**Mutagenesis of the nucleocapsid protein of Nipah virus involved in capsid assembly**

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The nucleocapsid protein of Nipah virus produced in *Escherichia coli* assembled into herringbone-like particles. The amino- and carboxy-termini of the N protein were shortened progressively to define the minimum contiguous sequence involved in capsid assembly. The first 29 aa residues of the N protein are dispensable for capsid formation. The 128 carboxy-terminal residues do not play a role in the assembly of the herringbone-like particles. A region with amino acid residues 30–32 plays a crucial role in the formation of the capsid particle. Deletion of any of the four conserved hydrophobic regions in the N protein impaired capsid formation. Replacement of the central conserved regions with the respective sequences from the Newcastle disease virus restored capsid formation.

Nipah virus (NiV), the second member of the genus *Henipavirus* (Wang et al., 2000, 2001), has been associated with several outbreaks of viral encephalitis in humans in South-east Asia. The first and largest NiV outbreak in Malaysia ended a year after its identification in 1998. During this period, more than 100 human lives were claimed and the pig industry in the country was paralysed. Pig farms were forced to close and millions of pigs were culled in order to control the viral outbreak (Chua et al., 1999, 2000). Two years later, an outbreak in Bangladesh alerted the world to the reoccurrence of this virus (Hsu et al., 2004; Harcourt et al., 2005). Shocking news were reported in 2004 from India where the mortality rate of the infected patients was more than 70% (Diederich & Maisner, 2007). The spread of the virus from human to human was suspected, and no pig intermediary was reported (Chadha et al., 2006). Several species of flying foxes, in the genus *Pteropus*, have been identified as the natural reservoirs of NiV (Eaton et al., 2006).

NiV has a single-stranded negative-sense RNA of approximately 18.2 kb which encodes six major structural proteins: nucleocapsid (N), phospho- (P), matrix (M), fusion (F), glyco- (G) and large (L) proteins (Wang et al., 2001). The most abundant structural protein, N, and the genomic RNA constitute the core helical nucleocapsid structure of NiV. The genomic RNA is associated with the N, P and L proteins to form the ribonucleoprotein complex (Diederich & Maisner, 2007).

Structural and functional studies of the N protein of paramyxoviruses have been the main focus for years, due to its crucial functional role during the replication of the genomic RNA. Transmission electron microscopic studies revealed that all the paramyxovirus nucleocapsids are helical in structure, but there are significant differences among the virus genera (Bhella et al., 2002) such as Sendai virus (SeV; Myers et al., 1999), measles virus (MeV; Bhella et al., 2004) and respiratory syncytial virus (RSV; MacLellan et al., 2007). This is most likely due to the differences in the primary structure of the N proteins of these viruses which determine the tertiary and quaternary structures of the nucleocapsids.

For members of the genus *Henipavirus*, the region of the N protein involved in capsid assembly has yet to be identified. We have previously reported that the recombinant N protein of NiV self-assembled into herringbone-like particles when it was expressed in *Escherichia coli* in the absence of other viral proteins (Tan et al., 2004). To further explore the minimum contiguous sequence of the amino acid residues involved in capsid formation, a series of deletions from the amino- and carboxy-termini of the N protein were produced in *E. coli*. The results showed that the amino terminus of the N protein played an important role in the assembly of the capsid, while the hypervariable 128 carboxy-terminal residues were dispensable in the formation of the nucleocapsid. Amino acid sequence...
alignments of the nucleocapsid proteins of viruses from the family Paramyxoviridae, including Hendra virus (HeV) and NiV, revealed four highly conserved regions (Kho et al., 2001). These regions are relatively hydrophobic and are believed to be involved in NiV N–N interactions. In the present study, the role of these regions in capsid assembly was studied by deletion mutagenesis and replacement with the respective sequences from the Newcastle disease virus (NDV).

A series of amino- or carboxy-terminal deletion mutants were constructed as described (Tan et al., 2004) by using the primers listed in Supplementary Table S1, available in JGV Online. Fig. 1(a) shows the amino-terminal truncated mutants. The carboxy-terminal truncated mutants are shown in Fig. 1(b). The internal deletion mutants (Fig. 1c) were constructed using the PCR method as described by Saiki et al. (1988) and Szewczyk et al. (2007).

In order to further investigate the role of the four conserved regions in capsid assembly, these regions were replaced individually with the respective sequences from the nucleocapsid (NP) protein of NDV strain AF2240 (Kho et al., 2001) using the PCR method as described in Supplementary Table S1. Recombinant plasmids were isolated from the putative clones and verified by restriction mapping and confirmed by DNA sequencing. These N mutants were produced as fusion proteins, containing the myc epitope and His-tag at their carboxy-termini.

