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Influenza virus NS1 protein binds cellular DNA to block transcription of antiviral genes

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Abstract

Influenza NS1 protein is an important virulence factor that is capable of binding double-stranded (ds) RNA and inhibiting dsRNA-mediated host innate immune responses. Here we show that NS1 can also bind cellular dsDNA. This interaction prevents loading of transcriptional machinery to the DNA, thereby attenuating IAV-mediated expression of antiviral genes. Thus, we identified a previously undescribed strategy, by which RNA virus inhibits cellular transcription to escape antiviral response and secure its replication.

1. Introduction

Influenza A viruses (IAVs) are important human pathogens that cause global epidemics and pandemics (www.who.int). It is estimated that IAVs are responsible for up to 500,000 deaths a year [1]. The successful recovery from viral infection largely depends on efficient activation of innate and adaptive immune responses [2–4]. Innate immune responses are triggered by cellular Toll-like receptors (TLR3 and TLR7), which recognize viral patterns upon IAV entry in the cell [4–6]. These pattern recognition receptors (PRRs) activate transcription of interferon genes (IFNB1, IL28A, IL29, IL28B, IFNG, IFNA1, IFNA2, and IFNW1), whose products mediate expression of interferon stimulated genes (ISGs) [7–10]. Following transcription of ISGs, their protein products, such as RIG-I (DDX58), MD5 (IIFH1), and PKR (EIF2AK2) recognize viral RNA and its replication intermediates to trigger activation of innate immune responses and apoptosis, as well as to inhibit protein synthesis [11–13]. In addition, the ribonucleases encoded by other ISGs (OASL, OAS1, OAS2) degrade viral RNA [14–16]. Moreover, E3-ligases and ubiquitin-like molecules encoded by HERC5, Trim25, and ISG15 ISGs modify influenza and cellular proteins to alter their cellular functions [17,18]. Simultaneously, cytokines produced from IL1B, IL8, IL6, CXCL10, CCL5 and some other ISGs are secreted from infected cells to alarm bystander uninfected cells of a viral infection as well as to attract immune cells to the site of infection [2,4,19]. Some of these cytokines are processed into secretory forms by the inflammasome, which is activated by IFN-inducible GTPases, such as GBP1, GBP4, GBP5, MX1 and MX2 [20–22]. Moreover, COX2, IDO and 25HC encoded by PTGS2, IDO, and CH25H ISGs catalyse the production of prostaglandin H2, lymurenine, and oxysterol 25-hydroxycholesterol, respectively, which act as immunomodulators [19,23–25]. Thus, antiviral response consist of transcriptional, post-transcriptional, translational and post-translational events resulting in the clearance of infection.

To counteract the cellular defence and secure viral replication, IAV utilizes its non-structural NS1 protein, which is synthesized by infected cells only few hours after infection. NS1 interacts with replication intermediates of viral RNA to hinder these molecules from recognition by cellular PRRs [26]. It also binds RIGI, PKR, TRIM25, ISG15, GBP1 and other ISG products, to inhibit their functions [27,28]. However, NS1 interactions with these host antiviral proteins are virus- or host cell-specific [27–30]. We hypothesized that NS1 could also block the
transcription of innate antiviral genes by binding cellular dsDNA to prevent the loading of cellular transcriptional machinery (Fig. 1A and B). Indeed, we demonstrate that NS1 binds cellular dsDNA, antagonizes RNA polymerase II (Pol II) recruitment to the DNA and, consequently, inhibits the transcription of IFNs and ISGs. Thus, our study offers a previously undescribed mechanism, by which RNA virus manipulates cellular transcription to downregulate the antiviral responses.

