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Mini Review: A Glimpse of Nonstructural Protein 1 of Influenza A H1N1

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Abstract: The nonstructural protein 1 (NS1) is encoded by influenza A viral RNA segment 8. It is expressed abundantly in infected cells and showing unique regulative properties to establish infection in its host. The NS1 protein possesses two functional domains which are dsRNA-Binding Domain (RBD) and Effector Domain (ED). Its multifunctional nature causes it to be potent to evade host immune-response. NS1 protein interacts with RNA and is responsible to trigger the interferon-based antiviral response through a series of intracellular pathways including activation of host cell PI3K (phosphatidylinositol 3-kinase) growth factor and inhibition on cellular stimulated genes of PKR (protein kinase R). In addition, this nascent protein is able to block the active site of nucleolin and hold back post-transcriptional nuclear export of mRNAs. The cloning and expression of NS1 protein can be considered as the fundamental source to study the function of this gene for future research and application.

Key words: Nonstructural protein 1, H1N1, influenza A

INTRODUCTION

Influenza A viruses are from the family of Orthomyxoviridae (Bouvier and Palese, 2008) and normally spherical in shape with 80 to 120 nm in diameter (Donatelli *et al*., 2003). There are eleven types of genetically-distinct proteins translated by eight negative sense lipid-bounded RNA segments (Bouvier and Palese, 2008). The segments encode structural and non-structural proteins that play an important role to enhance virulence. Viral transmission has been identified in a wide spectrum of genetically-distinct hosts, either in animals or human. Influenza A virus could undergo a series of reassortment events; thus, re-emergence of the virus strain may occur. Nowadays, the influenza A virus subtype H1N1 has shown its global pathogenic potential.

In the influenza A virion, the NS1 protein is encoded by segment 8, the shortest viral RNA segment. This 26 kDa protein is specifically assembled by at least 230 amino acids (Lin *et al*., 2007). The NS1A protein is known as cell-associated protein, noticeably, it is virulent and expresses abundantly in the nucleus of the newly-infected cells (Li *et al*., 1998). Shaw *et al*. (1981) reported that NS1A protein can be detected by indirect immunofluorescence on the surfaces of infected mouse cells as early as 4 hours after infection with the influenza A/WSN (H1N1) strain.

Generally, NS1A protein interacts with the cellular factors and further modulates the host-cell process; hence, its innate properties often attract research interest. The pathogenic influenza disease is considerably exacerbated by NS1 protein and other viral proteins, neither of which can be excluded from contributing the virulence alone during the viral infection.

NS1A PROTEIN: PROTEIN STRUCTURAL STUDY

The influenza A NS1 viral protein consists of two functional domains which are known as dsRNA-Binding Domain (RBD) and Effector Domain (ED), suggesting that they are essential for intracellular and extracellular interaction. The RNA-binding domain, which is corresponding to the N-terminal end, plays an important role to disarm the interferon-based defense system of the host while the effector domain located at C-terminus is responsible to inhibit the viral polyadenylation activity (Lin *et al*., 2007).

Observations via X-ray crystallography had aided many research groups to determine and refine the three-dimensional structure of NS1 protein domain. The RNA-Binding Domain (RBD) consists of 73 amino acids and this basically forms a symmetrical homodimer with each monomer made up of three α-helix chains (Xia *et al*., 2009; Chien *et al*., 2004). A hypothetical working model of

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NS1A RBD built by Lin *et al*. (2007) depicted that the RBD is in a straddling position electrostatically stabilized by its phosphate backbone during binding to double-stranded (ds) RNA. In addition, the antiparallel helices 2 and 2' strands reside on the two identical monomers of NS1A RBD, with a few arginine and lysine side chains twinning around them and these are believed to be involved in the dsRNA binding (Lin *et al*., 2007).

