Structure of the dengue virus glycoprotein non-structural protein 1 by electron microscopy and single-particle analysis

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The flavivirus non-structural protein 1 (NS1) is a glycoprotein that is secreted as a soluble hexameric complex during the course of natural infection. Growing evidence indicates that this secreted form of NS1 (sNS1) plays a significant role in immune evasion and modulation during infection. Attempts to determine the crystal structure of NS1 have been unsuccessful to date and relatively little is known about the macromolecular organization of the sNS1 hexamer. Here, we have applied single-particle analysis to images of baculovirus-derived recombinant dengue 2 virus NS1 obtained by electron microscopy to determine its 3D structure to a resolution of 23 Å. This structure reveals a barrel-like organization of the three dimeric units that comprise the hexamer and provides further insights into the overall organization of oligomeric sNS1.

INTRODUCTION

The genus Flavivirus comprises many important human pathogens including dengue (DENV), yellow fever (YFV), Japanese encephalitis (JEV), West Nile (WNV), Tick-borne encephalitis (TBE), St Louis encephalitis (SLEV) and Murray Valley encephalitis (MVEV) viruses. Clinical outcomes of infection vary from asymptomatic to self-limiting febrile illness through to encephalitis or meningitis, haemorrhage and shock that can be fatal (Chappell et al., 2008; Rossi et al., 2010; Schmidt, 2010). Flaviviruses are small, enveloped viruses with a positive-sense RNA genome that encodes three structural (C, prM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Lindenbach et al., 2000; Schmidt, 2010). Flaviviruses are small, enveloped viruses with a positive-sense RNA genome that encodes three structural (C, prM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Lindenbach & Rice, 2003). The non-structural protein, NS1, is a glycoprotein whose structure and mechanistic function has remained elusive since it was first identified in 1970 as a soluble complement fixing (SCF) antigen in dengue-infected patient sera (Brandt et al., 1970; Smith & Wright, 1985).

All flavivirus NS1 genes share a high degree of homology and are 1056 nt in length encoding a 352 aa long polypeptide (Deubel et al., 1988; Mackow et al., 1987; Mandl et al., 1989; Wright et al., 1989). The DENV NS1 monomer has a predicted molecular mass of between 46 and 50 kDa, depending on its glycosylation status and exists in multiple oligomeric forms. It can be found associated with cell membranes (mNS1), both within cells and at the cell surface, or as a secreted extracellular species (sNS1) (Mason, 1989; Smith & Wright, 1985; Westaway & Goodman, 1987; Winkler et al., 1988). Intracellular NS1 plays an essential role in virus replication and has been shown to co-localize with dsRNA and other components of viral replication complexes in viral-induced membranous compartments referred to as vesicle packets (Mackenzie et al., 1996; Panyasrivanit et al., 2009; Westaway et al., 1997). However, the precise function of this protein in viral replication has yet to be determined. Secreted and cell surface-associated NS1 are highly immunogenic and both the protein itself, as well as the antibodies it elicits have been shown to contribute to either protection or pathogenesis as well as being implicated in innate immune evasion (Avirutnan et al., 2006; Falgout et al., 1990; Henchal et al., 1988; Schlesinger et al., 1987; Sun et al., 2007). sNS1 binds to a wide range of cells via interaction with cell surface glycosaminoglycans (Avirutnan et al., 2007) and has been shown to interact with a number of different host cell proteins (Avirutnan et al., 2010; Chua et al., 2005; Chung et al., 2006b; Krishna et al., 2009; Kurosu et al., 2007).
Despite the key role that NS1 plays in both viral replication and engagement with the host, little is known about its tertiary structure and hence, the molecular basis of these interactions. It is known that the secreted form of NS1 is hexameric (Crooks et al., 1994) and that this hexamer comprises three detergent-stable dimers (Flamand et al., 1999). Furthermore, all flavivirus NS1 species contain 12 conserved cysteine residues that form six intra-chain disulphide bonds (Blitvich et al., 2001; Wallis et al., 2004). In combination with mAb epitope-mapping studies, these findings have led to a basic understanding of discrete domains within NS1 (Chung et al., 2006a). However, ongoing efforts by a number of groups, including ours, to crystallize this species have been unsuccessful to date. The elucidation of high-resolution NS1 structures would provide much needed impetus to research on its function in viral replication and the basis of its binding with host cell components. During the preparation of this manuscript a structure for DENV-1 hexameric NS1, based on cryo-electron microscopy (EM) single-particle analysis (SPA) was published, providing the first step towards this goal (Gutsche et al., 2011). These authors identified sNS1 as a lipoprotein particle with the oligomeric units forming an open-barrel protein shell. Here, we have used single-particle image processing and supporting biochemical analyses to provide further insights into the quaternary architecture of baculovirus-expressed recombinant DENV-2 sNS1.

