

EB virus-encoded RNAs are recognized by RIG-I and activate signaling to induce type I IFN

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Epstein–Barr virus (EBV)-encoded small RNAs (EBERs) are nonpolyadenylated, untranslated RNAs, exist most abundantly in latently EBV-infected cells, and are expected to show secondary structures with many short stem-loops. Retinoic acid-inducible gene I (RIG-I) is a cytosolic protein that detects viral double-stranded RNA (dsRNA) inside the cell and initiates signaling pathways leading to the induction of protective cellular genes, including type I interferons (IFNs). We investigated whether EBERs were recognized by RIG-I as dsRNA. Transfection of RIG-I plasmid induced IFNs and IFN-stimulated genes (ISGs) in EBV-positive Burkitt's lymphoma (BL) cells, but not in their EBV-negative counterparts or EBER-knockout EBV-infected BL cells. Transfection of EBER plasmid or *in vitro*-synthesized EBERs induced expression of type I IFNs and ISGs in RIG-I-expressing, EBV-negative BL cells, but not in RIG-I-minus counterparts. EBERs activated RIG-I's substrates, NF- κ B and IFN regulatory factor 3, which were necessary for type I IFN activation. It was also shown that EBERs co-precipitated with RIG-I. These results indicate that EBERs are recognized by RIG-I and activate signaling to induce type I IFN in EBV-infected cells.

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Introduction

Epstein–Barr virus (EBV) is widespread in the human population, is the causative agent of infectious mononucleosis, and is associated with various malignancies, including Burkitt's lymphoma (BL), nasopharyngeal carcinoma, gastric carcinoma, Hodgkin's lymphoma, and lymphomas in immunosuppressed patients (Rickinson and Kieff, 2001). It efficiently infects human resting B-lymphocytes *in vitro* and transforms

them into indefinitely proliferating lymphoblastoid cell lines (LCLs). LCLs express 12 EBV gene products, including six EBV nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNA-LP), three latent membrane proteins (LMP1, LMP2A, and LMP2B), BamH1-A rightward transcripts (BARTs), and two EBV-encoded small RNAs (EBER1 and EBER2) (Kieff and Rickinson, 2001).

EBERs are the most abundant viral transcripts in cells with latent EBV infection (Rymo, 1979). EBERs are nonpolyadenylated, untranslated RNAs either 167 or 172 nucleotides long (Rosa *et al*, 1981; Glickman *et al*, 1988), and are structurally highly conserved (Arrand *et al*, 1989). Several reports have described growth-stimulatory activities of EBERs (Komano *et al*, 1999; Kitagawa *et al*, 2000; Ruf *et al*, 2000; Yamamoto *et al*, 2000; Iwakiri *et al*, 2003; Yang *et al*, 2004; Iwakiri *et al*, 2005). They are known to make complexes with several cellular proteins, such as RNA-activated protein kinase PKR (Clarke *et al*, 1991), ribosomal protein L22 (Toczyski and Steitz, 1991; Toczyski *et al*, 1994), and La (Lerner *et al*, 1981). Therefore, EBERs may exert various biological effects through their direct interaction with the cellular proteins. Among them, the significance of the interaction between EBERs and PKR, a key mediator of the antiviral effect of type I interferon (IFN- α and IFN- β), has been most extensively studied (Sharp *et al*, 1993; Yamamoto *et al*, 2000; Nanbo *et al*, 2002). We have shown that in BL cells, EBERs confer resistance to IFN- α -induced apoptosis by binding PKR and inhibiting its phosphorylation (Nanbo *et al*, 2002). Moreover, we have shown that EBERs induce the expression of various cellular cytokines—interleukin 10 (IL-10) in B-lymphocytes (Kitagawa *et al*, 2000), IL-9 in T-lymphocytes (Yang *et al*, 2004), and insulin-like growth factor 1 (IGF-1) in epithelial cells (Iwakiri *et al*, 2003, 2005)—each of which acts as an autocrine growth factor. However, the mechanisms by which EBERs induce these cytokines remain to be elucidated.

Retinoic acid-inducible gene I (RIG-I) is a cytosolic protein that detects viral double-stranded RNA (dsRNA) inside the cell and initiates signaling pathways leading to the induction of protective cellular genes, including type I IFNs and inflammatory cytokines. RIG-I contains an N-terminal caspase recruitment domain (CARD) and a C-terminal DExD/H-box RNA helicase domain (Yoneyama *et al*, 2004). The helicase domain is responsible for dsRNA recognition, and the CARD domain activates downstream signals, resulting in the activation of transcription factors, NF- κ B and IFN regulatory factor 3 (IRF-3). RIG-I functions independently of toll-like receptor 3 (TLR3), which is involved in the recognition of viral dsRNA on the cell surface and induction of type I IFN responses. In latently EBV-infected cells, EBERs are expressed abundantly (Rymo, 1979) and are expected to form a stem-loop structure by intramolecular base-pairing, giving rise to dsRNA-like molecules (Rosa *et al*, 1981; Glickman *et al*, 1988). Thus, we hypothesized that EBERs were recognized by RIG-I as dsRNA and activated RIG-I signaling in EBV-infected cells.

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Hence, we analyzed the interaction of EBERs and RIG-I using BL-derived Mutu, Akata, and Daudi cell lines (Klein *et al*, 1968; Gregory *et al*, 1990; Takada *et al*, 1991). They were originally EBV-positive, and we isolated EBV-negative cell clones from these three BL cell lines (Shimizu *et al*, 1994; Nanbo *et al*, 2002). We here report that in BL cells, EBERs are recognized by RIG-I and activate signaling to induce type I IFN and IFN-stimulated genes (ISGs).

