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LIIS KARO-ASTOVER

Studies on the Semliki Forest virus replicase protein nsP1

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to by their Roman numerals from this point forward.

I Žusinaite E., Tints K., Kiiver K., Spuul P., **Karo-Astover L.**, Merits A. and Sarand I. (2007). Mutations at the palmitoylation site of non-structural protein nsP1 of Semliki Forest virus attenuate virus replication and cause accumulation of compensatory mutations. J Gen Virol 88:1977–1985.

II **Karo-Astover L.**[*], Šarova O.[*], Merits A. and Žusinaite E. (2010). The infection of mammalian and insect cells with SFV bearing nsP1 palmitoylation mutations. Virus Res 153:277–287.
[*] These authors contributed equally to this work.

III Lulla V.[*], **Karo-Astover L.**[*], Rausalu K., Merits A. and Lulla A. (2013). Presentation overrides specificity: probing the plasticity of alphaviral proteolytic activity through mutational analysis. J. Virol. 87:10207–10220.
[*] These authors contributed equally to this work.
This thesis also contains unpublished data.

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Author's contribution:

I Sequencing, detecting compensatory mutations, and creating the constructs

II Performing all experiments with mammalian cells and creating the constructs

III Creating the constructs and performing the *in vitro* translation experiments, pulse-chase experiments, and growth curves

ABBREVIATIONS

aa	amino acid residue
C	capsid protein
CHIKV	Chikungunya virus
CPV	cytopathic vacuole
CSE	conserved sequence element
DI RNA	defective interfering RNA
EGFP	enhanced green fluorescent protein
Enh	capsid enhancer sequence
icDNA	infectious cDNA
NLS	nuclear localization signal
Ns	non-structural
nsP	non-structural protein
Nt	nucleotide
NTPase	nucleotide triphosphatase
IFN	interferon
IRES	internal ribosomal entry site
MOI	multiplicity of infection
PAC	puromycin acetyl-transferase
piRNA	PIWI-interacting RNA
PIWI	P-element-induced wimpy testis, genes for maintaining incomplete differentiation in stem cells
Rluc	*Renilla* luciferase
RNAi	RNA interference
SFV	Semliki Forest virus
Sg	subgenomic
SINV	Sindbis virus
siRNA	small interfering RNA
UTR	untranslated region
VEEV	Venezuelan equine encephalitis virus
VRP	virus replicon particle
Wt	wild type

I INTRODUCTION

Alphaviruses (family *Togaviridae*) cycle between susceptible vectors and vertebrate hosts in nature. Although their evolutionary origin has not been confirmed, some sequence analysis suggests that alphaviruses may initially have arisen from a parasite-borne plant virus and that the mobility of the vector contributed to the divergence of the genus. At least two transoceanic inductions of alphaviruses must have occurred in the past [1].

Since their isolation, alphaviruses have served as a model system for studying the structure and functions of enveloped viruses of animals. Semliki Forest virus (SFV) was among the first alphaviruses to be discovered, and SFV was isolated in 1942 from mosquitoes in Uganda [2]. Other alphaviruses, including Sindbis virus (SINV) and Chikungunya virus (CHIKV), have been subsequently isolated from mosquitoes [3] and mammals [4].

According to their geographic distribution, alphaviruses have been divided historically into Old World and New World viruses. Old World alphaviruses, including SFV, SINV and CHIKV, primarily cause fever and rash but can also induce arthritic symptoms and severe joint pain. New World alphaviruses, including Venezuelan Equine Encephalitis virus (VEEV), tend to cause encephalitis [1]. The laboratory strains of SFV and SINV are non-pathogenic to humans; however, these viruses, as well as a number of other alphaviruses, are lethal to mice at a certain age [5–7]. Several strains and isolates of a single virus, the virulence of which can differ significantly, have been discovered. For example, in contrast to the widely used laboratory SFV4 strain [8], the replication of SFV strain A7 that was isolated from Mozambique mosquitoes in 1959 [5] is restricted by the age of neurons [9]. Very few amino acid (aa) substitutions are responsible for the more neuroinvasive phenotype of SFV4 [10]. In addition, the virulence of alphaviruses depends on their ability to overcome the host's innate immune response. Interferon (IFN)-competent animals can limit the spread of SFV and protect some organs and tissues from being infected [11]. Thus, both genetic determinants of the virus and the competent IFN response of the host contribute to the outcome of the alphaviral infection.

2. REVIEW OF THE LITERATURE

2.1 An overview of alphaviruses

SFV is one of the most well studied alphaviruses, mainly due to the simplicity of its genome, its high biosafety [12] and its significant potential for use in biotechnology. Like all alphaviruses, SFV is a positive-strand RNA virus. Its genome is approximately 11.5 kb in length, is capped at the 5' end, and has a poly(A) tail at the 3' end [13]. The SFV genome encodes four nonstructural (ns) proteins (nsP1-4), the viral components of the viral replicase, and five or six structural proteins that are needed for the formation of the capsid and the glycoprotein-containing envelope [14]. The structural proteins are expressed from the subgenomic (sg) RNA that is synthesized from the sg promoter. In contrast, ns-polyprotein P1234 or P123 and P1234 polyproteins (for some SFV strains such as A7 [10]) are directly translated from the genome. Two polyproteins are synthesized for the viruses and strains, which have a leaky in-frame stop codon present between the nsP3 and nsP4 coding regions. Gradual autocatalytic cleavages in the ns-polyprotein are performed by the nsP2 protein or the nsP2 region of ns-polyproteins [15,16]. In early infection, P1234 is autocatalytically cleaved to P123 and nsP4 [17]. This complex synthesizes the complementary negative-strand RNA and is therefore named an early replication complex [14,18]. Subsequent cleavages in the ns-polyprotein provide the late replicase complex that consists of individual nsPs. This complex is prone to synthesize RNA of positive polarity – new genomic RNA and sg RNAs. The late replicase complex is generally incapable of synthesizing RNA of negative polarity; thus, due to changes in the ns-polyprotein processing pattern the synthesis of negative-strand RNA stops at the late stages of alphavirus infection [19]. Structural gene products do not participate in replication and can be replaced with any sequence of interest up to 9 kb in length. This property has been used for the development of SFV-based replicon vectors [8].

SFV infects different vertebrate and insect cell lines. In vertebrate cells, the SFV infection leads to prominent changes in cellular structures and inhibition of different host biosynthesis processes, and it results in cell death by apoptosis [20,21]. This type of infection reflects *in vivo* infections in vertebrates that are characterized by high viremia and can lead to the death of the host [22]. However, infection in mosquito vectors is quite different. In mosquitoes, the infection starts in the midgut of the insect and then spreads to other organs. After the virus reaches the salivary glands, the infected vector begins spreading the virus with each blood meal [23,24]. Infection in the mosquito is persistent and does not have a major impact on the viability of the vector. In addition, only limited cell death, mostly occurring during the early phase of infection, can be observed in alphavirus-infected, cultivated mosquito cells [25]. The early phase of infection in mosquito cells is relatively similar to the infection in vertebrate cells; this phase is characterized by the active production of progeny virions [26]. After this phase, the persistent infection is established [25]. During alphaviral

infection in invertebrate cells, a large amount of defective interfering (DI) RNA genomes are synthesized [27,28]; the DI RNA genomes may have a role in the establishment of the persistent infection. However, other studies have identified RNA interference (RNAi) as one of the most prominent antiviral mechanisms used by insects and insect cells to restrict alphavirus infection [29]. Other studies have clearly indicated that RNAi does not represent the only defense against alphaviruses, and some other mechanisms protect insects from alphavirus infection equally well [28,30–32]. As the infection of insect cells is restricted by multiple different antiviral mechanisms, alphaviruses must be fit to infect these cells. Indeed, mutations in alphaviral replicase proteins that affect viral replication in vertebrate cells minimally or not at all significantly reduce the infectivity in insect cells or cause a temperature-sensitive phenotype of the virus [14,33–35]. Thus, the requirements for efficient replication in mosquito and vertebrate cells are diverse, and viral nsPs may have different roles in distinct host cell types.

2.2 Alphaviral infection cycle

Virion

The alphaviral virion is a spherical particle that is approximately 68 nm in diameter [36]. The virion has a T=4 type of geometry [37,38]. Thus, the genomic RNA is covered by a core of 240 molecules of capsid protein (C) that are organized in pentameric and hexameric structures. The core containing the virus genome (nucleocapsid) is surrounded by a lipid bilayer derived from the host cell membrane [37,39]. The lipid bilayer itself is further covered by a glycoprotein lattice that almost completely hides the membrane, and distinct glycoprotein spikes that protrude from the particle can be observed [40]. The spikes are composed of all three structural glycoproteins (E3, E2 and E1) in an SFV virion [41,42] or only E2 and E1 in the virions of most other alphaviruses [36,39] and are hollow on the inside [40]. One spike contacts a single monomer of the capsid protein [39,43], and this contact occurs via the cytoplasmic domain [41] of the E2 protein [44]. This mechanism ensures a 1:1 molar ratio between the capsid proteins and the E1 and E2 glycoproteins [37]. Three glycoprotein spikes form a trimeric complex. Thus, in total, 240 copies of each glycoprotein, which are organized into 80 heterotrimeric complexes, are present in each virion.

Binding and entry

Alphaviruses have a broad host range and use a number of different cellular receptors. The identity of the receptors depends on the host and tissue type to be infected. Receptors that enable the infection of a certain host are not always involved in infecting other hosts. In addition, alphaviruses may use several receptors in a single cell line. Therefore, the identity of alphaviral receptors remains unclear. For SFV, class I major histocompatibility antigens are thought to be involved in the infection of human and murine cells [45]. SINV can use laminin receptors for promoting its infection in mammalian and insect cells

[46]. However, attachment to C-type lectins [47], heparan sulphate [48,49] and a divalent metal ion transporter (natural resistance-associated macrophage protein) have also been demonstrated [50]. In chicken cells, SINV can use a 63 kDa protein for entering the cells [51]. The viral factor (anti-receptor) needed for efficient binding on the plasma membrane of the cell is the glycoprotein E2 [52,53].

After binding to the receptor, alphaviruses enter the cell by clathrin-dependent endocytosis (Fig. 1). The virus is engulfed into endocytic vesicles [54] and fuses its membrane with vesicle membranes to release its nucleocapsid into the cytosol. This process is aided by a decrease in pH that occurs as endocytic vesicles mature; however, the success of the fusion also depends on the lipid composition of the endosome membrane. Cholesterol has been shown to mediate viral binding to target membranes, while sphingolipids assist the actual fusion event [55,56]. The participation of monovalent cations has also been observed [57]. When these molecules are present in the target membrane, a pH lower than 6 [56,58] induces membrane fusion that is completed within less than one minute at 37 °C [40,59].

The fusion mechanism is relatively well understood. In response to acidic conditions, the spikes (E1:E2 heterodimers) in the virus envelope dissociate [60]. The E2 portion moves away from the center of the trimeric complex, whereas E1 slides toward the center, the entire spike region elongates [40], and E1 forms homotrimers [61]. The fusion peptide residing in E1 becomes exposed [59,61], and the membranes are fused [62]. The exact details of these processes have been revealed by using the three-dimensional structure of alphaviral glycoprotein envelope [63,64].

Through membrane fusion, the nucleocapsid is released into the cytosol, where the RNA is uncoated due to the contacts between the C protein and ribosomes [65–67]. The C proteins remain associated with ribosomes, whereas viral RNA is engaged in translation and becomes associated with cellular membranes [65]. C protein cleavage may also contribute to the uncoating of RNA in the cytosol [68].

Thus, the initial steps of infection in vertebrate cells are relatively well described. Nevertheless, other mechanisms of entry may also exist [69]. As is the case with many aspects of alphavirus infection, their binding and entry into insect cells are poorly understood.

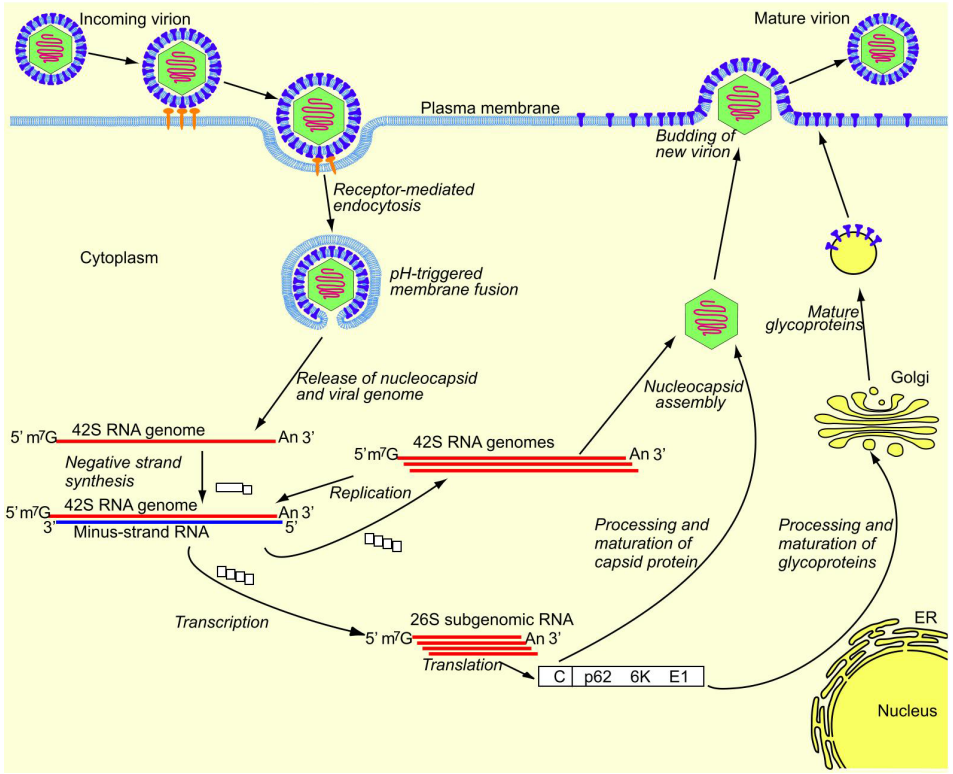

Figure 1. Schematic representation of the alphavirus infection cycle. Modified from [246], with permission. Alphavirus infection begins by the binding of a virion to a cellular receptor and entrance into the cell by receptor-mediated endocytosis. The endosomes fuse with lysosomes, resulting in a decrease in pH. The low pH induces a membrane fusion event, and the nucleocapsid is released into the cytoplasm. Upon nucleocapsid disassembly, the viral genome is released into the cytoplasm and becomes translated by ribosomes. The cleavage products of ns-polyprotein P1234 form a virus-specific part of the alphavirus replicase. The synthesis of negative-strand RNA and new positive-strand RNAs is associated with the cytoplasmic membranes, which are represented primarily by lysosomal membranes in the late stage of infection. After the synthesis and cleavage of the structural polyprotein, the viral glycoproteins are transported to the plasma membrane, whereas capsid protein surrounds the newly synthesized viral genomes. The formed nucleocapsids are transported to the plasma membrane, where the capsid protein associates with the cytoplasmic tails of the E2 glycoproteins. The binding event triggers the budding of the virion.

