New Virulence Determinants Contribute to the Enhanced Immune Response and Reduced Virulence of an Influenza A Virus A/PR8/34 Variant

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The identification of amino acid motifs responsible for increased virulence and/or transmission of influenza viruses is of enormous importance to predict pathogenicity of upcoming influenza strains. We phenotypically and genotypically compared 2 variants of influenza virus A/PR/8/34 with different passage histories. The analysis revealed differences in virulence due to an altered type I interferon (IFN) induction, as evidenced by experiments using IFNAR−/− mice. Interestingly, these differences were not due to altered functions of the well-known viral IFN antagonists NS1 or PB1-F2. Using reassortant viruses, we showed that differences in the polymerase proteins and nucleoprotein determined the altered virulence. In particular, changes in PB1 and PA contributed to an altered host type I IFN response, indicating IFN antagonistic properties of these proteins. Thus, PB1 and PA appear to harbor previously unknown virulence markers, which may prove helpful in assessing the risk potential of emerging influenza viruses.

Keywords. influenza A virus; virulence; type I interferon; polymerase complex; virulence determinants.

Despite intensive research in the last century, influenza A viruses (IAVs) still represent a severe threat to mankind and are among the most dangerous respiratory pathogens. With 3–5 million hospitalizations and up to 500,000 fatalities annually, as estimated by the World Health Organization, these viruses are not only a devastating burden to global health but also significantly impact the economy. Besides seasonal outbreaks, which are especially dangerous for the elderly and immunocompromised individuals, IAVs hold the potential to cause pandemics with several million victims, as evidenced by outbreaks such as the Spanish flu in 1918–1919 [1]. Although healthcare has dramatically improved since this time, IAV is still discussed as a potential causative agent for upcoming, life-threatening pandemics [2]. Pandemic outbreaks such as the 2009 H1N1 swine flu pandemic and the ongoing incursions into humans of highly pathogenic avian influenza viruses of subtype H5N1 (or recently also H7N9) clearly highlight the need to learn more about the interplay of influenza viruses and their hosts.

Infection with IAV results in an early and strong activation of cellular innate immune responses, dominated by the type I interferon (IFN) system, being the most prominent host antiviral defense mechanism able to restrict viral replication efficiently. Ablation of type I IFN signaling in mouse embryonic fibroblasts (MEFs) derived from IFNAR−/− or STAT1−/− mice results in strongly enhanced replication of IAV, whereas wild-type MEFs are nearly resistant to infection [3, 4]. This early control of viral replication by the type I IFN...
response appears to be a crucial factor for the later outcome of infection. In IAV-infected IFNAR−/− or STAT1−/− mice, the deficiency in type I IFN signaling causes a fulminant systemic infection [3]. For H5N1 IAV, the ability to replicate in extrapulmonary organs is strongly dependent on the early type I IFN response, as even infection with the low-pathogenic H5N1 A/Hong Kong/486/97 (HK/486) strain—primarily restricted to the respiratory tract and generally not lethal—results in a fatal systemic infection in IFNAR-deficient mice [5].

To ensure efficient replication, IAVs have evolved several strategies counteracting these host immune responses. For example, the nonstructural protein 1 (NS1) inhibits induction of the type I IFN response via interaction with RIG-I [6, 7] and TRIM25 [8]. Furthermore, it has recently been shown that NS1 inhibits IFN-induced signaling by inhibiting expression of the type I IFN receptor subunit IFNAR1 and inducing expression of the suppressor of cytokine (SOCS) 3 [9]. In addition, NS1 inhibits cellular proteins with antiviral activities such as protein kinase R [10, 11], blocking cellular messenger RNA (mRNA) processing via binding to CPSF30, [12] but is likewise involved in the enhancement of viral mRNA translation [13].

Beside NS1, PB1-F2 is also described as a type I IFN antagonist that seems to inhibit IFN induction by interfering with the mitochondrial antiviral signaling (MAVS) protein [14, 15]. Additionally, 2 recent studies suggest that, at least in vitro, the viral polymerases, predominantly the PB2 subunit, also inhibit type I IFN induction by interacting with MAVS [16, 17].

In the current study, we discovered new IAV virulence determinants by characterizing a specific variant of strain A/PR/8/34 (PR8), PR8M, which has a different passage history than the wild-type virus PR8WT. Our data demonstrate that the amino acid differences identified within the polymerase proteins and the nucleoprotein (NP) strongly affect virulence and that alterations in PB1 and PA significantly impact the host type I IFN response. These data shed new light on viral strategies for counteracting the type I IFN response and assessing the life-threatening potential of emerging viruses.