Results

RIG-I induces type I IFN in EBV-infected cells, but not in EBV-uninfected counterparts

To examine the RIG-I signaling pathway in the EBV-infected cells, we first used BL-derived Mutu cells (Gregory *et al*, 1990). They originated from an EBV-positive BL cell line and from which EBV-negative subclones have been isolated (Nanbo *et al*, 2002). EBV-positive Mutu cells (Mutu(+)) and EBV-negative Mutu cells (Mutu(-)) were transfected with a plasmid carrying green fluorescence protein (GFP)-tagged RIG-I or a control plasmid carrying only the GFP gene. The efficiency of transfection and expression of RIG-I in both Mutu(+) and Mutu(-) cells were checked at 24 h post-transfection by fluorescence and immunoblot assay (Figure 1A). Expression of RIG-I resulted in the activation of IFN- α , IFN- β , and ISG56 in Mutu(+) cells (Figure 1B), but not in Mutu(-) cells (Figure 1C). EBV-positive Mutu cells retained BL-type EBV expression (termed type I latency) that was characterized by expression of a restricted set of EBV latent genes, including EBNA1, BARTs, and EBERs (Nanbo *et al*, 2002). Among these latently expressed genes, we envisioned that EBERs would be responsible for RIG-I activation, because it had been demonstrated that EBERs functioned as dsRNA-like molecules (Glickman *et al*, 1988; Clarke *et al*, 1991) and that RIG-I specifically recognized dsRNA (Yoneyama *et al*, 2004).

RIG-I induces type I IFN in EBER-expressing cells

To test our hypothesis that EBERs were responsible for RIG-I activation, we used Akata cells infected with EBER-knockout EBV (EBER(-) EBV) or EBER-reintroduced EBV (EBER(+) EBV). The reasons for choosing Akata cells were that they were BL-derived cells with type I latency, and both EBER(-) EBV- and EBER(+) EBV-infected cell clones were available (Yajima *et al*, 2005). Transient expression of RIG-I resulted in the induction of IFN- β and ISG56 in EBER(+) EBV-infected Akata cells, but not in EBER(-) EBV-infected Akata cells (Figure 2A).

To further confirm that EBERs were responsible for RIG-I activation, we analyzed RIG-I function in EBV-negative Akata cells stably expressing EBERs and in control Akata cells carrying the neomycin resistance gene (Komano *et al*, 1999). Transient expression of RIG-I resulted in the expression of IFN- β and ISG56 in EBER-expressing Akata cells (Akt/EBER), but not in control Akata cells (Akt/Neo^r) (Figure 2B). Knockdown of RIG-I by siRNA resulted in downregulation of basal expression of IFN- β in EBER(+) EBV-infected Akata cells (Figure 2C) and also in EBER-expressing Akata cells (Figure 2D), but not in their negative counterparts. These results clearly demonstrated that EBERs activate RIG-I-mediated signaling cascades.

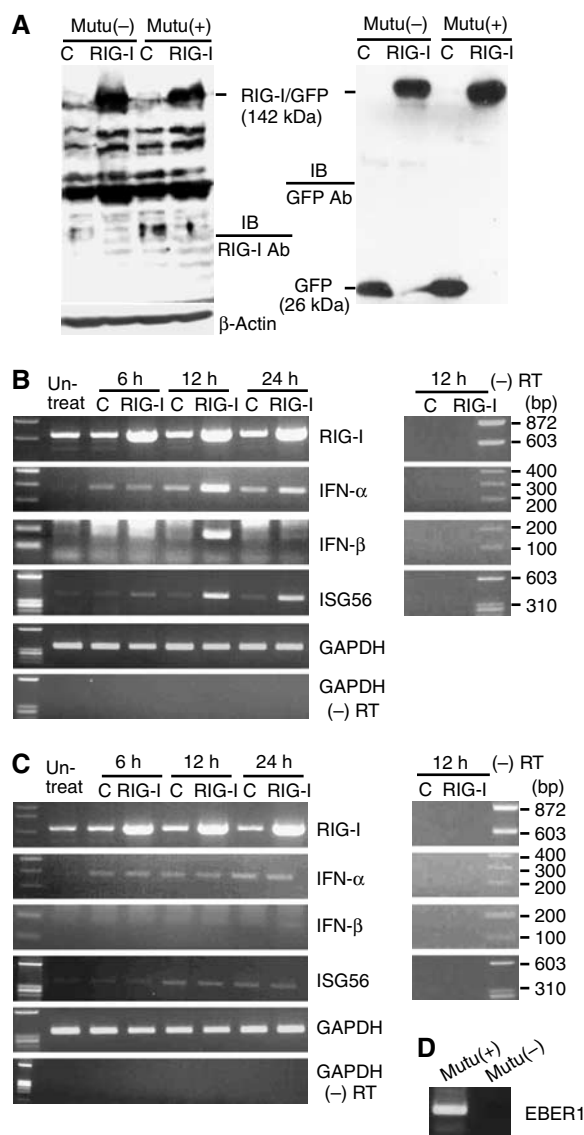


Figure 1 RIG-I induces type I IFN in EBV-infected cells. EBV-positive Mutu cells (Mutu(+)) and EBV-negative Mutu cells (Mutu(-)) (5×10^6 each) were transfected with 30 μ g of GFP-tagged RIG-I plasmid or control GFP plasmid, and expression of IFNs and ISG56 was examined at the designated time by RT-PCR. (A) Immunoblot analysis for detection of RIG-I. The blots were probed with anti-RIG-I antibody (left panel) and anti-GFP antibody (right panel). Cell lysates were prepared after 24 h of transfection and 20 μ g of protein sample was loaded per slot. (B) RT-PCR analysis of expression of IFN- α , IFN- β , and ISG56 in Mutu(+) cells. (C) RT-PCR analysis of expression of IFN- α , IFN- β , and ISG56 in Mutu(-) cells. (D) RT-PCR analysis of EBER1 expression in Mutu(+) and Mutu(-) cells.

