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# Secretion of Nonstructural Protein 1 of Dengue Virus from Infected Mosquito Cells: Facts and Speculations

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**ABSTRACT** Dengue virus nonstructural protein 1 (NS1) is a multifunctional glycoprotein. For decades, the notion in the field was that NS1 is secreted exclusively from vertebrate cells and not from mosquito cells. However, recent evidence shows that mosquito cells also secrete NS1 efficiently. In this review, we discuss the evidence for secretion of NS1 of dengue virus, and of other flaviviruses, from mosquito cells, differences between NS1 secreted from mosquito and NS1 secreted from vertebrate cells, and possible roles of soluble NS1 in the insect flavivirus vector.

**KEYWORDS** dengue virus, dengue, flavivirus, NS1 protein, mosquito cells, glycosylation patterns, secretory pathways

Members of the genus *Flavivirus* are responsible for several mosquito-borne life-threatening viral diseases in humans; examples are West Nile virus (WNV), St. Louis encephalitis virus (SLEV), Japanese encephalitis virus (JEV), yellow fever virus (YFV), and, with a greater impact due their prevalence and repercussions on public health, dengue virus (DENV) and Zika virus (ZIKV) (1). It is estimated that nearly half of the world's population is at risk of contracting infections with these viruses. DENV is transmitted by mosquitoes of the genus *Aedes*, mainly of the species *A. aegypti*. The urban cycle of transmission begins once an adult female bites a viremic human, acquiring the circulating virus along with the blood meal. When an infected mosquito bites a healthy human, it retransmits the virus, completing the transmission cycle (2). Dengue is the most important mosquito-borne virus affecting human health and is endemic in over 100 countries of the tropical and subtropical regions of the planet (1). Levels of dengue disease burden are difficult to estimate, but at least 350 million people are at risk of contracting the disease. There is not yet a specific antiviral treatment available for dengue, and the live-attenuated vaccine for dengue that was licensed in some countries is now under reevaluation, as evidence of an increase of the incidence of severe illness was found in dengue-naive vaccine recipients. Therefore, it is clear that dengue infection remains a major threat for human health. Moreover, it has been realized that vaccination alone is not sufficient to control dengue and that sustainable vector control measurements are required.

The DENV genome encodes 3 structural proteins, namely, membrane, capsid, and envelope (M, C, and E), and 7 nonstructural proteins (nonstructural protein 1 [NS1], NS2A, NS2B, NS3, NS4B, NS4B, and NS5). The replication cycle of DENV initiates once the virus recognizes its receptor on the surface of the cell membrane and is subsequently internalized in endosomes. Once released in the cytoplasm, the genomic RNA is translated by ribosomes of the endoplasmic reticulum (ER) into a single polyprotein. Subsequently, by the action of viral and cellular proteases, structural and nonstructural proteins are generated, which then participate in the process of viral RNA replication and virion assembly. Virus replication takes place in structures derived from intracellular

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membranes. The immature viral particle enters the classical ER secretory pathway and then transits to the Golgi complex, where the pre-M protein is cleaved by the cellular protease furin, causing its maturation to protein M. In this way, mature viral particles are formed and are subsequently secreted by exocytosis (3). Although extensive research on DENV and the disease it causes exists, information about the virus life cycle in its arthropod host remains less thoroughly understood. In particular, the role in the insect of the NS1 protein has not been fully investigated. NS1 has a plethora of functions in the cell and a critical role in protection and pathogenesis, hence the importance of increasing our understanding of the mechanisms of action of this protein. Current knowledge, with an emphasis on the biology and trafficking of NS1 in mosquito versus vertebrate cells, as well as on the role of NS1 in the DENV insect hosts, is discussed here.