MATERIALS AND METHODS

Viruses, Cells, and Infection Conditions

The human recombinant influenza virus A/Puerto-Rico/8/34 (H1N1) (PR8WT) was generated using the pHW2000-based reverse genetic system (a kind gift of Dr Robert G. Webster, Memphis, Tennessee) [18]. The virus variant PR8M was taken from the virus strain collection of the Institute of Virology, Giessen, Germany.

Cultivation and infection procedures for the A549, Madin-Darby canine kidney (MDCK), and DF-1 cells was conducted as described earlier [19, 20]. Normal human bronchial epithelial (NHBE) cells (Lonza) were cultivated following the manufacturer’s protocol.

Generation of Recombinant Influenza Viruses

For generation of recombinant PR8 mutant viruses, the reverse pHW2000-based genetics system was used. The mutations depicted in Table 1 were introduced by site-directed mutagenesis and confirmed by sequencing. Primer sequences will be provided upon request.

Generation of recombinant viruses was performed as described earlier [19]. MDCK cells were used for virus titer determination by plaque assay and for preparation of virus stocks.

Western Blotting and Antibodies

Cell lysis was performed as described earlier [19]. To monitor induction of the type I IFN response, STAT1 phosphorylation was analyzed with a phospho-specific anti-STAT1 (pY701) mouse monoclonal antibody (mAb; BD). Viral proteins were detected by Western blot analysis using an anti-NS1 mouse mAb (clone NS1-23-1; developed at the Institute of Molecular Virology, Muenster, Germany), an anti-M1 mouse mAb (Serotec), and an anti-NP mouse mAb (Serotec). Anti-ERK2 (C-14) rabbit pAb (Santa Cruz) and anti-Stat1 rabbit pAb (BD) were used in Western blot analysis for loading controls. Protein bands were visualized by standard enhanced chemiluminescence reaction.

Standard Plaque Titration Assay

Supernatants of infected cells were collected at the indicated times postinfection and used to quantify the number of infectious virus particles (plaque titers) in the samples as described elsewhere [19].

| Table 1. Sixteen Amino Acid Substitutions Revealed by Genotypic Characterization |
|--------------------------|------------------------|--------------------------|
| Viral Protein | Amino Acid Exchange (PR8WT/PR8M) | |
| PB2 | V560I | |
| PB1 | E398G | S524G |
| | | I563R |
| PB1-F2 | . . . | |
| PA | P275L | E351K |
| | | D682N |
| HA | S123R | G276D |
| | | M288I |
| | | I349F |
| NP | S287N | G349S |
| NA | . . . | |
| M1 | . . . | |
| M2 | . . . | |
| NS1 | K55E | E26G |

New Influenza A Virus Virulence Marker • JID 2014:209 (15 February) • 533
For the luciferase reporter gene assay, A549 cells were transfected with either the pTATA-luc-4x-IRF-3 construct, containing a luciferase reporter gene under the control of 4 copies of the IRF-3 binding PRD1/III motif of the IFN-β promoter [21] or the pG-L–IFN-β–luc construct, which has the luciferase gene controlled by the IFN-β promoter (both 0.15 µg per 12 wells). Twenty-four hours posttransfection, cells were infected with the indicated viruses for 6 hours. Harvesting and measurement of luminescence were performed as described earlier [22].

To generate pcDNA3-NS1, protein expression plasmids of PR8WT (NS1 K55) and PR8M (NS1 E55) and PR8WT- and PR8M-derived NS1 open reading frames were linked to a KOZAK-sequence and an EcoRI restriction site and inserted into pcDNA3. Transfection of DNA was performed as described above. Transfection of RNA was performed with Lipofectamine 2000 following the manufacturer’s protocol.

Quantitative Reverse Transcription Polymerase Chain Reaction

The isolation of total RNA, reverse transcription of mRNA, and quantitative reverse transcription polymerase chain reaction (qRT-PCR) were performed as described earlier [23]. Relative RNA levels were determined after 40 cycles of amplification using the 2-ΔΔCT method [24]. The housekeeping gene gapdh served as an internal standard. The analysis of the relative amounts of the 3 viral RNA species, viral RNA (vRNA), complementary RNA (cRNA), and mRNA, was performed as described in Kawakami et al [25]. All primer sequences will be provided upon request.

