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LIUBOV CHERKASHCHENKO

New insights into alphaviral nsP2 functions

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New insights into alphaviral nsP2 functions

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the three original papers, listed below:

I. **Cherkashchenko L.**, Rausalu K., Basu S., Alphey L., Merits A. Expression of Alphavirus Nonstructural Protein 2 (nsP2) in Mosquito Cells Inhibits Viral RNA Replication in Both a Protease Activity-Dependent and –Independent Manner. Viruses 2022 Jun 17;14(6):1327. https://doi.org/10.3390/v14061327.

II. Lello L.S., Miilimäe A., **Cherkashchenko L.**, Omler A., Skilton R., Ireland R., Ulaeto D., Merits A. Activity, Template Preference, and Compatibility of Components of RNA Replicase of Eastern Equine Encephalitis Virus. J Virol. 2022 Dec 19:e0136822. https://doi.org/10.1128/jvi.01368-22.

III. Liu X., Mutso M., **Cherkashchenko L.**, Zusinaite E., Herrero L.J., Doggett S.L., Haniotis J., Merits A., Herring B.L., Taylor A., Mahalingam S. Identification of Natural Molecular Determinants of Ross River Virus Type I Interferon Modulation. J Virol. 2020 Mar 31;94(8):e01788-19. https://doi.org/10.1128/JVI.01788-19.

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My personal contribution to the papers:

I. Responsible for the experimental part, data analysis, preparation of the figures and writing the manuscript.

II. Designed and constructed the set of plasmids of three-component EEEV *trans*-replicase.

III. Involved in the experimental design, conducted pulse labeling of proteins in infected cells and performed analysis of nonstructural polyprotein processing using *in vitro* translation.

LIST OF ABBREVIATIONS

AUD	alphavirus unique domain (same as ZBD)
aa	amino acid (residues)
CD2AP	CD2 Associated Protein
CHIKV	chikungunya virus
CMV	human cytomegalovirus
CP	capsid protein
CPE	cytopathic effect
CPV	cytopathic vacuole
CSE	conserved sequence element
DdRpII	DNA-dependent RNA polymerase II
dsRNA	double-stranded RNA
ECSA	East/Central/South African (CHIKV genotype)
EEEV	Eastern equine encephalitis virus
EGFP	enhanced green fluorescent protein
EILV	Eilat virus
FHL1	four and a half LIM domains 1
FXR	protein from fragile X syndrome family
G	genomic (RNA or promoter)
G3BPs	Ras-GTPase-activating protein (GAP)-binding proteins
GAGs	glycosaminoglycans
HSC70 (90)	heat-shock protein 70 (90)
HS	heparan sulfate
HVD	hyper variable domain
IFN	interferon
IFIT1	interferon-induced protein with tetratricopeptide repeats 1
IRES	internal ribosome entry site
ISV	insect specific virus
JAK-STAT	Janus kinase-signal transducer and activator of transcription
LBD	ligand-binding domain
LDLRAD3	low density lipoprotein receptor class A domain containing 3
MD	macro domain
MAYV	Mayaro virus
NAP1L1	nucleosome assembly protein 1-like 1
NC	nucleocapsid
nsPs	non-structural proteins
NTPase	nucleotide triphosphatase
NTD	N-terminal domain
ONNV	O'nyong-nyong virus
ORF	open reading frame
PKR	protein kinase R
PS	packaging signal
RC	replicase complex

RI	replication intermediate
RRV	Ross River virus
RSE	repeated sequence element
RTPase	RNA triphosphatase
SG	subgenomic (RNA or promoter)
SH3KBP1	SH3 domain-containing kinase-binding protein 1
SINV	Sindbis virus
SFV	Semliki Forest virus
SL	stem loop
SIE	superinfection exclusion
TF	trans-frame protein
Ubi	polyubiquitin promoter
UTR	untranslated region
VLDLR	very low-density lipoprotein receptor
VEEV	Venezuelan equine encephalitis virus
VRP	virus replicon particle
wt	wild type
ZBD	Zinc-binding domain (same as AUD)

1. INTRODUCTION

Viruses are noncellular parasites that can infect a huge variety of hosts, including bacteria, algae, fungi, plants, insects, and vertebrates, including humans. The main feature of viruses is their ability to adapt to the host cell environment and change and use cellular resources to perform their own replication. Viruses are classified based on their origin, hosts, and replication cycles. The current taxonomy (March 2022) recognizes 6 realms, 10 kingdoms, 17 phyla, 2 subphyla, 39 classes, 65 orders, 8 suborders, 233 families, 168 subfamilies, 2,606 genera, 84 subgenera, and 10,434 species of viruses (https://ictv.global/taxonomy).

Alphaviruses (realm *Riboviria,* kingdom *Orthornavirae,* phylum *Kitrinoviricota,* class *Alsuviricetes,* order *Martellivirales,* family *Togaviridae,* genus *Alphavirus*) are positive-strand RNA viruses. Most alphaviruses known to date are arboviruses; they infect their vertebrate host *via* arthropod vectors (invertebrate host). Currently, alphaviruses are one of the major medical concerns worldwide. The genus *Alphavirus* includes important human pathogens such as chikungunya virus (CHIKV), o'nyong-nyong virus (ONNV), Ross River virus (RRV), Sindbis virus (SINV) and Eastern equine encephalitis virus (EEEV). The largest outbreaks have been reported for CHIKV infection, with more than 500,000 recorded cases (1). There are no antiviral drugs or licensed vaccines currently available which protect against CHIKV or any other alphavirus infection. The high pathogenicity of many alphaviruses and the lack of suitable research tools hamper our understanding of the mechanisms involved in virus replication and interactions with hosts and vectors.

The key to understanding the biology and pathogenesis of alphaviruses lies in studying the viral genome replication process carried out by virus-encoded nonstructural (ns) proteins (nsPs), representing replicase subunits and involved in every stage of RNA replication as well as in other processes crucial for virus infection. Biological, biochemical, and biophysical studies have been applied to reveal the functions of individual nsPs as well as their synergistic effects. Among the alphaviral nsP proteins, nsP2 can be viewed as a central player and is therefore extensively studied. nsP2 is a multifunctional protein with four enzymatic activities involved in the RNA replication process. In addition, it also plays an important role in the modulation of antiviral response, development of cytopathic effect (CPE), and virion formation (2, 3). Therefore, mutations introduced into nsP2 result in different effects on viral infection, from virus attenuation (including the generation of a noncytotoxic phenotype) to the complete lack of RNA synthesis and virus infectivity.

The aim of this work was to analyze the properties of components of the alphavirus RNA replicase. The focus was on nsP2 of CHIKV, SINV and RRV and on the RNA replicase of the poorly studied EEEV. This work was intended to discover mechanisms underlying the ability of nsP2 to interfere with infection by competing (superinfecting) virus and to analyze how the functions of nsP2 can be altered by introducing the substitution(s) into the protein sequence. Our findings

revealed new properties of the alphavirus nsP2 protein. Simultaneously, our findings shed light on the formation of virus replicase as the result of sequential processing of the replicase precursor (ns polyprotein) by the wild type (wt) and modified nsP2 proteins of homologous and heterologous alphaviruses. In addition, this work allowed us to map the substitutions in nsP2 that are involved in the regulation of the IFN response. We also obtained novel information regarding RNA replication of EEEV, including previously unnoted requirements of replicase formation and template RNA recognition. These novel findings enhance our understanding of the details of the alphaviral RNA replication mechanisms and interactions between different alphaviruses infecting the same cell. Thus, the current study contributes to the discovery of approaches for the prevention of alphaviral infection.

2. LITERATURE OVERVIEW

2.1. Alphaviruses

The family *Togaviridae* (order *Martellivirales*) represents a group of enveloped, positive-strand RNA viruses. Currently, it contains a single genus: *Alphavirus.* The genus *Rubivirus,* which until recently also belonged to the family *Togaviridae,* was removed to separate the family *Matonaviridae* (order *Hepelivirales*) because based on the current taxonomy (and understanding of viral biology), it is only very distantly related to alphaviruses. Currently, the viruses most closely related to alphaviruses (also belonging to the order *Martellivirales*) are different plant viruses from the families *Bromoviridae, Closteroviridae, Virgaviridae, Endornaviridae, Kitaviridae,* and *Mayoviridae*. The genus *Alphavirus* includes 32 recognized virus species (ICTV, March 2022; https://ictv.global/taxonomy). Most of these viruses are arboviruses, i.e., they are transmitted between vertebrate hosts (including humans) by arthropod vectors, often by *Aedes albopictus* and *Aedes aegypti* mosquitos (4, 5). Based on the disease manifestations and geographical distribution, alphaviruses are divided into Old World and New World alphaviruses. The Old World alphaviruses (e.g., CHIKV, ONNV, SINV, RRV, Semliki Forest virus (SFV), etc.) mainly cause fever, polyarthralgia, myalgia, rash, and headache, while New World alphaviruses (e.g., EEEV, Western equine encephalitis virus (WEEV), and Venezuelan equine encephalitis virus (VEEV)) are mainly responsible for neurological syndromes (6, 7, 8). Based on the similarity of the genome structure and antigenic cross-reactivity of viral structural proteins, arthropod-transmitted alphaviruses are divided into 8 antigenic complexes (serogroups): Barmah Forest, Eastern equine encephalitis, Middleburg, Ndumu, Semliki Forest, Venezuelan equine encephalitis, Trocara and Western equine encephalitis complexes. Based on the amino acid identities of their proteins (42.1–67.1%) and specific ecological niches, the Alaskan harbor porpoise alphavirus and the Southern elephant seal virus were proposed to be included as members of a new antigenic complex named the Marine Mammal Virus Complex (5, 9, 10). In addition, the genus *Alphavirus* also includes fish-infecting Salmon pancreas disease virus as well as an increasing number of insect-specific viruses (ISVs) lacking vertebrate hosts and capable of infecting only mosquitoes. Of note, Eilat virus (EILV) was the first ISV belonging to the genus *Alphavirus,* and several more have been recently discovered (11, 12).

For more than two decades, many outbreaks associated with alphaviruses such as CHIKV, SINV, Mayaro virus (MAYV), and RRV have been recorded. It has been established that changes in climate, demographic and social aspects affect the distribution, ecology, and transmission of alphaviruses (13, 14). Currently, the epidemiological situation has become more global due to the adaptation and colonization of *Aedes albopictus*, one of the main alphaviral transmission vectors, in the majority of Southern European countries (15). A high transmission rate of alphaviruses can lead to the emergence of new, more virulent strains. For example, evidence of increased virulence associated with changes in genome sequences has been documented for the originally avirulent VEEV subtype ID (16).

The natural life cycle of arboviruses consists of the infection of mosquitoes and vertebrates (17). The transmission occurs in sylvatic (between mosquitos and wild animals/birds), rural (between mosquitoes and domestic animals) and urban (between mosquitos and humans) cycles. CHIKV was first isolated in 1952 in Tanzania from a patient's serum, but was not recognized as a pathogen of major concern until the early 2000s. CHIKV has an evolutionary history of several hundreds of years, and according to phylogenetic analysis, four major lineages of CHIKV can be identified: East/Central/South African (ECSA), West African, Indian Ocean (IOL) and Asian (Figure 1). The ECSA lineage is, with respect to its sequence and biological properties, very similar to the IOL (18), i.e., they belong to the same genotype. It is known that the Asian lineage of CHIKV is spreading in an urban cycle, where the virus is mostly disseminated by *Aedes aegypti*, while CHIKV in Africa is typically circulating in the sylvatic cycle and is mostly transmitted by *Aedes furcifer* and *Aedes africanus* mosquitoes (19, 20). However, under certain circumstances, this virus may also establish an urban cycle, a phenomenon that can lead to massive virus outbreaks. This ability makes CHIKV currently the most medically important member of the *Alphavirus* genus. Since 2004, massive CHIKV outbreaks have been reported in Africa, parts of Asia, in the Indian Ocean islands and, since 2013, in the Americas. It is assumed that such outbreaks have also occurred in the past but were not recognized as CHIKV infections, probably due to the similarity of symptoms with dengue fever. The closest relative of CHIKV is ONNV, which has high sequence similarity and very similar antigenic properties and causes similar (albeit milder) symptoms. ONNV displays, however, major differences in transmission, as it cannot be transmitted by *Aedes* mosquitoes; instead, it is transmitted by *Anopheles* (21, 22).

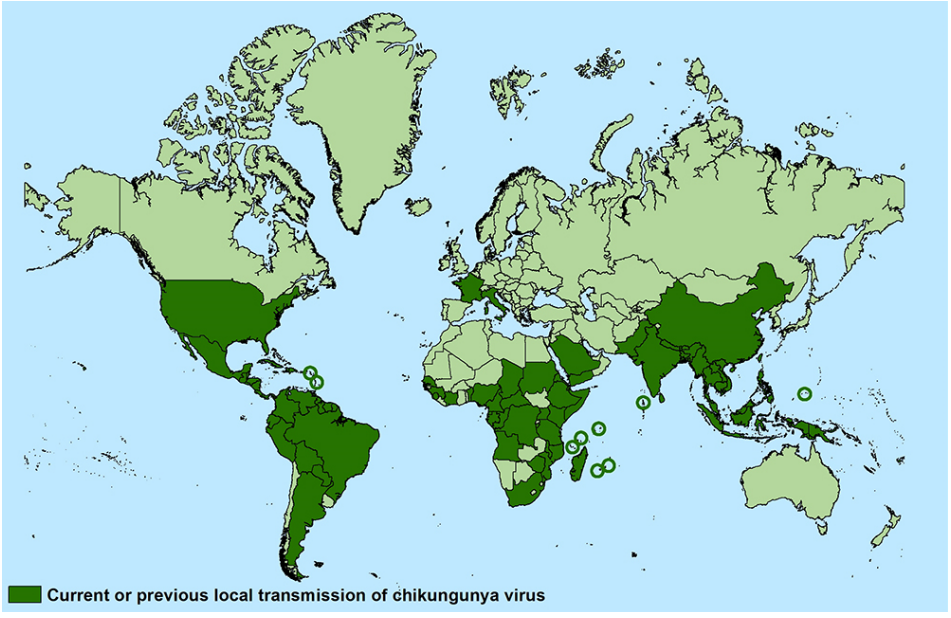

Figure 1. Worldwide distribution of CHIKV (as of October 30, 2020). The countries and territories where CHIKV outbreaks have been registered are marked in dark green (https://www.cdc.gov/chikungunya/geo/index.html).

To date, alphaviruses remain among the biggest threats for many tropical regions, especially due to the efficient adaptation of new transmission vectors (mosquito species), a phenomenon allowing the enhancement of virus spread and, in some cases, the establishment of an urban transmission cycle. The risk of diseases is also enhanced due to cocirculation with other arboviruses that use the same transmission vectors and cause similar symptoms (for example, dengue virus (DENV), yellow fever virus (YFV), and Zika virus (ZIKV)). These factors affect the efficiency of methods for the diagnosis and treatment of arbovirus infections (23, 24, 25).

2.1.1. Alphavirus virion

The alphavirus virion (Figure 2) is approximately 70 nm in diameter and exhibits icosahedral symmetry (T=4). It consists of genomic RNA, the lipid envelope and two distinct protein layers. The inner structure of the virion, the nucleocapsid, contains a single copy of genomic RNA enclosed in the capsid formed by 240 molecules of capsid protein (CP). The envelope is derived from the host cell membrane and carries viral E1 and E2 glycoproteins, which are type 1 membrane-spanning proteins and are arranged in the form of 80 spikes (26). Each spike structure contains three E1/E2 heterodimers. E1/E2 proteins cover the surface of the virion so tightly that the envelope is not exposed.

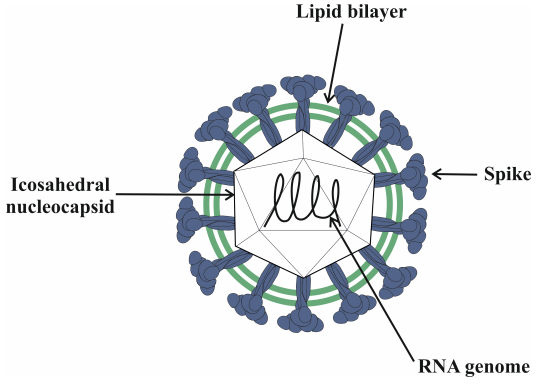

Figure 2. Schematic structure of alphavirus virion. The virion has T=4 symmetry and a diameter of approximately 70 nm. The nucleocapsid core is surrounded by a lipid bilayer with embedded glycoprotein spikes composed of trimers of E1/E2 heterodimers.

2.1.2. Alphavirus genome organization

Alphaviruses have a positive-strand RNA genome approximately 12 kb in length. At the 5’ end, their genomic (G) RNA has a type 0 (7-methyl-GpppA) cap and a 3’ poly(A) tail. G RNA (Figure 3) contains two open reading frames (ORFs) flanked by untranslated regions (UTRs). The first ORF is translated from G RNA and encodes a polyprotein named P1234 or two polyproteins (P123 and P1234)

that are precursors of nsPs. The second ORF is translated from subgenomic (SG) RNA that is synthesized during the infection; it encodes six structural proteins that are also translated in the form of polyprotein precursors. The 5' and 3' UTRs are located at the ends of the genome. Depending on the species, the length of the alphavirus 5' UTR varies between 26–85 nt, while the 3' UTR is longer, typically 87–723 nt in length (27, 28). The third UTR, also named the intergenic region, is located between ORFs and typically has a length of approximately 50 nt. The alphavirus genome contains four conserved *cis*-acting sequence elements (CSEs) which are crucial for viral RNA synthesis (29, 30):

1. CSE1 corresponds to the first 44 nt located at the 5' end of the genome (31). It contains (in the complementary orientation) the promoter for G RNA synthesis. The stem–loop (SL) structure formed by CSE1 is known as one of the determinants of virus pathogenicity due to its ability to mask the type 0 cap structure of alphaviruses from sensors of the host cell and avoid blocking genome translation by IFIT1 (interferon-induced protein with tetratricopeptide repeats 1) (32, 33).
2. CSE2 represents a 51 nt sequence located within the region encoding nsP1. It forms two SL structures, sometimes referred to as SL3 and SL4. Recent studies indicate that CSE2 is not essential for RNA synthesis in mammalian cells but affects RNA replication in mosquito cells. For instance, it has been shown that in mosquito cells, the 51 nt CSE2 is able to increase the production of viral positive- and negative-strand RNAs (34, 35, 36).
3. CSE3 is 24 nt in length and is located at the 3' end of ORF1, sometimes including a few nucleotides from the intergenic region, depending on the virus. In complementary orientation, it represents a promoter for SG RNA transcription (37). The efficiency of SG RNA synthesis initiation depends on the recognition of the SG promoter sequence by viral and possibly host proteins. Mutations introduced into the SG promoter sequence have been shown to inhibit SG RNA synthesis and reduce CPE development in cell culture (38, 39).
4. CSE4 is 19 nt in length and is located immediately upstream of the 3' poly(A) tail (40). CSE4 is a part of the promoter that initiates negative-strand RNA synthesis; another part of this promoter is located at the 5' end of the genome (41). Three subregions in CSE4 were proposed: a 3'-distal region (–19 to –14), a central region (–13 to –7), and a 3'-proximal region (–5 to –1). Of note is that the residues at the –6 and –14 positions were considered as delineating residues. Experimentally, it was shown that mutations in the 3'-distal region of CSE4 of SINV did not prominently alter the efficiency of negative-strand RNA synthesis (–19 to –14). The changes in negative-strand RNA synthesis have been shown to be predominantly caused by mutations or/and deletions in the central and 3'-proximal regions due to their involvement in the initiation of this process. In addition to intact CSE4, 11 to 12 residues of poly(A) have been shown to be required for efficient negative-strand RNA synthesis (42, 43, 44). It was recently discovered that CSE4 and poly(A) can form an RNA pseudoknot that is similar to the human telomerase pseudoknot; mutations that alter the stability of pseudoknot also diminish SINV replication (45).

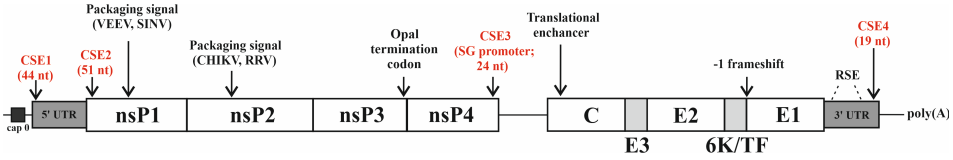

Figure 3. Alphavirus genome organization. Localization of conserved *cis*-acting sequence elements (CSE1-4) is shown in red. Other RNA structures (packaging signals, translational enhancer and RSEs) that are not conserved among all alphaviruses are marked in black. The localization of the –1 frameshift site in the 6K/TF region is indicated. Schematic is not to scale.

In addition to the SL at the 5' end of the CHIKV genome, several more SLs were discovered in the region located in the 5' end of the region encoding nsP1 and named SL85, SL102, SL165, SL194, and SL246 (note that SL165 and SL194 correspond to the abovementioned SL3 and SL4 located in CSE2). Surprisingly, silent mutations disturbing SL165 and SL194 reduced CHIKV titers in human but not in mosquito cells. Thus, it is possible that host-specific effects of mutations in this region also depend on the virus or, perhaps, on the type of introduced mutations (deletions *versus* synonymous changes). The precise function of these RNA structures is under investigation, but presumably, they are involved in the establishment of highly ordered RNA-RNA interactions in the viral genome (32, 34).

The alphavirus genome contains other functional RNA structures that mediate viral replication. Repeated sequence elements (RSE) approximately 40 to 60 nt in length were identified in the 3' UTR. Presumably, the main function of RSE is interaction with the host proteins that consequently determine the host specificity of the virus (46, 47). Genomes of most alphaviruses contain the packaging signal (PS) – RNA structures that ensure efficient packaging of G RNA into the virus particles. It has been shown that the PS of VEEV and SINV is present within the nsP1 coding sequence; however, it was proven that the functionality of PS does not depend on its position in the genome. The PS of alphaviruses belonging to the SFV clade was found within the nsP2 encoding sequence (48, 49). However, it remains unclear what impact, if any, the PS exerts on virion formation, as a very recent study revealed that this element is not required for SFV or CHIKV virion formation. Instead, multiple CP binding sites enriched in G RNA regions but not present in SG RNA (i.e., found only in the 5' UTR and ORF1 region of G RNA) were found to promote SFV replication and G RNA packaging (50).

Another *cis* element has been detected in the region encoding CP of several alphaviruses. This element, called the translational enhancer, is located downstream of the AUG codon of ORF2, and its presence increases the level of SG RNA translation. It was shown that the enhancer is functional only in infected cells, but not in cells transfected with *in vitro* synthesized SG RNAs (51, 52). Thus, its function is not to increase translation *per se* but to allow synthesis of structural proteins at the late stage of infection, when translation of cellular mRNAs is inhibited by the virus. In addition to the translational enhancer, two more *cis*-elements that regulate the translation of viral proteins are present in alphavirus genomes.

One of them serves to facilitate translational read-through of the opal terminator codon often present at the end of the sequence encoding nsP3 (53), while the other is located in the region encoding structural proteins and causes a –1 ribosomal frameshift, resulting in the synthesis of the sixth structural protein of alphaviruses named TF (transframe) (54).

2.2. Overview of alphavirus infection cycle

2.2.1. Binding and entry

Alphaviruses are famous for their ability to replicate in a wide range of invertebrate and vertebrate hosts, such as flies, mosquitos, fishes, birds, and mammals (including humans); alphavirus RNA replication can occur even in the nematode *Caenorhabditis elegans*. Thus, the main obstacle for alphavirus infection is not the inability to synthesize viral RNAs but the ability of viruses to enter the cells, spread within the organism and overcome host defenses. Arbovirus members of the genus *Alphavirus* are capable of productive infection, at least in some invertebrate vectors and vertebrate hosts, indicating that these viruses can bind to and enter corresponding cells. Conversely, the presence of the variety of attachment factors and receptors on the surface of the target cells explains the broad range of species susceptible to alphavirus infection (55). Viruses use attachment factors that contribute to their concentration on the host cell surface, ensuring more efficient spread of infection, as well as factors that are directly required for entry (virus receptors) (56, 57). A number of relatively nonspecific attachment factors have been described. One of the most well-studied attachment factors is heparan sulfate (HS), which belongs to the large glycosaminoglycan complex (GAGs). In the presence of HS, the infectivity of alphaviruses in cell cultures increases. In contrast, the *in vivo* virulence of alphaviruses that have an increased ability to bind HS is often decreased, most likely due to poor release of virions and/or rapid clearance of virions from the blood (58, 59, 60). Two other proteins – ATP synthase subunit β present on the cell membrane and HSC70 (heat-shock protein 70) – can also serve as attachment factors for CHIKV entry. HSC70 is involved in the uncoating of clathrin-coated vesicles, a process that takes place during endocytosis and therefore facilitates alphavirus entry into the host cell. The other factors described to be favorable for attachment of alphavirus virions are prohibitin (PHB), TIM-1 (T-cell immunoglobulin and mucin 1) and DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin) (55, 61).

