Identification of specific residues in avian influenza A virus NS1 that enhance viral replication and pathogenicity in mammalian systems

Pumaree Kanrai,1† Ahmed Mostafa,1,2 Ramakanth Madhugiri,1 Marcus Lechner,3 Esther Wilk,4 Klaus Schughart,4 Leena Ylösmäki,5 Kalle Saksela,5 John Ziebuhr1 and Stephan Pleschka1

1Institute of Medical Virology, Justus Liebig University Giessen, Schubertstrasse 81, 35392 Giessen, Germany
2Center of Scientific Excellence for Influenza Viruses, National Research Center (NRC), 12311 Dokki, Giza, Egypt
3Department of Pharmaceutical Chemistry, Philipps University Marburg, Marbacher Weg 6, 35037 Marburg, Germany
4Department of Infection Genetics, Helmholtz Centre for Infection Research, Inhoffenstrasse 7, 38124 Braunschweig, Germany
5Department of Virology, University of Helsinki, PO Box 21 (Haartmaninkatu 3) 00014, Finland

Reassortment of their segmented genomes allows influenza A viruses (IAV) to gain new characteristics, which potentially enable them to cross the species barrier and infect new hosts. Improved replication was observed for reassortants of the strictly avian IAV A/FPV/Rostock/34 (FPV, H7N1) containing the NS segment from A/Goose/Guangdong/1/1996 (GD, H5N1), but not for reassortants containing the NS segment of A/Mallard/NL/12/2000 (MA, H7N3). The NS1 of GD and MA differ only in 8 aa positions. Here, we show that efficient replication of FPV-NSGD-derived mutants was linked to the presence of a single substitution (D74N) and more prominently to a triple substitution (P3S+R41K+D74N) in the NS1MA protein. The substitution(s) led to (i) increased virus titres, (ii) larger plaque sizes and (iii) increased levels and faster kinetics of viral mRNA and protein accumulation in mammalian cells. Interestingly, the NS1 substitutions did not affect viral growth characteristics in avian cells. Furthermore, we show that an FPV mutant with N74 in the NS1 (already possessing S3+K41) is able to replicate and cause disease in mice, demonstrating a key role of NS1 in the adaptation of avian IAV to mammalian hosts. Our data suggest that (i) adaptation to mammalian hosts does not necessarily compromise replication in the natural (avian) host and (ii) very few genetic changes may pave the way for zoonotic transmission. The study reinforces the need for close surveillance and characterization of circulating avian IAV to identify genetic signatures that indicate a potential risk for efficient transmission of avian strains to mammalian hosts.

INTRODUCTION

Due to the low fidelity of the viral RNA-dependent RNA polymerase (RdRp), the genomic RNAs of influenza A viruses (IAV) readily accumulate genetic changes and their segmented genome facilitates genetic reassortment upon co-infection of the same host with different IAV (Chen & Deng, 2009; Nelson & Holmes, 2007). As a result, IAV strains with novel properties may emerge at any time and may be associated with high pathogenicity and/or efficient human-to-human transmission. Currently, avian IAV (AIV) strains belonging to the H5 (Vogl et al., 2007), H7 (Fouchier et al., 2004; Gao et al., 2013; Ostrowsky et al., 2012; Tweed et al., 2004) and H9 (Butt et al., 2005; Cheng et al., 2011; Peiris et al., 1999) subtypes are of particular concern because they are highly prevalent in birds and able
to infect humans directly. They may cause severe and even fatal disease but, thus far, have not (or extremely rarely) been transmitted between humans. Nevertheless, by further adaptation or reassortment with other IAV subtypes, specific AIV could gain the ability to transmit more easily between humans causing a major epidemic or even pandemic.

In order to cross existing species barriers between birds and mammals, AIV must acquire adaptive mutations to overcome specific host restrictions and/or optimize virus–host interactions required for efficient viral replication in the new host (Mänz et al., 2013). Several viral proteins have been identified to play important roles in adaptation to new hosts. For example, the viral haemagglutinin (HA) (Chuttinimitkul et al., 2010; Hulse et al., 2004; Matrosovich et al., 2000, 2004; Rogers et al., 1983; Yamada et al., 2006), the NS1 (Hatta et al., 2001; Imai et al., 2010; Seo et al., 2002) and the viral RdRp (for a recent review, see Cauldwell et al., 2014; Mänz et al., 2013).

There is increasing evidence that the NS1 protein is an important factor involved in enhancing IAV polymerase activity in specific hosts. In addition to its many other functions (for a review, see Hale, 2014; Hale et al., 2008), NS1 has been implicated in the regulation of viral RNA synthesis (Falcón et al., 2004; Min et al., 2007; Shimizu et al., 1994; Wang et al., 2010; Wolstenholme et al., 1980), possibly through interactions with the viral ribonucleoprotein (RNP) complex. In line with this, NS1 can be co-immunoprecipitated with specific viral polymerase subunits and nucleocapsid protein (NP) from lysates of infected cells (Marion et al., 1997b) and expression of truncated forms of NS1 affects the production of viral RNA (vRNA), but not of cRNA and viral mRNA in infected cells, suggesting a role of NS1 in the regulation of viral genome replication (Falcón et al., 2004). Furthermore, the NS1 protein of a human IAV was shown to inhibit the antiviral activity of the cellular protein kinase R (PKR) and to modulate the time course of viral RNA synthesis in mammalian cells (for a review, see Hale et al., 2008; Min et al., 2007). In addition, NS1 interacts with CPSF30, as part of a macromolecular complex that also contains the viral polymerase and NP (Kuo & Krug, 2009). Together, these studies suggest a direct interaction between NS1 and the RNP complex. This hypothesis is further supported by data showing that NS segments derived from specific H5N1-type AIV strains in the genetic background of a reassortant AIV (FPV, A/FPV/Rostock/34, H7N1) improve virus reproduction and RNA synthesis in mammalian cells (Wang et al., 2010). In order to determine the specific NS1 subdomains and amino acids residues required to confer this new phenotype, the present study characterized genetically engineered FPV reassortants and establishes a critical role for the amino acids substitutions P3S+R41K+D74N expressed in mutated forms of NS1 in optimizing viral mRNA and protein production in mammalian cells without compromising replication in avian cells. Importantly, we also show that the single mutation, D74N, when introduced in the (wild-type) NS1 protein of FPV already possessing S3S+K41, allows this FPV-NS<sub>WT,D74N</sub> mutant to replicate and cause disease in a mammalian host <i>in vivo</i> (in mice).