## DENV NS1: A MULTIFUNCTIONAL PROTEIN

Flavivirus NS1 has been assigned a diversity of functions, which include roles in viral replication as well as active participation in the pathogenesis of infection and in the generation of protection (4). Because NS1 is a ubiquitous protein among flaviviruses, the functions and interactions described today are the products of a collection of data from NS1 studies from different members of the flavivirus genus. NS1 is a 352-amino-acid (aa) glycoprotein, with a molecular mass of between 46 and 54 kDa, depending on its glycosylation profile, with two N-glycosylation sites at positions 130 and 207 (5). NS1 is translated as a part of a viral polyprotein directly from viral RNA and is translocated toward the lumen of the endoplasmic reticulum (ER). NS1 is cleaved from the viral polyprotein by proteolysis in the ER lumen and then dimerizes. In infected vertebrate cells, dimeric NS1 follows several trafficking routes: (i) it serves as an anchor on the luminal face of the ER membrane, as part of the replicative virus complexes; (ii) it locates on the external face of the plasma membrane (PM) in vertebrate cells, following a still-unknown route; and (iii) it continues through the classic intracellular route of glycoprotein secretion (ER-Golgi network), in which 3 NS1 dimers come together to form a hexamer that is then secreted into the extracellular space as soluble NS1 (sNS1) (4).

The first experimental evidence about the tridimensional structure of secreted sNS1 was obtained by cryo-electron microscopy as previously described by Gutsche et al. (5). sNS1 is organized as an open-barrel structure formed by the noncovalent assembly of three dimers (5). Notably, secreted sNS1 was found to be a bona fide lipoprotein, with a central channel rich in lipids that resembles the lipid composition of high-density lipoproteins (HDL). The resolution of the crystal structure of the dimer showed that NS1 has three structural domains, a hydrophobic $\beta$-roll (aa 1 to 29) followed by an external $\alpha/\beta$ wing domain (aa 38 to 151) and a central $\beta$-ladder containing an extended $\beta$-sheet (aa 181 to 352) (6). In the hexamer structure, the hydrophobic $\beta$-roll faces the inside, conforming to the hydrophobic hole of the barrel. Application of the methodological advances in X-ray crystallography of the past few years to structural studies of DENV and WNV proteins contributed to the elucidation of the structure of ZIKV proteins (7, 8). The structure of ZIKV sNS1, as also seen with the NS1 of DENV, has a hexameric assembly, the product of the union of three dimers, in the form of a hollow barrel, with the internal channel rich in lipids. However, it was reported that, unlike the NS1 of DENV and WNV, the dimer of ZIKV NS1 presents extended membrane interactions and a unique electrostatic potential map, both on the inner and outer sides, which could favor its interaction with other proteins in the host in a manner different from that seen with those bound by sNS1 of DENV and WNV (7).

Proteomic studies have demonstrated that NS1 has many diverse interactions with host proteins and that its multifunctional nature suggests the presence of selective pressure for adaptation. In infected cells, dimeric DENV NS1 fulfills a variety of intracellular roles, interacting with several proteins involved in different biological processes, such as translation, glycolysis, transport, stress response, regulation of energetic metabolism, cell structural modifications, and virus assembly (4). On the other hand, extracellular sNS1 has been implicated in the evasion of the immune response and in

various mechanisms of pathogenesis through its interaction with host proteins, including the complement system; destabilization of tight junctions; and glycocalyx disruption (9, 10). However, and despite the conserved sequences and features of NS1 from different flaviviruses, it has been suggested that NS1 has particular functions for each flavivirus. Due to the multifunctional and antagonistic participation of NS1 during the infection process, and despite the structural similarities of various NS1 proteins, it is important to delve more deeply into the functional differences between NS1 from DENV and ZIKV, due to the differences in the pathologies caused by each virus.

## DENV NS1 SECRETION FROM INFECTED MOSQUITO CELLS

NS1 is a relatively well-conserved protein, encoded only by mosquito-borne flaviviruses, and yet there is evidence to support the idea that each individual flavivirus NS1 has unique characteristics that can be affected by the individual context of the infection and host. One remarkable example is sNS1 secretion. NS1 secretion is a phenomenon that has been well documented during infection in vertebrates. Soluble NS1 is used as an early diagnostic marker during DENV infection, because of the large amounts (up to micrograms per milliliter) circulating in patient serum during the acute phase of the disease (11). In addition, high sNS1 levels have been associated with the development of severe dengue (12), although others have failed to find such a correlation (11, 13).

