Published in final edited form as:

Nat Rev Microbiol. 2008 September ; 6(9): 699–708. doi:10.1038/nrmicro1928.

Architects of Assembly: roles of *Flaviviridae* nonstructural proteins in virion morphogenesis

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Abstract

Viruses of the *Flaviviridae* family, including hepatitis C, dengue, and bovine viral diarrhoea, are responsible for significant morbidity and mortality worldwide. Recent advances in understanding virion assembly have uncovered commonalities among distantly related members of this family. We discuss the emerging hypothesis that physical virion components are not alone in forming the infectious particle, but that nonstructural proteins are intimately involved in orchestrating morphogenesis. Pinpointing the roles of *Flaviviridae* proteins in virion production could reveal new avenues for antiviral therapeutics.

Introduction

The *Flaviviridae* family is a group of small, enveloped RNA viruses, consisting of the genera *Hepacivirus*, *Flavivirus*, and *Pestivirus*, and including numerous significant pathogens. Approximately 120 million people worldwide are currently infected with hepatitis C virus (HCV), the prototype member of the *Hepacivirus* genus. These individuals are at risk of developing cirrhosis, hepatocellular carcinoma and extrahepatic disease. The genus *Flavivirus* includes vector-borne disease agents such as yellow fever virus (YFV), dengue virus (DEN), and West Nile virus (WNV). Many of these pathogens are resurging and spreading to new environments and are responsible for significant morbidity and mortality. Pestiviruses, including bovine viral diarrhoea virus (BVDV) and classical swine fever virus (CSFV), are pathogens of animals and are of significant financial concern to the livestock industry (reviewed in ¹).

The *Flaviviridae* genome is a single-stranded RNA molecule, which, upon introduction to the cell, is recognized as a messenger RNA and translated by the host cell machinery to yield a polyprotein (Fig 1). Processing by viral and cellular enzymes releases the individual viral gene products (Fig 2). Structural proteins, which comprise the virion, consist of core (capsid in flaviviruses, C) and the envelope glycoproteins. Most of the nonstructural proteins associate to form the replicase complex, which catalyzes RNA accumulation in close association with modified cytoplasmic membranes^{2,3,4}. NS3 and its cofactor NS4A, or NS2B (flaviviruses), is the main viral protease; NS3 also has helicase and nucleoside triphosphatase (NTPase) activities, which are essential for replication. NS5B and NS5 (flaviviruses) constitute the viral RNA-dependent RNA polymerases. NS1, a secreted protein unique to flaviviruses, plays a role early in replication. HCV and pestivirus NS4B and NS5A proteins, as well as flavivirus NS2A, NS4A and NS4B proteins, have as yet undefined but essential roles in RNA accumulation (reviewed in ¹).

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Many of the features of *Flaviviridae* replication have been elucidated using viral genomic cDNA clones. Transcription of the genomic RNA *in vitro*, followed by its transfection into cultured cells is sufficient to launch a full infectious cycle for many flavivirus and pestivirus isolates (see ^{5, 6} for examples). In contrast, HCV genomes have been extremely difficult to propagate in culture. Emergent mutations and adapted cell lines were able to coax modest levels of RNA replication, but failed to produce progeny virions^{7,8}. In a recent and major advance, a unique patient isolate, termed Japanese fulminant hepatitis 1 (JFH-1) was found to replicate robustly and produce infectious HCV in cell culture^{9,10,11}. This breakthrough has allowed the previously enigmatic processes of HCV assembly, infectivity, and entry to be investigated for the first time.

Production of infectious virus

The enveloped virions of the *Flaviviridae* are thought to arise by budding of the C protein and associated genomic RNA into endoplasmic reticulum (ER)-derived membranes that are studded with virus envelope glycoproteins (Fig 3). Although morphogenesis is expected to commence by binding of C to RNA, a packaging signal has not been identified for any member of the *Flaviviridae*. Nucleocapsids are rarely observed in infected cells, and detailed cryo-electron microscopy image reconstructions of members of the genus *Flavivirus* have indicated that these virions do not contain an ordered nucleoprotein interior^{12,13,14,15,16}. The basic C protein might instead aggregate with the RNA to neutralize and condense it within the particle^{17,18}, forming a complex that does not make contacts with the envelope¹⁴. This suggests that the glycoproteins, which form a highly ordered herringbone-like array on the surface of the mature virion^{12,13}, drive morphogenesis through their lateral associations¹⁹. Consistent with this, subviral particles that contain no C or RNA, are produced in natural flavivirus infections and can be induced by overexpression of the glycoproteins in cells²⁰. It is not known whether HCV or pestivirus infection also results in subviral particle production.