Similar to NS1A RBD, the NS1A Effector Domain (ED) also appears as a dimeric subunit and structurally formed into a folded bundle which is rich in β-sheets with few α-helices (Lin *et al*., 2007). It extensively forms a hydrophobic network which acts as an interaction platform for both secondary structures. Apart from that, the N-terminus of two symmetrical monomers in the NS1A ED is most likely to form an intervening groove into which the RBD is attached (Lin *et al*., 2007). The importance of Trp187 in effector domain dimeric assembly was realized (Hale *et al*., 2008). Furthermore, it was observed that the residue alternation on the region of binding pocket adjacent to the side chain of Trp187 could affect the binding of specificity factor (CPSF30) (Xia *et al*., 2009). Besides that, the NS1 protein may perform a high replication and undergo a series of regulations to develop the viral activity (Hale *et al*., 2008). The NS1 protein may perhaps undergo genetic-alternations, suggesting its short segment allows insertion to take place along the heterologous sequences (Garcia-Sastre *et al*., 1998). The nucleotide truncation may occur to generate a potent protective immunity against challenge with the wild-type virus (Talon *et al*., 2000).

NS1A PROTEIN: ITS INNATE BIOPHYSICAL CHARACTERIZATION

The NS1A protein is identified as one of the important virulence factors in viral infection. Despite its crucial role in inhibiting host antiviral responses, this protein also takes part in cellular signaling pathway. However, its innate properties alone are not essential for it to do that. Therefore, it is required to carry out multi-functions to ensure efficient viral infection (Table 1). Evidence has demonstrated that the NS1 protein is involved in a series of viral and cellular activities such as viral protein synthesis, nuclear export by establishing interaction with either RNA or other accessory proteins (Lu *et al*., 1994; Burgui *et al*., 2003). Regardless which interaction NS1 participates in, it is believed that its protein properties correlate to its intracellular localization pattern (Melen *et al*., 2007).

NS1A PROTEIN: PROTEIN-RNA INTERACTION

Previous study has demonstrated that the NS1A protein is involved in the post transcriptional activity that inhibits the nuclear export of any viral mRNA molecules from the nucleus to the cytoplasm (Li *et al*., 1998). Qiu and Krug (1994) reported that such NS1 inhibition can be detected in the virus itself or in the host cell. Notably, the presence of poly-(A) tail is essential for this inhibition. It was reported that the RBD of NS1 protein binds specifically to 3' terminus of the poly-(A)-mRNA and high accumulation of NS1 in the nucleus will initiate the nuclear export blocking on both viral and cellular mRNA (Qiu and Krug, 1994). Generally, the pre-mRNA will associate with the Small Nuclear Ribonucleoprotein (snRNP) to form spliceosome and further lead to pre-mRNA splicing. However, the event is slightly different in influenza virus A because the RBD of NS1A protein will bind to the snRNA-mRNA complex, neither splicing nor other snRNA-protein interactions will occur subsequently (Lu *et al*., 1994). Therefore, through the RNA-binding

Table 1: Review on Multifunction of Influenza A NS1 Protein

Present study on multifunction of NS1A protein	Methodology: Viral Strain	Methodology: Technique	Reference
Activation of transcription factors: IFN-regulatory factors (IRF) that regulate IFN-β production	Influenza A/PR/8/34 (PR8) (H1N1)	*In vitro* systems of expressing the protein in mammalian cells	Garcia-Sastre *et al*. (1998)
Inhibition of the antiviral properties by binding with protein kinase R (PKR)	Influenza A/Udorn/72 virus	*In vitro* PKR assays; PCR mutagenesis	Li *et al*. (2006) and Min *et al*. (2007)
Post-transcriptional block to nuclear export of mRNAs	Influenza virus A/Victoria/3/75 strain	*In vitro* systems; transfected cell lines were analyzed by RNase protection assay	Qiu and Krug (1994)
Activation of the phosphatidylinositol 3- kinase (PI3K) pathway	Influenza A/PR/8/34(PR8)(H1N1), A/Victoria/3/75(H3N2)(Victoria) and A/Thailand/KAN-1/2004(H5N1)	Cell deaths were determined by fluorescence-activated cell sorter analysis	Ehrhardt *et al*. (2007)
Inhibition of the active site of nucleolin	Influenza A/Udorn/72 (H3N2)	GST (glutathione S-transferase) pull-down assay; laser confocal microscopy	Murayama *et al*. (2007)

mechanism, the nascent NS1 protein is also thought to inhibit the mRNA splicing of segment 8 from which it is encoded (Lu *et al*., 1994).

