Structural and Functional Analysis of NS1 and NS2 Proteins of H1N1 Subtype

Parveen Salahuddin and Asad U. Khan*

Distributed Information Sub-Centre, Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh 202002, India.

Abstract

Influenza A virus (H1N1), a genetic reassortment of endemic strains of human, avian and swine flu, has crossed species barrier to human and apparently acquired the capability of human to human transmission. Some strains of H5N1 subtype are highly virulent because NS1 protein inhibits antiviral interferon α/β production. Another protein NS2 mediates export of viral ribonucleoprotein from nucleus to the cytoplasm through export signal. In this paper, we have studied structure-function relationships of these proteins of H1N1 subtype and have determined the cause of their pathogenicity. Our results showed that non-conservative mutations slightly stabilized or destabilized structural domains of NS1 or NS1-dsRNA complex, hence slightly increased or decreased the function of NS1 protein and consequently enhanced or reduced the pathogenicity of the H1N1 virus. NS2 protein of different strains carried non-conservative mutations in different domains, resulting in slight loss of function. These mutations slightly decreased the pathogenicity of the virus. Thus, the results confirm the structure-function relationships of these viral proteins.

Key words: influenza A virus, H1N1, pathogenicity, NS2, NS1

Introduction

Influenza A virus causes annual epidemic and occasional pandemics due to its contagious nature and high mutation rate, possessing significant challenge to both human and animal health. In April 2009, a novel H1N1 virus was detected in several cases showing influenza-like illness in California and was subsequently recognized to be the cause of a major outbreak of respiratory disease in Mexico (1). The virus was found to be an H1N1 virus that was genetically and antigenically unrelated to human seasonal influenza viruses but genetically related to viruses circulating in swine (2-5). The virus was subsequently confirmed in patients from many countries around the world (6). The pandemic extends into 2010, although their number declined considerably (7). The virus is a reassortment of four known strains of influenza A virus, one endemic in human, one endemic in bird and two endemic in swine (8). This H1N1 virus has crossed species barrier to human and apparently acquired the capability of human to human transmission (9, 10), therefore this virus is currently of grave concern to public health authorities.

Influenza A virus belongs to the family of Orthomyxoviridae. It is a lipid-enveloped virus with a
negative single-stranded sense RNA genome consisted of eight separate segments. These segments code for eleven proteins (11, 12), including hemagglutinin (HA), neuraminidase (NA), matrix protein M1, ion-channel M2, non-structural proteins NS1 and NS2, nucleoprotein NP, pro-apoptotic protein PB1-F2, and RNA-dependent RNA polymerase PA, PB1 and PB2 (13).

Non-structural proteins NS1 and NS2 are coded by the eighth segment of viral RNA. The non-structural genes contain 890 nucleotides (nt) including open reading frames (14). The NS2 mRNA contains a 473 nt intron. NS1 and NS2 genes have two overlapping sequences: a 56 nt leader sequence containing initiation codon (AUG) before the intron, and a 210 nt sequence after the intron (14). In the infected cell, NS1 is localized in the nucleus and NS2 in the cytoplasm. NS1 is a multifunctional protein that plays an important role as a post-transcriptional regulator in the influenza virus life cycle: it binds virion RNA (15-17), poly(A)-containing RNA (18), U6 snRNA (19), and some viral and cellular proteins. Some strains of H5N1 subtype are highly virulent because NS1 protein inhibits antiviral interferon α/β production (20). NS1 protein contains 230-237 amino acids depending upon the strain, and has a molecular mass of approximately 26 kDa (21). It has two major domains (16, 17, 22): the first is an RNA-binding domain located in the N-terminal region (residues 1-73), which protects the virus against the antiviral state induced by IFN α/β by blocking the activation of 2'-5' oligo(A) synthetase/RNase L pathway; the second is a C-terminal domain (from residue 74 to the end), which is an effector domain containing several other domains, including cleavage and polyadenylation specificity factor (CPSF)-binding domain (residues 175-210), poly(A)-binding protein (PABP) domain (residues 218-225), nuclear localization signal 1 (residues 34-38), nuclear localization signal 2 (residues 211-216), nuclear export signal (residues 132-141) and e1F4G1 domain (residues 81-113) (20). The effector domain interacts with a large number of viral and cellular factors, such as the virus polymerase and ribonucleoprotein (RNP) (17), which are cellular proteins involved in translation and post-transcriptional processing of RNA (22-28). Such interactions may alter cellular gene expression (22, 25, 29-31), virus gene expression (32, 33), and may also block the signaling pathway mediated by Jun N-terminal protein kinase (34) and down-regulate apoptosis (35). Moreover, NS1 plays a crucial role in protecting the influenza virus against the cellular cytokine response (36, 37).

