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SHORT COMMUNICATION

The implication of amino acid mutations at flavivirus NS1-2A cleavage site on NS1' protein production

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ABSTRACT

Aims: The presence of a C-terminally extended form of NS1 (NS1' protein) has been previously reported in encephalitic flaviviruses, due to the presence of -1 programmed ribosomal frameshift at the N-terminal of NS2A protein. This present study is aimed to further confirm that the NS1' protein production is independent of the authentic cleavage at NS1-2A junction.
Methodology and results: Six different constructs (P1-Leu, P2-Asp, P3-Phe, P3-Leu, P3-Gly and P5-8 Ala) containing various mutations at conserved and variable amino acids at C-terminal of NS1 protein were generated by site-directed mutagenesis and analysed with transient polyprotein expression assay. While analysis on the NS1-2A cleavage of the mutants exhibited extremely poor to efficient cleavage ranging from 6-89%, significant amount of NS1' being expressed in all mutants irrespective of their NS1-2A cleavage outcome.
Conclusion, significance and impact study: In this analysis, we showed for the first time that the abolishment of the authentic NS1-2A cleavage in Murray Valley encephalitis virus (MVEV) did not impact on NS1' production. This observation extend on previous studies to show that NS1 and NS2A proteins are the product of NS1-2A cleavage which is catalysed by an unknown host protease while NS1' protein is a product of ribosomal frameshift, independent of the authentic cleavage at NS1-2A junction.

Keywords: Flavivirus, NS1', NS1-2A polyprotein cleavage

INTRODUCTION

Flaviviruses are small, enveloped viruses with approximately 500 Å in diameter with icosahedral symmetry (Murphy *et al*., 1968; Nishimura *et al.*, 1968; (Mukhopadhyay *et al*., 2003). The virion contains a positive-sense, single stranded RNA genome of approximately 11 kilobases (kb) in length and lacks a 3' polyadenylate tail with type I methylated cap, $m^7GpppAmpN_1$ at the 5' end (Stollar *et al*., 1967; Wengler and Gross, 1978; Cleaves and Dubin, 1979). The RNA genome encodes a single open reading frame (ORF) flanked by 5' and 3' untranslated region (UTR). Translation of the single ORF produces a polyprotein that is co- and post-translationally cleaved by the host protease and a viral-encoded proteinase producing at least 10 discrete proteins in the order NH_2-C-prM/M-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-COOH' (Castle *et al*., 1985; Rice *et al*., 1985; Wengler *et al.*, 1985; Castle *et al.*, 1986; Dalgarno *et al.*, 1986; Coia *et al.*, 1988; Speight *et al.*, 1988;). The polyprotein is organized with the structural proteins (C, prM/M and E) at the NH_2-terminal end and the non-structural proteins at the COOH-terminal end (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5).

The NS1' protein is often detected in cells infected with Japanese encephalitis virus (JEV) serogroup (Mason, 1989; Blitvich *et al*., 1999). It was initially thought that this NS1' proteins was derived from either glycosylation products of NS1 or due to a suboptimal cleavage with the cleavage site likely to reside at the beginning of NS2A protein (Falgout *et al*., 1989; Mason, 1989; Blitvich *et al*., 1999). The NS1' protein has later been shown by others as a product of ribosomal shift that occurs at a conserved slippery heptanucleotide motif at the N-terminus of NS2A protein and is stimulated by a downstream RNA pseudoknot structure (Firth and Atkins, 2009; Melian *et*

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al., 2010; Sun *et al*., 2012; Ye *et al.*, 2012; Young *et al*., 2013).

Thus, in this study the NS1' expression was compared to that of the production of NS1 and NS2A proteins in mutants with variable mutations at the C-terminal of NS1 or "octapeptide sequence" (P1 through to P8) yielding various NS1-2A cleavage efficiency.

MATERIALS AND METHODS

Cells

African green monkey kidney (COS-7) cells were cultured in Eagle's minimum essential medium (MEM; Invitrogen, USA) supplemented with 5% heat-inactivated fetal calf serum (FCS), 0.1 mM non-essential amino acids (Invitrogen, USA) and 100 U/mL penicillin-streptomycin (PSN; Invitrogen, USA), and were grown in a humidified 37 °C CO_2 incubator.

