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MARGUS VARJAK

Functional analysis of viral and host components of alphavirus replicase complexes

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Functional analysis of viral and host components of alphavirus replicase complexes

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on following publications that are referred to in the text by their Roman numerals:

I. **Varjak M**, Zusinaite E, Merits A. 2010. Novel functions of the alphavirus nonstructural protein nsP3 C-terminal region. Journal of Virology, 84: 2352–2364

II. Pohjala L, Utt A, **Varjak M**, Lulla A, Merits A, Ahola T, Tammela P. 2011. Inhibitors of alphavirus entry and replication identified with a stable Chikungunya replicon cell line and virus-based assays. PLoS One, 6(12): e28923

III. Panas MD, **Varjak M**, Lulla A, Eng KE, Merits A, Karlsson Hedestam GB, McInerney GM. 2012. Sequestration of G3BP coupled with efficient translation inhibits stress granules in Semliki Forest virus infection. Molecular Biology of the Cell, 23:4701–4712

IV. **Varjak M**, Saul S, Arike L, Lulla A, Peil L, Merits A. 2013. Magnetic fractionation and proteomic dissection of cellular organelles occupied by the late replication complexes of Semliki Forest virus. Journal of Virology, 87: 10295–10312

Author's contribution:

I. I designed and performed most of the experiments; I analyzed the data and wrote the manuscript.
II. I participated in the experimental design, performed the experiments and analyzed the data.
III. I participated in the experimental design, performed the experiments and analyzed the data.
IV. I designed and performed most of the experiments; I analyzed the data and wrote the manuscript.

ABBREVATIONS

aa	amino acid
ACLY	ATP citrate lyase
CHIKV	Chikungunya virus
CPV-I	type I cytopathic vacuoles
CSE	conserved structural elements
DMV	double-membraned vesicle
dsRNA	double-stranded RNA
EEEV	Eastern equine encephalitis virus
ER	endoplasmatic reticulum
FASN	fatty acid synthase
Fluc	luciferase from firefly (*Photinus pyralis*)
HCV	hepatitis C virus
hnRNP	heterogeneous ribonucleoprotein
kd	knock-down using siRNA
mRNP	messenger ribonucleoprotein complex
MOI	multiplicity of infection
NC	nucleocapsid
NLS	nuclear localization signal
NRAMP	natural resistance-associated macrophage protein
ns	non-structural
NTPase	nucleotide triphosphatase
PABP1	poly(A)-binding protein 1
Pat A	pateamine A
PB	processing body
p.i.	post-infection
PM	plasma membrane
RC	replication complex
RdRP	RNA-dependent RNA polymerase
Rluc	luciferase from sea pansy (*Renilla reniformis*)
RRV	Ross River virus
RTPase	RNA trisphosphatase
SFV	Semliki Forest virus
sg	subgenomic*
SG	stress granule*
SILAC	stable isotope labeling with amino acids in cell culture
SINV	Sindbis virus
SLiM	short linear interaction motif
TF	TransFrame protein
VEEV	Venezuelan equine encephalitis virus
VRP	virus replicon particle
WEEV	Western equine encephalitis virus

** please pay attention to lower- or uppercase lettering – sg vs SG*

1. INTRODUCTION

There are many viruses around us; curiosity on the one hand and the need for a cure on the other have urged man to discover and analyze these small but often dangerous pathogens. Alphaviruses are no exception- many of these viruses are pathogenic to humans, and infection with these viruses can cause serious illnesses, e.g., encephalitis and/or arthritis. In nature, blood-feeding insects often transmit human and animal pathogens; therefore, the most dangerous alphaviruses reside in tropical regions where mosquito species are responsible for the spread of Chikungunya virus, Ross River virus, and Venezuelan equine encephalitis virus.

In addition to increasing our knowledge of alphaviruses, investigation of these species (or viruses in general) has revealed important information about humans, e.g., how the host can be tricked and abused to fulfill the needs of the pathogens and how the host copes by launching an immune response to eradicate infection. Although alphaviruses have been investigated for several decades, numerous questions at the molecular, cellular and organism level have remained unanswered. The more complicated the biological level, the more difficult it is to perform studies and interpret the experimental data. Traditionally, Semliki Forest virus and Sindbis virus have been used as safe models for in vitro studies, cell culture and *in vivo* models (using rodent hosts and insect vectors).

The aim of this study was to investigate the alphavirus replication complex, to further characterize the viral components and to determine their interactions with host proteins. It was determined that non-structural protein 3, the most enigmatic replication complex protein, has a short half-life and attracts cellular stress granule components to the site of viral replication, causing the deregulation of stress granule function. Through analyzing the host-virus interaction, it was determined that alphavirus replication complexes interact or colocalize with several host proteins; however, not all of the interactions are beneficial to virus multiplication.

2. REVIEW OF LITERATURE

2.1. Positive-strand RNA viruses

According to the classical Baltimore classification of viruses, viruses with RNA genomes are divided into several groups based on their genome organization and mode of replication. There are RNA viruses with double-stranded RNA genomes, viruses with negative polarity RNA genomes and those with positive polarity RNA genomes. The viruses with positive polarity RNA genomes represent the largest group of known viruses. Positive strand RNA viruses are the most important viruses in terms of animal (and human) pathogenicity, and most plant viruses contain this type of genome (Knipe and Howley, 2007). Based on the sequence analysis of virus-encoded RNA-dependent RNA polymerases (RdRp) and the genome organization, positive-strand RNA viruses were divided into three main groups: the picorna-like, flavi-like and alphavirus-like superfamilies (Koonin and Dolja, 1993). Members of an individual superfamily can differ significantly. For example, the virion structure can vary from icosahedral to helical, it can be non-enveloped or enveloped, and the host range and species can be very different. In addition, within the same superfamily, the replicase proteins can exhibit significant rearrangements, such as acquisitions and deletions. A number of these differences can be explained by the high mutation rate of the viral RdRp and/or by the high frequency of recombination. Because RdRp and RNA virus replicase complexes do not generally exhibit proof-reading activity, the error rate of RdRp is in the range of 10^{-5} to 10^{-3} mutations per nucleotide in one round of replication (Domingo et al., 1997). If the average size of a positive-strand RNA virus genome is 10 kb, the new copy of the genome can contain as many as 10 differences from the parental genome.

In infected cells, the genome of positive-strand RNA viruses behaves in a manner similar to mRNA and viral proteins, which are needed for the initial steps of infection, are translated directly from the genome. This process is often achieved through the expression of polyprotein precursors, which are processed into individual proteins by the viral and host proteases. According to current knowledge, all eukaryotic positive-strand RNA viruses exhibit a common and intriguing feature: the genomic RNA is targeted to intracellular membranes together with the replicase proteins, which results in the formation of specific membrane-bound replication sites (virus replication organelles). These organelles contain virus replication complexes (RC), which are formed from the viral and host RNAs and proteins and cellular lipids. Therefore, RC formation causes significant alterations in the lipid and protein content of targeted cellular membranes (Miller and Krijnse-Locker, 2008) (Table 1). Depending on the virus, the Golgi complex, endoplasmic reticulum (ER), peroxisomes, mitochondria, plasma membrane (PM) and/or lysosomes can be targeted by the viral components and used as sites for RC formation. Electron microscopy images demonstrate that the RCs from positive-strand RNA viruses form small membranous invaginations that resemble ‘mini-organelles’ and are termed,

depending on the virus, spherules or double-layered membrane (DMV) vesicles (Table 1). The size, structure and presumably the composition of these mini-organelles can differ; nevertheless, all are composed of membranes, which separate the viral replication machinery from the cytoplasm. Accordingly, most of these structures contain a connecting pore, allowing the inflow of molecules needed for viral RNA synthesis and the outflow of nascent RNAs (Denison, 2008; den Boon and Ahlquist, 2010).

Table 1. Examples of important pathogenic positive-strand RNA viruses

Family	Virus	Size of genome	Host	Type of membrane modification	Original cellular membrane
Coronaviridae	SARS	~30kb	Humans, bats	DMV	ER
Picornaviridae	Poliovirus	~8kb	Humans	DMV	ER, Golgi, lysosomes
Togaviridae	Sindbis virus	~11kb	Animals (humans)	Spherule	Lysosome
Flaviviridae	Hepatitis C virus	~10kb	Humans	DMV	ER
Nodaviridae	Flock house virus	~5kb	Insects	Spherule	Mitochondria
Bromoviridae	Brome mosaic virus	~9kb	Plants	Spehrule	ER

2.2. Alphaviruses

The family *Togaviridae*, along with several plant and animal viruses, is a member of the superfamily of alpha-like viruses. *Togaviridae* consists of two virus genera, the genus Alphavirus and genus Rubivirus. The sole known member of the genus Rubivirus is the Rubella virus, which lacks an insect vector, and its only host is human. The genus Alphavirus has more than 30 members, including several that are pathogenic to humans and animals (Strauss and Strauss, 1994). Alphaviruses are distributed worldwide and have been grouped historically into New World and Old World alphaviruses.