2. Materials and methods

2.1. Viruses and cells

Influenza A/WSN/33(H1N1) viruses expressing wild type (WSNWT) or R38A/K41A mutant NS1 (WSNRK/AA) were generated using WSN eight-plasmid-based reverse genetics system in HEK and Vero cells as described previously [31]. We sequenced the viral NS1 genes of WSNRT/AA and WSNWT viruses to verify the authenticity of the mutations. Viruses were titrated in Madin-Darby canine kidney epithelial (MDCK) cells using plaque assay as described [32]. We obtained smaller plaques and approximately 100 times lower titres for WSNRK/AA virus than for WSNWT (Fig. S1). The viruses were stored at –80 °C. MDCK, human embryonic epithelial cells (HEK293T) and African green monkey kidney epithelial cells (Vero) were grown in Dulbecco modified Eagle’s medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2 mM L-glutamine (Lonza; Basel, Switzerland), 50 U/ml penicillin-streptomycin mix (PenStrep, Lonza) and 10% fetal bovine serum (FBS; Gibco, Paisley, UK). Human telomerase reverse transcriptase-immortalized retinal pigment (RPE) cells were grown in DMEM-F12 medium supplemented with 50 U/ml PenStrep, 2 mM L-glutamine, 10% FBS, and 0.25% sodium bicarbonate (Sigma-Aldrich). The cells were propagated at 37 °C in 5% CO2.

2.2. Transfection of RPE cells with siRNA

RPE cells were cultured to 80% confluence in 24 well plates and transfected with 100 nM siGenome SMARTpool or ON-TARGETplus non-targeting control siRNA (Table S1; Dharmacon, Lafayette, CO, USA) using Lipofectamine® RNAiMAX Reagent (Thermo Fisher Scientific). Importantly, some of these siRNA have been validated in previous [33,34] and in the present study (Fig. S2). 24 h after transfections, the cells were infected with WSNWT, WSNRK/AA viruses or mock infected. 8 h after infection, the levels of genes and proteins of interest were assessed using RT-qPCRs and immunoblotting, respectively.

2.3. Infection of RPE cells with WSNWT and WSNRK/AA viruses

The growth medium of the RPE cells was changed to growth medium (VGM) containing 0.2% BSA (Sigma-Aldrich), 2 mM L-glutamine, 0.348% NaHCO3 and 1 μg/ml 1-1-tosylamido-2-phenylethyl chloromethyl keto-trypsin (TPCK)-trypsin (Sigma-Aldrich) in DMEM-F12. The cells were infected with WSNWT, WSNRK/AA viruses or mock.

2.4. Gene expression profiling

RNA was extracted from WSNWT, WSNRK/AA, or mock-infected RPE cells at 8 h.p.i. using RNeasy Plus mini kit (Qiagen, Germany). Gene expression profiling was done using Illumina Human HT-12 v4 Expression BeadChip Kit according to manufacturer’s recommendation as described previously [35]. Raw microarray data were normalized using the BeadArray and Limma packages from Bioconductor suite for R. Normalized data were further processed using a variance and intensity filter. Genes differentially expressed between samples and controls were determined using the Limma package. Benjamini-Hochberg multiple testing correction testing method was used to filter out differentially expressed genes based on a q-value threshold (q < 0.05). Filtered data were sorted by logarithmic fold change (log2FC). The gene-expression data was deposited to Gene Expression Omnibus (GEO accession number: GSE65699). Gene set enrichment analysis was performed using open-source software (www.broadinstitute.org/gsea).