Recently, it became clear that DENV NS1 is also secreted from infected insect cells. For decades, it was believed that NS1 of flaviviruses was secreted only from infected vertebrate cells and not from infected insect cells (4, 11). This notion was widely adopted after publication of observations reported by Mason (14), who did not detect NS1 in the culture fluid of 3 mosquito cell lines infected with Japanese encephalitis virus (JEV), while sNS1 was readily detected in culture fluids from infected Vero cells. The inability of mosquito cells to produce complex oligosaccharides was the rationale put forward to explain the absence of NS1 in the insect cell culture supernatant (14). However, the presence of NS1 in supernatants of C6/36 cells (derived from *Aedes albopictus*) infected with different DENV serotypes was readily observed using either commercial enzyme-linked immunosorbent assay (ELISA) kits or Western blotting as reported by several authors (15–17). NS1 has also been detected in the cell supernatant of C6/36 cells persistently infected with DENV (18). In agreement, DENV NS1 expressed in the baculovirus system was also detected in Sf9 insect cell culture supernatants (6, 19). In later discussions, it was pointed out that the presence of flavivirus NS1 in the supernatant of infected mosquito cell cultures was due to cell lysis and did not represent true secretion. This notion has persisted in the field and has been extrapolated to the NS1 proteins of all flaviviruses (20). Recently, it was clearly demonstrated by Alcalá et al. (21) that DENV NS1 is indeed secreted efficiently by infected mosquito cell. NS1 was detected, using a commercial enzyme immunoassay (EIA), in supernatants from C6/36 and Aag2 (derived from *Aedes aegypti*) cell cultures infected with DENV serotype 2 (DENV2) or DENV4, starting at 6 h postinfection (hpi), with a continuous concentration increase up to 24 h hpi. Nevertheless, no association was observed between the presence of NS1 in the cell supernatants and cell lysis, with infected cells showing an average cell survival rate of 97% at 24 hpi (21). Additionally, it was shown that the DENV NS1 secreted from mosquito cells is also hexameric and likely presents the same open-barrel structure as that observed in sNS1 derived from vertebrate cells. These results were soon independently confirmed by Thiemmeca et al. (22) who, in addition to reporting efficient secretion of multimeric NS1 from infected mosquito cells, observed that the NS1 secreted from mosquito cells was functional and capable of complement fixation. In addition, the presence of sNS1 was also observed in the supernatants of C6/36 cells infected with a wild-type strain of yellow fever virus (23) and in the cell supernatant of insect cells expressing recombinant NS1 of ZIKV (24). On the other hand, Youn et al. (25) observed that C6/36 cells infected with WNV did not secrete NS1 but that the introduction of 2 DENV amino acid substitutions at positions 10 and 11 (RQ10NK) into the WNV NS1 sequence resulted in the accumulation of WNV NS1 in the supernatants of infected C6/36 cells. These observations suggest that the secretion

of NS1 from infected insect cells is not exclusive to DENV but that differences in amino acid sequences among flaviviruses dictate the capacity of NS1 proteins to be secreted from mosquito cells.

The host protein interactions with all four DENV serotypes may either be serotype specific or serotype independent. In the case of NS1, the secretion patterns seen with the same DENV strain are different depending on the host. For example, it has been established that, depending on the infecting DENV strain, the amount of sNS1 is variable in patients, animal models, and vertebrate cells in culture (11, 13, 26). Infections of two different insect cell types with DENV2 and DENV4 at the same multiplicity of infection yielded different amounts of sNS1 in supernatants and showed marked differences in the secretion kinetics in comparison with Vero cells infected in parallel (22). This result suggests that virus-host specific processes and interactions also occur in insect cells and that, despite the genetic similarity between the members of the *Flavivirus* genus and the conservation of NS1 genes and structures, it is important to conduct studies of the same process using various strains before extrapolation of results to the whole genus. Once it has been established that DENV NS1 is effectively secreted from insect cells, a challenge is to elucidate how the secretion process is carried out despite the inherent metabolic limitations of these lines.

