The dengue virus non-structural protein 1 (NS1) is secreted from infected mosquito cells via a non-classical caveolin-1-dependent pathway

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Abstract

Dengue virus NS1 is a glycoprotein of 46–50 kDa that is conserved among flaviviruses, associates as a dimer to cell membranes and is secreted as a hexamer to the extracellular milieu. Recent evidence showed that NS1 is secreted efficiently from infected mosquito cells. To explore the secretory route of NS1 in mosquito cells, infected cells were treated with brefeldin A (BFA) and methyl-beta-cyclodextrin (MJCD). The results showed that MJCD, but not BFA, significantly reduced the release of NS1. Moreover, silencing the expression of caveolin-1 (CAV1; a key component of the caveolar system that transports cholesterol inside the cell), but not SAR1 (a GTPase that participates in the classical secretory pathway), also results in a significant reduction of the secretion of NS1. These results indicate that NS1 is released from mosquito cells via an unconventional secretory route that bypasses the Golgi complex, with the participation of CAV1. In agreement with this notion, differences were observed in the glycosylation status between secreted NS1 and E proteins. Classical mechanics and docking simulations suggested highly favoured interactions between the caveolin-binding domain present in NS1 and the scaffolding domain of CAV1. Finally, proximity ligation assays showed direct interaction between NS1 and CAV1 in infected mosquito cells. These findings are in line with the lipoprotein nature of secreted NS1 and provide new insights into the biology of NS1.

INTRODUCTION

Non-structural protein 1 (NS1) is a conserved protein in the genome of flaviviruses that use insects as vectors (reviewed in [1, 2]). The gene encoding the dengue virus (DENV) NS1 is 1056 nucleotides in length, corresponding to a protein of 356 amino acids. DENV NS1 has a molecular weight of between 45 and 55 kDa and two N-linked glycosylation sites at positions Asn137 and Ans205 [3]. Once the NS1 is translated, as part of a polyprotein, it is proteolytically processed by the viral protease complex NS2B-NS3 and an as yet unidentified host protease to generate monomers that rapidly dimerize inside the lumen of the rough endoplasmic reticulum [1, 4]. In infected vertebrate cells, NS1 dimers are trafficked by mechanisms that are not yet fully understood to different cell locations, including vesicular compartments within the cell [membrane-associated NS1 (mNS1)] and the outer face of the plasma membrane [plasma membrane-associated NS1 (pNS1)], or secreted into the extracellular medium in soluble form [soluble NS1 (sNS1)] [5–8]. It has been shown that NS1 that is associated with intracellular and plasma membranes remains as a dimer, but the secreted form found in the extracellular medium or in serum of patients is hexameric [9–11]. In addition, it has been proposed that soluble hexameric NS1 may ‘bind back’ and attach to the plasma membrane of infected or uninfected cells [12]. Recently, we [11, 13] and others [14–16] have found evidence indicating that DENV NS1 is also secreted efficiently as a hexamer from infected mosquito cells.

Intracellularly, the NS1 protein is reported to act as a cofactor in viral replication [17, 18]. It is presumed that through its interaction with NS4A/NS4B, and due to its location into the lumen of ER, West Nile virus NS1 serves as a scaffolding protein that is necessary for the stability of the viral replication complex [19]. It has also been proposed that NS1 is required in the later steps of the viral replication cycle, because its interaction with the structural proteins E and pr-
M in ER lumen is crucial for the generation of infectious dengue viral particles [20].

The crystal structure of the DENV NS1 dimer shows three main domains, designated as (i) the ‘β-roll’ hydrophobic core (aa 1–29), which is located on the inside of the hexamer; (ii) the ‘wing’ domain (aa 38–151), which is located on the outside of the hexamer; and (iii) the β-ladder domain, which contains an extensive β-folded sheet and the so-called ‘spaghetti loop’ (aa 181–352), both of which located outside the hexamer [21]. The hexameric secreted form of this protein is a lipoprotein particle with an open barrel shape, 10 nm in diameter, consisting of three dimers of the protein with a prominent central channel rich in lipids [22]. The lipid composition of NS1, as determined by biochemical and nuclear magnetic resonance analysis, revealed a composition reminiscent of human plasma high-density lipoprotein (HDL), which includes triglycerides, cholesterol esters and phospholipids in an equimolar ratio [22].

DENV NS1 protein interacts with a variety of proteins involved in different biological processes, such as translation, glycolysis, transport and response to stress [23]. It has also been reported that NS1 may act as an important modulator of cellular energy metabolism, because it is capable of interacting with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to increase glycolytic activity in vitro [24].

