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The non-structural (NS1) protein of influenza A virus associates with p53 and inhibits p53-mediated transcriptional activity and apoptosis

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ABSTRACT

NS1 protein of influenza A virus is involved in regulating the apoptosis of infected cells. We found that exogenously expressed NS1 was able to associate with the tumor suppressor p53 that plays an essential role in regulating apoptosis of influenza A virus-infected cells. Exogenous expression of NS1 resulted in inhibition of p53-mediated transcriptional activity and apoptosis. The p53 inhibitory domain of NS1 was located between amino acids 144 and 188. This domain is necessary for NS1 to inhibit p53 activity, but it requires additional region(s) to cooperatively exert this inhibitory function.

1. Introduction

Influenza A virus (IAV) is a cytolytic virus that is highly infective in humans, causing approximately 500,000 deaths worldwide per year [1], and many animal species. The genome of IAV consists of eight single-stranded, negative-sense viral RNA segments, which encode 11 known proteins. Segment 8 encodes two non-structural proteins, NS1 and nuclear export protein (NEP) [2]. NS1 is a non-essential virulence factor that has multiple accessory functions including suppression of host immune and apoptotic responses [3]. NS1 is involved in regulating the apoptosis of infected cells, but its role in apoptosis is contradictory, as NS1 has been described to have both pro- and anti-apoptotic functions [4–7]. An intriguing hypothesis is that NS1 contributes temporally to both 'early' suppression of apoptosis and 'late' induction of cell death [3].

Tumor suppressor p53, a major cellular defense against tumor development, plays an important role in mediating apoptosis in response to stress, including virus infection [8]. Infection of IAV induces the activation of p53, and the activated p53 plays an essential role in regulating apoptosis of the infected cells [9]. In a previous study, we found that p53 was activated in a biphasic pattern with a first transient elevation at the beginning phase, a nadir at the early phase, and a second elevation at the middle-late phase of IAV infection. The nadir of p53 activity correlated with the onset of NS1 expression [10]. We therefore speculated that the expression of NS1 affects p53 activity. The findings from this study show that NS1 interacts with p53 and inhibits p53-mediated transcriptional activity and apoptosis.

2. Materials and methods

2.1. Plasmid construction

The cDNA encoding viral NS1, NEP and M1 of a strain of influenza A/Swine/Jiangsu/2/2006 (H3N2 subtype) was amplified by RT-PCR and cloned into p3xFLAG-CMV-7.1 vector, resulting in generation of Flag-tagged protein expression plasmids Flag-NS1-wt, Flag-NEP and Flag-M1, respectively. Plasmids engineered to express GFP- or Flag-tagged human p53 (GFP-p53 or Flag-p53) were constructed by inserting the full-length human p53 cDNA into pEGFP-C1 or p3xFLAG-CMV-7.1 vector, respectively. A series of plasmids expressing Flag-tagged deletion mutants of NS1 were generated by modified PCR-based site-directed mutagenesis [11] using Flag-NS1-wt as the template.

2.2. Antibodies

The commercial antibodies employed in this study were an anti-Flag monoclonal antibody (M2, Sigma, St. Louis, MO, USA), an anti-p53 polyclonal antibody (FL-393, Santa Cruz Biotechnology, Santa Cruz, CA, USA), an anti-GFP polyclonal antibody (ab6556, Abcam, Cambridge, MA, USA), an anti-β-actin monoclonal antibody (AC-15, Sigma), a horseradish peroxidase-conjugated

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goat anti-rabbit IgG (sc-2004, Santa Cruz Biotechnology) and a goat anti-mouse IgG antibody (sc-2005, Santa Cruz Biotechnology). An anti-NS1 polyclonal antibody was generated in our laboratory (unpublished data).

2.3. Cells, transfection and luciferase assay

Vero, Saos-2 and H1299 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum. Cells were transfected using Lipofectamine™ 2000 (Invitrogen), according to the manufacturer's protocol. For the luciferase assay, cells were transiently co-transfected with the indicated plasmids, p53-Luc reporter plasmid (Stratagene, San Diego, CA, USA) and control plasmid Renilla luciferase pRL-TK (Promega, Madison, WI, USA). The firefly luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega), according to the manufacturer's protocol, and normalized to Renilla luciferase activity.

