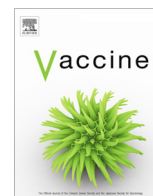Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Review

The role of capsid in the flaviviral life cycle and perspectives for vaccine development

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ABSTRACT

The arthropod-borne flaviviruses cause a series of diseases in humans and pose a significant threat to global public health. In this review, we aimed to summarize the structure of the capsid protein (CP), its relevant multiple functions in the viral life cycle and innovative vaccines targeting CP. The flaviviral CP is the smallest structural protein and forms a homodimer by antiparallel α-helixes. Its primary function is to package the genomic RNA; however, both steps of assembly and dissociation of nucleocapsid complexes (NCs) have been obscure until now; in fact, flaviviral budding is NC-free, demonstrated by the subviral particles that generally exist in flavivirus infection. In infected cells, CPs associate with lipid droplets, which possibly store CPs prior to packaging. However, the function of nuclear localization of CPs remains unknown. Moreover, introducing deletions into CPs can be used to rationally design safe and effective live-attenuated vaccines or noninfectious replicon vaccines and single-round infectious particles, the latter two representing promising approaches for innovative flaviviral vaccine development.

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1. Introduction

The emergence or reemergence of flaviviruses, including dengue virus (DENV), Zika virus (ZIKV), yellow fever virus (YFV), Japanese encephalitis virus (JEV), West Nile virus (WNV), and tick-borne encephalitis virus (TBEV), has posed an enormous threat to global public health in recent decades. Most of these viruses are arthropod-borne viruses (i.e., arboviruses) that are transmitted to vertebrate hosts through the bites of infected mosquitoes or ticks, causing diseases in animals and humans. Among them, DENV is the most important human pathogen, with approximately 390 million infections every year [1].

Flaviviruses are a group of enveloped positive-sense RNA viruses. The viral shell is formed by 180 copies of glycosylated E and M proteins, which are anchored to a lipid bilayer; within the lipid bilayer, the core of the viral particle is the nucleocapsid complex (NC), which consists of capsid proteins (CPs) and viral genomic RNA (vRNA) [2–6]. The viral genome is approximately 11,000 nucleotides in length and contains a single open reading frame encoding a polyprotein of approximately 3400 amino acids. After cleavage by the viral NS3 protease (with cofactor NS2B) and host signalase, the polyprotein generates three structural proteins (C, prM, and E) and seven nonstructural proteins (NS1, NS2A/2B, NS3, NS4A/4B, and NS5) [7].

Over the past decades, great advances have been made to understand flaviviruses, but the CP-related processes, viral encapsidation and uncoating are still unclear. Although vaccines for YFV, JEV, and TBEV have obtained successes and progress has been made in DENV vaccine development, there are still some aspects that are less than satisfactory, and there are still no available vaccines for WNV and ZIKV. Vaccines play a key role in preventing flavivirus infection and controlling viral spread, considering that there is no specific treatment available for the diseases caused by flaviviruses; thus, vaccine development is a consistently important issue in flavivirus research.

2. The functional and structural flexibility of flavivirus capsid proteins

The mature CP is approximately 100 amino acids in length. Although the amino acid sequences of CPs are not very conserved among the *Flavivirus* genus (Fig. 1A), similar structures and properties are shared by all flavivirus CPs. Approximately one-quarter of the residues are basic and mainly cluster at the N-terminus and C-terminus; this property is in accordance with their function in the vRNA package. Moreover, there is a characteristic region predominately composed of hydrophobic residues in all flavivirus CPs, called the "central hydrophobic domain" (Fig. 1A) [8], which has been proven to play an essential role in viral assembly [9].

The structures of the DENV, WNV (Kunjin subtype), ZIKV and JEV CPs have been resolved by different methods [10–15]. All the determined structures reveal that CP is dimeric (Fig. 1B) and that each monomer contains 4 distinct α-helices (termed α1-α4, they are connected by loops), but its N-terminus (approximately 20 aa) is intrinsically disordered [11,14]. The homodimers are connected mainly by extensive hydrophobic interactions and hydrogen bonds [10,13,14]. All flavivirus CP homodimers contain at least two layers of contact interface. The α2-α2′ interface forms the bottom of a hydrophobic cleft (Fig. 1B) that is crucial for viral association with biological membranes [10] and lipid droplets (LDs) (Fig. 1C) [16]. The longest helix pair, α4-α4′, is at the bottom of the homodimer and forms an interface with a high density of positive charges, and this interface is proposed as the vRNA-binding site (Fig. 1C) [10,13]; the α4-α4′ pairs also affect protein stability and overall conformation [17]. The N-terminus and α1-helix of CPs are very flexible among the *Flavivirus* genus, which may help them to adopt different conformations for various physiological processes [12,15]. The α1 of ZIKV CP is much shorter than that of other flavivirus CPs but has a unique long pre-α1 loop. The orientation of the α1 of DENV CP is different from that of others [14]. The third layer of the contact interface on the top of the dimer structures, which is formed by antiparallel (WNV and JEV) α1-α1′ [12,14] or (ZIKV) α1-α1′ with pre-α1-pre-α1′ loops [11,13,15] that occlude the α2-α2′ hydrophobic cleft and form a closed conformation, except the DENV CP dimer. By contrast, the α1 and α1′-helices of the DENV CP dimer form an open conformation without pairing, exposing the α2-α2′ hydrophobic cleft (Fig. 1B). However, it should be noted that the only structure of the DENV CP was solved by NMR in solution, which is relatively less stable than the structures of the ZIKV, WNV and JEV CPs in crystals. A subsequent study confirmed that the DENV α2-α2′ interface is in conformation exchange in the free state, interconverting between an open and a closed state regulated by the flexible N-terminus [18]. Whether other flavivirus CPs also employ the same mechanism for different biological processes is still unclear. In addition, tetrameric and hexameric arrangements are also observed in the WNV [12] and ZIKV [13] CP crystal structures, respectively.

