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Small non-coding RNAs encoded within the herpes simplex virus type 1 latency associated transcript (LAT) cooperate with the retinoic acid inducible gene I (RIG-I) to induce beta-interferon promoter activity and promote cell survival

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Abstract

The herpes simplex virus type 1 (HSV-1) latency-associated transcript (LAT) is abundantly expressed in latently infected trigeminal ganglionic sensory neurons. Expression of the first 1.5 kb of LAT coding sequences restores wild type reactivation to a LAT null HSV-1 mutant. The anti-apoptosis functions of the first 1.5 kb of LAT coding sequences are important for wild type levels of reactivation from latency. Two small non-coding RNAs (sncRNAs) contained within the first 1.5 kb of LAT coding sequences are expressed in trigeminal ganglia of latently infected mice, they cooperate to inhibit apoptosis, and reduce the efficiency of productive infection. In this study, we demonstrated that LAT sncRNA1 cooperates with the RNA sensor, retinoic acid inducible gene I (RIG-I), to stimulate IFN- β promoter activity and NF- κ B dependent transcription in human or mouse cells. LAT sncRNA2 stimulated RIG-I induction of NF- κ B dependent transcription in mouse neuroblastoma cells (Neuro-2A) but not human 293 cells. Since it is well established that NF- κ B interferes with apoptosis, we tested whether the sncRNAs cooperated with RIG-I to inhibit apoptosis. In Neuro-2A cells, both sncRNAs cooperated with RIG-I to inhibit cold-shock induced apoptosis. Double stranded RNA (PolyI:C) stimulates RIG-I dependent signaling; but enhanced cold-shock induced apoptosis. PolyI:C, but not LAT sncRNAs, interfered with protein synthesis when cotransfected with RIG-I, which correlated with increased levels of cold-shock induced apoptosis. LAT sncRNA1 appeared to interact with RIG-I in transiently transfected cells suggesting this interaction stimulates RIG-I.

Keywords

Latency associated transcript (LAT); HSV-1; RIG-I; Cell survival; Apoptosis

1. Introduction

Acute herpes simplex virus type 1 (HSV-1) infection is initiated in mucocutaneous epithelium [reviewed in (Jones, 1998, 2003)]. High levels of lytic cycle viral gene expression and infectious virus occur during acute infection. Despite a vigorous immune response during acute infection, HSV-1 efficiently establishes lifelong latency in sensory neurons. In contrast to productive infection, abundant lytic cycle viral gene expression and shedding of infectious virus does not occur in latently infected neurons. Latent HSV-1 periodically reactivates from latency resulting in virus transmission and occasionally recurrent disease.

Mice, rabbits, or humans latently infected with HSV-1 express abundant levels of the latency-associated transcript (LAT) in latently infected sensory neurons (Croen et al., 1987; Deatly et al., 1987, 1988; Krause et al., 1988; Mitchell et al., 1990; Rock et al., 1987; Stevens et al., 1987; Wagner et al., 1988a, 1988b). The primary LAT transcript is 8.3 kb and splicing yields a stable 2 kb LAT and unstable 6.3 kb LAT (Deatly et al., 1988; Rock et al., 1987; Zwaagstra et al., 1990). The 2 kb LAT can be further spliced in infected neurons (Mador et al., 1995). The 2 kb LAT is not capped or poly-adenylated because it is a stable intron (Farrell et al., 1991; Krummenacher and Zabolotny, 1997). The LAT locus also encodes numerous micro-RNAs (miRNA) (Cui et al., 2006; Jurak et al., 2010; Umbach et al., 2008, 2009). Two small non-coding RNAs, 62 nt and 36 nt long, are expressed from the first 1.5 kb of LAT coding sequences (LAT sncRNA1 and sncRNA2) (Peng et al., 2008). LAT sncRNA1 and sncRNA2 are not miRNAs because the mature miRNA band that migrates between 21 and 23 nucleotides is not detected, they lack certain structural features of miRNAs, and both sncRNAs have the potential to form complex secondary structures. It is unlikely that LAT sncRNA1 and sncRNA2 were detected using procedures described for the HSV-1 encoded LAT miRNAs (Jurak et al., 2010; Umbach et al., 2008) because RNA species migrating between 17 and 30 nucleotides were size selected and then deep-sequencing performed.

In general, HSV-1 LAT null mutants do not reactivate from latency as efficiently as LAT expressing strains [reviewed by (Jones, 1998, 2003; Wagner and Bloom, 1997)]. Expression of the first 1.5 kb of LAT coding sequences (LAT nucleotides 1–1499) restores wild type levels of reactivation to a LAT null mutant (Inman et al., 2001; Jin et al., 2003; Perng et al., 1996a, 1996b). LAT reduces apoptosis in infected tissue culture cells (Jin et al., 2004), and promotes neuronal survival in TG of infected rabbits (Perng et al., 2000) and mice (Ahmed et al., 2002; Branco and Fraser, 2005). Plasmids expressing LAT interfere with caspase 8- and caspase 9-induced apoptosis (Ahmed et al., 2002; Henderson et al., 2002; Inman et al., 2001; Jin et al., 2003; Peng et al., 2003; Perng et al., 2000). Inhibiting apoptosis is a crucial function of LAT because three anti-apoptosis genes (Jin et al., 2005, 2008; Mott et al., 2003; Perng et al., 2002) restore the wild type reactivation phenotype to a LAT null mutant. In transient transfection studies, the LAT sncRNAs inhibit cold-shock induced apoptosis in mouse neuroblastoma cells and productive infection (Shen et al., 2009). LAT also interferes with expression of infected cell protein 4 (ICP4) (Chen et al., 1997; Garber et al., 1997) and ICP0 (Chen et al., 1997; Garber et al., 1997; Mador et al., 1998).

