Differential RNA silencing suppression activity of NS1 proteins from different influenza A virus strains

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The NS1 gene of influenza A virus encodes a multi-functional protein that plays an important role in countering cellular antiviral mechanisms such as the interferon (IFN), protein kinase R and retinoic acid-inducible gene product I pathways. In addition, NS1 has recently been shown to have RNA interference (RNAi) or RNA silencing suppression (RSS) activity. This study analysed the IFN antagonistic activity of NS1 and the RSS activity for several influenza subtypes: H1N1, H3N2, H5N1 and H7N7. It was shown that the various NS1 proteins were capable of inhibiting the activation of an IFN-responsive promoter. However, differential RSS activity was measured among the NS1 variants. The NS1 protein of strain A/WSN/33 (H1N1) was most potent in suppressing short hairpin RNA-mediated gene silencing. In contrast, NS1 proteins of the highly pathogenic H5N1 strains A/VN/1194/04 and A/HK/156/97 were most potent in complementing the RSS function of the human immunodeficiency virus type 1 Tat protein. These results show that the ability of NS1 to suppress RNAi varies among influenza strains and is likely to contribute to differences in viral replication capacity and pathogenicity.

INTRODUCTION

Innate antiviral immune responses act as the first line of defence against invading pathogenic viruses. In mammals, the interferon (IFN) pathway plays a key role in this innate antiviral response. In order to replicate, most viruses have developed ways of counteracting the IFN pathway. The mechanism by which influenza A virus blocks innate immune responses is still under investigation. An important viral factor is the multifunctional NS1 protein, which is able to modulate several antiviral pathways. For example, NS1 blocks 2’-5’-oligoadenylate synthetase-mediated activation of RNase L (Min & Krug, 2006) and limits the induction of IFN-β (Wang et al., 2000; Ludwig et al., 2002). Furthermore, NS1 binds to and inhibits the cytoplasmic dsRNA sensor retinoic acid-inducible gene product 1 (Guo et al., 2007; Mibayashi et al., 2007) and blocks protein kinase R-mediated inhibition of protein synthesis (Bergmann et al., 2000; Min et al., 2007). In addition to its IFN antagonistic activities, NS1 has recently been shown to actively suppress RNA silencing in plant, insect and mammalian cells (Bucher et al., 2004; Delgadillo et al., 2004; Li et al., 2004; Haasnoot et al., 2007).

RNA interference (RNAi) facilitates the sequence-specific degradation of RNA and serves as an antiviral mechanism in plants, fungi and animals (Kasschau & Carrington, 1998; Schott et al., 2005; Wilkins et al., 2005; Segers et al., 2007). Similar to the IFN response, antiviral RNAi is triggered by viral dsRNA replication intermediates. These virus-specific dsRNAs are processed by the RNAi machinery into small interfering RNAs (siRNAs) that are incorporated into the RNA-induced silencing complex and act catalytically to cleave viral RNAs. To overcome antiviral RNAi responses, many plant and insect viruses encode RNA silencing suppressor (RSS) proteins that enable them to replicate at higher titres. In addition to the influenza A virus NS1 protein, several other factors encoded by mammalian viruses exhibit RNAi suppression activity. These can either be proteins such as vaccinia virus E3L, hepatitis C virus (HCV) core, primate foamy virus type 1 Tas, human immunodeficiency virus type 1 (HIV-1) Tat and the Ebola virus VP35, or RNAs such as the adenovirus virus-associated RNAs I and II (Li et al., 2004; Lu & Cullen, 2004; Andersson et al., 2005; Bennasser et al., 2005; Lecellier et al., 2005; Wang et al., 2006; Haasnoot et al., 2007). For many RSS proteins, including NS1, this RSS activity depends on the ability to bind dsRNA.
The finding that mammalian viruses encode RSS factors is one indication that the mammalian RNAi pathway has a role in antiviral defence responses. In contrast to plants and insects, virus-specific siRNAs have been difficult to detect in mammals. Instead, it has been shown for some viruses that cellular microRNAs (miRNAs), which normally regulate cellular gene expression, are involved in antiviral responses by targeting viral mRNAs (Lecellier et al., 2005; Huang et al., 2007; Otsuka et al., 2007; Triboulet et al., 2007). More recently, accumulation of virus-specific small RNAs has in fact been reported for a number of mammalian viruses, including HCV, Sindbis virus and HIV-1 [Parameswaran et al., 2008; Kuan-Teh Jeang (Laboratory of Molecular Microbiology, NIAID, National Institutes of Health, Bethesda, MD, USA) personal communication]. A role for antiviral RNAi in mammals is further supported by the fact that knockdown of components of the RNAi pathway results in increased replication of HIV-1 and vesicular stomatitis virus (Otsuka et al., 2007; Triboulet et al., 2007). Similarly, we have reported that the level of Sindbis virus and adenovirus replication can be significantly increased by suppressing RNAi in cells via stable expression of influenza A virus NS1 protein (de Vries et al., 2008). Another study showed that Dicer is involved in protection against influenza A virus infection (Matskevich & Moelling, 2007). These combined findings support the idea that RNAi, either siRNA or miRNA based, is part of the innate immune system in mammals.