It was assumed that the functional CP requires a highly organized arrangement, and major mutations that transform its structure abrogate its functions [19]. Unexpectedly, the research team of W. Mandl showed that TBEV CP tolerated large internal deletions, and the generated mutants could be divided into three groups according to their viability in cell culture [9,19,20]. For the first group, mutant RNA could generate viable infectious viruses, albeit viral growth was correspondingly decreased [20]. For the second group, the viral production of mutants carrying large deletions was completely or nearly abolished, but pseudorevertants could easily be restored by spontaneous second-site mutations in the capsid gene [9]. For the third group, viral infection spread was irreplaceably abolished by deletions that were too large; these mutants became noninfectious replicons that secreted only subviral particles (SVPs) [19,21]. Consistent with TBEV CP, it was demonstrated that YFV CP, with nearly 40 aa in the N-terminus or 27 aa in the C-terminus, was still functional in RNA packaging [22]. Similar properties were also observed in WNV; when even 36 or 37 residue deletions were introduced into CP by reverse genetics technology, these mutants were still viable [23]. These results together suggest the remarkable flexibility of flaviviral CPs in both structure and function. However, all of these viable mutants or pseudorevertants displayed attenuated viral growth kinetics in cell culture compared with those of the wild type, without influences on RNA replication and translation, but producing substantial amounts of SVPs [9,20,22,23].

3. Capsid protein maturation and the anchor sequence

Post-translation, CP anchors in the ER membrane via an internal signal peptide sequence (known as the anchor) at its C-terminus. The anchor spans the ER membrane and directs the translocation of the downstream prM protein. Cleavage of the C-prM junction employs a coordinated two-step proteolytic process by viral NS2B3 and signalase at the two termini of the anchor, and the signalase cleavage of prM in the lumen remains inefficient until NS2B3 cleavage occurs [24–29].