Requirements for replication

The RNA synthesis of alphaviruses is asymmetric and temporarily regulated [19]. Three types of viral RNAs are present in an alphavirus-infected cell: the genomic RNA of positive polarity (42S for SFV), the corresponding chain of negative polarity and the sg RNA with positive polarity [70]. Only the genome length RNAs are associated with the replication process. The replication of alphaviruses occurs in association with cellular membranes [71] and will be addressed in detail in later chapters.

The SFV genomic and sg RNAs have a 5' terminal cap structure [72] and a poly(A) tail [70]. These elements are required for their stability and translation but are also essential for efficient replication and transcription of the virus genome. Three untranslated regions (UTR) are found in alphavirus genomes: one at each end of the genome and one in the junction region between the structural and ns-polyprotein coding sequences. Inside the UTRs, two conserved sequence elements (CSE) have been identified. In total, the alphavirus genome contains four CSEs that are numbered according to their position from the 5' end of the genome. One of these CSEs (CSE2) is located inside the coding sequence of nsP1, and another (CSE3) is typically located at the 3' part of the coding sequence of nsP4 (Fig. 2). These elements interact with host and viral proteins to ensure the correct replication and transcription of viral RNA. CSE1 is 40 nt in length and has a stem-loop structure [73]. This element is required for initiating both negative- and positive-strand RNA synthesis and is considered to be the core promoter for RNA synthesis [74]. CSE2 is located approximately 150 nt from the 5' end of the genome. CSE2 is 51 nt in length (hence, this element is also called the 51 bp conserved sequence element), and its secondary structure contains two stem-loops [73]. This element enhances negative- and positive-strand RNA synthesis but is not absolutely required for virus replication in mammalian cells [74]. Transcription of the sg RNA is initiated on the sg promoter, which contains CSE3 that spans from 19 nt upstream to 2 nt downstream of the starting site of sg RNA synthesis. However, the minimal essential sg promoter is somewhat longer (-19 to +5) [75]. The final conserved element is CSE4, which directly precedes the poly(A) tract. CSE4 is located in the 3' UTR of the genome and is required for the initiation of negative-strand RNA synthesis [76,77]. A 19-nt portion of the element is shown to be required for defective interfering RNA replication [77]. During negative-strand synthesis, CSE4 and CSE1 are brought into contact by host cell proteins [74]. In several alphaviruses, an AU-rich segment and a repeating sequence element are present upstream of CSE4. In contrast to CSEs, this region is rather tolerant to smaller modifications that affect virus viability only in certain cell types [30]. Therefore, the region preceding CSE4 is thought to affect the host specificity of the alphavirus.

Figure 2. Alphavirus genome and location of sequences required for replication, gene expression and virion formation. The genomic RNA is capped at the 5' end and polyadenylated at the 3' end. The CSEs in the figure are represented by black boxes and numbers. PS indicates the packaging signal residing in the SFV nsP2 coding region. The asterisk represents the in-frame stop codon present in some SFV strains and in genomes of most other alphaviruses. The arrow represents the start site of the sg promoter; CSE-3 resides inside the sg promoter element. A translation enhancer element (Enh) is located in a region encoding the N-terminus of the capsid protein. R-AU represents the repeated and AU-rich sequences hypothesized to bind host specific factors. The sizes in the figure are not proportional to their actual sizes in the virus genome to allow a clearer presentation of the short CSEs.

Aside from the elements required for replication, additional RNA sequences that are important for alphavirus infection have been described. For example, the first 102 nt from the capsid protein-encoding region comprise a translation enhancer for SFV [78]. This element represents a highly stable RNA secondary structure that is not found in the genomes of all alphaviruses. Another important element is the packaging signal that contains several stem-loop structures and resides in the nsP1 or nsP2 coding region in SINV or SFV, respectively [79,80].

Virion formation and budding

Translation of the sg RNA begins in the cytoplasm of an infected cell using free ribosomes [81]. The first protein encoded by the sg RNA is the C protein, which is cleaved autoproteolytically from the structural polyprotein immediately after its synthesis is completed [82]. The remaining structural polyprotein is the precursor for glycoproteins. While still being translated, this polyprotein is translocated to the endoplasmic reticulum, where it is inserted into the membrane according to the signal sequences within it [83–85] and processed by a signal peptidase [84,86] into three parts: p62 (the precursor of E3 and E2), 6K and E1. The glycoproteins are heavily modified by glycosylation [87] and palmitoylated [88]. Either before or during transport to the Golgi apparatus, primary spike complexes between the p62-E1 proteins are formed [89] In the trans-Golgi network, the last structural polyprotein precursor, p62, is cleaved into E3 and E2 [90]. This cleavage that is performed by a cellular furin protease [91] destabilizes the structure of the spikes. Once introduced into the virion membrane, the spikes can mediate the fusion between the virion membrane and endosomal membranes. This process is essential for infecting the next cell [92]. Subsequently, the mature glycoprotein complexes are inserted into the plasma membrane via exocytosis [93].

The formation of nucleocapsids that contain genomic RNA and capsid proteins, glycoprotein processing and transport occur simultaneously. Most alphaviruses, including SINV, have a conserved secondary RNA structure that acts as the packaging signal located in the nsP1 coding region [79,94]. The corresponding region in SFV and CHIKV does not have any effect on genome encapsidation [94]. Rather, the packaging signal for these viruses is represented by the conserved RNA secondary structure located in the nsP2 coding region [80]. Because the packaging signal resides in the ns-polyprotein coding region in both cases, the sg RNA is not included in the virions. C protein dimerization is promoted by RNA folding [95]. The packaging signal interacts with a 68 aa lysine and arginine-rich fragment residing in the N-terminal region of the capsid protein [79]. The resulting capsid protein-genomic RNA complex forms a T=4 nucleocapsid that moves to the cell membrane, where the capsid protein interacts with the long cytoplasmic tail of the E2 protein [96].

For SFV, the usual path of exit from infected vertebrate cells is budding from the plasma membrane [93]. The RNA-containing nucleocapsid needs to contact E2 directly to begin the budding process [96,97]. Virion exit from the infected cell has been shown to depend on cholesterol. Cells depleted of cho-

lesterol produce remarkably less viral particles than unmodified cells [98,99]. In addition, the interactions between E1/E2 and their transmembrane domains strongly affect budding efficiency [100].

Similar to the entry process, possible alternatives to this general exit pathway exist. Primarily, the formation of alphavirus virions in insect cells does not occur only on the plasma membrane. Instead, formation can occur on cytoplasmic membranes as well, and matured virions are then released by exocytosis [101]. A similar process may also occur in some vertebrate cells, such as neurons.

2.3 Individual nonstructural proteins

Although the primary role of the alphaviral ns-proteins is mediating RNA replication and transcription, a large fraction of the synthesized ns-proteins does not localize in the replication complexes. Instead, significant amounts of these proteins are dispersed throughout the entire cell. Each of these proteins has a distinct location, and these proteins conduct distinct functions apart from their roles in RNA replication [102]. Subcellular locations of the ns-proteins outside the replicase complexes are summarized in table 1.

Table 1. Subcellular locations of the ns-proteins in an infected cell

Protein	Known localization outside replicase complexes
nsP1	Filopodia-like extensions, plasma membrane
nsP2	Nucleus, diffusely in cytoplasm
nsP3	In punctate structures in cytoplasm
nsP4	In punctate structures in cytoplasm before rapid degradation

NsP1 of SFV consists of 537 aa residues and has a molecular mass of 64 kDa [103]. This protein has been shown to participate in negative-strand RNA synthesis [104–107]. NsP1 is also important for capping viral genomic and sg RNAs due to its guanine-7-methyltransferase [108] and guanylyltransferase activities [109,110]. Capping is vital for the virus because mutations that abolish the enzymatic activities required for the capping function render the corresponding RNA genomes non-infectious [111]. NsP1 is also thought to participate in sg RNA synthesis, as stable expression of nsP1 in a transgenic host cell has been shown to cause delayed accumulation of structural proteins [112].

NsP1 is the only ns-protein that interacts directly and strongly with cellular membranes [113,114], more specifically with membranous anionic phospholipids [115]. When expressed in a cell in the absence of other ns-proteins, nsP1 localizes to the plasma membrane, causing the induction of filopodia-like

structures void of F-actin [114,116]. Some quantities of the transmembrane protein CD44 and the plasma membrane protein ezrin have also been found on the filopodia in addition to nsP1 [116]. The filopodia-like structures are also characteristic of SFV-infected cells; however, the significance of these structures is not known. In contrast, when nsP1 is expressed in the form of uncleavable P123 or P1234 polyprotein, nsP1 directs (together with nsP3) the localization of the ns-polyprotein to cytoplasmic membranes [117].

The binding of nsP1 to membranes occurs in two steps: first, the N-terminal and central parts of nsP1 mediate binding to the plasma membrane [115,118], and second, the interaction is fortified by the palmitoylation of Cys residues located in the C-terminal part of the protein [118]. The first binding step is mediated by a stretch of staggered positively charged and hydrophobic aa residues with the sequence G^{245}STLYTESRKLLRSWHLPS^{263}V [115], which is organized into an amphipathic helix. The corresponding sequence is conserved among alphaviruses, and the binding that is mediated by this element is essential for viral replication [118,119]. Furthermore, similar sequences can be found in proteins homologous to nsP1 in other viruses belonging to the superfamily of alphavirus-like viruses [120].

The amphipathic membrane binding peptide in the middle portion of nsP1 is prone to forming interactions with negatively charged parts of the membrane phospholipids, such as phosphatidylserine [118]. A synthetic peptide with this sequence competes with nsP1 for binding to negatively charged phospholipids. The sequence also acts as a weak membrane localization signal because two or more copies of this peptide can direct enhanced green fluorescent protein (EGFP) to nonspecific membranous fractions in the cell [119]. Mutations in the conserved hydrophobic residues in the amphipathic helix not only affect the binding of nsP1 to liposomes and membranes but also strongly diminish the enzymatic activity of nsP1, especially the methyltransferase activity [115,120]. The introduction of point mutations into the amphipathic helix region often leads to the selection of secondary compensatory mutations located near or distant from the helix [119]. This finding supports the idea of a central role for the helix in mediating contacts between different portions of the replicase. However, as with many functions of alphavirus-encoded proteins, the requirement for membrane attachment of nsP1 appears not to be absolute. Thus, nsP1 from SINV is enzymatically active even in the absence of membranes [121].

The second binding step is mediated by the post-translational palmitoylation of cysteine residues 418-420 in the CC^{419}C sequence in nsP1 of SFV, and this modification strongly binds the protein to membranes [118,122]. The palmitoylated form of nsP1 causes the decomposition of actin stress fibers [116]. However, the palmitoylation step is not absolutely required for SFV. Mutant nsP1, in which the CC^{419}C sequence is replaced with non-palmitoylated Ala residues (AA^{419}A sequence), exhibits 40% of the guanylyltransferase and methyltransferase activities of wt nsP1 [118]. NsP1 that is not palmitoylated does not induce the formation of filopodia-like structures [118,122] and the mutant nsP1 tends to localize on cytoplasmic membranes. A virus carrying such a mu-

tation is also viable, although this virus has a lower growth rate than the wt virus [122] and is less pathogenic. This virus does not cause plaque formation in certain cell lines, and the virus cannot be found in the brains of infected mice even though it causes blood viremia [122]. However, the rescued virus does not represent the original mutant but is rather a mixture of viruses with different pseudoreversions (unpublished data from our research group). These second-site mutations arise rapidly for the palmitoylation-deficient SFV mutants, which have extremely low original infectivity [123]. The defect, which is responsible for the low infectivity and the selection of pseudoreversions, was due to the inability of the mutant protein to interact with nsP4. Thus, second-site compensatory mutations, which restore the nsP1:nsP4 interactions and, in some cases, the induction of filopodia-like structures, were selected [123]. This result agrees with several previously published co-immunoprecipitation analyses that demonstrated that nsP1 contacts nsP4 directly [33,124], and nsP3 probably indirectly with the aid of cellular proteins [102,117]. Thus far, neither direct nor indirect contacts between mature nsP1 and nsP2 have been described.