After budding, *Flaviviridae* particles are transported through the cellular secretory pathway to the extracellular milieu. For the genus *Flavivirus*, egress coincides with a massive, acid-induced, reorganization of the glycoproteins, from trimeric spikes^{15,21} to an array of dimers that completely cover the lipid bilayer in a relatively smooth proteinaceous coat^{12,13,22}. This conformational change allows cleavage of the prM envelope protein by furin in the *trans* Golgi network^{22,23}. prM functions to protect the fusogenic E protein during transit through the acidic compartments of the secretory pathway²⁴ and its processing, followed by dissociation of the pr fragment upon particle release²², results in mature virions that are primed for low-pH triggered entry into a naïve target cell²⁴. The maturation of particles of the genera *Hepacivirus* and *Pestivirus* seems to be fundamentally different from that of flaviviruses. Intracellular infectious virus can be released from HCV-²⁵ and pestivirus-²⁶ infected cells, suggesting that these virions mature immediately or rapidly after their formation; post-assembly cleavage events analogous to flavivirus prM processing do not seem to be involved. Despite undergoing low-pH dependent entry, HCV and BVDV particles are acid resistant, indicating that unknown factors during uptake, rather than during egress, prime the envelope proteins for fusion^{27,28}. Although HCV virions are infectious soon after they are formed, they undergo physical modification during egress, decreasing in density as they pass through the secretory pathway²⁵. The finding that very low-density lipoprotein (VLDL) components form essential interactions with HCV during morphogenesis has led to speculation that HCV particles might be closely associated with, or even internalized in, lipid-rich host structures during virus assembly and secretion^{29,30,31}.

Nonstructural proteins orchestrate assembly

Most nonstructural proteins have essential roles in replication, and it was long thought that these replicase proteins were responsible solely for catalyzing the accumulation of genomic

RNA molecules that could subsequently be packaged by the structural proteins. This canonical division of labour was initially supported by the ability of subgenomes that lack the structural region to efficiently undergo RNA synthesis^{7,8,32,33}. Whereas replication is independent of the structural proteins, however, packaging of genomes into infectious particles seems to require more than just the physical components of the virion. This Review examines the emerging hypothesis that many *Flaviviridae* nonstructural proteins are directly and intimately involved in infectious virion morphogenesis, and might orchestrate this complex process at specialized sites of assembly.

Roles for nonstructural proteins in the production of infectious virus *p7* membrane protein

One of the first *Flaviviridae* nonstructural proteins found to function in infectious virus production was the pestivirus product p7 (Fig 4)³⁴. This small hydrophobic protein, which consists of two membrane-spanning helices and a conserved basic cytosolic loop^{35,36}, has a homologue in the hepacivirus but not the flavivirus genome^{35,37,38}. Deletion of p7 does not affect RNA replication, but completely abrogates the production of infectious pestivirus or HCV particles^{34,39,40}. Incomplete processing of p7 by signal peptidase results in an accumulation of uncleaved E2-p7, an envelope protein precursor that might be incorporated into virions as a minor component^{36,37,38,41,42}. This fusion protein, however, is not essential for BVDV³⁴ or HCV³⁹ infectivity, and neither p7 nor E2-p7 has been detected in pestivirus particles³⁶; studies of HCV virions are ongoing.

Similarities have been noted between p7 and viroporins, a class of small, hydrophobic, viral proteins that have ion channel activity *in vitro*, and are involved in enhancing infectivity (Box 1)^{34,35,43}. HCV and BVDV p7 proteins oligomerize into ion conductive pores in various artificial systems^{43,44,45,46}. Although the stoichiometry and specificity of the channels are not known, it has been proposed that HCV p7 forms hexameric^{43,47} or heptameric complexes⁴⁸ with a preference for cation conduction^{43,48,49}. Conserved basic residues within the p7 cytosolic loop are involved in ion channel activity^{44,48} and were found to be essential for infectious pestivirus production³⁴, required for induction of HCV viraemia in chimpanzees⁵⁰, and important for optimal growth of HCV in tissue culture^{39,40}. Mutations of residues within the proposed interior of the pore, however, did not drastically affect infectious titres^{39,40}.

