Biological: Full-length

Dengue virus-induced autophagosomes and changes in endomembrane ultrastructure imaged by electron tomography and whole-mount grid-cell culture techniques

Shobha Gangodkar¹, Preksha Jain¹, Nishikant Dixit¹, Kanjaksha Ghosh²
and Atanu Basu^{1,*}

¹Electron Microscopy Group, National Institute of Virology (ICMR), 20A Dr Ambedkar Road, Pune 411001, India and ²National Institute of Immunohematology (ICMR), 13th Floor KEM Hospital, Parel, Mumbai, India

*To whom correspondence should be addressed. E-mail: basua@icmr.org.in

Abstract The biogenesis events and formation of dengue virus (DENV) in the infected host cells remain incompletely understood. In the present study, we examined the ultrastructural changes associated with DENV-2 replication in three susceptible host cells, C6/36, Vero and SK Hep1, a cell line of human endothelial origin, using transmission electron microscopy, whole-mount grid-cell culture techniques and electron tomography (ET). The prominent feature in C6/36 cells was the formation of large perinuclear vacuoles with mature DENV particles, and on-grid whole-mount examination of the infected Vero cells showed different forms of DENV core structures associated with cellular membranes within 48 h after infection. Distinct multivesicular structures and prominent autophagic vesicles were seen in the infected SK Hep1 cells when compared with the other two cell lines. ET showed the three-dimensional organization of these vesicles as a continuous system. This is the first report of ET-based analysis of DENV-2 replication in a human endothelial cell line. These results further emphasizes the strong role played by intracellular host membranes–virus interactions in the biogenesis of DENV and strongly argues for the possibility of targeting compounds to block such structure formation as key anti-dengue agents.

Keywords electron microscopy, dengue, electron tomography, on-grid cell culture

Received 28 January 2010, accepted 14 July 2010, online 12 August 2010

Introduction

The earliest electron microscopic observation of dengue viruses (DENV) probably dates back to 1948 by Sabin and Schleisinger on mouse brain-derived virus particles and later in 1954 by Reagan and Brueckner in cell culture [1]. These studies, although limited by many technical hurdles like specimen processing and imaging artefacts, pioneered the use of electron microscopy (EM) in DENV research. Subsequently, over the last five decades, the ultrastructure and morphogenesis of DENV have been

studied extensively and many facets of DENV replication in host cell and particle organization of the virion have begun to emerge [2,3]. Despite much research on DENV morphology, a review of the present knowledge on morphogenesis events of DENV replication and assembly in the host cell remain, to a great extent, incompletely understood. The backbone of these lacunae resides with the fact that synchronized time-lapse imaging of DENV-infected cells to monitor the progress of the virus replication events using EM-based techniques have

been compromised by methodology limitations, although molecular and analytical cell biology tools and fluorescence microscopy have given insights into virus replication biology [3]. However, several novel techniques of EM like electron tomography (ET) and cryoelectron microscopy have recently emerged to visualize virus–host interactions in 3D, giving better insights into replication events [4,5].

In the present study, we used a combination of conventional transmission electron microscopy (TEM) methods along with on-grid cell culture and ET to study the nature of cytoplasmic membrane alterations seen in DENV-2-infected cell lines from different origins – including a human endothelial cell line, SK Hep1 [6].

Materials and methods

Cells and virus

Vero E6 cells were grown in minimum essential medium (MEM; HiMedia Ltd, India) supplemented with 10% foetal bovine serum (GIBCO, USA), 1% L-glutamine, penicillin and streptomycin (HiMedia Ltd, India). Cells were grown in six-well tissue culture plates (Nunc, USA) in a culture volume of 1 ml and under 5% CO₂ at 37°C. The C6/36 cells were taken from the cell repository of the National Institute of Virology (NIV) in Pune and grown in Mitsuhasi and Maraorosch insect cell growth medium (HiMedia Ltd, India) supplemented with penicillin G, streptomycin, yeastolate and 10% tryptose phosphate broth at 29°C. The third cell line we used in this study was the SK Hep1, a cell line of human endothelial origin [6] and susceptible to DENV (unpublished data). The SK Hep1 cells were obtained from the National Center for Cell Sciences in Pune and grown in MEM supplemented with 10% foetal bovine serum, 1% L-glutamine, streptomycin and penicillin solution (HiMedia Ltd, India). The virus used in the present study was the Tr1751 strain of DENV-2 virus obtained from the NIV virus repository.

