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Secreted dengue virus nonstructural protein NS1 is an atypical barrel-shaped high density lipoprotein

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ABSTRACT

Dengue virus (DENV) causes the major arboviral disease of the tropics, characterized in its severe forms by signs of hemorrhage and plasma leakage. DENV encodes a nonstructural glycoprotein NS1 that associates with intracellular membranes and the cell surface. NS1 is eventually secreted as a soluble hexamer from DENV-infected cells and circulates in the bloodstream of infected patients. Extracellular NS1 has been shown to modulate the complement system and to enhance DENV infection, yet its structure and function remain essentially unknown. By combining cryo-electron microscopy analysis with a characterization of NS1 amphipathic properties, we show that the secreted NS1 hexamer forms a lipoprotein particle with an open-barrel protein shell and a prominent central channel rich in lipids. Biochemical and NMR analyses of the NS1 lipid cargo reveals the presence of triglycerides, bound at an equimolar ratio to the NS1 protomer, as well as cholesteryl esters and phospholipids, a composition evocative of plasma lipoproteins involved in vascular homeostasis. This study suggests that DENV NS1, by mimicking or hijacking lipid metabolic pathways, contributes to endothelium dysfunction, a key feature of severe dengue disease.

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INTRODUCTION

DENV (genus *Flavivirus*, family *Flaviviridae*) is responsible for the major arthropod-borne viral human disease of the tropics (1). It is estimated that 50-100 million dengue cases occur annually, ranging from mild fever to life-threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (2). The number of severe forms can exceed half a million per year, and lead to tens of thousand deaths (1). DHF is associated to thrombocytopenia, coagulopathy, acute inflammation, frequent hepatomegaly, and most importantly, plasma leakage to which the risk of fatal hypovolemic shock (DSS) is associated (3-5). It has been proposed that an inadequate immune response is the major cause of severe clinical manifestations (6-8). Soluble mediators produced during the acute phase of the disease likely play a pivotal role in vascular permeability, as suggested by the rapid recovery of most DHF patients (9-11).

There is accumulating evidence that flavivirus NS1, a ~50 kDa nonstructural glycoprotein, participates to different stages of the virus life cycle. Part of NS1 resides in virally-induced intracellular organelles where it plays an essential role in viral replication (12-16). The protein, possibly modified by glycosylphosphatidylinositol (GPI), also associates to lipid rafts at the plasma membrane and mediates a signaling pattern common to GPI-anchored proteins in the presence of specific antibodies (17-19). NS1 is eventually secreted by DENV-infected mammalian cells (20-22) and released in the blood stream of infected individuals (23, 24). The protein is detectable in plasma from the onset of fever up to the first days of convalescence, at

concentrations that can exceed several $\mu\text{g/mL}$ (23-25). The amount of NS1 circulating in human sera appears significantly higher in patients who developed DHF rather than dengue fever (26), although it is not clear whether this effect is a cause or a consequence of plasma leakage. *In vitro*, the protein binds cell surface glycosaminoglycans (27) and is targeted to late endosomes upon entry into target cells (28). Pre-incubation of hepatocytes with soluble NS1 enhances subsequent infection by a homologous strain of DENV (28). In addition, both soluble and cell-surface-associated NS1 are capable of modulating complement activation pathways through the formation of immune complexes or binding to host proteins such as the regulatory protein factor H, complement factor C4 or clusterin (29-32).

In this paper, we investigated the structure/function relationship of DENV NS1. We determined a low-resolution three-dimensional reconstruction of the hexamer, which appears as an open barrel with a wide central channel. We identified that specific lipids associate to the NS1 particle, in particular triglycerides, cholesteryl esters and phospholipids that likely fit into the central cavity. These results point to striking similarities between DENV NS1 and high density lipoproteins (HDL) involved in vascular homeostasis.

