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Review

The flavivirus NS1 protein: Molecular and structural biology, immunology, role in pathogenesis and application as a diagnostic biomarker

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ABSTRACT

The flavivirus nonstructural glycoprotein NS1 is an enigmatic protein whose structure and mechanistic function have remained somewhat elusive ever since it was first reported in 1970 as a viral antigen circulating in the sera of dengue-infected patients. All flavivirus NS1 genes share a high degree of homology, encoding a 352-amino-acid polypeptide that has a molecular weight of 46–55 kDa, depending on its glycosylation status. NS1 exists in multiple oligomeric forms and is found in different cellular locations: a cell membrane-bound form in association with virus-induced intracellular vesicular compartments, on the cell surface and as a soluble secreted hexameric lipoparticle. Intracellular NS1 co-localizes with dsRNA and other components of the viral replication complex and plays an essential cofactor role in replication. Although this makes NS1 an ideal target for inhibitor design, the precise nature of its cofactor function has yet to be elucidated. A plethora of potential interacting partners have been identified, particularly for the secreted form of NS1, with many being implicated in immune evasion strategies. Secreted and cell-surface-associated NS1 are highly immunogenic and both the proteins themselves and the antibodies they elicit have been implicated in the seemingly contradictory roles of protection and pathogenesis in the infected host. Finally, NS1 is also an important biomarker for early diagnosis of disease. In this article, we provide an overview of these somewhat disparate areas of research, drawing together the wealth of data generated over more than 40 years of study of this fascinating protein.

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1. Introduction

Flaviviruses are small, enveloped viruses with a positive–sense RNA genome. The flavivirus genus 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. Disease associated with these viruses varies from asymptomatic infection and self-limiting febrile illness to encephalitis or meningitis or haemorrhage and shock, which can be fatal (Malavige et al., 2004; Chappell et al., 2008; Guzman et al., 2010;

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Ross, 2010; Rossi et al., 2010). The flavivirus genome encodes for 3 structural (C, prM and E) and 7 nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Lindenbach and Rice, 2003).

A soluble complement fixing (SCF) antigen was first reported for dengue virus in 1970 in the serum and brain extracts of infected mice (Brandt et al., 1970a,b). This antigen, later recognized as the nonstructural protein NS1 (Smith and Wright, 1985), was subsequently found in the blood of dengue-infected patients (Young et al., 2000). The SCF antigen was originally referred to as gp48, based on its molecular weight, as determined by SDS–PAGE analysis. It was later renamed NS1 after the sequencing of the YFV genome in 1985 placed the gene encoding this protein as the first of the nonstructural proteins (Rice et al., 1985). All flavivirus NS1 genes share a high degree of homology and are 1056 nucleotides in length, encoding a 352-amino-acid polypeptide (Mackow et al., 1987; Deubel et al., 1988; Mandl et al., 1989; Wright et al., 1989).

NS1 has a molecular weight of 46–55 kDa, depending on its glycosylation status, exists in multiple oligomeric forms and is found at different cellular locations: either cell-membrane-associated (mNS1), in vesicular compartments within the cell or on the cell surface, and as a secreted lipid-rich, extracellular (nonvirion) species (sNS1) (Smith and Wright, 1985; Westaway and Goodman, 1987; Winkler et al., 1988; Mason, 1989; Gutsche et al., 2011). Intracellular NS1 plays an essential cofactor role in virus replication and has been shown to co-localize with dsRNA and other components of replication complexes (Mackenzie et al., 1996; Westaway et al., 1997). However, the precise function of this protein in replication has yet to be elucidated. Secreted and cell-surface-associated NS1 are highly immunogenic, and both the protein and the antibodies it elicits have been implicated in disease pathogenesis (Schlesinger et al., 1987; Henchal et al., 1988; Falgout et al., 1990; Avirutnan et al., 2006; Sun et al., 2007).

This review aims to bring together an extensive and sometimes confusing body of literature on this unusual flavivirus protein. NS1 has been implicated in a multitude of roles, ranging from eliciting a protective immune response in infected hosts to playing a direct role in pathogenesis. Specifically, we will review the current state of knowledge of the structure and trafficking of NS1 within and from the infected cell, its proposed role in viral replication, potential as a vaccine candidate, value in diagnostic applications and its role in pathogenesis *in vivo*, through the interaction of NS1 itself or the antibodies it elicits, with an ever-increasing number of host cell targets (see also Muller and Young, 2011).

2. Expression, post-translational processing and trafficking

Following flavivirus entry and uncoating, the viral genome provides the template for the first round of translation from the single viral RNA open reading frame (Fig. 1). NS1 is translocated into the lumen of the ER via a signal sequence corresponding to the final 24 amino acids of E (Falgout et al., 1989) and is released from E at its amino terminus via cleavage by the ER resident host signal peptidase (Nowak et al., 1989). NS1 is cleaved at its C-terminus from the downstream NS2A by an as yet, unidentified ER resident host cell protease (Falgout and Markoff, 1995). The last 8 amino acids of NS1 have been reported to be necessary for cleavage to take place with evidence of the ER resident protease recognising the octapeptide motif L/M-V-X-S-X-V-X-A at the end of the NS1 protein (Chambers et al., 1990; Hori and Lai, 1990; Pethel et al., 1992; Falgout and Markoff, 1995). In a recombinant vaccinia virus expression system it was found that 70% of NS2A was required to mediate effective cleavage (Hori and Lai, 1990). However, only 26 amino acids at the N-terminus of NS2A were required for NS1/2A cleavage using recombinant baculovirus expression in insect (*Sf*9) cells (Leblois and Young, 1995). Further studies are required to fully explore this apparent anomaly but conformational constraints imposed by variably truncated NS2A may be responsible.

We had earlier thought that an ideal candidate for cleavage activity at the NS1-NS2A junction might be the ER resident glycosyl-phosphatidylinositol (GPI) transamidase, following our finding of a GPI-anchored form of NS1 (Jacobs et al., 2000). However, while virus replication was indeed reduced in mutant cell lines defective for GPI addition, the NS1-NS2A junction was still efficiently cleaved (White and Young, unpublished observations). The protease responsible and the exact role of downstream NS2A sequences in the efficiency of cleavage remain to be determined.

The hydrophilic monomer that is released from the viral polyprotein contains 12 cysteines that form 6 discrete disulfide bonds that are thought to be important for both the structure and function of NS1 (Mason et al., 1987; Leblois and Young, 1993; Blitvich et al., 2001; Wallis et al., 2004). The role of these disulfide bonds in stabilization and correct folding of the monomer is reflected in mutagenesis studies that showed that the last 3 cysteines were essential for NS1 maturation, secretion and the formation of oligomeric species (Pryor and Wright, 1993). Using mass spectrometry, the first 3 and all 6 disulphide bonds were determined for MVE (Blitvich et al., 2001) and DENV-2 (Wallis et al., 2004) NS1 respectively. The disulfide bonds were determined to link in the following arrangements: C1/C2, C3/C4, C5/C6, C7/C12, C8/C10 and C9/C11 (Fig. 3A).

Following cleavage in the ER (Fig. 1, step 1), NS1 is first glycosylated by the addition of high-mannose carbohydrates (Fig. 1, step 2) (Winkler et al., 1988; Pryor and Wright, 1994). This hydrophilic monomer rapidly dimerizes (20–40 min) (Winkler et al., 1988, 1989), acquiring a partially hydrophobic nature, as demonstrated by the separation of dimeric NS1 into both membrane and aqueous phases in Triton X-114 phase-separation experiments (Winkler et al., 1989). This newly acquired hydrophobicity is thought to be the major factor in NS1 becoming associated with the ER membrane (Fig. 1, step 3). However, the nature and location of this hydrophobic component has yet to be fully identified.

Oligomerization of NS1 is a common feature of all flaviviruses, with the stable dimeric form of NS1 first identified in SDS–PAGE analyses of infected mammalian and mosquito cell cultures (Winkler et al., 1988; Chambers et al., 1989; Mason, 1989). This dimer is resistant to treatment with both non-ionic and ionic detergents, however they can be dissociated by heat or acid (pH 2.2–3) treatment (Winkler et al., 1988; Falconar and Young, 1990). Recombinant expression studies have shown that multimeric species spontaneously form in the absence of other viral proteins, indicating that NS1 contains all the information needed to drive oligomerization (Parrish et al., 1991; Pryor and Wright, 1993; Leblois and Young, 1995). Mutations of the octapeptide cleavage motif that leave the NS1/NS2A junction intact do not affect dimer formation (Parrish et al., 1991; Pryor and Wright, 1993) and indeed the unique NS1′ of some encephalitic flaviviruses that carries a carboxy-terminal extension derived via a ribosomal frame-shift is also dimeric (Mason, 1989; Melian et al., 2009). In Kunjin virus and MVE, a single amino acid substitution at residue 250 from proline to leucine has been shown to result in a loss of detectable dimers, suggesting a role for this C-terminal region of the protein in the dimerization process (Fig. 3A). Despite the apparent loss of dimer formation, the resulting monomeric NS1 was still secreted. While these findings suggest that dimerization may not be essential for viral infectivity, this mutation did correlate with retarded virus growth and reduced virulence in mice (Hall et al., 1999; Clark et al., 2007).