The NS2 protein was previously thought to be presented only in infected cells, until it was shown to exist in the viral particle (38). It is also referred to as nuclear export protein (NEP) because it plays an important role in mediating the export of viral RNP from nucleus to the cytoplasm through export signal and via interaction with Crm1 protein (39, 40). The NS2 protein is involved in viral assembly by interacting with the M1 protein (41). Recently, it has been proposed that NS2/NEP protein regulates influenza virus transcription and replication that is independent of its viral RNP export function (42). NS2 protein consists of 121 amino acid residues and has a molecular mass of approximately 15 kDa (43). Important domains of NS2 protein have been reported, such as the influenza M1 protein binding region (residues 59-116) and nuclear export signal (residues 11-23) (39, 44, 45). Except for the first nine amino acids, these NS1 and NS2 proteins do not bear structural relationship for the rest of polypeptide chain containing several domains with different function. In view of significant role of these proteins in virus life cycle, we have selected NS1 and NS2 proteins of different strains with the main objective to identify mutations in different structural domains of NS proteins and thus to deduce their structure-function relationships and consequently determine the exact cause of pathogenicity of H1N1 virus. Furthermore, we have also analyzed mutations in the antigenic sites of NS proteins.

Results and Discussion

Sequences with more than 90% identity show similar structure and function. In the present study, we have compared the sequences of NS1 and NS2 of H1N1 strains ACR08506 and ACR08507, respectively, with those of other strains showing identity greater than 90%. We have identified mutations in different domains of these proteins and deduced their structure-function relationships.
Structure-function analyses of NS1 protein

Previous studies have shown that high levels of interferon α/β and its mRNA were produced in cells infected with a recombinant A/wisconsin/33 (AWSN/33) containing a mutation in RNA-binding domain (46, 47). The experimental studies on recombinant A/Udorn/72 virus revealed that the RNA-binding domain of NS1A protein does not inhibit the influenza A virus induced by synthesis of interferon-β mRNA; however, it is required for the production of influenza A virus against antiviral state induced by interferon-β. The NS1 protein antagonizes the interferon α/β production by inhibiting 2′-5′ oligo(A) synthetase/RNase L pathway (48).

The sequence of NS1 protein of strain ACR08506 was compared with the sequences of NS1 of other strains, resulting in 99.54% to 99.08% identity, and mutations were identified in different structural domains of NS1. Eighteen sequences of NS1 were selected in this study (Table 1). A large number of strains (ACT68236, ACQ76393, ACR67137, ACT79148, ACT10313, A/Bethesda/SP508/2009(H1N1) ACT79188, A/New York/3184/2009(H1N1) ACT83860, A/Italy/85/2009(H1N1) ACT79188, A/Canada-NS/RV1538/2009(H1N1) ACQ89930, A/Texas/15/2009(H1N1) ACQ83304 and ACT86044) showed mutations in the RNA-binding domain (Table 2).

Table 1  NS1 sequence dataset from human source

| Strain                                | Accession No. | Country   |
|---------------------------------------|---------------|-----------|
| A/New Mexico/04/2009(H1N1)            | ACR08506      | Mexico    |
| A/Kobe/1/2009(H1N1)                   | ACR54020      | Japan     |
| A/Utsunomiya/1/2009(H1N1)             | ACS92605      | Japan     |
| A/Canada-MB/RV2013/2009(H1N1)         | ACT68236      | Canada    |
| A/Nebraska/02/2009(H1N1)              | ACQ76393      | USA       |
| A/North Dakota/04/2009(H1N1)          | ACR67137      | USA       |
| A/Brawley/40081/2009(H1N1)            | ACT79148      | USA       |
| A/Italy/85/2009(H1N1)                 | ACT10313      | Italy     |
| A/Bethesda/SP508/2009(H1N1)           | ACT79188      | USA       |
| A/Caen/2716/2009(H1N1)                | ACS91361      | France    |
| A/New York/3460/2009(H1N1)            | ACT83860      | USA       |
| A/Stockholm/34/2009(H1N1)             | ACT21950      | Russia    |
| A/Canada-SK/RV1767/2009(H1N1)         | ACT68220      | Canada    |
| A/Canada-NS/RV1538/2009(H1N1)         | ACQ89930      | Canada    |
| A/Texas/15/2009(H1N1)                 | ACQ83304      | USA       |
| A/New York/3184/2009(H1N1)            | ACQ77453      | USA       |
| A/New York/3501/2009(H1N1)            | ACT86044      | USA       |
| A/Minnesota/02/2009(H1N1)             | ACQ76353      | USA       |

Table 2  Amino acid mutations in NS1 protein of different strains at specific positions and their location in different domains

