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The R35 residue of the influenza A virus NS1 protein has minimal effects on nuclear localization but alters virus replication through disrupting protein dimerization

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ABSTRACT

The influenza A virus NS1 protein has a nuclear localization sequence (NLS) in the amino terminal region. This NLS overlaps sequences that are important for RNA binding as well as protein dimerization. To assess the significance of the NS1 NLS on influenza virus replication, the NLS amino acids were individually mutated to alanines and recombinant viruses encoding these mutations were rescued. Viruses containing NS1 proteins with mutations at R37, R38 and K41 displayed minimal changes in replication or NS1 protein nuclear localization. Recombinant viruses encoding NS1 R35A were not recovered but viruses containing second site mutations at position D39 in addition to the R35A mutation were isolated. The mutations at position 39 were shown to partially restore NS1 protein dimerization but had minimal effects on nuclear localization. These data indicate that the amino acids in the NS1 NLS region play a more important role in protein dimerization compared to nuclear localization.

Introduction

The RNA genome of influenza A virus is replicated in the nucleus of infected cells (Taylor et al., 1977). The influenza NS1 protein has been shown to localize to the nucleus and cytoplasm of infected cells through two identified nuclear localization sequences (NLS) as well as a nuclear export sequence (NES) (Melen et al., 2007; Han et al., 2010; Forbes et al., 2013). A classic monopartite NLS, NLS1, is found between amino acids 34 and 41 and is highly conserved among influenza A virus strains. It contains four basic amino acids, R35, R37, R38, and K41 (Greenspan et al., 1988; Melen et al., 2007). Of these, R35, R38, and K41 have been shown to decrease interaction with importin-α if mutated to alanine (Melen et al., 2007). The second NLS (NLS2), is bipartite, located between amino acids 219 and 232 and is virus strain specific (Greenspan et al., 1988; Melen et al., 2007, 2012). The NES is located between amino acids 138 and 147 (Li et al., 1998). A virus strain specific nucleolar localization (NoLS) signal has also been identified which overlaps with NLS2 (Melen et al., 2007, 2012; Zhu et al., 2013).

The NS1 protein is divided into two functional domains (Qian et al., 1994). The first 73 amino acids form the RNA binding domain while amino acids after position 74 form the effector domain (Qian et al., 1995). The RNA binding domain interacts with at least four RNA targets: dsRNA, polyA sequence, U6snRNA, and u6atac snRNA (Wang et al., 1999) and it is important for antagonizing the innate immune response in both the cytoplasm and the nucleus (reviewed in Hale et al., 2008). Arginine at position 38 is critical for binding RNA and mutating this amino acid to alanine results in loss of RNA binding (Wang et al., 1999; Melen et al., 2007). This loss in RNA binding results in viruses that are more sensitive to exogenous interferon beta treatment and virus-infected cells produce more type I interferon and cytokines than wild type virus-infected cells (Wang et al., 1999; Donelan et al., 2003; Min and Krug, 2006; Newby et al., 2007). NS1 interaction with RNA also requires that the protein form a dimer (Nemeroff et al., 1995; Wang et al., 1999) and a number of amino acids in the effector domain (Wang et al., 2002) and the RNA binding domain (Wang et al., 1999) contribute to protein dimerization. Of particular interest for dimerization is arginine at position 35 which is also part of NLS1 (Melen et al., 2007).

Consistent with having both NLS and NES signals, the NS1 protein has functions in both the nucleus and cytoplasm and can traffic between the compartments (Melen et al., 2007; Han et al., 2010; Forbes et al., 2013). In the nucleus, NS1 inhibits cellular mRNA maturation through interactions with multiple host factors. Interactions with cleavage and polyadenylation specificity factor (CPSF) and polyadenine binding protein II (PABPII) prevent cleavage and polyadenylation of cellular mRNAs (as reviewed in Hale et al., 2008), while interactions with the mRNA splicing machinery and nuclear export factors further limit cellular mRNA maturation and export

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(Satterly et al., 2007). NS1 also colocalizes with ND10 in nuclear dots and interacts with nucleolin (Sato et al., 2003; Murayama et al., 2007). In the cytoplasm, NS1 interacts with a number of cellular pathways. Some of these pathways, including 2′5′OAS/RNase L, Rig-I/Trim25 and PKR inhibit host anti-viral responses (Donelan et al., 2003; Li et al., 2006; Min and Krug, 2006; Kochs et al., 2007; Min et al., 2007; Opitz et al., 2007; Gack et al., 2009). NS1 also interacts with eIF4GI, PABPI, hnRNP-f and hStaufen to increase viral protein synthesis (Lee et al., 2009; reviewed in Hale et al., 2008).

To study the role of the influenza NS1 N terminal NLS on virus replication, a series of mutations were introduced into four basic amino acids (R35, R37, R38 and K41) in the NLS1 and recombinant influenza A/WSN/33 viruses encoding these mutated proteins were rescued and characterized. Viruses encoding the R37A, R38A and K41A mutations were recovered and had minimal effects on virus replication and NS1 nuclear localization. Recombinant viruses with the R35A mutation were only rescued when spontaneous, second site mutations at position D39 arose. Our data indicate that the amino acids comprising the NS1 NLS1 have minimal effects on NS1 nuclear localization, but at least one, R35, plays an important role in protein dimerization. We demonstrate that second site mutations at position D39 are able to correct for a loss in dimerization caused by the R35A mutation, thereby restoring virus replication.