While attachment factors facilitate the “first contact” of the virus particle with the host cells, it is the cellular receptor (or receptors) that specifically interacts with viral E2 glycoprotein that allows specific binding of virions and their subsequent internalization. Thus, the presence of the corresponding receptors on the cell surface determines the susceptibility of the target cells to virus infection. The identity of receptors used by alphaviruses is not yet fully known. There are several reasons for this. First, alphaviruses may use different receptors for binding to

mosquito and vertebrate cells. Second, alphaviruses can use multiple receptors so that knockout of the main receptor (if known) does not completely block binding and entry of the virus. Finally, different alphaviruses use different receptors. Overall, the picture is complicated, but several important pieces of this puzzle have been recently revealed. Thus, one of the receptors for SINV is natural resistance-associated macrophage protein (NRAMP) and its homolog in mosquitoes. The main receptor for CHIKV, RRV, and MAYV is matrix remodeling-associated protein 8 (Mxra8) (62, 63). According to recently published data, the very low-density lipoprotein receptor (VLDLR) was considered and later tested as a potential receptor for a few alphaviruses (SFV, EEEV, and SINV) (64, 65), and LDLRAD3 has been very recently identified as a receptor for VEEV (66, 67, 68).

From the alphavirus side, there is much less variation – components of the glycoprotein spike of the virion fulfill both target cell binding and entry (membrane fusion) functions. E2 glycoprotein is the receptor-binding molecule (anti-receptor). It has three immunoglobulin-like domains (domains A, B and C), a stem region (domain D) located close to the membrane, a transmembrane domain and a C-terminal endodomain (33 aa) that is mainly responsible for the interaction of E2 with CP, ensuring efficient virion formation and budding (69, 70, 71). Domains A and B of CHIKV E2 have been shown to be involved in the recognition of Mxra8, the main cellular receptor for CHIKV and related alphaviruses.

Internalization of alphaviral particles occurs via clathrin-mediated endocytosis (72, 73). The clathrin-coated vesicles are delivered to the early endosomes of the host cells. The E1 glycoprotein contains a fusion peptide necessary for the initiation of fusion with the host cell membrane. To liberate the fusion activity of E1, which is blocked by its association with E2, conformational rearrangements occur under low pH conditions in endosomes, leading to removal of E2 and establishment of the homotrimeric form of E1 (74). E1 also has the ability to form ion-permeable pores, which contribute to the fusion of the virus envelope with the endosomal membrane of the host cells. This mechanism allows for the free flow of K^+ ions between endosome and cytoplasm environments, which contributes to the establishment of low-pH conditions necessary for disassembly of the viral nucleocapsid (29). As all of these events are essential for virus entry but detrimental for new virion formation and release, alphaviruses have a simple but elegant mechanism to prevent these events at late stages of infection. Namely, at these stages, E2 exists in the form of a p62 precursor (E3+E2) that forms heterodimers with E1 (and these in turn form trimers). The E3 part of p62, as well as individual E3 bound to E2, plays a major role in the stabilization of the E1-E2 dimer and prevents premature exposure of a fusion loop (75). Only after the release of virions from the cell, E3 dissociates, whereupon the virus particle becomes ready for infection of the next cell.

The disassembly of the nucleocapsid occurs due to the interaction of CP with the 60S ribosomal subunit, resulting in the release of the RNA genome that is subsequently used as mRNA for translation of the ns polyproteins (76, 77) (Figure 4).

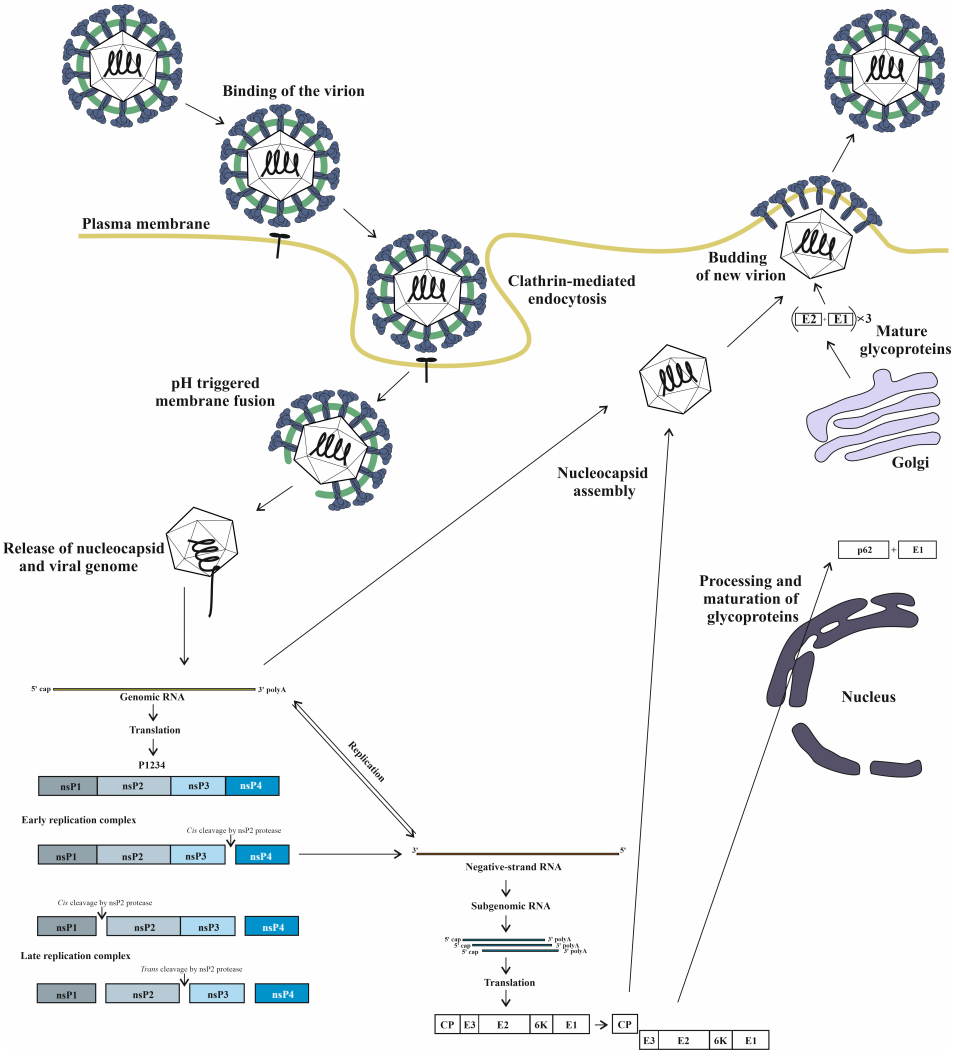

Figure 4. Schematic overview of the alphavirus infection cycle. E2 recognizes the receptor(s) on the cell surface, and bound virions are internalized by clathrin-mediated endocytosis. Under acidic conditions, the viral envelope is fused with endosomal membranes; the nucleocapsid is released to the cytoplasm, where uncoating of the RNA genome occurs. The alphavirus genome serves as mRNA to produce the ns polyprotein P1234, which is sequentially processed. The processing products first form early replicase complexes synthesizing negative-strand RNA and then are converted to late replicase complexes synthesizing G and SG RNAs. SG RNA is used as mRNA to translate structural proteins. Synthesis of CP occurs in the cytoplasm. CP is released from structural polyproteins by its protease activity and interacts with G RNAs, resulting in the formation of nucleocapsids. Precursors of glycoproteins are synthesized by membrane-bound ribosomes in the ER, where they bind to membranes and undergo initial processing. The processing products p62 (E3+E2) and E1 form dimers that are transported to the Golgi apparatus, where the final processing event, the cleavage of p62 precursor, takes place. E1 and E2 heterodimers form trimeric spikes that are delivered to the plasma membrane, where they interact with nucleocapsids. The release of newly formed virions occurs by budding.

2.2.2. Alphavirus RNA replication

Based on existing knowledge, all alphaviruses use a similar strategy of replicase protein expression and RNA replication. Incoming G RNA serves as mRNA for translation of precursors of nsPs; typically, two polyproteins – P123 and P1234 – are synthesized. Synthesis of the prevalent (~90%) P123 occurs due to the presence of the opal termination codon located at the end of the nsP3-encoding region; synthesis of P1234 requires read-through of the terminator, and therefore, its amount is significantly lower: only 10–20% from ns polyproteins (78, 79). However, for some alphaviruses or their strains (CHIKV, SFV, and ONNV), P1234 is the only translation product, as there is no opal termination codon in the nsP3-encoding region (80). The presence or absence of the terminator codon has relatively little impact on the alphavirus phenotype, and it is possible that both variants with and without a terminator coexist in the pool of G RNAs of an alphavirus; the variants with a terminator may be dominant during propagation in insect cells, while in mammalian hosts, the variants without a terminator have an advantage. Similarly, the loss of terminator may be one of the adaptations for accelerated virus growth in cell culture. The relatively low impact of different levels of nsP4 production may be due to an additional mechanism reducing the abundance of this replicase protein (see 2.3.4)

Individual nsPs of alphaviruses are generated by processing P123 and P1234 by protease activity located in the nsP2 region. Processing is well regulated, and only one processing pathway is known to result in the formation of functional replicase complexes (RCs). Therefore, during the first stage, the cleavage of P1234 should occur at the site between nsP3 and nsP4 (hereafter referred to as the 3/4 site; similar naming is used for other sites as well). This processing event occurs *in cis* (although the 3/4 site is also cleavable *in trans*). The cleavage products P123 and nsP4 form a short-lived early replication complex that synthesizes negative-strand RNA (81, 82); most likely, only one negative strand is synthesized per G RNA molecule. As G RNA of alphaviruses serves both as a template for synthesis of P1234 and a template for negative-strand RNA synthesis, it is clear that at some stage before the start of negative-strand RNA synthesis, the translation must be stopped. The mechanism by which this occurs, as well as the mechanisms by which the template RNA is recognized by alphavirus replicase proteins, are not well understood. Negative-strand RNA is not released as an individual molecule; instead, double-stranded RNA (dsRNA, so-called replication intermediate (RI)) containing negative- and positive-strand RNAs is formed. The synthesis of negative-strand RNA is associated with host-cell membranes, but the exact architecture of these structures is poorly defined due to the short-lived nature of early replicase (83). However, it is clear that RI is not exposed to the host cell cytoplasm; instead, it is hidden in forming membranous vesicles (84). The subsequent *cis*-cleavage of the 1/2 site is a delayed event that is probably required to allow the formation of vesicles and the completion of RI synthesis. This cleavage results in the formation of the nsP1+P23+nsP4 complex (85, 86) capable of synthesizing G and SG RNAs. However, its role in infection is unclear,

as the final cleavage between nsP2 and nsP3 occurs almost immediately. Unlike the previous two processing events, this is a *trans* cleavage that results in the formation of a stable late replication complex consisting of all four individual nsPs; this complex is unable to synthesize negative-strand RNA. Instead, it uses the negative strand of RI to synthesize multiple G RNAs and SG RNAs (Figure 5). The formation of replicase complexes occurs at early stages of infection (up to 3–6 h post-infection) and is altered, most likely because the accumulation of mature nsP2 causes a switch in the P1234 processing pattern. Namely, at a later stage of infection, free nsP2 present in infected cells cleaves P1234 (possibly already during its synthesis) into P12 and P34 polyproteins that are not capable of forming functional RCs (87).

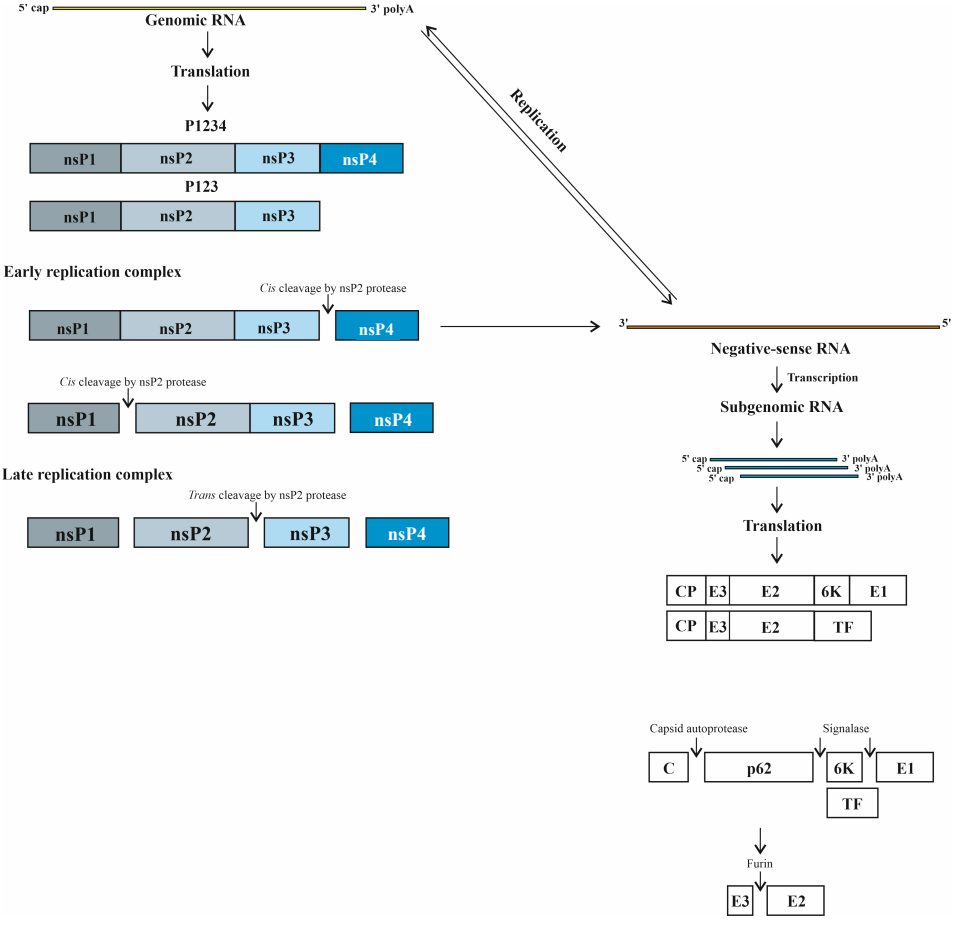

Figure 5. Scheme of alphavirus protein expression. Translation products of G RNA (P1234 and P123 polyproteins) are cleaved into mature nsP1-4 by well-controlled processing events. SG RNA is transcribed using the SG promoter on the negative-strand RNA. Translated structural polyproteins are processed into six individual structural proteins. Schematic is not to scale.

Cryo-immuno-electron microscopy analysis showed that processed nsPs and nascent RNA template are packed in spherules: membranous bulb-like vesicles with an approximate diameter of 50 nm. These structures have been shown to serve as sites for RNA replication (88, 89). It was demonstrated that spherule formation requires the presence of P123+nsP4 and RNA template and that the actual size of the spherules directly depends on the length of the RNA template (90, 91, 92, 93). Later, it was found that the minimal requirement for spherule-like structure formation is the presence of nsP1, nsP4 and uncleaved P23 (93). Very recently, the fine architecture of these complexes was revealed. It was found that the core of the RNA replicase consists of 12 molecules of nsP1, one molecule of nsP4 and one molecule of nsP2 placed at the neck of the spherule and forming a membrane-bound ring structure. This structure is associated with another cytoplasmic ring structure that most likely consists of nsP3 and host factors interacting with nsP3 (94). The formation of spherules occurs at the plasma membrane (95, 96). For some alphaviruses (such as SFV), these complexes are subsequently internalized, forming replication organelles called type 1 cytopathic vacuoles (CPVs) (95, 97, 98). CPVs are altered endosomal and lysosomal structures that contain multiple spherules. Their formation occurs after the establishment of early replication complexes and the beginning of negative-strand RNA synthesis (99, 100).

2.2.3. Expression of structural proteins, virion assembly and budding

Transcription using the SG promoter results in the production of SG RNA, a type of mRNA for the translation of structural polyproteins (101, 102). This translation is started in the cytoplasm by free ribosomes. After synthesis and autocatalytic release of CP, the E3 peptide, which is responsible for translocation of the rest of the structural polyprotein to the ER, becomes exposed. Translation is continued on ER membranes, resulting in E3-E2-6K-E1 or, due to frameshift events, E3-E2-TF polyproteins that are processed by cellular enzymes into p62 precursor (E3+E2), 6K/TF, and E1 proteins (103, 104, 105). The interaction of p62 with E1 leads to the formation of heterodimers; three of these heterodimers are assembled into immature spikes lacking fusion activity. Then, in the *trans*-Golgi network, p62 is cleaved by cellular furin peptidase into E3 and E2 (106).

CP recognizes a specific packaging signal(s) in G RNA, resulting in the formation of NC (107, 108). Presumably, the newly formed NC and mature spikes are delivered to the cellular plasma membrane by type II cytopathic vacuoles (CPV-II). At the plasma membrane, the cytoplasmic tail of the E2 and CP interaction leads to virion formation and release by budding (109, 110). Of note is that the process of assembly and budding of alphaviral particles also depends on the host cell type (in mosquito cells, budding occurs in internal membranes), temperature and pH of the environment (111). Once virions are released from cells, E3 dissociates from spikes (though for some alphaviruses, it remains associated

with virions), activating the fusogenic potential of virions. The precise functions of 6K and TF proteins in virion formation and release are not well understood; deletion of this region reduces virus titers but does not inhibit replication, virion formation or release. One of the studies proposed that 6K is involved in assembly of the spikes and in interactions between E2 and CP, thus ensuring the correct budding process (112, 113).

2.3. Nonstructural proteins of alphaviruses

2.3.1. nsP1

nsP1 (~60 kDa) is a multifunctional membrane-binding protein possessing S-adenosyl-L-methionine (SAM)-dependent methyltransferase (MTase) and m^7GTP transferase (GTase) activities necessary for capping of viral positive-strand RNAs (114, 115). It also serves as the membrane anchor of viral RNA replicase. In the spherules, nsP1 is located in the neck region, forming a monotopic membrane-associated dodecameric ring structure (Figure 6) (116, 117).

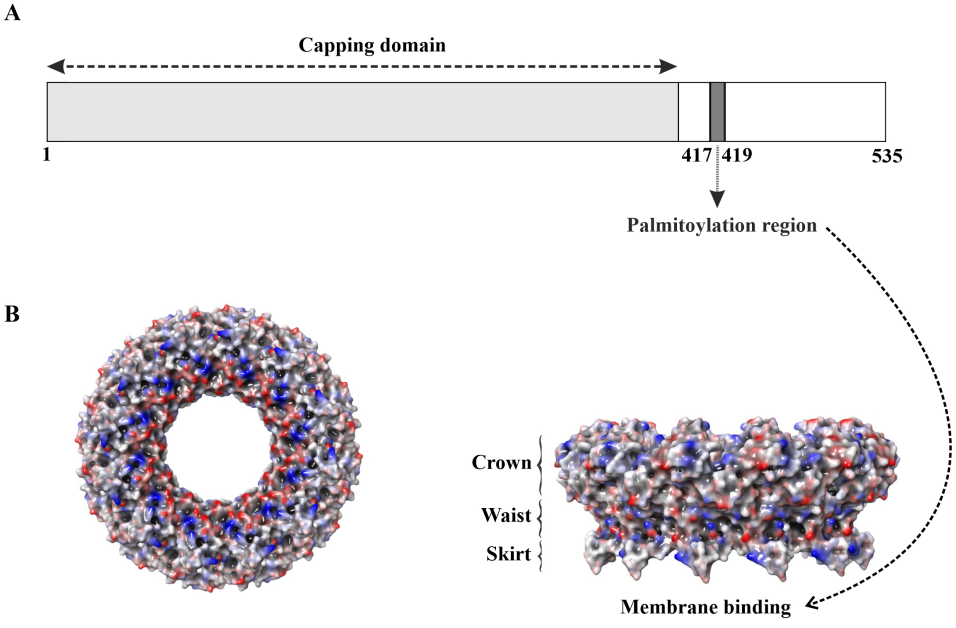

Figure 6. (A) Schematic representation of the nsP1 of CHIKV. The conserved region required for MTase and GTase activities (capping domain) and the region responsible for palmitoylation and membrane binding are shown. **(B) nsP1 forms membrane-bound ring-like structures (PDB ID:6Z0V).** The dodecameric ring structure of CHIKV nsP1 (depicted on the left side) is represented as a molecular surface coloured by electrostatic potential (blue – positive; red – negative); three regions (the crown, waist and skirt) are shown on the right side. At the bottom, membrane-binding spikes are indicated with dashed lines.

nsP1 has no transmembrane domain, and our understanding of the mode of its interaction with the membrane has changed over time. Approximately 20 years ago, it was found that nsP1 contains a sequence of approximately 20 aa in length that, in the corresponding synthetic peptide, forms an amphipathic helix (118). Furthermore, such a peptide can bind to membranes, and mutations in this region of nsP1 are often detrimental to both the enzymatic activities of nsP1 and alphavirus RNA replication. However, while the importance of this region is well supported by experimental evidence, its role in membrane binding in the context of full nsP1 remains uncertain, as it seems not to have contacts with the membrane in the 3D structure of nsP1. The binding of nsP1 to membranes depends on their cholesterol and phospholipid compositions. The interaction with phospholipids has been shown to affect the MTase and GTase functions of nsP1. The membrane binding affinity of SFV nsP1 is increased by palmitoylation of three cysteine residues (^{418}CCC^{420}) (119), and the contacts of these modified residues with the cellular membrane are also observed in the 3D structure of the protein. However, while nsP1 palmitoylation is universal for alphaviruses, its extent and importance are not. SINV nsP1 has only one palmitoylated cysteine residue, and palmitoylation is dispensable; in contrast, SFV lacking nsP1 palmitoylation survives only by acquiring adaptive mutations in nsP1 (120). For some alphaviruses, such as CHIKV, the lack of nsP1 palmitoylation is fatal, as it completely abolishes the activity of CHIKV RNA replicase. Palmitoylation of cysteine residues is also necessary for membrane remodeling (formation of filopodia-like structures) by nsP1 (121, 122). However, the exact role of remodeling of the host cell membranes in alphavirus replication is still not clear.

Modification of the viral genome by adding a cap-0 (m7GpppA) structure at the 5’ end is an important step in the alphavirus infection cycle necessary for protection of the viral RNAs from degradation, for their efficient translation and for avoiding host cell defense (123). The capping mechanism of alphaviruses differs from the cellular mechanism and is performed in three steps. During the first step, 5′ γ-phosphate is removed from the RNA by the triphosphatase activity of nsP2. In the next step, the MTase activity of nsP1 transfers a methyl group from S-adenosyl methionine to GTP, resulting in the m^{7}GTP structure. In the third step, m^{7}GTP is hydrolyzed by the GTase activity of nsP1 and forms the m^{7}GMP-nsP1 complex, after which m^{7}GMP is transferred to the 5′ end of G or SG RNA (124, 125). Capping domains similar to those in alphavirus nsP1 have also been found in rubiviruses, hepeviruses and many plant viruses (126). According to the newly reported results, a portion of alphavirus RNA does not undergo capping, and as a result, both capped and noncapped RNAs are produced and packaged into virions. Presumably, the noncapped RNAs are involved in the activation of the type I interferon (IFN) response (127).