RIG-I-expressing stable clone recognizes EBERs

EBV-negative Daudi cells were transfected with the plasmid carrying GFP-tagged RIG-I or control plasmid carrying the GFP gene, and cell clones stably expressing RIG-I/GFP or GFP were isolated. The reasons for choosing EBV-negative Daudi cells to make stable clones were that they did not express TLR3 (data not shown), they were also BL-derived (Klein *et al*, 1968), and EBV-negative derivatives were available (Nanbo *et al*, 2002). The expression of RIG-I in the stable clone was analyzed by immunoblot assay using an anti-GFP antibody (Figure 3A). To express EBERs, we used EKS10

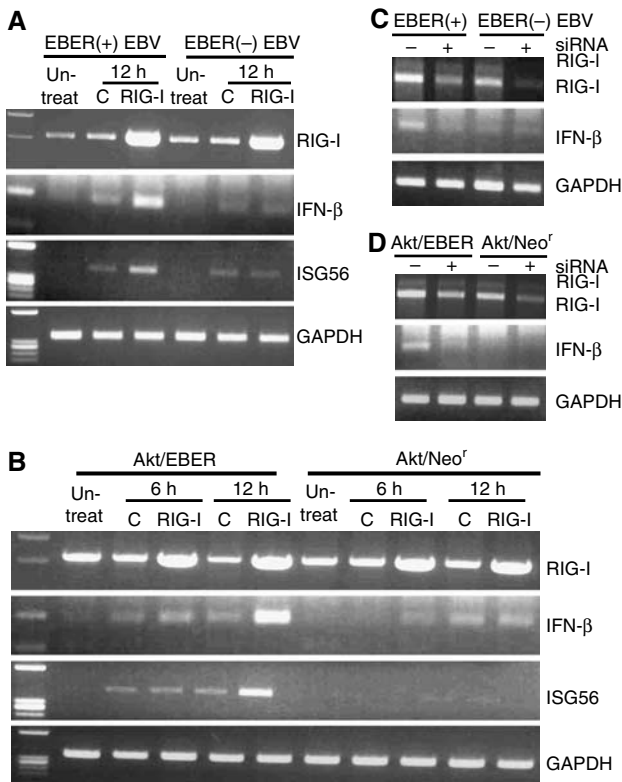


Figure 2 RIG-I induces type I IFN in EBV-expressing cells. BL-derived, EBV-positive and EBV-negative Akata cells (5×10^6 each) were transfected with 30 μ g of GFP-tagged RIG-I plasmid or GFP plasmid, and expression of IFN- β and ISG56 was examined at the designated time by RT-PCR. (A) Expression of IFN- β and ISG56 in Akata cells infected with EBV-knockout EBV (EBV(-) EBV) or EBV-reintroduced EBV (EBV(+) EBV). After 12 h of transfection of the RIG-I plasmid or control plasmid, cells were subjected to RT-PCR analysis. (B) Expression of IFN- β and ISG56 in Akata cells stably transfected with EBV plasmid (Akt/EBER) or in those with control plasmid carrying the neomycin resistance gene (Akt/Neo^r). After 6 and 12 h of transfection of the RIG-I plasmid or GFP plasmid, cells were subjected to RT-PCR analysis. (C) EBV-positive EBV-infected and EBV-negative EBV-infected Akata cells were transfected with 100 nM of RIG-I siRNA or control siRNA. After 24 h, expression of RIG-I and IFN- β was checked by RT-PCR. (D) EBV-expressing Akata cells and control cells were also transfected with 100 nM of RIG-I siRNA/control siRNA and the expression of RIG-I and IFN- β was examined at 24 h post-transfection.

plasmid containing 10 tandem repeats of the *SacI/EcoRI* subfragment from the *EcoRI* K fragment of Akata EBV DNA (Komano *et al*, 1999), and transfected it into the RIG-I-expressing cell clone and control cell clone. Transient expression of EBERS resulted in the induction of IFN- β only in RIG-I-expressing clones. The induction of IFN- β peaked as early as 6 h after transfection, suggesting that EBERS readily interacted with RIG-I after being expressed from the transgene (Figure 3B).

Both EBV1 and EBV2 activate RIG-I to induce type I IFN

EBERS consist of EBV1 and EBV2. They have different primary nucleotide sequences and secondary structures (Rosa *et al*, 1981; Glickman *et al*, 1988). Thus, it is possible that EBV1 and EBV2 behave differently when they activate RIG-I. To resolve this issue, we synthesized both EBV1 and EBV2 by *in vitro* transcription and purified them as

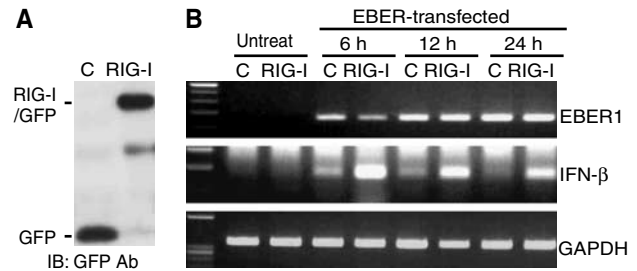


Figure 3 EBERS induce type I IFN in RIG-I-expressing cell clones. EBV-negative Daudi cells were transfected with the plasmid carrying GFP-tagged RIG-I or control plasmid carrying the GFP gene only, and cell clones stably expressing RIG-I/GFP or GFP were isolated. They (5×10^6 each) were transfected with 30 μ g of EBV plasmid, and expression of type I IFN was examined at the designated time by RT-PCR. (A) Immunoblot analysis for detection of RIG-I. The blots were probed with the anti-GFP antibody. Protein samples amounting to 20 μ g was loaded per slot. (B) RT-PCR analysis for detection of IFN- β .