The retinoic acid-inducible gene I (RIG-I) is a cytosolic RNA sensor, which when activated, stimulates production of type I IFNs (IFN- α/β) and inflammatory cytokines (Yoneyama et al., 2004). RIG-I contains a N-terminal caspase recruitment domain (CARD) and a C-terminal DExD/H-box RNA helicase domain (Yoneyama et al., 2004). The helicase domain recognizes viral dsRNA, and the CARD domain activates downstream signaling through the mitochondrial antiviral signaling protein (MAVS) (Yoneyama and Fujita, 2009). The C-terminal regulatory domain of RIG-I interacts with the N-terminal CARD domain, preventing its association with MAVS. RNA binding to the C-terminal helicase induces conformational changes and exposes the CARD domain, which promotes an interaction with MAVS. The interaction between RIG-I and MAVS activates two transcription factors, nuclear factor kappa B (NF- κ B) and IFN regulatory factor 3 (IRF3). NF- κ B and IRF3 induce transcription of type I IFN and other innate immune modulatory genes (Yoneyama et al., 2004). *In vitro*, RIG-I recognizes RNAs containing a 5'-triphosphate moiety and partially double-stranded regions (Hornung et al., 2006; Kato et al., 2008; Pichlmair et al., 2006; Schlee et al., 2009b; Takahashi et al., 2008). In the context of a viral infection, RIG-I preferentially associates with shorter viral RNAs that contain 5'-triphosphates and/or regions that resemble double stranded RNA (dsRNA) (Baum et al., 2010). RIG-I stimulates innate immune responses independent of toll-like receptor 3 (Alexopoulou et al., 2001).

In this study, we demonstrated that LAT sncRNA1 cooperates with RIG-I to consistently stimulate IFN- β promoter activity and NF- κ B dependent transcription. LAT sncRNA2 stimulated NF- κ B dependent transcription as efficiently as LAT sncRNA1 in mouse neuroblastoma (Neuro-2A) cells, but not in human 293 cells. LAT sncRNA1 and sncRNA2, but not PolyI:C, cooperated with RIG-I to interfere with cold shock induced apoptosis in Neuro-2A cells. PolyI:C, but not the LAT sncRNAs, interfered with protein synthesis in transfected Neuro-2A cells, which correlated with the ability of PolyI:C to enhance cold-shock induced apoptosis.

2. Results

2.1. LAT sncRNA1 stimulate IFN- β promoter activity in the presence of RIG-I

The first 1.5 kb of LAT coding sequences encode two small non-coding RNAs (sncRNAs) that are expressed in trigeminal ganglia of latently infected mice (Peng et al., 2008; Shen et al., 2009) (Fig. 1C and D). We predicted that the sncRNAs might interact with and regulate the RNA sensor (RIG-I) because they have the potential to be folded into dsRNA (Peng et al., 2008; Shen et al., 2009) and they are relatively short RNAs. To test this prediction, human cells (293) were cotransfected with a plasmid that expresses a LAT sncRNA, a reporter construct that is driven by the human IFN- β promoter, and RIG-I. The plasmid expressing sncRNA1, but not sncRNA2, cooperated with RIG-I to increase IFN- β promoter activity approximately 10-fold (Fig. 2). The induction of IFN- β promoter activity by LAT sncRNA1 and RIG-I was significantly different than RIG-I or RIG-I cotransfected with LAT sncRNA2 ($P < 0.05$). In the absence of RIG-I, sncRNA1 consistently stimulated IFN- β promoter activity approximately 3-fold. LAT sncRNA2 did not stimulate IFN- β promoter activity any better than the empty vector. The constitutively active RIG-I construct, N-RIG-I, stimulated higher levels of IFN- β promoter activity in transfected cells compared to the wt

RIG-I construct. As expected, RIG-I efficiently stimulated IFN- β promoter activity when cotransfected with double stranded RNA (PolyI:C) in 293 cells (data not shown) and primary bovine testicle cells (da Silva and Jones, 2012b).