Old World alphaviruses, e.g., Chikungunya virus (CHIKV), Semliki Forest virus (SFV), Ross River (RRV), and Sindbis (SINV), are found in Europe, Asia, Africa, and Australia. Infections with Old World alphaviruses often cause rash, fever, and arthritis. New Worlds alphaviruses (e.g., Venezuelan equine encephalitis virus (VEEV), Eastern equine encephalitis virus (EEEV), and Western equine encephalitis virus (WEEV)) are spread throughout South and North America, and infection with these viruses in humans and domestic animals typically leads to encephalitis.

Alphaviruses can replicate in invertebrate vectors and in vertebrate hosts (e.g., birds and mammals; fish-infecting alphaviruses have also been described). In nature, most alphaviruses are spread by blood-sucking arthropods, e.g., mosquitos from the *Aedes* and *Culex* genera; therefore, alphaviruses belong to the group of arboviruses (an abbreviation for arthropod-borne). In insects, alphaviral infections are largely asymptomatic and result in persistent lifelong infection. In vertebrates, either the infection tends to be acute and short-lived and ends with the death of the host or the pathogen is removed from the organism by the immune system. However, for several Old World alphaviruses, notably for CHIKV, chronic symptoms, likely associated with chronic infection, have been described. The different types of infection are also observed in *in vitro* systems. In cell culture, the acute infection of mosquito cells is limited and is converted to persistent infection without detriment to the host cell; however, the infection of vertebrate cells is usually associated with rapid cell death (Strauss and Strauss, 1994).

SFV and SINV viruses are the most studied members of their genus. In contrast to several other alphaviruses, such as WEEV, EEEV, VEEV and the recently re-emerging CHIKV, SFV and SINV are not typically associated with serious human illness (Strauss and Strauss, 1994; Schuffenecker et al., 2006) and are therefore considered safe model systems. In tissue culture, SFV and SINV replicate in a wide range of cells of invertebrate and vertebrate origin, in which they grow into high titers. In addition to tissue culture, mice and rats are typical choices for investigating the course of SFV and SINV infection at the organism level. The availability of SINV and SFV infectious cDNA (Rice et al., 1987; Liljeström et al., 1991) has allowed the use of reverse genetic approaches. These studies have demonstrated multiple important aspects of the alphavirus infection cycle (such as RNA replication, transcription, and viral polyprotein processing) and several basic cellular processes (Jose et al., 2009). However, although members of the same genus, SFV and SINV are different viruses and not all of their biological properties are identical. In addition to basic studies, viral expression vectors have been designed based on both the SFV and SINV genomes. These vectors have been used in biotechnological studies and represent promising tools for anti-cancer treatment and vaccine development (Riezebos-Brilman et al., 2006; Johansson et al., 2012).

2.3. Virion and genome organization

The SFV virion is spherical in shape and is enveloped; the diameter of the virion is 70 nm. Beneath the envelope is the nucleocapsid (NC), with a diameter of 30 nm. The NC is composed of 240 capsid (C) protein monomers that are tightly connected to each other, and the symmetry type is T=4. The N-terminal portion of the C protein is rich in positively charged amino acid (aa) residues and is bound to the genomic RNA. The single genomic RNA strand measuring

11.5 kb is located within the nucleocapsid (Strauss and Strauss, 1994; Jose et al., 2009).

The envelope of the alphavirus virion is derived from the plasma membrane (when the virus is produced in vertebrate cells) or the endomembranes (when the virus is produced in insect cells). The virions produced in mammalian cells have been investigated in detail, and the envelope of these virions is rich in steroids and sphingolipids. The viral-encoded proteins consist of 240 copies of E1-E2 heterodimers (both E1 and E2 proteins contain several membrane-spanning domains), and three E1-E2 dimers form a spike complex; therefore, each virion contains 80 spike complexes. In addition to E1 and E2, lower numbers of smaller proteins (E3, 6K and TransFrame (TF)) are present in alphavirus virions, and the abundance of these proteins differs in different alphaviruses. E2 plays an important role in binding to the host cell and is important for virion formation because it is bound to C-protein (Jose et al., 2009). Cryo-electron microscopy-based analysis has generated a significant amount of information regarding the structures of the SFV, SIN, and VEEV virions (Paredes et al., 1993; Mancini et al., 2000; Zhang et al., 2011); recently, the structures of the CHIKV membrane proteins have been resolved using X-ray crystallography, and the atomic structure of the virion has been reconstructed (Voss et al., 2010).

The genomic RNA (also referred as 42S RNA for SFV and 46S RNA for SINV) of alphaviruses contains two open reading frames, which encode 10 proteins in total. The 5' two-thirds of the genome encode the non-structural (ns) proteins, designated nsP1, nsP2, nsP3, and nsP4, and form the virus-specific part of the viral replicase. The 3' one-third of the genome is responsible for synthesizing structural proteins. The precursor or precursors of the ns-proteins are translated directly from the genomic RNA. For synthesis of the structural proteins, subgenomic mRNA (sgRNA, also referred as 26S RNA in SFV) is required. The 26S RNA is not included in the virion and is synthesized only in infected cells from an internal promoter located on the minus-strand of a double-stranded RNA replicative intermediate (Levis et al., 1990; Strauss and Strauss, 1994) (see also figure 1). The sequence of the 26S RNA overlaps with the last one-third of the 42S RNA. Both the genomic RNA and sgRNA contain a cap0 structure at the 5' end and a 3' poly(A) tail (Strauss and Strauss, 1994).

There are four known conserved structural elements (CSE) in the genomic RNA of all alphaviruses. Approximately the first 44 nucleotides from the 5' end of the genome form CSE 1, which is thought to act as a promoter to synthesize genomic RNA from the negative strand and as a co-promoter for synthesis of the negative-strand from the positive-strand template. The second CSE is located slightly downstream of CSE 1 in the nsP1 coding region. This element, termed CSE 2, measures 51 nucleotides and facilitates both negative- and positive-strand RNA synthesis (Ou et al., 1983; Frolov et al., 2001). Additionally, it has been shown that CSE 2 is crucial for alphavirus replication in insect cells, whereas the role CSE 2 plays in vertebrate cells is smaller (Fayzulin and Frolov, 2004). The third CSE is located at the junction of the regions coding for

ns and structural proteins. CSE 3 overlaps with the region encoding the C-terminus of nsP4 and extends to a short non-coding region located upstream of a structural open reading frame. CSE3 is also referred to as a subgenomic promoter because it is essential for the synthesis of sg mRNA. The minimal length of the sg promoter in SINV and most alphaviruses is 24 nt (positions -19 to +5 with respect to the transcription start site); however, to be fully active, the sg promoter must measure 112 nt (Levis et al., 1990). Curiously, the minimal sg promoter in SFV is longer than in most alphaviruses (Rausalu et al., 2009). CSE 4 is located almost at the 3' end of the genome (immediately upstream of the poly(A) tail) and is 19 nt in length. CSE 4 functions (together with CSE 1) in the synthesis of the negative RNA strand; the site of negative strand synthesis initiation is located at the 3' end of CSE 4 (Hardy, 2006).