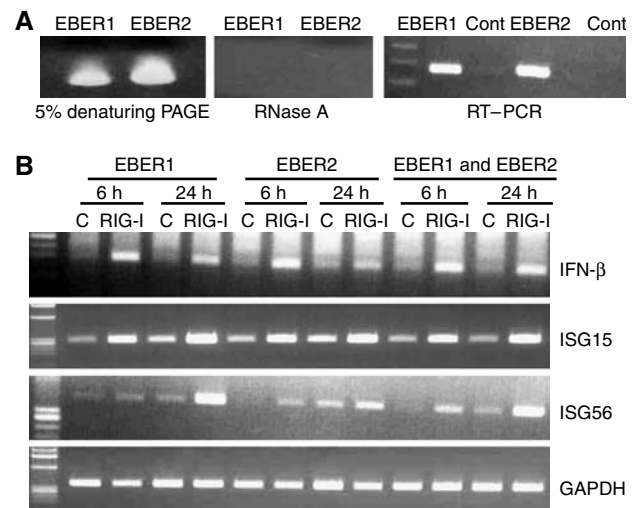


Figure 4 Both EBV1 and EBV2 activate RIG-I to induce type I IFN. (A) Analysis of *in vitro*-synthesized EBV1 and EBV2 in 5% denaturing PAGE before (left panel) and after (middle panel) RNase A treatment. RT-PCR analysis of EBV1 cDNA with EBV1 and EBV2 primers (right panel) and also EBV2 cDNA with EBV2 and EBV1 primers (right panel). (B) RT-PCR analysis of expression of IFN- β , ISG56, and ISG15 after transfection of EBV1 and EBV2. RIG-I or GFP- (5×10^6 each) expressing stable clones were transfected with 30 μ g of EBV1 or EBV2, separately or in combination (1:1). Expression of IFN- β and ISGs was examined after 6 and 24 h of transfection.

described previously (Sumpter *et al*, 2005). Analysis of purified *in vitro*-synthesized EBV1 and EBV2 in 5% denaturing PAGE (7 M urea) revealed a single band (Figure 4A, left panel), which was abolished after digestion with RNase A, indicating the purity of RNA and the absence of DNA contamination (Figure 4A, middle panel). It was also confirmed that *in vitro*-synthesized EBV1 and EBV2 were specifically amplified by EBV1- and EBV2-specific primers, respectively (Figure 4A, right panel).

Next, we transfected *in vitro*-synthesized EBV1 and EBV2 separately, as well as in combination (EBERS), into EBV-negative Daudi cell clones stably expressing RIG-I/GFP or GFP. Transfection of either EBV1 or EBV2 alone (30 μ g each) or together (EBV1 and EBV2; 15 μ g each) could

induce expression of IFN- β , ISG56, and ISG15 in RIG-I-expressing cell clones, but not in control cell clones (Figure 4B). EBER1 and EBER2 had identical effects on IFN- β and ISG15 induction, whereas induction of ISG56 appeared to be greater for EBER1 than EBER2.

EBERs activate NF- κ B and IRF-3

NF- κ B and IRF-3 are downstream signals that are activated by RIG-I and are necessary for RIG-I-mediated type I IFN activation (Yoneyama *et al*, 2004; Li *et al*, 2005; Sumpter *et al*, 2005).

To determine the involvement of NF- κ B in the induction of type I IFN by EBERs, we transfected *in vitro*-synthesized EBERs or polyI:C (as a positive control) into RIG-I-expressing EBV-negative Daudi cells and control cells. EBERs, as well as polyI:C, activated NF- κ B, as evidenced by enhanced phosphorylation of NF- κ B in RIG-I-expressing cells (Figure 5A, left panel), but not in control cells (Figure 5A, right panel). NF- κ B reporter assay also expressed similar results (Figure 5B). To further confirm that EBERs activated NF- κ B to induce type I IFN, RIG-I-expressing, EBV-negative Daudi cells were first transfected with an I κ B- α plasmid, a specific inhibitor for NF- κ B. At 36 h post-transfection, the cells were further transfected with *in vitro*-synthesized EBERs, and expression of IFN- β was examined at 8 h after the transfection of EBERs. The results demonstrated that transfection of I κ B- α plasmid decreased IFN- β induction by EBERs in a dose-dependent

manner (Figure 5C). The efficiency of the I κ B- α plasmid to block NF- κ B activation was confirmed by reporter assay, in which I κ B- α plasmid blocked luciferase expression from the NF- κ B promoter (Figure 5D).

To examine whether IRF-3 was activated by EBERs in RIG-I-expressing cells, RIG-I-expressing, EBV-negative Daudi cells were transfected with *in vitro*-synthesized EBERs or polyI:C (as a positive control). Immunoblot analysis with native PAGE indicated that EBERs, as well as polyI:C, phosphorylated IRF-3, as evidenced by detection of the phosphorylated IRF-3 in RIG-I-expressing cells, but not in control cells (Figure 5E). Knockdown of IRF-3 by siRNA resulted in downregulation of IFN- β and ISG56 induction by EBERs in RIG-I-expressing cells (Figure 5F).

These results suggested that the induction of type I IFN by EBERs could be mediated by activation of the RIG-I signaling pathway through NF- κ B and IRF-3, in agreement with other dsRNA-mediated signaling reported earlier (Yoneyama *et al*, 2004; Li *et al*, 2005; Sumpter *et al*, 2005).

EBER is recognized by full-length RIG-I

RIG-I consists of an RNA-binding helicase domain and a signal-transducing CARD domain. RIG-I recognizes dsRNA through its helicase domain (Yoneyama *et al*, 2004). To analyze the functional interaction of EBERs with the helicase domain and CARD domain of RIG-I, we established two EBV-negative Daudi cell clones stably expressing the deletion mutants of RIG-I, as described previously (Yoneyama *et al*, 2004). The first cell clone expressed an N-terminal CARD