However, some caution needs to be taken in drawing conclusions about the oligomeric nature of this mutant form of NS1 from these results. The presence of dimers was determined by either

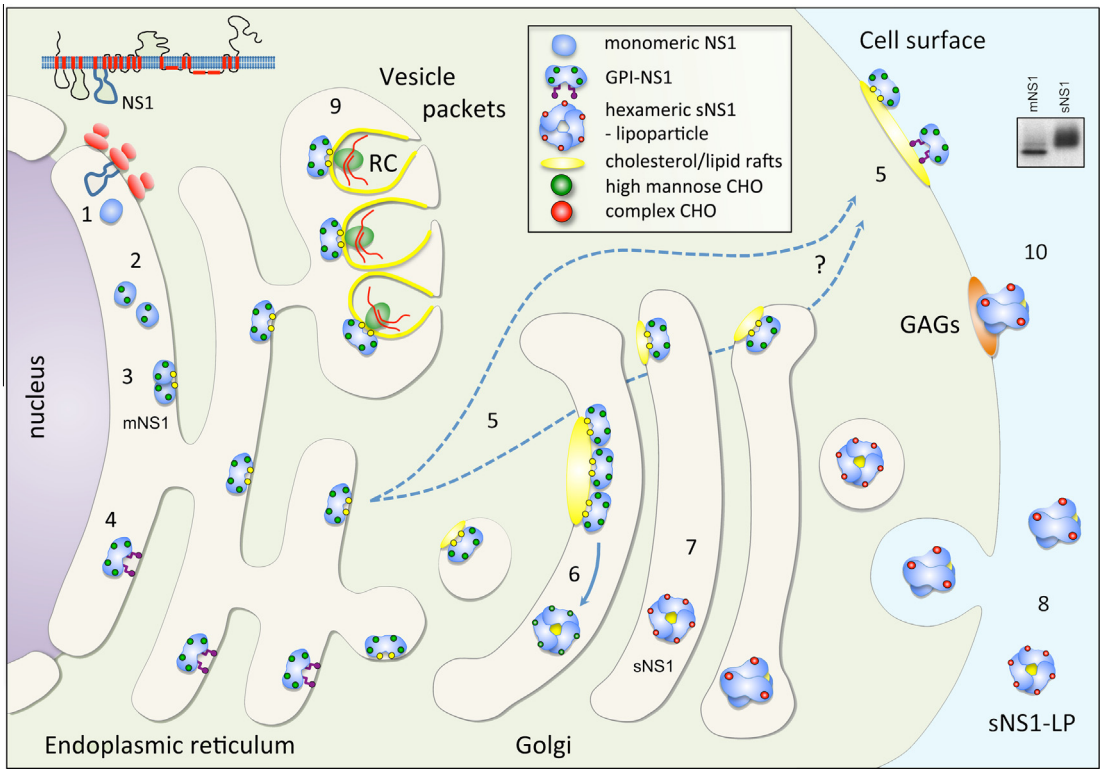

Fig. 1. Schematic summary of NS1 trafficking in mammalian cells. NS1 is initially expressed in association with the endoplasmic reticulum (ER) as part of a long polyprotein that is encoded by a single open reading frame of the viral RNA genome. A signal sequence at the C-terminus of the virion glycoprotein E targets NS1 to the lumen of the ER, and co-translational cleavage within the ER lumen at both its N and C-terminus generates a hydrophilic monomeric subunit (1). This monomer is modified by the addition of high-mannose carbohydrate moieties at multiple sites (2) and rapidly forms a dimeric species, leading to the acquisition of a hydrophobic character (mNS1) and resulting in membrane association (3). A subset of dengue virus NS1 acquires a glycosyl-phosphatidylinositol (GPI) anchor in the ER as a consequence of the recognition of a GPI addition signal at the N-terminus of NS2A (4). Both mNS1 and GPI-anchored NS1 are trafficked to the cell surface via an unknown pathway, where they have been shown to associate with lipid rafts (yellow membrane highlights) (5). A portion of NS1 trafficks from the ER through the Golgi, where dimeric units associate to form soluble hexamers (6), although it is also possible that these hexamers are formed earlier in the ER. The soluble hexamers acquire a lipid cargo which is stored in a central channel of the hexameric assembly (graphically represented by a yellow interior). Passage of this soluble hexameric lipoparticle (sNS1) through the Golgi results in one of the high-mannose carbohydrate moieties on each monomer being trimmed and processed to a complex carbohydrate form (7), which is then secreted from the cell (8). The difference in carbohydrate composition of the mNS1 and sNS1 forms is clearly seen in their differing migration profile on SDS–PAGE analysis (inset). An alternative pathway for mNS1 from the ER sees a subset of the high-mannose form becoming associated with vesicle packets (VP), where it co-localises with other nonstructural viral proteins that comprise the viral replication complex (RC, see Fig. 2) (9). Some of the cell-surface-associated NS1 may be previously secreted NS1 that has bound directly to cell-surface glycosaminoglycans (GAGs) (10). The oligomeric nature of the various cell-bound forms of NS1 remains largely unknown.

SDS–PAGE analysis without sample heating or by reactivity in fixed infected cell monolayers with a MAb previously characterized as binding only to dimeric NS1 on immunoblots. Native NS1 normally retains its dimeric status when separated on SDS–PAGE in the absence of heating, however the possibility that the proline to leucine mutation merely results in lower affinity interactions between individual monomers that are then disrupted by exposure to SDS treatment cannot be excluded. Furthermore, the reactivity of a MAb to a fixed cell substrate may not entirely reflect the native form of the protein. Gel filtration and/or cross-linking studies of untreated secreted NS1 would need to be performed on this mutant to adequately answer the question of whether or not the oligomeric form of NS1 is essential for viral replication.

A role for this region of NS1 in dimerization was supported in a recent study examining viable NS1 insertion sites for immunogenic epitopes (Rumyantsev et al., 2010). A 56-amino-acid insertion of an influenza T cell epitope (M2e) at NS1 residue 236 was tolerated in a live chimeric JEV vaccine candidate (ChimeriVax-JE). However, while NS1 secretion was retained, NS1 no longer formed dimers, highlighting the importance of this region in the dimerization process (Rumyantsev et al., 2010).

Following dimerization, NS1 is trafficked to three separate destinations: sites of viral replication within the cell, the cell surface and secreted into the extracellular space (Fig. 1, steps 9, 5 and 8 respectively). The majority of cell-associated NS1 co-localises with dsRNA and other nonstructural proteins involved in genome replication in structures referred to as vesicle packets (Fig. 1 step 9 and Fig. 2) (Mackenzie et al., 1996; Lindenbach and Rice, 1997, 1999; Khromykh et al., 1999), while a small proportion of cell-associated NS1 is also found at the infected cell surface (Fig. 1, step 5) (Winkler et al., 1989; Schlesinger et al., 1990). In mammalian cells, another component of expressed NS1 is trafficked through the Golgi via the secretory pathway, where exposed carbohydrate moieties are trimmed and processed to more complex sugars and then secreted from the cell as a soluble hexamer (Fig. 1 steps 7 and 8) (Crooks et al., 1990, 1994; Flamand et al., 1999).

Recent reports of higher-order oligomers of molecular weight >675 kDa observed in gel filtration column profiles (Somnuke et al., 2011; Youn et al., 2012) are most likely misfolded and aggregated proteins, or may be NS1 bound to host proteins, as Lin et al. (2012) noted at least 5 different host proteins that bind to NS1 using mass spectrometry analysis. Notably, NS1 is not secreted from infected insect cells (Mason, 1989) despite one report suggesting that it is (Ludert et al., 2008). This latter report is based on NS1 detection in media harvests using a sensitive NS1 capture ELISA assay (Ludert et al., 2008). These experiments were not quantitative and were performed relatively late in infection, suggesting that detection of NS1 in the media was most likely the re-

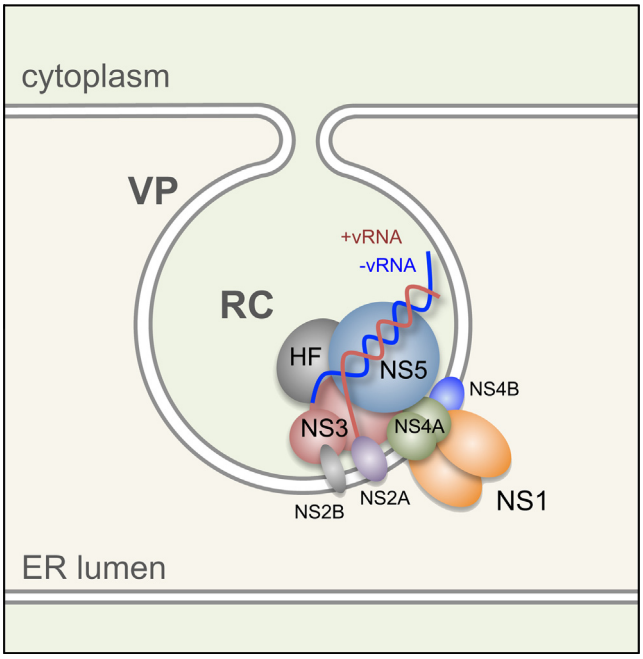

Fig. 2. Schematic of the dengue virus replication complex. This hypothetical model was derived from a consensus view of the available flavivirus replication literature. The replication complex (RC) resides inside membranous vesicle packets (VP) induced within infected cells. The replication complex has been variably shown to comprise all 7 nonstructural proteins, NS1, NS2A, NS2B/NS3, NS4A, NS4B and NS5 along with selected host factors (HF) that are required for efficient replication. NS1 is located within the ER lumen, and its engagement across the VP membrane with the RC is therefore thought to occur via interactions with NS4B and NS4A. Direct evidence of a physical connection has now been reported for NS4B, but not yet for NS4A. Positive-sense vRNA is transcribed via a double-stranded intermediate from a negative-sense template by the polymerase encoded in the C-terminal domain of NS5. The NS3 helicase plays a key role in this process, along with the MTase activity of the N-terminal domain of NS5 that provides a 5′-methylated cap to generate a mature viral genomic RNA. This genomic RNA is then transported out of the VP through a pore that connects the VP with the ER membrane, perhaps with the assistance of NS2A, which has been shown to bind vRNA.

sult of liberation from infected cells undergoing lysis. Extensive pulse-chase experiments early during infection, where minimal cell lysis has occurred has failed to demonstrate any significant secretion from insect cells (Mason, 1989; our own observations). It is also worth noting that, while intracellular, membrane-associated NS1 has been represented in Fig. 1 as a dimeric species, and indeed this is the form often attributed to it in the literature, there is still no direct experimental evidence for this assumption.

The hexameric form of NS1 is a high density lipoprotein that is held together by weak hydrophobic interactions that are readily disrupted by detergent treatment (Flamand et al., 1999; Gutsche et al., 2011) and so the detergent based lysis that is required to solubilize infected and/or transfected cells for analysis would not be compatible with the recovery of these higher oligomer forms. Therefore, while it is clear that NS1 is at least dimeric within the infected cell, the exact higher order oligomeric nature of intra-cellular NS1 awaits experimental confirmation. Despite this reservation, one early study employing cross-linking of intact yellow fever virus infected cells indicated that surface associated NS1 likely exists in a dimeric, and not hexameric form (Schlesinger et al., 1990).