RESULTS

Three-dimensional (3D) organization of the DENV NS1 hexamer

To get insights into the NS1 structure/function relationship, we sought to characterize the 3D organization of the secreted hexamer. We analyzed an authentic NS1 protein purified from the extracellular medium of Vero cells infected with DENV serotype 1 (DENV-1). Numerous crystallization trials failed to yield diffraction quality crystals. Using cryo-electron microscopy (cryo-EM), we obtained a 3D reconstruction of the DENV-1 NS1 hexamer. The resolution of the reconstruction (at about 3.0 nm) allows to visualize the protein as an open barrel with a 32 point-symmetry, approximately 10 nm in diameter and 9 nm in height, featuring a prominent central channel running along the molecular 3-fold axis (Fig. 1). Three 2-fold symmetric twisted rods, corresponding to the dimeric subunits, form the walls lining the channel. The lateral interactions between the dimeric building blocks take place along a fairly thin area representing at most 5 nm². Each rod is made of two ellipsoidal lobes, which most likely correspond to the individual protomers (Fig. 1 and Fig. S2). The central channel has an estimated volume of 80 nm³ with triangular openings of about 9 nm² at each end, rotated by 40 degrees about the 3-fold axis (Fig. 1C).

The DENV NS1 dimeric subunits behave as membranous proteins in a Triton X-114 detergent phase partitioning assay.

The very narrow interfaces observed between the dimeric subunits, together with the previously reported instability of the NS1 hexamer in nonionic detergents (20, 33)

and its resistance to high molarities of salt or to chelating agents (Fig. S3), indicated that the dimers are essentially held together by weak hydrophobic interactions. Further elements localizing within the channel are likely to be necessary to hold the dimeric rods together, for instance amphiphilic molecules such as lipids, as inferred by the dual behavior of the protein in a TX-114 detergent phase partitioning assay (Fig. 2). Whereas soluble and membranous proteins segregate in the aqueous and detergent phases, respectively, the detergent-treated NS1 partitions into both phases, with a higher proportion of protein retained in the detergent fraction (Fig. 2A). The DENV E protein, which contains a transmembrane anchor, remains exclusively in the detergent phase, as expected (Fig. 2A). NS1 recovered from the detergent-rich phase is essentially dimeric, as observed by treatment of the corresponding fraction with chemical cross-linker dimethylsuberimidate (DMS) and analysis of the resulting products by SDS/PAGE and Coomassie blue staining or mass spectrometry (Fig. 2B and 2C, respectively). In contrast, NS1 from the aqueous phase maintains its characteristic hexameric pattern with DMS treatment (Fig. 2B, 2C). Thus, the soluble hexamer is composed of amphipathic dimeric subunits that behave as membrane proteins upon dissociation. This indicates that dimeric precursors likely interact with lipid membranes prior to hexamer assembly, possibly dragging lipids out of the membrane during the oligomeric transition.

The NS1 protein is secreted as a lipoprotein particle rich in triglycerides.

To investigate the presence of lipids in the DENV NS1 particle, we subjected NS1 to treatment with organic solvent and separated the lipid moiety on thin layer chromatography (TLC). A predominant species, well resolved on the TLC plate (Fig. 3A, arrow), was recovered and analyzed by nuclear magnetic resonance (NMR, Fig. 3B). The ^1H NMR spectrum displays the characteristic signals of triglycerides (TG), including peaks at 5.17, 4.17 and 4.03 ppm related to protons of the glycerol moiety, and the corresponding cross peaks in 2D double quantum filtered correlation spectroscopy (DQF-COSY) (Fig. 3B). This was corroborated by gas-liquid chromatography (GLC) analysis of NS1-associated TG (Fig. 3C). TG molecules are formed by three fatty acid chains that can all be different or all alike. Their length and degree of saturation is variable, although aliphatic chains with 16, 18 and 20 carbon atoms, which may contain one or two double bonds, are most frequently observed. GLC analysis of fatty acids derived from DENV-1 NS1 TG shows two major peaks corresponding to saturated palmitic acid (16:0) and unsaturated oleic acid (18:1), as well as minor polyunsaturated palmitoleic acid (16:1) and linoleic acid (18:2), and other peaks that likely correspond to background signals (Fig. 3C). Compared to authentic NS1 recovered from the supernatant of DENV-1 infected Vero cells, recombinant DENV-2 NS1 produced in *Drosophila* S2 cells also contains TG that display a homogeneous fatty acid profile composed of palmitic acid and stearic acid (18:0) (Fig. 3C). TG molecules extracted from control HDL particles are formed by palmitic, oleic and linoleic acid (Fig. 3C).

