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Minireview

Structure and Function of the NS1 Protein of Influenza A Virus

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Abstract The avian influenza A virus currently prevailing in Asia causes fatal pneumonia and multiple organ failure in birds and humans. Despite intensive research, understanding of the characteristics of influenza A virus that determine its virulence is incomplete. NS1A protein, a non-structural protein of influenza A virus, was reported to contribute to its pathogenicity and virulence. NS1A protein is a multifunctional protein that plays a significant role in resisting the host antiviral response during the influenza infection. This review briefly outlines the current knowledge on the structure and function of the NS1A protein.

Key words influenza A virus; NS1A protein; RNA-binding domain; effector domain; dimer; resistance; antiviral response

Influenza A viruses, which have been isolated from a wide variety of avian and mammalian species, are the common pathogens with high variability that cause acute respiratory disease. Highly pathogenic strains of influenza A virus have emerged occasionally in recent history, producing pandemics such as the one in 1918, which resulted in the death of 20 to 40 million people worldwide [1,2]. In 1997, an influenza A virus H5N1 was transmitted from birds to humans in Hong Kong, and resulted in six deaths among 18 infected persons [3]. The highly virulent H5N1 subtype avian influenza viruses caused disease outbreaks in poultry in China and seven other east Asian countries during late 2003 and early 2004. These viruses were extremely pathogenic to humans in Thailand and Vietnam (killing 34 of 43 infected persons), however, their ability to transmit from person to person was limited [4,5]. On 24 March 2006, the World Health Organization reported 105 fatalities among 186 human cases, corresponding to a death rate of more than 50% for known infections [6]. Furthermore, there were many people who might have contracted avian influenza but were not diagnosed because their symptoms were too mild [7]. Some authorities proposed that the H5N1 subtype influenza A viruses wildly spreading in Southeast Asia in these years are strong candidates for causing the next flu pandemic if they acquire an efficient ability for human-to-human transmission [8,9].

It is still not clear why H5N1 subtype influenza A virus is so virulent, but some contributing factors have been identified. The influenza A virus-encoded non-structural (NS) protein 1 is one of them [10]. The results obtained with recombinant mutant viruses indicated that the NS1 protein contributes to the virulence of influenza viruses during infection primarily by allowing the viruses to disarm the interferon (IFN)-based defence system of the host cell [4,10]. Additional studies showed that the lethal H5N1 strains have one of two different mutations in the NS1 protein: a point mutation, D92E; or a deletion of residue 80–84. These mutations were linked to increased virulence, cytokine resistance or both [11,12].

Therefore, in this review, we will discuss what is currently known about the structure of the NS1 protein of influenza A virus (NS1A protein) and how it interacts with cellular targets and contributes to virulence during influenza viral infection.

Overview

Introduction of NS1A protein

The genome of influenza A viruses consists of eight

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single-stranded negative sense RNA segments that encode 10 or 11 viral proteins, depending on the strain. All of the proteins are structural proteins except for NS1 and PB1-F2. The NS1 protein is translated from the colinear transcript of segment 8, which also encodes nuclear export protein (NS2 protein) from a spliced mRNA. The NS1 protein is designated as non-structural because it is synthesized in infected cells, but is not incorporated into virions [13].

The NS1A protein is a multifunctional protein that participates in both protein-protein and protein-RNA interactions. It binds non-specifically to double-stranded RNA (dsRNA) and to specific protein targets. Folding of proteins into highly ordered structures is especially critical for carrying out their multiple functions. In addition, multifunctional proteins usually show a modular organization, with different domains responsible for different functions. Two important domains have been described in this 26 kDa NS1A protein accomplishing its multiple functions (**Fig. 1**): the N-terminal structural domain (RNA-binding domain, RBD), which protects the virus against the antiviral state induced by IFN-α/β, primarily by blocking the activation of the 2′-5′-oligo(A) synthetase/RNase L pathway; and the C-terminal structural domain (effector domain), which inhibits the maturation and exportation of the host cellular antiviral mRNAs by binding cleavage and polyadenylation specificity factor (CPSF) and inhibiting poly(A)-binding protein (PAB II) function. The effector domain is crucial for the function of the RBD [14,15]. It was revealed that dimerization of these two domains is essential for NS1A protein to interact with RNA or cellular proteins (**Fig. 2**).