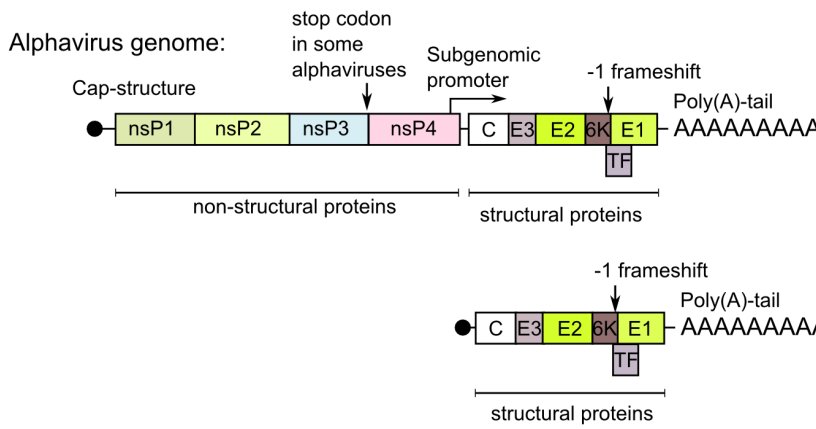

Figure 1. Alphavirus genome organization. The alphavirus genome has positive polarity, a cap-structure at the 5' end and a poly(A)-tail at the 3' end; the genome acts as the mRNA for synthesizing the ns-polyprotein. A number of alphaviruses contain an in-frame stop codon near the end of the nsP3 coding region, and read-through of the codon occurs with a frequency of approximately 10%. The subgenomic promoter region is required for synthesizing capped subgenomic mRNA, which in turn is required for the translation of structural proteins. A signal for a '-1' ribosomal frame shift is located in the region encoding the 6K protein; if the ribosomal frame shift occurs, the TF protein is synthesized.

In addition to the conserved sequence elements needed for RNA replication or transcription, SFV and SINV also contain other important RNA structures. First, the 5′ end of the capsid gene encoding the first 34 aa residues has been shown to contain a translational enhancer that is needed for the efficient synthesis of structural proteins in infected cells in later phases (Frolov and Schlesinger, 1994; Sjöberg et al., 1994). Second, the sequence encoding the 6K protein contains the -1 ribosomal frame-shift signal (Firth et al., 2008) that results in synthesis of the structural TF protein. Third, SINV and likely a number of SFV strains contain specific signals facilitating the read-through of a

termination codon located close to the end of the sequence encoding the nsP3 protein (Firth et al., 2011). Finally, a region encoding non-structural proteins contains a packaging signal for the alphavirus genome; in this regard, SFV and SINV clearly differ. The packaging signal in the SINV genome is located in the region encoding the nsP1 protein and shares similarity with the packaging signals of other alphaviruses (Frolova et al., 1997). In contrast, the packaging signal in SFV (and possibly a number of related alphaviruses, such as CHIKV) is located in the region encoding the nsP2 protein and has a different organizational pattern (White et al., 1998).

2.4. Alphavirus replication cycle

2.4.1. Alphavirus entry

The first steps of alphavirus entry have been investigated in great detail (reviewed by Leung et al., 2011). The alphavirus entry is a receptor-mediated process, and the E2 protein is an antireceptor responsible for virion binding to the cell surface (Tucker and Griffin, 1991; Smith et al., 1995). Alphaviruses can infect a large number of different cells; however, the cell surface receptors are currently unknown for most of the members of this genus. In SINV, the laminin receptor may be the high-affinity attachment receptor (Wang et al., 1992), and it has been proposed that the binding of virions is dependent on heparan sulfate (Klimstra et al., 1998). Recently, an alternative receptor, NRAMP (natural resistance-associated macrophage protein), was shown to facilitate SINV entry into both *Drosophila* and mammalian cells (Rose et al., 2011). However, NRAMP is not the universal receptor for alphaviruses because it is not required for entry of the Ross River virus. To date, no receptor for SFV has been identified.

Cell-bound virions are internalized via clathrin-dependent endocytosis (Helenius et al., 1980; DeTulleo and Kirchhausen, 1998). As endocytosis proceeds, the endosomes mature and the intravesicular pH becomes acidic, causing conformational changes within the envelope. The E1-E2 heterodimers are destabilized, leading to exposure of the E1 fusion peptide that was previously shielded by E2. Subsequently, the fusion peptide is inserted into the endosomal membrane, which leads to trimerization of the E1 proteins and eventually to the fusion of the virion envelope and endosome (Kielian and Helenius, 1985; Wahlberg et al., 1992; Bron et al., 1993; Justman et al., 1993). The process of fusion is dependent on the presence of sphingolipids and cholesterol (Kielian et al., 2010). The NC is then released into the cytoplasm, where it becomes disassembled. It has been shown that the capsid proteins from NCs become bound to ribosomes (Singh and Helenius, 1992); accordingly, genomic RNA is liberated, and viral ns proteins are translated.

2.4.2. Expression of ns-proteins, replicase complex formation and genome replication

In alphaviruses, similar to all positive-strand viruses, the released genomic RNA is used as a template to produce the viral components of RNA replicase (Figures 1 and 2). In SFV, the replicase proteins are expressed in the form of a precursor ns-polyprotein. It has been shown that this mode of expression is crucial for the subsequent formation of replication complexes (Strauss and Strauss, 1994; Salonen et al., 2003).

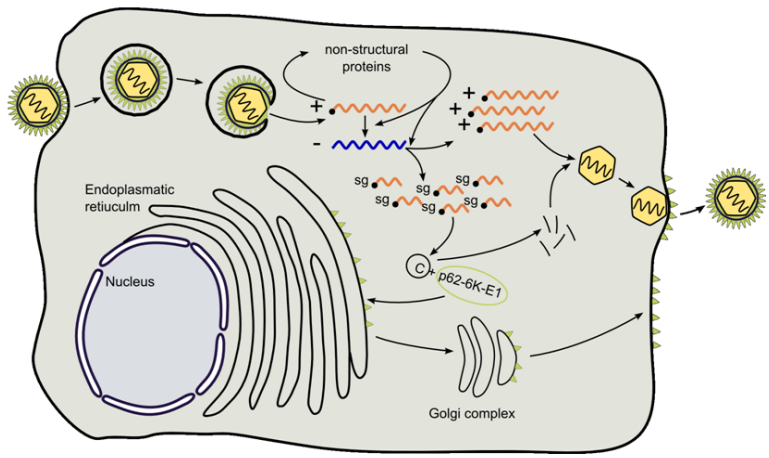

Figure 2. Alphavirus replication cycle. Following the binding to the cellular receptor, the virion enters the cell via endocytosis. Acidification of endosomes leads to the structural rearrangement of envelope proteins, resulting in fusion of the virion envelope with the endosomal membrane. The nucleocapsid is released into the cytoplasm, followed by liberation of the genomic RNA that is used to synthesize the ns-polyproteins required for the synthesis of negative strand RNA and the formation of replication complexes (RCs). RCs are active in making new genomic and subgenomic RNAs. Subgenomic RNAs are translated to generate structural proteins. A capsid protein resides inside the cytoplasm, where it binds genomic RNA; other structural proteins are transported via the endoplasmic reticulum and Golgi complex to the plasma membrane, where virion budding occurs.

The synthesized ns-polyprotein is cleaved into processing intermediates and then into individual ns-proteins in a well-controlled manner at the region corresponding to the nsP2 protein (Vasiljeva et al., 2003; Lulla et al., 2006). In the majority of alphaviruses (e.g., SINV, VEEV and even certain SFV strains), the nsP3 coding region contains an opal termination codon (UGA) in the 3' region; therefore, these viruses express mainly a shorter P123 polyprotein. However, in approximately 10-20% of cases, terminator read-through occurs, and a full-length P1234 is synthesized. P1234 contains the last 6 aa residues of nsP3 (part of the cleavage site between nsP3 and nsP4) as well as the full nsP4

sequence (Li and Rice, 1993; Strauss and Strauss, 1994; Lulla et al., 2006). Therefore, in most alphaviruses, two nsP3 isoforms that differ in the presence or absence of the 6 C-terminal aa residues are present in infected cells. However, in most SFV isolates (such as SFV4 and SFV L10) and CHIKV isolates from recent outbreaks (such as LR2006OPY1, SGP011 and IND91), the opal codon is absent, and only P1234 is synthesized. Consequently, these viruses express only a single form of nsP3, which corresponds to the longer version of the nsP3 in SINV and VEEV.

