ELSEVIER

www.elsevier.nl/locate/farmac

Il Farmaco 56 (2001) 33–36

IL FARMACO

# Characterisation of inter- and intra-molecular interactions of the dengue virus RNA dependent RNA polymerase as potential drug targets

Subhash G. Vasudevan [a,*], Magnus Johansson [a], Andrew J. Brooks [a], Lyndon E. Llewellyn [b], David A. Jans [c]

[a] *Department of Biochemistry and Molecular Biology, James Cook University, Townsville, Qld., 4811, Australia*
[b] *Australian Institute of Marine Science, PMB No. 3, Townsville, Qld. 4810, Australia*
[c] *Division of Biochemistry and Molecular Biology, John Curtin School of Medical Research, Canberra, ACT 2601, Australia*

**Abstract**

Our research is directed towards enhancing the understanding of the molecular biology of dengue virus replication with the ultimate goal being to develop novel antiviral strategies based on preventing critical inter- or intra-molecular interactions required for the normal virus life cycle. The viral RNA-dependent RNA polymerase (NS5) and the viral helicase (NS3) interaction offers a possible target for inhibitors to bind and prevent replication. In this study the yeast-two hybrid system was used to show that a small region of NS5 interacts with NS3, and also with the cellular nuclear transport receptor importin-β. Furthermore, intramolecular interaction between the two putative domains of NS5 can also be detected by the yeast two-hybrid assay. We have also modified the colony lift assay for the β-galactosidase reporter activity in intact yeast cells which reflects the strength of interaction between two proteins to a microtiter plate format. This assay offers a unique opportunity to screen for small molecule compounds that block physiologically important interactions. 

*Keywords:* Protein interactions; Nuclear localisation signal; Yeast two-hybrid assay; Molecular recognition; High throughput screening

## 1. Introduction

Dengue virus is a member of the *Flaviviridae*, which includes family members that are associated with human diseases such as yellow fever, Japanese encephalitis and hepatitis C. The family members are small, enveloped, single-stranded (ss), positive polarity RNA viruses. Dengue is a mosquito borne disease. There are four serotypes of dengue virus, Den 1–4, but dengue type-2 virus is the most prevalent. Following primary infection, lifelong immunity develops, preventing repeated assault by the same serotype. However, the non-neutralising antibodies from a previous infection or maternally acquired antibodies are thought to complex with a different serotype from a subsequent infection and cause dengue haemorrhagic fever/dengue shock syndrome, which can be fatal [1].

The ~11 kb ss (+) RNA genome of the dengue virus acts directly as a template for the synthesis of the virus proteins. A single translation initiation site leads to 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 ten 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 cyclisation motifs that are important for the synthesis of new RNA [1]. In flavivirus genome replication a dsRNA known as the replicative form (RF) is the recycling template for the synthesis of new plus strand RNA.

The replication of the RNA requires at least two virus encoded multidomain non-structural proteins,

* Corresponding author.
*E-mail address:* subhash.vasudevan@jcu.edu.au (S.G. Vasudevan).

0014-827X/01/$ - see front matter 
PII: S0014-827X(01)01014-X

NS3 (helicase) and NS5 (RNA-dependent RNA polymerase; RdRP). RNA synthesis is blocked when antibodies to either NS3 or NS5 are present in a replication assay [2]. NS5 is the largest of the ten flavivirus proteins at 104 kDa (900 amino acid residues), the amino terminal region of which is predicted to be an S-adenosyl methionine transferase (SAM) based on the similarity to the methyltransferases from a wide variety of species. The carboxyl terminal region of NS5, from residue 455 onwards, contains eight highly conserved sequence motifs that have been recognised in many RdRPs and includes the tripeptide 'GDD' found in all polymerases (POL) [3,4].

NS3 is composed of 618 amino acids and is the helicase involved in an unwinding step during replication of the virus genome. The N-terminal one-third (167 amino acid residues) has the characteristic serine protease domain (PD) 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 (HD) which consists of nucleotide binding, nucleotide triphosphatase (NTPase) and RNA binding motifs. Conserved sequences for the NTPase catalytic motif places dengue NS3 in the DEXH box (Asp-Glu-Xaa-His) superfamily of helicases.

