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Epstein-Barr Virus BRLF1 Inhibits Transcription of IRF3 and IRF7 and Suppresses Induction of Interferon-β

Gretchen L. Bentz[1,4], **Renshui Liu**[1,4], **Angela M. Hahn**[5], **Julia Shackelford**[3,4], and **Joseph S. Pagano**[2,4,*]

[2]Departments of Medicine and Microbiology and Immunology, The University of North Carolina, Chapel Hill, North Carolina USA

[3]Department of Cellular Biology, The University of North Carolina, Chapel Hill, North Carolina USA

[4]Lineberger Comprehensive Cancer Center, The University of North Carolina, Chapel Hill, North Carolina USA

Abstract

Activation of interferon regulatory factors (IRFs) 3 and 7 is essential for the induction of Type I interferons (IFN) and innate antiviral responses, and herpesviruses have evolved mechanisms to evade such responses. We previously reported that Epstein-Barr Virus BZLF1, an immediate-early (IE) protein, inhibits the function of IRF7, but the role of BRLF1, the other IE transactivator, in IRF regulation has not been examined. We now show that BRLF1 expression decreased induction of IFN-β, and reduced expression of IRF3 and IRF7; effects were dependent on N- and C-terminal regions of BRLF1 and its nuclear localization signal. Endogenous IRF3 and IRF7 RNA and protein levels were also decreased during cytolytic EBV infection. Finally, production of IFN-β was decreased during lytic EBV infection and was associated with increased susceptibility to superinfection with Sendai virus. These data suggest a new role for BRLF1 in the ability of to evade host innate immune responses.

Keywords

Epstein-Barr Virus; Rta; BRLF1; Type I interferons; antiviral response; Interferon Regulatory Factor; IRF3; IRF5; IRF7; immune response

Introduction

Interferon (IFN) regulatory factors (IRFs) are a family of transcription factors that play a critical role in the regulation of IFN-stimulated genes (ISGs) as well as the induction of the Type I IFNs, including IFN-α and IFN-β. The human IRF family, which consists of 9 members (IRF1-9), is defined by a highly conserved amino-terminal DNA-binding domain characterized by a five-tryptophan residue repeat (Eason et al., 1999) that allows the IRFs to bind, as

*Corresponding author: Dr. Joseph S. Pagano, Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, CB# 7295, Chapel Hil, NC, 27599, Phone: 919- 966-5907, FAX: 919-966-9673, jspagano@med.unc.edu.
[1]Contributed equally
[5]Present Address: School of Allied Health and Life Sciences, University of West Florida, Pensacola, Florida USA

homodimers or heterodimers, to consensus GAA and AANNNGAA motifs found in IFN-stimulated response elements (ISREs), including promoters of the Type I IFNs and ISGs (Paun and Pitha, 2007). This interaction mediates how IRFs exert critical effects on ISG expression, cellular growth, cellular differentiation, and innate immune responses (Barnes et al., 2002; Nguyen et al., 1997; Paun and Pitha, 2007; Tamura et al., 2008).

Of all the members of the IRF family, IRF3 and IRF7 are considered to be the key regulators of the expression of Type I IFNs (Honda et al., 2006; Honda and Taniguchi, 2006; Honda et al., 2005; Paun and Pitha, 2007; Sakaguchi et al., 2003; Zhang and Pagano, 1997; Zhang and Pagano, 2002). While IRF3 is responsible for the early phase of Type I IFN induction, IRF7 is now understood to be the master regulator of all Type I IFN-dependent responses (Honda et al., 2006; Honda et al., 2005), and together they are critical elements in the activation of host innate immune responses, particularly in response to infection by different pathogens, including viruses. Virus-infected cells produce a mixture of Type I IFNs, but fibroblasts and epithelial cells synthesize predominantly IFN-β, whereas leukocytes, macrophages, and dendritic cells mainly express IFN-α (Malmgaard, 2004). Together, the production of both IFN-α and IFN-β has important immune-modulatory consequences, specifically through enhancing antigen presentation in virally infected cells leading to their destruction (Malmgaard, 2004) as well as through regulation of cytokines released by the infected cells (Abele and Tampe, 2004; Luft et al., 1998; Malmgaard, 2004). In addition to the antiviral effects of the Type I IFNs, ISGs are also important for host innate immune responses through their ability to inhibit viral replication through degradation of RNA and inhibition of protein translation (Sen and Sarkar, 2007).

The ability of IRF3 and IRF7 to regulate the expression of both Type I IFNs and ISGs points to the importance of these transcription factors in controlling viral infection and virus replication. Viruses, including herpesviruses, have evolved mechanisms through which they can circumvent the activation of IRF3 and IRF7 and block innate responses. For example, binding and entry of herpesviruses into cells promotes activation of IRF3 and IRF7 (Grandvaux et al., 2002; Li et al., 2004; Means et al., 2002; Miller et al., 2002; Pollara et al., 2004). However, despite this initial activation of cellular antiviral responses accumulation of IFNs and ISG transcripts is inhibited following viral replication (Grandvaux et al., 2002; Li et al., 2004; Means et al., 2002; Miller et al., 2002; Pollara et al., 2004).

Specific examples can be found in each *Herpesviridae* subfamily. For the α-herpesviruses, the immediate-early protein, ICP0, of bovine herpesvirus I and herpes simplex virus inhibits the activity of IRF3 by recruiting activated IRF3 and inducing its degradation (Melroe et al., 2004; Melroe et al., 2007; Saira et al., 2007). ICP0 also inhibits IRF7 transactivation activity (Saira et al., 2007) as well as inactivates the Jak/Stat signaling pathway, thereby impeding the expression of the Type I IFNs (Eidson et al., 2002; Harle et al., 2002; Lin et al., 2004; Melroe et al., 2004; Melroe et al., 2007; Pollara et al., 2004; Saira et al., 2009). The β-herpesvirus human cytomegalovirus (HCMV) encodes a protein, pp65, that subverts the activation of IRF3 by inhibiting its nuclear accumulation and regulating innate immune responses (Abate et al., 2004). The IE protein 1 of the related β-herpesvirus HHV-6 also inhibits the nuclear localization of IRF3 leading to decreased IFN-β production (Jaworska et al., 2007). For the γ-herpesviruses, human herpesvirus-8 (Kaposi's sarcoma herpesvirus; KSHV) and the related rhesus rhadinovirus encode a cluster of IRF homologous genes, called viral IRFs (vIRFs), which cannot bind to ISREs but suppress expression of the Type I IFNs by forming heterodimers with cellular IRFs and repressing their ability to transactivate promoters (Barnes et al., 2002). We have shown that Epstein-Barr virus (EBV) IE protein Zta (BZLF1) physically interacts with IRF7, inhibiting its ability to activate the IFN-α, IFN-β, and Tap2 promoters (Hahn et al., 2005). The EBV tegument protein LF2 also interacts with IRF7, inhibiting its ability to bind to and activate the IFN-α promoter (Wu et al., 2009). In addition, EBV BGLF4, the viral PK,

interacts with IRF3 and reduces the amount of active IRF3 recruited to ISREs and thus inhibits induction of the Type I IFNs (Wang et al., 2009a). These findings led us to ask whether other EBV proteins might regulate the activity of IRFs and inhibit innate immune responses.