Virus infection

Vero E6, C6/36 and SK Hep1 cells were grown to a confluency of >80% as observed by inverted phase-contrast microscopy (Eclipse T2000, Nikon Corp.,

Japan) and exposed to 1×10^3 LD₅₀ dose of DENV-2 for 1 h with gentle shaking at 37°C and under 5% CO₂. The virus-containing media was washed off with two changes of media and the cells were grown in complete media at 37°C and 5% CO₂.

Culturing cells on EM grids

Vero E6 and C6/36 cells were cultured directly on EM grids using the earlier method of Hyatt *et al.* [7] with modifications. Briefly, 400-mesh nickel EM grids (Ted Pella, Switzerland) were provided with formvar support, carbon coated and sterilized by floatation (coated side down) over 3% glutaraldehyde under ultraviolet light. The grids were then removed, rinsed thoroughly with sterile deionized water and used for culturing cells. The surface of the formvar support was further conditioned for cell adhesion by treatment with poly-L-lysine (Sigma Chemicals, USA) or alcian blue (Sigma Chemicals, USA). Cells grown to more than 80% confluency were made into a single-cell suspension, seeded directly on the coated grids and cultured with complete media under 5% CO₂ and at 37°C. Cells were stained with propidium iodide to image the nuclei and layered-growth morphology on the grid-support film. The SK Hep1 cells were not cultured on-grid but in conventional six-well plastic culture plates (Nunc, USA) using the growth conditions described earlier [8]. The grid-cell cultures were processed for TEM as described earlier [7].

Immunofluorescence microscopy for detection of viral antigens

To detect DENV replication in the infected cells, an indirect immunolabelling method for fluorescence microscopy (IFA) was used. Briefly, cells were fixed in chilled methanol:acetone (1:1) followed by rehydration with 0.05 M Tris/NaCl (wash buffer) and blocking buffer (0.05 M Tris/NaCl + 5% normal human serum) incubation for 20 min each. Cells were then incubated with mice anti-dengue hyperimmune serum (1:50 dilution), washed and probed with a goat anti-mouse IgG-FITC (Sigma Chemicals, USA) for 30 min. The cells were then washed with wash buffer and counterstained with Evans blue stain. All incubations were carried out at room temperature. Slides were mounted with standard mountant and examined

under epifluorescence imaging with ultraviolet illumination in an inverted microscope (Nikon Eclipse 2000, Nikon Corp., Japan). DENV-2 virus infected and uninfected C6/36 cells were used as positive and negative controls.

Electron microscopy

Whole-mount specimen processing

The on-grid whole-mount cultures were fixed with 3% glutaraldehyde in 0.2 M sodium cacodylate buffer pH 7.2 by floating the grids on a drop of fixative, cell side down. Grids were then washed with two changes of 0.2 M sodium cacodylate buffer, dehydrated through a graded ethanol series, air dried and negative stained with 1% uranyl acetate or phosphotungstic acid as described earlier [7,9].

Plastic embedding and ultramicrotomy

Infected and control cells were harvested using a cell scraper, pelleted by centrifugation and processed for embedding in EPON 812 as described earlier [10]. Ultrathin sections were cut using glass knife in an ultramicrotome (Ultracut R, Leica, Austria) and the 70- to 100-nm thin sections collected on 400-mesh copper grids. Staining was done using uranyl acetate and contrasted by Reynold's lead citrate.

Imaging

The cells were imaged under 120 KeV operating voltage and low beam current conditions in a goniometer stage of a transmission electron microscope (Tecnai 12 BIOTWIN™, FEI Co., Netherlands). Images were recorded using a side-mounted CCD camera (Megaview III, Olympus Imaging, Germany).

