Epstein–Barr virus Rta-mediated transactivation of p21 and 14-3-3σ arrests cells at the G1/S transition by reducing cyclin E/CDK2 activity

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Many herpesviral immediate-early proteins promote their robust lytic phase replications by hijacking the cell cycle machinery. Previously, lytic replication of Epstein–Barr virus (EBV) was found to be concurrent with host cell cycle arrest. In this study, we showed that ectopic expression of EBV immediate-early protein Rta in HEp-2 cells resulted in increased G1/S population, hypophosphorylation of pRb and decreased incorporation of 5-bromo-2′-deoxyuridine. In addition, EBV Rta transcriptionally upregulates the expressions of p21 and 14-3-3σ in HEp-2 cells, 293 cells and nasopharyngeal carcinoma TW01 cells. Although p21 and 14-3-3σ are known targets for p53, Rta-mediated p21 and 14-3-3σ transactivation can be detected in the absence of p53. In addition, results from luciferase reporter assays indicated that direct binding of Rta to either promoter sequences is not required for activation. On the other hand, a special class of Sp1-responsive elements was involved in Rta-mediated transcriptional activation on both promoters. Finally, Rta-induced p21 expression diminished the activity of CDK2/cyclin E complex, and, Rta-induced 14-3-3σ expression sequestered CDK1 and CDK2 in the cytoplasm. Based on these results, we hypothesize that through the disruption of CDK1 and CDK2 activities, EBV Rta might contribute to cell cycle arrest in EBV-infected epithelial cells during viral reactivation.

INTRODUCTION

Epstein–Barr virus (EBV) is classified as a member of the gamma herpesvirinae with a dsDNA genome of 172 kbp. EBV infection is associated with various human malignancies of lymphoid and epithelial cell origin (Crawford et al., 1979; Epstein et al., 1964; Henle & Henle, 1976; Imai et al., 1994; Niedobitek & Young, 1994). Generally, EBV maintains itself as a latent episome in a proliferative cell and converts to a lytic replicating genome when the host exits from the last cell cycle (Feederle et al., 2007; Sun & Thorley-Lawson, 2007; Tao et al., 1995). The reactivation of EBV from latency can be induced by various chemicals (Takada, 1984; zur Hausen et al., 1978) or by ectopic expression of the immediate-early transcription activators Zta (aka BZLF1, ZEBRA and IE1) or Rta (aka BRLF1 and IE2) (Countryman & Miller, 1985; Ragoczy et al., 1998; Zalani et al., 1996). Furthermore, Zta and Rta activate each other and function synergistically to complete a full productive replication cycle (Feederle et al., 2000). Expression of Rta in nasopharyngeal carcinoma (NPC) cells is potentially useful in early diagnosis (Feng et al., 2000).

The cell cycle is an ordered, tightly regulated process involving multiple checkpoints that ensure a faithful round of cell duplication. However, these fine-tuned processes are often perturbed by an invading virus once a lytic replication cycle is initiated (Flemington, 2001). Small DNA viruses, such as SV40 and papilloma virus, encode
limited viral gene products and trigger an S phase cell cycle so that a copious environment for viral replication is guaranteed (Hiscott & Defendi, 1979). On the other hand, large DNA viruses, such as herpesviruses, usually encode their own enzymes necessary for supporting viral replication. Thereby, during lytic replication of a herpesvirus, progression of the cell cycle might need to be blocked so that a less competitive environment for viral replication is maintained (DiMaio & Coen, 2001). For instance, infection by herpes simplex virus type 1 causes cell cycle blockage at the G1/S boundary (de Bruyn Kops & Knipe, 1988; Ehmann et al., 2000). In addition, the human cytomegalovirus has evolved mechanisms to facilitate viral DNA replication at the expense of the host’s nuclear DNA synthesis in cells arrested at G0/G1 (Sanchez & Spector, 2008). Finally, chemically induced EBV reactivations also displayed a strong bias in cell populations at the G1 phase (Rodriguez et al., 2001).