NsP2 in SFV consists of 798 aa residues and has a molecular mass of 86 kDa [103,125]. In SFV-infected cells, approximately half of the synthesized nsP2 is localized in the nucleus, mainly in the nucleolus [102,126]. This localization pattern is presumably due to the presence of a $PRR^{649}R$ sequence, which is thought to function as a nuclear localization signal (NLS). However, the activity of this signal also depends on the surrounding protein context. For nucleolar targeting of SFV nsP2, an additional sequence element located between aa residues 470-489 is necessary [127]. Mutations in the NLS that result in the cytoplasmic localization of nsP2 are not lethal to the virus. Such recombinant viruses replicate well in cell culture but are less pathogenic in mice. This reduced pathogenicity is likely due to the inability of such viruses to interfere sufficiently with host cell antiviral responses, most importantly with the expression and secretion of type I IFN [128]. In addition to the specific suppression of host antiviral gene expression, nsP2 in Old World alphaviruses also participates in shutting down host cell transcription in general. Therefore, nsP2 is considered to be the main determinant for the cytotoxicity caused by SFV and SINV [20,129]. NsP2 also causes the degradation of the catalytic subunit of mammalian (but not insect) RNA polymerase II [130].

The N-terminal half of nsP2 is an Mg^{2+}-dependent RNA triphosphatase [131] and nucleotide triphosphatase (NTPase) [132]. SFV nsP2 contains the universal NTP binding motifs $GVPGSGK^{192}S$ and $YVDEAF^{255}A$ [133,134]. The affinity of nsP2 to the RNA substrate is approximately 30-fold higher than to NTPs [131]. The full-length SFV nsP2 has also RNA helicase activity [135]. In contrast to the NTPase and RNA triphosphatase activities, both the N-terminal and C-terminal domains of nsP2 are required for the RNA helicase activity. Recent unpublished studies from our laboratory have shown that this indeed is the case.

The crystal structure of the C-terminal portion of nsP2 has been resolved for several alphaviruses, including VEEV [136]. The C-terminal region of nsP2

contains a papain-like protease domain and a methyltransferase-like domain. The latter is enzymatically inactive because some essential structural elements are missing. However, nsP2 is a well-known atypical Cys-protease that has His^{548} and Cys^{478} (numbering according to SFV nsP2) in its catalytic center [136,137]. The substitution of Cys^{478} with Ala results in an protease-inactive nsP2 [16], and ns-polyproteins containing this mutation have been used as substrates for nsP2 in several studies. Three other conserved amino acids in nsP2 coding region are Trp^{549}, Asn^{600} and Asn^{605}; mutations in these residues either inactivate the enzyme completely or significantly impair its activity. In addition, the Gly residue at the penultimate position upstream of the scissile bond of the cleavage site is absolutely required for nsP2 protease activity; replacement of this Gly residue with a Glu residue renders the corresponding substrate non-cleavable [138]. This Gly residue is conserved in all cleavage sites in SFV ns-polyproteins (See III, Fig. 2a for clarification).

The protease activity of nsP2 is resistant to some common inhibitors of Cys proteases but is sensitive to others. Its activity is dependent on some divalent cations: Cu^{2+} and Zn^{2+} inhibit protease activity completely; Co^{2+} and Ni^{2+} partially inhibit its activity; and Ca^{2+}, Mg^{2+}, and Mn^{2+} do not influence protease activity. Thus far, the only known substrates for nsP2 are the ns-polyproteins from the virus. A recombinant protein with the protease domain can cleave the ns-polyprotein between nsP3 and nsP4 (the 3/4 site; hereafter similar abbreviations are used for other cleavage sites) and cleaves the 1/2 site less efficiently [139,140]. These cleavages are independent of the free N-terminus of nsP2 and can also be performed by the nsP2-containing polyprotein [139,140]. In contrast, the cleavage of the 2/3 site can be performed only by nsP2 with a free and native N-terminus [141]. These results and previous mutational analyses suggest that interdomain interactions occur between the C-terminal protease and the N-terminal domain of nsP2 [139,142].

NsP3 in SFV consists of 482 aa residues and has a molecular mass of 61 kDa [103]. The protein is essential for synthesizing RNA with negative [104,114] and positive polarity [114]. NsP3 is the only ns-protein that is phosphorylated in infected mammalian cells [143]. When expressed alone, nsP3 is dispersed in punctate granules throughout the cytoplasm of the cell. In immunofluorescence microscopy, these granulated structures appear similar to the virus replication sites (CPVs), but the structures lack the fine ultrastructural characteristics of CPVs and do not co-localize with lysosomal membranes [117,144]. Thus, nsP3 is not the only factor required for the formation of CPVs that are required for RNA replication. However, the nsP3 part of the P123 polyprotein directs the future viral components of the replication complex to the endosomal membranes in mammalian cells [117].

The N-terminal one third of nsP3 is a macro-domain that is conserved among alphaviruses, coronaviruses and rubella virus [145]. The three-dimensional structure of this domain has been resolved for many alphaviruses. The macro-domain of nsP3 in CHIKV and VEEV has been shown to bind ADP ribose [146]. However, this property is not conserved because the same domain

from SFV and SINV does not bind ADP ribose. Instead, this domain can bind poly-ADP ribose and poly(ADP) [147], indicating its likely role in RNA binding. The N-terminal macrodomain of nsP3 functions, albeit very poorly in SFV, as a phosphatase for ADP-ribose 1-phosphate [146,148]. The significance of this activity for the virus is not known.

The middle portion of nsP3 is conserved only among alphaviruses. The three-dimensional structure of this region is only known for SINV [149]; however, the sequence similarity between these domains of different alphaviruses suggests that the fold is conserved for all of them. The most prominent function of this domain is binding Zn^{2+}, and this function is crucial for virus infectivity [149].

The C-terminal portion of nsP3 is highly variable both in sequence and in length. Nevertheless, for all known alphaviruses, this region contains Ser and Thr residues that can be phosphorylated [143]. NsP3 has no kinase activities; therefore, this region must be phosphorylated by cellular kinases. Viruses with mutated nsP3 that cannot be phosphorylated are viable but less pathogenic in mice [150]. NsP3 is degraded quickly in the cell when not stabilized by other components of the replicase complex; this degradation occurs due to the presence of a specific degradation signal in the C-terminus. If the degradation signal with residues immediately upstream are removed, an individually expressed nsP3 becomes stable and localizes to filamentous stretches inside the cytoplasm [144]. In contrast to the first domains of nsP3, the C-terminal domain lacks a definite three-dimensional structure. Numerous studies have indicated the role of this domain in interactions with several host proteins, including G3PB (Ras-GAP Src-homology 3 domain binding proteins) [151] and amphiphysins [152].

NsP4 is the catalytic subunit of the alphavirus replicase [153]. In SFV, nsP4 consists of 614 aa residues and has a molecular mass of 68 kDa [103]. On its own, nsP4 is the most unstable ns-protein of alphaviruses [103]; nsP4 is rapidly degraded by the N-end rule [154]. The reason for this degradation is the N-terminal Tyr residue in nsP4. This residue is essential for the viability of the virus; a non-aromatic residue at this position severely impairs replication [155].

When expressed alone in a cell, nsP4 stabilized by the presence of a non-native N-terminal Met residue is distributed in the cytoplasm in a punctuate manner [102]. Whether this localization occurs for native nsP4 is unclear because the protein is too unstable to be detected (unpublished data from our laboratory). In some strains of SFV and most other alphaviruses (including SINV), nsP4 is produced by the read-through of an opal termination codon located near the 3' end of the nsP3 encoding sequence [156]. Even if other termination codons are inserted in place of opal, nsP4 is produced [157]. This result is due to the specific RNA sequence located near the read-through codon [158]. This mechanism, together with the rapid degradation of individual nsP4, ensures that the amounts of nsP4 in infected cells are much lower than any other ns-protein. In contrast, most SFV strains have an arginine codon at the position of the termination codon, and the amount of expressed nsP4 is equal to other ns-proteins

[133]; however, nsP4 that is not included in formed replicase complexes is rapidly degraded.

In vitro, nsP4 possesses *de novo* RNA synthesis activity [153] and terminal nucleotide transferase activity [159] with a strong preference for adding adenosine nucleotides [160]. Together with the finding that the site of negative-strand RNA synthesis initiation is located at last C-nucleotide in CSE4 [161], this result indicates that alphaviruses do not use a poly(U) template for synthesizing the poly(A) tails of positive-strand RNAs; instead, such tails are synthesized by the terminal transferase activity of nsP4.

NsP4 has a relatively long N-terminal domain that is conserved among alphaviruses but lacks homology with any other known protein sequence [153]. This sequence is likely involved in interactions with other ns-proteins, including nsP1, and host factors [33,124]. To synthesize RNA of negative polarity, nsP4 with this N-terminal region and the presence of non-processed P123 are required. The synthesis of positive-strands depends on other factors; nsP4 alone is capable of synthesizing genomic RNAs, while nsP2 is also required for sg RNAs [153].

2.4 The replicase complex: membrane binding, replication and transcription.

The expression of four ns-proteins as individual proteins in the same cell cannot lead to successful replication complex formation. However, when the ns-proteins are expressed in the form of P1234 (or P123 plus nsP4 with a native N-terminus), the formation of replicase complexes and RNA replication is observed if a suitable template is provided [162,163]. This requirement implies that the formation of replicase complexes depends on viral RNAs, the interactions between ns-polyproteins and their cleavage process and cellular components (such as membranes).

2.4.1 Formation of replicase complexes (spherule) and replication organelles (CPV).

The replication of alphaviruses in mammalian cells occurs in association with cellular endosomal membranes, where small membranous invaginations, called spherules (approximately 50 nm in diameter), are formed [71,102]; [164]. Spherules are located at the outer membrane of large vesicles (up to 2 μm in diameter); these virus replication organelles are also called cytopathic vacuoles type I (CPV). CPVs contain multiple spherules, each of which contains all replicase proteins and viral RNA [114]. Thus, a spherule represents the replication complex of alphaviruses. The inner volume of each spherule is connected to the cytoplasm with an electron-dense structure resembling a neck [102,117,165]. The neck is thought to be the route that newly synthesized viral RNAs use to

enter the cytoplasm and the route through which NTP substrates are transported to the spherule.

RNA synthesis coincides with spherule formation at the plasma membrane, and no spherule formation can be observed in the absence of RNA replication [162,163]. Though the spherules are initially formed at plasma membranes, the spherule-containing membranes are subsequently internalized with the aid of phosphatidylinositol 3-kinase and the actin network to form neutral vesicles that undergo short, fast and multidirectional movements. Larger vesicles that are formed by the fusion of vesicles with more acidic compartments use microtubules to move long distances. Ultimately, CPVs are formed, which tend to localize around the nucleus in SFV-infected cells [166]. For these processes, the coordinated action of several ns-polyproteins and nsPs are needed. NsP1 anchors the replication complex to membranes [115,118,119], while nsP3 is important for transport to the lysosomal compartment [117]. The spherule-containing structures can also move and merge within a cell and bud and invaginate from the plasma membrane [102].

2.4.2 Replication and transcription

Viral genomic RNA is the matrix for the translation of ns-polyproteins [81,103,125], which are gradually cleaved by nsP2 [16]. First, the early replicase synthesizes the negative-strand of RNA. This strand is used as a template by the late replicase, which synthesizes new genomic and sg RNAs [14,18,167]. The conversion from the early to late replication complex is mediated by the sequential cleavage of ns-polyproteins and their acquired conformations. The combination of P123 and nsP4 preferably synthesizes negative-strand RNA (early replicase), while the late replicase complex that consists of fully cleaved ns-proteins synthesizes positive-strand RNA [14,103]. The artificially stabilized cleavage intermediate that consists of nsP1, nsP4 and P23 is capable of synthesizing RNA strands with both positive and negative polarity [14].

The negative-strand RNA lacks a poly(U) tract in its 5’ end, although the corresponding positive-strands contain a 3’ poly(A) sequence [70,161]. Similarly, negative-strand RNA lacks the 5’ cap structure but has one extra, non-templated G residue at the 3’ end [168]. Analysis of RNA synthesis intermediates has revealed that the negative-strand does not exist as a free ssRNA molecule; instead, the negative-strand forms a duplex with the positive-strand of RNA [169,170]. Several forms of dsRNAs, designated replication intermediates, can be purified from alphavirus-infected cells. The synthesis of negative-strand RNA generally stops approximately 4 h post-infection [19]; however, continuous negative-strand RNA synthesis is observed in certain mutant viruses carrying mutations in the nsP2 coding region [170,171]. As with all alphavirus RNAs, the synthesis of negative-strand RNA also depends on nsP4; mutations in the nsP4 coding region may render a virus temperature-sensitive, which means that the virus has a reduced negative-strand RNA synthesis rate and im-

paired virion release at the restrictive temperature [172]. Defects in alphavirus negative-strand RNA synthesis can also arise from mutations in the nsP3 coding region [107,173] or nsP1 portion [106]; the latter is likely due to impaired interactions between nsP1 and nsP4 [33,124]. Thus, the type of RNA synthesized in an alphavirus-infected cell depends on the number of scissile bonds that have been cleaved in a polyprotein and on the presence of mutations in several replicase protein coding sequences. Furthermore, mutations that modify the RNA synthesis ability of an early or late replicase have been described. First, SINV [174] and SFV (III) can synthesize positive-strand RNAs even if P123 cannot be processed; only one (SINV) or two (SFV) point mutations in the ns-polyprotein coding regions are required to re-gain this activity. Second, several temperature-sensitive mutants of SFV and SINV can re-activate negative-strand RNA synthesis at the restrictive temperature [175,176]. These findings suggest that ns-polyprotein processing does not have a decisive role in determining strand synthesis specificity; other factors may have equally or more important roles.

