Alleles A and B of non-structural protein 1 of avian influenza A viruses differentially inhibit beta interferon production in human and mink lung cells

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Non-structural protein 1 (NS1) counteracts the production of host type I interferons (IFN-α/β) for the efficient replication and pathogenicity of influenza A viruses. Here, we reveal another dimension of the NS1 protein of avian influenza A viruses in suppressing IFN-β production in cultured cell lines. We found that allele A NS1 proteins of H6N8 and H4N6 have a strong capacity to inhibit the activation of IFN-β production, compared with allele B from corresponding subtypes, as measured by IFN stimulatory response element (ISRE) promoter activation, IFN-β mRNA transcription and IFN-β protein expression. Furthermore, the ability to suppress IFN-β promoter activation was mapped to the C-terminal effector domain (ED), while the RNA-binding domain (RBD) alone was unable to suppress IFN-β promoter activation. Chimeric studies indicated that when the RBD of allele A was fused to the ED of allele B, it was a strong inhibitor of IFN-β promoter activity. This shows that well-matched ED and RBD are crucial for the function of the NS1 protein and that the RBD could be one possible cause for this differential IFN-β inhibition. Notably, mutagenesis studies indicated that the F103Y and Y103F substitutions in alleles A and B, respectively, do not influence the ISRE promoter activation. Apart from dsRNA signalling, differences were observed in the expression pattern of NS1 in transfected human and mink lung cells. This study therefore expands the versatile nature of the NS1 protein in inhibiting IFN responses at multiple levels, by demonstrating for the first time that it occurs in a manner dependent on allele type.

INTRODUCTION

Type I interferons (IFN-α and -β) establish a first line of defence and are initiated within hours of viral and eukaryotic host cell interaction. Intracellular dsRNA generated either as an intermediate product during viral replication or as synthetic dsRNA (poly I: C) introduced into the cell exogenously, is considered a proximal inducer of type I IFNs. The binding of dsRNA to helicases [retinoic-acid-inducible protein 1 (RIG-I)/melanoma differentiation-associated protein 5 (Mda5)] or Toll-like receptors (TLR), initiates a series of events culminating in the activation of interferon regulatory factors 3 and 7 (IRF3 and IRF7) and nuclear factor-κB (NF-κB) transcription factors (Hornung et al., 2006). Activated IRF3, IRF7 and NF-κB induce the transcription of IFN-α and IFN-β which then initiate a positive feedback-production loop by binding to the IFN-α/β receptor (IFNAR), leading to the transcription of more than 300 IFN-stimulated genes (ISGs), which are characterized by an IFN stimulatory response element (ISRE) (Randall & Goodbourn, 2008). Despite the fact that eukaryotes have type I IFN as an inducer of an efficient antiviral system, viruses have also evolved strategies to counteract IFN actions for their successful replication. The non-structural protein 1 (NS1) of influenza A viruses is one well-characterized example of a multifunctional protein with IFN-antagonistic actions.

The NS1 protein is composed of an N-terminal RNA-binding domain (RBD, aa 1–73), which is known to interact with RNAs of several species, and a C-terminal effector domain (ED, aa 74–230), which primarily mediates interactions with cellular proteins but also facilitates stabilization of the RBD (Hale et al., 2008). Both of these domains are involved in the regulation of various cellular activities in infected cells, of which mediation of IFN-antagonistic
actions are well established and studied. Arginine at position 38 and lysine at position 41, in the N-terminal RBD, determine the binding capacity of NS1 to dsRNA and, ultimately, its sequestration. A polyubiquitination is required for the interaction of RIG-I with its downstream partner, mitochondrial antiviral signalling protein, which is induced by the ubiquitin E3 ligase tripartite motif containing protein 25 (TRIM25). Recently, it has been reported that NS1 interacts with TRIM25, leading to inhibition of RIG-I sensing (Gack et al., 2009; Munir, 2010) and hence blocking downstream signalling that leads to IFN-β production.

