The Interdomain Region of Dengue NS5 Protein Interacts with NS3 and Host Proteins

by

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Abstract

Although dengue virus genome replication occurs in the cytoplasm of infected cells, it has been shown that the NS5 protein (RNA-dependent RNA polymerase) is hyperphosphorylated at a late stage in infection and localized to the cell nucleus. A 37 amino acid sequence of NS5 (residues 369-405) was shown to contain a functional nuclear localization signal (NLS) that interacted with the cellular nuclear transport factor, importin α/β heterodimer. Further studies using the yeast two-hybrid system revealed that the NS5 region (residues 320-368) immediately adjacent to the NLS contained an importin β-binding site that abuts or overlaps the binding site for the NS3 protein (protease/helicase). The importin β-binding site has also been shown to be a functional NLS (bNLS). Intriguingly, when both bNLS and NLS (residues 320-405) were present, the fused β-galactosidase protein did not accumulate in the nucleus. Here we provide a review of our studies on the NS5 interdomain region and compare it to other members of the Flavivirus genus in order to highlight the importance of this region as a possible target for developing broad-acting antiviral agent against dengue and other mechanistically-related viruses.

Keywords: NS5 protein, protein interactions, NS3 protein, nuclear localization signal.

Introduction

The Flaviviridae family contains three genera, Hepacivirus, Flavivirus and Pestivirus that are small, enveloped, single-stranded (ss) and positive polarity RNA viruses. The dengue virus, of which there are four distinct serotypes (DEN 1-4), belongs to the Flavivirus genus. The DEN-2 virus is the most prevalent and frequent cause of epidemics in many parts of the world. In Australia, dengue epidemics have occurred sporadically in the north-eastern part of the continent (North Queensland) and detailed
accounts of epidemic outbreaks of dengue in this region date back to 1898\(^{(1)}\). Since the 1990s the frequency of outbreaks of dengue fever have escalated, mirroring the situation in the neighbouring regions and elsewhere in the world\(^{(2)}\). Although dengue virus is not endemic to North Queensland, the widespread prevalence of the mosquito vector, *Aedes aegypti*, results in small outbreaks (after introduction by international travellers) that are controlled by the excellent public health measures in Australia. However, the 1992/1993 dengue epidemic in the North Queensland towns of Townsville and Charters Towers was quite widespread and more than 1000 cases of dengue were reported. The DEN-2 strain TSV01 was isolated from a Townsville patient in 1993\(^{(3)}\) and its complete nucleotide sequence has been obtained recently (Genbank accession no: AY037116).

The ~11 kb ss (+) RNA genome of dengue virus is capped at the 5' end but not poly-adenylated and upon uncoating, serves directly as a template for the synthesis of the virus proteins. A single translation initiation site leads to the production of a precursor polyprotein that is arranged NH\(_3\)-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-COO-. Host signal peptidases and viral proteinases co-translationally and post-translationally process the polyprotein into at least 10 viral proteins; the three structural proteins C, prM and E that form the virion particle, and the seven non-structural proteins, NS1 to NS5, that function in the virus life cycle. The untranslated terminal regions account for less than 5% of the genome, and complementary elements in these regions form stem-loop structures and cyclization motifs that are important for the synthesis of new RNA\(^{(4)}\). In Flavivirus genome replication the membrane-associated synthesis of minus-strand produces a dsRNA known as the replicative form (RF), which is the recycling template for the synthesis of new plus strand RNA. To achieve this, the viral proteins NS5 and NS3 act in concert through protein-protein interactions within the Replicative Complex (RC) that includes other viral NS proteins. The details of the interactions within the RC are poorly understood and our laboratory has chosen to focus on the protein-protein interactions of NS3 and NS5 using the observation that NS5 is localized to the nucleus\(^{(5)}\) as a handle.

### Structure and function of NS5 and NS3

NS5, the largest of the ten flavivirus proteins at 104kDa (900 amino acid residues), is a multidomain protein with at least two domains that contain enzyme activities that are crucial for the replicative cycle of the virus. The N-terminal region is associated with the RNA capping reaction that puts a cap 1 structure (\(^{5}\)Me\(\text{G}\)\(^{5}\)pp\(^{5}\)N\(^{5}\)Me) on the plus strand RNA genome and the C-terminal contains the eight highly conserved sequence motifs (I to VIII) that have been recognized in many RNA-dependent RNA polymerases (RdRPs) and includes the tripeptide “GDD” found in all polymerases (POL)\(^{(6,7)}\). Interestingly the capping activity involves three enzymatic steps, a 5’-terminal RNA triphosphatase, guanylyltransferase, and RNA methyltransferase. The first of these, 5’-terminal RNA triphosphatase activity, is contained within NS3 and the methyltransferase (MTase) activity has only recently been demonstrated for the first time in a recombinant truncated NS5 protein comprising residues 1-296. The 3D structure of the NS5\(_{\text{MTase}}\) domain has been solved\(^{(8)}\).
Located between the two enzymatic domains is the region that we have characterized to be a “hot-spot” for protein interactions with cellular importin proteins and viral NS3(8,9,10) (Figure 1A).