Infection with EBV produces both lytic and latent infections. The initiation of the lytic cycle, either via primary infection or following reactivation of viral replication from the latent state, is controlled by the IE proteins BZLF1 (Zta) and BRLF1 (Rta). EBV Rta is a 605-amino-acid (aa) protein with no known cellular homologs. The N-terminal region of Rta contains a DNA-binding domain (aa 1-280) that coincides with a dimerization domain (aa 1-232) (Manet et al., 1993; Manet et al., 1991). The mid-region of Rta contains the nuclear localization sequence (NLS), which is responsible for the localization of Rta in the nucleus (Hsu et al., 2005). The C-terminal region of Rta contains the transcriptional activation domain that interacts with TATA-binding protein and TFIID (Manet et al., 1993; Manet et al., 1991). While EBV Rta mainly functions as a transcriptional inducer of early and late viral genes, it also interacts with several cellular proteins and affects the activities of host cells to facilitate viral replication (Adamson et al., 2000; Darr et al., 2001; Li et al., 2004). To date, no known immunomodulatory function has been uncovered for Rta, and knowledge of the mechanisms by which EBV escapes innate immune responses is still incomplete.

Here we show that EBV Rta can downregulate the transcription of IRF3 and IRF7 resulting in decreased protein expression and thereby modulate Type I IFN responses to virus infection. Endogenous levels of these IRFs, but not IRF5, are reduced during reactivation of the viral lytic cycle in EBV-infected cells. Finally, endogenous levels of Type I IFN, specifically IFN-β, are decreased following EBV reactivation and coincide with increased susceptibility of the EBV-infected cells to superinfection with Sendai virus. These findings suggest that EBV can avert suppression of viral replication by Type I IFNs by downregulation of IRF3 and IRF7.

Methods and Materials

Reagents and antibodies

N-carbobenzoxyl-L-leucinyl-L-norleucinal (MG132), mammalian ubiquitin, rabbit ubiquitin-activating enzyme (E1), and recombinant human UBC H6 were purchased from Boston Biochem, Inc. (Cambridge, MA). Protease-inhibitor cocktail was from Roche. The nuclear preparation kit was from Thermo Scientific (Rockford, IL). Antibodies against IRF3, IRF5, IRF7, GAPDH, γ-tubulin, ubiquitin, GFP and lamin were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). EBV Rta antibodies were obtained from Argene (Varilhes, France). HA mAb 12CA5, FLAG mAb M2, Anti-FLAG affinity beads and anti-HA affinity beads were from Sigma-Aldrich (St. Louis, MO). cIAP1 was expressed in 293T cells and purified as previously described (Yang et al., 2000).

Plasmids and constructs

Flag-tagged full-length IRF3 and IRF5 cDNAs were inserted into the mammalian expression vector pCMV-2. pCMV2-Flag-tagged IRF-7 expression plasmid was a gift from Dr. George Miller. pCI-GFP-Rta and pCI-GFP-Rta mutant plasmids were kindly provided by Dr. Tsuey-Ying Hsu (Hsu et al., 2005). pcDNA3-HA-Rta full-length plasmid was provided by Dr. Shih-Tung Liu (Chang et al., 2004).

Transfections and luciferase assay

293T cells were maintained in DMEM (Cellgro, Mediatech; Manassas, VA) supplemented with 10% fetal bovine serum (FBS; Cellgro). Transient transfection were performed in 6-well plates with Lipofectamine 2000 (Invitrogen; Carlsbad, CA) according to the manufacturer's protocol, and 0.2 μg reporter plasmid, 0.1 μg Renilla luciferase plasmid (pRL-TK [Promega]),

and 1 μg expression plasmid used unless otherwise noted. At 24 h after transfection, cells were harvested for luciferase assays as described before (Hahn et al., 2005; Ning et al., 2003; Ning et al., 2005a; Ning et al., 2005b) with dual luciferase assay reagent (Promega); relative light units were normalized to the activity of Renilla luciferase and used as internal control. Representative data of three experiments done in triplicate are shown.

In some experiments, cells were infected with Sendai virus (50 HA units/ml; a gift from Dr. Mark Heise, University of North Carolina at Chapel Hill) 16 hours post-transfection. Eight hours after infection, cells were collected for analysis.

Immunoblotting

The harvested cell pellets were separated into cytoplasmic extract and nuclear fractions by the method described (Smirnova et al., 2000) or lysed with 0.5% CHAPS in 20 mM HEPES (pH 7.4), 10 mM KCl, 1 mM MgCl2, and 1 mM dithiothreitol (all Sigma-Aldrich). The modified Bradford method was used to quantify protein concentration in supernatant fluids. Cellular proteins and pre-stained molecular mass markers were subjected to SDS-PAGE and transferred to nitrocellulose membrane. Membranes were blocked with 5% nonfat milk in TBST; incubated with antibodies to ubiquitin γ-tubulin, lamin, GFP, Flag or HA (1:2000) for 2 h; washed with TBST and stained with peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:5000). Immunoreactivity was visualized by enhanced chemiluminescence (ECL kit; Santa Cruz).

Semi-quantitative RT-PCR

293T cells were plated in 6-well plates at a density of 7.5×10^5 the day before experiments. Cells were transfected with 2 μg of plasmids expressing EBV Rta or empty vector pCMV. 14 hours post-transfection, cells were stimulated with 500U/ml of Type I IFN (Biomedical Lab). RNA was harvested 24 hours post-transfection using the Qiagen RNeasy Kit. Total RNA was reverse-transcribed with ABI PRISM RT kit. The cDNA was amplified by PCR reaction with specific sense and anti-sense primers for IRF3, IRF5, IRF7, and actin. PCR products were fractionated on 0.8% agarose gels, stained with ethidium bromide, and bands detected by UV.