All the N mutants were successfully expressed in E. coli BL21 (DE3) cells at 25 °C after being induced with IPTG for 3–5 h, as analysed by Western blotting using anti-myc monoclonal antibody (Invitrogen) as a probe (data not shown). Briefly, cells were harvested and resuspended in 25 mM HEPES [pH 7.6; 10 ml (g cells)⁻¹], and then lysed by sonication. The lysate was treated with RNase (5 μg ml⁻¹) and DNase I (5 μg ml⁻¹) for 1 h at 4 °C and the cell extract was recovered by spinning at 16 000 g for 20 min at 4 °C. The mutated recombinant N proteins were precipitated by ammonium sulphate (40 % saturation) and dialysed against Tris-NaCl buffer (50 mM Tris, 100 mM NaCl).
NaCl; pH 8.0). The dialysed samples were layered on a 10–70% sucrose gradient and centrifuged as described by Tan et al. (2004). Fractions containing the purified proteins were pooled and concentrated using Viva Spin Concentrators (Vivascience) with 50–100 kDa cut-off. A drop (~15 μl) of each purified protein was applied to the 200-mesh carbon-coated Formvar copper grids and stained with 2% uranyl acetate. Visualization up to ×150 000 was made via an energy-filter transmission electron microscope (EFTEM; LEO 912AB).

A total of seven amino- and 16 carboxy-terminal deletion mutants were constructed (Fig. 1a and b). All these N mutants were purified and detected by anti-myc antibody in Western blot analysis (data not shown), indicating that the amino-terminal end deletion mutants were successfully produced in E. coli. Fig. 2(a) shows that mutants N27–532fus and N30–532fus assembled into herringbone-like particles while the longer deletion mutants, N33–532fus, N41–532fus and N54–532fus, aggregated into spherical particles with heterogeneous sizes. Deletion of the 129 aa from the carboxy terminus of the N protein (mutant N1–403fus) completely abolished the formation of the herringbone-like particles (Fig. 2b). This mutant aggregated into spherical particles with different sizes. A larger deletion beyond 129 aa (mutants N1–402fus and N1–401fus) further confirmed the inhibition of capsid assembly (data not shown). On the other hand, deletion of 128 aa or less from the carboxy terminus (mutants N1–404fus, N1–405fus, N1–410fus, N1–414fus, N1–420fus, N1–426fus, N1–452fus, N1–480fus and N1–506fus) did not impair formation of the herringbone-like structure.

A comparison of the predicted nucleocapsid amino acid sequences of NiV [GenBank accession no. AJ564621 (AbuBakar et al., 2004)] and NDV AF2240 [AF284646 (Kho et al., 2001)] revealed four highly conserved regions (Fig. 3), which consist predominantly of hydrophobic residues in the middle of the primary structure. These regions are predicted to be involved in N–N interactions for members of the paramyxoviruses (Heggeness et al., 1981; Kho et al., 2001). These four highly conserved regions are designated region 1 (R1), region 2 (R2), region 3 (R3) and region 4 (R4). In order to investigate the role of

Fig. 2. Electron microscopic observation of sucrose gradient-purified NiV N mutants; (a) amino-terminal deletion N mutants, (b) carboxy-terminal deletion N mutants and (c) substituted conserved region N mutants.
these four conserved regions, another eight mutants were constructed: four mutants with one of these conserved regions removed individually (namely, N\textsubscript{275–326} fus, N\textsubscript{252–275} fus, N\textsubscript{282–314} fus and N\textsubscript{322–342} fus). Fig. 1c) and another four mutants having these conserved regions replaced with the respective regions from NDV (Figs 1d and 3). All these mutants (N\textsubscript{AR1 fus}, N\textsubscript{AR2 fus}, N\textsubscript{AR3 fus} and N\textsubscript{AR4 fus}) were successfully produced in E. coli.

Electron microscopic analysis revealed that none of the internal deletion N mutants (N\textsubscript{167–182} fus, N\textsubscript{252–275} fus, N\textsubscript{282–314} fus and N\textsubscript{322–342} fus) was able to assemble into herringbone-like particles (data not shown). This suggests that the highly conserved hydrophobic regions are required for capsid assembly. Fig. 2(c) shows that mutants N\textsubscript{AR1 fus} and N\textsubscript{AR4 fus}, with the conserved regions R1 and R4 replaced by those from NDV did not assemble into herringbone-like structures. Instead, these mutants aggregated predominantly into spherical structures with different sizes. This implies that the R1 and R4 regions are likely to be involved in the NiV N capsid assembly. On the other hand, mutants N\textsubscript{AR2 fus} and N\textsubscript{AR3 fus} still assembled into herringbone-like particles (Fig. 2c), demonstrating that the R2 and R3 regions are interchangeable with those from NDV and the differences in the amino acid residues in these regions (Fig. 3) are unlikely to be involved in NiV capsid formation.