2.5. Quantitative PCR

Quantitative PCRs were done on the Lightcypher 480 using Fast SYBR Green Master Mix (Roche, USA). The following sets of primers were used for detection of specific genes or cDNA: EML4 (forward: 5′-TGGC TTCATGCAAACCTT-3′, reverse: 5′-AATCCCTCAGCTCATCC-3′), IFNB1 promotor (forward: 5′-GTCACTAGTACCAGTAGAGAGAG-3′) and reverse: 5′-CTTGGGAGAAAGCAAAGGAAAG-3′) and exon (forward: 5′-GCGGATTGGTTGATG-3′; reverse: 5′-GCAAAGGTTCTCAACAAAT-3′), IFNA1 (forward: 5′-CATCCAAAGCAGCAAGCAAG-3′), IFNA16 (forward: 5′-AGCCACACCGCCCTTCC-3′), IL29 (forward: 5′-TCACCTGTTTCTTCCT-3′), CXCL1 (forward: 5′-GACCAACACCGCCCTTCC-3′), IL16 (forward: 5′-CGGGTTGTAGGTGATG-3′; reverse: 5′-CGTCAGCAGAGGAGAAG-3′), and reverse: 5′-CGTCAGCAGAGGAGAAG-3′), and reverse: 5′-GTTGTAAGGCGGCT-3′). The relative gene expression differences were calculated as described previously [35].

Fig. 1. Influenza NS1 through R38 and K41 may bind dsDNA to inhibit transcription of antiviral genes. A. Novel hypothetical mechanism of action of influenza NS1 against antiviral responses in influenza A virus-infected cells. IAV infection stimulates the transcription of IFNs and ISGs, which inhibit virus replication. Newly synthesized influenza NS1 protein blocks transcription of IFNs and ISGs by binding to cellular dsDNA. Representative PRRs, IFNs and ISGs are shown. IAV – influenza A virus, PRRs – pattern recognition receptors, IFNs-interferon genes, ISGs- interferon stimulated genes. B. Hypothetical model of NS1 RBD-dsDNA complex. The structures of NS1 RBD (PDBID: 2ZKO) and B-form dsDNA (4W9M) were used to build this model.
previously [36] and the results were represented as relative units (RU). Technical triplicates of each sample were performed on the same qPCR plate and non-templates and non-reverse transcriptase samples were analysed as negative controls. Statistical significance (p < 0.05) of the quantitation results was evaluated with t-test. Benjamini-Hochberg method was used to adjust the p-values.

2.6. Immuno-fluorescence analysis (IFA)

RPE cells were infected with WSNWT (moi 1) or WSNRK/AA (moi 1) viruses or they were mock-infected. After 10 h of infection the cells were fixed with 4% paraformaldehyde (PFA) in phosphatebuffered saline (PBS), then permeabilized and blocked in the BP buffer (10% Bovine serum albumin (BSA) and 0.1% Triton X-100 in PBS) supplemented with 5% goat serum (Life Technologies, USA). Primary rabbit anti-NS1 antibodies (1:250 dilution in 5% BSA-TBST; Abcam, 8WG16), goat anti-NS1RK/AA protein as described previously[40].

2.7. Chromatin immunoprecipitation

ChiP experiments were performed on RPE cells infected with WSNWT (moi 1), WSNRK/AA (moi 1) or mock as previously described (34). Briefly, after cross-linking with formaldehyde at room temperature, chromatin was prepared, sonicated on ice using BIORUPTOR (Diagenode, Philadelphia, PA, USA) and pre-cleared. Samples were incubated with the antibody and then pulled down using protein G Sepharose beads. After extensive washes the protein-DNA complexes were eluted, the cross-linking was heat-reverted. DNA was purified with QIAquick PCR purification kit (Qiagen) and quantified by PCR with primers targeting promoter or exon region of IFNB1 gene was performed.