Box 1

Viroporins

Viroporins are an expanding group of small (60–100 residue) hydrophobic viral proteins that demonstrate oligomerization and membrane permeabilization activities *in vitro* and in cell-based artificial systems. Viroporins, which can be incorporated in or excluded from the virion, are important for the infectivity of many diverse viruses (reviewed in ¹⁰⁴). For the majority of viroporins, the relationship between *in vitro* pore formation and *in vivo* ion channel activity has not been firmly established, and the importance of ion conduction in the viral life cycle is not clear.

A strict correlation between ion channel activity and an *in vivo* mechanism of action has, however, been demonstrated for the influenza A virus viroporin, M2 (reviewed in ^{104, 105, 106}). M2 is a homotetrameric integral membrane protein that is present in the virus envelope in small amounts. The ability of M2 to selectively conduct protons in a low-pH-activated manner has been demonstrated in planar lipid bilayers, amphibian oocytes, and mammalian cells; a specific inhibitor of *in vitro* ion conductance, amantadine, blocks influenza A virus growth, with resistance mutations mapping to M2^{105,106}. The M2 protein has dual functions in promoting influenza A virus infectivity. During virus egress, intracellular M2 increases the pH of the Golgi lumen, preventing premature activation of the HA envelope protein.

During virus entry, M2 in the virion acidifies the particle interior to facilitate genome uncoating. This concordance of evidence has established that ion conduction by M2 is essential for influenza A virus propagation^{105, 106}.

In contrast, the HIV-1 viroporin Vpu does not seem to invoke an *in vivo* ion channel activity. Vpu is an integral membrane protein that is excluded from virus particles and accomplishes two distinct roles in HIV-1 infectivity by interactions with host factors (reviewed in ^{104, 105, 107}). Vpu binding to intracellular CD4 targets the virus receptor for degradation. This allows free passage of the envelope precursor, gp160, to the sites of budding, as well as favours virus dissemination to naïve cells. In a recently defined independent role, Vpu colocalizes with and antagonizes tetherin, an antiviral host protein that prevents release of nascent particles from the cell surface¹⁰⁸.

Viroporins have been reported for many other virus families, including *Flaviviridae* (p7), *Coronaviridae* (E), *Picornaviridae* (2B), and *Togaviridae* (6K); their roles include virus assembly, budding, and egress (reviewed in ^{104, 109}). It is not known whether these viroporins function by conducting ions, by interacting with host proteins, or by additional undefined mechanisms. As more is uncovered about the roles of small hydrophobic proteins in virus assembly and infectivity, the focus will likely shift from their similar behaviour *in vitro* to their diverse mechanisms *in vivo*, and the classification of viroporin may diverge into functionally defined subsets.

By analogy to the influenza A virus viroporin M2, an ion channel activity might protect fusogenic glycoproteins in progeny virions from low-pH mediated inactivation during their passage through the Golgi⁴⁴. Although HCV and pestivirus particles transit the low-pH compartments of the secretory pathway, the finding that these virions are acid-resistant until post-release priming suggests that such protection may be unnecessary^{27,28}. Deletion of p7 from either BVDV or HCV blocks an early event in virus assembly, before the formation of infectious intracellular particles^{34,39,40}, while mutations in the HCV p7 cytosolic loop seem to decrease virion release⁴⁰. This small protein might therefore influence several steps of infectious virus production; indeed multiple roles have been reported for other viroporins. As with the majority of viroporins, it is still unclear how the ion channel and oligomerization properties of p7 relate to its functions in progeny virus assembly and release.

NS2 autoprotease

NS2 is a membrane-associated cysteine autoprotease that mediates cleavage of its own carboxyl terminus from NS3^{51,52,53,54,55}. Homologues of this enzyme are present in hepaciviruses and pestiviruses, but not flaviviruses. After liberation of NS3, NS2 is dispensable for RNA replication^{7,8,32,53,56}, but is essential for progeny particle production (Fig 4)^{39,57, 58}. HCV NS2 functions early in morphogenesis, before the assembly of an infectious intracellular virus³⁹. This function requires at least the protease domain of NS2, but not its catalytic activity, nor the presence of the uncleaved NS2-3 precursor³⁹. In contrast, pestivirus NS2 functions only within the context of uncleaved NS2-3^{57,58}; the post-cleavage form of NS2 does not appear to play any role for pestivirus growth in cell culture⁵⁸. The function of pestivirus uncleaved NS2-3 in morphogenesis does not require the protease activity of NS2, nor the protease, helicase, or NTPase activities of NS3, although cleavage of NS3 from its cofactor, NS4A, is necessary^{57,58}.

