

Structural basis for suppression of a host antiviral response by influenza A virus

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Influenza A viruses are responsible for seasonal epidemics and high mortality pandemics. A major function of the viral NS1A protein, a virulence factor, is the inhibition of the production of IFN- β mRNA and other antiviral mRNAs. The NS1A protein of the human influenza A/Udorn/72 (Ud) virus inhibits the production of these antiviral mRNAs by binding the cellular 30-kDa subunit of the cleavage and polyadenylation specificity factor (CPSF30), which is required for the 3' end processing of all cellular pre-mRNAs. Here we report the 1.95-Å resolution X-ray crystal structure of the complex formed between the second and third zinc finger domain (F2F3) of CPSF30 and the C-terminal domain of the Ud NS1A protein. The complex is a tetramer, in which each of two F2F3 molecules wraps around two NS1A effector domains that interact with each other head-to-head. This structure identifies a CPSF30 binding pocket on NS1A comprised of amino acid residues that are highly conserved among human influenza A viruses. Single amino acid changes within this binding pocket eliminate CPSF30 binding, and a recombinant Ud virus expressing an NS1A protein with such a substitution is attenuated and does not inhibit IFN- β pre-mRNA processing. This binding pocket is a potential target for antiviral drug development. The crystal structure also reveals that two amino acids outside of this pocket, F103 and M106, which are highly conserved (>99%) among influenza A viruses isolated from humans, participate in key hydrophobic interactions with F2F3 that stabilize the complex.

antiviral drug discovery | bird flu | vaccine engineering | virology | X-ray crystallography

The NS1 protein of human influenza A viruses (NS1A protein) is a small, multifunctional protein that participates in both protein-RNA and protein-protein interactions. Its N-terminal RNA-binding domain binds double-stranded RNA (dsRNA) (1–3). By identifying the replication defect of a recombinant influenza A/Udorn/72 (Ud) virus that encodes an NS1A protein lacking dsRNA-binding activity, it was established that the primary role of NS1A dsRNA-binding activity is the inhibition of the IFN- α/β -induced oligo A synthetase/RNase L pathway, and that NS1A dsRNA-binding activity has no detectable role in inhibiting the production of IFN- β mRNA or inhibiting the activation of protein kinase R (PKR) (4, 5). The rest of the NS1A protein, which is referred to as the effector domain, has binding sites for several cellular proteins, including: the cellular 30-kDa subunit of the cleavage and polyadenylation specificity factor (CPSF30), a cellular factor required for the 3' end processing of cellular pre-mRNAs, thereby inhibiting the production of all cellular mRNAs, including IFN- β mRNA (6–10); p85 β , resulting in the activation of phosphatidylinositol-3-kinase signaling (11–14); and PKR, resulting in the inhibition of PKR activation (15).

Of these multiple protein binding sites on the NS1A protein, only the dsRNA-binding site has been structurally characterized (3, 16–18). These structural studies have revealed key features of the NS1A dsRNA-binding site that can be targeted for the development of antivirals directed against influenza A virus (18). Here we describe the structure of the interface between CPSF30 and the NS1A protein, a molecular interaction that suppresses host antiviral responses. Specifi-

cally, we report the 1.95-Å resolution X-ray crystal structure of the effector domain of the human influenza Ud NS1A protein in complex with a domain of CPSF30 comprising its second and third zinc (Zn) finger motifs (F2F3). We used the F2F3 domain of CPSF30 because it has been established that this domain binds efficiently to the Ud NS1A protein, and that expression of F2F3 in virus-infected cells leads to the inhibition of Ud virus replication and increased production of IFN- β mRNA, presumably by occupying the CPSF30 binding site on the NS1A protein and hence blocking the binding of endogenous CPSF30 to this site (19, 20).