Induction of Akata cells

Akata cells, an EBV latently infected cell line derived from Burkitt's lymphoma, were maintained in RPMI (Cellgro) supplemented with 10% heat-inactivated FBS. For induction of lytic infection, 3.0×10^7 cells were collected and divided into three samples. Each sample was plated at a density of 2×10^6 cells per ml of medium. F(AB′)2 fragment to human IgG (100 μg/ml; MP Biomedicals, LCC., Solon, OH) was used to cross-link cell surface IgG (Gershburg et al., 2004; Takada and Ono, 1989; Zacny et al., 1999). Cells were incubated for one hour at 37°C with 5% CO_2 with occasional mixing, at which time cell density was adjusted to 1×10^6 cells per ml of medium. At 0, 24, and 48 hours after reactivation, cells were collected. Half of each sample was used for Western blot analyses of expression of IRF3, IRF5, IRF7, Rta, and EA-D. GAPDH was used as loading control. Total RNA was harvested from the remaining half of the sample, and semi-quantitative RT-PCR performed. Samples were separated on 2% agarose gels to examine RNA expression of IRF3, IRF5, IRF7, IFN-β, and Rta. Densitometry was performed with relative RNA or protein expression normalized to GAPDH expression. Data are shown as fold change (relative to 0 hours post-induction) ± standard deviation of experiments performed in triplicate.

In some experiments, Akata cells (induced and left uninduced) were infected with Sendai virus (50 HA units/ml) 24 hours after reactivation. 24 hours after infection, supernatant fluids were collected and used for plaque assays of Sendai virus replication.

Plaque Assays

Plaque assays were performed as previously described in LLC-MK_2 cells (Touzelet et al., 2009). Briefly, cells were grown to confluence in six-well plates. Samples were serially diluted in DMEM and left to absorb for 1 hour at 37°C. Cells were washed and overlaid with 3 ml DMEM with 1% agar (Bacto), 1.5 mg.ml BSA (Sigma), and 1.2 μg/ml trypsin (Sigma). Five days later, agar was removed, cells were stained with crystal violet, and plaques were counted. Data are shown as fold change in plaque-forming units (PFU)/ml (relative to uninduced) ± standard deviation of experiments performed in triplicate.

Results

Rta negatively regulates IFN-β promoter activity

IFN promoter-reporter activity is inhibited during γ-herpesvirus lytic infection (Hahn et al., 2005; Manet et al., 1993). We have reported that the EBV IE transactivator Zta inhibits IFN promoter activity (Hahn et al., 2005), and others have shown KSHV ORF50/Rta downregulates IFN activity (Manet et al., 1993). Therefore, we investigated whether EBV Rta could inhibit IFN responses by similar or different mechanisms. To determine whether IFN-β expression is downregulated by Rta, luciferase activity from the IFN-β promoter-reporter construct, an established target of IRFs (Lin et al., 2000; Yang et al., 2004), was assayed in 293T cells.

IFN-β reporter construct was transfected into 293T cells with control vector or plasmid encoding EBV Rta. Following transfection, cells were infected with Sendai virus, which induces a robust antiviral response. IFN-β promoter activity was measured by relative luciferase activity, normalized to renilla-luciferase expression. Upon Sendai virus infection, transfected cells showed a 46-fold increase in relative IFN-β promoter activity (Figure 1A). Co-expression of EBV Rta strongly suppressed this increased promoter activity, returning it to basal levels and indicating that EBV Rta protein strongly inhibits Sendai virus-induced IFN-β promoter activity.

To examine the biological relevance of these findings more directly, endogenous IFN-β RNA levels were also examined (Figure 1B). 293T cells were transfected with control vector or EBV Rta and mock- or Sendai virus-infected 16 hours after transfection. Semi-quantitative RT-PCR was performed on RNA harvested 24 hours after transfection (eight hours after infection). Results revealed a trend similar to that observed with the reporter assays in which Sendai virus infection strongly induced the production of IFN-β RNA, and EBV Rta expression greatly inhibited this response. These results suggest that EBV Rta is capable of regulating the activation of the IFN-β promoter and in turn the production of IFN-β, thus regulating Type I IFN responses.

Because IRF3 and IRF7 play central roles in the production of Type I IFN, including IFN-β, during virus infection (Honda et al., 2006; Honda and Taniguchi, 2006; Honda et al., 2005; Paun and Pitha, 2007; Sakaguchi et al., 2003; Zhang and Pagano, 1997; Zhang and Pagano, 2002), we next examined whether EBV Rta affected transcriptional activities of IRF3 and IRF7 by IFN-β reporter assays, in which IRF3 or IRF7 were co-expressed with the viral protein. The results showed that overexpression of IRF3 and IRF7 significantly ($P < 0.05$) increased transactivation of the IFN-β promoter (Figures 1C and 1D). EBV Rta expression abrogated the increased transcriptional activity of IRF3 and IRF7 (Figures 1B and 1C) but did not affect basal IFN-β promoter activity. These data suggest that EBV Rta can suppress induction of IFN-β by down-regulating the activities of IRF3 and IRF7.

EBV Rta negatively regulates levels of IRF3 and IRF7 proteins

To decipher how EBV Rta suppressed the transcriptional activity of IRF3 and IRF7, we first examined whether it altered their expression along with that of IRF5, an IRF family member involved in signal transduction events triggered by virus infection that activate Toll-like receptors (Barnes et al., 2002; Malmgaard, 2004; Takaoka et al., 2005). 293T cells were transfected with Flag-tagged IRFs and RTA expression plasmids, and IRF and Rta expression was analyzed 24 hours later. High levels of Flag-tagged IRF3, IRF5, and IRF7 were detected (Figure 2A). Co-expression of EBV Rta consistently coincided with decreased expression levels of IRF3 and IRF7 while IRF5 levels were not altered (Figure 2A). Thus, EBV Rta selectively down-regulates the expression of IRF3 and IRF7, the main regulators of the Type I IFNs.

Rta downregulates IRF3 and IRF7 expression in the cytoplasm and nucleus independent of its localization

The phosphorylation of the IRFs, including IRF3 and IRF7, and their subsequent nuclear translocation are important steps in their transcriptional activation (Nguyen et al., 1997; Paun and Pitha, 2007). We next determined if EBV Rta affects the activation of IRF3 and IRF7 in addition to regulating their expression levels. Cytoplasmic and nuclear extracts were collected 24 hours after transfection and analyzed by Western blot analyses. IRF3 and IRF7 were detected in both cytoplasmic and nuclear extracts (Figure 2B), suggesting that they are activated by phosphorylation resulting in their nuclear translocation. Co-expression of EBV Rta resulted in reduced expression of IRF3 and IRF7 in both the cytoplasmic and nuclear extracts (Figure 2B), but the proteins were still detected in both extracts. These data confirm that the expression of EBV Rta inhibits the expression of IRF3 and IRF7 and suggests that Rta does not affect the activation, phosphorylation, and nuclear translocation of these IRFs.