Many studies have shown that paramyxovirus nucleocapsid proteins self-assemble into herringbone-like particles when expressed in a heterologous system in the absence of the viral proteins (Buchholz et al., 1993; Curran et al., 1993; Bankamp et al., 1996; Krishnamurthy & Samal, 1998; Seal et al., 2002; Kho et al., 2003). However, the contiguous regions of the N protein required for capsid assembly are poorly characterized in Henipavirus. In the present study, we found that mutants N\textsubscript{237–332 fus} and N\textsubscript{300–332 fus} were able to self-assemble into herringbone-like structures (Fig. 2a). However, N\textsubscript{33–332 fus} which had an additional removal of 3 aa, did not exhibit any capsid formation. This suggests that the first 29 aa from the amino terminus do not play an important role in the assembly of the NiV capsid; whereas the 3 aa at positions 30 to 32 are required for capsid formation. These findings have not been reported so far. Deletion mutagenesis of other paramyxoviruses, Sendai virus (Buchholz et al., 1993; Curran et al., 1993), measles virus (Bankamp et al., 1996) and bovine respiratory syncytial virus (Krishnamurthy & Samal, 1998), revealed that the residues at the amino terminus of their N proteins are indispensable for capsid assembly. These findings are in contrast to the results of our present study where the deletion of 29 aa from the amino terminus of the NiV N protein did not impair formation of the herringbone-like particles. Besides, deletion from the carboxy-terminus revealed that the first 404 aa residues from the amino terminus are sufficient for the assembly of the capsid. In general, like for other members of the paramyxoviruses, it can be concluded that the amino-terminal end of the NiV N protein contributes more than the carboxy-terminal end to capsid assembly.

Based upon our earlier study on the amino acid sequences of paramyxoviruses’ nucleoproteins (Kho et al., 2001), we hypothesized that the four highly conserved regions are involved in the NiV N–N interactions for formation of the capsid. Hence, attempts were made to delete these regions and replace them with the respective conserved sequences from NDV. The self-assembly ability of the four different internal deletion N mutants (N\textsubscript{167–182} fus, N\textsubscript{252–275} fus, N\textsubscript{282–314} fus and N\textsubscript{322–342} fus), covering the conserved regions of the NiV N protein, was investigated. However, none of these mutants was able to assemble into herringbone-like particles. This demonstrates that the conserved regions play a very important role in capsid assembly and that disruption of any of them totally abolishes formation of capsid.

The replacement of conserved region R1 with the respective NDV sequence abolished the formation of herringbone-like particles, suggesting that residues 169E, 172I and 175L are most likely to be involved in capsid assembly. Mutant N\textsubscript{AR4 fus} with its fourth conserved region (R4) substituted with the NDV sequence, also did not form herringbone-like particles, indicating that residues 326G, 329L and 340T play an important role in the capsid assembly. We speculated that the disruption of these amino acids (169, 172, 175, 326, 329 and 340) has affected the assembly of the N monomers into herringbone-like particles. On the other hand, self-assembly ability was not affected by replacing the two central conserved regions (R2 and R3) with their respective sequences from NDV (mutants N\textsubscript{AR2 fus} and N\textsubscript{AR3 fus}), suggesting that the non-conserved residues in R2 (amino acids 253S, 256G, 260E and 265A) and R3 (amino acids 286E, 289S, 293T, 299L, 302T, 305A, 308T, 310T, 313L, 320K, 323E, 327D, 329L and 340T) play an important role in the capsid assembly.
303E, 304I, 306P, 307R and 312V) are not critical in NiV capsid assembly. However, site-directed mutagenesis of these residues will be necessary to provide a more precise mapping of the exact residues involved in capsid assembly. In addition, it could not be ruled out that the replacement of the conserved regions led to conformational changes in the substitution mutants which then affected capsid formation. The nucleoproteins of paramyxoviruses interact with RNA molecules, but the role of the latter in the assembly of NiV nucleocapsid is still unclear.

The nucleocapsid serves as a template for transcription and replication of paramyxovirus genomes (Lamb & Kolakofsky, 2001). The L protein associates with the P protein to form the polymerase complex (Holmes & Moyer, 2002) which binds to the nucleocapsid (Buchholz et al., 1994). One of the binding sites of the P protein was mapped to the 29 aa carboxy-terminal region of the N protein of members of the genus Henipavirus (Chan et al., 2004). In this study, we have mapped two regions critical for self-polymerization of NiV N. The minimum contiguous sequence of N protein involved in capsid assembly was located in residues 30–404.

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