This crystal structure reveals an NS1A:F2F3 tetrameric complex with two F2F3 binding pockets. The NS1A amino acids comprising the F2F3 binding pocket are highly conserved among human influenza A viruses, strongly suggesting that this CPSF30 binding pocket is used by all human influenza A viruses to suppress the production of IFN- β mRNA. This binding pocket is a potential target for the development of antivirals directed against influenza A virus. The crystal structure also shows that the interaction surface between NS1A and F2F3 extends beyond the primary F2F3 binding pocket alone, and that two amino acids in the NS1A protein, Phe at position 103 and Met at position 106, play key roles in stabilizing the tetramer. Although F103 and M106 are highly conserved (>99%) in the NS1A proteins of human influenza A viruses (21), a few prominent human influenza A viruses encode NS1A proteins with different amino acid residues at these positions. The biological properties of these few virus variants, however, reinforce the importance of NS1A protein-mediated CPSF30 binding for circulating human influenza A viruses.

Results and Discussion

The Crystal Structure Reveals a Tetrameric NS1A:F2F3 Complex. The Ud NS1A effector domain construct used in our experiments (amino acid residues 85–215) was identified by generation and assessment of the expression levels and solubility of 64 different NS1A constructs [supporting information (SI) Table S1]. This Ud NS1A (85–215) effector domain construct comprises 80% of the effector domain and is well-ordered in solution, as determined by its NMR spectra (Fig. S1). The 61-residue F2F3 tandem Zn-finger construct of CPSF30 comprises ~30% of its full-length sequence, and is active *in vivo* in blocking interactions between full-length Ud

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 2RHK).

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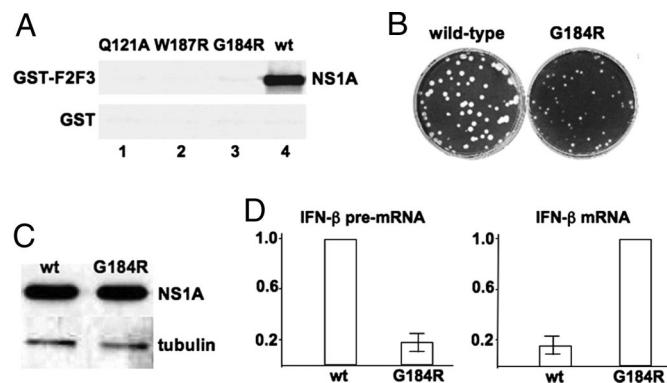


Fig. 2. Effects of amino acid substitutions in the NS1A protein on its interaction with CPSF30 and on its function in influenza A virus-infected cells. (A) GST-pulldown assay. GST-F2F3 or GST were mixed with equal amounts of the WT or indicated mutant ³⁵S-labeled full-length NS1A protein of Ud, which were prepared as described in *SI Materials and Methods*. The labeled proteins eluted with glutathione from GST-F2F3 or GST were resolved by SDS-polyacrylamide gels, which were analyzed by exposure to X-ray film. (B) Plaque sizes of the WT and G184R mutant Ud viruses in Madin-Darby canine kidney (MDCK) cells. (C) The G184R mutation in the Ud NS1A protein does not affect the amount of the NS1A protein synthesized in MDCK cells infected with 5 pfu/cell. Immunoblots of cell extracts collected at 6 h after infection were carried out with either anti-NS1A or antitubulin antibody. (D) Quantitative RT-PCR measuring amounts of IFN-β pre-mRNA (Left) and IFN-β mRNA (Right) in WT and G184R Ud-infected cells. Pre-mRNA results were normalized to WT, and mRNA results were normalized to G184R data. The results show the average and standard deviation for the relative levels of G184R pre-mRNA and WT mRNA from three different virus infections.

3D structure of the complex is distant from, and not in contact with, the NS1A effector domain (see Fig. S2). These results demonstrate that the S94 substitution does not disrupt the structure of F2F3, and that [S94]-F2F3 and [P94]-F2F3 can form complexes with NS1A (85-215) with similar structures. Most importantly, as described below, the [S94]-F2F3:NS1A (85-215) crystal structure accurately predicts effects of single-site mutations on specific functions of the NS1A protein in virus-infected cells, verifying the biological validity of this crystal structure.

The F2F3 Binding Pocket on the NS1A Protein. The crystal structure identifies the F2F3 binding pocket on the surface of NS1A (Fig. 1 C and D). This largely hydrophobic pocket, primarily defined by amino acid residues K110, I117, I119, Q121, V180, G183, G184, and W187, interacts with aromatic side chains of residues Y97, F98, and F102 of the F3 Zn finger of the corresponding F2F3 molecule.

