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Epstein–Barr virus BRLF1 inhibits transcription of IRF3 and IRF7 and suppresses induction of interferon-β

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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 with the ability to evade host innate immune responses.

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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 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, 2005; Honda and Taniguchi, 2006; Paun and Pitha, 2007; Sakaguchi et al., 2003; Zhang and Pagano, 1997, 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, 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

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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, 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, 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, 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, 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 down-regulation of IRF3 and IRF7.

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 293 T cells.

IFN-β reporter construct was transfected into 293 T 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 (Fig. 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 (Fig. 1B). 293 T cells were transfected with control vector or EBV Rta and mock- or Sendai virus-infected 16 h after transfection. Semi-quantitative RT-PCR was performed on RNA harvested 24 h after transfection (8 h 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, 2005; Honda and Taniguchi, 2006; Paun and Pitha, 2007; Sakaguchi et al., 2003; Zhang and Pagano, 1997, 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 (Fig. 1C and D). EBV Rta expression abrogated the increased transcriptional activity of IRF3 and IRF7 (Fig. 1B and C) 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). 293 T cells were transfected with Flag-tagged IRFs and RTA expression plasmids, and IRF and Rta expression was analyzed 24 h later. High levels of Flag-tagged IRF3, IRF5, and IRF7 were detected (Fig. 2A). Co-expression of EBV Rta consistently coincided with decreased expression levels of IRF3 and IRF7 while IRF5 levels were not altered (Fig. 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

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