All three EBV Rta functional domains are required for its ability to regulate IRF3 and IRF7 expression

To examine the mechanism by which EBV Rta regulates the expression of IRF3 and IRF7, the contributions of the different functional domains of Rta that mediate its function as a transcriptional activator were examined. Three main Rta domains are the amino terminus, which contains a DNA-binding domain (aa 1 to 280), a dimerization domain (aa 1 to 232), and a transcriptional activation domain in the carboxy-terminus which contains a nuclear localization signal (Manet et al., 1993; Manet et al., 1991). EBV Rta (pGFP-Rta wt (GFP-tagged Rta (aa 1-605)), Rta domain mutants (pGFP-Rta ΔNLS (mutation of the NLS); pGFP-Rta ΔN (aa 100-605); pGFP-Rta ΔC (aa 1-441)), or empty vector were co-expressed with flagged-tagged IRF3 or IRF7. GFP-Rta wt, GFP-Rta ΔN, and GFP-Rta ΔC localized to the nucleus, while GFP-Rta ΔNLS was only detected in cytoplasm (data not shown), confirming that the NLS is required for the nuclear translocation of Rta. IRF3, IRF7, and all forms of Rta were expressed at expected molecular sizes (Figure 3). High levels of Flag-tagged IRF3 and IRF7 were detectable when co-expressed with empty GFP vector, but levels were significantly down-regulated when co-expressed with GFP-Rta. Deletion of the N- or C-termini or mutation in the NLS sequence of Rta completely abrogated its ability to alter expression of IRF3 and IRF7. Together, these data demonstrate that both the DNA-binding and dimerization domains of the N-terminus and the C-terminal transactivation domain are required for EBV Rta to regulate expression of IRF3 and IRF7. In addition, the finding that the NLS is essential for Rta-dependent IRF down-regulation suggests that Rta may regulate their expression at the transcriptional level.

Rta expression reduces endogenous RNA levels of IRF3 and IRF7 but not IRF5

Rta is a known transcription factor (Ragoczy et al., 1998; Ragoczy and Miller, 1999), so we tested whether it could regulate the expression of the IRFs at the transcriptional level by assay of endogenous levels of IRF RNAs by semi-quantitative RT-PCR. 293T cells, transfected with EBV Rta or vector control, were stimulated with Type I IFN, RNA extracted and levels of IRF3, IRF5, IRF7, and actin, which was used as a loading control, were measured. Levels of IRF3, IRF5, IRF7 RNA were readily detectable (Figure 4A). Over-expression of EBV Rta resulted in reduced IRF3 and IRF7 RNA levels while IRF5 RNA levels remained unchanged (Figure 4A).

To confirm these results, 293T cells were also transfected with EBV Rta or vector control and infected with Sendai virus (50 HA units/ml). RNA was extracted and semi-quantitative RT-PCR performed to examine the expression of endogenous levels of IRF3, IRF5, and IRF7 (Figure 4B). The data showed that Sendai virus slightly increased expression of these two IRFs while IRF5 levels remained the same. Together, these results demonstrate that EBV Rta selectively regulates the expression of IRF3 and IRF7 at the transcriptional levels, which in turn accounts for the decreased protein levels observed (Figures 2 and 3).

Endogenous IRF3 and IRF7 RNA and protein levels are diminished during viral reactivation

Finally, we examined the expression of IRF3 and IRF7 in the viral lytic cycle. Akata cells are latently EBV-infected Burkitt's lymphoma cells in which viral reactivation can be induced by cross-linking surface B-cell receptors with IgG (Gershburg et al., 2004; Takada and Ono, 1989; Zacny et al., 1999). Cells were collected 0, 24, and 48 hours after induction and divided so that RNA could be extracted for RT-PCR and protein analyzed by Western blot analyses.

IRF3, IRF5 and IRF7 RNA were all readily detectable in Akata cells at the time of induction (Figure 5A). 24 and 48 hours later, RNA levels for IRF3 and IRF7 were decreased but IRF5 levels remained unchanged. IRF3 and IRF7, but not IRF5, protein levels decreased in a similar fashion (Figure 5B). As a control for viral reactivation, we also examined the expression of Rta and EA-D, an EBV early gene (Figure 5B). Densitometry of relative IRF RNA and protein levels normalized to relative GAPDH RNA and protein levels confirmed the trend observed in IRF3 and IRF7 expression. These data demonstrate that EBV Rta inhibits the expression of IRF3 and IRF7 at the transcriptional level resulting in decreased protein expression.

The biological relevance of these data were also investigated by examining the endogenous expression of IFN-β following Akata cell induction as well as by examining the susceptibility of uninduced and induced Akata cells to Sendai virus superinfection. First, semi-quantitative RT-PCR was performed to examine RNA levels of IFN-β in Akata cells at 0, 24, and 48 hours post induction (Figure 6A). Densitometry of relative IFN-β RNA levels normalized to relative GAPDH RNA levels showed that there is a significant ($P < 0.05$) decrease in IFN-β expression by 48 hours post induction, which coincides with decreased IRF3 and IRF7 expression. Second, Akata cells were induced or left uninduced for 24 hours and then infected with Sendai virus (50 HA units/ml) for 24 hours. Cell supernatant fluids were collected and used to quantitate Sendai virus replication by plaque assays on LLC-MK_2 cells. The data showed that induced Akata cells were significantly ($P< 0.05$) more susceptible to Sendai virus superinfection when compared with uninduced Akata cells (Figure 6B). Specifically, Sendai virus reached titers 13-fold greater in induced Akata cells compared to uninduced cells. Together, these data suggest that EBV Rta contributes to immune evasion and inhibition of Type I IFN responses during lytic infection through regulation of IRF3 and IRF7 expression.

Discussion

Protection from consequences of viral infection depends on innate immune responses. To evade such responses, herpesviruses have evolved mechanisms by which they inhibit the central activators of Type I IFN responses, IRF3 and IRF7 (Abate et al., 2004; Barnes et al., 2002; Eidson et al., 2002; Hahn et al., 2005; Harle et al., 2002; Jaworska et al., 2007; Lin et al., 2004; Pollara et al., 2004; Saira et al., 2009; Wu et al., 2009). Relatively little has been reported on how EBV performs such maneuvers. Recently three EBV products expressed at early stages of productive viral infection, Zta, LF2, and BGLF4, have been reported to interact with IRF7 and inhibit the induction of IFN-β (Hahn et al., 2005; Wu et al., 2009). Here we shown that EBV Rta, the second major EBV IE protein, down-regulates the activity of IRF3 and IRF7 by a different mechanism. Specifically, EBV Rta suppressed IFN-β promoter activity and RNA levels induced by Sendai virus by reducing levels of IRF3 and IRF7 RNA and protein, which are ordinarily expressed during viral infection. Suppression of these IRFs was selective in that IRF5 was not affected. In addition, endogenous IRF3 and IRF7 RNA and protein levels were reduced during lytic EBV replication, and coincide with decreased levels of IFN-β RNA and increased susceptibility of the EBV-infected cells to superinfection with Sendai virus.