The lack of polar residues in the c-region of the anchor sequence indicates that it is not a typical signal sequence, and a PQAQA mutant of the c-region with polar residues dramatically uncoupled the coordinated proteolytic process [25]. However, the PQAQA mutant displayed impaired growth kinetics because enhanced signalase cleavage of anchor-prM increased the assembly and release
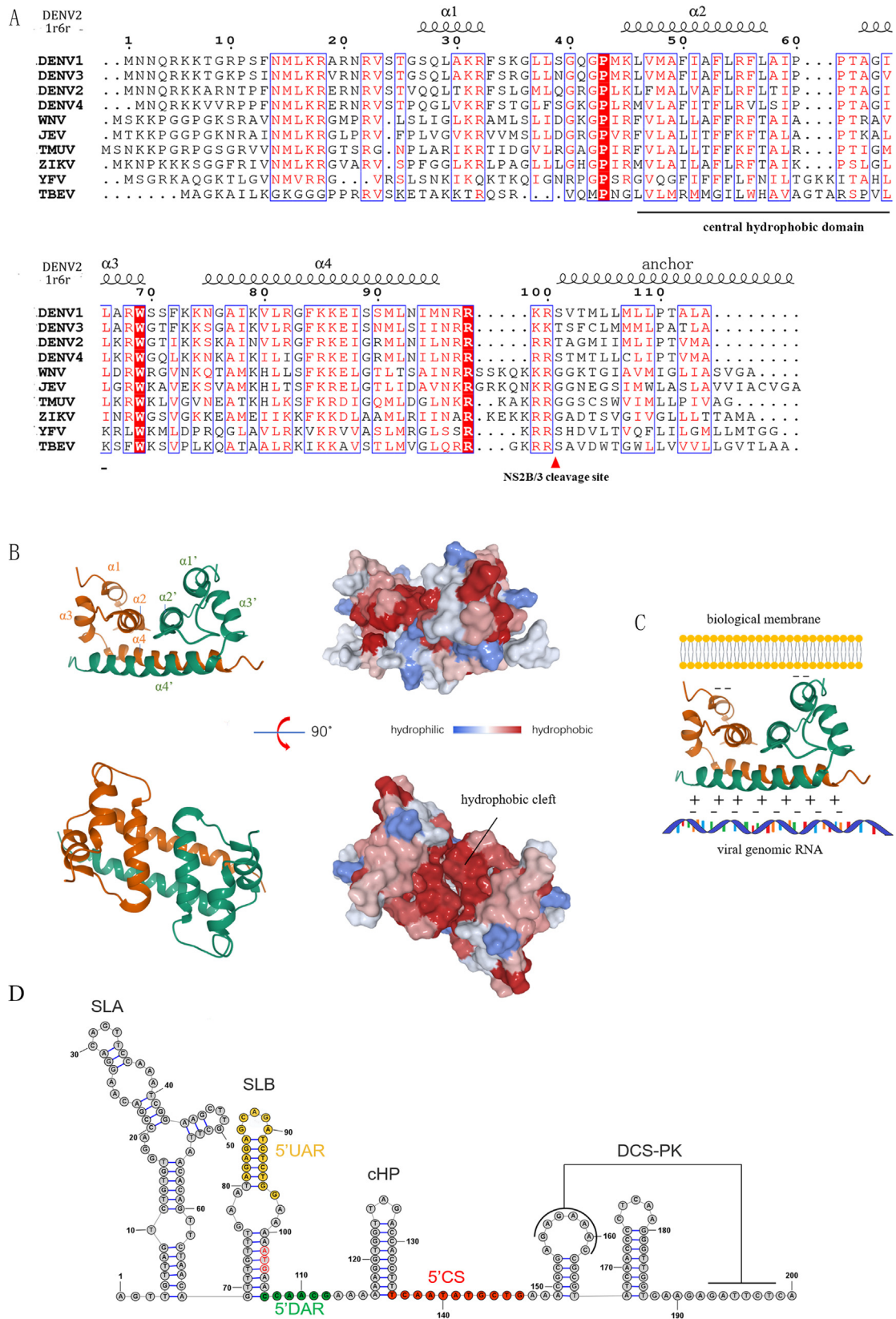
Fig. 1. The structures of flaviviral capsid proteins. (A) The multiple sequence alignments among flaviviral capsid proteins, produced by CLUSTALW. The secondary structure of the DENV2 capsid protein is indicated at the top, and the "central hydrophobic domain" of the DENV4 capsid protein is shown at the bottom. (B) The dimeric structure of the DENV2 capsid protein (1R6R.pdb), with each monomer marked in green and brown, respectively. (C) The model of capsid protein binding to vRNA and the biological membrane. (D) The secondary structure of 1–200 nt of DENV4 genome. 5'UTR:1–101 nt; capsid gene: 102–200 nt. The 5'UAR, 5'DAR and 5'CS elements are highlighted in yellow, green and red, respectively. DCS-PK is also indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of SVPs at the expense of infectious virion production [28,29]; for YFV, the PQAQA mutant is even lethal for viral production [27]. This finding indicates that sequential cleavage at the C-prM junction facilitates infectious particle assembly. In addition, the anchor also contributes to E protein stability to prompt assembly of infectious particles [30], and a cleavable anchor in context is more efficient than separate mature CP for particle assembly [29,31]. Interestingly, a noncleavable C-anchor could not be utilized for packaging the MVEV replicon, but that of YFV and ZIKV could, suggesting that the accessibility of the anchor cleavage site to the NS2B3 protease is virus specific [29,32,33]. Only mature CPs are packaged into virions in flavivirus infection; surprisingly, a recent study reported that unprocessed C-anchor proteins could be packaged into DENV2 pseudoviruses [34]. In summary, the sequential cleavage of the C-prM junction is pivotal for efficient flavivirus assembly, and this process is controlled by sequence elements in the CP anchor.

4. The conserved RNA secondary structures in the capsid gene

Flavivirus CP is involved in vRNA replication in a special manner. Although RNA sequences are not conserved among divergent flaviviruses, there are several conserved RNA secondary structures in the 5′-terminus of the CP-encoding sequence that are necessary for vRNA replication. (1) The AUG codon of CP starts from the stem loop B (SLB) structure, and a partial 5′-upstream AUG region (UAR)-flanking stem (UFS) is located in the CP coding sequence [35]. (2) The 5′ downstream AUG region (5′DAR) and (3) 5′ cyclization sequence (5′CS) [36–38]. (4) A stable hairpin known as capsid-coding hairpin (cHP) is surrounded by the 5′DAR and 3′CS [39,40]. (5) The downstream 5′ CS pseudoknot (DCS-PK) element contains sequences complementary to a region in the 3′UTR [41–43]. Therefore, these *cis*-elements must be taken into consideration when studying CP functions or constructing subgenomic replicon tools and inserting markers between the viral 5′UTR and CP coding sequence.

