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. 1. Crystal structure of F2F3:NS1A (85-215) complex. (A) Gel filtration data demonstrating complex formation between NS1A (85-215) and F2F3. Traces show chromatographic profiles on a Superdex G75 column for NS1A (85-215) alone (red) and the complex of NS1A (85-215) with [S94]-F2F3 (blue). Inset shows calibrated gel filtration data for (A) [S94]-F2F3 (~10 kDa), (B) NS1A (85-215) alone (~27 kDa), and (C) [S94]-F2F3:NS1A complex (~47 kDa). Similar results were obtained by static light scattering analysis of effluent fractions from size exclusion chromatography, as described in *SI Materials and Methods*: (A) 15 ± 3 kDa, (B) 25 ± 5 kDa, and (C) 48 ± 5 kDa. The molecular mass expected for the tetrameric complex observed in the crystal structure is 49,250 Da, which is in good agreement with these light scattering and gel filtration data. The elution times for isolated NS1A (85-215) (single chain calculated molecular mass 15,943 Da) and [S94]-F2F3 (single chain calculated molecular mass 8,682 Da) domains differ when loaded at different protein concentrations, suggesting that these molecules form weak homodimers under these solution conditions. Calibration standards (A–D) are described in *SI Materials and Methods*. (B) Two NS1A effector domains (green and red) and two F2F3 domains (blue and yellow) of CPSF30 form the tetramer. Some NS1A' amino acid residues that function in complex formation are highlighted in cyan. (C) F3-binding pocket on NS1A (85-215). A hydrophobic pocket on the NS1A surface binds to the F3 Zn finger of F2F3. Both chains of NS1A in the head-to-head dimer interact with each F2F3 molecule. (D) Expanded view of the F3-binding pocket. The NS1A amino acid residues labeled in red interact with the aromatic side chains of residues Y97, F98, and F102 of the F3 Zn finger of F2F3.

NS1A and full-length human CPSF30 (19). The complex between NS1A (85-215) and F2F3 was formed, purified by gel filtration (Fig. 1A), and crystallized. The structure of this F2F3:NS1A (85-215) complex was then determined using selenomethionine (Se-Met) multiwavelength anomalous diffraction (MAD) techniques (22) and refined at 1.95-Å resolution, to R_{work} and R_{free} of 0.210 and 0.234, respectively (Table S2). The chain fold of this Ud NS1A domain is similar to that reported for the uncomplexed PR8 NS1A effector domain (PDB ID 2GX9) (23). Interestingly, the Zn fingers of F2F3 (Cys-X₇/X₈-Cys-X₅/X₄-Cys-X₃-His) are structurally similar to the C3H Cys-X₈-Cys-X₅-Cys-X₃-His Zn-finger domains of human TIS11d, which binds class II AU-rich elements in the 3' untranslated regions of target mRNAs to regulate mRNA turnover (24). This structural similarity suggests a possible RNA-binding function for these Zn-finger domains of CPSF30.

The structure of the F2F3:NS1A (85-215) complex reveals an unexpected mode of interaction between the NS1A protein and CPSF30. The complex is a tetramer, in which two F2F3 molecules wrap around two NS1A effector domains that are interacting with each other in a head-to-head orientation (Fig. 1B). The F2F3-binding surface has contributions from both chains of NS1A (85-215) in the head-to-head orientation (Fig. S2). The surface area of one NS1A (85-215) molecule is $\sim 5,600$ Å², of which $\sim 1,680$ Å² participates in intermolecular interactions, while for each F2F3

molecule, $\sim 1,310$ Å² of $\sim 4,300$ Å² of surface area takes part in tetramer formation.