Electron tomography

Plastic sections (100–110 nm) were obtained by ultramicrotomy and placed in a high-tilt tomography holder (FEI Co., Eindhoven, Netherlands) that was optimized as per the software calibration protocols (Automated Electron Tomography Software, FEI Co., Eindhoven, Netherlands). Digital images were recorded using the automated tomography acquisition software, XPLORE 3D™ (FEI Co., Eindhoven, Netherlands). Single-axis tilt series was recorded between $\pm 60.0^\circ$ at 1° interval. The camera binning fac-

tor was 2. The raw data were stored in an integrated media storage platform (Terastation, Buffalo Co., USA) and subsequently processed for analysis. The tomograms were aligned using the INSPECT 3D™ software (FEI Co., Eindhoven, Netherlands) as per the software data analysis flow and 20 iteration cycles of SIRT volume reconstruction done. The reconstructed volume data file was subsequently visualized in 3D using the AMIRA™ Visualization Package (Version 5.2.0, Visage Imaging, GmbH, Berlin, Germany) by manually selecting areas of interests and 3D volume rendering was done at different slices in x , y and z planes.

Results

Optimization of the EM grid support for cell growth

The use of alcian blue treatment of the carbon-coated formvar support significantly enhanced cell adhesion and growth densities when compared with the poly-L-lysine treatment and examined as cells per grid square by light microscopy (Fig. 1a). The viability of cells was also not affected in the presence of nickel grids, but copper grids did not yield suitable cell seed monolayers on the support film. Adequate density of cells grown on the formvar support could also be imaged with propidium iodide staining (Fig. 1b).

Cytopathic effect after virus infection and expression of viral antigens

DENV-2 infected changes in C6/36 cells included moderate round and progressive clumping of the cells with increasing post-exposure time (Fig. 1c). The earliest detectable morphological changes (Fig. 1d) and presence of viral antigens in the cells (Fig. 1d) could be observed within 48 h post-infection day (p.i.d.) and maximum after 72 h. In the Vero E6 cells grown in conventional plastic wells, cytopathic effect (CPE) appeared initially in the form of microplaques after 48 h and rapidly merged, leading to degeneration of the cell sheet by 72 h p.i.d. In the SK Hep1 cells, the CPE was not acute but showed moderate rounding of cells and long 'spindle-like' elongated structures on the adherent cells by 48 h. p.i.d. DENV-2 antigens could be detected by IFA in all cell lines (data not shown).

Fig. 1. Vero E6 cells grown on support film of nickel grids as whole-mount cultures. (a) A representative low-power image of Vero E6 cells showing confluent growth and no cytotoxic effect. (b) Propidium iodide staining of the cells showing optimum growth density on the support film. (c) Representative phase-contrast image showing rounding and aggregation of the cells after DENV-2 virus exposure (shown in circles). (d) Representative images of DENV-2 antigen-positive cells imaged by immunofluorescence. Magnification scale bars are embedded into each figure.

Electron microscopy imaging

Ultrastructural changes

A variable spectrum of ultrastructural changes was observed after DENV-2 infection in the different cell lines. Prominent, large perinuclear vacuoles containing amorphous electron-dense material and mature 40- to 45-nm mature flavivirus particles could be observed in the C6/36 cells within 48 h after infection (Fig. 2a-c). In some fields, these vacuolar structures were seen to have communications with an adjacent vacuole filled with a reticular meshwork (Fig. 2c). Negative stained imaging of cell-free culture supernatants also showed the presence of mature enveloped flaviviruses, suggesting the release of virus particles from infected cells (Fig. 2d). The CPE seen in Vero E6 cells was more abrupt, with degenerative changes prominent within 72 h. Ultrastructural examination of the cells also showed extensive injury in the form of cytoplasmic degeneration (data

not shown). The DENV-2-infected SK Hep1 cells showed ultrastructural changes as chromatolysis, degenerative necrosis (Fig. 3a), morphologically distinct autophagosomes and cytoplasmic membrane alterations (Fig. 3b) and dilated endoplasmic reticulum (ER) (Fig. 3c).

Whole-mount imaging

Examination of the whole mount and negative stained Vero E6 cells infected with DENV-2 virus revealed abundant virus particles associated with cell membrane-derived structures (Fig. 4 Panel A). Interestingly, the structure of these DENV-2 particles imaged in the 48-h post-infection cells were not enveloped but showed a capsid morphology that was icosahedral and a size range of 30–40 nm. Importantly, from a single field where a large number of particles could be imaged (Fig. 4 Panel A), individual particles revealed different core morphologies of the nucleoprotein structures (Fig. 4 Panel B).