2.8. Protein electrophoresis and immunoblotting

Cells were lysed with a 2 × Laemmli loading buffer (4% sodium dodecyl sulphate, 20% glycerol, 10% β-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl, pH 6.8). Proteins were resolved in 4–20% gradient SDS-polyacrylamide gel (Biorad, Hercules, USA) at 150 V for 50 min. The gels were stained using Coomassie blue or immunoblotted. For immunoblotting, proteins were transferred from SDS-PAGE onto Immobilon-P membranes (Millipore, MA, USA). The membranes were blocked with 5% non-fat milk or 5% BSA (Sigma-Aldrich) in TBST, stained with different primary antibodies overnight, followed by secondary HRP-conjugated antibody labelling and detection by chemiluminescence. The primary antibodies used in this study were anti-RNA polymerase II CTD repeat YSPTSPS antibody (1:1000 dilution in 5% BSA-TBST; Abcam, 8WG16), goat anti-TLR3 (1:1000 dilution in 5% BSA-TBST; sc6892, SantaCruz), rabbit anti-MAVS (1:1000 dilution in 5% BSA-TBST; from I. J. laboratory), guinea pig anti-MDA5 (1:250 dilution in 5% milk-TBST; from I. J. laboratory), and rabbit anti-IAV NS1 antibody (1:5000 dilution in 5% BSA-TBST; from I. J. laboratory). The secondary HRP antibodies (anti-guinea pig, anti-goat and anti-rabbit) were from Dako and used as 1:1000 dilution. To confirm equal sample loading the membranes were stripped and labelled with anti-GAPDH antibody (1:1000 dilution in 5% milk-PBST, Santa Cruz, sc-47724).

2.9. Enzyme-linked immunosorbent assay (ELISA)

The levels of CXCL10, TNFα, and IFNA in the cell supernatants were assayed with ELISA (PBL Interferon Source) as described previously [37].

2.10. Mass spectrometry

Five 175 cm² plates of RPE cells were infected with WSNWT (moi 1) or WSNRK/AA (moi 3) viruses or were left mock-infected. 10 h post infection the media was removed. 400 µl of lysis buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl and 1% Triton-X100) was added to each plate. The lysates were collected and centrifuged at 14,000 rpm at 4C for 10 min. Insoluble fractions were resuspended in 2 ml buffer containing 20 mM Tris-HCI, pH 7.5, 100 mM NaCl and 0.1 mg/ml RNase A (Qiagen) and incubated on ice for 1 h. The mixtures were centrifuged at 14,000 rpm at 4C for 10 min. Insoluble fractions were resuspended in 2 ml lysis buffer and centrifuged again to remove remaining RNAse and cleaved RNA. Insoluble fractions were resuspended in 2 ml buffer containing 20 mM Tris-HCl, pH 7.5 and 600 mM NaCl. Insoluble fractions were obtained by centrifugation, resuspended in 2 ml buffer containing 20 mM Tris-HCI, pH 7.5 and 100 mM NaCl and sonicated for 20 min (2 s pulse/ 2 s pause; amplitude 25%, 20 kHz, 750 W). Proteins were resolved on SDS page.

Identification and quantification of proteins of “insoluble fraction” was done using quadruplex iTRAQ (isobaric tag for relative and absolute quantitation) labelling combined with liquid chromatography-tandem mass spectrometry (LC–MS/MS) analysis as described previously [38]. In brief, protein alkylation, trypsin digestion and labelling of the resulting peptides were done according to manufacturer’s instructions (AB Sciex). Labelled peptides were fractionated by strong cation exchange chromatography and each fraction containing labelled peptides was analysed twice with nano-LC–ESI-MS/MS using Ultimate 3000 nano-LC (Dionex) and QSTAR Elite hybrid quadrupole time-of-flight–MS (AB Sciex). MS data were acquired automatically using Analyst QS 2.0 software. Protein identification and relative quantitation was performed using ProteinPilot 4.0 software (AB Sciex). Data files from both technical replicates of an iTRAQ sample set were processed together. The search database was a self-built combination of Uniprot Human protein sequences and Uniprot ssRNA negative-strand virus sequences (both form the release 55.0, 02/08). The search criteria were: cysteine alkylation with MMTS, trypsin digestion, biological modifications allowed, thorough search and detected protein threshold of 95% confidence (Unused ProtScore > 1.3). Additionally, automatic bias correction was used. False discovery rates were calculated using a concatenated normal and reversed sequence database.