The C-terminal ED of the NS1 protein suppresses the 3’ end processing of pre-mRNA by binding with two proteins called cleavage and polyadenylation specificity factor (CPSF30) and poly(A)-binding protein (PAB) II, which are otherwise required for the post-transcriptional modifications of pre-mRNA. Inhibition of 3’ end processing leads to nuclear retention of cellular mRNAs including the IFN-β mRNA (Fortes et al., 1994). A stretch of residues from 81 to 113 in the ED forms a trimeric complex with the contribution of two cellular proteins: eIF4G1 and PABI. This complex recruits the eIF4F translation initiation factor and stimulates the translation of viral mRNA (Burgui et al., 2003).

The NS1 protein has been categorized into two distinct gene pools based on putative amino acid sequences of the influenza A viruses NS gene, which were initially referred to as alleles A and B (Treanor et al., 1989). A few years later, in an attempt to study the evolutionary trend between alleles, Ludwig et al. (1991) revealed, based on available sequences, that allele B exclusively contains the strains from avian influenza viruses, while allele A comprises strains from both avian and mammalian (human, equine and swine) origin with one exception. A/equine/Jilin/1/1989/H3N8 was found to belong to allele B despite being a mammalian virus (Guo et al., 1992). We have recently noted that a virus originating from swine (A/Swine/Saskatchewan/18789/2002/H1N1) also belongs to allele B (Zohari et al., 2008b). It is interesting to observe that the equine virus caused high mortality and was transmitted to a huge number of horses in China, while the virus that caused disease in swine was isolated from a pig in a farm without spreading to other pigs. The transmission of both these viruses is believed to have originated from an avian source since all the eight genome segments are avian-like. Consistent with the higher evolutionary rate of mammalian than avian influenza viruses, it was observed that the NS1 proteins from allele A, but not from allele B undergo continuous selection pressure (Ludwig et al., 1991). Recently, a computational study indicated that human viruses are more closely related to NS genes of allele A than of allele B (Chen et al., 2006). Further, it has been demonstrated that an avian virus (A/FPV/Rostock/34, H7N1) containing an allele A NS1 replicates efficiently in mammalian cells, while reassortants containing an allele B NS gene were shown to replicate poorly in the upper and lower respiratory tract of squirrel monkeys (Treanor et al., 1989). However, recently Ma et al. (2010) have shown that A/FPV/Rostock/34 (H7N1), if carrying allele B of A/Goose/Guangdong/96 (H5N1), had infectivity in mice and replicated more efficiently in human and mouse cell lines than wild-type H7N1. These examples clearly dictate that the NS1 protein can act as an essential determinant of influenza virus pathogenesis in a species-specific manner. Recently, we provided evidence and established a concept that NS1s from two influenza viruses, one representing allele A [(A/mink/Sweden/84 (H10N4)] and the other allele B [(A/chicken/Germany/N/49 (H10N7)] differ in regulation of IFN-β production (Zohari et al., 2010a).

Taken together and given that allele A NS1 protein differs from allele B NS1 protein by over 30% of their amino acids, it is plausible to hypothesize that the level of virulence and pathogenicity of influenza A viruses in mammalian species might be allele dependent. In line with this, we have already reported that highly pathogenic avian influenza viruses of human origin mainly contain allele A NS1 protein (Zohari et al., 2008a). The present study focuses on the structural and functional correlations between NS1 proteins of alleles A and B avian influenza viruses to circumvent the host immune response. In particular, we were interested in determining if influenza NS1 proteins of alleles A and B may differ in their ability to inhibit IFN-β production.

## RESULTS

### Inhibition of poly I:C induced activation of IFN-β promoter is more effective by allele A than allele B NS1 protein

An initial screening of several NS1s from various avian influenza isolates was carried out to investigate whether NS1 inhibition of IFN-β activation is allele dependent, by using an ISRE reporter. For this purpose, we transfected the cells with pISRE-Luc reporter plasmid and stimulated with poly I:C, as an evaluation system. The NS1 proteins from all influenza isolates was carried out to investigate whether NS1 inhibition of IFN-β activation is allele dependent, by using an ISRE reporter. For this purpose, we transfected the cells with pISRE-Luc reporter plasmid and stimulated with poly I:C, as an evaluation system. The NS1 proteins from all influenza viruses in this screening, except for PR8, inhibited the ISRE-induced luciferase expression in human A549 cells compared with ISRE control at variable levels (Fig. 1). Allele A NS1 proteins significantly inhibited ISRE promoter activation, which ranged from 58 to 87% and based on the level of inhibition can clearly be differentiated from allele B influenza viruses.