2.3.2. nsP2

The largest alphavirus-encoded nsP2 protein (~90 kDa) has multiple functions. The protein consists of several domains (Figure 7) that form two relatively independent regions: the N-terminal region with RNA triphosphatase (RTPase) and nucleotide triphosphatase (NTPase) activities and the C-terminal region containing a papain-like cysteine protease domain and enzymatically nonfunctional S-adenosyl-L-methionine-dependent RNA methyltransferase subdomain (128, 129). These regions are connected via a flexible linker (130), and the presence of both of these regions is needed for the fourth enzymatic activity of nsP2: RNA helicase activity (131) (Figure 7A, B).

The N-terminal helicase region of nsP2 belongs to helicase superfamily 1. The alphaviral helicase consists of two RecA-like domains with canonical NTPase/helicase motifs responsible for the binding and hydrolysis of NTPs (132, 133). The N-terminal domain of nsP2 has a unique fold and binds RNA using stacking interactions between aromatic amino acid residues and RNA nucleobases (133). It has been demonstrated that the N-terminal part of nsP2 plays a crucial role in causing the CPE in mammalian cells and affects the processing of the 2/3 site in ns polyproteins (131). Unlike other viruses with a positive-strand RNA genome, the alphavirus nsP2 helicase region represented by the first 470 N-terminal aa does not possess the unwinding activity necessary for separation of the RNA duplex or secondary RNA structures; the presence of the C-terminal protease part of nsP2 is critical for this function. The functioning of the alphaviral helicase also requires the presence of the full-length nsP4 protein (134, 135).

Another feature of alphaviral nsP2 is its NTPase/RTPase activity. The NTPase activity was first described for nsP2 of SFV (134) and is most likely used to generate energy essential for RNA helicase activity. The RTPase activity is, as described above, responsible for removal of the γ-phosphate from the 5′ end of the RNA during the initial step of the capping reaction (136).

The C-terminal part of nsP2 is a papain-like cysteine protease crucial for the sequential processing of P123 and P1234 polyproteins. The active site is formed by a catalytic dyad containing conserved Cys and His residues (137, 138, 139). Replacement of catalytic Cys with Ala resulted in complete abolishment of the nsP2 protease activity. In the case of SINV, the Trp residue immediately following the His residue is also needed for the correct functioning of nsP2 (140, 141). As described above (see 2.2.2), the cleavage of P1234 by nsP2 occurs at three sites. Cleavage of the 3/4 site activates the RNA polymerase function of nsP4 (96, 142). Interestingly, for some alphaviruses, cleavage of the 3/4 site occurs only in the early stage of infection (143), likely indicating that for these viruses, the protease cleaving the 3/4 site is P123 or P23, but not an individual nsP2. The cleavage of the 1/2 site occurs in *cis,* and according to previous studies, processing of this site is an important (though not strictly required) step for the initiation of positive-strand RNA synthesis (144). The 2/3 site cleavage is carried out in *trans,* since the C-terminal part of nsP2, and consequently the scissile peptide bond of the 2/3 site, is located too far away from the active site of the nsP2 protease to be processed in a *cis* reaction (145, 146).

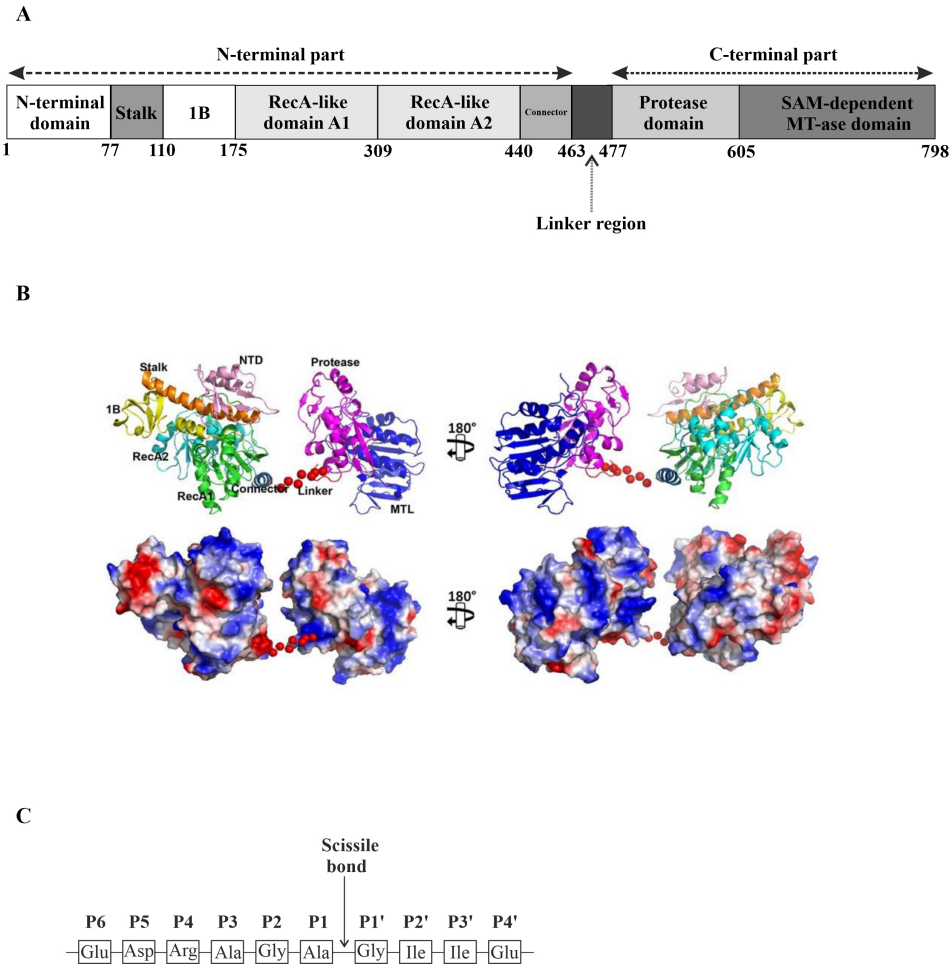

Figure 7. (A) Overview of the structural organization of the nsP2 of CHIKV. The N-terminal part of nsP2 consists of the N-terminal domain (NTD), Stalk and 1B regions and two RecA-like domains. It is responsible for NTPase and RTPase activities. The C-terminal part of nsP2 contains protease and methyltransferase-like (MTL) domains, which are crucial for proteolytic processing of alphavirus ns polyproteins. The N- and C-terminal parts of nsP2 are connected by a flexible linker region. **(B) 3D structure of CHIKV nsP2.** NTD, together with Stalk and 1B domains, serves as a cover for the ssRNA-binding groove (adapted from 130). **(C) Schematic representation of the cleavage site of the nsP2 protease.** The amino acid residues residing around the scissile bond of the CHIKV 3/4 cleavage site are shown, and designations of cleavage site residues are according to Schechter and Berger nomenclature.

The processing of the 2/3 site can only be performed by full-length nsP2 with the native N-terminus and requires the presence of the macrodomain of nsP3 in the substrate; hence, it is considered to be driven by macromolecular assembly rather than recognition of a short cleavage site sequence (146). As individual nsPs are not capable of performing negative-strand RNA synthesis, the 2/3 site cleavage should

represent a “point of no return” for functional RC formation (146). However, contradictory to this, Dé and colleagues showed that temperature-sensitive mutations in nsP4 and nsP2 may lead to the production of negative-strand RNAs at late (stable) stage of replication (147). The reproducibility of existing data and elucidation of how it fits within the current understanding of alphavirus replicase structure and the RNA replication process remain unclear.

The mode of cleavage of each site in P1234 is determined by the properties of the enzyme and those of the cleavage site. Thus, unlike other viruses expressing replicase in the form of a polyprotein precursor, the type of cleavage (*cis* or *trans*) does not generally change during alphavirus infection, with the sole exception being processing of the 3/4 site in P34, which becomes uncleavable during the late stage of infection for some alphaviruses. Nevertheless, the accumulation of nsP2 in infected cells has an impact on the processing pattern (143, 148): when there is no or little nsP2, P1234 is processed first to P123 and nsP4 (pathway leading to RC formation), while high levels of nsP2 favor the processing of P1234 into P12 and P34 (nonreplicative pathway). Thus, the presence and amount of nsP2 affect RC formation. Coherently, mutations introduced in the sequences of the 1/2 or the 2/3 sites alter the dynamics of P123 processing and replication efficiency. Thus, substitution of conserved Gly534 (P2 residue of the 1/2 site of CHIKV) to Val abolishes cleavage at the 1/2 site and has a prominent negative effect on 2/3 site cleavage; Gly1332 (P2 residue of the 2/3 site) to Val substitution abolishes 2/3 site cleavage (Figure 7C). These mutations have a negative impact on virus replicase formation in mammalian cells and result in a temperature-sensitive phenotype of the virus; however, in insect cells, such mutations boost RNA replication (87, 93, 149). The effect observed in insect cells is due to increased stability of P123 and P23 intermediates ensured by slower processing of the precursors, thus leading to more efficient RC formation.

Overall, the facts mentioned above suggest that nsP2 protease and its interaction with cleavage sites in P1234 represent major determinants for efficient virus replication (147). Therefore, it is not surprising that several studies have demonstrated that nsP2 is an important virulence factor in alphavirus infection. However, this is not solely because of the role of nsP2 in alphavirus RNA replicase formation. nsP2 does have additional nonenzymatic activities important for the suppression of host cell transcription and antiviral responses. The cytotoxicity of nsP2 of Old World alphaviruses is caused by its ability to translocate into the nucleus of infected vertebrate cells, where it causes degradation of a catalytic subunit of DNA-dependent RNA polymerase II (RPB1), leading to transcriptional shutdown. Mutation of SINV nsP2 at position 726 (Pro726 to Gly) resulted in reduced CPE in vertebrate cells, reduced virus replication and abolished inhibition of JAK-STAT signaling (150, 151). Mutation introduced to the analogous position of nsP2 of CHIKV (Pro718 to Ser or Gly) has similar, albeit less prominent, effects on the virus – inhibition of the JAK-STAT signaling pathway was significantly reduced, but the cytotoxic effect of virus infection was maintained (152, 153). Mutations affecting aa residues 679–688 of nsP2 of SINV (or aa residues 670–680 of nsP2 of CHIKV) also resulted in a similar effect: corresponding

viruses could not cause RPB1 degradation and shut down host transcription. Interestingly, in these cases, the viral replication efficiency was not reduced (154, 155). These observations show that nsP2, by its nature, is the key protein in the development of CPE in vertebrate cells infected by Old World alphaviruses. More detailed studies revealed that the inhibition of host cell transcription and translation by alphaviruses are separate events and that only the former is clearly associated with the nsP2 protein (156).

2.3.3. nsP3

nsP3 (~60 kDa) is the least understood ns protein of alphaviruses. It is not a part of the RNA replicase core and is probably not directly involved in alphaviral RNA synthesis. However, it is still absolutely essential for viral RNA synthesis. It also exhibits a large number of additional functions. Among other things, it has been established that nsP3 contributes to the vector specificity of CHIKV and ONNV and increases SFV neurovirulence in mouse models (157, 158). In infected cells, nsP3 exists in the form of different complexes. Some of these are RCs; nsP3 is likely the main component of the amorphous cytoplasmic ring and contributes to interactions between viral and host components. At the same time, a fraction of nsP3 forms large cytoplasmic aggregates with unknown functions (159, 160).

Structurally, nsP3 contains three domains: the N-terminal macro domain, the alphavirus unique domain (AUD, also known as zinc binding domain, ZBD) and the C-terminal hypervariable domain (HVD). According to the literature, macro domains similar to those in nsP3 have been found in human proteins as well as in proteins encoded by many RNA viruses (rubella virus, hepatitis E virus, coronaviruses). Furthermore, the crystal structures of CHIKV and VEEV macro domains were found to be similar to the domain found in the proteome of *Escherichia coli* (161). Sequences of macro domains of alphaviruses are highly conserved. Not surprisingly, known functions of the alphavirus macro domain are conserved as well: it serves for binding of ADP-ribose and dephosphorylation of ADP-ribose-1″-phosphate and removes ADP-ribose from mono-ADP-ribosylated (MARylated) substrates. The latter activity can impede the host antiviral response. Recent studies have revealed that mutations in the putative ADP-ribose binding site decreased CHIKV nsP3 ADP-ribosyl hydrolase activity and resulted in virus attenuation and inhibition of virus replication (162, 163). Another study demonstrated that point mutation at position 68 in the active site of the macro domain of SINV nsP3 prevented nsP3 phosphorylation and had a negative impact on negative-strand RNA synthesis (164). In addition to the functions described above, the macro domain is required for the cleavage of the 2/3 site by alphaviral nsP2 (146) (Figure 8A, B).

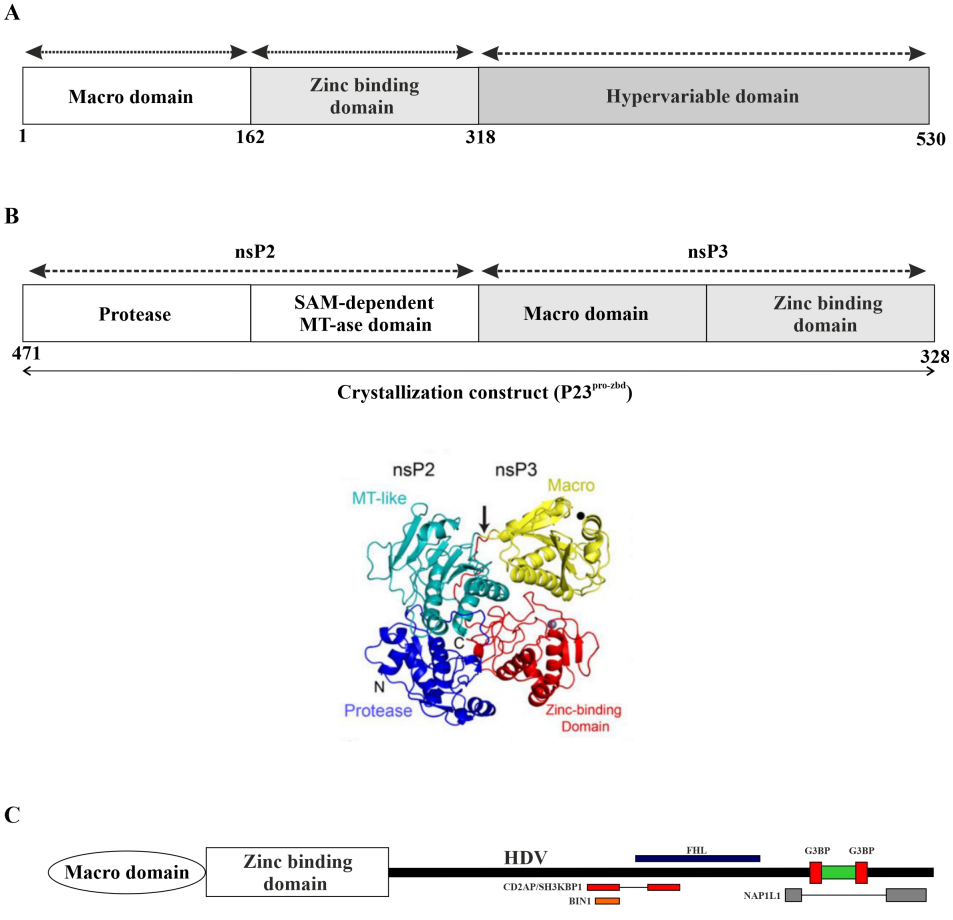

Figure 8. (A) Overview of the structural organization of nsP3 of CHIKV. Three domains are displayed: a highly conserved macro domain, a zinc-binding domain (= alphavirus unique domain) and an unstructured hypervariable domain. **(B) 3D structure of the first two domains of nsP3 of SINV.** The schematic above shows the localization of the ns polyprotein used for structural analysis. In the resolved precleavage structure (i.e., with the 2/3 site not cleaved) of the nsP2 protease and nsP3 macro + zinc binding domain region, the scissile bond is shown by an arrow. The zinc-binding domain was found to have tight contacts with the protease region of nsP2 (adapted from 165). **(C)** Localization of the previously identified binding sites for host cellular proteins in the HVD of CHIKV nsP3.

AUD occupies the central region of nsP3 and binds Zn^{2+} ions due to the presence of a coordination site containing four conserved Cys residues (165). Any interruptions of the integrity of this domain lead to defects in negative-strand RNA production, processing of the ns polyprotein, RC formation and neurovirulence in mice (166). Nevertheless, these functions were not attributed only to the ZBD. Most likely, this indicates that the ZBD functions in synergy with both the macro domain and HVD, but the specific mechanisms of their interactions remain unclear.

HVD is located at the C-terminus of nsP3. As its name implies, this region is highly variable both in length and sequence among different alphaviruses. Unlike the first two domains of nsP3, the HVD is intrinsically disordered (167) – a property common for domains of viral proteins involved in extensive interactions with host-encoded proteins. The nsP3 HVD also has multiple phosphorylation sites (mostly, if not exclusively, Ser and Thr residues) that allow the protein to assume different phosphorylated states during infection (168, 169). Alterations in phosphorylation status have been shown to exert different effects on the replication of alphaviruses. For SFV, removal of phosphorylation sites decreased virus pathogenicity in mice but had minimal effects on RNA synthesis and virus growth rate *in vitro*. At the same time, mutational analysis for VEEV showed that changes in the phosphorylation sites did not affect virus growth or RNA synthesis in mammalian cells, but had a prominent negative impact on replication in mosquito cell lines (170). In contrast, elimination of phosphorylation sites from the HVD of CHIKV is lethal for a virus (171), possibly because some important host proteins can only bind to the phosphorylated sites in the HVD (172). HVD contains a number of short linear interaction motifs that bind host proteins, including amphiphysins (173), CD2AP and SH3KBP1 (164, 174), FHL1 (175, 176), and NAP1L1/NAP1L4 (172). Thus, specific motifs in the nsP3 HVD serve as a central location for interactions with the host proteins necessary for efficient infection. However, while alphavirus replication requires interaction with at least one of these components, none of them is absolutely essential for virus infectivity (177). This is, however, not the case for G3BP1 and G3BP2 (or Rin in mosquitoes) proteins bound by HVD of Old World alphaviruses or FXR proteins bound by HVD of New World alphaviruses (178). These proteins are found in the cellular stress granules, the main function of which is to assist the arrest of mRNA translation. Alphaviruses have "elaborated their own method" to overcome this blockage: once nsP3 interacts with G3BPs, stress granules fail to form properly (179, 180). However, this is not the only – and not even the main – role of the nsP3:G3BP interaction. It was demonstrated that lack of G3BP proteins in the cells blocks replication of CHIKV (178, 181) but not VEEV; conversely, knockout of FXR proteins has no impact on CHIKV replication, but blocks that of VEEV (178). It has been found that the ability of nsP3 to bind G3BPs is important for all Old World alphaviruses and their strains, but to different extents; CHIKV is especially susceptible to G3BP depletion. The molecular basis of this is not yet fully understood. However, it has been demonstrated that in the absence of G3BPs, CHIKV cannot initiate the synthesis of negative-strand RNAs (182). It is also interesting that unlike other alphaviruses, the HVD of nsP3 of EEEV can bind both G3BPs and FXR proteins, and depletion of either of these proteins does not affect its replication (183) (Figure 8C). Taken together, it can be concluded that HDV mediates various interactions with infected host cells, contributing to the role of nsP3 as one of the most important factors in alphavirus pathogenesis (184, 185).

nsP3 also has functions unrelated to viral RNA replication. It has been shown that in SINV-infected vertebrate cells, nsP3 (but not nsP2) is responsible for shutdown of host cell translation (155). nsP3 is capable of activating the

phosphatidylinositol-3-kinase-Akt-mTOR signaling pathway (186) to direct proviral metabolic changes in infected cells (187). An interesting fact about nsP3 was discovered by Varjak and colleagues: they demonstrated that the C-terminal region of SINV and SFV nsP3 contains a degradation signal and that an individual nsP3 protein, released after processing, is degraded during the early replication stages, while the same effect was not observed when nsP3 was expressed as a part of P123. At the same time, several studies suggested that nsP3 of SFV is involved in the stabilization of nsP4 (184, 188).

2.3.4. nsP4

The nsP4 protein (~70 kDa) consists of the N-terminal domain (NTD) and the C-terminal RNA-dependent RNA polymerase (RdRp) domain (Figure 9A). nsP4 is the first ns protein that is released from P1234 and performs the synthesis of alphaviral RNAs as an RNA-dependent RNA polymerase (189). nsP4 contains the active site motif GDD (Gly-Asp-Asp), which is highly conserved among viral RNA polymerases. Its alteration to the GAA sequence (Gly-Ala-Ala) completely inactivates the polymerase activity of nsP4 and abolishes viral RNA synthesis (190). The analysis of the structure of nsP4 has been complicated due to the low stability and poor solubility of recombinant nsP4. Only during 2022 were the structures of the RdRp domains of nsP4 of SINV and RRV resolved. In general, the structures of these two enzymes are similar to each other and to other viral RdRps: characteristic right-hand folds were revealed (Figure 9B). There are also specific features not observed in RdRps of other viruses: the thumb subdomain of nsP4 shows poor structural homology with its counterparts from other RdRps, and its C-terminal region is folded in a different orientation without β-sheet formation. These structural differences, in contrast to the closest viral RdRp homologs, present the alphavirus nsP4 as an α-helix-rich structure. In contrast to the nsP4 of RRV (Figure 9), the nsP4 of SINV did not form the encircled right-hand fold. This may, however, not be a real difference between these two enzymes, as nsP4 of SINV formed dimers (probably as crystallization artifacts), while nsP4 of RRV did not. It was also observed that several regions of the RdRp domain were not visible in the crystal structure, that the NTD was intrinsically disordered, and that the structure of nsP4 was highly dynamic in solution (191). These features are consistent with the poor solubility and activity of an individual nsP4 and indicate that in infected cells, nsP4 must be stabilized, very likely through interactions with other nsPs. The revealed structure of the core of the RNA replicase (Figure 10) confirmed these assumptions: nsP4 is stable in complex with nsP1 and nsP2, it has high enzymatic activity, and all of its regions, including the NTD and C-terminal tail, appear to be folded. The position of nsP4 in the replicase core is also highly logical – it occupies the central pore of the nsP1 dodecameric ring and interacts with the N-terminal domain of nsP2. In addition, channels potentially used for the transport of substrates and RNA transcripts were visible upon this interaction (94).

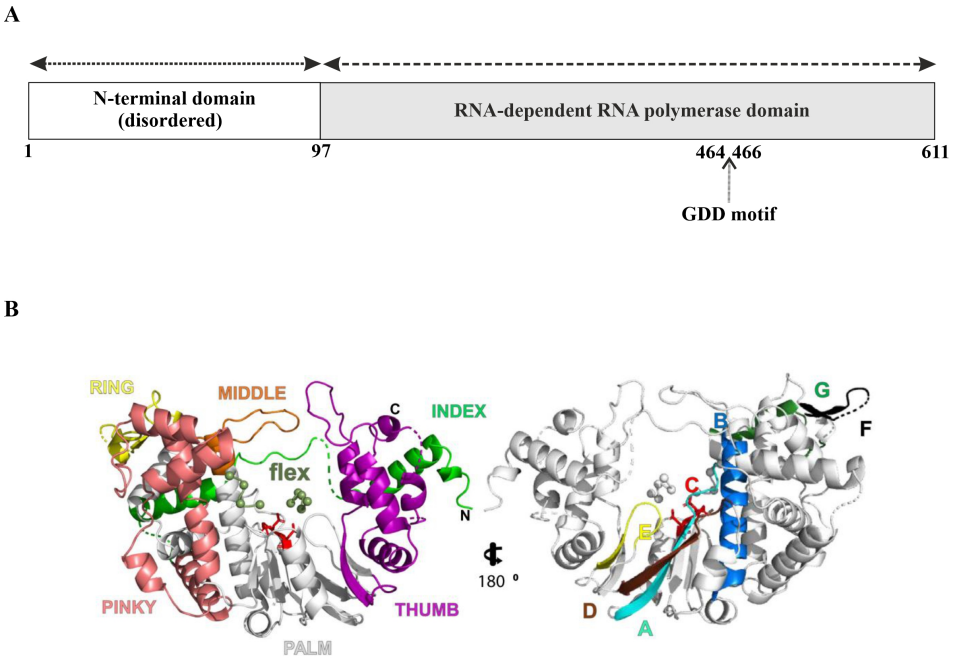

Figure 9. Overview of the structural organization of the nsP4 of CHIKV. (A) nsP4 contains 2 domains. The N-terminal part is unique for alphaviruses. The C-terminal RdRp domain contains a conserved GDD motif and is organized similarly to RdRps of other RNA viruses. **(B) 3D structure of the RdRp region of nsP4 of RRV.** The RdRp domain of nsP4 has a right-hand fold with fingers (index, middle, ring and pinky), palm and thumb sub-domains. The index finger forms contacts with the thumb, resulting in a unique closed conformation. Several regions (flex) appear to have dynamic folds and are not visible in the X-ray structure. The N-terminal region of nsP4 (not shown) is also highly dynamic and, for individual nsP4 proteins, appears to be intrinsically disordered (adapted from 191).