2.11. In vitro assays

Wild type (NS1WT) and R38A, K41A mutant (NS1RK/AA) of NSI of influenza A/chicken/Nigeria/O10/2007(H5N1) virus were produced in E. coli BL21(DE3) cells and purified to homogeneity as described previously [39]. Importantly, we also attempted but did not succeed to purify wild type proteins of A/WSN/1933, A/Aden/1972, and many other IAV strains because they were insoluble when overexpressed in E. coli BL21(DE3) cells.

Run-off transcription assay was performed using highly-purified TFIIH, TFIIIE, TFII F, TBP, RNA polymerase II and NS1WT or NS1RK/AA protein as described previously [40].

EMSA assay was performed with recombinant purified NS1WT or NS1RK/AA proteins and dsDNA fragments as described previously [41]. Briefly, the synthetic 199 bp-long dsDNA (DNA–199) was produced by PCR using two oligonucleotides (forward 5'-ATGGTACAAAACAGTGGTCA-3', reverse 5'-CTTCCATTATTAGCTCTTC-3') and pHW188-NS plasmid as a template (29). The synthetic 76 bp-long dsDNA (DNA–SELEX) was produced as described in [42]. Hundred ng of dsDNA was incubated with purified recombinant proteins for 15 min on ice. 10 × loading buffer (20 mM Tris–HCl pH 8.6, 50 mM NaCl, 10% glycerol) was added to the samples and the samples were resolved in 1% agarose gel containing ethidium bromide in a TAE buffer (40 mM Tris-base, 20 mM acetic acid, 1 mM EDTA). Protein–DNA–SELEX complex were excised from the gel, and DNA was purified using Qiaquick gene extraction kit.
The DNA was sequenced with SR3 primer (5′-GTTCAGAGTTC
TACAGTC-3′) using ABI3730xl DNA Analyzer and standard Sanger
method, adjusted for sequencing of short fragments.

Microscale thermophoresis assay was performed with recombinant
highly-puriﬁed NS1WT or NS1RK/AA proteins and ﬂuorescently labeled
synthetic 199 bp-long dsDNA (DNA-199). DNA-199 was produced by
PCR using oligonucleotides containing a cyanine ﬂuorophore covalently
linked to the 5′ ends and pHW188-NS plasmid as a template. 10 pM of
Cy5-labeled synthetic dsDNA was incubated with different concentra-
tions of puriﬁed proteins for 5 min on ice. Differences in thermophoretic
properties of free and protein-bound dsDNA were determined using
Monolith NT.115 instrument (NanoTemper Technologies, Munich,
Germany).

3. Results

3.1. NS1 inhibits transcription of antiviral genes in virus-infected cells

It has been previously shown that NS1 through R38 and K41
residues binds dsRNA to sequester it from recognition by PRRs [15,43,
44]. We hypothesized that via the same residues, NS1 could also bind
dsDNA to inhibit transcription of IFNs and ISGs (Fig. 1B). To test this
hypothesis, we used wild-type inﬂuenza A/WSN/33(H1N1) virus
(WSNWT) and its variant (WSNRK/AA), which expresses NS1 protein
with R38A and K31A [31]. We infected human RPE cells with WSNWT
or WSNRK/AA viruses, and used the uninfected cells (mock) as a control.
8 h post infection we analysed the expression of cellular genes using
dNA microarray. We found that infection with WSNWT virus acti-
vated the expression of 33 genes more than eight-fold, whereas
WSNRK/AA virus induced expression of 88 genes over the same
fold, including 31 transcripts that were the same as those up-
regulated by WSNWT (Fig. 2A). We validated our microarray results
using RT-qPCR and ELISA (Fig. 2B and C). These results suggested
that WSN virus via R38A and K41 residues of NS1 was able to
down-regulate the transcription of antiviral genes in infected
human RPE cells.