As noted above, membrane association likely follows the acquisition of a hydrophobic character following dimerization early after synthesis (Mason, 1989; Winkler et al., 1989; Noisakran et al., 2008a). Through unknown mechanisms, perhaps mediated by the local concentration of this form of NS1 on cholesterol-rich lipid rafts and/or lipid droplets (Noisakran et al., 2008a), it is possible that a proportion of dimers are able to dissociate from the ER or Golgi membranes and associate instead with two other dimeric units via this same hydrophobic domain, thereby dragging lipid cargo out of the membrane and forming a HDL-like lipoprotein (Gutsche et al., 2011). This association would sequester their inherent hydrophobicity to form the soluble hexameric species (Fig. 1, step 6; Flamand et al., 1999). However, it is also possible that both membrane association and soluble hexamer formation occur immediately following dimerization and acquisition of hydrophobicity, with the fate of individual dimers being solely dependent on NS1 concentration. As the infection progresses, higher local concentrations of NS1 may lead to a greater likelihood of dimeric units partnering with others on the ER membrane. Pulse-chase labelling experiments have shown that the formation and secretion of sNS1 is significantly delayed following the initial synthesis of mNS1, a finding that would support either of these scenarios (Mason, 1989). Further studies are required to clarify the details of this early stage in NS1 maturation.

Glycosylation of a viral nonstructural protein is somewhat unusual, given that this post-translational modification is usually restricted to virion surface proteins. Furthermore, a range of different glycosylation patterns are seen for NS1 that are dependant on the infecting flavivirus, the host cells they infect as well as their different cellular locations. NS1 from all serotypes of DENV, JEV and YFV contain two conserved glycosylation sites, at positions Asn 130 and Asn 207 (Smith and Wright, 1985; Mason et al., 1987; Zhao et al., 1987; Flamand et al., 1992; Pryor and Wright, 1994). With the addition of carbohydrate moieties, monomeric NS1 migrates on SDS–PAGE with molecular weights of between 49–55 kDa, depending on the level of processing and complex sugar addition. In mammalian cells, NS1 exists in two major forms. The membrane-associated form of NS1 (mNS1) migrates with a MW of approximately 49 kDa as a sharp band on SDS–PAGE as it contains only high mannose carbohydrate additions (Post et al., 1991). The second, secreted form of NS1 (sNS1) migrates on SDS–PAGE some 3–6 kDa larger than mNS1 as a smear from 52–55 kDa, due to the additional trimming and processing of the high mannose carbohydrate at Asn 130 with a heterogeneous mix of complex sugars (Fig. 1, inset). For some members of the JEV subgroup, including WNV, SLEV and MVEV, an additional linkage site at Asn 175 is also processed to a complex form (Dalgarno et al., 1986; Trent et al., 1987; Coia et al., 1988; Mandl et al., 1989; Blitvich et al., 1999, 2001).

The addition of complex carbohydrates indicates the passage of secreted NS1 through the Golgi compartment where trimming of high mannose and the addition of more complex sugars occurs (Winkler et al., 1988; Mason, 1989). Insect cells do not possess the required glycosylation machinery to process NS1 to the complex carbohydrate form. In these cells, as noted above, NS1 is not secreted, but instead accumulates, suggesting an association between complex carbohydrate addition and secretion (Mason, 1989; Flamand et al., 1999). This is supported by mutagenesis studies that have found that removal of either or both glycosylation sites in DENV, WNV or YFV results in decreased NS1 secretion, as well as reduced neurovirulence in mice, small-plaque phenotype, decreased virus yields, reduced cytopathology and depressed RNA accumulation (Pryor and Wright, 1994; Muylaert et al., 1996; Crabtree et al., 2005; Tajima et al., 2008; Somnuke et al., 2011). The importance of correct NS1 glycosylation for virus replicative capacity and the potential of this post-translational modification as a target for antiviral drug design was recently demonstrated in a study of the effects of the α-glucosidase inhibitor, Celgosivir (Rathore et al., 2011). Treatment of cells harbouring a dengue virus subgenomic replicon (lacking the structural genes, prM and E) with Celgosivir, was shown to result in mis-folding of NS1 and impair replicative efficiency (Rathore et al., 2011). Somewhat surprisingly,

NS1 devoid of carbohydrate additions appears to be trafficked efficiently to the cell surface (Youn et al., 2010; Somnuke et al., 2011). Taken together, these results indicate that glycosylation is important for NS1 maturation, at least in terms of its secretion, role in viral RNA replication and virulence of disease.

The hexameric nature of the secreted form of NS1 (sNS1) was first identified in the media harvests of TBEV-infected mammalian cells and later confirmed in DENV- and WNV-infected cells (Fig. 1 step 8 (Crooks et al., 1990, 1994; Flamand et al., 1999; Chung et al., 2006a). Secretion kinetics appear to be different for different flaviviruses, with TBEV NS1 secreted within 45 min of expression, whereas NS1 from JEV and YFV has been shown to take up to 2 h before it is detected in media harvests (Lee et al., 1989; Mason, 1989). A short motif at the N-terminus of NS1 (residues 10 and 11) has recently been identified in a comparative study of WNV and DENV that may explain some of this variation in secretion versus cellular retention (Youn et al., 2010). WNV NS1 was shown to accumulate at higher relative levels on the infected cell surface than DENV NS1 and revealed a distinctive reticular staining pattern in immunofluorescence analyses, with DENV NS1 showing a more diffuse surface distribution. In contrast, DENV NS1 was more efficiently secreted into the infected cell media. The authors suggest that this motif may mediate the differential binding of the respective NS1 species to an ER resident host cell protein and so influence the subsequent pathway of maturation to predominantly cell membrane association or secretion (Youn et al., 2010). However no direct evidence for such a host protein interaction was presented in this report. It could equally be argued that differences in specific residues at this location may directly influence the efficiency of hexamer formation, so that increased cell membrane association versus secretion may simply be a loss-of-function mutation, rather than reflecting binding to a host cell membrane protein. Further studies are required to clarify the mechanistic role of this motif in flavivirus replication, but it certainly adds to the growing list of phenotypic differences now identified between the various flavivirus NS1 species.

Gel filtration studies have shown that hexameric NS1 has a molecular weight of 310 kDa and a Stokes radius of 64.4 Å. This form of NS1 is held together by weak hydrophobic interactions and will dissociate in the presence of non-ionic detergents to the more stable dimeric subunits that can only be dissociated to monomers following heat or acid treatment (Crooks et al., 1994; Flamand et al., 1999). Cross-linking experiments, using dimethylsuberimidate (DMS) or BS^3 and SDS–PAGE analysis have shown that hexameric NS1 denatures preferentially to tetramers, dimers and monomers, suggesting that the hexamer is made up of a trimer of dimers (Flamand et al., 1999; Gutsche et al., 2011; Muller et al., 2012c). As noted above, secretion of NS1 has been attributed in part to the differential glycosylation processing that occurs in mammalian cells. After NS1 dimerises and moves through the secretory pathway, the high-mannose carbohydrate at Asn 130 is trimmed and processed to a complex carbohydrate. The second carbohydrate addition site at Asn 207 is sterically protected from processing in the oligomeric form,and so retains its high-mannose carbohydrate moiety (Flamand et al., 1999).

Detailed analysis of the composition of this secreted hexameric form of NS1 recently revealed that it carries a significant lipid cargo (Gutsche et al., 2011). Equimolar amounts of triglycerides along with mono- and diacylglycerol, cholesterol, cholesteryl ester, phosphatidylcholine, phosphatidyl-ethanolamine and sphingomyelin were detected in purified sNS1 preparations. The tight association of these cellular lipids suggest a mechanism for hexamer formation. Once dimeric NS1 becomes membrane-associated, clustering at lipid droplets/lipid rafts/triglycerides causes disruption of the membrane with a group of three dimers pinching off to form the hexamer, dragging a lipid cargo out of the membrane in the process (Gutsche et al., 2011). This model is supported by the reduction in sNS1 secretion from cells treated with lipid-modulating drugs (Gutsche et al., 2011). An analysis of lipid content for recombinant sNS1 expressed and purified from cells grown in serum-free media subsequently revealed a much lower lipid cargo, suggesting that there is flexibility in the amount of lipid sequestered within this lipoparticle form of sNS1(Muller et al., 2012c). Given the extensive membrane reorganisation that occurs during flavivirus infection, it is tempting to speculate that one of the functions of NS1 is to contribute to the modulation of cellular lipid content characteristic of infected cells and perhaps involvement in recruitment of cholesterol/triglycerides to the replication complex.

No high-resolution structural information is currently available for any form of NS1, with ongoing efforts by a number of groups, including ours, to crystallize this species being unsuccessful to date. The recent finding that secreted NS1 contains a variable lipid core may go some way to explaining this lack of success. Recently however, single-particle analysis has been employed to generate low-resolution 3D structures for dengue virus type 1 and 2 sNS1. Both reconstructions revealed a right-handed, barrel-like structure comprising three asymmetrically aligned rods, each presumably a dimeric subunit (Fig. 3). The dengue virus type 1 (native) structure was found to be in an open barrel configuration with D3 symmetry measuring 10 nm by 7.5 nm and with a central cavity approximately 4.5 nm in diameter (Fig. 3C). The dengue virus type 2 NS1 expressed using a recombinant baculovirus system was found to be a longer, more slender closed barrel configuration with C3 symmetry measuring 7 nm by 10 nm, and also with a central cavity of approximately 4.5 nm (Fig. 3D). These structural differences, coupled with the variable lipid content, suggests a degree of flexibility for hexameric sNS1 that may serve to accommodate a variable lipid cargo while still retaining overall oligomeric structural integrity. Both groups put forward speculative dimer and monomer arrangements within the overall structure, but these cannot yet be unequivocally assigned (Gutsche et al., 2011; Muller et al., 2012c).

The overall structural topography of sNS1 can also be inferred from antigenic epitope competition mapping with monoclonal antibodies (Henchal et al., 1987; Hall et al., 1990; Young, 1990; Chung et al., 2006b; Muller et al., 2012c), as well as the localization of their binding site using synthetic linear peptides (Falconar et al., 1994; Huang et al., 1999; Wang et al., 2009; Chen et al., 2010) and recombinantly expressed fragments (Putnak and Schlesinger, 1990; Chung et al., 2006b). The most comprehensive of these to date was an analysis of WNV NS1 using a panel of 22 MAbs that identified what appear to be three separate structural domains (Fig. 3B), a finding that is entirely consistent with the earlier reports (Chung et al., 2006b). Furthermore, antibody competition mapping and binding to recombinantly expressed sub-fragments showed that some epitopes overlap more than one fragment suggesting that domains at either end of the NS1 sequence may be in physical proximity (Chung et al., 2006b; Muller et al., 2012c). A schematic representation of these findings, in combination with other known structural features of the NS1 oligomer, is shown in Fig. 3 (Chung et al., 2006b). The final resolution of the quaternary structure of sNS1 is keenly awaited.

