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Influenza NS1 interacts with p53 and alters its binding to p53-responsive genes, in a promoter-dependent manner

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ABSTRACT

The interplay between influenza A viruses (IAV) and p53 has only been reported in a limited number of studies, mainly focusing on the antiviral role of p53. We investigated the impact of IAV infection on p53 stability and transcriptional activity. Our results indicate that IAV-induced stabilization of p53 only partially correlates with modulation of p53 transcriptional activity measured during infection. Moreover, we show that the viral non-structural protein 1 (NS1) is able to inhibit p53 transcriptional activity, in a promoter-dependent manner. Based on these data, we propose that NS1 may contribute to p53-mediated cell fate decision during IAV infection.

Structured summary of protein interactions:
p53 physically interacts with **NS1** by anti bait coimmunoprecipitation (View interaction)

1. Introduction

Influenza A viruses (IAV) belong to the *Orthomyxoviridae* family of enveloped viruses that contain a segmented genome of single-stranded negative sense RNA. This family comprises some of the few RNA viruses that undergo replication and transcription within the host cell nucleus using the nuclear machinery to support their propagation [1]. Several studies have shown that IAV are able to modulate and/or hijack several cellular networks and signalling pathways [2,3]. The viral non-structural protein 1 (NS1) is involved in multiple aspects of IAV/host interactions that together inhibit a broad range of cellular functions, notably those related to the antiviral response, and also interferes with host mRNA export and splicing [4]. Indeed, NS1 is believed to be important for regulation of the host cell cycle and was recently shown to be involved in the IAV-induced G0/G1 cell cycle arrest [5]. Moreover, NS1 seems to play an ambivalent role in the regulation of apoptosis during IAV infection, based on contradictory results from studies reporting either its pro- or anti-apoptotic functions [6–8].

In response to stress, the transcription factor p53 rapidly accumulates in the nucleus where it regulates gene expression to maintain genomic and cellular integrity [9]. The numerous genes regulated by p53 are involved in several biological processes, including cell cycle arrest (e.g. P21/CDKN1A), apoptosis (e.g. BAX) or senescence [9]. The interplay between influenza viruses and p53 has only been reported in a limited number of studies which mainly highlight the role of p53 as an antiviral protein [10,11]. Moreover, we recently showed that p53 isoforms are involved in the regulation of these p53-dependent antiviral properties [12].

Most of the studies dedicated to IAV and p53 have described an increase in p53 protein levels during infection [11,13–15]. However, it is still not clear whether this accumulation is correlated with the activation of p53 and consecutive transactivation of its target genes. Wang et al. have suggested a possible interaction between NS1 and p53 leading to the inhibition of p53-mediated transcriptional activity [16]. Interestingly, results from our previous study based on transcriptional profiling of IAV-infected human cells, showed a massive downregulation of the p53 pathway, mostly its downstream part, in response to IAV infection [11].

Based on these observations, we further investigated the impact of IAV infection on p53 stability and transcriptional activity in human lung epithelial cells, with a systematic comparative focus

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on NS1. Our results indicate an IAV-induced increase in p53 stability that is only partially correlated with the modulation of p53 transcriptional activity during IAV infection. Moreover, they show that NS1 is able to inhibit p53 transcriptional activity by altering its binding to target genes, in a promoter-dependent manner.

2. Materials and methods

2.1. Cell line, virus and infection

Human lung epithelial A549 cells (ATCC CCL-185, wild type p53) and H1299 (ATCC CRL-5803, p53 null) were grown at 37 °C in DMEM supplemented with 10% heat-inactivated foetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin sulphate. Influenza virus A/Moscow/10/99 (H3N2) was propagated in MDCK cells (ATCC CCL-34) in EMEM supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin sulphate and 1 mg/mL trypsin. The virus was then titrated to determine the 50% tissue culture infective dose (TCID50) in MDCK cells, as previously described [17]. Sub-confluent A549 cells were then infected with the influenza virus at multiplicities of infection (MOI) of 0.1 or 2 for 1 h in a minimal volume of DMEM supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin sulphate and 0.5 μg/mL trypsin (infection medium) at 37 °C. Fresh infection medium was then added to the cells before their incubation at 37 °C for different lengths of time.

2.2. Transfection and transactivation assay

Transient transfections were performed using TransIT-LT1 reagent (Mirus), according to the manufacturer's instructions. For the transactivation assay, A549 cells were transfected with 1 μg firefly luciferase reporter vectors. Transfection efficiency was normalised using 100 ng of Renilla luciferase plasmid. After 24 h, cells were infected with influenza virus A/Moscow/10/99 at a MOI of 0.1 or 2 before being harvested at different time points for further analysis. Alternatively, cells were co-transfected with a pCI-empty or pCI-NS1-H3N2 expression plasmid and then harvested at 24 h post-transfection. Luciferase activity was measured in whole cell extracts using the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's instructions. Three independent experiments were performed in triplicate. The different reporter vectors used were pG13-luc, with a firefly luciferase gene under the control of thirteen copies of the p53-binding consensus sequence (5′- CCAGGCAAGTCCAGGCAGG- 3′ [18]), and p21-luc, Mdm2-luc and Bax-luc, with the firefly luciferase gene under the control of the complete (p21) or partial (Mdm2 and Bax) promoter sequence of the corresponding genes [18–20].

