Antiviral Research 127 (2016) 79–89

Contents lists available at ScienceDirect

Antiviral Research

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

Protein Phosphatase-1 regulates Rift Valley fever virus replication

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ARTICLE INFO

Article history:
Received 8 October 2015
Received in revised form
21 December 2015
Accepted 14 January 2016
Available online 20 January 2016

Keywords:
L polymerase
Phosphorylation
Protein phosphatase 1
Antiviral agent
Rift valley fever virus
Bunyaviridae

ABSTRACT

Rift Valley fever virus (RVFV), genus *Phlebovirus* family *Bunyaviridae*, is an arthropod-borne virus endemic throughout sub-Saharan Africa. Recent outbreaks have resulted in cyclic epidemics with an increasing geographic footprint, devastating both livestock and human populations. Despite being recognized as an emerging threat, relatively little is known about the virulence mechanisms and host interactions of RVFV. To date there are no FDA approved therapeutics or vaccines for RVF and there is an urgent need for their development. The Ser/Thr protein phosphatase 1 (PP1) has previously been shown to play a significant role in the replication of several viruses. Here we demonstrate for the first time that PP1 plays a prominent role in RVFV replication early on during the viral life cycle. Both siRNA knockdown of PP1α and a novel PP1-targeting small molecule compound 1E7-03, resulted in decreased viral titers across several cell lines. Deregulation of PP1 was found to inhibit viral RNA production, potentially through the disruption of viral RNA transcript/protein interactions, and indicates a potential link between PP1α and the viral L polymerase and nucleoprotein. These results indicate that PP1 activity is important for RVFV replication early on during the viral life cycle and may prove an attractive therapeutic target.

1. Introduction

Members of the Bunyaviridae family are among the most widespread viruses in the world. Rift Valley fever virus (RVFV), genus *Phlebovirus* family *Bunyaviridae*, is an arthropod-borne-virus whose cyclic epidemics have had devastating economic effects on livestock populations throughout much of sub-Saharan Africa (Pepin et al., 2010). In humans, RVFV causes Rift Valley fever (RVF), which is characterized by a mild to moderate febrile illness. In a small percentage of patients retinitis with visual impairment, hemorrhagic liver necrosis, and permanent neurological damage can occur due to inflammation of the spinal cord and meninges (Pepin et al., 2010; Ikegami and Makino, 2011). Past outbreaks have been a source of concern as RVFV has proven adept in its ability to break past traditional geographic barriers, escaping continental Africa into the Arabian Peninsula and Madagascar (Ikegami and Makino, 2011; Rolin et al., 2013). The recent geographical expansion of West Nile virus and yellow fever virus are particularly troubling as RVFV is capable of being transmitted through similar arthropod vectors and has been isolated from up to 40 different species of mosquitoes in the field (Sang and et al., 2010). Due to its increasing spread, host susceptibility, and in particular vector plasticity and ease of aerosolization, the CDC has listed RVFV as an emerging infectious disease and a Category A priority pathogen. Despite being recognized as a significant and emerging threat, there are currently no FDA licensed vaccines or therapeutics for RVF and there is an urgent need for their development.

RVFV contains a tripartite single stranded negative sense RNA genome composed of a large (L), medium (M), and small (S) segment. The viral RNA-dependent RNA polymerase or L protein (238 kDa) is encoded on the L segment, while the M segment encodes the precursors for the two glycoproteins Gc (56 kDa) and Gn (54 kDa), the nonstructural NSm protein, and a 78 kDa glycoprotein (Pepin et al., 2010). The S segment codes for the viral nucleoprotein

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http://dx.doi.org/10.1016/j.antiviral.2016.01.007
0166-3542/

N (26 kDa) and the nonstructural protein NSs (31 kDa) (Pepin et al., 2010). While the nonstructural proteins are replication dispensable; they do play a significant role in the pathogenesis of the disease *in vivo* (Pepin et al., 2010; Ikegami and Makino, 2011). The NSs protein in particular has been established as a major virulence factor due to its ability to suppress the host's innate immune response (Pepin et al., 2010; Ikegami and Makino, 2011).

Viruses are intraobligate cellular parasites reliant on manipulating host cellular signaling pathways in order to facilitate infection and viral replication. Host targeted therapeutics have become increasingly popular as they provide novel targets and insights to existing pathogens, along with a decreased likelihood of viral adaptation. Our previous studies have demonstrated that RVFV infection results in the phosphorylation of a large number of host signaling proteins such as ataxia telangiectasia mutated (ATM), p53, p38, extracellular signal regulated kinase (ERK), and other mitogen-activated protein kinases (MAPK) (Austin and et al., 2012; Baer and et al., 2012a; Popova and et al., 2010; Narayanan and et al., 2011). The phosphorylation of viral and host proteins can play a critical role in regulating both viral replication and the host response to an invading pathogen.

