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Effect of Rta protein of Epstein-Barr virus on the cell cycle in HeLa cells

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Summary. – Epstein-Barr virus (EBV) replication and transcription activator (Rta) is an immediate-early transcription factor that mediates the switch from latent to lytic infection. DNA viruses often modulate the function of critical cell cycle proteins to maximize the efficiency of virus replication. Here we have examined the effect of Rta on cell cycle progression. Cell cycle analysis revealed that Rta induced HeLa cells in G0/G1-phase to reenter the S-phase. Analysis of the expression pattern of a key set of cell cycle regulators revealed that expression of Rta inhibited the expression of Rb and p53 and induced the expression of E_2F_1. These findings suggest that Rta plays an active role in redirecting HeLa cell physiology through an Rta-mediated cell cycle transformation.

Keywords: Epstein-Barr virus; Rta; Rb; p53; E2F-1; cell cycle; HeLa cells

Introduction

EBV is a gammaherpesvirus that infects approximately 85% of human population. It causes infectious mononucleosis and is also associated with nasopharyngeal carcinoma, Burkitt's lymphoma, Hodgkin's disease, gastric cancer and post-transplant lymphoproliferative disease (Cohen, 2000; Swanton and Jones, 2001; Young and Murray, 2003; Dolcetti, 2007; Brady *et al.*, 2008). EBV displays two distinct phases in its life cycle: latency and lytic replication. It utilizes two separate classes of genes that carry out very distinct functions in its life cycle (Klein, 1994). Many viruses manipulate the host cell environment, in particular cell cycle progression, as a mechanism to enhance viral replication. Small DNA tumor viruses push cells into the S phase (Song, 2000), while herpesviruses have been found to arrest cells in all phases of the cell cycle (Ehmann *et al.*, 2000). The relationship between the EBV lytic program and the cell cycle has not been elucidated so far.

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Abbreviations: EBV = Epstein-Barr virus; Rta = replication and transcription activator

Rta, encoded by BRLF1, which is expressed within 2 hrs after application of an inducing stimulus (Packham *et al.*, 2001), is a transcription factor expressed by EBV during the immediate-early stage of the lytic cycle. The BRLF1 gene is expressed as a 4.0-kb mRNA that is controlled from a promoter immediately upstream of the BRLF1 ORF. The mRNA is alternatively spliced into a 3.3-kb message encoding the Rta. Rta is a 605-amino acid protein. The N-terminus of Rta contains an overlapping DNA binding and dimerization domain (Manet *et al.*, 1991). The transcription activation domain is found in the C-terminal region of the protein. Rta is known to have many biological functions. Rta binds to a GC-rich motif known as the Rta-responsive element (RRE) found in viral promoters of some EBV lytic genes, such as BMRF1, BMLF1, and BALF2. However, Rta also activates the transcription of viral genes, which lack an RRE in their promoter. For example, Rta activates them indirectly by stimulating cell-signaling pathways, involving phosphatidylinositol 3-kinase (PI3-K) (Darr *et al.*, 2001) and p38 mitogen activated protein kinase (Adamson *et al.*, 2000). Rta may even activate certain viral genes through the modulation of E_2F_1 (Liu *et al.*, 1996).

In addition to its effects on EBV gene transcription, Rta profoundly affects the host cell environment. For example,

Rta forms a complex with Sp1 and MCAF1 on an Sp1-binding site to regulate several host genes (Chang *et al.*, 2005). Rta also initiates a cellular senescence program and promotes growth arrest (Yu *et al.*, 2009). These effects of Rta on the host cell environment presumably serve to enhance the efficiency of lytic virus replication. Many viruses manipulate the host cell environment, in particular cell cycle progression, as a mechanism to create optimal conditions for viral replication. However, the effects of EBV on the cell cycle progression are complex. Previous studies have provided evidence that enforced expression of Zta could induce cell cycle arrest in several tumor cell lines. However, the relationship between Rta expression and the cell has received only limited attention (Staudt and Dittmer, 2007). Here we demonstrate that Rta promotes G_0/G_1 to S transition in HeLa cells. To further define the molecular mechanisms involved in the event, we investigated the expression profiles of cell cycle regulators in cells ectopically expressing Rta. Our study focuses on laying a solid foundation for further research on the function of Rta.