Our findings also demonstrate that the mechanism whereby EBV Rta causes evasion of Type I IFNs is quite different from that used by its KSHV homolog (KSHV Rta, ORF50). Specifically, EBV Rta does not influence the activation and nuclear translocation of the IRFs (Figure 2B), whereas KSHV Rta inhibits these events (Yu et al., 2005). Furthermore, KSHV Rta functioned as an ubiquitin E3 ligase for IRF7 resulting in its ubiquitination and proteasome-mediated degradation (Yu et al., 2005). However, we could not detect EBV Rta E3 ligase activity with the substrates tested (Supplemental Figure 1).

We define a novel mechanism by which EBV Rta contributes to immune evasion. Rta has been shown to bind directly and indirectly to several promoters (Adamson et al., 2000; Darr et al., 2001; Hsu et al., 2005; Kenney et al., 1989; Quinlivan et al., 1993), and we show expression of Rta expression resulted in decreased endogenous IRF3 and IRF7 RNA and protein levels, but not IRF5 levels. Because Rta also inhibited overexpression of IRF3 and IRF7, it is possible that Rta affects expression by binding to GC-rich regions of these genes or by affecting RNA elongation. However, this mechanism has not been reported for Rta or any IRFs.

Not only herpesviruses but other viruses can suppress innate immune responses by inhibiting the activity of IRF3 and IRF7 (Abate et al., 2004; Barnes et al., 2002; Barnes et al., 2004; Eidson et al., 2002; Hahn et al., 2005; Harle et al., 2002; Lin et al., 2004; Pollara et al., 2004; Saira et al., 2009; Wang et al., 2009a; Wu et al., 2009). Human papillomavirus and hepatitis B virus, encode proteins that prevent the activation of IRF3 (Wang et al., 2009b) or the expression of IRF7 (Xu et al.), respectively, resulting in suppression of the Type I IFNs. Other quite different viruses, including the RNA viruses Ebola, hepatitis C, and rotavirus all are united by encoding proteins that inhibit the activation of IRF3 (Baril et al., 2009; Basler et al., 2003; Foy et al., 2003; Hartman et al., 2008a; Hartman et al., 2008b; Myskiw et al., 2009). This list is still growing, and it is likely that most viruses have mechanisms by which they evade the activation of the Type I IFNs to allow virus replication to occur. A possible common link between DNA and RNA virus infection and regulation of the innate immune responses is the ability of the virus to regulate Toll-like receptor (TLR) signal transduction events (Schroder and Bowie, 2007; Severa and Fitzgerald, 2007).

The data presented here disclose how EBV Rta affects IRF3 and IRF7 expression and induction of IFN promoters thus regulating expression of the Type I IFNs; similar effects have also been observed with other members of the IRF family. IRF1 is involved in antiviral immune responses mediated by IFN-γ (Paun and Pitha, 2007), and while it does not have a role in virus stimulation

of the Type I IFNs, it binds to a transcription enhancer complex in the IFN-β promoter region (Thanos and Maniatis, 1995). IRF2 can also bind to this complex and regulate IFN-β expression (Paun and Pitha, 2007). IRF9 has distinct antiviral effects in that it binds STAT1/2 heterodimers forming another complex, IFN-stimulated growth factor 3, which binds ISREs (Fu et al., 1992; Improta et al., 1994). Examination of the effect of EBV Rta on the expression of IRF1, IRF2, and IRF9 revealed that EBV Rta suppressed expression of IRF1 and IRF3, but not IRF9, at the transcriptional level (Supplemental Figure 2), resulting in decreased protein expression and decreased transactivational activity (data not shown). The basis for these selective effects on IRFs is unknown. EBV Rta is well known for transactivational activity on EBV promoters (Ragoczy et al., 1998; Ragoczy and Miller, 1999) as well as a cellular promoter (Li et al., 2004), so it is possible that Rta directly exerts its inhibitory effect on the promoters of IRF1, 2, 3, and 7 and does not bind to the promoters of IRF5 and IRF9. Some support for this idea comes from our data that the NLS, the DNA-binding and dimerization domains, and the transactivating domain, which interacts with TATA-binding protein and TFIID, are required for the observed Rta-mediated response (Figure 3). Together, these findings suggest a specific mechanism by which EBV Rta regulates how select IRFs subvert the activation of interferon responses.

We propose that EBV has multiple methods that target different facets of the IFN response and suppresses its induction. For example, Zta can counteract an inflammatory response via the inhibition of IRF7 activity but not through the down-regulation of IRF7 expression (Hahn et al., 2005). Recently, a role for two EBV early proteins, LF2 and BGLF4, in regulating the host IFN- mediated immune response based on their ability to regulate the function of IRF7 and IRF3, has been uncovered (Wang et al., 2009a; Wu et al., 2009). While EBV LF2 interacts with IRF7, inhibiting its ability to bind to and activate the IFN-α promoter (Wu et al., 2009), EBV BGLF1 interacts with IRF3 reducing the amount of active IRF3 recruited to the ISREs (Wang et al., 2009a). Accordingly, we propose that following viral reactivation, upon expression of the EBV IE proteins Zta and Rta, Zta interacts with endogenous IRF7 and possibly IRF3, inhibiting their activity. At the same time Rta decreases the transcription of IRF3 and IRF7 resulting in decreased protein expression. By the time the EBV early proteins, BGLF4 and LF2, are expressed, only limited amounts of the IRFs are present in the cell, and these viral proteins interact with and inhibit the remaining IRF3 and IRF7. This multi-pronged mechanism ensures that the Type I IFN response is inhibited and allows viral replication to occur. The role of additional immediate-early (BHRF1), early, and late proteins in this complex response remains to be examined, but it is likely that other viral proteins contribute to the observed antiviral response.