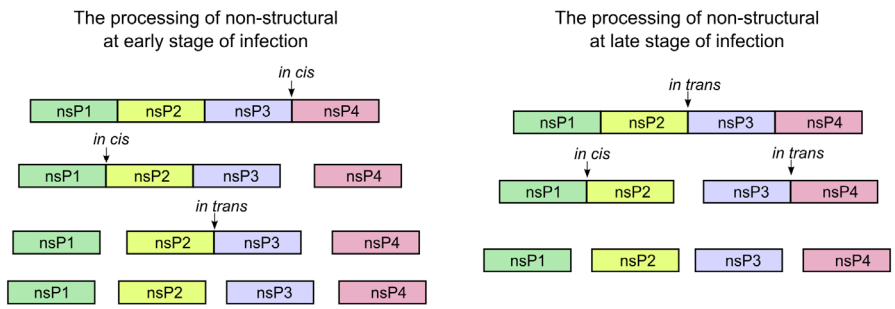

Figure 3. Processing of ns-polyprotein by the protease activity of nsP2 region. Left, the processing of ns-polyprotein at the early stages of infection. Initial cleavage occurs *in cis* and results in P123+nsP4, an early replicase that is active in negative strand synthesis. Cleavage of P123 *in cis* yields nsP1+P23. The final cleavage of P23 occurs *in trans*, and the replication complexes are active only in the synthesis of genomic and subgenomic RNAs. Right, processing order in the late stages of infection. Because the cleavage of P1234 occurs between nsP2 and nsP3, there is no early replicase (P123 + nsP4) and, therefore, no synthesis of negative strands.

The formation of functional replicase complexes is regulated by processing of P1234. To generate a functional replicase, the processing events must proceed as follows: First, the synthesized ns-polyprotein P1234 is cleaved (most likely *in cis*) into P123 and nsP4, activating the catalytic activity of nsP4, which together with P123 forms the early replication complex (Fig. 2, 3). The early replicase is effective in synthesizing negative strand RNAs; however, this replicase does not make the plus strands (or makes them with low efficiency). The double-stranded RNA (dsRNA) molecules are formed from the negative and positive strand templates. Several different forms of these dsRNAs can be purified from alphavirus-infected cells (replication intermediates and replication forms). Negative strand synthesis occurs early in the infection process. In late infection (4 to 6 hours post-infection, depending on conditions), negative strand synthesis ceases, likely because of the switch to the P1234 processing pathway (Vasiljeva et al., 2003). The early replicase complexes are short-lived (they are likely capable of a single round of negative-strand RNA synthesis) and are rapidly converted into the late replicase complexes. The cleavage between nsP1

and nsP2 occurs *in cis*, yielding nsP1, P23, and nsP4 (Lemm et al., 1994; Shirako and Strauss, 1994; Vasiljeva et al., 2003). These proteins are effective in synthesizing both negative- and positive-strand RNAs; however, in a wt alphavirus infection, the complex is extremely short-lived (cannot be detected using 5' pulses, indicating a half-life less than one minute), making it unlikely that this replicase complex plays a significant role in wt virus infections.

The P23 processing occurs *in trans* (Vasiljeva et al., 2003) and leads to the formation of a stable later replicase complex that is composed of individual nsP1, nsP2, nsP3 and nsP4 proteins. According to current knowledge, the cleavage of P23 marks the point of no return because it transforms the replication complex into the late form and prevents the subsequent synthesis of negative-strand RNAs. The late replicase is capable of synthesizing only positive-strand, genomic and sgRNAs (Lemm et al., 1994; Shirako and Strauss, 1994; Vasiljeva et al., 2003; Lulla et al., 2012). Positive-strand RNAs are synthesized from the negative strand (or rather from the dsRNA intermediate) template. The synthesis of positive strands continues until the death of the infected cells. Genomic RNAs interact with the capsid protein, and they are packed into new virions; subgenomic mRNAs are used as templates for structural proteins.

In the late stages of infection, the order of ns-polyprotein processing is altered (Fig. 3). It is likely that processing occurs because of the accumulation of free nsP2 in the cytoplasm of infected cells. Free nsP2 is responsible for the rapid cleavage of P1234 (likely before the protein is fully translated) at the cleavage site between nsP2 and nsP3, generating P12 and P34. Thereafter, the P12 is self-cleaved to yield nsP1 and nsP2. P34 remains uncleaved (in SINV) or is processed by nsP2 (in SFV) into nsP3 and nsP4. No combination of these proteins and cleavage intermediates is capable of forming new replication complexes that can synthesize negative strands (Vasiljeva et al. 2003); the released nsPs exhibit other functions in infected cells and/or become degraded (see chapter 2.5 for details).

Alphavirus replicase complexes are always associated with modified intracellular membranes. In electron microscopy images, the complexes have the appearance of small sac-like invaginations termed spherules, representing sites of replication and transcription (Froshauer et al., 1988; Kujala et al., 2001). The spherules first appear on the plasma membrane of infected cells, and RNA and ns-proteins, synthesized in form of P1234 precursor, are needed for this process (Frolova et al., 2010; Spuul et al., 2010). Next, the spherules are internalized through endocytosis, and a step-by-step transportation and fusion process causes the spherules to bind to modified endosomes and lysosomes. Eventually, large static cytoplasmic vesicles are formed, referred to as type I cytopathic vacuoles (CPV-I), and the diameter of these structures is 0.6-2 μm (Strauss and Strauss, 1994). The inner environment of a spherule is connected to the cell cytoplasm via a narrow channel with a diameter of 8 nm. Each spherule likely contains one dsRNA molecule and an unknown number of viral ns-proteins; additionally, several host proteins are bound to the spherules (Frolova et al., 2010; Spuul et al., 2010). Currently, the stoichiometry of the ns-

proteins, viral RNA and host components in the early and late alphavirus replicase complexes is unknown.

The positive RNA strand synthesis is coupled with their use, and there is a functional link between the synthesis of genomic RNAs and their packaging into nucleocapsids. Similarly, the translation of sgRNAs occurs near spherules. The sgRNA is used as a template to synthesize the structural polyprotein in the form of C-p62(E3E2)-6K-E1. The C protein is cleaved autocatalytically from the polyprotein (Choi et al., 1991). This autocleavage step exposes the signal peptide at the beginning of the E3-region, which leads to binding to the ER, and the remaining polyprotein is inserted into the ER as it is synthesized (Garoff et al., 1990). The p62-6K-E1 protein contains several membrane-spanning regions (Fig. 4). In the ER, the structural polyprotein is processed and modified; it is heavily glycosylated, palmitoylated and cleaved by cellular proteases into individual p62, 6K and E1 proteins. The p62-E1 heterodimer is formed and transported from the ER to Golgi, and during transport, p62 is cleaved into E3 and E2 (Liljeström and Garoff, 1991b; Strauss and Strauss, 1994). The E2-E1 heterodimers are transported to the plasma membrane to participate in virion formation. It has been shown that in a number of alphaviruses (such as SFV, CHIKV, and VEEV), E3 is also incorporated into virions, whereas this does not occur in the majority of alphaviruses (including SINV). The functions of the 6K protein are largely unknown; however, this protein can be deleted without deleterious effects on viron formation. The 6K protein, which is incorporated into virions in smaller amounts, likely affects the interactions between E2 and E1 (Jose et al., 2009). During the translation of SFV structural proteins, a ribosomal frame-shift can occur in the sequence of 6K producing the transframe (TF) protein (Firth et al., 2008). The TF protein can also be packaged into virions; however, the functions of this protein are unclear (Snyder et al., 2013) and are likely associated with virion formation.

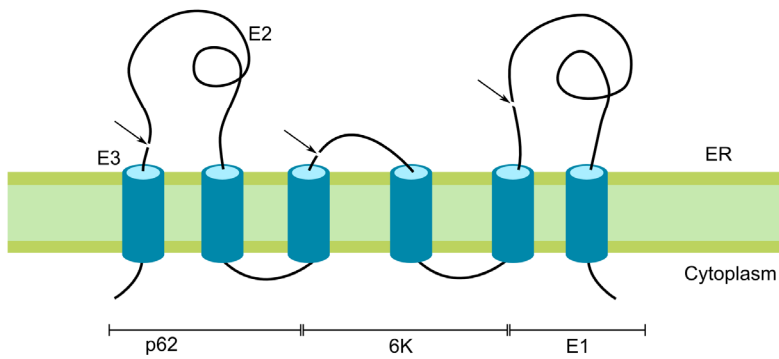

Figure 4. Arrangement of structural proteins (except capsid protein) in cellular membranes. The structural proteins are synthesized as a single polyprotein precursor. The capsid protein is cleaved autocatalytically from the precursor, liberating the ER-targeting signal at the beginning of the E3 protein. In the ER, p62-6K-E1 is cleaved into p62, 6K and E1. p62 is processed into E3 and E2 in the Golgi complex (cleavage sites are indicated by arrows).

