Short Communication

The C terminus of NS1 protein of influenza A/WSN/1933(H1N1) virus modulates antiviral responses in infected human macrophages and mice

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Non-structural protein NS1 of influenza A viruses interacts with cellular factors through its N-terminal RNA-binding, middle effector and C-terminal non-structured domains. NS1 attenuates antiviral responses in infected cells and thereby secures efficient virus replication. Some influenza strains express C-terminally truncated NS1 proteins due to nonsense mutations in the NS1 gene. To understand the role of the NS1 C-terminal region in regulation of antiviral responses, we engineered influenza viruses expressing C-terminally truncated NS1 proteins using A/WSN/33(H1N1) reverse genetics and tested them in human macrophages and in mice. We showed that a WSN virus expressing NS1 with a 28 aa deletion from its C terminus is a more powerful inducer of antiviral responses than the virus expressing full-length NS1, or one with a 10 aa truncation of NS1 in vitro. Thus, our findings suggest that the C-terminal region of NS1 is essential for regulation of antiviral responses. Moreover, viruses expressing truncated NS1 proteins could be good vaccine candidates.

Human influenza A viruses mutate rapidly and cause seasonal epidemics and occasional pandemics, imposing a constant health threat and economic burden. Influenza A viruses belong to the family Orthomyxoviridae and have segmented ssRNA genomes, which encode haemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), matrix proteins (M1 and M2), non-structural protein (NS1), nuclear export protein (NS2), polymerase subunits (PA, PB1 and PB2) and auxiliary proteins, such as PA-X and PB1-F2. The influenza A virus genome is protected by NP and polymerase subunits, which together form viral ribonucleoprotein particles (vRNPs). The vRNPs are encapsulated in an M1 shell. The shell is surrounded by a lipid bilayer. HA, NA and M2 are embedded in the lipid bilayer. Upon infection, influenza A viruses express the NS1 proteins, which antagonize antiviral responses to ensure efficient virus replication (Müller et al., 2012; Ayllon and García-Sastre, 2015; Hale et al., 2008).

Human influenza A viruses are classified into subtypes based on the structures of HA and NA (e.g. H1N1, H3N2, H2N2). Influenza strains of the H1N1 subtype caused two global influenza pandemics (‘Spanish flu’ in 1918 and ‘swine flu’ in 2009) and many influenza epidemics (http://www.cdc.gov).

Analysis of available influenza A/H1N1 viral genomes revealed that the viruses sporadically acquire nonsense and missense mutations, which result in truncations or extensions of the C terminus of the viral NS1 protein (Fig. 1a) (Dundon and Capua, 2009; Jalovaara et al., 2014; Lakspere et al., 2014). It was shown that truncations and extensions of the C terminus of viral NS1 could modulate virus pathogenicity (Jackson et al., 2008; Kong et al., 2015; Soubies et al., 2010).

To address a possible role of the NS1 C terminus in regulation of antiviral responses, we generated two viruses (WSN-220 and WSN-202) expressing C-terminally truncated NS1...
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(a) A/Fort Monmouth/1/1947 (H1N1) RFA
A/Puerto Rico/8/1934 (H1N1) RFAWR
A/New York/644/1955 (H1N1) RFAWR
A/California/10/1978 (H1N1) RFAWR
A/Alabama/096-0455/2007 (H1N1) RFAWR
A/Boston/02/2009 (H1N1) RFAWR
A/Helsinki/p14/2009 (H1N1) RFA
A/California/07/2009 (H1N1) RFAWR
A/Helsinki/544/2014 (H1N1) RFAWR

(b) WSN-202 RFA
WSN-220 RFAWR
WSN-230 RFAWR

(c) WSN-230 WSN-220 WSN-202

(d) Log10 p.f.u. ml⁻¹

(e) NS1 RNA in cell lysates (log10 RU)

(f) NS1 RNA in cell culture media (log10 RU)

(g) WB NP β-Actin NS1

Phosphorylation site
ISGylation site
SUMOylation site
PDZ
proteins using the reverse genetics system of A/WSN/33(H1N1) (Hoffmann et al., 2000), as well as a Phusion site-directed mutagenesis kit (Life Technologies) and specific primer pairs 5′-(Pho)AGATCGGAGGAAG and 5′-(Pho)CTGTAGGTGTTCAAGACT to generate the WSN-202 virus, and 5′-(Pho)AAATTAGCATGTGGTGAAGA and 5′-(Pho)GTTCGGCAGTTACGGTTT to generate the WSN-220 virus] (Fig. 1b). We also reconstituted WT virus (WSN-230) expressing full-length NS1. The A/WSN/33(H1N1) strain was chosen because it is probably the best characterized cell-culture- and mouse-adapted A/H1N1 strain, and it is genetically more stable than A/H1N1pdm09 viruses (Jalovaara et al., 2014). The positions of nonsense mutations were chosen based on the sequence alignments of NS1 C-terminal regions. In particular, WSN-220 virus was designed to express NS1 lacking the PDZ-binding domain, and WSN-202 virus was designed to express NS1 lacking the PDZ-binding domain, as well as phosphorylation, SUMOylation and ISGylation sites (Fig. 1b). It should be noted that nonsense mutations introduced into NS1 RNA did not affect the sequence of NS2 RNA, which is expressed from the same viral genetic segment as NS1.