Most avian influenza subtypes do not cause severe outbreaks in poultry and wild birds, but some H5 and H7 subtypes do regularly cause such outbreaks and actually form a threat for public health. Although influenza virus pathogenicity is a multigenic trait, it has been shown that the NS1 protein is an important virulence determinant in avian, swine and human influenza viruses (Seo et al., 2002; Li et al., 2006; Jackson et al., 2008). Studies have also shown that plant viral RSS proteins are important virulence/pathogenicity factors (Cronin et al., 1995; Ding et al., 1995). Similarly, the RSS activity of NS1 may contribute to viral pathogenicity. Therefore, we set out to determine the IFN antagonistic activity and the RSS activity of NS1 proteins of both pathogenic and non-pathogenic influenza A virus strain. We have shown here that all tested NS1 variants had potent IFN antagonistic properties, whereas differential RSS activity was measured. Interestingly, NS1 proteins derived from highly pathogenic influenza viruses could efficiently replace the previously described RSS function of the HIV-1-encoded Tat protein and thus support virus production. These data support a role for RNAi in antiviral responses and a contribution of NS1 RSS activity to influenza virus pathogenicity.

**METHODS**

**Construction of expression plasmids.** NS1 genes from influenza A virus strains A/WSN/33, A/PR/8/34, A/NL/178/95, A/NL/213/03, A/HK/156/97, A/VN/1194/04, A/CH/NL/1/03 and A/NL/33/03 and the green fluorescent protein (GFP) gene were cloned using Gateway technology (Invitrogen) into the mammalian expression vector pEF5/FRT/V5-DEST with the constitutive human EF1α promoter.

**Cell culture and transfections.** Human embryonic kidney (HEK293T) cells were grown as a monolayer in Dulbecco’s minimal essential medium (DMEM; Gibco-BRL) supplemented with 10 % fetal calf serum (HyClone), minimal essential medium with non-essential amino acids, penicillin (100 U ml⁻¹) and streptomycin (100 μg ml⁻¹) at 37 °C and 5 % CO₂. One day before transfection, cells were trypsinized, resuspended in DMEM and seeded in 24-well plates at a density of 1.5 × 10⁵ cells per well. At the time of transfection, the cells were 60–70 % confluent. Transfection was performed at least three times using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer.

For the luciferase RNAi suppression assay, cells were transfected with 100 ng of the luciferase-expressing plasmid pGL3 (Promega) and 20 ng of the expression plasmid shLuc, which encodes a short hairpin RNA (shRNA) against luciferase under the control of the U6 promoter. Cells were lysed at 2–3 days post-transfection in 150 μl 1× Passive Lysis Buffer (Promega) by shaking for 30 min at room temperature. The cell lysate was cleared by centrifugation for 5 min at 1500 r.p.m. in an Eppendorf centrifuge 5810R (microtitre plate rotor A-2-MTP) and luciferase expression was measured with 5 μl supernatant in a luciferase reporter assay system (Promega).