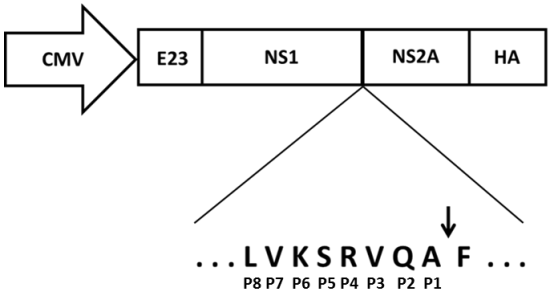

Figure 1: Design and expression of plasmid expressing ns1 and ns2A genes. The pRc/CMV.NS1-2A.HA plasmid contains an N-terminal signal sequence consisting of the last 23 codons of MVEV E protein and HA tag at the C terminus. Shown below is the last 8 codons of NS1 (octapeptide sequence) and the first codon of NS2A.

Eukaryotic expression plasmids

To generate plasmid pRc/CMV.NS1-2A.HA (7345 bp) encoding the MVEV NS1 and NS2A proteins (Figure 1), complementary deoxyribonucleic acid (cDNA) corresponding to the *ns1* and *ns2A* genes was cloned into pRc/CMV (Invitrogen, USA) using *Hind*III/*Xba*I sites. The NS1 protein was preceded by its authentic signal peptide and the NS2A protein was C-terminally fused to an influenza virus hemagglutinin (HA) epitope tag (Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala-Gly), which allows recovery of NS2A by immunoprecipitation with monoclonal antibody (mAb), 12CA5 (Wilson *et al*., 1984). To introduce amino acid substitutions in the octapeptide region, a fusion polymerase chain reaction (PCR) approach was employed (Lee and Lobigs, 2008) using the upstream primer, 5'-ACTGGATTGAGAGTGGACTCAATG-3', and downstream primer, 5'-CTGATCAGCGAGCTCTAGCATTTAAGGTGA-3', in combination with the corresponding mutagenesis primers (sequences will be provided upon request). Construction of mutant derivatives from plasmid, pRc/CMV.NS1-2A.HA, was by double digestion of mutagenized fragments (932 bp) and the corresponding *ns1-ns2A* gene region in the wild-type (wt) plasmid with restriction enzymes, *Ppu*MI and *Xba*I, and replacement of the wt with a mutated cDNA fragment. Ligation mixtures were transformed into *Escherichia coli* MC1061.1 cells, and the *ns1-ns2A* gene sequences verified by sequence analysis using BigDye v.3 (Applied Biosystems, USA) at the Biomolecular Resources Facility in the John Curtin School of Medical Research in accordance with the manufacturer's protocol.

Transient expression in COS-7 cells, metabolic labelling, immunoprecipitation and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Transfection of eukaryotic expression plasmid DNA into COS-7 cells was performed by the DEAE-dextran method, as described previously (Lee *et al.*, 2010). Metabolic labelling of proteins with Trans ^{35}S-label (MP Biomedicals, USA), lysis with radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.2 mM EDTA) containing a mammalian protease inhibitor cocktail (GBiosciences, USA), immunoprecipitation with 2 µL anti-HA epitope tag mAb (12CA5; 1.5 mg/mL) or anti-NS1 antibody (4G4) (Clark *et al*., 2007), and SDS-PAGE were as described previously (Stocks and Lobigs, 1998), with the variation of using a shorter labelling period (0.5 h followed by a 0.5 h chase) to prevent degradation of the NS2A protein. Following electrophoresis, gels were fixed by incubation for 30 min in 200 mL of 20% acetic acid, thoroughly rinsed in distilled water and dried on Whatman 3M paper using a gel dryer. Dried gels were placed in contact with a Photo-Imager screen (Fuji Film, Japan) for 3 to 4 days before the screen was scanned using a Fuji Film FLA/LAS or Typhoon FLA 9000 instrument. Image analysis for protein band quantitation was performed using Multi Gauge version 2.0 (Fuji Film, Japan).