While the catalytic activities of NS2 and uncleaved NS2-3 are not required for infectious particle production, these enzymes, or the cleavages that they perform, are essential for replication. This dichotomy suggests that regulated expression or activation of these nonstructural proteins might allow switching from RNA synthesis to virion production, a hypothesis illustrated by pestivirus growth⁵³. BVDV NS2-3 cleavage, and thus RNA

replication, is absolutely dependent on a cellular cofactor termed *J*-domain protein interacting with viral protein (Jiv)^{59,60}. A single molecule of Jiv binds two sites on NS2, one encompassing the catalytic center and the other close to the substrate region, likely positioning these elements to facilitate processing^{59,61}. After cleavage, NS2 remains bound to Jiv, leading to gradual depletion of this limiting co-factor and eventually preventing further rounds of NS2-3 processing⁵⁹. Titration of Jiv therefore results in the accumulation of uncleaved NS2-3, a plateau of RNA replication, and the commencement of infectious virus production⁵³. Interestingly, this regulation of NS2-3 processing is also linked to the ability of BVDV to establish persistent infection. While coordination of RNA replication and virus assembly through NS2-3 cleavage does not seem to occur during HCV growth in cell culture, controlled processing might impact chronic infection *in vivo*.

There is evidence that the function of HCV NS2 in infectious progeny assembly requires additional nonstructural proteins. Studies of chimeric genomes encoding sequences from diverse HCV isolates indicated that regions of compatibility within NS2 are major determinants of infectious titre^{62,63}. Specifically, genotypic matching of the amino terminal helix of NS2, p7, and the structural proteins, coupled with pairing of the remainder of NS2 and the replicase proteins led to optimal infectivity^{62,63}. These results hint that NS2 might form multiple functional associations, including interactions with p7, as well as possible independent connections with replicase proteins, such as NS3^{62,63}. Indeed, physical interactions between HCV NS2 and NS3 have been reported⁶⁴. Concerted action of NS2 and NS3 might therefore be a common feature of hepacivirus and pestivirus progeny morphogenesis, with possible physical association of the two HCV proteins and a requirement for the pestivirus uncleaved NS2-3 precursor.

NS2A

The viruses of the genus *Flavivirus* do not encode an autoprotease equivalent to NS2. Instead, two hydrophobic proteins that span the membrane several times are encoded upstream of NS3 in the polyprotein. One of these, NS2B, is the NS3 protease cofactor¹, the other, NS2A, is an essential component of the replicase (Fig 4)⁶⁵. Processing within the NS2A-2B-3 region is carried out by the viral protease, NS3, with its NS2B cofactor. NS2A is also cleaved by the viral protease at a cryptic internal site, creating a truncated form of the protein termed NS2A α ⁶⁶. Mutational analysis revealed that NS2A α itself is dispensable for replication and infectivity, but that a basic residue at the cleavage site (K190) is essential for production of infectious YFV⁶⁷. Mutation of K190 did not affect subviral particle secretion, suggesting that NS2A plays an important role in the incorporation of genomic RNA into the budding virion⁶⁷. Residues in NS2A (I59 and T149) have also been implicated in the production of infectious Kunjin virus (KUN)^{68,69}.

NS2A interacts with other nonstructural proteins, including NS3⁷⁰. Interestingly, second-site changes in the helicase domain of NS3 were found to suppress the assembly defect caused by mutation of the NS2A α cleavage site⁶⁷. These findings suggest that, similar to HCV and the pestiviruses, physical associations between the multifunctional NS3 enzyme and an upstream membrane associated protein are essential for infectious flavivirus morphogenesis. Unlike HCV NS2 and pestivirus uncleaved NS2-3, which are not required for RNA replication, NS2A colocalizes with replication complexes⁷⁰ and has an essential, but poorly understood, role in the process of RNA accumulation⁶⁵. Purified NS2A binds RNA, leading to the hypothesis that this integral membrane protein might shuttle genomic substrates out of membrane bound replication complexes to the sites of packaging^{68,70}.