RESULTS

Chemical cross-linking, size-exclusion chromatography (SEC), mAb epitope mapping and EM were performed on recombinant, baculovirus-derived NS1 to confirm that its biophysical and antigenic properties were similar to that of its native counterpart (Figs 1 and 2).

Biochemical analysis of recombinant sNS1

SDS-PAGE analysis confirmed the purity of recombinant sNS1 preparations and that this species migrated as a dimer

![Fig. 1. Analysis of recombinant sNS1. (a) SDS-PAGE analysis of purified sNS1 showing the detergent stable dimer and heat labile monomer. (b) Immunoblot of secreted recombinant NS1 cross-linked with BS3 at 0, 1, 5 and 20 mM cross-linker concentrations. All samples were boiled prior to separation on a SDS-PAGE gel. (c) SEC profile of nickel-purified recombinant sNS1. The elution positions of standard proteins used for molecular mass estimation (- - -) are superimposed on the sNS1 trace. (d) Competition maps of captured anti-NS1 mAb binding of 35 S-sNS1 from Vero cells (top panel) and Sf9 cells (bottom panel) in the presence of secondary competitor mAb. Primary mAbs (1°) are listed horizontally and secondary mAbs (2°) are listed vertically. ■ <30 % binding, □ <30–50 % binding, □ 51–150 % binding.]
that was detergent-stable, but heat sensitive (Fig. 1a). This detergent-stable characteristic of native NS1 was first identified by Winkler et al. (1988). Cross-linking with increasing concentrations of bis(sulfosuccinimidyl) suberate (BS3) (Thermo Scientific) additionally showed that as with its native counterpart, recombinant sNS1 is hexameric (Fig. 1b). The observed conversion of monomer, primarily through dimer and tetramer intermediates to the hexameric form, as cross-linker concentration increases (Fig. 1b) supports the model that the native sNS1 hexamer is likely to consist of a trimer of trimers (Flamand et al., 1999). The fact that trimers did not comprise a major species further supports this hypothesis.

SEC was used to purify sNS1 following nickel-affinity chromatography and provided further evidence for the hexameric arrangement of sNS1. The molecular mass of sNS1 was estimated by comparison to a regression curve constructed from retention times of standard proteins eluted from the Superdex 200 column used to purify sNS1 (representative trace shown in Fig. 1c). NS1 harvested and purified from the baculovirus-infected cells routinely eluted with a retention volume of between 10.6 and 13 ml, corresponding to an approximate molecular mass of 250–350 kDa. Compared with the predicted molecular mass of six NS1 monomers (approx. 300 kDa) and given the uncertainty in monomer molecular mass due to the extent of glycosylation, this result is consistent with a hexameric sNS1 species.