The amount of nsP4 in the infected cells is significantly lower than the amount of the other nsPs (129, 192). First, this is a consequence of the presence of a termination codon preceding the nsP4 coding region present in most alphaviruses. Second, the N-terminal Tyr residue of nsP4, which is absolutely conserved in all alphaviruses, is known as a destabilizing amino acid. According to the N-end rule, the presence of a destabilizing residue leads to the proteasomal degradation of individual nsP4. Incorporation of the protein into the RC makes it stable and prevents its destruction (193); indeed, within the RNA replicase core structure, the N-terminal residue of nsP4 is not exposed. Substitutions of the Tyr residue with any other amino acid, except for other aromatic residues or His, resulted in a lethal outcome for SINV or the appearance of second-site mutations and/or reversions in the genome (194). Again, the structure of the RNA replicase core and that of the nsP2 helicase domain provides a plausible explanation for this phenomenon – alphavirus nsPs can interact with RNA and NTPs using stacking interactions between aromatic amino acid residues and nucleobases. Taking into

account the localization of the N-terminus of nsP4 in the RNA replicase core, it seems that the aromatic group of Tyr (or other aromatic amino acids) is likely used to establish a stacking interaction with GTP, the nucleotide used to prime the synthesis of negative-strand RNA.

It was shown for SINV that nsP4 binds to both G and SG promoters. While an individual nsP4 can recognize the G promoter, recognition of the SG promoter occurs only synergistically with other nsPs. Li and Stollar demonstrated that R331A and R332A substitutions in nsP4 abrogated SG RNA synthesis, specifically the binding of replicase proteins to the SG promoter, without having any impact on G RNA synthesis. This indicates that recognition sites for binding of nsP4 to G and SG promoters are different (195, 196). This also indicates that nsP4 has a specific – and perhaps a leading – role in template RNA recognition. This assumption was supported by recent data. It was observed that the functional RCs of alphaviruses can often be formed by using heterologous combinations of nsP4 and P123 expressed from separate plasmid constructs. For viruses belonging to the SFV complex, it was observed that the heterologous combinations of P123 and nsP4 preferred template RNA that originated from the same virus as nsP4 (197). It was also observed that the compatibility of P123 and nsP4 was often asymmetric; for example, P123 of SFV formed an active replicase with nsP4 of SINV, while the complex formed by P123 of SINV and nsP4 of SFV had virtually no replicase activity. Thus, interactions between P123 (and/or its processing products) and nsP4, which are essential to activate the RdRp activity of nsP4, are likely complex. Some studies have also demonstrated that the intact N-terminal region of nsP4 guarantees the recognition of the rearrangement of nsPs during polyprotein processing events (switching from negative- to positive-strand RNA synthesis) (198). Additionally, the N-terminus of nsP4 was shown to be essential for the recognition of the promoter sequence at the 3' end of the RNA genome as well as for interaction with other nsPs, especially with nsP1 (199, 200). All of these data fit well with the recently revealed structure of the alphavirus RNA replicase core (Figure 10).

In addition to RdRp activity, nsP4 also has terminal adenylyl transferase (TATase) activity. This is required for the synthesis and/or repair of the poly(A) sequence of alphavirus positive-strand RNAs (201, 202), an essential function as negative-strand RNAs of alphaviruses lack poly(U) tails at the 5' ends (203). Truncated versions of nsP4 lacking 97 N-terminal aa (Δ97nsP4) have TATase activity to catalyze the addition of adenosine residues to the 3' end of the RNA in a template-independent manner. In the same experiments, Δ97nsP4 was unable to synthesize negative-strand RNA, indicating the crucial role of the NTD of the nsP4 protein in this process (191, 199). A very recent study also demonstrated that the ability to add adenosine residues in a template-independent manner depends on the presence of nsP2 (94).

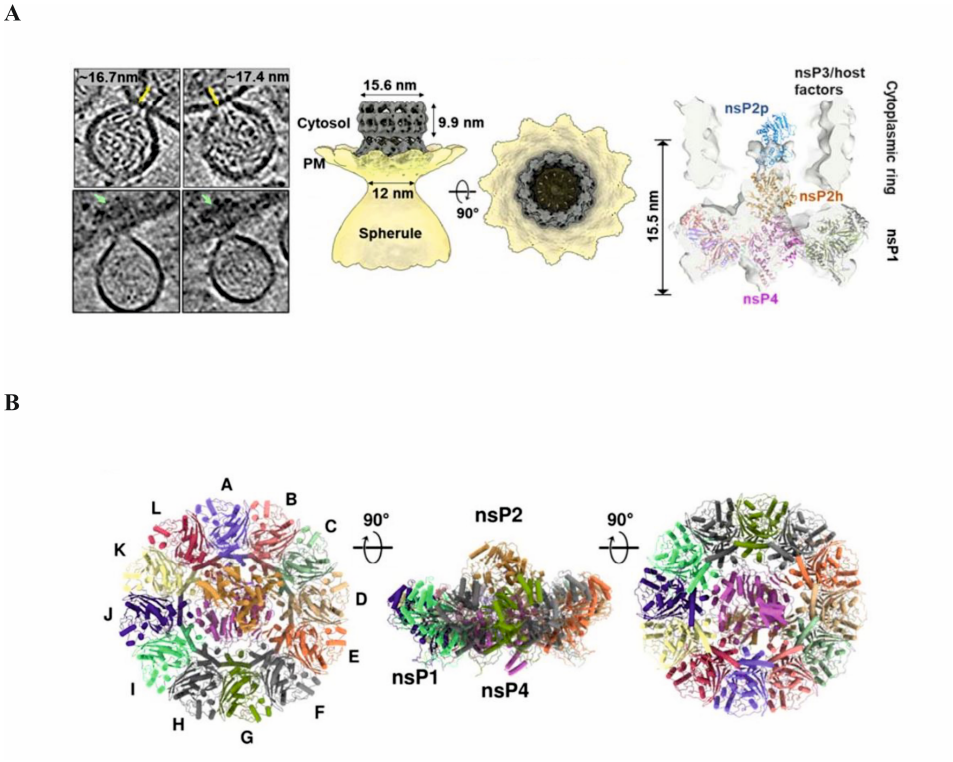

Figure 10. (A) Architecture of the alphavirus replicase complex in CHIKV-infected cells. From left to right are shown examples of cryo-EM images of spherules, resolved structures of the neck of the spherule and a structural view of two ring-like structures present in the neck of the spherule (adapted from 94). **(B) High-resolution structure of the alphavirus RNA replicase core**. The core consists of 12 subunits (A-L) of nsP1, one subunit of nsP4 located in the pore of the nsP1 ring and one subunit of nsP2 interacting with nsP4 via its N-terminal part. Note that in the replicase core, nsP4 is completely folded. The NTD of nsP4 folds between the nsP1 ring and RdRp domain of nsP4, and the C-terminus of nsP4 localizes to the spherule side of the complex (adapted from 94).

2.4. Alphavirus replicon vectors and marker viruses

Working with highly pathogenic viruses requires the use of high biosafety level laboratories. In addition, experiments involving genome mutagenesis (such as construction of chimeric viruses) also cause biosafety concerns. Scientifically more important is that infectious viruses are not always the best model to be used: the ability of a virus to spread in cell culture makes it difficult to focus on specific stages of the infection cycle, and the ability of the virus to revert or generate compensatory mutations complicates analysis of the mutant genomes. One way to circumvent these obstacles is to use various subviral test systems. As one type of these systems, alphavirus-based replicon vectors represent valuable tools for investigating different aspects of viral infection. In addition, they also have

potential for vaccine development and gene therapy applications. The ability to replicate with a high rate, to infect various host cells and the possibility of generating high titer stocks of virus replicon particles (VRPs) are among the advantages of these vectors (201, 202). At the same time, the utilization of any models for conducting various studies faces intrinsic limitations, as these tools may not be applicable in some *in vitro* and many *in vivo* experiments.

Alphavirus replicon vectors are self-replicating RNAs that lack the ability to produce infectious virions. They contain all necessary components for RNA replication – 5' and 3' UTRs, SG promoter(s), and ORFs for ns proteins. Additionally, they may include ORF(s) for marker protein(s) but lack structural protein coding sequences. Due to the presence of nsP2- and nsP3-encoding regions, replicons of the Old World alphaviruses are cytotoxic to vertebrate cells – a property that is advantageous for some (mostly basic) studies and problematic for others (often applied). Introduction of the specific mutations was shown to be successful to limit their cytotoxic effects. Most of the changes related to the construction of noncytopathic replicons were made by introduction of the mutations in nsP2, as this protein is responsible for shutting down cell transcription. As a result, the replicons became noncytopathic, in turn leading to the establishment of persistent infection in mammalian cells with or without changing the kinetics of RNA replication (206). Currently, numerous studies have indicated that specific mutations in other nsPs (nsP1, nsP3 and nsP4) can also contribute to the establishment of the noncytopathic properties of alphaviral replicons for vertebrate cells (207).

In general, the process of constructing alphavirus replicons includes replacement of the region encoding structural proteins with the sequence of a gene of interest; in some cases, additional elements regulating the expression of foreign genes (IRES, duplicated SG promoters, translational enhancer) are also used. Overall, once delivered to the cell, the alphavirus replicon mimics a virus genome except that it is unable to form infectious progeny. The problem with such tools is their delivery to the cells *via* transfection. Transfection may not be efficient for some cell types (often invertebrate cells) and may damage other cells. For some studies, such as analysis of virus entry and establishment of infection, transfection is simply not acceptable, as its mechanism is completely different from natural virion binding, entry and activation of the virus genome. Such obstacles can be overcome by packaging replicon RNAs into VRPs. For this purpose, constructs expressing the structural proteins CP and viral glycoproteins (together or separately), termed "helper RNAs", are delivered to highly susceptible and easily transfectable cells (often BHK-21 cells) together with replicon RNA using the transfection procedure (204, 208). Thus, after transfection, the replicon triggers not only its own replication/transcription, but also replication of helper RNA(s) and synthesis of corresponding SG RNAs. This ensures synthesis of structural proteins and packaging of replicon RNA (but not helper RNAs, due to the lack of packaging signal) into VRPs. Newly produced VRPs are released into the growth medium in a way similar to alphavirus virion release (Figure 11). VRPs obtained using this method resemble natural virions except for one important difference –

they are capable only of a single round of infection, as new particles cannot be formed in cells infected with VRPs. All of the approaches mentioned above ensure the safety of the experiments (209, 210, 211). The advantage of using a replicon-based system is the possibility of introducing one or several genes of interest and studying different aspects of alphavirus replication in the context of a lack of cell-to-cell spread of infection.

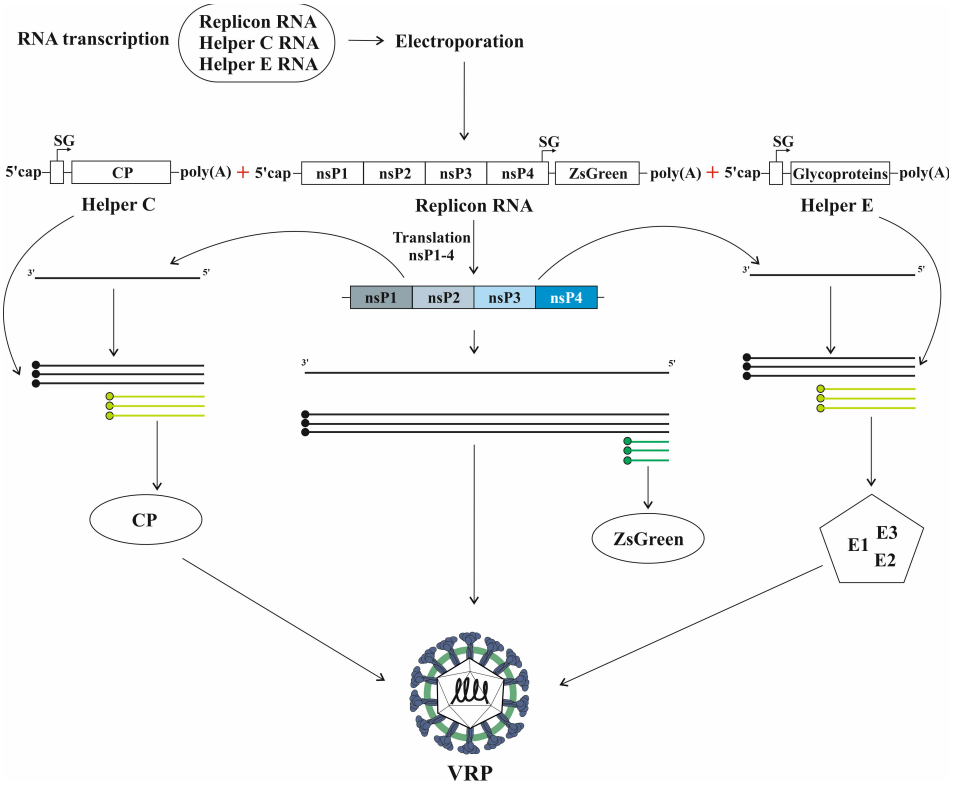

Figure 11. An overview of the strategy used for packaging replicons (generation of VRPs). In replicons, the structural protein region of the virus genome is replaced with the sequence encoding the ZsGreen marker. In the helper constructs, most of the ns region has been deleted, and the SG promoter is used to express SG RNAs encoding capsid proteins (CP) or envelope glycoproteins (E). SP6 – promoter for RNA polymerase of bacteriophage SP6 used for *in vitro* transcription. To package replicons into VRPs, cells are cotransfected with *in vitro* transcribed capped RNAs corresponding to the replicon and helper constructs. The replicon RNA is used as mRNA for translation of nsPs that form RCs, which performs replication of the replicon and helper RNAs – synthesis of negative strands and then new full-length and SG RNAs form the SG promoter. SG RNAs from the helper constructs encode CP and precursors of envelope glycoproteins, while SG RNA from replicons expresses ZsGreen. In the last step, produced structural proteins interact with newly synthesized replicon RNAs that are packed into VRPs and released from transfected cells.

Aside from replicons, alphavirus genomes carrying sequences encoding easily detectable reporters are frequently used for the investigation of different aspects of alphavirus infection. There are multiple ways to prepare these constructs, often called marker viruses. The sequences encoding reporters can be inserted in the part of the genome encoding ns or structural proteins, but are most often inserted between these regions or downstream of the structural region. In these cases, the use of duplicated SG promoters is necessary to ensure the expression of structural proteins or the reporter (Figure 12). As always, there are certain limitations. The marker(s) cannot be too large (or too numerous) due to the limited size of the genome that can be packaged into icosahedral NCs. When markers are introduced into the ns region of the genome, insertions can be performed only in certain positions of nsP1, nsP2 or nsP3; otherwise, they cause instability of the viral genome and a decrease in virulence or nonfunctionality in some cell lines (212). In the case of the insertion of EGFP into nsP2 or nsP3, the expression of nsPs can be tracked, and their subcellular localization depending on the stage of infection can be monitored (213, 214). Most commonly, a duplicated version of the SG promoter is used to control and enhance the expression of a foreign sequence such as EGFP or any other marker gene (215). The practical use of marker viruses is different from that of replicons, as marker viruses do not require helper RNAs and are able to infect/spread in multiple cells at the same time. There are biosafety concerns similar to those for natural viruses. One of the key problems with these tools is their low (or modest) genetic stability – as insertion of markers reduces the speed of viral RNA replication and efficiency of virion formation, these reporter-harboring viruses cannot compete with viruses that have lost marker (often by random deletion). Thus, once such viruses are formed (which invariably occurs), they outcompete the marker virus within a few passages.

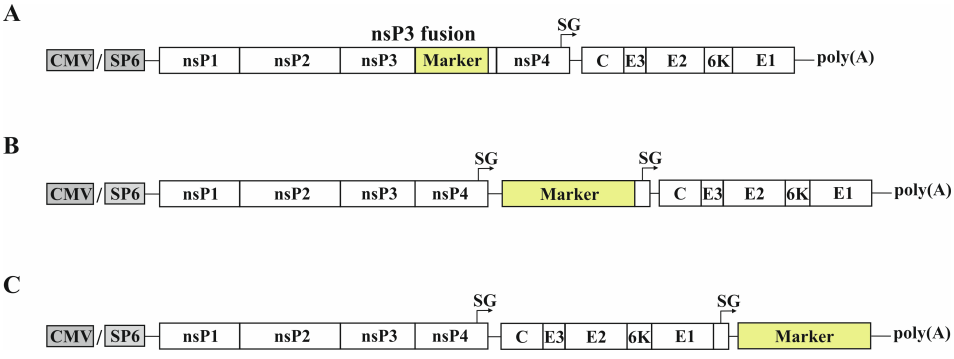

Figure 12. Alphavirus genomes with inserted marker genes. (A) Alphavirus infectious cDNA (icDNA) clone with a sequence encoding a fluorescent marker inserted into the region encoding nsP3. **(B)** Alphavirus icDNA clones with sequences encoding fluorescent markers inserted (upper) downstream of the native SG promoter or (lower) downstream of the duplicated SG promoter placed after the structural region. **(C)** For the former design, a duplicated SG promoter is used to express the viral structural proteins. SP6 – a promoter for RNA polymerase of bacteriophage SP6 used for *in vitro* transcription; CMV – an immediate early promoter for human cytomegalovirus used for transcription with cellular RNA polymerase II in the nucleus of transfected cells.

In summary, replicon vectors and marker viruses have been applied to investigate the functions related to different nsPs and the impacts of mutations on the virus replication rate and/or replicase complex formation (168, 169). However, both tools have intrinsic limitations, and technologies/approaches allowing improvement of tools for studies of virus RNA replication and/or analysis of virus infection *in vivo* are still needed.

2.5. Alphavirus *trans*-replicase system

Alphavirus G RNA serves as an mRNA for translation of replicase proteins and as a template used by replicase formed by these proteins. Thus, the expression of nsPs is tightly coupled with RNA replication (i.e., less replication produces fewer nsPs, which in turn results in even less replication…). Accordingly, in the context of alphavirus infection, the effect of mutation(s) in the nsPs on the RNA replication rate is difficult to evaluate. Furthermore, it is challenging or impossible to differentiate between direct effects (changing the activity of RNA replicase) and indirect effects (for example, those affecting host cell antiviral responses), as both ultimately result in reduced RNA replication. This becomes even more complex because mutations in some key proteins – such as nsP2 – can exert both direct and indirect effects on viral infection. Finally, all systems, where virus nsPs are produced from replicating RNA templates, have intrinsic problems related to adaptations, reversions of the introduced mutations, pseudoreversions and/or introduction of second-site compensatory mutations in the mutated genome or replicon during the RNA replication process. Consequently, a method for estimating alphaviral RNA replication/transcription levels without changes in the production of replicase proteins would be highly appropriate for conducting basic alphavirus studies.

Alphavirus replicase is highly active *in trans* – it is not restricted to use of its own mRNA for subsequent replication; it can find and use any suitable RNA in the cells. This property has been known for nearly 50 years, as passaging of alphaviruses in cells gives rise to defective interfering particles (216). The RNAs packaged in such particles cannot encode replicase; instead, they use replicase synthesized by coinfecting normal virus. To do so, the defective genome should contain only *cis*-active sequences needed for RNA replication. The same property of alphavirus replicase is used in replicon/helper RNA systems (Figure 11): helper RNA does not encode for replicase proteins and uses nsPs produced by the replicon RNA for its replication and transcription. These properties were used for the development of alphavirus *trans*-replicase systems (Figure 13A) (190, 217), allowing uncoupling of replicase protein expression and viral RNA synthesis.

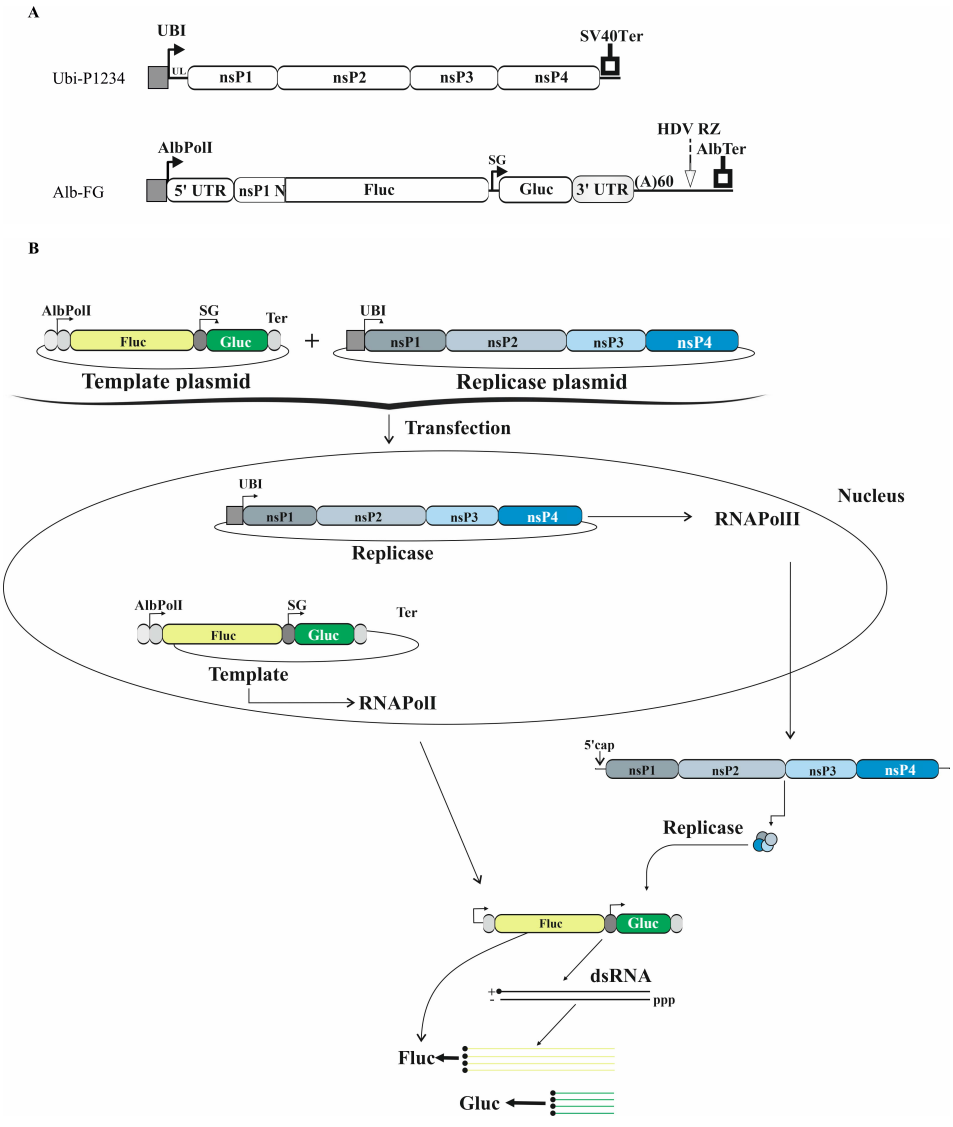

Figure 13. *Trans*-replicase assay in mosquito cells. (A) Schematic representation of plasmids used for the expression of P1234 of an alphavirus (Ubi-P1234) and replication-competent template RNA encoding the Fluc (genomic) and Gluc (subgenomic) markers (Alb-FG). Designations are the same as for **Figure 1, Publication I. (B) Principle of the *trans*-replicase assay.** Cells are co-transfected with plasmids expressing mRNA encoding P1234 and a plasmid expressing RNA template. The RNA transcribed from the P1234 expression plasmid cannot be replicated due to the lack of essential *cis*-sequences and serves only for translation of replicase proteins. The plasmid containing the sequence of template RNA is transcribed by RNA polymerase I; accordingly, a corresponding transcript lacks a 5' cap and is poorly translated. When the template RNA is recognized by replicase proteins, RCs are formed, and RNA replication is initiated. The latter results in the synthesis of multiple copies of the capped full-length template RNAs used for the expression of Fluc (marker of replication activity); in addition, capped SG RNAs used for the expression of Gluc (marker of transcription activity) are synthesized. The activity of virus replicase is typically estimated by comparison of Fluc and Gluc activities of the replicase under investigation and polymerase-inactive replicase.