Conclusion

We have shown here, for the first time, that endogenous RNA and protein levels of IRF3 and IRF7 are down-regulated during the course of lytic EBV infection. The mechanism by which Rta regulates the ability of the IRFs to induce IFN production (by transcriptional regulation of IRF expression) is unique when compared with the way other viral proteins regulate IFN responses, namely, by degrading the IRFs or directly inhibiting their transactivational activity and thus circumventing host immune responses. The fact that EBV invokes its two principal IE lytic-cycle transactivators, Rta and Zta, and two early lytic-cycle proteins in the suppression of IFN responses emphasizes the biological importance of these events for successful viral replication.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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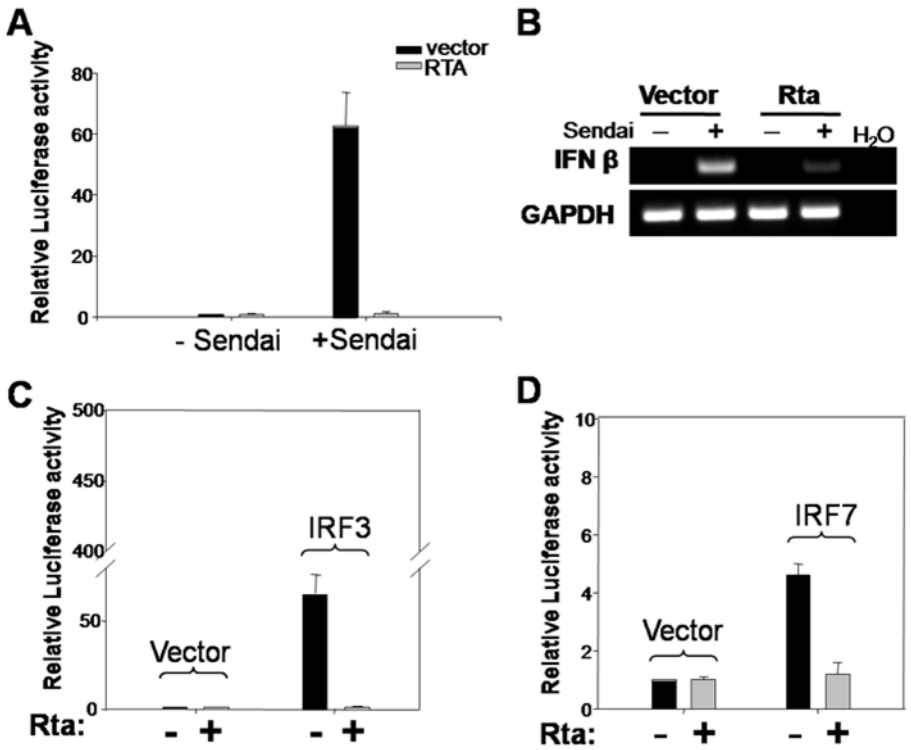

Figure 1. EBV Rta regulates IFN-β promoter activity

A) 293T cells were transfected with EBV Rta or vector control along with IFN-β reporter plasmid and Renilla luciferase plasmid. Cells were mock-infected or infected with Sendai virus (50 HA units/ml) 16 hours post transfection. Eight hours later (24 hours post transfection), cells were harvested for firefly luciferase assays and normalized to Renilla luciferase expression. **B)** 293T cells were transfected with EBV Rta or vector control and Sendai virus infected 16 hours post transfection. 24 hours post transfection, cells were collected and RNA harvested for semi-quantitative RT-PCR to examine IFN-β and GAPDH expression. 293T cells were transfected with the vector control or **C)** IRF3 or **D)** IRF7 in the presence or absence of EBV Rta.or the vector control and luciferase assays were performed 24 hours post transfection. Data are shown as the fold change (relative to vector control) ± standard deviation of experiments performed in triplicate.

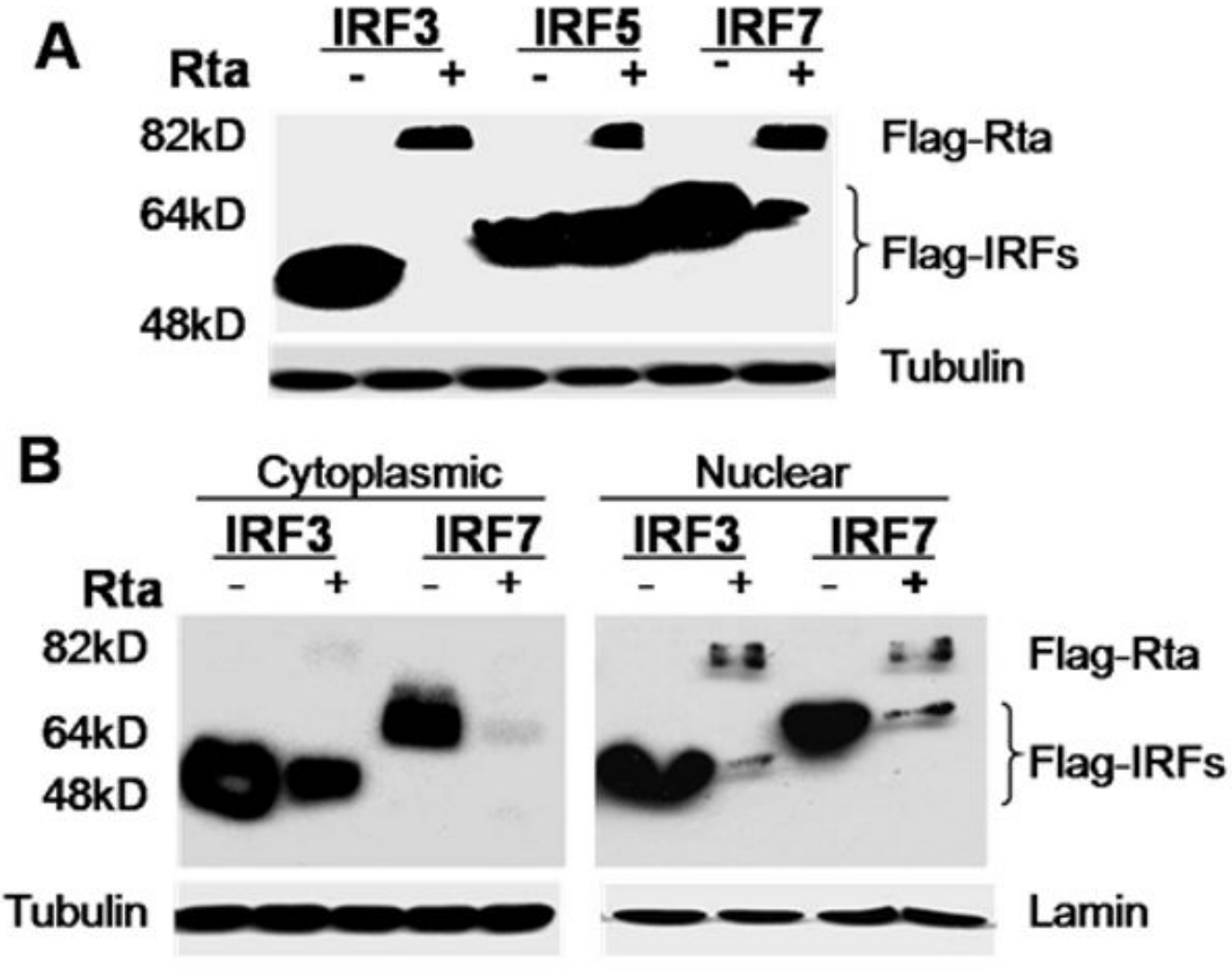

Figure 2. EBV Rta negatively regulates IRF3 and IRF7 protein expression but does not alter IRF5 expression

293T cells were transfected with Flag-IRF3, Flag-IRF5, Flag-IRF7, and/or Flag-EBV Rta as indicated. **A)** 48 hours post transfection whole cell lysates were collected and Western blot analyses performed examining Flag expression/exogenous protein expression. **B)** 48 hours post transfection, cytoplasmic extracts and nuclear extracts were collected and Western blot analyses performed examining Flag expression in the two extracts. Tubulin and lamin were used as loading controls.