The NS1A protein is one of the virally encoded IFN antagonists. It has been proposed that NS1A protein plays a significant role in resisting the cellular immune response during the viral life cycle and is essential for a viable infection by multiple mechanisms. However, the mechanisms have yet to be clarified. In order to gain insights into the NS1A protein function during influenza virus infection, structural analyses and functional studies have been carried out on the N-terminus and C-terminus.

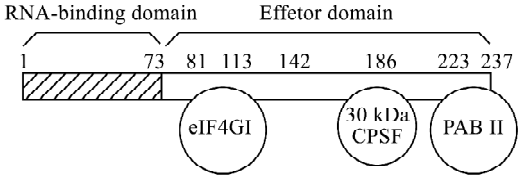

Fig. 1 Binding sites of cellular proteins on the domains of the NS1A protein

CPSF, cleavage and polyadenylation specificity factor; eIF4GI, eukaryotic initiation factor 4GI; NES, nuclear export signal; PAB II, poly(A)-binding protein.

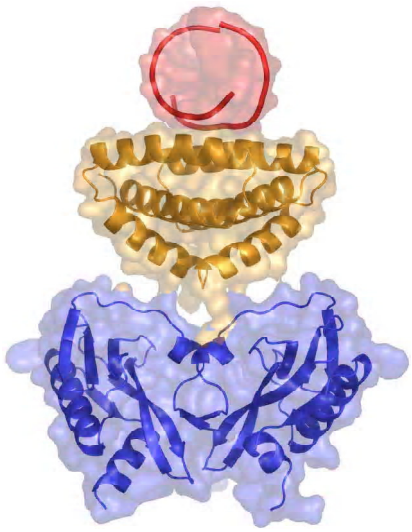

Fig. 2 Model for the dimer of the NS1A protein

The red area represents RNA, the gold area represents the RNA-binding domain dimer, and the blue area represents the effector domain dimer. Reproduced from Bornholdt and Prasad [16].

Formation of NS1A protein

The influenza A virus RNA segment 8, which contains 890 nucleotides, directs the synthesis of two mRNAs in infected cells. One is colinear with the viral RNA segment and encodes for NS1 protein of 230 amino acids; the other is derived by alternative splicing from the NS1 mRNA and translated into nuclear export protein of 121 amino acids. Starting with the initiation codon for protein synthesis, the nuclear export protein mRNA leader sequence encodes for 10 amino acids which would be the same as those at the N-terminus of the NS1 protein, whereas the rest of the coding sequences are translated from different open reading frames [17] (**Fig. 3**).

N-terminus of the NS1A Protein

Structure of the N-terminus

The dsRNA-binding domain of the NS1A protein is located at its N-terminal end. An N-terminal structural domain, which comprises the first 73 amino acids of the intact protein NS1A(1–73), possesses all of the dsRNA binding activities of the full-length protein [19]. Nuclear magnetic

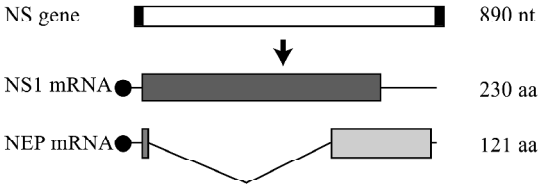

Fig. 3 Arrangement of the NS1 and nuclear export protein (NEP) mRNAs of the influenza A virus

The intact NS gene is represented as a white box flanked by black squares that represent the non-coding regions of the gene. Thin lines at the ends of the mRNAs represent untranslated regions. The 5′ cap structures (black circles) and poly(A) tails [(A)] in the mRNAs are shown. The open reading frame of the NS1 protein is represented as a gray box. The specific NEP open reading frame is shown as a hatched box. The NEP mRNA derived from the NS gene is a spliced product of the NS1 mRNA, as indicated by the V-shaped line. Reproduced from Garcia-Sastre *et al*. [18]. aa, amino acids; nt, nucleotides.