Protein phosphatase 1 (PP1) is a well characterized and conserved Ser/Thr phosphatase holoenzyme comprised of a regulatory subunit, and one of three highly homologous catalytic subunits PP1α, PP1γ, or PP1β/δ (Bollen et al., 2010). The catalytic subunits interact with over 200 regulatory proteins including components of the MAPK signaling cascade such as ERK1/2, c-Jun N-terminal kinase (JNK), and p38 (Bollen et al., 2010). PP1 catalytic subunits typically bind to their regulatory subunits through a combination of short binding motifs, such as through the well-established RVxF motif and the recently identified SILK, MyPhoNE, SpiDoC and iDoHA motifs (Heroes and et al., 2013). Numerous studies have shown that PP1 plays a prominent role in viral replication affecting the antiviral response, signal transduction, cell cycle checkpoint control, RNA splicing, and protein synthesis (Zeng and et al., 2009; Nekhai et al., 2007a; Brown et al., 1994; Modrof et al., 2002; Phosphorylation of Marbur (2001)). While the significance of cellular kinases for RVFV has been partially described (Carsillo et al., 2010; Bakre and et al., 2013; Kim and et al., 2013; Chu and Yang, 2007), relatively little is known about the impact of cellular phosphatases on viral replication.

PP1 has previously been shown to play a significant role in the viral replication of papovavirus, adenovirus, human immunodeficiency virus 1 and 2 (HIV-1 & HIV-2), and Ebola virus (EBOV) (Zeng and et al., 2009; Nekhai et al., 2007a; Brown et al., 1994; Modrof et al., 2002; Phosphorylation of Marbur (2001)). For EBOV, the switch between viral transcription and replication is dependent on the ability of PP1 to regulate the phosphorylation status of the viral polymerase cofactor VP30, which when phosphorylated at two N-terminal serine clusters promotes viral replication at the expense of viral transcription (Modrof et al., 2002; Martinez and et al., 2008; Biedenkopf et al., 2013a).

We recently developed small molecules that were efficient in inhibiting HIV-1 (Ammosova and et al., 2014a; Nekhai et al., 2007b) as well as EBOV infection in a PP1 dependent manner (Ilinykh and et al., 2014a). Initially, we developed a library of small molecules that were designed to specifically bind to the RVxF binding site of PP1 and identified the small molecule, 1H4, which inhibited HIV-1 transcription (Ammosova and et al., 2014a). We further modified the 1H4 compound and identified compound 1E7-03, a tetrahydroquinoline derivative, which efficiently inhibited HIV-1 (Ammosova and et al., 2014a) and also efficiently inhibited transcription of the EBOV genome and replication of viral particles (Ilinykh and et al., 2014a).

Our previous studies indicated that RVFV relies on a large number of cellular phosphosignaling events in order to regulate the cellular environment to facilitate viral replication (Austin and et al., 2012; Baer and et al., 2012a; Popova and et al., 2010; Narayanan and et al., 2011). As PP1 is a critical regulator of numerous pathways utilized by RVFV, a compound screen of small molecule PP1 inhibitors was run against RVFV infected cells and found to inhibit viral replication (data not shown). Based on our previous studies and drug screenings, we hypothesize that PP1 activity may be important for RVFV replication through interaction with a viral substrate, and may potentially play a role in regulating the viral life cycle.

2. Materials and methods

2.1. Viral infections and drug treatments

The MP-12 strain of RVFV, a live attenuated strain derived from the ZH548 strain, was generated by 12 serial passages in MRC5 cells in the presence of 5-fluorouracil resulting in 25 nucleotide changes across the three viral genome segments (Rift Valley fever virus. rMP-12-LV5 is a recombinant strain derived from MP-12 with a V5 tag inserted between amino acid position 1852 and 1853 of the viral L protein. The rMP-12-NSsdel (ΔNSs) strain completely lacks the NSs ORF. For the rMP-12-NSsLuc (rMP-12-Luc) strain the NSs ORF was replaced with a renilla luciferase reporter (Ikegami and et al., 2009). rMP-12-Flag (NSs-Flag) has a C-terminal Flag-tagged NSs (Ikegami and et al., 2009). Control experiments were performed with Influenza A/California/2009 (H1N1), obtained from BEI Resources (ATCC), in murine Darby canine kidney (MDCK) cells.

During viral inoculation, the growth media was removed, cells washed with phosphate buffered saline (PBS without calcium and magnesium), overlaid with a viral inoculums diluted in growth media, and incubated for 1 h at 37 °C at 5% CO_2. Following the 1 h incubation, infectious supernatants were removed, cells washed with PBS, and growth media replaced. Unless otherwise noted, cells were pre- and post-treated during the course of an infection with either dimethyl sulfoxide (DMSO) or the PP1 targeting small molecule compound 1E7-03 (Ammosova and et al., 2014a). DMSO amounts were equalized and matched to corresponding drug treatment volumes and always less than 0.1% of final sample volume. Okadaic acid was used at a final concentration of 5 nM. Plaque assays were performed as previously described (Baer and et al., 2012b; Baer and Kehn-Hall, 2014).

2.2. Cell culture

Human small airway lung epithelial cells (HSAECs) were isolated from an anonymous donor and grown in Ham's F12 medium according to the vendor's protocol (Cambrex Inc., Walkersville, MD). MDCK, 293T cells and Vero cells were cultured using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% glutamax [(American Type Culture Collection (ATCC)]. BSR-T7 cells, which stably express T7 RNA polymerase, were grown in minimal essential medium (MEM) supplemented with 10% FBS, 1% non-essential amino acids, 1% penicillin/streptomycin, 1% glutamax and 1 mg/ml G418 (Habjan et al., 2008). Cells were maintained at 37 °C and 5% CO_2.

2.3. Western blot analysis

Cells were collected as previously described using a mixture of T-PER reagent (Pierce, IL), 2 × Tris-glycine (sodium dodecyl sulfate) SDS sample buffer (Novex, Invitrogen), 33 mM dithiothreitol (DTT), and protease/phosphatase inhibitor cocktail tablets (1 × Halt