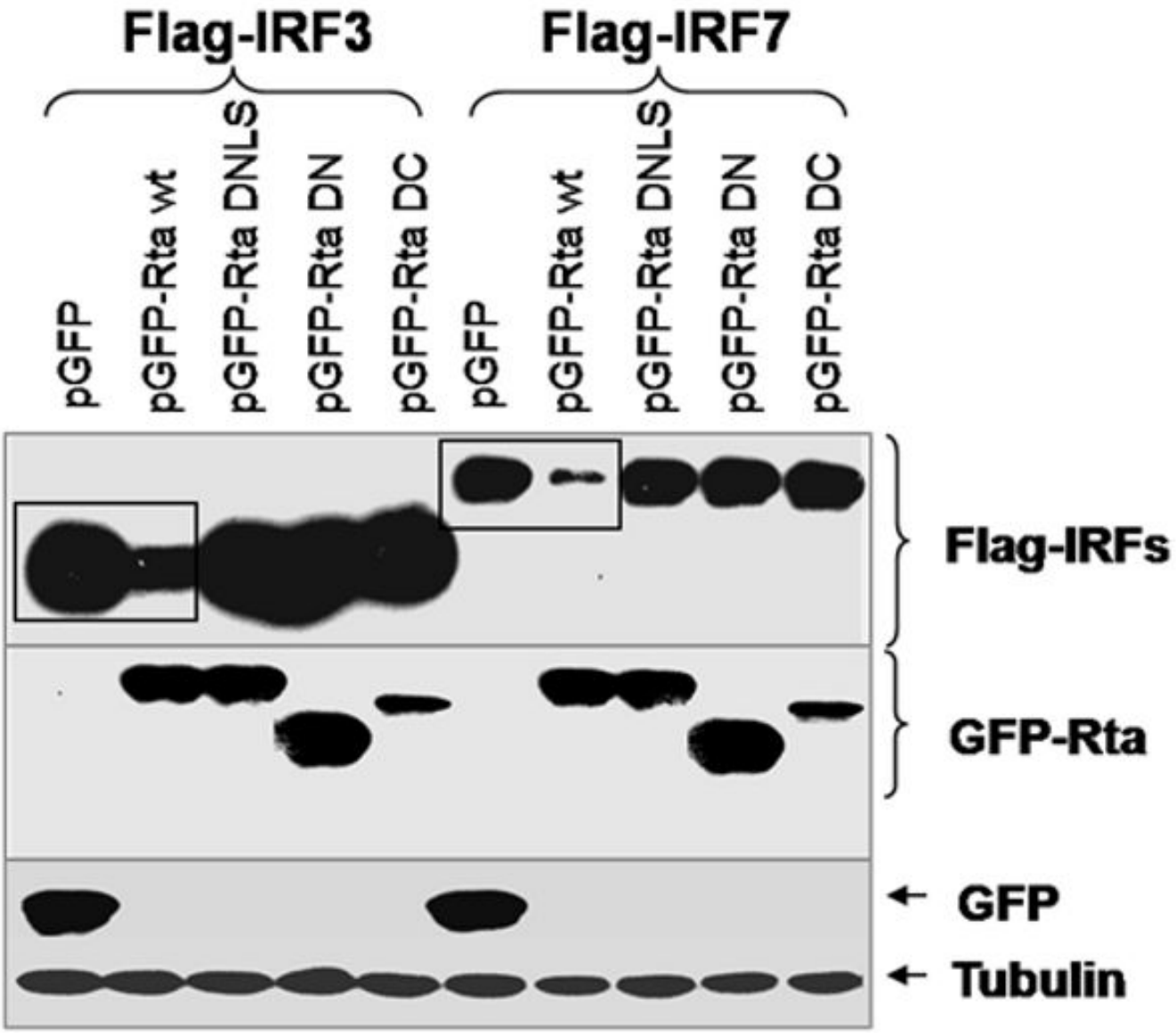

Figure 3. All classified EBV Rta function domains are required for the regulation of IRF3 and IRF7 expression

293T cells were transfected with Flag-IRF3, Flag-IRF7, and GFP-EBV Rta or select GFP-EBV Rta mutants as indicated. 48 hours post transfection whole cell lysates were collected and Western blot analyses performed examining Flag and GFP expression. Tubulin was used as a loading control.