Taken together, the data suggest that the amino acids S3+K41+N74 in the NS1 protein may allow (specific strains of) AIV to establish productive infections in mammals. This study adds to our understanding of factors and genetic traits that may indicate changes in host range and, thus, helps to determine potential risks of cross-species transmission and, more generally, to predict potential threats for human and animal health associated with specific circulating AIV strains.

RESULTS

Critical roles of specific NS1 residues for FPV replication in mammalian cells

As reported previously (Wang et al., 2010), the viral replication characteristics in mammalian cells of reassortants AIV A/FPV/Rostock/34 (FPV, H7N1) carrying an NS segment from the highly pathogenic avian influenza virus (HPAIV) strain A/Goose/Guangdong/1/1996 (GD, H5N1) or the low pathogenic avian influenza virus (LPAIV) A/Mallard/NI/12/2000 (MA, H7N3) varied greatly. While the reassortant FPV-NS<sup>G</sup> GD replicated to high titres, FPV-NS<sup>MA</sup> GD replicated significantly less efficiently. By contrast, in avian cell culture systems, both reassortants replicated to similar levels. The results suggested that NS1<sup>G</sup> GD has specific features that contribute to mammalian adaptation of AIV (Wang et al., 2010). The NS segments of GD and MA belong to the same NS allele (allele B) and differ by as few as 8 aa residues (Fig. 1a, available in the online Supplementary Material). To identify specific subdomains in the NS1<sup>G</sup> GD that might be responsible for the superior replication efficiency of FPV-NS<sup>G</sup> GD in mammalian cells, we generated chimeric NS segments cloned into the appropriate vRNA expression vector (s). The two constructs contained the 5’-terminal half of the NS1<sup>G</sup> GD coding sequence fused to the 3’-terminal half of the NS1<sup>MA</sup> coding sequence (pPol-I-NS-GD/MA) and vice versa (pPol-I-NS-MA/GD) (Fig. 1a). We then generated FPV-derived reassortants containing the desired chimeric NS segments, which were used to infect A549 cells (m.o.i. of 0.001) and to determine viral growth characteristics (Fig. 1b). We observed that FPV-NS<sup>G</sup> GD and FPV-NS<sup>G/MA</sup> GD replicate efficiently to similar titres at 6, 24 and 48 h and with a minor difference at 36 h post-infection (p.i.) (P<0.05), while FPV-NS<sup>MA/GD</sup> compared to FPV-NS<sup>G/MA</sup> GD replicated to significantly (P<0.001) lower titres (ca. 2 logs). The differences in virus titres between the two pairs of viruses were consistent with the plaque sizes observed in the mammalian MDCK-II (Madin–Darby canine kidney) cells. While FPV-NS<sup>G/MA</sup> and FPV-NS<sup>G</sup> produced large plaques of similar size in MDCK-II cells, FPV-NS<sup>MA/GD</sup> and FPV-NS<sup>MA</sup> produced significantly smaller plaques (P<0.001) (Fig. 2a, b). Taken together, the data led us to conclude that one or more residue(s) in the N-terminal region of the GD NS1 (S3, K41, N74, M98 and S153) are
Fig. 1. Identification of the functional comparison of NS1-GD and NS1-MA proteins’ replication kinetics. (a) Schematic representation of chimeric NS1 constructs. ‘GD/MA’ contains the N-terminal region of GD (blue) and the C-terminal region of MA (pink), and the chimeric NS1 protein ‘MA/GD’ contains the N-terminal region of MA and the C-terminal region of GD. (b) Replication kinetics of reassortant FPV-NSGD (blue), FPV-NSMA (red), FPV-NSGD/MA (green) and FPV-NSMA/GD (black) expressing different forms of wild-type and chimeric NS1, respectively, in A549 cells. Cell culture supernatants of infected cells (m.o.i. of 0.001) were collected at the indicated time points p.i. and virus titres were determined by focus assay using MDCK-II cells. Virus titres are given as mean±SEM (n=3). Statistical analysis was performed using two-way ANOVA, followed by Bonferroni post hoc test. (c) Cells were infected (m.o.i. of 0.001) with FPV-NSGD (blue), FPV-NSMA (red), FPV-NSMA_D74N (green) and FPV-NSMA_P3S+R41K+D74N (black), respectively. Virus titres at the indicated time points p.i. were determined by focus assays using MDCK-II cells. They are given as mean±SEM (n=3). Statistical analysis was performed using two-way ANOVA, followed by Bonferroni post hoc test.
involved in supporting viral growth of FPV-NS^{GD} and FPV-NS^{GD/MA} in mammalian cells. To our knowledge, none of these residues has previously been identified as a replication/host range-associated determinant (Hatta et al., 2001; Li et al., 2005; Seo et al., 2002; Yamada et al., 2010).