The NS1 lipid moiety is similar to the lipid cargo of high density lipoproteins.

In addition to TG, we were able to isolate cholesteryl ester (CE) molecules from the NS1 hexamer of DENV type-1 and -2. Sterol esters were isolated from recombinant NS1 preparations, separated on TLC plate and saponified. The corresponding sterol fractions were found to be identical to standard cholesterol both by GLC and mass spectrometry (Fig. 4). Other lipid species were identified by TLC and GLC, including mono- and diacylglycerol, phosphatidylcholine (PC) and phosphatidyl-ethanolamine (PE). We estimated the number of lipid molecules by comparing the amount of fatty acid chains (fatty acid methyl esters recovered by transesterification of NS1-associated lipids) to a defined amount of a C17 standard on GLC. By this method, we found that each NS1 hexamer binds 6 TG molecules (i.e. one per protomer), twice as many mono- and diacylglycerol molecules, 16-33 CE and 18-27 phospholipids (Table 1). Cholesterol and sphingomyelin (SM) were also observed on TLC but not quantified. Overall, the NS1 lipid composition is very similar to that of HDL, although HDL show a higher lipid:protein weight ratio (Table 1). Accordingly, the NS1 hexamer is denser than what would be expected for an HDL particle of similar size (1.20-1.23 g/mL for the NS1 hexamer in comparison to 1.063-1.12 g/mL for 9-12 nm wide HDL) and rather fits within the class of very high density lipoproteins (VHDL, 1.21-1.25 g/mL), close to soluble proteins (1.26-1.28 g/mL). Of note, we found that the hexameric organization and lipid content is identical for two different DENV serotypes of the NS1 protein (Table 1 and Fig. 3C).

Modeling lipid organization within the DENV NS1 channel.

We calculated that about 70 lipid molecules could be extracted from a single NS1 hexamer particle, representing a total volume of roughly 75 nm^3 based on the specific volumes of the various lipid species (34, 35). These numbers do not take into account molecules of cholesterol and SM that are part of the lipid cargo. Depending on the lipid composition, and the presence of cholesterol in particular (35), lipid compaction events can occur, suggesting that the whole NS1 lipid cargo can fit well into the 80 nm^3 channel. TG and CE probably constitute the central lipid core while charged lipids such as PC, PE and SM rather occupy the outer layers, at either end of the NS1 channel. As described above, we estimate that there are between 18-27 phospholipids in total (i.e. 9-13 per channel opening). Considering an average surface area of 0.5 nm^2 per phospholipid (34, 35), these can fill about 60% of the 9 nm^2 triangular surface present at each end of the NS1 channel (Fig. 1), leaving extra space for other polar lipids, such as sphingolipids and glycolipids. Altogether, the amount of lipids extracted from the NS1 particle is compatible with the dimensions of the central channel.

DISCUSSION

The flavivirus nonstructural protein NS1 has long been reported to undergo a complex maturation process. On the one hand, it is attached to intracellular membranes and the surface of infected cells, on the other, it is secreted in the extracellular medium and circulates in the serum of infected patients. In this study, we used a combination of biochemical and structural approaches to investigate the organization and composition of NS1 released by DENV-infected cells.

We obtained a cryo-electron microscopy reconstruction of the secreted form of NS1, which reveals a barrel-like hexameric particle of about 10 nm in diameter, in which the three dimeric rods interact along narrow lateral surfaces and form a wide central channel (Fig. 1). The channel was a most unexpected finding and we investigated its possible contribution to NS1 structure/function. As the contact areas between the dimers appeared insufficient to maintain the hexameric state of NS1 in solution (36), and were not consistent with the high stability of the protein in an aqueous environment, we searched for the presence of stabilizing elements that would localize within the channel. The dual behavior of the NS1 protein in a TX-114 detergent phase partitioning assay, in which the dimeric subunits partition to the detergent phase just as membrane proteins while the NS1 hexamer remains in the aqueous phase, indicated that amphiphilic molecules such as lipids could possibly be present (Fig. 2). Lipids could indeed be isolated from purified hexamer preparations and we observed a heterogeneous population by TLC with one predominant species identified by NMR as TG (Fig. 3). Other NS1-associated lipid species included mono-

and diacylglycerol, cholesterol, CE, PC, PE and SM, an overall lipid composition thus very close to that of endogenous HDL circulating in plasma.