Results

Rescue of recombinant viruses with mutations in NS1 NLS1

The A/WSN/33 NS1 protein only encodes an amino terminal NLS (NLS1). In order to study the effects of these amino acids on influenza virus replication, the basic amino acids in NLS1 were mutated to alanine, generating plasmids encoding NS1 R35A, R37A, R38A, and K41A (Table 1, top half "NLS"). A virus with the NS1 R38A mutation (rWSN NS1 R38A), which disrupts both NLS1 and RNA binding, was previously isolated (Newby et al., 2007). The rWSN NS1 R37A and rWSN NS1 K41A viruses were successfully rescued and plaque purified. The rWSN NS1 R35A virus was not isolated in three separate rescue attempts, but recombinant viruses with amino acid substitutions at position 39 of NS1 (D39A, D39N, or D39Y, Table 1, bottom half collectively referred to as "D39X") in addition to the R35A mutation were isolated. The mutations were all the result of single nucleotide changes from wild type GAU (D) to G**C**U (A), **A**AU (N) or **T**AU (Y).

Nuclear localization mutations have minor effects on virus replication in MDCK cells

To study the effect of alanine substitutions in the NS1 NLS1 on virus replication, MDCK cells were infected at a multiplicity of infection (MOI) of 0.001 and virus production was monitored every 12 h by TCID50 assay (Fig. 1A). There were no differences in replication between any of the rescued viruses (Fig. 1A). As another measure of virus replication, virus plaque diameter

Table 1
Influenza NS1 sequence and mutations.

Virus		Sequence[a]	Rescued
NLS	rWSN	^{33}ldrlrrdqksl^{43}	Yes
	rWSN NS1 R37A	----a------	Yes
	rWSN NS1 R38A	-----a-----	Yes
	rWSN NS1 K41A	--------a--	Yes
	rWSN NS1 R35A	--a---------	**No**
D39X	rWSN NS1 R35A D39A	--a---A----	Yes
	rWSN NS1 R35A D39N	--a---N----	Yes
	rWSN NS1 R35A D39Y	--a---Y----	Yes

[a] Bold lowercase letters indicate mutations introduced by plasmid mutagenesis. Capital letters indicate mutations that arose during the rescue of recombinant viruses.

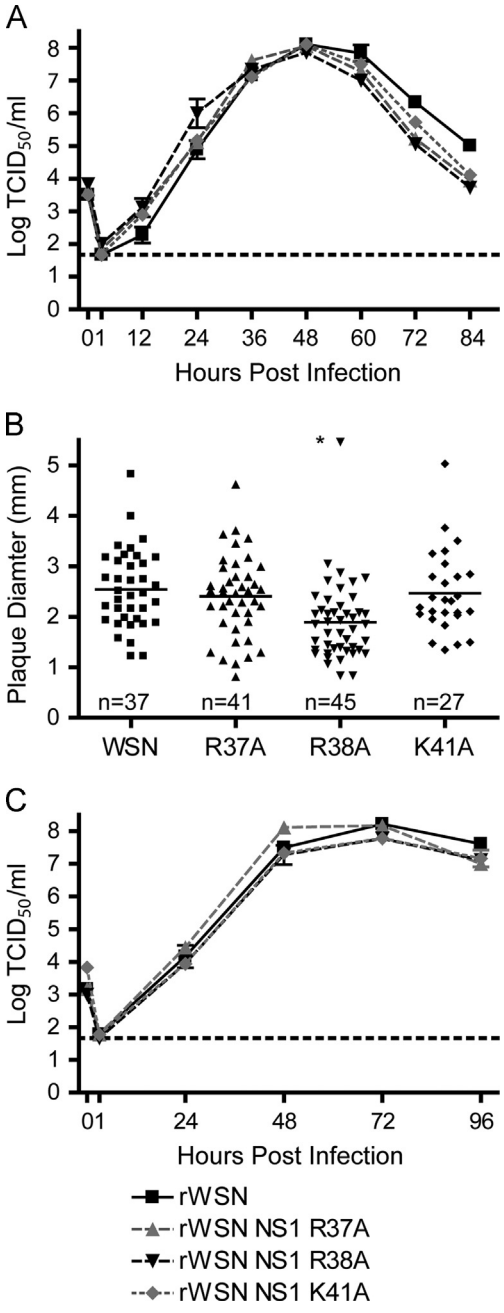

Fig. 1. NS1 NLS viruses replicate to high titers on MDCK and Vero cells lines. (A) MDCK cells were infected with rWSN or rWSN NS1 NLS viruses at a MOI of 0.001 $TCID_{50}$/cell. Supernatants were collected every 12 h and virus titers were analyzed by $TCID_{50}$ assay. The limit of detection is indicated by a dotted line at 1.67 and mean and standard error of the mean are graphed. There were no statistical differences in virus replication (two-way ANOVA). (B) MDCK cells were infected with serial dilutions of rWSN or rWSN NS1 NLS viruses and incubated under 1% agarose and media for three days then fixed. Plaque diameter was measured using ImageJ. Individual plaques are graphed and mean diameter is indicated with a solid horizontal line. Significant differences relative to rWSN are indicated with an asterisk (*), $p < 0.01$, one-way ANOVA and a Bonferroni multiple comparison post test. (C) Vero cells were infected with rWSN or rWSN NS1 NLS viruses at a MOI of 0.001 $TCID_{50}$/cell. Supernatants were collected every 24 h and virus titers were analyzed by $TCID_{50}$ assay. The limit of detection is indicated by a dotted line at 1.67 and mean and standard error of the mean are graphed. There were no statistical differences in virus replication using a two-way ANOVA and a Bonferroni multiple comparison post test.