**Effects of amino acid substitutions in the NS1^{MA} N-terminal domain in FPV-NS^{MA} replication in A549 cells**

To further assess the potential role(s) of the 5 aa in FPV-NS^{GD} (S3, K41, N74, M98 and S153) in supporting adaptation to mammals, we generated five constructs, each containing a single codon replacement in the NS1^{MA} coding sequence. Additionally, we produced a set of constructs with two or more amino acid substitutions: P3S+R41K, P3S+R41K+D74N, P3S+R41K+D74N+I98M and P3S+R41K+D74N+I98M+D153S. All nine FPV-NS^{MA}-derived mutants could be rescued, and their growth characteristics were determined in mammalian (human A549) and avian (quail QT6) cells and compared to those of FPV-NS^{GD} and FPV-NS^{MA} (Table S1). We found that the mutant containing the single substitution D74N (FPV-NS^{MA_D74N}) showed improved replication, which was further strongly enhanced by the triple substitution P3S+R41K+D74N in the NS1-MA protein (FPV-NS^{MA_P3S+R41K+D74N}), compared with FPV-NS^{GD} (P<0.05), while most other single or combined substitutions enhanced viral replication in A549 cells to a lesser extent or not at all (Fig. 1, Table S1). Compared to the parental viruses, both FPV-NS^{MA_D74N} and FPV-NS^{MA_P3S+R41K+D74N} replicated with similar efficiencies in QT6 cells, suggesting that the observed ‘adaptation’ to the mammalian system in the two mutants did not compromise their replication in avian cells (Figs 1c and 2b, Table S1). Furthermore, we studied the replication kinetics of FPV-NS^{GD}, FPV-NS^{MA}, FPV-NS^{MA_D74N} and FPV-NS^{MA_P3S+R41K+D74N} in Calu-3 (mammalian, human), MDCK (mammalian, dog) and LMH (avian, chicken) cells. The combined data revealed statistically significant growth differences at 36 h p.i. between FPV-NS^{MA} compared with the other three viruses [FPV-NS^{GD} (P<0.001), FPV-NS^{MA_D74N} (P<0.05) and FPV-NS^{MA_P3S+R41K+D74N} (P<0.001)] irrespective of the specific type of mammalian cells used (A549, Calu-3 and MDCK-II). By contrast, there were no statistically significant differences in viral growth between these four viruses in avian cells (LMH and QT6) at all time p.i. (Fig. 1c). To further corroborate our growth kinetics data, we determined plaque sizes produced in MDCK-II cells at 48 h p.i. (Fig. S2a, b). FPV-NS^{MA_P3S+R41K+D74N} produced plaques that were similar in size to those observed for FPV-NS^{GD}, but significantly bigger than those observed for FPV-NS^{MA} (**P<0.001). FPV-NS^{MA_D74N} produced intermediate-size plaques, which were significantly larger compared with FPV-NS^{MA} (**P<0.01), but significantly smaller than those observed for FPV-NS^{GD} (***P<0.001) and FPV-NS^{MA_P3S+R41K+D74N} (***P<0.001). The data suggest that the (combined) presence of three substitutions (P3S+R41K+D74N) in the NS1^{MA} strongly enhances viral replication in mammalian cells, while the presence of the single substitution D74N has clearly detectable (but less profound) effects in this assay.

These results lead us to suggest that the triple substitution P3S+R41K+D74N in the NS1^{MA} protein affects the function of NS1 in a way that ultimately leads to enhanced replication of a representative AIHV in mammalian cells.

**D74N and P3S+R41K+D74N substitutions in the NS1^{MA} increase viral RNA accumulation**

Previous studies implicated NS1 in the regulation of viral RNA synthesis (Falcón et al., 2004; Min et al., 2007; Shimizu et al., 1994; Wang et al., 2010; Wolstenholme et al., 1980) and suggested that NS1 may interact with RNPs in the chromatin fraction of infected cells (Robb et al., 2011). This prompted us to investigate possible effects of NS1 on the activity of the viral polymerase. Therefore, we determined the accumulation of vRNA, cRNA and mRNA of the NP segment in single-cycle replication (m.o.i.=2) by primer extension analysis using total RNA isolated at different time points p.i. from human A549 and avian QT6 cells infected with FPV-NS^{GD}, FPV-NS^{MA}, FPV-NS^{MA_D74N} and FPV-NS^{MA_P3S+R41K+D74N}, respectively. Quantification (Fig. 2a) revealed that, compared with FPV-NS^{GD}, FPV-NS^{MA} showed a delayed production of all viral RNA species in A549 cells. By contrast, in virus mutants carrying single or combined amino acid substitutions in their (MA-derived) NS1 (FPV-NS^{MA_D74N}, FPV-NS^{MA_P3S+R41K+D74N}), levels and kinetics of RNA accumulation were comparable to that of FPV-NS^{GD}. Especially viral mRNA accumulation was up to 6 h p.i. strongly delayed in FPV-NS^{MA}-infected A549 cells compared with cells infected with FPV-NS^{GD} and the two NS-MA-derived mutants (FPV-NS^{MA_D74N}, FPV-NS^{MA_P3S+R41K+D74N}), where viral mRNA became detectable as early as 2 h p.i. and peaked at 4 h p.i. (Fig. 2a, lower panel). Nevertheless, it should be noted that the final level of vRNA produced by FPV-NS^{MA_P3S+R41K+D74N} is lower than the level of FPV-NS^{MA}. Using NA segment-specific primers demonstrated similar RNA accumulation levels, indicating that the effect caused by the different NS segments on the production of RNA species is presumably not segment specific (Fig. S3). Quantification of viral RNA accumulation in the avian QT6 cells revealed less profound differences for vRNA and mRNA production between the four viruses. There was no early peak of viral mRNA with any of the viruses (Fig. 2b, lower panel). Larger differences were observed in cRNA production. Here FPV-NS^{MA_P3S+R41K+D74N} showed the highest level and FPV-NS^{GD} showed the lowest level, while those of FPV-NS^{MA} and FPV-NS^{MA_D74N} were intermediate. Nevertheless, the kinetics showed the same trend of an increase between 2 and 4 h p.i. that flattens off at later time points, resembling the picture in A549 cells.

Taken together, this data leads us to suggest that the respective viral polymerases produce similar levels of mRNA in avian, but not in mammalian cells. NS1^{MA} with the single
(D74N) or the triple substitution (P3S+R41K+D74N) leads to an increase of mRNA accumulation of FPV-NS\textsuperscript{MA} in mammalian (A549) cells, comparable to FPV-NS\textsuperscript{GD}.

D74N and P3S+R41K+D74N in the NS1-MA protein lead to increased viral protein concentrations in mammalian cells

We next asked whether the differences in viral mRNA accumulation translate into different amounts of viral proteins. Cells were infected with either FPV-NS\textsuperscript{GD}, FPV-NS\textsuperscript{MA}, FPV-NS\textsuperscript{MA,D74N} and FPV-NS\textsuperscript{MA,P3S+R41K+D74N}, respectively, and total RNA was isolated at the indicated time points p.i. Viral RNA and 5S rRNA (loading control) were detected using appropriate primers. The accumulation of specific RNA species in the course of infection was quantified (n=3) and is shown in the bottom panels (a.u., arbitrary units). RNA levels were normalized using the 5S rRNA control.

