A Single Amino Acid Substitution in the Novel H7N9 Influenza A Virus NS1 Protein Increases CPSF30 Binding and Virulence

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Although an effective interferon antagonist in human and avian cells, the novel H7N9 influenza virus NS1 protein is defective at inhibiting CPSF30. An I106M substitution in H7N9 NS1 can restore CPSF30 binding together with the ability to block host gene expression. Furthermore, a recombinant virus expressing H7N9 NS1-I106M replicates to higher titers in vivo, and is subtly more virulent, than the parental virus. Natural polymorphisms in H7N9 NS1 that enhance CPSF30 binding may be cause for concern.

Since early 2013, zoonotic transmission of a novel avian-origin H7N9 influenza A virus in eastern China has led to at least 441 human infections and 122 deaths (1). Sequence analyses and functional studies have identified several mammalian adaptive polymorphisms in PB2 (2, 3), PA (4), hemagglutinin (HA), and neuraminidase (NA) (5, 6) that may be responsible for promoting replication and pathogenicity of this virus in humans. Nevertheless, it is clear that avian-origin H7N9 has yet to adapt to humans: HA retains its preference for avian receptors (7–10), and H7N9 is unable to transmit efficiently between humans. Identifying sequence changes in H7N9 that may evolve naturally and contribute to future human-to-human transmission, or that may alter H7N9 virulence, is critical.

Novel H7N9 virus NS1 is an IFN antagonist in human and chicken cells. NS1 is a multifunctional virulence factor that plays a major role in antagonizing host interferon (IFN) responses during infection (reviewed in reference 11). Notably, this property can vary in efficiency between virus isolates and between the species of origin of the host cell (12–16). We tested the ability of H7N9 NS1 to limit production of beta IFN (IFN-β) in human and avian cells and compared the results to those from a panel of NS1 proteins from seasonal human influenza viruses or other avian H5N1 viruses that have sporadically infected humans since 1997 (Fig. 1). Human 293T, or chicken DF-1, cells were transfected with an IFN-β promoter-driven firefly luciferase (FF-Luc) reporter construct together with expression plasmids for the NS1 proteins of interest (or glutathione S-transferase [GST] as a control). For analysis of novel H7N9, we tested NS1 proteins derived from two different human H7N9 strains (A/Shanghai/1/2013 [Sh/1] and A/Shanghai/2/2013 [Sh/2]), along with the NS1 protein from a closely related avian H9N2 virus, A/chicken/Dawang/1/2011 (Dw/11). Subsequent infection with a defective interfering genome (DI)-rich Sendai virus (SeV) preparation induced robust amounts of IFN-β promoter-driven FF-Luc activity in GST-expressing human 293T cells but not in cells expressing any of the

FIG 1 The H7N9 NS1 protein is an IFN antagonist in human and chicken cells. Human 293T (A) or chicken DF-1 (B) cells were cotransfected for 16 h with a pCAGGS expression plasmid encoding the indicated NS1 protein (or GST) together with a FF luciferase (FF-Luc) IFN-β promoter reporter plasmid (p125Luc). After infection with a DI-rich SeV preparation for a further 12 h, FF-Luc activity was determined. Results represent the means and standard deviations of triplicate values (normalized to GST + SeV) obtained in a single experiment and are representative of results of two independent experiments. The NS1 sequences (containing silent splice acceptor mutations to prevent NEP/NS2 expression [12]) were derived from A/Texas/36/1991 (Tx/91; human seasonal-like H1N1 virus), A/Wyoming/03/2003 (Wy/03; human seasonal-like H1N1, previously 2009 pdmH1N1), A/Hong Kong/156/1997 (HK/97; representative of the 1997 H5N1 outbreak), A/Vietnam/1203/2004 (VN/04; representative of the 2004 H5N1 outbreak), A/Shanghai/1/2013 (Sh/1; human H7N9), A/Shanghai/2/2013 (Sh/2; human H7N9), and A/Chicken/Dawang/1/2011 (Dw/11; avian H9N2 with NS1 closely related to H7N9 NS1).
CPSF30 binding and inhibition of host gene expression. This notion and is an additional mechanism by which most human influenza viruses attenuate host antiviral responses (20–22). The inhibition of cellular pre-mRNA processing during infection contributes to the global posttranscriptional inhibition of general host gene expression. Human 293T cells were cotransfected with a pCAGGS expression plasmid encoding the indicated NS1 protein (or GST) together with a constitutively active IFN induction cascade. Experiments were performed as for panel B, using 293T lysates overexpressing FLAG-RIG-I (D), V5-TRIM25 (E), or HA-Riplet (F). Western blotting was performed using appropriate anti-tag antibodies. (D to F) Binding of NS1 to cellular factors involved in the IFN induction cascade. Precipitates eluted after extensive washing were analyzed by SDS-PAGE and Western blotting using anti-NS1 and anti-FLAG antibodies. (C) NS1-mediated inhibition of host gene expression. Human 293T cells were cotransfected with a pCAGGS expression plasmid encoding the indicated NS1 protein (or GST) together with a constitutively active Renilla-luciferase plasmid. Luciferase activity was determined 24 h posttransfection. Results are given as the means and standard deviations of triplicate values normalized to GST. Statistical significance (**) was determined using the Student t test. (D to F) Binding of NS1 to cellular factors involved in the IFN induction cascade. Experiments were performed as for panel B, using 293T lysates overexpressing FLAG-RIG-I (D), V5-TRIM25 (E), or HA-Riplet (F). Western blotting was performed using appropriate anti-tag antibodies.