2.2. LAT sncRNA1 cooperates with RIG-I to consistently stimulate NF- κ B dependent transcription

The cellular transcription factor NF- κ B is also activated by RIG-I, and NF- κ B is important for stimulating IFN- β promoter activity, reviewed in (Yoneyama and Fujita, 2009). Consequently, studies were performed to test whether LAT sncRNAs had an effect on NF- κ B dependent transcription in the presence of RIG-I. A luciferase reporter gene containing a simple promoter and 5 consensus NF- κ B binding sites (p5X-NF- κ B) was used to measure NF- κ B dependent transcription. LAT sncRNA1 stimulated NF- κ B dependent transcription approximately 8-fold in 293 cells when cotransfected with RIG-I (Fig. 3A). LAT sncRNA2 stimulated NF- κ B dependent transcription approximately 3-fold when cotransfected with RIG-I, which was significantly less ($P < 0.05$) than the effect seen with sncRNA1 or just RIG-I cotransfected with empty vector. In the absence of RIG-I, LAT sncRNA1 stimulated NF- κ B dependent transcription less than twofold. As expected, the constitutively activated RIG-I deletion construct (N-RIG-I), but not wt RIG-I, stimulated NF- κ B dependent transcription approximately fourfold in 293 cells.

As a comparison to the results obtained in 293 cells, the studies were repeated in mouse neuroblastoma cells (Neuro-2A). LAT sncRNA1 and sncRNA2 stimulated NF- κ B dependent transcription at least 6-fold when cotransfected with RIG-I (Fig. 3B), which was significantly different compared to RIG-I cotransfected with empty vector ($P < 0.5$). In contrast to 293 cells, LAT sncRNA2 consistently activated NF- κ B dependent transcription at slightly higher levels relative to LAT sncRNA1; but the difference was not significantly different ($P > 0.05$). In the absence of RIG-I, their effect was nominal indicating that the ability of LAT sncRNAs to stimulate NF- κ B dependent transcription was dependent on abundant levels of RIG-I. As expected, PolyI:C induced NF- κ B dependent transcription but the effect was less than the LAT sncRNAs. In contrast to 293 cells, the N-RIG-I construct was unable to stimulate NF- κ B dependent transcription in Neuro-2A cells. These studies indicated that LAT sncRNA1 induced NF- κ B dependent transcription when cotransfected with RIG-I in 293 and Neuro-2A cells. LAT sncRNA2 stimulated NF- κ B dependent transcription in Neuro-2A cells, but not in 293 cells.

2.3. LAT sncRNAs stimulate cell survival following cold shock induced apoptosis

Additional studies were performed to test whether RIG-I stimulates the anti-apoptosis functions of LAT sncRNAs. The rationale for this study is NF- κ B promotes cell survival (Foehr et al., 2000; Goodkin et al., 2003; Mattson and Meffert, 2006), LAT sncRNA1 interferes with apoptosis in transiently transfected Neuro-2A cells (Shen et al., 2009), and LAT sncRNA1 cooperated with RIG-I to stimulate NF- κ B dependent transcription in 293 and Neuro-2A cells. Furthermore, LAT sncRNA2 enhances the anti-apoptosis properties of sncRNA1 (Shen et al., 2009).

Neuro-2A cells were chosen for these studies because unlike 293 cells they are sensitive to cold shock induced apoptosis (Shen et al., 2009; Shen and Jones, 2008). Furthermore, cold shock may have relevance to the HSV-1 latency-reactivation cycle because cold stress can induce recurrent herpetic keratitis in squirrel monkeys (Varnell et al., 1995). Plasmids expressing the respective LAT sncRNAs, RIG-I, and a CMV β -Gal expression plasmid were transfected into Neuro-2A cells and cold-shock induced apoptosis performed as previously described (Carpenter et al., 2007; Shen et al., 2009; Shen and Jones, 2008). The β -Gal co-transfection assay accurately measures the effects of various genes on apoptosis (Ciacci-Zanella et al., 1999; Henderson et al., 2002; Inman et al., 2001; Jin et al., 2003; Perng et al., 2000) because a known apoptosis stimulator reduces the number of β -Gal⁺ cells. Comparing changes in the number of β -Gal⁺ Neuro-2A cells after cold shock induced apoptosis among cultures treated with anti-apoptosis genes versus those treated with negative controls are identical to differences in DNA laddering, the number of sub-G₁ levels of DNA, and trypan blue exclusion (Shen et al., 2009). At 36 h after transfection, cells were starved in 2% fetal calf serum for 12 h, cultures were incubated on ice for 1 h, and cultures were then returned to 37 °C for 3.5 h. Neuro-2A cells (Fig. 4A) or Neuro-2A cells placed on ice for 1 h contain little to no detectable DNA laddering or cell death as judged by a decrease in the number of β -Gal⁺ cells (Shen et al., 2009; Shen and Jones, 2008). However, extensive DNA laddering, indicative of apoptosis, occurs when Neuro-2A cells are returned to 37 °C for 3 or 6 h (Shen et al., 2009; Shen and Jones, 2008).

When LAT sncRNA1 or sncRNA2 were cotransfected with the RIG-I expression plasmid, the number of β -Gal⁺ Neuro-2A cells was significantly increased ($P < 0.05$) after cold shocking cultures and then returning them to 37 °C for 3.5 h relative to cells transfected with an empty expression vector and RIG-I (Fig. 4A and B). PolyI:C, in contrast to the LAT sncRNAs, reduced the number of β -Gal⁺ Neuro-2A cells when cotransfected with RIG-I. No significant differences were observed when comparing the number of β -Gal⁺ Neuro-2A cells treated with PolyI:C relative to cells treated with PolyI:C and transfected with RIG-I (Fig. 4B and C). In the absence of the RIG-I expression plasmid, LAT sncRNA1 and LAT sncRNA2 increased the number of β -Gal⁺ cells approximately 3-fold. A previous study (Shen et al., 2009) found that LAT sncRNA2 had little effect on inhibiting apoptosis; whereas this study demonstrated that it inhibited cold shock apoptosis. Although Neuro-2A cells are immortalized, they exhibit different growth properties as they are passaged, which affects their sensitivity to cold shock induced apoptosis suggesting this influenced the results we obtained with LAT sncRNA2. In summary, these studies demonstrated that LAT sncRNA1 and sncRNA2 cooperated with RIG-I to inhibit cold shock induced apoptosis in Neuro-2A.