Reconstituted FPV polymerase activity in the presence of different NS1 proteins

We next sought to investigate potential effects of the different NS1 proteins on the viral polymerase activity employing a transient, plasmid-based expression system to measure RdRp activity of \textit{in vitro} reconstituted RNPs (Pleschka \textit{et al.}, 1996) in the presence of co-expressed NS1 variants. Cells were transfected with plasmids to express the (i) FPV-PB1, -PB2, -PA and -NP, (ii) a vRNA-like transcript encoding the CAT (chloramphenicol acetyltransferase) gene (Pleschka \textit{et al.}, 1996) and (iii) the appropriate NS1 protein. Western blot analysis confirmed similar NS1 (NS1\textsubscript{FPV}, NS1\textsubscript{GD}, NS1\textsubscript{MA}, NS1\textsubscript{MA,D74N} and NS1\textsubscript{MA,P3S+R41K+D74N}) expression levels in transfected cells (Fig. 4a). As a marker for viral mRNA synthesis by the viral polymerase, CAT activity was determined (Fig. 4b). The FPV RdRp was found to be significantly more active in human 293T cells that co-expressed NS1\textsubscript{GD} compared with cells that co-expressed wild-type NS1\textsubscript{FPV} (**P<0.01) or NS1\textsubscript{MA} (**P<0.001). Similarly, co-expression of NS1\textsubscript{MA,D74N} resulted in a significant increase in CAT activity as compared with the wild-type NS1\textsubscript{MA} (**P<0.01) and a slight increase compared with the wild-type NS1\textsubscript{FPV} (*P<0.05). Co-expression of NS1\textsubscript{MA,P3S+R41K+D74N} resulted in even higher CAT activity.

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\caption{Viral RNA accumulation. vRNA, mRNA and cRNA levels were determined by primer extension. A representative experiment using an NP segment-specific oligonucleotide is shown. (a) A549 and (b) QT6 cells were infected (m.o.i. of 2) with FPV-NS\textsuperscript{GD}, FPV-NS\textsuperscript{MA}, FPV-NS\textsuperscript{MA,D74N} and FPV-NS\textsuperscript{MA,P3S+R41K+D74N}, respectively, and total RNA was isolated at the indicated time points p.i. Viral RNA and 5S rRNA (loading control) were detected using appropriate primers. The accumulation of specific RNA species in the course of infection was quantified (n=3) and is shown in the bottom panels (a.u., arbitrary units). RNA levels were normalized using the 5S rRNA control.}
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http://jgv.microbiologyresearch.org 2139
compared with NS1<sub>MA</sub> (**P<0.01), wild-type NS1<sub>FPV</sub> (***P<0.001) and surprisingly, even higher compared with NS1-GD (**P<0.01) (Fig. 4).

The data led us to conclude that NS1 variants carrying the single/triple substitution(s) significantly enhance the activity of the avian FPV RdRp in mammalian cells in the absence of other viral proteins, suggesting that specific molecular features of NS1 may affect (directly or indirectly) AIV (FPV)-encoded polymerase activities in mammalian cells.

Prevalence of N74 and/or S3+K41+N74 residues in IAV NS1 proteins

To assess the prevalence of the amino acid residues S3, K41 and N74 in the NS1 of circulating IAV strains, we determined the conservation of these residues in NS sequences (n=32 310) available in the ‘NCBI Influenza Virus Resource Database’ (Bao et al., 2008). N74 and the combination S3+K41+N74 were present in only 0.1–2 % of the currently available sequences of human, avian and other isolates, the only exception being isolates obtained from swine in which
these substitutions were identified in about 10% of all sequences (Fig. 5). Although the number of virus isolates encoding N74 and/or S3+K41+N74 in their NS1 gene was small (1.51%; 488 out of 32310 sequences), this group included viruses with proven record of successful cross-species transmission. For example, swine H1N1 isolates (collected 1976–1979) that had been transmitted from birds to swine, as well as independent cases of the 1997 H5N1–(collected 1976 http://jgv.microbiologyresearch.org 2141–1981), 1997 H7N9–isolates A/Fujian/1/2013, all of which transmitted from birds to humans. Furthermore, in the 2009 pandemic H1N1 virus, representing another example of cross-species transmission from swine to humans. Interestingly, the S3+K41 combination is strongly conserved in human, avian, swine and other IAV (approximately 94–96% of all virus isolates) (Fig. 5), suggesting that, in some cases, the introduction of just one more substitution (N74) may profoundly affect viral replication in mammalian hosts. To further explore this possible scenario, we decided to study potential effects of N74 in the context of an NS1 that already contains S3+K41.

**N74 in NS1 enhances viral RNA synthesis in the FPV wild-type strain**

As shown above (Figs 2 and 3), the single (D74N) or the triple (P3S+R41K+D74N) substitution(s) in the NS1MA (allele B), when introduced into the FPV genetic background, was sufficient to enhance viral mRNA and protein accumulation in mammalian cells. To determine whether this effect is specific for this particular virus or rather reflects a more general mechanism that also applies to other NS1 proteins (allele A or other wild-type AIV NS1), we generated an FPV mutant expressing the cognate NS1 protein with a D74N replacement. Importantly, the NS1 of FPV-WT already carries S3 and K41. Based on our previous experiments, we hypothesized that this mutant may show a phenotype similar to that of FPV–NSWT_P3S+R41K+D74N. As predicted, infection of A549 cells with FPV–NSWT_D74N led to increased viral mRNA and protein levels (Fig. 6a, b) compared with wild-type FPV, while no such differences were observed in QT6 cells infected with these two viruses (Fig. 6b). In line with this, the FPV–NSWT_D74N replicated to significantly higher titres compared with FPV–NSWT in A549 cells at 24 and 36 h p.i. (**P<0.01), while, in QT6 cells, FPV–NSWT and FPV–NSWT_D74N replicated to similar titres (Fig. 6c). The data correspond very well to the data presented above and provide evidence that, in different genetic backgrounds, NS1S3+K41+N74 may support AIV adaptation to mammalian hosts.