Materials and Methods

Cells. B95-8 cells (EBV-secreting marmoset cell line; Key Medical Laboratory for Tumor Immunology of Shandong Province) were cultured in RPMI 1640 medium (Gibco). HeLa cells (human cervical carcinoma cell line; Key Medical Laboratory for Tumor Immunology of Shandong Province) were maintained in DMEM (Gibco). The cells were supplemented with 10% fetal bovine serum (Gibco) and grown at 37°C in a humidified atmosphere containing 5% CO_2.

Plasmid construction. Total RNA was isolated from B95-8 cells using the TRIzol reagent (Invitrogen), according to the manufacturer's instructions. Reverse transcription was performed with the Moloney murine leukemia virus reverse transcriptase (Promega). The BRLF1 cDNA was amplified by PCR with Pyrobest DNA polymerase (Takara) using the primers 5′-CGGCTAGCCA CCCATGAGGCC TAAAAAGGATGGCTT-3′ (sense) and 5′-GA AAGATCTCTAAAATAAGCTGGT GTCAAAAATAG-3′ (antisense). The reaction conditions were as follows: 95°C/5 mins; 30 cycles of 94°C/30 secs, 57°C/40 secs, and 72°C/2 mins; 72°C/7 mins. The PCR product was ligated into the *Nhe*I and *Bgl*II sites of pIRES2-EGFP (Clontech Laboratories Inc) to form pIRES2-EGFP-Rta.

Transfection. Transfection of HeLa cells was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer′s protocol. Briefly, cells were seeded at 1.5×10^5 cells per well in 12-well plates one day prior to the transfection. Cells were transfected with a total of 1 μg of DNA and harvested at 48 hrs post-transfection.

Cell cycle analysis. 1×10^6 cells were collected, washed with PBS, fixed with 70% cold ethanol for at least 18 hrs, washed with PBS, incubated with 0.1 mg/ml RNase A (Calbiochem) and stained with propidium iodide (Sigma) (20 μg/ml in PBS) for 30 mins at 37°C. Cell cycle analysis was carried out with a FACS (BD Biosciences).

Western blot analysis. Cells were lysed in RIPA buffer (50 mmol/l Tris (pH 8), 150 mmol/l NaCl, 0.1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 1 mmol/l phenylmethylsulfonyl fluoride and Complete protease inhibitor (Roche). The protein concentration was measured spectrophotometrically at 562 nm by using the BCA protein assay reagent (Pierce, Rockford). The cellular lysates were subjected to SDS-PAGE and proteins were electroblotted to PVDF (Millipore). The blots were blocked for 30 mins in TBS containing 5% milk powder and then incubated with the antibodies against Rb (MBL), E_2F_1 (Santa Cruz), p53 (Santa Cruz), Rta (Argene) overnight at 4°C and washed three times in TBST buffer. The blots were then incubated with HRP-conjugated secondary antibodies (Zhongshan Golden Bridge) for 1 hr at room temperature before being washed three times in TBST buffer. Bands were detected by the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

Results

Characterization of the recombinant plasmid pIRES2-EGFP-Rta

To determine the effect of Rta on the cell cycle progression, we first generated the construct expressing Rta protein. BRLF1 cDNA was prepared as template to amplify Rta gene by PCR. An approximately 1818 bp long DNA band was observed using 1.5% agarose gel electrophoresis (Fig.1a). The recombinant plasmid pIRES2-EGFP-Rta was digested by *Nhe*I and *Bgl*II. Approximately 1818 bp long DNA band was observed in the positive clone using 1.5% agarose gel electrophoresis (Fig. 1b).