Although the aforementioned cell backgrounds and experimental designs varied, many herpesviral immediate-early proteins play a role in cell cycle arrest. Human cytomegalovirus immediate-early 2 protein inhibits cellular DNA synthesis (Petrik et al., 2006; Wiebusch & Hagemeier, 2001) and induces premature senescence (Noris et al., 2002). Human herpesvirus simplex virus ICP0, a virion associated immediate-early product, elicits cell cycle arrest in G1 by both p53-mediated and p53-independent pathways (Hobbs & Deluca, 1999). In EBV, Zta arrests cell cycle progression is cell type dependent: a G0/G1 blockage was observed in HeLa cells and normal human fibroblasts (Cayrol & Flemington, 1996; Wu et al., 2003), whereas an S-phase-like stage was observed in keratinocytes, gastric cancer cells and B-cells (Kudoh et al., 2003; Mauser et al., 2002). By contrast, the role of Rta in cell cycle control has received less attention. In one experimental design, re-examined the roles of Rta in cell cycle regulation by establishing Tet-on Rta inducible systems in NPC, 293 and HEp-2 cells. In these epithelial cells, Rta invariably modulated the levels of signature G1 arrest molecules that were followed by striking induction of the cellular senescence marker, SA-β-Gal (Chen et al., 2009 and unpublished data). In addition, results from microarray analysis confirmed that the cell cycle regulators altered by Rta were expressed at the transcriptional level (Chen et al., 2011). Here, we chose p21 and 14-3-3σ for further mechanistic studies because they are key CDK inhibitors that control cell cycle progression. We found that although p21 and 14-3-3σ are known p53 targets, the Rta-mediated inductions of p21 and 14-3-3σ can be independent of p53. Surprisingly, deletion or mutation of Rta-responsive elements (RREs) in the promoter regions of p21 and 14-3-3σ did not significantly diminish the induction activities, indicating that Rta may employ an indirect mechanism in activating CDK inhibitor promoters.

RESULTS

Rta activated p21 and 14-3-3σ at the transcriptional level in various epithelial cells

Previously, we found that when Rta was provided ectopically in NPC-TW01 cells, 293 cells or HEp-2 cells, the cell growth rates were significantly reduced as compared with those of the vector controls. To comprehensively delineate the host genes that could be modulated by Rta, which in turn resulted in decelerating cell growth, we established Tet-on inducible systems in 293 and NPC-TW01 cells (termed 293TetER and TW01TetER, respectively), followed by genomewide transcriptome analysis. There were 120 genes commonly modulated by Rta in these two inducible cell lines (Chen et al., 2011). Eleven upregulated and 11 downregulated genes are shown in Table 1. Among these, the induction of FASN and MERTK were consistent with a previous microarray study (n=5000), in which primary human keratinocytes were infected with an adenovirus vector expressing Rta (Li et al., 2004a, b), suggesting that some of the transcriptional acts imposed by Rta might be conserved in different cell types. In addition, Rta similarly modulated many cell cycle regulatory genes in both cell types, indicating an active role for Rta in regulating the cell cycle (Chen et al., 2011). Because p21 (CDKN1A) and 14-3-3σ (SFN) are key CDK inhibitors involved in G1 (el-Deiry et al., 1993) and G2 (Hermeking et al., 1997) phase cell cycle arrest, we chose these two genes as examples to investigate how Rta functions to modulate the levels of cell cycle regulators. It bears to note that although Rta-induced expression of p21 and 14-3-3σ was invariably detected at the protein level (detailed below), the induction of p21 and 14-3-3σ transcripts in TW01TetER cells was not significant (Table 1). This could be due to post-translational controls, or, due to inherent low sensitivity of microarray analysis. With this in mind, quantitative RT-PCR was conducted to measure the relative levels of p21 and 14-3-3σ transcripts in the same batch of RNAs submitted for microarray analysis. As shown in Fig. 1, expression of Rta resulted in the induction of p21 and 14-3-3σ transcripts in both 293 and TW01 cells (1st and 2nd panels). Specifically, Rta induced a twofold increase of p21 and a threefold increase of 14-3-3σ in TW01TetER relative to the doxycycline-treated control cells (Fig. 1, 2nd panel). Furthermore, transient-expression of Rta in HEp-2 cells also gave rise to an evident increment of p21 (3.7-fold) and 14-3-3σ (4.3-fold) mRNAs (Fig. 1, 3rd panel) at the transcriptional level. Together, these results suggested that cellular p21 and 14-3-3σ promoters are accessible and activated by EBV Rta.

Ectopic expression of Rta in HEp-2 cells increased G1/S population and decreased DNA synthesis

Previously, we showed that long-term doxycycline-treated 293TetER cells and TW01TetER cells with sustained Rta expressions exhibited irreversible cell cycle arrest followed by
Table 1. Fold-changes of representative genes commonly regulated by EBV Rta in Rta-inducible nasopharyngeal carcinoma (TW01TetER) and 293 (293TetER) cells