## NS1 SECRETION PROCESS IN MOSQUITO CELLS

The presence of DENV sNS1 in serum samples from patients and in the supernatant of infected vertebrate and insect cell cultures has been demonstrated by several groups (11, 21, 22, 27). However, the intracellular secretion process involved in NS1 reaching the extracellular medium has not been as thoroughly studied and its analysis is hence based on many assumptions. This secretion process can be evaluated indirectly by determining the glycan composition on the secreted form of the NS1 or directly by analysis of specifically intracellular molecular interactions.

### (i) NS1 MOLECULAR INTERACTIONS AND LOCALIZATION

Several interactions between NS1 and different intracellular molecules have been reported. There are some interactions that have been reported that can help generate hypotheses about the transit of NS1 to the plasma membrane (PM) and its subsequent secretion. After obtaining evidence of DENV NS1 secretion from insect cells, Alcalá et al. (28) explored the secretory route of sNS1 using various approaches, such as treatment with specific drugs and gene silencing. The treatment of C6/36 or Aag2 cells with brefeldin A (BFA), a drug known to disrupt the *cis* and *trans* cisternae of the *trans*-Golgi network (TGN), inhibited the secretion of virions in cells infected with either DENV2 or DENV4, while the secretion of sNS1 remained unaffected. As expected, BFA treatment of infected vertebrate cells significantly inhibited cell release of both virions and NS1. Identical results were obtained when these experiments were repeated in DENV-infected C6/36, Aag2, and BHK21 cells treated with the *cis*-Golgi network-disrupting drug golgicide A (R. Rosales and J. E. Ludert, unpublished data). Moreover, silencing of the expression of SAR1 protein (a GTPase necessary for the formation of the coat protein II [COPII] complex of the classical secretory pathway) in C6/36 cells did not inhibit secretion of sNS1, while virion secretion was significantly reduced. In contrast, both treatment with methyl-beta-cyclodextrin (M$\beta$CD) and silencing of expression of caveolin 1 (CAV1), a main component of caveolae but also a protein involved in cholesterol transport inside cells, reduced the sNS1 supernatant levels significantly, without affecting virion release. Molecular dynamics analysis predicted a favorable interaction between NS1 and CAV1, and proximity ligation assays confirmed the interaction between NS1 and CAV1 in infected C6/36 cells fixed at 6 and 24 hpi. On the basis of these results, it was proposed that, whereas virions are released from mosquito cells following the classical secretory pathway, NS1 is released from mosquito cells via an unconventional secretory route that bypasses the Golgi complex and includes the participation of CAV1 (28). Given the observation that there are flaviviruses whose only hosts are mosquitoes (mosquito-only flaviviruses), it is likely that DENV was also once

a mosquito-only flavivirus. Thus, it is reasonable to speculate that secretion of NS1 through the highly efficient classical secretory pathways represents a gain of function acquired by DENV after jumping into vertebrate hosts, which resulted in enhanced secretion of this protein. And yet, additional experiments are necessary in order to fully dissect the route of secretion of NS1 in mosquito cells and to identify additional molecules, if any, which may also be involved in NS1 secretion from mosquito cells.

Differences have also been observed between vertebrate and mosquito cells regarding the location of NS1. Notably, in vertebrate cells, NS1 is located on the external face of the plasma membrane, either because it is exported there, by an as-yet-unknown traffic route, or because it "binds back" from the supernatant (4). However, Alcalá et al. (21), by using three different methodological approaches, concluded that NS1 was not present on the plasma membrane of infected mosquito cells. Given the high levels of secreted NS1 in mosquito cells observed by Alcalá et al. (21) and others, this observation is in line with data previously obtained with WNV and DENV chimeras, suggesting that there is a reciprocal relationship between efficient secretion and plasma membrane location (25). In addition, NS1 is expressed in vertebrate cells as a glycosylphosphatidylinositol (GPI)-linked form that allows binding of the protein to internal membranes and to the outer face of the plasma membrane (29); however, metabolic labeling of NS1 expressed in mosquito cells showed very low levels of GPI-linked forms compared with HeLa cells (29). The reasons for the inefficient addition of GPI to NS1 in mosquito cells are unknown. Uninfected insect cells are able to synthesize GPI-anchored proteins, and yet inhibition of GPI biosynthesis has been shown in insect cells infected with baculovirus (30). The inhibition in GPI addition to proteins observed in baculovirus-infected cells was attributed to the blocking of de-N-acetylation of GlcNAc-PI into GlcNH2-PI (31). It would be interesting to determine if this phenomenon is reproduced in DENV-infected insect cells. The structure and composition of the GPI anchor are known to play key roles in determining the trafficking of proteins from the ER to the cell surface, via the Golgi apparatus (32, 33). Thus, the absence of a GPI tail in the NS1 protein produced in insect cells may be connected to the nonclassical secretory route followed by NS1 in insect cells. Moreover, this would also explain the absence of NS1 from the outer plasma membrane of insect cells. Interestingly, it has been established that the acquisition of a GPI anchor may confer to proteins a variety of physiological roles in cell signaling that modulate different intracellular processes (32). Thus, the absence of a GPI anchor in the NS1 protein produced in mosquito cells suggests that NS1 plays different roles in the vertebrate and insect host.