Analysis of the amino acid sequences of the NS1 proteins indicated that allele A NS1s differed by about 30% of amino acids from that of allele B, including the presence of substitution at two critical regions (Fig. 2): aa position 221 (K in allele A and Y in allele B) at the C terminus, which is crucial for nuclear localization signal (NLS) and nucleolar localization signal (NoLS) (Han et al., 2010), and aa position 103 (F in allele A and Y in allele B) that has been characterized as critical for binding to CPSF30 (Kochs et al., 2007). Since both of these substitutions were found to be located in functionally critical regions of NS1, it was of interest to find out whether they could contribute to phenotypical differences between alleles A and B.
Two subtypes (H6N8 and H4N6), each comprised of isolates having both alleles A and B group of NS1 protein, were chosen for further analysis. These viruses were identical regarding other viral proteins (with few amino acid differences at uncharacterized positions) except for the NS gene segment. The level of inhibition of the ISRE-containing promoter was significantly higher in allele A (77% for H6N8 and 71% for H4N6) compared with allele B ($P<0.05$) (Fig. 3a) and this trend appeared to be dose dependent (data not shown). In line with a previous study (Hayman et al., 2006), an exception was observed with the PR8 NS1 (allele A) that was unable to inhibit the ISRE-containing promoter. On the contrary, the ISRE promoter was stimulated in the presence of PR8 NS1 in A549 cells (Fig. 3a).

Next, we thought to test the sensitivity of the system in natural influenza virus infection. After 24 h of transfection with pISRE reporter plasmid, A549 cells were infected with influenza viruses (both alleles of H6N8 and H4N6 along with PR8) at an m.o.i. of 5. After 10 h of infection, luciferase was extracted and measured. The trend between alleles A and B influenza viruses was comparable with the observations made with NS1 transfection system in A549 cells (Fig. 3b). However, contrary to this system, allele B influenza viruses from both subtypes showed a profound inhibition (50% in H6N8 and 49% in H4N6). Notably, PR8 also showed a comparable inhibition of ISRE promoter activity under viral infection as reported earlier (Hayman et al., 2006).

The results of the reporter assay proposed that NS1 proteins are involved in differential blocking of signal transduction pathways that lead to activation of the ISRE promoters. Considering that these roles might be species specific, we further examined these pathways in mink lung cells (MiLu). Contrary to A549 cells, both alleles were consistently better in blocking the induction of ISRE promoter in MiLu where allele B showed an inhibition of 60 and 58% in H6N8 and H4N6 subtype of influenza virus, respectively (Fig. 3c), and allele A showed an inhibition of 97 and 96% in H6N8 and H4N6, respectively.

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**Fig. 1.** Blocking of IFN induction by NS1 proteins from several avian influenza A viruses. A549 cells were co-transfected with the pISRE signalling plasmid and NS1 expression plasmid of different influenza viruses for 24 h. Luciferase activity was measured 24 h post-stimulation with poly I:C. The data show the relative luciferase units (RLUs) ± SD of three independent experiments performed in duplicate. Allele A NS1s from different isolates showed significantly higher inhibition of ISRE promoter compared with allele B NS1s, *P<0.05* (Student’s t-test). NS1 proteins used for further analysis are represented with white bars, and NS1 proteins from other viruses are shown with grey bars.

**Fig. 2.** Alignment of the amino acid sequence for the isolates used in this study. Left panel: a total of 71 (~30%) amino acids differ in both the RBD and ED. Box indicates the point of mutation while the NLS1 (aa 34–38), NES+ regulatory sequence (aa 134–161) and NLS2 (aa 216–230) are underlined. Up headed arrow indicates the NS1 chimeras’s break points. Right panel: the structures of both alleles A and B of H6N8 were predicted using I-TASSER based on multiple-threading alignments, as described previously (Roy et al., 2010) and aligned in MacPymole. The amino acids having similar properties were assigned the same colour.
Notably, under influenza virus infection, allele A of both isolates completely blocked the ISRE promoter activity in MiLu cells, while allele B NS1s were weak in this inhibition compared with allele A (Fig. 3d). These results validate our system although we had the question at this moment whether the differential signalling was because of a difference in ISRE transcription or due to the difference in IFN production, up- or downregulated by NS1.