The values in 293TetER are denoted as mean fold-change \( \pm \) SD from two biological replicates; the values in TW01TetER are fold-changes from a cell clone expressing lower amount of Rta (low) and a cell clone expressing high amount of Rta (high). Details for the experimental design and data process procedures are referred to Gene Expression Omnibus (GEO) under accession number GSE24587.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>293TetER</th>
<th>TW01TetER (low/high Rta)</th>
<th>Gene description</th>
</tr>
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<tbody>
<tr>
<td>Upregulated genes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDKN1A</td>
<td>2.2 ( \pm ) 0.01</td>
<td>-1.4/1.0</td>
<td>Cyclin-dependent kinases 1A (p21)</td>
</tr>
<tr>
<td>CORO1A</td>
<td>7.4 ( \pm ) 0.28</td>
<td>2.8/8.7</td>
<td>Coronin, actin-binding protein, 1A</td>
</tr>
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<td>FASN</td>
<td>2.2 ( \pm ) 0.03</td>
<td>-1.1/2.2</td>
<td>Fatty acid synthase</td>
</tr>
<tr>
<td>H1F0</td>
<td>3.8 ( \pm ) 0.02</td>
<td>1.5/2.4</td>
<td>H1 histone family, member 0</td>
</tr>
<tr>
<td>MERTK</td>
<td>8.3 ( \pm ) 0.22</td>
<td>3.1/21.5</td>
<td>c-mer proto-oncogene tyrosine kinase</td>
</tr>
<tr>
<td>MYOM2</td>
<td>5.0 ( \pm ) 0.46</td>
<td>1.3/4.4</td>
<td>Myomesin (M-protein) 2, 165 kDa</td>
</tr>
<tr>
<td>SFN</td>
<td>14.3 ( \pm ) 0.32</td>
<td>1.6/1.6</td>
<td>Stratifin (14-3-3-( \sigma ))</td>
</tr>
<tr>
<td>SLC25A45</td>
<td>2.1 ( \pm ) 0.05</td>
<td>10.5/25.4</td>
<td>Solute carrier family 25, member 45</td>
</tr>
<tr>
<td>TP53INP1</td>
<td>2.2 ( \pm ) 0.09</td>
<td>1.4/4.4</td>
<td>Tumour protein p53 inducible nuclear protein 1</td>
</tr>
<tr>
<td>TRIM43</td>
<td>3.5 ( \pm ) 1.03</td>
<td>2.0/2.0</td>
<td>Tripartite motif-containing 43</td>
</tr>
<tr>
<td>TRIM52</td>
<td>2.3 ( \pm ) 0.13</td>
<td>2.5/4.5</td>
<td>Tripartite motif-containing 52</td>
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<tr>
<td>Downregulated genes</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CALB1</td>
<td>-8.3 ( \pm ) 0.02</td>
<td>-1.8/-2.7</td>
<td>Calbindin 1, 28 kDa</td>
</tr>
<tr>
<td>CCND2</td>
<td>-3.1 ( \pm ) 0.03</td>
<td>1.1/-3.3</td>
<td>Cyclin D2</td>
</tr>
<tr>
<td>CD44</td>
<td>-2.8 ( \pm ) 0.00</td>
<td>-2.4/-3.9</td>
<td>CD44 molecule (Indian blood group)</td>
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<tr>
<td>LRIG3</td>
<td>-2.4 ( \pm ) 0.01</td>
<td>-2.2/-6.0</td>
<td>Leucine-rich repeats and Ig-like domains 3</td>
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<tr>
<td>LYPD1</td>
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<td>-1.6/-2.9</td>
<td>LY6/PLAUR domain containing 1</td>
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<tr>
<td>MYC</td>
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<td>-1.7/-4.5</td>
<td>v-myc myelocytomatosis viral oncogene homologue</td>
</tr>
<tr>
<td>RRS1</td>
<td>-2.6 ( \pm ) 0.04</td>
<td>-1.1/-2.5</td>
<td>RRS1 ribosome biogenesis regulator homologue</td>
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<td>-1.3/-2.0</td>
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<tr>
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<td>Small nucleolar RNA, C/D box 14E</td>
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<td>SNORD45B</td>
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<td>Small nucleolar RNA, C/D box 45B</td>
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<tr>
<td>SOX4</td>
<td>-3.1 ( \pm ) 0.00</td>
<td>-1.2/-2.1</td>
<td>SRY (sex determining region Y)-box 4</td>
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</table>

Cellular senescence (Chen et al., 2009, 2011). Here, we used the HEp-2 cell line as an additional cell background to validate Rta-induced cell growth arrest. At a transfection rate \( \sim 80\% \), a prominent G1 population enrichment (58 vs 74\%) was observed at 24 h after transfection in cells transiently transfected with Rta-expression plasmid (pRTS15) as compared with those with vector control (pSG5) (Fig. 2a, left panel). Results from Western blot analysis showed that overexpression of Rta in HEp-2 cells concurrently augmented the level of p21, reduced the phosphorylation states of pRb and the concentration of cyclin A (Fig. 2a, right panel). In comparison, Rta altered less in the levels of cyclin D3, cyclin E and CDK2. Since p21 and pRb are gatekeepers of the G1 to S phase transition, these results are in agreement with a G1 population accumulation in Rta-expressing cells. Furthermore, when the transfected cells were released from aphidicolin-synchronized early S phase, a blockage of G1 to S transition (68 vs 29\%) was evidently detected in Rta-expressing cells relative to the vector controls (Fig. 2b, R4), indicating that expression of Rta is closely associated with impedance in S phase transition. Finally, to validate that in the present system the S phase progression is linked to DNA synthesis, transfected cells were subjected to 5-bromo-2'-deoxyuridine (BrdU) incorporation assays. As expected, while 16\% of cells transfected with vector control (pSG5) were detectable by BrdU incorporation, only 6\% of cells transfected with Rta expression plasmid (pRTS15) were BrdU positive (Fig. 2c). Thus, results from DNA content analysis, Western blot analysis and BrdU incorporation assay all indicated that a G1/S arrest was induced in Rta-expressing HEp-2 cells. In addition, because irreversible cell cycle arrest is a preceding event before cellular senescence (Blagosklonny, 2006), the 9 day doxycycline-treated HEp2TetER cells were subjected to senescence-associated \( \beta \)-galactosidase (SA-\( \beta \)-Gal) activity assay. As expected, a strong SA-\( \beta \)-Gal activity was evidently detected in doxycycline-treated, Rta-expressing cells (Fig. 2d, Dox), suggesting that Rta-induced cell cycle arrest contributed to cellular senescence.