Mouse Experiments

Ethics Statement

All mouse studies were performed in compliance with animal welfare regulations (permission numbers Az. 8.87–50.1036.09.007 and 84–02.04.2012.A413 by the LANUV, Germany) or approved by the Animal Care and Use Committee at St Jude Children’s Research Hospital. Six- to 8-week-old BALB/c, C57Bl/6 (both Harlan-Winkelmann), DBA/J2 (Jackson Laboratory), and C57Bl/6 IFNAR−/− (kind gift of Dr Ulrich Kalinke, Hannover, Germany) mice were used for the mouse studies. For infection, the mice were anesthetized by intraperitoneal injection of 250 µL of a 1:1 mixture of 2% xylazin (Ceva) and 10% ketamine (Ceva) diluted 1:10 in phosphate-buffered saline (PBS) or by

Figure 1. The type I interferon (IFN) response is strongly induced upon infection with PR8M. A, A549 cells were infected for 8 hours with PR8WT or PR8M (multiplicity of infection [MOI] as indicated) or left uninfected. Phosphorylated STAT1 (Y701) was detected by Western blot analysis; viral protein synthesis was visualized in M1, NS1, and NP Western blot analysis. Equal amounts of protein were verified using STAT1 and ERK2 antibodies. B, At 24 hours after transfection with pTATA-luc-4xIRF3 (IRF3-luc) or pGL–IFN-β–luc (IFN–β-luc), A549 cells were infected with PR8WT or PR8M (MOI = 5) for 6 hours or were left uninfected, and afterward were harvested and assayed for luciferase activity. Depicted are fold changes compared to uninfected samples. C, A549 cells were infected with PR8WT or PR8M (MOI = 5) for 8 hours or left uninfected. Messenger RNA (mRNA) levels of ifnβ and mxa were measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and depicted as fold changes compared to uninfected samples. D and E, A549 cells were transfected with viral RNA (vRNA) extracted from PR8WT virions or PR8M virions (0.5 µg per 12 wells) or left untransfected. D, After 6 hours’ incubation, phosphorylated STAT1 (Y701) was detected by Western blot analysis. Equal protein amounts were verified using a STAT1 antibody. E, After a 6-hour incubation, the level of IFN-β mRNA was measured by qRT-PCR and depicted as the fold change compared to the uninfected samples.
inhalation of 2.5% isoflurane (Baxter Healthcare). Mice were infected intranasally with the indicated viruses and plaque-forming units (PFU) in a 50-µL volume and monitored daily for disease symptoms, body weight, and survival. Due to animal welfare restrictions, mice were killed upon a body weight loss of 25%.

**Determination of Virus Titers and Cytokine and Chemokine Levels in Mouse Lungs**

Quantification of infectious virus particles in infected mouse lungs was performed as described earlier [23]. For analyzing cytokine and chemokine levels in total mouse lungs, animals were killed at the indicated time points postinfection and lungs were collected in Trizol. For isolation of RNA, a modification of the RNAzol method was used [26]. Reverse transcription and qRT-PCR reactions were again conducted as described in [22].

The housekeeping gene gapdh served as an internal standard. Primer sequences are available upon request.

**RESULTS**

**Strong Induction of the Type I IFN Response Upon Infection With the PR8 Variant PR8M**

A major prerequisite for successful replication of RNA viruses such as influenza is to efficiently circumvent the host type I IFN response. An initial comparison of a recombinant influenza virus strain, A/Puerto Rico/8/34, obtained from St Jude Children’s Research Hospital (PR8WT), with a variant that was independently passaged at the University of Giessen/Germany (PR8M), revealed that PR8M was a much stronger inducer of the type I IFN response.

To confirm our initial observations and unravel the underlying genotypic differences, we first compared both virus variants with regard to induction of the type I IFN response in more detail. As a general readout, we analyzed phosphorylation of STAT1, which is provoked by virally induced and secreted IFN-α and IFN-β via their receptor IFNAR.

Upon infection with the recombinant wild-type virus PR8WT, only a marginal induction of the type I IFN response was detectable. In contrast, infection with PR8M resulted in a strong type I IFN response indicated by phosphorylation of STAT1 in human lung epithelial cells (Figure 1A).