NsP2 is the enzyme that performs the gradual cleavage of the ns-polyprotein. To achieve the formation of a functional replicase, the processing of the ns-polyprotein must be highly regulated. First, the rapid cleavage of the 3/4 site occurs, most likely *in cis* (Fig. 3). This step is followed by a much slower cleavage of the 1/2 site, which only occurs *in cis* (except in the presence of a large excess of free nsP2 in a test-tube reaction). The final step of the ns-polyprotein processing is the extremely fast cleavage of the 2/3 site *in trans*. The latter occurs only when the N-terminal part of nsP2 has been released [16,138]. The main determinants for the 3/4 site cleavage are 4 aa residues preceding (P-side) and one aa residue following (P'-side) the scissile bond [15]. The 2/3 site cleavage is driven by the correct macromolecular assembly rather than by the sequence of the cleavage site; both the macro-domain of nsP3 and the N-terminal portion of nsP2 are responsible for placing the scissile bond correctly in the cleavage pocket [141]. As the amount of free nsP2 increases late in the infection, the cleavage of P1234 at the 2/3 site dominates over other polyprotein cleavages [177], resulting in the cessation of early replicase complex formation.

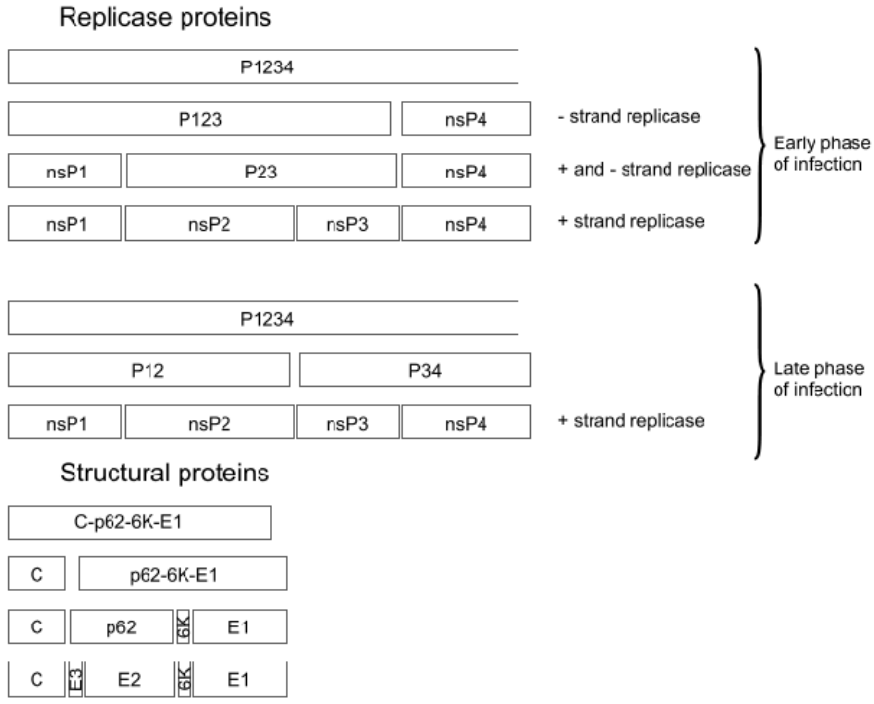

Figure 3. Schematic representation of cleavages of ns- and structural polyproteins. The ns-polyprotein P1234 is cleaved differently during the early and late phases of infection. During the early phase, processing begins with the release of nsP4 from P1234; this cleavage is performed *in cis* and results in the P123 + nsP4 complex that can synthesize negative-strand RNA. The subsequent *in cis* cleavage between nsP1 and P23 is followed by a rapid *trans* cleavage of the site between nsP2 and nsP3. These cleavages convert early replicase to the late replicase, which loses its ability to synthesize RNA with negative polarity but can synthesize positive-strand genomes and sg RNAs. During the later phase of infection, free nsP2 is abundantly present in the cytoplasm of the infected cell. This protein can cleave P1234 *in trans* at the cleavage site between nsP2 and nsP3, resulting in the presence of P12 and P34. These polyproteins can neither form replicase complexes nor synthesize RNA of any polarity; therefore, the formation of new replicase complexes is halted. P12 and P34 are processed into mature ns-proteins, which have specific functions not directly related to viral RNA synthesis.

The C protein is released from the structural polyprotein by autoproteolysis, which occurs immediately after this part of the polyprotein is synthesized. P62, 6K, trans-frame protein (not shown in the figure) and E1 are released by cleavages performed by cellular proteases; these cleavages occur during the translocation of the corresponding part of the polyprotein through the endoplasmic reticulum. p62 is cleaved into E3 and E2 during the transport from the endoplasmic reticulum to the plasma membrane.

Alphavirus replication complexes are formed only if nsP1, nsP2 and nsP3 are expressed in the form of the polyprotein P123(4) [178]. The forming replicase complex can engage any RNA with the correct CSEs. During native infection, the involved RNA is most often the RNA genome that was used for the translation of the ns-polyprotein (template present *in cis*); however, templates

presented *in trans*, such as DI RNA or helper RNA, can be used to trigger spherule formation and start replication. The size of spherules and the length of RNA engaged in replication are directly correlated [179].

Alphaviral sg RNA is transcribed using the negative-strand RNA as a template [86,156], and it corresponds to the last one third of the genome [180]. The synthesis of sg RNA depends on nsP2 [181], which directs the polymerase toward sg RNA synthesis when bound to the negative-strand RNA [169]. In infections with some temperature-sensitive mutants, the mutant nsP2 detaches from the template at the restrictive condition. This detachment results in the cessation of sg RNA synthesis, but the synthesis can be restarted when cells are returned to the permissive temperature [169]. Similarly, some mutations in the nsP2 coding region render the virus temperature-sensitive due to a decrease in the amount of sg RNA synthesized under restrictive conditions [182].

For several alphaviruses, a 21-nt CSE2 has been identified as a major part of a minimal promoter for sg RNA synthesis; the promoter is generally 24 nt (from -19 to +5) long [75]. This sequence exhibits lower activity in directing sg RNA synthesis than the complete promoter, which is considerably longer [183]. This difference may be due to the presence of several enhancing sequences in the complete promoter. Such elements may contribute to the efficient transcription of the sg RNA in mammalian and mosquito cell lines [184]. The length of the minimal sg promoter is not identical among different alphaviruses. To achieve sg RNA synthesis, the SFV sg promoter needs to be longer, spanning from 26 nt upstream to 20 nt downstream of the starting site of sg RNA synthesis [185]. The differences in sg promoter length are thought to be a consequence of the G residue at the -1 position of the SFV sg promoter; in SINV and most other alphaviruses, this position is occupied by an U residue [186].

2.5 Structural proteins and their expression

SFV structural proteins are translated as a single C-E3-E2-6K-E1 polyprotein from the sg RNA. The E3-E2 part of the polyprotein has the longest half-life and is also called p62 according to its molecular mass [81,86,125,187]. The translation of structural proteins is activated by a translation enhancer, which is an element encoded by the first 102 nt of the capsid protein-encoding sequence [78]. The enhancer is functional only in wt SFV-infected vertebrate cells [188].

After the cleavage of the C protein from the N-terminal part of the structural polyprotein, the N-terminus of p62 is released [83] and functions as a membrane translocation signal. The binding to membranes is required for correct folding, processing and modification of the remaining structural proteins. Alphaviruses encode three glycoproteins: E3, E2 and E1 [189]. Several translocation signals are required for the correct folding of the glycoproteins into the membranes. First, the N-terminal part of p62 begins the translocation into the endoplasmic reticulum membranes, where p62 becomes glycosylated [83]. Second, the C-terminal segment of p62 is required to initiate 6K and E1 translo-

cation [84]. Third, the hydrophobic peptide in the C-terminus of the 6K protein is required for stopping and anchoring 6K and E1 protein translocation [85]. The cleavage events between p62/6K and 6K/E1 are likely performed by a cellular enzyme signal peptidase [84,86]. As indicated above, p62 is cleaved at the end of the viral structural protein maturation process. p62 forms complexes with E1, and the final cleavage occurs just before these complexes arrive at the plasma membrane [90]. The structure of the carbohydrate component of all the glycoproteins is strongly dependent on the host. SINV virions formed in mosquito cells have mannose sugars added to the glycoproteins, whereas several glycans are preferred for the same virions formed in vertebrate cells [190,191].

Capsid protein (C; 33 kDa) is the only structural protein with enzymatic activity. Capsid protein is an autoprotease that removes itself from the structural polyprotein [82]. The protease domain found in the highly conserved C-terminus of the capsid protein [192] shares homology with the conserved sequences of serine proteases [193]. The C-terminal part of the capsid protein also binds to the cytoplasmic region of E2 [44], while its N-terminal region, which is rich in positively charged aa residues, binds viral RNA [79]. In this manner, the capsid protein participates in the formation of a two-layered protection for the virus genome.

E3 is an 11 kDa glycoprotein [189,194] that mediates the proper folding of p62 [83]. E3 remains peripherally associated with the major spike proteins [195] and modulates particle assembly and spike activation for viral entry [196]. p62:E1 complexes are stable at a low pH, which is important for viral protein transport through the acidic cellular compartments. However, if the cleavage of p62 is blocked, highly stable virions that are also insensitive to a low pH are formed and the effectiveness of the viral infection is impaired [92].

E2 is an approximately 51 kDa transmembrane glycoprotein [189] that is also palmitoylated. This modification is essential for the efficient budding of the virions [197]. E2 assists the correct localization of other glycoproteins into the membrane [83] and is responsible for binding mature virions to cellular receptors during the early steps of infection. The binding is mediated by the tip of E2 in its trimeric conformation [52]. E2 also contains the major epitopes to which neutralizing antibodies bind [51].

6K is a 60 aa residue-long hydrophobic transmembrane protein [84]. 6K can be translated in two forms: the shorter 6 kDa protein and the recently discovered longer trans-frame 8 kDa protein (synthesized via ribosomal frame shifting occurring with 10-18% effectiveness). The trans-frame protein is more abundant in SFV virions [198], although to a lower extent than glycoproteins. Both proteins are acylated [199], and the trans-frame protein is more heavily palmitoylated than the shorter form of the protein [198].

Although present in virions, the exact function of the protein is not known; deletion of 6K and/or its trans-frame part of the protein decreases virus release but does not interfere with spike protein complex formation or with transport to the cell membrane [187]. The signal sequence present in the C-terminus of the alphaviral 6K protein contributes to the correct localization of the E1 protein

[85] and enhances the budding process [200]. In addition, the protein can form ion channels [201], which is thought to be a contributing factor to the budding process. However, both the 6K and trans-frame proteins are essential for alphavirus pathogenesis *in vivo* [202].

E1 is an approximately 50 kDa transmembrane glycoprotein [189] that is palmitoylated [203]. Palmitoylation of E1 contributes to the stability of the budding virions [204]. E1 is responsible for the acid-induced fusion of lysosomal and virion membranes during virus entry [60,62,205]. The E1 resident peptide is also proposed to have a role in the formation of ion-permeable pores, which are important for the acidification of cytoplasmic vacuoles and, thus, the infection process [206].

2.6 Alphaviruses in mosquito cells

Mosquitoes are the arthropod vectors for most alphaviruses. In general, the infection in mosquito cells and especially living mosquitoes is much less understood than the infection in vertebrate cells and organisms. Several factors are common for the replication of alphaviruses in both mosquito and vertebrate cells. One of these factors is the requirement for cholesterol in the membranes of the infected cells. Mosquito cells depleted of cholesterol are resistant to SFV infection, a defect that has been attributed to the inhibition of membrane fusion events [207]. The dependence on cholesterol has also been demonstrated on virion exit stages [99]. Other factors that are similar between the infection processes in different hosts include cytoplasmic replication and the order of the processes associated with RNA replication.

As described previously in the above sections, a number of important differences can be found between infections in different host cells. For example, virion budding can occur on both the plasma membrane and cytoplasmic vesicles in mosquito cells [101]. Virus survival in mosquito cells depends on the native sequences and functions of the viral ns-proteins and CSEs. In general, mutations in these regions affect virus fitness in the mosquito more severely than in vertebrate cells, although examples of the opposite are also known. Thus, certain changes in the viral 3' UTR or nsP4 coding sequences affect viral RNA release in mosquito cells to a greater extent than in their vertebrate counterparts [30,208]. However, the majority of known differences between infections in vertebrate and insect hosts are related to virus-host interactions.

Much of the research on alphaviruses and their vectors has been conducted using cell lines derived from mosquito larvae. Furthermore, alphaviruses also infect fruit fly cells (*Drosophila melanogaster*) reasonably well, and this model organism can be used for *in vivo* infections as well. Depending on the cell line, significant morphological changes and cytopathic effects can appear during the infection, but eventually all insect cell lines exhibit a cured phenotype [101]. The typical infection can be described as follows. The initial acute phase of replication is characterized by the infection of all the cells in the cell culture (at

least if a high MOI is used) and a high level of viral progeny production. However, this phase is followed by a persistent phase, and only approximately 2% of cells remain infected at 48 hours post-infection. The persistent phase is associated with a sharp decrease in virion production [26]. The low levels of extracellular virus production are essential for maintaining persistent infection [209] because a constant re-infection of cured cells by the virus emerging from infected cells occurs during this phase.

The molecular mechanisms for generating persistent infection in mosquitoes are not fully understood. Some evidence suggests that the persistent infection is regulated by the inhibition of negative-strand RNA synthesis, which itself results from changes in the ns-polyprotein processing pattern [167]. In cell clones with fewer virus-induced cytopathic effects, less RNA with negative polarity is synthesized [28]. However, recent studies have shown a leading role of the general immune signaling pathways, RNA interference (RNAi) [29] and PIWI-interacting RNAs (piRNAs) [210] in controlling the alphavirus infection in mosquitoes.

The activation of some innate immune signaling pathways in mosquito cells before infecting them with SFV reduces subsequent virus replication. However, the viral infection itself does not activate these pathways. Despite the inability to interfere with the already activated immune signaling pathways, the virus can reduce cellular gene expression in mosquito cells and, while doing so, likely suppress gene activation before an immune response can be established [211].