NS3 and NS5 have been previously shown to form complexes in vivo in dengue 2 infected monkey kidney (CV-1) cells and recombinant vaccinia virus co-infected HeLa cells [5]. Furthermore, in vitro evidence for interaction of the two proteins has been obtained by co-immunoprecipitation with antisera against NS3 or NS5, and also by the binding of NS3 to His-tagged NS5 that was immobilised on Ni-NTA affinity beads [5].

## 2. Results and discussion

Athough dengue virus replicated in the cytoplasm of infected cells, it has been noted that some virus-encoded proteins accumulated in the nucleus or nucleolus. The precise role of this targeting is not clear, however a hyperphosphorylated form of NS5 that does not interact with NS3 has been located in the nucleus. Another lymphotrophic virus, yellow fever virus, was also localised to the nucleus and similarly phosphorylated, suggesting that these processes are probably functionally important in the virus life cycle and perhaps in viral pathogenesis as well [6].

Since the nuclear pore complex does not permit the entry by passive diffusion of proteins $>45$ kDa, a short non-cleavable peptide sequence called the nuclear localisation signal (NLS) is required for the active nuclear import of large proteins. These consist of either a single cluster of positively charged amino acids similar to the SV40 large tumour antigen (T-ag) or two clusters of positively charged residues that are separated by a 10–12 amino acid residue spacer as exemplified by nuclear targeted proteins such as the steroid hormone receptors for glucocorticoid and progesterone [7]. Importin-α binds the NLS region of nuclear targeted proteins and importin-β then binds to importin-α to enhance the former interaction. The complex consisting of the import proteins and the nuclear targeted protein are transported through the nuclear pore in a Ran-GTP dependent process [7]. For dengue-2 NS5 we demonstrated using in vitro and in vivo assay systems that the 37 amino acid interdomain linker of NS5 (residues 369–405, Fig. 1) contains a nuclear localisation sequence (NLS) which is capable of targeting β-galactosidase to the nucleus [8]. We showed that the linker was recognised by NLS-binding importin-α/β complex with an affinity similar to that of the bipartite NLS of the retinoblastoma protein.

In order to characterise further the linker region with respect to interaction between NS3 and NS5, we firstly confirmed that bacterially expressed NS3 and NS5 cloned from the Townsville isolate (TSV01) behaved similarly in a pull-down assay as proteins generated from the prototype New Guinea C strain (NOC) (data not shown). Next, the cDNA encoding NS5 and NS3 were fused in-frame with the BD or AD of the Ga14 yeast two-hybrid system (Y2H) system 2 (Clontech) plasmids, pAS2-1 (BD) or pACT2 (AD). The Y2H assay detects protein–protein interactions *in vivo* in yeast by taking advantage of the modular nature of the transcription factor Gal4. Briefly, a protein of interest is fused to a DNA binding domain (BD), while a second protein is fused to a transcription activation domain (AD). If the two proteins interact, the chimeric complex couples the BD domain bound at an upstream sequence element to the AD domain which activates transcription of a reporter gene(s) e.g. *lacZ* or *HIS3*. The Y2H system has also previously been shown to be suitable for mapping interactions between the polymerase and helicase for the positive strand, brome mosaic virus [9].

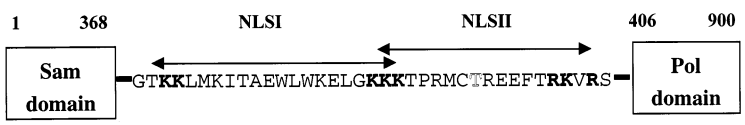


Fig. 1. The NS5 domain structure. The linker region containing two bipartite NLS sequences is shown schematically, with the linker region amino acid sequence highlighted in the single letter code where the basic residues are in bold type and the CK2 site threonine is outlined [8]. The two putative bipartite NLSs are indicated.