**Rta could induce p21 expression and G1/S arrest without the participation of p53**

Because both p21 and 14-3-3\( \sigma \) are known transcriptional targets of p53, it is possible that p53 and Rta may function synergistically to induce these two genes. To test this hypothesis, HEp-2 cells were cotransfected with Rta- together
with p53-specific short interfering (si) RNA expression plasmids, followed by flow cytometry and Western blot analysis. To our surprise, depletion of p53 in HEp-2 cells produced almost no effect on Rta-mediated G1/S arrest (not shown) or the inductions of p21 and 14-3-3σ (Fig. 3a, compare lane 2 to lanes 4–6), suggesting that p53 and Rta might act independently to transactivate these two cellular promoters. The same phenomenon was also observed in H1299 cells, a p53-null lung cancer epithelial cell line, in which Rta was able to cause an increased G1/S population (14 vs 74% in Fig. 3b, R4) as well as p21 and 14-3-3σ expressions (Fig. 3b, right panel). Interestingly, these results reveal the difference between Rta and Zta in p21 induction: the former can function in the absence of p53, while the latter requires p53 (Cayrol & Flemington, 1996). In addition, it is envisaged that Rta would be more responsible for p21 synthesis than Zta if the cellular p53 were to be dysfunctional or deleted, as reported in most undifferentiated NPC cells (Crook et al., 2000).

**Sp1-responsive elements present in p21 and 14-3-3σ promoters were involved in Rta-mediated transcriptional activation**

To identify the potential RREs (Gruffat & Sergeant, 1994) in the p21 and 14-3-3σ genes, the upstream sequences of p21 (2.4 kbp) and 14-3-3σ (820 bp) were cloned in front of the luciferase coding region, yielding p21-Luc and p14-3-3σ-Luc, respectively. Of note, sequence inspection of the two promoters revealed two p53 sites (in p21-Luc), two RREs (one in p21-Luc and one in p14-3-3σ-Luc) and a number of Sp1-binding sites, as illustrated in Fig. 4. The deletion of p53 sites in p21-Luc (p21-Sp1/d2) showed little effect in Rta-induced transactivation of the p21 promoter, reinforcing that p53 was not involved. Unexpectedly, mutations of the RRE sites in either p21-Luc (p21-Sp1/d2) or p14-3-3σ-Luc (∆RRE) also had little influence in reporter assays (Fig. 4), indicating that direct Rta binding onto these promoter regions might not be essential. These results suggest that Rta might utilize an indirect mechanism, which involved either activation of signalling pathways (Adamson et al., 2000; Darr et al., 2001; Hsu et al., 2005; Lee et al., 2008) or interaction with other DNA-binding proteins (Chang et al., 2005; Ragoczy & Miller, 2001), to transactivate p21 and 14-3-3σ promoters. On the other hand, mutations of the third Sp1 site in p21-Luc (i.e. p21 Sp1/m3 and p21 Sp1/m1m3) or the first two (overlapped) Sp1 sites in p14-3-3σ-Luc (p14-3-3σ-Sp1/m1) dramatically abolished both the Rta-induced activity and the basal transcriptional activity on these promoters (Fig. 4a, b). Thus, these two Sp1 elements seemed to be essential for promoter responsiveness. Next, a commonly used Sp1 inhibitor, mithramycin A (which prevents Sp1 binding to GC-rich boxes in DNA), was used to test whether Rta-mediated activations were dependent on Sp1. As shown in Fig. 4(c), results from the mithramycin A inhibition analysis correlated well with those from the luciferase reporter assays. It is noteworthy that (i) the Rta-induced expression of p21 and 14-3-3σ was inhibited by mithramycin A in a dose-dependent manner (10–30 nM); (ii) higher dose of mithramycin A (50–100 nM) increases the basal activities of 14-3-3σ but not that of p21. These results resemble those from luciferase reporter assays of which mutations of Sp1-3 or Sp1-4 sites in p14-3-3σ-Luc indeed increased reporter activities (Fig. 4b). The underlying mechanism regarding this phenomenon awaits further investigation. Finally, when the two pieces of sequence were