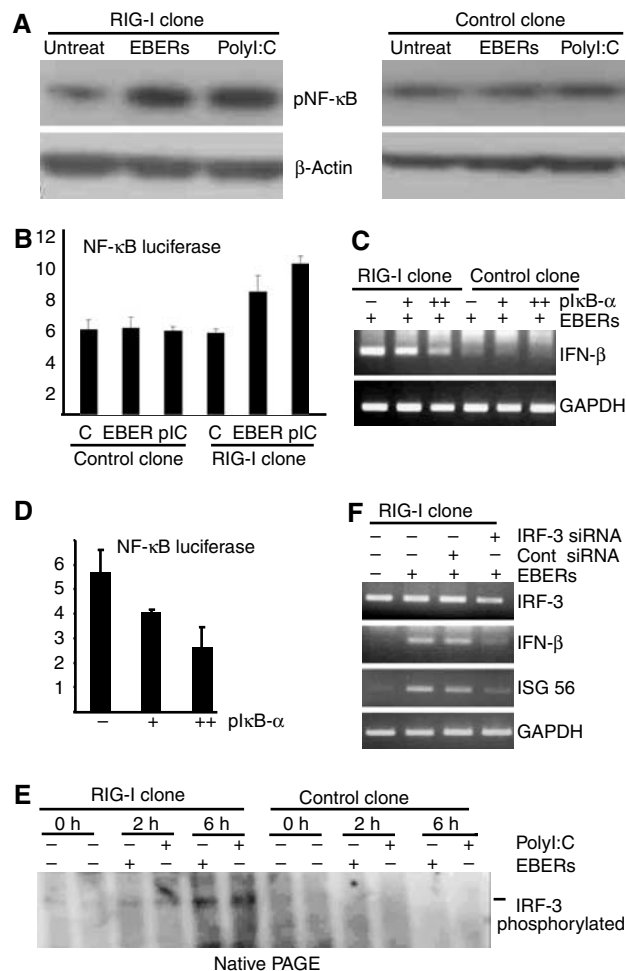


Figure 5 EBERs activate NF- κ B and IRF-3. (A) Phosphorylation of NF- κ B in RIG-I- or GFP-expressing stable clone was analyzed after they (5×10^6 cells each) were transfected with $30 \mu\text{g}$ of *in vitro*-synthesized EBERs (1:1) or polyI:C (positive control). After 6 h of transfection, whole-cell lysates ($20 \mu\text{g}$ of protein for each sample) were subjected to immunoblot analysis for detection of phosphorylated NF- κ B. (B) NF- κ B reporter assay. NF- κ B plasmid was transfected into both RIG-I-expressing Daudi cell clone and control clone and after 36 h of transfection, EBERs or polyI:C were transfected. After 12 h of EBER or polyI:C transfection, luciferase activity was measured in cell lysates (Figure 5B). Results are shown as the means \pm standard errors from three independent experiments. (C) Effect of I κ B- α , a specific inhibitor of NF- κ B, on EBER-induced phosphorylation of NF- κ B. RIG-I-expressing stable clones (5×10^6 cells) were first transfected with $10 \mu\text{g}$ (+) or $20 \mu\text{g}$ (++) of I κ B- α plasmid. After 36 h, they were further transfected with $30 \mu\text{g}$ of *in vitro*-synthesized EBERs (1:1), and expression of IFN- β was examined by RT-PCR after 8 h of transfection. (D) Inhibition of NF- κ B activation by I κ B- α plasmid. RIG-I-expressing stable clones (5×10^6 cells) were transfected with $10 \mu\text{g}$ of pNF- κ B/luc (firefly luciferase) and 100 ng of pCMV/luc (*Renilla* luciferase as an internal control), along with $10 \mu\text{g}$ (+) or $20 \mu\text{g}$ (++) of I κ B- α plasmid. After 36 h, luciferase activity was quantified in cell lysates using a Dual-Luciferase reporter assay system (Promega). Results are shown as the means \pm standard errors from three independent experiments. (E) Phosphorylation of IRF-3 in EBV-negative Daudi cells stably expressing RIG-I. RIG-I- or GFP- (5×10^6 cells each) expressing stable clones were transfected with $30 \mu\text{g}$ of *in vitro*-synthesized EBERs ($15 \mu\text{g}$ each) or polyI:C (positive control). After 2 and 6 h of transfection, whole-cell lysates ($30 \mu\text{g}$ of protein for each sample) were separated with native PAGE gel (8%) to analyze the phosphorylation of IRF-3 by EBERs or polyI:C. The blot was probed with anti-phospho-IRF-3 antibody. This antibody reacted with phosphorylated IRF-3, but not with unphosphorylated IRF-3. (F) RIG-I-expressing stable clone was treated with 100 nM of IRF-3 siRNA or control siRNA. After 24 h, cells were transfected with $30 \mu\text{g}$ of *in vitro*-synthesized EBERs, and 6 h later, expression of IRF-3, IFN- β , and ISG56 was examined by RT-PCR.

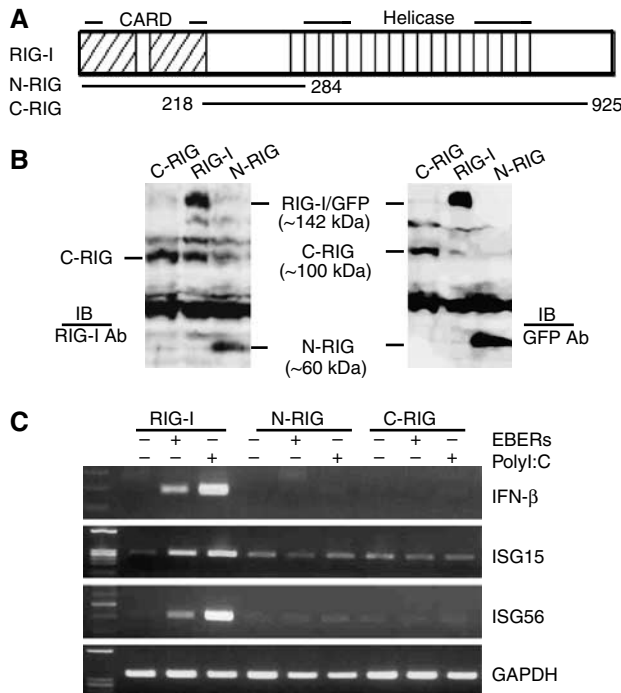


Figure 6 EBER is recognized by full-length RIG-I. (A) Schematic diagram of mutant plasmids containing the CARD domain (1–284 aa) or the helicase domain (218–925 aa). (B) Immunoblot analysis for detection of mutant RIG-I expressions in EBV-negative Daudi cell clones stably transfected with the deletion plasmids containing GFP-tagged CARD domain (N-RIG) or GFP-tagged helicase domain (C-RIG). Expression of N-RIG and C-RIG was detected by anti-RIG-I antibody (left panel) and also after reprobing with anti-GFP antibody (right panel). (C) Expression of type I IFN and ISGs in EBV-negative Daudi cells stably transfected with the deletion plasmids of RIG-I. The cells (5×10^6 cells each) were transfected with $30 \mu\text{g}$ of EBERs or polyI:C, and after 6 h, subjected to RT-PCR for detection of IFN- β , ISG15, and ISG56.

