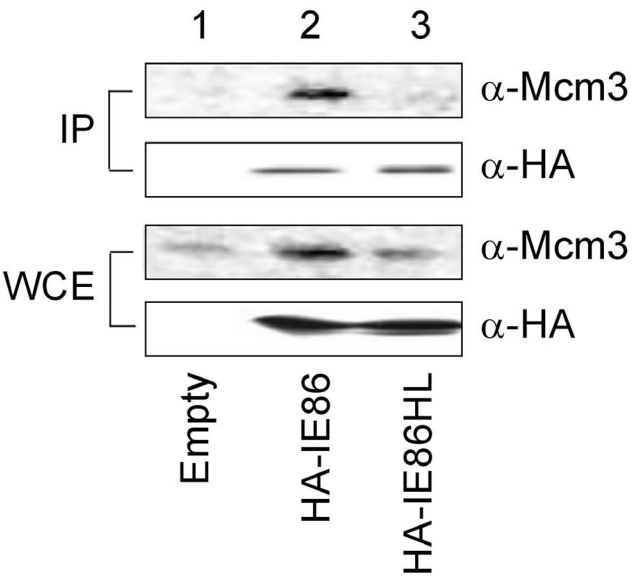

Fig. 1

Western blot analysis of IE86 binding to Mcm3 in U373MG cells

U373MG cells were transfected with an empty vector (lane 1), a vector expressing either HA-IE86 (lane 2) or HA-IE86HL (lane 3). WCE and immunoprecipitated (IP) proteins were visualized using α-Mcm3 or α-HA antibodies.

which fails to inhibit cell cycle progression in HFFs and contains the amino acid his in positions 446 and 452 in a putative zinc finger domain converted to leu, did not bind to the Mcm3 in a yeast two hybrid assays (data not shown). To determine whether IE86 protein binds to Mcm3 in permissive U373MG cells, the cells were transfected with an expression vector for HA epitope-tagged IE86 or IE86HL. WCEs were immune precipitated with anti-HA agarose beads and Mcm3 binding was assessed by Western blot analysis. Mcm3 bound to IE86 was co-immunoprecipitated with IE86, but not with IE86HL in permissive U373MG cells (Fig. 1). As previously reported, the IE86, but not IE86HL protein induced the expression of Mcm3 (Song and Stinski, 2002).

IE86 does not inhibit cellular DNA synthesis in U373MG cells

We determined the effect of the IE86 protein on cellular DNA synthesis using U373MG cells. Serum-starved U373MG cells were infected with 10 PFU/cell of either Ad-IE86 or Ad-GFP. Cells at low density were grown in high serum medium with BrdU to allow re-entry into the cell cycle. After 18 hrs, the cells were fixed and treated with α-IE86 or α-BrdU antibodies for immunofluorescence