**Viral pathogenicity in mice infected with FPV–NSWT_D74N**

Collectively, the in vitro data described above indicate that the combined presence of S3+K41+N74 in the NS1 may help AIV to overcome host restrictions and replicate efficiently in mammalian cells. To validate this hypothesis in an in vivo system, we used the strictly avian FPV–WT, which has a low pathogenicity for mice that survive the infection (Ma et al., 2010), as well as the single substitution mutant FPV–NSWT_D74N in mouse infection experiments. C57BL/6J mice were infected intranasally with 2×10⁴ p.f.u. per mouse of FPV–WT and FPV–NSWT_D74N, respectively, and body weight and survival rate were monitored for 14 days p.i. (Fig. 7a, b). Mice infected with FPV–NSWT_D74N showed a dramatic decrease in body weight of up to 30% at day 8 p.i. and only one mouse survived this infection beyond day 9 p.i. In contrast, FPV–WT caused only a loss of body weight of up to 20% and mice that recovered (9/10), regained...
100% of their initial body weight. The results provide compelling evidence for the critical importance of N74 along with S3+K41 for AIV replication and pathogenicity in mammals.

**N74 in NS1 increases the propagation of a wide range of IAVs**

To confirm and extend our finding we introduced amino acid N74 into the NS1 of other IAVs that are known to (i) have S3 and K41 in their NS1 protein and (ii) replicate poorly in mammalian cells/hosts.

First, we used SC35, a virus that was originally derived from A/Seal/Massachusetts/1/80 (H7N7) by serial passages in chicken embryo cells, thereby acquiring a multi-basic cleavage site in its HA (Li et al., 1990), which rendered the virus 100% lethal for chickens. A549 cells were infected with SC35 (NS allele A) and the mutant SC35-NS\textsubscript{G74N}, respectively, and viral titres and viral RNA accumulation were determined. Infection with SC35-NS\textsubscript{G74N} resulted in a significantly (***P<0.001) increased virus titres at 24 h and 36 h p.i. and increased accumulation for all viral RNA species compared with that of SC35 (Fig. 8a, b). The data again corroborate our conclusion that the residues S3, K41 and N74 in the NS1 strongly increase viral genome replication and transcription, thereby contributing to improved virus propagation in a mammalian host.

Second, we introduced the N74 codon into the NS1 of the 2009 pandemic influenza virus (H1N1pdm09) (Fig. 8c) and a human isolate of an H5N1-type HPAIV (A/Thailand/1 (KAN-1)/2004, KAN-1) with a genetically engineered monobasic HA cleavage site (Fig. 8d), respectively, both of which carrying S3+K41 in their NS1, and analysed the replication characteristics of the resulting mutants in A549 cells. The results demonstrated once again that genetically diverse viruses with S3, K41 and N74 in the NS1 display superior replication kinetics in human A549 cells compared with the parental wild-type viruses that only possess S3+K41.

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**Fig. 6.** Effect of N74 in the NS1 of FPV on the RdRp activity and the virus titre. Effects of the single replacement, D74N, in the FPV NS1 were studied with respect to viral RNA and protein accumulation in infected cells and virus reproduction. (a) A549 and QT6 cells were infected (m.o.i. of 2) with wild-type FPV (FPV-WT) or mutant FPV (FPV-NS\textsubscript{WT,D74N}), respectively. At the indicated time points p.i., vRNA, mRNA, cRNA and 5S rRNA (loading control) levels were determined by primer extension analysis. A representative experiment using an NP segment-specific oligonucleotide is shown. (b) A549 and QT6 cells were infected (m.o.i. of 2) with FPV-WT and FPV-NS\textsubscript{WT,D74N}, respectively. At the indicated time points p.i., total cells lysates were prepared and viral NS1 and NP expression levels were analysed by immunoblotting. Cellular β-actin was used as a loading control. (c) A549 and QT6 cells were infected (m.o.i. of 0.001) with FPV-WT and FPV-NS\textsubscript{WT,D74N}, respectively, and at the indicated time points p.i. virus titres were determined. Virus titres are given as mean±SEM (n=3). Statistical analysis was performed using two-way ANOVA, followed by Bonferroni post hoc test. Significant differences are indicated (*=P<0.05, **=P<0.01, ***=P<0.001, ns= not significant).
DISCUSSION

We have previously shown that the NS segments of the AIVs A/Goose/Guangdong/1/1996 (GD, H5N1) or A/Mallard/NL/12/2000 (MA, H7N3) in the genetic background of the AIV A/FPV/Rostock/34 (FPV, H7N1), affect FPV replication characteristics in mammalian cell culture, but not prominently in avian cell culture (Ma et al., 2010; Wang et al., 2010). The two NS1 proteins (NS1MA, NS1GD) differ at only eight positions (Fig. S1). The replication capacity of these FPV reassortants in mammalian cells critically depends on the NS segment, with FPV-NSGD replicating to titres almost 2 log10 higher compared with FPV-NSMA and producing larger plaques compared with FPV-NSMA (Figs S2a, b, 3a–c). By contrast, in avian cells, FPV-NSGD and FPV-NSMA, which both contain NS segments from avian viruses, replicate to similar titres, suggesting that, in the genetic background of the avian FPV, NS segments from other AIV are generally well tolerated and have similar effects on viral replication (Fig. 1b). A possible link between IAV pathogenicity in mammalian systems and specific NS segments was discussed in another study showing that the human IAV A/WSN/33 (H1N1) containing the NS segment of the 1918 IAV (H1N1) was attenuated in mice compared with the wild-type strain (Basler et al., 2001). It was suggested that the attenuation in mice is related to the avian origin of the 1918 NS1 gene and that interactions of the NS1 with specific host cell factors might play a role in viral pathogenesis. This data suggests that functional effects resulting from NS segment exchanges may be linked to both the host tropism of the donor strain and the specific host system in which the recipient strain is being characterized.