NS3 multifunctional enzyme

To date, the only clearly understood mechanism of action for a nonstructural protein in *Flaviviridae* progeny virus production is the proteolytic role of the NS3 protease from the genus *Flavivirus* (Fig 4). This enzyme, together with the NS2B cofactor, mediates cleavage of the flavivirus C protein from its membrane anchor, and is therefore essential for maturation of a major virion component. Processing of C on the cytosolic side of the ER membrane by the flaviviral protease is essential for subsequent cleavage of C from the envelope protein, prM, by the host enzyme signal peptidase in the ER lumen^{71,72}. This regulated sequence of cleavages ensures that processing of prM from the polyprotein is delayed, and budding is stalled, until genomic RNA substrates accumulate through replication; the secretion of the flavivirus glycoproteins, E and prM, as immunogenic subviral particles is thereby minimized^{71,72,73}. Inspection of C protein sequences from the genera *Pestivirus* and *Hepacivirus* revealed the presence of conserved NS3 protease recognition sites in the corresponding membrane anchors^{57,74}. Subsequently, however, it was found that a host enzyme, signal peptide peptidase, processed both proteins^{75,76}. Although NS3 might interact with C without initiating proteolysis, studies of HCV have shown that mutation of the putative cleavage site residues in C does not drastically affect virus titres⁷⁷. It is not known if other mechanisms delay budding for these viruses.

While a proteolytic role for NS3 in assembly might be unique to flaviviruses, the discovery of an essential function for pestivirus uncleaved NS2-3 that is independent of its known enzymatic activities, suggested analogous additional roles for NS3 proteins of the *Flavivirus* and *Hepacivirus* genera^{57,58}. This hypothesis was supported by the finding that NS3 of the flavivirus, KUN, can function *in trans* for C processing and RNA replication, but must be supplied *in cis* for infectious virus to be produced^{78,79}. YFV NS3, which is not absolutely required *in cis*⁸⁰, was recently directly implicated in flavivirus morphogenesis⁸¹. A single tryptophan residue in the helicase domain is dispensable for RNA replication but essential for nucleocapsid incorporation and possibly other aspects of particle specific infectivity⁸¹. By complementation *in trans*, this function of NS3 in infectious virus assembly was shown to be independent of its protease, helicase and NTPase activities⁸¹. NS3 has also been implicated in the production of HCV progeny, as adaptive mutations in this region have been found to augment infectious titres of viruses passaged in cell culture^{63,82}. For a minimally infectious intergenotypic chimera, titres were specifically increased by the emergence of a point mutation in the NS3 helicase domain⁶³. This mutation rescued infectious virus production without affecting RNA replication, or the known catalytic activities of NS3 (M. Yi and S. Lemon, personal communication).

Protease cofactor

Cofactors NS4A and NS2B (flavivirus) contribute to the structure of NS3, anchor the enzyme to the membrane, are required for the majority of NS3-mediated processing events, and can modulate its helicase activity (reviewed in ¹). Although the known enzymatic activities of NS3 are not required for infectious pestivirus production, the presence of the NS4A cofactor significantly increases the activity of the essential precursor, uncleaved NS2-3, in this process (Fig 4)^{57,58}. NS4A can be functionally supplied *in trans* to uncleaved NS2-3, but it cannot be recruited from an active replication complex, and fragments or full-length NS4A sequences that cannot be cleaved from the NS2-3 precursor are highly inhibitory to its function^{57,58}. This requirement for available NS4A might indicate that a specific conformation of the NS3 protease is essential for the function of uncleaved NS2-3 in virion morphogenesis. Alternatively, NS4A could bind and recruit additional essential factors to the sites of assembly⁵⁸. The flavivirus protease cofactor, NS2B, is also involved in infectious virus production by facilitating NS3-mediated cleavage of C^{71,72}. Additional functions of NS3 in assembly and infectivity, however,

do not require *cis*-encoded NS2B⁸¹. It remains to be tested whether the emerging role for HCV NS3 in progeny virus production will include a requirement for NS4A.

NS5A phosphoprotein

The zinc-binding NS5A proteins of HCV and the pestiviruses^{83,84} have essential but undefined roles in RNA replication; no enzymatic activity has been ascribed to them (Fig 5). For both genera, these proteins are basally phosphorylated on serines by what seems to be the same cellular kinase(s)^{85,86}; HCV NS5A is also a substrate for casein kinase I (CKI)- α -mediated hyperphosphorylation^{85,87}. A regulatory role for NS5A phosphorylation has long been suspected, and was supported by observations that adaptive mutations⁷, engineered mutations⁸⁸, or kinase inhibitors⁸⁹ that decrease hyperphosphorylation increase RNA replication but abolish HCV infectivity in chimpanzees⁹⁰. The finding that hyperphosphorylation inhibited the ability of NS5A to bind an essential cellular component of the replicase, hVAP-A, suggested that this modification might serve as a switch to break apart the replication complex and allow infectious virus assembly to begin^{91,92}.