resonance (NMR) solution and X-ray crystal structures of NS1A(1–73) have shown that it forms a symmetric homodimer in solution. Each polypeptide chain of the NS1A(1–73) domain consists of three α-helices: residues Asn4-Asp24 (helix 1), Pro31-Leu50 (helix 2), and Ile54-Lys70 (helix 3) [20]. A biophysical experiment [21] showed that the arginine side chain at position 38 (Arg38) and possibly the lysine side chain at position 41 (Lys41) in the second α-helix are the only two amino acid side chains that are required for the dsRNA binding activity of the intact dimeric protein. The NS1A(1–73) domain is almost totally α-helical and forms a symmetrical homodimer with a unique six-helical chain fold. This six-helical chain fold has a novel dimeric structure that differs from that of the predominant class of dsRNA-binding domains, which are found in a large number of cellular proteins. The antiparallel helices 2 and 2′ of this symmetrical homodimer play a central role in binding the dsRNA target. According to the gel filtration and sedimentation equilibrium measurements, the dimeric NS1A(1–73) domain binds to the 16 bp synthetic dsRNA duplex with a 1:1 stoichiometry, yielding a complex with an apparent dissociation constant (K_d) of approximately 1 μM. In the NS1A(1–73)-dsRNA complex, both NS1A(1–73) and dsRNA are similar to their free forms, indicating that little or no change in the conformations of either the protein or its A-form dsRNA target occurs as a result of binding. Furthermore, studies of the interaction between NS1A(1–73) and different double-stranded nucleic acids indicate that NS1A(1–73) recognizes canonical A-form dsRNA, but does not bind to dsDNA or dsRNA-DNA hybrids, which feature B-type or A/B-type intermediate conformations, respectively. According to these results, a hypothetical working model

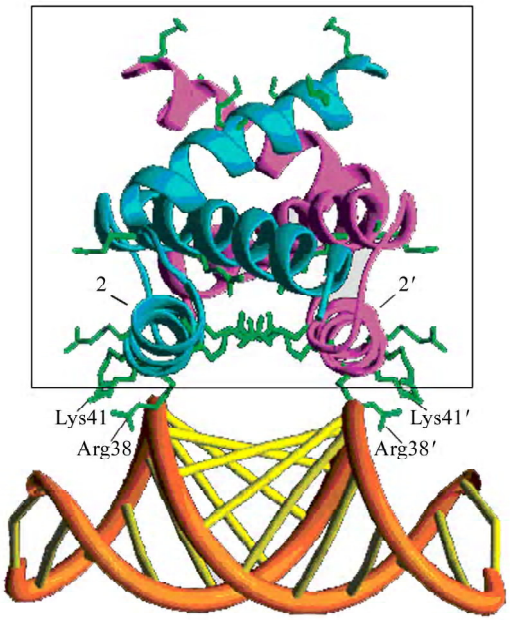

Fig. 4 Hypothetical working model of NS1A(1−73) binding to double-stranded (ds)RNA

The two monomers of the RNA-binding domain dimer are colored blue and pink. All side chains of Arg and Lys residues are colored green. Phosphate backbones and base pairs of dsRNA are shown in orange and yellow, respectively. The antiparallel helices 2 and 2′ are indicated. Reproduced from Chien CY *et al*. [20].

has been formulated. In this model, NS1A(1–73) sits astride the minor groove of A-form RNA with a few amino acids in the helix 2-helix 2′ face forming an electrostatically stabilized interaction with the phosphodiester backbone. This mode of dsRNA binding differs from that observed for any other dsRNA-binding protein (**Fig. 4**). But this picture is just a working model, consistent with the available data but useful only for the purpose of designing experiments to test the implied hypotheses.

Function of the N-terminus

The function of the dsRNA-binding activity of the NS1A protein during influenza A virus infection has not been elucidated yet. Some of the previously held theories have been disproved by new findings. In the early studies, NS1A protein inhibited the activation of protein kinase RNA-regulated (PKR) by sequestering dsRNA through the dsRNA-binding domain of the NS1A protein [22–24]. However, it was reported recently that the inhibition was realized by the direct binding of NS1A protein and the N-terminal 230 amino acid region of PKR, for which the dsRNA-binding domain is not responsible [25]. In another aspect, previous studies reported that high levels of IFN-α/β and its

