Virology 392 (2009) 11–15

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Rapid Communication

RIG-I activation inhibits *ebolavirus* replication ☆

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ARTICLE INFO

Article history:
Received 26 May 2009
Returned to author for revision 16 June 2009
Accepted 18 June 2009
Available online 23 July 2009

Keywords:
Ebolavirus
RIG-I

ABSTRACT

Hemorrhagic fever viruses are associated with rapidly progressing severe disease with high case fatality, making them of public health and biothreat importance. Effective antivirals are not available for most of the members of this diverse group of viruses. A broad spectrum strategy for antiviral development would be very advantageous. Perhaps the most challenging target would be the highly immunosuppressive filoviruses, *ebolavirus* and *marburgvirus*, associated with aerosol infectivity and case fatalities in the 80–90% range. Here we report that activation of evolutionarily conserved cytosolic viral nucleic acid sensor, RIG-I can cause severe inhibition of *ebolavirus* replication. These findings indicate that RIG-I-based therapies may provide an attractive approach for antivirals against Ebola hemorrhagic fever, and possibly other HF viruses.

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Introduction

The genus *Ebolavirus* contains five virus species; *Zaire*, *Sudan*, *Cote d'Ivoire*, *Reston*, and *Bundibugyo ebolavirus* (Sanchez et al., 2007; Towner et al., 2008). These viruses have been identified in various regions of sub-Saharan Africa and the Philippines, and are classified as biosafety level 4 (BSL-4) pathogens and select agents based on their association with high case fatality, human-to-human transmission. The high genetic and antigenic diversity of these viruses coupled with the sporadic nature of the disease outbreaks pose considerable challenges for the development of effective preventive vaccines and antiviral therapies.

Normally, cell recognition of pathogen-associated molecular patterns leads to activation of an innate and adaptive immune responses which contribute to blocking virus replication and spread (Bowie and Unterholzner, 2008; Takeuchi and Akira, 2009). However, profound immunosuppression and high virus titers, reaching 10^9 pfu/ml in some cases, are hallmarks of fatal Ebola hemorrhagic fever cases (Sanchez et al., 2004; Towner et al., 2004). While some negative sense RNA viruses avoid activation of innate immune responses by modifying their RNA 5′ ends during virus transcription (Habjan et al., 2008), *ebolavirus* uses alternate mechanisms involving the virus VP24 and VP35 proteins. VP24 has been shown to interfere with the IFN signaling pathway by blocking the translocation of the phosphorylated STAT-1 to the nucleus (Reid et al., 2006). VP35 has been shown to be involved in the block of many different steps of the host immune response. These include directly inhibiting RIG-I function (Cárdenas et al., 2006), IRF-3 phosphorylation, dimerization and nuclear translocation (Basler et al., 2003), and impairment of the function of IKKe and TBK-1 kinases (Prins et al., 2009). In addition, VP35 has been shown to block PKR activation (Feng et al., 2007) and suppression of RNA silencing (Haasnoot et al., 2007). These observations suggest that an intervention which tips the initial balance between host innate immune response versus virus replication and suppression of innate immunity might serve as an effective antiviral strategy.

Earlier studies have demonstrated that recognition of viral RNA by cytoplasmic pathogen sensors such as Retinoic Acid Inducible Gene-I (RIG-I) is critical in the activation of the type I interferon-dependant antiviral innate immune response. RIG-I consists of two N-terminal CARD domains and a C-terminal helicase domain. Functional analysis has revealed that the N-terminal CARD domains are sufficient to induce type I interferon induction (Yoneyama et al., 2004). Furthermore, 5′ PPP-ssRNA (Hornung et al., 2006; Pichlmair et al., 2006; Saito et al., 2008), or dsRNA (Takahasi et al., 2008) are known to serve as ligands that activate RIG-I. Here we tested the hypothesis that stimulation of host cell innate immune responses by 5'PPP-RNA dependent RIG-I activation or by expression of constitutively active N-terminal domain of RIG-I, may be an effective antiviral strategy for *ebolavirus*.