Dengue virus NS1 has been shown to bind to a wide variety of cells via a charge interaction with glycosaminoglycans (GAG), heparin sulphate and chondroitin sulphate E, although the amino acid sequence of flavivirus NS1 does not contain any obvious GAG binding motifs (Fig. 1 step 10 and Fig. 4, step 2) (Avirutnan et al., 2007). NS1 binds strongly to epithelial and fibroblast cells in culture with considerable variability in binding to endothelial cells (human dermal, lung microvascular and aortic endothelial cells) (Avirutnan et al., 2007). Dengue virus sNS1 has also been shown to display a tropism for hepatocytes, both *in vitro* and *in vivo* (Alcon-LePoder et al., 2005). Following internalization by endocytosis, it accumu-

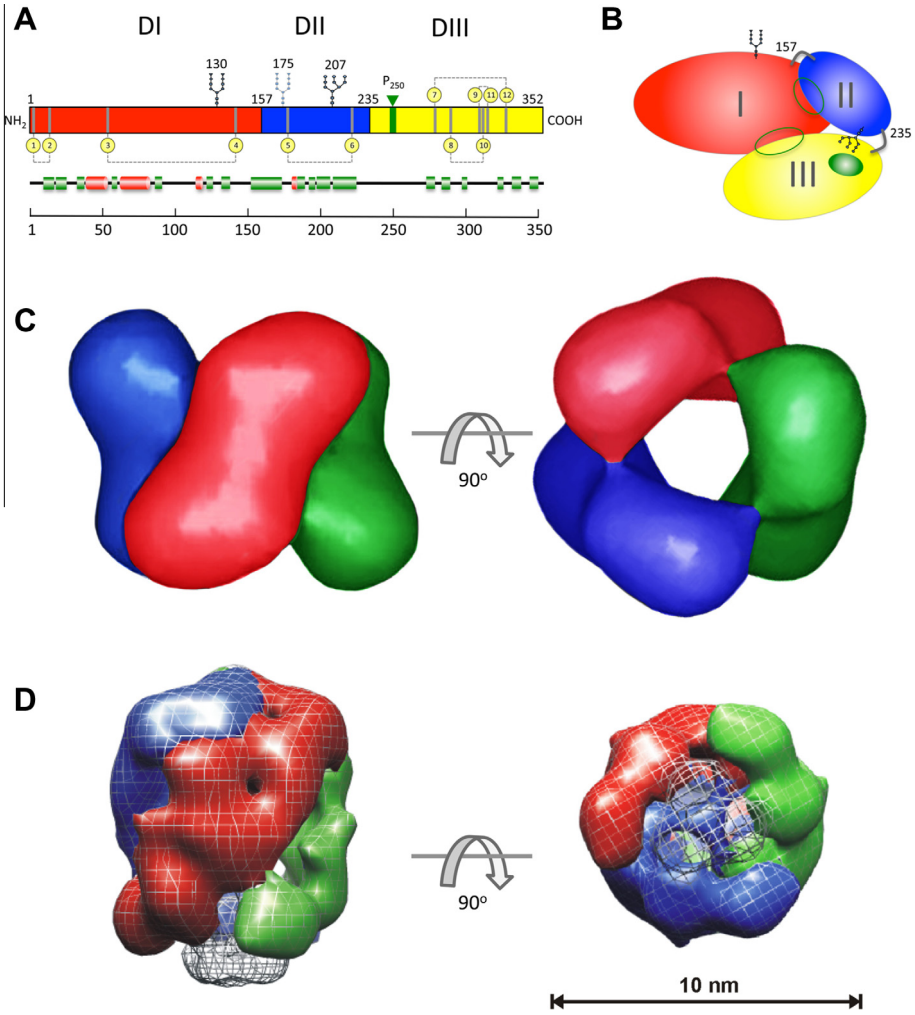

Fig. 3. Antigenic model and 3D reconstructions of sNS1. (A) Linear representation of NS1 highlighting the three structural fragments, Domain I (red), Domain II (blue) and Domain III (yellow) identified for WNV NS1. Six disulfide linkages for all 12 conserved cysteines are shown with yellow spheres, and the two conserved N-linked glycosylation sites are shown in black (complex CHO at Asn130 and high mannose CHO at Asn207). A third carbohydrate addition site at position 175 that is found in the NS1 species of the JE serocomplex of flaviviruses is shown in grey. Destabilization of dimers by a naturally occurring mutation of residue 250 (green arrowhead) in WNV and MVE suggests that the dimerization domain is located in this region. A consensus secondary-structure prediction based on an alignment of more than 40 flavivirus sequences and showing putative β sheets (green cylinders) and α helices (red cylinders) is shown below. (B) Proposed tertiary arrangement of the three structural domains of an NS1 monomer, based on overlapping epitope reactivity by monoclonal antibodies (schematically represented by the green ovals). (C) Cryo-EM 3D reconstruction of native DENV-1 sNS1 in an isosurface representation (Gutsche et al., 2011; reproduced with permission). (D) Negative stain EM 3D reconstruction of recombinant DENV-2 sNS1 in an isosurface representation (Muller et al., 2012; reproduced with permission). The left panel in each shows a side view of the hexameric particle down a twofold axis. The right panels show the particles rotated through 90°, with a view down the central channel. The three dimeric NS1 subunits that comprise the hexamer are colored red, blue and green for clarity. Each dimeric rod-like structure of the DENV-1 reconstruction comprises two lobes, likely representing individual NS1 monomers. Each dimer of the DENV-2 recombinant sNS1 shows additional topographical detail. Both particles were found to harbor a lipid cargo, with a comparison of the structures suggesting that there may be considerable flexibility in the way the dimeric units interact.

lates within late endosomes and can be detected for at least 48 h without degradation (Alcon-LePoder et al., 2005). The pH of late endosomes is reported to be around pH 5.5 and at this pH, NS1 has been shown to be stable in its dimeric form (Winkler et al., 1988; Falconar and Young, 1990; Alcon-LePoder et al., 2005). It was also found that treatment of hepatocytes with NS1 leads to enhanced virus production (Alcon-LePoder et al., 2005). Whether cellular binding and/or endosomal accumulation are responsible were not addressed and hence a mechanism for this enhanced infection remains unclear.

The range of cells identified as substrates for NS1 binding and the number of protein partners characterized as binding different flavivirus NS1 species suggests that NS1 may be an inherently "sticky" protein that forms interactions via non-specific as well as specific charged and hydrophobic interactions (Chua et al., 2005; Chung et al., 2006a; Avirutnan et al., 2007; Kurosu et al., 2007; Noisakran et al., 2008b; Wilson et al., 2008). The acquisition of hydrophobicity by the dimeric form, its lipid content and the relative fragility of hexameric NS1 suggests that interpretation of some of this binding data needs to be treated with some caution with many of the interactions identified requiring further confirmation. There is also growing evidence that many of the host-cell-derived partners identified for NS1 may be specific to individual flaviviruses, suggesting a greater diversity in how different flaviviruses utilize their respective NS1 species than previously thought (Chung et al., 2006a; Krishna et al., 2009).

NS1 encoded by DENV-1 to 4 and JEV have recently been shown to associate with detergent resistant lipid rafts in infected cells (Lee et al., 2008; Noisakran et al., 2008a). Similar observations were made for NS1 expressed in cells transfected with recombinant NS1 constructs (Lee et al., 2008; Noisakran et al., 2008a). Mammalian cell membranes comprise a lipid bilayer made up

