The influenza NS1 protein modulates RIG-I activation via a strain-specific direct interaction with the second CARD of RIG-I

Received for publication, October 9, 2019, and in revised form, November 30, 2019 Published, Papers in Press, December 16, 2019, DOI 10.1074/jbc.RA119.011410

Alexander S. Jureka‡1, Alex B. Kleinpeter†, Jennifer L. Tipper‡, Kevin S. Harrod§, and Chad M. Petit†2
From the †Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham School of Medicine, Birmingham, Alabama 35294 and the ‡Department of Anesthesiology and Perioperative Medicine, Division of Molecular and Translational Biomedicine, University of Alabama at Birmingham School of Medicine, Birmingham, Alabama 35294

Edited by Craig E. Cameron

A critical role of influenza A virus nonstructural protein 1 (NS1) is to antagonize the host cellular antiviral response. NS1 accomplishes this role through numerous interactions with host proteins, including the cytoplasmic pathogen recognition receptor, retinoic acid–inducible gene I (RIG-I). Although the consequences of this interaction have been studied, the complete mechanism by which NS1 antagonizes RIG-I signaling remains unclear. We demonstrated previously that the NS1 RNA-binding domain (NS1RBD) interacts directly with the second caspase activation and recruitment domain (CARD) of RIG-I. We also identified that a single strain-specific polymorphism in the NS1RBD (R21Q) completely abrogates this interaction. Here we investigate the functional consequences of an R21Q mutation on NS1’s ability to antagonize RIG-I signaling. We observed that an influenza virus harboring the R21Q mutation in NS1 results in significant up-regulation of RIG-I signaling. In support of this, we determined that an R21Q mutation in NS1 results in a marked deficit in NS1’s ability to antagonize TRIM25-mediated ubiquitination of the RIG-I CARDs, a critical step in RIG-I activation. We also observed that WT NS1 is capable of binding directly to the tandem RIG-I CARDs, whereas the R21Q mutation in NS1 significantly inhibits this interaction. Furthermore, we determined that the R21Q mutation does not impede the interaction between NS1 and TRIM25 or NS1RBD’s ability to bind RNA. The data presented here offer significant insights into NS1 antagonism of RIG-I and illustrate the importance of understanding the role of strain-specific polymorphisms in the context of this specific NS1 function.

The influenza A virus (IAV)3 is a serious public health concern that causes annual epidemics and occasional pandemics, resulting in significant levels of morbidity and mortality each year (1, 2). The ability of IAV to adapt to various hosts and undergo genetic reassortment ensures constant generation of unique strains with unknown degrees of pathogenicity, transmissibility, and pandemic potential. Currently, our knowledge of the precise combination of factors that drives the emergence of strains with pandemic potential is incomplete. However, several proteins expressed by IAV have been identified as key determinants of virulence during infection. One such protein encoded by IAV is nonstructural protein 1 (NS1).

NS1 is highly multifunctional and ranges from 215–237 amino acids in length, depending on the strain from which it is derived. During infection, NS1 plays a critical role in antagonizing the cellular innate immune response (3–5). It consists of two independently folding functional domains: the N-terminal RNA-binding domain (NS1RBD) and the C-terminal effector domain (NS1ED). Both domains facilitate the immunosuppressive action of NS1 through interactions with numerous host proteins involved in the cellular innate immune response (3–5). Specifically, NS1 is well-known for its abrogation of the type I IFN response through interactions with pathogen recognition receptors and their activation partners, such as retinoic acid inducible gene I (RIG-I) and the E3 ubiquitin ligase TRIM25 (6–11).

RIG-I is an ATP-dependent cytoplasmic helicase whose primary function is to induce an antiviral signaling cascade in response to RNA virus infections (12, 13). It contains two N-terminal caspase activation and recruitment domains (2CARDs), a central helicase domain consisting of three subdomains (Hel-1, Hel-2i, and Hel-2), a linker domain (Br), and a regulatory C-terminal domain. RIG-I is predominantly activated by short 5′-triphosphorylated RNAs (5’ppp dsRNAs), such as those produced by the partial complementarity in the 5′ and 3′ UTRs of IAV genomic RNAs (13–16). Therefore, RIG-I is considered the main cytoplasmic sensor of IAV infection (17). Briefly, RIG-I undergoes a significant conformational change upon recognition of IAV RNAs that results in exposure and subsequent ubiquitination of the N-terminal 2CARDs by TRIM25 (18, 19). Upon ubiquitination, RIG-I translocates to the mitochondria, where it participates in a CARD–CARD...
Modulation of RIG-I activation by the influenza NS1 protein

interaction with the mitochondrial antiviral signaling protein (MAVS, also known as IPS-1, VISA, and CARDIF) (20–22). This results in recruitment of the kinases IKK and TBK1, which function to phosphorylate the antiviral transcription factor IRF3 (23–25). Upon phosphorylation, IRF3 translocates to the nucleus and promotes transcription of type I IFNs, such as IFN-α/β.

Ubiquitination of the RIG-I 2CARDs by the E3-ubiquitin ligase TRIM25 is a critical step in activation of the RIG-I signaling pathway (20, 26, 27). This step in the signaling pathway is inhibited during influenza infection by a direct interaction between NS1 and TRIM25 (6, 26). Although it is has been shown that this interaction is a critical mechanism through which NS1 antagonizes RIG-I 2CARD ubiquitination (6, 26), it has not been shown whether additive antagonistic effects could result from additional direct interactions between NS1 and RIG-I. Recently, we reported that the NS1RBD from 1918H1N1 IAV (A/Brevig Mission/1/1918) interacts directly with the second CARD of RIG-I and that a naturally occurring mutation in the NS1RBD (R21Q) abrogated this direct interaction (8). Because of the critical role the CARDs play in RIG-I activation, we hypothesized that this direct interaction would enhance the ability of NS1 to inhibit ubiquitination of the RIG-I 2CARDs. Furthermore, this inhibition would be independent of other previously studied NS1 interactions involving TRIM25 (6–11) and dsRNA structures (3) also known to inhibit activation of RIG-I.

In this study, we demonstrate that the naturally occurring R21Q mutation in NS1 (NS1R21Q) significantly impacts NS1’s ability to control RIG-I signaling. Specifically, we used reverse genetics to generate WT A/Puerto Rico/8/1934 IAV (rPR8WT) and a mutant IAV encoding the R21Q mutation in NS1 (rPR8R21Q) to test the effects of the mutation on aspects of the viral life cycle. We observed that rPR8R21Q induced significantly more IFN3 phosphorylation and IFN-β expression compared with rPR8WT. These data indicate that rPR8R21Q is a significantly more potent RIG-I activator compared with rPR8WT. Further analysis revealed that NS1R21Q is significantly less efficient at inhibiting TRIM25-dependent ubiquitination of the RIG-I 2CARDs, a critical step in activation of RIG-I signaling. Additionally, we determined that the R21Q mutation markedly diminished the ability of NS1 to interact with the RIG-I 2CARDs. Finally, we confirmed that the R21Q mutation did not have an effect on any of the other NS1 functions known to inhibit the RIG-I pathway. Taken together, this is the first study to identify a natural polymorphism in the NS1RBD that mitigates its ability to control RIG-I 2CARD ubiquitination by TRIM25. This study demonstrates that strain-specific polymorphisms in NS1 not only have a significant impact on its ability to efficiently antagonize RIG-I activation but that the effects of these polymorphisms may be conditional to species-specific activation of RIG-I.