5. The subcellular distribution of capsid protein

Although current studies on flaviviruses have shown that the flaviviral assembly process does not exhibit a necessary step occurring in the cell nucleus, it has been well demonstrated that many mosquito-borne flavivirus CPs partially localize in the cell nucleus [44–48]; in the cytoplasm, in addition to localizing in the ER, DENV CP and ZIKV CP have also been demonstrated to accumulate on LDs [13,16], but the link between the functional importance and the subcellular distribution of CPs is still unclear.

5.1. Nuclear localization of capsid protein in viral production

Flavivirus CPs localized in the nucleus independent of viral infection, and overexpression of only the capsid gene resulted in its nuclear localization and colocalization with nucleoli [45,49]. Although the molecular weight of flavivirus CPs is relatively low, they are actively transported into the nucleus but not by simple diffusion [44,46]. Interestingly, the nuclear localization signals (NLSs) of flavivirus CPs are heterogeneous [44–46,49–51]. It has also been demonstrated that the nuclear localization of flavivirus CPs is functionally correlated with virus production [45,49,51]. It is worth stressing that the key sites of the predicted NLS are usually basic residues, which play a role in RNA binding for CPs. However, it is still to be solved to uncouple the correlation of nucleolar localization and RNA association in present studies.

5.2. Lipid droplet association of capsid protein

Both the DENV and ZIKV CPs were observed accumulating on the surface of LDs in infected cells [13,16]. It can be speculated that the CP accumulation on LDs may be a common feature in flavivirus infection, but further verification is needed. Experimental evidence indicated that the hydrophobic α2-helix and the positively charged N-terminal region participate in the interaction with LDs [46,52], but these regions are also responsible for ER membrane association and RNA binding, respectively. Thus, studies to define the significance of LD-associated CPs during viral infection are complicated, although the numbers of LDs and viral replication affect each other [16]. It is possible that LDs may serve as a reservoir of CPs in early infection and are then mobilized to the ER membrane for particle morphogenesis when needed [16]. However, the functional significance of CP accumulation on LDs still deserves further study.

6. The role of capsid protein in nucleocapsid formation

One of the most important roles of CPs in the viral lifecycle is the formation of the NC, and the CP dimer is believed to be the building block for NC assembly [53]. Previously resolved cryo-EM structures of both mature and immature DENV virions revealed that the core lacks a well-formed protein organization [2,54]. According to a recent 9-Å-resolution structure of immature ZIKV, the NC core was observed close to the transmembrane domains of the E and prM proteins [55]. The NC core of immature Kunjin virus (KUNV) was also shown to be positioned asymmetrically with respect to the glycoprotein shell [56]. Therefore, these results indicate that the icosahedral axes of the CP shell may not coincide with the axes of the dominant outer glycoprotein shell, thus causing poor reconstruction of the CP shell.

6.1. RNA-binding activity of capsid protein

To assemble the NC, CP must bind to vRNA. Based on the structures of flavivirus CP dimers, the positively charged α4-α4′ interface is proposed as the main RNA binding site [10]. In fact, *in vitro* studies have shown that the N-terminal region of CP also binds to RNA [15,57], and the N-terminal region is essential for viral particle assembly [58]. The CP dimers showed no specificity toward different nucleic acids *in vitro* [13,17]; however, only positive-sense vRNA is packaged in flaviviral virions, and a study *in vitro* showed that the DENV CPs and ZIKV CPs associate with vRNA at specific sites rather than in a random manner [59].

6.2. Nucleocapsid assembly and viral budding

The encapsidation signals for flavivirus genomes have not been identified to date; thus, the specific mechanism of NC formation remains unclear. The nucleocapsid-like particles (NLPs) can be assembled *in vitro* but are larger than the authentic NC, which may be attributed to cellular or viral components participating in NC formation *in vivo* [17,60]. The formation of CP-only particles is unlikely due to the highly positive charge of the CP dimer, which is neutralized by the packaged genome [17]. In cells, the NC may be assembled in a near-neutral environment within the ER membranous compartment, which is physically linked to vesicles and contains the prM-E proteins [61]. It is believed that viral encapsidation and NC incorporation into viral particles is a coordinated process, supported by the fact that the NC has never been isolated from infected cells. Thus, the NC assembly process and the NC structure are still elusive.

Previous structural studies observed a clearly low-density gap between the NC and lipid bilayer [2,3,54,62]; in addition, unlike

in coronavirus and hepatitis B virus, the cytoplasmic sides of the flaviviral prM and E proteins on ER membranes are extremely short and are devoid of a distinct NC-binding domain [20]. These traits suggest that the NC and outer glycoproteins may not directly interact. However, recently determined structures at higher resolutions showed that the NC is asymmetrically positioned with respect to the outer glycoprotein shell in both immature KUNV [56]; however, NC is still positioned concentrically with the outer glycoprotein shell in mature virions [6,56], suggesting a rearrangement of the NC during viral maturation. According to these structural studies, a model for flaviviral budding was put forward: the NCs interact with glycoproteins at the beginning of viral budding and form an immature virion with an eccentrically positioned NC core; during viral maturation, the conformational rearrangements of outer glycoproteins possibly result in loosening of NC-glycoprotein interactions and release of the NC core to the center [56]. Recent studies have also proposed a general model for flavivirus virion assembly. In this model, the transmembrane protein NS2A plays a central role in orchestrating virion assembly [63].