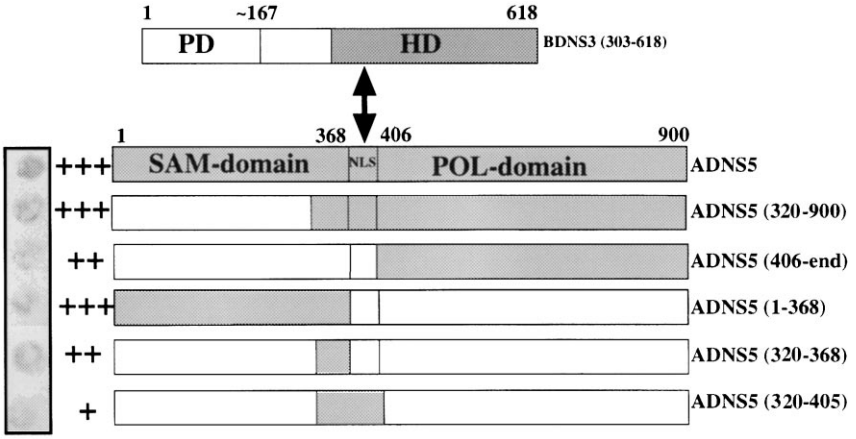


Fig. 2. The Y2H assay to examine the interaction between NS3 and NS5 proteins from dengue type 2. Filter lift assay showing the β-galactosidase activities of the yeast transformants, where the strength of interaction activity is indicated by increased blue colour development (visual scoring of intensity is indicated by +). Schematic representation of the constructs assayed by the Y2H assay are shown.

The plasmid constructs that directed the synthesis of BDNS3 and ADNS5 were used in co-transformation of *Saccharomyces cerevisisiae* strains Y190 or Y187, however no reporter (β-galactosidase) activity was detected in the transformants indicating that the full-length fusion proteins did not interact. On the other hand the reporter assay indicated that truncated NS3 construct BDNS3 (303–618) interacted strongly with ADNS5. Based on Western blot studies using monoclonal antibodies against Ga14 BD and AD, the lack of the interaction between full-length NS3 and NS5 in the Y2H system can most likely be attributed to proteolytic cleavage of NS3 (around residue 150) liberating the NS3 C-terminus from the Ga14 BD to compete with the full length protein for NS5. This is consistent with the fact that NS3 (residues 303–618) interacts with full-length NS5 as well as with various truncations of NS5 that were constructed using defined PCR primers to generate the mini-gene fragment (shaded in grey in Fig. 2) [10]. Most importantly, the NS3 binding site on NS5 is close to the interdomain linker region that contains the NS5-NLS that was previously characterised.

Next we examined the interaction of full length importin-β (nuclear transport receptor) or the N-terminal truncated importin-β (262–876) both fused to BD against ADNS5. No reporter activity was detected using full length BDimportin-β with ADNS5 (data not shown). Previous studies have shown that the full-length BDimportin-β and ADimportin-α do not interact in the Y2H system, because the N-terminal domain of importin-β can bind RanGTP and prevent importin-α/importin-β interaction [11]. In contrast, BDimportin-β (262–876), which lacks the ability to bind Ran, shows strong interaction with importin-β. Interestingly, BDimportin-β (262–876) also exhibited an interaction with ADNS5, implying that the NS5 nuclear import might be mediated directly by importin-β. Most intriguingly, the Y2H interaction assays with a range of NS5 deletion constructs indicated that NS3 and importin-β interactions with NS5 occurs within the same small region that is immediately N-terminal to the NS5-NLS linker region. Although previous in vitro studies had indicated that NS5-NLS is recognised with reasonably high affinity by the importin-α/β complex [8], several recent reports indicate that there are a number of different pathways by which nuclear targeted proteins can be transported to the nucleus in addition to the well characterised importin-α/β heterodimer mediated pathway [7]. The ability of importin-β to interact directly with NS5 independently of importin-α suggests that the latter may be transported to the nucleus by the importin-β mediated pathway. This direct interaction of importin-β with NS5 independent of importin-α was confirmed biochemically in pull-down assays [10].

The interaction of the Sam and Pol domains of NS5 was examined in the Y2H system by co-transformation of yeast strain with plasmid that directed the synthesis of BDNS5 (1–405) together with ADNS5 (full length), ADNS5 (320–900) or ADNS5 (406–900). Reporter activity was detected only in the strain containing the two non-overlapping segments of NS5, i.e. BDNS5 (1–405) and ADNS5 (406–900) (data not shown). Similarly when plasmid encoding BDNS5 (1–368) was co-transformed together with either ADNS5, ADNS5 (320–900) or ADNS5 (406–900) encoding plasmids, interaction was only observed with ADNS5 (320–900) (data not shown). Together these results indicated that the NS5-NLS region is also important for intramolecular domain interactions of NS5 (Fig. 3).