**Allele A NS1 proteins of H6N8 and H4N6 suppress the IFN-β mRNA transcription and IFN-β protein secretion more than allele B NS1 proteins**

To find out whether the difference in IFN-β promoter induction is due to a difference in endogenous IFN-β synthesis and its secretion, we applied an ELISA to measure the level of expressed IFN-β protein and real-time PCR to measure the level of transcribed mRNA for the IFN-β gene. Cell culture supernatants containing expressed IFN-β protein were collected from NS1-transfected A549 cells at 0, 2, 4, 8, 16 and 24 h post-poly I:C stimulation and were subjected to ELISA. The results showed that cells expressing allele A-type NS1 were poorer IFN-β producers than allele B (Fig. 4a). Further, the peak of IFN production was observed at 16 h of stimulation and then it plateaued for the rest of the experiment, which was consistent with the results of the ISRE luciferase assay at the same time points (Fig. 4b). These results further strengthen the validity of the poly I:C stimulation system as a reliable method to mimic viral infection. Here, we were able to conclude that the difference between alleles A and B was due to a difference in the mechanism(s) involved in IFN production.
in the presence of NS1 and not due to signalling from the IFN receptors.

Next, the qRT-PCR analysis clarified that the difference in IFN-β protein expression was due to a difference in the IFN-β mRNA transcription. We observed a 1.3- and 2.2-fold increase in the level of IFN-β mRNA for alleles A and B in H6N8 and a 1.1- and 2.6-fold increase for alleles A and B in H4N6 (Fig. 4c). These results indicated that the underlying difference resides in differential NS1 suppression of IFN induction (pre-transcription IFN suppression).

**Homogeneous expression and accumulation of alleles A and B NS1 proteins in A549 cells**

In order to clarify that the observed differences were not caused by insufficient expression, the level of expressed NS1 proteins was evaluated. A549 cells were transfected with NS1 constructs from both H6N8 and H4N6 isolates including the NS1 construct from PR8. The cells were lysed 18 h post-transfection and Western blotting was performed. The NS1 proteins from all isolates were expressed in high quantity and the level of allele A NS1 was comparable to NS1 protein of allele B (Fig. 5a). We further used a different and highly sensitive technique to quantify the level of accumulation of NS1 in transfected cells.

Consistent with the blotting, the in situ proximity ligation assay (PLA) showed that the expressed protein was homogeneously accumulated in the cytoplasm and nucleus (Fig. 5b) and there was no detectable difference between alleles in terms of NS1 production as measured by the quantification of expressed NS1 proteins using BlobFinder software (Fig. 5c). Thus, the results indicated that the low inhibition of IFN-β induction in the presence of allele B NS1 protein was not due to a difference in allele B NS1 protein expression and accumulation in the cells.

**Alleles A and B NS1 proteins localized differentially in subcellular compartments**

We next asked whether the difference in the timing of nuclear localization of NS1 proteins influence their functional properties since the concern of nuclear localization pattern-dependent functional differences has recently been highlighted (Han et al., 2010; Volmer et al., 2010). For this reason, A549 and MiLu cells were transfected with NS1 construct of both alleles of H6N8 and H4N6 and were fixed at 2, 4, 6, 12 and 18 h post-transfection and incubated with specific anti-NS1 antibodies. Analysis by in situ PLA showed that the NS1 protein was not readily detectable at 2 h post-transfection for any of the isolates including PR8 NS1 (data not shown).
shown). From 6 h onwards, NS1 protein formed clear and characteristic accumulation spots in the nucleus and cytoplasm of cells transfected with both alleles and PR8 (Fig. 6 and data not shown). In fact, these spots were visible by 4 h in allele A H6N8 and in PR8, in contrast to allele B H6N8. Generally, NS1 appeared to be consistently spread throughout the nucleus and cytoplasm at 6 h post-transfection and afterwards, albeit the initial difference at 4 h post-transfection. In MiLu, the nuclear accumulation of NS1 was comparatively delayed and localization was observed at 6 h of transfection without any apparent difference between isolates (Fig. 6). Interestingly, NS1 protein localization in MiLu substantially differed from the patterns observed in human A549 cells. In MiLu, NS1 was observed only in the nucleus especially at late time points of 12 and 18 h (data not shown), whereas in human A549 cells it was localized both in the cytoplasm and the nucleus from 4 h post-transfection and onwards. These results showed that the cellular protein transport machineries of diverse cell types differentially regulate the localization signals of the NS1 protein.
The F103Y and Y103F substitutions of NS1 proteins do not appear to influence alleles A and B differential IFN-β production