Effect of Rta on the cell cycle

The pIRES2-EGFP-Rta was used, along with an EGFP marker that was translated from an IRES sequence, to facilitate monitoring of the transfection efficiency. High efficiency of transfection was achieved. Fig. 2a shows that HeLa cells were efficiently transfected and green signals from EGFP were observed clearly. The data in Fig. 2b demonstrates that the transfection with pIRES2-EGFP-Rta resulted in the efficient expression of Rta.

As shown in Fig. 3a and b, a significant enrichment of S phase cells was observed in the cells expressing Rta. After 48 hrs, 53% of pIRES2-EGFP-Rta-expressing cells were in S phase, versus only 30% of cells transfected with the pIRES2-EGFP vector. Moreover, the percentage of the cell population in G_0/G_1 phase was decreased (61% versus 39%). Hence, there was an obvious leakage of G_0/G_1 phase. These results support

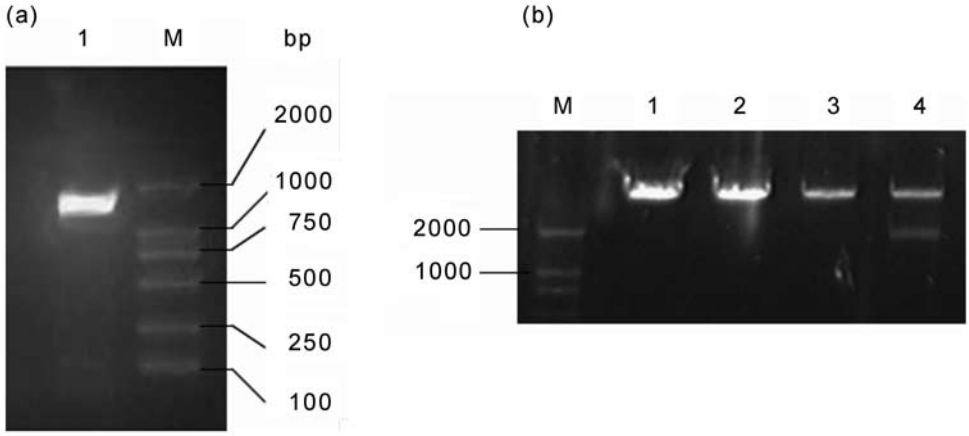

Fig. 1

Construction of the plasmid expressing Rta

(a) PCR amplification of Rta gene. Rta gene (1), DNA size marker (M). (b) Digestion of the plasmid construct with *Nhe*I and *Bgl*II. Empty plasmid (1–3), the plasmid construct (4).

