Conformational plasticity of the influenza A virus NS1 protein

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During infection, the influenza A virus non-structural protein 1 (NS1) interacts with a diverse range of viral and cellular factors to antagonize host antiviral defences and promote viral replication. Here, I review the structural basis for some of these functions and discuss the emerging view that NS1 cannot simply be regarded as a ‘static’ protein with a single structure. Rather, the dynamic property of NS1 to adopt various quaternary conformations is critical for its multiple activities. Understanding NS1 plasticity, and the mechanisms governing this plasticity, will be essential for assessing both fundamental protein function and the consequences of strain-dependent polymorphisms in this important virulence factor.

Introduction

Non-structural protein 1 (NS1) is a remarkably multifunctional virulence factor encoded by influenza A viruses. Its roles in regulating viral RNA metabolism, restricting host immune responses (typified by IFN) and manipulating cellular homeostatic mechanisms [e.g. phosphoinositide 3-kinase (PI3K)] are well established (Engel, 2013; Hale et al., 2008c; Krug & Garcia-Sastre, 2013). Many of the key RNA–protein and protein–protein interactions underpinning known NS1 functions during infection have been elucidated (see Fig. 1a for an overview), and many more are likely to be identified in the future given that some NS1 activities are host or strain specific.

Several crystal and nuclear magnetic resonance (NMR) structures for both full-length and individual domains of NS1 are available (Bornholdt & Prasad, 2006, 2008; Carrillo et al., 2014; Chien et al., 1997; Hale et al., 2008a; Liu et al., 1997; Xia et al., 2009). These structures are invaluable for designing new structure-based mutational studies and for improving interpretations of previous experiments. However, although the secondary and tertiary structures of NS1 domains have been established, it has been difficult to identify biologically relevant quaternary structures of NS1. Here, I rationalize recent structural and biophysical data with studies of NS1 function. I focus on interactions that NS1 makes with viral and cellular factors, and highlight the use of structural biology to address NS1 multifunctionality. NS1 is far more dynamic than previously appreciated, and conformational plasticity at the quaternary level likely contributes to NS1 occupying a large functional space.

Basics of the NS1 protein dimer

NS1 proteins naturally vary in length between strains, with their sequences being 202–237 aa (Hale et al., 2008c). NS1 is divided into four regions including two distinct globular domains. The first ~73 aa of NS1 constitute a unique N-terminal dsRNA-binding domain (RBD). A short interdomain linker region (LR) then connects the RBD to the effector domain (ED), which encompasses residues 88–202. The remaining residues of NS1 form the C-terminal ‘tail’ (CTT) (Fig. 1a). Length variability maps to the LR and the CTT: LRs can be 10–15 aa, while CTTs are typically 11–33 aa.

RNA-binding domain

The RBD is a dimer comprising three α-helices from one monomer that interlock with three α-helices from another monomer in a six-helical symmetrical arrangement (Chien et al., 1997; Liu et al., 1997). Helix-2 from each monomer aligns to form antiparallel ‘tracks’ consisting of several highly conserved basic residues, including the key arginine-38 residue, that directly interact with the dsRNA backbone (Cheng et al., 2009) (Fig. 1b). Bioinformatics analysis of the interface between the two monomers suggests that the RBD is a very stable, if not obligate, homodimer (Kerry et al., 2011a), implying that the minimal unit of full-length NS1 is also a dimer.

Linker region

Only recently have crystal structures been obtained that permitted visualization of the LR connecting the RBD to the ED (Carrillo et al., 2014). Within the LR, immediately after the RBD, is a short type I β-turn made up of residues 74–77. This β-turn causes the remainder of the extended random-coil LR structure to fold back such that hydrophobic residues of the LR sit in a groove on the ‘underside’ of the RBD dimer. The LRs from each NS1 monomer cross over one another before extending into independent globular EDs, thereby forming an ‘intertwined’ full-length NS1 homodimer (Carrillo et al., 2014) (Fig. 1b).
ED and C-terminal 'tail'

Each ED monomer within the full-length NS1 dimer adopts the same unique fold, whereby several $\beta$-strands form a twisted antiparallel $\beta$-sheet crescent around a long, central $\alpha$-helix (Bornholdt & Prasad, 2006). At the C-terminal end of the ED is a short $\alpha$-helix (residues 195–202), which extends into the CTT (Fig. 1b). Although the CTT was present in many of the constructs used for determining NS1 structures, its precise structure has not been resolved. This suggests that the CTT is intrinsically
disordered in its native state (Hale et al., 2008a), but could adopt a stable conformation upon binding an interactor, or after post-translational modification. This structural property, combined with its high degree of sequence and length variation, likely contributes to the diverse functional versatility of the CTT, and may be an example of how NS1 is still ‘exploring’ its interactions with particular host processes to improve replication fitness or species adaptation.