Fig. 2. Representative transmission electron micrographs showing ultrastructural changes in DENV-2-infected C6/36 cells. (a) A low-power image showing a large number of cells in the field with cytoplasmic vacuolation. (b) Profile of single DENV-2-infected C6/36 cell showing reticular meshwork in a cytoplasmic vacuole (vac) and an adjacent vacuole with electron-dense material (arrow). (c) Higher magnification of the same showing the presence of virus particles marked v (shown in red rectangular boxes and indicated with white arrows). M indicates the junction between the vacuoles. N denotes the nucleus of the cells in (b) and (c). D denotes electron-dense granules. (d) Negative stained image of a group of DENV-2 (arrows) detected in supernatant of infected C6/36 cells. The red circle marks a single virus particle showing complete envelope projections. Magnification scale bars are embedded in all micrographs. This figure is available in black and white in print and in colour at JEM online.

Electron tomography

The 3D reconstructions of the areas showing typical autophagosomal structures and flavivirus-associated vesicular proliferations are shown in Fig. 5. The autophagosomes and vesicular membrane structures were seen to be continuous after 3D reconstructions. Importantly, similar to the recent report of Welsch *et al.* [18], these vesicles had pores with the opening towards the nucleus (Fig. 5a). Complete isosurface volume rendering in 3D showed the autophagosome structures continuous with the virus-derived vesicular membrane structures when imaged at different sectional planes (Fig. 5a).

Discussion

Early studies on DENV antigens in infected cells had documented evidence of a characteristic, intense,

perinuclear staining that faded as a gradient into the background cytoplasm [11,12]. This pattern was not only for DENV but also seen with other Group B arboviruses [13] and laid the foundation towards conceptually understanding the alterations in host cell membrane architecture that forms a crucial framework in the biogenesis events of DENV and other flaviviruses – an area that has come under intense scrutiny in the recent years.

Using different EM techniques studying DENV replication in infected cells, a striking observation was the formation of distinct membrane structures in these cells [11,14–18] in the form of convoluted membrane, double membrane-bound vesicles and occasional membrane tubules. Immunolabelling and antigen localization studies further showed the presence of both viral RNA and non-structural proteins (NS1, NS3 and NS4) co-localizing in

Fig. 3. Representative ultrastructural changes in SK Hep1 cells infected with DENV-2. (a) Areas of typical cytoplasmic degeneration D are shown within the rectangle, and chromatolysis C are indicated by arrows. N denotes the nucleus. (b) A typical early autophagosome formation with endomembrane changes (arrow) shown within the rectangle. (c) A field showing dilated ER. (d) A representative profile of an uninfected SK Hep1 cell. All magnification bars are embedded into the micrographs. This figure is available in black and white in print and in colour at JEM online.

these structures formed in insect and Vero cells [17,18].

Findings from our present study further strengthen these earlier observations that unique cytoplasmic membrane alterations constitute a key host feature in DENV-infected cells. Interestingly, with the DENV-2 Tr1751 strain studied by us, the cytoplasmic vacuolar morphology was rather large and similar to the earlier morphology described by Cardiff *et al.* [11] and consistent with other reports of DENV-2-associated ultrastructural changes in C6/36 cells [19]. Although we did not use immunolabelling, distinct 40 to 50-nm mature virions could be seen along with reticular electron-dense material in these vacuoles of DENV-2-infected C6/36 cells by 24 h post-infection presumably representing viral replicosome complexes, an important point that needs emphasis on the ultrastructural changes observed

in Vero E6 cells after DENV-2 infection. The grid-cell culture studies showed evidence of 40-nm flavivirus core-like structures associated with cell-free membrane detected by negative staining within 48 h post-infection and corresponding ultrathin sections showed drastic degenerative changes. This needs further study in conjunction with analytical cellular fractionation studies to establish the origin of these entities and differentiate them from possible artefacts. Takasaki *et al.* had earlier reported the rapid degenerative morphology in Raji cells infected with DENV-2 [20], but to the best of our knowledge, direct on-grid whole-mount cell culture studies on DENV-infected cells by TEM has not been published and can provide information on the fine structure of both assembly and cytoskeletal alterations in the host, as shown earlier with the Akabane virus [7].

Fig. 4. Membrane-associated DENV-2 particles released from whole-mount Vero E6 cells. Panel A shows a membrane structure associated with 40-nm DENV-2 core-like particles in random distribution (arrows). None of these particles have detectable envelope projections. Panel B (a–f) shows higher-magnification images of the single virions from the same field showing the presence of core structures (arrows) having varied organizations (magnification scale bar = 200 nm in Panel A and 10 nm in Panel B).