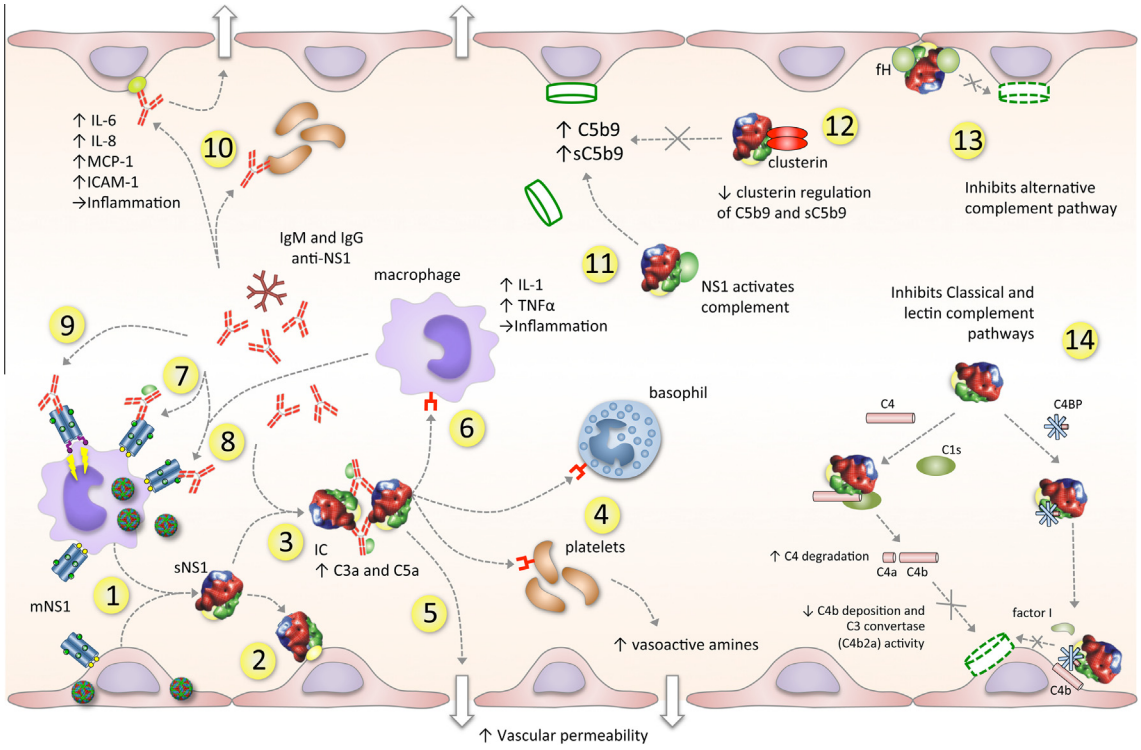

Fig. 4. Schematic of flavivirus NS1 engagement with host-cell components. The production of sNS1 and mNS1 from flavivirus infected cells and their interaction with selected host cell components is depicted. NS1 is expressed on the surface of, and secreted from, flavivirus-infected cells (1). Circulating sNS1 can subsequently bind to the surface of both infected and uninfected cells via charged interactions with GAGs, heparin sulphate and chondroitin sulphate E (2). The consequences of that binding are yet to be fully determined. In dengue virus secondary infections, an anamnestic IgM and IgG antibody response to NS1 during the acute phase of disease can lead to the formation of immune complexes (3) that are capable of triggering a range of inflammatory processes, including the activation of complement (green) to generate the anaphylatoxins C3a and C5a. ICs also act on basophils and platelets via Fc receptor engagement to release vasoactive amines (4) that can cause endothelial cell retraction and increase vascular permeability. This in turn may lead to IC deposition (5), inducing platelet aggregation and further complement activation. Binding of ICs to macrophages leads to their activation and the release of cytokines, further increasing the inflammatory response (6). NS1-specific antibody binding to cell surface-exposed NS1 targets infected cells for complement-mediated lysis (7) and/or complement-independent phagocytosis (8). NS1-specific antibody binding of cell surface-exposed GPI-anchored NS1 may also mediate activation of infected cells (9). A role for auto-immune, cross-reactive anti-NS1 antibodies in pathogenesis has also been proposed. These auto-antibodies have been shown to bind to host determinants on the surface of both platelets and endothelial cells, resulting in the release of inflammatory cytokines and nitric oxide, leading to inflammation and/or apoptosis. In the absence of antibody, circulating sNS1 has been shown to modulate/inhibit complement pathways through its interaction with various complement components (11–14). sNS1 has been shown to activate complement directly resulting in increased formation of the membrane attack complex C5b9 and sC5b9 (11) while its interaction with the complement inhibitory factor, clusterin is thought to result in the increased formation of sC5b9 in the serum of infected patients (12). sNS1 has also been shown to display an immune evasion function through its binding to the alternative complement pathway regulatory protein, fH (13). Its interaction with the classical complement pathway involves at least two separate mechanisms (14). Interaction with C1s promotes cleavage of C4 to C4b, promoting its degradation, with the resulting decrease in the deposition of C4b and C3 convertase, leading in turn to a decrease in the terminal membrane attack complex. NS1 has also been found to interact with C4BP, recruiting its cofactor activity for factor I-mediated cleavage and inactivation of any C4b that does make its way to the infected cell surface. The diverse array of ways in which NS1 and NS1-specific antibodies engage with the host as depicted in this schematic, is by no means exhaustive.

mainly of three different types of lipids; phosphoglycerides, sphingolipids, and sterols (Simons and Ikonen, 1997, 2000; Ikonen, 2001). Lipid rafts are detergent-resistant microdomains (DRM) that are enriched in cholesterol and sphingolipids, accumulating in a liquid-ordered arrangement (Simons and Ikonen, 1997, 2000; Ikonen, 2001). DRMs have the ability to both include as well as exclude proteins and are known to cluster macromolecules involved in signal transduction, cholesterol homeostasis, lipid sorting and protein trafficking (Simons and Ikonen, 1997, 2000; Ikonen, 2001). Recently, cholesterol has been implicated in flavivirus entry, RNA uncoating and replication, with NS1, NS3 and NS5 all being found to be associated with lipid rafts (Lee et al., 2008; Heaton et al., 2010).

Sequence analysis clearly reveals that NS1 is essentially a hydrophilic protein that lacks a traditional membrane spanning anchor domain. So the molecular basis of NS1 membrane-association, lipid raft associated or otherwise, has remained an intriguing and unanswered question since this association was first identified two decades ago (Winkler et al., 1989). The first report of a possible mechanism for membrane-association came with the identification of a glycosyl-phosphatidylinositol (GPI)-linked form of dengue NS1 (Jacobs et al., 2000) that was subsequently confirmed by others (Jacobs et al., 2000; Noisakran et al., 2007, 2008a). This was the first viral encoded protein identified to be expressed with a GPI-anchor, but has so far only been identified for DENV (Fig. 1, step 4) (Jacobs et al., 2000). In this study, the amino terminus of NS2A was shown to contain a hydrophobic region that could act as a signal sequence for GPI anchor addition (Jacobs et al., 2000). This post-translational modification takes place in the ER following cleavage of a carboxy-terminal hydrophobic signal sequence and the covalent addition of a preformed GPI precursor. GPI-anchored NS1 is then targeted to the cell surface, where it is lipid raft associated (Jacobs et al., 2000; Noisakran et al., 2007, 2008a). Intriguingly, the addition of anti-NS1 antibodies that bind to GPI anchored NS1 at the cell surface results in signal transduction, as evidenced by tyrosine phosphorylation of cellular proteins (Jacobs et al., 2000).

While the consequences of the induction of this particular signal transduction pathway on virus replication remain unknown, the interaction of a cell-surface-bound, GPI-anchored viral antigen with specific antibodies that are anamnestically elicited *in vivo* during secondary infection suggests a novel mechanism of cellular activation that may contribute to the pathogenesis of human den-

gue disease (Fig. 4, step 9) (Jacobs et al., 2000). However, this and other reports showing that NS1 expression on its own, in the absence of downstream sequences, retains its hydrophobic properties and is still found in association with the cell surface (Fig. 4, step 1) (Fan and Mason, 1990; Jacobs et al., 2000; Youn et al., 2010) indicates that GPI-anchoring is not the only mechanism of membrane-association. Furthermore, the observation that phospholipase C digestion of intact dengue virus infected cells removes only a small component of NS1 displayed at the cell surface suggests that GPI-anchoring most likely contributes only a small fraction of cell surface associated NS1 (Jacobs et al., 2000). The region of NS1 that acquires a hydrophobic character after dimerization and that is most likely responsible for the majority of membrane association remains unknown. As noted above, sequence variation in a short motif (specifically at residues 10 and 11) in the N-terminal region of WNV and DENV NS1 has recently been identified as influencing the differential targeting of NS1 to either the cell surface or for secretion (Youn et al., 2010). While the contribution to membrane association of this motif has yet to be fully explored, the authors clearly demonstrate that variation within this motif strongly influences the level of cell surface expression.

Flaviviruses in the JE serocomplex express an additional form of NS1 with a carboxy-terminal extension, designated NS1′ (Mason, 1989; Blitvich et al., 1995, 1999; Chen et al., 1996; Melian et al., 2009). NS1′ is a 52–53 kDa species that is expressed in both mosquito and mammalian cells as a cell associated oligomer. As with their NS1 counter-parts, NS1′ from mosquito cells is retained in the cell while NS1′ from infected mammalian cells is found in both cell-associated and secreted forms (Mason, 1989; Blitvich et al., 1999; Firth and Atkins, 2009). NS1′ has the same glycosylation pattern as NS1, with NS1′ retained in mammalian cells comprising high mannose carbohydrate additions, while secreted NS1′ contains additional complex carbohydrate additions (Mason, 1989).

For many years, NS1′ was thought to be the product of alternative cleavage of the viral polyprotein, consisting of full-length NS1 fused to the N-terminus of NS2A. Recently however, bioinformatics analysis suggested that expression of NS1′ may actually be the result of the presence of a conserved pseudoknot in the 5' end of the NS2A nucleotide sequence that is preceeded by a conserved slippery heptanucleotide motif (Firth and Atkins, 2009; Melian et al., 2009). This possibility was quickly confirmed experimentally (Firth and Atkins, 2009; Melian et al., 2009). This slippery heptanucleotide sequence and pseudoknot is conserved in the JE serocomplex flaviviruses and is a classical -1 ribosomal frame-shift motif. The ribosome frame shift occurs between codons 8 and 9 of NS2A and results in the addition of 52 extra amino acids. While the function of this novel NS1 species remains unknown, there is experimental evidence suggesting involvement in neurovirulence, with ablation of NS1′ resulting in partial attenuation of viral neuroinvasiveness (Melian et al., 2009). The frame shift leading to the alternative NS1′ is terminated by a stop codon, and therefore no further expression of downstream genes occurs. Whether the observed association with neuroinvasiveness is due to NS1′ itself or a change in the ratio of structural to nonstructural proteins remains to be elucidated (Melian et al., 2009).

3. NS1 as a cofactor in virus replication

While the exact functional involvement of NS1 in the viral replication cycle remains elusive, many studies have identified that NS1 plays an essential cofactor role in viral RNA replication (Mackenzie et al., 1996; Lindenbach and Rice, 1997, 1999; Westaway et al., 1997; Khromykh et al., 2000). NS1 was initially thought to be involved in virus assembly and maturation, as its secretion profile largely mirrored that of E and prM (Rice et al., 1986; Lee et al., 1989; Mason, 1989). However, it was also noted that in pulse-chase experiments a substantial component of expressed NS1 after extended chase periods remained cell-associated. Since these initial findings, this cell-associated NS1 was shown to localise to sites of viral RNA replication, in close association with vesicle packets and cytoplasmic vacuoles in Vero and C6/36 cells respectively (Mackenzie et al., 1996).

This co-localization with dsRNA, and not to sites of virus assembly as initially suspected, suggested a role in RNA replication as a component of the viral replication complex (Mackenzie et al., 1996; Westaway et al., 1997). However, given the location of NS1 on the lumenal side of the ER derived vesicular membrane it is physically separated from the viral replication machinery and so is unlikely to contribute to RNA replication directly (see Fig. 2). Rather, it has been suggested that along with transmembrane replicase components it may fulfil a structural role, helping to anchor the replication complex to the membrane. *Trans*-complementation and mutagenesis experiments have further shown that whatever role NS1 does play it does so early in RNA replication (Muylaert et al., 1996, 1997; Lindenbach and Rice, 1997; Butrapet et al., 2000; Liu et al., 2006; Suzuki et al., 2007). The complementation studies found that homologous NS1 supplied in *trans* could complement a defective YFV or WNV (Kunjun) genome, resulting in recovered viral RNA synthesis and replication (Lindenbach and Rice, 1997; Khromykh et al., 1999). This *trans*-complementation of NS1 was found to be species-specific, as DENV-2 NS1 was not able to complement a defective YFV genome (Lindenbach and Rice, 1999). However, a genetic screen for suppressor mutants that were able to overcome this species specific interaction identified a single point mutation, Asn-42-Tyr in the NS4A gene that then enabled rescue of the defective YFV genome (Lindenbach and Rice, 1999).