Despite the notable homology of their lipid content, DENV NS1 and HDL particles have a fundamentally different protein organization. While HDL particles are composed of narrow ribbons of apolipoprotein A1 that tie up a large lipid bundle (37-39), the NS1 hexamer consists of a thick protein shell organized as an open barrel that can only accommodate a much smaller lipid cargo compared to HDL of similar size. Accordingly, the density of the NS1 hexamer (1.20-1.23 g/mL) rather corresponds to the smallest subclass of HDL particles (i.e., less than 7 nm in diameter). We estimate that over 70 lipid molecules associate to an NS1 hexamer, which is in agreement with the amount of lipids that could theoretically fit into the central channel (35). According to a model of lipid distribution in lipoprotein particles (34), we hypothesize that TG, along with CE, preferentially constitute the central core of the lipid cargo, around which polar lipids (PC, PE, SM in particular) can pack, their charged heads facing the aqueous environment at either opening of the NS1 channel.

The mechanism by which NS1 acquires its lipid cargo appears to involve an initial interaction of NS1 dimers with intracellular membranes. Since recombinant NS1, lacking the putative glycosylphosphatidylinositol (GPI)-anchor signal present in the downstream NS2A coding region (17, 19), shows no difference in lipid binding capacity in comparison to native NS1, a GPI modification cannot account for NS1 attachment to membranes. Early studies following the folding process of NS1 in the endoplasmic reticulum (ER) indicated that the NS1 monomer is water-soluble and

becomes membrane-associated once the protein dimerizes (40). We propose that lipid-binding sites form during the dimerization process itself, possibly at the dimer interface (i.e. between the two lobes of the dimer, Fig. 1). The 1:1 molar ratio of TG to NS1 (i.e., six TG molecules per NS1 hexamer, Table 1) suggests that NS1 dimers would bind specifically the hydrophilic head of the lipid. As intracellular TG essentially accumulates between the two leaflets of the ER membrane, from which cytosolic lipid droplets (LD) arise (41, 42), one possibility is that NS1 dimers insert into the hemi-membrane, thus gaining access to the neutral lipid pool. Such insertion would locally destabilize the organization of lipids, favoring the association of three NS1 dimers around a lipid cargo and its release from the membrane by pinching off, as pictured in Fig. S1. The association of secreted NS1 with TG links the DENV cycle to the biogenesis of LD within the infected cell. This corroborates a recent report indicating that the interaction of the DENV capsid (C) protein with LD is essential for viral replication and virus particle assembly (43), thus raising the question as to whether C and NS1 cooperate in any way to hijack nascent LD and support intracellular viral processes.

The discovery that DENV NS1 carries lipids in the extracellular milieu (Fig. S1) also has important pathophysiological implications. Lipoproteins are known to play a key role in vascular homeostasis and defects in lipoprotein functions can affect coagulation and predispose to vascular inflammation and thrombosis (44, 45). By diverting certain lipids from their original fate, NS1 has the potential to interfere with the biogenesis of endogenous lipoprotein particles (46) or to deregulate the intracellular

lipid sensing machinery during entry into target cells (47, 48). In line with our observations, several reports show that DENV-infected patients who developed DHF/DSS present a decrease of high- and low-density lipoprotein content in plasma, and altered levels of cholesterol and triglycerides (49-51). Moreover, the nutritional status of children appears to impact the risk of developing a fatal DHF/DSS (52, 53). The NS1 lipoprotein particle can also directly modulate host response by binding to factors of the complement system (29, 32), either as part of a viral escape mechanism or as a mean to exacerbate inflammation (54, 55).