| Accession No. | Mutation at specific position | Location of mutation in domains | Hydrophobicity change (Kyte & Doolittle) |
|---------------|------------------------------|---------------------------------|----------------------------------------|
| ACR54020      | V178→I178                    | CPSF-binding domain             | +1.656 → +1.689                         |
| ACS92605      | R204→K204                    | CPSF-binding domain             | −1.478 → −1.411                         |
| ACT68236      | L33→F33                      | RNA-binding domain              | −0.600 → −0.711                         |
| ACQ76393      | D12→E12                      | RNA-binding domain              | +0.822 → +0.822                         |
| ACR67137      | D24→N24                      | RNA-binding domain              | −0.867 → −0.867                         |
| ACT79148      | I111→T111                    | e1F4G1-binding domain           | −0.656 → −0.689                         |
| ACT10313      | I112→M112                    | e1F4G1-binding domain           | +0.156 → +0.133                         |
| ACT79188      | A57→T57                      | RNA-binding domain              | +0.611 → +0.333                         |
| ACT83860      | F34→S34                      | RNA-binding domain              | −0.711 → −0.710                         |
| ACT21950      | G189→D189                    | CPSF-binding domain             | −1.000 → −1.344                         |
| ACT68220      | D55→G55                      | RNA-binding domain              | +0.567 → +0.911                         |
| ACQ89930      | D97→G97                      | e1F4G1-binding domain           | −0.989 → −0.644                         |
| ACQ83304      | G45→R45                      | RNA-binding domain              | −1.589 → −2.044                         |
| ACR77453      | D92→G92                      | e1F4G1-binding domain           | −0.533 → −0.189                         |
| ACT86044      | I123→V123                    | RNA-binding domain              | −0.656 → −0.689                         |
| ACQ76353      | R88→C88                      | e1F4G1-binding domain           | −0.578 → +0.200                         |
The biophysical study has demonstrated that two basic amino acids, Arg38 and Lys41, are the only amino acids that are required for the dsRNA binding (49). The actual crystal structure of NS1 in complex with dsRNA is not solved yet. So a detailed view of amino acids that participate in dsRNA binding is not available. However, we can predict which amino acid participates in dsRNA binding by identifying the nature of amino acid mutations and their consequences on the protein function.

The NS1 protein of strain ACT68236 carried non-conservative mutation in RNA-binding domain where Leu33 was replaced by Phe33. The conformation of RNA-binding domain was not altered. In the mutant strain, an aromatic Phe33 probably formed hydrophobic interaction with ribose ring of dsRNA, which leads to increase in its binding. Hence, the complex was stabilized and the virus propagated in the host and increased its pathogenicity. This observation is validated by recent experimental findings that showed recombinant influenza A virus expressing RNA-binding defective NS1 protein induced high levels of interferon-β production and was attenuated in mice (46).

NS1 of strain ACQ76393 showed mutation at Asp12→Glu12. The Asp12 forms an electrostatic interaction with Arg26 at the dimer interface (50, 51). Since this is a synonymous mutation (Asp12 to Glu12), it did not abolish electrostatic interaction with Arg26. Hence the structure as well as dimerization of NS1 was not affected and it remains binding to dsRNA. The interferon α/β was not synthesized; therefore, this strain was virulent. In ACR67137 strain, there was Asp24→Asn24 conservative substitution that did not change the structure or microenvironment of RNA-binding domain of NS1 protein. Hence, mutant strain binds dsRNA normally and it was pathogenic in nature.

NS1 of strain ACT79188 carried double mutations, one is non-conservative mutation Ala57→Thr57 and the other is conservative mutation I123→V123. The non-conservative mutation occurred in RNA-binding domain, where an aromatic Phe34 was mutated to polar Ser34. There was a loss of hydrophobic interaction that occurred between Phe34 and ribose ring of dsRNA, but gain of hydrogen bonding between Ser34 and OH of ribose. Since hydrogen bonding is a weak interaction compared with hydrophobic interaction, therefore NS1 of this strain binds weakly to dsRNA. The interferon α/β was produced. Consequently this strain shows weak pathogenicity.

In strain ACQ83304, a non-conservative mutation occurred, where Gly45 was substituted by Arg45 in RNA-binding domain. Recent study suggests that Gly45 is one of the conserved amino acids that enhanced the stability of NS1-dsRNA complex (52). However, in the mutant strain Arg45 also enhanced the stability of NS1-dsRNA complex by forming an electrostatic interaction with phosphate backbone of dsRNA. Therefore, this strain was pathogenic. These results imply that besides Arg38 and Lys41, other amino acids like Phe33, Thr57, Gly55 and Arg45 of NS1 protein might also contribute to dsRNA binding by stabilizing the structural domains of NS1 or complex of NS1-dsRNA. Hence these amino acids facilitate pathogenicity in viral strains.

In NS1 of strain ACR77453 of H5N1 subtype, mutation Asp92 to Gly92 occurred in eIF4G1-binding domain. Recent studies showed that Asp92→Glu92 mutation in NS1 protein of H5N1 subtype is associated with increase in mRNA binding hence increase in virulence, cytokine resistance or both (53, 54). A molecular modeling study (55) showed that Asp92→Glu92
mutation in NS1 protein of H5N1 subtype could cause weakened hydrogen bonding interactions of the carboxylate side chain of Glu with other residues such as Ser195 and Thr197. Thus, these two residues exist in a free state and are available for interactions with host targets or for phosphorylation, which thus increased the pathogenicity of this mutant (55). In NS1 of the strain ACR77453 of subtype H1N1, Asp92 with a longer side chain was substituted by Gly92 with a smaller side chain. This leads to loss of hydrogen bonding interaction between short side chain of Gly and polar Ser195 and Thr197. Therefore, these two residues Ser195 and Thr197 exist in free state and undergo phosphorylation or interact with host target. Thus, this increased the pathogenicity of ACR77453 strain.