We assembled recombinant WSN-202, WSN-220 and WT WSN-230 viruses in HEK cells and propagated them in Vero cells. We obtained similar titres for all three viruses (Jalovaara et al., 2014). Infected MDCK cells were infected with WSN viruses at an m.o.i. of 0.01 for the indicated times. Cell culture supernatants were collected, and virus titres were determined in MDCK cells. The titres are plotted. Error bars indicate SD from three experiments. (e, f) Macrophages were infected with WSN viruses at an m.o.i. of 0.01 following the procedure described by Denisova et al. (2014). Error bars indicate SD from three measurements. RU – relative units. (g) Macrophages were infected with WSN viruses at an m.o.i. of 2. Total cell lysates were obtained at the indicated time points and analysed by Western blotting (WB) using primary guinea pig anti-NS1 (diluted 1 : 2000), rabbit anti-NP (1 : 500) and mouse anti-β-actin (1 : 5000; Sigma-Aldrich) antibody following the procedure described by Denisova et al. (2012).

Fig. 1. Polymorphism of the C-terminal region of NS1 proteins of human influenza A/H1N1 viruses. (a) Alignment of C-terminal regions of NS1 proteins of 6967 H1N1 viruses, including 5424 influenza strains of H1N1pdm09 lineage. Sequences were obtained from the Influenza Virus Resource database. Numbers in parentheses on the right refer to the number of influenza strains with the corresponding sequence length. Functional motifs are highlighted. The numbers labelling the top of the figure indicate the amino acid positions of NS1 proteins. (b) Schematic representation of C-terminally truncated NS1 proteins expressed by genetically engineered WSN viruses. Functional sites are shown in colours as in (a). (c) Genetically engineered WSN viruses produced in HEK/Vero cells were titrated on MDCK cells. Representative plaque assays are shown. (d) MDCK cells were infected with WSN viruses at an m.o.i. of 0.01 for the indicated times. Cell culture supernatants were collected, and virus titres were determined in MDCK cells. The titres are plotted. Error bars indicate SD from three experiments. (a, f) Macrophages were infected with WSN viruses at an m.o.i. of 0.01 for the indicated times. Cells and cell culture supernatants were collected, and total NS1 RNA was quantified by quantitative reverse-transcription (RT)-PCR following the procedure described by Denisova et al. (2014). Error bars indicate SD from three measurements. (e, f) Macrophages were infected with WSN viruses at an m.o.i. of 0.01 following the procedure described by Denisova et al. (2014). We found that WSN-202, WSN-220 and WSN-230 viruses (Jalovaara et al., 2014). The WSN-202 and WSN-220 viruses enhanced production of IL-6 and IL-8 in comparison with WSN-230 viruses following procedures described by Denisova et al. (2014). WSN-230, WSN-220 and WSN-202 viruses induced expression of 94, 110 and 158 cellular genes, respectively, compared with non-infected cells (Fig. 2a, b). Importantly, the majority of differentially expressed genes are involved in antiviral responses (e.g. MX1, MX2, ISG15, IFIT1, IFIT2, OAS1, CCL8, CXCL10, IL6 and IL8). We concluded that the C-terminal region of NS1 can modulate expression of immune-related genes in infected cells.

To monitor the effect of NS1 C-terminal truncations on antiviral responses, we infected macrophages with WSN-202, WSN-220 or WSN-230 virus at an m.o.i. of 2. After 8 h, we extracted total RNA and analysed expression of cellular genes as described previously (Denisova et al., 2014). WSN-230, WSN-220 and WSN-202 viruses induced expression of 94, 110 and 158 cellular genes, respectively, compared with non-infected cells (Fig. 2a, b). Importantly, the majority of differentially expressed genes are involved in antiviral responses (e.g. MX1, MX2, ISG15, IFIT1, IFIT2, OAS1, CCL8, CXCL10, IL6 and IL8). We concluded that the C-terminal region of NS1 can modulate expression of immune-related genes in infected cells.

We next analysed cytokine production by infected macrophages following procedures described by Denisova et al. (2014). The WSN-202 and WSN-220 viruses enhanced production of IL-6 and IL-8 in comparison with WSN-230 (Fig. 2c). However, CCL4 levels were lower in the medium of WSN-202- and WSN-220-infected cells. These results indicate that the C-terminal region of NS1 affects cytokine production in infected cells.