The first *trans*-replication assay was elaborated for SFV (217, 218). Multiple changes have been applied to improve the efficiency of the assay, such as codon optimization of replicase-expressing sequences, selection and optimization of *cis*-active sequences included in constructs expressing template RNAs for these replicases, and selection of suitable promoters for replicase and template RNA synthesis (217). Many studies have shown that the efficiency of this system depends on the choice of promoters and on the cell lines selected for experiments. Several promoters have been tested: the T7 RNA promoter, which requires co-expression of T7 TNA polymerase, the immediate early promoter of human cytomegalovirus (CMV) for mammalian cells, the *Aedes aegypti* polyubiquitin promoter (Ubi) for mosquito cells (149, 190) and, for template RNAs, promoters of human, hamster or mosquito RNA polymerase I (219).

The general principle of the *trans*-replicase system is simple but elegant (Figure 13B). The plasmid encoding the replicase proteins produces mRNA that encodes the same proteins as viral G but lacks *cis*-acting elements crucial for RNA replication, thus making it unable to replicate. On the other hand, a suitable RNA template is transcribed from another plasmid delivered to the cell together with the replicase-coding plasmid. This RNA template contains necessary *cis*-elements, which interact with nsP1-4 provided *in trans* (220). Once the replicase proteins and the template RNA "find each other", the synthesis of negative-strand RNA is initiated, which in turn leads to spherule formation and subsequent amplification of template RNA (replication) and production of a truncated version of SG RNA (transcription). This simple but robust system has been applied for the investigation of RC formation and the dependence of spherule size on the length of RNA templates, for the analysis of minimal requirements of the replication competence of RNA templates, for tagging different replicase proteins and for the study of the effects of mutations introduced into replicase proteins on viral RNA synthesis in cell culture (92, 93, 190, 217).

G and SG RNA versions synthesized in a *trans*-replicase system can be detected and quantified directly using Northern blotting or RT–qPCR. These methods are, however, laborious and/or expensive. Therefore, the template RNA used in the *trans*-replicase system is designed to express easily detectable reporters whose expression is increased (boosted) in the presence of replicase expressed from the corresponding plasmid (Figure 13). In an ideal system, the background level of reporters should be undetectable (no expression in the absence of active replicase); in reality, this is not achievable. Therefore, to eliminate the background activity generated by the reporters (typically, luciferases), a proper negative control for the *trans*-replication assay is needed. For construction of the plasmid expressing inactivated polymerase (negative control), the conserved GDD motif in the active center of nsP4 was replaced with GAA. Another issue is to reduce the natural background level of marker expression. This may not be necessary for a marker placed under the SG promoter, as its expression from nonreplicating template RNA is very low; corresponding activation requires template replication and SG RNA synthesis. For the marker placed under the G promoter, it is more challenging, as its expression can also occur from nonreplicating (cellular RNA polymerase

generated) template RNAs. Indeed, numerous experiments have pointed out that the increase in the expression of such markers in the presence of an active replicase is difficult or impossible to detect (149, 190). This has been circumvented by the use of the promoters of T7 RNA polymerase or RNA polymerase I: corresponding RNA polymerases produce uncapped transcripts that are poorly translated; in contrast, copies generated by a replicase (G RNAs) have a 5' cap and are therefore more efficient mRNAs for reporter expression (Figure 13B). In general, good results are obtained in mammalian cells; however, in mosquito cells, these approaches are somewhat less efficient (190, 219, 220). Therefore, additional approaches for reducing the background level of reporter expression and/or increasing replicase-mediated G RNA synthesis are needed for these cells.

2.6. The phenomenon of superinfection exclusion during alphavirus infection

Numerous studies have been dedicated to revealing the mechanisms of RNA replication, virus–host interactions and changes induced in host cells during alphavirus infection. Another aspect of alphavirus infection that has been studied over decades is the specificity of the interaction between homologous or heterologous viruses. The results of these investigations revealed the existence of the superinfection exclusion (SIE) phenomenon (221). SIE, also called homologous interference, is a process by which the infection of a cell with the "first" virus eliminates the ability of the next "incoming" (superinfecting) virus to establish infection in the already-infected cell. SIE is a common phenomenon found in many virus groups and families. Mechanisms causing SIE are diverse, from removal of virus receptors from the surface of the infected cell to the direct inhibition of gene expression and genome replication of a superinfecting virus.

For alphaviruses, SIE was described nearly 50 years ago (222). Despite the numerous studies dedicated to the study of SIE caused by alphaviruses, its nature is rather poorly understood, and available data are controversial. The reasons behind the lack of exact knowledge include limited understanding of events occurring during the very early stages of infection as well as the diversity of alphaviruses and their hosts (especially considering that SIE is established both in infected vertebrate and invertebrate cells). What is known can be summarized as follows. SIE is established very rapidly. Upon infection, the genome of the first virus is translated and replicated, while the genome of the superinfecting virus is translated, but RNA replication does not occur. The efficiency of SIE is both time- and virus type-dependent. In early studies, Johnston *et al.* showed that primary infection with SINV blocked the replication of the second (homologous) virus within the first 15 minutes, and the maximum inhibition was achieved as early as 60 minutes postinfection. Very similar results were obtained by Singh and colleagues for SFV (223, 224). It was also observed that infection with SINV caused SIE for homologous virus (SINV) infection; however, SINV-infected cells

remained infectible and supported replication of EEEV, a heterologous alphavirus (225). A study performed using *Aedes albopictus* cells revealed that cells infected with CHIKV, SINV or SFV maintained SIE against homologous virus indefinitely. However, at the stage when persistent infection was established (at 8–10 days postinfection), the cells infected with CHIKV supported limited (10–50-fold reduced) replication of heterologous alphaviruses, such as SINV. However, more detailed analysis showed that only 8–10% of cells in such cultures were superinfected with SINV. This result indicated that SINV gained the ability to replicate in a limited number of cells rather than the ability to replicate (albeit poorly) in every cell in the culture (225). Another aspect that has been shown to affect SIE is the multiplicity of infection (MOI). In the case of using a 10-fold higher MOI for superinfecting SFV compared to the first virus, SIE was not observed. Only when the interval between the first infection and superinfection was increased to 3 hours was the superinfecting virus not capable of replicating even when used at high MOI (224). This may indicate that SIE at early stages of infection relies on different mechanisms compared to SIE at later stages of infection, i.e., alphaviruses can cause SIE using multiple mechanisms.

Considering the limited and controversial data, it is not surprising that the exact mechanism of SIE in alphavirus infection is not clear. It has been hypothesized that SIE may represent an outcome of competition for the receptors presented on the host cell surface; however, there is little evidence supporting this theory. Studies dedicated to the analysis of the mechanism(s) of SIE caused by alphaviruses revealed two common features: 1) SIE is mostly related to the blockage of RNA replication and 2) it has a cell-intrinsic nature (223, 226, 227, 228). On the other hand, experimental data for SFV showed that homologous interference was caused by multiple factors and at different stages of infection, whereas it has been shown for CHIKV that SIE does not truly depend on the activity of nsP2 or any other nsPs (229). These observations lead to the conclusion that SIE involves multiple mechanisms and may serve to reduce the possible recombination events between two viral genotypes. Thus far, the hypothesis supported by the largest amount of experimental data is that the establishment of SIE is caused by the presence of nsP2 encoded by the first virus in the cytoplasm of infected cells. After processing, nsP2 is released from polyprotein, but only a relatively small fraction of it is included in the RNA replicase core, where nsP1 and nsP2 are present with 12:1 stoichiometry (94). Thus, the bulk of nsP2 accumulates inside the infected cells and is located partly in the cytoplasm, and partly in the nucleus (152, 230). The “free” nsP2 found in the cytoplasm exhibits protease activity and may be responsible for restricting superinfection with homologous or heterologous viruses by performing cleavage of the P234 of the second virus at a specific position (presumably the 2/3 site) or at a time which is not compatible with the pathway leading to the formation of functional RCs. This mechanism was found to be responsible for SIE in SINV infection and partially responsible for SIE in SFV infection (225).

3. AIMS OF THE STUDY

Previous studies have revealed the complexity and elegance of alphavirus replication strategies. However, despite the considerable amount of existing data, the full diversity of the mechanisms and/or viral/host factor interactions involved in alphavirus replication is far from being fully understood. Processing of ns polyprotein is one of the key requirements for RNA replication that can influence the outcomes of alphavirus infection at very different levels. The processing is strictly regulated and depends on the alphavirus nsP2, a multifunctional enzyme with RNA helicase, NTPase, triphosphatase and protease activities. In addition to the aforementioned functions, nsP2 has been shown to be one of the determinants necessary for proper binding of viral RNA to RCs and virions for modification of the intracellular environment, including inhibition of the innate antiviral immune response and host cell transcription. Multiple functions of nsP2 are connected with each other, making the investigation of the precise roles of this protein in the virus infection process very complicated.

To investigate the involvement of nsP2 in different poorly understood aspects of alphavirus infection, the following aims were proposed:

1. To study the functional impacts of the presence of the WT or protease-inactive form of nsP2 of CHIKV and SINV on the activities of RNA replicases of homologous and heterologous alphaviruses in mosquito *Aedes albopictus* cells and analyze the mechanism(s) of nsP2-mediated inhibition, formation and functioning of alphavirus RCs (**I**).
2. To demonstrate the role of nsP2 and its protease activity in the development of SIE during alphavirus infection using mosquito cells and a replicon vector model (**I**).
3. To investigate the formation of the replicase complex of EEEV and the impact of stoichiometry of its ns proteins on the activity of EEEV RNA replicase (**II**).
4. To analyze the role of nsP2 in RRV infection, including its involvement in the shutdown of host cell protein synthesis (**III**).

4. MATERIALS AND METHODS

A complete description of the materials and methods used for the accomplishment of the experimental part of this study is available in the corresponding sections of publications I, II and III. A brief overview and rationale behind the choice of the methods used are provided below.

Construction of nsP2 expression plasmids (Publication I)

Sequences encoding 10 C-terminal amino acid residues of nsP1 and full-length nsP2 of CHIKV (isolate LR2006OPY1) or nsP2 of SINV (isolate Toto1101) were optimized according to codon usage of *Aedes aegypti*, and the cryptic splicing sites present in these sequences were also removed. This allows us to avoid the expression of the aberrant forms of nsP2 (from transcripts spliced using cryptic sites) that may have unpredictable effects on alphavirus replicase formation and function. Corresponding synthetic DNAs were cloned between the *Aedes aegypti* polyubiquitin promoter (one of the strongest promoters known for mosquitoes) and the transcription terminator of the hsp70 gene from *Drosophila melanogaster* in the pB-HR5/IE1 plasmid. DsR also contains a cassette for the expression of the red fluorescent protein dsRed. This marker is important for the detection of transfected cells, as, in general, the transfection efficiency of mosquito cells is relatively low, and using higher amounts of transfection reagents tends to damage the cells. The obtained plasmids were used to generate expression constructs for mutant versions of these proteins; changes in sequences encoding nsP2 proteins were made using site-directed mutagenesis and subcloning procedures.

Analysis of nsP2 expression using Western blotting (Publication I)

Mosquito C6/36 cells grown in 6-well plates were transfected with 800 ng of plasmid expressing nsP2 of CHIKV or nsP2 of SINV using Lipofectamine LTX and the Plus Reagent. The reagents and transfection conditions were selected based on the results of the preliminary tests that revealed this transfection reagent to be reasonable, effective and harmless to mosquito cells. Cells were harvested at 12, 18, 24, 36 and 48 h posttransfection, lysed with 1× Laemmli buffer and boiled for 10 min. Proteins were separated by SDS–PAGE in 10% gels and transferred to polyvinylidene difluoride membranes followed by staining with primary anti-CHIKV nsP2 (raised against recombinant protein corresponding to residues 1–470 of nsP2 of CHIKV) or anti-SINV nsP2 (raised against full-length recombinant nsP2 of SINV) polyclonal antisera. Secondary antibodies were conjugated to fluorescent infrared dyes, allowing detection of expressed proteins using the LI-COR Odyssey Fc imaging system. In another experiment, C6/36 cells were transfected with plasmids expressing the mutant forms of nsP2 of CHIKV or SINV; transfection was performed, and samples were analyzed 48 h posttransfection as described above.

Trans-replicase assay (Publication I)

In this assay, the expression constructs of CHIKV P1234, its variants harboring mutations blocking cleavage at the 1/2 site or at the 2/3 site and a plasmid expressing P1234 harboring an inactivating mutation in the RNA polymerase active site of nsP4 were used as tools to express CHIKV replicase proteins. A plasmid for the expression of the replication-competent template RNA of CHIKV containing *Firefly* luciferase (Fluc) and *Gaussia* luciferase (Gluc) markers also harbored a promoter and terminator for *Aedes albopictus* RNA polymerase I. Briefly, in this construct, most of the ns region was substituted by the sequence of Fluc, and all regions encoding structural proteins were replaced with those for Gluc. The markers were used to allow easy and accurate measurement of the synthesis of the full-length RNA (a template for Fluc expression) as well as the truncated version of SG RNA (a template for Gluc expression) (Figure 13A). Thus, in essence, the RNA transcribed from the template-expressing plasmid represents a defective interfering RNA with very low translation of the Fluc marker (due to lack of a 5' cap) and even lower expression of Gluc (due to lack of a 5' cap and the region encoding Gluc being the second ORF in the RNA). At the same time, the alphavirus replicase-generated RNAs are not only more abundant than transcripts made by RNA polymerase I, but are also capped at the 5' ends, and in the shorter transcript, ORF for Gluc is the first ORF in that RNA (Figure 13B). Selection of this design was based on previous studies which revealed that such constructs produce low background and high replicase-driven boost of marker protein expression. The same principles were also used in replicase and template expression plasmids of other alphaviruses.

Our preferred format of *trans*-replication assays was based on the use of cells grown in 96-well plates (approximately 35,000 cells/well): these conditions limited the amounts of necessary (and expensive) transfection reagents and took full advantage of the sensitivity of the measurement of luciferase activities. At the same time, we observed that to obtain clear reads and reproducible results, relatively large amounts of expression plasmids were needed: the transfection mixture contained 440 ng of the P1234-encoding plasmid, 440 ng of plasmid expressing the corresponding template and 440 ng of plasmid encoding nsP2 of CHIKV or SINV or the mutant version thereof. In experiments aiming for the analysis of an effect of co-expression of SINV and CHIKV nsP2 on the RNA replicase from heterologous alphavirus, the matching pairs of P1234 and template RNA expression plasmids were used, and the amount of protease expression plasmids was increased to 1,600 ng. Because high amounts of plasmids may potentially negatively impact the cells, we mimicked the conditions used in control experiments where the protease-expressing plasmid was substituted by the same amount of irrelevant ("dummy") plasmid DNA encoding only dsRed. The transfected cells were harvested at 48 h posttransfection, and Fluc and Gluc activities were measured (Figure 14). To allow statistical analysis and ensure reproducibility of the results, all *trans*-replication experiments were performed with at least three biological replicates.

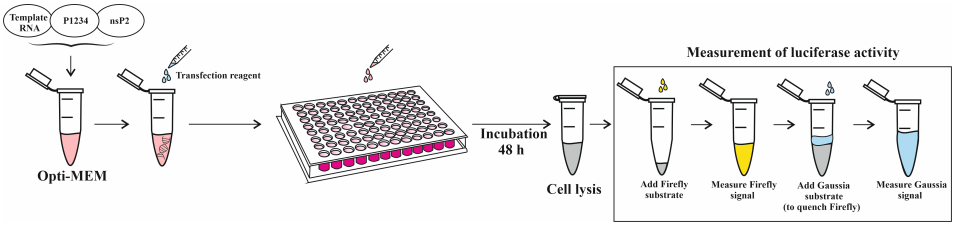

Figure 14. Schema of experiments used for analysis of the effect of the expression of free nsP2 on the activity of *trans*-replicases in C6/36 cells. Note that labels above the tube shown to the left of the schematic indicate molecules expressed from plasmids used to transfect C6/36 cells.

Packaging of CHIKV replicons into virus replicon particles (Publication I)

Our laboratory has developed several systems for obtaining CHIKV virus replicon particles (VRPs). From those, a system based on the use of a single helper RNA is the most efficient (resulting in the highest VRP yield). However, this system suffers from the possibility of infectious virions arising due to copy-choice recombination between replicons and helper RNAs. As such contamination was unacceptable for this study, a two-helper system for CHIKV replicons (231) was used (Figure 11). The plasmid pSP6-CHIKVRepl-ZsGreen was constructed by cloning the sequence encoding the ZsGreen marker under the control of the SG promoter in the CHIKV replicon vector; ZsGreen was chosen due its bright green fluorescence, which is easy to detect and distinguish from the red fluorescence of the dsRed marker expressed by nsP2-expression plasmids. pSP6-CHIKVRepl-ZsGreen and helper plasmids were linearized, and DNA was purified and transcribed *in vitro* using a mMESSAGE mMACHINE SP6 transcription kit. Then, 8×10^6 BHK-21 cells were transfected *via* electroporation with 1 µg of RNA transcripts corresponding to the replicon and each helper RNA. Compared to the standard protocol, these amounts are much lower: if the aim is to obtain maximal yield of VRPs, up to 100 µg of RNA transcripts can be used. Here, however, a lower amount of RNA was favored to reduce the possibility of nonhomologous recombination between the delivered RNAs. The transfected cells were seeded on a 60-mm plate. After incubation of the plates at 37 °C for 48 h, supernatants containing VRPs were harvested and clarified by centrifugation. For determination of the titer, different dilutions of VRP stock were made in L-15 medium and used for infection of C6/36 cells. Infected cells were incubated for 16 h at 28 °C and harvested, and the percentage of cells expressing ZsGreen was determined using flow cytometry. The aim was to determine the amount of VRPs necessary to achieve infection of 30–40% cells. This value was selected because the corresponding number of fluorescent cells can be accurately measured by FACS, and most importantly, under these conditions, the majority of fluorescent cells are infected by a single VRP. RNA replication is initiated by ns proteins translated from a single replicon RNA delivered by VRP.

Infection of transfected cells with VRPs and flow cytometry assay (Publication I)

C6/36 cells were grown on 12-well plates at a density of approximately 350,000 cells/well and transfected with 3 µg of plasmids expressing nsP2 of CHIKV, SINV or their protease inactivated mutants and dsRed marker. The control cells were transfected with the empty expression vector pB-HR5/IE1.DsR expressing only dsRed marker. At 48 h posttransfection (selected as a time with high-level expression of nsP2 and dsRed), cells were infected with CHIKV VRPs at a multiplicity of infection of approximately 0.2 VRP/cell and incubated for 16 h at 28 °C. Afterward, the medium was removed, and the cells were fixed by incubation at room temperature in 10% formalin for 30 min. Cell analysis was performed using an Attune NxT Acoustic Focusing Cytometer. For detection of the ZsGreen fluorescence signal, a 488 nm laser with a 493–490 nm filter was used, while detection of dsRed was performed using a 561-nm laser with a 612–627 nm filter. A scatter plot was used to determine the percentage of cells expressing ZsGreen and/or dsRed in the prepared samples (Figure 15). For each sample, 30,000 events were recorded. The experiment was performed in triplicate and repeated two times.

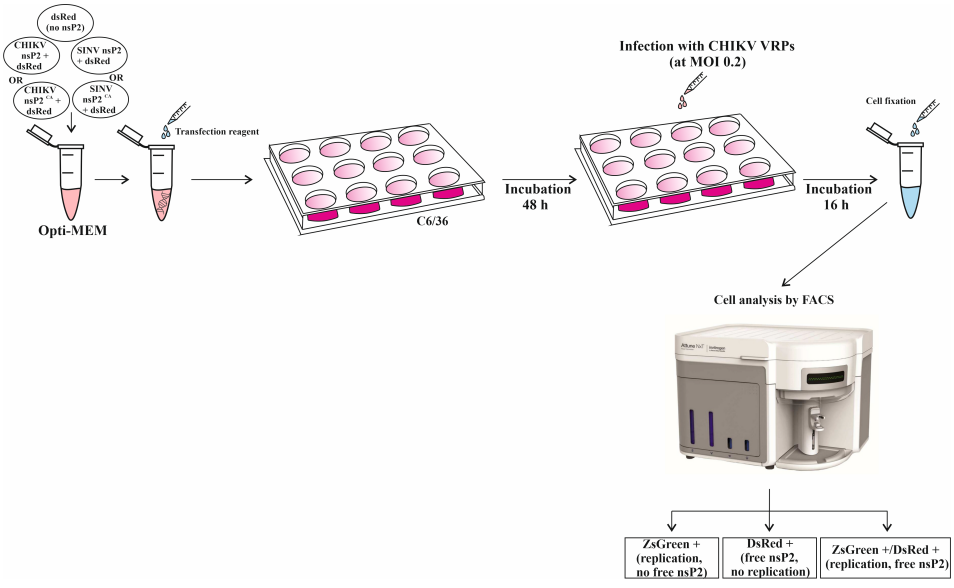

Figure 15. Schema of the experiment used for analysis of the effect of the expression of free nsP2 on the infection of C6/36 cells by CHIKV VRPs containing ZsGreen-expressing replicons. Note that labels above the tube shown to the left of the schematic indicate molecules expressed from the plasmids used to transfect C6/36 cells. The expression vector for free nsP2 also expresses dsRed. The efficacy of suppression of VRP infection was analyzed by measuring the reduction in double-colored cells (expressing dsRed and ZsGreen markers) depending on the presence of nsP2, its origin (CHIKV or SINV) and the presence or absence of its protease activity.

Design and use of three-component *trans*-replicase of Eastern equine encephalitis virus (Publication II)

Basic constructs of the EEEV *trans*-replicase system (plasmids for expression of P1234, its polymerase-dead version and suitable template RNA) were constructed previously. Two-component EEEV *trans*-replicase consisting of the expression vectors for EEEV P123 and nsP4 (expressed in the form of ubi-nsP4 to ensure the critical N-terminal Tyr residue of nsP4) was constructed in our lab by Laura Sandra Lello and was found to be highly active. However, for several alphaviruses (such as SINV and SFV), the P123 component can be further split between two expression plasmids, one for nsP1 and another for P23. The latter should have an N-terminal ubiquitin fusion to ensure the correct N-terminal residue of nsP2, which is, depending on the virus, Gly or Ala (but never Met). There is also an additional requirement – the P23 precursor must be uncleavable. The reason for this is simple: if no measures are taken for stabilization of P23, then it is almost instantly processed into nsP2 and nsP3 and fails to contribute to formation of the functional RCs. There are two ways to stabilize P23 – this can be done either by inactivation of protease activity of nsP2 or by making the site between nsP2 and nsP3 uncleavable by introduction of a Gly to Val (or Gly to Ala) mutation at the P2 position of the cleavage site. Here, we chose the former approach. The EEEV nsP1 plasmid was prepared by using PCR and the CMV-P1234 template; as a result, the region encoding nsP2-nsP4 was removed. CMV-P2^{CA}3-EEEV was generated by replacing the nsP1 region in CMV-P123-EEEV with a region encoding ubiquitin; the Cys residue in the active site of nsP2 protease was replaced with Ala using PCR-based mutagenesis and subcloning.

A *trans*-replicase assay was utilized to reveal the activities of the three-component EEEV *trans*-replicase and to determine the optimal ratio of the nsP4 expression plasmid to the nsP1 and P2^{CA}3 expression plasmids. The efficiency of the three-component system was analyzed based on analysis of luciferase marker expression (encoded by the HSPolI-FG template plasmid) or based on a flow cytometry assay (detection of ZsGreen encoded by the HSPolI-FZsG template plasmid) (Figure 16).