mRNA were produced in cells infected with a recombinant influenza A/Wisconsin/33 (A/WSN/33) virus expressing an NS1A protein with a mutated RNA-binding domain [26,27]. However, a new study has shown that this mutant WSN NS1A protein is located in the cytoplasm, rather than the nucleus of infected cells, and the phenotype of this mutant WSN virus is due to the mislocalization of the mutant NS1A protein rather than to the loss of NS1A dsRNA-binding activity. Mutant NS1A protein expressed by recombinant A/Udorn/72 virus could localize in the nucleus of the infected cells for the second nuclear localization signal. The experiment using this recombinant A/Udorn/72 virus revealed that the RNA-binding activity of the NS1A protein does not have a role in inhibiting the influenza A virus-induced synthesis of IFN-β mRNA, but is required for the protection of influenza A virus against the antiviral state induced by IFN-β. This protection primarily involves inhibiting the IFN-α/β-induced 2′-5′-oligo(A) synthetase/RNase L pathway [28].

Besides type I IFN, the NS1A protein is also involved in the inhibition of other pro-inflammatory cytokines, such as tumor necrosis factor-α, interleukin (IL) 6, chemokine (CC motif) ligand 3 (CCL3), macrophage inflammatory protein-1 alpha (MIP-1α), IL1β and IL18. NS1A protein regulates the production of pro-inflammatory cytokines in infected macrophages through the function of both N- and C-terminal domains. Moreover, the N-terminal part of the NS1 protein appeared to be crucial for the inhibition of IL1β and IL18 production, whereas the C-terminal part was important for the regulation of IFN-β, tumor necrosis factor-α, IL6 and CCL3 (MIP-1α) production in influenza A virus-infected human macrophages [29]. Recently, it was suggested that NS1A protein restricts the production of IL1β in influenza A virus-infected macrophages at the post-translational level. As caspase-1 is a key enzyme for the post-translational processing of pro-IL1β and pro-IL18, Stasakova *et al*. presumed that the NS1A protein, through the function of its N-terminal domains, might control caspase-1 activation, thus repressing the maturation of pro-IL1β-, pro-IL18- and caspase-1-dependent apoptosis in infected primary human macrophages. As a result, virus-induced apoptosis is delayed, but the exact mechanism is not yet known in detail [29].

Another function of NS1A protein is to inhibit the splicing of pre-mRNA in virus-infected cells by binding to a specific stem bulge in one of the spliceosomal small nuclear (sn) RNAs, U6 snRNA, a key component of the catalytic core within the spliceosome [30]. The possible mechanism is that this stem bulge of U6 snRNA forms an A-form structure, which was like dsRNA in solution, allowing NS1A(1–73) to form a complex with U6 snRNA similar to the one formed between NS1A(1–73) and the 16 bp synthetic dsRNA fragment [21].

The dsRNA binding domain of the NS1A protein can also bind to the 5′ untranslated region of viral mRNAs and poly(A) binding protein 1 (PABP1). The eukaryotic initiation factor 4GI (eIF4GI) binding domain is located in the middle of the NS1A protein, a region close to PABP1 interacting domain. Accordingly, it is reasonable to infer that the NS1A interactions with eIF4GI and PABP1, as well as with viral mRNAs, could promote the specific recruitment of the viral mRNA translation initiation complexes, thus enhancing the translation of the viral mRNA [31].

In the recent experiment investigating the ability of a selection of human influenza A viruses to block IFN-β production in cultured cell lines, Hayman *et al*. found the variation between these virus strains [32]. Although the NS1A proteins of different strains behaved similarly with respect to their abilities to block dsRNA signaling, the efficiency and mechanism by which the IFN response is blocked are strain-specific. Moreover, the intracellular localization of NS1A proteins is strikingly various among these virus strains. Further studies are needed for the remaining questions, such as whether these variances are related to either the structure or the function of the N-terminus of the NS1A protein, and what the mechanism is.