6.3. Nucleocapsid-free subviral particles

SVPs are routinely generated as a byproduct in flavivirus-infected cell cultures; secreted particles contain only the M and E proteins and lipid membrane but lack the NC core. Similar recombinant SVPs can be produced by coexpressing the prM and E proteins in cell culture. The assembly and budding of these particles occur in an NC-independent manner; however, these particles undergo the same maturation and transport process as whole virions [64]. Two distinct sizes of recombinant SVPs were observed in TBEV; however, smaller SVPs (approximately 30 nm in diameter) were far more prevalent than the larger SVPs (approximately 50 nm in diameter) [65]. A structural study revealed that the orga-

nization of the E protein is different in SVPs than in the whole virus [66], but SVPs show hemagglutination and fusion activities similar to those of whole virions and have been proven to be excellent immunogens with protective capability [28,67,68].

7. The uncoating of nucleocapsid

In viral infection, how the NC is dissociated and how vRNA is released to enable protein synthesis are still unknown steps in flavivirus biology because there is little research data for this process to date. Tracing the fate of DENV CP after viral entry, we found that CP was degraded after internalization by a ubiquitin proteasome-dependent process; however, mutational analysis revealed that DENV CP ubiquitination occurs at noncanonical residues but not Lys residues [69]. A deletion mutation study of ZIKV CP using an infectious clone indicated that the α3-helix may also affect NC uncoating steps [33]. These data indicate that both viral and host factors are possibly involved in the viral NC uncoating process; however, as one of the most understudied viral processes in flaviviruses, further attention is urgently needed to clarify the molecular mechanism of the flaviviral uncoating process.

8. Rational vaccine design targeting capsid protein

Due to increasing transnational communication and climatic and socioeconomic changes, emerging and reemerging flaviviruses have caused an ever-growing threat to global public health; a recent example of this issue is the rapid and wide spread of ZIKV throughout the Americas since 2015 [70]. There is currently no efficient medical treatment against these important viral diseases; thus, safe and efficacious vaccines are of great importance for preventing viral infection and spread. In many respects, flavivirus vac-
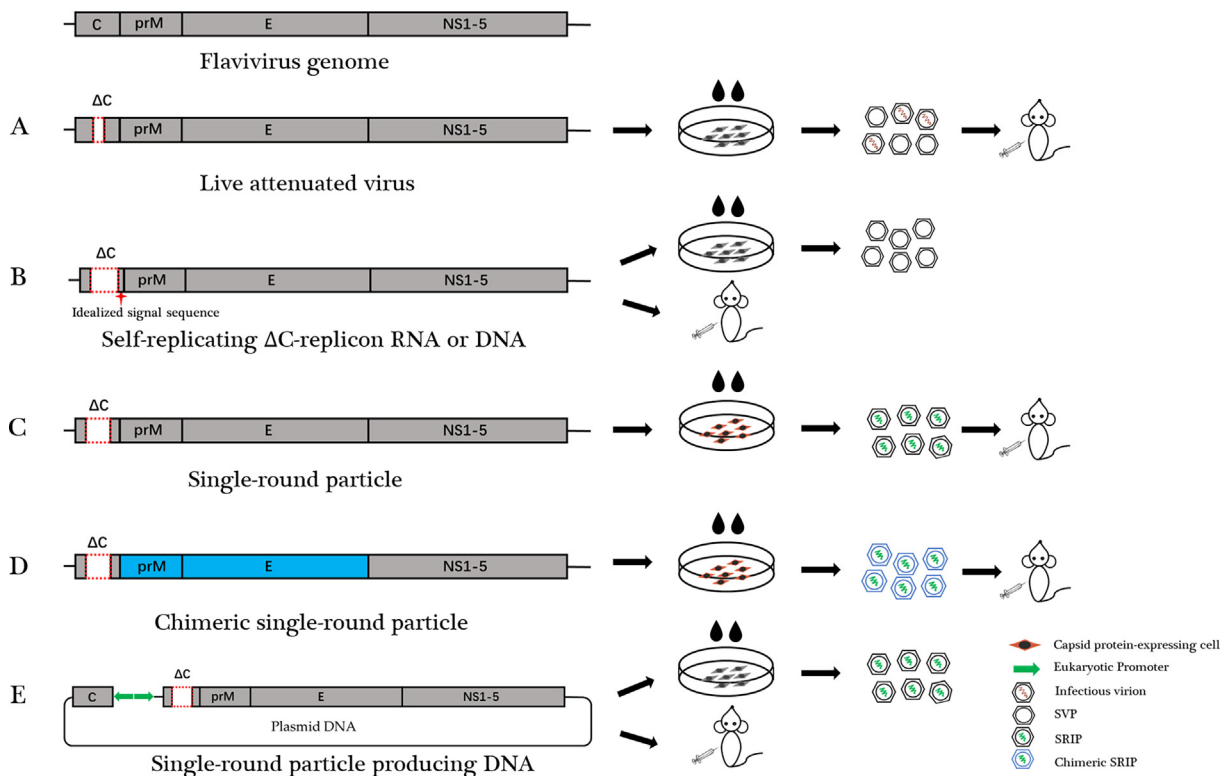
Fig. 2. Rational vaccine design targeting capsid proteins. (A) A schematic diagram of live-attenuated viruses with capsid deletions. (B) A schematic diagram of ΔC replicon vaccines. (C) A schematic diagram of ΔC replicons based on single-round particles. (D) A schematic diagram for chimeric SRIPs. (E) A schematic diagram for an SRIP-producing DNA vaccine. SRIP: single-round infectious particle.