In line with the previous study, the NS1 protein was detected abundantly in the nucleus of infected cell at early phase of post infection (Li *et al*., 1998). In addition, its whereabouts in the cytoplasm was also documented (Greenspan *et al*., 1988). The distribution of NS1 proteins in the infected cells is specified by the viral infection duration and the expression level of NS1 protein (Li *et al*., 1998). The intracellular localization of this nascent protein involves its own Nuclear Localization Sequence (NLS) which is varied in quantity among the virus strains. Normally, the influenza virus type A NS1 protein possesses at least one nuclear localization sequence (NLS) (Greenspan *et al*., 1988). The Nuclear Localization Sequence (NLS) of the NS1A protein, which ought to show consensus sequence with the helices 2 and 2' of RBD, is highly conserved among the influenza A virus strains (Melen *et al*., 2007; Murayama *et al*., 2007). To mediate nuclear import, the NLS of NS1A protein is deployed to interfere with dsRNA and simultaneously bind to cellular protein to form dsRNA-α/β importin complex prior to translocation into nucleus (Bornholdt and Prasad, 2008). The subsequent release of α-importin in the nucleus will mediate the sequestration of dsRNA (Melen *et al*., 2007).

Nucleolin is recognized as a multi-functional RNA-binding-nucleolar protein, involving in the co-localization with NS1 protein. It accumulates abundantly in the nucleolus and is found to be proportional to the cytoplasm. It is considered that the accumulation of nucleolin in the nucleus will affect the host cellular function and further lead to apoptosis during the viral infection cycle (Hiscox, 2002), suggesting such an event is due to blocking of the active site on nucleolin by NS1 (Murayama *et al*., 2007). According to the study by Murayama *et al*. (2007), the NS1 protein interacts with the nucleolin via its RNA-Binding Domain (RBD) at N-terminus. Melen *et al*. (2007) has revealed that the NS1 protein containing NLS mutant, which had undergone site-directed mutagenesis at amino acid residue 38 and 41 residing on RBD of NS1A protein, was not detected in nucleoli. Despite of amino acid composition at C-terminus region, the viral strain may affect the nuclear and nucleolar targeting mechanism of NS1 protein in the infected cell (Murayama *et al*., 2007).

As an interferon antagonist, the NS1 protein is prerequisite to conquer or defend against those assorted antiviral activities triggered by its host (Xia *et al*., 2009). Evidence has shown that the host antiviral activity is initiated by NS1 dsRNA binding (Garcia-Sastre, 2006, 1998). The IFN-stimulated genes encoding both the Protein Kinase R (PKR) and 2'-5' oligoadenylate synthetase (OAS), are activated in its transcription in the presence of dsRNA, resulting in subsequent release of IFN. The activation of NS1-mediated OAS may enhance the IFN-β synthesis (Hale *et al*., 2008). Presumably, such association between NS1 RBD and IFN-stimulated genes could inhibit the translation process and RNA degradation in the infected cell (Garcia-Sastre *et al*., 1998). Accordingly, the NS1 protein most likely will sequester the dsRNA during the viral infection to escape from detection of host innate immune system (Garcia-Sastre, 2006).

NS1A PROTEIN: PROTEIN-PROTEIN INTERACTION

It was shown that the NS1A protein establishes protein-protein interaction with other viral proteins. Previous studies have revealed that the NS1 protein interacts directly with the viral polymerase subunits in the infected cell (Marion *et al*., 1997). It was suggested that such interaction may give advantages to the post-transcription mechanism (Marion *et al*., 1997). The trimeric polymerase complex, which is formed by PB2, PB1 and PA proteins of influenza A virus, is essential to interact with NP protein for virus replication and transcription (Elton *et al*., 2006). Despite this, Marion *et al*. (1997) has discussed that there was no positive result detected on the association between NS1 protein with one of the polymerase subunits, suggesting that binding of NS1 protein only occurs when the polymerase complex was formed.

Besides that, the NS1 protein is reported to show interaction with other poly-(A)-binding proteins including PABPI and PABPII, even though there is no consensus sequence found on NS1 RBD with the poly-(A)-binding protein (Qiu and Krug, 1994). The NS1 protein usually shows a direct association with translation initiation factor eIF4G1 and further recruits the PABPI in both viral and cellular pathway; thereby, initiating the viral mRNA translation (Burgui *et al*., 2003). Data collection has revealed that the effector domain of NS1A protein targets the poly-(A)-binding protein II, PABPII, both *in vitro* and *in vivo*. Furthermore, the interaction of NS1 protein and the cellular PABPII with the Cleavage and Polyadenylation Specificity Factor (CPSF) is essential to block the PABPII protein from binding with pre-mRNA.