2.4. LAT sncRNAs stimulate RIG-I activity but do not interfere with expression of Flag-tagged proteins

The finding that LAT sncRNAs promoted cell survival when transfected with RIG-I but PolyI:C enhanced apoptosis when transfected with RIG-I suggested that the mechanism by which PolyI:C stimulated RIG-I dependent signaling pathways was different than the LAT sncRNAs. In general, dsRNA (PolyI:C for example) induces the IFN- β signaling pathway and protein synthesis is inhibited, reviewed in (Clemens and Elia, 1997; Katze et al., 2002;

Yoneyama and Fujita, 2009). It is also well established that inhibiting protein synthesis enhances apoptosis (Coxon et al., 1998; Marissen and Patel, 1998; Martin et al., 1990) suggesting that PolyI:C, but not the LAT sncRNAs, interfered with protein synthesis in Neuro-2A cells and enhanced cold-shock induced apoptosis.

Studies were consequently conducted to test whether LAT sncRNAs interfered with protein synthesis when cotransfected with RIG-I. Since not all Neuro-2A cells are transfected with a plasmid that expresses a LAT sncRNA, we were concerned it would not be possible to compare total protein synthesis in the respective samples cells. Consequently, we included in the transfection mixture a plasmid that expresses a Flag tagged protein, which allowed us to examine the effect that PolyI:C or the LAT sncRNAs has on steady state levels of the respective Flag-tagged proteins. In the presence of PolyI:C and RIG-I, protein levels of the Notch3 intercellular domain (Notch3) or the BHV-1 ICP27 protein were reduced compared to cells transfected with just an empty vector (Fig. 5). Neuro-2A cells cotransfected with plasmids expressing LAT sncRNA1 or LAT-sncRNA2 and RIG-I expressed similar levels of Notch3 or bICP27 when compared to cells transfected with an empty expression vector and plasmids expressing Notch3 or bICP27. In summary, this study suggested that PolyI:C, but not the LAT sncRNAs, reduced Notch3 or bICP27 steady state protein levels in transfected Neuro-2A cells.

2.5. LAT sncRNA1 is associated with RIG-I

Although the results presented above suggested that LAT sncRNA1 consistently stimulated RIG-I dependent signaling in 293 and Neuro-2A cells, they do not allow us to conclude whether this effect was direct or indirect. When RIG-I interacts with short dsRNA, the RIG-I signaling pathway is activated (Kato et al., 2008; Schlee et al., 2009b; Schmidt et al., 2009; Yoneyama et al., 2004). If LAT sncRNA1 directly activates the RIG-I signaling pathway, one would predict that LAT sncRNA1 would be stably associated with the RIG-I protein. Conversely, if LAT sncRNA1 indirectly activated the RIG-I signaling pathway, it would not be expected to stably associate with RIG-I.

To test whether LAT sncRNA1 was associated with RIG-I, 293 cells were cotransfected with RIG-I, N-RIG-I or an empty vector plus a plasmid expressing LAT sncRNA1. As previously reported (da Silva and Jones, 2012b), immunoprecipitation of RIG-I or N-RIG-I with a FLAG monoclonal antibody efficiently precipitated the RIG-I or N-RIG-I protein (Fig. 6A). As expected, the IgG heavy chain (HC) and light chains (LC) were also readily detected. N-RIG-I lacks the RNA binding domain of RIG-I and thus LAT sncRNA1 would not be expected to associate with N-RIG-I. 48 h after transfection, 293 cells were lysed and RIG-I/RNA or N-RIG-I/RNA complexes were immunoprecipitated (IP) with a FLAG-antibody (Fig. 6A). Following IP, RNA associated with RIG-I or N-RIG-I was recovered by acid phenol extraction and converted into cDNA by performing RT-PCR using adapter specific primers. The resulting cDNA was subsequently used as a template for PCR with primers specific for the LAT sncRNA1 as described in the material and methods. In several independent studies, we found that this procedure led to multiple bands in all of the lanes (Fig. 6B) suggesting that non-specific amplification of RNAs associated with RIG-I occurred. To test for specific amplification of LAT sncRNA1, a Southern blot was

performed using a ^{32}P -labeled DNA fragment containing the full-length LAT sncRNA1 as a probe (Fig. 6C). The probe recognized a band of approximately 200 bp that was amplified from cDNA originating from the RIG-I/RNA complex from cells cotransfected with RIG-I and LAT sncRNA1 (Fig. 6C). In contrast, the ^{32}P -labeled LAT sncRNA1 probe did not recognize a specific band in the RIG-I IP from cells transfected with N-RIG-I or the empty vector. Although this fragment appears to be larger than expected, there appears to be more than one adapter that was added to LAT sncRNA1 during the ligation process. Furthermore, LAT sncRNA1, perhaps due to its potential secondary structure, migrates larger than expected. Similar studies were unable to detect a stable association between LAT sncRNA2 and RIG-I in 293 cells (data not shown).