The NS1 of some strains showed mutation at CPSF-binding domain (Table 2). In strains ACR54020 and ACS92605, conservative mutations occurred in CPSF-binding domain, where a hydrophobic amino acid is substituted by another hydrophobic amino acid (V178→I178) and a basic amino acid is substituted by another basic amino acid (R204→K204). These mutations did not change either structural or microenvironmental property of CPSF-binding domain. Therefore, NS1 proteins of these two strains bind cellular protein CPSF. Hence in these two mutants, adenylates 3' mRNA and interferon α/β was inhibited. Therefore, these strains were pathogenic. This observation is supported by an experimental study, which showed that influenza A virus NS1 protein binds to the cellular 30 kDa subunit of CPSF and inhibits 3' end formation of cellular pre-mRNAs and therefore is pathogenic (25).

NS1 of strains ACS91361 and ACT21950 carried non-conservative mutation in CPSF-binding domain (Table 2). Therefore, both the structural stability and microenvironment of CPSF-binding domain was slightly changed. The NS1 protein binds weakly to CPSF protein. This leads to low levels of adenylates of the 3'-pre-mRNA including interferon α/β. Hence these strains were less pathogenic.

The rest strains ACT79148, ACT10313, ACT83860, ACQ89930 and ACQ76353 carried non-conservative mutations in e1F4G1 domain of NS1. The NS1 of these strains binds weakly to e1F4G1. Hence, translation of viral mRNA was slowed. Therefore, these strains showed weak pathogenicity.

Earlier study has demonstrated that the role of C-terminal of NS1 protein in vivo is to stabilize and/or facilitate NS1 dimer or multimer formation; therefore it promotes RNA binding function of NS1 N-terminal domain (56). Mutations observed here in CPSF-binding domain or e1F4G1 domain influenced dimerization or multimerization of NS1, hence it affected RNA binding function of NS1 protein. These studies showed that the consequences of non-conservative mutations in structural domains of different NS1 strains were varied. It either slightly stabilized or destabilized the structure or complex of NS1 hence enhanced or reduced the pathogenicity of the H1N1 virus. The structure of NS proteins of some strains was slightly altered, therefore they were less pathogenic. Thus, this study showed that NS proteins of this pandemic strain H1N1 caused mild severity.

**Antigenicity of NS1 protein**

Table 3 shows the antigenicity profile of NS1 in strain ACR08506. Ten antigenic sites have been predicted by computer program “Antigenic”. This antigenicity profile was found common in all strains studied except in ACT68236, which showed mutation Leu33→Phe33. Therefore, antigenic segment 29-35 was missing from this strain. This is a major change that occurred at the antigenic sites of this strain, which may be one of the causes of antigenic variations in NS1 protein (Table 3). It is more pathogenic since antibody binding site is lost and viral neutralization may not take place. Other strains (ACT79148, ACT10313 and ACT83860) showed minor changes at the antigenic sites (100-122). Strains ACT79198 and ACT68220 carried mutations in antigenic segment (55-68). Strains ACR54020, ACQ76393 and ACQ76353 showed mutations in antigenic segments 174-184, 8-18 and 80-90, respectively. Hence, all of these strains escaped antibody binding and viral neutralization phenomenon. The internal proteins like NS1 or NS2 do not mutate as fast as surface proteins HA and NA. Most recently, recombinant influenza viruses with truncated or mutated NS1 proteins have been used as promising live-attenuated virus vaccines.
Table 3  Predicted antigenic sites in NS1 protein

| Amino acid position | Antigenic segment | No. of amino acid residues | Score |
|---------------------|-------------------|---------------------------|-------|
| 108-122             | RQKIIIGPLCVRLDQA  | 15                        | 1.184 |
| 8-18                | SFQVDCFLWHI       | 11                        | 1.158 |
| 55-68               | ETATLVGKQIVEWI    | 14                        | 1.141 |
| 174-184             | VKNAVGVLIGG       | 11                        | 1.140 |
| 141-149             | LETLILLRA         | 9                         | 1.133 |
| 154-169             | GAVGIEISPLPGH     | 16                        | 1.115 |
| 80-90               | TIASVPTSRYL       | 11                        | 1.093 |
| 126-139             | KNIVLKANFSVFN     | 14                        | 1.092 |
| 29-35               | DAPFLDR           | 7                         | 1.066 |
| 192-197             | VRVSEN            | 6                         | 1.061 |
| 29-35 (ACT68236)    | DAPFLDR (Antigenic segment missing) | – | – |

(57-61). Thus, recombinant vaccines containing antigenic epitopes of internal proteins NS would be an effective measure in preventing the rapid spread of H1N1 virus infection.