Results

Previously, we demonstrated that an R21Q mutation in the NS1RBD abrogated its ability to interact with the second CARD domain of RIG-I. Residue 21 is distal to other residues in the NS1RBD (Arg35, Arg38, and Lys41) that are known to have functions in RNA binding and NS1 cellular localization (Fig. 1) (28–30). Disrupting these residues (Arg35, Arg38, and Lys41) by mutation is well-known to cause significant defects in viral fitness, including decreased replication and inability to control the cellular antiviral response. What is not known, however, is the effect of the R21Q mutation on the viral life cycle, as mutations in this region of the NS1RBD have yet to be studied.

The R21Q mutation has no effect on the overall structure of the NS1RBD

Given that residue 21 is in an unstudied region of the NS1RBD, we first wanted to ensure that there were no gross structural changes in the NS1RBD associated with the R21Q mutation. To assess any potential structural changes caused by the mutation, we used NMR to analyze the 1H-15N heteronuclear single-quantum coherence (HSQC) spectra of both the WT NS1RBD (RBDWT) and the R21Q mutation (RBDR21Q). Both HSQC spectra showed a single set of dispersed peaks, indicative of a well-folded protein with a single, major conformation (Fig. 2A). Because the NS1RBD is a symmetric homodimer, only 72 amide resonances were expected and ultimately observed (not including the side-chain amides of glutamine and asparagine) for both the RBDWT and RBDR21Q. We then obtained resonance assignments for RBDR21Q using three-dimensional NMR experiments such as HNCACB and CBCA(CO)NH for further analysis. High-quality triple-resonance spectra allowed backbone resonance assignments to be obtained for 70 of the 72 (97%) possible assigned residues of the RBDR21Q. Analysis of the 13Cα chemical shifts indicated that each monomer of the RBDR21Q mutant is composed of α1-turn-α2-turn-α3 (Fig. 2B) (31). This six-helix dimeric structure of the RBDR21Q homodimer is consistent with both 13Cα chemical shift analysis of the RBDWT (Fig. 2B) and our previously solved solution structure of the 1918H1N1 NS1RBD.

Characterization of rPR8WT and rPR8R21Q replication and NS1 cellular localization

Because the NS1 R21Q mutation had not been characterized previously, it was critical to determine whether the mutation impacted basic aspects of the viral life cycle, such as IAV replication and NS1 cellular localization. To accomplish this, we used a previously described IAV reverse genetics system (32) to generate and rescue recombinant A/Puerto Rico/8/1934
Modulation of RIG-I activation by the influenza NS1 protein

viruses encoding WT NS1 (rPR8WT) and R21Q NS1 mutant (rPR8R21Q). Upon infecting adenocarcinomic human alveolar basal epithelial (A549) cells at m.o.i. 0.01, we observed no significant differences in viral replication between rPR8WT and rPR8R21Q (Fig. 3A). Similarly, we observed no significant change in the cellular localization of NS1 in A549 cells infected with rPR8WT or rPR8R21Q 12 h post-infection (m.o.i. 2) (Fig. 3B). Given that R21Q is a naturally occurring mutation, it is not surprising that replication and intracellular localization are not affected; numerous viruses capable of productive infection in humans also possess a Gln at position 21. However, the observation of similar replication kinetics does not adjudicate the question of whether the two viruses will result in differential activation of the RIG-I signaling pathway upon infection, nor is it prognostic of potential differences in virulence between the two viruses, as influenza replication levels do not necessarily correlate with virulence (33, 34).

**Infection with rPR8R21Q results in significantly increased IRF-3 phosphorylation and IFN-β expression compared with rPR8WT infection**

Activation of the RIG-I pathway results in phosphorylation of the antiviral transcription factor IRF3, which then translocates to the nucleus and induces transcription of type I interferons (e.g. IFN-β). IAV NS1 has been shown previously to inhibit IRF3-mediated signaling through its antagonism of the RIG-I pathway (9, 35). Based on our previous data demonstrating that NS1R21Q is unable to interact with the second CARD of RIG-I, we hypothesized that rPR8R21Q would be less efficient at antagonizing RIG-I signaling. To test this hypothesis, we infected A549 cells with rPR8WT and rPR8R21Q at m.o.i. 0.01, 12, 24, 48, and 72 h post-infection, supernatants were collected (n = 6) and titrated on MDCK cells using standard plaque assays. A, A549 cells were infected with rPR8WT (black) or rPR8R21Q (lavender) at m.o.i. 0.01, 12, 24, 48, and 72 h post-infection, supernatants were collected (n = 6) and titrated on MDCK cells using standard plaque assays. B, A549 cells were infected with each rPR8WT or rPR8R21Q at m.o.i. 5. Cells were fixed 12 h after infection and stained with an anti-NS1 antibody (NS1) and 4',6-diamidino-2-phenylindole (DAPI, nuclei). Localization was visualized using an Olympus FV1000 confocal microscope. Scale bars = 10 μm.

**Figure 3. Infection with rPR8WT and rPR8R21Q demonstrates similar replication kinetics and NS1 intracellular localization.**

A, A549 cells were infected with rPR8WT (black) or rPR8R21Q (lavender) at m.o.i. 0.01, 12, 24, 48, and 72 h post-infection, supernatants were collected (n = 6) and titrated on MDCK cells using standard plaque assays. B, A549 cells were infected with each rPR8WT or rPR8R21Q at m.o.i. 5. Cells were fixed 12 h after infection and stained with an anti-NS1 antibody (NS1) and 4',6-diamidino-2-phenylindole (DAPI, nuclei). Localization was visualized using an Olympus FV1000 confocal microscope. Scale bars = 10 μm.

The R21Q mutation in NS1 results in increased IFN-β promoter activation

Although increased IRF3 phosphorylation and IFN-β induction during infection suggest that rPR8R21Q is less efficient at antagonizing RIG-I signaling, it does not provide specific information regarding the mechanistic underpinnings by which NS1 antagonizes the RIG-I pathway. To overcome this limitation, we designed a series of experiments to probe how NS1WT and NS1R21Q affect specific steps of the RIG-I activation pathway. In addition to our hypothesis that NS1 directly inhibits ubiquiti-
nation of the RIG-I 2CARDs, NS1 has been shown previously to suppress two additional steps in the RIG-I signaling pathway. These steps include NS1 potentially shielding dsRNA structures from cellular factors responsible for detecting viral infection (3) as well as inhibition of RIG-I ubiquitination through a direct interaction with TRIM25 (6, 26). Completion of these experiments would result in a more mechanistic understanding of the phenotypic differences observed between rPR8WT and rPR8R21Q.

We first sought to determine whether the difference in the ability of rPR8WT and rPR8R21Q to inhibit IRF3 phosphorylation and IFN-β induction during viral infection could be recapitulated in a plasmid-based system. This was accomplished by transfecting HEK293T cells with a reporter construct containing a minimal IFN-β promoter driving firefly luciferase expression (p125-Luc), NS1WT or NS1R21Q expression constructs, and a Renilla luciferase control vector. IFN-β promoter activity was stimulated by infection with the Cantell strain of Sendai virus, a well-known RIG-I agonist (14, 36), or cotransfection with a construct encoding GST-2CARDs. It is important to note that overexpression of the RIG-I 2CARDs alone induces IFN-β promoter activity (27). In agreement with our virus results and previously published reports, overexpression of NS1WT resulted in a marked decreased in IFN-β promoter activity in cells stimulated with Sendai virus (Fig. 5A) and RIG-I GST-2CARDs (Fig. 5B). Compared with NS1WT, overexpression of NS1R21Q led to significantly increased IFN-β promoter activity under the same conditions. This suggests that introduction of the R21Q mutation reduces the efficiency of this antagonism because all other amino acids that have been demonstrated to significantly affect this pathway (Arg38, Lys41, Glu96, and Glu97) remain intact (6).