domain of RIG-I (N-RIG) tagged with GFP, and the second cell clone expressed a C-terminal helicase domain (C-RIG) tagged with GFP (Figure 6A). The expressions of N-RIG and C-RIG in stable clones were checked by immunoblot assay using anti-RIG-I antibody and also with anti-GFP antibody (Figure 6B). Next, we transfected *in vitro*-synthesized EBERs ($15 \mu\text{g}$ each of EBER1 and EBER2) into N-RIG-expressing and C-RIG-expressing cell clones. The results indicated that deletion of the helicase domain, as well as the CARD domain, abrogated RIG-I function to induce IFN- β and ISGs (Figure 6C). Transfection of polyI:C also revealed the same result (Figure 6C). Thus, we concluded that both CARD domain and helicase domain of RIG-I were necessary to activate IFN signaling that is triggered by EBERs.

EBERs co-precipitate with RIG-I

Next, we examined whether EBERs could co-precipitate with RIG-I. As anti-RIG-I antibody, which was used in this study, was not available for immunoprecipitation, we used GFP-tagged full-length RIG-I to examine the binding of EBERs with RIG-I. The full-length RIG-I plasmid (RIG-I/GFP) or control plasmid (containing only the GFP gene) was transfected into Mutu cells infected with EBER-positive EBV (Mutu(+) cells). The same set of plasmids was also transfected into EBV-negative Mutu cells (Mutu(-)) and also EBV-negative Mutu

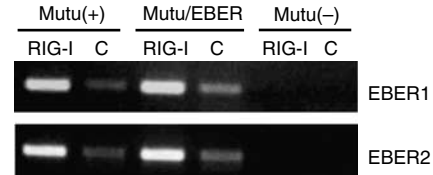


Figure 7 Binding assay for detection of association of EBERs with RIG-I. The full-length RIG-I plasmid (RIG-I/GFP) or control plasmid (containing the GFP gene only) was transfected into EBV-negative Mutu cells (Mutu(-) cells), EBER-positive EBV-infected Mutu cells (Mutu(+) cells), and EBER-transfected Mutu cells (Mutu/EBER cells). After 48 h, cells were treated with UV irradiation, digested with RNases, and subjected to immunoprecipitation with the anti-GFP antibody. RNA was isolated from immunoprecipitates, and EBER1 and EBER2 were measured by RT-PCR.

cells stably expressing EBERs (Mutu/EBER cells). After 48 h of transfection, cells were treated with UV irradiation and then with RNases to remove unbound RNAs, and subjected to immunoprecipitation with anti-GFP antibody. RNA was isolated from immunoprecipitates, and the amounts of EBER1 and EBER2 were measured by RT-PCR. The result revealed that EBERs co-precipitated along with RIG-I when the cells were transfected with RIG-I/GFP and subjected to immunoprecipitation (Figure 7). By contrast, little EBERs were found in the precipitates when the cells were transfected with the control plasmid. Thus, we concluded that EBERs bound RIG-I or were at least part of the same complex.

RIG-I recognizes EBERs in the early phase of EBV infection

The results described above indicated that RIG-I-expressing cells could be activated by EBERs to induce type I IFN and ISGs. We speculated, therefore, that in primary EBV infection, newly synthesized EBERs could be recognized by RIG-I and activate the induction of type I IFN. To test this hypothesis, we used EBV-negative Daudi cell clones stably expressing RIG-I/GFP or GFP. They were infected with EBER(+) EBV or EBER(-) EBV (Yajima *et al*, 2005), and induction of IFN- β was examined at the designated time of EBV infection. The results indicated that IFN- β was induced by EBER(+) EBV infection in RIG-I-expressing cells, but not in control cells (Figure 8). On the other hand, IFN- β was not induced by EBER(-) EBV infection in either RIG-I-expressing cells or control cells (Figure 8), although both EBER(+) EBV and EBER(-) EBV infected the cells with similar infectivities as revealed by the expression of EBNA2. These results, considered together, indicated that EBERs were recognized by RIG-I in the early phase of EBV infection resulting in the induction of type I IFN in BL cells.

Discussion

It is well established that RIG-I is activated by dsRNA that is synthesized as a replicative intermediate during replication of RNA viruses, including Newcastle disease virus, vesicular stomatitis virus (Yoneyama *et al*, 2004), and hepatitis C virus (Sumpter *et al*, 2005). Here, we demonstrated that EBV-encoded, non-coding RNAs, EBERs, were recognized by RIG-I and activated its downstream signaling, leading to the induction of type I IFN and other IFN-inducible genes in

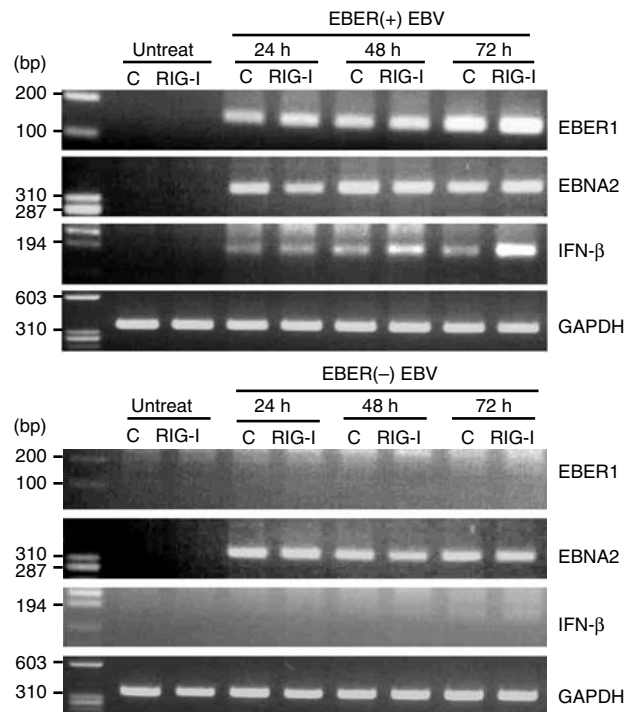


Figure 8 RIG-I recognizes EBVs in the early phase of EBV infection. EBV-negative Daudi cell clones stably expressing RIG-I/GFP or GFP were infected with EBV(+) EBV or EBV(-) EBV, and expression of EBV1, EBNA2, and IFN- β was examined at the designated time of EBV infection by RT-PCR.