Structure-function analyses of NS2 protein

The NS2 protein is involved in virus assembly through its interaction with M1 protein (41). It has M1 protein binding region and nuclear export signal. The crystal structure of C-terminal (M1-binding domain) NS2 showed that helical hairpin is amphipathic in nature, with one face hydrophobic and the other face hydrophilic (45). The hydrophobic face contains 16 of 21 hydrophobic residues whereas the hydrophilic face has 15 of 19 charge residues. Six glutamate side chains cluster near the centre of hydrophilic face surrounding Trp78. The hydrophobic face of one helix is buried against second hairpin helical structure. Hence, there are large numbers of glutamates present near Trp78. These glutamates will form electrostatic interactions with basic nuclear localization signal (NLS) of M1 protein.

We compared the sequence of NS2 protein of strain ACR08507 with NS2 proteins of other strains, resulting in 99.52% to 98% identity. Twelve sequences of NS2 of H1N1 subtype were selected (Table 4). There are several strains having mutation in M1-binding region (Table 5), including ACR54980, ACR56358, ACT67144, ACQ84455, ACQ83305, ACS91365, ACR67131, ACQ89931 and ACP52563.

Since Trp78 is surrounded by a large number of glutamates at the charge surface of M1-binding region, presence of mutated basic amino acid in strains ACQ84455 and ACQ83305 decreased the acidity around Trp78, and the two mutated basic amino acids Lys63 and Lys67 did not form electrostatic interaction with basic NLS of M1 protein (45). Therefore, virus assembly was slightly inhibited and the NS2 proteins of these two strains are less virulent.

NS2 of strain ACS91365 showed mutation in M1-binding region at position 89 (Ala89→Thr89). The crystal structure of C-terminal domain (M1-binding region) of NS2 protein showed that eight residues forming an interhelical turn are well ordered, including two hydrophobic residues that are partially buried between the helices (45). In ACS91365 strain, mutation occurred in an interhelical region where hydrophobic Ala89 is substituted by polar Thr89. The

Table 4  NS2 sequence dataset from human source

| Strain                                | Accession No. | Country |
|---------------------------------------|---------------|---------|
| A/New Mexico/04/2009(H1N1)            | ACR08507      | Mexico  |
| A/Shanghai/1/2009(H1N1)               | ACR54980      | China   |
| A/Guangdong/03/2009(H1N1)             | ACR56358      | China   |
| A/CastillaLaMancha/GP369/2009(H1N1)   | ACT67144      | Spain   |
| A/Korea/01/2009(H1N1)                 | ACQ84455      | Korea   |
| A/Texas/15/2009(H1N1)                 | ACQ83305      | USA     |
| A/New York/3203/2009(H1N1)            | ACR77484      | USA     |
| A/Paris/2670/2009(H1N1)               | ACS91365      | France  |
| A/Az/irica/07/2009(H1N1)              | ACR67131      | USA     |
| A/Canada-NS/RV1538/2009(H1N1)         | ACQ89931      | Canada  |
| A/California/06/2009(H1N1)            | ACP52563      | USA     |
| A/Texas/19/2009(H1N1)                 | ACS72585      | USA     |
Table 5  Amino acid mutations in NS2 protein of different strains at specific positions and their location in different domains

| Accession No. | Mutation at specific position | Location of mutation in domains | Hydrophobicity change (Kyte & Doolittle) |
|---------------|------------------------------|---------------------------------|------------------------------------------|
| ACR54980      | R86→K86                      | M1-binding domain               | −1.422 → −1.356                         |
| ACR56358      | E67→D67                      | M1-binding domain               | −2.211 → −2.211                         |
| ACT67144      | K64→R64                      | M1-binding domain               | −3.178 → −3.222                         |
| ACQ84455      | E63→K63                      | M1-binding domain               | −3.178 → −3.222                         |
| ACQ83305      | E67→K67                      | M1-binding domain               | −2.211 → −2.256                         |
| ACR77484      | M16→I16                      | Nuclear export signal           | +0.144 → +0.433                         |
| ACS91365      | A89→T89                      | M1-binding domain               | −1.611 → −1.889                         |
| ACR67131      | A115→V115                    | M1-binding domain               | −0.433 → −0.167                         |
| ACQ89931      | A115→T115                    | M1-binding domain               | −0.433 → −0.711                         |
| ACP52563      | A189→E189                    | M1-binding domain               | −1.611 → −2.200                         |
| ACS72585      | G22→E22                      | Nuclear export signal           | −0.889 → −1.233                         |

Table 6  Predicted antigenic sites in NS2 protein of different strains

| Accession No. | Amino acid position | Antigenic segment | No. of amino acid residues | Score |
|---------------|---------------------|-------------------|---------------------------|-------|
| ACR08507      | 99-112              | FMQALQLLLLVEQE    | 14                        | 1.178 |
|               | 37-43               | SLKIYRD           | 7                         | 1.064 |
| ACQ84455      | 55-60               | LHYLQ (Gain of antigenic segment) | 6                     | 1.095 |
| ACR77484      | 10-15               | QDILMR (Gain of antigenic segment) | 6                     | 1.057 |
| ACS91365      | 54-59               | DLHYLQ (Gain of antigenic segment) | 6                     | 1.095 |
| ACR67131      | 112-118             | EIRVFSF (Gain of antigenic segment) | 7                     | 1.142 |
| ACQ89931      | 54-59               | DLHYLQ (Gain of antigenic segment) | 6                     | 1.095 |
| ACP52563      | 54-59               | DLHYLQ (Gain of antigenic segment) | 6                     | 1.095 |