Antigenic competition mapping of sNS1

Antigenic mapping of sNS1 purified from both DENV-2-infected Vero cells and recombinant, baculovirus-infected Sf9 cells was performed using seven mAbs specific for conformational (5F10.3, 1E2.3 and 2A5.1) or linear (1A12.3, 1H7.4, 5H4.4 and 5B9.3) epitopes (Falconar & Young, 1991). The precise binding sites of the linear sequence reactive antibodies used in this mapping study have previously been determined by PEPSCAN analysis (Falconar, 1993; Falconar et al., 1994). 1H7.4 and 5H4.4 bind to aa 25–33, 1A12.3 binds to aa 112–120 and 5B9.3 binds to aa 249–257. Chequerboard competition maps of the seven mAbs, reacted against metabolically labelled native or recombinant sNS1, are shown in Fig. 1(d). All mAbs showed efficient self-competition thus providing an inbuilt control for the assay. Cross-competition between 1H7.4 and 5H4.4 was expected for antibodies that recognize the same sequence, while cross-competition between 5B9.3 and 5F10.3 and between 1E2.3 and 2A5.1 identified two further unique epitopes. In addition, a one-way competition in binding of 5B9.3 by the mAbs 1H7.4 and 5H4.4 was seen in both epitope maps. Competition for binding by antibodies that recognize epitopes more than 200 residues apart suggests an overlapping epitope, resulting from either the structural juxtaposition of these linear distant sites or similar conformational modifications following antibody binding. Overall, the high degree of correlation between competition maps incorporating both linear and conformational mAbs suggests that our recombinant sNS1 is correctly folded. Taken in the context of the combined biochemical and biophysical analyses, these data indicate that recombinant sNS1 is essentially equivalent in size, oligomeric structure and antigenicity to native sNS1, making it an excellent candidate for further analysis by single-particle EM.

EM of NS1

Baculovirus-derived recombinant sNS1 was purified by SEC and pre-screened by negative-stain transmission EM to assess particle heterogeneity and suitability for subsequent analysis. A representative micrograph is shown in Fig. 2(a). A relatively homogeneously sized distribution of globular particles was observed with a diameter of approximately 10 nm. For selected particles (Fig. 2b, top two panels) a distinctive, striated appearance was discernible that was consistent with the barrel-like oligomeric structure reported recently for native DENV-1 sNS1 (Gutsche et al., 2011). Particles apparently oriented end-on were also seen (Fig. 2b, lower two panels). Additional preparations were then imaged and a full dataset was collected for 3D structure determination using SPA.

Image processing and 3D reconstruction

A combination of 2D image processing tools was initially used to generate bias-free class averages, representing 2D projection views of NS1. These were used to evaluate possible point symmetries associated with the structure. Classification of particle images according to the symmetry profile of their rotational power spectra identified clusters of images with strong tendencies towards twofold [see
Supplementary Fig. S1(a–c), available in JGV Online and threefold [see Supplementary Fig. S1(d–f)] rotational symmetry, consistent with a model of a trimer of dimers. Maximum-likelihood analysis was used to subclassify and align the images rotationally and translationally within each cluster. The aligned images were then averaged to generate high signal-to-noise projection images representative of each image cluster. For rigour, an image cluster with a moderate tendency towards sixfold rotational symmetry was also analysed in this way [see Supplementary Fig. S1(g–i)].

A number of 3D reconstructions were next generated under the imposition of differing molecular point symmetries that were compatible with both the putative symmetries identified in the 2D projection images of the complexes, and with its already determined hexameric structure. The highest order (D6) as well as most likely subsymmetries (C6, D3 and C3) consistent with these observations was imposed. Maximum-likelihood analysis-derived threefold and sixfold symmetrical class averages were used to generate threefold and sixfold symmetrical starting models using the STARTCSYM procedure in EMAN (Ludtke et al., 1999). Subsequently, these starting models were used to arrive at four independent 3D reconstructions, refined to convergence under the imposition of C3 (refined from the threefold starting model), D3 (threefold starting model), C6 (sixfold starting model) or D6 molecular point symmetry (sixfold starting model).