The budding of new virions in infected vertebrate cells occurs in the plasma membrane. The formation of nucleocapsids likely starts in the cytoplasm near replicase complexes; the newly synthesized genomic strands are packed into the capsid. The packaging signal in SFV is located in the genomic RNA in the region encoding the nsP2 protein and is required for interactions between the RNA and the C protein (White et al., 1998). This process leads to the multimerization of C proteins, and an icosahedral NC is formed. The interaction between "the pocket" in the C protein and the C-terminal domain of E2 is required to trigger the budding and release of virions (Zhao et al., 1994; Jose et al., 2009).

2.5. Individual properties of ns-proteins in alphaviruses

All nsPs are required for alphavirus replication; each protein plays a unique and specific role during infection. At least three of the nsPs are also present outside of replicase organelles; this nsP fraction also plays a significant role in virus infection (Fig. 5).

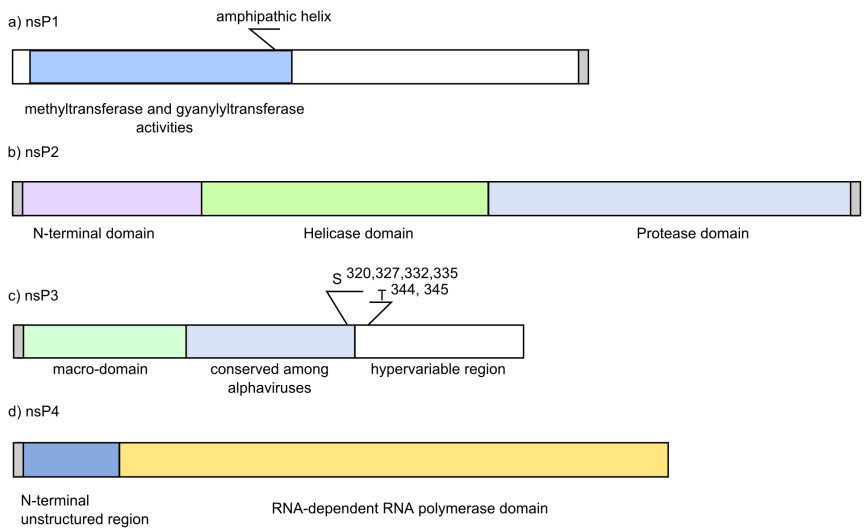

Figure 5. Schematic representation of ns-proteins of alphaviruses a) nsP1, b) nsP2, c) nsP3, and d) nsP4. Regions at the N-terminus and C-terminus of proteins (if present) needed for recognition by nsP2 to perform P1234 polyprotein cleavage are depicted in grey.

nsP1 (size 537 aa) is the only membrane-binding protein in the replicase and is involved in the synthesis of the cap-structure of the virus genome and sgRNA (Fig. 5A). As part of a polyprotein and in the context of the mature alphavirus

replicase, nsP1 is tightly bound to the membrane (Salonen et al., 2003). The binding of nsP1 to the membrane is crucial for the viral replicase because the other virus-specific components in this complex lack this property. The most important membrane anchor in nsP1 is a 22-aa peptide, which forms an amphipathic alpha-helix and is located between aa 245 and 264 in SFV. Because one side of the helix is hydrophobic and the other side is hydrophilic, this 22-aa peptide is localized partially inside the cellular membrane. Binding with the anionic membrane phospholipids is absolutely required for the enzymatic activity of nsP1; point mutations in the helix region, which prevent binding to the membrane, are lethal to the virus (Ahola et al., 1999; Spuul et al., 2007). However, this feature may not be universal for all alphaviruses; all nsP1s contain amphipathic helixes, but nsP1 in SINV is also enzymatically active in the absence of anionic lipids (Tomar et al., 2011)

In addition to the amphipatic helix, three consecutive cysteines in SFV nsP1 are post-translationally palmitoylated (region 418-420 aa); this modification further strengthens the membrane binding but is not required for the enzymatic activities of nsP1 or the viability of the virus. When nsP1 in SFV is palmitoylated, it induces the formation of filopodia-like structures on the plasma membrane via an unknown mechanism (Laakkonen et al., 1996, 1998). The functions of these structures are unknown; however, they may play a role in cell-to-cell transmission of SFV. The palmitoylation site clearly overlaps with other functional determinants of the virus; deletions and substitutions in the palmitoylation region disrupt the interaction between nsP1 and nsP4 and therefore virus replication. However, the virus recovers from these mutations through the accumulation of second-site compensatory mutations that restore the nsP1 – nsP4 interactions and virus replication. Interestingly, all known compensatory changes were located in nsP1; however, none of the changes restored palmitoylation of the protein (Zusinaite et al., 2007). The importance of the nsP1 interactions with nsP4 has been demonstrated in several studies. Studies using temperature-sensitive mutants of SFV and SIN have suggested that nsP1 regulates negative strand synthesis via interactions with nsP4 (Shirako et al., 2000; Lulla et al., 2008).

The enzymatic functions of nsP1 are required for capping genomic and sg mRNAs. The first reaction in nascent RNA capping is performed by nsP2, which exhibits RNA triphosphatase (RTPase) activity, whereas the next two reactions are performed by nsP1 (Mi and Stollar, 1991; Laakkonen et al., 1994). First, nsP1, which is a guanylyltransferase, forms a covalent complex with GMP. Second, nsP1 transfers a methyl group from S-adenosyl-methionine to the nsP1-GMP complex (methyltransferase activity), generating nsP1-m^7GMP complexes (Ahola and Kääriäinen, 1995). These reactions are conserved throughout the entire alphavirus-like superfamily of viruses and clearly differ from the reactions used for the synthesis of the cellular cap structure, in which the GMP residue is transferred to the RNA, after which it is methylated. To date, the enzyme that performs the final reaction of alphavirus cap synthesis (transfer of m^7GMP from nsP1 to RNA molecule) has not been identified.

nsP2 is the largest ns protein (size 799 aa). Consistent with its size, this protein also has the largest number of known enzymatic and non-enzymatic functions (Fig. 5B). nsP2 consists of two different functional regions, each of which likely contains more than one domain. The N-terminal portion of nsP2 (aa residues 1-470) exhibits nucleotide triphosphatase (NTPase) and RNA trisphosphatase (RTPase) activities (Rikkonen et al., 1994a; Vasiljeva et al., 2000). The RTPase function of nsP2 is needed for cap-structure generation (see also nsP1 above), and the NTPase activity is required for helicase activity; helicase motifs have been identified in the N-terminal portion of the protein (Koonin & Dolja, 1993). However, the N-terminal of nsP2 is unable to unwind dsRNAs, indicating that a number of the sequences required for this activity are located in another region of the protein. Indeed, full-length nsP2 has been shown to function as a helicase. However, the role of RNA helicases in the replication of positive-strand RNA viruses is unclear. It is not known if nsP2 unwinds the viral dsRNA replication intermediate and/or is involved in the unwinding of secondary structure elements in the viral RNA genome (Gomez de Cedrón et al., 1999).

The C-terminal portion of nsP2 consists of two distinct domains but exhibits only one known enzymatic activity. The first domain in this portion of the protein is a protease domain, which is homologous to papain-like proteases. The nsP2 protease is the only protease required for processing P1234 fully, and the catalytic cysteine residue is located at position 478 (Merits et al., 2001). The structures of VEEV protease (Russo et al., 2006), SINV protease (Shin et al., 2012) and CHIKV protease (Cheung et al., 2011) have been determined via X-ray crystallography. In all three proteases, the papain-like domain is followed by a methyltransferase-like domain. The methyltransferase-like domain is not active as a methyltransferase; however, it plays an obvious role in the protease activity of nsP2 and may be important for other enzymatic activities of the protein.

Several criteria must be met for proteolytic cleavage by nsP2 to ensure that the cleavages occur in a particular order. The aa sequences surrounding both sides of the processing site are important; however, the structural placement of the nsP2 domains and other replicase proteins is also important (Vasiljeva et al., 2003; Lulla et al., 2006, 2012).