Out of the isolates tested in this study, allele A NS1 carried amino acids Phe103 and Met106, whereas allele B NS1 possessed Tyr103 instead (Fig. 2). Therefore, to test the contribution of the amino acid at position 103 in post-transcriptional pre-mRNA processing, we exchanged this position in allele A with allele B (Phe103 to Tyr103) and vice versa for allele B. The luciferase activity evaluated for the ISRE promoter in A549 cells transfected with mutant clones did not differ significantly from the wild-type, though weak variation was observed in A549 cells (Fig. 7a). Similarly, in MiLu cells these substitutions also non-significantly altered the ISRE promoter activity (Fig. 7b).

Characterization of domains for differential blocking of IFN induction

To sort out the genetic basis of the difference between allele A and B NS1 proteins in blocking IFN production, we engineered chimeric NS1 proteins, which carry the RBD of allele A and the ED from allele B (H6N8 chiNS1 A/B) and vice versa (H6N8 chiNS1 B/A). We also constructed plasmids carrying either the RBD or ED from alleles A and B of strain H6N8 (a schematic presentation is shown in Fig. 8a). Results based on IFN-induced ISRE gene expression in both A549 and MiLu cells indicated that the ability to block IFN-induced ISRE signalling is mapped into the ED, whereas the RBD alone is unable to block ISRE promoter activity (Fig. 8b, c). Surprisingly, the chimeric NS1 (H6N8 chiNS1 A/B) showed higher inhibition of ISRE gene expression in relation to the wild-type and H6N8 chiNS1 B/A in A549 and MiLu (Fig. 8b, c). These results indicated that the RBD of allele A in combination with the ED of allele B is a potent inhibitor of IFN-induced ISRE gene expression.

DISCUSSION

In the present study, we observed the anti-IFN properties of NS1 by comparing the capacities of alleles A and B NS1 proteins from various avian influenza strains. We have previously provided further evidence for the essential role of NS1 in the early stages of influenza virus infection in mammalian cells where expression of NS1 from two influenza viruses, one representing allele A [A/mink/Sweden/84 (H10N4)] and the other allele B [A/chicken/Germany/N/49 (H10N7)], appeared to have contributed to the virulence of the virus in mink by helping the virus to evade the innate immune responses (Berg et al., 1990; Zohari et al., 2010a, b).

In line with our previous report, here we confirmed that NS1 proteins from allele A of H6N8 and H4N6 isolates show enhanced ability to inhibit the IFN-β production compared with allele B, and we attempted to study the underlying mechanism at the molecular level. A comparison of putative amino acid sequences revealed a difference of 71 aa (~31 %) between alleles A and B of both isolates. Most of the previously characterized and important amino acids for the functionality of NS1 were found to be identical between NS1 from alleles A and B of both strains and could therefore not explain the discrepancies (Hale et al., 2008; Li et al., 2001, 2006; Nemeroff et al., 1998; Wang et al., 1999; Zohari et al., 2008b). In this study, we used a Firefly luciferase reporter gene under the control of the IFN-β and poly I:C stimulation as a model system. This system is based on the functional interference of the NS1 protein, excluding the possible involvement of other viral proteins in IFN-β inhibition as has recently been demonstrated for PB2 of influenza A viruses infection (Graef et al., 2010; Iwai et al., 2010).