Both NS1 itself, either secreted or plasma membrane-associated, as well as the antibodies against NS1, have been implicated in the pathogenesis of severe dengue (reviewed in [25]). Soluble NS1 can bind to the cell plasma membrane or be endocytosed and participate in mechanisms of signal transduction favouring viral replication [26, 27]; NS1 also binds to several components of the complement pathway that inhibit its activation [28–30]. Recently, it has been reported that sNS1 may induce plasma leakage by promoting glycolcalyx degradation and endothelial barrier dysfunction [31, 32]. Additionally, sNS1 acts as a pathogen-associated molecular pattern (PAMP), because it is able to activate murine macrophages and mononuclear cells from human peripheral blood (PBMCs) via the Toll 4 (TLR4) receptor, allowing the induction and release of proinflammatory cytokines and chemokines [33]. These observations corroborate the previously observed association between high levels of sNS1 circulating in the serum of patients and the severity of the clinical picture [34, 35]. Meanwhile, anti-NS1 antibodies have been implicated in pathogenesis by molecular mimicry mechanisms involving the direct recognition of platelets or endothelial cells [36–38]. Interestingly, it has also been reported that anti-NS1 antibodies are protective against infection in suckling mice [39].

NS1 protein is the only viral non-structural protein secreted by DENV-infected vertebrate and mosquito cells [10, 11]. Despite the prominent role attributed to soluble NS1 and anti-NS1 antibodies in the pathology of severe dengue, little is known about the secretory pathways followed by sNS1 in infected cells, especially infected mosquito cells, where the secretion process was only recently discovered. The glycosylation patterns of sNS1 suggest that in vertebrate cell sNS1 follows a classical secretion route, passing through the ER and Golgi complex to reach the extracellular medium [1]. In this work, we explore the secretory pathways of NS1 in infected mosquito cells; the results indicate that in contrast to vertebrate cells, in mosquito cells sNS1 follows a non-classical secretory route that is dependent of the presence of caveolin-1 (CAV1). Moreover, evidence is presented that indicates direct interaction between DENV NS1 and CAV1.

RESULTS

Brefeldin A treatment does not affect the secretion of NS1 in mosquito cells

Brefeldin A (BFA) is a fungal macrocyclic lactone that is widely used as an inhibitor of protein traffic in the mammalian endomembrane system [40]. BFA inhibits trafficking from the endoplasmic reticulum and causes disassembly of the Golgi apparatus – both organelles involved in the classical protein secretion pathway [41]. Thus, if treatment of infected cells with BFA is able to inhibit the secretion of NS1, this will be an indication that this protein is being secreted by the classical pathway. To evaluate the effect of BFA on NS1 secretion, cell monolayers of C6/36HT, Aag2 or Vero cells were infected DENV2 or DENV4 at a multiplicity of infection (m.o.i.) of 3. At 6 h p.i. BFA was added at concentrations of 7 µg ml−1 in C6/36HT and Aag2 cells, and 0.1 µg ml−1 in Vero cells. These BFA concentrations had previously been tested for 24 h on the corresponding monolayers and found to be non-toxic to the cell cultures, using an MTS assay (Fig. S1a, available with the online Supplementary Materia). At 24 h p.i. the supernatants were collected and the presence of soluble NS1 was determined by Platelia. As shown in Fig. 1, BFA treatment had no effect on

![Fig. 1. Secretion of NS1 in mosquito and vertebrate cells infected with dengue virus and treated with brefeldin A. Levels of NS1 were measured in cell supernatants collected at 24 h p.i. using a commercial ELISA. Results represent the mean of at least three experiments, with standard deviations indicated; P<0.005. Dark grey bars, control untreated cells; light grey bars, brefeldin A-treated cells.](image-url)
NS1 secretion levels from C6/36HT or Aag cells infected either with DEVN2 or DENV4. However, significant reductions (35 and 55% for DENV4 and DENV2, respectively) in the levels of NS1 were observed in the supernatants of infected Vero cells treated with BFA compared to the untreated control cells. It is known that the secretion of virions occurs through the classical pathway (reviewed in [42]). Thus, to corroborate that the BFA concentrations used were effective, viral titres were measured in supernatants of treated cells and untreated cells that had also been collected at 24 h p.i. The results showed an up to 3 log reduction in the viral yields from treated insect or eukaryotic cells in relation to untreated cells (Table 1), indicating that the BFA concentrations used for each cell type were effective in disturbing the classical secretory pathway. Taken together, these data indicate that the DENV NS1 protein is mainly secreted through a BFA-insensitive pathway from mosquito cells and by a classical pathway from Vero cells.