EBV-infected B-lymphocytes. This is the first report showing that DNA virus-encoded RNAs are recognized by RIG-I.

It is known that in viral infection, IFN- β is first expressed and then the induced IFN- β activates the expression of IFN- α (Levy *et al*, 2003). This will be the reason why IFN- β is expressed earlier than IFN- α in our studies.

The primary sequence similarity between EBV1 and EBV2 is only 54%, but both EBV1 and EBV2 show striking similarity in their secondary structures (Rosa *et al*, 1981; Glickman *et al*, 1988). Activation of RIG-I by both EBV1 and EBV2 highlights the importance of double stranded-like structures of EBVs. EBVs have extensive structural similarity to adenoviruses VA1 and VA2 and cell U6 small RNAs, which are small nonpolyadenylated RNAs like EBVs (Rosa *et al*, 1981). It would be interesting to test whether VAs and U6 RNAs are recognized by RIG-I and induce type I IFN signaling.

Production of type I IFN by EBVs induces apoptosis of EBV-infected cells. To evade IFN attack, EBV has developed several protective viral genes, including BHRF1, LMP1, and EBVs (Henderson *et al*, 1991, 1993; Nanbo *et al*, 2002, 2005). BHRF1 protein, a viral homolog of Bcl-2, protects EBV-infected cells from apoptotic cell death. LMP1 has been shown to induce Bcl-2, thus protecting EBV-infected cells from apoptosis. Moreover, we have shown that EBVs counteract PKR activation, which is a key mediator of the antiviral effect of type I IFN. PKR is induced by IFN and its autophosphorylation is stimulated by viral dsRNA, thereby activating two cellular substrates, eIF-2 α and I κ B- α , leading to the apoptosis of virus-infected cells (Moran *et al*, 2005). We have shown that EBVs bind PKR, inhibit its phosphorylation, and confer resistance to IFN- α -induced apoptosis in

EBV-infected cells. These findings, together with the present findings, indicate that EBV evades antiviral activities of IFN through activation of protective viral genes, and suggest that EBV latent infection is maintained under the balance of actions of these viral and cellular gene products.

Another aspect of the functions of RIG-I that we have not dealt here is the induction of various inflammatory cytokines. It is well known that activation of RIG-I results in production of type I IFN as well as inflammatory cytokines such as IL-6, IL-8, and IL-12p40 (Foy *et al*, 2005; Kato *et al*, 2005). Previously, we have reported that EBVs induce transcription of various cytokines, including IL-10 (Kitagawa *et al*, 2000), IL-9 (Yang *et al*, 2004), and IGF-1 (Iwakiri *et al*, 2003, 2005), and that the produced cytokines act as autocrine growth factors. However, the mechanism of cytokine induction by EBVs is still not known. It is very likely, and is worth testing, that EBVs induce transcription of these cytokines through activation of RIG-I.

Further studies will be needed to elucidate the overall picture of the interaction of EBVs and RIG-I and to uncover strategies by which EBV evades and/or utilizes this surveillance mechanism to establish stable infection not only in BL cells but also in other EBV-associated malignancies.

Materials and methods

Cells and culture

EBV-negative and EBV-positive Mutu cells (Gregory *et al*, 1990) and EBV-negative Daudi cells (Nanbo *et al*, 2002) were maintained in RPMI 1640 medium containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Akata cells infected with EBV-knockout EBV or EBV-reintroduced EBV (Yajima *et al*, 2005) and Akata cells stably transfected with the EBV plasmid or control plasmid with the neomycin resistance gene (Komano *et al*, 1999) were maintained in the same medium with the addition of 500 μ g/ml of G418 (Sigma, St Louis, MO).

Plasmids, transfection, and establishment of stable clones

Human RIG-I cDNA was amplified from LCL and cloned into pSG5/EGFPN1 vector, thereby making a fused RIG-I expression construct (pSG5/EGFPN1/RIG-I) with the GFP gene tagged at the C-terminus. RIG-I deletion constructs containing only the CARD domain (N-RIG) or helicase domain (C-RIG) with GFP gene at the C-terminus were also made as described earlier (Yoneyama *et al*, 2004). EKS10 plasmid, containing 10 tandem repeats of EBV1 and EBV2, was made as described previously (Komano *et al*, 1999). pNF- κ B/luc (encoding firefly luciferase) and pCMV/luc (encoding *Renilla* luciferase as an internal control) were obtained commercially (BD Biosciences, San Jose, CA), and pI κ B- α , a specific inhibitor of NF- κ B, was a kind gift of Bill Sugden (Mitchell and Sugden, 1995). Endotoxin-free plasmid DNA was prepared by using an endo-free Mega Prep kit (Invitrogen). For transient or stable expression of plasmids, we performed electroporation. Stable transfectants expressing full-length RIG-I (RIG-I), helicase-deleted RIG-I (N-RIG), CARD-deleted RIG-I (C-RIG), and GFP clones were selected by culturing with 1000 μ g/ml of G418 (Sigma).

Virus production and infection

For EBV-positive EBV and EBV-negative EBV production, we followed the protocol described earlier (Yajima *et al*, 2005).

For EBV infection, EBV-negative Daudi cells stably expressing RIG-I (RIG-I/GFP) or GFP (10^6 cells each) were suspended in 1 ml virus soup and incubated for 90 min at 37°C with continuous gentle mixing. Then, the cells were washed with serum-free medium, resuspended in 2 ml of growth medium, and incubated for 24, 48, and 72 h in 12-well cell culture plates.