Hence, the elongation of poly-(A) tail is ruled out. The CPSF, with its hydrophobic binding site is detected as residing on the effector domain of NS1 (Xia *et al*., 2009), is required for the 3' end processing of all cellular pre-mRNA. In an X-ray crystallography study, the effector domain of NS1A protein was observed forming tetramer complex with the F2F3 zinc finger motif of CPSF30 (Das *et al*., 2008; Xia *et al*., 2009), suggesting that the binding may suppress the production of IFN-β mRNA (Das *et al*., 2008).

Once viral attack occurs, the infected cell will automatically release IFN and constitute the neighbouring cells with immune defense against the viral infection. In response, the host cell will produce large amount of enzyme known as protein kinase R (PKR). The NS1 protein apparently inhibits the IFN pathway by activating the interferon regulatory factor 3 (IRF-3) (Talon *et al*., 2000). The IRF-3 could evoke the antiviral response under association with other transcription factor within the nucleus. In recent studies, the PKR was believed to be phosphorylated by dsRNA (Bergmann *et al*., 2000; Chien *et al*., 2004) as well as its cellular activator protein (Li *et al*., 2006). In addition, the NS1 protein was supposed to inhibit PKR activation by fighting it for dsRNA in the infected cell but no apparent pathway was determined (Li *et al*., 2006). Recent studies has revealed that the binding of PKR to the NS1 protein was able to inhibit the PKR activation pathway (Min *et al*., 2007) but Li *et al*. (2006) has demonstrated that the RBD of NS1 protein was not essential to mediate such interaction.

In general, PI3K/Akt (phosphatidylinositol 3-kinase) has certain impact on the cell growth as well as cellular signaling (Ehrhardt *et al*., 2007; Shin *et al*., 2007). It has been reported that the P13K activity was reduced in the truncated NS1 protein. Therefore, it can be concluded that the NS1 protein is responsible to activate the PI3K signaling response and further inhibit the viral apoptosis during the virus infection cycle (Ehrhardt *et al*., 2007). In addition, the gene profiling analysis accompanied by reverse genetics has demonstrated that the truncated NS1 gene established its effect on gene expression in the culture of infected human lung epithelial cells (Geiss *et al*., 2002).

NS1A PROTEIN: PROTEOMIC STUDIES-CLONING AND EXPRESSION PROFILES

To date, cloning techniques are normally used to study specific genes and determine the biological process accordingly. The nature of NS1 protein which is correlated to the virulence activity has been recorded. To gain more insight of its role in viral life cycle, the cloning and expression of NS1 protein were carried out purposely for further characteristic-identification and functional-analysis. The NS1 clone is further applied in crystallographic study which could give advantages to hypothetical prediction on dimeric interface (Bornholdt and Prasad, 2008; Xia *et al*., 2009).

Apart from that, many papers or studies adopt different transformation methods. Regardless of which method was used in transformation, the bacterial strain, *E. coli,* is the most commonly used as expression host. However, some studies have transformed their plasmid into yeast, *Saccharomyces cerevisiae* (Ward *et al*., 1994). Frequently, isopropyl-β-D-thiogalactoside (IPTG) at different concentrations was used as inducer in the cloning studies to allow maximum gene expression (Table 2). Recent studies have documented that the NS1 protein was detected as expressing in both soluble and insoluble fraction. Typically, the expressed NS1 fusion protein was detected at 26 kDa (Manasatienkij *et al*., 2008; Ma *et al*., 2009). However, Liu *et al*. (2003) has documented that the NS1 fusion protein appeared at 30 kDa molecular weight. Birch-Machin *et al*. (1997) has demonstrated that the designated NS1 present in NS1-N (1-385 nucleotide) and NS1-C (313-692 nucleotide) were established in larger molecular weight.