2.4. Apoptosis detection

H1299 cells were transiently transfected with the indicated plasmids and incubated for 14 h. The transfectants were double-stained with FITC-annexin V and propidium iodide using the FITC-Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, California, USA), and were subjected to FACS analysis. For the TUNEL assay, H1299 cells grown on coverslips were transiently co-transfected with the indicated plasmids and incubated for 16 h. The apoptotic cells were stained using the In Situ Cell Death Detection Kit, TMR red (Roche, Mannheim, Germany), and examined under a fluorescence microscope.

2.5. Immunoprecipitation and Western blot analysis

Immunoprecipitation and Western blot analysis was performed as described previously [12].

3. Results

3.1. NS1 inhibited the transcriptional activity of p53

Vero cells were transfected with plasmid Flag-NS1-wt, Flag-M1, Flag-NEP or Flag-vector in the presence of a p53-Luc reporter plasmid (p53-Luc) that contains 14 tandem repeats of the p53 consensus binding sites. The expression of Flag-tagged proteins was confirmed by Western blot analysis (Fig. 1A). The expression of either Flag-M1 or Flag-NEP had no effect on the luciferase activity, whereas the expression of Flag-NS1-wt significantly reduced the luciferase activity (approximately threefold), compared with the Flag-vector (Fig. 1B). No remarkable change of p53 in protein level was observed in all transfectants (Fig. 1A). Next, Vero cells were transfected with increasing amounts of Flag-NS1-wt in the presence of p53-Luc (Fig. 1C). The luciferase activity of the transfectants was significantly reduced by the increasing amount of Flag-NS1-wt expression in a dose-dependent manner (Fig. 1D). To further confirm these results, p53 null Saos-2 cells were co-transfected with Flag-p53 and Flag-NS1-wt in the presence of p53-Luc (Fig. 1E). Expression of Flag-p53 alone resulted in a significant enhancement of the luciferase activity compared with the Flag-vector, whereas the enhanced luciferase activity was significantly reduced by co-expression of Flag-NS1-wt. Co-expression of Flag-NEP had no inhibitory effect on the enhancement of luciferase activity. These results suggested that NS1 inhibited the transcriptional activity of p53.