There are many gaps in our knowledge concerning the NS1 secretion process in both vertebrates and insect cells that remain to be filled. For example, the exact composition of the NS1-CAV1 complex that travels from the ER to the plasma membrane is unknown, as is the trafficking route followed by dimeric NS1 to reach the outer face of the plasma membrane in infected vertebrate cells. It will also be necessary to evaluate other molecular mechanisms that are possibly involved, such as vacuolar secretion or the involvement of the cytoskeleton. Different secretion pathways result in different NS1 structural or chemical modifications that may have a role in specific effects of the extracellular forms of NS1 and in transmission to the next host.

### (ii) sNS1 GLYCOSYLATION

The conventional pathway for protein secretion involves the progression from the ER to the Golgi cisternae, known as the classical secretion pathway. This is a very efficient pathway that is used by the virion to reach the extracellular space in both vertebrate and mosquito cells. During intracellular transit, nascent proteins undergo several modifications, primarily acquiring oligosaccharide chains specific to the compartment transited by the protein. Asn-linked glycosylation begins with the *en bloc* transfer in the ER of a lipid-linked oligosaccharide ($Glc_3Man_9GlcNAc_2$) from a dolichol carrier to specific asparagine residues (NxS/T) of the protein, via an oligosaccharyltransferase. As the glycoprotein transits the Golgi complex, compartment-specific glycosi-