To validate the biological relevance of the binding pocket, site-specific Ud NS1A protein variants were designed and evaluated for their effect on CPSF30-binding. Because the viral NS2/NEP protein and NS1A are coded for by the same region of the viral genome, but in a different translation frame (21), substitutions were selected such that the amino acid sequence of NS2/NEP would not be affected when a NS1A-mutant recombinant influenza A virus was generated (described below); for example, only Arg substitutions are possible at the positions G184 or W187 of NS1A without affecting the NS2/NEP sequence. Either of these two Arg substitutions, or substitution of Ala for Q121, eliminated detectable binding of the full-length Ud NS1A protein to F2F3 in GST-pulldown experiments (Fig. 2A), confirming our hypotheses based on the crystal structure that these three amino acids are required for the formation of the F2F3:NS1A complex. To ascertain if the G184R substitution alters the structure of the NS1A protein, [G184R]-NS1A (85-215) was cloned, purified, and characterized. This amino acid substitution has little or no effect on the overall fold of NS1A (85-215), as indicated by amide circular dichroism and two dimensional (¹H-¹⁵N)-HSQC NMR spectra (Fig. S4).

We next assessed the role of the CPSF30-binding pocket during virus infection by generating a recombinant Ud virus that expresses a NS1A protein with a G184R mutation. This recombinant virus forms plaques only ~25% the size of WT plaques (Fig. 2B), and during multiple cycle growth (at low multiplicity of infection) the recombinant replicates 20-fold slower than WT; for example, at 24 h after infection the titers of recombinant and WT are 1.9×10^5 and 3.8×10^6 pfu/ml, respectively. Attenuation of the G184R virus is not because of a reduction in the amount of the NS1A protein synthesized in G184R-infected cells (Fig. 2C). To determine whether this attenuation is because of reduced suppression of pre-mRNA processing, the relative amounts of IFN-β pre-mRNA and IFN-β mRNA in WT- and G184R-infected cells were determined by quantitative RT-PCR (Fig. 2D). A substantial amount of unprocessed IFN-β pre-mRNA was detected in WT virus-infected cells, verifying that the Ud virus activates transcription of the IFN-β gene via the activation of interferon regulatory factor (IRF)-3 and other transcription factors (6, 9, 26). Approximately 20% as much IFN-β pre-mRNA accumulated in G184R-infected cells, whereas the amount of mature IFN-β mRNA was approximately five times more than that in WT-infected cells. Consequently, the processing of IFN-β pre-mRNA, which is largely blocked in WT virus-infected cells, occurs much more efficiently in G184R virus-infected cells. This functional analysis demonstrates *in vivo* the biological significance of the tetrameric [S94]-F2F3:NS1A (85-215) complex structure, and particularly the importance of the CPSF30 binding pocket. It also provides definitive evidence for the essential role of NS1A-CPSF30 binding in the inhibition of IFN-β mRNA production during infection with influenza A/Udorn/72 virus.

Of the eight amino acid residues identified in the CPSF30-binding pocket of the Ud NS1A protein by this crystal structure, six are almost completely (>98%) conserved among influenza A viruses isolated from humans (21), strongly suggesting that this CPSF30 binding site is used by all human influenza A viruses to suppress the production of IFN-β mRNA. These residues are also conserved in H5N1 viruses isolated from humans and in the pandemic 1918 virus (A/Brevig Mission/1/18). The exceptions are I119 and V180, at the edge of the pocket shown in Fig. 1D, which in some sequences are replaced by similar hydrophobic Met and Ile residues, respectively, preserving the hydrophobicity of the pocket.

The Role of F103 and M106 of the NS1A Protein in Stabilizing the Tetrameric Complex. The interaction surface between NS1A and F2F3 in the tetrameric complex extends beyond the primary F2F3 binding pocket shown in Fig. 1D. Two NS1A amino acids outside the binding pocket, F103 and M106, are also critically involved in formation of the tetrameric complex (Fig. 3A and B, and Fig. 4). As illustrated in Fig. 4, the side chain of residue M106 is positioned at the tetrameric epicenter and interacts with the side chain of M106' of the NS1A' (molecule II) and with residues in both the F2F3 and F2F3' domains. The aromatic side chain F103 of NS1A (molecule I) interacts extensively with hydrophobic residues L72', Y88', and P111' of F2F3' (molecule II). Residues F103 and M106 are required for the tight binding of F2F3 *in vitro*. Thus, for example, no observable binding between F2F3 and an Ud NS1A protein with simultaneous F103L and M106I substitutions occurs *in vitro* GST pull-down experiments (Fig. 3C).