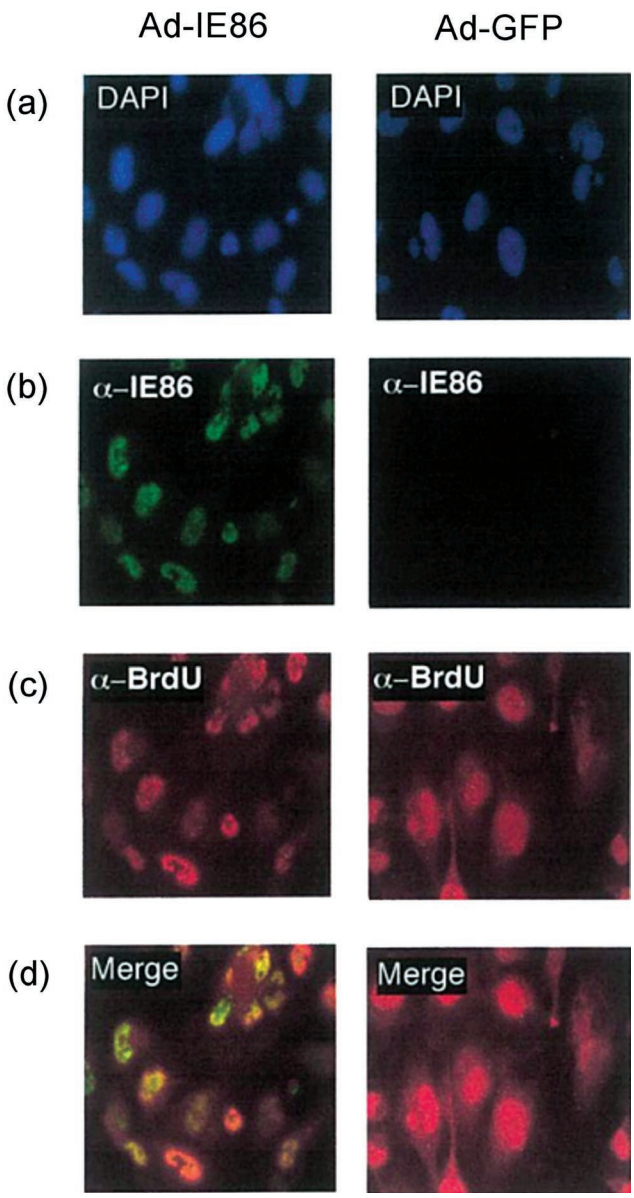

Fig. 2

Effect of IE86 on cellular DNA synthesis in U373MG cells

U373MG cells were infected with either Ad-IE86 or Ad-GFP, labeled with BrdU and subjected to immunofluorescence assay using α-IE86 (b) or α-BrdU (c) antibodies. Nuclei were visualized with DAPI (a). Merged images b and c (d).

assay. In U373MG cells, the IE86 protein appeared in the nucleus as expected and did not inhibit cellular DNA synthesis in contrast to the result found in HFFs (Fig. 2) (Song and Stinski, 2005). Therefore, the IE86 protein utilizes such a mechanism(s) to inhibit cellular DNA synthesis in HFFs cells, which is non-functional or is absent in U373MG cells.

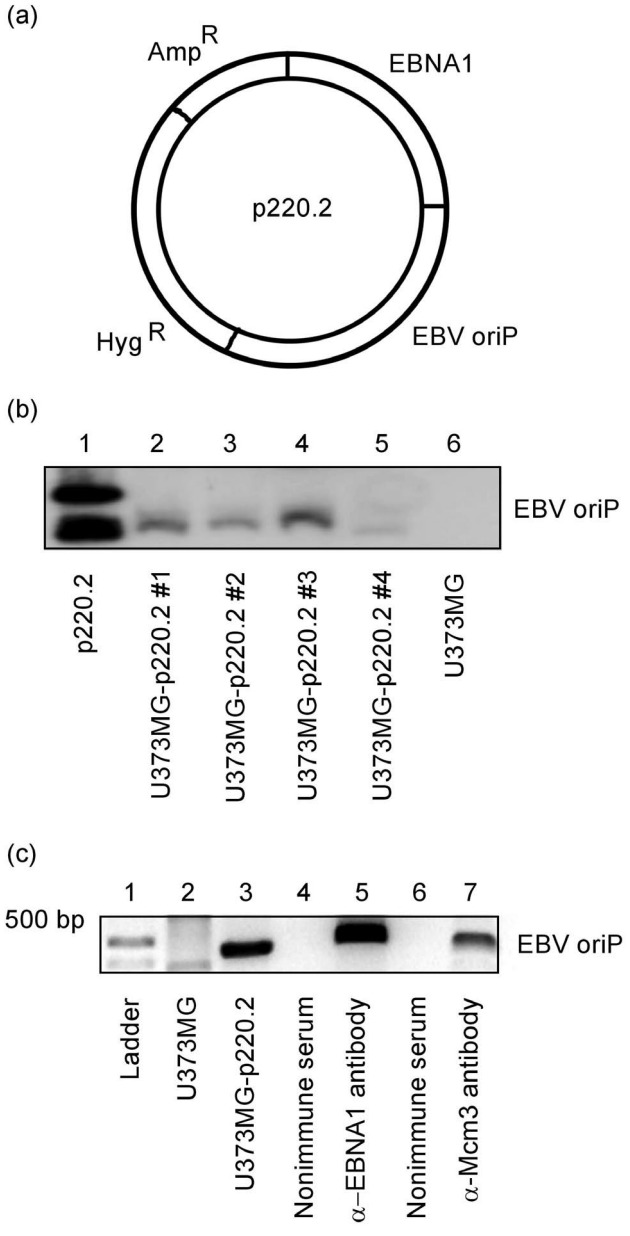

Fig. 3

Generation of U373MG-p220.2 cells and detection of EBNA1 and Mcm3 binding to EBV oriP

(a) Plasmid p220.2. (b) Detection of oriP in U373MG-p220.2 cells by gel electrophoresis and Southern blot hybridization. Plasmid p220.2 (lane 1), U373MG-p220.2 cell clones 1-4 (lanes 2–5), U373MG cells (lane 6). (c) Detection of the binding of EBNA1 and Mcm3 to oriP in U373MG-p220.2 cells. The cell chromatin was precipitated by α-EBNA1 or α-Mcm3 antibodies and subjected to PCR for oriP. DNA size marker (lane 1), non-precipitated U373MG cell chromatin (lane 2), non-precipitated U373MG-p220.2 cell chromatin (lane 3), U373MG-p220.2 cell chromatin precipitated by α-EBNA1 antibodies (lane 5), α-Mcm3 antibodies (lane 7) or non-immune serum (lanes 4 and 6).