The R21Q mutation decreases the ability of NS1 to inhibit ubiquitination of RIG-I 2CARDs by TRIM25

Our previous study showing that the R21Q mutation abrogated the interaction between the NS1RBD and the second CARD domain of RIG-1 led to our hypothesis that NS1R21Q would be less efficient at blocking ubiquitination of the RIG-I 2CARDs by TRIM25. To test this hypothesis, we used pulldown

**Figure 4.** Infection with rPR8R21Q results in significantly increased IRF3 phosphorylation and IFN-β expression. A, A549 cells were infected with rPR8WT or rPR8R21Q at m.o.i. 2 for 6, 12, and 24 h. At each respective time point, monolayers were lysed and probed by Western blotting for total IRF3, phosphorylated IRF3 (pIRF3), IAV NS1, RIG-I, and β-actin. B, the ratio of pIRF3 to IRF3, quantified in triplicate for each time point. C, A549 cells were infected at m.o.i. 2. Cell monolayers were lysed, and total RNA was collected 6, 12, and 24 h post-infection. IFN-β mRNA expression was determined using qPCR, with -fold change values calculated via the ΔΔCT method. D, IFN-β levels in supernatants harvested from A549 cells infected with rPR8WT or rPR8R21Q (m.o.i. 2) were determined 6, 12, and 24 h post-infection using an IFN-β-specific ELISA. Data were analyzed using Student’s t test, and error bars represent the mean ± S.E. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; NS, not significant. All data are representative of three independent experiments, with rPR8WT indicated in black and rPR8R21Q indicated in lavender.
studies in which 293T cells were transfected with plasmids encoding GST-fused human RIG-I 2CARDs (GST-2CARDs), TRIM25, and either NS1WT or NS1R21Q. The GST-2CARDs fusion protein was pulled down using GSH-agarose beads and visualized by Western blotting to assess the relative ubiquitination level of 2CARDs in the presence of either NS1WT or NS1R21Q. Use of the GST-2CARDs fusion protein as a method to determine the effect of viral proteins on RIG-I 2CARD ubiquitination has been described previously (6, 20, 26, 37). Consistent with previous studies (6, 26), there was a marked decrease in GST-2CARD ubiquitination in the presence of NS1WT (Fig. 6A). We also observed that NS1R21Q was less efficient at inhibiting ubiquitination of 2CARDs by TRIM25 relative to NS1WT, confirming our hypothesis (Fig. 6B). However, the ability to inhibit 2CARD ubiquitination was not completely abrogated, as there was a significant decrease in ubiquitination of 2CARDs in the presence of NS1R21Q relative to the GFP control. From these data, it is clear that the R21Q polymorphism significantly decreases the ability of NS1 to inhibit ubiquitination of RIG-I 2CARDs.

The data presented so far provides strong evidence that NS1R21Q is a less efficient RIG-I antagonist because of its inability to control RIG-I 2CARD ubiquitination. However, it is important to confirm that the molecular basis of the observed differences is due to NS1R21Q being deficient in binding to the RIG-I 2CARDs. To accomplish this, 293T cells were transfected as above, and GST-2CARD was pulled down. Probing for 3×FLAG-NS1WT and NS1R21Q in pulldown fractions revealed that, indeed, NS1WT is capable of interacting with the RIG-I 2CARDs, but this interaction is markedly diminished for the NS1R21Q mutant (Fig. 6, C and D). In addition, the interaction between NS1WT and the RIG-I 2CARDs remained unchanged in the absence of TRIM25 (Fig. 6C, sixth and seventh lanes), indicating a direct interaction between NS1WT and RIG-I 2CARDs. To ensure that the observed interaction between NS1 and 2CARDs was not due to nonspecific interactions with GST or the GA beads, we incorporated the necessary controls into the GST pulldown experiments (Fig. 6E). These data agree with our previous study showing that NS1WT is capable of interacting directly with the second CARD of RIG-I, and that the R21Q mutation disrupts the interaction. Furthermore, the inability of NS1R21Q to bind the RIG-I 2CARDs as efficiently as NS1WT provides a straightforward explanation for the observed differences in ubiquitination of 2CARDs. Specifically, direct interaction between NS1WT and RIG-I 2CARDs could physically obstruct ubiquitination of the second CARD of RIG-I either by TRIM25 or its ability to bind free-floating ubiquitin chains. In contrast, NS1R21Q binding to RIG-I 2CARDs is largely abolished relative to NS1WT, resulting in significantly less suppression of RIG-I 2CARD ubiquitination.

The R21Q mutation has no effect on the interaction between NS1 and TRIM25

Although we demonstrated that the R21Q mutation in NS1 inhibits its ability to suppress ubiquitination of RIG-I 2CARDs, other potential mechanisms underlying the differences in IRF3 phosphorylation and IFN-β induction observed during rPR8WT and rPR8R21Q infection remain. Consequently, it was necessary to determine the effect of the mutation on other known mechanisms by which NS1 interferes with RIG-I activation to obtain more mechanistic insight. One example of how NS1 interferes with activation of the RIG-I pathway is through its direct interaction with TRIM25 (38). It was therefore neces-
necessary to determine whether our initial observations were independent of this interaction or whether this interaction contributed to the mitigation of NS1 function observed for the R21Q mutation. To test this, 293T cells were transfected with HA-TRIM25 along with either a GFP control vector, 3xFLAG-NS1WT, or 3xFLAG-NS1R21Q. TRIM25 was immunoprecipitated using HA-agarose 36 h after transfection. Analysis of the immunoprecipitated fractions revealed that the R21Q mutation in NS1 has no effect on its ability to bind TRIM25 (Fig. 7). This observation offers critical insight into the molecular underpinnings of our initial observation that the R21Q mutant NS1 is less efficient at suppressing RIG-I activation. These data, combined with the data in Fig. 4, suggest that the R21Q mutation in NS1 specifically affects NS1’s ability to bind the RIG-I 2CARDs independent of its TRIM25 binding function. Furthermore, these data support our hypothesis that the enhanced RIG-I activation and induction of type I interferons are due to NS1R21Q’s deficiency in binding 2CARDs and not the interaction between NS1 and TRIM25.

**The R21Q mutation has no effect on the interaction between NS1 and RNA**

Another potential mechanism by which NS1 antagonizes the RIG-I pathway is through its binding of dsRNA structures. It has been posited that NS1 binds dsRNA structures to shield its presence in the cell from host proteins that have evolved to induce the antiviral signaling cascade in response to RNA virus infection (3). Assessing the R21Q mutation’s effect on this interaction would allow us to interrogate another possible contributor to the differences we observed between rPR8WT and rPR8R21Q infection. We employed fluorescence polarization anisotropy to measure the binding affinity of a dsRNA structure to both the WT NS1RDDB and the R21Q NS1RDDB mutant. For this analysis, we chose to use a previously described dsRNA (39) to determine its binding affinity to the NS1RBD (Fig. 8A). Analysis of the isotherms indicated that the R21Q mutation had no effect on the ability of NS1 to bind the dsRNA structure, eliminating it as a potential contributor to our observed phenotype (Fig. 8B). Specifically, the binding affinities (K_d) to the dsRNA structure for NS1WT and NS1R21Q were measured to be 27 ± 0.95 nM and 28.6 ± 1.2 nM, respectively. These data reinforce our hypothesis that the differences in IRF3 phosphorylation and IFN-β induction observed during rPR8WT and rPR8R21Q infection are due to direct inhibition of ubiquitination of 2CARDs by NS1.