![Fig. 1. Validation of Rta-mediated p21 and 14-3-3σ transcriptional activations by quantitative RT-PCR assays. (top and middle) Same batches of RNAs subjected to microarray analysis, see Table 1, were used for 293TetER cells and TW01TetER cells, respectively. (bottom) RNAs were extracted from HEp-2 cells transiently transfected with vector control (pSG5) or Rta-expressing plasmid (pRTS15). Error bars depict SD of three PCR assays. Two independent experiments were performed, similar results were obtained. One set of data is shown.](image-url)
aligned, a highly conserved 13 bp motif (92% identity) that encompasses the essential Sp1 sites in p21-Luc and p14-3-3σ-Luc were revealed (Fig. 4, bottom panel). These results suggest that a special class of Sp1 recognition element was involved in Rta-mediated p21 and 14-3-3σ expressions.

**Inactivation of CDK1 and CDK2 via Rta-induced p21 and 14-3-3σ**

The cell cycle regulatory activities of p21 and 14-3-3σ act primarily by disturbing CDK1 and CDK2 activities at appropriate stages. To examine whether Rta-induced p21 would impede the function of the G1/S transition regulator, i.e. CDK2/cyclin E complex, an *in vitro* immunoprecipitation (IP)-kinase assay was performed using histone H1 as a substrate. First, similar quantities of CDK2/cyclin E complex were observed in cells transfected with vector control or Rta-expressing cells (Fig. 5; CDK2 in lanes 3 and 4; cyclin E in lanes 5 and 6). Next, Rta-induced p21, but not Rta itself, was detected in the CDK2/cyclin E IP complexes

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**Fig. 2.** Rta-mediated p21 expression contributed to G1/S phase arrest. (a) (left) HEp-2 cells transfected with vector control (pSG5) or Rta-expression plasmid (pRTS15) were analysed by flow cytometric analysis of cell cycle distribution. An increase in G1 arrest was detected in Rta-expressing cells (58.30 vs 73.97%). (right) Western blot analysis of cell cycle checkpoint proteins p21, phosphorylated pRb, cyclins A, D3, E and CDK2 in HEp-2 cells transfected with vector control (pSG5) or Rta-expression plasmid (pRTS15); β-actin served as a loading control. (b) HEp-2 cells transfected with pSG5 or pRTS15 were synchronized with aphidicolin (1 μg ml⁻¹) for 24 h followed by flow cytometric analysis of cell cycle distribution at the time of drug release (R0) and 4 h after drug release (R4). A prominent G1/S arrest was detected in Rta-expressing cells (68.46%). (c) BrdU incorporation assays of HEp-2 cells (mock-PE) transiently transfected with vector (pSG5-PE, pSG5-BrdU-PE) or Rta expression plasmid (pRTS15-BrdU-PE). Prior to harvest, two sets of the cells (pSG5-BrdU-PE and pRTS15-BrdU-PE) were labelled for 30 min with BrdU and stained with PE-conjugated anti-BrdU antibody followed by flow cytometric analysis. The unlabelled, vector transfected control (pSG5-PE) was included to determine the background fluorescence under transfection conditions. The BrdU fluorescence is represented on the x axis, and SSC-H is represented on the y axis. (d) Strong SA-β-Gal activity was detected in day 9 doxycycline-treated HEp2TetER cells (+Dox), but not in the untreated cells (Control) (x200 magnifications).
(lanes 4 and 6). Finally, the presence of p21 severely reduced the kinase activity of CDK2/cyclin E towards the substrate histone H1 (lane 3 vs 4; lane 5 vs 6). Thus, Rta-induced p21 was a potent CDK2 inhibitor and the results also explained the hypophosphorylation status of pRb in Rta-expressing cells observed previously (Fig. 2a, right panel). To assess whether Rta-induced 14-3-3s would perturb CDK1 or CDK2 activities, the subcellular localizations of CDK1, CDK2 and 14-3-3s were examined in the inducible HEp2TetER cells using an immunofluorescence assay. As shown in Fig. 5(b), whereas CDK1 and CDK2 were found more homogenously in the control groups, the two kinases were clearly entrapped and colocalized with 14-3-3s in the cytoplasmic compartments of doxycycline-treated, Rta-expressing cells. These results strongly suggest that Rta-induced 14-3-3s blocked the functions of CDK1 and CDK2 by sequestering them in the cytoplasm.

DISCUSSION

Viruses are obligate intracellular parasites. Their replication and genomic expression depend on the host’s machineries. Here, we propose that EBV Rta may cooperate with the host transcription factor Sp1 to upregulate two CDK inhibitors, p21 and 14-3-3s. The Rta-induced p21 formed a complex with CDK2/cyclin E, which resulted in a noteworthy loss in kinase activity. Simultaneously, the Rta-induced 14-3-3s was very likely to inactivate certain functions of CDK1 and CDK2 by mislocating them in the cytoplasm. Because p21 and 14-3-3s are key gatekeepers of cell cycle progression, thus Rta may actively take control of the cell cycle machinery and advance the lytic replication cascade.