In vertebrates, dsRNA often triggers the induction of antiviral genes (for example, type I interferons); dsRNA may also trigger the production of small interfering RNAs (siRNA) [212]. In vertebrate cells, the existence and role of siRNAs that are generated in response to alphavirus infection are unclear. In contrast, such siRNAs are used in the RNAi pathway in both mosquito cells and mosquitoes [213,214]. For alphaviruses, the dsRNA replication intermediate is used for generating siRNAs, confirming the historical finding that the emergence of dsRNAs in an alphavirus infection can be detected in insect cells [32]. Furthermore, using massive parallel sequencing, it has been revealed that some sequences in the viral genome are more frequently present in siRNAs than others. However, these so-called “hot-spot” siRNAs are less potent in inducing the silencing of virus replication than less abundant (“cold spot”) derived siRNAs [215]. Thus, the virus appears to allow the host defense systems to react against the sequences that counteract replication to a smaller extent, whereas sequences with a vast influence on replication are thereby protected.

Gap junctions are present between individual cells in mosquito cell culture [216]. These junctions allow the spread of siRNAs from infected cells to uninfected neighboring cells. Such cell-to-cell spreading of the RNAi response suppresses SFV infection in mosquito cell cultures because the virus is unable to reduce this effect. However, if a viral suppressor of RNAi from another virus is expressed in these cells, SFV replication and particularly its spread in cell cultures is enhanced [217]. Most likely, a similar effect also occurs in infected mosquitoes.

piRNAs regulate RNA silencing in the cell [218]. This mechanism was discovered in testis cells from the fruit fly and is thought to offer protection from transposable elements, thus ensuring genome stability [31,219]. In contrast to siRNA-induced gene silencing, the piRNA pathway is independent of Dicer-mediated production from the dsRNA [212,218]. PIWI proteins are found in germ cells and are thought to reduce vertical virus transmission [210]. In mosquitoes, PIWI-like proteins have also been found in somatic cells [220]. Research on CHIKV suggests that the inducer of the piRNA pathway may be dsRNA, similar to the siRNA pathway [220]. However, other evidence suggests that ssRNA is required for the activation of the piRNA pathway in SFV-infected cells [210]. Regardless of the mechanism of induction, SFV infection can trigger piRNA production in mosquito cells, and virus production is enhanced if the expression of piRNA pathway proteins is knocked down [210].

2.7 Alphaviral vectors

Alphavirus vectors can be potentially used for vaccine construction, central nervous system disease therapy and anti-cancer therapy. These vectors are characterized by their high biosafety, easy manipulation techniques and broad host range. Thus far, primarily animal models and alphavirus replicon vectors have been tested for such applications [221]. The use of replicon vectors is facilitated by the fact that foreign sequences of interest with lengths up to 9 kb can be inserted into a vector without affecting the formation of virus-replicon particles (VRPs) [222].

Alphavirus vectors can be used for the transient expression of foreign proteins [221]. The highest expression levels can be achieved when the sequence of a translation enhancer is fused to the 5' end of the coding sequence of the foreign protein [188]. These vector systems have been developed for several alphaviruses, including SFV [187], SINV [223,224] and others. Because an infection by replicon vectors of Old World alphaviruses is cytotoxic to the vertebrate host, a long-term and persistent replication can only be achieved when mutations in the viral part of the vector render it non-cytopathic [223,225].

Three different approaches are used for the transient use of alphavirus-based systems: VRPs, layered DNA-RNA vectors and replication-competent vectors [221]. The VRPs are designed to undergo a single round of replication because sequences coding for structural proteins are not included in the particle [8]. Layered vectors represent plasmids in which production of the replicon RNA is achieved by a cytomegalovirus early promoter placed upstream of a replicon cDNA. The primary problem with layered DNA-RNA vectors is their low transfection efficiency when compared to infection with VRPs. However, the immune responses obtained using this type of vector are much higher than those from DNA-based vaccines [226]. Replication-competent vectors express the transgene(s) in addition to viral genes, either via a duplicated sg promoter [185],

via an inserted internal ribosomal entry site (IRES) [227] or due to the insertion of the transgene sequence into the viral polyprotein coding regions [228,229].

In a replicon-VRP system, the structural polyprotein coding region is substituted with the sequence of interest. The packaging of these RNAs is achieved by their co-transfection with a helper RNA, which is capable of replication and encodes structural proteins [8]. However, only the replicon RNA is packaged into the particle because the signal required for encapsidation lies within the ns-polyprotein coding region [79,80]. The probability of recombination between replicons and helper RNA represents a significant concern because this event may lead to the formation of an infectious virus. To diminish the recombination risk, split-helper systems have been developed. In this case, the regions encoding structural proteins are provided from two different helpers [230]. In addition, mutations that attenuate the virus should the recombination event take place can also be inserted into the vector backbone [231]. Alternatively, the replication of the recombinant virus, resulting from a recombination event, can be blocked by the introduction of target sequences of cellular miRNAs [232].

For replication-competent alphavirus vectors, several additional issues need to be considered. The duplicated sg promoter and sequences expressed using this element are frequently removed from the vector genome because these sequences provide no selective advantage for the virus and instead slow down virus multiplication [185]. Other approaches that can solve or release the instability issue have been proposed. One possibility is inserting an IRES element instead of an additional copy of the sg promoter. Unfortunately, this solution does not work equally well for all alphaviruses [185], and other adaptive mutations that ensure efficient replication emerge (Volkova et al., 2008). Thus, the outcome of IRES induction cannot be predicted easily. Inserting foreign sequences into viral polyprotein coding regions also results in more stable constructs. However, this technique is also the most labor-consuming approach because viral proteins that are vital to the virus are affected by transgene insertion. To find the suitable insertion sites and develop insertion strategies, random mutant libraries have been screened [233]. Alternatively, an insertion strategy may be based on the data obtained for the processing of ns [228] or structural polyproteins [229].

3 RESULTS AND DISCUSSION

This PhD thesis summarizes research aiming to analyze the functions of the SFV replicase protein nsP1 during infection in vertebrate and insect cells. The importance of the release of nsP1 from the ns-polyprotein and the requirements for the processing of the 1/2 cleavage site are also analyzed.

The aims of this study were as follows:

1. to assess the influence of the defect in nsP1 palmitoylation on SFV infection in mammalian cells;
2. to compare the effects observed for palmitoylation-deficient mutants in mammalian cells to those revealed in insect cells;
3. to reveal the requirements for the release of nsP1 from the polyprotein precursor; and
4. to analyze the importance of nsP1 release on virus replication and the impact of this event on the antiviral innate immune response.

3.1 NSP1 palmitoylation is dispensable for SFV replication (I)

3.1.1 Design of palmitoylation-deficient SFVs

It has previously been reported that nsP1 palmitoylation is not essential for SFV viability [122]. In contrast to these results, SFV replicon vectors harboring corresponding mutations in the nsP1 coding region were characterized by extremely low VRP production, indicating the presence of a serious replication defect. These data were in conflict with the previous report, and therefore, the effects of nsP1 palmitoylation on SFV viability were analyzed in a detailed manner. Three different mutants were constructed on the basis of an infectious pSP6-SFV4 clone [187] (I, Fig. 1). In SFV4mut3A, the three Cys residues in positions 418-420 in nsP1 were substituted with Ala residues; the corresponding residues were deleted in SFV4Δ3; and a large deletion including these Cys residues and four aa residues preceding the normally palmitoylated cysteine residues was generated in SFV4Δ7. The same mutations were also inserted into a SFV1 replicon [8], which also contained the EGFP coding sequences under the control of a sg promoter, and into an expression plasmid encoding individual nsP1.

Transfection of BHK-21 cells with obtained replicons confirmed that less than 1% of cells transfected with transcripts of SFV1mut3A/d1EGFP or SFV1Δ3/d1EGFP expressed EGFP; in contrast, more than 90% of cells transfected with the wt replicon transcripts were EGFP-positive. Thus, these mutations in the palmitoylation site caused serious replication defect(s). Interestingly, however, the results for SFV1Δ7/d1EGFP were different; this mutation had only a moderate effect on replication as 40% of the cells transfected with

the corresponding transcripts expressed EGFP (I, Fig. 1, table). Therefore, it was hypothesized that the palmitoylation of nsP1 *per se* was not required for SFV infectivity and that the replication of SFV1mut3A/d1EGFP or SFV1Δ3/d1EGFP was hampered due to some other defects caused by these (but not by Δ7) mutations.

3.1.2 Characterization of palmitoylation-deficient SFVs

An infectious center assay and growth curve experiments were used to assess the effect of diminished replication on virus viability and growth. Compared to wt SFV4, the infectivity of the SFV4mut3A and SFV4Δ3 genomes was diminished more than 20,000-fold (I, Fig. 1, table). The drastic decrease in infectivity resulted in a delayed release of progeny virions and, as a result, the yield of mutant viruses was considerably lower than that of SFV4 (I, Fig. 2a). Similarly, the infectivity of SFV4Δ7 was diminished, but to a much lesser extent, approximately 40-fold, and the decrease in virus release was less pronounced (I, Fig. 1 table, Fig. 2a). Upon transient expression, none of the mutant nsP1s were palmitoylated (Fig. 4b). Taken together, these results confirm that only a moderate effect on replication can be directly attributed to the lack of nsP1 palmitoylation. Hence, the serious defect in infectivity of SFV4mut3A and SFV4Δ3 must have another cause. The guanylyltransferase activities of the mutant nsP1s did not differ considerably from wt nsP1 (I, Fig. 4a), indicating that the synthesis of cap structures was not affected by the mutations. Therefore, the observed defect in infectivity of SFV4mut3A and SFV4Δ3 cannot be attributed to the known enzymatic functions of nsP1 and lies elsewhere.

The analysis of the subcellular localization of transiently expressed mutant nsP1 proteins in HeLa cells revealed that the wt nsP1 was localized mainly to the plasma membrane and caused an extensive induction of filopodia-like extensions, in agreement with previous reports. In contrast, nsP1-Δ3, nsP1-Δ7 and nsP1-mut3A did not induce the formation of filopodia-like extensions. Furthermore, the intracellular localization of nsP1-Δ3 and nsP1-mut3A was clearly altered, as these proteins were not located solely on the plasma membranes but were also found in the cytoplasm of the transfected cells (I, Fig. 5a-d).

Next, it was investigated whether the progeny viruses obtained from the cells transfected with mutant genomes were also impaired in their ability to grow in BHK-21 cells. It turned out that the differences between the mutant and wt viruses had disappeared (I, Fig. 2b). This data clearly indicates that during propagation in transfected cells, the fitness of the mutant viruses was largely restored. The most obvious reason for this result could be the reversion of mutated sequences. However, sequencing of mutant virus stocks revealed that a reversion did not occur. Therefore, the increase in fitness must be attributed to some other changes, most likely to the creation of second-site compensatory mutations.

3.1.3 Identification and functional significance of compensatory mutations

In general, RNA viruses tend to have multiple ways to compensate for any specific defect. Occasionally, one of these changes is more favorable than other changes. Therefore, one change quickly becomes dominant and can be revealed by sequencing viral stocks. In other cases, none of the multiple changes is frequent enough to be detected using this approach, and massive parallel sequencing is required. However, at the time of this study (2005), such methods were not available. Therefore, to circumvent the problem of the possible mixed nature of the original stocks, plaque purification was used. Four isolates from each mutant stock were obtained and analyzed for the presence of possible second-site mutations in the ns-region.

This analysis revealed three independent single aa changes (P181Q, L234F and Q357L; hereafter referred to as mut3A-1, 3A-2 and 3A-3, respectively) for the isolates purified from the primary stocks of SFV4mut3A and two double mutations (M124V+A197D and ΔG224+T352S; hereafter referred to as Δ3-1 and Δ3-2, respectively) for the isolates purified from the primary stocks of SFV4Δ3. All these mutations were located in the nsP1 coding region (I, Table 1). In addition, two single aa changes in the nsP4 coding region, E127V and L583I, were revealed for the isolates purified from the primary stocks of SFV4Δ3 (unpublished results). All the analyzed viruses contained at least some of these mutations, strongly suggesting that these changes may be responsible for the increased fitness of the mutant viruses. In contrast, no second-site mutations were discovered in the isolates of SFV4Δ7. Therefore, the sufficiently high infectivity of SFV4Δ7 likely prevented the fixation of a certain pseudoreversion because the growth advantage of such genotypes over SFV4Δ7 was not significant.

The identified mutations were subsequently introduced into SFV4mut3A and SFV4Δ3, and the infectivities of the obtained constructs were assayed. The introduction of the compensatory changes found in nsP1 restored the infectivity of the mutant genomes to the levels similar to wt SFV4 (I, Table 1). In addition, no delays in virus release were observed, and only a minor diminution in the amounts of the released virions could be detected (I, Fig. 3). Thus, the introduced compensatory changes indeed restored the fitness of the viral genomes encoding the palmitoylation-deficient nsP1. Furthermore, when the L234F mutation, which was found in the SFV4mut3A background, was introduced into the SFV4Δ3 and SFV4Δ7 clones, the mutation restored the infectivity of SFV4Δ3 but did not increase the infectivity of SFV4Δ7 (I, Table 2). This finding suggests that the Δ3 and mut3A mutations have a similar effect on nsP1, whereas the Δ7 mutation causes a different defect.

In contrast to the compensatory mutations found in nsP1, the second-site mutations found in the nsP4 coding region failed to rescue the infectivity of SFV4Δ3 or SFV4mut3A (unpublished). This result indicates that the changes in

nsP4 may function together with some other mutations, for example, mutations that are located in the 5' UTR or 3' UTR, which were not sequenced. Nevertheless, the emergence of second-site mutations in the nsP4 coding region was highly coherent with previous reports that these two proteins interact with each other [102,117]. Therefore, compensatory changes in the nsP4 coding region that are capable of restoring the defect caused by mutations in the nsP1 coding region may exist. Massive parallel sequencing techniques could provide an insight into this issue.