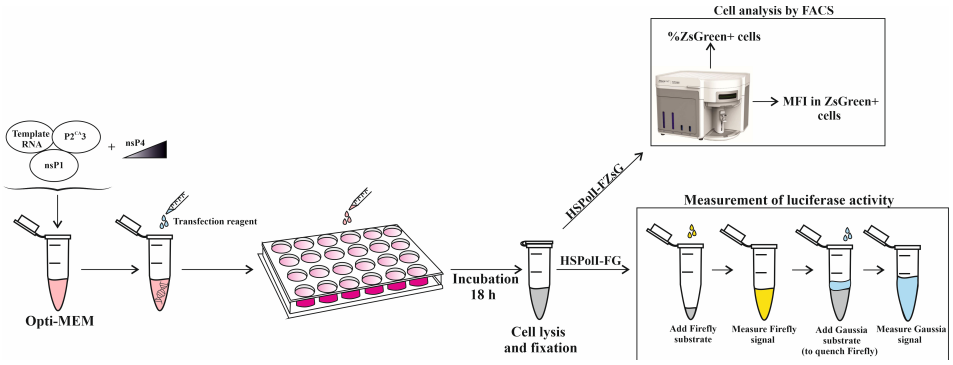

Figure 16. Schema of experiments used for analysis of the effect of the ratio of nsP1+P2^{CA}3 components of RC to the nsP4 component using three-component EEEV *trans*-replicase. Note that labels above the tube shown to the left of the schematic indicate molecules expressed from plasmids used to transfect C6/36 cells; in some experiments, the plasmid used for the expression of nsP4 was used at different concentrations. In experiments based on the use of HSPolI-FG template RNA-encoding plasmids, the activities of *trans*-replicase were estimated by measuring the activities of expressed Fluc and Gluc reporters. In experiments based on the use of HSPolI-FZsG template RNA-encoding plasmids, the efficacy of functional replicase formation was estimated by analysis of the percentage of ZsGreen-positive cells, and the activity of *trans*-replicase-mediated transcription was measured by analysis of the mean fluorescence intensity (MFI) in ZsGreen-positive cells.

Analysis of the shutdown of cellular translation in cells infected by RRV T-48, its mutant variants and chimeras based upon it (Publication III)

Analysis of sequence RRV 2548, an isolate of RRV from Australian mosquitoes, revealed several nonsynonymous differences compared to the prototype strain of RRV, RRV T-48. Three mutations found in the region encoding nsP1, and four mutations found in the region encoding nsP2 were introduced into the infectious clone RRV T-48 using site-directed mutagenesis, resulting in 7 mutant viruses. In addition, the chimeric viruses designated M12 and M34 were prepared by replacing the nsP1/nsP2 or nsP3/nsP4 regions of RRV T-48 with corresponding fragments of RRV 2548. The panel of these nine icDNA clones was prepared by collaborators at Griffith University.

To analyze whether the introduced mutations and swaps affect the ability of RRV to cause cellular translation shutdown, we used metabolic labeling of proteins in BHK-21 cells infected with [^{35}S]Met and [^{35}S]Cys. This approach allows us to measure the expression of host and virus proteins over a time course of infection and provides much clearer images than an alternative approach based on the use of puromycin and its detection using Western blotting. Briefly, cells were infected with RRV T-48 and mutant variants at high MOI. At selected time points (4, 8, 12, and 24 h postinfection), infected cells were starved for Met and Cys for 30 min and labeled with 50 μCi[^{35}S]Met and [^{35}S]Cys for another 30 min. Afterward, cells were lysed in SDS gel loading buffer, and labeled proteins were separated using SDS–PAGE followed by drying and autoradiography (Figure 17).

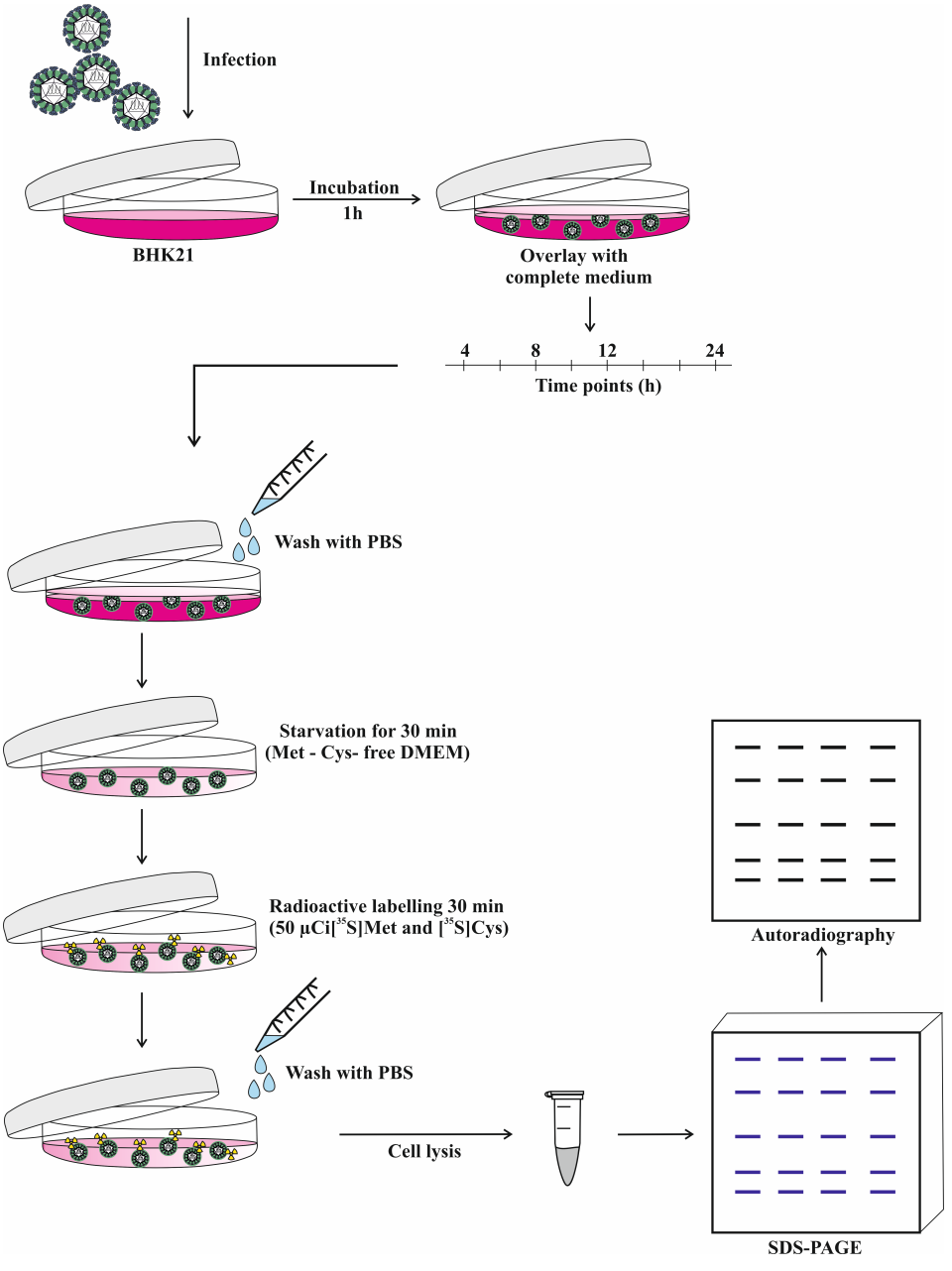

Figure 17. Schema of the experiment used for the analysis of RRV-induced shutdown of cellular protein synthesis and expression of viral structural proteins.

Analysis of processing of RRV P1234 using in vitro translation (Publication III)

In vitro translation and radiolabeling were employed to evaluate the impacts of the substitutions found in the nsP1/nsP2 region of RRV 2548 on P1234 processing. This approach was chosen because the antibodies against nsPs of RRV were not available, i.e., the only way to detect and identify viral nsPs and their polyprotein precursors was the synthesis of radiolabeled proteins and subsequent analysis of their electrophoretic mobility. The speed of ns polyprotein processing can be analyzed by measuring the ratio of the mature nsP2 to its precursor, P123 polyprotein (theoretically, P1234 could be used instead of P123, but due to very rapid cleavage of the 3/4 site, the amount of P1234 tends to be very low, and the corresponding band is difficult to detect). A panel of icDNAs including 7 RRV point mutants and RRV T-48 (control) was used as a template. *In vitro* translation was performed using the TnT-SP6-coupled rabbit reticulocyte system. The time of translation was 45 minutes, followed by 5 minutes of treatment with cycloheximide and 5 minutes of treatment with RNaseA. These times were chosen based on previous experience with CHIKV and SFV ns polyproteins (149, 168), as they are sufficient for translation of detectable amounts of viral ns polyproteins and their partial, but incomplete, processing. The obtained images were visualized with a Typhoon phosphorimager. The intensities of the P123 precursor and mature nsP2 (unlike comigrating nsP1 and nsP3, nsP2 is larger and can therefore be easily identified) bands were quantified using ImageQuant TL software (Figure 18).

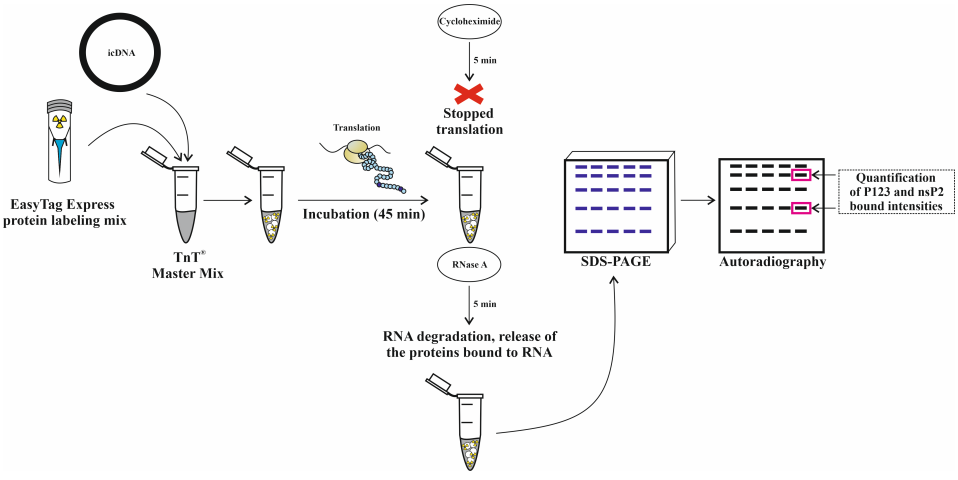

Figure 18. Schema of the experiment used for analysis of processing of different variants of RRV polyproteins.

5. RESULTS AND DISCUSSION

5.1. Expression of individual nsP2 in *Aedes albopictus* cells interferes with formation and/or activity of RNA replicases of homologous and heterologous alphaviruses (Publication I)

5.1.1. Expression of individual nsP2 of CHIKV or SINV inhibits the activity of corresponding *trans*-replicases and reduces the efficiency of infection by CHIKV VRPs

The formation of alphavirus RNA replicase occurs *via* sequential processing of P1234 and P123 by protease activity residing in the nsP2 region. While the first and the second cleavage occur *in cis,* the final cleavage – that of the 2/3 site – occurs *in trans* (Figure 4). Thus, this cleavage can be performed by an individual (free) nsP2 previously formed by processing of ns polyproteins of the virus or, in the case of superinfection, processing of ns polyproteins of the first virus. The existence of free nsP2 is an inevitable consequence of the stoichiometry of the alphavirus RNA replicase core, which contains 12 subunits of nsP1 but only one molecule of nsP2 (94). The presence of free nsP2 in infected cells was already noted in many earlier studies that also revealed that a free form of nsP2 localizes almost equally in the cytoplasm and nucleus of mammalian cells. Free nsP2 does not participate directly in RNA replication; instead, it has numerous nonreplicative functions generally related to the shutdown of host cell metabolism and antiviral responses. It has also been presumed, but not directly proven, that free nsP2 is one of the factors (or even the main one) responsible for the establishment of SIE. In this study, we intended to examine in depth the role of free nsP2 as a factor modulating the formation/activity of RNA replicases of homologous and heterologous alphaviruses.

Theoretically, any free nsPs present in a cell may act either as an inhibitor or activator for alphavirus RNA replicase. Inhibitory properties have been coherently described for an individual nsP3 (188), while the presence of excess nsP4 has been shown to activate the RNA replication of numerous alphaviruses (197). Free nsP2 can also inhibit alphaviral RNA replication by different mechanisms. Acceleration of P1234 processing and the activating pathway resulting in the formation of P12 and P34 (as reviewed in Section 2.2.2) interferes with RC formation. However, this is not the only option, as free nsP2 can also inhibit RNA replicase formation by binding crucial host factors, triggering host cell shutdown and/or altering cellular responses to virus infection. nsP2 of a heterologous alphavirus can also act as a dominant negative factor competing with homologous nsP2 for position in the RNA replicase core. The same may be the case for different mutant variants of nsP2. Thus, there are multiple possibilities. Furthermore, the potential mechanism of action of free nsP2 likely depends on virus species, as the

nonreplicative functions of alphavirus nsPs are less conserved than replicative ones. Finally, the effects of free nsP2 on alphavirus infection in vertebrate and mosquito cells can also be different. Altogether, the universal answer to what nsP2 does and how it functions (other than being a component of the RNA replicase core) in alphavirus-infected cells may not even exist.

To narrow the focus of the study and avoid problems caused by the high cytotoxicity of free nsP2 in mammalian cell lines, we selected *Aedes albopictus* (C6/36) cells as an object to assess the impact of free nsP2 on alphavirus replicase activity and the development of SIE-like conditions in the cells. These cells were selected due to ease of handling and lack of a functional siRNA pathway. This allowed us to detect the nsP2 protein-mediated effects that are not masked by inhibitory effects caused by the production and activity of siRNAs. C6/36 cells are also suitable for alphavirus *trans*-replicases, and their variants harboring mutations in processing sites of P1234 have high activities in C6/36 cells (149). However, several aspects of work with mosquito cells pose challenges. Compared to mammalian cells, they are far less studied, and tools suitable for working with these cells are limited and generally of moderate/low efficiency. Among other factors, transfection of C6/36 cells with plasmid DNAs is relatively inefficient – usually no more than 50% are successfully transfected – and attempts to increase transfection efficiency using different approaches and reagents typically result in damage to the cells. The low transfection activity did not allow the use of the simplest experimental schema: transfection of cells with the nsP2 expression plasmid and subsequent infection with an alphavirus. In this case, more than 50% of cells remain nontransfected, allowing the virus to replicate without any hindrance. Clearly, this would almost completely mask any inhibitory effect occurring in successfully transfected cells: at most, twofold replication inhibition may be obtained (i.e., complete inhibition in all cells successfully transfected with the nsP2 expression plasmid, but fully active replication in cells that remained untransfected and did not express free nsP2). The most obvious way to solve this problem is using stably transfected cell lines, but repeated attempts to obtain such cells in our lab failed. Namely, transgenic cell lines were easy to obtain, but none of them had detectable expression levels of nsP2 – most likely, it was silenced by mosquito cells (the mechanism behind this inhibition was not studied in detail, but it was assumed to be caused by the siRNA pathway). As a way out, a *trans*-replication-based approach was pursued: two plasmids of the *trans*-replication system (expressing template and P1234, respectively, Figure 13) were co-transfected with a plasmid for the expression of free nsP2. In general, co-transfection results in the codelivery of all three plasmids: cells are either transfected with all three plasmids or with none of them. Furthermore, to keep the number of cells that received only two *trans*-replicase plasmids and no nsP2 expression plasmid, the latter was used in molar excess. Another aspect favoring the use of the *trans*-replicase-based approach relies on previous studies performed in our lab. We have generated numerous state-of-the-art tools, including a combination of P1234 and RNA template encoding plasmids, where transcription is driven by *Aedes aegypti* polyubiquitin (Ubi) and *Aedes albopictus* RNA polymerase I

(AlbPolI) promoters, respectively. These plasmids have been tested in mosquito cells (111) and developed for numerous alphaviruses. The system is compatible with plasmids expressing free nsP2 of CHIKV or SINV (WT or mutated versions) (**Publication I, Table I**) that were prepared for this study. It should, however, be noted that the use of a *trans*-replication system for this study was not without problems – the nature of co-transfection dictates that the effector (nsP2) and its target (replicase system) must be delivered to the cells simultaneously (subsequent transfection would not ensure codelivery of plasmids). This contrasts with the real SIE, where the viruses enter the cell consecutively. It can be assumed that this limitation will likely reduce the efficiency of suppression of *trans*-replicase activity, as all proteins/RNAs are expressed at the same time, i.e., a pool of free nsP2 does not form before the start of the expression of P1234. On the other hand, a molar excess of the nsP2 expression plasmid could ensure a high level of nsP2 expression that should partly or completely compensate for this effect. The number of unknowns dictates the need for the use of adequate controls. In the current study, nsP2 lacking protease activity (carrying a Cys to Ala mutation in the protease active site) was used as a negative control in all assays. This allowed us to estimate the "real" impacts of CHIKV and SINV nsP2 (WT or mutant versions harboring different substitutions) on P1234 processing and, thus, RC formation. In addition, the use of nsP2 lacking protease activity allowed for the detection of potential protease-activity-independent inhibitory effects caused by co-expression of free nsP2.

Once the assay conditions were optimized, the obtained results became highly reproducible, and the experiments became rather straightforward. It was found that individually expressed protease-inactive nsP2 of CHIKV did not significantly decrease the replication of the template RNA of CHIKV in mosquito cells. In contrast, the impact of co-expression of WT nsP2 of CHIKV was profound: the replication/transcription activity of CHIKV RNA replicase was significantly reduced (**Publication I, Figure 2B**). These findings clearly highlighted the importance of the protease activity of nsP2 as one of the "key players" necessary for the inhibition of CHIKV RNA replication under the experimental conditions mimicking SIE. It has also been recently demonstrated that replacement of Glu515 of CHIKV nsP2 with the Val residue increases the protease activity of nsP2, resulting in accelerated processing of P1234 at the 1/2 site and dramatic inhibition of RNA replicase functioning (149). When this mutation was introduced into the CHIKV nsP2 expression construct and the plasmid was used in *trans*-replicase, both replication and transcription activities were further (compared to co-expression of WT nsP2) reduced. On the one hand, this result seems to correspond with the prediction. However, on the other hand, the increased activity of the mutant nsP2 mostly causes acceleration of 1/2 site processing that (as in the experiments performed in the previous study) occurs predominantly, if not exclusively, *in cis*. This contrasts with the situation in our experiment, where co-expressed free nsP2 can cleave target(s) in P1234 only *in trans*. Thus, it was assumed that the observed enhanced effect may be caused by factors other than increased protease activity. To verify whether the more potent inhibitory effect of this mutant form of nsP2

can indeed be attributed to its increased protease activity, an additional mutation was added (YA). Substitution of Tyr161 to Ala in the N-terminal region of nsP2, which disrupts the helicase activity of nsP2 and abolishes RNA synthesis and alphavirus replication (133), was added to nsP2 harboring a Glu515 to Val substitution. Experiments performed with this double mutant showed that its inhibitory effects on replication/transcription rates of *trans*-replicase were more similar to those of WT nsP2 and not to the nsP2 with Glu515 to Val substitution alone, i.e., the additional inhibitory potential was lost. A substitution in the RNA helicase part of nsP2 is very unlikely to affect the protease activity of the enzyme, as residue 161 is distant from the residues located very close to the N-terminus of nsP2, which affects the protease activity of SFV nsP2 (145), or from residue 228, which affects the processing of the ns polyprotein of SFV/CHIKV chimeras (245). Thus, most likely, these data imply that the profound suppression of *trans*-replicase activity observed in the presence of nsP2 with Glu515 to Val substitution alone was not due to accelerated cleavage of the 1/2 site (or 2/3 site) in P1234. The elevated inhibitory potential of this nsP2 mutant form may be due to the direct dominant effect on the CHIKV RNA replicase. Data from the experiments performed using P1234 polyproteins harboring mutations in nsP2 cleavage sites (see below, 5.1.2) are in line with this assumption.

The findings obtained using one alphavirus often do not apply to another, especially if these viruses do not belong to the same complex of alphaviruses. Our laboratory has a large collection of nsP2 and P1234 mutants of SFV. However, SFV belongs to the same complex as CHIKV and has very similar ns polyprotein processing requirements. For these reasons, SINV, belonging to a different complex, was selected as a second model. The processing requirements of SINV P1234 have not been studied in detail, but existing data suggest that they must differ at least to some degree from those of CHIKV and SFV. Namely, at the late stage of infection, SINV nsP2 cannot cleave the 3/4 site, resulting in the accumulation of P34 in infected cells. In addition, mutation in nsP2 of SINV resulting in increased speed of 1/2 site processing has been described (137). Despite all differences between CHIKV and SINV, it was found that the co-expression of free nsP2 and *trans*-replicase of SINV resulted in an effect very similar to that obtained for CHIKV. Expression of WT nsP2 of SINV prominently reduced the activities of *trans*-replicase (**Publication I, Figure 2C**). Mutation increasing the protease activity of nsP2 of SINV clearly increased this effect, while mutation in the active site of nsP2 protease essentially abolished it.

We also attempted to analyze the potential impacts of mutations affecting the cytotoxicity or subcellular localization of nsP2 on its ability to inhibit CHIKV and SINV *trans*-replicases. It was found that these mutations result in rather minor changes, and the impact of the mutations on inhibitory properties is very small (if present at all). There are two mutually nonexclusive explanations for this. First, neither of these properties (cytotoxicity or subcellular localization) are related to the ability of nsP2 to induce SIE-like inhibition. This seems rather unlikely because more prominent cytosolic localization of nsP2 should, in theory, favor cleavage of newly translated P1234. Second, both of these phenomena have been

described for mammalian cells and may not be relevant for mosquito cells. Indeed, alphavirus infection and nsP2 expression are not toxic to these cells, and very recent data from our collaborators (Reitmayer *et al.,* submitted for publication) revealed that in mosquito cells, nsP2 does not exhibit nuclear localization.

As mentioned above, one of the drawbacks of *trans*-replicase as a system for mimicking SIE is the need for co-transfection of cells with the nsP2 expression plasmid and plasmids of the *trans*-replicase system. Separation of these transfections in time will inevitably lead to an increased number of cells transfected only with *trans*-replicase plasmids, and thus a reduction in the observed inhibitory effects at the cell culture level. Analysis based on the use of single cells was not available due to a lack of necessary equipment. In theory, cells not transfected with nsP2 expression plasmids can be eliminated using cell sorting; however, this process tends to damage cells and can significantly impact virus infection on its own. Therefore, we selected an approach based on the use of a two-color system – red for nsP2 expression and green for RNA replication. For *trans*-replication, this approach is not suitable, as RNA replication is established in a rather small fraction of transfected C6/36 cells (220), making the changes in the numbers of cells positive for both markers (red+green) difficult to detect (due to the small number of these cells). The more efficient – and likely also more biologically relevant – system is based on transfection of cells with nsP2 expression plasmids (or control plasmid) and subsequent infection of cells with VRPs containing packaged replicon RNA that can establish one-cycle infection and expresses green marker (in our case, ZsGreen). The use of a replicon allows the infection of the desired fraction of transfected cells, and the reduction in the number of double-marker-positive cells is easy to measure (Figure 16). In this setup, replicons and corresponding VRPs are preferable to full viruses harboring marker genes because the latter produce infectious progeny that will ultimately infect all cells in culture and establish stable infection. The experiments performed with CHIKV VRPs confirmed findings made by *trans*-replicases (**Publication I, Figure 3**). The magnitudes of the observed effects were different, most likely because of the different designs of the experiment, but the trends were nonetheless the same. Transfection of cells with a plasmid expressing WT CHIKV nsP2 prominently reduced their ability to become infected by CHIKV VRPs; the effect was reduced but not eliminated when nsP2 harboring a mutation in the protease active site was used. The expression of WT or protease-inactive nsP2 of SINV had an almost identical and relatively mild impact on the ability of cells to become infected by CHIKV VRPs. These data indicate that the inhibition was independent of protease activity. However, at least under the conditions used, the protease-activity-dependent effects of nsP2 of SINV could be too small to be detected.