Mutated amino acid Thr89 is polar in nature therefore it is not buried between the helices. Hence, the conformation and microenvironment of M1-binding domain was slightly changed. It binds weakly to M1 protein and consequently slightly inhibits virus assembly and causes mild pathogenicity. In strain ACQ89931, mutation occurred in helix C2 where hydrophobic Ala115 was replaced by polar Thr115. Due to the presence of polar Thr in hydrophobic surface of M1-binding domain, the microenvironment of this domain was changed. Thus, hydrophobic interaction occurring between helix C2 and C1 of same or different monomer was slightly inhibited (45). This leads to slight inhibition of virus assembly and the strain may be less virulent.

NS2 of ACP52563 strain was found to be substituted from hydrophobic Ala189 to charged Glu189 in the hydrophobic surface of M1-binding domain. The hydrophobicity of the surface decreased slightly, hence it interacts weakly with hydrophobic surface of same or other monomer of NS2 (45). This may again lead to slight inhibition of virus assembly and weak pathogenicity.

Recent findings indicate that methionine at positions 14, 16, and 19 and leucine at position 21 of the sequence are important for nuclear export sequence (NES) function (40). In the strain ACR77484 amino acid, Met at position 16 was substituted by Ile in NES region. Therefore, export of vRNP was slightly inhibited. In ACS72585 strain, hydrophobic Gly22 was substituted by an acidic Glu22 in NES region. The NS2 of this strain is not easily exported through nuclear membrane because of charged amino acid at position 22 (Glu22). Hence, these strains showed weak virulence.

Antigenicity of NS2 protein

Table 6 shows the antigenicity profile of NS2 protein. Two antigenic sites appeared in ACR08507 strain. A third antigenic site also appeared in other strains.
(ACQ84455, ACR77484, ACS91365, ACR67131, ACQ89931 and ACP52563). These antigenic sites are present at the N and C terminal of protein. These regions are a little bit more exposed in most of the proteins. Recently, recombinant influenza viruses with truncated or mutated internal protein like NS1 have been used as promising live-attenuated virus vaccines (60, 61). Hence these antigenic segments of NS2 are important targets for drug and antiviral vaccine developments.

Conclusion

Our studies conclusively showed that the consequences of non-conservative mutations occurring in different structural domains of NS1 of H1N1 strains were varied. It either slightly stabilized or destabilized structural domains of NS1, hence slightly increased or decreased the function of NS1 protein and consequently enhanced or reduced the pathogenicity of the H1N1 virus. NS2 proteins of different strains carrying non-conservative mutations showed a slight alteration in structure and/or microenvironment of domains, hence the function of virus was slightly inhibited and consequently decreased the pathogenicity of the virus. Thus, these studies justify the structure-function relationships of the viral NS1 and NS2 proteins.

Materials and Methods

The NS1 and NS2 protein sequences were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/), subjected to protein-protein blast (http://www.ncbi.nlm.nih.gov/BLAST/), and aligned by ClustalW multiple alignment tool using weight matrix GONNET for proteins (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html). These aligned sequences were analyzed for mutations in amino acids at specific positions in important structural domains. Hydrophobicity values were obtained from the tool ProtScale at ExPASy server by selecting Kyte & Doolittle hydrophobicity scale (http://www.expasy.org/tools/protscale.html). These proteins were also subjected to antigenic analysis using program “Antigenic” (http://liv.bmc.uu.se/cgi-bin/emboss/antigenic).

Acknowledgements

This work was supported by the DBT grant sanction No. BT/PR7507/BID/07/201/2006 to AUK. Authors are also grateful to Prof. M. Saleemuddin for providing the internal fund for this project.

Authors’ contributions

PS collected the dataset, performed data analyses and drafted the manuscript. AUK designed the study, supervised the project and co-wrote the manuscript. Both authors read and approved the final manuscript.

Competing interests

The authors have declared that no competing interests exist.