In conclusion, our study identifies a novel class of lipoprotein particle, exemplified by the barrel-like DENV NS1 protein. The organization of the NS1 hexamer around a “soft” lipid core explains at least in part the lack of success in the crystallization efforts and the limited resolution of the cryo-EM reconstruction. The striking similarities of the NS1 lipid moiety with that of HDL suggests that NS1 has the potential of interfering with the vascular system and inducing important physiological disorders during its circulation in infected patients. The NS1 properties reported here are common to different DENV serotypes, opening promising new therapeutic avenues to fight dengue disease, such as interfering with NS1 secretion or targeting its hydrophobic channel.

EXPERIMENTAL PROCEDURES

Three-dimensional reconstruction of the DENV NS1 hexamer. Cryo-electron microscopy analysis of the NS1 protein sample was carried out with a Philips CM12 transmission electron microscope using a LaB6 filament at 120 kV. Images were analyzed with EMAN and EM software packages. Characteristic class averages representing different orientations of the particles on the micrograph were used to calculate an initial 3D reconstruction, which was further refined, as described in the Supporting Information section, to obtain a final electron density map to a resolution of about 3 nm.

Triton X-114 detergent phase partitioning assay. Triton X-114 detergent phase partitioning was performed on purified preparations of NS1 using a pre-condensed preparation of Triton X-114 at a 1% final concentration. Following an overnight incubation at 4°C, potential aggregates were pelleted by high speed centrifugation (insoluble fraction) before separating the detergent from the aqueous phase at 30°C. In a set of experiments, proteins from the aqueous and detergent phases were further cross-linked with dimethylsuberimide (DMS) at 4°C. Proteins were analyzed by SDS-PAGE and Coomassie Blue staining or by mass spectrometry on a PBS II mass reader (Ciphergen Biosystems, Inc.). The DENV envelope protein E was used as a control transmembrane protein and detected by Western Blotting.

Characterization of the NS1 lipid moiety. Potential lipid components were extracted using a standard solvent extraction procedure. Lipid were analyzed by TLC and stained with iodine or dichlorofluoresceine. Different lipid classes were trans-esterified for their characterization by GLC. Fatty acid methyl esters (FAMES) were analyzed on an Agilent Technologies chromatograph model 6890 equipped with a BPX 70 fused silica capillary column (60mx 0.25mm i.d., 0.25 μ m film thickness). One of the predominant lipid species was also subjected to NMR (see Supplemental Methods). The NMR spectra were acquired on a Bruker 600MHz spectrometer equipped with a triple resonance z-axis gradient cryoprobe at 298K, in CDCl₃.

Full experimental procedures and associated references are available as Supporting Information.

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Author Contributions M.F., I.G. and F.A.R. designed the experiments and analyzed the data. I.G., M.F., F.C., J.E.V., J.S., E.G., F.M., and E.L. performed experiments. J.d'A., P.C. and T.K. provided tools, materials and reagents. M.F., F.A.R. and I.G. wrote the paper.

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FIGURE LEGENDS

Fig. 1. Cryo-EM analysis of DENV-1 secreted NS1. (A) Representative field showing a preparation of NS1 purified from DENV-1-infected Vero cell supernatants. (B) Characteristic class averages of NS1 used to produce an initial 3D reconstruction. (C) 3D reconstruction of NS1, in an isosurface representation. The upper left panel displays a view down the 3-fold molecular axis. The lower-left panel is a view down the 2-fold axis relating two dimeric subunits (90 degrees rotation as indicated). The lower-right panel shows the NS1 particle further rotated by 180 degrees about the 3-fold axis, down the 2-fold axis relating protomers within a dimeric subunit. The intradimer contacts are much more extensive than the interdimer ones. Note the large central channel of roughly 80 nm³ running along the 3-fold axis of the molecule.

Fig. 2. The NS1 hexamer is composed of amphipathic dimeric subunits. (A) Purified DENV-1 NS1 was treated with the nonionic detergent TX-114 at a 1% final concentration (Tot). Aggregated or insoluble material was pelleted in the cold at high-speed centrifugation (Ins) before separating the aqueous (Aq) and detergent (Det) phases. Proteins from the two phases were analyzed by SDS-PAGE and Coomassie blue staining (left panel). The transmembrane DENV-1 envelope protein E, used as a control, was detected by immunoblotting (right panel). (B-C) Oligomeric state of TX-114 partitioned NS1. The protein present in the initial preparation (Tot) and the two phases (Aq and Det) was chemically cross-linked with DMS as described in

Supplementary Methods and analyzed by (B) SDS-PAGE and Coomassie blue staining or (C) SELDI-TOF mass-spectrometry. The stars indicate irrelevant peaks corresponding to double-charged species (2H).