In infected cells, approximately 25% of the nsP2s are associated with replicase organelles, whereas 25% of the nsP2s are located diffusely throughout the cytoplasm and may be crucial for switching P1234 processing to the late pathway (P1234 to P12 and P34) and the possibility of superinfection exclusion. Of the nsP2s in the cell, 50% are transported to the nucleus. Furthermore, when nsP2 is expressed *outside of the P1234 context*, the protein is located almost exclusively in the nucleus (Rikkonen et al., 1994b). The mechanism(s) of nuclear transport of nsP2 is controversial; SINV nsP2 lacks a classical nuclear localization signal (NLS) (Frolov et al., 2009), whereas the PRRRV sequence in SFV nsP2 (position 647-651 aa) is assumed to function as an NLS. It has been demonstrated that mutating arginine residues to aspartate residues in this

sequence results in the cytoplasmic localization of nsP2 and also affects the cytotoxicity of SFV and polyprotein processing (Fazakerley et al., 2002; Tamm et al., 2008).

In Old World alphaviruses, nsP2 is the main viral component responsible for the cytopathogenicity of the infection. Even the expression of a single nsP2 can lead to the shut-down of cellular transcription and translation; these effects are also observed in the context of virus infection, in which these changes favor the synthesis of viral macromolecules (Gorchakov et al., 2005; Garmashova et al., 2006). The mechanisms of nsP2-mediated translational shutdown are unclear; however, it has been demonstrated that nsP2 interacts with several ribosomal proteins, and it is not known if and how this interaction affects translation. Recently, the mechanism of transcriptional shutdown has been demonstrated. It was determined that in vertebrate but not invertebrate cells, nsP2 causes the degradation of RNA polymerase II (Akhrymuk et al., 2012). Curiously, the New World alphavirus nsP2 lacks this ability; instead, CP is responsible for the cellular transcription block (Garmashova et al., 2007). In addition to the inhibition of cellular macromolecule synthesis, the nsP2 in Old World alphaviruses is also active in the innate immune response (Breakwell et al., 2007).

nsP3 (size 482 aa) has been relatively enigmatic for a long time (Fig. 5C). The protein can be divided into three regions; the first 160 amino acids form a structurally conserved macro-domain that is conserved among alphaviruses, rubiviruses, hepeviruses and coronaviruses (Koonin and Dolja, 1993). Macro domains are found in proteins from bacteria, archae and eukaryotes. The crystal structures of the macro domain in CHIKV, VEEV and SINV have been determined; the CHIKV and VEEV macro domains are active adenosine diphosphoribose 1''-phosphate phosphatases. This activity was undetectable in the macro domain in SFV nsP3, suggesting that this function is not needed for virus replication. The alphaviral macro domain can bind ADP-ribose, poly-ADP-ribose and RNA, and binding to the RNA might be the true function of the nsP3 macro-domain (Malet et al., 2009; Neuvonen and Ahola, 2009). The C-terminal region of the macrodomain (or the residues immediately downstream of the domain) represents an important determinant for processing the cleavage site between nsP2 and nsP3 (Lulla et al., 2012)

The second nsP3 region is similar in length to the first region, and based on the sequence similarity only conserved among alphaviruses (Strauss and Strauss, 1994). For a long time, nothing was known about the functions of this region. Recently, however, this region was crystallized as part of the SINV nsP2-nsP3 polyprotein (Shin et al., 2012). It was demonstrated that this region binds zinc ions, the crystal structure of the SINV polyprotein revealed that in the region beginning at the protease portion of nsP2 to the end of the zinc-binding domain of nsP3, the zinc-binding domain makes contact with the nP2 protease region at multiple sites, and site-specific mutagenesis demonstrated that these interactions are essential to the virus. Finally, the 3-D structure of this domain suggests that, similar to the macro domain, this region participates in the accommodation of the RNA molecule.

The third region of nsP3 is represented by hypervariable sequences of different lengths. Unlike the first two domains, this region of nsP3 is intrinsically unstructured. Despite the lack of overall sequence similarity within this region, the region contains areas of functional similarities. First, the region contains a large number of short linear motifs (often repeated more than once) shared between different, but usually not all, alphaviruses. These elements likely bind to different sets of interacting cellular proteins. The identity of these cellular proteins is unknown, although amphiphysins have been shown to interact with the proline-rich element in nsP3 (Neuvonen et al., 2011). Furthermore, it has been shown that the cellular proteins bound to this sequence are different in New World and Old World alphaviruses. Additionally, a duplicated sequence motif at the end of C-terminus of VEEV nsP3 is essential for efficient VEEV replication in different cell lines (Foy et al., 2012, 2013). nsP3 represents the only alphavirus phosphoprotein, containing a cluster of phosphorylated serine and threonine residues at the junction between the second and third domain (Vihinen and Saarinen, 2000; Vihinen et al., 2001). In SFV, there are 16 phosphorylation sites in total, which are located in a 50-aa region, and six of these residues ($S^{320, 327, 332, 335}$ and $T^{344, 345}$) account for the majority of the nsP3 phosphorylation. The elimination of the phosphorylation sites has a relatively minor effect on replication in mammalian SFV and VEEV (Vihinen et al., 2001; Foy et al., 2013); however, in Sindbis, phosphorylation plays a role in negative strand synthesis (Dé et al., 2003).

Whereas nsP1 is needed for anchoring of the replicase complex proteins to the plasma membrane, nsP3 is required for targeting the replicases to the endolysosomal membranes. When expressed alone, nsP3 localizes to cytoplasmic non-membranous granules of variable sizes. When nsP3 is expressed as part of the P123 polyprotein, it triggers the re-localization of viral proteins from the plasma membrane to endolysomal membranes, which have an appearance similar to CPVs except that spherule structures are not formed (Salonen et al., 2003). This ability to form different complexes does not represent an artifact of recombinant protein expression and can be observed in alphavirus infection. In SINV, it has been demonstrated that a fraction of nsP3 can be found in replication organelles, whereas the remaining proteins form different complexes located in the vicinity of the nuclear envelope (Gorchakov et al., 2008). The different complexes exhibit different functions, and correspondingly, the cellular partners of nsP3 vary depending on the type of complex.

nsP4 (size 614 aa) is the alphavirus RNA-dependent RNA-polymerase (RdRp) (Fig. 5D). The C-terminal portion of the protein exhibits sequence homology with other known RdRps, including the conserved RdRp motif GDD. The first 100 (approximately) N-terminal aa of nsP4 exhibit no similarity with known sequences from different viruses and cells and are conserved only among alphavirus proteins. The function of these aa is unknown; however, genetic evidence suggests that these sequences might be involved in interactions with other ns-proteins (Rupp et al., 2011). Although nsP4 is responsible for synthesizing new RNA strands, it only acquires this function following previous

contact with other ns-proteins (Rubach et al., 2009). This observation suggests that correct folding of nsP4 occurs only if the other ns-proteins (presumably in the form of P123) are present. Once folded correctly, nsP4 separates from the other ns-proteins and can synthesize genomic positive- and negative-strand RNAs, but not sg mRNAs.

The levels of nsP4 in alphavirus-infected cells are relatively low for the following two reasons: 1) in most alphaviruses, an opal stop codon is located near the end of nsP3; therefore, P1234 can be only synthesized if stop-codon read-through occurs (Strauss and Strauss, 1994). 2) A peculiar feature of nsP4 is that the first aa is always a Tyr residue. According to the N-end rule, Tyr is a destabilizing aa, and the individual nsP4 is degraded rapidly by proteasomes unless it becomes incorporated into replicase complexes in which it is protected from degradation (de Groot et al., 1991). Moreover, the Tyr residue at the N-terminus of nsP4 is required for polymerase activity; the virus replicates efficiently only if this aa position is occupied by Tyr, and the only acceptable substitutions are other aromatic residues or histidine. Other mutations are lethal for the virus (the Met residue is somewhat tolerated) and result in the selection of genomes with reversions or second-site mutations found in nsP4, nsP1 and the 5'-terminal region of the RNA genome (Shirako and Strauss, 1998). These findings indicate that the N-terminal Tyr residue is involved in the recognition of conserved sequence elements required for replication, and this function is performed in conjunction with other replicase proteins.

The catalytic activity of nsP4 was investigated in a deletion mutant lacking the first 97 aa (del97nsP4). Following expression in *E.coli*, the purified del97nsP4 enzyme lacks RdRp activity but retains terminal adenylyltransferase activity, a function likely required for the maintenance and repair of the poly(A)-tail at the end of genomic and sg mRNAs (Tomar et al., 2006). Indeed, it is known that alphavirus can repair truncated poly(A) sequences at the end of the genome; furthermore, RNAs lacking complete poly(A) sequences are infectious and acquire these sequences during replication (Raju et al., 1999). It is likely that the poly(A) sequences in alphavirus positive strands are always synthesized using this nsP4 adenylyltransferase activity; recent evidence demonstrated that the synthesis of the negative strand is initiated at the first nucleotide upstream of the poly(A) sequence (Hardy, 2006).