Taken together, the accumulated data showed that co-expression of the individual (free) nsP2 plays a major role in suppression of viral RNA synthesis by *trans*-replicase and alphavirus replicon vectors. The *trans*-replicase-based assay was found to possess higher sensitivity. There were both protease-activity-dependent and protease-activity-independent factors that resulted in suppression of

replicase activities. The balance between these factors depended on the virus, mutations introduced into nsP2, the type of assay used (*trans*-replicase or VRP infection) and, to a degree, the conditions of the assay (including the number of transfected cells and amounts of plasmids used for transfection). Considering the findings obtained in the current study, we can presume that alteration of RC formation and/or functioning by the co-expressed individual nsP2 is just one side of a coin and that indirect effects of nsP2 expression may also have a certain impact on alphavirus RNA synthesis.

5.1.2. Blocking of processing sites in P1234 reduces or eliminates sensitivity of CHIKV *trans*-replicase to co-expression of an individual nsP2 (Publication I)

To result in the formation of functional RNA replicase, the processing order of the junctions in P1234 should be as follows: first 3/4, then 1/2 and finally the 2/3 site (142). The strict order and precise timing of processing events also means that its disturbance, i.e., premature processing and/or altered processing order of P1234 of superinfecting virus, could be the basis for SIE. The most plausible explanation is that processing starts by *trans*-cleavage of the 2/3 site; this cleavage is also influenced by processing of the 1/2 site, as the 2/3 site in P123 is a less suitable substrate for nsP2 than the 2/3 site in P23. Hence, the role of ns polyprotein processing in the establishment of SIE-like effects can be confirmed by the use of ns polyprotein precursors made uncleavable at the 1/2 or 2/3 site by replacement of the conserved P2 Gly residue with the Val residue. This approach was also used in this study. However, it should be mentioned that such protease site mutations exert a significant impact on the activity of CHIKV replicase in mosquito cells. Thus, Bartholomeeusen et al. described that the Gly to Val mutation at the P2 positions of 1/2 or 2/3 cleavage sites resulted in more efficient RNA replication in mosquito cells; the impact of the mutation at the 2/3 site was more profound than that of the 1/2 site mutation (149). This effect may, depending on experimental conditions, enhance or diminish the impact resulting from co-expression of nsP2. Indeed, if replication levels are increased 10-fold or more by a processing site mutation, it may be more difficult for co-expressed nsP2 to suppress such elevated activity. However, another possibility exists: achievement of such high levels of RNA replication is likely demanding, and even relatively minor disturbances in cells may reduce the efficiency of such high-level RNA replication. Thus, the data obtained using *trans*-replicases with processing-deficient P123 regions must be interpreted with care.

Consistent with previous observations, the introduced cleavage site mutations prominently boosted the expression of marker proteins encoded in template RNA. The activation (compared to WT replicase) was smaller for P1GV234 (harboring Gly534 to Val substitution that blocks cleavage of the 1/2 site) and more prominent for P12GV34 (harboring Gly1332 to Val substitution that blocks cleavage at the 2/3 site) (**Publication I, Figure 2A**). Importantly, it was observed that both

P1GV234 and P12GV34 mutations diminished or eliminated the negative impact of co-expression of WT nsP2 on the activities of CHIKV *trans*-replicase. The inhibition in the case of the P1GV234 polyprotein was modest; in contrast, co-expression of WT nsP2 had almost no effect on the activities of the *trans*-replicase using the P12GV34 polyprotein. In the latter case, the processing of P1234 into P12 and P34 (normal products of the late nonreplicative processing pathway) was prevented. Thus, these data indicate an important role of the 2/3 cleavage site in SIE. In the case of P1GV234, the cleavage of 2/3 site is possible but, as its efficiency depends on the release of the N-terminus of P23 polyprotein (prevented by Gly534Val substitution), the efficiency of such a cleavage is reduced, which most likely represents the real reason for reduced sensitivity to WT nsP2 expression. The role of the cleavage of the 1/2 site on its own is likely minimal (if it exists at all), as this site is effectively cleavable in P12GV34. To confirm this, we constructed and used a polyprotein containing both cleavage site mutations (P1GV2GV34). It was found that the sensitivity of the corresponding replicase to nsP2 co-expression resembled that of the P12GV34 replicase. Thus, the premature cleavage of the 2/3 site (or, more precisely, processing that starts from cleavage of the 2/3 site) is indeed a key event in the establishment of SIE-like inhibition of replication by individual nsP2. Interestingly, while the impact of co-expression of WT nsP2 on the replication activity of P1GV234- and P12GV34-based *trans*-replicases was eliminated, some negative impact on transcription activity remained. It is possible that co-expression of nsP2 could not inhibit RNA replicase formation (most likely, the protein was unable to shut down the synthesis of negative-strand RNA synthesis) but had some ability to interfere with the synthesis of SG RNA. This is consistent with the observation that nsP2 might be a factor affecting the ability of alphaviruses to synthesize SG RNAs. This may be possible, as in contrast to the mature RNA replicase core containing 1 subunit of nsP2, the immature (early) cores should contain more: 12 subunits in the case of P1GV234 and likely more than 1 for P12GV34. Therefore, it seems plausible that free and individual nsP2 can interfere with P12/P123 or P23 polyproteins present in these immature replicase complexes and affect their ability to produce SG RNAs.

Most of the mutant forms of nsP2 had similar or reduced impacts on the activities of processing-deficient *trans*-replicases. The exception is nsP2 carrying the Glu515 to Val substitution, which had a significant negative influence on the replication/transcription levels of P1GV234- and P12GV34-type *trans*-replicases, albeit clearly smaller compared to its effect on WT (P1234) *trans*-replicase. Most likely, this indicates that the protease-activity-dependent effect of nsP2 with Glu515 to Val substitution was lost (or reduced), while the protease-activity-independent effect was preserved (**Publication I, Figure 2B**).

Overall, the obtained data revealed that blocking the 1/2 or 2/3 cleavage sites in P1234 inhibited the ability of the co-expressed WT nsP2 to suppress *trans*-replicase activity in a protease-activity-dependent manner. It has been confirmed that the abolition of alphavirus RC assembly/activity directly depends on the ability of co-expressed nsP2 to interfere with ns polyprotein processing in the *trans*-

replicase assay, and presumably with that of superinfecting alphavirus. It is also evident that the *trans*-processed 2/3 site is the main target for the protease activity of nsP2, while the block of cleavage of the 1/2 site most likely functions by making the 2/3 site in the same polyprotein less accessible for the protease activity of free nsP2.

5.1.3. The impact of co-expression of individual nsP2 of CHIKV or SINV on the activity of *trans*-replicases of heterologous alphaviruses (Publication I)

The primary sequence of nsP2 shows many variations, especially when alphaviruses belonging to different complexes are compared. Thus, the aa identity between CHIKV and SINV nsP2 is approximately 44%, while the similarity between nsP2 of CHIKV and VEEV is approximately 42%. At the same time, the 3D structures of nsP2 protease regions of VEEV, SINV and CHIKV are very similar. Thus, despite the differences in the proteins' primary sequences, they retain similar functional properties necessary for polyprotein processing (235). The "relationships" between the protease and cleavage sites in ns polyprotein are very complex and cannot be described simply as a key fit to a lock. The highly coordinated manner of ns polyprotein processing and its efficiency are difficult to explain due to the dissimilar aa composition of the cleavage sites. Furthermore, our data indicate that the key for SIE is recognition and processing of P1234 at the 2/3 site by free nsP2. The 2/3 site is the only cleavage site in ns polyprotein processing, which absolutely depends on the macromolecular mechanism of the assembly of nsP2 with nsP3 (MD). It is also the only site not cleavable by the nsP2 protease alone or by full-length nsP2 harboring extra aa residue(s); the perfectly natural N-terminal part of the enzyme and macrodomain in the substrate are strictly required (146). One can assume that such complex requirements make cleavage of this site inefficient and/or that the cleavage cannot be performed by heterologous nsP2. The reality is, however, the opposite: processing of the 2/3 site in the infected cells is very rapid, and there is clear evidence that the 2/3 site in SFV ns polyprotein can be cleaved by nsP2 of SINV, but not the other way around (146). Thus, cleavage of the 2/3 site is important for establishment of SIE, and the site in P1234 of one alphavirus can, at least in some cases (though not always), be processed by nsP2 of another alphavirus. Accordingly, one could expect that expression of nsP2 of one alphavirus can restrict replication of heterologous alphaviruses as well, albeit to different extent (it is important to note the existence of protease-activity-independent inhibition). To verify whether this is the case, we used co-expression of nsP2 (WT or protease inactive) of either CHIKV or SINV in C6/36 cells transfected with *trans*-replicases of alphaviruses belonging to different complexes, namely, *trans*-replicases of SFV, RRV, MAYV, EILV, VEEV and EEEV. We excluded *trans*-replicases of ONNV and BFV from this study, as data from previous studies performed in our lab revealed that the replicases of these viruses have low activity in *Aedes albopictus* cells (220).

As expected, due to the smaller impact of the co-expression of an individual nsP2 of CHIKV or SINV on the *trans*-replicase of heterologous virus than homologous virus, a larger amount of nsP2-expressing plasmids was used in these experiments. As a consequence, a protease-activity-independent effect of nsP2 expression on the activity of CHIKV *trans*-replicase became clearly evident; however, it was overshadowed by the effect caused by co-expression of WT nsP2 (**Publication I, Figure 4A**). Interestingly, co-expression of the protease-inactive nsP2 of CHIKV also inhibited replicases of other alphaviruses; the largest reduction of transcription activity was observed for *trans*-replicase of MAYV, while the smallest inhibition was observed for *trans*-replicases of SFV and insect-specific EILV. The extent of inhibitory effect showed little, if any, correlation with the relationship between viruses: for example, SFV and MAYV are closely related to CHIKV, while EILV is not. Thus, our data not only demonstrated that such downregulation was not directly caused by the protease activity of nsP2, but also that downregulation is unlikely to be attributed to an effect caused by altering the stoichiometric ratio of the replicase subunits. More likely, the effect was mediated by a cell or resulted from multiple factors. Data from similar experiments performed using protease-inactive nsP2 of SINV support this hypothesis. It was observed that co-expression of this protein had only a modest effect on the transcriptional activity of *trans*-replicases of heterologous alphaviruses; the largest impact was, again, on *trans*-replicase of MAYV, while *trans*-replicase of CHIKV was affected to the smallest extent (**Publication I, Figure 4B**). Such effects are difficult to explain if one assumes that nsP2 of heterologous alphavirus interferes directly with subunits of replicase of another virus. In this case, one could expect to observe a correlation between an inhibitory effect and evolutionary relationship between the involved viruses. In contrast, if the effect is mediated by cells, one could expect that viruses will not be ranked by sequence similarity, but rather by sensitivity to these cell-mediated effects. The highest sensitivity of MAYV to co-expression of the protease inactive nsP2 of CHIKV or SINV seems to be in line with this hypothesis. It can also be speculated that a profound effect caused by the nsP2 of CHIKV may occur because Aedes mosquitoes are natural vectors for CHIKV, but not for SINV. Furthermore, the high sensitivity of the *trans*-replicase of MAYV to the co-expression of heterologous nsP2 may result because this virus is naturally transmitted by Hemagogus mosquitoes and not well adapted to Aedes, making it more vulnerable. At the moment, all of these ideas represent speculations that may or may not be correct. What is certain is that a mechanism – or more likely mechanisms – behind nsP2 protease-activity-independent inhibition of alphavirus replication represents an interesting topic for further studies.

As expected, the co-expression of WT nsP2 had, at least in the majority of cases, a more prominent inhibitory effect on *trans*-replicases of heterologous viruses. Interestingly, again, the effects caused by nsP2 of CHIKV and SINV were not identical. In general, the additive effect of the protease activity of CHIKV nsP2 was rather small, with the exception of the *trans*-replicases of CHIKV and SFV (**Publication I, Figure 4A**). There are at least two plausible explanations for this.

First, a very prominent protease-activity-independent effect makes the additional inhibition (due to protease activity) more difficult to observe. Second, it is likely that CHIKV nsP2 is not efficient in *trans*-cleavage of 2/3 sites in ns polyproteins of heterologous alphaviruses (SFV being the only exception). On the other hand, WT nsP2 of SINV appeared to be a stronger inhibitor of *trans*-replicases of different alphaviruses, including that of insect-specific EILV (**Publication I, Figure 4B**). It is highly likely that this is an indicative feature of the ability of nsP2 of SINV to process 2/3 sites in ns polyproteins of SFV (previously confirmed) and other alphaviruses, including EILV. The plausible exceptions in this case are VEEV and CHIKV: with respect to their *trans*-replicases, the WT nsP2 of SINV was only a minimally more potent inhibitor than its protease-inactive form. For VEEV, this may be explained by the evolutionary distance between these viruses and/or the relatively low efficiency of VEEV *trans*-replicase in Aedes cells, making the detection of reduced activity more challenging. The data for CHIKV are consistent with those obtained from previous experiments and seem to indicate that SINV nsP2 either does not cleave the 2/3 site in CHIKV P1234 or that this cleavage does not occur early enough/efficiently enough to affect the formation of CHIKV replicase complexes. Again, these ideas require additional verification. On the mechanistic site, the detection of products of P1234 processing would be useful. More importantly, however, testing the biological relevance of these findings using in vivo (live mosquito) models are necessary.

Taken together, the extent of inhibition of replication of homologous or heterologous alphaviruses by nsP2 of CHIKV and SINV was significant, possibly due to the significant differences in the protease activity toward different substrates (sequences of processing sites and/or their presentation) and utilization of different protease-activity-independent mechanisms. Co-expression of SINV with nsP2 predominantly resulted in a protease-activity-dependent negative effect, while the expression of nsP2 of CHIKV nsP2 was characterized mostly by causing protease-activity-independent reduction of *trans*-replicase activity (Figure 19). The ability of nsP2 of SINV to cause inhibition of multiple alphaviruses in a protease-activity-dependent manner opens the possibility to exploit this for the development of mosquitoes unable to become infected by or unable to transmit multiple alphaviruses, a possibility currently under investigation in our laboratory and by our collaborators. An individual nsP2 of CHIKV is an equally potent inhibitor of alphavirus RNA replication but is likely a less efficient tool for this approach: much of the inhibition caused by the expression of this protein originates from the protease-activity-independent mechanism(s) and may be difficult, if possible, to reproduce in living mosquitoes. Indeed, if the effect is at least partly cell-mediated, it may result in excessive penalty costs for insects expressing CHIKV nsP2 at levels high enough to cause wide-spectrum resistance against alphavirus infection.

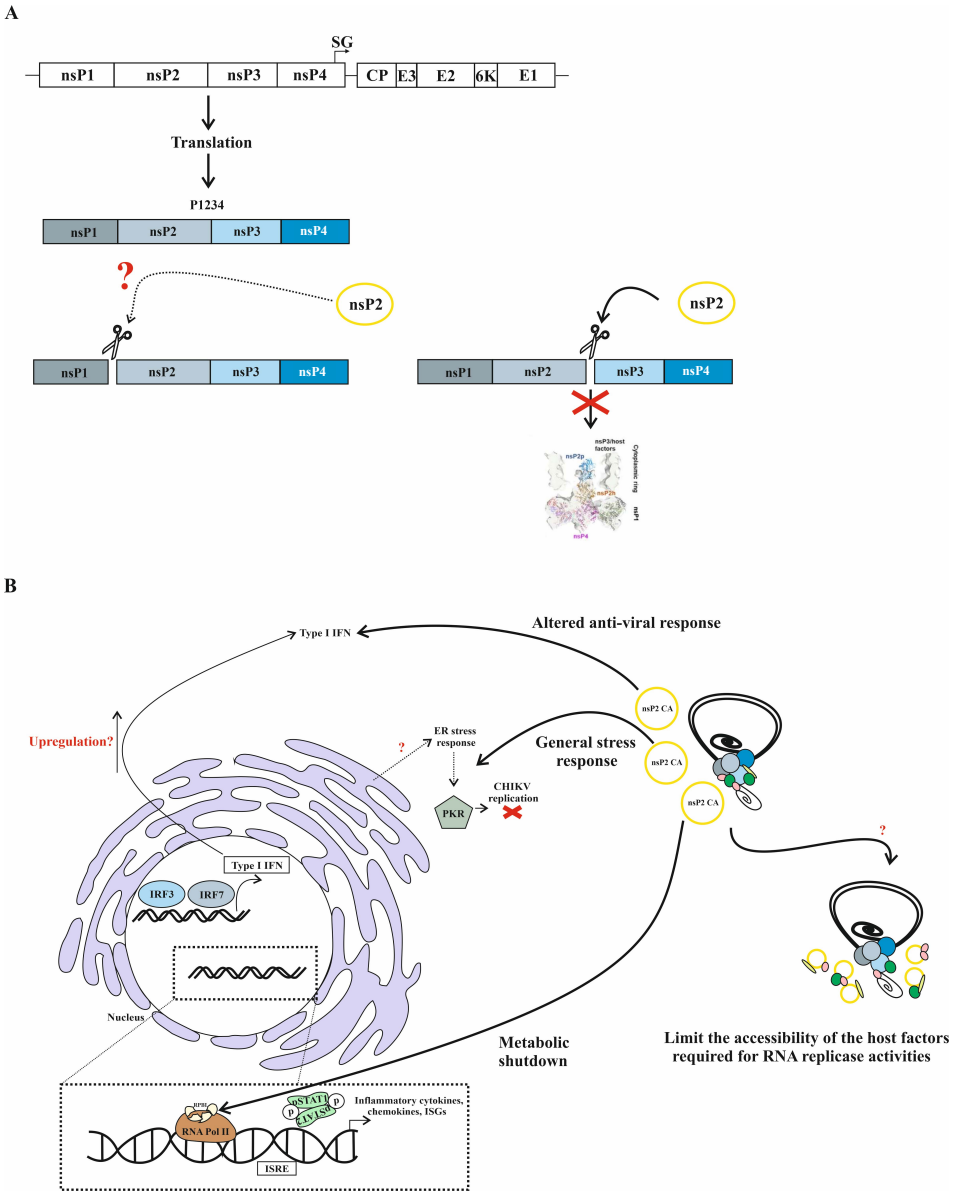

Figure 19. Schematic representation of the mechanisms of nsP2-mediated inhibition of alphavirus replication. (A) Mechanism of the reduction in RNA replication due to the protease activity of nsP2. Free nsP2 cleaves the P1234 replicase precursor expressed from the genome of superinfecting virus or from mRNA transcribed from the *trans*-replicase plasmid at the 2/3 site, resulting in P12 and P34 polyproteins. These as well as their cleavage products (mature nsPs) are unable to form functional replicase complexes. Premature cleavage of P1234 at the 1/2 site is less likely, as this site is normally processed in cis. **(B)** Overview of the possible mechanisms of protease-activity-independent suppression of alphaviral RNA replicase activity by free nsP2. nsP2 lacking protease activity can affect the functioning of the replicase complex by binding essential host factors, triggering cellular antiviral responses, modulating the stress response or causing general metabolic changes in cells, including shutdown of transcription, by inducing the degradation of RNA polymerase II (in vertebrate cells). In turn, these effects can reduce the activity of viral RNA replicase.

5.2. Activity of Eastern equine encephalitis virus RNA replicase depends on the molar ratio of nsP1:P23:nsP4 components (Publication II)

New World alphaviruses differ from Old World alphaviruses not only in their geographical distribution but also by specificity of virus–host interaction, virulence and distinct features of pathogenesis. EEEV is a medically important pathogen. Its infection may lead to the development of life-threatening neurologic diseases such as meningitis and/or encephalitis in humans (244). The factors contributing to the different pathogenicity of Old World and New World alphaviruses (including EEEV) remain largely unknown. However, as RNA replication is a central event for the existence of any RNA virus, it is obvious that all biological properties of the virus (including pathogenesis) are directly or indirectly affected by this process. For this reason, a study aiming to characterize the requirements for EEEV RNA replicase formation, its characterization and the development of systems for its practical application (including a test system avoiding/ minimizing the use of highly pathogenic EEEV) was performed in our laboratory. As in the studies described in Publication I, we applied a *trans*-replicase system to study EEEV RNA replication. Several versions of EEEV *trans*-replicase were developed, including different templates (expressing luciferases or fluorescent marker proteins) and different constructs for replicase polyproteins expressing either full P1234 (one-component replicase), P123 and nsP4 (two-component replicase), or nsP1, uncleavable P23 and nsP4 (three-component replicase) (**Publication II, Figure 4**). These systems allow 1) validation of the functionality of "artificially" reassembled RCs; 2) evaluation of the compatibility of heterologous templates with replicase proteins of EEEV; 3) evaluation of the compatibility of EEEV replicase proteins with those from heterologous alphaviruses; and 4) the use of two- or three-component *trans*-replicases to analyze the impacts of different molar ratios of replicase components (P123:nsP4 or nsP1:P23: nsP4) on the activity of EEEV RCs.

Previous studies have shown that the *trans*-replicase systems of alphaviruses are generally highly active in mammalian cell lines (220). In the current study, the same was confirmed for the *trans*-replicase of EEEV, which was found to be among the most active systems developed in our laboratory. It has been previously described that the two-component *trans*-replicases of alphaviruses studied thus far are highly active and that active replicases can also be formed using heterologous combinations of P123 and nsP4 components (i.e., P123 from one alphavirus and nsP4 from another alphavirus). However, the number of highly efficient heterologous combinations is limited, and the formation of efficient heterologous replicases is often not reciprocal. The replicase formed by P123 of one virus and nsP4 from another alphavirus may be active, while the reverse combination (nsP4 from the first virus and P123 from the second one) may have no or very low activity (197). Again, it was found that the properties of P123 and nsP4 of EEEV fit this pattern. In contrast to two-component *trans*-replicases, the

three-component *trans*-replicases of alphaviruses have considerably lower activities, and for some viruses (such as RRV), no activity could be detected at all. Here, we found that the three-component *trans*-replicase of EEEV has a very low ability to synthesize full-length positive-strand RNAs (mRNAs for Fluc) but a decent ability to synthesize SG RNAs (depending on the type of template used, these are mRNAs for Gluc or ZsGreen; **Publication II, Figure 4B, C, D**). Thus, two- and three-component *trans*-replicases of EEEV can be used to estimate the optimal ratios of EEEV P123 (or nsP1+P23) to nsP4 components needed for active replicase formation.

It has previously been observed that the activities of two-component *trans*-replicases of CHIKV, SINV, BFV, VEEV, and EILV all increase if the plasmid encoding nsP4 is used in molar excess compared to the plasmid expressing P123. At high nsP4:P123 expression plasmid ratios, the activities either plateaued or continued to increase. For CHIKV, it was also found that the increase was mostly due to a stronger signal (i.e., more active replication) in cells positive for RNA replication; the number of cells where replication was initiated did, however, decline if the plasmid expressing nsP4 was provided in large excess (197). This shows that an excess of nsP4 is not per se beneficial for alphavirus replication and provides reasons for the methods these viruses use to downregulate nsP4 levels (use of translational readthrough, degradation of the nsP4 using proteasomal pathway). It also opens an interesting possibility – can nsP4 also be used as a factor for SIE? Even if nsP4 does not have such a function in the case of natural viruses, maybe it can be achieved artificially by overexpression of nsP4 or its mutant versions? Such an approach can be a rather powerful method for the generation of broad-spectrum resistance, as alphaviruses from different complexes can use nsP4 proteins from a large number of different alphaviruses to build functional replicases (197). Thus, if nsP4 can be used, it can possibly also be misused. With this possibility in mind, the following experiments were performed using EEEV *trans*-replicases.

First, different ratios of plasmids expressing P123 and nsP4 of EEEV were tested. In contrast to the previously studied alphaviruses, it was found that the 1:1 ratio of P123 to nsP4 expression plasmids resulted in the highest *trans*-replicase activity (**Publication II, Figure 3**). The same was observed for the nsP1+P23 to nsP4 expression plasmid ratio (**Publication II, Figure 4C**). These findings seemingly contrast with those for other alphaviruses and could indicate that the defined ratio of EEEV P123 (or nsP1+P23) to nsP4 is crucial for the high activity of the two- or three-component *trans*-replication systems of EEEV. Second, using a template expressing ZsGreen via SG RNA, it was found that this difference from CHIKV (and presumably other alphaviruses) *trans*-replicase was due to EEEV replicase activity in replication-positive cells. For CHIKV, higher amounts of the nsP4 expression plasmid resulted in increased replication; for EEEV, the same resulted in a reduction in replication. The number of cells where replication was initiated also declined (**Publication II, Fig. 3E, 4E**), although the *trans*-replicases of EEEV and CHIKV (197) were similar to each other.