Analysis of recombinant reassortant FPV with chimeric NS variants (Fig. 1a) or mutated NS1MA demonstrated that 5 aa in the N-terminal part of NS1GD may be connected to the enhanced propagation of FPV-NSGD in MDCK-II cells (Figs 1b and S2). We were further able to identify three specific residues (S3, K41 and N74) out of the five that appear to be responsible for adaptation to mammalian hosts (Fig. 1), suggesting a specific role for these residues (and, more generally, the NS1) in the adaptation of AIV to mammalian hosts. Improved viral replication efficiency in mammalian cells was evident from the increased virus titres (Fig. 1) and viral gene expression levels (mRNA and protein synthesis) (Figs 2 and 3). Our studies further revealed that distinct properties of the NS1 may affect the levels and time course of viral RNA (particularly mRNA) accumulation (compare FPV-NSMA and FPV-NSGD in Fig. 2) and there is initial evidence that (some of) these effects may be cell type or host specific (Fig. 1b). The fact that the final level of vRNA produced by the stronger replicating FPV-NSMA_P3S+R41K+D74N (Fig. 2a) was lower than the vRNA level of FPV-NSMA_P3S+R41K+D74N (Fig. 2a), which is reflected in the increased production of viral proteins (Fig. 3a) contributes to the enhanced virus titres. RNP reconstitution experiments in cell culture in the presence of different NS1 proteins further supported this hypothesis as the reporter gene expression by the FPV-WT RdRp activity was greatly increased when the co-expressed NS1MA contained the aforementioned D74N or P3S+R41K+D74N substitutions.
It should be noted that here we applied an adapted transfection protocol, which is less toxic and more sensitive compared with previous experiments that could not demonstrate this effect (Wang et al., 2010). The NS1-dependent increase in transcriptional activity of the transiently expressed RdRp in human 293T cells was consistent with the mRNA and protein accumulation data obtained for virus-infected A549 cells (Figs 2, 3 and 4). Together, these data suggest that NS1<sup>MA</sup> lacks functional properties required for optimal viral RdRp activity of FPV in mammalian cells and that the amino acid residues at positions 3, 41 and 74 affect the RdRp activity, possibly by direct or indirect interactions with other viral and/or cellular factors. This hypothesis is in line with previous studies that have linked the NS1 protein to regulation of viral RNA synthesis and/or interactions with components of the polymerase complex (Falcón et al., 2004; Min et al., 2007; Rameix-Welti et al., 2009; Robb et al., 2011; Shimizu et al., 1994; Wang et al., 2010; Wolstenholme et al., 1980).

It remains to be studied whether the increased protein levels in mammalian cells infected with FPV-NS<sup>MA</sup> variants compared with infection with the parental FPV-NS<sup>MA</sup> (Figs 2 and 3) merely reflect increased viral mRNA levels or whether other mechanisms are involved, for example, an increased translation efficiency and/or stability of viral mRNAs in cells expressing specific NS1 variants. It was shown that the N-terminal half of the NS1 protein is sufficient to enhance viral mRNA translation (Marion et al., 1997a). As the P3S+R41K+D74N substitutions are part of binding domains for RNA and cellular factors involved in translation (e.g. Poly(A)-binding protein, PABP), we cannot exclude that specific interactions with these factors are affected, which may have consequences for viral protein translation and/or stability.

At this stage, it is not clear how the substitutions at positions 3, 41 and 74 affect the structure and the (multiple) functions of the NS1 N-terminal and linker domain to promote efficient IAV replication in mammalian cells. The residue at position 41 is located within the RNA-binding domain (RBD) (Wang et al., 1999). Two basic amino acid residues in this region (R38 and K41) were previously reported to be involved in suppressing host IFN induction and were shown to affect IAV virulence in mice (Donelan et al., 2003). Furthermore, K41 was suggested to be a part of a nuclear localization signal (NLS1) (Melén et al., 2007) and may contribute to a surface that interacts with the NP in

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**Fig. 8.** Effect of N74 in the NS1 of genetically diverse IAV on RdRp activity and the virus titre. (a) A549 cells were infected (m.o.i. of 0.001) with SC35 and SC35-NS<sup>G74N</sup>. At the indicated time points p.i. virus titres were determined. (b) A549 cells were infected (m.o.i. of 2) with SC35 and SC35-NS<sup>G74N</sup>. At the indicated time points p.i., NP-specific vRNA, mRNA and cRNA and 5S rRNA (loading control) were determined by primer extension analysis using appropriate primers. (c) A549 cells were infected (m.o.i. of 0.001) with pH1N1 (A/Giessen/6/2009) or pH1N1-NS<sup>G74N</sup>. (d) A549 cells were infected (m.o.i. of 0.001) with H5N1 (A/Thailand/1(KAN-1/2004) or H5N1-NS<sup>T74N</sup>. At the indicated time points p.i. virus titres were determined. Virus titres are given as mean±SEM (n=3). Statistical analysis was performed using two-way ANOVA, followed by Bonferroni post hoc test. Significant differences are indicated (*=P<0.05, **=P<0.01, ***=P<0.001).
viral RNP complexes, the latter interaction possibly affecting RdRp activity (Robb et al., 2011). Moreover, the N-terminal domain of NS1 was shown to bind to the host RNA helicase DD2X1. This helicase binds PB1, and thereby inhibits RdRp activity. Interactions of NS1 with DD2X1 may release PB1 from this complex, and thereby stimulate viral RNA synthesis and, subsequently, protein expression (Chen et al., 2014). Also, NS1 can interact with RNA helicase A in an RNA-dependent manner involving K41, which supports viral replication (Lin et al., 2012). Residue N74 is located in a linker region (LR) that connects the NS1 N- and C-terminal domains and LR has been implicated in viral replication, pathogenesis and host range (Dundon & Capua, 2009; Li et al., 2011; Long et al., 2008; Seo et al., 2002), probably by controlling the structural flexibility and spatial orientation of the functional (sub)domains in the N- and C-terminal regions of the protein (Carrillo et al., 2014; Hale, 2014).