Importantly, we obtained similar results using human macrophages
previously [31]. In particular, infection of human macrophages with
WSNWT virus up-regulated the expression of 57 IFNs and ISGs more
than eight-fold, whereas WSNRK/AA virus infection activated the expres-
sion of 93 genes (>8-fold), including 32 of WSNWT-up-regulated genes.
These data suggested that WSN virus NS1 protein, via R38A and K41
residues was able to control the transcription of antiviral genes in
both infected human macrophages and RPE cells. However, human
macrophages derived from donors with different genetic and epigenetic
background possessed slightly different responses to infection with
WSN virus (Fig. S3) [29,31,45]. To exclude these variations, we used
only RPE cells in our next experiments.

Gene set enrichment analysis showed that the majority of differen-
tially expressed genes were involved in antiviral responses. To conﬁrm
this, we suppressed the expression of some of these genes using speciﬁc
siRNAs before infection. We demonstrated that IFNB1, EIF2AK2 (PKR),
IDO, BAMBI, CH25H, DDX60L, OAS1, OAS3, PIGC2 (COX2), and IFIH1
(MDA5) as well as TLR3 and MAVS (used as controls) were necessary

Fig. 2. Inﬂuenza NS1 through R38 and K41 inhibits transcription of antiviral genes. A. RPE cells were mock-, WSNWT-, or WSNRK/AA-infected. 10 h post infection cells were collected, total
RNA was isolated and subjected to genome-wide transcription proﬁlling. Three independent experiments were performed. Differentially expressed genes were selected (p < 0.05) and
shown on a heatmap. Each cell is colored according to the average of the log2-transformed and quantile-normalized expression values (log2 fold change N 3 and b −3) of the triplicate
samples with the average of mock controls subtracted. B. RPE cells were treated as for panel A, total RNA was isolated at 10 hpi, and the expression of 5 antiviral (CCL5, IL6, IFNA16,
NFKB1 and OAS1) and one housekeeping (EML4) gene was analysed using RT-qPCRs. The data points are mean values and error bars represent the SD from three independent
experiments. Statistically signiﬁcant (p < 0.05) differences in gene expression between virus- and mock-infected cells are indicated with asterisks. C. Cells were treated as for panel A,
cell culture supernatants were collected at 24 h post-infection, and cytokine levels were determined using ELISA.
to limit transcription and replication of viral M1 RNAs upon WSNWT and WSNRK/AA virus infections (Fig. 3A). Interestingly, TLR3 but not TLR7 was required for transcription of IFNB1, which triggered the expression of a set of ISGs in response to IAV infection (Fig. 3B). Thus, IAV via R38 and K41 of NS1 attenuated transcription of antiviral IFNs and ISGs.

3.2. NS1 locates to chromatin to suppress transcription of antiviral genes

Next we asked how does NS1 control the transcription of antiviral genes. We first investigated the cellular localization of NS1WT and NS1RK/AA. We infected RPE cells with WSNWT or WSNRK/AA viruses and after 10 h post infection examined the distribution of NS1WT and NS1RK/AA by immunofluorescence. These experiments showed that both NS1WT and NS1RK/AA were located mainly in the nucleus of infected cells (Fig. 4A).

We then purified NS1-associated factors from mock-, WSNWT-, and WSNRK/AA-infected RPE cells. Cells were lysed with Triton X-100, and cell extracts were subjected to fractionations, high-RNAse A and high-salt treatments followed by SDS-PAGE, immunobloting, and agarose gel electrophoresis analysis. Surprisingly, the majority of NS1WT and NS1RK/AA were found in insoluble fraction together with cellular DNA, RNA Pol II and histones (Fig. 4B). This indicates that NS1 can be co-purified with chromatin factors from virus-infected cells.

We next extracted proteins from the insoluble fractions and analysed them by quantitative mass spectrometry (iTRAQ LC–MS/MS). We found that histones H3.2, H1.2, H1.5, H2A.1D and other chromatin-associated proteins were enriched in NS1WT and NS1RK/AA-containing insoluble fractions in comparison to mock (Fig. 4C; Table S2). Of note, some differences in the levels of H2A1D, YBOX-1, H3.2 and other proteins were seen between NS1WT- and NS1RK/AA-containing fractions. These data suggest that WSNWT and WSNRK/AA infections alter protein composition of chromatin fractions.