---

**Figure 6.** NS1R21Q is less effective at inhibiting RIG-I 2CARD ubiquitination and exhibits diminished interaction with RIG-I 2CARD. A, 293T cells were transfected with expression plasmids encoding the indicated combination of TRIM25, GFP (control), RIG-I 2CARDs fused with GST (GST-2CARDs), 3xFLAG-NS1WT, and 3xFLAG-NS1R21Q. 36 h post-transfection, GST-2CARDs was pulled down, and ubiquitinated GST-2CARDs (red arrows) was visualized by Western blotting. B, densitometric analysis was used to quantify the ratio of ubiquitinated GST-2CARDs to free GST-2CARDs. NS1WT is indicated in black, and NS1R21Q is indicated in lavender. C, 293T cells were transfected with the indicated combination of expression plasmids. 36 h post-transfection, GST-2CARDs was pulled down and free 2CARDs, ubiquitinated 2CARDs, 3xFLAG-NS1WT, and 3xFLAG-NS1R21Q in whole-cell lysates (WCL) as well as pulldown fractions were visualized by Western blotting. D, 293T cells were cotransfected with either 3xFLAG-NS1WT or 3xFLAG-NS1R21Q along with a GST expression vector or GFP control. All samples were subjected to pulldown with GSH-agarose, and pulldown fractions were probed for 3xFLAG and analyzed by Western blotting. Data were analyzed using Student’s t test and error bars represent the mean ± S.E., *p < 0.05.

**Figure 7.** The R21Q mutation has no effect on TRIM25 binding. 293T cells were transfected with pHA-TRIM25 along with a GFP control vector, p3xFLAG-NS1WT, or p3xFLAG-NS1R21Q. 36 h post-transfection, lysates were harvested and subjected to immunoprecipitation (IP) with αHA-agarose (Pierce, 26181). HA-TRIM25, 3xFLAG-NS1WT, and 3xFLAG-NS1R21Q in whole cell lysates (WCL) and immunoprecipitated fractions were visualized by Western blotting.
In vivo characterization of the R21Q mutation in the mouse model

Given our observations that the R21Q mutation decreases the ability of NS1 to inhibit ubiquitination of RIG-I 2CARDs by TRIM25, we sought to characterize the effect of the R21Q mutation in vivo. Two groups of 8- to 12-week old C57BL/6 mice were inoculated intranasally with 100 pfu of rPR8WT or rPR8R21Q. Each group of mice was monitored for 14 days post-infection for mortality (Fig. 9A), weight loss (Fig. 9B), changes in body temperature (Fig. 9C), and signs of illness (Fig. 9D). Following Institutional Animal Care and Use Committee (IACUC) guidelines, when body weight was reduced to 75% of the starting weight, mice were deemed moribund and were euthanized. At the end of the study, 60% and 50% of the mice infected with the rPR8WT and rPR8R21Q viruses, respectively, were euthanized by day 11 post-infection. In addition, both viruses caused nearly identical percentages and rates of weight loss that stopped on day 8. However, mice infected with rPR8R21Q regained this weight ~30% faster than mice infected with rPR8WT, weighing an average 99% of their starting weight 12 days post-infection. Mice infected with rPR8R21Q also experienced less severe clinical signs of illness compared with those infected with rPR8WT. Taken together, the in vivo data suggest that the rPR8R21Q virus may be moderately less pathogenic compared with rPR8WT, albeit at levels not determined to be statistically significant. We note that the mechanism of RIG-I activation is fundamentally different when comparing humans and mice (26). It is this species-specific difference in RIG-I activation that offers a potential explanation for the lack of significant effect in vivo despite our observation that rPR8R21Q results in significantly increased IRF3 phosphorylation, type I interferon induction, and TRIM25-dependent ubiquitination of RIG-I 2CARDs relative to rPR8WT. The potential species-specific nature of the R21Q mutation will be addressed under “Discussion.”

Discussion

To date, several strain-specific polymorphisms in IAV NS1 have been described to have significant effects on NS1’s function as an innate immune antagonist. However, the strain-specific polymorphisms that have been described previously are primarily involved with the ability of NS1 to bind RNA and its interaction with CPSF30 (30-kDa subunit of the cleavage and polyadenylation specificity factor) (28, 40–42). Although both of these interactions play a large part in the overall ability of NS1 to control the host cellular antiviral response, NS1 takes part in a large number of other interactions where the effect of strain-specific polymorphisms is not well-understood. In this study, we investigated whether a previously described (8) strain-specific polymorphism at residue 21 in the IAV NS1 has a measurable effect on its ability to inhibit RIG-I signaling (Fig. 10). Using reverse genetics, we demonstrated that infection with rPR8R21Q results in significantly increased IRF3 phosphorylation and type I interferon compared with rPR8WT. In agreement with these results, we observed that NS1R21Q results in significantly increased TRIM25-dependent ubiquitination of the RIG-I 2CARDs, a critical step in the activation of RIG-I. Furthermore, we observed that NS1WT is indeed capable of interacting with the RIG-I 2CARDs in a TRIM25-independent manner and that an R21Q mutation in the NS1RBD significantly interferes with this interaction. Finally, we determined that the R21Q mutation had no observable effect on the ability of NS1 to bind dsRNA structures or TRIM25. Collectively, these data indicate that NS1R21Q is less efficient at blocking ubiquitination

Modulation of RIG-I activation by the influenza NS1 protein

Figure 8. The R21Q mutation has no effect on RNA binding. A, graphical depiction of a previously described dsRNA structure (39) that was used to determine the effect of the R21Q mutation on the known interaction between the NS1RBD and RNA. B, fluorescence polarization measurements of increasing concentrations of NS1WT and NS1R21Q in the presence of 5’-FAM-labeled shDM03 (5 nM) determined that the binding affinities of NS1WT (black) and NS1R21Q (lavender) to shDM03 were 27 ± 0.95 and 28.6 ± 1.2 nM, respectively. Change in polarization was calculated by subtracting FP measurements of shDM03 buffer-only controls from experimental FP measurements. All data are representative of three independent experiments. Error bars represent the mean ± S.E. Binding affinities were determined by fitting data to one-site-specific binding with a Hill slope (GraphPad).

Figure 9. In vivo characterization of the R21Q mutation. A–D, groups of 20 mice (10 female and 10 male) were inoculated intranasally with 100 pfu of rPR8WT (black) and rPR8R21Q (lavender). Survival (A), weight loss (B), temperature (C), and clinical observations (D) were monitored for 14 days post-infection.
of RIG-I 2CARDs, leading to more potent activation of the innate immune response. The effect of the R21Q mutation on these three specific functions of NS1 was assessed in this work. These assessments are indicated in the schematic by their respective steps in the RIG-I activation pathway. A, NS1 is posited to antagonize RIG-I activation by shielding the presence of dsRNA structures from host proteins by directly interacting with these dsRNA structures (3). The structural basis of dsRNA recognition by NS1 of IVA is illustrated (28) (PDB: 2ZKO). As indicated in Fig. 8, the R21Q mutation has no effect on the ability of the NS1NBD to bind dsRNA. B, NS1 was previously determined to interact directly with the second CARD of RIG-I, with the R21Q mutation abolishing this interaction (8). Additional data presented in this work supports this observation (Fig. 6C) and indicate that the R21Q mutation also reduces NS1’s ability to inhibit ubiquitination of 2CARDs (Fig. 6, A and B). A hypothetical model of the interaction between the NS1NBD and CARD2 is illustrated, using previously solved structures of 2CARDs (PDB: 4NQK) (49) and the NS1NBD (PDB: 2N74) (8). The most critical position of 2CARD ubiquitination (Lys172) is indicated in yellow. C, TRIM25 is the E3 ubiquitin ligase responsible for Lys172-specific polyubiquitination of 2CARDs of RIG-I at position Lys172. NS1 interacts directly with TRIM25 to inhibit RIG-I ubiquitination, suppressing activation of the RIG-I pathway (38). The structural basis of NS1-mediated TRIM25 (PDB: 5NT2) inhibition is illustrated. As indicated in Fig. 7, the R21Q mutation does not abolish the interaction between NS1 and TRIM25.

