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Dengue virus capsid protein interacts specifically with very low-density lipoproteins

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Abstract

Dengue affects millions of people worldwide. No specific treatment is currently available, in part due to an incomplete understanding of the viral components' interactions with host cellular structures. We tested dengue virus (DENV) capsid protein (C) interaction with low- and very low-density lipoproteins (LDL and VLDL, respectively) using atomic force microscopy-based force spectroscopy, dynamic light scattering, NMR and computational analysis. Data reveal a specific DENV C interaction with VLDL, but not LDL. This binding is potassium-dependent and involves the DENV C N-terminal region, as previously observed for the DENV C-lipid droplets (LDs) interaction. A successful inhibition of DENV C-VLDL binding was achieved with a peptide drug lead. The similarities between LDs and VLDL, and between perilipin 3 (DENV C target on LDs) and ApoE, indicate ApoE as the molecular target on VLDL. We hypothesize that DENV may form lipoviroparticles, which would constitute a novel step on DENV life cycle.

From the Clinical Editor: Using atomic force microscopy-based force spectroscopy, dynamic light scattering, NMR, and computational analysis, these authors demonstrate that dengue viral capsid proteins (DENV C) bind to very low density lipoprotein surfaces, but not to LDLs, in a potassium-dependent manner. This observation suggests the formation of lipo-viroparticles, which may be a novel step in its life cycle, and may offer potential therapeutic interventions directed to this step.

Key words: Dengue virus capsid protein; Lipoproteins; Single-molecule studies; AFM-based force spectroscopy; Dynamic light scattering

Dengue virus (DENV) causes a mosquito-borne disease affecting millions of people.[1,2] With around 390 million new infections per year worldwide,[3] DENV infection results in 500,000 severe hospitalization cases and over 20,000 deaths every year.[1,2] Although the disease vectors, *Aedes spp.* mosquitoes,[4,5] were originally confined to tropical and sub-tropical climates, they are now spreading to more temperate regions, including North America and Europe, where the first cases of autochthonous infection were detected in 2010.[1,2,6–8] A recent outbreak in the Madeira island (Portugal) resulted in over 2000 autochthonous infections.[9,10] No specific and effective treatment is currently available for DENV infection,[1] in part due

Abbreviations: AFM, atomic force microscopy; ApoE, apolipoprotein E; Cα RMSD, α-carbons root mean squared deviation; DENV, dengue virus; DENV C, dengue virus capsid protein; D_H, hydrodynamic diameter; DLS, dynamic light scattering; HCV, hepatitis C virus; HSQC, heteronuclear single quantum coherence; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; LD, lipid droplet; LVP, lipoviroparticles; NMR, nuclear magnetic resonance; VLDL, very low-density lipoprotein.

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to an incomplete understanding of the molecular basis underlying the viral life cycle, namely the interactions between the viral components and host features.[11]

DENV is a member of the *Flaviviridae* family, which includes other important human pathogens such as West Nile virus, yellow fever virus and hepatitis C virus (HCV).[11,12] These viruses are known to infect the host liver, causing relevant lipid metabolism impairments,[13–15] namely, liver steatosis, increased number and size of intracellular lipid droplets (LDs), and disordering of the intracellular membrane network. Furthermore, lipoprotein bloodstream levels are altered in the most severe cases of DENV infection.[16,17] Rather than simply affected by the viral infection, blood lipoproteins seem to play a critical role in the flaviviruses' life cycles.[18] Recent studies provided evidence that HCV virions actually fuse with premature very low-density lipoproteins (pre-VLDL) during viral assembly and maturation, in the endoplasmic reticulum lumen.[19,20] This process leads to the formation of lipoviroparticles (LVP), which are released to the bloodstream as normal lipoproteins.[19,20] LVP are lipoprotein-like structures enriched in triglycerides that contain a viral nucleocapsid core and, at the surface, viral envelope (E) proteins, as well as human apolipoproteins (Supplementary Material, Table S1).[19,20] As such, they follow the low-density lipoproteins receptor (LDLR) pathway for cell entry, being highly infectious and able to evade host defenses.[18–20] Other *Flaviviridae* species were found to infect cells through the LDLR pathway, namely hepatitis G virus and bovine viral diarrhea virus.[18]