Silencing SAR1 protein does not affect NS1 protein secretion from mosquito cells

In order to corroborate the results obtained in experiments with BFA, we chose to silence the SAR1 protein, which is a GTPase that participates actively in the classical secretory pathway as part of the COPII vesicles [43]. C6/36HT cells were transfected with a commercial mix containing three SAR1-specific silencers (Qiagen). Silencing was evaluated by confocal microscopy. The levels of NS1 were measured in the supernatants of DENV4-infected cells (m.o.i.=3) collected at 12 h p.i., at a time when a reduction of over 85% reduction was observed for SAR1 expression in transfected cells compared with cells transfected with an irrelevant siRNA used as control (Fig. 2a, b). The results showed no differences in the levels of NS1 between the supernatants collected from SAR1-silenced cells and irrelevant siRNA-treated (control) cells (Fig. 2c). However, a very strong (nearly 5 log) reduction in viral titre was observed in the supernatants of SAR1-silenced cells compared to the controls (Fig. 2d), which confirmed the effectiveness of SAR1 silencing for the inhibition of protein secretion by the classical pathway. These findings, together with those obtained in Table 1.

Table 1. Mean virus yield (n=3) at 24 h p.i. from dengue virus-infected cells treated or not treated with brefeldin A.

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Fig. 2. Secretion of NS1 and virus yield in mosquito cells silenced for SAR1 expression. C6/36HT cells were transfected with an siRNA specific for SAR1, or an irrelevant siRNA as a control. Silencing efficiency was determined by confocal microscopy (a) and expressed as mean fluorescence intensity (arbitrary units) as determined by the ZEN lite 2012 program (Carl Zeiss), with a minimum of 10 cells per field and 10 fields analysed (b). At 24 h p.t. cells were infected with DENV and the levels of NS1 and virus yield in supernatents collected at 12 h p.i. were determined by commercial ELISA (c) and plaque assay (d). The (b), (c) and (d) results represent the mean of at least three experiments, with standard deviations indicated; P≤0.005.
experiments with BFA, support the notion that NS1 secretion is carried out by an alternative route in C6/36HT cells.

**Sequestering cholesterol reduces NS1 secretion from mosquito cells**

To investigate possible alternative pathways followed by secreted NS1 in mosquito cells, DENV4-infected C6/36HT cells were treated at 6 h p.i. with methyl-β-cyclodextrin (MβCD), which is a compound known to sequester cholesterol within the cell. The secreted NS1 hexamer is a lipoprotein and previous work carried out in HEK293 cells showed that MβCD affected DENV2 NS1 secretion [22]. In mosquito cells, our results indicate an approximately 34% reduction in the supernatant NS1 levels for those cells treated with 3 μM, and one of 45% for cells treated with 6 or 9 μM MβCD, compared to non-treated cells (Fig. 3a). The MβCD concentrations used to treat the cells were not toxic, as determined by an MTS assay (Fig. S1b). Moreover, no reduction in viral yield was observed for any of the MβCD concentrations tested (Fig. 3b). These results suggest that reducing intracellular cholesterol negatively affects NS1 secretion, but not virion secretion, in infected C6/36HT.

**Silencing CAV1 protein reduces NS1 protein secretion from mosquito cells**

Synthesized *de novo* cholesterol is transported from the ER to the plasma membrane without passing through the Golgi via a fast pathway that is insensitive to BFA [44-46]. Although little is known about the molecular machinery and trafficking pathways mediating cholesterol transport from the ER to the plasma membrane, it has been proposed that CAV1 is a potential mediator of this function [44]. Based on the localization of NS1 in membrane lipid rafts [47], its lipoprotein composition [22] and our results with MβCD, we hypothesized that NS1 may be following lipid metabolic pathways for secretion by silencing CAV1. We tested this hypothesis by silencing CAV1 using a commercial pool of three siRNAs specific for CAV1 (Qiagen) to transfect C6/36HT cells. At 12 h p.t. C6/36HT cells were infected with DENV4 at m.o.i.=3 and at 12 h p.i. supernatants were collected to determine the NS1 levels and viral titres. The levels of CAV1 expression in cells transfected with specific siRNAs, or an irrelevant siRNA used as a control, were determined by confocal microscopy (Fig. 4a, b). A reduction of nearly 85% in the level of NS1 secretion was observed in the cells silenced for CAV1 compared to control cells (Fig. 4c). At the same time, no differences were observed in the virus titres in the supernatant of the silenced versus control cells (Fig. 4d), indicating that CAV1 silencing did not affect the secretion of viral particles. These data are evidence of the importance of CAV1 in the transport of NS1 to the extracellular medium and suggest that NS1 shares this cholesterol pathway for secretion in mosquito cells.