| Virus                        | Strain in this study | NS1 amino-acid | CPSF30 binding | Gain-of-function phenotype | Reference |
|------------------------------|----------------------|----------------|----------------|--------------------------|-----------|
| Seasonal H1N1 (pre-2009)     | Tx/91                | F M K D D      | Strong         | n/a                      | n/a       |
| Seasonal H3N2 (seasonal 2009+) | Wy/03               | F M K E D      | Strong*        | n/a                      | n/a       |
| Pandemic H1N1 (2009+         | Cal/09              | F M R E G      | Weak           | Decreased virulence      | Hale et al., JVI, 2010 |
| H5N1 (pre-2004)              | HK/97                | L I K D D      | Weak           | Increased virulence      | Spesock et al., JVI, 2011 |
| H5N1 (post-2004)             | VN/04                | F M K D D      | Strong         | n/a                      | n/a       |
| H7N9                         | Sh/1                 | L I K D D      | Weak           | Increased virulence      | This study |

A single I106M substitution in the H7N9 NS1 protein specifically restores efficient CPSF30 binding and inhibition of host cell gene expression. A summary of binding affinities for related H3N2 viruses (14) and is an additional mechanism by which most human influenza viruses attenuate host antiviral responses (20–22). The inhibition of cellular pre-mRNA processing during infection and is an additional mechanism by which most human influenza viruses attenuate host antiviral responses (20–22). This property of NS1 is not conserved in all strains (14, 23), and variation in CPSF30 binding has been reported to arise when viruses are adapted to replicate in certain new host species (24, 25). There are two notable examples of naturally occurring influenza viruses that have infected humans but which encode NS1 proteins unable to inhibit CPSF30: highly pathogenic avian-origin 1997 H5N1 virus (23) and the swine-origin 2009 pandemic H1N1 (pdmH1N1) virus (12). The defect in CPSF30 binding maps to slightly different amino acid positions in the 1997 H5N1 and 2009 pdmH1N1 NS1 proteins (summarized in Fig. 2A), and opposite phenotypes have been identified for “gain-of-function” substitutions that restore CPSF30 inhibition in these two viruses: for 1997 H5N1, gain of CPSF30 binding promotes systemic spread of the virus and increases virulence (26), while for 2009 pdmH1N1, gain of CPSF30 binding slightly decreases replication and virulence (12). Such phenotypic differences may be due to the disparate amino acid substitutions required to restore binding to or the distinct genetic constellations of the two avian- or swine-origin viruses. Certainly,
the impact on replication and virulence of modulating CPSF30 binding affinity is complex and is seemingly unpredictable between strains (12, 24–28).

Intriguingly, we observed that the novel H7N9 NS1 protein has the same amino acid polymorphisms that weaken CPSF30 binding as the 1997 H5N1 NS1 protein (Fig. 2A). We tested the coprecipitation of FLAG-tagged CPSF30 with bacterially expressed 6His-tagged wild-type (WT) Sh/2 NS1, as well as with L103F, I106M, or L103F/I106M double-mutant (DM) variants that we predicted might have a different CPSF30-binding profile. Only a small amount of FLAG-CPSF30 could be precipitated by NS1-WT or NS1-L103F (Fig. 2B). However, FLAG-CPSF30 bound effi-
Table 1: Characterization of H7N9-based NS1-WT and NS1-I106M viruses in vivo