Recently, it has been proposed that HCV infectious particle assembly takes place at membrane-associated lipid droplets. These are fatty intracellular deposits at which active replication complexes and envelope proteins congregate in a C-dependent manner⁹³. Mutations within the amino-terminal domain of NS5A (residues 99–104) were found to impair recruitment of the replicase to lipid droplets, while deletions in the carboxy-terminal region of NS5A disrupted its colocalization with C on these structures⁹⁴; both types of mutations decreased virus titres, suggesting a direct role for NS5A in infectious virus production^{93,94}. An independent mutational analysis of NS5A identified a single residue (S457) that is dispensable for RNA replication but essential for infectious particle assembly⁹⁵. Substitution of this serine with alanine abolished infectious titres, while mutation to aspartic acid, which mimicks phosphorylation, rescued the production of progeny virus⁹⁵. The aspartic acid mutant, but not the wild-type genome, produced normal levels of infectious virus in the presence of casein kinase II (CKII) inhibitors⁹⁵. This evidence, along with the finding that the serine to aspartic acid change slightly decreased RNA replication, suggested that CKII-dependent phosphorylation of NS5A S457 might mediate a switch between replication and assembly. Although it is not clear whether this serine is part of the basal or hyperphosphorylated subsets, mutations at this position seemed to reduce hyperphosphorylation⁹⁵. These studies provide compelling evidence that NS5A is a central regulator of virion morphogenesis. Temporally regulated phosphorylation of this protein might influence the localization of replication complexes or impact the partitioning of nascent positive-strand RNA molecules between the competing fates of translation, replication, and encapsidation. Modification of NS5A might also disrupt the replicase machinery, thereby releasing genomes for infectious virus formation.

RNA-dependent RNA polymerase

The flavivirus genome does not encode a homologue of NS5A; its NS5 protein has RNA-dependent RNA polymerase (RdRp) and methyltransferase activities, which are important for RNA synthesis and genome capping respectively (Fig 5). Despite no sequence similarity to NS5A, NS5 has a zinc-binding motif⁹⁶ and is phosphorylated by a putative NS5A-kinase⁸⁶, suggesting functional homology between these proteins⁸⁶. Evidence that DEN NS5 localizes and interacts with NS3 only when hypophosphorylated implies that this protein might also mediate a phosphorylation-dependent relocalization, or disruption, of the replicase complex⁹⁷. Studies of KUN have revealed that the polymerase activity of NS5 is a prerequisite for assembly, since replication-incompetent genomes were not substrates for incorporation into virions⁹⁸. Pestivirus and hepacivirus RdRp activity is encoded in NS5B. Although it is not known if these genomes must replicate in order to be packaged, BVDV NS5B has been directly implicated in morphogenesis. Insertion of an epitope tag in the carboxy-terminal region of the

polymerase was found to abolish infectious virus production while not affecting RNA replication⁹⁹.

These data suggest that NS5A, NS5B, and NS5 (flaviviruses) might be central to the regulation and coupling of RNA synthesis and virion morphogenesis, a link that likely depends on physical connections between membranous sites of replication and assembly^{93,98}. Immuno-electronmicroscopy of KUN-infected cells has visualized flavivirus structural proteins at modified membranes proximal to vesicle packets that are thought to be the sites of RNA replication¹⁰⁰. Similar membranous structures have been observed in pestivirus-infected, as well as in HCV-infected cells^{3,4}. HCV replication and assembly seem to converge at lipid droplets⁹³, a unique adaptation that may position budding particles for egress in association with VLDL. Hepatocytes are among the few cell types that secrete lipoproteins, suggesting that utilization of this pathway may dictate the highly restricted tropism of this virus³¹. Coupling of replication and virion formation at membranous intracellular structures might be envisioned to limit the propagation of defective genomes, and may be essential in the context of the highly error-prone RdRp activities⁹⁸. Direct transfer of RNA from an active replication complex to an assembling particle might also provide a mechanism for specific genome encapsidation in the absence of a packaging signal⁹⁴.

Nonstructural proteins and structural proteins

Nonstructural factors might be expected to form transient physical interactions with virion components in order to orchestrate their assembly, indeed it is possible that replicase proteins are incorporated into virus particles at levels below the current limits of detection. Flavivirus NS3 is known to interact with C, as it cleaves the hydrophobic anchor from this structural protein⁷². Passaging of assembly-defective HCV genomes has revealed multiple genetic interactions between structural and nonstructural sequences. Intergenotypic incompatibility of the C-NS2 region with the replicase proteins could be overcome by a compensatory mutation in E1 that acts synergistically with changes in NS2 or NS3⁶³, while genomes with deleterious mutations in C were rescued to various degrees by emergent mutations in p7, NS2 and NS3⁸². Evidence for physical interactions between NS2 and E2¹⁰¹, as well as NS5A and C¹⁰², has also been uncovered. The functional cooperation between NS5A and C in the recruitment of structural and replicase components to lipid droplets as a prerequisite for infectious HCV assembly has emphasized the importance of these associations⁹³.