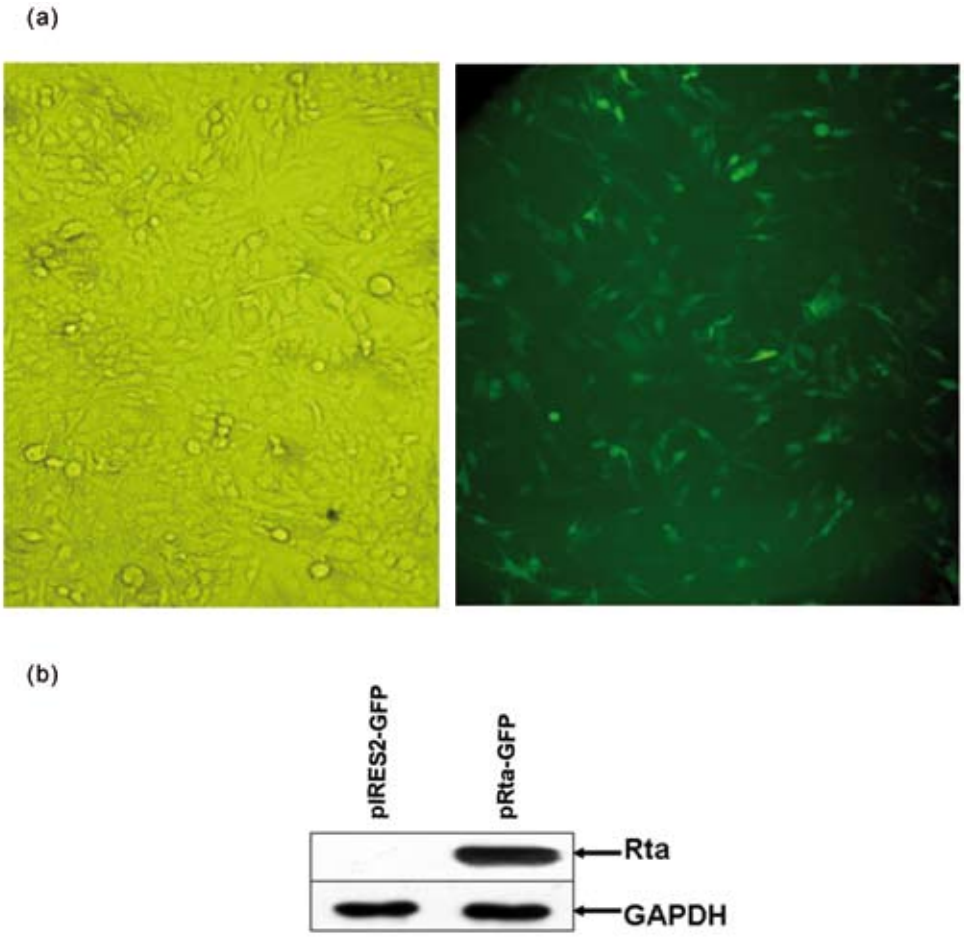

Fig. 2

Rta expression in HeLa cells

Fluorescent microscopy (a) and Western blot analysis (b) 48 hrs post transfection with the plasmid construct or empty plasmid.