Initially, we chose HEp-2 as an additional cell model to validate Rta-mediated cell cycle arrest because it originated from larynx carcinoma. Unfortunately, by DNA profiling analysis, ATCC recently identified a panel of HeLa-contaminated cell lines and HEp-2 was one of them. However, we believe that the impact of HPV18 E6 or E7 on Rta-mediated cell cycle regulation in HEp-2 cells could be minimal because these phenomena were also observed in other HPV-ve cell backgrounds, and vector controls were included in every experiment. Still, we cannot completely exclude the possibility that HPV oncoproteins may interact with Rta and impose synergistic effects on cell cycle regulation. More experiments are required to resolve this possibility.

In contrast to EBV latent proteins that drive the host cells to proliferate indefinitely (Arvanitakis et al., 1995; Parker et al., 2000; Sinclair et al., 1994; Vereide & Sugden, 2011), EBV Zta and Rta need to comply with their ‘immediate-early’ roles during lytic phase replication, i.e. to halt cells from further progression so that a less competitive
environment for virus replication can be maintained (DiMaio & Coen, 2001; Flemington, 2001; Op De Beeck & Caillet-Fauquet, 1997). Indeed, accumulating evidence indicates that Zta and Rta seem to have evolved diverse mechanisms in perturbation of cell cycle progression (Chen et al., 2009; Guo et al., 2010; Swenson et al., 1999; Wu et al., 2003). Because Rta is an evolutionarily conserved master switch in all gamma herpesviruses, we chose Rta as a working model for further in-depth investigation. Rta-mediated transcriptional regulation of the EBV genome can be distinguished by two modes: direct binding or indirect binding. Direct binding requires the presence of RREs in the promoter sequences. Indirect binding could be further divided into two classes: one class involves cellular signalling pathways and the other class requires additional DNA-binding factors such as Sp1. The relative abundance of Sp1 and Sp3 in a cell was suggested to be decisive in auto-stimulating the expression of Rta (Ragoczy & Miller, 2001). Interestingly, we noticed that the regulatory region responsible for Rta auto-stimulation (Fig. 5 in reference Ragoczy & Miller, 2001) also harbours an Sp1-binding site that shares 90% identity with the one we identified in the present study (GTCCCGCCYC in Fig. 4). Furthermore, Chang et al. (2005) proposed that MCAF1 is the mediator that bridges Rta and Sp1 in p21 activation. Thus, our results are consistent with these

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**Fig. 4.** An Sp1-responsive element was involved in Rta-mediated transactivation of p21 and 14-3-3-\(\alpha\) promoter sequences in HEp-2 cells. (a) A schematic drawing of the p21-Luc reporter plasmid (2.4 kb upstream sequences) is shown on the top. The known p53-binding sites and hypothetical Rta- or Sp1-responsive elements (RRE and Sp1, respectively) are denoted. The relative luciferase activities and activation folds of each p21-Luc derivative plasmid in response to Rta expression are depicted on the right for each plasmid. Only reporter plasmids mutated at the Sp1-3 element (\(\star\)) lost their Rta responsiveness. (b) A schematic drawing of p14-3-3-\(\alpha\)-Luc (820 bp upstream sequences) is shown on the top. The hypothetical Rta- or Sp1-responsive elements are denoted. The relative luciferase activities and activation folds of each p14-3-3-\(\alpha\)-Luc derivative plasmid in response to Rta expression are depicted on the right. Only reporter plasmid with an Sp1-1/2 site mutation (\(\star\)) lost Rta responsiveness. (c) The importance of Sp1 in Rta-mediated p21 induction was evidenced by Sp1 inhibitor mithramycin A treatment. HEp-2 cells transfected with vector control (pSG5) or the Rta-expression plasmid (pRTS15) were treated with the indicated concentrations of mithramycin A. At 10–30 \(\mu\)M, decreased expression levels of p21 and 14-3-3-\(\alpha\) were associated with increasing amounts of mithramycin A, reinforcing a role for Sp1 in Rta-mediated p21 and 14-3-3-\(\alpha\) expressions. (d) Comparison of p21 and 14-3-3-\(\alpha\) promoter sequences. Predicted RREs are underlined with arrowheads depicting orientations. A 13 bp consensus motif containing the Sp1-3 site in p21 and the Sp1-1/2 in 14-3-3-\(\alpha\) are indicated by rectangles. Mutations introduced in reporter plasmids p21 Sp1/m3 or p14-3-3-\(\alpha\)-Luc Sp1/m1 are indicated by stars.
previous findings and further delineate a 13 bp motif that may be the principal responsive element in the Rta/Sp1-mediated transcriptional activation. It will be of great interest to catalogue and validate how many additional host and viral genes are regulated by the same process exerted by Rta and Sp1 in EBV+ve cell backgrounds.