Nowadays, high resolution protein purification is used to produce relatively pure NS1 protein for further proteomic study. Significantly, the NS1 purity of more than 95% (Wang *et al*., 2008; Ma *et al*., 2009) can be obtained through Ni-NTA purification (Wang *et al*., 2008). In addition, the NS1 fusion protein can also be purified by using chitin affinity chromatography (Ma *et al*., 2009) or glutathione S-transferase affinity column (Birch-Machin *et al*., 1997). Ward *et al*. (1994) has tried the induction by copper sulfate ($CuSO_4$) on NS1 protein, designing to enhance the expressed NS1 gene to release the toxin in the infected cell. Concluded from the result, the toxicity could affect the cell growth but it was considered vital in yielding nuclear localization signal.

For immunogenicity study, the NS1 protein reacted significantly with NS1 monoclonal antibody of influenza virus in ELISA or Western blotting tests (Liu *et al*., 2003; Wang *et al*., 2008; Ma *et al*., 2009). Apart from that, Birch-Machin *et al*. (1997) had analyzed NS1 activity by ELISA assay and to their surprise, the NS1 protein was detected in the infected cells in their experiment

Table 2: Review on a series of cloning and expression work of influenza NS1 protein

	Experimental design		
NS1 gene source	Plasmid construction	Host (induction control conditions)	Research outcome
A/equine 2/Suffolk/89 (H3N8) (Birch-Machin *et al*., 1997)	Amplified NS1 gene was cleaved with *Bam*HI and cloned into pGEX-3X or pUC13	*E. coli* TG1 (1 mmol L^{-1} of IPTG at 37°C)	The NS1 fusion proteins were expressed in 52.6 kDa (NS1), 41.1 kDa (NS1-N) and 40.7 kDa (NS1-C); the C-terminus half of the protein acted as antigenic determinant.
A/Vietnam/1203/2004 (H5N1) (Bornholdt and Prasad, 2008)	NS1 mutant was cleaved with *Eco*RI -*Xho*I and cloned into pET-46 EK/LIC	*E. coli* BL21(DE3) (0.05 mmol L^{-1} of IPTG at 25°C)	NS1 protein structure was determined using molecular replacement techniques
A/chicken/Beijing/2/97 (H9N2) (Liu *et al*., 2003)	Amplified NS1 gene was cleaved with *Eco*RI-*Xho*I and cloned into pET30c	*E. coli* BL21(DE3) (0.4 mmol L^{-1} of IPTG at 37°C)	The NS1 fusion protein was found in 30 kDa and it reacted significantly with NSI monoclonal antibody of influenza virus
A/PR/8/34 (H1N1) (Ma *et al*., 2009)	Amplified NS1 gene was cleaved with *Nde*I-*Xho*I and cloned into pTXB1	*E. coli* BL21(DE3) (1 mmol L^{-1} of IPTG at 37°C)	The NS1 fusion protein was found in 26 kDa, expressing in both soluble and insoluble fraction; its purity is more than 95%
A/Chicken/TH/KU14/04 (H5N1) (Manasatienkij *et al*., 2008)	RT-PCR amplified NS1 gene was cleaved with *Bam*HI and cloned into pQE80L	*E. coli* DH5α (1 mmol L^{-1} of IPTG at 37°C)	The 26 kDa NS1 fusion protein accumulated in inclusion bodies fraction and the highly pure NS1 gave significant result in Western blot
H7N7, H4N6, H12N5, H6N8, H5N1 (Metreveli, 2006)	RT-PCR amplified NS1 gene was cleaved with *Eco*RI-*Xba*I and cloned into pCDNA-3.1+	*E. coli* DH5α (Concentration of IPTG and temperature acted as independent variables)	The NS1 virulence activity was correlated with the host species
A/NWS/33 (Ward *et al*., 1994)	NS1 mutant, pT7T3.18U.NS(-) was cleaved with *Bam*HI-*Eco*RI and cloned into pYEULCBX	Yeast *Saccharomyces cerevisiae* (5 mmol L^{-1} of $CuSO_4$ at 30°C)	The 26 kDa NS1 fusion protein was found abundantly in inclusion; it was reactive to anti-NS1 antisera and was toxic to its expressing host
A/SW/SD/1/2003(H9N2) (Wang *et al*., 2008)	RT-PCR amplified NS1 gene was cleaved with *Nco*I-*Xho*I and cloned into pET-28a (+)	*E. coli* BL21(DE3) (5 mmol L^{-1} of lactose at 37°C)	The NS1 fusion was highly expressed its purity was more than 95%; it showed significant result with immunogenicity tests
A/Udorn/72 NS1A effector domain (Xia *et al*., 2009)	Polypeptide containing NS1 effector domain was cloned into pET46-EK/LIC	*E. coli* Rosetta 2(DE3) (1 mmol L^{-1} of IPTG at 25°C)	The X-ray structure of NS1 fusion proteins was determined; hypothetical study on dimeric interface