The unstructured N-terminus is required for interaction with other ns-proteins, particularly nsP1 (Shirako et al., 2000). Furthermore, studies have indicated that the N-terminal region might play an important role in recognition of the promoter at the 3' end of the genomic strand for negative strand synthesis (Rubach et al., 2009), although the presence of P123 is needed for correct folding of nsP4. Furthermore, chemical crosslinking has demonstrated that nsP4 contains distinct regions for the recognition of genomic (residues 531-538) and sg (residues 329-334) promoters on negative strands. Again, nsP4 alone recognizes the genomic promoter (Li and Stollar, 2004, 2007) but for the recognition of sg-promoters, other nsPs, particularly nsP2, are required. These requirements may explain the properties of early and late alphavirus replicase

complexes. ns-polyprotein processing affects the transition from negative strand synthesis to positive strand generation, likely reflecting re-arrangements resulting from the processing of the other replicase proteins, and the N-terminal domain of nsP4 acts as an important switch, recognizing these re-arrangements (Rupp et al., 2011).

2.6. Alphavirus vectors

Infectious cDNA clones have been generated for many alphaviruses; therefore, genetic manipulation of the viruses is feasible, and alphavirus-based expression vectors are widely used. The advantages of these vectors include the rapid production of high-titer vector stocks, a broad host range (from insect cells to primary mammalian cells), rapid RNA replication and high expression levels of proteins of interest. Alphavirus-based vectors are also characterized by their cytotoxicity to the host cells, the activation of the innate immune response and short-term expression of foreign proteins. Depending on the application, these properties of alphaviruses can be viewed as disadvantages or advantages, for example, for killing cancer cells (Riezebos-Brilman et al., 2006) or vaccine development. Therefore, many alphavirus-based vectors have been designed for vaccine development, gene therapy of central nerve system diseases and anti-cancer therapy (Atkins et al., 2008). In addition, alphavirus based-vectors are used frequently in basic research, for example, to investigate the spread of SFV in the nervous system (Fragkoudis et al., 2009).

Alphavirus vector designs can be divided into two basic categories, alphavirus replicon vectors and replication-competent vectors. In replicon vectors, the strategy is to replace the region encoding structural proteins with the foreign gene (Xiong et al., 1989; Liljeström and Garoff, 1991a). Replicon vectors are capable of replication but are defective in particle formation. This limitation can be circumvented using virus replicon-particle (VRP) technology. VRPs can be produced if the *in vitro* transcribed replicon RNA is transfected into susceptible cells together with helper RNAs carrying sequences essential for the expression of structural proteins. In transfected cells, replicon and helper RNAs act like genomic RNAs; the replicase complexes generated using replicon RNA can recruit helper-RNA, which is replicated and transcribed. Transcription results in the production of the mRNA for structural proteins, which are needed for packaging replicon RNAs and the formation and release of VRPs. In general, helper RNAs are designed to lack a RNA packaging sequence and are not included into the VRPs. Therefore, VRPs are limited to a single round of infection.

Typically, replication-competent vectors are designed by placing the expression cassette containing a duplicated sg promoter and the foreign gene of interest downstream of the region encoding the structural proteins. Alternatively, the foreign gene can be placed under the control of a native sg promoter, and the expression of a duplicated sg promoter is used to express the structural

region (Atkins et al., 2008). Another strategy is to place the sequence of interest into the region containing the ns- proteins or structural proteins; in this case, the foreign protein is synthesized together with the viral proteins. Depending on the design, the foreign protein can be cleaved (Thomas et al., 2003; Tamberg et al., 2007) or remain fused with the viral protein. Using this strategy, different luciferases and fluorescent proteins have been inserted successfully into the hypervariable region of nsP3, producing viable viruses at high titers. Viable SINV carrying green EGFP fused to nsP2 has also been generated. The placement of EGFP into nsP2 or nsP3 allows monitoring of the dynamics of viral ns-protein expression and the changes in the location of these proteins during infection; GFP-based immunoprecipitation can also be used to identify the host proteins that bind nsP2 or nsP3 (Frolova et al., 2006; Atasheva et al., 2007).

To generate a cleavable foreign protein in the ns-region, the protein must contain a short specific stretch of aa from the nsP3 C-terminus at its tail. Additionally, the foreign protein must contain a short stretch of aa from the N-terminus of nsP4 at its own N-terminus. To increase the stability of the foreign protein, the first Tyr residue of the nsP4 sequence is changed to Gly (this change does not negatively affect cleavage by the viral protease). These stretches of aa are needed for processing-site recognition by nsP2 (Tamberg et al., 2007). There is also an option to insert a foreign protein between the C and E3 protein, in that case the 2A autoprotease from foot-and-mouth disease virus must have been added to the C-terminus of the foreign protein. C protein autocatalytically cleaves itself off from the introduced marker and to liberate E3 from the protein of interest 2A autoprotease is required (Thomas et al., 2003).

2.7. Aspects of alphavirus-host interactions

Alphaviruses need components synthesized by the host (e.g., proteins, lipids, energy, *etc.*) for genome replication, gene expression, and the formation of new virions, among other processes. The outcome of infection depends on several criteria, e.g., the viruses must direct changes in the inner cellular environment in their preferred direction, cope with the innate adaptive immune responses, manage possible attacks by the adaptive immune system, and be ready to handle competitor viruses and virus "relatives". To deal with these factors, alphaviruses have developed their own specific means "to get what they want".

2.7.1. Shutdown of cellular transcription and translation

In infected vertebrate cells, alphaviruses inhibit the synthesis of cellular RNA and proteins; simultaneously, the synthesis of virus-specific components is maintained at a high level. It has been demonstrated that transcriptional and translational shut-down are independent events (Gorchakov et al., 2005). The shut-down of cellular macromolecule synthesis allows the allocation of cellular resources to the virus and limits the production of antiviral proteins, including

type I interferons. This is certainly important for the virus because the release of type I interferons leads to autocrine and paracrine stimulation of the cells, which results in an antiviral state and stops the virus from spreading further (Frolova et al., 2002).

The role of nsP2 in Old World alphaviruses in the suppression of cellular antiviral responses has been known for some time; however, the mechanism of action is unknown. It has been determined that at the early phases of infection, nsP2 causes the degradation of the catalytic subunit Rpb1 of the RNA polymerase II complex. In the presence of nsP2, Rbp1 is ubiquitinated and therefore rapidly degraded, resulting in the cessation of host mRNA transcription (Akhrymuk et al., 2012). The downregulation of transcription leads to reduced type I interferon production (Gorchakov et al., 2005). Interestingly, in New World alphaviruses, a different mechanism is used; the capsid protein binds importin-alpha/beta and export receptor RCM1, and the complex accumulates at nuclear pores, eventually causing transcriptional shutdown (Garmashova et al., 2007).

Translational shut-down occurs partially because of the cellular defense mechanism. It is assumed that viral dsRNA synthesized in alphavirus-infected cells is recognized by PKR. This results, similar to many other viruses,in the phosphorylation of eIF2α and the inhibition of cellular translation initiation (Gorchakov et al., 2004; Barry et al., 2009). However, for alphaviruses, the PKR dependent shutdown of translation is not the only mechanism, or even the main mechanism, through which viruses achieve their goal. In SINV- or SFV-infected cells, the active synthesis of viral envelope proteins leads to their accumulation in the ER, which results in an unfolded protein response. In turn, this process activates one of the ER stress sensors, PERK kinase. Similar to PKR, PERK phosphorylates eIF2α. Alphaviruses likely use other mechanisms to achieve the shutdown of translation; however, these mechanisms are largely unknown.

A possible hypothesis is that the phosphorylation of eIF2α does not occur at the very beginning of infection; therefore, there is enough time for the translation of ns-proteins. However, the translation of ns-proteins remains efficient for many hours after the shutdown of cellular mRNA translation; therefore, translation must be less sensitive to inhibition. Furthermore, the translation of sgRNAs remains active until cell death. The most common hypothesis is that the 5’ end of this mRNA, although it contains a cap-structure, is translated in a cap-independent manner. Furthermore, the beginning of the coding sequence of the capsid protein folds into a stable secondary structure (the capsid enhancer), which stalls ribosomes in the infected cells and directs them to the correct site for the initiation of translation (Ventoso et al., 2006). Furthermore, it has been shown that for SINV at least, viral RNA translation is coupled with transcription (Sanz et al., 2007). However, the universality of these effects is uncertain. The sg RNAs in New World alphaviruses likely lack capsid enhancer structures, yet they are actively translated.