The most striking observations came from the SK Hep1 cells, a human endothelial cell line presumably of hepatic endothelial origin [6]. These cells were susceptible to DENV-2 infection and showed formation of distinct autophagosome-like structures near virus-induced membrane alterations. Interestingly, DENV-2-induced autophagy has been recently reported in Huh7 cells [21] (which interestingly is a human hepatoma cell line). Importantly, although conventional TEM imaging showed these structures as isolated entities, ET reconstructions and 3D imaging of a typical field in a thick section volume dramatically showed continuity within these membrane systems. Importantly, distinct pores within these structures opening towards the nuclear side also argue in favour of the autophagosomes being a part of the virus-derived membrane structures. The detailed 3D organization of DENV-2-induced cytoplasmic membrane alterations have been recently worked out by Welsch *et al.* using ET [18]. This study clearly demonstrated that the DENV-2-induced membrane structures are part of the modified ER network.

Similar studies using ET as a major tool have also showed the close association of virus replisomes with cyto-organelle membranes. The SARS corona virus replication Factor A and RNA was shown to associate with the outer mitochondrial membrane and ER-derived networks [22], thus re-shifting the targets of understanding intracellular flavivirus assembly sites as suggested earlier by Murray *et al.* [23].

In summary, our study shows that DENV-2-associated alterations in cytoplasmic membrane compartment of host cells varies in presentation but is a consistent feature. The important observation of the same in the endothelial cell line SK Hep1 and its reconstruction in 3D with electron tomography for the first time suggest that autophagosome vesicles could also be a continued network of DENV-2-mediated membrane alterations. This provides credible platform for the possibility of developing new-generation compounds to target destabilization of these structures as a potential blocker of DENV replication in host cells and, therefore, a possible therapeutic agent in dengue disease.

Fig. 5. Isosurface volume reconstruction of a representative cytoplasmic area by ET showing autophagy and endomembrane changes in a DENV-2-infected SK Hep1 cell. (a) 3D rendering of the endomembrane structures showing distinct pores in endomembrane vesicles (v) (white arrows) in the scaffold of membrane structures. The red rectangle highlights the representative area of interest and the smaller blue rectangle within it shows the continuity of the endomembrane system with the nuclear envelope (arrow). (b) An inset of the conventional transmission electron micrograph of the same field from where the tomograms were acquired. N denotes the nucleus. Magnification bars are embedded in both micrographs = 0.5 µm.

Concluding remarks

This is the first report of ET-based analysis of DENV-2 replication in a human endothelial cell line. The findings of the present study strongly argue in favour of the development of characteristic and unique DENV-induced cytoplasmic membrane bodies in infected host cells. The observation of autophagosome structures in DENV-2-infected human endothelial cell line SK Hep1 being continuous with the virus-altered membrane system is a novel feature. Further molecular analysis on the biogenesis of such structures could define potential and novel antiviral targets for drug development.

Supplementary material

A supplementary movie S1 showing the endomembrane reconstruction in 3D with open pores in DENV-2 infected SK Hep1 cells is available at *Journal of Electron Microscopy* online.

Funding

This study was supported through intramural funds from ICMR New Delhi and from the Department of Biotechnology Grant BT/PR/6032/Med/14/732/2005.

Acknowledgements

We would like to sincerely thank our colleagues at the Repository of National Institute of Virology, Pune for kindly supplying us with the DENV-2 Tr1751 strain and Mr Amit Mandal of ICON Analytical Company and FEI for the software support.