The validity of the four models was first assessed by manual inspection of the consistency between the class averages and reference projection images derived from the 3D molecular reconstructions. Poor correlation between class averages and reference projections was considered indicative of an incorrectly refined model. For the C6 and D6-symmetrised reconstructions a large number of class averages were of poor quality (incomplete, poor stain ring surrounding the class average). This was particularly true for reference projections close to the major symmetry axis and many reference projections had few or no matching particle images. The C6 and D6 reconstructions were therefore at this point excluded from further analysis.

To determine whether the images of hexameric NS1 were most consistent with a C3 or D3 molecular structure, reconstructions generated under the imposition of both these point symmetries were further analysed, in particular by testing the effect of relaxing the applied molecular symmetry to lower orders. The principle of this test was that if the correct symmetry had been applied, the determined 3D structure would remain stable after the symmetry was relaxed. If however the applied symmetry was too high, relaxing the symmetry constraint was expected to result in marked structural changes to the model. The C3 reconstruction was therefore refined free of any imposed symmetry (i.e. C1 point symmetry) and the D3 reconstruction was refined under the imposition of C3 symmetry. While subtle changes were observed when the C3 reconstruction was relaxed, overall the structure remained relatively stable (see Supplementary Fig. S2a, available in JGV Online), supporting the conclusion that the C3 symmetry was correct. This was particularly evident when the results were considered in the context of the D3 reconstruction, which exhibited more appreciable changes in structure following relaxation to C3. Notably, the orthogonal twofold symmetry (which distinguishes D3 point symmetry from C3) was lost almost immediately. This indicated that the imposition of C3 molecular point symmetry was the highest symmetry order that could be imposed to generate a 3D reconstruction consistent with our raw particle data. This differs from the DENV-1 sNS1 structure presented by Gutsche et al. (2011) which was refined with D3 rather than C3 symmetry imposed. The final converged, C3 reconstruction is shown in Fig. 3 and has a resolution of 23 Å as judged by the 0.5 Fourier shell correlation criterion (see Supplementary Fig. S3, available in JGV Online).

**DISCUSSION**

We have shown that recombinant, baculovirus-derived sNS1 retains both the biophysical and antigenic characteristics of its native hexameric counterpart. Purified sNS1 incubated with increasing concentrations of BS6 identified progressively cross-linked species from a monomer through to hexamer in a pattern similar to that observed for native DENV sNS1 (Flamand et al., 1999). The dominant species observed in the BS6 cross-linked samples were dimers, tetramers and hexamers, supporting the hypothesis that the hexamer is assembled as a trimer of dimers. SEC provided a molecular mass estimate for secreted NS1 of 250–350 kDa. This experimentally determined molecular mass range matches the expected molecular mass of a hexameric complex (~300 kDa, 6 × ~50 kDa monomers).

The representative linear and conformational mAbs chosen for this limited epitope study, confirmed that recombinant and native sNS1 exhibited very similar patterns of cross competition. The seven mAbs tested survey the length of the NS1 molecule, suggesting a high level of confidence that the recombinant form of baculovirus-derived sNS1 is antigenically equivalent to native sNS1 and is thus a suitable candidate for structural studies.

The molecular mass of sNS1 puts it at the lower end of what can be considered the ‘suitable molecular weight range’ for single-particle EM, with molecular reconstruction of protein assemblies smaller than 500 kDa generally accepted as challenging, despite some notable accomplishments (Chou et al., 2006; Jawhari et al., 2006). It was therefore essential to perform a comprehensive 2D and 3D image processing analysis that identified and considered all plausible point symmetries that may have been consistent with the true 3D structure of secreted, hexameric NS1. Multiple starting models were generated and refined under the imposition of differing symmetry constraints in order to minimize the imposition of incompatible or overestimated symmetry during reconstruction (Ludtke et al., 1999).
the four 3D reconstructions evaluated (C3, D3, C6 and D6), the C3-symmetrisation reconstruction was ultimately identified as the reconstruction that best reflected the 3D structure of sNS1. This conclusion is based on the fact that: (i) it yielded the highest symmetry reconstruction that remained stable under relaxed symmetry, (ii) it maintained consistency between class averages and reference projections and (iii) reflected a structure having a wide spatial distribution of reference projections.