Figure 11. Strain-specific polymorphisms at position 21 in NS1. A, sequence logo describing sequence conservation at position 21 (highlighted in lavender) in NS1. B, percent residue identity as a function of host and serotype derived from multiple alignments of NS1 sequences obtained from the Influenza Research Database.
NS1 sequences indicated that arginine is the predominant amino acid at position 21 (Fig. 11A). However, this predominance was not maintained when sequences were analyzed as a function of host species or serotype (Fig. 11B). NS1 proteins derived from serotypes that usually result in seasonal epidemics (H1N1/H3N2) have a clear disparity in the identity of residue 21. NS1 proteins derived from H1N1 serotypes predominantly have an Arg, whereas those derived from H3N2 strains predominantly have a Gln at position 21. Additionally, residue 21 identity in IAV subtypes generally regarded as highly pathogenic (H5N1/H7N9) is heavily biased toward Arg. It is plausible that these differences are the result of species-specific adaptation, as it has already been determined that NS1’s interference with RIG-I ubiquitination differs significantly between humans and mice (26). It is this difference in RIG-I activation that offers a potential explanation for our in vivo data. Specifically, it has been reported previously that NS1 does not antagonize the RIG-I signaling cascade in mice by preventing 2CARD ubiquitination during IAV infection (44, 45). Instead, NS1 prevents ubiquitination of RIG-I in regions of the protein distal to the 2CARDs, which ultimately leads to RIG-I activation in mice (26). This is because activation of human RIG-I requires Lys63-linked ubiquitination of Lys172 by TRIM25 ubiquitin E3 ligase (20), whereas murine RIG-I lacks Lys172 in 2CARDs (26). Because the R21Q mutation specifically abrogates the NS1 RBD’s interaction with 2CARD of RIG-I (8), it is likely that the species-specific differences in RIG-I activation underlie the marginal differences observed in the murine infection model used in this study.

Going forward, we believe it will be important to address this question by assessing how efficiently NS1 proteins from multiple strains of IAVs isolated from multiple species are able to antagonize RIG-I 2CARD ubiquitination. Of particular interest for future studies will be to determine how differential antagonism of RIG-I 2CARD ubiquitination contributes to virulence during IAV infection. Taken together, this study demonstrates that NS1 is capable of additional sequence-specific interactions with RIG-I to facilitate its antagonism of the cellular antiviral response. It stresses the importance of how strain-specific polymorphisms in NS1 can affect its ability to antagonize the host cellular immune response in ways that are yet to be appreciated. This knowledge provides a solid foundation for further investigation into the structural and molecular mechanisms that define how these strain-specific polymorphisms in NS1 affect its ability to inhibit the RIG-I pathway. Understanding strain-specific differences in coping with the host antiviral response may better prepare us to predict the pandemic potential of IAV and aid in the development of vaccines and antivirals.

**Experimental procedures**

**Plasmids and cloning**

The pEBG-GST and pGST-2CARD plasmids were kind gifts from Dr. Michaela Gack, and their construction and purpose have been described previously (20, 26, 27). The HA-TRIM25 construct was generated by RT-PCR from total RNA isolated from primary normal human bronchiolar epithelial cells with the following primers: forward, 5’-GAT CAT GCT AGC ATG GCA GAG CTG TGC CCC CTG-3’; reverse, 5’-GAT CAT GAA TTC CTG AGA GGA GCA GAT GGA-3’. TRIM25 was inserted into an existing vector (Addgene, 18712) using the Nhel and EcoRI restriction sites, giving TRIM25 an N-terminal HA tag. The p125-Luc construct was a kind gift from Dr. Takashi Fujita, and its construction has been described previously, pCMV-rLuc was a kind gift from Dr. Mengxi Xiang (University of Alabama at Birmingham). Construction of the influenza reverse genetics plasmids for the A/Puerto Rico/8/1934 strain has been described previously (32). The R21Q mutant NS1 segment was generated using inverse mutagenesis PCR with the following primers: forward, 5’-Phos-CTT TGG CAT GTC CGC AAA CAG GTT GCA GAC CAA GAA CTA-3’; reverse, 5’-Phos-AAA GCA ATC TAC CTG AAA GCT TGA CAC AGT GTT TGG ATC-3’. 3×FLAG-NS1WT and 3×FLAG-NS1R21Q expression constructs were generated by amplifying NS1 from the IAV reverse genetics plasmids used to rescue the viruses to ensure continuity between overexpression and infection studies. NS1WT and NS1R21Q were cloned into the p3×FLAG vector (Sigma) between the EcoRI and Smal restriction sites using the following primers: forward, 5’-GAT CAT GAA TTC AAT GGA TCC AAA CAC TGT GTC AAG CTT-3’; reverse, 5’-GAT CAT CCC GGG GTG AAA CTT ACC TAA TTG TTC CCG CCA T-3’.

**NMR spectroscopy**

NMR experiments were carried out at 25 °C using Bruker Avance III spectrometers equipped with TCI cryoprobes operating at 600 MHz and 850 MHz 1H frequencies. All NMR data were processed using NMRPipe (46) and analyzed using NMR-View (47) software that was compiled on Linux workstations. Backbone 1H, 13C, and 15N resonances were assigned using standard triple-resonance assignment experiments: HNCACB and CBCA(CO)NH (48).

**Viruses and infections**

Recombinant A/Puerto Rico/8/1934 (rPR8) viruses were rescued as described previously (32). Stocks of rPR8WT and rPR8R21Q were generated by low-m.o.i. propagation on Madin-Darby canine kidney cells (MDCK) in 1× serum-free (SF) eagle’s minimum essential medium (EMEM) supplemented with 3 μg/ml 1-1-tosylamido-2-phenylethyl chloromethyl ketone–treated trypsin (Worthington Biochemical). Stock IAVs were titered using traditional plaque assays on MDCKs. All recombinant viruses were verified by Sanger sequencing. A549 type 2 alveolar epithelial cells were infected with rPR8WT and/or rPR8R21Q at the given m.o.i. for 1 h in SF EMEM. After the 1-h adsorption, the inoculum was removed and replaced with fresh SF EMEM. Sendai virus (Cantell strain, ATCC, VR-907) was propagated in 10-day-old SPF chicken embryos in accordance with established IACUC protocols. Stock Sendai virus was titered using hemagglutination assays.

**Pulldown and immunoprecipitation**

For GST pulldown experiments, 293T cells were transfected with a control vector, pEBG-GST or pGST-2CARDs, along with pHA-TRIM25 and either 3×FLAG-NS1WT or 3×FLAG-NS1R21Q. 36 h after transfection, cells were lysed in GST lysis buffer.
buffer (50 mM Tris, 200 mM NaCl, and 1% Triton X-100 (pH 7.5)), and an equal amount of protein was added to GSH-agarose (GA) beads (Pierce, 16100). Lysates were incubated with the GA beads for 2 h at room temperature in a tube rotator. After incubation, the beads were collected at 1000 x g for 2 min and washed with GST wash buffer (50 mM Tris and 200 mM NaCl (pH 7.5)). Washing was repeated three times. Bound protein was eluted in 1 x Laemmli buffer with β-mercaptoethanol by heating at 95 °C for 5 min. Samples were then assayed by immunoblot analysis.