Estimation of NS1-2A cleavage efficiency

NS1-2A cleavage efficiency was calculated after normalising for the number of Met and Cys residues in uncleaved NS1-NS2A (30) and NS2A (14). The percentage cleavage efficiency was determined as the radioactivity (expressed as photostimulated luminescence, PSL) in the NS2A band divided by the sum of that in NS2A and the NS1-NS2A precursor bands using the formula: Cleavage (%) =

$$\left\lfloor \frac{NS2A}{NS2A+\left(NS1-NS2A_{precursor}\right)} \right\rfloor \times 100$$

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Table 1: Effect of mutations introduced into the MVEV octapeptide sequence on NS1-2A cleavage.

Octapeptide residue(s) (position and amino acid in wild-type)	Construct (position and mutation)	% cleavage (mean ± SEM)
P1 (Ala)	P1-Leu	6 ± 1
P2 (Gln)	P2-Asp	89 ± 4
P3 (Val)	P3-Phe	7 ± 3
	P3-Leu	23 ± 3
	P3-Gly	83 ± 1
P5 (Ser), P6(Lys), P7(Val), P8(Leu)	P5,6,7,8-Ala	28 ± 4

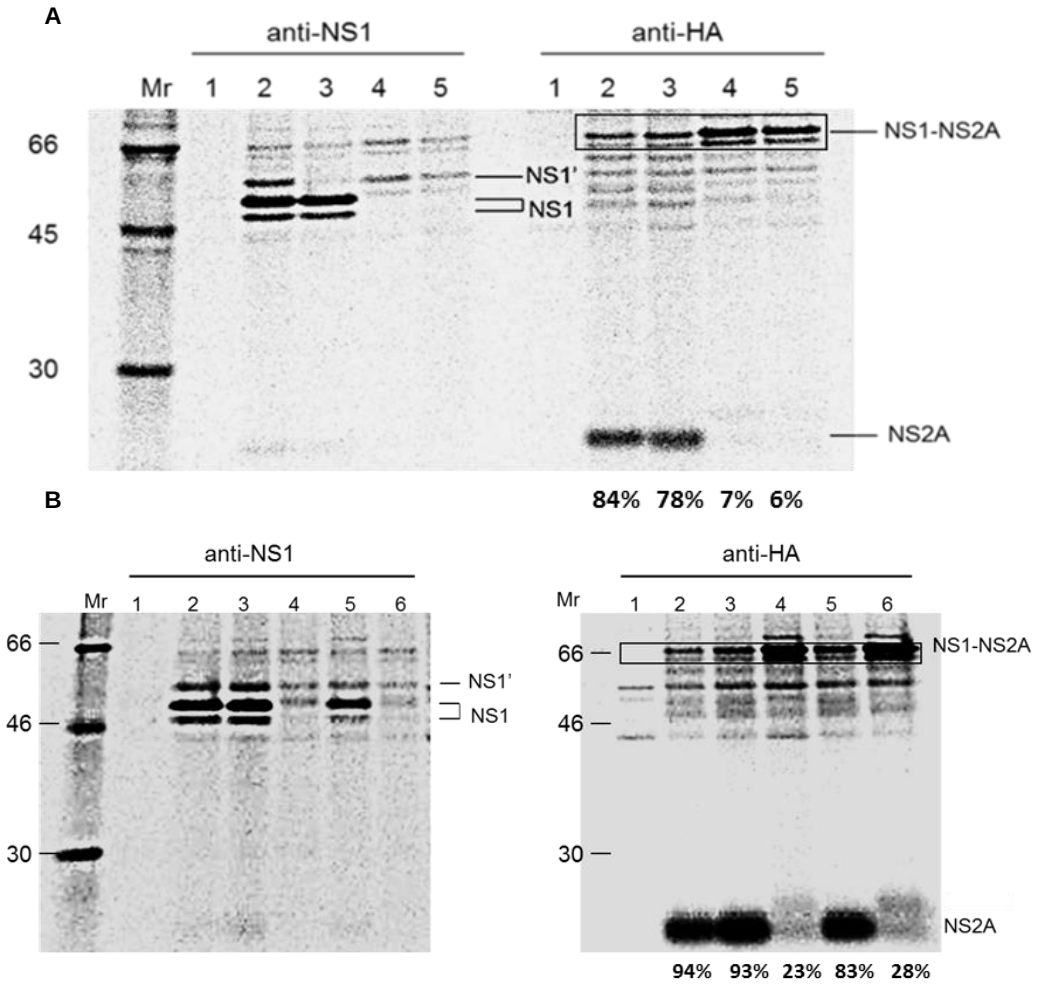

Figure 2: Analysis of NS1' production in NS1-2A cleavage defective mutants. A, Detection of NS1, NS1' and NS2A in lysates of (1) COS-7 cells mock transfected or transfected with (2) plasmid DNA of wt,(3) NS2A A30P, (4) P1-Leu and (5) P3-Phe. B, COS-7 cells were (1) mock transfected or transfected with plasmid DNA of: (2) wt, (3) P2-Asp, (4) P3-Leu, (5) P3-Gly, or (6) P5-8 Ala. Transfected cells were labelled with ^{35}S-methionine at 48 h post transfection, immune precipitated with anti-NS1 or anti-HA antibody and subjected to 12.5% SDS-PAGE. In both figures, the numbers on the left indicates the size of the ^{14}C-labelled marker proteins in kDa. The location of NS1', NS1, NS1-2A precursor and NS2A are shown on the right.

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RESULTS AND DISCUSSION

The NS1' expression was compared to that of the production of NS1 and NS2A proteins in mutants with variable mutations at the C-terminal of NS1 (P1 through to P8) following transient protein expression assay (Table 1). In this study, the COS-7 cells were transfected with plasmid DNA encoding wt MVEV NS1-2A, a construct with a non-conservative changes from Ala to Leu (P1-Leu), a construct with Gln to Asp substitution at P2(P2-Asp), two constructs with non-conservative substitution from Val to an amino acid with bulky hydrophobic side chain, Leu or Phe (P3-Leu or P3-Phe), a construct with substitution from Val to small, aliphatic amino acid, Gly (P3-Gly), a construct with quadruple Ala mutations from P5 through to P8(P5-8 Ala),and NS2A A30P. The NS2A A30P is a mutant with an Ala to Pro substitution at residue 30 of NS2A that results in the abolition of the formation of NS1' (Melian *et al.*, 2010).