The convergence of the interaction site(s) for NS3, importin-β and NS5 intramolecular domain interaction within a small region of NS5, suggests that this region may be a possible drug target. We have adapted the

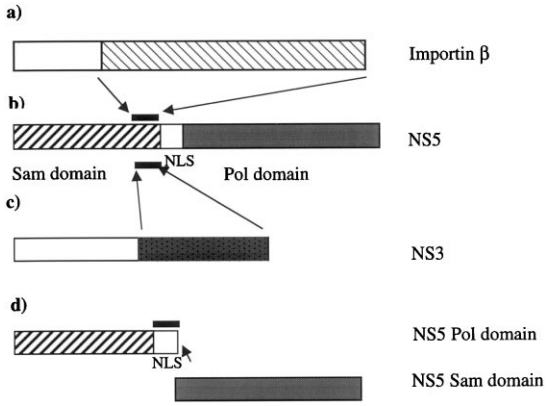


Fig. 3. Schematic diagram showing the convergence of the intermolecular and intramolecular interaction sites within a small sequence region in NS5 spanning from around residue 320 to 405. The shaded regions of (a) importin-β (242–868) and (c) NS3 (303–638) interact with the NS5 region (b) that is immediately N-terminal to NS5-NLS indicated by a solid black line (residue 320–368). Intramolecular interaction between NS5 Sam domain and Pol domain (d) maps within the NS5-NLS (368–405).

colony lift assay to a 96-well plate format. In this format the assay represents an attractive method to identify protein–protein interaction rapidly and to carry out high-throughput screening to identify small molecules that block specific interactions. Parallel studies using complementary biochemical and virological techniques will be carried out to understand the functional consequences of the different competing NS3/NS5/importin interactions.

## References

[1] T.J. Chambers, C.S. Hahn, R. Galler, C.M. Rice, Flavivirus genome organization, expression, and replication, Annu. Rev. Microbiol. 44 (1990) 649–688.
[2] A.I. Bartholomeusz, P.J. Wright, Synthesis of dengue virus RNA in vitro: initiation and the involvement of proteins NS3 and NS5, Arch. Virol. 128 (1993) 111–121.
[3] E.V. Koonin, The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses, J. Gen. Virol. 72 (1991) 2197–2206.
[4] E.V. Koonin, Computer-assisted identification of a putative methyltransferase domain in NS5 protein of flaviviruses and-2 protein ofreovirus, J. Gen. Virol. 74 (1993) 733–740.
[5] M. Kapoor, L. Zhang, M. Ramachandra, J. Kusukawa, K.B. Ebner, R. Padmanabhan, Association between NS3 and NS5 proteins of dengue virus type 2 in the putative RNA replicase is linked to differential phosphorylation of NS5, J. Biol. Chem. 270 (1995) 19100–19106.
[6] A. Buckley, S. Gaidamovich, A. Turchinskaya, E.A. Gould, Monoclonal antibodies identify the NS5 yellow fever virus non-structural protein in the nuclei of infected cells, J. Gen. Virol. 73 (1992) 1125–1130.
[7] D.A. Jans, C.K. Chan, S. Huebner, Signals mediating nuclear targeting and their regulation: application in drug delivery, Med. Res. Rev. 18 (1998) 189–223.
[8] J.K. Forwood, A. Brooks, L.J. Briggs, C.Y. Xiao, D.A. Jans, S.G. Vasudevan, The 37-amino-acid interdomain of dengue virus NS5 protein contains a functional NLS and inhibitory CK2 site, Biochem. Biophys. Res. Commun. 257 (1999) 731–737.
[9] E.K. O'Reilly, J.D. Paul, C.C. Kao, Analysis of the interaction of viral RNA replication proteins by using the yeast two-hybrid assay, J. Virol. 71 (1997) 7526–7532.
[10] M. Johansson, A.J. Brooks, D.A. Jans, S.G. Vasudevan, A small region of dengue virus-encoded RNA-dependent RNA polymerase, NS5, confers interaction with both the nuclear transport receptor importin-β and the viral helicase, NS3, J. General Virology 82 (2001) 735–745.
[11] A. Herold, R. Truant, H. Wiegand, B.R. Cullen, Determination of the functional domain organization of the importin alpha nuclear import factor, J. Cell Biol. 143 (1998) 309–318.