Analyzing the activation of interferon regulatory factor 3 (IRF3) and the transcriptional activity of the IFN-β promoter revealed a clear induction of the type I IFN system in PR8M-infected cells compared to uninfected or PR8WT-infected cells (Figure 1B). Additionally, measurements of the mRNA levels of ifnβ and the interferon-dependent gene (ISG) mxa also illustrated the induction of the host type I IFN response upon PR8M infection (Figure 1C). Interestingly, analysis of stimulatory properties of purified vRNA from PR8WT vs PR8M revealed no differences (Figure 1D and 1E).

**Figure 2.** PR8M exhibits strongly reduced virulence in an in vivo mouse model. A and B, Five Balb/c mice per group were infected intranasally with 10^2.5 plaque-forming units (PFU)/mouse of PR8WT or PR8M. A, Mice were monitored for survival for 15 days. Kaplan–Meier survival curve analysis was performed using the log-rank test. B, Virus titers in infected lungs were determined at 2 and 3 days postinfection (p.i.) by standard plaque titration assay. Each dot represents the result from 1 mouse; gray bars indicate the mean of all mice per group. C, Ten Balb/c mice per group were infected intranasally with 10^2.5 PFU/mouse of PR8WT or PR8M or phosphate-buffered saline (PBS) for control. One and 2 days after infection, total lungs were isolated and the level of interferon (IFN) β messenger RNA (mRNA) was measured using real-time polymerase chain reaction. Depicted are fold changes compared to PBS-inoculated mice. The housekeeping gene gapdh served as internal standard. Each dot represents the result from 1 mouse; gray bars indicate the mean of all mice per group.

These data demonstrate that the type I IFN-inducing potential in host cells is much stronger to PR8M in contrast to PR8WT.
Virulence of PR8\textsubscript{M} Is Reduced in an In Vivo Mouse Model and Dependent on Type I IFN Signaling

To assess virulence and replication fitness of both viruses in vivo, we infected BALB/c mice and monitored body-weight loss and lethality upon infection for 15 days. Using an intranasal infectious dose of $10^{3.5}$ PFU/mouse, infection with PR8\textsubscript{WT} resulted in 100% lethality, which was in strong contrast to infection with PR8\textsubscript{M}: here, all mice survived (Figure 2A). Additionally, we measured the accumulation of infectious progeny virus in the mouse lung. Consistent with reduced virulence, we found a statistically significant reduction in the amounts of progeny virus in the lungs of mice infected with PR8\textsubscript{M} in comparison to PR8\textsubscript{WT}-infected animals at all investigated time points (Figure 2B).

To clarify whether the striking reduction in virulence of PR8\textsubscript{M} is due to altered induction of the host type I IFN response, we analyzed the expression of \textit{ifn}\textbeta\textsubscript{β} in mouse lungs infected with PR8\textsubscript{WT} or PR8\textsubscript{M} at different time points (day 1 and 2 postinfection). Here, on day 1 postinfection, only infection with PR8\textsubscript{M} resulted in a weak but statistically significant increase in the \textit{ifn}\textbeta mRNA level in comparison to mock- or PR8\textsubscript{WT}-infected mice. On day 2 postinfection, infection with PR8\textsubscript{WT} as well as PR8\textsubscript{M} both resulted in clearly elevated IFN-\textbeta mRNA levels when compared to PBS control mice. Again, a significantly elevated expression level of \textit{ifn}\textbeta was measured upon infection with PR8\textsubscript{M} (Figure 2C), although overall virus accumulation was strongly reduced (Figure 2B).

To verify whether the enhanced induction of type I IFN is the cause of the reduced pathogenicity of PR8\textsubscript{M}, we investigated virulence in IFN\textsubscript{AR\textsuperscript{−−}} mice. Using an intranasal infectious dose of $10^{3}$ PFU/mouse of PR8\textsubscript{WT}, all wild-type animals (C57Bl/6) died, in contrast to 20% lethality after infection with PR8\textsubscript{M} (Figure 3A, left panel). In IFN\textsubscript{AR\textsuperscript{−−}} mice, the lethality of PR8\textsubscript{M} was strongly enhanced to 100% (Figure 3A, right panel), clearly indicating the interdependence of increased type I IFN and the diminished virulence of PR8\textsubscript{M}. Analysis of the quantity of infectious virus particles of the PR8\textsubscript{M} virus revealed a statistically significant increase (3.5-fold) in lungs of IFN\textsubscript{AR\textsuperscript{−−}} mice (Figure 3B). In contrast, no significant changes in viral lung titers in PR8\textsubscript{WT}-infected wild-type compared to IFN\textsubscript{AR\textsuperscript{−−}} mice were detected. These data suggest the influence of a functional type I IFN system on the replication capability of PR8\textsubscript{M}, whereas the replication of PR8\textsubscript{WT} is only marginally affected, if at all. Taken together, our data clearly demonstrate that the enhanced type I IFN response is responsible for the decreased virulence of PR8\textsubscript{M}.