Results and discussion

A 19mer 5'PPP-RNA which had previously been shown to be a particularly potent inducer of RIG-I, was produced by T7 RNA

☆ The findings and conclusions in this report are those of the authors and do not necessarily represent the views of Centers for Disease Control and Prevention.

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0042-6822/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.virol.2009.06.032

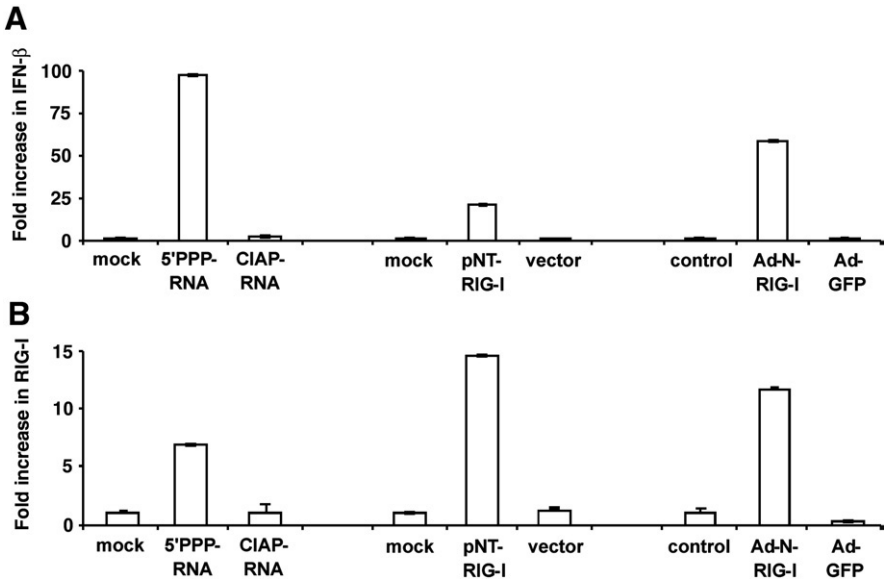

Fig. 1. RIG-I activation upregulated IFNβ and RIG-I mRNA expression. A549 cells were transfected for 24 h with 5′PPP-RNA, CIAP-RNA, pNT-RIG-I or control vector or infected for 24 h with Ad-N-RIG-I or control Ad-GFP at an MOI of 300 for 24 h. The cells were then infected with *ebolavirus* at an MOI of 0.5 for 48 h, total cellular RNA was extracted from each experimental group and real time PCR reactions were conducted. The RIG-I primers were designed to detect endogenously induced RIG-I and would not detect the transiently expressed N-terminal RIG-I by pNT-RIG-I plasmid or Ad-N-RIG-I infection. The threshold cycle number for cDNA was normalized to that of β-actin mRNA, and the resulting value was converted to a linear scale. For RT-PCR analysis, data from three independent experiments from RNA isolated from duplicate cultures were tested in duplicate. All data points fell into a normal distribution and there were no outliers.

polymerase *in vitro* synthesis as described previously (Hornung et al., 2005; Kim et al., 2004). In addition, we over-expressed the N-terminal CARD domain of RIG-I using either a plasmid construct (pNT-RIG-I) or a recombinant Adenoviral vector (Ad-N-RIG-I). The construction of the pNT-RIG-I has been described previously (Yoneyama et al., 2004). For the construction of Ad-N-RIG-I, cDNA encoding EGFP and N-RIG-I under separated CMV promoters was inserted in non-replicating human Adenovirus serotype 5 vector. The replication deficient Ad-N-RIG-I was included in this study for its high efficiency RIG-I gene delivery and its possible future use in *in vivo* experiments.