3. Discussion

These studies suggested that expression of LAT sncRNA1 and sncRNA2 play a role in the latency-reactivation cycle because they regulate RIG-I dependent signaling pathways, which consequently enhances cell survival. Both sncRNAs are located within the first 1.5 kb of LAT coding sequences. It is well established that the first 1.5 kb of LAT coding sequences restore the high wt reactivation phenotype to a LAT null mutant in small animal models (Perng et al., 1994, 1996a, 2001). Additional genetic studies support a role for expression of the LAT sncRNAs in the latency-reactivation cycle. For example, a recombinant virus that expresses just the first 811 bases of LAT coding sequences, and thus lacks the coding sequences for LAT sncRNA1 and sncRNA2, has reduced reactivation from latency (Drolet et al., 1999; Inman et al., 2001). Recombinant viruses containing more extensive LAT deletions reactivate from latency like a LAT null mutant (Drolet et al., 1999; Inman et al., 2001).

RIG-I recognizes RNA of various lengths and appears to prefer dsRNA with or without 5'-triphosphates (Baum et al., 2010; Hornung et al., 2006; Pichlmair et al., 2006; Schlee et al., 2009a, 2009b; Schmidt et al., 2009; Yoneyama and Fujita, 2009). RIG-I has structural similarity to DICER, an RNase III-type nuclease, that mediates RNA interference (Zou et al., 2009). Dicer requires dsRNA binding protein partners, PACT for example, for maximal activity (Kok et al., 2007; Lee et al., 2006). RIG-I also directly binds to PACT, and this interaction facilitates innate antiviral responses (Kok et al., 2011). Due to the complexity by which RIG-I recognizes its substrates, it is not clear how LAT sncRNA1 stimulated RIG-I dependent signaling or why LAT sncRNA2 cooperated with RIG-I to stimulate NF- κ B dependent transcription in neuro-2A cells but not in 293 cells. It seems reasonable to predict that cell type specific proteins, in part, regulate the RNA sensing functions of RIG-I and how RIG-I signals after being activated. Certain BHV-1 sequences derived from the latency related gene expressed from the pSilencer 2.1-U6 neo do not cooperate with RIG-I and stimulate IFN- β promoter activity (da Silva and Jones, 2012b) indicating that over-expression of any GC-rich sncRNA is not sufficient for this activity. For example, PolyI:C, but not LAT sncRNA1 or sncRNA2, reduced expression of proteins encoded by a plasmid when cotransfected with PolyI:C. Furthermore, LAT sncRNA1 and sncRNA2 promoted cell survival in Neuro-2A cells whereas PolyI:C enhanced cold-shock induced apoptosis.

Type I IFN (IFN- α and IFN- β) induction can induce apoptosis (Chawla-Sarkar et al., 2003; Leaman et al., 2002; Takao et al., 2003) or promote cell survival (Yang et al., 2000). Type I IFN promotes cell survival, in part by activating NF- κ B (Yang et al., 2005) and the threonine/serine protein kinase Akt (Yang et al., 2001). Further evidence that NF- κ B promotes cell survival comes from studies demonstrating that NF- κ B stimulates expression of c-FLIP (Benayoun et al., 2008), Bcl-2 family members (Bcl-X and Bfl-1/A1) (Lee et al., 1999), and the inhibitor of apoptosis (IAP) family members (Salvesen and Duckett, 2002). Interestingly, NF- κ B can also promote cell survival and neurite process formation in nerve growth factor-stimulated rat pheochromocytoma cells, PC12 (Foehr et al., 2000). Recent studies provide evidence that RIG-I protects neurons from axonal damage and decreases inflammation in the central nervous system (Dann et al., 2012) suggesting the LAT sncRNAs promote the repair of damaged neurons after infection. LAT, directly or indirectly, stimulates Akt activity in mouse neuroblastoma cells (Li et al., 2010) providing evidence that the LAT sncRNAs inhibited cold shock induced apoptosis by stimulating NF- κ B dependent transcription and perhaps Akt signaling.