Among their many functions, p21 and 14-3-3s are best known as CDK inhibitors. Cellular CDK is a family of Ser/Thr protein kinases of which CDK1, 2, 4 and 6 are known to be involved in cell cycle progression. Recently, Sato & Tsurumi (2010) elegantly delineated an intricate mechanism by which EBV Zta selectively blocks the downstream signalling pathways of S-phase CDK activities in B-cells and HeLa cells. Here, we showed that in Rta-expressing HEp-2 cells the excessive p21 reduced the activity of CDK2 and the increased 14-3-3s entrapped CDK1/CDK2 in the cytoplasm (Fig. 5). Hence, our results strongly suggest that Rta may impede CDK1 and CDK2 activities that in turn result in cell growth arrest during EBV lytic phase replication. Interestingly, a recent study of human cytomegalovirus showed that inhibition of CDK1 and CDK2 activities relieves the block of major immediate-early gene expressions in S/G2 cells (Zydek et al., 2010). Therefore, the actions and counteractions between host CDKs and virus immediate-early proteins may share certain similarities among herpesviruses. More spatiotemporal studies of lytic replication at different stages of cell cycle will help to disclose a unifying theme and to provide means in the control of herpesviral replication-associated pathogenesis.

**METHODS**

**Cell culture.** HEp-2 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % FBS. HEp2TetER is a doxycycline inducible, EBV Rta conditional expression cell line created by Virapower system (Invitrogen) according to the previously published procedure (Chen et al., 2009). HEp2TetER was maintained in DMEM containing 10 % Tet-free serum, 5 μg blasticidin-S·HCl ml⁻¹ and 200 μg zeocin ml⁻¹. All cells were grown at 37 °C in a humidified incubator containing 5 % CO₂.

**Plasmids.** pRTS15 is an EBV Rta expression plasmid derived from pSG5 (Gao et al., 1998). Expression plasmids encoding p53- and E7 siRNA have been described previously (Hsu et al., 2005). p21-Luc was created by cloning a 2.4 kb HindIII fragment of pWWP-Luc (el-Deiry et al., 1993) into pGL3-basic (Promega). p21-Sp1/d2, p21-Sp1/d3, p21-Sp1/m1, p21-Sp1/m3 and p21-Sp1/m1m3 were generated by PCR method using p21-Luc as template. p14-3-3s-Luc was constructed by PCR-cloning of an 844 bp upstream sequences of 14-3-3s coding region (NCBI gene id 2810, 2820~22) in front of luciferase gene in pGL2-basic (Promega). Other deletional or mutational plasmids derived from p14-3-3s-Luc were constructed by using 'single-primer PCR' procedures described previously (Makarova et al., 2000). All reporter plasmids have been sequence-verified for desired mutations. The primer sequences for each construct are listed in Supplementary Table S1 (available in JGV Online).
Antibodies. Antibodies used in this study were purchased from various companies: cyclin-A, -D3, -E, CDK2, pRb, 14-3-3ε and Sp1 were from Santa Cruz Biotechnology; EBV Rta was from Argene Inc.; p53 and p21 were from NeoMarker; β-actin was from Sigma; PE-conjugated anti-BrdU was from BD Pharmingen.

Transfection and luciferase assay. Cells were seeded with 5 × 10⁴ cells per well in a 24-well plate. For each well, cells were transfected with a total of 1 μg DNA including a transfection control renilla luciferase reporter (pRL-EF1α). Cell lysate were harvested at 24 h post-transfection and subjected to Dual-Glo luciferase assay (Promega) using Orion L microplate luminometer (Berthold). Data are presented as the means ± SD from triplicate assays. Two independent experiments were performed.

Cell synchronization. Cells were grown to near confluence before exchange with serum-free media. After serum starvation for 24 h, 1.5 × 10⁵ cells were replated on a 6 cm dish and grown in complete medium for 24 h. The cells were transfected with pSG5 or pRTS15 using Transfast (Promega) according to the manufacturer’s instructions. Three hours after transfection, 1 μg aphidicolin (Sigma) ml⁻¹ was added to the culture in order to synchronize cells at the G1/S boundary. Twenty-four hours after aphidicolin synchronization, cells were washed three times with PBS before harvest (R0); or refed with complete medium for an additional 4 h before harvest (R4).

Flow cytometric analysis. Aliquots of trypsinized cells (1 × 10⁶) were washed with PBS, fixed in ice-cold 75 % ethanol and stored at −20 °C until all samples from different time points were collected. Prior to flow cytometric analysis, the fixed cells were repelled by centrifugation, permeabilized in PBS containing 0.2 % Triton X-100 at room temperature for 10 min, and resuspended in PBS containing 10 μg RNase A ml⁻¹ and 10 μg propidium iodide ml⁻¹. After the cell mixtures were incubated in the dark for 30 min, flow cytometric analysis was performed using a fluorescence-activated cell sorter (FACScan; Becton Dickinson). The cell cycle profile was analysed using the CellQuest software and ModFit 2.0 software.