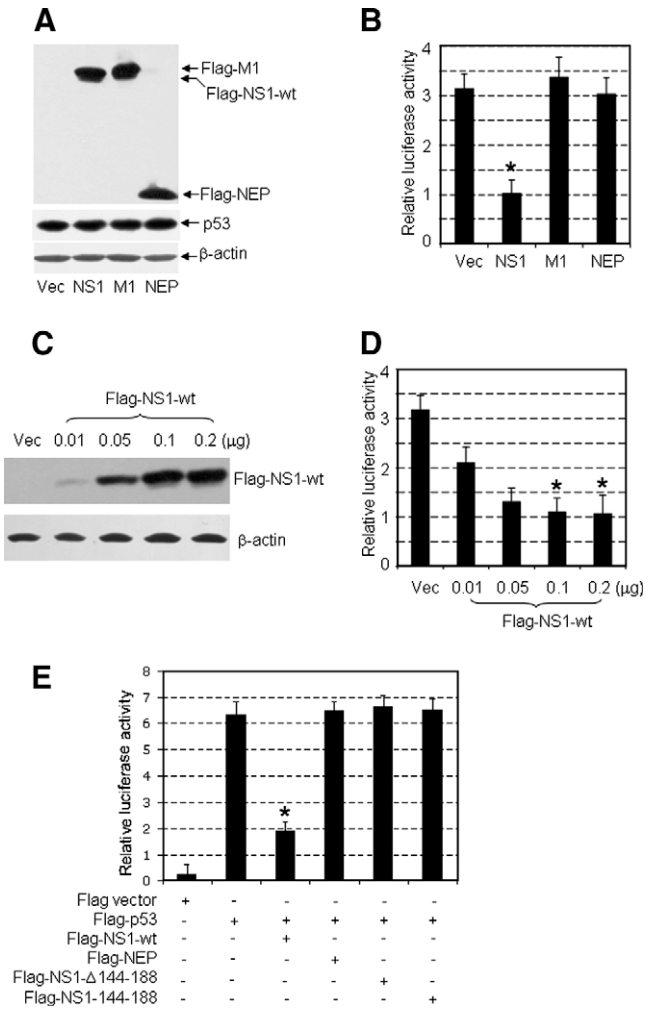

Fig. 1. Inhibitory effect of NS1 on p53-mediated transcriptional activity. (A) Vero cells were transiently transfected with plasmid Flag-NS1-wt, Flag-M1, Flag-NEP or Flag-vector (Vec) in the presence of p53-Luc and incubated for 24 h. Protein expression was detected by Western blot analysis using the indicated antibodies. (B) The luciferase activity of lysates prepared from (A) was analyzed. (C) Vero cells were transiently transfected with increasing amounts of Flag-NS1-wt in the presence of p53-Luc. The expression of Flag-NS1-wt was detected by Western blot analysis. (D) The luciferase activity of lysates prepared from (C) was analyzed. (E) Saos-2 cells were transiently co-transfected with a combination of the indicated plasmids in the presence of p53-Luc and incubated for 24 h. The luciferase activity of lysates prepared from the transfectants was analyzed. Results are presented as the mean ± standard error from three independent experiments. $^{*}p < 0.001$ compared to cells transfected with the empty vector.

3.2. Mapping of the p53 inhibitory region of NS1

To map the domain of NS1 required for inhibiting p53 transcriptional activity, we generated a series of plasmids expressing Flag-tagged deletion mutants of NS1 (Fig. 2A). Vero cells were transfected with these plasmids in the presence of p53-Luc. As shown in Fig. 2B, expression of Flag-NS1-wt significantly reduced the luciferase activity, consistent with the results illustrated in Fig. 1. Expression of the deletion mutants lacking the region between amino acids 11 and 228 (Flag-NS1-Δ11/228), 81 and 228 (Flag-NS1-Δ81/228) or 121 and 228 (Flag-NS1-Δ121/228) had no inhibitory effects on the luciferase activity, while the deletion mutant lacking the region between amino acids 189 and 228 (Flag-NS1-Δ189/228) or 11 and 144 (Flag-NS1-Δ11/144) had moderate inhibitory effects on the luciferase activity. These results suggested that the p53 inhibitory domain of NS1 was probably located