In contrast, such F103L and M106I substitutions in the NS1A protein reduce, but do not completely eliminate, its binding to CPSF30 in infected cells because other viral proteins bind to and stabilize the NS1A:CPSF30 complex (20). This phenomenon was observed with the NS1A protein of the 1997 pathogenic H5N1 influenza A/Hong Kong/483/97 (HK97) virus, which contains L (instead of F) at 103 and I (instead of M) at 106. The HK97 NS1A protein does not bind to F2F3 *in vitro* (20), like the mutant Ud NS1A protein that contains L103 and I106 (see Fig. 3C), but does bind CPSF30 to a significant extent *in vivo* when it is expressed in a virus that also encodes the other internal HK97 proteins (20). The

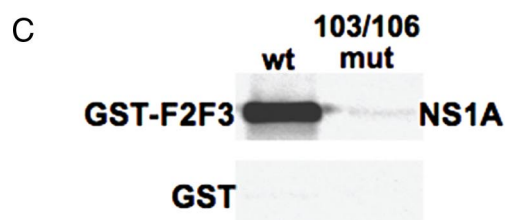
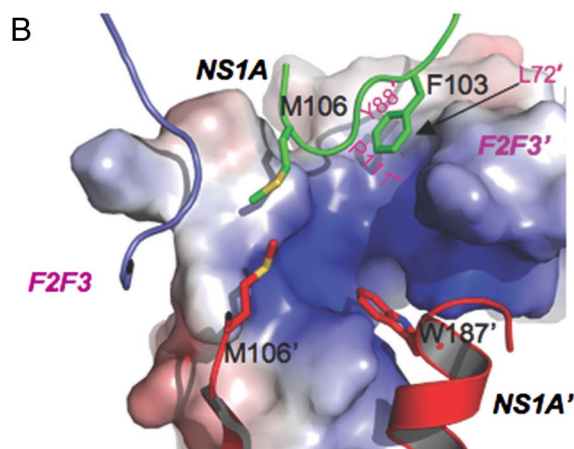
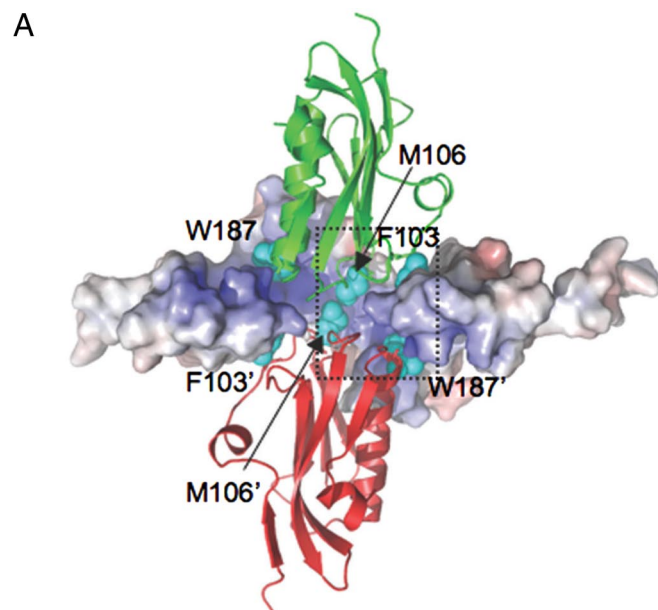


Fig. 3. Structural role of F103 and M106 in formation of the tetrameric complex. (A) Molecular graphics showing how two F2F3 molecules (represented as the electrostatic potential surface) wrap around two NS1A (85-215) molecules. The head-to-head interaction of NS1A molecules forms a docking surface for F2F3 binding. The side chain of residue M106 is critically positioned at the tetrameric epicenter and interacts with the other three molecules. (B) Expanded regions showing the structural environments of amino acid residues F103 and M106 of NS1A. The aromatic side chain of NS1A F103 interacts primarily with the hydrophobic amino acid residues L72', Y88' and P111' that are present on the surface of F2F3'. (C) GST-pulldown assay. GST-F2F3 or GST was mixed with the WT or 103/106 mutant [F103L, M106I]-NS1A protein, and analyzed as described in the legend of Fig. 2A.