**Secreted NS1 and E proteins show different glycosylation status**

Although the oligosaccharide processing pathway in mosquitoes has diverged from that in vertebrates, glycoproteins produced in mosquito cells are also subjected to N-linked glycosylation in the ER and to trimming processes in the Golgi complex [48]. Nascent glycoproteins are sensitive to EndoH and become resistant after the transit of the Golgi complex [48]. Secreted NS1 and virion-associated E protein are N-linked glycosylated in the ER, since they are both sensitive to the action of PNGase F. However, while the secreted E protein is resistant to Endo H digestion, as expected, secreted NS1 still remains Endo H-sensitive. These results indicate that in mosquito cells secreted NS1 bypasses the Golgi complex in its transit from the ER towards the extracellular milieu.

**Interaction of CAV1 and NS1 proteins**

To further study the interactions between CAV1 and DENV NS1, an *in silico* analysis was performed to search for the presence of caveolin-1-binding domains (CBD) in the sequence of NS1 [49]. The CBD FXXFXXXXW (where X represents any amino acid) was identified between residues 160 and 168 of the NS1 protein, and found to be well conserved among all four DENV serotypes, and interestingly, also in ZIKV. CBDs were not
found in any other non-structural protein of DENV (NS2A, NS2B, NS3, NS4A, NS4B or NS5), or in any other NS1 protein sequence of a phylogenetically related flavivirus, such as yellow fever, Japanese encephalitis, St Louis encephalitis, Murray Valley encephalitis or tick borne disease (Fig. S2).

Fig. 4. Secretion of NS1 and virus yield in mosquito cells silenced for CAV1 expression. C6/36HT cells were transfected with an siRNA specific for CAV1, or an irrelevant siRNA as a control. Silencing efficiency was determined by confocal microscopy (a) and expressed as mean fluorescence intensity (arbitrary units) (b). At 24 h p.t. cells were infected with DENV and the levels of NS1 and virus yield in supernatants collected at 12 h p.i. were determined by commercial ELISA (c) and plaque assay (d). The (b), (c) and (d) results represent the mean of at least three experiments, with standard deviations indicated; *P<0.005.

Fig. 5. Glycosylation status of NS1 and virion-associated E protein secreted from infected mosquito cells. Cells supernatants were collected at 24 h p.i. and subjected or not subjected to digestion with glycosidases (PNGase F and EndoH). The relative mobility of NS1 and E proteins was analysed by Western blot. Relative band migration was determined using the Image Lab program and expressed as arbitrary units (a.u.). Approximate molecular weights for NS1 and E are indicated. Experiments were carried out three times and typical results are shown.
Proteins that interact with CAV1 usually recognize a region in the CAV1 sequence located between residues 82 to 101 known as the caveolin-1 scaffolding domain (CSD) [49, 50]. The likelihood of the CBD of NS1, the full NS1 dimer, or the full NS3 monomer used as a control interacting with the CSD was predicted using classical mechanic simulations. The negative energy values (eV) obtained in the simulations predict very favourable interactions between the CSD and the CBD domain of NS1 or the full-length NS1 dimer, while, given the positive energy value, no thermodynamically favoured interactions at all are predicted between the CSD and the NS3 protein used as a negative control (Table S1).

Finally, experimental evidence of the direct interaction between the DENV NS1 protein and CAV1 in infected mosquito cells was obtained through in situ proximity ligation assays in DENV4-infected C6/36HT cells. The assay revealed CAV1 and NS1 interactions in the cytoplasm and around the cell nuclei in cells fixed at 6 and 24 h.p.i. (Fig. 6a). The observed PLA signal agrees with the expected sub-cellular localization of the two proteins alone. Meanwhile, no signal was observed when an anti-E Mab was used instead of the anti-NS1 Mab, or in the other negative or technical controls run in parallel (Fig. 6a). The quantification of PLA signals confirmed the observed differences in signal for NS1 and CAV1 interactions versus the controls (Fig. 6b). Overall, the in silico and in vitro assays indicated that CAV1 interacts with NS1 in DENV-infected mosquito cells.

**DISCUSSION**

Previous evidence obtained by us [11, 13] indicated that NS1 is secreted efficiently as a hexamer from DENV-infected mosquito cells. Thus, the main objective of this work was to shed light on the traffic route followed by NS1 in mosquito cells to reach the extracellular space. In general, little is known about the pathways that NS1 follows from the ER until its different destinations [1]. The classical pathway from the ER to the Golgi complex represents the principal route followed by proteins to be secreted (reviewed in [50]). However, treatment of mosquito cells from two different species that had been infected with two DENV serotypes with BFA suggested that the classical pathway is not involved in the secretion of NS1 from infected mosquito cells (Fig. 1). The glycosylation status of the sNS1 (Fig. 5), which remains Endo H-sensitive, also supports the notion that the transit of NS1 is secreted from the ER until secretion bypasses the Golgi complex. This observation was corroborated in C6/36HT cells by experiments in which the expression of SAR1, a major component and modulator of the classical secretory pathway, was silenced (Fig. 2). Conversely, a significant decrease in the secretion of NS1 was observed in Vero cells treated with BFA, or silenced for SAR1, analysed in parallel as controls (Figs 1 and 2). In addition, a decrease in viral titres was observed in all cells when treated with BFA or silenced for SAR1, indicating that the treatments had a disturbing effect on the classical pathway.