cine development has historically achieved success, such as the most successful live-attenuated vaccine YF-17D and many other live-attenuated or inactivated vaccines against YFV, JEV and TBEV infection [71]. However, concerns about safety have been raised regarding adverse reactions observed with YF-17D and inactivated JEV vaccines [72,73]. In addition, there are still no available vaccines for human against WNV and ZIKV, and the only licensed DENV vaccine is also less than satisfactory for its efficacy and safety [71,74]. Hence, innovative vaccine development must meet the stringent need for safety, efficacy and cost effectiveness and face the challenge of the complicated pathogenesis of some flaviviruses [19]. Manipulation of reverse genetics technology has identified CP as a target for flaviviral vaccine development; both live-attenuated and new-style replicon vaccines can be generated by introducing different sizes of deletions into the CP gene of the viral genome (Fig. 2). We summarize these reports here, and the potential benefits are discussed.

8.1. Live-attenuated vaccines with capsid deletions

As mentioned above, the flaviviral CPs display significant flexibility in structure and function. Introducing different sizes of deletions into CP resulted in various degrees of attenuation of viral replication *in vitro*. Do deletions in CP also affect flaviviral virulence *in vivo*? Assessing the 50% lethal dose (LD_{50}) and the 50% infective dose (ID_{50}) of TBEV ΔC mutants in mouse models, it was found that a small deletion mutant (CΔ28-31) exhibited virulence indistinguishable from that of the wild type, but a viable mutant with a large deletion (CΔ28-35) in the first group was highly attenuated, with a much higher LD_{50} than the wild-type virus ($\geq 10^{6.5}$ PFU versus $10^{0.9}$ PFU) [20]; the pseudorevertants C(Δ28-48/Du78-85) and C(Δ28-48/Q70L) were equally attenuated ($LD_{50} \geq 10^{6.5}$ PFU) but more infective than the mutant CΔ28-35 (ID_{50} of 10 PFU versus $10^{2.6}$ PFU), so a larger attenuation index was achieved (up to $10^{5.5}$, calculated from the ratio of the LD_{50}/ID_{50}) [9,19]. More importantly, upon inoculation with any of the mutants, all the seroconverted mice survived a challenge with a lethal dose of wild-type virus; in other words, the 50% protective dose was equal to the ID_{50}, indicating that all of these mutants elicited a protective immunity and are ideal and highly efficient immunogens [9,19,20]. In addition to TBEV, DENV2 ΔC mutants that were tested *in vivo* also showed significant attenuation in suckling mice and efficiently induced high antibody titers in adult mice [75]. Additionally, ΔC pseudorevertants of WNV did not cause disease but could induce protective immunity even at doses of 10^1–10^6 FFU [76]. Interestingly, a study showed that a CΔ61-71 mutant had abolished ZIKV infectious virion production that was then restored by adaptive mutations (prM-E21K and NS2B-E27G) only in BHK21 cells but not in other cell lines (indicate complex interactions that apparently occur between structural and non-structural proteins during virus replication and/or assembly), making this live virus function like a single-round infectious particle (SRIP) *in vivo* and safely inducing strong immunity protection against vertical transmission in mice [33]. These data indicate that engineering deletions into the capsid gene of flaviviruses via reverse genetics is a feasible approach for generating efficient live-attenuated vaccine candidates.

The flaviviral live-attenuated vaccines are characterized by their high efficiency in seroconversion and protection in animals. The classical live-attenuated vaccines were generated by continuous passaging of the virus in cell culture or animal tissue to accumulate various mutations, finally achieving the aim of significant attenuation but immunogenicity *in vivo*. The admirable example is the YF-17D vaccine, which was developed by 176 passages of the Asibi strain in mouse embryo tissue and chicken embryo tissue, which markedly reduced its viscerotropism and neurotropism [77].