Amino Acid Substitutions in the Polymerase Proteins and NP Can Be Linked to Virulence

Sequence analysis of the complete genome of both viruses identified 16 gene alterations leading to amino acid changes in all viral proteins except PB1-F2, M1, and M2 (Table 1). To identify single gene segments responsible for the reduced virulence observed with PR8\textsubscript{M}, we generated a set of 7:1 reassortant viruses carrying 7 segments of PR8\textsubscript{WT} and different single gene segments of PR8\textsubscript{M}.

Analysis of virulence in an in vivo mouse model revealed that exchange of either segment 1 (PB2), 2 (PB1), 3 (PA), or 5 (NP) resulted in dramatic decreases in virulence: although all mice died upon PR8\textsubscript{WT} infection, at least 4 of 5 mice survived when infected with mutants carrying the segment encoding for PB2, PB1, PA, or NP of PR8\textsubscript{M}. In contrast, exchange of segments 6 (NA) or 8 (NS) affect virulence only marginally (Figure 4A).

Taken together, our data show that the reduced virulence of PR8\textsubscript{M} is due to amino acid changes at specific sites in the polymerase proteins and NP.

Two recent studies demonstrated initial indications for IFN antagonistic properties of the polymerase proteins [16, 17]. To analyze the impact of these different gene products toward...
alteration of the IFN response and virulence, we created a reassortant virus carrying the backbone of PR8WT and the PB2, PB1, PA, and NP genes of PR8M (PR8PPP NP). Analysis of the type I IFN responses after infection with the reassortant virus PR8PPP NP compared to PR8WT revealed a clearly increased IFN induction at the level of STAT1 phosphorylation in A549 and primary NHBE cells (Figure 4B) as well as ISG expression (Figure 4C). Accumulation of the different influenza RNA species (Figure 4D) was comparable, indicating that altered type I IFN inhibitory properties are responsible for the alterations.

As for the parental strain PR8M, the pronounced increase in type I IFN responses upon infection with PR8PPP NP was accompanied by a strongly reduced virulence, as evidenced by increased survival in Balb/c and DBA mice (Figure 5A) and
reduced progeny virus titers in mouse lungs (Figure 5B). Again, the interdependence of increased type I IFN and the diminished virulence of PR8M could be verified in IFNAR−/− mice (Figure 5C).

Thus, our experiments identified the polymerase proteins and NP as sufficient for the increased type I IFN response as well as the reduced virulence of the parental virus PR8M.

**PB1 and PA Mediate Type I IFN Inhibitory Properties**

To clarify the impact of each single protein, we analyzed phosphorylation of STAT1 as a measure of induction of type I IFN by different reassortant virus mutants. Infection with virus mutants carrying either segment 2 (PB1) or 3 (PA) of PR8M resulted in enhanced STAT1 phosphorylation, although not reaching the levels of phosphorylation induced by PR8PPP NP. This suggested additive effects of viral proteins PB1 and PA on induction of the type I IFN response (Figure 6A). Indeed, the combination of segments 2 (PB1) and 3 (PA) of PR8M together resulted in an enhanced STAT1 phosphorylation comparable to the phosphorylation level induced by PR8PPP NP (Figure 6B). Further analysis revealed a subset of 4 amino acid positions involved in the inhibitory properties mediated by PB1 and PA as shown by the virus mutant PR8 PB1 E398G S524G I563R PA E351K (PR8hIFN; Figure 6C).

**DISCUSSION**

In the present study, we characterized the IAV variant PR8M, which differs dramatically in induction of the type I IFN response and virulence compared to a wild-type PR8 strain, PR8WT. We identified the polymerase proteins and NP as major contributors to these differences and showed that PB1 and PA may play prominent roles in inhibiting host type I IFN signaling.

In general, 2 scenarios are possible to explain the increased type I IFN response upon infection with PR8M: (1) the type I IFN-inducing potential of PR8M is enhanced compared to PR8WT, or (2) the viruses differ in their type I IFN antagonistic properties.