Taken together, these results indicate that NS1 uses an alternative non-classical secretory route for secretion from mosquito cells, but follows a classical pathway in vertebrate cells. The ER–Golgi system is an extremely accurate and efficient molecular machinery for exporting proteins; however, other unconventional or alternative protein secretion routes also exist [51]. For example, cholesterol synthesized de novo in the ER is predominantly transported to the cell surface by a Golgi-independent route. This alternative route depends on the capacity of CAV1 to bind cholesterol molecules; it has been postulated that a caveolin-1/caveolar system acts as a cholesterol ‘delivery device’ to transport cholesterol to the plasma membrane when CAV1 is present [44]. The results obtained in cells treated with MβCD or cells silenced for the expression of CAV1 indicate that in mosquito cells NS1 is secreted using this alternative pathway. NS1 secretion was reduced when cholesterol dynamics were perturbed by MβCD treatment (Fig. 3) or by CAV1 knockdown (Fig. 4), suggesting that NS1 requires the cholesterol secretion pathway. These results are in line with the lipoprotein nature of secreted hexameric NS1, which is rich in cholesterol and other lipids [22], and suggest that NS1 might usurp and follow lipid traffic pathways to exit the cell. In this sense it is worth noting that in Vero cells BFA treatment does not completely ablate NS1 secretion, suggesting that perhaps in vertebrate cells not all NS1 is secreted by a classical pathway. Moreover, the treatment of HEK293 cells with MβCD reduces NS1 secretion in similar manner to that observed by us in mosquito cells, without affecting intracellular NS1 levels [22]. Very few non-structural viral proteins are secreted from infected cells. However, one of the best studied secreted non-structural proteins is the rotavirus NSP4 protein, which is secreted in a truncated form by a BFA-insensitive route in infected MA-104 cells [52], and also as oligomeric glyco-lipoprotein by a BFA sensitive route in infected Caco-2 cells [53, 54]. Thus, the use of alternative secretory pathways, presumably lipid-related, for NS1 secretion in vertebrate cells is an open question that deserves further investigation.

Mosquito cells are not able to synthesize cholesterol de novo from simple compounds and obtain the required sterols from the medium [55]. However, these lipids need to be transported between cell compartments to the plasma membrane or secreted to the extracellular medium to maintain the intracellular lipid balance. On the other hand, the non-effect observed on viral titres in mosquito cells treated with MβCD, which is at variance with results obtained in vertebrate cells [56, 57], is consistent with previous results showing that the use of mosquito cells depleted in cholesterol had no effect on DENV replication [58].

In silico analysis indicates that NS1 presents CBD that is well preserved in all four DENV serotypes and absent from other DENV non-structural proteins. The presence of a CBD per se may not necessarily guarantee that a particular protein binds to CAV1, since nearly 30% of the cellular proteins reported to date have some CAV1 binding domain.
but they are not significantly enriched in the CAV1 interactome [49]. This is due in part to aromatic domains that form part of the CBD, which are usually hidden inside the proteins, making it unlikely that they may be interactions with CAV1 [49]. Also, proteins that lack the CBD, such as the dengue virus NS3 or the rotavirus NSP4, can interact with CAV1 using other regions [57, 59]. However, as shown in Fig. 7, in the dimeric NS1 structure the CBD is well exposed and locates between $\beta$-sheets 8 and 9, as part of the $\beta$-connector subdomain, a region with a strong hydrophobic character, predicted to be used by the NS1 dimer to anchor to the ER membrane [21].

Although the structure of the full-length CAV1 is unknown, recent modelling data predict that the CSD, either fully helical or partially unstructured, inserts into the inner leaflet of the lipid bilayer in a stable U-shaped conformation that is oriented almost vertically to the lipid bilayer [50, 60]. Thus, presumably the NS1 dimer located inside the ER lumen and CAV1 located in the cytoplasm will interact and touch within the ER membrane. The penetration of both proteins into the lipid bilayer seems compatible with this supposition and interaction of proteins through membranes was observed previously for other viral proteins. For example, Huang et al. [61] reported that the HIV gp-41 core