The F2F3 domain of human CPSF30 used in our work (amino acid residues 60-120) has a Ser at position 94, compared to the published sequence of CPSF30 (25), which has a Pro at this position. It is not clear if this single nucleotide variant (CCC to TCC) is a naturally occurring polymorphism or is a result of the cloning process. In any case, [S94]-F2F3 is biologically active in blocking CPSF30 binding by the NS1A protein *in vivo*, as it is the same molecule that was used to demonstrate that F2F3 expression in virus-infected cells inhibits virus replication and increases virus-induced production of IFN- β mRNA (19, 20). Gel filtration data demonstrate that [S94]-F2F3 binds Ud NS1A (85-215), forming a tetrameric complex with a molecular mass of ~ 48 kDa (see Fig. 1A) similar to that obtained using [P94]-F2F3. Attempts to crystallize the purified [P94]-F2F3:NS1A (85-215) complex provided only tiny crystals, while crystals of the [S94]-F2F3:NS1A (85-215) complex, with similar morphology, are significantly larger and suitable for X-ray crystallography. As illustrated in Fig. S3, the S94 residues in both F2F3 molecules of the complex have proline-like backbone conformations ($\phi = -72^\circ$; $\psi = 172^\circ$), essentially identical to the conformation reported for residue P94 ($\phi = -70^\circ$; $\psi = 173^\circ$) in the solution NMR structure of the isolated [P94]-F2F3 molecule (PDB accession code 2D9N). In any case, the location of residue 94 in the

A

B

C

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

M106, that stabilize the F2F3:NS1A tetrameric structure are also almost completely conserved (>99%) among human influenza A viruses (21). Interestingly, the properties of two prominent influenza A viruses, H5N1 HK97 and PR8, that encode NS1A proteins with different amino acids at positions 103 and 106, reinforce the importance of the CPSF30 binding site on the NS1A protein. The HK97 NS1A protein contains different hydrophobic amino acids at positions 103 and 106, and its binding to CPSF30 is stabilized in infected cells via the interaction of the viral polymerase with the NS1A:CPSF30 complex (20), demonstrating that a supplementary viral mechanism is used in infected cells to ensure that the NS1A:CPSF30 complex is formed. The PR8 NS1A protein has a hydrophilic (S) amino acid at position 103 that essentially eliminates CPSF30 binding affinity, but PR8 virus uses a unique strategy to suppress the production of IFN- β mRNA, namely suppressing IRF-3 activation by an undetermined mechanism. This PR8 strategy has been selected against during replication in humans, in competition with influenza A viruses that both activate IRF-3 and require CPSF30 binding by the NS1A protein to suppress the production of mature IFN- β mRNA. This selection further demonstrates the crucial importance of NS1A protein-mediated CPSF30 binding for circulating human influenza A viruses. Considering that F2F3 expression in cells inhibits the replication of influenza A viruses with no apparent effect on the cells (19), the intermolecular interfaces characterized here at atomic resolution, particularly the interface between the CPSF30 F3 finger and specific conserved amino acid residues of NS1A (see Fig. 1), are candidate target sites for the development of small-molecule antiviral drugs.

Materials and Methods

NS1A effector domain NS1A (85-215) and the F2F3 (60-120) fragments of CPSPF30, were cloned into modified pET21c and pET14c (Novagen) vectors (35). The constructs were verified by DNA sequence analysis and expressed in *E. coli* BL21(DE3) cells containing the rare tRNA expression plasmid pMGK. The over-expressed proteins were purified as described in [SI Materials and Methods](#). The crystals of the purified [S94]-F2F3:NS1A complex were obtained by hanging drop vapor diffusion against the well solution containing 0.1 M sodium acetate pH 5.5, 0.5 M KNO₃, and 10% sucrose at 20°C. The structure was solved by Se-Met MAD technique (22) and refined at 1.95 Å resolution to final R_{work} and R_{free} of 0.210 and 0.234, respectively. Details of the X-ray crystallography methods are presented in the [SI Materials and Methods](#). Recombinant Ud viruses were generated from cloned DNA as described in the [SI Materials and Methods](#). IFN- β pre-mRNA and IFN- β mRNA in virus-infected cells were measured by real-time quantitative RT-PCR as described in the [SI Materials and Methods](#).

Note Added in Proof. While this paper was in press, another X-ray crystal structure of an apo-NS1A effector domain was published (37), in which the NS1A dimer interface differs from that previously reported (as depicted in Fig. S5b). The dimer interface in this new structure involves residues which contribute to the F3-binding pocket in our structure (e.g., Trp-187).

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