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

Maria Anastasina [a,d,1], Nicolas Le May [b,1], Andrii Bugai [c,1], Yu Fu [a], Sandra Söderholm [d], Lana Gaelings [a], Tiina Ohman [d], Janne Tynell [e], Suvi Kyttänen [a], Matjaz Barboric [c], Tuula A. Nyman [d,f], Sampsa Matikainen [c], Ilkka Julkunen [e,g], Sarah J. Butcher [d], Jean-Marc Egly [b,*], Denis E. Kainov [a,*]

[a] Institute for Molecular Medicine Finland (FIMM), University of Helsinki, Helsinki 00290, Finland
[b] Department of Functional Genomics and Cancer, Institute for genetics, molecular and cellular biology (IGBMC), Strasbourg 67404, France
[c] Medicum, Department of Biochemistry and Developmental Biology, University of Helsinki, Helsinki FIN-00014, Finland
[d] Institute of Biotechnology, University of Helsinki, 00014, Finland
[e] National Institute for Health and Welfare (THL), Helsinki 00271, Finland
[f] Institute of Clinical Medicine, Sognsvannsveien 20, Rikshospitalet, 0372 Oslo, Norway
[g] Department of Virology, University of Turku, Turku 20014, Finland

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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*), MDA5 (*IFIH1*), 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*, *ISG20*) 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, kynurenine, and oxysterol 25-hydroxycholesterol, respectively, which act as immuno- and neuromediators [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

* Corresponding authors.
E-mail addresses: egly@igbmc.fr (J.-M. Egly), denis.kainov@helsinki.fi (D.E. Kainov).
[1] These authors contributed equally.

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transcription of innate antiviral genes by binding cellular DNA 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 (WSN^{WT}) or R38A/K41A mutant NS1 ($WSN^{RK/AA}$) 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 $WSN^{RK/AA}$ and WSN^{WT} 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 $WSN^{RK/AA}$ virus than for WSN^{WT} (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% CO_2.

2.2. Transfection of RPE cells with siRNA

RPE cells were cultured to 80% confluency 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 WSN^{WT}, $WSN^{RK/AA}$ viruses or mock infected. 8 h after infection, the levels of genes and proteins of interest were analysed using RT-qPCRs and immunoblotting, respectively.

2.3. Infection of RPE cells with WSN^{WT} and $WSN^{RK/AA}$ viruses

The growth medium of RPE cells was changed to the virus growth medium (VGM) containing 0.2% BSA (Sigma-Aldrich), 2 mM L-glutamine, 0.348% $NaHCO_3$ and 1 μg/ml l-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin (TPCK)-trypsin (Sigma-Aldrich) in DMEM-F12. The cells were infected with WSN^{WT}, $WSN^{RK/AA}$ viruses or mock.

2.4. Gene expression profiling

RNA was extracted from WSN^{WT}-, $WSN^{RK/AA}$- 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-Hocberg 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 (log_2Fc). The gene-expression data was deposited to Gene Expression Omnibus (GEO accessory 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 Lightcycler 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 TTCAGTGCAACTCTT-3′, reverse: 5′-AATCTCCATCACTGCCCATC-3′), IFNB1 promotor (forward: 5′-GTCAGTAGAATCCACGGATACAG-3′ and reverse: 5′-CTTGGGAGAAAGCAAAGGAAAG-3′) and exon (forward: 5′-GCCGCATTGACCATCTATGA-3′ and reverse: 5′-GCCAGGAGGTTCTCAA CAATAG-3′), IFNA1 (forward: 5′-ATGGCAACCAGTTCCAGAAG-3′, reverse: 5′-CATCCCAAGCAGCAGATGAA-3′), IFNA16 (forward: 5′-GACT CACTTCTATAACCACCACAA-3′, reverse: 5′-TAGTGCCTGCACAGGTAAAC-3′), IL6 (forward: 5′-TCATCACTGGTCTTTTGG-3′, reverse: 5′-CTCTGG CTTGTTCCTCAC-3′), CXCL1 (forward: 5′-TGAGCATCGCTTAGGAGA-3′, reverse: 5′-AGGACAGTGTGCAGGTAG-3′), and IL29 (forward: 5′-AGGC TGAGCTGGCCCTGA-3′, reverse: 5′-GGTGTGAAGGGGCTGGTC-3′). The relative gene expression differences were calculated as described

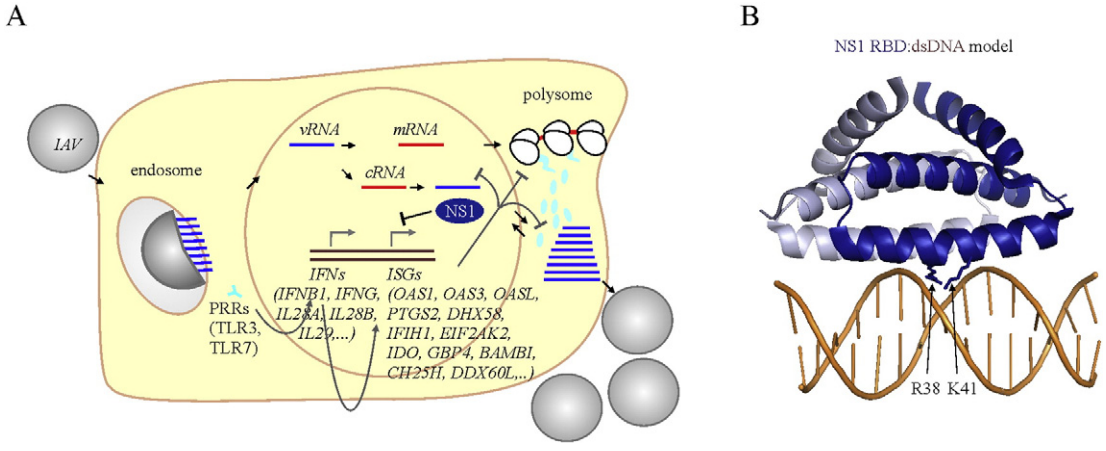

Fig. 1. Influenza NS1 through R38 and K41 may bind dsDNA to inhibit transcription of antiviral genes. A. Novel hypothetical mechanism of action of influenza NS1 against antiviral responses in influenza A virus-infected cells. IAV infection stimulates the transcription of IFNs and ISGs, which inhibit virus replication. Newly synthetized 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.