**Higher-order NS1 multimerization occurs via ED ‘helix–helix’ interactions**

NS1 multimerization is essential for some of its functions, not least dsRNA binding, in which only the dimeric RBD can interact with dsRNA (Wang et al., 1999). *In vitro* studies using purified full-length NS1 protein have shown that NS1 exists predominantly as a dimer at low total protein concentrations (Bornholdt & Prasad, 2008; Carrillo et al., 2014; Nemeroff et al., 1995). This is likely mediated by the stable RBD self-association (Aramini et al., 2011; Kerry et al., 2011a) (Fig. 1b). There is no evidence to suggest that full-length NS1 exists as a monomer, neither *in vitro* nor *in vivo*. Higher-order multimerization of full-length NS1 *in vitro* can be observed with increasing protein concentration (Aramini et al., 2011; Bornholdt & Prasad, 2008; Carrillo et al., 2014), and cell-based assays indicate that such multimerization is dependent upon the ED (Nemeroff et al., 1995). Indeed, isolated NS1 ED can self-associate to form dimers in solution (Aramini et al., 2011, 2014; Bornholdt & Prasad, 2006; Carrillo et al., 2014; Hale et al., 2008a; Kerry et al., 2011a; Xia et al., 2009; Xia & Robertus, 2010).

The precise way in which ED monomers form homotypic interactions has been difficult to establish. In crystal lattices of either full-length NS1 or isolated EDs, a range of ED–ED interactions have been observed (Bornholdt & Prasad, 2006, 2008; Carrillo et al., 2014; Hale et al., 2008a; Kerry et al., 2011a, b; Xia et al., 2009). One of these interfaces forms the ‘helix–helix’ dimer, where the highly conserved tryptophan-187 (W187) residues from each ED monomer reciprocally pack into hydrophobic pockets bounded by the long, central z-helix of the opposing ED monomer (Fig. 2a). Several bioinformatics, biochemical and structural assays show that this ‘helix–helix’ dimer is the predominant ED dimeric form in isolation (Aramini et al., 2011, 2014; Carrillo et al., 2014; Hale et al., 2008a; Kerry et al., 2011a; Xia & Robertus, 2010). However, in the context of purified full-length NS1 at low protein concentrations, 19F NMR studies suggest that W187 is solvent exposed and does not participate in any intra- or intermolecular interactions (e.g. Fig. 1b). Nevertheless, at higher protein concentrations, or if the ‘helix–helix’ ED dimer is artificially stabilized by disulphide bonds, W187 appears buried (Aramini et al., 2014). This, together with evidence from dynamic light scattering experiments where full-length wild-type, but not a W187A mutant, NS1 precipitates at high protein concentrations (Aramini et al., 2011), suggests that the predominant higher-order oligomer of NS1 is via ED–ED ‘helix–helix’ interactions. Thus, full-length NS1 oligomers can be generated by ‘chains’ of NS1 monomers linked to one another by alternating stable RBD–RBD and ED–ED ‘helix–helix’ interactions (Fig. 2a).

**Higher-order NS1 multimers potentiate dsRNA binding and virulence**

Biophysical studies recognized that the NS1 RBD dimer alone has a relatively low affinity for dsRNA (Chien et al., 2004), and it was unclear whether the RBD was sufficient to antagonize dsRNA-activated antiviral effectors such as 2′–5′-oligoadenylate synthase (Min & Krug, 2006). However, full-length NS1 has a higher affinity for dsRNA than the RBD alone, and this affinity can be ablated by mutating W187 (Aramini et al., 2011; Ayllon et al., 2012; Kerry et al., 2011a). In addition, the IFN-antagonistic and virulence properties of the RBD can be enhanced by fusing it to a heterologous non-viral dimerization domain, suggesting that it is simply ED quaternary structure that normally promotes RBD function (Wang et al., 2002). Thus, given that RNA-binding activity has not been detected in the ED, a plausible explanation is that NS1 oligomers mediated by ED ‘helix–helix’ interactions allow cooperative binding of full-length NS1 to long dsRNA (Fig. 2a). Cryo-electron microscopy of purified NS1 in the presence of dsRNA revealed long filamentous structures, and the first crystal structure of full-length NS1 identified tubular structures (Bornholdt & Prasad, 2008), observations both consistent with the concept of cooperatively bound oligomeric NS1. Furthermore, paracrystalline arrays of NS1 and RNA have been detected in the cytoplasm of infected cells (Yoshida et al., 1981). It is therefore tempting to speculate that NS1 ‘encapsidates’ dsRNA in multimeric structures as a means to counteract host dsRNA-activated responses (Aramini et al., 2011; Bornholdt & Prasad, 2008). Indeed, mutational analysis of W187 revealed the importance of NS1 oligomerization for virus replication and virulence *in vivo* (Ayllon et al., 2012). Determining the precise quaternary orientations of full-length NS1 in complex with dsRNA remains an important goal, despite the availability of a RBD–dsRNA structure (Cheng et al., 2009).