2.3. p53 stability assay

For the p53 half-life experiments, previously transfected or infected cells were treated with 50 μM cycloheximide (CHX). Total protein lysates were harvested at different time-points during a 1 h period and were analysed by western blot [12] to determine relative protein levels (RPL) by densitometry. RPLs between Mock and NS1-transfected or infected cells were subjected to statistical analysis (Student's t test, statistical significance $P < 0.05$).

2.4. Chromatin immunoprecipitation assay (ChIP)

ChIP experiments were performed as previously described [21,22]. Briefly, 2×10^6 H1299 cells were seeded onto a 15 cm plate and co-transfected with 4 μg of pSV-p53 and 4 μg of pCI-empty or pCI-NS1-H3N2 expression plasmid. After 24 h, cells were fixed with 1% formaldehyde for 10 min at room temperature. The cells were then scraped and washed with ice-cold PBS, and then sonicated with salmon sperm DNA and protease inhibitors. Immunoprecipitation was carried out using an anti-p53 monoclonal antibody (DO-1) and Dynabeads (Invitrogen). Mouse IgGs were used as negative controls. A mouse anti-NS1 monoclonal antibody (Santa-Cruz ref sc-130568) was used to control a possible binding of NS1 to promoter regions. The amount of total input DNA per ChIP was adjusted to 25 μg. After immunoprecipitation and DNA purification, samples were analysed by real-time quantitative PCR (RT-qPCR), using specific primers and probes for p21 and Bax. In parallel, the same quantitative analysis was performed on Input DNA. The results were expressed as a percentage of total input DNA. The specific primers and probes used for p21 and Bax were the same as those described by Kaeser and Iggo [21].

3. Results

3.1. IAV infection increases p53 stability

To investigate the impact of influenza infection on p53 stability, we mock-infected or infected A549 cells with influenza virus A/Moscow/10/99 (H3N2) at an MOI of 2 or 0.1. At 4, 24 or 48 h post infection (hpi), we then studied p53 stability by monitoring its levels over a 1 h period post treatment with 50 μM cycloheximide (CHX) (Fig. 1A). In all conditions tested, the mock-infected cells displayed a loss of around 80% of the RPL of p53 in 1 h, thereby reflecting a p53 half-life of approximately 15–20 min. In the H3N2-infected cells, however, the RPL of p53 consistently remained higher than in mock-infected cells, the difference of which was particularly marked at the later time-points. For example, at 24 and 48 hpi, the 1 h stability of p53 was almost complete (p53 RPL of 0.91, P-value <0.001) or complete, respectively (Fig. 1A). These first observations suggested a marked increase in p53 stability during IAV infection, even from 4 hpi (Fig. 1A).

3.2. IAV NS1 expression contributes towards p53 stability

To further investigate the potential involvement of NS1 in this virally-induced stabilization of p53, we transfected A549 cells with either an empty plasmid (pCI-empty) or a plasmid expressing NS1 from the H3N2 strain (pCI-NS1-H3N2). We used the same method as above to evaluate the endogenous p53 stability (Fig. 1B) and western blot to assess the expression of NS1, p53 and its targets (Fig. 1C) 36 h post-transfection. The CHX assay revealed a slight increase in p53 stability in the NS1-expressing cells. For example, at 30 min, the RPL of p53 measured in the NS1-expressing cells was more than two times higher than levels measured in pCI-empty transfected cells (p53 RPL of 0.44 *versus* 0.21, P-value <0.05) (Fig. 1B). Interestingly, stabilization of p53 in the NS1-expressing cells did not reach the same level as that measured in the infected cells, even with higher concentrations or longer kinetics of the transient expression of NS1 (data not shown). Not only does this finding support the contribution of NS1 towards the stabilization of p53, it also suggests that NS1 may not be the only determinant.

3.3. NS1 interacts with p53

To investigate the potential interaction between p53 and NS1, we transfected H1299 cells (p53 null) with an empty plasmid (pCI-empty) or one expressing NS1 (pCI-NS1-H3N2), along with a plasmid expressing p53 (pSV-p53). After 48 h, we analysed the cell lysates using a co-immunoprecipitation assay (co-IP) with an anti-p53 polyclonal antibody (CMI), or control IgG (Fig. 1D). Western blot displayed a band corresponding to NS1 only with the