Lung fibroblast (A549) cells were chosen for the study, based on their known high susceptibility to *ebolavirus* infection, ease of transfection, and robust innate immune responses. The cells were transfected with 5′PPP-RNA, plasmid expressed N-terminal RIG-I (pNT-RIG-I), or infected with Ad-N-RIG-I at an moi of 300, 24 h prior to infection with *Zaire ebolavirus* at an moi of 0.5. RNA which had been calf intestinal alkaline phosphatase (CIAP) treated to remove the 5′PPP, or vector alone, or control Ad vector lacking the N-terminal RIG-I ORF (Ad-GFP) were used as controls. At 48 h post *ebolavirus* infection, cell supernatants were collected and cell monolayers were lysed with either RNA extraction buffer (Tripure) or protein lysis buffer.

The expression of RIG-I and IFN-β mRNAs were examined by real time RT-PCR (Fig. 1). Cells transfected with 5′PPP-RNA showed a 100-fold increase in IFN-β mRNA and an approx. 10-fold increase in RIG-I mRNA. This induction was specific to 5′PPP-RNA as mock-transfected or cells transfected with RNA lacking the 5′PPP showed no induction. *Ebolavirus* infection alone did not induce RIG-I in either mock-transfected cells or cells transfected with RNA lacking the 5′PPP (Fig. 1). Cells transfected with pNT-RIG-I exhibited a 20-fold increase in IFN-β mRNA and a 15-fold increase in RIG-I mRNA. The induction was specific to pNT-RIG-I as mock-transfected cells, or cells transfected with vector alone, showed no induction. Similarly, infection of cells with Ad-N-RIG-I resulted in a 60-fold increase for IFN-β mRNA and a 15-fold increase on RIG-I mRNA over the Ad-GFP control. These results demonstrate that even in the presence of highly immunosuppressive *ebolavirus* infection RIG-I mediated innate immune responses can be stimulated with 5′PPP-RNA or by over-expression of RIG-I.

Similar results were seen at the protein level. For instance, stimulation of RIG-I protein expression was observed in cells transfected with 5'PPP-RNA (lanes 2 and 5), or plasmid (pNT-RIG-I) (lane 8) or Adeno vector (Ad-N-RIG-I) (lane 11) over-expressing the N-terminal domain of RIG-I (Fig. 2). *Ebolavirus* infection slightly reduced the level of RIG-I in comparison to uninfected cells (compare lanes 5 and 2).

Induction of the RIG-I has been shown previously to reduce viral replication for other negative strand RNA viruses such us vesicular stomatitis and influenza viruses (Kato et al., 2006; Kumar et al., 2006). To examine whether the increased RIG-I induction observed in the treated cells infected with *Zaire ebolavirus* resulted in inhibition of virus replication, we measured virus protein expression levels in the cell lysates (Fig. 3A) and quantitated virus release in the cell (Fig. 3B). Using an antibody specific for *Zaire ebolavirus* VP35, a striking reduction in virus protein expression (relative to mock treated cells) was observed in the 5′PPP-RNA transfected cells (lane 2), and in the

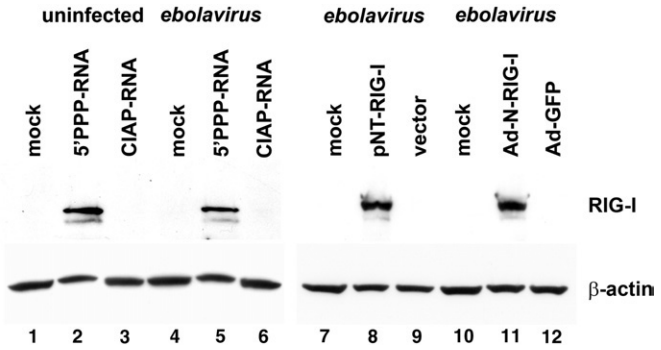

Fig. 2. Sustained induction of RIG-I in *Zaire ebolavirus* infected cells. A549 cells in duplicate cultures for each treatment were transfected for 24 h with 5′PPP-RNA, CIAP-RNA, pNT-RIG-I or vector alone or infected for 24 h with Ad-N-RIG-I or control Ad-GFP at an MOI of 300 for 24 h. The cells were then infected with *ebolavirus* at an MOI of 0.5 for 48 h and lysed. Equal quantities of solubilized protein were resolved in SDS-PAGE gel, transferred to nitrocellulose membrane and probed with anti-RIG-I rabbit polyclonal antibody. As a loading control the same membrane was stripped and probed with anti-β-actin antibody.