C-terminus of the NS1A Protein

Structure of the C-terminus

The C-terminus of the NS1A protein mainly contains three functional domains: eIF4GI, the 30 kDa subunit of CPSF (CPSF30), and the PAB II binding domain (**Fig. 1**). The biophysical study [16] on the NS1A effector domain showed that the NS1A effector domain forms dimers in solution. Each monomer consists of seven β-strands and three α-helices. Six of the β-strands form an antiparallel twisted β-sheet, but not the last one (**Fig. 5**). Six of the β-strands surround a central long α-helix, which is held in place through an extensive network of hydrophobic interactions between the twisted β-sheet and the α-helix. This structure is believed to be a novel fold that can be best described as an α-helix β-crescent fold, as the β-sheet forms a crescent-like shape about the α-helix (**Fig. 6**). The CPSF30 binding domain is at the base of the largest α-helix. The nuclear export signal is exposed on the opposite side of the dimer, in a valley that runs diagonally across each monomer. Asp92, whose mutation to

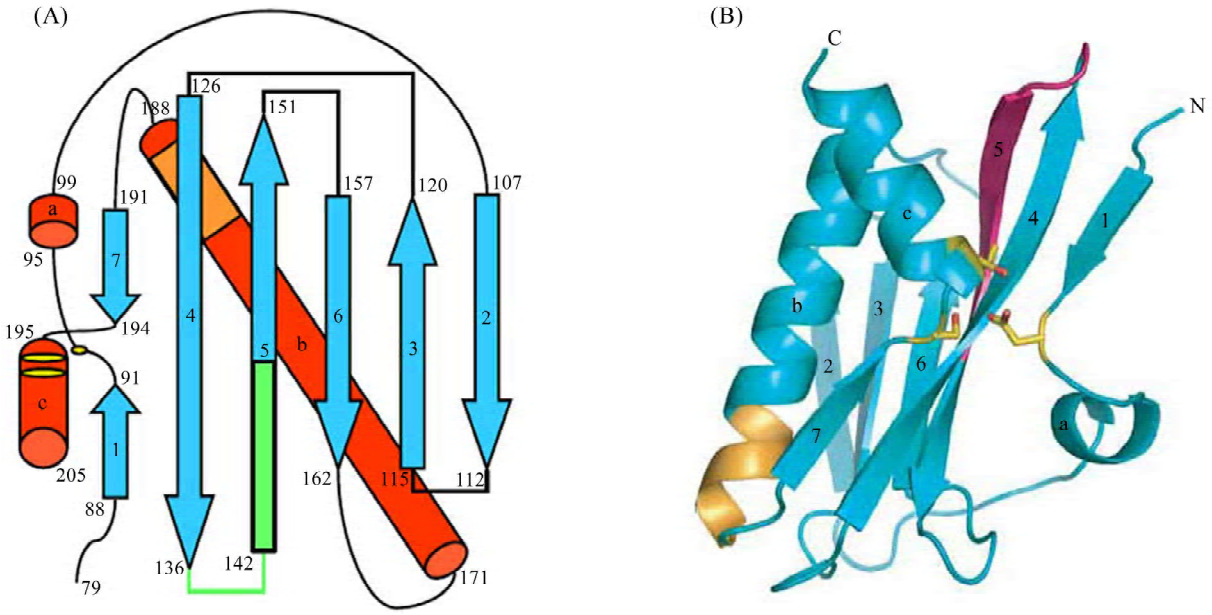

Fig. 5 Topology diagram (A) and hypothetical model (B) of the C-terminus monomer of the NS1A protein

The cleavage and polyadenylation specificity factor binding site is shown in orange, purple shows the nuclear export signal, and yellow indicates Asp92, Ser195 and Thr197. The β-strands (blue) are numbered 1–7, and the α-helices (red) are marked a, b and c. The N-terminus and C-terminus are also shown. Reproduced from Bornholdt and Prasad [16].

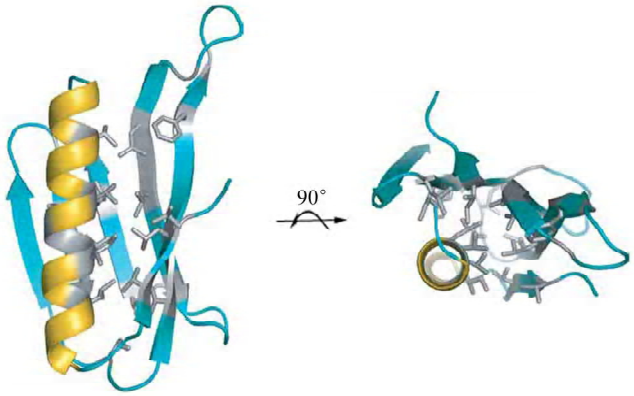

Fig. 6 α-helix β-crescent fold in the C-terminus monomer of the NS1A protein

Extensive hydrophobic interactions between the α-helix and the surrounding crescent-shaped antiparallel β-sheet are shown in two orientations. Reproduced from Bornholdt and Prasad [16].

glutamate is linked to increased virulence and cytokine resistance in certain H5N1 strains, is located in the bottom of a structurally dynamic cleft and is involved in strong hydrogen-bonding interactions with Ser195 and Thr197, shown in **Fig. 5**. The eIF4GI and the PAB II binding domains are not shown in these figures.