As shown in Figure 2A, immunoprecipitation with anti-NS1 Ab showed NS1 and NS1' production in cells transfected with wt plasmid DNA and as expected, no NS1' was detected for NS2A A30P. Parallel analysis by immunoprecipitation with anti-HA further confirms this observation, whereby the expression of NS2A was comparable for wt and NS2A A30P. On the other hand, very low levels NS1 were detected in immunoprecipitates from cells transfected with P1-Leu and P3-Phe and interestingly, descent amount of NS1' being expressed in these mutants. Complete absence of NS2A protein was observed for lysates from cells transfected with P1-Leu and P3-Phe, indicating abolishment of NS1-2A cleavage for these mutants. This observation was also seen in another 4 mutants (P2-Asp, P3-leu, P3-Gly and P5-8 Ala) with NS1-2A cleavage efficiency of moderate to efficient (23-93 %) (Figure 2B) where reduction in NS1' is always present irrespective to substitutions made at NS1-2A cleavage site.

Data shown in this study corroborates the observation shown by others that NS1' is a product of ribosomal shift that occurs due to a presence of -1 programmed ribosomal shift at N-terminal of NS2A protein (Firth and Atkins, 2009; Melian *et al.*, 2010; Sun *et al*., 2012; Ye *et al*., 2012; Young *et al*., 2013). This was also confirmed by two studies that a single nucleotide mutation in NS2A (A90P in Kunjin virus and G66A in Japanese encephalitis-live vaccine virus, SA 14-14-2) could abolish the formation of NS1' by disrupting the downstream frameshift-stimulating RNA pseudoknot structure (Melian *et al*., 2010; Ye *et al*., 2012). Taken together, it was evident from this analysis that the abolishment of NS1-2A cleavage did not impact on NS1' production which confirms that NS1' production is independent of NS1-2A cleavage.

CONCLUSION

In conclusion, the NS1' production is confirm to be a product of ribosomal frame shift, stimulated by a conserved pseudoknot structure downstream of NS2A. NS1' production is independent of the NS1-2A polyprotein processing, and this has been demonstrated by the transient protein expression assay using plasmid constructs containing variable mutations at NS1-2A cleavage site. It is observed that the NS1' protein is always expressed despite of abolishment of NS1-2A cleavage.

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