but not found in the virus-vaccinated animals (Birch-Machin *et al*., 1997) It was shown that the infection can be tracked through host antibody reaction with the existence of NS1.

REFERENCES

Bergmann, M., A. Garcia-Sastre, E. Carnero, H. Pehamberger, K. Wolff, P. Palese and T. Muster, 2000. Influenza virus NS1 protein counteracts PKR-mediated inhibition of replication. J. Virol., 74: 6203-6206.

Birch-Machin, I., A. Rowan, J. Pick, J. Mumford and M. Binns, 1997. Expression of the nonstructural protein NS1 of equine influenza A virus: Detection of Anti-NS1 antibody in post infection equine sera. J. Virol. Methods, 65: 255-263.

Bornholdt, Z.A. and B.V.V. Prasad, 2008. X-ray structure of NS1 from a highly pathogenic H5N1 influenza virus. Nature, 456: 985-988.

Bouvier, N.M. and P. Palese, 2008. The biology of Influenza viruses. Vaccine., 56: D49-D53.

Burgui, I., T. Aragon, J. Ortin and A. Nieto, 2003. PABP1 and eIF4GI associate with influenza virus NS1 protein in viral mRNA translation initiation complexes. J. General Virol., 84: 3263-3274.

Chien, C.Y., Y. Xu, R. Xiao, J.M. Aramini, P.V. Sahasrabudhe, R.M. Krug and G.T. Montelione, 2004. Biophysical characterization of the complex between double-stranded RNA and the N-terminal domain of the NS1 protein from influenza A virus: Evidence for a novel RNA-binding mode. Biochemistry, 43: 1950-1962.

Das, K., L.C. Ma, R. Xiao, B. Radvansky and J. Aramini *et al*., 2008. Structural basis for suppression of a host antiviral response by influenza a virus. Nat. Acad. Sci. United States Amer., 105: 13093-13098.

Donatelli, I., L. Campitelli, S. Puzelli, C. Affinito, M.A. de Marco, M. Delogu and G. Barigazzi, 2003. Influenza viruses: Structure and interspecies transmission mechanisms. Vet. Res. Commun., 27: 115-122.

Ehrhardt, C., T. Wolff, S. Pleschka, O. Planz and W. Beermann *et al*., 2007. Influenza A Virus NS1 Protein Activates the PI3K/Akt Pathway To Mediate Antiapoptotic Signaling Responses. J. Virol., 81: 3058-3067.

Elton, D., P. Digard, L. Tiley and J. Ortin, 2006. Structure and Function of the Influenza Virus RNP. In: Influenza Virology: Current Topics, Kawaoka, Y. (Ed.). Caister Academic Press, England, pp: 1-36.

Garcia-Sastre, A., 2006. Antiviral response in pandemic influenza viruses. Emerg. Infect. Dis., 12: 44-47.

Garcia-Sastre, A., A. Egorov, D. Matassov, S. Brandt and D. Levy, 1998. Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. Virol., 252: 324-330.

Geiss, G.K., M. Salvatore, T.M. Tumpey, V.S. Carter and X. Wang *et al*., 2002. Cellular transcriptional profiling in influenza A virus-infected lung epithelial cells: The role of the nonstructural NS1 protein in the evasion of the host innate defense and its potential contribution to pandemic influenza. Proc. Nat. Acad. Sci. United States Amer., 99: 10736-10741.

Greenspan, D., P. Palese and M. Krystal, 1988. Two nuclear location signals in the influenza virus NS1 nonstructural protein. J. Virol., 62: 3020-3026.

Hale, B., R. Randall, J. Ortin and D. Jackson, 2008. The multifunctional NS1 protein of influenza A viruses. J. General Virol., 89: 2359-2376.