During the preparation of this manuscript a parallel SPA study based on cryo-EM was published (Gutsche et al., 2011). Gutsche et al. (2011) present a similar, threefold symmetrical right-handed barrel-like structure and organisational assembly for native sNS1 purified from DENV-1-infected Vero cells. Notably, the structure of recombinant DENV-2 sNS1 reported here appears closed at one end of the barrel (Fig. 3), compared with the open barrel structure reported for DENV-1 sNS1 (Gutsche et al., 2011). This apparent difference is reflected in the molecular symmetry of the two structures – the DENV-1 structure has a reported D3 symmetry, while our DENV-2 structure has C3 symmetry, the latter being indicative of an asymmetry between the two ends of the barrel. NS1 is known to bind to phospholipid membranes and in its secreted form is essentially a lipoprotein particle (Gutsche et al., 2011) and so the most likely explanation for this difference is the presence of asymmetrically bound hydrophobic lipid material, resulting in an asymmetrical stain-exclusion pattern from the central channel. Combined with recent observations of NS1 association with lipid rafts (Noisakran et al., 2008), this structure may therefore provide clues to the region of NS1 involved in membrane association. To confirm the association of lipids with our purified preparations of recombinant DENV-2 sNS1, we performed TLC analysis (Supplementary Fig. S4, available in JGV Online). Associated phospholipid was indeed detected, albeit at a significantly lower molar ratio-to-protein content than that
found for DENV-1 sNS1 (Gutsche et al., 2011). This discrepancy may reflect inherent differences between these two DENV strains. However, Gutsche et al. (2011) showed similar lipid content for sNS1 purified from both DENV 1- and 2-infected cell cultures, suggesting that it is more likely a consequence of the differing expression systems. It is worth noting that the recombinant baculovirus-derived sNS1 used in the current study was expressed using serum-free conditions, which may contribute to the lower level of lipid content for this secreted species.

Presented in Fig. 3 is our SPA reconstruction (Fig. 3a) along with an interpretation of its quaternary structure (Fig. 3b) that is consistent with both the established biophysical properties of hexameric NS1 and the D3 symmetrized DENV-1 structure of Gutsche et al. (2011), while offering additional insights into its molecular architecture at the domain level. The structure comprises three symmetrically equivalent dimers, which may represent the detergent stable dimer identified in Fig. 1. These putative dimers consist of a pair of pseudo-symmetrically arranged subunits (circumscribed by the dashed black lines in Fig. 3b), each of which comprises three globular domains that may correspond to the three antigenic domains previously described for NS1 (Chung et al., 2006a; Muller & Young, 2011). It is known that residues in the C-terminal region of NS1 contribute to dimerization (Hall et al., 1999) and so the close spatial proximity of one of the globular domains from each monomer in the centre of the barrel suggests that this may constitute the C-terminus of the protein. This arrangement also places this putative C-terminal domain next to the opposite, presumably N-terminal domain (identified by the white dashed line in Fig. 3b) of a subunit from an adjacent dimer, consistent with antigenic mapping studies presented here (Fig. 1d) and reported previously (Chung et al., 2006a).

Although this model of the subunit arrangements within hexameric sNS1 is speculative it is entirely consistent with the known biophysical and antigenic properties of NS1. However, as with the structure proposed by Gutsche et al., the location of proposed NS1 monomers or dimers within the SPA reconstruction cannot be unequivocally assigned at this stage.

Despite similar organizational elements, the structure reported here reveals more extensive lateral interactions between the three proposed dimeric subunits (Fig. 3) than those reported by Gutsche et al. (2011). The limited contact made by these subunits in the latter structure lead this group to propose that it was the lipid content that stabilizes the sNS1 hexameric assembly in aqueous environments (Gutsche et al., 2011). This structural difference, coupled with the lower lipid content of our structure suggests a degree of flexibility for hexameric sNS1 that may serve to accommodate a variable lipid cargo while still retaining overall oligomeric structural integrity. This structural flexibility may offer one further explanation for the failure of extensive efforts to crystallize this form of the protein. The overall dimensions of the hexamer are comparable with previous descriptions (Flamand et al., 1999; Gutsche et al., 2011), while the absence of sixfold symmetry is consistent with the proposal that sNS1 is a trimer of dimers.