We also tested whether the NS1 C terminus affects cellular signalling events in infected cells. We analysed the phosphorylation status of several cellular phospho-proteins implicated in virus infection as described by Denisova et al. (2014). Infection with WSN-220, and especially with WSN-202, induced phosphorylation of STAT3, JNK, HSP60, AMPKα1 and Akt, by comparison with WSN-230 (Fig. 2d). These results indicate that the NS1 C-terminal region also modulates cellular signalling events in infected cells.
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Fig. 2. Influenza A viruses expressing NS1 proteins of different lengths differentially affect cellular transcription, cytokine production and signalling in macrophages. (a) Macrophages were mock, WSN-202, WSN-220 or WSN-230 infected (m.o.i. of 2). At 8 h p.i., cells were collected and total RNA was isolated and subjected to genome-wide gene expression profiling using a human HT-12 V4 expression array (GEO accession number GSE62127). A heat map of the genes affected by infection is shown. The heat map represents normalized expression data on a logarithmic scale (log2 fold change >2) as compared with non-infected cells. (b) A Venn diagram of gene sets transcriptionally upregulated (> fourfold) at 8 h p.i. with different viruses is shown. The numbers in brackets indicate the amounts of the transcriptionally upregulated genes (> 4 folds). The numbers on the Venn diagram indicate the amounts of shared genes transcriptionally upregulated in response to infection with different influenza viruses. (c) Macrophages were treated as in (a), cell culture supernatants were collected at 24 h p.i. and cytokine levels were determined using human cytokine array panel A. The relative intensities of spots were calculated by ImageJ software, and the values were normalized to reference spots (RS) and plotted. Error bars indicate SD from two experiments. The asterisks indicate statistically significant differences compared with the WSN-230 sample (n=6 per group, P<0.05, **P<0.01, ***P<0.001). (d) Macrophages were treated as in (a). Cells were collected after 8 h, and phosphorylation levels of kinases and their substrates were profiled using a human phosphokinase array. The relative intensities of spots were calculated in ImageJ, and the values were normalized to RS and plotted. Error bars indicate SD from two experiments. The asterisks over the columns indicate statistically significant differences compared with the WSN-230 sample (n=2, paired, two-tailed Student's t-test, *P<0.05, **P<0.01, ***P<0.001).

Fig. 3. The NS1 C terminus affects cytokine expression and mortality and morbidity of infected mice. (a) Relative body weights of specific-pathogen-free housed BALB/c mice after challenge with 7 × 10³ p.f.u. WSN-230 (corresponding to 1 × LD₅₀ for this virus) or with 7 × 10⁴ p.f.u. WSN-202 virus. Relative body weights were calculated as the ratio between the body weight of the indicated day and the body weight at the time of infection. The graph shows the mean relative body weight and SD of the indicated groups (n=6 per group, P<0.01 at day 3, P≤0.001 at day 4, P≤0.0001 at day 5–8; P values were calculated by two-way ANOVA Bonferroni’s multiple comparison test). (b) Kaplan–Meier survival curves of BALB/c mice challenged with 7 × 10³ p.f.u. (1 × LD₅₀) WSN-230 or WSN-202 (n=6 per group, P=0.019, log-rank Mantel–Cox test). The data are from two independent experiments. (c) Viral titres from mouse lung homogenates at day 3 in each treatment group (n=6 mice). The horizontal lines represent the average of viral titers from 6 mice. (d) Cytokine levels in mouse lung homogenates at day 3 in each treatment group (n=6 mice) were determined using mouse cytokine array panel A kit (n=2). Error bars indicate SD from two experiments.
We next investigated the role of the NS1 C terminus in regulation of antiviral responses in infected mice. We focused on a 28 aa deletion of NS1 because WSN-202 virus showed clear differences from WSN-230 and WSN-220 in vitro. We challenged female BALB/c mice with WSN-202, WSN-230 or mock infections (six mice per group) following a published procedure (Kakkola et al., 2013). Mice infected with WSN-202 experienced only moderate weight loss and completely recovered from infection, in contrast to WSN-230-infected mice (Fig. 3a, b). We next performed another experiment in which we infected female BALB/c mice with WSN-202, WSN-230 or mock (six mice per group), and at day 3 p.i., we sacrificed the mice and collected the lungs. Lungs were homogenized, and virus titres and cytokine levels were measured as described previously (Kakkola et al., 2013). We found significantly lower viral loads in the lungs of WSN-202-infected mice in contrast to WSN-230-infected animals, indicating that C-terminal truncation of NS1 attenuates replication of influenza WSN virus in mice (Fig. 3c).

We also observed a significant imbalance in cytokine production in WSN-202-infected mice in comparison with WSN-230-infected mice (Fig. 3d). This imbalance probably resulted from an ability of the immune system to clear the virus, eventually leading to recovery of the WSN-202-infected animals. These results indicate that the NS1 C terminus regulates antiviral responses and contributes to virus pathogenicity in infected mice.

Thus, our results suggest that the NS1 C terminus regulates antiviral responses in infected human macrophages and mice. Our results support previous studies, which demonstrated that not only functional motifs but also the physical length of the NS1 C terminus region modulates virus replication and pathogenicity (Jackson et al., 2008; Kong et al., 2015; Soubies et al., 2010).

The C-terminal region of NS1 accommodates or eliminates functional motifs involved in virus–host interplay and thereby provides evolitional plasticity for influenza A viruses. This viral property could be utilized to improve the efficacy of live-attenuated influenza vaccines, i.e. naturally occurring or genetically engineered influenza strains that encode NS1 with altered C-terminal regions could be used as vaccine candidates (Richt and García-Sastre, 2009).

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