NS3, is composed of 618 amino acids, plays a critical role in virus protein maturation and replication. The N-terminal one-third (167 amino acid residues) has the characteristic serine protease domain that requires NS2B for cleaving the polyprotein at NS2A-NS2B, NS2B-NS3, NS3-NS4A and NS4B-NS5 junctions. The remainder of NS3 forms the helicase domain which consists of nucleotide binding, nucleotide triphosphatase (NTPase) and RNA binding motifs. Previously, a truncated 50kDa fragment of NS3 amino terminal region (designated NS3') with a potential cleavage site located in the helicase domain(11,12) has been found in flavivirus-infected mammalian cells, but not in infected mosquito cells. A biological significance for the autolysis has not been established, although it has been speculated to regulate the helicase activity.

**Nuclear localisation of NS5**

Whilst it is well known that the Flavivirus RNA replication occurs in the cytoplasm, a hyperphosphorylated form of dengue NS5 that does not interact with NS3 has been located in the cell nucleus(13). Similar phosphorylation and nuclear localization of NS5 has been reported for another lymphotropic Flavivirus(13), Yellow Fever virus, suggesting that these processes are probably functionally important in the virus life-cycle and perhaps in viral pathogenesis. Since the nuclear pore complex does not permit the entry by passive diffusion of proteins >45kDa, a short non-cleavable peptide sequence called the nuclear localization signal (NLS) is required for the active nuclear import of large protein-protein complexes mediated by cytosolic NLS-binding proteins and other factors by a number of different pathways(6,10,14).

**Functional NLSs in NS5 and interaction with NS3**

Since NS5 functions in the cytoplasm of infected cells we argued that the NLS must be masked at the early stage when the proteins are assembled into the RC. For this reason the best candidate for a functional NLS was predicted to occur within residues 369-405. We demonstrated both in vivo (microinjection) and in vitro that the 37 amino acid sequence from NS5 residues 369-405 when genetically fused to the normally cytoplasmic β-galactosidase (Mr~500kDa as a tetramer) and fluorescently labelled (to render it visible by confocal laser scanning microscopy, CLSM), can function as a NLS(8). NS5(369-405)-β-gal contained two potential bipartite NLSs, so in order to narrow down the functional NLS, site-directed mutagenesis of the positive charge amino acid clusters (boxed in Figure 1A) as well as truncated constructs NS5 (369-391)- β-gal and NS5(386-405)- β-gal were constructed. In vitro studies with these constructs showed that the functional NLS that interacts with cellular importin α/β heterodimer is within NS5 residues 369-389 which we refer to as a/bNLS(10).

Previous evidence for the NS3-NS5 interaction includes the demonstration of complexes in vivo in dengue 2 infected monkey kidney (CV-1) cells and recombinant vaccinia virus co-infected HeLa cells and co-immunoprecipitation with antisera against NS3 or NS5, and also by the
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binding of NS3 to His-tagged NS5 that was immobilized on Ni-NTA affinity beads\(^5\). In addition to demonstrating biochemically by pull-down assays that bacterially-expressed NS3 and NS5 can interact with each other, we used a genetic screen for protein-protein interaction, the yeast two-hybrid (Y2H) system, to show that the C-terminal region of NS3 (residues 303-618) interacted specifically with NS5 residues 320-368\(^9\). As this region of NS5 is N-terminal to the a/bNLS, we examined the interaction of various NS5 constructs with importin \(\alpha\) and importin \(\beta\) separately\(^9\) and also as heterodimers using a yeast three-hybrid system (unpublished). These studies showed that NS5 (320-368) interacted strongly with importin \(\beta\) (Kd 23.5 nM) but not at all with importin \(\alpha/\beta\) heterodimer. Interestingly, NS3 competed with Importin \(\beta\) for the same site. Furthermore, we demonstrated that NS5(320-368)-\(\beta\)-gal accumulated in the nucleus to the same extent as NS5 (369-391)-\(\beta\)-gal or NS5(369-405)-\(\beta\)-gal albeit at a slower rate\(^1\)\(^0\), indicating that a second functional NLS that we refer to as bNLS is located in the NS5 interdomain region.