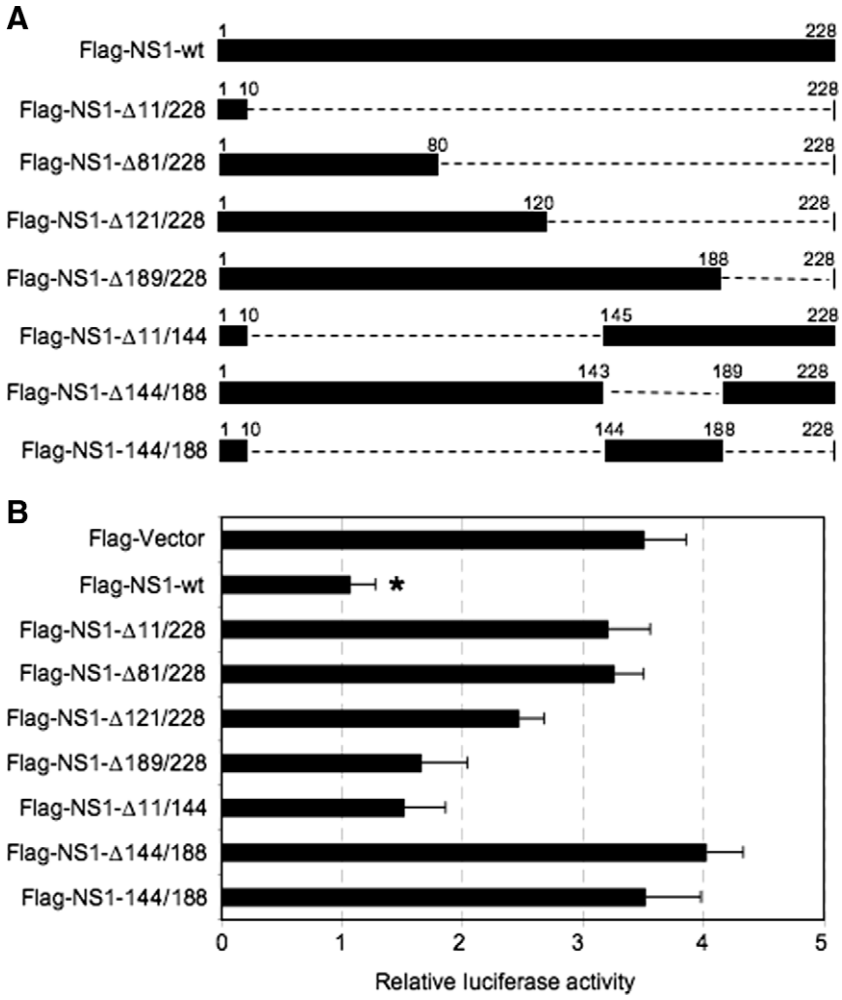

Fig. 2. Mapping of the p53 inhibitory region of NS1. (A) Schematic representation of Flag-tagged wild-type NS1 and deletion mutants. The numbers indicate amino acid positions. (B) Vero cells were transiently transfected with the indicated plasmids in the presence of p53-Luc and incubated for 24 h. The luciferase activity of lysates prepared from the transfectants was analyzed. Results are presented as the mean ± standard error from three independent experiments. $^{*}p < 0.001$ compared to cells transfected with the empty vector.

in the region between amino acids 144 and 188. We then generated a deletion mutant lacking the region between amino acids 144 and 188 (Flag-NS1-Δ144/188), and a deletion mutant remaining the regions between amino acids 1–10 and 144–188 (Flag-NS1-144/188) (Fig. 2A), and analyzed the effects of these deletion mutants on p53 transcriptional activity. As expected, Flag-NS1-Δ144/188 lost the ability to inhibit the luciferase activity (Figs. 1E and 2B), suggesting that the region between amino acids 144 and 188 was the inhibitory domain required for inhibition of the transcriptional activity of p53. However, surprisingly, we also found that Flag-NS1-144/188 had no inhibitory effect on the luciferase activity (Figs. 1E and 2B). These results suggested that the inhibitory domain alone was unable to inhibit the transcriptional activity of p53, which probably requires additional region(s) to cooperatively exert the inhibitory function.

3.3. NS1 inhibited p53-mediated apoptosis

Flag-NS1-wt or Flag-NS1-Δ144/188 was co-transfected with Flag-p53 into p53 null H1299 cells to examine the effect of NS1 on p53-mediated apoptosis. Expression of Flag-p53 alone resulted in an increase of approximately 10-fold in the number of FITC-annexin V positive cells (apoptotic cells) (Fig. 3A), suggesting that p53 was able to induce apoptosis of the H1299 cells used in this study. However, the number of FITC-annexin V positive cells dropped significantly when Flag-p53 was co-expressed with Flag-NS1-wt. As expected, expression of Flag-NS1-Δ144/188 had no effect on p53-induced apoptosis (Fig. 3A). These findings were further confirmed by the results of a TUNEL assay. The expression of Flag-NS1-wt remarkably reduced the number of TUNEL-positive cells (apoptotic cells), as indicated by red fluorescence (Fig. 3B, p53+NS1-wt panel), compared with cells transfected with Flag-53 alone (p53+vec panel). Expression of Flag-NS1-Δ144/188 had no effect on p53-induced apoptosis (p53+NS1-Δ144/188 panel). Taken together, these results suggested that NS1 inhibited p53-mediated apoptosis.