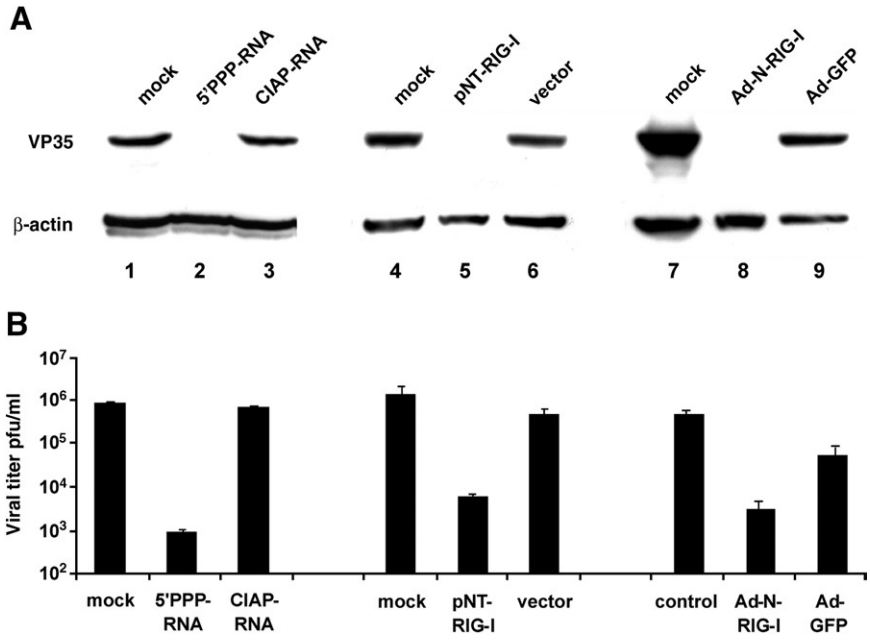

Fig. 3. RIG-I activation inhibited *ebolavirus* protein expression, and virus release. A549 cells were processed as described in Fig. 1. At 48 h post infection supernatants were collected and the cells were treated with protein lysis buffer. Total protein lysates were analyzed by SDS-PAGE followed by transfer to nitrocellulose membrane. *Ebolavirus* VP35 protein was detected using a VP35 specific monoclonal antibody (aVP35-1). As a loading control, the same membrane was stripped and probed with anti-β-actin antibody (Panel A). The collected supernatants were analyzed for *ebolavirus* replication (Panel B).

RIG-I over-expressing cells transfected with pNT-RIG-I (lane 5) or infected with Ad-N-RIG-I (lane 8). More significantly, we demonstrated a more than 1000-fold inhibition of infectious virus yield from infected cells treated with the 5′PPP-RNA (Fig. 3B). Similarly, over-expression of the N-terminal RIG-I by plasmid transfection or Adenovirus vector infection resulted in a more than 500-fold decrease of *ebolavirus* titers compared to the mock infected cells. It was noted that a modest (10-fold) decrease in *ebolavirus* replication was also seen in the control Ad-GFP (lacking the RIG-I ORF) infected cells relative to the mock treated cells. The reason for this is unclear, but appears unrelated to the more potent levels of RIG-I mediated virus inhibition.