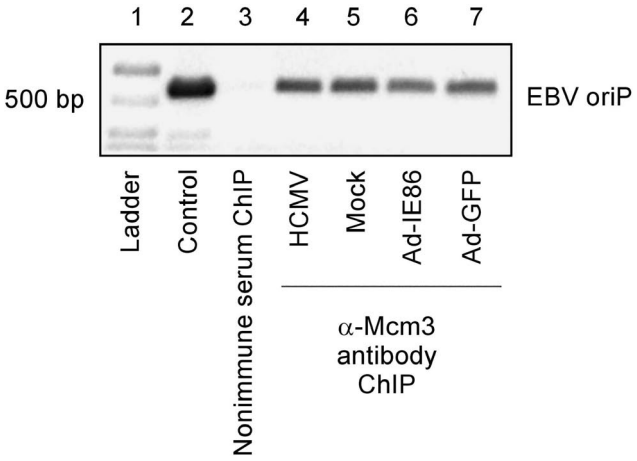

Fig. 4

Effect of HCMV or IE86 on the binding of Mcm3 to EBV oriP in U373MG-p220.2 cells detected by PCR

U373MG-p220.2 cells were mock-infected (lane 5) or infected with HCMV (lane 4), Ad-IE86 (lane 6) or Ad-GFP (lane 7) and subjected to ChIP and PCR for oriP. DNA size marker (lane 1), positive control (lane 2), negative control (lane 3).

Generation of U373MG-p220.2 cells carrying EBV oriP and expressing EBNA1

To determine the effect of HCMV infection or IE86 protein on the binding of Mcm3 to the replication origin, U373MG cells carrying an autonomously replicating plasmid with EBV oriP was generated. U373MG cells were transfected with plasmid p220.2 (Fig. 3a) containing the EBV oriP, EBNA1 gene and hygromycin B resistance gene. The cellular DNAs of hygromycin B resistant U373MG cells were fractionated by electrophoresis and analyzed by the Southern blot hybridization. Hygromycin B resistant U373MG cells contained plasmid p220.2 and migrated at the same size as purified plasmid p220.2 (Fig. 3b). These data indicated that U373MG hygromycin B-resistant cell clones (U373MG-p220.2) were carrying the plasmids p220.2 that contained EBV oriP.

EBNA1 and Mcm3 bind to EBV oriP in U373MG-p220.2 cells

We determined whether EBNA1 or Mcm3 combined with the oriP in U373MG-p220.2 cells by ChIP assay. Chromatin of U373MG-p220.2 cells was precipitated by α-EBNA1 or α-Mcm3 antibodies, and the immunoprecipitated DNA was amplified by PCR to detect the EBV oriP as described in the Materials and Methods. α-EBNA1 or α-Mcm3 antibodies brought down the EBV oriP sequences in ChIP assay

(Fig. 3c). These data indicated that EBNA1 or Mcm3 were bound to the EBV oriP in U373MG-p220.2 cells.

HCMV or IE86 does not inhibit the binding of Mcm3 to EBV oriP in U373MG-p220.2 cells

Since the IE86 protein bound to Mcm3 (Fig. 1), we examined whether the IE86 protein was able to inhibit the binding of Mcm3 to EBV oriP in U373MG-p220.2 cells. Serum starved U373MG-p220.2 cells were mock-infected, HCMV-infected or infected with 10 PFU/cell of either Ad-IE86 or Ad-GPF. After 12 hrs incubation with high serum medium, the cellular chromatin was precipitated with α-Mcm3 antibodies. Immunoprecipitated DNA was amplified by PCR to detect EBV oriP. Neither HCMV nor the viral IE86 protein inhibited the binding of Mcm3 to the EBV oriP in U373MG-p220.2 cells (Fig. 4).