3.4. NS1 associated with p53

The function of NS1 relies on its ability to participate in a multitude of protein–protein interactions [3]. We therefore speculated that NS1 associates with p53. H1299 cells were co-transfected with GFP-p53 and Flag-NS1-wt, and subjected to an immunoprecipitation assay. The advantage of this approach is that it distinguishes exogenously expressed GFP-p53 from the immunoglobulin G heavy chain that migrates very closely to native p53. As expected, Flag-NS1-wt immunoprecipitated GFP-p53 (Fig. 4), suggesting that NS1 was able to associate with p53 *in vivo*. In addition, although

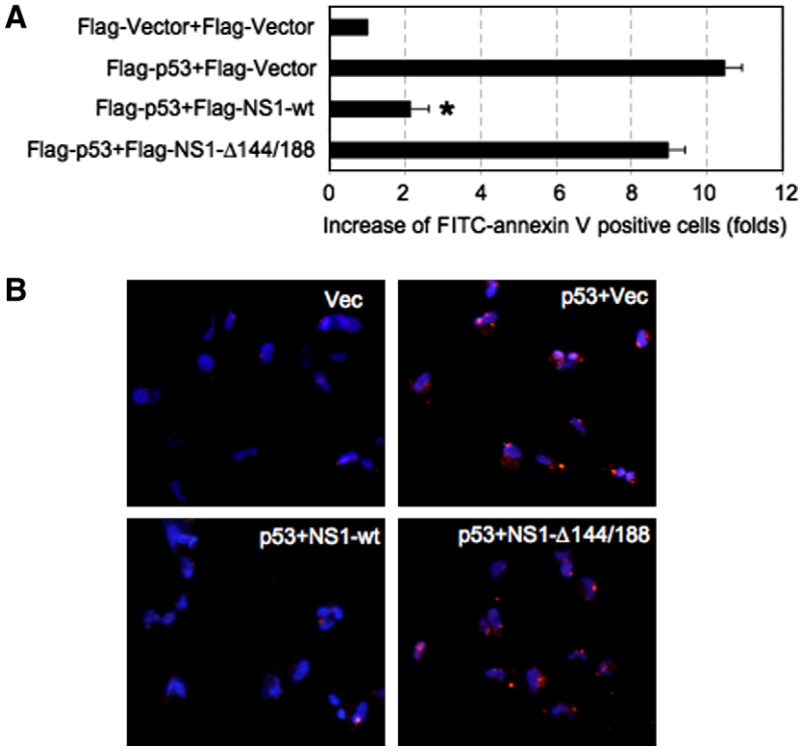

Fig. 3. Inhibitory effect of NS1 on p53-mediated apoptosis. (A) Flag-p53 was co-transfected into H1299 cells with Flag-NS1-wt or Flag-NS1-Δ144/188 at a 1:10 molar ratio. Apoptosis was assessed by FACS analysis after double staining with FITC-annexin V and propidium iodide. The proportion of FITC-annexin V positive cells (apoptotic cells) of Flag-p53 transfectants was normalized to that of Flag-vector transfectants. Results are presented as the mean ± standard error from three independent experiments. $^{*}p < 0.001$ compared to cells transfected with a combination of Flag-p53 and Flag-vector. (B) H1299 cells were transfected with Flag-vector (Vec), or a combination of Flag-p53 and Flag-vector (p53+Vec), Flag-p53 and Flag-NS1-wt (p53+NS1-wt), or Flag-p53 and Flag-NS1-Δ144/188 (p53+NS1-Δ144/188) at a 1:10 molar ratio. The apoptotic cells (red) were stained with a TUNEL assay kit, and merged with DNA images stained with 4-6-diamidino-2-phenylindole (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