However, this traditional method is usually time consuming; it is unpredictable whether the obtained viruses are attenuated and still can elicit protective immunity. In addition, because multiple mutations spread throughout the viral genome, it is toilsome to clarify the molecular mechanism responsible for the attenuated phenotype, limiting the assessment of the risk for spontaneous reversions to a virulent phenotype. However, using reverse genetics tools, we can rationally design the viral genome to obtain a desired phenotype in a relatively short time, when the desired phenotype and its significance for safety and immunogenicity is well understood, along with the specific molecular determinants behind them. Introducing deletional mutations into the flaviviral capsid gene on the basis of a well-understood molecular mechanism is a unique way to design and generate live-attenuated vaccine candidates, and this approach has several benefits. (1) The successful application of this approach in TBEV, DENV2 and WNV, in addition to the relatively conserved structures of CPs among flaviviruses, suggests that this approach can be a universal approach for different flaviviruses, although there are possibly intricate differences in the length and location of deleted mutations. (2) With regard to safety, the deleted mutations are very stable, especially large deletions, and it is almost impossible to revert to a wild-type sequence. Although risks can arise from intermolecular RNA recombination events between the genomes of closely related viruses, there are few reports on flaviviruses; thus, RNA combination is not a major risk because of the very low frequency [78]. In addition, CP deletions in combination with other well-understood mutations, such as point mutations in the E protein and other different principles, further improve the safety [19,76]. (3) C-deleted viruses contain authentic structural proteins (prM/E) and nonstructural proteins, so they induce only specific immune responses, which should be considered more advantageous than chimeric viruses [19,33]. In addition, C-deleted viruses are highly immunogenic, which is a general advantage of live vaccines; with only a single-dose vaccination, protective immunity can be elicited [9,76]. In summary, introducing deletions into the flaviviral capsid gene is a promising approach for novel flaviviral live-attenuated vaccine development (Summarized in Table 1).

8.2. Capsid-deleted replicon vaccines and single-round infectious particles

Although live vaccines are highly efficacious, there are still risks of rare vaccine-associated diseases, especially in immunodeficient individuals. To address this issue, limiting the infectivity of a vaccine by disabling its viral encapsidation process is an elegant solution. This goal can be easily achieved by introducing very large internal deletions into CP to generate noninfectious ΔC-replicons, therefore opening a new approach for flaviviral vaccines. A self-amplifying ΔC-replicon that is able to produce highly immunogenic SVPs may be delivered as RNA, DNA, or virus-like particles (VLPs) [79].

As a proof of concept, this approach was first verified with TBEV [21]. A 62-residue deletion in TBEV CP (Δ28-89) irreversibly abolished infectious virion formation but maintained RNA replication and promoted SVP production. By engineering supplementary mutations that uncouple the coordinated cleavage events in the C-prM junction (see previous section), an idealized mutant C (Δ28–89)-S that liberates significantly more SVPs was created. Mouse experiments showed that this replicon mRNA vaccine (delivered by a gene gun) induced a broad humoral and cellular immune response comparable to that of a live vaccine and protected mice from challenge; even a single immunization induced long-lasting (more than 1 year) and high titers of neutralizing antibodies [80]. Then, a DNA-based noninfectious ΔC replicon for WNV was also tested in mice, and it induced a high-quality immune

Table 1
Attenuated flaviviruses generated by C-deletion.

Species	Deletion size[a]	Minimal protective dose	Animal model	Reference
TBEV	C(Δ28-43)	$10^{2.6}$ PFU	mouse	[20]
TBEV	C(Δ28-48/Q70L) and	~10 PFU	mouse	[9]
	C(Δ28-48/Du78-85)			
WNV	C(Δ40-75/D39E) and C(Δ51-87)	~10 PFU	mouse	[23,76]
DENV	C(Δ41–49), C(Δ42–51)	ND[b]	mouse	[75]
ZIKV	C(Δ63-71) + prM-E21K + NS2B-E27G	10^5 FFU	mouse	[33]

[a] The deleted amino acids in capsid proteins are highlighted with underscores.
[b] ND: not determined.

response by intramuscular injection and effective protection of animals, similar to those of the virus or infectious DNA, while the magnitude was relatively lower [81]. Therefore, these studies demonstrated the enormous potential of flavivirus ΔC replicons as a new type of nucleic acid vaccine.

Nevertheless, in the early study of nucleic acid vaccines, booster immunizations are often necessary for providing sufficient protection against virulent strain challenges due to the low delivery efficiency of DNA and RNA *in vivo*. To overcome this barrier, one advisable alternative is viral delivery, which delivers replicon RNA via VLPs [79]. The ΔC replicon RNA can be packaged into VLPs by trans-complemented CPs to form SRIPs (or pseudoviruses). True to the SRIP name, these particles can invade cells and release the replicon RNA to the cytoplasm, followed by initial RNA replication but inability to form infectious virions because of the lack of a functional copy of the structural gene in the genome [82–84]; however, the cells infected by SRIP-packaged ΔC replicon RNA should produce a mass of SVPs. To date, different expression systems have been used to package the ΔC replicons of WNV [85], YFV [86] and DENV2 [87,88] to produce SRIPs.