Fig. 3. The NS1 hexamer carries a lipid cargo rich in triglycerides. (A) Thin layer chromatography of extracted lipids colored by iodine vapor. Lane 1: PC (flash) and PE (rhodamine-labeled, pink) used as markers, Lane 2: lipids associated to DENV-1 NS1. One predominantly represented lipid species (arrow) was extracted for further analysis. (B) Identification of triglyceride (TG) by 600 MHz NMR 1D proton and 2D COSY-DQF spectra. Characteristic NMR signals and the corresponding protons of the TG moiety are connected by arrows. The cross-peaks between the glycerol protons signals (at 5.17, 4.17 and 4.03ppm) are circled, and the connectivities traced. Vinylic to allylic protons correlations are boxed. Aliphatic and methyl protons are found around 1.2 and 0.8ppm, respectively, as expected. (C) GLC analysis of fatty acid derivatives from trans-esterified triglycerides. Triglycerides associated to DENV-1 and DENV-2 rNS1 are composed of three saturated and unsaturated aliphatic chains of 16 or 18 carbon atoms long. HDL is shown as a control.

Fig. 4. Identification of NS1-associated cholesteryl ester by GLC-MS. (A, B) Gas chromatogram of silylated sterols. In A, free cholesterol was used as a standard. In B, the NS1-related sterol derivatives were obtained from the sterol ester population that was eluted from the TLC plate and saponified. The sterol fraction was further separated

from fatty acids by TLC, silylated and analyzed by GLC. (C) Identification of the NS1-related sterol derivative by electron impact mass spectrometry. The table presents the retention times (RT) as observed in *A* and *B*, as well as the m/z ratios of the characteristic ion fragments. The values obtained for the NS1-related sterol derivative and cholesterol by GLC-MS analysis are identical, suggesting that the NS1-associated sterol ester is essentially cholesteryl ester.