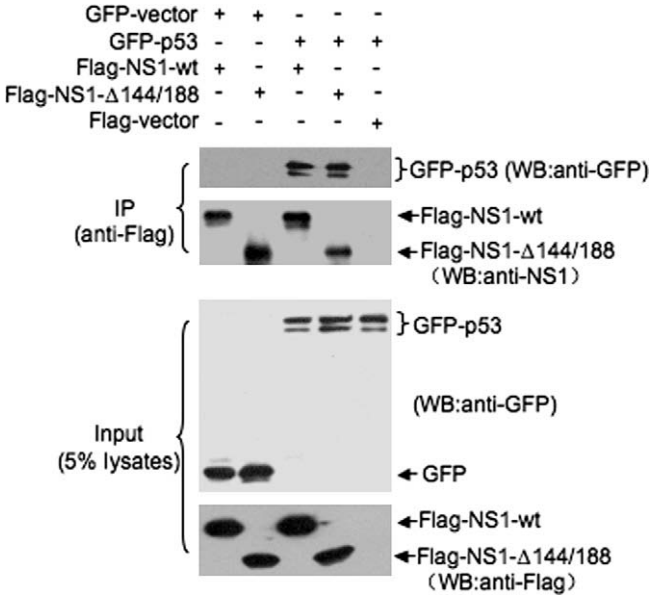

Fig. 4. Detection of the interaction between NS1 and p53. H1299 cells were transfected with a combination of the indicated plasmids and incubated for 16 h. The cell lysates prepared from the transfectants were subjected to immunoprecipitation using anti-Flag antibodies. The immunoprecipitates were immunoblotted with the indicated antibodies. The cell lysates were included as a loading control. IP, immunoprecipitation. WB, Western blot.

Flag-NS1-Δ144/188 lost the ability to inhibit p53-mediated transcriptional activity and apoptosis, it was able to immunoprecipitate GFP-53 (Fig. 4). The double band of p53 may be due to the antibody recognizing epitopes on two p53 isoforms [13]. These results indicated that NS1 associates with p53 and that the p53 inhibitory domain and the p53 interaction domain are probably located in different regions of the NS1 protein.

4. Discussion

Apoptosis is a host defense mechanism against viral infection, therefore, both the host cell and the invading virus compete to control apoptosis [14]. p53 plays an essential role in regulating apoptosis of IAV-infected cells [9]. We therefore speculated that p53 is one of cellular molecules targeted by IAV to control the apoptosis of infected cells. In this study, we found that NS1 was able to associate with p53 (Fig. 4) and inhibit p53-mediated transcriptional activity (Figs. 1 and 2) and apoptosis (Fig. 3), suggesting that p53 is a target molecule for IAV to control the apoptosis of infected cells.

The p53 inhibitory domain of NS1 was located between amino acids 144 and 188. Deletion of this domain (Flag-NS1-Δ144/188) resulted in the inability of NS1 to inhibit p53-mediated transcriptional activity and apoptosis (Figs. 1–3). However, the p53 inhibitory domain alone (Flag-NS1-144/188) had no such inhibitory effects, suggesting that the p53 inhibitory domain requires additional region(s), presumably a binding domain(s), to cooperatively exert its inhibitory function. This view was further supported by the observation that the deletion mutant Flag-NS1-Δ144/188, which was unable to inhibit p53-mediated transcriptional activity and apoptosis, was able to associate with p53 (Fig. 4).

The role of apoptosis in the replication of IAV is contradictory [15–17]. An intriguing hypothesis is that appropriately timed

apoptosis controlled by IAV is important for efficient viral replication [17]. Although we found that NS1 associated with p53 and inhibited p53-mediated transcriptional activity and apoptosis, the transcriptional activity of p53 in IAV-infected cells is only transiently decreased at the early phase of viral infection, which is timely correlated with the onset of NS1 expression, and then increased again regardless of the expression of NS1 [10]. These observations indicate the complexity of regulation of p53 activity during IAV infection. Indeed, we have observed that the nucleoprotein of IAV, which is expressed later than NS1, competes with NS1 to enhance p53 activity (our unpublished data).

In addition to regulating the apoptosis of infected cells, NS1 also plays an important role in counteracting the interferon (IFN)-mediated antiviral response [3]. The biological significance of p53 targeting by NS1 is probably not limited to the inhibition of apoptosis, but may also contribute towards impairing host innate antiviral responses because the activated p53 in IAV-infected cells is able to transactivate IFN regulatory factor 9, a central component of the IFN-stimulated gene factor 3 complex [10], and is necessary for activation of the IFN-stimulated response element [9].

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