Conclusions

While the formation of an infectious virus certainly requires concerted action by the structural proteins, evidence highlighted here underscores the importance of nonstructural proteins in this complex process. All *Flaviviridae* seem to share some common requirements for infectious virus production: coordination of the multifunctional NS3 protein with upstream hydrophobic sequences, and the coupling of replication to assembly through the functions of a replicase phosphoprotein and the RdRp. Nonstructural proteins that are involved in these processes might mediate localization of active replication complexes to specialized sites of assembly, regulate the release of RNA from the replicase, or ensure genome incorporation into a budding particle. Additional nonstructural proteins that are absent from the flavivirus genome might address unique requirements of HCV and pestivirus morphogenesis. The apparently coordinated roles of p7 and NS2 could participate in the initiation of virion assembly, mediate glycoprotein organization and budding, or ensure particle maturation, processes that may have diverged mechanistically from the flaviviruses.

Once thought to function solely in the accumulation of viral RNA, *Flaviviridae* nonstructural proteins are now emerging as integral players in the process of virion morphogenesis. With almost every nonstructural protein now implicated in progeny virus production, much work

remains in assigning their specific roles and elucidating their mechanisms of action. Ultimately it may be possible to exploit these essential functions in the development of antiviral drugs to treat the devastating infections caused by this group of viruses.

Acknowledgments

We thank Timothy Tellinghuisen, Richard Kuhn, Chinmay Patkar, Stanley Lemon, and MinKyung Yi for sharing pre-publication results. We acknowledge Timothy Tellinghuisen for critical reading of the manuscript. Work on infectious virus assembly in CMR's laboratory is supported by PHS grants (CA057973, AI072613, AI075099), the Starr Foundation, and the Greenberg Medical Research Institute.

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Fig 1. Life cycle of the *Flaviviridae*
See text for details.

Fig 2. Processing and putative topologies of the *Flaviviridae* polyproteins

The positive-sense RNA genomes of the *Flaviviridae* are translated to yield a single hypothetical polyprotein, which is processed by viral and cellular enzymes. The proposed topologies of the viral proteins with respect to the endoplasmic reticulum (ER) membrane, and the enzymes involved in their liberation from the polyprotein are indicated¹. Structural proteins are shown in yellow, nonstructural proteins are shown in green (required for infectious virus production) or blue (have not been implicated in infectious virus production).

Fig 3. Proposed pathways of *Flaviviridae* assembly and egress

Flaviviridae particles arise through envelopment of the nucleoprotein complex by an endoplasmic reticulum (ER) membrane displaying the viral glycoproteins (E1 and E2, hepacivirus; E¹_{ns}, E1, and E2, pestivirus; prM, E, flavivirus). Virions then transit from the ER lumen to the cell surface via the secretory pathway. **Hepacivirus** morphogenesis is believed to take place in association with membrane-apposed lipid droplets, fatty deposits at which replication complexes also accumulate⁹³. HCV particles appear to become associated with very low-density lipoproteins (VLDL) during assembly or egress^{29,30,31}. **Pestivirus** particles are also proposed to form in the ER before transport through the secretory pathway³. HCV and pestivirus virions are infectious immediately or rapidly after budding^{25,26}. **Flavivirus** particles

are immature until acid-induced rearrangement of the envelope protein E, and furin-mediated cleavage of glycoprotein prM to M in a late Golgi compartment^{22,23}. The formation of capsid and RNA-deficient subviral particles is a hallmark of flavivirus infections.

Fig 4. Required features of the p7-NS2-3-4A or NS2A-2B-3 polyprotein regions

The multifunctional enzyme NS3 and its hydrophobic neighbours in the polyprotein are emerging as conserved requirements for *Flaviviridae* progeny virion production. The determinants of this region involved in proteolysis, RNA replication, and infectious virus production are shown. Pro, protease; Hel/NTP, helicase/nucleoside triphosphatase; yellow, hydrophobic sequences involved in membrane association; orange, zinc-coordination motifs. Green, required region; blue, implicated through adaptive mutations; dark grey, of unknown importance; white circle, enzymatic activity required; black circle, enzymatic activity not required; grey circle, enzymatic activity of unknown importance; black arrow head, site of processing by indicated catalytic activity; white arrow head, cryptic site of processing by