For HA-specific immunoprecipitation, 293T cells were transfected with pHA-TRIM25 and either a GFP control vector, p3×FLAG-NS1WT, or p3×FLAG-NS1R21Q. 36 h post-infection, cells were lysed in Tris-buffered saline lysis buffer (50 mM Tris, 200 mM NaCl, and 1% Triton X-100 (pH 7.5)), and an equal amount of protein was added to anti-HA-agarose beads (Pierce, 26181). Lysates were incubated with anti-HA beads for 6 h at 4 °C. After incubation, the beads were collected at 12,000 x g for 3 s and washed with Tris-buffered saline wash buffer (50 mM Tris, 200 mM NaCl, and 0.05% Tween 20 (pH 7.5)). The washing steps were repeated three times. Beads were resuspended in 2 x Laemmli buffer without β-mercaptoethanol and heated at 95 °C for 5 min. Samples were then assayed by immunoblot analysis.

IFN-β promoter activity assays

293T cells were transfected with p125-Luc and either a GFP expression vector, 3×FLAG-NS1WT, or 3×FLAG-NS1R21Q along with pcMV-rLuc as a control for transfection efficiency. 36 h after transfection, cells were infected with 200 hemagglutinating units of Sendai virus for 16–18 h. After infection, luminescence was measured using a Dual-Luciferase assay (Promega) on a Cytation 5 microplate reader (Biotek).

Confocal microscopy

A549 cells were grown on coverslips and infected at 80% confluence with rPR8WT or rPR8R21Q at m.o.i. 5. 12 h after infection, the coverslips were fixed with 4% paraformaldehyde for 15 min at 25 °C. The fixed cells were then blocked and permeabilized in PBS containing 5% goat serum and 0.5% Triton X-100 for 1 h at 25 °C. Cells were then stained with an anti-NS1 primary antibody (Kerafast EMB005) at 1:200 dilution in PBS containing 5% goat serum and 0.3% Triton X-100 for 2 h at 25 °C and subsequently washed five times with PBS and 0.1% Tween 20 (PBST). Cells were then stained with an Alexa Fluor 488–conjugated goat anti-mouse IgG2b secondary antibody (Life Technologies, A21141) for 30 min at 25 °C, followed by five PBST washes. Cells were then treated with 4,6-diamidino-2-phenylindole (1:1000 in PBST) for 10 min at 25 °C and washed three times with PBST. Finally, coverslips were mounted onto microscope slides using Permafluor Aqueous Mounting Medium (Thermo Scientific) and sealed using nail polish. Images were acquired using an Olympus FV1000 confocal microscope and Olympus Fluoview FV10 AW 4.2 software. All images were acquired at 100 magnification with an additional x1.3 digital zoom.

Western blots and ELISA

Whole-cell lysates (GST lysis buffer or M-Per, Pierce, 78501) were separated by SDS-PAGE (Bio-Rad Citeron TGX) and transferred to nitrocellulose membranes according to the manufacturer’s protocols (Bio-Rad). After blocking in 5% non-fat dry milk in TBST (10 mM Tris, 150 mM NaCl, and 0.5% Tween 20 (pH 8)) for 1 h, the membrane was washed once with TBST and incubated with antibodies against GST (CST, 2624), FLAG (Sigma, X), β-actin (SCBT, 47778), IAV NS1 (SCBT, 130568), total IRF3 (CST, 11904), phospho-IRF3 (CST, 4947), and RIG-I (CST, 3743) in TBST at 4 °C overnight. Membranes were washed three times in TBST for 15 min, and membranes were incubated with HRP-conjugated anti-mouse or anti-rabbit secondary antibodies for 1 h at room temperature in 5% nonfat dry milk. Blots were washed as before and developed with Clarity ECL (Bio-Rad). Blots were imaged on a Chemi-Doc imager. ELISA for IFN-β in cell culture supernatants was done following the manufacturer’s instructions (PBL Assay Science). Densitometry of western blots was analyzed using Bio-Rad Image Lab.

Quantitative PCR

A549 cells were lysed with buffer RLT (Qiagen), and total RNA was extracted using the Qiagen RNeasy Plus kit with guide DNA eliminator columns. 60 ng of total RNA was used per reaction. Real-time analysis for IFN-β and β-actin was performed with the iTaq One-Step SYBR qPCR kit (Bio-Rad) following the manufacturer’s instructions. Relative IFN-β mRNA values were normalized according to β-actin expression levels. The -fold change of mRNA expression was calculated using the ΔΔCT method (Bio-Rad CFX Manager). The specificity of each amplicon was determined by analyzing its corresponding melting curve. The sequences of primers used in qPCR reactions were as follows: IFN-β, 5′-TGG GAG GCT GTA ATA CTG CCT CAA-3′ (forward) and 5′-TCT CAT AGA TGG TCA ATG CGG CGT-3′ (reverse); β-actin, 5′-ACC AAC TGG GAC GAC ATG GAG AAA-3′ (forward) and 5′-TAG CAC AGC CTG GAT AGC AAC GTA-3′ (reverse).

Fluorescence polarization

Fluorescence polarization (FP) experiments were carried out using purified WT and R21Q mutant 1918H1N1 NS1RBDs and 5′-6-FAM–labeled short hairpin RNA (shDM03) (39). Purification of the NS1RBDs was carried out as described previously (8). 5′ 6-FAM–labeled shDM03 was commercially synthesized (IDT). Prior to binding experiments, NS1RBDs and 5′ 6-FAM shDM03 were dialyzed against FP buffer (50 mM Tris-HCl, 100 mM NaCl, and 1 mM EDTA (pH 8)) to ensure buffer matching across samples. FP experiments were conducted with a constant concentration of shDM03 (5 nM) in the presence of increasing concentrations of NS1RBDs. When mixed, samples were incubated at room temperature for 1 h, and then FP measurements were made on a Victor X5 microplate reader (PerkinElmer Life Sciences) using the FP-fluorescein protocol with excitation and emission at 485 and 535 nm, respectively, and a counting time of 1 s. Change in polarization was determined by subtracting FP measurements of shDM03 buffer-only controls from experimental samples.
Male and female 8- to 12-week-old C57BL/6 mice were obtained from Taconic Biosciences (Rensselaer, NY). Mice were intranasally inoculated with 100 pfu of either A/PR8/1934-NS1WT or A/PR8/1934-NS1R21Q. Mice were monitored daily for 14 days post-infection for changes in weight, temperature, and clinical signs. Percent changes in clinical criteria were determined by comparison with preinfection values for each mouse. Mice were euthanized by intraperitoneal injection of Euthasol (200 mg kg⁻¹) when deemed moribund, according to IACUC guidelines.

Acknowledgments—We thank Dr. Michaela Gack for kindly providing the pGST-2CARD and pEBG-GST empty vector, Dr. Adolfo Garcia-Sastre for providing the PR8 reverse genetics constructs, and Dr. Takashi Fujita for kindly providing the p125-Luc vector. We also thank the Placzek laboratory for technical assistance with collecting the fluorescence polarization anisotropy data. The Bruker 850-MHz and 600-MHz magnets used in this work were funded by NCI, National Institutes of Health Grants 1P30 CA-13148 and 1P30 CA-13148 and NCRR Grant 1S10 RR022994 – 01A1.