F103L and M106I mutations weaken, but do not prevent, complex formation *in vivo* because cognate HK97 internal proteins interact with, and hence substantially stabilize, the CPSF30: HK97 NS1A complex in infected cells (20). Recent experiments show that such

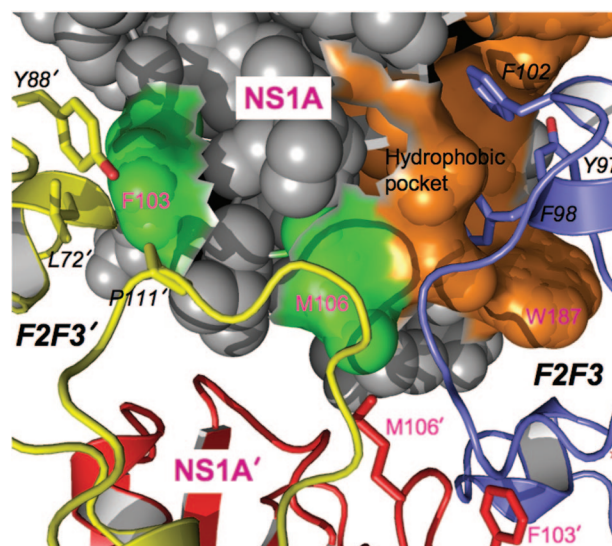


Fig. 4. Molecular graphic showing the locations of NS1A residues F103 and M106 with respect to the F3-binding pocket. The tetrameric interface extends beyond the hydrophobic pocket of the NS1A effector domain (orange) which binds one F2F3 molecule (blue). Residues M106 and F103 (green surfaces) of the same NS1A effector domain interact with both the F2F3 (blue) and F2F3' (yellow) molecules. The M106 sidechain of this NS1A effector domain also interacts with the M106' side chain of the second NS1A' molecule (red) at the tetrameric epicenter.

binding of CPSF30 to the HK97 NS1A protein requires the HK97 polymerase complex (PB1, PB2, PA, and NP), but not the HK97 M protein, and that the viral polymerase complex is actually part of the CPSF30:NS1A complex in infected cells, even when the NS1A protein has the optimum F103 and M106 amino acids (R.-L.K. and R.M.K., unpublished data).

Despite encoding a NS1A protein with less than optimum CPSF30 binding, the HK97 virus was pathogenic in birds, humans, mice, and ferrets (27, 28). Consequently, pathogenicity does not require a fully functional NS1A protein, and other viral proteins are sufficient to confer a pathogenic phenotype, consistent with a large body of literature indicating that pathogenicity/virulence is polygenic; that is, it cannot be ascribed to a single specific viral gene, but rather requires a combination of several, but not necessarily all, viral genes (29–31). Nonetheless, attenuated CPSF30 binding by the HK97 virus is suboptimal for virulence: changing L103 to F and I106 to M results in not only a 20-fold enhancement in virus replication in tissue culture (20), but also an even larger 250-fold, enhancement of virulence in mice (L.-M. Chen, R.T. Davis, R.-L.K., M. Malur, R.M.K., R.O. Donis, unpublished data). Thus, enhanced CPSF30 binding because of these two amino acid changes leads to enhanced influenza virulence in mice, demonstrating the importance of the intermolecular interactions involving the highly conserved F103 and M106 amino acids of the NS1A protein in the virulence of influenza A viruses.