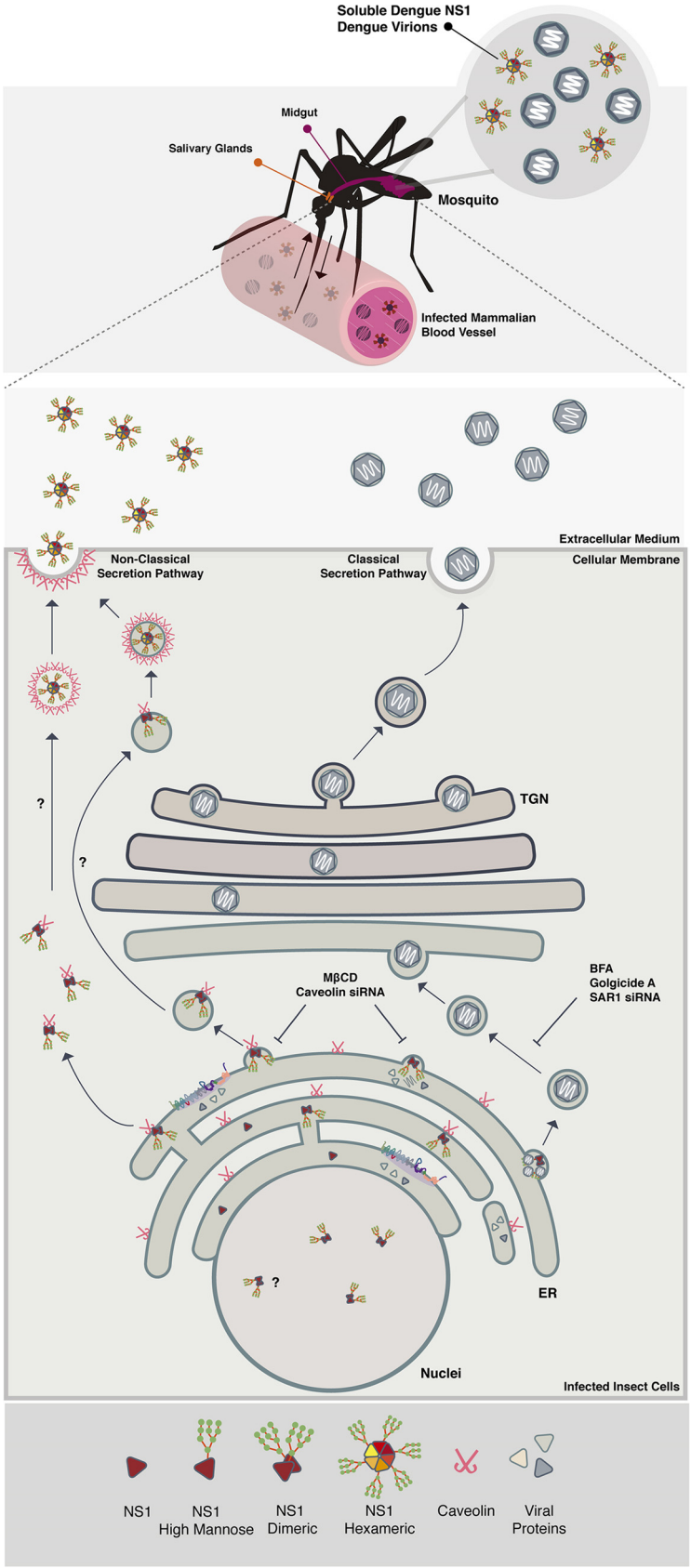


**FIG 1** In infected mosquito cells, NS1 and virions travel to the extracellular media following different traffic routes. After synthesis and proteolytic processing, NS1 rapidly dimerizes and becomes part of the

(Continued on next page)

dases trim the mannose residues before glycosyltransferases extend glycans to produce larger complex structures, usually sialylated structures in vertebrate cells (34). Insect cells are capable of recognizing the universal N-glycosylation sequence; however, unlike those of vertebrate cells, most glycan structures from insect cell proteins have only terminal mannose residues, with either paucimannose or high-mannose structures. One explanation for this difference is the negligible activities of N-acetylglucosaminyl-, galactosyl-, and sialyltransferases, responsible for assembly of the terminal sequences of N-glycans of mature mammalian glycoproteins, deterring the insect cells from yielding complex glycans. A role of the N207 glycan in the secretion of recombinant DENV2 sNS1 was previously reported, whereas the N130 glycan has been reported to be involved in the NS1 dimer stabilization (35). The requirement of N-linked glycosylation for NS1 secretion in vertebrate cells was previously demonstrated (25), and Thiemmeca et al. (22) showed that this was also true for sNS1 from insect cells, as treatment with tunicamycin significantly reduced the amount of sNS1 found in the supernatant. However, processing of N-glycans to form complex structures in the Golgi complex is not essential for NS1 or protein secretion (25). N-Glycosylation profiles have been used to infer the secretion route of sNS1 of flavivirus. DENV sNS1 proteins secreted from vertebrate cells have N-glycosylation profiles that reflect the processing in the ER and Golgi network, as can be concluded from the complex glycosylation and resistance to endo-$\beta$-*N*-acetylglucosaminidase H (Endo-H) treatment (36). The N-glycosylation profile of the DENV sNS1 protein from insect cells contains terminal mannose residues that can be removed by PNGase F treatment, as expected from insect glycoproteins (22, 28). However, the sNS1 secreted from insect cells remains sensitive to Endo-H treatment (22, 23), while the virion-associated E protein is not (28), in agreement with the notion that in mosquito cells, virions travel through the Golgi complex, while the NS1 transits via a different secretory route that bypasses the Golgi complex. In vertebrates, the role of sNS1 in the enhancement of DENV infection is well established; however, the specific molecular elements by which sNS1 favors infection are unknown (11, 22). Due to the known importance of glycans in the interaction of viral proteins with the host and in the triggering of various responses, it is important to elucidate and compare the specific N-glycosylation profiles of the NS1 proteins generated in mosquito and vertebrate cells, in order to gain additional knowledge on the secretion routes and also to better understand NS1 interactions and functions.