Taken together, our data indicate the existence of the optimal ratio of EEEV P123 to nsP4. Due to the lack of corresponding antibodies, it was not possible to determine the optimal ratio in *trans*-replicase-transfected cells in a manner similar to that in EEEV-infected cells. It is also not clear if the different behavior of EEEV (compared to other studied alphaviruses) represents a specific property of the virus or whether it simply reflects higher stability of EEEV nsP4 in cells that allows optimal (and subsequently nonoptimal) concentrations of nsP4 to be reached in transfected cells with lower amounts of nsP4 expression plasmids. An excess of nsP4 per se cannot increase or decrease the activity of RCs, as each of them contains only one subunit of nsP4 (94). Thus, the number of RCs per cell most likely varied. Indeed, if the nsP4 of EEEV is more stable than its counterparts from other alphaviruses, it has more time to find and interact with nsP1 (or P123) and likely also with template RNA, stabilize and form a platform for assembly of functional RC. However, it is also clear that the excess EEEV nsP4 in cells hampers the formation of RCs. There are multiple possibilities for how this can happen – an excess of nsP4 may interfere with the formation of the correct dodecameric structure of nsP1: if the formation of too many complexes is initiated, there would not be enough nsP1 to complete their formation. It is also possible that excess nsP4 (not bound to the RC core) competes for the binding of nsP2. Even more likely is that an excess of nsP4 may bind template RNA and reduce its availability for forming RCs. Of course, the effect may be cell mediated. These aspects merit both functional and structural studies. Once the mechanism or mechanisms behind the observed effect are clear, an expression of nsP4 – or more likely some enzymatically inactive but highly competitive form of it – can be used to inhibit the formation of functional RCs and to generate artificial virus resistance. It is also conceivable that such an approach can be combined with approaches based on the use of nsP2 as a restriction factor for alphavirus infection to achieve a stronger phenotype and/or to widen the range of viruses for which replication/transmission will be restricted (Figure 20).

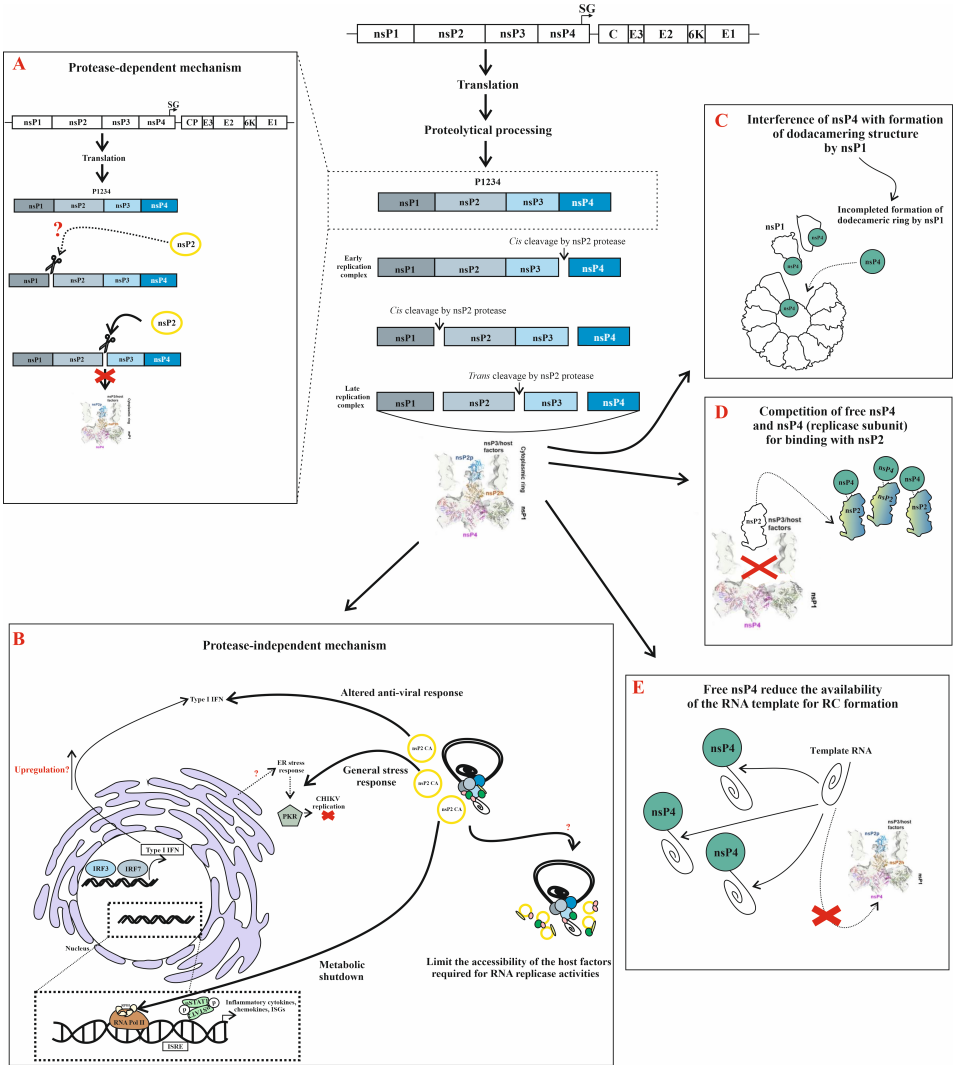

Figure 20. Overview of the multitargeted approaches by which alphavirus nsP2 and/or nsP4 can be used to restrict infection by homologous and heterologous alphaviruses. nsP2 can suppress replicase formation by a protease-activity-dependent mechanism **(A)** or by protease-activity-independent mechanisms **(B)**. Excess nsP4 may cause disturbance of the formation of dodecameric ring structures of nsP1 **(C)**, attachment of nsP2 to nsP1:nsP4 complexes **(D)** and/or binding of suitable template-RNAs, making them inaccessible for the formation of RCs **(E)**. Several of these approaches can be combined to achieve enhanced inhibitory effects.

5.3. Determinants located in nsP2 are important for the pathogenicity of the Ross River virus (Publication III)

The main focus of previous experiments in this thesis was the investigation of nsP2 as one of the factors regulating viral RNA replication. However, the functions of this protein extend far beyond viral RNA replication and involve interactions not only with other nsPs, but also with multiple host factors, such as ribosomal proteins, hnRNPs, and HSP90 (236, 237, 238). Importantly, this protein is one of the major determinants of the virulence of Old World alphaviruses, allowing them to overcome host antiviral responses (153, 239, 240). This clearly occurs through the use of not only a single mechanism, but a combination of several, from induction of general shutdown of transcription in vertebrate cells by targeting Rbp1 to proteasomal degradation, inhibition of cellular translation by utilization of protein kinase R (PKR)-dependent and/or PKR-independent pathways (241), counteraction of the type I interferon (IFN) response, counteraction of the unfolded protein response (246, 247), and likely other antiviral mechanisms of the cell. The efficiency of nsP2 as a protease also impacts antiviral responses, as the increased stability of early replicase likely results in overproduction of negative-strand RNAs (or, more accurately, double-stranded RNAs called replication intermediates) that act as inducers of antiviral signaling (248). In this study, we analyzed the impacts of aa substitutions found in nsP1 and nsP2 of a natural isolate of RRV on the formation of replicase proteins of the virus, virus-mediated shutdown of host metabolism and activation/suppression of the type I IFN response.

Similar to other alphaviruses, RRV is a potent modulator of the type I IFN response. A previous study in our lab revealed that host cell antiviral responses can be modulated by a single mutation introduced into the 1/2 site of the RRV ns polyprotein; this mutation increases the stability of P123 and leads to overproduction of double-stranded RNAs that act as agonists of type I IFN (249). Our collaborators in Australia also collected several naturally-occurring RRV strains from Australian mosquitos. When these were used to infect type I IFN response competent mammalian cells, few isolates showed a highly elevated capability to induce an IFN response (or were unable to suppress the type I IFN response) that resembled the above-described mutant virus. This observation has triggered a logical question: is the similar phenotype caused by the same molecular mechanism? To answer this question, the ns regions of the genome (nsP1-4 coding sequences) of these virus isolates were sequenced. P1234 of an isolate named RRV 2528 that induced the highest levels of type I IFN was found to contain numerous differences compared to P1234 of RRV T-48, a prototype strain of the virus characterized by a low level of IFN induction. The largest number (eight substitutions) was found in nsP2. This may indicate an important role of nsP2 in induction/counteraction of type I IFN or be simply incidental – nsP2 is the largest ns protein of alphaviruses, and therefore it is not unexpected that numerous aa differences map to this region. To determine the significance of these differences, the following experiments were performed.

First, our collaborators swapped nsP1-nsP2 or nsP3-nsP4 regions of RRV T-48 with the corresponding regions from RRV2528 and analyzed the obtained viruses, M12 and M34, for their ability to induce the expression of type I IFN. It was found that both recombinants induced more type I IFN than RRV T-48, and for M12, the levels of IFN were even higher than those induced by RRV 2528 infection. Thus, the most important determinants of type I IFN induction of RRV 2528 reside in the nsP1-nsP2 region. This region contains, in addition to 8 substitutions in nsP2, 4 substitutions in nsP1 (**Publication III, Table 3**). Based on their locations in the protein and their occurrence in other RRV isolates, 7 substitutions in the nsP1-nsP2 region of RRV 2548 (V186A, E402A, and R522Q in nsP1 and A31T, N219T, S580L, and Q619R in nsP2) were selected for further analysis. Each of these changes was introduced into the RRV T-48 sequence, and the ability of the corresponding recombinant viruses to induce type I IFN was analyzed. All viruses harboring a substitution in nsP1 induced IFN at levels comparable to RRV T-48 and much lower than RRV 2528. This indicates that these changes are not, at least alone, responsible for the elevated type I IFN induction. In contrast, all four viruses with substitutions in nsP2 induced type I IFN at levels similar to that of RRV 2528, indicating that nsP2 is indeed the key determinant for the induction and/or suppression of the type I IFN response. The remaining question was as follows: what is the mechanism behind this effect?

First, we analyzed whether the differences in type I IFN expression can be caused by differences in the general shutdown of host cell protein synthesis. The reasoning was that nsP2 is responsible for host cell transcriptional shutdown and is capable of affecting cellular translation either by depletion of the pool of cellular mRNAs or by affecting their translation by causing activation of PKR. Therefore, the translational shutdown in cells infected with RRV T-48 was compared to that in cells infected by the above-described mutant viruses. The experiment showed that there was no significant difference between RRV T-48 and mutant viruses, indicating that none of the mutations in nsP1 or nsP2 had a profound effect on the general shutdown of host protein synthesis (**Publication II, Figure 9A**). Next, we aimed to determine whether the differences were due to slower processing of P123, which is known to result in elevated type I IFN expression (248). Due to the lack of antibodies specific to the nsPs of RRV, the analysis was performed using in vitro translation. Somewhat unexpectedly, it was found that the processing of P123 encoded by viruses containing the above-listed substitutions in nsP1 or nsP2 was not significantly different from that of P123 encoded by RRV T-48 (**Publication III, Figure 9B**). This finding suggests that neither the basal protease activity of nsP2 nor the pattern of P123 processing was substantially affected by the introduced changes. Taken together, these findings demonstrated the existence of other mechanisms of regulation of IFN production, which function independently from the general host transcriptional/translational shutdown and synthesis of viral RNA molecules acting as agonists for the type I IFN system (although we did not analyze these RNAs directly, their synthesis correlated with processing of P123, which was found not to be affected). Thus, the differences between the nsP2 proteins of RRV T-48 and RRV 2528 are not

related to the general functions of nsP2. This implies that another, more fine-tuned mechanism (or mechanisms) is altered by the introduced mutations. For example, the mechanism could interfere with antiviral signaling, as alphaviruses are known to overcome the antiviral response by suppressing JAK-STAT signaling. This suppression downregulates the induction of interferon-stimulated genes (ISGs) (239, 243). To test this hypothesis and other similar hypotheses, additional experiments extending beyond the scope of the performed study are necessary.

To summarize, the experimental data proved the involvement of the nsP2 of RRV 2528 in the high type I IFN-inducing phenotype. The effect was mapped to numerous (at least 4) changes in nsP2 that act in parallel or in an additive manner. However, none of these mutations altered the processing of P123 of RRV or affected the general shutdown of infected cell protein synthesis. This indicates the role of one or more fine-tuning mechanisms used by nsP2 of RRV to interfere with host cell antiviral responses and thus modulate the infection process of an alphavirus.

6. CONCLUSIONS

Over the last decades, intensive studies of factors/activities responsible for multiple aspects of alphavirus infection have been performed. In particular, our understanding of the structures and functions of viral RNA replicase and its components has significantly increased. Novel findings emphasize that one of the first and most essential events in alphavirus infection is processing of the ns polyprotein carried out by its nsP2 region and an individual nsP2; this process not only ensures the release of functional replicase subunits, but also determines whether these proteins form the active RCs. Thus, it is increasingly evident that nsP2 is one of the "main driving forces" of successful RNA replication. Furthermore, due to its versatile functions and various activities, nsP2 is involved in other aspects of infection: it is one of the key determinants associated with activation as well as counteracting of antiviral response in infected cells. Therefore, different modifications of the protein, including point mutations, often drastically impact alphavirus infection. However, many of the precise mechanisms of P2 action remain enigmatic. What is clear is that nsP2 does not act alone, and that its activities are modulated by other components of viral replicase.

The current study allowed us to identify and confirm new functions and properties of nsP2 and alphavirus RNA replicase. The general conclusions of this study can be presented as follows:

1. Alphavirus *trans*-replicase systems can be applied as a tool for studies of the functions of nsP2 associated with inhibition of RC formation/activity. These functions are essential for SIE and are likely related to those used to regulate RNA replication in alphavirus-infected cells. Using *trans*-replicase systems of CHIKV and SINV, it was found that the key event in SIE is targeting of replicase precursor (P1234) by an individual nsP2 protein and that this ability of nsP2 can be altered by mutations present in its functionally important regions.

2. It was found that the synthesis of an individual (free) nsP2 in mosquito cells has an inhibitory effect on alphavirus RC formation/functionality. This is not, however, a result of a single mechanism but results from a combination of an nsP2 protease-activity-dependent mechanism and protease-activity-independent mechanisms. The level and dominant mode of inhibition of alphavirus RNA replication depend on the virus, source of free nsP2 and substitutions present in this protein. The protease-activity-mediated mechanism is important for suppression of replication of matching virus and relies mostly on the ability of nsP2 to cleave the 2/3 site in ns polyprotein. For some viruses, such as SINV, it is also a dominant mechanism used to suppress the formation of RNA replicases of heterologous alphaviruses.

3. nsP2 of SINV and CHIKV can inhibit the formation/activity of RNA replicase of heterologous alphavirus using protease-activity-independent mechanisms; for nsP2 of CHIKV, this is the dominant mechanism to suppress the activity of

RNA replicases of heterologous alphaviruses. The precise details of the protease-activity-independent mode of action of nsP2 remain unknown; however, it is clear that this property can be enhanced by the introduction of certain mutations into nsP2. It is likely that the protease-activity-independent inhibitory effect originates not from a single mechanism but from several mechanisms.

4. The *trans*-replicase of highly pathogenic EEEV was found to be highly active in human and mosquito cells. Splitting the construct of EEEV P1234 expression into two (P123 and nsP4) or three (nsP1, P23 and nsP4) expression constructs allowed the analysis of the requirements of active RC formation. It was found that the activity of the EEEV RNA replicase depends on the correct ratio of the P123 (or nsP1+P23) component to the nsP4 component and that in contrast to previously studied alphaviruses, an excess of nsP4 reduced the activity of the EEEV RNA replicase. The reduction was due to the decrease in the number of cells in which RNA replication was initiated, as well as to the reduced RNA replicase activity in such cells. It remains unclear whether this property is unique to the EEEV RNA replicase.

5. The natural isolate RRV 2528 was found to be a prominent inducer of type I IFN expression. This property was associated with specific amino acid substitutions in the nsP2 encoded by this isolate. None of these substitutions or their combination affected the ability of RRV to induce shutdown of cellular protein synthesis or the level of viral structural protein expression. Similarly, no effect on the processing of P1234 was detected. Taken together, these findings indicate that excessive type I IFN induction was not due to a lack of the ability to induce the general shutdown of cellular gene expression or due to defects in RC formation.

SUMMARY IN ESTONIAN

Uudsed vaated alfaviiruste poolt kodeeritud nsP2 valgu funktsioonide kohta

Alfaviirused (sugukond *Togaviridae*) on sfäärilise ümbrisega virionide ja positiivse polaarsusega RNA genoomiga viirused, mis enamasti levivad lülijalgsete vektorite vahendusel. Paljud alfaviirused on olulised inimeste patogeenid, sealhulgas ulatuslikke puhanguid põhjustav Chikungunya viirus (CHIKV), Austraalias leviv Ross River viirus (RRV), Ameerikas leviv ida hobuste entsefaliidi viirus (EEEV) ja ka Eestis leiduv Sindbis viirus (SINV). Alfaviiruste põhjustatud haigused sõltuvad viiruse liigist ja selle päritolust, kus üldistatult põhjustavad Vana Maailma alfaviirused palaviku, löövet ja artriiti, samal ajal kui Uue Maailma alfaviirused on sageli kõrge patogeensusega põhjustades neuroloogilisi haiguseid, sealhulgas entsefaliiti.

Alfaviiruste RNA genoom kodeerib nelja viiruse RNA replikaasi allühikut, mis toodetakse liitvalgust eellase (polüproteiini) kujul. Liitvalk lõigatakse neljaks valmis replikaasi valguks viiruse nsP2 proteaasi abil. Viimastel aastakümnetel läbi viidud uurimistööd on võimaldanud välja selgitada viiruse replikaasi kompleksi kui ka selle komponentide peamised funktsioonid ning meie arusaamine viiruse valkude struktuuridest on oluliselt täienenud. Ka need uurimised on näidanud, et alfaviiruse infektsiooni üks esimesi ja kõige olulisemaid sündmusi on replikaasi valkude eellase lõikamine valmis valkudeks. Eelvalgu lõikamised toimuvad kindlas järjekorras ja on kindlalt ajastatud, mis määrab ära selle, kas vabanevad valgud moodustavad toimiva RNA replikaasi või mitte. Seega kujutab eelvalgu lõikamise protsessi läbi viiv nsP2 endast RNA replikatsiooni käivitamise ja efektiivsuse regulaatorit. Samas on tegemist multifunktsionaalse valguga, mis on lisaks RNA sünteesi reguleerimisele oluline nii raku viirusvastaste kaitsereaktsioonide aktiveerimisel kui ka nende mahasurumiseks. Sellest tulenevalt mõjutavad muudatused nsP2 valgus, sealhulgas punktmutatsioonid valgu funktsionaalselt olulistes regioonides, oluliselt viiruse bioloogilisi omadusi. Seejuures ei ole mitmete nsP2 aktiivsuste taga seisvad molekulaarsed mehhanismid lõpuni selged. Küll aga on selgunud, et nsP2 funktsioone mõjutavad interaktsioonid alfaviiruse ülejäänute replikaasi valkude nsP1, nsP3 ja nsP4-ga.

Käesoleva uurimistöö raames selgitati välja ja analüüsiti mitmeid nsP2 valgu ja RNA replikaasi seni tundmatuid funktsioone. Töö peamised tulemused saab kokku võtta järgnevalt:

1. alfaviiruste genoomide alusel konstrueeritud *trans*-replikatsiooni süsteemid võimaldavad uurida nsP2 valgu funktsioone, mis on seotud viiruse RNA replikaasi kompleksi moodustamisega, aga ka selle moodustamise blokeerimise ja/või selle aktiivsuse mahasurumisega. Viimati nimetatud aktiivsused on olulised viiruste superinfektsiooni blokeerimisel ja tõenäoliselt ka viiruse replikatsiooni ajalisel reguleerimisel. CHIKV ja SINV *trans*-replikatsiooni süsteeme

kasutades tehti kindlaks, et superinfektsiooni blokeerimise puhul on võtmesündmuseks ühe viiruse replikaasi eellase lõikamine teise viiruse nsP2 poolt ja et seda mõjutavad mutatsioonid nii sihtmärk-liitvalgus kui ka nsP2 valgus;

2. leiti, et individuaalse nsP2 süntees sääse (vektorputuka) rakkudes surub maha alfaviiruse replikatsiooni aktiivsust. Seda nähtust ei põhjusta ainult nsP2 proteaasne aktiivsus – olulised on ka nsP2 proteaassest aktiivsusest sõltumatud mehhanismid. See, milline on konkreetsete mehhanismide osakaal, sõltub nii viiruse RNA replikaasi kui ka selle moodustamist inhibeeriva nsP2 valgu päritolust. Homoloogse viiruse replikatsiooni mahasurumisel domineerib nsP2 proteaassest aktiivsustest sõltuv mehhanism, kus nsP2 poolt teostatud replikaasi eelvalgu lõikamine 2/3 saidist välistab funktsionaalse RNA replikaasi moodustumise. Mõne alfaviiruse (SINV) puhul on nsP2 proteaassest aktiivsusest sõltuv mehhanism ka peamiseks mehhanismiks millega surutakse alla heteroloogsete alfaviiruste replikaaside moodustamist;

3. nii SINV kui ka CHIKV nsP2 valk takistab heteroloogsete alfaviiruste RNA replikatsiooni ka proteaassest aktiivsusest sõltumatute mehhanismide abil, kus CHIKV nsP2 puhul on need peamisteks meetoditeks heteroloogsete alfaviiruste replikatsiooni mahasurumisel. Selle inhibeerimise täpne põhjus on hetkel teadmata, küll aga näitasime, et erinevad mutatsioonid nsP2 valgus mõjutavad taolise inhibeerimise efektiivsust. Need andmed viitavad sellele, et proteaassest aktiivsusest sõltumatu replikatsiooni inhibeerimine saavutatakse tõenäoliselt mitmete paralleelsete mehhanismide abil;

4. väga patogeensele ja seetõttu vähe uuritud EEEV-le konstrueeritud *trans*-replikatsiooni süsteem osutus kõrgelt efektiivseks nii inimese kui ka sääse rakkudes. Samuti osutus võimalikuks EEEV replikaasi eelvalku (P1234) kodeeriva järjestuse jagamine kaheks (P123 + nsP4) ja kolmeks (nsP1+P23+ nsP4) komponendiks. See võimaldas uurida kas ja kuidas replikaasi komponentide vahekord mõjutab EEEV RNA replikaasi aktiivsust. Läbi viidud katsed näitasid, et erinevalt seni uuritud alfaviirustest sõltub EEEV RNA replikaasi aktiivsus teda moodustavate komponentide optimaalsest vahekorrast ja et RNA replikaasi katalüütilise allühiku (nsP4) ülehulk vähendab EEEV RNA replikaasi aktiivsust. Selline aktiivsuse langus tuleneb sellest, et väheneb nii rakkude hulk milles RNA replikatsioon aktiveeritakse, kuid ka RNA replikatsiooni aktiivsus nendes rakkudes. Hetkel pole veel selge kas tegemist on EEEV RNA replikaasi unikaalse omadusega või leidub ka teisi sarnaste omadustega alfaviiruste replikaase;

5. uuriti RRV loodusliku isolaadi RRV 2528 omadusi. Leiti, et see viiruse isolaat põhjustab väga tugevat tüüp-I interferoonide vastust. Viiruse genoomi analüüs tõi välja, et RRV 2528 erineb teistest vähemal määral interferoonide tootmist indutseerivatest RRV isolaatidest mitmete mutatsioonide poolest, millest paljud paiknevad just nsP2 valgus. Näitasime, et need mutatsioonid mõjutavad

RRV nakkuse käigus toimuvat interferoonide tootmist, kuid ei mõjuta viiruse replikaasi eelvalgu protsessimist valmis valkudeks, viiruse võimet suruda maha raku valgusünteesi ega viiruse struktuurvalkude sünteesi. Seega ei tulene suurenenud tüüp-I interferoonide vastus defektist RRV 2528 RNA replikatsioonikomplekside moodustamisel või võimetusest maha suruda raku üldist geenieksprессiooni.

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