The viral delivery approach of the ΔC-replicon was first applied for YFV and WNV [32], the ΔC-replicons can be easily packaged into VLPs, and the titer reached approximately 10^8 SRIPs/ml [32]. A single-dose intraperitoneal immunization of mice with 3×10^4 WNV SRIPs completely protected the mice from a lethal challenge with a virulent WNV strain [32]. In a subsequent study, a more efficient and safer cultivation system was developed for the production of second-generation WNV SRIPs, which was named RepliVAX WN [85]. Single immunization with RepliVAX WN elicited strong protective immunity against WNV disease in mice and hamsters, and durable protective immunity in hamsters lasted for 6 months [85,89]; in mice, WNV-specific IgG antibody responses and vigorous and specific CD4$^+$ and CD8$^+$ T cell responses were detected after immunization and induced a Th1-biased immune response, similar to live-attenuated vaccines and different from inactivated vaccines [90,91]; importantly, long-lived plasma cells secreting WNV-specific IgG antibodies and CD8$^+$ memory T cell responses were detected at 8 months post immunization [92]. Furthermore, in comparison with that of live-attenuated vaccines and inactivated vaccines in mice and hamsters, the immunogenicity of RepliVax vaccines (for WNV and YFV) was comparable in terms of both the magnitude and durability of the response [90]. Based on the RepliVax pseudoviruses, different single-cycle chimeric flavivirus vaccines were generated for JEV, DENV2, and TBV by replacing the native prM-E genes of C-deleted replicons with those of other flaviviruses [90,93–96]. All of these constructs revealed good capacity to induce a potent and durable immune response in animal models, and it is important that their immunogenicity was not significantly affected by preexisting immunity against the vector backbone [80,96]. There is also an investigation of an SRIP-producing DNA vaccine. This vaccine is based on a C(Δ18–100) replicon cDNA of KUNV, and a separate but complete CP is encoded in reverse orientation controlled by a second cytomegalovirus (CMV) promoter in the same plasmid. After transfection, both the replicon RNA and CP are produced in the cell, therefore forming secreted SRIPs that deliver the ΔC replicon to adjacent cells, and both DNA-transfected and SRIP-infected cells contain the ΔC replicon, which produces SVPs, resulting in an enhanced immune response [31,97].

Both ΔC replicon nucleic acid vaccines and their derived SRIP strategy appear promising for innovative flaviviral vaccine development, especially SRIPs because of their great capacity to provide protective immunity in animal models after only a single immunization, and they can be conveniently propagated and produced at high titers in a stable cell line; moreover, they are reliably safe without causing spread infection. However, SRIPs suffer from a high cost of production and storage and are difficult to purify [79]. Currently, the issues of mRNA instability, inefficient *in vivo* delivery and the difficulty of manufacturing are no longer barriers in the widespread implementation of mRNA vaccines. On the contrary, mRNA vaccines have several beneficial features over inactive and live-attenuated viruses, as well as DNA-based vaccines, such as being safer, being more efficient and having the potential for rapid, low-cost scalable manufacturing (for a review, see [98,99]), so ΔC replicon mRNA vaccines are also very promising alternatives to conventional flaviviral vaccines. In addition to having safety and efficiency similar to those of SRIPs, they are more suitable for meeting the challenges of rapid development and large-scale deployment for emerging virus vaccines [99].

9. Summary

Over the past decades, although we have made important advances in our understanding of key steps in the flaviviral life cycle and their application to development of novel vaccines, many aspects of the viral life cycle remain obscure. Flaviviral CP plays a crucial role in viral assembly. We have learned about structures, posttranslational cleavage and maturation, and the subcellular distribution of flaviviral CP; however, how the NC is assembled and dissociated, how the NC should be incorporated into viral particles and the process of NC uncoating are not well explained to date. Perhaps structural studies on the NC in the future will aid in our understanding of these aspects.

Due to the lack of specific treatments for flavivirus-related diseases, vaccines are the most powerful measures to prevent flaviviral infection and viral spread. Although vaccines have achieved success with respect to YFV, TBEV and JEV, there is still room for improvement concerning safety, efficiency, and low cost. As a viable approach, C-deleted genome-generated live-attenuated viruses are efficient and convenient approaches with several advantages over traditional methods for attenuation of viruses, but safety concerns for live viruses still exist. By contrast, the non-infectious ΔC replicon displays significant potential. Both viral delivery (SRIPs) and nonviral delivery (nucleic acid vaccine) of

the ΔC replicon have potential, and mRNA vaccines of the ΔC replicon are a particularly promising alternative approach [79,99]. However, to meet the need of public health emergencies caused by novel flaviviruses (suggested by ZIKV), the mRNA vaccines of the ΔC replicon can be rapidly put into use, which is also an advantage.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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