References
1. Thompson, W. W., Comanor, L., and Shay, D. K. (2006) Epidemiology of seasonal influenza: use of surveillance data and statistical models to estimate the burden of disease. J. Infect. Dis. 194, 582–591 CrossRef Medline
2. Molinari, N. A., Ortega-Sanchez, I. R., Messonnier, M. L., Thompson, W. W., Wortley, P. M., Weintraub, E., and Bridges, C. B. (2007) The annual impact of seasonal influenza in the US: measuring disease burden and costs. Vaccine 25, 5086–5096 CrossRef Medline
3. Ayllon, J., and Garcia-Sastre, A. (2015) The NS1 protein: a multitasking virulence factor. Curr. Top Microbiol. Immunol. 386, 73–107 Medline
4. Hale, B. G., Randall, R. E., Ortin, J., and Jackson, D. (2008) The multifunctional NS1 protein of influenza A viruses. J. Gen. Virol. 89, 2359–2376 CrossRef Medline
5. Krug, R. M. (2015) Functions of the influenza A virus NS1 protein in antiviral defense. Curr. Opin. Virol. 12, 1–6 CrossRef Medline
6. Gack, M. U., Albrecht, R. A., Urano, T., Inn, K. S., Huang, I. C., Carnero, E., Farzan, M., Inoue, S., Jung, J. U., and Garcia-Sastre, A. (2009) Influenza A virus NS1 targets the ubiquitin ligase TRIM25 to evade recognition by the host viral RNA sensor RIG-I. Cell Host Microbe 5, 439–449 CrossRef Medline
7. Guo, Z., Chen, L. M., Zeng, H., Gomez, J. A., Plowden, J., Fujita, T., Katz, J. M., Donis, R. O., and Sambharoo, S. (2007) NS1 protein of influenza A virus inhibits the function of intracellular protein sensor, RIG-I. Am. J. Respir. Cell Mol. Biol. 36, 263–269 CrossRef Medline
8. Jureka, A. S., Klempeter, A. B., Cornilescu, G., Cornilescu, C. C., and Petit, C. M. (2015) Structural basis for a novel interaction between the NS1 protein derived from the 1918 influenza virus and RIG-I. Structure 23, 2001–2010 CrossRef Medline
9. Mibayashi, M., Martínez-Sobrido, L., Loo, Y. M., Cárdenas, W. B., Gale, M., Jr., and García-Sastre, A. (2007) Inhibition of retinoic acid-inducible gene I-mediated induction of beta interferon by the NS1 protein of influenza A virus. J. Virol. 81, 514–524 CrossRef Medline
10. Opitz, B., Rejaib, A., Dauber, B., Eckhard, J., Vanzing, M., Schmeck, B., Hippenstiel, S., Suttrop, N., and Wolff, T. (2007) IFNβ induction by influenza A virus is mediated by RIG-I, which is regulated by the viral NS1 protein. Cell Microbiol. 9, 930–938 CrossRef Medline
11. Pichlmair, A., Schulz, O., Tan, C. P., Näsland, B. T., Liljestrom, P., Weber, F., and Reis e Sousa, C. (2006) RIG-I-mediated antiviral responses to single-stranded RNA bearing 5’-phosphates. Science 314, 997–1001 CrossRef Medline
12. Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., Akira, S., and Fujita, T. (2004) The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat. Immunol. 5, 730–737 CrossRef Medline
13. Yoneyama, M., Onomoto, K., Jogi, M., Akaboshi, T., and Fujita, T. (2015) Viral RNA detection by RIG-I-like receptors. Curr. Opin. Immunol. 32, 48–53 CrossRef Medline
14. Rehwinkel, J., Tan, C. P., Goubau, D., Schulz, O., Pichlmair, A., Bier, K., Robb, N., Vreede, F., Barclay, W., Fodor, E., and Reis e Sousa, C. (2010) RIG-I detects viral genomic RNA during negative-strand RNA virus infection. Cell 140, 397–408 CrossRef Medline
15. Schlee, M., Roth, A., Hornung, V., Hagmann, C. A., Wimmenauer, V., Barchet, W., Coch, C., Janke, M., Mihailovic, A., Wardle, G., Juranek, S., Kato, H., Kawai, T., Poeck, H., Fitzgerald, K. A., et al. (2009) Recognition of 5’ triphosphate by RIG-I helicase requires short blunt double-stranded RNA as contained in panhandle of negative-strand virus. Immunity 31, 25–34 CrossRef Medline
16. Hornung, V., Ellegaard, J., Kim, S., Brzózka, K., Jung, A., Kato, H., Poeck, H., Akira, S., Conzelmann, K. K., Schlee, M., Endres, S., and Hartmann, G. (2006) 5’-Triphosphate RNA is the ligand for RIG-I. Science 314, 994–997 CrossRef Medline
17. Baum, A., Sachidanandam, R., and García-Sastre, A. (2010) Preference of RIG-I for short viral RNA molecules in infected cells revealed by next-generation sequencing. Proc. Natl. Acad. Sci. U.S.A. 107, 16303–16308 CrossRef Medline
18. Ramanathan, A., Devarkar, S. C., Jiang, M., Miller, M. T., Khan, A. G., Marcotrigiano, J., and Patel, S. S. (2016) The autoinhibitory CARD2-Hel2i interface of RIG-I governs RNA selection. Nucleic Acids Res. 44, 896–909 CrossRef Medline
19. Kolakofsky, D., Kowalinski, E., and Cusack, S. (2012) A structure-based model of RIG-I activation. RNA 18, 2118–2127 CrossRef Medline
20. Gack, M. U., Shin, Y. C., Joo, C. H., Urano, T., Liang, C., Sun, L., Takeuchi, O., Akira, S., Chen, Z., Inoue, S., and Jung, J. U. (2007) TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. Nature 446, 916–920 CrossRef Medline
21. Jiang, X., Kinch, L. N., Brautigam, C. A., Chen, X., Du, F., Grishin, N. V., and Chen, Z. J. (2012) Ubiquitin-induced oligomerization of the RNA sensors RIG-I and MDA5 activates antiviral innate immune response. Immunity 36, 929–973 CrossRef Medline
22. Hou, F., Sun, L., Zheng, H., Skauog, B., Jiang, Q. X., and Chen, Z. J. (2011) MAVS forms functional prion-like aggregates to activate and propagate antiviral innate immune response. Cell 146, 448–461 CrossRef Medline
23. Kawai, T., Takahashi, K., Sato, S., Coban, C., Kumar, H., Kato, H., Ishii, K. J., Takeuchi, O., and Akira, S. (2005) IPS-1, an adaptor triggering RIG-I and Mda5-mediated type I interferon induction. Nat. Immunol. 6, 981–988 CrossRef Medline
24. Seth, R. B., Sun, L., Ea, C. K., and Chen, Z. J. (2005) Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kB and IRF 3. Cell 122, 669–682 CrossRef Medline
25. Xu, L. G., Wang, Y. Y., Han, K. I., Li, L. Y., Zhai, Z., and Shu, H. (2005) VISA is an adapter protein required for virus-triggered IFN-β signaling. Mol. Cell 19, 727–740 CrossRef Medline
26. Rajsbaum, R., Albrecht, R. A., Wang, M. K., Maharaj, N. P., Versteeg, G. A., Nistal- Villán, E., García-Sastre, A., and Gack, M. U. (2012) Species-specific inhibition of RIG-I ubiquitination and IFN induction by the influenza A virus NS1 protein. PLoS Pathog. 8, e1003059 CrossRef Medline
27. Gack, M. U., Kirchhoffer, A., Shin, Y. C., Inn, K. S., Liang, C., Cui, S., Myong, S., Ha, T., Hopfner, K. P., and Jung, J. U. (2008) Roles of RIG-I N-terminal tandem CARD and splice variant in TRIM25-mediated anti-
Modulation of RIG-I activation by the influenza NS1 protein

viral signal transduction. Proc. Natl. Acad. Sci. U.S.A. 105, 16743–16748 CrossRef Medline

28. Cheng, A., Wong, S. M., and Yuan, Y. A. (2009) Structural basis for dsRNA recognition by NS1 protein of influenza A virus. Cell Res. 19, 187–195 CrossRef Medline