An additional feature of this reconstruction that distinguishes it from the DENV-1 sNS1 structure is the presence of density within the channel, clearly visible emanating from three locations on the inside of the barrel and meeting in the centre (Fig. 3a). It has been known for some time that the second carbohydrate addition at Asn207 is protected from trimming and processing during transit of oligomeric NS1 through the Golgi prior to secretion (Post et al., 1991) and that mutagenesis of this site to ablate carbohydrate addition has a dramatic effect on assembly and secretion efficiency (Crabtree et al., 2005; Pryor & Wright, 1994). It is tempting to speculate that this density may represent these carbohydrate moieties, visible only in this structure perhaps because the SPA was performed on negatively stained images. We await with interest the crystal structure determination of NS1 and its subsequent docking into these new SPA structures.

**METHODS**

**Expression and purification of NS1.** Recombinant NS1 was expressed by infecting a 2 l culture of Sf9 cells at a density of 1 × 10⁶ cells ml⁻¹ in SF900II media with recombinant baculovirus (Leblois & Young, 1995) at an m.o.i. of 1 following incubation at 28 °C on a shaker until cell viability reached 80% (typically for 24–72 h). The medium was collected and prepared for ultrafiltration by centrifugation at 1000 g for 15 min in a Beckman JA-14 centrifuge. The clarified harvest was filtered through a 0.22 μm Seritofil filter (Millipore), buffer exchanged twice and concentrated in column binding buffer (300 mM NaCl, 50 mM NaH₂PO₄.H₂O, 10 mM imidazole pH 8) through a cross-flow system with two 50 kDa cut-off Viva Slice cassettes (Sartorius). The concentrated protein solution was bound to 3 ml pre-equilibrated Superflow Ni-NTA agarose (Qiagen) in a 50 ml tube for 1 h on a rotor at room temperature. Following binding, the nickel agarose was packed into a column and washed with 30 ml wash buffer (300 mM NaCl, 50 mM NaH₂PO₄.H₂O, 20 mM imidazole pH 8). Protein was eluted in 1 ml fractions in elution buffer (300 mM NaCl, 50 mM NaH₂PO₄.H₂O, 250 mM imidazole pH 8). After elution, samples were routinely analysed by SDS-PAGE in order to determine the efficiency of recovery.

Nickel-agarose purified recombinant protein was further purified by SEC. Samples were first concentrated as described previously. The concentrated protein was applied to a Superdex 200 10/300 gel filtration column (Pharmacia) attached to an AKTA FPLC (GE Healthcare). The column was equilibrated and washed with one column volume of 20 mM Tris, 300 mM NaCl pH 8. The concentrated protein was injected and eluted in one column volume (24 ml) with 20 mM Tris, 300 mM NaCl pH 8. Fractions (24 × 1 ml) were collected for SDS-PAGE analysis and further studies. For molecular mass estimation the column was calibrated against a mixture of thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa) and ovalbumin (45 kDa).

**BS⁵⁰ cross-linking of proteins in solution.** Purified NS1 was cross-linked with BS⁵⁰, a nearest neighbour cross-linker with an 11.4 Å (8 atoms) spacer arm and an amine-reactive NHS-ester at each end. 0, 1,
5, 10 and 20 mM concentrations of cross-linker were added to NS1 in PBS and samples incubated for 2 h at 37 °C. Following incubation, SDS-PAGE loading buffer was added to the samples, boiled for 2 min and then analysed on 4–20 % pre-cast SDS-PAGE gradient gels (Bio-Rad). Separated proteins were transferred to a nitrocellulose membrane, probed with NS1 specific antibodies and visualized by chemiluminescence.