The interface of the effector domain dimer is formed by the first N-terminal β-strand of each monomer. These β-strands run antiparallel to each other along the dimeric interface (**Fig. 7**). This dimer has a concave surface, flanked by the N-terminus, rich in acidic residues providing both surface and charge complementarity for the RNA-

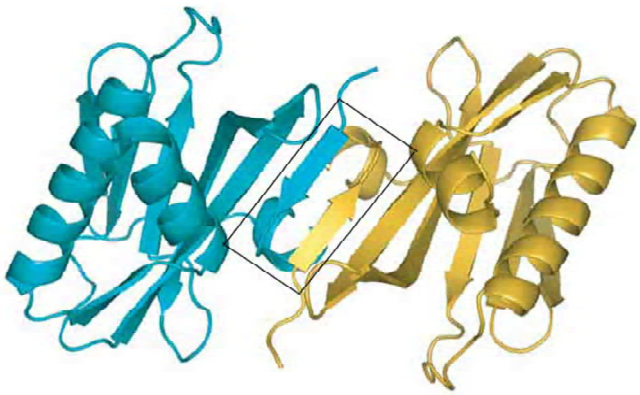

Fig. 7 Model of the effector domain dimer of NSIA protein

Dimer structure is shown with the two monomers colored blue and gold. The interface of the effector domain dimer is boxed off. From Bornholdt and Prasad [16].

binding domain, shown in **Fig. 2**.

Function of the C-terminus

As described previously, there are binding sites for eIF4GI, CPSF30 and PAB II in the C-terminus of the NS1A protein, and the interaction between eIF4GI and NS1A protein is associated with enhancement of the translation of the viral mRNA. It is also mentioned that the dsRNA-binding activity of the NS1A protein is not related to the inhibition of the synthesis of IFN-β mRNA. Nevertheless, the level of the IFN-β does decrease in virus-infected cells. Why? It has already been identified that NS1A protein binds and inhibits the function of two cellular proteins that are essential for the 3′-end processing of cellular pre-mRNAs, CPSF30 and PAB II by way of its effector domain, thereby inhibiting the production of mature cellular mRNAs, including IFN-β mRNA [33]. The binding to CPSF30 and the resulting inhibition of 3′-end processing of cellular pre-mRNAs is mediated by amino acid 144 of the NS1A protein, as well as by amino acids 184 to 188 (the 186 region). These two regions interact with the second and the third zinc finger (F2F3) of CPSF30 which contains five C3H zinc finger repeats in all [34,35] [**Fig. 8(A)**]. More significantly, constitutively expressing epitope-tagged F2F3 in the nucleus could effectively block the binding of full-length endogenous CPSF30 and NS1A protein, thereby inhibiting the replication of the influenza A virus in culture cells. These results suggest that small chemical compounds directed against the CPSF30 binding site of the NS1A protein would be expected to inhibit the replication of all strains of influenza A virus [34] [**Fig. 8(B)**]. Amino acids 215 to 237 of the NS1A protein have been identified as the binding site for PABII. Binding of NS1A and PABII, which facilitates the elongation of oligo(A) tails during the generation of the 3′ poly(A) ends of mRNAs, prevents PAB II from properly extending the poly-A tail of pre-mRNA within the host cell nucleus, and blocks these pre-mRNAs exporting from the nucleus [36]. It was also reported that another role of the C-terminal of the NS1A protein *in vivo* is to stabilize and/or facilitate formation of NS1 dimers and multimers and, therefore, to promote the RNA binding function of the NS1 N-terminal domain [15].