To our knowledge, there is no information on specific functions of S3, which is part of the RBD. Substitutions may thus affect the RNA-binding properties of NS1. Although, we do not know the structural and functional consequences of the respective NS1 substitutions that confer this significant phenotypic change in mammalian systems, we believe that the three substitutions cause only minor structural changes in NS1, but might affect specific binding sites for viral or cellular RNA and/or proteins or, in the case of D74N, the orientation and spatial flexibility of the two NS1 subdomains. Importantly, these presumed structural changes do not appear to affect NS1 protein functions in avian cells, suggesting that the overall structure is preserved in the triple mutant.

Interestingly, a very high percentage (94–96%) of IAV field isolates encodes NS1 proteins with S3+K41 (Fig. 5). We now show that three genetically diverse viruses (H1N1pdm09, KAN-1 and SC35) that already contain S3+K41 in their NS1 replicate more efficiently in mammalian cells than the parental (wild-type) viruses, when N74 is introduced (Figs 6, 7 and 8), once again illustrating that very few changes in NS1 may result in improved viral replication in mammalian hosts. To further validate this conclusion in vivo we infected mice with wild-type FPV (FPV-WT), which is pathogenic for mice, but already contains S3+K41 in its NS1, as well as with FPV-NS<sup>WT,D74N</sup>, containing the additional D74N substitution (Fig. 7). Our data demonstrate that this single replacement gives rise to an FPV with profoundly increased pathogenicity in vivo.

Notably, N74 not only increases the replication efficiency in mammalian cells of non-adapted AIV (FPV, SC35) but also of ‘human’ IAV (H1N1pdm09, KAN-1) with relatively recent zoonotic transmission to humans (Figs 6c and 8). The four viruses that we studied here in vitro and/or in vivo are not closely related, but share the S3+K41 ‘signature’ in their NS1 protein. FPV (H7N1) was isolated in 1934, A/Seal/Massachusetts/1/80 (H7N7) was originally isolated in 1980 and subsequently adapted to chickens (SC35) in 1990, KAN-1 was isolated in 2004 and H1N1pdm09 was isolated in 2009. In all cases, the introduction of N74 into the NS1 increased the viral replication efficiency in mammalian cells (FPV-NS<sup>WT,D74N</sup>, SC35-NS1<sup>G74N</sup>, H5N1-NS1<sup>T74N</sup> and SOIV-H1N1-NS<sup>S74N</sup>) and the pathogenicity in mice (FPV-NS1<sup>WT,D74N</sup>) (Figs 6, 7 and 8).

The fact that many avian viruses carry S3+K41 in their NS1, but are lacking N74, might be explained in part by the observation that according reassortant viruses did not show a specific selection advantage in avian systems (Figs 1, 2, 3 and 6). This is supported by the circumstance that although the number of virus isolates encoding S3+K41+N74 in their NS1 gene was small, this group included viruses that successfully transmitted to a mammalian host, such as swine H1N1 isolates, which crossed the species barrier from birds to swine in 1976–1979 or from swine to humans causing the 2009 pandemic. Furthermore, independent cases of H5N1 and H7N9 viruses that were transmitted from bird to humans in 1997 and 2013, respectively.

In conclusion, the study adds to our knowledge of genetic traits and single substitutions in specific viral proteins that might indicate changes in host tropism and/or pathology. Interestingly, the NS1 signature S3+K41+N74 that is suggested to be linked to efficient replication in human cells does not appear to have detrimental effects on viral replication in avian systems, suggesting that IAV may evolve specific variants of viral proteins that promote efficient replication in genetically diverse hosts. Following reassortment with other IAV, such variants might greatly facilitate transmission to new (mammalian) hosts because they do not require further adaptation. Extensive global surveillance and genome sequencing programmes established over the past few years, will hopefully provide suitable tools and databases to identify potential risks of zoonotic transmission associated with specific AIV strains.

**METHODS**

**Cells, virus infection, titration and plaque assay.** Human embryonic kidney (HEK) 293T cells (constitutively expressing the SV40 large T antigen), A549 cells (human alveolar epithelial cells) and MDCK-II (Madin–Darbin canine kidney) cells, QT6 (quail fibroblast) cells and LMH (chicken hepatocellular carcinoma) cells were maintained as previously described (Wang et al., 2010). Calu-3 (cultured human airway epithelial) cells were maintained in Eagle’s minimum essential medium (EMEM, Invitrogen) supplemented with 10% FCS (PAA Laboratories GmbH) and antibiotics.

Infections and virus titration (by focus forming assay) were performed as previously described (Ma et al., 2010). Plaque sizes were determined via standard plaque assay. The diameters of plaques were measured for 50 plaques and experiments were done in triplicate.

**Plasmids.** Expression plasmids pcDNA3.1-FPV-PB1, -PB2, -PA and -NP and pHMG-PB1, -PB2, -PA and -NP encode the polymerase and NP proteins of influenza virus A/FPV/Rostock/34 (FPV, H7N1) (Ma et al., 2010) and A/Puerto Rico/8/34 (PR8, H1N1) (Pleschka et al., 1996). The expression plasmids pcDNA3.1-NS1-FPV, pcDNA3.1-NS1-GD and pcDNA3.1-NS1-MA encode the NS1 proteins.
of the influenza virus FPV, A/Goose/Guangdong/1/1996 (GD, H5N1) and A/Mallard/NI/12/2000 (MA, H7N3), respectively (Ma et al., 2010). Further plasmids used were (i) pPol-I-VP2, -PB1, -PA, -HA, -NP, -NA, -M and -NS, expressing the vRNAs of the respective FPV segments (Wagner et al., 2000), (ii) pBD-NS and pPol-I-NS-MA expressing the NS vRNA of GD and MA (Ma et al., 2010), (iii) the pPol-I-NS-GD/MA and -NS-MA/GD plasmids (encoding the N-terminal sequence of the NS1 and the C-terminal sequence of the NS2A segment or, vice versa), (iv) pHW2000-VP2, -PB1, -PA, -HA, -NP, -NA, -M and -NS containing the viral genome sequences of SC35 (H7N7) or A/Thailand/1(KAN-1)/ 2004 (H5N1, KAN-1), with the latter encoding an HA with a monobasic (‘low pathogenicity’) cleavage site as previously described (Auewarakul et al., 2007; Mostafa et al., 2015; Schmier et al., 2015) and (v) pMPcclIP-VP2, -PB1, -PA, -HA, -NP, -NA, -M and -NS containing the viral genome sequences of A/Giesens/6/2009 (H1N1pdm09) (Mostafa et al., 2013). Mutations in pCDNA3.1-NS1-MA, pPol-I-NS-MA, pHW2000-NS1-SC35, -NS1-HSNI and -NS1-H1N1 were generated and confirmed as previously described (Mostafa et al., 2015) using specific oligonucleotide primers (sequences available upon request).