**Competition ELISA.** Competition assays were based on a method described by Lopez et al. (1986) with the following modifications (Lopez et al., 1986). Each competition experiment was performed in quadruplicate and 50 µl was used per well, unless otherwise stated. Immulon 4 microtitre plates (Pacific Diagnostics) were coated with 10 µg Protein A (Pharmacia) in coating buffer overnight at 4 °C. After washing the plates with PBS 0.05 % Tween-20 (PBS.T), 150 µl blocking solution (PBS containing 1 % gelatin) was added to each well and left at room temperature for 1 h. The plates were washed with PBS.T and then 18 µg rabbit anti-mouse IgG (H and L) in PBS.T/0.25 % gelatin was added to bind to the Protein A for a further 1 h at 37 °C. Following four washes with PBS.T, the primary mAb (diluted 1/100 in PBS.T/0.25 % gelatin) was added and incubated at 37 °C for 1 h. Plates were washed as before and immunofluorinity purified 35S-Met/Cys-labelled NS1 (2000 c.p.m. per well) was combined with a 1/10 dilution of the secondary mAb in PBS.T/0.25 % gelatin and immediately added to the plate followed by incubation for a further 1 h at 37 °C. Finally the plate was washed four times with PBS.T prior to the addition of lysis buffer (2 % SDS, 1 % 2-mercaptoethanol in SDS-PAGE sample buffer) to each well. After 10 min incubation at room temperature the lysis buffer was removed individually from each well, added to 1 ml scintillation fluid (Optiphase HiSafe II; Wallac) and counted in a B-scintillation counter (LKB). A set of positive controls were run with each set of experiments and binding was expressed as a percentage of NS1 binding in the absence of competing antibody, i.e.

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\text{Binding} (\%) = 100 \times (\text{average c.p.m in the presence of competing antibody})/(\text{average c.p.m in the absence of competing antibody})
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Binding between 0 and 30 % was defined as strong competition, 31–75 % was defined as partial competition, 76–150 % as no competition and greater than 150 % was defined as indicating enhanced binding.

**Sample preparation and EM.** Baculovirus-derived recombinant sNS1 at a concentration of ~100 µg ml⁻¹ in PBS was applied to glow discharged, carbon-coated copper mesh grids, negatively stained with 1 % uranyl acetate and analysed by transmission EM in a Philips Tecnai 12 TEM at a high tension voltage of 120 kV and magnification of ×68 000. EMs were recorded to Kodak SO-163 film.

**Image processing and 3D reconstruction.** Micrographs recorded by EM were digitized at a pixel size of 6.3 µm (corresponding to 0.93 Å at the specimen level) using a Nikon Super Coolscan with recommended settings (Taype et al., 2005). Micrographs exhibiting astigmatism or drift were excluded prior to semi-automated particle picking using Swamp (Woolford et al., 2007). The initial dataset of approximately 5700 particles was stringently refined, removing particles with poor staining, poor contrast or suffering other obvious defects, ultimately yielding a ‘clean’ dataset of 3523 particles for subsequent image processing.

Symmetry evaluation and the calculation of non-biased, reference-free class averages was performed using a collection of image processing routines within the EMAN and Xmipp software packages (Ludtke et al., 1999; Scheres et al., 2005; Sorzano et al., 2004). The presence of rotational symmetry in raw particle images was detected by clustering particles into a kernel density estimator self-organizing map (KerDenSOM) based on the symmetry profile of rotationally averaged power spectra calculated for each particle image. Initial models were built from maximum-likelihood analysis-derived reference-free class averages using the STARTCSYM program. 3D structure refinement was carried out using the EMAN REFIN program in a stepwise, iterative manner with each round of refinement reducing the sampling angle and each step iterated to pseudoconvergence as determined by Fourier shell correlation (Ludtke et al., 2001). To reduce noise, the final model was band pass filtered with a low pass cut-off of 20 Å and a high pass cut-off of 200 Å.

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