| Virus [rSh/1 (6 + 2)] | MLD50 (PFU) | Dose (PFU) | % survival (no. of survivors/total no. tested) | Median day of death (range) | Mean maximum % wt loss (range) | Median no. of days with indicated wt loss (range) |
|----------------------|-------------|------------|-----------------------------------------------|----------------------------|-------------------------------|-----------------------------------------------|
| NS1-WT               | 10.8        | 2          | 100 (15/15)                                   | NA                         | 8.1 (0.4 to 17.4)             | 0 (0 to 3)                                  |
|                      |             | 10         | 65 (13/20)                                    | 11 (10 to 11)              | 20.5 (2.5 to 25)              | 5 (0 to 8)                                  |
|                      |             | 50         | 15 (3/20)                                     | 9 (8 to 10)                | 24.6 (19.5 to 25)             | 10 (4 to 11)                                |
|                      |             | 250        | 0 (0/5)                                       | 9 (8 to 9)                 | >25 (>25)                    | 10 (10 to 11)                               |
|                      |             | 1,250      | 0 (0/5)                                       | 7 (7 to 8)                 | >25 (>25)                    | 12 (11 to 12)                               |
| NS1-I106M            | 4.22        | 2          | 100 (15/15)                                   | NA                         | 13.5 (1.3 to 21.8)            | 2 (0 to 4)                                  |
|                      |             | 10         | 20 (4/20)                                     | 10 (9 to 11)               | 22.8 (2.2 to 25)             | 8.5 (0 to 10)                               |
|                      |             | 50         | 0 (0/20)                                      | 8 (8 to 9)                 | >25 (>25)                    | 10 (10 to 11)                               |
|                      |             | 250        | 0 (4/4)                                       | 7.5 (7 to 8)               | >25 (>25)                    | 11 (11)                                    |
|                      |             | 1,250      | 0 (5/5)                                       | 7 (6 to 7)                 | >25 (>25)                    | 12 (11 to 12)                               |

*NA, not applicable.*

An H7N9-based virus expressing NS1-I106M shows enhanced replication and virulence in vivo. To test whether increased affinity of H7N9 NS1 toward CPSF30 would affect viral replication and pathogenicity in vivo, we determined 50% mouse lethal dose (MLD50) values for the WT and NS1-I106M viruses in 6- to 8-week-old C57BL/6 mice (Jackson Laboratory, ME). All procedures were performed in accordance with the IACUC guidelines of Icahn School of Medicine at Mount Sinai, and animals showing >25% weight loss were considered to have reached the experimental endpoint and were humanely euthanized. MLD50 values were subsequently calculated according to the method of Reed and Muench (the data are summarized in Table 1). Even in the highly virulent rSh/1 (6 + 2) background, the NS1-I106M mutation led to a modest ~2.5-fold increase in MLD50 and mice infected with the NS1-I106M virus exhibited greater overall mortality (Fig. 3E) and morbidity (as determined by the duration and extent of weight loss and the day of death; Table 1) than mice infected with the WT virus. We speculate that the virulence-enhancing impact of H7N9 NS1-I106M may be even more pronounced in the context of non-PR8 glycoproteins, where the respective WT virus would have a much higher MLD50.

To assess replication in vivo, mice were intranasally infected with 500 PFU of each virus, and lungs were excised on days 2 and 4 postinfection. Following homogenization and centrifugation (10,000 × g, 5 min, 4°C), the supernatants were used to determine...
the viral titer. The NS1-1106M virus replicated to titers >5-fold higher than those of the WT by day 2 (P = 0.0056), while titers at day 4 were similar (Fig. 3F). Notably, qRT-PCR analysis of lung homogenates from independently infected mice suggested a trend for the NS1-1106M virus to induce less IFN-β mRNA than the WT virus; however, this difference was not statistically significant (Fig. 3G).

Concluding remarks. Continued zoonotic transmission of H7N9 to humans is a significant cause for concern given the mild to lethal human respiratory disease the virus causes and the fear that it may yet acquire human-to-human transmission capability. Here, we characterize the H7N9 NS1 protein as an efficient IFN antagonist. Nevertheless, H7N9 NS1 is defective in binding CPSF30 and is consequently unable to block host cell gene expression. We identify the single 1106M natural polymorphism found in non-H7N9 strains as a potential gain-of-function mechanism by which the H7N9 NS1 could acquire CPSF30 binding and provide evidence that this substitution promotes virus replication and virulence in vivo. These results parallel those found with the 1997 H5N1 virus and the laboratory strain PR8, where similar substitutions enhanced CPSF30 binding and virulence (23, 26, 28). Although polymorphisms in H7N9 NS1 that might restore CPSF30 binding have yet to be identified in the sequenced strains that are available, our study highlights the importance of continued surveillance to monitor potential natural gain-of-function mutations in H7N9 NS1 that may impact pathogenicity.

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