Unlike the NS1A protein of the HK97 virus, all of the NS1A proteins of H5N1 viruses isolated from humans since 2003 contain the optimum F103 and M106 amino acids (20, 21). The origin of the selective pressure favoring replacement of F for L at 103 and of M for I at 106 in human H5N1 NS1A proteins is not known. Surprisingly, in 1999 to 2002, a period during which no H5N1 viruses were isolated from humans, the vast majority of the H5N1 viruses isolated from avian species encoded NS1A proteins with F and M at these positions (21). It is likely that these avian H5N1 viruses were the source of the H5N1 viruses that were transmitted to humans in 2003. It is not known what caused this change in the avian H5N1 NS1A protein, in light of the fact that the identities of the

amino acids at positions 103 and 106 in the NS1A proteins of other types of avian viruses (e.g., H9N2 and H6N1) showed considerable variability from 1997 to 2006 (21).

F103 and M106 are highly conserved in seasonal (H1N1, H3N2, H2N2) human influenza A viruses. Of the 2,284 seasonal influenza A viruses isolated from humans since 1933, 2,276 (99.6%) contain F103 and M106 (21). Only four seasonal viruses have encoded a NS1A protein with Leu instead of the consensus Phe at position 103 (21) and these viruses, like the H5N1 HK97 virus (20), presumably bind CPSF30 suboptimally in infected cells. Only five seasonal viruses have encoded an NS1A protein with a hydrophilic amino acid (S) at position 103, namely, three viruses isolated in 1934 to 1936 (including influenza A/PR/8/34), one virus isolated in 1954 (A/Leningrad/54), and one virus isolated in 1976 (A/New Jersey/76) (21). The absence of such viruses since 1976 shows that influenza A viruses encoding NS1A proteins with S103 are selected against during replication in humans.

Our F2F3:Ud NS1A structure predicts that a hydrophilic residue at position 103 in the NS1A protein should attenuate CPSF30 binding (see Figs. 3B and 4), and indeed the A/PR/8/34 (PR8) NS1A protein that contains S103 does not bind CPSF30 *in vitro* (32). In addition, the PR8 NS1A protein does not inhibit cellular gene expression in infected cells (32), indicating that it does not bind CPSF30 in infected cells (which we have confirmed). The 2.1 Å resolution crystal structure of the PR8 NS1A effector domain is a dimer, stabilized by intermolecular β -sheet interactions (23). As illustrated in Fig. S5, the oligomer orientations in the PR8 NS1A structure are completely different from the F2F3-assisted head-to-head dimer of Ud NS1A effector domains observed in the F2F3:NS1A (85-215) complex structure. The Ud NS1A dimer formation is mediated by extensive hydrophobic interactions with two F2F3 molecules, whereas the PR8 NS1A dimer interface primarily involves hydrogen bonds between two small β -strands (β 1). The Ud NS1A effector domain also forms weak oligomers at higher concentrations and in the absence of F2F3 (as described in the legend to Fig. 1). However, these interactions are weak and could readily dissociate to form other oligomeric states with appropriate binding partners, such as the F2F3-stabilized head-to-head dimer seen in the Ud NS1A:F2F3 complex.

Why is CPSF30 binding by the PR8 NS1A protein not required during virus infection? Previous studies have reported that PR8 virus infection does not activate transcription factor IRF-3 (32, 33). In one set of experiments, IRF-3 activation was assayed by determining whether the large amount of the NS1A protein that accumulated after 12 h of influenza A virus infection inhibited the IRF-3 dimerization induced by a subsequent 6 h superinfection by Sendai virus (32). Based on this assay, it was claimed that the NS1A protein of PR8 virus, as well as the NS1A proteins of other influenza A viruses, inhibits IRF-3 activation. However, it was not established that the effect of such accumulated NS1A protein on subsequent Sendai virus-induced IRF-3 activation accurately mirrors the actions of the NS1A protein on IRF-3 activation induced by influenza A virus itself at earlier times of infection. To address this issue, we directly assayed IRF-3 activation in influenza A virus-infected cells by measuring the formation of the homodimer of IRF-3 that results from the phosphorylation-dependent activation of IRF-3 (6, 29). After infection of cells for 7 h with the Ud virus, ~50% of the endogenous IRF-3 migrates in the position of the activated dimer (Fig. 5, lane 2), confirming our previous study (6). This activated dimeric IRF-3 functions to activate high level transcription of the IFN- β gene, as documented here by the accumulation of a substantial amount of unprocessed IFN- β pre-mRNA in infected cells (see Fig. 2B). In fact, we have found that IRF-3 is activated by influenza A viruses expressing NS1A proteins encoded by many other influenza A virus strains [e.g., two H5N1 viruses (HK97; A/Vietnam/1203/04) and the 1918 virus] (data not shown). In contrast, the PR8 virus does not activate IRF-3 (see Fig. 5, lane 3), confirming previous studies by others (32, 33). Consequently,