The region of NS5 from residues 320 to 405 were compared in order to extend our experimental findings for DEN-2 sequences (Figure 1B). Interestingly, a 20 amino acid sequence within the bNLS is almost completely conserved amongst all the flaviviruses, including the cell fusing agent virus (CFAV) that is tentatively classified as a member of this family\(^1\)\(^0\). In a previous genetic study with Kunjin virus replicons, Khromykh and colleagues\(^1\)\(^5\) speculated that this region may be important for NS3 binding and we confirmed this experimentally\(^9,10\). We showed by 3D modelling that within the 20 amino acid region is a short peptide that is very similar to the N-terminal peptide of importin \(\alpha\) that binds to importin \(\beta\) to form the heterodimer\(^10\). Furthermore, the functional relevance of the NS5 interdomain region and its potential value in producing rationally designed attenuated strains for use as vaccine was demonstrated by alanine scanning mutagenesis of different charged residue (within NS5 residues 320-405) in a full-length infectious clone of DEN-4, which showed that the region was critical for virus viability\(^16\).

![Figure 1 (A): Schematic diagram of the domain organization of NS5.](image)

The region of NS5 from residues 320 to 405 were compared in order to extend our experimental findings for DEN-2 sequences (Figure 1B). Interestingly, a 20 amino acid sequence within the bNLS is almost completely conserved amongst all the flaviviruses, including the cell fusing agent virus (CFAV) that is tentatively classified as a member of this family\(^1\)\(^0\). In a previous genetic study with Kunjin virus replicons, Khromykh and colleagues\(^1\)\(^5\) speculated that this region may be important for NS3 binding and we confirmed this experimentally\(^9,10\). We showed by 3D modelling that within the 20 amino acid region is a short peptide that is very similar to the N-terminal peptide of importin \(\alpha\) that binds to importin \(\beta\) to form the heterodimer\(^10\). Furthermore, the functional relevance of the NS5 interdomain region and its potential value in producing rationally designed attenuated strains for use as vaccine was demonstrated by alanine scanning mutagenesis of different charged residue (within NS5 residues 320-405) in a full-length infectious clone of DEN-4, which showed that the region was critical for virus viability\(^16\).
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TSV01
NGC
Den1-WestPac
Den3-H87
Den4-814669
Yellow Fever
Banzi
Edgew Hill
West Nile
Kunjin
St. Louis
JEV
Powassan
Langat
TBEV
Louping ill
Sepik
Rio Bravo
CFA

The residues highlighted in dark boxes indicate amino acid identity and those in light shaded boxes signify amino acid similarity for the sequences compared.

Model for nuclear transport of NS5

A schematic model (Figure 2) for the series of events that may occur in a dengue virus-infected cell that leads to the transport of NS5 to the nucleus is proposed here.

First, the RC is like a protein machine where virus and probably also some host proteins interact to replicate the viral genome. The interactions of NS3 and NS5 have been localized to the helicase domain of NS3 and the bNLS of NS5 (9,10). Mutations within bNLS as demonstrated by the changes to residues 356/357 (16) affected full-length virus viability, probably because the critical interaction between NS3 and NS5 in the RC may have been disrupted. However, during the replication of wild-type virus the NS3 protein has been known to autoproteolyse within the helicase domain as mentioned earlier. This event probably leads to the dislodgement of NS3 from bNLS by importin β since the affinity for this interaction is in the low nM range. Importin β is able to transport NS5 to the nucleus, but analogous to the situation in hepatitis C virus where NS5A is thought to sequester cellular importin proteins, we suggest that the transport of NS5 by importin β may also retain the protein in the cytoplasm. The slow rate of nuclear transport using bNLS compared to a/bNLS somewhat supports this notion (10). The bound importin β probably recruits importin α, and since the binding affinity of the N-terminal peptide of importin α for importin β is in the very low nM range (17), the formation of the importin heterodimer is promoted. The phosphorylation of NS5 at this stage leads to conformational change that exposes the a/b NLS sequence. This is then bound strongly by the importin α/β heterodimer and located rapidly to the nucleus where it probably carries out functions that remain to be
discovered. Nevertheless, our characterization of the nuclear localization of dengue NS5 has led to a clear definition of the interdomain region of this multidomain protein and opens the door to further studies that will be necessary for understanding the details of the interactions between NS3 and NS5.

NS5 is represented as two domains linked by the bNLS and a/bNLS region. The two humps in the bNLS indicate the conserved residues that are probably important for the importin β interaction\(^{10}\). NS3 is also indicated as a two domain protein with the helicase domain shown to interact with the bNLS region. The Kd values for protein interactions of Importin b with bNLS and Importin α/β with the a/b NLS (the value in parenthesis is for interaction with NS5 residues 369-405). Once importin b binds the bNLS site after dislodgement of NS3 importin a is recruited to the site. The importin α/β heterodimer does not bind bNLS\(^{10}\), however it binds a/b NLS with nm affinity. This complex is then transported to the nucleus.
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