Using a model system, we were able to demonstrate that there were significant differences between NS1s of different strains of influenza A viruses where allele A but not allele B NS1 efficiently suppressed the activation of the IFN-β promoter and IFN-β gene expression. It has previously been shown that the ED of NS1 is not only responsible for the interaction with cellular proteins but is also essential for the stability and dimerization of the protein (Donelan et al., 2003; Guo et al., 2007; Hale et al., 2008; Kochs et al., 2007; Wang et al., 2002). Consistent with this, we also observed that the ED, either alone or in fusion with the RBD, was able to inhibit IFN-induced ISRE gene expression.
expression (Hayman et al., 2006). Since the dimerization of the NS1 protein is required for the functionality of the RBD, it was not surprising that the RBD alone was completely non-functional in terms of inhibition of IFN-β production. This demonstrated that the two domains are probably functionally interactive in a co-operative manner to result in their overall IFN antagonism properties. It is also likely that the difference between the two NS1 proteins influences the three-dimensional structure and dimerization of the NS1 protein, which affects the function of NS1 in the suppression of IFN-β promoter activation. Notably, the RBD contains a difference of eight continuous amino acids (21RFADQELG28 and 21LLSMRDMC28) between alleles A and B NS1 proteins and the difference between the ED of alleles A and B was observed to be 44 aa (28%). These amino acid differences most likely reflect differences in the respective protein function and would be interesting to study in terms of the biological significance in the context of viral infections.

We have confirmed that alleles A and B differ in their abilities in IFN-β protein synthesis, IFN-β mRNA transcription and ISRE promoter activation. However, IFN responsiveness in influenza virus infection is a polygenic trait and NS1 counteracts IFN expression through different ways at multiple steps. It has been recently demonstrated that the expression of NS1 induces upregulation of the suppressor of cytokine signalling (SOCS)1 and SOCS3, which are potent janus kinase/signal transducer and activator of transcription protein (JAK/STAT) inhibitors (Jia et al., 2010). Since alleles A and B differ in the suppression of the ISRE promoter, it is plausible to hypothesize that this difference is due to the interactions and inhibition of the JAK/STAT pathway, downstream to IFN receptors, which might reflect the additional effects of the immune evasion potential of different NS1s. The biological implications and the mechanisms underlying the differences between alleles A and B NS1 proteins in counteracting the IFN-α/β response warrant further investigation.

The amino acid sequence also revealed that both alleles differ at aa site 103, which is characterized as essential for the interaction of NS1 with CPSF30 (Kochs et al., 2007). Tyrosine (Y) has been seen in allele B of H6N8 and H4N6 at position 103 and phenylalanine (F) in allele A of both isolates. Consistent with a previous report (Twu et al., 2006), reversal of amino acid at this position did not change the abilities of any of the NS1s to inhibit IFN-β induction.

All of the NS1s in this study have inhibited the activation of the ISRE promoter with an exception of PR8 (PR8/34), for which the effect was not evident when NS1 proteins were either exogenously expressed (this study) or in the context of viral infection (Hayman et al., 2006). A/PR8/34 is a well-studied virus and considered a prototype for influenza

![Fig. 8.](https://example.com/fig8.png)
virus (Kochs et al., 2007). It is conceivable from our functional comparison of five different NS1 molecules that the abilities of alleles A and B NS1s to inhibit IFN production can be compared with the A/PR8/34 NS1 as a positive control since the role of A/PR8/34 NS1 in our study is in accordance with the results demonstrated in other studies (Hayman et al., 2006; Kochs et al., 2007).

Recently, two reports have highlighted the correlation between functionality and nuclear localization of NS1. One report demonstrated that the same NS1 showed differential nuclear localization patterns in a species-specific manner (Volmer et al., 2010). Another report demonstrated that aa 221 in the C terminus was crucial for NLS and NoLS (Han et al., 2010). Sequence analysis of all NS1s in this study revealed that allele A of both H6N8 and H4N6 strains has lysine (K), while allele B has tyrosine (Y), whereas PR8 has glutamic acid (E) at position 221, apart from other differences in both NES and NLS. It is likely that the observed earlier nuclear localization of allele A NS1 protein might interfere with the processing and export of newly synthesized mRNA. A similar pattern has been observed for A/Udorn/72 and A/Victoria/3/75, to which allele A shows high sequence similarity (Hayman et al., 2006; Qian et al., 1994). It is further demonstrated that accumulation of A/Victoria/3/75 NS1 in the nucleus of COS-1 cells results in the nuclear retention of mRNA (Fortes et al., 1994; Portela et al., 1985).