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**Fig. 6.** In situ proximity ligation assay for NS1, E and CAV1 in mosquito cells infected with dengue virus. (a) Cells were fixed at 6 or 24 h p.i. and processed following the manufacturer’s instructions. Positive interactions between NS1 and CAV1 are visualized with red dots. Cell nuclei were counterstained with DAPI. Neg Cx, infected cells fixed at 24 h p.i. incubated solely with secondary antibodies. Mock, mock-infected cells incubated with primary and secondary antibodies. Experiments were carried out three times (except for NS1 staining at 6 h p.i.) and typical results are shown. Scale bars represent 10 µm. (b) Assay quantification. The relationship for the integrated density per cell was determined for each interaction tested. The relations were calculated by field in three images per condition from three independent experiments. The differences between the NS1 and CAV1 interactions measured at 6 and 24 h p.i. were not significant ($P<0.01$). The bars indicate standard deviations. a.u. F µm$^2$, arbitrary units of fluorescence per area (µm$^2$) per cell.
(specifically the CBD) interacts with CAV1 through the plasma membrane. A model simulation of the interactions of CAV1 and NS1 is presented in Fig. 7. Theoretical calculations based on the frontier orbitals HOMO and LUMO for the CAV1 scaffolding domain and the NS1 caveolin-1 binding domain fragments predict strongly favourable electrostatic interactions between both proteins (data not shown). The likelihood of NS1 and CAV1 interactions was well supported by classical mechanical and docking simulations, which predicted that very low energies would be required for both proteins to interact. These theoretical interactions were experimentally confirmed in infected cells by in situ PLA (Fig. 6).

CAV1 is the main lipid raft protein and is essential for caveolae formation, yet many caveolae-independent roles have been proposed for CAV1 [61]. Both the CAV1 N-terminus and the C-terminus are located in the cytoplasm, while the CSD, located inside the membrane, has been implicated in membrane attachment, oligomerization, cholesterol binding and interactions with several cellular and viral proteins [49, 60–63]. The process followed by the NS1 to reach the plasma membrane and the cellular factors that may be involved in the hexamerization of the secreted form of the protein are still unknown. Binding of NS1 to the CSD may not only facilitate the transport of NS1 to the extracellular space, but also seed NS1 hexamer formation, as a scaffold protein. Of note, substitution of F160A, disrupting the CBD, results in decreased virus and RNA synthesis without affecting the membrane-binding capacity of the NS1 dimer [21], suggesting that NS1 and CAV1 interactions may play an important role in viral replication.

The evidence indicating that NS1 shares the lipid secretory pathway and interacts with CAV1 raises questions about the consequences for cell physiology and the regulation of lipid transport. Moreover, considering the importance that has been attributed to the soluble form of NS1 in severe dengue pathogenesis, the secretory route of NS1 is becoming an attractive target for the design of antiviral compounds, making it worthwhile to study the secretion process of NS1 in both insect and vertebrate cells in detail.

METHODS

Viral stocks

DENV serotype 4 (DENV4) strain 4H241 and DENV serotype 2 (DENV2) strain 16681, generously provided by Dr Richard Kinney (CDC, Fort Collins, CO, USA), were propagated in suckling mice brain as previously described [64]. Virus titres in mice brain homogenates were determined by plaque assay in BHK-21 as described by Ludert et al. [13].

Cell lines

C6/36HT cells (derived from C6/36 Aedes albopictus cells but adapted to grow at 34 °C), kindly provided by Dr Goro Kuno (CDC, Puerto Rico, USA), were grown in MEM, supplemented with 7% foetal calf serum, nonessential amino acids, vitamins, 0.370 g l⁻¹ sodium bicarbonate, 50 U ml⁻¹ of penicillin and 50 µg ml⁻¹ of streptomycin at 34 °C. Previous results indicate that C6/36HT cells behave as regular C6/36 cells in relation to DENV replication or biology [11, 13, 15]. Aag2 cells, a cell line derived from Aedes aegypti embryos [65], kindly provided by Dr Isabel Salazar (Instituto Politécnico Nacional, Mexico), were grown in
Schneider’s medium supplemented with 7% foetal calf serum (Invitrogen) and 50 U ml\(^{-1}\) of penicillin and 50 µg ml\(^{-1}\) of streptomycin at 28 °C. Vero cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Advanced GibcoRL) supplemented with 8% foetal calf serum (Gibco-BRL), 2 mM L-glutamine, 1.5 g l\(^{-1}\) sodium bicarbonate, 50 U ml\(^{-1}\) of penicillin and 50 µg ml\(^{-1}\) of streptomycin. BHK21 cells were grown in minimal essential medium (MEM; Gibco-BRL) supplemented with 10% foetal calf serum (Gibco-BRL), 2 g/l sodium bicarbonate, 50 U ml\(^{-1}\) of penicillin and 50 µg ml\(^{-1}\) of streptomycin. Vero and BHK-21 cell lines were grown and maintained in a 5% CO\(_2\) atmosphere.