Next we investigated the consequences of the altered histone composition on NS1 interaction with specific gene regions during IAV infection. For this, RPE cells were mock-infected or infected with either WSNWT or WSNRK/AA viruses for 8 h. NS1- and Pol II-associated DNA was extracted from chromatin fractions of infected and non-infected cells. The promoter and exon regions of IFNB1 were analysed using q-PCR. We found that NS1WT was enriched on the promoter and exon region of IFNB1 in WSNWT infected cells, whereas Pol II was enriched on the same IFNB1 regions in WSNRK/AA infected cells (Fig. 4D). These results suggest that wild type NS1 can prevent the association of Pol II with IFNB1 gene during virus infection. Altogether, these results may indicate that upon IAV infection PRRs mediated chromatin remodelling to activate the expression of antiviral genes and suppress general transcription, and NS1 upon expression suppresses transcription of some antiviral genes, depending on chromatin context.

Fig. 3. SiRNA experiment revealed several cellular factors, which restrict both WSNWT and WSNRK/AA virus replication in RPE cells. A, B. RPE cells were transfected with specific or control siRNA. 24 h later cells were infected with WSNWT (moi 1) or WSNRK/AA (moi 1) viruses or mock-infected. Expression levels of (A) viral M1 and (B) cellular IFNB1 gene were analysed 10 h later by RT-qPCRs. Statistically significant (p < 0.05) differences in gene expression between non-targeted siRNA control and targeted siRNA condition are indicated with asterisks.
3.3. Purified recombinant NS1 through R38 and K41 binds synthetic dsDNA and inhibits Pol II transcription in vitro

To further analyse the mechanisms of NS1 binding to DNA, we performed EMSA experiments with purified recombinant proteins (Fig. 5A). For this experiment, we produced linear synthetic dsDNA (DNA-SELEX), which contained central random 16 N base pair (bp) region flanked by defined 30 bp sequences. 16 N bp region was chosen because NS1 RBD dimer was shown to occupy 16 bp of A-form dsRNA [46]. EMSA showed that NS1WT but not NS1RK/AA retarded the migration of dsDNA (Fig. 5B). We analysed the possible sequence-specificity of NS1 interaction with its target DNA using Sanger sequencing, i.e. we sequenced NS1-interacting DNA. We found that NS1 binds DNA independently of...
sequence of central region, suggesting that the interaction was not sequence-specific (Fig. 5C).

We then determined the dissociation constant (K_d) of NS1WT and dsDNA fragment (DNA-199). EMSA showed that NS1WT retarded the migration of DNA-199 probe in a concentration-dependent manner with micromolar K_d (Fig. 5D). Microscale thermophoresis assay revealed that the K_d for NS1WT was 11.1 ± 0.7 μM, whereas the K_d for NS1RK/AA was 100 μM (Fig. 5E). Interestingly, the K_d for NS1WT-dsDNA complex was comparable to that of NS1WT-dsRNA complex reported previously [39,46–48]. These results suggested that in vitro NS1 binds dsDNA non-specifically with micromolar affinity, and that the residues R38 and K41 of NS1 are essential for the binding.

To address whether NS1 through R38 and K41 can inhibit transcription in vitro, we purified recombinant NS1WT or NS1RK/AA proteins (Fig. 4A) and added them to in vitro run-off transcription assays containing naked AdMLP DNA template. We found that NS1WT but not NS1RK/AA inhibited the in vitro synthesis of RNA in a concentration-dependent manner (Fig. 4F). To further understand how NS1 was able to block transcription, we set up two different experimental conditions. We first pre-incubated NS1 with dsDNA, and then added pre-initiation complex (PIC) and Pol II before the reaction started (Fig. 4G, incubation 1). Alternatively, we pre-incubated NS1 with PIC and Pol II, and then added dsDNA (Fig. 4G, incubation 2). In both experimental settings, NS1WT but not NS1RK/AA inhibited the in vitro RNA synthesis. We concluded that NS1 inhibits in vitro transcription by binding to DNA, which prevents the loading of PIC and Pol II.