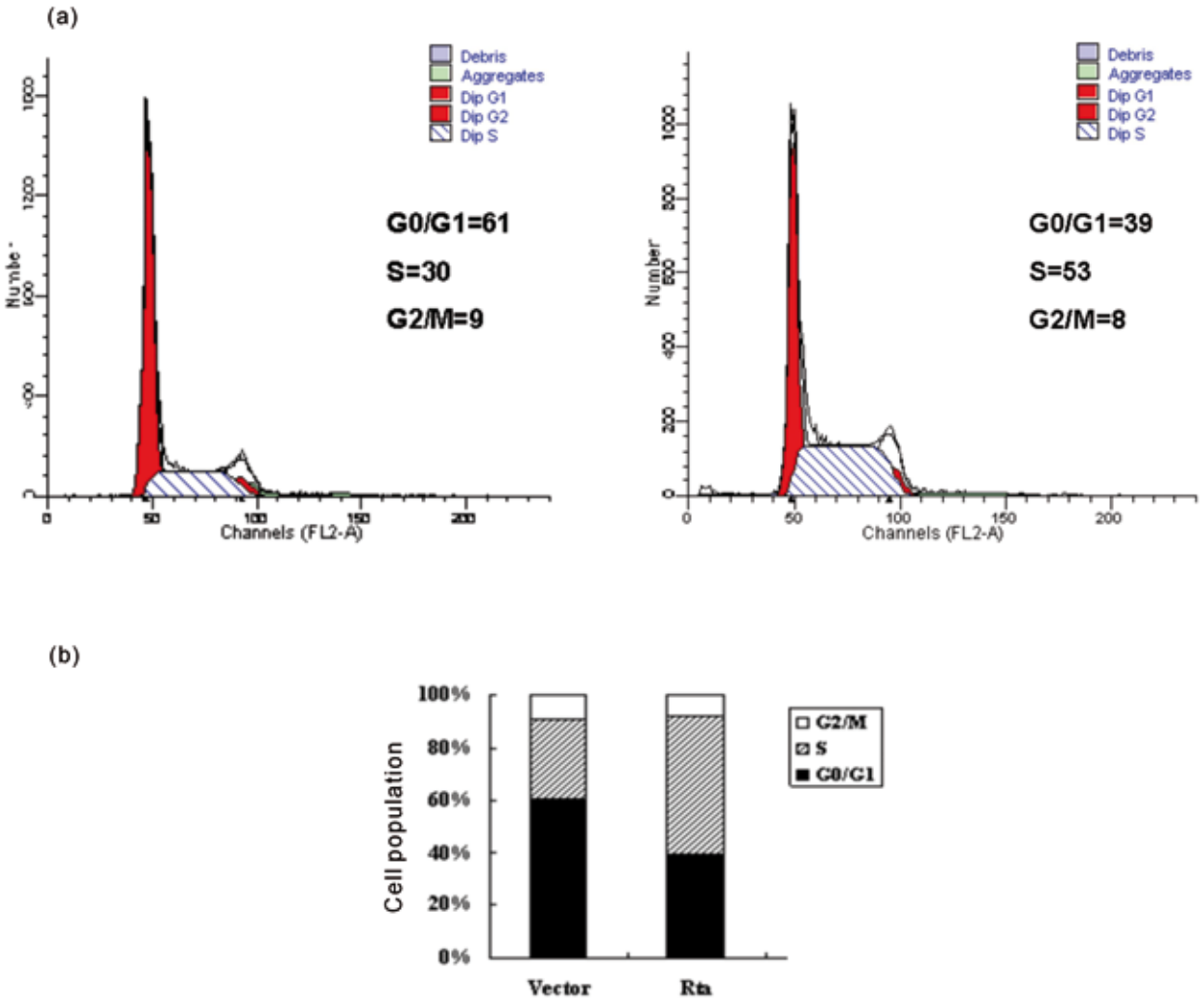

Fig. 3

Effect of Rta on the cell cycle

Flow cytometry of HeLa cells at 48 hrs post transfection with the plasmid construct or empty plasmid.

the conclusion that G_0/G_1 phase arrested cells may reenter the cell cycle following the Rta expression.

Effect of Rta on cell cycle regulators

The entry into the S phase is controlled, in part, by the level of active E_2F_1. Therefore, to understand the molecular basis of the altered cell cycle progression, the effect of Rta on the expression of E_2F_1 in cells was examined by Western blot analysis. Since Rta has been previously shown to interact directly with Rb, we also examined the effect of Rta expression on the level of Rb. As shown in Fig. 4, the expression of Rta prominently resulted in the disappearance of Rb. Surprisingly, the level of p53 was also down-regulated by Rta. Notably, the level of E_2F_1 was significantly elevated concurrent with the Rta expression.

Discussion

EBV has two phases of infection: a predominant latent state and an inducible lytic state. It utilizes two separate classes of genes that carry out very distinct functions in its life cycle. The EBV latency-associated gene expression is associated with cell cycle progression. The relationship between the EBV lytic program and the cell cycle has so far not been elucidated. EBV reactivation from latency into the lytic cycle can be initiated by two immediate-early viral proteins,

Zta and Rta. It was reported that the EBV lytic protein Zta arrested cells in G0/G1 (Cayrol and Flemington, 1996a) or G2/M (Mauser *et al.*, 2002a), even G1/S (Kudoh *et al.*, 2003). On the other hand, only a few reports were related to the Rta in controlling the cell cycle. Our data revealed that Rta-expressing cell population reenters S phase. To testify the correlation of Rta expression with S phase progression, we inspected the alteration of the expression profiles of a key set of cell cycle regulators in HeLa cells. Our data demonstrated that the expression of Rta substantially interfered with Rb, E_2F_1 and p53 regulatory machinery in HeLa cells, strongly inhibiting the expression of Rb and p53 and inducing the expression of E_2F_1.