## CONCLUSIONS AND PERSPECTIVES

The new evidence demonstrating that DENV NS1 is secreted at high levels from infected mosquito cells reversed a long standing paradigm in the flavivirus field. Moreover, the NS1 from other flaviviruses that are important pathogens of humans, such as Zika virus and yellow fever virus, also seems to be secreted from mosquito cells. Similarities between vertebrate-secreted and mosquito-secreted NS1 proteins, such as the glycosylated and hexameric nature of the secreted product and its capacity to fix complement, have been found. Yet, the NS1 protein produced in mosquito cells has important differences from the NS1 protein produced in vertebrate cells such as the

**FIG 1** Legend (Continued)
replication complex(es) as a scaffolding protein. Yet another portion of the protein becomes attached to caveolin, presumably by interactions between the caveolin binding domain present in the "greasy finger" loops of NS1 and the scaffolding domain present in caveolin. Bound to caveolin, NS1 travels to the extracellular space, bypassing the Golgi complex. Yet the nature of the NS1-CAV complex is unknown. Bypassing the Golgi complex implies that sugars bound to NS1 are not further processed to form complex carbohydrates. The interaction with caveolin and the lack of a GPI anchor are presumably key determinants in keeping NS1 from entering COPII vesicles and therefore from entering the classical secretory route. NS1 is also detected inside the nucleus, but nothing is known about what drives NS1 into the nucleus. Meanwhile, virions are released following a classical secretory route. In contrast, in vertebrate cells, both virions and NS1 are released following a classical secretory route. Also in vertebrate cells, but not in mosquito cells, NS1 is found on the outer phase of the plasma membrane. Important questions are raised about the role of soluble NS1 produced inside the mosquito and also present in the mosquito saliva along with infecting virions. siRNA, small interfering RNA. (Courtesy of José Luis Zambrano; reprinted with permission.)

lack of a GPI tail, the absence of NS1 attachment to the plasma membrane, and the secretory routes taken. These differences suggest particular functions for NS1 in each host and highlight the peculiarities of the replicative cycles of a single virus in two very different host cells (Fig. 1).

The importance of mosquito saliva in modulating DENV infection in humans is well recognized (37). The interesting observation reported by Thiemmeca et al. (22) that NS1 is present in infected mosquito saliva calls for new experiments where the role of NS1, along with mosquito saliva proteins, in virus transmission is evaluated. It is known that NS1 is produced at high levels in infected *Aedes aegypti* mosquitoes (38–40), although formal proof that NS1 is actually secreted *in vivo* by cells in the mosquito is lacking. However, the presence of NS1 in the mosquito saliva suggests that this is the case. Keeping in mind that NS1 is a protein present only in mosquito-borne arboviruses, the observation that NS1 is secreted at high levels by infected mosquito cell lines prompts important questions regarding the functional role of soluble NS1 produced inside the mosquito vector. The importance of NS1 in the blood meal intake for the successful establishment of a viral infection in the mosquito is starting to be understood (24). However, in contrast to the role played by soluble NS1 present in the sera of infected vertebrates, we know nothing of the functional importance of soluble NS1, intrinsically produced inside the mosquito, during the dengue virus replicative cycle or in the spread of the infection inside the vector. Does the capacity to open tight junctions reported for NS1 in vertebrate cells (10) also apply to the mosquito? And if so, how does it impact the establishment or spread of the infection in the mosquito? Do the levels of sNS1 inside the mosquito affect the intrinsic incubation period? Is sNS1 a modulator of the innate immunity in the mosquito? Does it facilitate viral infection in the mosquito, as has been observed in liver cells (11)? These questions are also presumably relevant to other mosquito-borne flaviviruses such as Zika virus and yellow fever virus. Experiments involving the localization of extracellular soluble NS1, as well as the disruption or enhancement of NS1 secretion in the mosquito, are required. There are clearly still many questions to be answered in relation to the passage of the virus through the insect vector. Determining the answers to these and other relevant questions will be a challenge and a focus for many in the field.

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