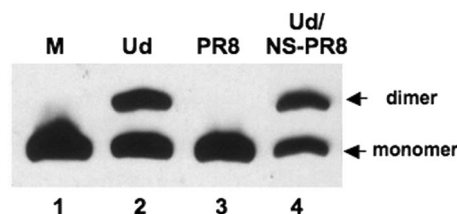


Fig. 5. PR8 virus infection, unlike infection by Ud virus, does not activate IRF-3. HEL299 cells were either mock-infected (M, lane 1), infected with 5 pfu/cell of Ud virus (Ud, lane 2), infected with 5 pfu/cell of PR8 virus (PR8, lane 3), or infected with 5 pfu/cell of a recombinant Ud virus in which the Ud NS gene was replaced by the PR8 NS gene (Ud/NS-PR8, lane 4). At 7 h after infection, cell extracts were prepared, subjected to electrophoresis on a 7.5% native gel, and IRF-3 monomers and dimers were detected by Western immunoblotting using rabbit anti-IRF-3 antibody (36). An immunoblot with anti-NS1A antibody confirmed that equivalent amounts of the NS1A protein were synthesized in Ud, PR8, and Ud/NS-PR8 virus-infected cells (lanes 2–4). Further details are provided in the *SI Materials and Methods*.

because IRF-3 is not activated in PR8 virus-infected cells, and the resulting activation of IFN- β gene expression does not occur, CPSF30 binding by the PR8 NS1A protein is not required in PR8 infected cells.

To determine whether this lack of IRF-3 activation is caused by the PR8 NS1A protein itself, we generated a recombinant Ud virus that expresses the PR8 instead of the Ud NS1A protein. In cells infected by this recombinant virus, IRF-3 is activated (Fig. 5, lane 4), demonstrating that the PR8 NS1A protein does not suppress IRF-3 activation and that other mechanisms are responsible for the lack of IRF-3 activation in PR8 virus-infected cells. This recombinant virus is attenuated: during multiple cycle growth the recombinant replicates 40-fold more slowly than WT; that is, at 24 h after infection the titers of the recombinant and Ud are 2×10^5 and 8×10^6 pfu/ml, respectively. Despite encoding an NS1A protein that is defective in CPSF30 binding (32), as well as defective in another function (34), the PR8 virus is pathogenic in mice (31), again attesting to the polygenic nature of pathogenicity (29–31).

Conclusions

The X-ray crystal structure of the Ud NS1A (85-215):F2F3 complex described here provides unique insights into the binding interface between NS1A and CPSF30 and its linked suppression of a crucial host antiviral response. These insights are not anticipated by the available structures of the F2F3 fragment alone (PDB ID 2D9N) or of the PR8 NS1A effector domain that does not bind F2F3 or CPSF30 (23, 32). The key structural features include two symmetric F2F3 binding pockets that are formed at a protein–protein interface in the tetrameric structure of the Ud NS1A:F2F3 complex. The two Ud NS1A effector domains in this complex interact with each other in a head-to-head orientation, which is unexpected and different from the extended β -sheet dimer interface observed in the PR8 NS1A effector domain (23). As illustrated in Fig. S6, this head-to-head dimer structure is also compatible with the known dimeric structure of the N-terminal RNA-binding domain (16, 17). The Ud NS1A:F2F3 structure also explains the roles of NS1A residues F103 and M106 in stabilizing the functional complex and their strong evolutionary conservation.

Based on these insights, we can conclude that CPSF30 binding by the NS1A protein is the primary, if not the only, mechanism by which circulating human influenza A viruses suppress the production of IFN- β mRNA in infected cells. The X-ray crystal structure presented here reveals the atomic details underlying this binding process. Significantly, six of the amino acids comprising the F2F3/CPSF30-binding pocket are almost completely (>98%) conserved among human influenza A viruses, including H5N1 viruses and the 1918 virus (21). In addition, the two NS1A residues, F103 and