indicated catalytic activity. **NS2-mediated autoproteolysis** of its carboxy terminus from NS3 is an essential step in hepacivirus and pestivirus polyprotein processing. The protease domain of NS3 is required for the catalytic activity of HCV NS2; the involvement of the zinc-binding site, but not the enzymatic activity, of NS3 suggests a structural contribution^{51,52,55}. Pestivirus NS2 protease activity requires only two residues of NS3^{53,61}, but depends on the entire NS2 membrane-associated domain⁵³ and on a cellular cofactor, Jiv^{59,60}. **NS3 protease activity** is present in the amino-terminal domains of the hepacivirus, pestivirus, and flavivirus proteins; NS3 also possesses helicase/NTPase activity. HCV and pestivirus NS4A and flavivirus NS2B proteins are essential cofactors for NS3-mediated proteolysis¹. **Replication** requires the protease and helicase/NTPase functions of NS3. HCV and pestivirus NS2 proteins are not involved in RNA accumulation after release of NS3^{7,8,32}; flavivirus NS2A and NS2B are essential⁶⁵. **Infectious virus production** by HCV and pestivirus genomes requires the p7 protein^{34,39,40}. HCV morphogenesis also depends on a function of NS2 that is independent of its protease activity³⁹, and a role for NS3 has been suggested by emergent mutations^{63,82}. Infectious pestivirus production requires the uncleaved NS2-3 precursor protein in conjunction with NS4A, again without a requirement for the known enzymatic activities of NS2 or NS3^{57,58}. Flavivirus morphogenesis requires NS2A, likely in cooperation with NS3^{67,68,81}; in a second, proteolytic role, NS3 and its cofactor NS2B are important for capsid protein processing^{71,72,73}.

Fig 5. Required features of the NS5A-5B or NS5 regions

The RNA-dependent RNA polymerase and a replicase phosphoprotein have been recently implicated in *Flaviviridae* progeny virus production. The determinants of this region involved in RNA replication and infectious virus production are shown. RdRp, RNA-dependent RNA polymerase; MT, methyltransferase; bar, region of basal phosphorylation; star, site of regulated or hyperphosphorylation. Yellow, hydrophobic sequences involved in membrane association; orange, zinc-coordination motifs. Green, required region; blue, implicated through adaptive mutations; dark grey, of unknown importance; white circle, enzymatic activity required; grey circle, enzymatic activity of unknown importance. **Replication** requires the activities of NS5A and NS5B or NS5 proteins. Regions within the carboxy-terminal domains of HCV NS5A are not essential^{88,94,103}, but binding to a cellular factor, hVAP-A is required^{91,92}. Although many HCV NS5A serines are dispensable, it is unclear if RNA replication is entirely independent of basal phosphorylation⁸⁸. The exact sites of phosphorylation and relevance of these modifications to pestivirus and flavivirus growth are not known. **Infectious virus production** may involve the hyperphosphorylated form of HCV NS5A^{7,88,89,92}; casein kinase II (CKII)-dependent modification of a serine in the carboxy-terminal region is required⁹⁵. Flavivirus NS5 is required to replicate the RNA genome before its incorporation into progeny

virions⁹⁸ and changes in pestivirus NS5B have been shown to specifically abolish infectious virus production⁹⁹.

Table 1*Flaviviridae* nonstructural proteins involved in virion morphogenesis

	Required for RNA replication	Function/Step of infectious virus production	References
Hepacivirus Hepatitis C virus			
p7	–	Infectious virus assembly Egress	39' 40
NS2	–	Infectious virus assembly	39' 62
NS3	+		63' 82, M. Yi and S. Lemon, personal communication
NS4A	+		By analogy to pestivirus
NS5A	+	Lipid droplet association of replicase complex Infectious virus assembly	93 94' 95
NS5B	+		By analogy to pestivirus
Pestivirus Bovine viral diarrhoea virus Classical swine fever virus			
p7	–	Infectious virus assembly	34
Uncleaved NS2–3	–		57' 58
NS4A	+		57' 58
NS5A	+		By analogy to HCV
NS5B	+		99
Flavivirus Yellow fever virus Dengue virus West Nile virus Kunjin virus			
NS2A	+	Genome encapsidation	67' 68' 69
NS2B	+	Cofactor for capsid cleavage	71' 72
NS3	+	Capsid cleavage Genome encapsidation	71'72'73 67' 78' 79' 81
NS5	+	Coupling replication and assembly	98