This rescue mutation was the first evidence of a genetic interaction between NS1 and NS4A. The mutation in NS4A is located on the cytoplasmic side of induced viral membranes while NS1 is found on the lumenal side. Therefore it has been proposed that either this mutation in NS4A has an effect on the conformation of the lumenally displayed regions of NS4A, or that NS1 and the lumenal peptide of NS4A may induce a conformational change in the region around amino acid 42 of NS4A resulting in recovery of RNA replication (Lindenbach and Rice, 1999). Further supportive biochemical evidence was identified using recombinant NS4A fused to glutathione-S-transferase. Column bound NS4A was found to interact with all the proteins that are proposed to make up the flavivirus replication complex including NS1 (Mackenzie et al., 1998; Welsch et al., 2009).

Genetic and biochemical evidence that NS4B also interacts with NS1 has recently been reported (Youn et al., 2012). Substitution of DENV sequences into WNV NS1 at the same site previously identified as playing a role in cell membrane association (RQ10NK) was shown to enhance NS1 secretion and the generation of a small-plaque phenotype and reduced viral replication. Within two passages, variable plaque phenotypes emerged. with sequencing revealing the emergence of compensatory mutations NK10YK and NK10KK. In the revertant containing the YK mutation, a rescue mutation was also found in NS4B (F86C), providing evidence of a genetic interaction between NS1 and NS4B. Subsequent co-immunoprecipitation and mass spectrometry analyses identified a physical interaction between NS1 and NS4B (Youn et al., 2012). NS4B contains three transmembrane domains with the N-terminal portion of the protein residing in the luminal side of the ER (Miller et al., 2006). A reasonable hypothesis is that NS1 fulfills its membrane stabilizing, structural role for the replication complex via a physical interaction with regions of NS4A and/or NS4B displayed within the lumen of the ER (Fig. 2). Identification of the protein domains involved in these interactions would provide promising targets for antiviral drug design.

4. NS1 engagement with host innate and adaptive immunity

Flavivirus infection of both animal models and humans results in the circulation of sNS1 in sera during the acute phase of the disease (Young et al., 2000; Alcon et al., 2002; Macdonald et al., 2005; Chung and Diamond, 2008). Indeed, during dengue virus infection, sNS1 may accumulate to very high levels with up to 50 μg/ml being detected in some patient sera (Young et al., 2000; Libraty et al., 2002; Alcon-LePoder et al., 2006). The *in vivo* function of this secreted viral protein has been the subject of many investigations, with emerging evidence that NS1 engages with the host in a multitude of different ways. These include the paradoxical ability to elicit both a protective (Schlesinger et al., 1985, 1987; Schlesinger et al., 1986) and a potentially pathogenic immune response (Henchal et al., 1987; Falconar, 1997; Chang et al., 2002) as well as contributing directly to the disease process through its interaction with different cell types (Falconar, 1997; Chang et al., 2002; Alcon-LePoder et al., 2005; Avirutnan et al., 2006, 2007) or via binding to a range of specific host proteins (Chung et al., 2006a; Kanlaya et al., 2010).

Circulating NS1 has also been identified as an important diagnostic marker of infection (Young et al., 2000; Alcon et al., 2002; Libraty et al., 2002). Collectively, these studies have revealed that NS1 displays a remarkable diversity of engagement with various components of the innate and adaptive arms of the host immune response. It is also becoming clear that many of these interactions may be specific to individual flaviviruses, suggesting that they may each have evolved separate strategies to utilize this secreted and cell surface bound protein. A schematic summary of some of these host interactions is presented in Fig. 4.

4.1. NS1 as a vaccine immunogen

NS1 is a major viral immunogen, which is not a surprising observation for a protein that circulates in relatively high concentrations in the sera of individuals during the acute phase of infection. In primary dengue infection, relatively low anti-NS1 IgM and IgG responses are elicited from 2 and 9 days respectively, post onset of symptoms (Huang et al., 1999; Shu et al., 2003, 2004) while in secondary dengue infection, an anamnestic antibody response results in a rapid rise in anti-NS1 antibodies early during the acute phase of disease (Falkler et al., 1973; Kuno et al., 1990; Churdboonchart et al., 1991). As NS1 is not a component of the virion, these are not neutralizing antibodies. However in the mid 1980s, using some of the first flavivirus specific monoclonal antibodies (MAbs) to be generated, the somewhat surprising discovery was made that selected MAbs against this nonstructural protein were able to afford solid protection to mice against a lethal viral challenge in passive protection studies (Schlesinger et al., 1985; Gould et al., 1986). A direct correlation was noted between those MAbs that fixed complement and those that afforded protection, suggesting that the likely mechanism of protection was via complement-mediated lysis of infected cells following antibody recognition of cell-surface-bound NS1 (Schlesinger et al., 1985) (Fig. 4, step 7).

Since these initial experiments with YFV, passive administration of NS1-specific MAbs against a range of flaviviruses has also been shown to provide varying levels of protection against homologous virus challenge (Schlesinger et al., 1985, 1986; Henchal et al., 1988). Although there is a wealth of evidence confirming a role for complement mediated lysis of infected cells (Schlesinger et al., 1985, 1987; Schlesinger et al., 1986; Falgout et al., 1990; Krishna et al., 2009), protection does not always correlate with the ability of a MAb to fix complement, indicating that other mechanisms are also involved (Henchal et al., 1988; Young, 1990; Schlesinger et al., 1993; Jacobs et al., 1994; Chung et al., 2006b, 2007; Diamond et al., 2008). Recent studies using complement and specific Fc-γ receptor knock-out mice along with WNV specific anti-NS1 MAbs of varying isotype, have shown that protection in mice can be mediated by phagocytosis and clearance of infected cells through Fc-γ receptor I and/or IV recognition of cell-surface NS1-bound antibodies of the IgG2a subclass (Chung et al., 2006b, 2007; Diamond et al., 2008) (Fig. 4, step 8). These studies provide an explanation for the earlier observation of complement-independent protection afforded mice challenged with YFV, specifically by IgG2a isotype anti-NS1 MAbs (Schlesinger et al., 1993) along with a more complete understanding of the role of anti-NS1 antibodies in providing protection against flaviviruses in general.

One of the major concerns for any vaccine strategy against the dengue viruses is the potential priming of antibody-dependent enhancement (ADE), an important and accepted risk factor for the development of the more severe disease outcomes of dengue infection, dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). The potential benefit to vaccine design of a protective immunogen that circumvents the induction of virion-reactive antibodies, and therefore avoids the potential risks of ADE, was recognized early (Gibson et al., 1988). Consequently, there was an immediate explosion in reports describing various immunization strategies, using NS1 either alone or in combination with other viral proteins, each of which provided evidence of either partial or complete protection of mice or monkeys from lethal virus challenge (Schlesinger et al., 1985, 1987; Schlesinger et al., 1986; Cane and Gould, 1988; Despres et al., 1991; Falconar and Young, 1991; Lieberman et al., 2007; Lin et al., 2008b).

Although overtaken to some degree by live attenuated chimeric virus vaccine candidates, there is renewed interest in NS1 as a component of second-generation subunit vaccines for dengue as well as other flaviviruses (Krishna et al., 2009; Miller, 2010). Many strategies have been employed for the delivery of NS1 as a vaccine candidate, including immunization with recombinant or native protein subunit (Schlesinger et al., 1985, 1987; Gould et al., 1986; Gibson et al., 1988; Calvert et al., 2006), live recombinant vaccinia virus (Falgout et al., 1990; Hall et al., 1996), defective recombinant adenovirus (Jacobs et al., 1992, 1994; Timofeev et al., 1998), recombinant baculovirus derived (Zhang et al., 1988; Qu et al., 1993; Eckels et al., 1994), naked DNA (Lin et al., 1998; Wu et al., 2003; Timofeev et al., 2004; Costa et al., 2007) and peptide-(Volpina et al., 2005) based approaches In addition to complement-dependent and independent antibody-mediated protection, NS1 has also been identified as a target of cell-mediated immunity, indicating that both arms of the immune response are likely to play a role (Murali-Krishna et al., 1995; Green and Rothman, 2006; Gao et al., 2008).

Progress towards the development of NS1 as a viable vaccine candidate will not be without significant hurdles. One of the major stumbling blocks will be the fact that, at least for dengue NS1, in addition to eliciting protective antibody responses as outlined above, it also appears to display epitopes that elicit auto-antibodies (discussed below) that cross-react with platelets (Fig. 4, step 4) and components of the extracellular matrix (Falconar, 1997; Chang et al., 2002) as well as bind to and induce damage to endothelial cells (Fig. 4, steps 4 and 10 (Lin et al., 2002). Indeed, in mouse model studies, some passively administered MAbs have been shown to act either alone or synergistically to enhance disease (Falconar, 1997, 2008; Henchal et al., 1988). Whether these limitations apply to all of the flaviviruses remains to be determined, although preliminary evidence suggests that they may be flavivirus-specific (Krishna et al., 2009). Nevertheless, any vaccine based on NS1 will probably require a delivery and formulation strategy that ensures the induction of antibodies of the correct subclass (Chung et al., 2007; Diamond et al., 2009) as well as a degree of epitope specific-

ity that avoids the induction of auto-antibodies (Cheng et al., 2009).

4.2. NS1, complement and host-cell protein-binding partners

It has been 40 years since a series of landmark papers first described the dengue virus soluble complement fixing (SCF) antigen in infected mice and cell culture (Brandt et al., 1970a, 1970b; McCloud et al., 1970; Russell et al., 1970; Smith et al., 1970). This SCF antigen was quickly identified as a secreted nonstructural viral protein with a molecular weight of 39–43 kD (Cardiff et al., 1971), later to be confirmed as sNS1. It was immediately seen as a potential player in the pathogenesis of severe disease, given the reported association between high levels of complement consumption and dengue shock syndrome (DSS) (Russell et al., 1969). These studies led to an expansion of interest in the role of complement pathway engagement by dengue viruses in infected patients and in particular, the role of immune complexes in potentiating the severity of disease (Russell et al., 1970; Bokisch et al., 1973a; Sobel et al., 1975; Theofilopoulos et al., 1976; Ruangjirachuporn et al., 1979). The measurement of a wide range of complement factors in comparative studies of both dengue haemorrhagic fever and dengue fever patients highlighted the involvement of augmented complement activation in the pathophysiology of disease, with decreased levels of C3 found in patients with more severe disease (Bokisch et al., 1973b).