References

1. Neumann, G., et al. 2009. Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature 459: 931-939.
2. Brown, I.H. 2000. The epidemiology and evolution of influenza viruses in pigs. Vet. Microbiol. 74: 29-46.
3. Olsen, C.W. 2002. The emergence of novel swine influenza viruses in North America. Virus Res. 85: 199-210.
4. Webby, R.J., et al. 2000. Evolution of swine H3N2 influenza viruses in the Unites States. J. Virol. 74: 8243-8251.
5. Gordon, S.M. 2009. Update on 2009 pandemic influenza A (H1N1) virus. Cleve. Clin. J. Med. 76: 577-582.
6. Shinde, V., et al. 2009. Triple-reassortant swine influenza A (H1N1) in humans in the United States, 2005-2009. N. Engl. J. Med. 360: 2616-2625.
7. Centers for Disease Control and Prevention (CDC). 2010. Update: influenza activity-United States, August 30, 2009-March 27, 2010, and composition of the 2010-11 influenza vaccine. Morb. Mortal. Wkly. Rep. 59: 423-430.
8. Smith, G.J., et al. 2009. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature 459: 1122-1125.
9. Butler, D. 2009. Swine flu goes global. Nature 458: 1082-1083.
10. Cohen, I. and Enserink, M. 2009. Infectious diseases. As swine flu circles globe, scientists grapple with basic questions. Science 324: 572-573.
11. Kobasa, D. and Kawaoka, Y. 2005. Emerging influenza viruses: past and present. Curr. Mol. Med. 5:791-803.
12. Obenauer, J.C., et al. 2006. Large-scale sequence analysis of avian influenza isolates. Science 311: 1576-1580.
Danishuddin, M., et al. 2009. Molecular interactions between mitochondrial membrane proteins and the C-terminal domain of PBI-F2: an in silico approach. J. Mol. Model. 16: 535-541.

Lamb, R.A., et al. 1980. Mapping of the two overlapping genes for polypeptides NS1 and NS2 on RNA segment 8 of influenza virus genome. Proc. Natl. Acad. Sci. USA 77: 1857-1861.

Hatada, E., et al. 1997. Binding of the influenza virus NS1 protein to model genome RNAs. J. Gen. Virol. 78: 1059-1063.

Marion, R.M., et al. 1997. The N-terminal half of the influenza virus NS1 protein is sufficient for nuclear retention of mRNA and enhancement of viral mRNA translation. Nucleic Acids Res. 25: 4271-4277.

Marion, R.M., et al. 1997. Influenza virus NS1 protein interacts with viral transcription-replication complexes in vivo. J. Gen. Virol. 78: 2447-2451.

Qiu, Y. and Krug, R.M. 1994. The influenza virus NS1 protein is a poly (A)-binding protein that inhibits nuclear export of mRNAs containing poly (A). J. Virol. 68: 2425-2432.

Qiu, Y., et al. 1995. The influenza virus NS1 protein binds to a specific region in human U6 snRNA and inhibits U6-U2 and U6-U4 snRNA interactions during splicing. RNA 1: 304-316.

Lin, D., et al. 2007. Structure and function of the NS1 protein of influenza A virus. Acta Biochim. Biophys. Sin. 39: 155-162.

Hale, B.G., et al. 2008. The multifunctional NS1 protein of influenza A viruses. J. Gen. Virol. 89: 2359-2376.

Qian, X.Y., et al. 1994. Two functional domains of the influenza virus NS1 protein are required for regulation of pre-mRNA splicing and blocks mRNA nuclear export of mRNAs containing poly (A). J. Virol. 68: 2425-2432.

Qiu, Y., et al. 1995. The influenza virus NS1 protein interacts with the cellular 30 kDa subunit of CPSF and inhibits U6-U2 and U6-U4 snRNA interactions during splicing. RNA 1: 304-316.

Aragon, T., et al. 2000. Eukaryotic translation initiation factor 4GI is a cellular target for NS1 protein, a translational activator of influenza virus. Mol. Cell Biol. 20: 6259-6568.

Burgui, I., et al. 2003. PABP1 and eIF4GI associate with influenza virus NS1 protein in viral mRNA translation initiation complexes. J. Gen. Virol. 84: 3263-3274.

Falcon, A.M., et al. 1999. Interaction of influenza virus NS1 protein and the human homologue of Staufen in vivo and in vitro. Nucleic Acids Res. 27: 2241-2247.

Nemeroff, M.E., et al. 1998. Influenza virus NS1 protein interacts with the cellular 30 kDa subunit of CPSF and inhibits 3’ end formation of cellular pre-mRNAs. Mol. Cell 1: 991-1000.

Wolff, T., et al. 1998. NS1-Binding Protein (NS1-BP): a novel human protein that interacts with the influenza A virus nonstructural NS1 protein is relocalized in the nuclei of infected cells. J. Virol. 72: 7170-7180.

Qian, X.Y., et al. 1995. An amino-terminal polypeptide fragment of the influenza virus NS1 protein possesses specific RNA-binding activity and largely helical backbone structure. RNA 1: 948-956.

Fortes, P., et al. 1994. Influenza virus NS1 protein inhibits pre-mRNA splicing and blocks mRNA nucleocytoplasmic transport. EMBO J. 13: 704-712.

Fortes, P., et al. 1995. Influenza virus NS1 protein alters the subnuclear localization of cellular splicing components. J. Gen. Virol. 76: 1001-1007.

Lu, Y., et al. 1994. The influenza virus NS1 protein: a novel inhibitor of pre-mRNA splicing. Genes Dev. 8: 1817-1828.

de la Luna, S., et al. 1995. Influenza virus NS1 protein enhances the rate of translation initiation of viral mRNAs. J. Virol. 69: 2427-2433.