In conclusion, functional and structural studies comparing IFN-β responses of alleles A and B NS1 proteins suggest a possible correlation with the virus's pathogenic and zoonotic potential. Since allele A, contrary to allele B of avian influenza viruses, is under continuous selection pressure to mutate, it is plausible that an IFN-abrogating activity associated with the virus could help to prevent high levels of type I IFNs, expected to be triggered by active virus replication. In this respect, NS1 appears to represent a unique case in influenza virus evolution and epidemiology where allele B can be an archaic edition of the NS1 protein and upon switching host to mammalian species, may change to allele A under strong selection pressure. Nevertheless, we further presented that NS1 proteins of low pathogenic avian influenza virus are potent inhibitors of IFN-β as an early anti-IFN response in mammalian cells. The comparatively different behaviour of PR8 from other strains was confirmed to be unsurprising because of its extensive adaptation through passages in eggs and mice over many years. Our study therefore highlights the versatile nature of NS1 in inhibiting the IFN responses. To our knowledge, it is the first time that such an evaluation has been done focusing on allele contribution in NS1-dependent inhibition of IFN.

METHODS

**NS1 expression and reporter plasmids.** Five isolates, designated A/mallard/Sw/412/05 (GenBank accession no. EU518721) allele A (H6N8-A), A/mallard/Sw/418/05 (GenBank accession no. EU518722) allele B (H6N8-B), A/mallard/Sw/795/05 (GenBank accession no. EU518757) allele A (H4N6-A), A/mallard/Sw/795/05 (GenBank accession no. EU518749) allele B (H4N6-B) and A/Puerto Rico/8/34 (GenBank accession no. CY040174) allele A (PR8-A) were thoroughly studied. Other isolates include H4N6 (A/mallard/Sw/754/05), H4N6 (A/mallard/Sw/807/05), H10N8 (A/mallard/Sw/229/03), H4N3 (A/mallard/Sw/825/05), H3N8 (A/mallard/Sw/465/05), H6N8 (A/mallard/Sw/424/05), H6N8 (A/mallard/Sw/796/05). All NS1s were amplified from full-length cDNA of segment 8 using primers NS1-Kpnl-Fw (5’-ATTCGGTACCAGCAGAAACCGGGTCTACAAAGG-3’); and NS1-XbaI-Rev (5’-TAACTCTCTGAGAATTCTAACAGGTTGTTTTTAT-3’). The resulting PCR products were digested with Kpnl and XbaI and cloned into their respective site in the mammalian expression vector pcDNA3.1+ (Invitrogen). Integrity of all the NS1 constructs was checked by X-ray with 32P radiolabelling (TNT T7 Quick Coupled Transcription/Translation System; Promega) and orientation by sequencing.

The Firefly luciferase reporter constructs (pISRE-Luc; Clontech), containing ISRE under the control of IFN-β, were used. The pTA-Luc plasmid containing the Renilla luciferase gene (Clontech) was used as an internal control.

**Generation of mutations and construction of chimeric NS1s.** To create single a amino acid exchange at position 103 (nucleotide position 308) in the NS1 of both alleles A and B, QuickChange Site-Directed Mutagenesis kit (Stratagene) was used with appropriate mutagenesis primers. Using the EcoRI site in the H6N8-A and H6N8-B, NS1 chimeric clones were generated. Clone H6N8 chnNS1 A/B encodes the RBD (aa 1–73) of H6N8-A and ED (aa 74–230) of H6N8-B, while clone H6N8 chiNS1 B/A contains the RBD (aa 1–73) of H6N8-B and the ED (aa 74–230) of the H6N8-A. The RBDs (H6N8-A-RNA and H6N8-B-RNA) and EDs (H6N8-A-ED and H6N8-B-ED) of each allele were cloned between XbaI and Kpnl restriction sites in pcDNA3.1 + .