DENV has not been reported to form typical LVP, as observed for other similar closely related viruses.[19,20] However, recent *in vitro* studies in cell lines showed that DENV may produce alternative viral structures, with a different morphology.[21] Briefly, it was reported that virus-induced vesicles can be formed in DENV infection, possibly from the plasma membrane or formed within multivesicular body compartments, which fuse at the cell surface, releasing the microvesicular content, possibly alternative DENV RNA-containing virions. This process shares striking resemblance to the hypothesized LVP based infection, which can also potentiate DENV infection and transmission, probably by shielding DENV from the immune system action. The process of LVP-mediated infection may indeed be a major advantage for DENV and similar flaviviruses' life cycles and, eventually, a widespread mechanism among them. Thus, the role of plasma lipoproteins in DENV infection deserves to be investigated in greater detail. Based on this, we hypothesize that, if DENV can form LVP, they should contain copies of the DENV Capsid protein (DENV C), forming the viral nucleocapsid, as it was previously established for HCV LVP.[19,20] For that to occur, it is reasonable to expect that the multifunctional DENV C (further described in Supplementary Material)[11,22–25] may interact specifically with human lipoprotein intrinsic components (either lipids or proteins).

We therefore tested the ability of DENV C to directly and specifically interact with human lipoproteins, isolated from blood plasma, employing atomic force microscopy (AFM)-based force spectroscopy, complemented with dynamic light scattering (DLS), nuclear magnetic resonance (NMR) and a computational analysis of the protein structure to correlate the variation in size of VLDL with the binding of DENV C. We also tested whether pep14-23, an amidated peptide previously shown to have inhibitory action against DENV C binding to lipid droplets,[24] could inhibit those interactions. The use of these combined methodologies exemplifies the advantages of employing in parallel different nanoevaluation techniques and the potential of this nanoscale approach to study protein-ligand interactions at the single-molecule level. The lipoproteins used were very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) (oxidized LDL were also tested; results in Supplementary Material Figure S3). For the force spectroscopy measurements, the AFM tip, chemically functionalized with DENV C,[22,26] was approached and retracted from the plasma lipoproteins, non-covalently immobilized on a solid surface, enabling the detection of binding/unbinding events, and their quantitative evaluation (for review, see Carvalho & Santos, 2012).[27]

Methods

DENV C expression and purification

DENV C from serotype 2, strain New Guinea, was expressed and purified as recently described.[22,24] After purification, Coomassie-stained SDS-PAGE gels showed only one band at ~13 kDa, consistent with the DENV C molecular weight and a purity level above 95%. The purified protein showed an UV–Vis absorption spectrum typical from tryptophan, which was used to calculate the protein concentration through the absorption at 280 nm with a molar absorption coefficient (ε_{280nm}) of 5500 M^{-1} cm^{-1}. The purified protein was shown to be properly folded, giving a characteristic α-helical circular dichroism (CD) spectrum (between 200 and 260 nm) and possessing, in the ^{15}N-^{1}H heteronuclear single quantum coherence (HSQC) spectra analysis, peaks with the proper chemical shifts of DENV C (shown in Supplementary Figure S4).[23,24]

Lipoprotein preparation for AFM measurements

Human lipoproteins (Kalen Biomedical LLC, Montgomery Village, MD, USA), isolated from human blood plasma by ultracentrifugation through a density (*d*) gradient, were kept at 4 °C in a solution with 154 mM NaCl, 5.6 mM Na_2HPO_4, 1.1 mM KH_2PO_4, and 0.34 mM EDTA, at pH 7.4. VLDL ($d \leq 1.006$), highly oxidized LDL, and LDL ($1.019 \leq d \leq 1.063$) were purchased at minimum total protein concentrations of 1 mg/mL (VLDL and oxidized LDL) or 5 mg/mL (LDL). Fifty microliters of lipoproteins solution were placed on thin freshly cleaved muscovite mica and allowed to deposit for 30 min at room temperature. Non-adherent lipoproteins were removed by 5 sequential washes with 20 mM Tris–HCl buffer, pH 7.4, containing 1 mM EDTA, 1 mM EGTA and 100 mM KCl (or 100 mM NaCl). Samples were loaded into the AFM apparatus and allowed to equilibrate for 10 min before measurements. In the experiments to test the inhibition by pep14-23 (H-NMLKRARNRV-NH2; Schafer-N, Copenhagen, Denmark),[24,28] the peptide was incubated for 15–20 min before measurements in solution with the deposited VLDL.

Functionalization of AFM tips

Force spectroscopy measurements were performed using DENV C functionalized silicon nitride AFM tips. For the