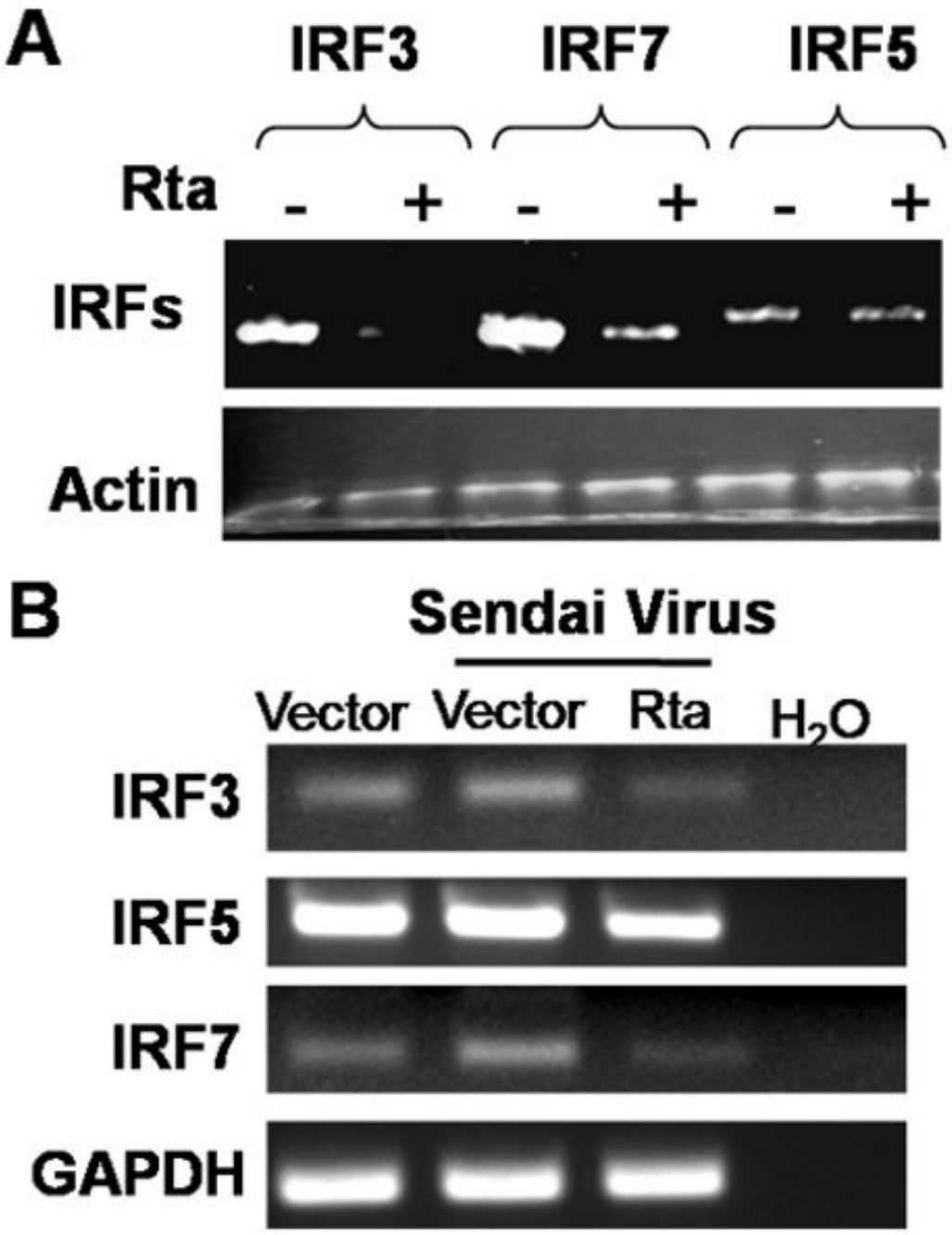

Figure 4. EBV Rta reduces endogenous RNA levels of IRF3 and IRF7

293T cells were transfected with vector control or EBV Rta as indicated. 14 hours post transfection, cells were **A)** stimulated with 500U/ml of Type I IFN or **B)** infected with Sendai virus (50 HA units/ml). Cells were collected and RNA harvested 24 hours post tranfection. Semi-quantitative RT-PCR was performed to detect expression of IRF3, IRF5, and IRF7. Actin and GAPDH were used as loading controls.

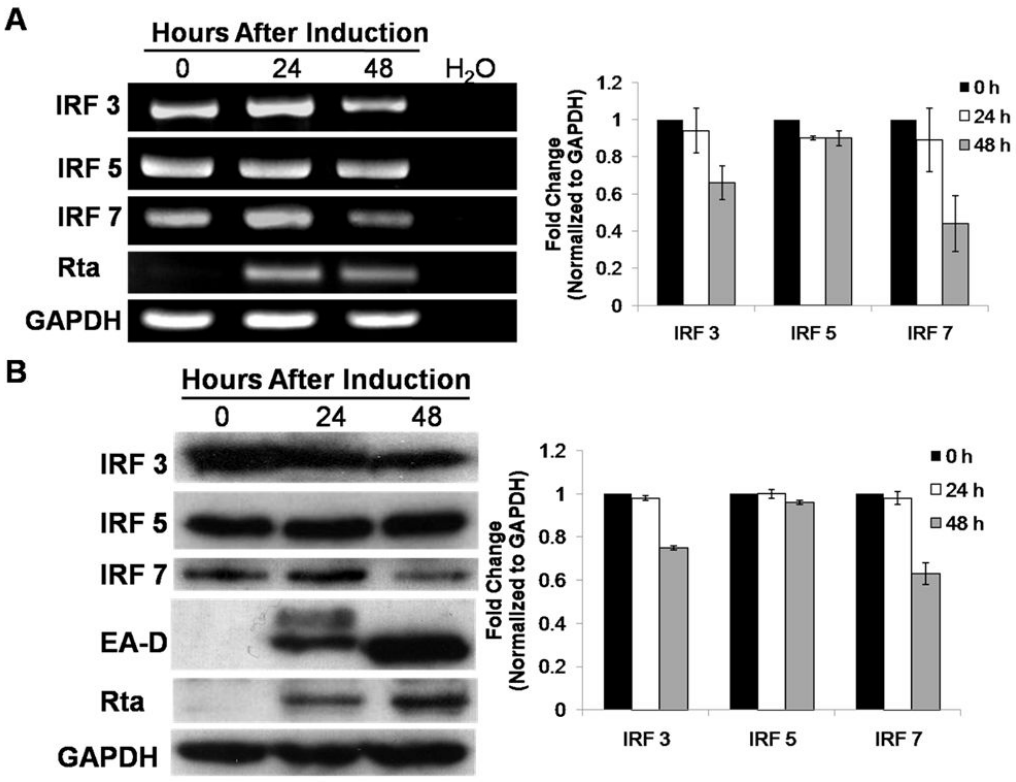

Figure 5. Endogenous IRF3 and IRF7 RNA and protein expression is reduced during EBV lytic infection

Viral reactivation was performed in Akata cells where the cells were incubated with F(AB′)2 fragment to human IgG for 0 hours (0 h), 24 hours (24 h), or 48 hours (48 h). **A)** Total RNA was harvested and semi-quantitative RT-PCR performed examining IRF3, IRF5, IRF7, and Rta RNA expression. **B)** Whole cell lysates were collected and Western blot analyses performed examining IRF3, IRF5, IRF7, Rta, and EA-D protein expression. Densitometry was performed in which relative RNA or protein expression was normalized to relative GAPDH expression. Data are shown as fold change (relative to 0 hour post induction) ± standard deviation of experiments performed in triplicate.

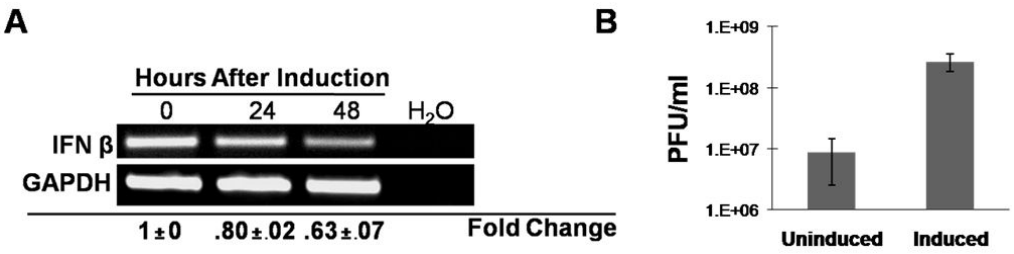

Figure 6. Endogenous IFN-β RNA levels decrease during EBV lytic infection and coincide with increased susceptibility to Sendai virus infection

Viral reactivation was induced in Akata cells as in Figure 5. **A)** Total RNA was harvested and semi-quantitative RT-PCR performed to detect IFN-β and GAPDH RNA at 0, 24, and 48 hours post induction. Densitometry was performed where relative IFN-β levels were normalized to relative GAPDH levels. Data are shown as the fold change (relative to 0 hour post induction) ± standard deviation of experiments performed in triplicate. **B)** 24 hours after induction (or mock-induction), Akata cells were infected with Sendai virus (50 HA units/ml). 24 hours after infection, supernatant fluids were collected for plaque assays on LLC-MK_2 cells. Viral titers were determined and data are shown as PFU/ml ± standard deviation of experiments performed in triplicate.