Despite the reported observations of a clear and direct correlation between disease severity, complement consumption and a rise in complement split-products C3a and C5a, with known effects on vascular integrity, much of the ongoing research effort from the mid-1980s was re-focussed on the role of activated lymphocytes and the overproduction of vasoactive cytokines (Kurane et al., 1994). As a consequence, the underlying *in vivo* mechanism of complement activation remained a matter of conjecture. However, the recent development of NS1 capture assays (Young et al., 2000; Alcon et al., 2002) and the discovery of remarkably high levels of circulating NS1 in patient sera that correlate with disease severity (Libraty et al., 2002; Avirutnan et al., 2006) has brought us full circle to the soluble complement-fixing antigen identified in 1970. Although other viral proteins may contribute, secreted NS1 is likely to be the major viral antigen responsible for immune complex formation and an important trigger of complement activation (Avirutnan et al., 2006; Nascimento et al., 2009) (Fig. 4, step 3 and 5).

In addition to contributing significantly to immune complex formation, NS1 has now been shown to bind a number of different complement pathway components as well as other host cell regulatory proteins. These include the complement regulation protein factor H (fH), complement inhibitory factor clusterin, complement proteins C4 and proC1s/C1s, hnRNP C1/C2, STAT3β, thrombin/prothrombin and has been shown to trigger the generation of C5b-9 and SC5b-9 complexes (Chua et al., 2005; Avirutnan et al., 2006, 2010; Chung et al., 2006a; Schlesinger, 2006; Kurosu et al., 2007; Noisakran et al., 2008b; Wilson et al., 2008; Krishna et al., 2009; Baronti et al., 2010; Lin et al., 2012). It has also been suggested that the WNV NS1 is able to antagonize TLR3 signalling (Wilson et al., 2008), although this finding has recently been questioned in a study that failed to confirm inhibition of TLR3 signalling by NS1 from three different flaviviruses: DENV-2, YFV and WNV (Baronti et al., 2010).

In dengue infection, sNS1 has been shown to activate complement directly (Fig. 4, step 11), while binding of NS1-specific antibodies to mNS1 on infected cell surfaces directs complement attack (Avirutnan et al., 2006). The consequent generation of membrane attack complexes (C5b-9) can trigger cellular activation and the production of inflammatory cytokines, and along with soluble membrane attack complexes (SC5b-9) (Fig. 4, step 11) is likely to contribute to the pathogenesis of severe dengue (Avirutnan et al., 2006). Indeed SC5b-9 levels in patient sera were found to follow similar kinetics to those of sNS1, and like NS1 correlated with disease severity (Avirutnan et al., 2006). Further evidence for NS1 involvement in the generation of soluble membrane attack complexes was suggested when NS1 was found to bind directly to the complement inhibitory factor clusterin, which inhibits the formation of the membrane attack complex (Kurosu et al., 2007). It was proposed that NS1 binding results in a reduction in circulating free clusterin and hence to an increase in SC5b-9 formation in the serum of infected patients Fig. 4 step 12) (Kurosu et al., 2007). However, in the case of dengue, this hypothesis needs to be considered in the context of a secondary infection, in which peak NS1 is only observed early during the acute phase of disease, with free NS1 rapidly disappearing from the circulation as a result of the anamnestic rise in cross-reactive anti-NS1antibodies. As a consequence, high levels of free NS1 are not co-incident with the onset of severe disease (Libraty et al., 2002). At a time of rising anti-NS1 antibody levels and the presence of NS1 in immune complexes (IC), IC deposition and complement activation are more likely to contribute to severe disease outcome, at least for dengue, than is free NS1 binding to complement regulating proteins.

It has recently been shown that NS1 may also display an immune-evasion function through inhibition of the classical/lectin complement pathways (Avirutnan et al., 2010). In co-immunoprecipitation studies, sNS1 was found to co-precipitate with the complement proteins C4 and C4b (Avirutnan et al., 2010). The authors found that sNS1 binds to proC1s/C1s and C4 which results in increased cleavage of C4 to C4a and C4b. They hypothesize that this limits the amount of C4 available, thus protecting virus from neutralisation (Fig. 4, step 14) (Avirutnan et al., 2010). Secreted NS1 from DENV, WNV and YFV have also recently been shown to bind to the complement regulatory protein C4 binding protein (C4BP) presenting additional mechanisms for inhibiting the classical and lectin complement activation pathways. NS1 binding of C4BP could lead to C4BP recruiting factor I to mediate cleavage of C4b in solution. Additionally, deposition of the sNS1–C4BP complex onto the cell surface could lead to inactivation of cell surface bound C4b thereby protecting infected cells from complement mediated lysis (Avirutnan et al., 2011) (Fig. 4, step 14).

Another proposed mechanism of virus-mediated protection against complement-directed killing of infected cells was suggested by the observation that WNV NS1 binds to factor H (fH), a circulating regulator of the alternative complement pathway (Chung et al., 2006a). Binding of circulating fH by sNS1 may lead to accelerated breakdown of C3bBb convertase with consequent reduced C3b deposition and a resulting reduction in the formation of the terminal C5b-9 membrane attack complex (Fig. 4 step 13) (Chung et al., 2006b; Schlesinger, 2006). However, it should be noted that this is not a generic property of flavivirus NS1 species as it has also been reported that JEV NS1 does not bind to fH (Krishna et al., 2009). While the antagonism of complement pathways by NS1 appears to be a common strategy employed by flaviviruses, the underlying differences in specific interacting host partners suggests that its contributing role in the pathogenicity of infection is likely to vary between different flaviviruses.

4.3. NS1 induction of autoantibodies and a potential role in pathogenesis

Despite the fact that most antibodies directed against NS1 have been found to provide some level of passive protection to mice from a lethal flavivirus challenge (Schlesinger, 1985; Schlesinger et al., 1986; Henchal et al., 1988), a small number have been shown to increase morbidity (Falconar, 1997, 2008; Henchal et al., 1988). Since these early observations, anti-NS1 antibodies have been

shown to cross-react with a wide range of host cell components including extracellular matrix, blood clotting and integrin/adhesion proteins, platelets as well as to ATP synthase β chain, protein disulfide isomerise (PDI), vimentin and heat shock protein on endothelial cells (Falconar, 1997, 2007; Lin et al., 2001, 2002, 2003, 2006; Sun et al., 2007; Cheng et al., 2009; Immenschuh et al., 2013). The induction of auto-antibodies relatively early in acute secondary dengue infections as part of the anamnestic antibody response that can bind platelets and uninfected endothelial cells has suggested a possible role for these antibodies in the endothelial cell dysfunction that underlies thaemorrhage and vascular leak in DHF/DSS patients (Sun et al., 2007).

Falconar (1997) was the first to show that anti-NS1 antibodies raised in mice were able to bind to common epitopes on human blood clotting, integrin/adhesion proteins as well as directly to human endothelial cells (Falconar, 1997). This binding to endothelial cells was subsequently found to induce apoptosis in a caspase-dependant manner, with the up-regulation of p53 and Bax inducing nitric oxide, leading to cell death (Lin et al., 2002). It has also been demonstrated that binding to endothelial cells by cross-reactive NS1 MAbs can lead to protein tyrosine phosphorylation and activation of NF-κB, resulting in an inflammatory response producing IL-6, IL-8, MCP-1 and increased expression of ICAM-1, followed by increased adhesion of PBMCs to endothelial cells (Lin et al., 2005, 2006). Lin et al. (2003) have also shown that dengue patient antibodies react with endothelial cells, and that there is an increase in anti-endothelial cell activity in patients suffering from acute DHF/DSS, when compared to patients with acute dengue fever (Lin et al., 2003).

In murine models, both passive administration of anti-NS1 antibodies and active immunization with DENV NS1 were shown to damage liver endothelial cells, resulting in increased serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (Lin et al., 2008a). Antibodies elicited by active immunization with DENV NS1 were found by histology to bind to liver vessel endothelium and passive administration of anti-NS1 antibodies was shown to lead to endothelial cell damage and monocyte infiltration (Lin et al., 2008a). This immune-mediated liver injury in mice provides supporting evidence that anti-NS1 antibody responses may also play a role in the liver damage characteristically seen in human dengue virus disease. Using a proteomic approach, a sequence motif located between amino acid residues 311–330 of NS1 has been identified that is shared with a number of host components including the ATP synthase β chain, PDI, vimentin and heat shock protein 60 (Cheng et al., 2009). Furthermore, sera from patients with DHF were shown to bind to these proteins (Cheng et al., 2009).

In a mouse model developed by (Wu-Hsieh et al., 2009), dengue virus infection of endothelial cells could be detected by 12 h after intra-dermal inoculation, and by 24 h post-infection, infiltration by TNF-α secreting macrophages had occurred (Fig. 4, step 6). Increased levels of circulating endothelial cells were also found along with the expression of inducible nitric oxide synthase (iNOS) and peroxinitrate, leading to apoptosis of cells just before haemorrhage was observed in this model (Yen et al., 2008). There is therefore now ample evidence that anti-NS1 antibodies not only have the potential to provide protection against flavivirus infection, but through their interaction with host cell components, may also exacerbate disease. These findings have clear implications for vaccine design, with strategies that include NS1 as a component immunogen, while taking into account the potential for inducing auto-antibodies. Some progress towards this goal has been made with the recent report that a C-terminally truncated recombinant NS1, with the cross-reactive motif identified above removed, induced antibodies in mice that had lower platelet-binding characteristics than antibodies induced by its full-length NS1 counterpart (Chen et al., 2009).