**NS1 conformational plasticity: spatial and host-factor dependence**

As inferred above, NS1 does not exist as an obligate higher-order multimer: *in vitro*, W187 can be exposed (‘helix open’) or buried (‘helix closed’) depending upon full-length NS1 protein concentration (Aramini et al., 2014). In cells, a mAb that recognizes an epitope including W187 fails to detect a large proportion of cytoplasmic NS1 by immunofluorescence microscopy during the first 8 h of infection, but readily detects nuclear NS1 (Kerry et al., 2011a). This suggests that W187 is exposed (‘helix open’) in a population of NS1 molecules in the nucleus, but not
exposed (‘helix closed’) in the cytoplasm, hinting that at certain stages of infection the majority of cytoplasmic NS1 is in a W187-mediated oligomeric state. This could correlate with NS1 multimerizing to sequester cytoplasmic dsRNA (Fig. 2a). Given that W187 can be detected throughout the cell at very late times post-infection (Kerry et al., 2011a), it

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**Fig. 2.** Conformational plasticity of the influenza A virus NS1 protein. (a) Cartoon depiction of a W187-mediated NS1 oligomer and its cooperative binding to dsRNA. NS1 monomers are coloured yellow and blue, and W187 is highlighted (generated using the crystal lattice observed in PDB ID 4OPA; Carrillo et al., 2014). A cartoon of long dsRNA (generated from PDB ID 2ZKO; Cheng et al., 2009) has been placed in close proximity to the near-parallel array of RBDs to represent cooperative dsRNA binding. Note that this is indicative only. (b) Crystal structure of the NS1 ED in complex with two zinc fingers of CPSF30 (F2F3). The complex is a tetramer of two EDs (coloured yellow and blue) and two F2F3 molecules (coloured red). W187 is indicated for reference. The figure was generated using PDB ID 2RHK (Das et al., 2008). (c) Crystal structure of the NS1 ED in complex with a portion of the inter-SH2 domain of p85β. A single ED monomer (yellow) is thought to interact with a single molecule of p85β (green). W187 and Y89 are indicated for reference. Figure generated using PDB ID: 3L4Q (Hale et al., 2010a). (d) Conformational dynamics at the ED ‘helix–helix’ interface. Surveying multiple ED–ED crystal structures reveals that the two ED monomers (coloured yellow and blue; only the long α-helices are shown from the blue EDs for simplicity) can rotate relative to one another while remaining in a dimeric state. The flexible dynamics of this interface, and its ability to break apart, allow NS1 to adopt several different quaternary structures. The figure was generated using PDB IDs 3D6R, 3O9U, 3OA9 and 3EE9 (Hale et al., 2008a; Kerry et al., 2011a; Xia et al., 2009).
appears that NS1 adopts different quaternary conformations in infected cells on a spatial and concentration-dependent basis. This ability is one mechanism by which NS1 might expose or shield particular binding epitopes in its tertiary structure, or even create or destroy new ones on the basis of particular homotypic quaternary associations: this principle is exemplified by known interactions of NS1 with cellular factors, which are discussed in the following sections.

**CPSF30**

NS1 proteins of many influenza A viruses bind and inhibit the cellular 30 kDa subunit of the cleavage and polyadenylation specificity factor (CPSF30) as one mechanism to suppress host antiviral gene expression (Noah et al., 2003). Notably, this interaction is primarily mediated by the same hydrophobic interface of the ED as that required for ‘helix–helix’ homodimerization (including W187) (Noah et al., 2003). Furthermore, in the crystal structure of the NS1 ED with two zinc-finger domains of CPSF30, the NS1 ED forms a novel ‘head-to-head’ quaternary homodimer structure utilizing loop residues 122–126, around which a dimer of the CPSF30 zinc-finger domains wrap (Das et al., 2008) (Fig. 2b). Thus, W187-mediated oligomerization of NS1 and NS1 binding to CPSF30 are structurally incompatible, indicating that a conformational change in NS1 must occur if the same NS1 molecules are to switch between binding CPSF30 and forming a stable dsRNA-bound oligomer.