There are currently no licensed vaccines and no effective antiviral drugs available for *ebolaviruses*. Several antiviral approaches are currently being investigated with variable success, including protein cleavage inhibitors (Ströher et al., 2007), antisense RNAs, and siRNAs (Spurgers et al., 2008; Warfield et al., 2006). Of these, the *ebolavirus* antisense phosphorodiamidate morpholino oligomers appear the most promising (Warfield et al., 2006). However, the cumulative data so far suggest that it is likely that a combination of different drugs may be necessary for effective treatment or to achieve short-term protection (Paragas and Geisbert, 2006). We show here that activation of RIG-I can very effectively suppress *ebolavirus* replication and shows considerable promise for use against these important public health and bioterrorism threats.

Previous studies of *ebolavirus* infected patients demonstrated that cases going on to survive infection had at approx. 2–3 log lower viremias at early times post onset of disease relative to cases going on to fatal outcome (Sanchez et al., 2004; Towner et al., 2004). Interferon treatment alone has shown only a 10- to 100-fold reduction of *ebolavirus* replication in cell culture (Jahrling et al., 1999). Our findings that 5′PPP-RNA treatment could reduce *ebolavirus* replication by 3 logs, suggests that these high levels of virus inhibition may be sufficient to impact disease outcome. This study suggests that developing small molecules that activate RIG-I may have therapeutic potential for the treatment of *ebolavirus* infected individuals or for prophylactic use to ensure short-term protection of disease outbreak response personnel and laboratory workers. Recent advances in the delivery of small RNA molecules (Castanotto and Rossi, 2009) suggest this approach is sufficiently promising to consider testing in animal models. Alternatively the replication deficient Adenovirus vectors expressing N-terminal RIG-I remains another viable approach on the path of developing short-term prophylactics for highly pathogenic HF viruses such as *Zaire ebolavirus*.

Materials and methods

Cells and viruses

Vero E6 and A549 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 100 U/ml penicillin, 100 U/ml streptomycin, and 10 mM HEPES buffer (complete) supplemented with 10% fetal bovine serum (FBS). The *Zaire ebolavirus* Mayinga strain stock preparation and virus titration were performed in Vero E6 cells. All work with infectious *ebolavirus* was performed in the biosafety level 4 laboratory at the Centers for Disease Control and Prevention. Samples were inactivated with 2×10^6 to 5×10^6 rd from a Gammacell irradiator when necessary. Production and quantification of type-5 adenoviral vectors expressing either EGFP under the control of CMV promoter or expressing both EGFP and FLAG-tagged human N-terminal RIG-I under the control of separate CMV promoter was carried out by Vector BioLabs, Inc.

In vitro synthesis of 5′PPP-RNA

In vitro transcribed RNAs were synthesized using T7 RNA polymerase. Using annealed complimentary oligo nucleotides, a DNA template was constructed that contains a T7 RNA polymerase promoter followed by the sequence of interest (AGCUUAACCUGUCCUUCAA) to be transcribed (Yoneyama et al., 2004). 20 pmol of the DNA template were incubated with 25 U T7 RNA polymerase, 40 U RNase inhibitor in a buffer containing 40 mM Tris–Hcl (pH 8.0), 10 mM DTT, 2 mM spermidine–HCl, 20 mM $MgCl_2$ and NTPs. DNA template was digested with DNase I and RNA was purified using phenol: chloroform extraction and ethanol precipitation. Size and integrity of RNA was analyzed by gel electrophoresis. Calf intestine alkaline

phosphatase (CIAP) treatment was performed to remove tri-phosphate groups from *in vitro* synthesized RNA. Briefly, 100 μg of *in vitro* transcribed RNA was treated with 150 U of CIAP for 3 h at 37 °C in a buffer containing 50 mM Tris–HCl (pH 8.0), 0.1 mM EDTA and 50 U of RNase inhibitor. RNA was purified as described above.