29. Melén, K., Kinnunen, L., Fagerlund, R., Ikonen, N., Twu, K. Y., Krug, R. M., and Julkunen, I. (2007) Nuclear and nucleolar targeting of influenza A virus NS1 protein: striking differences between different virus subtypes. J. Virol. 81, 5995–6006 CrossRef Medline

30. Greenspan, D., Palese, P., and Krystal, M. (1988) Two nuclear location signals in the influenza virus NS1 nonstructural protein. J. Virol. 62, 3020–3026 Medline

31. Wishart, D. S., and Sykes, B. D. (1994) The 13C chemical-shift index: a simple method for the identification of protein secondary structure using 13C chemical-shift data. J. Biomol. NMR 4, 171–180 Medline

32. Quinlivan, M., Zamarin, D., García-Sastre, A., Cullinane, A., Chambers, T., and Palese, P. (2005) Attenuation of equine influenza viruses through truncations of the NS1 protein. J. Virol. 79, 8431–8439 CrossRef Medline

33. Askovich, P. S., Sanders, C. J., Rosenberger, C. M., Diercks, A. H., Dash, P., Navarro, G., Vogel, P., Doherty, P. C., Thomas, P. G., and Aderem, A. (2013) Differential host response, rather than early viral replication efficiency, correlates with pathogenicity caused by influenza viruses. PLoS ONE 8, e74863 CrossRef Medline

34. Fan, S., Hatta, M., Kim, J. H., Halfmann, P., Imai, M., Macken, C. A., Le, M. Q., Nguyen, T., Neumann, G., and Kawaoka, Y. (2014) Novel residues in avian influenza virus PB2 protein affect virulence in mammalian hosts. Nat. Commun. 5, 5021 CrossRef Medline

35. Talon, J., Horvath, C. M., Polley, R., Basler, C. F., Muster, T., and Palese, P., and García-Sastre, A. (2000) Activation of interferon regulatory factor 3 is inhibited by the influenza A virus NS1 protein. J. Virol. 74, 7989–7996 CrossRef Medline

36. Baum, A., and García-Sastre, A. (2011) Differential recognition of viral RNA by RIG-I. Viruses 2, 166–169 CrossRef Medline

37. Sánchez-Aparicio, M. T., Feinman, L. J., García-Sastre, A., and Shaw, M. L. (2018) Paramyxovirus V proteins interact with the RIG-I/TRIM25 regulatory complex and inhibit RIG-I signaling. J. Virol. 92, e01960-17 CrossRef Medline

38. Koliopoulos, M. G., Lether, M., van der Veen, A. G., Haubrich, K., Hennig, J., Kowalinski, E., Stevens, R. V., Martin, S. R., Reis E Sousa, C., Cusack, S., and Rittinger, K. (2018) Molecular mechanism of influenza A NS1-mediated TRIM25 recognition and inhibition. Nat. Commun. 9, 1820 CrossRef Medline

39. Marc, D., Barbachou, S., and Soubieux, D. (2013) The RNA-binding domain of influenza virus non-structural protein-1 cooperatively binds to virus-specific RNA sequences in a structure-dependent manner. Nucleic Acids Res. 41, 434–449 CrossRef Medline

40. Cheng, J., Zhang, C., Tao, J., Li, B., Shi, Y., and Liu, H. (2018) Effects of the S42 residue of the H1N1 swine influenza virus NS1 protein on interferon responses and virus replication. Virol. J. 15, 57 CrossRef Medline

41. Dankar, S. K., Miranda, E., Forbes, N. E., Pelchat, M., Tavassoli, A., Selman, M., Ping, J., Jia, J., and Brown, E. G. (2013) Influenza A/Hong Kong/156/1997(H5N1) virus NS1 gene mutations F103L and M106I both increase IFN antagonism, virulence and cytoplasmic localization but differ in binding to RIG-I and CPSF30. Virol. J. 10, 243 CrossRef Medline

42. Dankar, S. K., Wang, S., Ping, J., Forbes, N. E., Keleta, L., Li, Y., and Brown, E. G. (2011) Influenza A virus NS1 gene mutations F103L and M106I increase replication and virulence. Virol. J. 8, 13 CrossRef Medline

43. Zeng, W., Sun, L., Jiang, X., Chen, X., Hou, F., Adhikari, A., Xu, M., and Chen, Z. J. (2010) Reconstitution of the RIG-I pathway reveals a signaling role of unanchored polyubiquitin chains in innate immunity. Cell 141, 315–330 CrossRef Medline

44. Oshiumi, H., Matsumoto, M., Hatakeyama, S., and Seya, T. (2009) Riplet/RNF135, a RING finger protein, ubiquitinates RIP1 to promote interferon-beta induction during the early phase of viral infection. J. Biol. Chem. 284, 807–817 CrossRef Medline

45. Oshiumi, H., Miyashita, M., Inoue, N., Okabe, M., Matsumoto, M., and Seya, T. (2010) The ubiquitin ligase Riplet is essential for RIG-I-dependent innate immune responses to RNA virus infection. Cell Host Microbe 8, 496–509 CrossRef Medline

46. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) NMRpipe: a multidimensional spectral processing system based on Unix pipes. J. Biomol. NMR 6, 277–293 CrossRef Medline

47. Johnson, B. A., and Blevins, R. A. (1994) NMRView: a computer program for the visualization and analysis of NMR data. J. Biomol. NMR 4, 603–614 CrossRef Medline

48. Oshiumi, H., Matsumoto, M., Hatakeyama, S., and Seya, T. (2009) Riplet/RNF135, a RING finger protein, ubiquitinates RIP1 to promote interferon-beta induction during the early phase of viral infection. J. Biol. Chem. 284, 807–817 CrossRef Medline

49. Oshiumi, H., Miyashita, M., Inoue, N., Okabe, M., Matsumoto, M., and Seya, T. (2010) The ubiquitin ligase Riplet is essential for RIG-I-dependent innate immune responses to RNA virus infection. Cell Host Microbe 8, 496–509 CrossRef Medline

50. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) NMRpipe: a multidimensional spectral processing system based on Unix pipes. J. Biomol. NMR 6, 277–293 CrossRef Medline

51. Johnson, B. A., and Blevins, R. A. (1994) NMRView: a computer program for the visualization and analysis of NMR data. J. Biomol. NMR 4, 603–614 CrossRef Medline

52. Oshiumi, H., Miyashita, M., Inoue, N., Okabe, M., Matsumoto, M., and Seya, T. (2010) The ubiquitin ligase Riplet is essential for RIG-I-dependent innate immune responses to RNA virus infection. Cell Host Microbe 8, 496–509 CrossRef Medline

53. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) NMRpipe: a multidimensional spectral processing system based on Unix pipes. J. Biomol. NMR 6, 277–293 CrossRef Medline

54. Johnson, B. A., and Blevins, R. A. (1994) NMRView: a computer program for the visualization and analysis of NMR data. J. Biomol. NMR 4, 603–614 CrossRef Medline

55. Oshiumi, H., Miyashita, M., Inoue, N., Okabe, M., Matsumoto, M., and Seya, T. (2010) The ubiquitin ligase Riplet is essential for RIG-I-dependent innate immune responses to RNA virus infection. Cell Host Microbe 8, 496–509 CrossRef Medline

56. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) NMRpipe: a multidimensional spectral processing system based on Unix pipes. J. Biomol. NMR 6, 277–293 CrossRef Medline

57. Johnson, B. A., and Blevins, R. A. (1994) NMRView: a computer program for the visualization and analysis of NMR data. J. Biomol. NMR 4, 603–614 CrossRef Medline