Enami, K., et al. 1994. Influenza virus NS1 protein stimulates translation of the M1 protein. J. Virol. 68: 1432-1437.

Ludwig, S., et al. 2002. The influenza A virus NS1 protein inhibits activation of Jun N-terminal kinase and AP-1 transcription factors. J. Virol. 76: 11166-11171.

Zhirkov, O.P., et al. 2002. NS1 protein of influenza A virus down-regulates apoptosis. J. Virol. 76: 1617-1625.

Hatada, E., et al. 1999. Mutant influenza viruses with a defective NS1 protein cannot block the activation of PKR in infected cells. J. Virol. 73: 2425-2433.

Seo, S.H., et al. 2002. Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. Nat. Med. 8: 950-954.

Richardson, J.C. and Akkina, R.K. 1991. NS2 protein of influenza virus is found in purified virus and phosphorylated in infected cells. Arch. Virol. 116: 69-80.

O’Neill, R.E., et al. 1998. The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins. EMBO J. 17: 288-296.

Neumann, G., et al. 2000. Influenza A virus NS2 protein mediates vRNP nuclear export through NES-independent interaction with hCRM1. EMBO J. 19: 6751-6758.

Schmitt, A.P. and Lamb, R.A. 2005. Influenza virus assembly and budding at the viral budzone. Adv. Virus. Res. 64: 383-416.

Robb, N.C., et al. 2009. NS2/NEP protein regulates transcription and replication of the influenza virus RNA genome. J. Gen. Virol. 90: 1398-1407.

Greenspan, D., et al. 1985. Expression on influenza virus NS2 nonstructural protein in bacteria and localization of NS2 in infected eukaryotic cells. J. Virol. 54: 833-843.

Ward, A.C., et al. 1995. Expression and analysis of the NS2 protein of influenza A virus. Arch. Virol. 140: 2067-2073.

Akarsu, H., et al. 2003. Crystal structure of the M1 protein-binding domain of the influenza A virus nuclear export protein (NEP/NS2). EMBO J. 22: 4646-4555.

Donelan, N.R., et al. 2003. A recombinant influenza A virus expressing an RNA-binding defective NS1 protein
induces high levels of beta interferon and is attenuated in mice. J. Virol. 77: 13257-13266.

47 Wang, X., et al. 2000. Influenza A virus NS1 protein prevents activation of NF-kappaB and induction of alpha/beta interferon. J. Virol. 74: 11566-11573.

48 Min, J.Y. and Krug, R.M. 2006. The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: inhibiting the 2'-5' oligo(A) synthetase/RNase L pathway. Proc. Natl. Acad. Sci. USA 103: 7100-7105.

49 Wang, W., et al. 1999. RNA binding by the novel helical domain of the influenza virus NS1 protein requires its dimer structure and a small number of specific basic amino acids. RNA 5: 195-205.

50 Chien, C.Y., et al. 1997. A novel RNA-binding motif in influenza A virus non-structural protein 1. Nat. Struct. Biol. 4: 891-895.

51 Liu, J., et al. 1997. Crystal structure of the unique RNA-binding domain of the influenza virus NS1 protein. Nat. Struct. Biol. 4: 896-899.

52 Yin, C., et al. 2007. Conserved surface features form the double-stranded RNA binding site of non-structural protein 1 (NS1) from influenza A and B viruses. J. Biol. Chem. 282: 20584-20592.

53 Seo, S.H., et al. 2004. The NS1 gene of H5N1 influenza viruses circumvents the host anti-viral cytokine responses. Virus Res. 103: 107-113.

54 Lipatov, A.S., et al. 2005. Pathogenesis of Hong Kong H5N1 influenza virus NS gene reassortants in mice: the role of cytokines and B- and T-cell responses. J. Gen. Virol. 86: 1121-1130.

55 Li, M. and Wang, B. 2007. Homology modeling and examination of the effect of the D92E mutation on the H5N1 nonstructural protein NS1 effector domain. J. Mol. Model. 13: 1237-1244.

56 Nemeroff, M.E., et al. 1995. The influenza virus NS1 protein forms multimers in vitro and in vivo. Virology 212: 422-428.

57 Richt, J.A., et al. 2006. Vaccination of pigs against swine influenza viruses by using an NS1-truncated modified live-virus vaccine. J. Virol. 80: 11009-11018.

58 Solorzano, A., et al. 2005. Mutations in the NS1 protein of swine influenza virus impair anti-interferon activity and confer attenuation in pigs. J. Virol. 79: 7535-7543.

59 Vincent, A.L., et al. 2007. Efficacy of intranasal administration of a truncated NS1 modified live influenza virus vaccine in swine. Vaccine 25: 7999-8009.

60 Richt, J.A. and García-Sastre, A. 2009. Attenuated influenza virus vaccines with modified NS1 proteins. Curr. Top. Microbiol. Immunol. 333: 177-195.

61 Steel, J., et al. 2009. Live attenuated influenza viruses containing NS1 truncations as vaccine candidates against H5N1 highly pathogenic avian influenza. J. Virol. 83: 1742-1753.