**Measurement of secreted NS1 protein**

The Platelia NS1 Ag (Bio-Rad, Hercules, CA, USA) commercial kit was used to determine the presence of soluble NS1 in cell supernatants. Platelia is a one-step sandwich format microplate enzyme immunoassay that uses monoclonal antibodies for antigen capture and revelation, and was developed for the qualitative or semi-quantitative detection of DENV NS1 antigen in human sera or plasma. The assay was carried out following the procedure indicated by the manufacturer.

**Viral infections**

All experimental infections, either with DENV2 or DENV4, were carried out at an m.o.i. of 3. Cell monolayers grown to about 80% confluence were washed once with Hank’s medium (Gibco-BRL) after the removal of growth medium. Viral inoculum was added and the cells incubated for 1 h at the appropriate growth temperature for each cell line. Afterwards, the inoculum was removed, the monolayer was washed twice with Hank’s medium, enough medium to cover the monolayer was added, and the cells were incubated at the growth temperature for the time required for each experiment.

**Immunofluorescence assays**

C6/36HT cells were grown on 13 mm diameter coverslips placed into 24-well plates. Once the cells reached confluence, they were either infected with DENV or mock-infected, and at different times post-infection stained for immunofluorescence. Briefly, cell monolayers were washed twice with PBS and fixed for 20 min with 4% paraformaldehyde (Merck, Darmstadt, Germany). The fixed monolayers were washed three times with PBS and permeabilized with cold acetone for 5 min. Following three washes with PBS, the cells were blocked for 1 h at room temperature with blocking solution (1X PBS, 10% FBS, 3% BSA and 10 mM glycine). Cells were incubated with primary antibodies in diluted solution (3% PBS, 1% BSA and 10 mM glycine in PBS) overnight at 4 °C. The primary antibodies used were a commercial goat anti-SAR1 polyclonal antibody (Thermo Scientific, Rockford, IL, USA; cat. PA1-9124) and a rabbit anti-CAV1 polyclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA; cat. 894). Afterward, slides were washed five times with PBS and incubated for 2 h at room temperature with anti-goat Alexa-647 or anti-rabbit Alexa-488 (Invitrogen, Waltham, MA, USA; cat. A-21446 and A-11034) in diluting solution as secondary antibodies. During assembly, the coverslips were inverted on 2 µl of the compound DAPI (4′-diamidino-2-phenylindole) to stain nuclei. The cover slips were sealed with nail polish and visualized in an LSM 700 confocal microscope (Carl Zeiss AG, Jena, Germany). Images were processed using the ZEN lite 2012 program (Carl Zeiss AG, Jena, Germany).

**Cell treatments with brefeldin A**

C6/36HT, Aag2 or Vero cell monolayers were grown in 24-well plates. Once the cells reached 80% confluence, they were infected or not infected with DENV (serotypes 4 or 2) at m.o.i. = 3. At 6 h p.i., the cell medium was replaced with new medium containing brefeldin A (BFA; Sigma-Aldrich, St Louis, MO, USA) at a concentration of 7 µg ml\(^{-1}\) for C6/36HT and Aag2 cells and a concentration of 1 µg ml\(^{-1}\) for Vero cells. Supernatants were collected 24 h p.i. and analysed for NS1 presence by Platelia ELISA, while viral progeny were determined by plaque assays. In some cases, the monolayers were used to evaluate cell viability using a commercial MTS assay (CellTiter 96 AQueous One Solution cell proliferation assay; Promega, Madison, WI, USA).

**Cell treatment with methyl-β-cyclodextrin**

C6/36HT cell monolayers were grown in 24-well plates. Once the cells reached 80% confluence, they were infected or not infected with DENV4 at m.o.i. = 3. The supernatants were removed at 6 h p.i. and new medium containing 3, 6 or 9 mM methyl-β-cyclodextrin (MBCD; Sigma-Aldrich) was added. Supernatants were collected 24 h p.i. and analysed for NS1 presence by Platelia ELISA, while viral progeny were determined by plaque assays. In some cases, the monolayers were preserved to evaluate cell viability using a commercial MTS assay.

**Silencing experiments**

C6/36HT cells were grown on 13 mm cover glasses placed in 24-well plates. Eighty per cent confluent monolayers were transfected with either 5 nM CAV1 or SAR1 siRNA (Qiagen, Germantown, MD, USA), 5 nM of control siRNA (Qiagen), or with no siRNA, using Hiperfect transfection reagent (Qiagen) according to the manufacturer’s instructions. Twelve hours after transfection, cells were infected with DENV4 at m.o.i. = 3 for 1 h at 34 °C; afterward, the inoculum was removed, the cells were washed, the medium was changed and the cells were incubated for 12 h at 34 °C. The supernatants were collected to determine the NS1 levels by Platelia ELISA and the viral progeny by plaque assay. In parallel, cell monolayers were treated to determine cell viability by MTS assay, used as indicated by the manufacturers (Promega, Madison, WI, USA). Silencing of CAV1 and SAR1 was evaluated by immunofluorescence assays using confocal microscopy.
Glycosylation status analysis