4. Discussion

Influenza NS1 protein is an important virulence factor and deciphering the mechanism by which NS1 antagonizes antiviral responses is critical for understanding of disease progression. Here we
demonstrated that influenza NS1 binds synthetic dsDNA in a sequence non-specific manner. Furthermore, we showed that this interaction inhibited the loading of transcriptional machinery on the synthetic DNA and thereby prevented the transcription reaction in vitro. In infected cells, NS1 inhibited Pol II recruitment to the exon and promoter regions of IFNβ1. This observation could be potentially expanded to other IFN genes and ISGs whose transcription was up-regulated in response to WSNR/AA in comparison to WSNWT and mock infections. In addition, our results indicate that IAV infection promoted chromatin remodelling, which could be associated with inhibition of general transcription and activation of expression of certain IFNs and ISGs. Influenza NS1 protein could, therefore, bind DNA of transcriptionally active genes and attenuate their expression. This may potentially lead to reduced expression of IFNs and ISGs, leading to compromised antiviral responses of infected cells.

Importantly, R38 and K41 residues of NS1 mediate an interaction of IAV NS1 with non-specific dsDNA and dsRNA, and the dissociation constants of these interactions are very similar [39,46–48]. This suggests that NS1 can bind both dsDNA/RNA via the phosphate backbone. Binding of NS1 to dsDNA/RNA can hinder them from loading of cellular transcription machinery and recognition by cellular PRRs, respectively.

Interestingly, our attempt to purify NS1 from infected cells revealed that the majority of NS1 was in insoluble fraction together with histones, transcription machinery and DNA. High salt and RNase treatments of these fractions, as well as R38A and K41A mutations in NS1, did not increase NS1 solubility. This indicates that additional transcriptional regulation mechanisms involving NS1 interactions with cellular factors may take place, which is in agreement with previous findings showing that NS1 non-conserved residues outside dsDNA/RNA binding site may interact with chromatin-associated factors [49,50].

The RNA/DNA-binding residues R38 and K41 of NS1 protein are evolutionary conserved among IAVs. Moreover, the corresponding NS1 residues are also conserved in influenza B viruses [51–53]. Thus, our findings point to a general strategy, by which influenza viruses can antagonize antiviral responses in infected cells to secure its replication. Other RNA viruses are also able to inhibit cellular transcription to secure their replication. For example, bunyamwera virus NS-S protein inhibits the phosphorylation of Pol II C-terminal domain, while Rift Valley Fever Virus NSs protein targets TFIIF to inhibit the transcription of cellular genes including those of antiviral genes [54,55]. However, to our knowledge, there are no data available except this study, that negative- or positive-sense RNA viruses can inhibit cellular transcription by direct binding of viral proteins to cellular DNA. Thus, our work provides a first example of such a mechanism that could be potentially exploited by other RNA virus families.

Finally, the influenza NS1-dsDNA interaction can be potentially exploited for treatment of IAV infection. In particular, small-molecular inhibitors of this interaction can potentially restore innate immune responses and inhibit virus replication. In addition, viruses expressing dsDNA binding deficient NS1 may display characteristics desirable for potential live-attenuated viral vaccines.

5. Conclusions

Host cell activates transcription of a set of antiviral genes in response to IAV infection. Our results suggest that the viral NS1 protein can inhibit transcription of some of these genes by binding to dsDNA and preventing the loading of cellular transcription machinery. Thus, IAV can exploit its NS1 protein to attenuate antiviral responses at transcriptional level to secure its replication.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagrm.2016.09.005.

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