The emergence of mutations in the nsP4 coding region (though without a prominent effect) implied that the primary defect caused by the Δ3 and mut3A mutations, but not the Δ7 mutation, may be the disruption of interactions between nsP1 and nsP4. The revealed second-site mutations in the nsP1 coding region may serve to restore the nsP1:nsP4 interaction. To test this possibility, nsP4 was co-expressed with different nsP1 mutants, and the interaction between these two proteins was analyzed using immunoprecipitation. This assay revealed that the Δ3 and mut3A mutations indeed resulted in the loss of an interaction with nsP4 and that this interaction was restored by all of the second-site mutations identified in the nsP1 coding region (I, Fig. 6). In contrast, none of these compensatory mutations restored the palmitoylation of nsP1 (I, Fig. 4b) or had a positive effect on the guanylyltransferase activities of the mutant proteins (in fact, the opposite was observed; I, Fig. 4a). The only additional effect, which was consistently observed, was a more extensive plasma membrane localization of palmitoylation-deficient nsP1s with second-site mutations (compared to nsP1-Δ3 and nsP1-mut3A; I, Fig. 5e-i). Based on these results, it was hypothesized that the plasma membrane localization of nsP1 is essential for its interaction with nsP4. Notably, the ability to induce the formation of filopodium-like extensions was restored by the majority of, but not all, compensatory changes. Thus, the ability to induce filopodium-like extensions is not a pre-requisite for the viability of the virus.

Taken together, it was found that although mutations in the nsP1 coding region that prevent its palmitoylation diminished the infectivity of the mutant genomes, these mutations did not block virus production. For SFV4mut3A and SFV4Δ3, virus production was associated with the accumulation of second-site compensatory changes. Primary mutations affected the plasma membrane localization of nsP1 and abolished its interaction with nsP4; compensatory changes restored these functions. Therefore, it can be concluded that palmitoylation is not required for the efficient reproduction of SFV but has a supporting role in the membrane association of nsP1, which is a pre-requisite for the formation of functional contacts with nsP4 and possibly other viral and host components that are important for replication.

This analysis was largely completed eight years ago and, thus, had some limitations. First, the exact role of the plasma membrane in replicase complex formation was not known at this time but was described several years later [163]. Therefore, some implications of the observed effects were overlooked;

no attention was given to the possible role of the plasma membrane association of nsP1 in the formation of replicase complexes. Second, due to relatively high costs, the plaque-purified isolates were not completely sequenced. These sequences include the first 20 nt of the virus genome and sequences encoding structural proteins. Therefore, some mutations that also contribute to restoring the infectivity of the virus may lie inside these regions. Third, it is likely that the full spectrum of compensatory changes capable of rescuing the infectivity of the mutant genomes was not revealed. The application of massive parallel sequencing may provide new insights into this question.

3.2 Mutations in nsP1 affect viral infection in mammalian and insect cells (I and II)

3.2.1 Mutations resulting in non-palmitoylated nsP1 render SFV replicons temperature-sensitive

The growth of SFV4Δ3 with potential compensatory mutations in the nsP4 coding region was restricted at 37 °C but not at 28 °C. Based on this finding, other mutant viruses containing mutations in nsP1 (I, Table 1) were tested for temperature-sensitive phenotypes in vertebrate (mammalian BHK-21) and invertebrate (mosquito C6/36) cells.

First, to allow easy quantification of SFV replication, the coding sequence of *Renilla* luciferase (Rluc) was cloned into mutant replicon vectors (the original Δ3 and mut3A and their combinations with compensatory changes in the nsP1 coding region). The marker expression of these replicons has been shown previously to be proportional to the level of replication and transcription of the replicon RNA [234]. For this experiment, the benefit of replicon vectors over the full virus genome was minimization of the impact of novel adaptive mutations because the replicons harboring such changes cannot spread in cell culture.

It was found that in BHK-21 cells transfected with SFV1-enhRluc-Δ3 and SFV1-enhRluc–mut3A transcripts, Rluc expression at the permissive temperature (28 °C) was reduced approximately 1000-fold when compared to the expression level achieved using wt SFV1-enhRluc RNA transcripts (II, Fig. 1). At the restrictive temperature (39 °C), Rluc expression in the SFV1-enhRluc-Δ3 transfected cells was further diminished and was over 10,000-fold lower than for the wt-vector transfected cells at the same conditions. This result indicates that the defect caused by the Δ3 mutation depends on the temperature. No similar effect was observed for SFV1-enhRluc–mut3A, which expressed approximately 1000-fold less Rluc compared with wt SFV1-enhRluc at both temperatures. As expected, the previously identified compensatory mutations increased the levels of Rluc expression by SFV1-enhRluc–mut3A and SFV1-enhRluc-Δ3 up to 100-fold; this increase was observed at both temperatures. Compensatory mutations had a greater enhancing effect on Rluc expression by SFV1-

enhRluc–mut3A than by SFV1-enhRluc-Δ3 (II, Fig. 1). A difference was not observed when full-length viral genomes were used (I), indicating that the method using combined replicon vectors with marker genes exhibits higher sensitivity than experiments based on the analysis of replication-competent viruses.

Transfection of C6/36 cells with *in vitro* synthesized RNA transcripts was found to be quite inefficient (several reagents and transfection procedures were tested). Therefore, the observed Rluc activities in mosquito cells were always significantly lower than those obtained in vertebrate cells. Nevertheless, clear differences between the constructs were also observed in C6/36 cells, and the overall outcome of the experiment was relatively similar in both cell lines. First, the decrease in Rluc expression levels achieved using replicons harboring Δ3 and mut3A mutations was up to 1000-fold at the permissive temperature (28 °C). Second, no further decrease was observed in the SFV1-enhRluc–mut3A transfected cells at the restrictive temperature (34.5 °C), whereas at the restrictive temperature, SFV1-enhRluc-Δ3-transfected cells expressed up to 10,000-fold less Rluc than cells transfected with wt SFV1-enhRluc. Thus, the Δ3 construct was found to have a temperature-sensitive phenotype in mosquito cells as well. Compensatory mutations that were originally selected in mammalian cells were found to affect Rluc production ambiguously. Combined with the original mut3A mutation, the compensatory changes increased marker protein expression up to 100-fold; the effect was nearly identical to that observed in BHK-21 cells. In contrast, combining compensatory changes with the Δ3 mutation further diminished Rluc expression levels at the permissive temperature (approximately 10-fold), while Rluc expression was essentially abolished at the restrictive temperature (II, Fig. 1). Thus, in the context of the SFV1-enhRluc-Δ3 replicon, the selected compensatory mutations displayed cell type-specific effects; an increase in replication levels was observed in mammalian cells, while the opposite effect was observed in insect cells. Thus, this analysis confirmed that the defects caused by the mut3A and Δ3 changes are similar but not identical.

3.2.2 Cytotoxic effects of mutant replicons in vertebrate cells

Previous reports have demonstrated that many mutations in the ns-protein coding region inhibit the ability of the virus to shut down host protein synthesis. When introduced into a replicon, these mutations reduce the virus' cytotoxicity and allow longer expression of the desired recombinant protein [129,225]. Therefore, one of the original reasons for creating SFV nsP1 palmitoylation-deficient mutants was to use these constructs to obtain novel vectors with prolonged transgene expression. Accordingly, an experiment to analyze the cytotoxicity of these replicons in BHK-21 cells was performed.

In this experiment, mutant replicons with the puromycin acetyl-transferase (PAC) gene under the control of a sg promoter were used. This method allows the survival of transfected cells to be followed; if the replicon is not cytotoxic

and expresses sufficient levels of PAC, then colonies can form in the presence of puromycin [235,236]. Hence, both the survival of transfected cells in a puromycin-containing medium and their ability to form antibiotic resistant colonies were analyzed. The phenotypes of all mutant replicons, except mut3A-2, were found to be indistinguishable from the wt replicon, indicating that their cytotoxicity was not reduced (II, Fig. 2). In addition, no colonies were formed with mut3A-2, and the transfected cells died rapidly in the presence of puromycin. This result indicates that the cells transfected with this mutant were unable to express PAC at levels sufficient to protect them from puromycin. Additionally, RT-PCR coupled with sequencing confirmed the presence of the replicon with the proper combination of mutations and the PAC sequence in the transfected cells. The reasons for these properties of SFV1- mut3A-2 are therefore not clear, especially considering that this effect was not observed in experiments with the SFV1-enhRluc–mut3A-2 replicon (II, Fig. 1). The only explanation that could be provided is that the combination of the 3A-2 mutation and the PAC gene influences the three-dimensional structure of the replicon RNA such that the translation of the ns-polyprotein and/or the transcription of sg RNA is hindered. Taken together, the mutant replicons designed in this study were not suitable vector candidates for prolonged gene expression.

3.2.3 The effect of mutations on viral infection in different cell lines

In a previous study (I), only the viability of the mutant viruses and their basic growth characteristics were analyzed. Here all mutant viruses with compensatory mutations in their nsP1 coding regions were confirmed to be viable and replicated to high titers in both mammalian and mosquito cell lines (II, Table 2). Although the differences in Rluc expression levels observed in the replicon amplification experiments were vast (II, Fig. 1), the differences in viral titers were relatively minor; no greater than a 5- to 50-fold decrease in virion production (compared to wt SFV4) was observed for any mutant. For most of the constructs, the titers obtained in mammalian cells and insect cells were similar; however, the SFV4mut3A-1, SFV4mut3A-3 and SFV4Δ3-2 constructs produced less infectious progeny in insect cells. This result indicates the existence of a mild host-specific defect in these mutants.

To determine whether mutations in the nsP1 coding region affect the ability of the virus to activate its protein synthesis and suppress the protein synthesis of the host, metabolic labeling experiments were carried out. BHK-21 cells were infected at an MOI 10 to obtain simultaneous infection of all cells and pulse-labeled every hour from 3 to 10 h p.i. The wt SFV4 infection resulted in intensive synthesis of viral structural proteins at 6 h p.i. and was followed by a gradual decrease in cellular protein expression levels (II, Fig. 3). The onset of structural protein synthesis in cells infected with any of the nsP1 palmitoylation-deficient viruses, except SFV4Δ3-1, was delayed by one hour; SFV4Δ3-1 began synthesizing viral structural proteins two to three hours later

than wt SFV4 (II, Fig. 3). All mutant viruses produced considerably less structural proteins when compared to the wt virus. The lowest level of structural proteins was detected for SFV4Δ3-1. This result correlates well with the finding that this mutant produces the lowest levels of infectious virus in BHK-21 cells (II, Table 2) and suggests that the lower yield of virus particles is most likely due to the decreased synthesis of structural proteins in this mutant.

3.2.4 Effect of the mutations on viral RNA synthesis

In addition to the availability of structural proteins, virion production depends on many other factors including the production of genomic RNA. Therefore, the synthesis of viral positive-strand RNAs in BHK-21 and C6/36 cells was analyzed using northern blotting.

Major differences in the viral RNA synthesis levels were revealed between different nsP1 palmitoylation-deficient viruses and the wt SFV4 in BHK-21 cells. The SFV4-Δ3-1 virus that produced the lowest levels of infectious virus (II, Table 2) also had the lowest rate of RNA production; the defect was most evident at 6 h p.i. (II, Fig. 4a). SFV4-Δ3-2 had the highest RNA synthesis levels among the analyzed mutants. Although the RNA levels of SFV4-Δ3-2 were diminished at 6 h p.i, they were comparable to the RNA levels of wt SFV4 at 12 h p.i. Because SFV4-Δ3-2 was one of the constructs that produced the lowest amount of infectious virus in BHK-21 cells, this finding was unexpected. Thus, it can be concluded that the low virion production was not a result of impaired RNA synthesis. Indeed, it has been recently shown that the levels of viral RNAs, the amount of synthesized structural proteins and the viral titers do not necessarily correlate with each other in VEEV mutants [237]. Nevertheless, the delay in structural protein synthesis observed for the mutant viruses in BHK-21 cells correlates reasonably well with the delayed genomic and sg RNA synthesis (II, Fig. 3 and 4a).

In C6/36 cells, the analysis of viral RNA synthesis was conducted at both the permissive and restrictive temperatures. At the permissive temperature, all viruses replicated efficiently, and only minor delays and reductions in genomic RNA synthesis levels could be detected at 48 h, a time point that has been reported to correspond to the onset of persistent infection [26] (II, Fig. 4b). Notably, however, all mutant viruses produced considerably less sg RNA (II, Table 3); this finding correlates well with the observed reduction of Rluc expression in the replicon experiment (Rluc is also expressed from mRNA synthesized using the sg promoter; II, Fig. 1). Interestingly, the presence of numerous RNAs with sizes shorter than the sg RNA was observed in C6/36 cells infected with the mutant viruses. These RNAs clearly represented DI RNA molecules. The emergence of DI RNAs in alphavirus-infected mosquito cells has been observed previously [238]; however, their abundance and the speed of their appearance (during a single round of infection) in this experiment indicate that their production was enhanced by mutations in the nsP1 coding region. At the restrictive

temperature, all tested viruses exhibited low levels of RNA synthesis with maximum levels of genomic RNA detected at 48 h p.i. (II, Fig. 4c). Abundant DI RNAs were detected at this temperature as well.

The existence of DI RNA molecules, combined with the finding that sg RNA production in insect cells is strongly affected, suggests that the interaction of the mutant replicase and the cis-acting elements in the virus genome was altered. Although the compensatory mutations restored the interactions between nsP1 and nsP4 in mammalian cells (I, Fig. 6), this result may not occur in insect cells. Therefore, additional passaging of mutant viruses in insect cells may be required to detect mutations that are specifically required for the efficient replication of nsP1 palmitoylation-deficient viruses in these cells.