In the context of the latency-reactivation cycle, we predict that the ability of the LAT sncRNAs to stimulate RIG-I dependent signaling is important during the establishment and maintenance of latency because it impairs HSV-1 replication and gene expression in sensory neurons. For example, the ability of LAT sncRNA1 to reduce HSV-1 replication efficiency (Shen et al., 2009) may be due to its ability to consistently stimulate IFN- β promoter activity in the presence of RIG-I. RIG-I is an IFN inducible gene (Cui et al., 2004; Kawaguchi et al., 2009), and consequently amplification of IFN signaling would dampen viral gene expression and enhance neuronal survival. During the maintenance of latency, low levels of viral gene expression periodically occur in a subset of latently infected neurons, which is defined as spontaneous molecular reactivation (Feldman et al., 2002). During spontaneous reactivation, IFN- β signaling and increased RIG-I protein levels may occur as a result of lytic cycle viral gene expression. Although RIG-I is IFN inducible, RIG-I protein expression is detected in many un-stimulated cell types (Kawaguchi et al., 2009) suggesting LAT encoded sncRNAs interact with RIG-I in the absence of viral gene activity and consequently enhances neuronal survival. Support for this prediction comes from the finding that LAT sncRNA1 stimulated IFN- β promoter activity even when RIG-I was not over-expressed. In summary, our studies suggest that interactions between RIG-I and LAT sncRNAs promote latency and neuronal survival by “sensing” viral activity during acute infection or spontaneous reactivation. To directly examine the role that LR sncRNAs play in the latency-reactivation cycle, it will be necessary to determine whether expression of the LAT sncRNAs influence the latency-reactivation cycle of HSV-1 in small animal models of infection.

4. Methods

4.1. Cells and transfection of these cells

Mouse neuroblastoma cells (Neuro-2A) and human embryonic kidney cells (293) were cultured in Earle's modified Eagle's (EMEM) medium supplemented with 10% fetal calf serum, penicillin (10 U/ml), and streptomycin (100 μ g/ml) in a humidified 5% CO₂ atmosphere at 37 °C.

Neuro-2A cells were transfected with the designated plasmids using TransIT Neural (MIR2145; Mirus) according to the manufacturer's instructions. Human 293 were transfected with the designated plasmids using Lipofectamine 2000 (Invitrogen, San Diego, CA) according to the manufacturer's instructions.

4.2. Plasmids

The human IFN- β chloramphenicol acetyltransferase (CAT) plasmid was obtained from Stavros Lomvards (Columbia University, NY) and contains sequences (positions -110 to -20) necessary for IFN- β activation. The LAT-encoded sncRNA1 and sncRNA2 constructs were cloned into pSilencer 2.1-U6 neo (Ambion) (Shen et al., 2009). The FLAG tagged RIG-I constructs; including the full length RIG-I (pFE-BOS RIG-I) and the constitutively active C-terminal deletion mutant RIG-I (pEF-BOS N-RIG-I) (Sumpter et al., 2005) were obtained from M. Gale (University of Washington), and are referred to as RIG-I and N-RIG-I, respectively. The p5X-NF- κ B-luciferase reporter construct (Alexopoulou et al., 2001) was obtained from R. Flavell (Yale University School of Medicine), and the *Renilla* luciferase (pRL-TK) reporter construct was purchased from Promega. Plasmids expressing a Flag-tagged infected cell protein 27 (bICP27) encoded by BHV-1 and the notch intercellular domain 3 (Notch3) were previously described (da Silva and Jones, 2012a; Workman et al., 2011).

4.3. Measurement of reporter gene expression

Chloramphenicol acetyltransferase (CAT) reporter assays were performed as previously described (da Silva and Jones, 2011, 2012b; Workman et al., 2012; Workman and Jones, 2010). Approximately 40 h after transfection, 293 cells were lysed by three freeze-thaw cycles in 250 mM Tris-HCl (pH7.4). CAT assays were performed with 0.2 μ Ci (7.4 KBq) 14 C-chloramphenicol (Amersham Biosciences, CFA754) and 0.5 mM acetyl coenzyme A (Sigma, A2181). Chloramphenicol and its acetylated forms were separated by thin-layer chromatography and CAT activity measured with a PhosphorImager (Molecular Dynamics, CA). Transfection experiments for CAT assays were repeated at least three times to confirm the results.

The NF- κ B luciferase reporter assays were performed in Neuro-2A and 293 cells as previously described (da Silva and Jones, 2012b). In brief, Neuro-2A and 293 cells seeded in 60 mm dishes containing EMEM plus 5% FCS at approximately 24 h before transfection. 2 h before transfection, EMEM with 5% FCS was replaced with fresh EMEM containing 0.5% FCS, which kept basal levels of NF- κ B promoter activity consistently low. Cells were then transfected with a plasmid containing the firefly-luciferase gene that is regulated by a simple promoter with 5 consensus NF- κ B binding sites (p5X-NF- κ B-luciferase), a plasmid encoding *Renilla*-luciferase under control of the herpesvirus TK promoter (pRL-TK) plus the indicated plasmids. 24 h after transfection, cells were harvested and subjected to the dual luciferase-assay by using a commercially available kit (Promega, E1910), according to the manufacturer's instructions.