**Generation of recombinant viruses.** Transfection and virus rescue of FPV-NS1<sup>WT</sup>, FPV-NS1<sup>MA</sup> and FPV-NS1<sup>MA</sup> were performed as previously described (Ma et al., 2010; Wang et al., 2010). To generate reassortant FPV-NS1<sup>MA</sup> viruses carrying the desired mutations in the NS segment, the pPol-I-NS-MA plasmid was omitted and replaced by the appropriate pPol-I-NS-MA mutant plasmid. To generate wild-type and mutant reassortants derived from SC35 (H7N7), KAN-1 (H5N1) and H1N1pdm09, we transfected a co-culture of 293T and MDCK-II cells with a mixture of eight plasmids encoding all eight influenza gene segments (Hoffmann et al., 2000).

**SDS-PAGE and Western blot analysis.** Cell lysis and Western blotting were performed as previously described (Pleschka et al., 2001) to detect NP and NS1 the membranes were incubated overnight at 4 °C with monoclonal mouse anti-NP antibody [1:500 dilution (Sigma)] and/or rabbit anti-NS1 serum [1:200 dilution (Sigma)] and subsequently with a 1:1000 dilution of goat anti-mouse and/or goat anti-rabbit serum conjugated to HRP (Santa Cruz Biotechnology) and by standard enhanced chemiluminescence (Fierce, Thermo Scientific). Documentation and quantification was done with Quantity One (Bio-Rad). To analyse NS1 expression in transfected cells, 293T cells (2×10<sup>6</sup> cells (3.5 cm well)<sup>−1</sup>) were grown overnight and transfected in triplicate with 1 µg each of pCDNA3.1-NS1-FPV, -NS1-GD, -NS1-MA, p-NS1-MA<sup>67SN</sup>, -NS1-MA<sup>P35K</sup>, p-NS1<sup>67SN</sup>, -NS1<sup>P35K</sup> or empty vector. Transfection was performed for 8 h as described previously (Mostafa et al., 2013). The cells were harvested 24 h post-transfection, pelleted and subjected to protein extraction as previously described (Mostafa et al., 2015). Total cell extracts were separated by SDS-PAGE and NS1 expression was analysed by Western blotting as described above.

**Reconstitution of the influenza virus polymerase.** To analyse RdRp activity (Wang et al., 2010), nearly confluent 293T cells seeded into 3.5 cm dishes were transiently transfected with TransIT 2020 (2 µl reagent per µg plasmid DNA) as described previously (Mostafa et al., 2013) and a plasmid mixture containing pCDNA3.1-FPV-PB1, -PB2, -PA (0.25 µg each) and -NP expression plasmids (1 µg), pPol-CaRT-RT (2.0 µg) (Pleschka et al., 1996) and pCDNA3.0-NS1 (2.0 µg) encoding wild-type FPV NS1, NS1<sup>67SN</sup>, NS1<sup>P35K</sup> and NS1<sup>MA</sup> variants or empty vector (2.0 µg). At 24 h post-transfection, cell extracts were prepared and tested for chloroamphenicol acetyltransferase (CAT) activity (1:1000 dilution) as described previously (Mostafa et al., 2015).

**Primer extension analysis.** To investigate viral genome replication and transcription primer extension analysis was performed as described previously (Fodor et al., 1998; Wang et al., 2010). Briefly, cells were infected (m.o.i.=2). After the indicated time points p.i., cells were processed as described (Wang et al., 2010). The gene-specific DNA primers used to analyse specific viral RNAs were as follows: NP vRNA (5′-ATGATGAGAGTGCCAGACC-3′, expected size: 181 nt), NP mRNA and cRNA (5′-ACCATCTCCCAACAGATGC-3′, expected size for mRNA: 135 nt, expected size for cRNA: 121 nt). A primer specific for cellular 55 rRNA (5′-TCCACGCGGTCTCCCACTCC-3′) was used as an internal control. RT products were detected and quantified as described previously (Wang et al., 2010).

**Mice infections.** C57BL/6j mice (Janvier, France) were maintained under specific pathogen-free conditions in the biosafety level 3 (BSL3) facility at the Helmholtz Centre for Infection Research, Braunschweig, Germany, according to the German animal welfare law. The protocol used in these experiments had been approved by the appropriate Ethics Committee (Permit Number: 33.9.42502-04-051/09). Female mice (10–12 weeks of age) were anesthetized by intraperitoneal injection with ketamin–xylazine solution [85 % NaCl (0.9 %), 10 % ketamine, 5 % xylazine; 200 µl (20 g body weight)<sup>−1</sup>] and then infected intranasally with a dose of 2×10<sup>4</sup> p.f.u. of the respective virus in 20 µl sterile PBS. A scoring from 1 (morbund) to 6 (very active) including parameters like general condition, activity, behaviour and weight was used for the daily monitoring. Mice with a score of less than 3 were euthanized and recorded as dead. The body weight loss (BWL) together with the scoring very well correlates with other pathological criteria (Dengler et al., 2014). Even though, a BWL of 20 % might not give information about mortality, it is just one criterion amongst others and mice were not killed before 30 % BWL. From our experience this correlates well with mortality. Otherwise, we are restricted by the German animal welfare law, which requires the mice to be killed at 30 % BWL.

**Biosafety.** All experiments with infectious virus were performed according to German regulations for the propagation of influenza viruses. All experiments involving highly pathogenic AI were performed in biosafety level 3 (BSL3) containment laboratories approved for such use by the local authorities.

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