Viral infection and real time RT-PCR

To assess 5′PPP-RNA induced RIG-I and IFNβ expression A549 cells (1×10^6 cells/well) in a 6-well tissue culture plate in duplicate cultures for each treatment were mock-transfected (control) or transfected with pEF-BOS (4 μg) or pEF-BOS-NT-RIG-I (4 μg), or 5′ PPP-RNA (2 μg), CIAP-RNA (2 μg) using lipofectamine 2000 or infected with either Adenoviral vector expression GFP or GFP and N-RIG-I at an MOI of 300:1 for 24 h. A549 cells were then infected with *ebolavirus* at an MOI of 0.5 for 48 h. Cells were then harvested and total RNA was extracted from each experimental group using Tri-Pure reagent. Real time PCR reactions were conducted, using 2 μg total cellular RNA and Superscript III One-Step RT-PCR (Invitrogen) according to the manufacturer's instructions. Real time PCR was performed with denaturation at 94 °C for 15 s, annealing at 56 °C for 30 s, and extension at 72°C for 30 s for a total of 45 cycles. Two sets of PCR assays were performed for each sample using the following primers specific for cDNA of each of the following genes.

IFNβ: forward 5′-TGG GAG GCT TGA ATA CTG CCT CAA-3′
reverse 5′-TCT CAT AGA TGG TCA ATG CGG CGT-3′
RIG-I: forward 5′-AAA CCA GAG GCA GAG GAA GAG CAA-3′
reverse 5′-TCG TCC CAT GTC TGA AGG CGT AAA-3′
β-actin: forward 5′-ACC AAC TGG GAC GAC ATG GAG AAA-3′
reverse 5′-TAG CAC AGC CTG GAT AGC AAC GTA-3′

The RIG-I primer set used was designed to amplify only the C-terminal domain of RIG-I (nt position 2335 to 2476). This is present in the endogenous RIG-I mRNA, but absent in the plasmid expressed N-terminal RIG-I.

Virus plaque assay

Plaque assays were set up in a laminar-flow safety cabinet in a BSL-4 laboratory on confluent monolayers of Vero E6 cells. Virus dilutions of 10^{-1} through 10^{-6} were adsorbed to the cells by plating 200 μl of a diluted specimen in duplicate onto the monolayer and incubating it for 1 h at 37 °C. The inoculum was removed and the monolayers were overlaid with a solution of 1% agarose (SeaKem ME; FMC), 2% fetal bovine serum, 2 mM L-glutamine, 15 mM HEPES (pH 7.5), 1× minimal essential medium without phenol red (GIBCO, Invitrogen, Life Technologies), and 1× antibiotic–antimycotic (GIBCO, Invitrogen, Life Technologies). The plates were then incubated for 7 to 8 days at 37 °C and then fixed overnight with 3% formaldehyde per well. Virus plaques were revealed by a 1-h incubation with a 1:1000 dilution of a rabbit anti-*ebolavirus* antibody followed by a second 1-h incubation with a 1:1500 dilution of a horseradish peroxidase-labeled goat anti-rabbit secondary antibody (BioRad). After thorough rinsing with deionized H_2O, 500 μl of True-Blue (KPL) substrate was added, and after 10 to 15 min, virus plaques were counted.

Immunoblot

Cells were washed with chilled PBS and then lysed in 100 μl of ice-cold lysis buffer (50 mM Tris–Cl, pH 8.0, 150 mM NaCl, 10% v/v glycerol, 1% v/v Triton X-100, 2 mM EDTA, 1 mM PMSF, 20 μM leupeptin containing aprotinin 0.15 μg/ml) for 20 min at 4 °C. The protein content of different samples was determined using a protein assay reagent (BioRad, Inc., CA, USA). Equal quantities of solubilized protein were resolved by 10% SDS-PAGE, blotted to nitrocellulose membrane and probed with the indicated primary antibodies, anti-VP35 and anti-β-actin (Sigma, St. Louis, MO, USA). Antibody signals were detected by chemiluminescence using secondary antibodies conjugated to horseradish peroxidase and an ECL detection kit (Amersham Biosciences, Inc., NJ, USA).

Acknowledgment

The work was supported by a grant from NVPO awarded to SS.

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