Analysis of DNA synthesis by BrdU staining. BrdU incorporation was used to determine the progression of the S phase. Before cells were harvested, 20 μM BrdU was added to the culture medium and incubated for 30 min. The cells were trypsinized and fixed with 70 % ethanol overnight at −20 °C. After centrifugation, the cells were washed with PBS, incubated with 1 ml 2 M HCl for 30 min at room temperature, and neutralized by 1 ml 0.1 M Na₂B₄O₇, pH 8.5, for 5 min. After centrifugation, the cells were resuspended in 50 μl 0.5 % Tween 20/PBS solution, followed by the addition of 20 μl anti-BrdU antibody and incubated for 30 min at 37 °C. The cells were washed with PBS and the percentage of cells incorporated with BrdU was measured by a FACScan flow cytometer.

Western blot analysis. Cells were rinsed twice with PBS, trypsinized and lysed with ice-cold RIPA buffer. Total cell lysates were clarified by centrifugation followed by BCA protein assay kit (Pierce) for the protein concentration. Cell lysates were resolved by 10 % SDS-PAGE. The protein bands were transferred electrophoretically onto Hybond- C super membrane. The blots were hybridized with indicated primary antibodies followed by three washes in TBS plus 0.05 % Tween 20 for 5 min each. The blots were hybridized with horseradish peroxidase-conjugated secondary antibodies followed by three washes in TBST for 5 min each. Blots were developed by using ECL detection reagent (Amersham).

IP-kinase assay. Five-hundred micrograms of protein lysate from each sample was incubated with indicated IP antibodies at 4 °C overnight. Immunocomplexes were captured by protein A-Sepharose beads (Pharmacia Biotech) at 4 °C for 2 h. The precipitates were washed three times with RIPA buffer containing protease inhibitors. The precipitates were washed twice with kinase buffer (50 mM HEPES, pH 7.5, 10 mM MgCl₂, 1 mM DTT, 10 mM β-glycerophosphate, 1 mM NaN₃ and 0.1 mM Na₃VO₄. The precipitates were resuspended in 30 μl kinase buffer containing 2.5 μg histone H1, 50 μM ATP, 5 μCi [α-³²P] ATP (Amersham Life Science) and incubated at 30 °C for 30 min. The reaction was stopped by adding 5 μl 6 × Laemmli sample buffer and resolved by 10 % SDS-PAGE. The gel was dried, exposed to an X-ray film and developed by autoradiography.

Immunofluorescence assay. Cells were resuspended in PBS and dropped onto multiple-well diagnostic microscope slides and fixed in methanol/acetone (1 : 1) at −20 °C for 20 min. Cells were permeabilized with 0.1 % Triton X-100 at room temperature for 20 min. The slide was incubated with indicated primary antibody at room temperature for 1 h, washed three times in PBS for 5 min each, and incubated with FITC-conjugated secondary antibody at room temperature for 1 h. After the PBS wash, the slide was incubated with Hoechst 33258 at room temperature for 20 min, washed with PBS, mounted in Vectashield medium and inspected by fluorescence microscopy.

Quantitative reverse transcription-PCR (qRT-PCR). Total RNA was extracted from healthy cells with ~90 % confluence by using Trizol reagent (Invitrogen). Reverse transcription of 1 μg RNA was performed in a 20 μl SuperScript III reaction mixtures (Invitrogen) according to the manufacturer’s instructions. One tenth of the resulting cDNAs were used for each qPCR composed of 4 μl diluted cDNA, 5 μl Power SYBR Green Master Mix (Applied Biosystems) and 1 μl primer mix (2 μM). The primers used in the present study were as follows: p21-forward (5'-AGCAGGGCTGAAGGTTCCA-3') and p21-reverse (5'-TCAGCCGGCGTTTGGAGTGG-3'); 14-3-3ε-forward (5'-GCCCTGCTGCTCTGATCG-3') and 14-3-3ε-reverse (5'-ACAGCCAGCAGACATGCTTC-3'); GAPDH-forward (5'-CAAGAAGTGTTGAAGCAGG-3') and GAPDH-reverse (5'-GCT-GTTGAGTCGAAGGACCC-3'). The reaction was conducted and detected by StepOnePlus Real-Time PCR system (Applied Biosystems).

SA-β-Gal staining. Staining of SA-β-Gal activity was performed as described previously (Chen et al., 2009). Briefly, cells were washed with PBS and fixed in 2 % formaldehyde and 0.2 % glutaraldehyde for 15 min at room temperature. After thorough washing with PBS twice, cells were incubated at 37 °C overnight with a staining solution containing 40 mM citric acid/sodium phosphate (pH 6), 150 mM NaCl, 2 mM MgCl₂, 1 mg X-Gal ml⁻¹, 5 mM potassium ferrocyanide and 5 mM potassium ferricyanide. Cells were viewed under bright-field illumination at ×200 magnification.

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REFERENCES