Hiscox, J.A., 2002. The nucleolus: A gateway to viral infection?. Arch. Virol., 147: 1077-1089.

Li, S., J.Y. Min, R.M. Krug and G.C. Sen, 2006. Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA. Virol., 349: 13-21.

Li, Y., Y. Yamakita and R.M. Krug, 1998. Regulation of a nuclear export signal by an adjacent inhibitory sequence: the effector domain of the influenza virus NS1 protein. Nat. Acad. Sci. United States Amer., 95: 4864-4869.

Lin, D., J. Lan and Z. Zhang, 2007. Structure and function of the NS1 protein of influenza A virus. Acta Biochimica et Biophysica Sinica, 39: 135-162.

Liu, J.h., Q.M. Wu, W.M. Shi and Y.P. Guo, 2003. Cloning and expression of the nonstructural protein (NS1) of the H9N2 chicken influenza virus. Virol. Sinica, 18: 503-505.

Lu, Y., X.Y. Qian and R.M. Krug, 1994. The influenza virus NS1 protein: A novel inhibitor of pre-mRNA splicing. Genes Develop., 8: 1817-1828.

Ma, F.L., Y.Q. Li and L.S. Zheng, 2009. Prokaryotic expression and purification of NS1 A virus subtype H1N1. Chinese J. Biol., 22: 221-225.

Manasatienkij, W., P. Lekcharoensuk and N. Upragarin, 2008. Expression and purification of NS1 protein of highly pathogenic avian influenza virus H5N1 in *Escherichia coli*. Kasetsart J., 42: 485-494.

Marion, R.M., T. Zurcher, S. de la Luna and J. Ortin, 1997. Influenza virus NS1 protein interacts with viral transcription-replication complexes *in vivo*. J. General Virol., 78: 2447-2451.

Melen, K., L. Kinnunen, R. Fagerlund, N. Ikonen, K.Y. Twu, R.M. Krug and I. Julkunen, 2007. Nuclear and nucleolar targeting of influenza a virus NS1 protein: striking differences between different virus subtypes. J. Virol., 81: 5995-6006.

Metreveli, G., 2006. Expression of influenza virus nonstructural protein 1 (NS1). Master of Science, Swedish University of Agricultural Sciences.

Min, J.Y., S. Li, G.C. Sen and R.M. Krug, 2007. A site on the influenza A virus NS1 protein mediates both inhibition of PKR activation and temporal regulation of viral RNA synthesis. Virol., 363: 236-243.

Murayama, R., Y. Harada, T. Shibata, K. Kuroda, S. Hayakawa, K. Shimizu and T. Tanaka, 2007. Influenza A virus non-structural protein 1 (NS1) interacts with cellular multifunctional protein nucleolin during infection. Biochem. Biophys. Res. Commun., 362: 880-885.

Qiu, Y. and R. Krug, 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.

Shaw, M.W., E.W. Lamon and R.W. Compans, 1981. Surface expression of a nonstructural antigen on influenza A virus-infected cells. Infect. Immunity, 34: 1065-1067.

Shin, Y.K., Q. Liu, S.K. Tikoo, L.A. Babiuk and Y. Zhou, 2007. Influenza A virus NS1 protein activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway by direct interaction with the p85 subunit of PI3K. J. General Virol., 88: 13-18.

Talon, J., M. Salvatore, R. O'Neill, Y. Nakaya and H. Zheng *et al*., 2000. Influenza A and B viruses expressing altered NS1 proteins: A vaccine approach. Nat. Acad. Sci. USA., 97: 4309-4314.

Wang, F., X. Yuan, Y. Wang, C. Zhang, L. Xu and S. Liu, 2008. Cloning, prokaryotic expression and antigenicity analysis of NS1 gene of H9N2 swine influenza virus. Agric. Sci. China, 7: 895-900.

Ward, A.C., A.A. Azad and I.G. Macreadie, 1994. Expression and characterization of the influenza A virus non-structural protein NS 1 in yeast. Arch. Virol., 138: 299-314.

Xia, S., A.F. Monzingo and J.D. Robertus, 2009. Structure of NS1A effector domain from the influenza A/Udorn/72 virus. Acta Crystallog. Sect. D: Biol. Cryst., 65: 11-17.