Figure 1

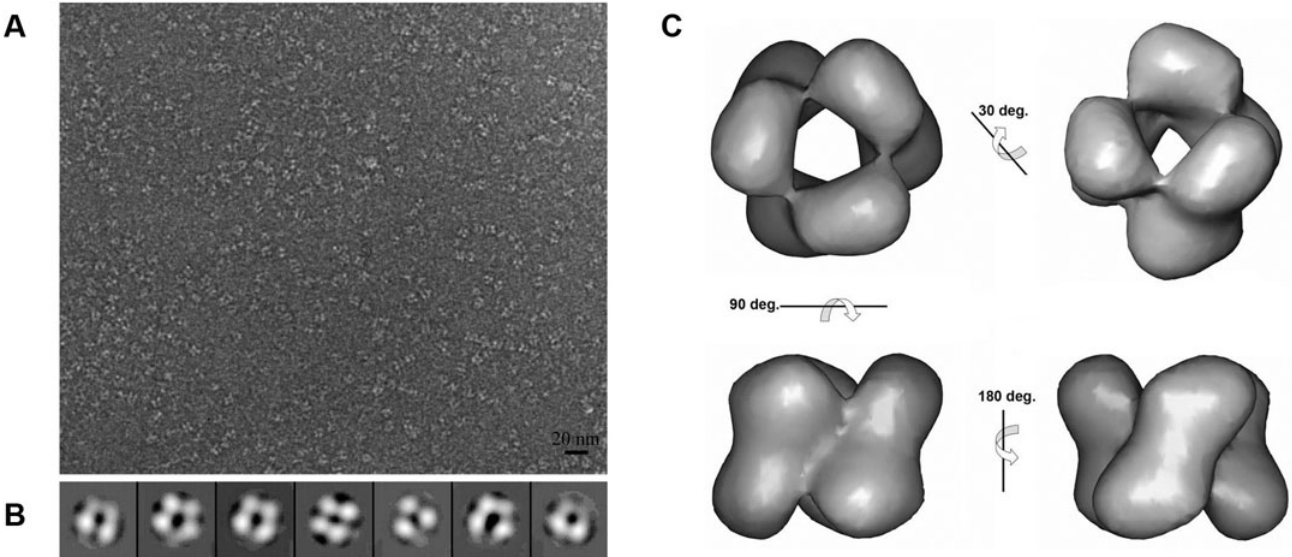


Figure 2

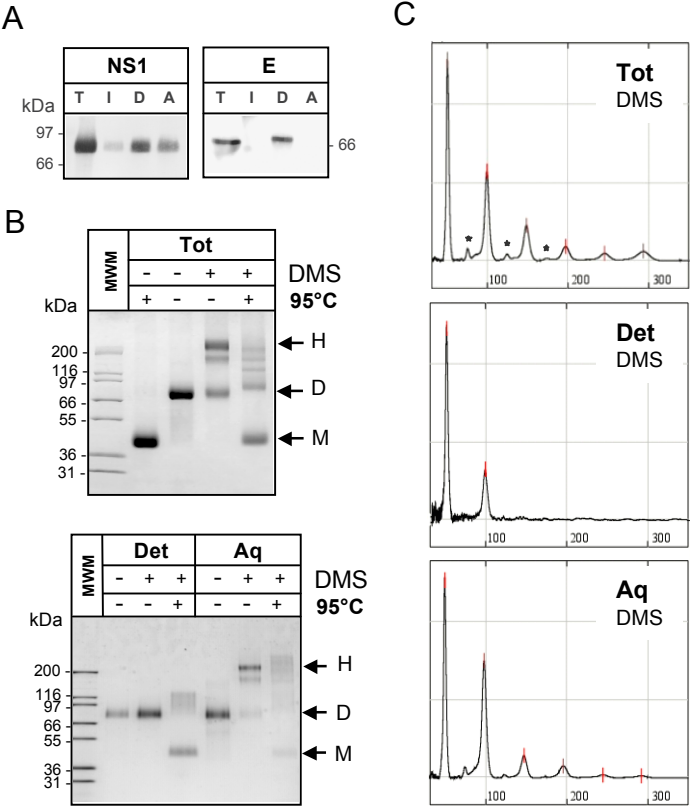


Figure 3

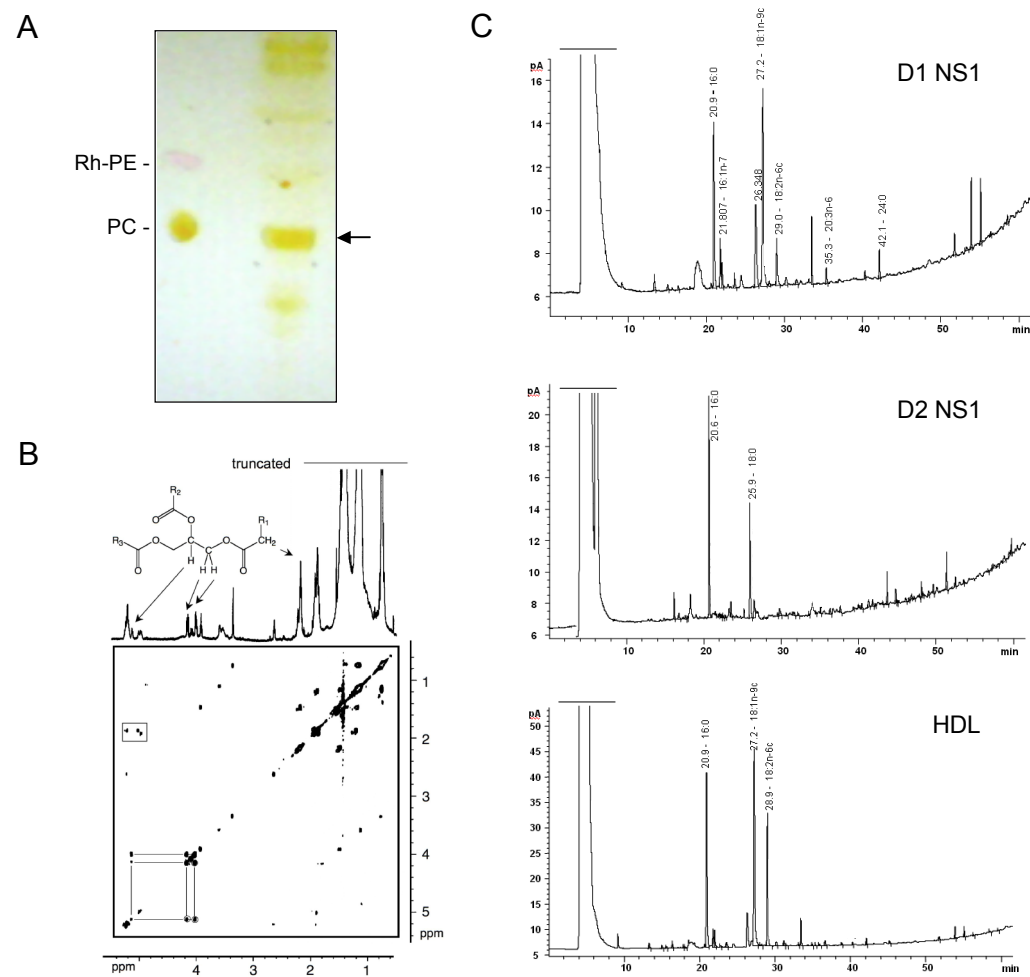


Figure 4

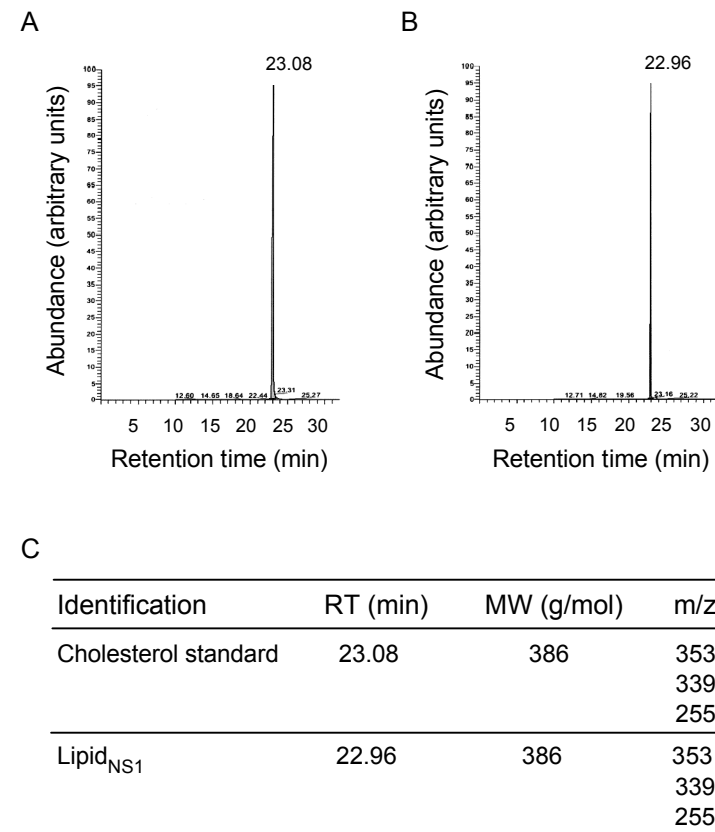


Table 1: Quantification by gas-liquid chromatography of different lipid species associated to the DENV-1 NS1 hexamer^{a,b}

	Triglycerides	Mono-/Diglycerides	Cholesterol Ester	Phospholipids
DENV-1 rNS1 ^c	5.3-4.0	7.9-9.8	11.4-23.3	12.5-19.1
HDL	21.9	11.8	98	63.4

^a nmol, relative values using a C17 fatty acid calibration standard

^b lipids recovered from 200 µg purified protein preparations (approximately 4.5 nM of the 50 kD NS1 protomer; See Supplemental Material)

^c values reported for two different purified NS1 preparations