In vitro synthesis of EBVs and RNA transfection

EBV1 and EBV2 were amplified from LCL (Akata) and cloned into pGEMT easy vector (Promega, Madison, WI). From pGEMT/EBV1

and pGEMT/EBER2, T7 promoter-tagged (at the 5' end) EBER1 and EBER2 were synthesized by PCR (primer pairs: EBER1, 5'-TGT AAT ACG ACT CAC TAT AGG GAC CTA CGC TGC CCT AGA GGT TTT GCT; 5'-AAA ACA TGC GGA CCA GC; EBER2, 5'-TGT AAT ACG ACT CAC TAT AGG GAC AGC CGT TGC CCT AGT GGT TTC GGA; 5'-AAA ACA CGG GAC AAG CCG AAT ACC). One microgram of purified DNA (PCR product) was used as a template per reaction, and *in vitro* transcription was carried out for 6 h following the manufacturer's protocol (Epicenter, Madison, WI). Purified EBER1 and EBER2 (3 µg each) before and after RNase A treatment were checked in 5% denaturing polyacrylamide gel and by RT-PCR. For RNA transfection, EBER1, EBER2, EBER1 and EBER2 together (1:1), or polyI:C (30 µg each) was transfected by electroporation.

RNA extraction and RT-PCR analysis

Reverse transcription was carried out with oligo-dT primer using total cellular RNA. For EBERs, gene-specific 3' end primers were used. One-tenth of cDNA was subjected to PCR using primers specific for IFN- α (5'-ATC CAG CAG ATC TTC AAT CT; 5'-AAG AAA AAG ATC TCA TGA TT; 35 cycles), IFN- β (5'-GAT TCA TCG AGC ACT GGC TGG; 5'-CTT CAG GTA ATG CAG AAT CC; 30 cycles), TLR3 (5'-TCA CTT GCT CAT TCT CCC TT; 5'-GAC CTC TCC ATT CCT GGC; 35 cycles), ISG15 (5'-GGT GGA CAA ATG CGA CGA AC; 5'-ATG CTG GTG GAG GCC CTT AG; 28 cycles), ISG56 (5'-TAG CCA ACA TGT CCT CAC AGA C; 5'-TCT TCT ACC ACT GGT TTC ATG C; 30 cycles), EBER1 (5'-AGG ACC TAC GCT GCC CTA GA; 5'-AAA ACA TGC GGA CCA GC; 18 cycles), EBER2 (5'-AGG GAC AGC CGT TGC CCT AGT GGT TTC GGA; 5'-AAA ACA GCG GAC AAG CCG AAT ACC; 18 cycles), RIG-I (5'-GCA TAT TGA CTG GAC GTG GCA; 5'-CAG TCA TGG CTG CAG TTC TGT C; 30 cycles), EBNA2 (5'-GCT GCT ACG CAT TAG AGA CC; 5'-CAG CAC TGG CGT GTG ACG TGG TGT AAA GTT; 35 cycles), IRF-3 (5'-CACAGCAGGAGGATTTCCGG; 5'-CCTG CGTATCAGAAGTAC; 26 cycles), and GAPDH (5'-GCC TCC TGC ACC ACC AAC TG; 5'-CGA CGC CTG CTT CAC CAC CTT CT; 20 cycles). Control reactions were performed in parallel in the absence of reverse transcriptase.

Immunoblot analysis

Cell lysates containing equal quantities of protein (20–30 µg) were analyzed in 8% SDS-PAGE gel. The membranes were treated with the primary antibodies for RIG-I (Imaizumi *et al*, 2004), GFP (BD Biosciences), phospho-NF- κ B p65 (Ser-536), and β -actin (Cell Signaling, Beverly, MA) at 4°C overnight, followed by reaction

with HRP-conjugated anti-rabbit (1:2000) or anti-mouse (1:5000) IgG antibodies (Amersham Biosciences, Buckinghamshire, UK).

For IRF-3 phosphorylation analysis, whole-cell lysates were prepared after 2 or 6 h of EBERs or polyI:C transfection into RIG-I-expressing or control cell clones. Cell lysate preparation and immunoblotting with an anti-phospho-IRF-3 antibody (IBL, Takasaki, Japan) were performed, as described previously (Mori *et al*, 2004), using 8% Tris-HCl native PAGE ready gel (Invitrogen). Protein bands were visualized using ECL Plus Western blotting detection reagents (Amersham Biosciences, UK).

Immunoprecipitation and RT-PCR

Co-precipitation of EBERs with RIG-I was analyzed by UV crosslinking and immunoprecipitation of RIG-I and EBER complexes. GFP-tagged RIG-I or GFP was transiently expressed in EBER(+) EBV-infected Mutu cells, EBER-expressing EBV-negative Mutu cells, and EBV-negative Mutu cells (5×10^6 cells each). After 48 h, cells were washed with pre-chilled PBS and UV irradiated. Cell lysates were treated with RNase T1 and RNase A to remove unbound RNAs and immunoprecipitated with anti-GFP antibody. Total RNA was isolated from the immunoprecipitants, and one-third of the RNA sample was subjected to 30 cycles of RT-PCR with EBER1- and EBER2-specific primers. The compositions of buffers and the methodology for cell lysate preparation, RNA extraction, and RT-PCR analysis were described previously (Nanbo *et al*, 2002).

RNA interference

For siRNA experiments, dsRNA duplexes composed of 25-nucleotide sense and antisense oligonucleotides were synthesized from Invitrogen. RNA oligonucleotides used for targeting RIG-I and IRF-3 were as follows: RIG-I sense 5'-UUAGGAUUCUCAUUGCUGG GAUCCC-3', antisense 5'-GGGAUCCCAGCAAUGAGAAUCCUAA-3' and IRF-3 sense 5'-UAAACGCAACCCUUCUUGCGGUUG-3' and antisense 5'-CAACCGCAAAGAAGGGUUGCGUUUA-3'. Lipofectamine RNAiMax (Invitrogen) was used for reverse transfection of cells following the manufacturer's protocol (Invitrogen).

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