Purified vRNA from PR8WT vs PR8M stimulates the type I IFN response equally, arguing against the hypothesis that PR8M has increased IFN-inducing capacities. These findings favor the
assumption that differences in the type I IFN inhibitory activities of proteins of the 2 viruses are responsible for the observed differences in the type I IFN responses and virulence.

The most prominent interferon antagonistic proteins of IAV are NS1 and PB1-F2 [14, 15, 27, 28]. The PB1-F2 amino acid sequence is identical in both viruses and NS1 only differs in 1 amino acid (position 55), which did not lead to different IFN inhibitory properties (Supplementary Figure 1). This supports the assumption that the altered antagonistic features of PR8M are most likely coupled to another gene product.

We found that the polymerase proteins and NP gene segments were most responsible for the differences in virulence and type I IFN induction, with the greatest impact being made by PB1 and PA. In vivo, the functional link between increased type I IFN induction and diminished virulence, as reported earlier [29], could be explored in IFNAR−/− mice. The absence of a functional IFN system resulted in a clear harmonization of the pathogenicity of both PR8 variants. This finally confirmed the interplay of increased type I IFN induction and reduced virulence as being responsible for the phenotype of PR8M. The differences in type I IFN expression 1 and 2 days upon infection are indicative of an early block of viral replication in PR8M-infected lung epithelia. This could be responsible for the reduced pathology of the virus. Nevertheless, an impact of the increased type I IFN response on more complex immune regulation functions such as immune cell recruitment and function [30, 31] cannot be ruled out as a mechanism of differential virulence, as massive infiltration by exudate macrophages and neutrophils is reported as a cause of immunopathological alterations in the lung, ultimately leading to death [32, 33].

The immune response to an infection with IAV is dependent on the host genetic background [34]. Our virus mutant showed the same reduced virulence in 3 mouse lines, arguing against a mouse strain-specific phenomenon. Also, the enhanced type I IFN response in primary human bronchiolar epithelial cells favors the assumption that the virulence-determining potential of the described amino acids is crucial in the human host.

The interferon antagonistic functionality of the polymerase proteins and NP act apart from simple regulation of replication, as the reassortant viruses showed comparable replication in vitro (Supplementary Figure 2) and accumulation of viral mRNA, cRNA, and vRNA. Although the functions of the polymerase proteins and NP in replication and transcription of viral RNA have been widely investigated [35–37], only rudimentary knowledge exists about the functions of the polymerase proteins in inhibiting antiviral signaling [16, 17], and the impact of these functions on virulence is not yet understood.

Interestingly, our genotypic analysis revealed 8 amino acid substitutions in these 4 proteins, none of which has so far been linked to type I IFN induction or virulence regulation. Some of these amino acid sites are located within functional domains, such as the PB1-binding domain of the PA protein [37] or the NP-1 and PB2-2 domain within the NP protein [38]. This suggests that interaction between individual polymerase proteins themselves and/or NP are affected and the cause of the described phenotype. A defective interaction between the viral proteins could also result in altered interaction of the polymerase complex with cellular factors resulting in reduced type I IFN inhibitory properties, as the PB2 subunit inhibits type I IFN induction via interacting with MAVS [16].

Figure 6. The type I interferon (IFN) induction is strongly dependent on PB1 and PA. A–C, A549 cells were infected for 8 hours with PR8WT or indicated virus mutants (multiplicity of infection [MOI] = 5) or left uninfected. Phosphorylated STAT1 (Y701) was detected by Western blot analysis; viral protein synthesis was visualized in M1, NS1, and NP Western blot analysis. Equal amounts of protein were verified using STAT1 and ERK2 antibodies.

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Comparison of consensus sequences of recent human influenza virus isolates revealed a distinct conservation of the described amino acid positions (Supplementary Table 1). Interestingly, not all viruses carry the amino acids responsible for an efficient type I IFN inhibition. Therefore, adaptation to the host immune system via mutation of these positions seems to be possible.

Summarizing our data, in this study we characterized PR8Xs, a variant of A/PR/8/34, which has a high type I IFN-inducing capacity and diminished pathogenicity. We were able to determine that the polymerase proteins and NP were tightly linked to induction of the host type I IFN signaling, in which PB1 and PA play prominent roles. Thus, our data favor the assumption that the polymerase proteins, most likely PB1 and PA, have as yet unknown IFN antagonistic functions and even single amino acid substitutions in these proteins can be identified as novel virulence determinants.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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