4.4. Cell survival studies

Cold shock induced apoptosis was performed as previously described (Shen and Jones, 2008; Shen et al., 2009). In brief, approximately 3×10^5 Neuro-2A cells were plated in six well culture plates containing EMEM with 10% FCS 24 h prior to transfection. Cells were cotransfected with a plasmid encoding the β -Galactosidase (β -Gal) gene (0.025 μ g), the RIG-I expression plasmid (1 μ g), and 2 μ g of pSilencer encoding a LAT sncRNA1 or sncRNA2. To maintain equal amounts of DNA for transfection, the empty pSilencer plasmid was used. After transfection for 24 h, cells were seeded in 24 well plates containing EMEM with 10% FCS and incubated for 12 h, and then EMEM with 2% FCS was added to the cultures. After 12 h in EMEM with 2% FCS, Neuro-2A cells were placed on ice for 1 h with the culture plates sealed with Parafilm. After 1 h on ice, the Parafilm was removed and plates were incubated at 37 °C for 3.5 h. Cells were then fixed and stained for β -Gal expression. To calculate the relative level of cell survival after cold-shock induced cell death, the number of β -Gal positive cells were counted as previously described (Ciacci-Zanella et al., 1999; Ciacci-Zanella and Jones, 1999; Henderson et al., 2002; Perng et al., 2000, 2002). The number of blue cells in cultures transfected with the empty vector plus β -Gal was set as 1. The number of blue cells in cultures transfected with RIG-I along with empty vector or the LAT sncRNAs constructs was divided by the number of blue cells in cultures transfected with the empty vector plus β -Gal. The results are the average of at least three independent experiments.

4.5. Western blots

Western blot analysis was performed as previously described (da Silva and Jones, 2011; da Silva et al., 2011; da Silva and Jones, 2012a, 2012b).

4.6. Identification of RIG-I-associated RNA in transfected 293 cells

Procedures to identify interactions between BHV-1 miRNAs encoded by the latency related gene and RIG-I were previously described (da Silva and Jones, 2012b). In brief, human 293 cells (4×10^6) were cotransfected with 5 μ g of full-length RIG-I plasmid, the C-terminal deletion mutant N-RIG-I or an empty FLAG expression vector, plus 5 μ g of plasmids expressing sncRNA1 or sncRNA2. 48 h after transfection cells were harvested in hypotonic buffer (10 mM Tris pH7.5, 10 mM KCl, 0.5 mM EGTA and 1.5 mM $MgCl_2$, plus protease inhibitor) and subjected to immunoprecipitation followed by isolation of RIG-I-associated RNA, as previously described (Chiu et al., 2009; da Silva and Jones, 2011). One hundred ng of RIG-I-associated RNA was used to amplify total sncRNAs using the Global MicroRNA Amplification Kit (SBI System Biosciences, catalog # RA400A-1) according to the manufacturer's instructions. One percent of the amplified cDNA served as a template for PCR-amplification with the forward primers specific for sncRNA1 (5'-GCCTGTGTTTTTGTGCCTGGCTC-3') or sncRNA2 (5'-CATTCTTGTTTTCTAACTATGTTCTCTG-3') and a reverse primer specific for the 3'-adaptor provided in the kit. The PCR reactions were electrophoresed in a 1.2% agarose gel. The DNA in the gel was blotted onto Hybond N+ (Amersham Biosciences), UV cross-linked, and probed at 55 °C with the full-length LAT sncRNA1 sequences. The probe was obtained by digestion of the pSilencer 2.1-U6 neo construct containing LAT sncRNA1 with

BamHI and HindIII to release LAT sncRNA1 sequences. LAT sncRNA1 was labeled at its 5'-termini with ³²P using polynucleotide kinase and ³²P-gamma-ATP. After autoradiography, radioactive bands were visualized using a PhosphorImager (Molecular Dynamics, CA).

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Fig. 1.

Schematic of HSV-1 and organization of the LAT locus. Panel A: The prototypic HSV-1 genomic structure is shown at the top. The viral repeat regions are shown as open rectangles. TRL is the terminal long repeat. IRL is the internal (or inverted) long repeat. TRS is the terminal short repeat. IRS is the internal (or inverted) short repeat. The unique long (UL) and the unique short (US) regions are each represented by a solid line, and the position of the LAT locus within the repeats is denoted. Panel B: The LAT region (one in each long repeat) is shown in expanded form. The primary 8.3 kb LAT is denoted as a long arrow. The stable 2 kb LAT is shown as a solid rectangle. The LAT TATA box is denoted by TATA, and the small arrow and +1 indicate the start of LAT transcription (genomic nucleotide 118801). The relative locations of mRNAs encoding ICP0 and ICP34.5 are shown for reference. Panel C: The position of certain restriction enzyme sites within the first 1.5 kb of LAT coding sequences and relative locations of the two LAT sncRNAs previously identified (Peng et al., 2008). Panel D: The nucleotide sequence and positions of LAT sncRNA1 and LAT sncRNA2 are presented.

Fig. 2.

LAT sncRNA1 induces IFN- β promoter activity when cotransfected with RIG-I. Human 293 cells (2×10^6) were transfected with a CAT reporter plasmid containing the human IFN- β promoter (1 μ g), RIG-I (1 μ g), and 2 μ g of a plasmid expressing the LAT sncRNA1 or sncRNA2. N-RIG-I (1 μ g) served as a positive control. Equivalent amounts of DNA were used for transfection by adding an empty expression vector (pSilencer 2.1-U6 neo). 2 days after transfection, cells were harvested and CAT activity measured. The results are shown as fold increase relative to cells transfected with the IFN- β CAT reporter plasmid plus the empty pSilencer plasmid (Empty), which was arbitrarily set as 1. The values are the average of three independent experiments. An asterisk denotes significant differences ($P < 0.05$) in cells transfected with the human IFN- β CAT reporter plasmid plus LAT sncRNA1 and the RIG-I expression plasmid relative to IFN- β promoter activity in cells transfected with LAT sncRNA2 plus RIG-I or RIG-I plus empty vector, as determined by the Student *t*-test.