References

- 1 Regan R L and Brueckner A L (1956) Studies of dengue virus by electron microscopy. *Am. J. Trop. Med. Hyg.* **5**: 809–811.
- 2 Pokydisheva E, Zhang Y, Battisi A J, Battor-Kelly C M, Chipman P R, Xiao C, Gregorio G G, Hendrickson W A, Kuhn R J, and Rossman M G (2006) CryoEM reconstruction of dengue virus in complex with carbohydrate recognition domain of DC-SIGN. *Cell* **3**: 485–493.
- 3 Bartenschlager R and Miller S (2008) Molecular aspects of dengue virus replication. *Future Microbiol.* **3**: 155–165.
- 4 Baumister W (2005) A voyage to the inner space of cells. *Protein Sci.* **14**: 257–269.
- 5 Frey T G, Perkins G A, and Ellisman M H (2006) Electron tomography of membrane-bound cellular organelles. *Annu. Rev. Biophys. Biomol. Struct.* **35**: 199–224.
- 6 Heffelfinger S C, Hawkins H H, Barrish J, Taylor L, and Darlington G J (1992) SK Hep1: a human cell line of endothelial origin. *In Vitro Cell. Dev. Biol.* **28A**: 136–142.
- 7 Hyatt A D and Eaton B T (1990) Virological applications of the grid-cell-culture technique. *Electron Microsc. Rev.* **3**: 1–27.
- 8 Cogger V C, Arias I M, Warren A, McMohan A C, Kiss D L, Avery V M, and Le Coutier D G (2008) The response of fenestrations, actin and caveolin 1 to vascular endothelial growth factor in SK Hep1 cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **295**: G137–G145.
- 9 Brenner S and Horne R W (1959) A negative staining method for high-resolution electron microscopy of viruses. *Biochim. Biophys. Acta* **34**: 103.
- 10 Bozolla J J and Russell L D (1992) Specimen preparation for transmission electron microscopy. In: *Electron Microscopy, Principles and Techniques for Biologists*, pp. 16–37 (Boston USA, Jones and Bartlett Publishers).
- 11 Cardiff R D and Lund J K (1976) Cytological localization of dengue 2 antigens: an immunological study with ultrastructural correlation. *Infect. Immun.* **7**: 809–816.
- 12 Cardiff R D and Lund J K (1976) Distribution of dengue 2 antigens by electron immunocytochemistry. *Infect. Immun.* **3**: 1699–1709.
- 13 Atchison R W, Ordonez J V, Sather G E, and Hammon W (1964) Fluorescent antibody complement fixation method for detection of dengue viruses in mice. *J. Immunol.* **94**: 936–943.
- 14 Barth O M (1982) Replication of dengue viruses in mosquito cell cultures – a model from ultrastructural observations. *Mem. Inst. Oswaldo Cruz* **87**: 565–574.
- 15 Barth O M (1999) Ultrastructural aspects of the dengue virus (flavivirus) particle morphogenesis. *J. Submicrosc. Cytol. Pathol.* **31**: 407–412.
- 16 Ng M L, Yeong F M, and Tan S H (1994) Cryosubstitution technique reveals new morphology of flavivirus-induced structures. *J. Virol. Methods* **49**: 305–314.
- 17 Mackenzie J M, Jones M K, and Young P R (1996) Immunolocalization of the dengue virus nonstructural protein NS1 suggests a role in viral RNA replication. *Virology* **220**: 232–240.
- 18 Welsh S, Miller S, Romero-brey I, Merz A, Bleck C K E, Walther P, Fuller S D, Antony C, Krijnse-Locker J, and Bartenschlager R (2009) Composition and three dimensional architecture of the dengue virus replication and assembly sites. *Cell Host Microbe* **5**: 365–375.

- 19 Pandey B, Ichinose A, and Igarashi A (1998) Electron microscopic examination of *Aedes albopictus* clone C6/36 cells infected with dengue virus 2 at elevated incubation temperature. *Acta Virol.* **42**: 35–39.
- 20 Takasaki T, Takada K, and Kurane I (2001) Electron microscopic study of persistent dengue virus infection: analysis of a cell line persistently infected with dengue 2 virus. *Intervirology* **44**: 48–54.
- 21 Lee Y, Lei H-Y, Liu M-T, Wang J-R, Chen S-H, Jiang-Shieh Y-F, Lin Y S, Yeh R M, Liu C C, and Liu H-S (2008) Autophagic machinery activated by dengue virus enhances virus replication. *Virology* **374**: 240–248.
- 22 Knoops K, Kikert M, Van den Wom S, Zevenhoven-Dobbe J, Van der Meer Y, Koster A J, Mommas A M, and Snijder E J (2008) SARS coronavirus replication is supported by a tubulovesicular network of modified endoplasmic reticulum. *PLoS Biol.* **6**: 1957–1974.
- 23 Murray C L, Jones C T, and Rice C M (2008) Architects of assembly: roles of flaviviridae nonstructural proteins in virion morphogenesis. *Nat. Rev. Microbiol.* **6**: 699–708.