Although a role in the pathogenesis of flavivirus infections for auto-antibodies elicited by epitopes on NS1 that are cross-reactive with host cell components is compelling, the dynamics of antibody kinetics over the course of infection also need to be taken into account. Most reports have provided solid *in vitro* and/or *in vivo* evidence for the binding of cross-reactive anti-NS1 antibodies to platelets and endothelial cells, as well as subsequent cellular damage and inflammatory activation (Falconar, 1997, 2007; Lin et al., 2001, 2002, 2003, 2006, 2008a; Sun et al., 2007; Cheng et al., 2009). However, few have commented on the apparent paradox of disease recovery during convalescence in the presence of the ongoing circulation of these otherwise damaging auto-antibodies. For example, auto-antibodies elicited by NS1 in dengue patients have been proposed as playing a major role in the endothelial dysfunction and consequent vascular leak that is characteristic of DHF/DSS patients experiencing secondary infections (Lin et al., 2008a). However, patient recovery from symptoms of vascular leak can often be quite dramatic (Mairuhu et al., 2004) and does not coincide with a sudden drop in circulating antibody. Furthermore, in primary infections of young infants experiencing DHF/DSS, high levels of circulating antibody are not found during the acute phase of the disease. So while these platelet and endothelial cell reactive antibodies may indeed play some role in the pathogenesis of flavivirus disease, their activity needs to be viewed more in the context of a wide range of other modulating host and viral risk factors.

5. NS1 as a diagnostic biomarker

Correct serological diagnosis of a flavivirus infection can be challenging, particularly in regions of the world where more than one flavivirus co-circulates, as the traditional serological approaches suffer from the relatively high antigenic cross-reactivity of the major virion envelope protein, E against which the majority of the antibody response is targeted. Furthermore, serological approaches, mostly based on IgM capture ELISA, not only suffer specificity problems from antigenic cross-reactivity but as they measure the patient immune response to infection, detection is not available early in course of the disease. Until recently, alternative laboratory diagnosis relied on virus isolation or RT–PCR, both of which present particular problems. Virus isolation requires lengthy culture, is expensive and has relatively poor sensitivity. RT–PCR, while accurate and ideal for early diagnosis, requires specialised equipment not always available in tropical settings where many flaviviruses predominate. As NS1 is secreted from infected cells, it was reasoned that this viral protein would be a suitable early surrogate biomarker for viraemia and/or infected cell mass in patients (Young et al., 2000). An NS1 antigen capture ELISA for DENV was developed which revealed that NS1 is secreted from the onset of symptoms in some infected individuals at high levels of up to 50 μg/ml (Young et al., 2000).

Early assessment of this assay by a number of groups (Young et al., 2000; Alcon et al., 2002; Libraty et al., 2002) led to the commercial development of NS1-capture ELISAs by several companies. Second-generation rapid assays are also now available for point-of-care diagnosis (Chaiyaratana et al., 2009; Hang et al., 2009; Zainah et al., 2009). All of these tests have now been subjected to a large number of field evaluations that have proven the value of the assay in the early diagnosis of dengue infection (Lemes et al., 2005; Xu et al., 2006; Kumarasamy et al., 2007a; Kumarasamy et al., 2007b; Simmons et al., 2007; Bessoff et al., 2008; Blacksell et al., 2008; Dussart et al., 2008; Lapphra et al., 2008; Ludert et al., 2008; Chaiyaratana et al., 2009; Hsieh and Chen, 2009; McBride, 2009; Phuong et al., 2009; Shu et al., 2009; Zainah et al., 2009; Arya et al., 2011a,b; Fry et al., 2011; Tricou et al., 2011; Anders et al., 2012; Blacksell et al., 2012a, 2012b; Gowri Sankar et al., 2012;

Gupta et al., 2012; Muller et al., 2012a). The commercial development and application of NS1 detection as a diagnostic tool has revolutionised dengue diagnosis. as it now provides a simple and relatively low-cost assay that has high sensitivity and specificity (Castro-Jorge et al., 2010; Lima Mda et al., 2010; Wang and Sekaran, 2010b). More recent studies have shown that NS1 detection may also be applicable to the diagnosis of other flavivirus infections (Chung and Diamond, 2008).

What makes NS1 such an ideal diagnostic marker is the fact that it is found at high levels in the blood of infected individuals very early in infection, typically from or before the onset of symptoms (Alcon et al., 2002; McBride, 2009; Bessoff et al., 2010; Thomas et al., 2010). It is detected before an antibody response is mounted and as early as viral RNA, with the latter leading to NS1 being referred to as a surrogate marker for viraemia. However, closer examination of the kinetics of NS1 and viraemia levels in individual patients often reveals differential profiles suggesting a more complex association (Libraty et al., 2002; McBride, 2009; Zainah et al., 2009; Tricou et al., 2010). Although NS1 should more accurately be referred to as a surrogate marker of infected cell mass, these differences may in part be due to differing mechanisms of clearance operating on virions and free NS1 protein.

In primary dengue infection, NS1 can be detected in patient serum or plasma samples taken as much as 9–12 days after disease onset (Alcon et al., 2002; Libraty et al., 2002; Xu et al., 2006). However, a complicating factor for NS1 detection in secondary dengue infections is the rapid anamnestic rise of serotype cross-reactive anti-NS1 antibodies. As a consequence of the formation of immune complexes and their likely clearance from circulation, NS1 is rarely detected in secondary-infected patients beyond 5–7 days after the onset of symptoms (Vazquez et al., 2010). Depending on the timing of clinical presentation, this may therefore result in a dengue-infected patient being tested as NS1-negative. This has led to some confusion in the field in the application of this assay for diagnosis of secondary-infected individuals, by far the majority of patients seen clinically in endemic countries. However, a negative result under these circumstances is not a failure of the assay, as defined by poor sensitivities reported in a number of recent publications, but merely an accurate assessment of the circulation of low levels of free NS1 (Blacksell et al., 2008; Datta and Wattal, 2010). While some efforts have been made to improve the sensitivity of the assay under circumstances where immune complex formation is likely to have occurred. by incorporating methods for immune complex disruption (Lapphra et al., 2008), it is imperative for accurate diagnosis of secondary-infected dengue patients that the NS1 assay is not used alone, but is complemented with the detection of dengue-specific IgM antibodies (Blacksell et al., 2008; Lima Mda et al., 2010; Osorio et al., 2010; Wang and Sekaran, 2010a; Fry et al., 2011). Diagnostic assays incorporating both these markers are now making their way into practical use.

An observation made relatively early in the determination of NS1 levels in dengue-infected patients was that high levels of NS1 early in infection appeared to correlate with the later onset of severe disease (Libraty et al., 2002). This finding has subsequently been confirmed in other studies (Avirutnan et al., 2006), offering the exciting prospect of including NS1 as an early prognostic biomarker of severe disease. In addition to the detection of NS1 itself, the antibodies that it elicits have also been used in developing useful diagnostic assays. The relative type-specificity of the antibody responses elicited has lead to the development of ELISA-based assays that are capable of determining infecting serotype, primary or secondary infection and able to differentiate JEV from dengue, a particular problem in many countries of South East Asia (Shu et al., 2000, 2001, 2002, 2003, 2004). Beyond the diagnosis of human dengue infection, NS1 rapid strips have recently been applied to the detection of dengue virus infection of mosquitoes (Tan et al., 2011; Muller et al., 2012b; Voge et al., 2013). These studies have shown that a single infected mosquito can be detected in a large pool of as many as 100 uninfected mosquitoes. In conjunction with small portable homogenizers, detection of DENV-infected mosquitoes in the field could become a routine part of vector surveillance measures (Muller et al., 2012b).

6. Conclusions

For a viral protein that has been the focus of research for more than two decades, it is remarkable that there is still so much we don't know about the function of NS1 in viral replication and disease. The post-translational modifications that give rise to multiple species of both cell-associated and secreted forms of NS1 have been identified. However we do not know the molecular mechanism of membrane association, nor the manner in which the hexamer is generated. Unequivocal evidence of a role for NS1 in viral RNA replication and an association with replication complexes has been documented. However, we do not know how NS1 is physically associated with the replication complex, nor its specific role in RNA replication. Specific interactions with a wide range of host cell components have been reported for both sNS1 and mNS1 that may play a significant role in the pathogenesis of flavivirus disease. However the molecular basis of these interactions and the level to which they are shared by different flaviviruses remains unclear.

DENV NS1 has been shown to display cross-reactive epitopes that are shared with a number of host cell components, and the auto-antibodies that are induced by these determinants in secondary infections are thought to contribute to the platelet and endothelial cell damage that leads to the vascular permeability characteristic of severe DHF/DSS. Circulating sNS1 itself has been shown to bind to different cell populations, and has also been proposed to contribute directly to the disease process. However, little is known about the dynamics of the appearance and interactions between these different species during the course of disease. The presence of both NS1 and the antibodies it induces during the acute phase of disease is unusual and further studies are required to determine the modulating effect each has on the other, and of course the contribution of their interaction, immune complexes, to the overall disease process.

In this article, we have sought to provide a comprehensive overview of the biochemical features of NS1, its use as a diagnostic tool and its role in pathogenesis and protection. Our hope is that it will provide insights into further research directions that may answer some of the many outstanding questions posed above. Of paramount importance is the resolution of the structure of NS1. The three-dimensional structure of monomeric and/or the biologically relevant dimeric and hexameric forms should provide extremely useful insight, not only into the primary replicative function of this species, but also into the reasons it is found in partnership with such a wide range of host-cell components.

Despite the many gaps in our knowledge of the structure and function of NS1, a picture has emerged of a key viral protein that is involved in many stages of the virus life cycle, from its essential role in viral RNA replication to its somewhat contradictory contribution to the induction of both protection and pathogenesis in the infected host. Its detection in infected patients has also turned out to be a very useful tool in early diagnosis. Perhaps one of the most intriguing aspects of this viral gene product can be found in a comparison with the genome coding strategy of the two related members of the *Flaviviridae* family, the hepaciviruses and pestiviruses. Members of these two genera do not encode an equivalent NS1 species. For a protein that is essential for replication in the flaviviruses, how is this function compensated in these other flaviviruses? Is this a gene that has been lost during the separate

evolution of these genera, or have the flaviviruses acquired this gene from their hosts during their evolution? It is certainly worth noting that, unlike the hepaciviruses and pestiviruses, most of flaviviruses cycle between two quite different hosts, insects and mammals. Does NS1 play a role in bridging the separate replicative requirements of two different cellular environments? Studies that directly compare the replication of these separate members of the flavivirus family may be quite revealing. Ongoing research into the flavivirus NS1 is bound to reveal more surprises and additions to the many aspects of this somewhat enigmatic protein. We eagerly await further developments.

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