**p85β**

NS1 binds directly to the inter-SH2 domain of the p85β isoform of PI3K and stimulates the lipid kinase activity of p85β-associated p110 to promote virus replication (Hale et al., 2008b). The crystal structure of NS1 ED in complex with the inter-SH2 domain, together with modelling of the NS1–PI3K heterotrimer and its association with membranes (Hale et al., 2010a), revealed that NS1 could not interact with PI3K at the plasma membrane in the form of a W187-mediated oligomer, nor with EDs in the ‘head-to-head’ quaternary configuration. Thus, although the inter-SH2 binding site on NS1 ED is distinct from the homodimerization/CPSF30 interface, and instead involves a groove in NS1 bounded by residues in the first short β-strand (e.g. tyrosine-89), a short z-helix (residues 95–99) and the 162–170 loop (Fig. 2c), it seems that only an additional conformation whereby the NS1 EDs are splayed out from the RBD dimer could effectively bind p85β.

**Properties of NS1 that permit conformational plasticity**

The ability of NS1 EDs to adopt different positions relative to the RBD in a dimeric NS1 unit, as well as to regulate NS1 oligomer conformations, is highly dependent upon two factors: (i) the ‘helix–helix’ ED-dimer interface, and (ii) LR length and flexibility. The ‘helix–helix’ ED-dimer interface area is small, suggesting a weak, transient interaction (Kerry et al., 2011a). Both 19F NMR studies and a survey of NS1 ‘helix–helix’ ED crystal structures also identified conformational heterogeneity at this interface: the two monomers can adopt a range of orientations relative to one another while remaining as a dimer (Aramini et al., 2014; Kerry et al., 2011a) (Fig. 2d). Furthermore, ‘fast’ interdomain dynamics as one monomer rotates around the other are probably permitted by highly conserved glycine residues in the interfacial long z-helices (Aramini et al., 2014; Kerry et al., 2011a). These dynamic properties of a weak interface likely potentiate conformational flexibility in NS1 oligomers and maintain the ED–ED interface in a readily dissociable, non-static state, primed to form other quaternary conformations.

Strikingly, around the year 2000, a naturally occurring deletion at residues 80–84 in the NS1 LR emerged in highly pathogenic H5N1 influenza A viruses. Several studies have now shown that this deletion increases the virulence of multiple influenza virus strains (Li et al., 2014; Long et al., 2008; Trapp et al., 2014), although the molecular mechanisms underpinning this phenotype are unknown. Crystallography has revealed that a shortened LR restricts the ability of EDs to adopt different positions relative to the RBD (Carrillo et al., 2014). This is likely to have significant impacts on multiple NS1 functions, although notably W187-mediated oligomerization is still possible. In addition, individual aa polymorphisms in the LR (e.g. at position 71) have been suggested to affect conformation (Carrillo et al., 2014). Although the properties of only a few LR aa substitutions have been studied previously (Li et al., 2011), a thorough structure–function analysis of the NS1 LR would be invaluable to determine how the LR impacts NS1 plasticity. This will be particularly pertinent for deciphering why the 80–84 aa LR deletion evolved and how precisely it modulates the virulence properties of NS1.

**Concluding remarks**

Understanding the structural biology underpinning NS1 functionality is key to deciphering the biological properties of this important virulence factor. NS1–interactor structures can act as a platform to predict new NS1 functions bioinformatically (de Chassey et al., 2013), and are essential for contributing to the rapid characterization of novel pandemic influenza viruses (Hale et al., 2010b). Furthermore, atomic resolution maps of both NS1–NS1 and NS1–host interactions are likely to facilitate structure-based antiviral drug design targeting critical and conserved NS1 domains, such as the dsRNA binding or the CPSF30/multimerization interfaces. Indeed, several small-molecule NS1 antagonists already exist (Engel, 2013), but their sites of interaction with NS1 have not been elucidated. Solving the structures of NS1 in complex with these inhibitors will help to define ligand-binding pockets, and may suggest new opportunities to improve efficacy.

Our knowledge of NS1 quaternary structures, and of their mechanism of regulation during infection, is limited.
Although a W187-mediated oligomer appears to be the predominant multimer (Aramini et al., 2011, 2014), the adoption of other quaternary conformations cannot be excluded (Carrillo et al., 2014). Indeed, multiple conserved ED–ED dimer interfaces have been observed in crystal lattices, but their in vivo relevance is unknown (Bornholdt & Prasad, 2006, 2008; Carrillo et al., 2014; Das et al., 2008; Kerry et al., 2011a, 2011b). The dynamic properties of NS1 may mean that it is constantly sampling all of the available functional space until an appropriate interactor is engaged, and a particular quaternary structure is formed. Future work is clearly warranted to explore the ubiquity and function of all potential NS1 quaternary structures, with a particular focus on improving our full-length NS1–interactor structural catalogue. To be effective, studies may have to integrate in vitro data from crystallography and biophysics with data from infected cells obtained using advanced fluorescence- or electron-microscopy techniques. In this regard, the development of new NS1 conformation-specific probes, such as mAbs (Kerry et al., 2011a), will be critical.

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