Although Rta has been recently shown to interact directly with Rb for inactivation (Zacny *et al.*, 1998), the Rta-induced activation of S phase entry may be primarily due to E_2F_1 induction rather than due to inactivation of Rb. First, E_2F_1 overexpression by itself is often sufficient to activate entry into S phase. Second, our results show an overall reduction in the Rb protein level in Rta-expressing cells. Rb degradation by Rta is worth considering, because Rta may mediate its degradation in a proteasome-dependent manner (Guo *et al.*, 2010).

p53 also blocks cell proliferation in part by inducing p21, a protein that binds and inhibits the cyclin/CDK complexes, which are required for progression through the cell cycle. The observed decrease in the level of p53 reflects the cellular response to S phase entry. We can also explain the simultaneous Rb and p53 decrease by assuming that Rta may regulate gankyrin, which is a dual-purpose negative regulator of Rb and p53 (Guillermina and Gerard, 2005).

Many viruses need to manipulate the host cell environment in order to optimize the conditions for viral replication. The Rta-positive cells had an increased number of cells in the S phase compared to the Rta-negative cells. Thus, Rta regulates the expression of cellular proteins associated with cell cycle progression. This suggests that an S-phase-like environment may be advantageous for efficient lytic EBV replication in some cell types, because Rta expression is sufficient to induce the switch from latent to lytic phase. Upon EBV reactivation, the growth arrest signals of Zta activate the cellular factors, and subsequent expression of Rta influences the checkpoint factors of cell cycle progression and stimulates S phase progression to support efficient viral replication. It will be important to determine how Zta and Rta interact in cells to create and maintain a cellular environment favorable for lytic EBV replication.

Together, our data propose a model for G_0/G_1 to S transition controlled by Rta (Fig. 5): The Rta carries out its functions through a complex series of interactions with multiple cell cycle control proteins.

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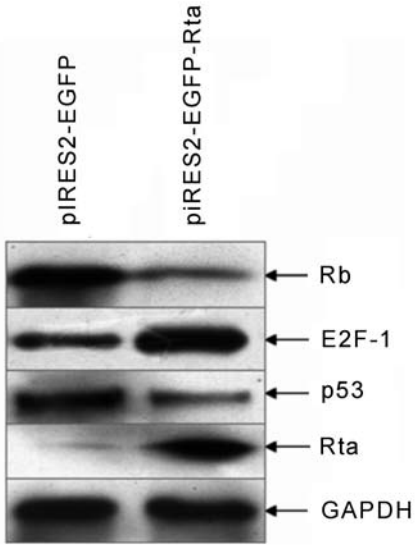

Fig. 4

Effect of Rta on cell cycle regulators

Western blot analysis of HeLa cells at 48 hrs post transfection with the plasmid construct or empty plasmid.

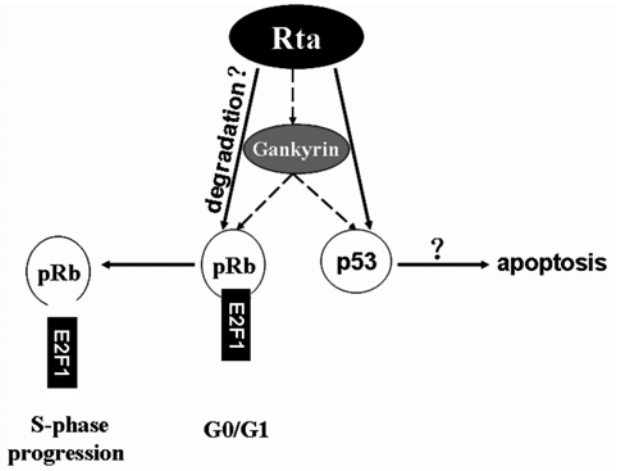

Fig. 5

Mode of action of Rta on the cell cycle

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