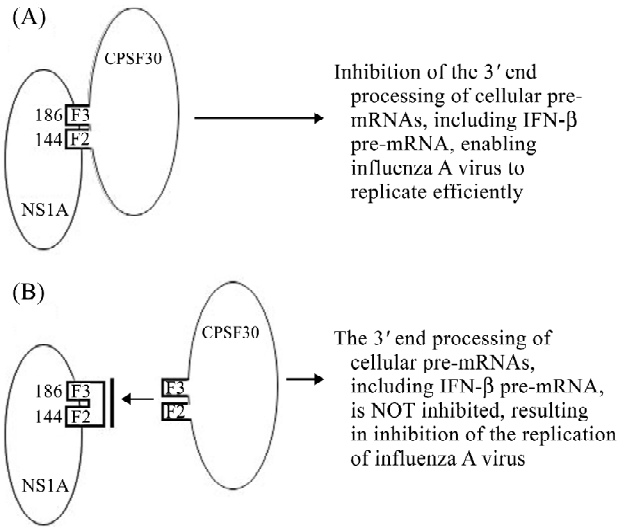

Fig. 8 Proposed mechanism for the selective inhibition of influenza A virus replication by the second and the third zinc finger (F2F3) fragment of cleavage and polyadenylation specificity factor (CPSF)30

(A) Inhibition of the 3′ end processing of cellular pre-mRNAs, including interferon-β pre-mRNA, enabling influenza A virus to replicate efficiently. (B) The 3′ end processing of cellular pre-mRNAs, including IFN-β pre-mRNA, is NOT inhibited, resulting in inhibition of the replication of influenza A virus.

The cytokine resistance conferred by the D92E mutation might be due to the increased affinity for dsRNA with this mutation [37]. Because of the proximity of Asp92 to the dimeric interface, this mutation might alter the stability or orientation of the RBD to affect its dsRNA binding affinity. However, the mutation D92E might lower the efficiency of NS1 phosphorylation. It is known that NS1 phosphorylation is required for the induction of apoptosis that allows viral ribonucleoprotein (vRNP) exporting from the nucleus. This mutation results in a virulent phenotype by prolonging the viral life cycle [16]. The deletion of residues 80–84 found in recent H5N1 strains could increase cytokine resistance by altering either the orientation or the stability of the RBD, or both, as these residues are parts of a flexible linker between the RBD and the effector domain [16,38].

The influenza A virus mutants expressing C-terminally truncated forms of the NS1 protein (NS1-81 and NS1-110) displayed a temperature-sensitive phenotype, with kinetics of virus replication in Madin-Darby canine kidney (MDCK) cells similar to those of wild-type virus when grown at 32 ºC, but were unable to replicate at the non-permissive temperature of 39 ºC [39]. These NS1 mutants have been identified to be attenuated in the murine system *in vivo*, but are able to induce efficient cellular and humoral immune responses. In addition, they contain an easily monitored genetic marker, which could grow efficiently in tissue culture, and did not produce disease but conferred protection in inoculated animals. Based on these properties, these temperature-sensitive viruses can be considered as potential master strains for preparation of attenuated live vaccines [40].

Prospects

As described previously, NS1A protein has various functions during influenza A virus infection through both its RNA-binding domain and effector domain, such as protecting influenza A virus against the antiviral state, inhibiting several kinds of pro-inflammatory cytokines, and blocking the maturation and exportation of the host cellular antiviral mRNAs. The crystal structures of the NS1 RNA-binding domain and effector domain indicate that NS1A protein functions as a dimer. In this dimer, the NS1 RNA-binding domain and effector domain form a six-helical chain fold and an α-helix β-crescent fold, respectively, which is unique. Together with the hereditary conservation, the NS1A protein is regarded as an appealing specific target against influenza A virus.

At present, the vaccines and antiviral drugs used to aim directly at the haemagglutinin (HA) and neuraminidase (NA) of influenza A virus have rendered prevention and treatment less predictably effective because of the viral antigenic mutation. Based on the above-mentioned data, it is feasible to develop live attenuated viral vaccines using the NS1A-mutational viruses [40], and design effective antiviral drugs to directly target some of the functional sites, such as the CPSF30 binding site [34]. It is also possible to explore the assisting function in tumor therapy using the recombinant virus expressing truncated NS1 protein [41].

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