**Cells, transfections, infections and luciferase assays.** A549 cells, a type II alveolar epithelial cell line from human adenocarcinoma (ATCC, CCL 185), were grown in Dulbecco’s modified Eagle’s Medium (DMEM), while MiLu were maintained in Eagle’s minimum essential medium with non-essential amino acids. These media were supplemented with 10% FBS, 2 mM glutamine, 100 U penicillin ml⁻¹, 100 µg streptomycin ml⁻¹ and incubated at 37 °C and 5% CO₂ in a humidified atmosphere.

Transfections of A549 and MiLu cells were performed using FuGENE6 reagent (Roche) in 24-well plates as instructed by the manufacturer. The day before transfection, 24-well plates were seeded with ~2.5 x 10⁴ cells per well to attain 80% confluency by the next day for transfection. Two hours before transfection the culture medium was changed with fresh medium containing 1% FBS without antibodies. A 0.45 µg quantity of each reporter plasmid and either NS1 expression plasmid or empty pcDNA3.1+ were transfected at a ratio of 1:3 per well. Twenty-four hours post-transfection, the cultures were stimulated by adding 10 µg poly I:C (Invivogen) ml⁻¹ in 100 µl FDMEM without serum or mock-treated. Each transfection experiment was repeated at least three times.

Twenty-four hours post-stimulation, the cells were lysed using One-Glo Luciferase Assay System (Promega) and luminescence was measured in a Varian VICTOR X multilabel counter (Varian Sverige AB), according to the manufacturer’s recommended protocol. For infections, A549 cells were transfected with ISRE reported plasmids for 24 h followed by infection with influenza viruses at an MOI of 5. Firefly luciferase activity was determined in the cell lysates using the luciferase assay system as described above.

**In situ PLA.** PLA was performed with the Duolink in situ PLA kit (Olink Biosciences) as recommended. A 0.15 µg quantity of either
PCR Master Mix kit (Agilent Technologies) as per manufacturer’s instructions. For quantitative measure of the expression level, Slides were visualized under standard immunofluorescence microscopy (Nikon) using suitable filters for Texas red (PLA) and DAPI (nuclear) staining. For quantitative measure of the expression level, slides were incubated in detection solution. followed by amplification of ligated probes using rolling cycle amplification. Finally, the cells were incubated in detection solution. Thereafter, all of the steps were followed as recommended by the manufacturer. Briefly, the probes were hybridized and the ends ligated to form initiation complexes.

Western blot analysis. All of the transfections for Western blot analysis were performed in 24-well plates and Western blotting was performed using primary antibodies raised against the C terminus of NS1 protein [anti-NS1 (ProSci)], as we described before (Zohari et al., 2010a).

Measurement of human IFN-β secretion by ELISA. A549 cells were seeded into six-well plates and after 24 h cells were either mock transfected or transfected with various NS1 expressing plasmids and incubated for the next 24 h. The cell supernatants were collected at 0, 2, 4, 8, 16 and 24 h post-poly I: C stimulation and human IFN-β was quantified using a commercially available VeriKine sandwich ELISA kit (PBL Biomedical Laboratories) according to the manufacturer’s instructions. The IFN titres for the samples were calculated by MasterPlex ReaderFit software.

Quantitative real-time PCR for IFN-β mRNA. A549 cells-seeded six-well plates were either mock treated or transfected with various NS1 expressing plasmids as described above. Twenty-four hours later, cells were stimulated with 10 μg poly I: C and human IFN-β was measured by extracting total RNA and reverse-transcribing using StrataScript RT (Stratagene). Real-time PCR was performed to measure the level of mRNA for IFN-β and β-actin using Brilliant II SYBR Green QRT-PCR Master Mix kit (Agilent Technologies) as per manufacturer’s instructions.

Statistical analysis. Data were analysed using Student’s t-test where P-value ≤ 0.05 indicates a significant difference.

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Recent advancements in influenza virus research have focused on the role of NS1 in regulating the immune response. NS1 is a multifunctional protein that targets the ubiquitin ligase TRIM25 to evade recognition by the host viral RNA sensor RIG-I. The NS1 protein of influenza A virus inhibits the function of intracytoplasmic pathogen sensor, RIG-I. This work was supported by a grant from the Swedish Research Council for the Environment, Agricultural Sciences and Spatial Planning.