3.2.5 Mutant nsP1 expression in mammalian and insect cells

The nsP1 expression levels in BHK-21 cells infected with the mutant viruses were similar to the nsP1 levels in cells infected with wt SFV4 (II, Fig. 5a). In infected insect cells, the amounts of expressed nsP1 were always relatively lower than in BHK-21 cells; however, the protein levels observed in cells infected with the mutant and wt SFV4 were similar (II, Fig. 5b).

In transfected mammalian cells, the defect in nsP1 palmitoylation disrupted the formation of filopodia-like extensions and directed nsP1 to localize intracellularly. Most second-site mutations restored the binding of nsP1 to the plasma membrane and the formation of filopodia (I, Fig. 5). In insect cells, wt and mutant nsP1s also localized to the plasma membrane, but mut3A-nsP1 and Δ3-nsP1 were additionally found to localize in the cytoplasm (II, Fig. 6). No diversities in localization were detected at different temperatures. Importantly, within the same insect cell culture that was transfected with the same expression plasmid encoding one mutant type of nsP1, nsP1 positive cells with and without filopodia-like structures were detected. Furthermore, the introduced compensatory mutations did not result in an increase in the number of filopodia-containing cells (II, Fig. 6). Filopodia-like structures that differed slightly in appearance from nsP1-induced filopodia were also found on the mock-transfected control cells. Thus, the formation of these structures is likely an intrinsic property of C6/36 cells. Because the cell cultures used in these experiments were not synchronized, this result may reflect the different behaviours of cells in distinct cell cycle stages. Although viral proteins produced in insect cells can be palmitoylated [88], neither the wt nsP1 nor the mutants were tested for their palmitoylation properties. Therefore, the ability of nsP1 to induce filopodia-like structures in insect cells cannot be clearly associated with the palmitoylation of the protein.

When expressed as a part of the P123 polyprotein, the intracellular localization of nsP1 changes; the localization is no longer exclusive to the plasma membrane, and a significant amount of nsP1 is found in intracellular vesicles [117]. These structures also contain other replicase proteins. It was found that in BHK-21 cells transfected with the P123 expression construct, all palmitoylation-deficient nsP1s co-localized with nsP3 at the perinuclear region (II, Fig. 7a). In mosquito cells, the co-localization of nsP1 and nsP3 in vesicles was

less apparent (II, Fig. 7b), most likely because of intrinsic differences in CPV formation in infected mosquito cells [162]. Taken together, these results indicate that the basic components of the replicase complexes (nsP1-nsP3) containing the palmitoylation-deficient nsP1 forms localized correctly in transfected cells.

To summarize, it can be concluded that in most cases the compensatory mutations found in BHK-21 cells enhanced virus production in mosquito cells as well, although the effect tended to be less pronounced than in mammalian cells. In insect cells, the viruses containing the corresponding mutations synthesized sg RNA at significantly lower levels than wt SFV and were prone to produce rapidly large amounts of DI RNAs. This result can be attributed to the altered interactions between the mutant replicase and the cis-acting elements of the SFV genome but not to the incorrect localization of the mutant replicase proteins and their complexes in insect cells.

An issue that these studies failed to resolve is the real biological significance of nsP1 palmitoylation and the reasons for its appearance in alphavirus evolution. The analysis of a SINV nsP1 palmitoylation-deficient mutant indicated that nsP1 palmitoylation is less important for this virus than for SFV [118,122]. In mammalian cells, the virus can survive without palmitoylation, and tighter membrane association appears to be dispensable for the virus. These experiments were performed exclusively in BHK-21 cells, which lack a functional interferon system. As another set of mutant SFV viruses behaved quite differently in an IFN-competent system (III), the behaviour of nsP1 palmitoylation-deficient viruses in other cell lines may have been quite different. However, the corresponding experiments were not performed in this study and no suppositions can be made.

Another interesting property associated with nsP1 palmitoylation is the induction of filopodia-like structures. This function was also restored by all but one (mut3A-2) of the compensatory mutations. Interestingly, the replicon containing the mut3A-2 mutation was also the only mutation that was unable to provide puromycin resistance (II, Fig. 2); therefore, the possible connection between these effects should be investigated. It is also possible that the ability to induce the filopodia-like structure is relevant primarily for *in vivo* infection. Previous work has suggested or shown (unpublished results from our research group) that alphaviruses can infect cells using other pathways in addition to the infection with virions. The formation of filopodia could mediate the cell-to-cell spreading of viral genomic material. Similar pathways have also been noted for other viruses, although the functioning of these pathways is generally poorly understood. For example, the hepatitis C virus can spread from cell to cell without virion formation [239]. The obvious advantages of this mechanism are the possibility to spread in the presence of neutralizing antibodies and to infect cells lacking suitable receptors for the binding of mature virions.

The presence of nsP1 on the filopodia-like structures may serve to modulate the immune response of the host. This idea has been suggested for CHIKV, which mediates the production of auto-immune antibodies and causes symp-

toms similar to rheumatoid arthritis [240]. Because nsP1 is located on the plasma membrane, nsP1 can cause the re-localization of host components by binding to the proteins normally found in other cellular compartments. If this re-localization results in their presence in the incorrect cellular location, it can trigger an immune response against these cells. It has been shown that SFV-induced CPVs contain increased amounts of antigens, such as different heterologous nuclear ribonucleoproteins that are normally present in the nuclei of cells [241]. Filopodia-like structures and their possible ability to mediate the secretion of replicase-containing vesicles would certainly enhance the presentation of such proteins. Further research involving *in vivo* experiments and the analysis of clinical samples from patients with chronic symptoms of CHIKV infection is needed to address these questions.

3.3 Alphavirus replication and interactions with the host innate immunity are regulated by the processing of the 1/2 cleavage site (III, unpublished)

The expression and correct processing of the alphavirus ns-polyprotein is essential for the viability of the virus. The production of free nsP4, either by processing the 3/4 cleavage site (natural expression) or *in trans* (in this case, the co-expression of P123 is also required), represents an absolute requirement for active replication complex formation [18,163]. If free nsP4 is not formed, no replication can be observed. However, the requirements for processing the remaining P123 are less strict. The virus can survive a significant delay in the processing of any of the two remaining cleavage sites (III, [141] or a complete blockage of the sites (III, [174]. On the other hand, the acceleration of cleavage with a hyper-activated nsP2 protease, has been reported to be lethal for SINV [242]. Manipulating the cleavage efficiency at the 2/3 site has little or no effect on alphavirus infectivity [15,141], III) and therefore the infectivity was thought to be controlled by the cleavage of the 1/2 site. It is known that the 1/2 site can be cleaved *in trans* by nsP2, but the efficiency is approximately 5000-fold lower than the processing of the 3/4 site [140]. Therefore, most of the 1/2 cleavages in infected cells and *in vitro* occur *in cis* [16,138]. Processing of the 1/2 site is also absolutely required for the subsequent processing of the 2/3 site [141], and differences in the processing speed of the 1/2 site have been shown to influence SINV neurovirulence in mice [243].

Thus, the 1/2 cleavage controls, at least in part, the stability of P123. What is more, the stability of P123 is an important determinant in the correct subcellular localization of alphaviral replicase complexes [117], ensuring that all replicase components are transported together to the plasma membrane, where replication complex formation occurs [162,163]. Thus, it can be assumed that all these processes are directly controlled by the cleavage of the 1/2 site.

3.3.1 Mutations accelerating 1/2 cleavage are detrimental for SFV replicase complex formation and for the infectivity of the corresponding recombinant genomes (unpublished)

Previous studies have identified the Arg residue at the P4 position in the 3/4 cleavage site (the 4th amino acid residue preceding the scissile bond in the cleavage site) as the major determinant for efficient processing of the site [15]. In the 1/2 cleavage site in SFV4, this position is occupied by a His residue (III, Fig. 2), but an Arg residue is retained in the A7/74 isolate of SFV [10]. Because both isolates are viable, this observation led to the hypothesis that the P4 residue in the 1/2 cleavage site cannot be the only determinant, and may not even be the decisive one, for its processing. Therefore, to artificially accelerate the cleavage event at this site, the native cleavage site with the sequence EYHAG^{531}A was substituted with LGRAG^{531}A, which is the sequence naturally occurring in the most actively cleaved 3/4 processing site of SFV4. It was found that the infectivity of the obtained recombinant RNA transcripts decreased drastically by almost 10,000-fold when compared to wt SFV4. Nevertheless, similarily to the nsP1 palmitoylation-deficient mutants (I), infectious progeny with growth characteristics relatively similar to wt SFV4 was obtained. Sequence analysis of these viruses revealed that the majority of the viruses had either reverted the P4 Arg residue to the native His residue or substituted it with Cys, Leu or Ser residues. All these substitutions significantly slowed down the accelerated processing of the P123 polyprotein, indicating that this change was the key defect caused by the LGRAG^{531}A mutation. Furthermore, another compensatory mutation that compensated for the defect caused by the LGRAG^{531}A mutation was identified in the protease part of nsP2. The properties of the corresponding mutants are currently under detailed analysis using *in vitro* and *in vivo* experiments. However, based on the presented data, it is clear that the delayed cleavage of the 1/2 cleavage site is crucial for viral replicase complex formation and virus infectivity.

3.3.2 The P5 residue controls the processing of the 1/2 cleavage site

The data presented above clearly indicate that the introduction of an Arg residue into the P4 position of the 1/2 site can accelerate its processing. However, this result does not indicate that this residue is the major determinant in controlling this cleavage event. While the mutant with the LGRAG^{531}A residues could only survive due to the accumulation of various adaptive mutations, the SFV A7 isolate is viable (P4 Arg) on its own. Thus, it is likely that other residues are also important for controlling the processing of the 1/2 site.

To highlight the importance of these aa residues in P1234 processing, the residues in LG^{528}R (P6-P4) were substituted with Ala residues (III, Fig. 2a). The corresponding single Ala substitutions were introduced into a construct designed for *in vitro* transcription and translation analyses; a construct in which all

three residues in the P6-P4 region (527-529) were substituted with Ala residues was also generated. This triple mutation was introduced into the DNA clone of SFV4 [187]; a construct with similar substitutions at other cleavage sites was also generated.

Using an *in vitro* transcription and translation system, it was found that the introduction of an Ala residue in the P5 position of the 1/2 cleavage site resulted in increased amounts of several cleavage intermediates, especially P12. Correspondingly, a decrease in the amounts of mature ns-proteins was observed (III, Fig. 1a). The same result was also observed for the triple $AA^{528}A$ mutant, but not for mutant polyproteins containing only the P4 Ala or P6 Ala substitutions. Thus, it was confirmed that the P5 position in the 1/2 cleavage site represents the major determinant for directing the release of nsP1.

3.3.3. Mutations in all cleavage sites reduce the infectivity of recombinant genomes

The transcripts of icDNA clones with a triple Ala mutation in the 1/2 cleavage site were highly infectious. Thus, in contrast to the cleavage accelerating LGR mutation in the 1/2 site, the mutation that decreased the cleavage rate (AAA) had little or no effect on the infectivity of the virus (III, Fig. 2b). Furthermore, the overall growth kinetics of the rescued virus resembled wt SFV4 (III, Fig. 3); the reduced plaque size of the mutant SFV4-1AAA virus was the only difference that could be detected. Consistent with the high infectivity of the mutant genome and efficient propagation of the corresponding virus, no reversions, pseudoreversions or common second-site mutations were detected in SFV4-1AAA infected cells. However, a drastic decrease in infectivity (more than 1000-fold) was observed when a RNA transcript of a construct containing three similarly mutated cleavage sites (SFV4-3xAAA) was analyzed. This effect cannot be attributed to mutations in any specific cleavage site because mutant genomes having three Ala residues in the 2/3 or 3/4 cleavage sites (P6-P4 positions) demonstrated similar infectivity and growth kinetics to wt SFV4. Therefore, the defect must be attributed to the combination of the 1/2 site mutation with other mutations. This combination of mutations not only altered the speed and efficiency of the processing of the cleavage sites in P1234 but also altered its processing pattern as a whole. SFV4-3xAAA produced minute plaques in infected BHK-21 cells and had a decreased rate of virion release (III, Fig. 3). However, after the first passage, SFV4-3xAAA was already capable of growing to high titers. This behaviour was similar to the viruses with palmitoylation-deficient forms of nsP1, suggesting that the initial defect must have been compensated by additional mutations. Nevertheless, no reversions or pseudoreversions in any of the mutated cleavage sites were observed when the SFV4-3xAAA virus was passaged five times in BHK-21 cells. Therefore, several individual viral clones were obtained using plaque purification techniques. Sequencing of these clones revealed a common second-site mutation leading to a

Q706R change in the nsP2 protein. When this mutation was introduced into the genome of SFV4-3xAAA (the resulting construct was designated as SFV4-3xAAA-Q706R), the infectivity of the obtained recombinant genome increased approximately 100-fold (III, 2b). Thus, the Q706R mutation compensated for the defects that arose due to the altered aa composition of the cleavage sites.

3.3.4 The effects of combined mutations on polyprotein processing

The reduced infectivity of SFV4-3xAAA (and the lack of a similar effect in mutants with changes in any of the individual cleavage sites) suggested that cleavages at different sites affect each other. To verify this effect directly, the processing of the mutant P1234 polyprotein containing mutations in all three sites was analyzed using an *in vitro* transcription and translation system. This analysis revealed that the levels of all fully processed nsPs were diminished (III, Fig. 4). In agreement with this result, the levels of unprocessed polyproteins, especially P34, were increased. Importantly, however, the introduction of the Q706R mutation modified the cleavage pattern of the mutant polyprotein, which now resembled wt P1234. These data suggest that the identified mutation restores the infectivity of the SFV4-3xAAA-Q706R transcripts by altering the processing of the mutant ns-polyprotein. The same analysis was also conducted in BHK-21 cells using a pulse-chase method. Because SFV4-3xAAA was genetically unstable, the analysis of P1234-3xAAA polyprotein processing without the Q706R mutation in virus-infected cells was not possible. The ns-polyprotein processing patterns of SFV4-3xAAA viruses with the compensatory Q706R mutation were found to closely resemble wt SFV4; the only detectable difference was the increased stability of P123 in cells infected with the mutant viruses (III, Fig. 5b). Thus, the differences in the ns-polyprotein processing pattern observed in the test tube reaction were clearly reproduced in the context of viral infection as well.