These data indicated that HCMV did not inhibit cellular DNA synthesis by inhibition of binding Mcm3 to the chromatin of U373MG-p220.2 cells. The inhibition of Mcm3 binding to the cellular origin of replication in HCMV-infected HFFs might be a cell-type specific event occurring in HFFs cells, but not in U373MG-p220.2 cells.

Discussion

HCMV infection activates quiescent HFFs to re-enter the cell cycle and induce cellular genes essential for the S-phase entry by inactivating the Retinoblastoma (Rb) family of proteins. However, HCMV infection inhibits cellular DNA synthesis and blocks cell cycle progression at the G_1/S interphase in human fibroblasts by a mechanism that is not clearly understood. By inhibiting cellular DNA synthesis, HCMV can utilize cellular machinery to replicate its own DNA without competing with cellular DNA synthesis.

By separating the chromatin fraction from the soluble fraction in HCMV-infected HFFs followed by Western blot analysis for Mcm proteins, HCMV was reported to inhibit the binding of Mcm proteins to chromatin (Biswas *et al*., 2003; Wiebusch *et al*., 2003). Biswas *et al.* (2003) reported that geminin that inhibited the pre-RC formation, was accumulated in HCMV-infected HFFs. However, the detailed mechanism of cellular DNA synthesis inhibition by HCMV is still unclear.

HFFs expressing the IE86 protein alone exhibit the cell cycle phenotype, which is similar to that of HCMV-infected cells (Murphy *et al*., 2000; Wiebusch and Hagemeier, 2001; Noris *et al*., 2002). The IE86 protein binds to cellular Rb protein and alleviates Rb-mediated repression of E2F responsive promoters to induce a cell cycle progression towards the S-phase (Hagemeier *et al*., 1994; Fortunato *et al*., 1997; Castillo and Kowalik, 2002; Kalejta and Shenk, 2002; Song and Stinski, 2002; Stinski and Song, 2002). However, the IE86 protein inhibits cellular DNA synthesis in HFFs and, in turn, cell division.

Since IE86 protein binds to the Mcm3, the hypothesis that the IE86 protein inhibits cellular DNA synthesis by blocking the binding of Mcm3 to the cellular origins of replication was tested. Using U373MG-p220.2 cells carrying an autonomously replicating plasmid with EBV oriP, the effect of HCMV infection or the viral IE86 protein on the Mcm3 binding to EBV oriP was determined using a ChIP assay. EBV oriP is an excellent model for the study of initiation of DNA replication, because its replication is dependent on the pre-RC. The quality of ChIP assay was controlled, because more Mcm3 proteins were bound to EBV oriP when the cells were treated with mimosine, a drug that inhibited cell cycle progression at the G_1/S transition (data not shown). Although the viral IE86 protein was bound to Mcm3, neither HCMV nor the viral IE86 protein inhibited Mcm3 from binding to EBV oriP in U373MG-p220.2 cells. The IE86 protein did not inhibit cellular DNA synthesis in U373MG cells, although it inhibited the cell cycle progression to the S-phase (Murphy *et al*., 2000; Song and Stinski, 2005). These data indicated that the function of the Mcm proteins is not inhibited by HCMV or the IE86 protein in U373MG cells and suggested that the inhibition of Mcm protein binding to cellular replication origins by HCMV in HFFs is a cell type-specific phenomenon that is not reproducible in U373MG-p220.2 cells.

The inhibition of Mcm binding to the chromatin in HFFs may be due to a cell cycle arrest in the late G_1 phase by HCMV, which does not occur in the U373MG-p220.2 cells. In HFFs, HCMV may inhibit cellular DNA synthesis by utilizing a cellular check point system(s) rather than specifically inhibiting the formation of pre-RC. The cellular check point system(s) may operate at the G_1/S transition in $p53^{+/+}$ cells and during the S-phase in the p53 mutant U373MG cells. We reported that the IE86 protein inhibited cellular DNA synthesis in $p53^{+/+}$ cells, but not in $p53^{-/-}$ cells (Song and Stinski, 2005). Our data further supported the hypothesis that HCMV or IE86 protein utilized the p53 pathway to inhibit cellular DNA synthesis in HFFs, because the p53 protein is mutated in U373MG cells.

Taken together, the IE86 protein binds to Mcm3, but it may not inhibit the function of Mcm3 in DNA replication. IE86 protein may possibly utilize Mcm proteins for viral replication and/or transcription. The function of Mcm3 binding to the IE86 protein and the check point system(s) affecting cell cycle progression in HCMV-infected cells require further investigation.

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