To compare the glycosylation patterns between secreted NS1 and virion-associated E protein, supernatants were collected from infected mosquito C6/36HT cells at 24 h p.i. After partial removal of the albumin from the supernatants using a commercial kit (Albumin/IgG removal kit; Pierce), aliquots of the supernatants were digested with endoglycosidase H (EndoH; Sigma-Aldrich), peptide-N4-(N-acetyl-betaglucoamnily) asparagine amidase (PNGaseF; Calbiochem), or left untreated at 37 °C overnight. The relative mobility of the proteins after the enzymatic treatment was assayed by Western blot using anti-NS1 mAb (Abcam) or anti-E mAb (Santa Cruz Biotechnology) as primary antibodies. An anti-mouse IgG peroxidase conjugate (Santa Cruz Biotechnology) was used a secondary antibody and the bands were visualized by chemiluminescence (SuperSignal West Femto; Thermo Scientific). The relative migration distance of the bands of interest was estimated using the Image Lab 4.1 program and expressed as arbitrary units.

Analyses of genomic sequences

To evaluate the presence, location and conservation of a putative caveolin-binding domain (CBD) in the amino acid sequences of the NS1 protein of different serotypes of DENV and other flaviviruses, alignments were made using the CLUSTAL W program and MEGA version 7.0. NS1 sequences were taken directly from GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

Classical mechanics simulations

The region corresponding to residues 82 to 101 (DGIW-KASFTTFTVTKYWFYR) containing the caveolin-1 scaffolding domain (CSD) and the region corresponding to residues 158 to 172 (YGFGMFTTNWMMKFR) containing the putative caveolin-1 binding domain (CBD) of NS1 of DENV4, plus the full DENV NS1 (MMDB ID: 117727) and NS3 (MMDB ID: 60353) protein structures, taken directly from the NCBI structure database (http://www.ncbi.nlm.nih.gov/structure), were used in these simulations. Since the 3D structure of CAV1 is unknown, the CAV1 CSD was modelled based on the relations of the integrated density per cell by the following equation:

$$
\Delta E_{ij} = E_{ij} - (E_i + E_j)
$$

where \( \Delta E_{ij} \) is the calculated interaction energy, \( E_{ij} \) is the energy of the interaction system (protein+cavelolin-1 scaffolding domain), \( E_i \) is the energy of the protein of interest in vacuum and \( E_j \) is the interaction energy of the caveolin-1 scaffolding domain in vacuum. With these definitions, a negative \( \Delta E_{int} \) value indicates very favourable interactions between proteins.

Protein interaction assays

Interactions between the CAV1 and NS1 proteins in infected C6/36HT cells were detected using the commercial kit Duolink (Sigma-Aldrich; cat. DUO92101) based on in situ proximity ligation assay (PLA). PLA is a valid alternative to traditional immunoassays that allows protein–protein interactions to be detected and localized in situ with high specificity and sensitivity. In addition, PLA offers the advantage that the protein–protein interactions are studied while the architecture of the cell is preserved, making information on the localization of the protein complexes in the cell available and enabling quantitation at single-cell resolution [66]. Briefly, C6/36HT monolayers were grown on microscope cover glasses, infected with DENV4 (m.o.i.=3), and at 24 h p.i., fixed with 4 % paraformaldehyde for 20 min at room temperature. Cells were permeabiled with cold methanol and blocked with blocking buffer for 30 min at 37 °C. Cells were incubated with mouse anti-NS1 mAb (Thermo Scientific, Rockford, IL, USA) and rabbit anti-CAV1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as primary antibodies, washed and then incubated with the PLA PROBES as secondary antibodies. After washing, the cells were subjected to ligation and amplification reactions following the manufacturer’s instructions, and the signals detected in a confocal microscope (Carl Zeiss AG, Jena, Germany) using a 63× immersion oil objective. The specificity of PLA was evaluated using negative controls, which consisted of infected cells incubated with secondary but no primary antibodies, mock-infected cells incubated with both primary and secondary antibodies, and infected cells where the mouse anti-NS1 primary mAb was replaced with a mouse anti-E mAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The PLA signals were quantified based on the fluorescence microscopy images. Image analysis was performed using Fiji ImageJ software, and quantitative measurements were determined based on the relation of the integrated density per cell by image. The values were determined considering the mean of three images per condition in three independent experiments.

Statistical analysis

The analysis of variance (ANOVA) one-tailed test was used to compare the mean OD values of secreted NS1 between groups.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

References

34. Libraty DH, Young PR, Pickering D, Endy TP, Kalayanarooj S et al. High circulating levels of the dengue virus nonstructural protein