Fig. 3.

LAT sncRNA1 stimulate NF-κB dependent transcription when cotransfected with RIG-I. Approximately 6×10^5 293 cells (Panel A) or Neuro-2A cells (Panel B) were transfected with the 5× NF-κB luciferase reporter construct (1 μg), pRL-TK (0.033 μg), RIG-I (1 μg) and 2 μg of the indicated LAT sncRNAs. N-RIG-I (1 μg) and Poly I:C (0.5 μg) served as positive controls. Cells were harvested at 24 h after transfection and the Dual-Luciferase assay performed. The data represents the firefly luciferase activity normalized relative to *Renilla* luciferase activity. The values are expressed as fold difference relative to cells transfected with the luciferase reporter vectors plus an empty vector (Empty), which was arbitrarily set as 1. The values are the average of three independent experiments. An asterisk denotes significant differences ($P < 0.05$) in cells transfected with the human IFN-β CAT reporter plasmid plus LAT sncRNA1 and the RIG-I expression plasmid relative to IFN-β promoter activity in cells transfected with RIG-I plus empty vector, as determined by the Student *t*-test.

Fig. 4.

LAT sncRNAs interfere with cold-shock induced apoptosis. Panel A: Neuro-2A cells were cotransfected with plasmids encoding the β -Galactosidase (β -Gal) gene (0.025 μ g), RIG-I (1 μ g), the sncRNA1 or sncRNA2 (2 μ g), or Poly I:C (0.5 μ g), as indicated. At 48 h after transfection, Neuro-2A cells were submitted to cold-shock induced apoptosis and subsequently recovered for 3.5 h at 37°C. As a control, Neuro-2A cells that were transfected with the β -Gal expression vector and pSilencer 2.1-U6 neo; but not subjected to cold-shock induced apoptosis were included. Cells were then fixed, stained for β -Gal expression, and representative pictures of the respective cultures shown. Panel B: The number of β -Gal positive cells (blue cells) was counted and the relative cell survival after cold-shock induced cell death was expressed as fold of induction relative to cells transfected with an empty vector plus the β -Gal plasmid, which is arbitrarily set as 1. The results are the average of three independent experiments. An asterisk denotes significant differences ($P < 0.05$) in cell survival following transfection with the RIG-I expression plasmid and LAT sncRNAs relative to cells transfected with RIG-I plus empty vector, as determined by the Student *t*-test. Panel C: The number of surviving blue Neuro-2A cells in mock transfected cells was compared to cells treated with PolyI:C or PolyI:C plus over-expression of RIG-I.

Fig. 5.

In contrast to PolyI:C, LAT sncRNAs do not interfere with protein synthesis. Western blot analysis comparing the levels of two proteins (Notch3 and bICP27) in the presence of Poly I:C or RIG-I cotransfected with plasmids expressing a LAT sncRNA expression plasmid. Neuro-2A cells were cotransfected with plasmids encoding Notch3 (1 μ g) or bICP27 (1 μ g), Poly I:C (0.5 μ g), RIG-I (1 μ g) and 2 μ g of the LAT sncRNAs (1 or 2). After 36 h, whole cell lysate was prepared. A total of 100 μ g of protein was separated in an 8% SDS-PAGE gel, and expression of Notch3 or Flag-tagged bICP27 detected by Western blot analysis using a rabbit anti-Notch3 polyclonal antibody (Santa Cruz) or a anti-Flag monoclonal antibody (Sigma) to detect Flag tagged bICP27, respectively. β -actin protein levels were analyzed in the respective samples as a loading control.

Fig. 6.

LAT sncRNA1 interacts with full length RIG-I. Panel A: Monolayers containing 4×10^6 293 cells were transfected with 5 μ g of an empty Flag-vector (Empty) or Flag-vectors expressing RIG-I or N-RIG-I along with 5 μ g of plasmids expressing the LAT sncRNA1. 48 h after transfection cells were harvested in hypotonic buffer and then a RIG-I IP performed using an anti-Flag monoclonal antibody. The efficiency of the IP was monitored by western blot (WB) analysis using the anti-Flag monoclonal antibody. The position of the RIG-I protein, N-RIG-I protein, heavy chain of IgG (HC), and light chain of IgG (LC) are denoted. The position of molecular weight markers (kD) is shown on the right side of the western blot. Panel B: The RNA associated with RIG-I was extracted by acid-phenol:chloroform extraction and subsequently converted into cDNA using a MicroRNA Amplification kit as described in material and methods. The resultant cDNA served as a template for PCR-amplification by the forward primers specific for the LAT sncRNA1 and the reverse primer specific for the 3'-adaptor. The resultant PCR products were electrophoresed on a 1.2% agarose gel and stained with ethidium bromide. Panel C: Southern blot analysis was performed using as a probe a 32 P-labeled DNA fragment digested from a plasmid containing the sequences encoding LAT sncRNA1. The arrow denotes the position of the LAT sncRNA1-specific band recognized by the probe. These results are representative of 2 independent experiments.