**Table 1.** Human influenza A virus isolates

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<tr>
<th>Virus</th>
<th>Subtype</th>
<th>Pathogenicity</th>
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<tbody>
<tr>
<td>A/WSN/33</td>
<td>H1N1</td>
<td>Laboratory-adapted strain (attenuated in humans)*</td>
</tr>
<tr>
<td>A/PR/8/34</td>
<td>H1N1</td>
<td>Laboratory-adapted strain (attenuated in humans)</td>
</tr>
<tr>
<td>A/NL/178/95</td>
<td>H3N2</td>
<td>Human strain</td>
</tr>
<tr>
<td>A/NL/213/03</td>
<td>H3N2</td>
<td>Human strain</td>
</tr>
<tr>
<td>A/HK/156/97</td>
<td>H5N1</td>
<td>Highly pathogenic avian influenza†</td>
</tr>
<tr>
<td>A/VN/1194/04</td>
<td>H5N1</td>
<td>Highly pathogenic avian influenza†</td>
</tr>
<tr>
<td>A/CH/NL/1/03</td>
<td>H7N7</td>
<td>Highly pathogenic avian influenza§</td>
</tr>
<tr>
<td>A/NL/33/03</td>
<td>H7N7</td>
<td>Highly pathogenic avian influenza§</td>
</tr>
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*Lethal in mice.
†Fatal human case.
§Mild conjunctivitis case.
For the complementation studies, 100 ng of the Tat-negative HIV-rtTA-Tatfs reporter virus was transfected with the indicated amounts of RSS plasmid. The total amount of DNA was brought to 1 μg using pBluescript (Stratagene). Two to three days after transfection, virus production was determined by measuring capsid protein (CA-p24) levels by ELISA.

RESULTS AND DISCUSSION

To investigate whether the RSS activity of the NS1 protein differs among influenza A strains, we selected eight viruses from different subtypes and with distinct pathogenicity properties (Table 1). The respective NS1 genes were cloned into the mammalian expression vector pEF5/FRT/V5-DEST under the transcriptional control of the constitutive EF1α promoter. Fig. 1(a) shows the amino acid sequence alignment of these NS1 proteins, and their phylogenetic relationship is plotted in Fig. 1(b), which also shows the subtype classifications.