3.3.5 Mutations in the P1234 processing sites restrict replication or rescued the virus in many cell lines

To estimate whether the compensatory changes obtained in BHK-21 cells were cell type-specific, the two plaque-purified isolates of SFV4-3xAAA (containing the common Q706R mutation) and the rescued SFV4-3xAAA-Q706R virus were used to infect other cell types. The productive infection of C6/36 cells with the mutant viruses was not possible, suggesting that the defects resulting from mutations had more severe effect(s) in mosquito cells. In contrast, efficient replication was observed in the human hepatocyte carcinoma Huh7 cell line. It can be therefore concluded that the compensatory changes are not strictly BHK-21-specific.

It was observed that in several cells lines, including the human HS633T fibroblast cell line, the SFV4-3xAAA-Q706R viruses replicated very poorly.

First, these cells are naturally less susceptible to SFV4 infection compared to BHK-21 cells; at least five-fold more wt SFV4 was needed to achieve the same level of infected cells. Furthermore, even at an MOI of 5, no more than 95% of the HS633T cells could be infected. Nevertheless, these effects could not explain the defect of SFV4-3xAAA-Q706R viruses because the wt SFV4 was capable of performing productive infection in these cells. However, the defects in establishing an infection was larger for mutant viruses; as little as 30-40% of the cells were infected using an MOI of 5. However, all mutant viruses were capable of replicating in HS633T cells, and the CPVs containing the SFV replicase complexes that are produced by the mutant viruses were even larger than those induced by a wt SFV4 infection (III, Fig. 6). Thus, AAA mutations in the cleavage sites (compensated by a Q706R change) had little effect on the formation of replication-associated structures in these cells. In contrast, the morphology of the CPVs was altered for a SFV mutant that expressed the uncleavable P123 (an E452A mutation was introduced into nsP4 to obtain higher levels of the virus as described in [174]). In this mutant, very small CPVs were observed.

3.3.6 The effect of cleavage site mutations on interferon production

The HS633T cell line has an intact interferon system [244]. Therefore, an infection in these cells mimics some aspects of the innate immune responses triggered in animals infected with alphaviruses. Growth curves of viruses obtained using these cells indicated that none of the constructed mutants were able to produce more infectious virions than was used to infect them (III, Fig. 7a). In cells infected with SFV4-1^2^3-E452A (^ represents a blocked cleavage site), a steady decline in the virus titer was observed, most likely indicating the lack of release of infectious progeny. In cells infected with SFV4-2^3 (another control virus used in this experiment) or with any of the SFV4-3xAAA-Q706R viruses, low amounts of viral progeny were produced. Cytotoxic effects, characteristic for wt SFV4 infection at 24 h p.i., were only observed in the SFV4-2^3-infected cells. Thus, in the presence of an IFN response, the SFV4-3xAAA-Q706R viruses failed to produce large amounts of progeny, and the infection was cleared, most probably by the innate immune response.

Thus, the introduced mutations likely caused a defect in the ability of SFV to suppress the IFN response. To verify this hypothesis directly, the release of IFNα/β into the growth medium of infected cells was analyzed. It turned out that the SFV4-1^2^3-E452A-infected cells produced the highest amounts of IFN, beginning as early as 4 h p.i. (III, Fig. 7b). This result is in agreement with the data observed for a SINV mutant with a similar design [174] and is thought to result from the inability of the uncleaved P123 to enter the nucleus and prevent the transcription of IFN genes. In contrast, the IFN release from wt SFV4-infected cells was negligible at 8 h p.i. Again, this result was expected because wt SFV4 has been shown to efficiently suppress type I IFN production [128].

After 8 hours, the production of type I interferon began to increase in wt SFV4-infected cell cultures; this production continued until the excessive death of the infected cell culture. The SFV4-3xAAA and SFV4-3xAAA-Q706R clones displayed an intermediate phenotype in IFN production. For cells infected with these viruses, IFN expression was detected at 8 h p.i.; however, high levels of IFN were not reached. The same result was observed in a parallel experiment designed to assess the influence of newly produced mutant virions on IFN production (in this experiment, the growth medium was not substituted with fresh medium, and only aliquots were collected). These data indicate that unlike wt SFV4, the SFV4-3xAAA and SFV4-3xAAA-Q706R viruses cannot suppress IFN induction and signaling efficiently enough. However, the defect in suppressing IFN production was not nearly as profound as in SFV4-1^2^3-E452A. The latter virus could not produce any free nsP2 and expressed exclusively P123. In contrast, in BHK-21 cells, no delay in nsP2 release was detected for SFV4-3xAAA viruses (III, Fig. 5B). If the same result would also be true for HS633T cells, then the most likely reason for a high level of IFN induction (compared to wt SFV4) might be in the Q706R mutation in nsP2 that can reduce its functioning in reducing IFN expression. The prolonged production of P123 might also result in the synthesis of excessive amounts of dsRNA. To distinguish between these possibilities, additional experiments would be needed. However, the data obtained in this study indicate that the IFN response has an important role in limiting the infection of SFV4-3xAAA and may limit the spread of such viruses in an organism. This hypothesis corresponds to previous observations made using SINV; these studies have shown that a mutation in the 1/2 cleavage site is one of the main determinants of its virulency and that the introduction of this mutation induced a high level of IFN production [245]. To test this hypothesis, *in vivo* experiments using different SFV strains, especially the most neurovirulent L10 strain engineered to contain cleavage site mutations, need to be performed.

SUMMARY

NsP1 is an essential component of the alphavirus replicase. Among other functions, nsP1 is involved in regulating the formation of a replicase complex and attaching the complex to host cell membranes.

The analysis of Semliki Forest virus (SFV) mutants with different palmitoylation-deficient versions of nsP1 in BHK-21 cells revealed that, in general, the mutant genomes had severely reduced infectivities. Some of the corresponding viruses had a temperature-sensitive phenotype. The main defect caused by most but not all mutations in the nsP1 palmitoylation site was disruption of the interactions between nsP1 and nsP4. This defect was rescued by second-site compensatory mutations identified in nsP1-encoding sequences in the plaque-purified isolates of rescued viruses. These mutations also led to the increased infectivity of the corresponding RNA genomes. In general, this effect was also observed in insect C6/36 cells, albeit to a lesser extent. In these cells, the impact of the original mutation on virus infection was more prominent, and the most affected step of virus infection was the production of subgenomic RNA (sg RNA). The second-site mutations had little effect on the sg RNA production defect in C6/36 cells. Instead, the mutant viruses triggered the formation of a large number of defective interfering genomes in insect cells. Therefore, it was proposed that in C6/36 cells, the mutations in the palmitoylation site of nsP1 also disrupted the connections between cis-acting elements of the viral genome and the replicase proteins, and this defect could not be restored by the second-site mutations selected in mammalian cells. The formation of filopodia-like structures, characteristic of vertebrate cells infected with wt SFV, was also affected by nsP1 mutations. Most of the identified compensatory mutations were found to contribute to the restoration of the formation of these structures. The filopodia-like structures were thought to be involved in the cell-to-cell spreading of the infection.

The analysis of the processing requirements of the 1/2 cleavage site in the SFV ns-polyprotein highlighted its importance as one of the major regulatory factors controlling the assembly of a functional replication complex. It was shown that the accelerated processing of this site severely diminished the infectivity of the corresponding mutant genome, while a reduction in the cleavage efficiency had only a minor effect. The processing of the 1/2 site depends mostly on the amino acid residue in the P5 position. The processing of the 1/2 site was slowed when the original P5 residue was replaced with an Ala residue. When this mutation was combined with mutations that reduce the cleavage efficiency in the 2/3 and 3/4 sites, the resulting virus genome had reduced infectivity and became genetically unstable. However, the defects in all three cleavages were efficiently rescued by a single second-site mutation (Q706R) in the nsP2 coding region. The resulting viruses were incapable of replicating in C6/36 cells and poorly infected cells with an intact IFN system. It was suggested that the prolonged expression of P123 that results from the mutation in the P5 position in the 1/2 cleavage site and the Q706R mutation in the nsP2

coding region contributes to the enhanced type I IFN production in these cells. Detailed studies using animal models are required to provide further information about the biological implications of these findings.

SUMMARY IN ESTONIAN

Semliki Forest viiruse replikaasivalgu nsP1 uurimine

Semliki Forest viirus (SFV) kuulub alfaviiruste perekonda sugukonnas *Togaviridae.* Alfaviirused on olulised patogeenid ning põhjustavad erinevaid vaevusi ka inimestele. Levik looduses toimub sääskede vahendusel. Lisaks tähtsusele patogeensuse tõttu on alfaviirused kasutusel ka bio- ning geenitehnoloogias ning seetõttu olulisteks uurimisobjektideks. Alfaviirustel on positiivse polaarsusega RNA genoom, millelt transleeritakse viiruse replikaasi subühikud (nsP1-nsP4). Antud töö põhiline uurimisobjekt nsP1 vastutab lisaks otsesele osalemisele replikatsioonil ja transkriptsioonil replikaasi sidumise eest peremeesraku membraanidele.

Käesolevas uurimistöös analüüsiti SFV mutante, mille nsP1 valku kodeerivat ala oli muudetud nii, et sünteesitavat valku rakkudes ei palmitüleerita. Kasutades imetajate rakke (BHK-21) leiti, et selliste viiruste genoomide infektsioonilisus oli järsult vähenenud ja osadel saadud viirustel oli temperatuuritundlik fenotüüp. Põhiline funktsionaalne defekt, mis kaasnes enamike mutatsioonidega nsP1 palmitüleerimise saidis, oli nsP1 ja nsP4 vaheliste kontaktide puudumine. Kasutades vabanenud viiruste plaag-puhastamist ja sekveneerimist selgitati välja, et viiruste infektsioonilisuse taastamise põhjuseks olid mutatsioonid, mis võimaldasid mutantsel nsP1 seonduda nsP4 valguga. Näidati, et sellised sekundaarsed mutatsioonid tõstsid oluliselt vastavate RNA genoomide infektsioonilisust BHK-21 rakkudes.

Üldjoontes esinesid sarnased efektid, ehkki vähemal määral, ka putukast pärinevates C6/36 rakkudes. Nendes rakkudes olid esialgsete mutatsioonide poolt põhjustatud defektid viiruse paljunemise juures suuremad kui imetaja rakuliinis. Kõige suuremaks efektiks oli subgenoomse RNA tootmise vähenemine. Kompensatoorsed mutatsioonid suurendasid viiruse infektsioonilisust C6/36 rakkudes väiksemal määral kui imetajate rakkudes ning tekkis suur hulk defektseid interfereeruvaid RNA genoome. Seetõttu võib oletada, et nsP1 palmitüleerimissaidi mutatsioonid takistavad C6/36 rakkudes viiruse genoomi *cis*-elementide ja replikaasivalkude vahelisi interaktsioone. Ilmselt osalevad nendes interaktsioonides ka raku valgud, mistõttu imetaja rakkudes paljundamisel tekkivad sekundaarsed mutatsioonid ei suuda putuka rakkudes tekkivaid defekte kompenseerida.

Lisaks leiti, et nsP1 palmitüleerimise puudumine takistab filopoodiumi-laadsete struktuuride moodustamist nakatatud rakkude plasmamembraanil. Tuvastati, et suurem osa analüüsitud kompensatoorsetest mutatsioonidest taastab vähemalt osaliselt viiruse võime selliseid struktuure moodustada. Sellest lähtudes pakuti välja hüpotees, mille kohaselt filopoodiumi-laadsed moodustised aitavad kaasa viirusinfektsiooni levimisele rakust rakku.

Mittestruktuurse polüproteiini 1/2 lõikesaidi analüüs näitas, et selle saidi lõikamine õigel ajal on üks olulisematest regulatoorsetest etappidest funktsionaalse replikaaskompleksi moodustumisel. Näidati, et 1/2 saidi lõikamise

kiirendamine vähendas olulisel määral vastavate mutantsete genoomide infektsioonilisust sama ajal kui lõikamise aeglustamisel oli väiksem mõju. Leiti, et 1/2 saidi lõikamine sõltub eriti tugevalt lõikesaidi P5 positsioonis asuvast aminohappejäägist. Selles positsioonis oleva aminohappejäägi asendamine alaniini jäägiga aeglustus liitvalgu lõikamise kiirust, kuid ei muutnud rekombinantse viiruse genoomi infektsioonilisust. Viiruse genoom, kuhu lisaks mutatsioonidele 1/2 lõikesaidis oli sisse viidud ka lõikamist takistavad mutatsioonid 2/3 ja 3/4 saitides, oli aga oluliselt vähenenud infektsioonilisusega ja moodustatud viirus oli geneetiliselt ebastabiilne. Leiti et kõiki sisse viidud defekte saab kompenseerida ühe täiendava mutatsiooniga (Q706R) nsP2 kodeerivas piirkonnas, mis taastas viiruse elujõulisuse ja liitvalgu korrektse lõikamise BHK-21 rakkudes. Samas ei suutnud selline viirus paljuneda C6/36 rakkudes ning tema paljunemine oli takistatud ka terviklikku interferoonvastust omavates imetajarakkudes. Nendest tulemustes lähtuvalt pakuti välja hüpotees, mille kohaselt mutatsioonid 1/2 lõikesaidis ja nsP2 valgus põhjustavad viiruse P123 liitvalgu eluea pikenemise, mis takistab viirusel võimet I tüüpi interferoonide tootmist efektiivselt blokeerida. Uuritud mutatsioonide bioloogiliste efektide põhjalikumaks uurimiseks on tarvis läbi viia loomkatseid.

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