The NS1 proteins were tested in a reporter-based RNAi assay, depicted in Fig. 2(a, left). This assay scored the ability of NS1 to suppress shRNA-mediated silencing of a
luciferase reporter gene. We co-transfected HEK293T cells with different plasmids: firefly luciferase reporter, a shRNA-expression vector against firefly luciferase (shLuc), a Renilla luciferase to control for transfection efficiency and increasing amounts of the NS1 construct. As a negative control, we transfected a GFP vector instead of the NS1 construct. As a positive control, we scored potent RNAi suppression by the HIV-1 Tat RSS protein. Luciferase expression was measured 3 days after transfection using a Dual-Luciferase Reporter kit (Promega). Firefly luciferase activities were normalized against Renilla luciferase activities and plotted. We observed a strong decrease in firefly luciferase expression in cells co-transfected with shLuc and a rebound upon NS1 expression as illustrated for the A/WSN/33 variant in Fig. 2(a, right). To compare the set of NS1 proteins directly, we calculated the relative RNAi suppression activity as the percentage of unsuppressed luciferase expression, which was set at 100 % (Fig. 3a). Only NS1 protein from the highly pathogenic A/WSN/33 strain was able to restore luciferase expression fully. Modest RSS activity was observed for the A/HK/156/97 strain. The other NS1 proteins did not exhibit RSS activity in this assay, consistent with initial studies on the A/PR/8/34 strain (Haasnoot et al., 2007). These results indicated that only some NS1 proteins are capable of overcoming shRNA-mediated silencing. To exclude putative post-transcriptional activities, we tested whether the set of NS1 proteins affected the expression of a stably expressed firefly luciferase reporter. HEK293–Luc cells were transfected with a set of NS1 expression plasmids and firefly luciferase expression was scored at 2 days post-transfection. We did not observe any effects of NS1 on luciferase expression compared with the control GFP-transfected cells (results not shown). This suggested that NS1 does not post-transcriptionally affect firefly luciferase reporter expression. An alternative method of measuring RSS activity is by functional complementation of an established RSS (Li et al., 2004). We recently showed that the Ebola VP35 protein is capable of suppressing RNAi in mammalian cells and that its RSS activity is functionally equivalent to that of the HIV-1 Tat protein (Haasnoot et al., 2007). To test whether NS1 proteins from different influenza strains could functionally complement the Tat RSS function, we used the Tat-negative HIV-rtTA-Tatfs reporter virus illustrated in Fig. 2(b, left). This reporter virus has a production defect in transfected HEK293T cells, which was rescued by NS1 of strain A/WSN/33 (Fig. 2b, right). To compare the complete set of NS1 proteins, we plotted the relative RSS activity as the virus production of HIV-rtTA-Tatfs in the presence of NS1, with the control HIV-rtTA-Tatwt set at 100 % (Fig. 3b). We observed RSS activity for the positive-control HIV-1 Tat protein and no RSS activity for the GFP control vector. The NS1 proteins of the subtype H1N1 strains (A/WSN/33 and A/PR/8/34) were able to complement the Tat RSS function. The A/WSN/33 protein was most potent at
low NS1 concentrations, whereas the A/PR/8/34 function was most active at higher NS1 concentrations. Of the viral strains tested, the NS1 proteins from the highly pathogenic H5N1 strains A/HK/156/97 and A/VN/1194/04 were most effective in suppressing RNAi. The NS1 proteins from the human H3N2 strains A/NL/178/95 and A/NL/213/03, the chicken strain A/CH/NL/1/03 and the human strain A/NL/33/03 (both H7N7) could not overcome the Tat RSS defect. Recent studies suggest intriguing links between the IFN response and the RNAi machinery (de Vries & Berkhout, 2008). The NS1 protein was first shown to act as an IFN antagonist (Garcia-Sastre et al., 1998; Wang et al., 2000) and was subsequently shown to influence the RNAi pathway (Bucher et al., 2004; Delgadillo et al., 2004; Haasnoot et al., 2007). We therefore also tested our set of NS1 genes for their ability to block transcriptional activation of the IFN-β promoter. For this, we used an IFN-β-luciferase (IFN-β–Luc) reporter that was first induced by poly(1:C) and subsequently suppressed by NS1 (Fig. 2c, left). HEK293T cells were co-transfected with this IFN-β–Luc construct, NS1 vector and the Renilla luciferase construct as an internal control. Luciferase expression was measured 3 days after transfection using a Dual-Luciferase Reporter kit. Firefly luciferase activities were normalized against Renilla luciferase activities and plotted. Poly(1:C) strongly induced firefly luciferase gene expression, but this induction was largely neutralized by NS1 of the A/WSN/33 isolate (Fig. 2c, right). For the complete set of NS1 genes, we calculated the relative suppression of the poly(1:C)-activated IFN-β promoter, compared with the uninduced [no poly(1:C) transfection] promoter activity, which was set at 100% (Fig. 3c). The negative GFP control and the HIV-1 Tat protein did not affect the IFN-β promoter. We observed that all NS1 variants were able to suppress the activated IFN-β promoter. The NS1 protein of strain A/WSN/33 was the most potent in this assay. In this study, we compared the capacity of the NS1 proteins of eight influenza A strains, representing four subtypes, to suppress RNAi in mammalian cells. We used different assay systems for this survey. Only the NS1 protein of strain A/WSN/33 could overcome shRNA-mediated silencing of a reporter gene in HEK293T cells. We note that the same NS1 variant did not suppress shRNA-mediated silencing in
The question arises of whether the RSS activity of the influenza NS1 protein contributes to viral pathogenicity. Interestingly, the highly pathogenic H5N1 strains A/HK/156/97 and A/VN/1194/04, which cause severe outbreaks in birds and are fatal in humans, were the most potent in functionally complementing the Tat RSS function. The two highly pathogenic avian H7N7 strains lacked this property: the chicken strain A/CH/NL/1/03, which caused a severe outbreak, and the A/NL/33/03 strain, which was transmitted to humans. However, neither of these strains causes a lethal infection in humans. It will be of interest to test whether all highly pathogenic H5N1 strains share the property of complementing the Tat RSS function, and whether this property is directly related to their pathogenicity. To test whether NS1 RSS activity is linked to the pathogenicity of influenza A viruses, one should ideally test this set of NS1 genes against the same viral background. One study showed that the NS gene of Hong Kong H5N1/97 viruses causes high pathogenicity when inserted in a viral background that is either pathogenic (such as PR/8 in mice) or non-pathogenic (such as PR/8 in pigs) (Lipatov et al., 2005). Moreover, replacement of the NS1 gene of A/PR/8/34 with that of A/WSN/33 enhanced viral pathogenicity. In conclusion, NS1 genes from different influenza A virus strains differentially suppress RNAi in mammalian cells, and this activity is not linked to IFN-β promoter inhibition. The role of NS1-mediated RNAi suppression in influenza A replication and its possible contribution to pathogenicity remain to be determined.

REFERENCES