2.7.2. Host factors associated with alphavirus RCs

Several studies have attempted to identify the host factors used by different positive strand RNA viruses to complete the infection cycle. In general, the approaches used for these investigations are based on the identification of physical interactions between viral proteins (or RNAs) and host proteins. Similarly, functional screens have been used, including whole genome searches using siRNA libraries and/or yeast two-hybrid screens. The general picture emerging from these studies is that host factors participate in different stages of the viral replication cycle, e.g., in template recruitment, the shift from translation to replication, replicase complex assembly, RNA synthesis, viral RNA stabilization, packaging and the regulation of these processes. Host proteins that affect viral infection in a negative manner have also been identified (Li and Nagy, 2011; Nagy and Pogany, 2012). These screening approaches demonstrated that the cellular factors directly or indirectly involved in virus replication tend to be dependent on the method used for their identification, and the list is long, consisting of hundreds of proteins. The mechanism(s) of action is known only for a very small number of these proteins.

Alphaviruses are not an exception to these rules; the list of cellular factors that interact with alphavirus nsPs and RNAs is slowly but steadily growing. In initial studies, SINV expressing a GFP-tagged nsP3 was used (Cristea et al., 2006; Frolova et al., 2006; Park and Griffin, 2009). This line of investigation was extended using SINV expressing a GFP-tagged nsP2 (Atasheva et al., 2007), and more recently, SINV expressing nsP4 was tagged with a FLAG-tag (Cristea et al., 2010). Immunoprecipitation was used to pull-down the tagged protein and the associated cellular proteins. These studies led to the identification of overlapping sets of cellular partners, including G3BP1, G3BP2, PARP-1, several hnRNPs, and 14-3-3 proteins.

Alternative methods to identify interaction partners for alphavirus replicase proteins produced different results. A number of studies were somewhat specific and resulted in the identification of one or more host factors. Therefore, comparing the cytoplasmic membrane fractions between mock-infected and infected cells, hnRNP K was identified as an interaction partner for alphavirus RCs. The hnRNP K protein was also demonstrated to interact with the sg RNA from SINV (Burnham et al., 2007). Another cellular protein, HuR, was shown to bind to the 3' untranslated regions (UTRs) of both genomic and sg RNA strands, protecting them from degradation (Sokoloski et al., 2010) (Dickson et al., 2012). It was also demonstrated that the C-terminal domain of nsP3 from SFV and CHIKV recruits amphiphysins to RCs; based on this observation, it has been hypothesized that this interaction facilitates spherule formation because amphiphysins exhibit membrane-bending capabilities (Neuvonen et al., 2011).

Other studies were performed on a genome-wide scale. Accordingly, a genome-wide yeast two-hybrid screening produced a list of cellular proteins that overlapped with the CHIKV nsP-interacting proteins (Bouraï et al., 2012). Clearly, the results of this study were biased towards the nsP2-interacting

proteins. Interestingly, however, few of the proteins overlapped with the interaction partners identified via pull-down experiments in nsP2-tagged SINV infected cells (Atasheva et al., 2007). In addition, replicase-bound nsP3 and nP3 located in cytoplasmic granules have been shown to interact with different sets of host proteins (Gorchakov et al., 2008), and the same likely applies to other nsPs.

Similar to other RNA viruses, the current information regarding the precise roles and functions of the identified host proteins in the context of alphavirus infection is limited. Furthermore, a thorough investigation of possible host factors has not been performed in New World alphaviruses. Therefore, many important host components remain to be identified, and almost all factors will require further analysis to characterize their roles in virus infection and identify their mechanisms of actions.

2.7.3. Stress granules, P-bodies and RNA viruses

Cells contain several types of RNA-containing non-membranous granules or aggregates, many of which are dynamic structures appearing/disappearing in response to different viral infections. Several studies using RNA viruses have been performed to elucidate the interactions of the virus with these granules.
Cells can control post-transcriptional gene expression through the formation of stress granules and/or processing bodies. Briefly, once the cells are stressed (e.g., by heat, chemicals, infection, etc.), translation ceases, and the polysomes are disassembled. The mRNAs released as a result of these processes are sorted and stored until their fate is determined (Kedersha and Anderson, 2007).

The typical mechanism of translational arrest is the same as that described for alphavirus-infected cells; eIF2α is phosphorylated, and translation initiation is inhibited. Alternative pathways include virus (such as poliovirus)-initiated cleavage of eIF4G or inhibition of eIF4E helicase activity (reviewed by Lloyd, 2012). Translation initiation complexes that also contain the ribosomal 40S subunit are stalled, and the messenger ribonucleoprotein complexes (mRNP) can be converted to stress granules (SG) in a step-by-step process. Not all of the SG components have been identified. However, it has been demonstrated that SGs contain the stalled initiation complexes, including mRNA transcripts, initiation factors eIF3, eIF4E, eIF4A, eIFG, eIF4B, small ribosomal subunits and poly(A)-binding protein 1 (PABP1). In addition, a number of the components involved in translational silencing, e.g., TIA-1, TIAR, and FAST, are SGs. Finally, several components affecting mRNA stability (promoting its decay), e.g., PMR1, TTP and proteins that are normally involved in RNA editing, splicing or localization, are found in stress granules. Despite their morphological integrity, SGs are dynamic structures; their components shuttle rapidly in and out of the SGs. Notably, the identification of typical *bona fide* SGs can be difficult because not all structures that look like and function as SGs contain all the components listed above. Therefore, several SG markers are used to identify structures as SGs. The overexpression of several proteins, e.g., TIA-

1, TIAR, G3BP, TTP, BRF1, and FXR1, nucleates the formation of SGs. The proteins bind stalled translation initiation complexes, and the primary aggregation is followed by a secondary aggregation resulting from protein-protein interactions; consequently, the mRNA-binding protein aggregates grow larger and are visible under light microscopy. SGs are composed of multiple semi-independent mRNP complexes. In the next round, the proteins that do not bind to the mRNA itself are recruited via a so-called piggyback process. Once the stress is relieved, the SGs and stalled translation initiation complexes are activated (Fig. 6).

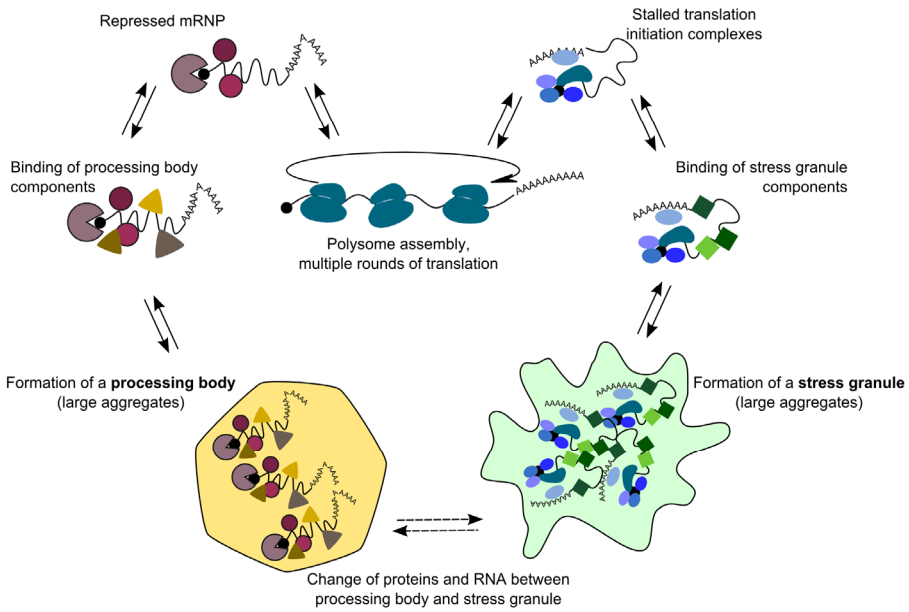

Figure 6. Interplay between polysomes, stress granules and processing bodies. In cells, as translation proceeds, polysomes are formed on the mRNA. When cells are stressed, translation is halted, and the ribosomes stall. Components of stress granules bind these complexes, and if the conditions are appropriate, smaller complexes aggregate into larger ones, leading to the formation of large stress granules visible under light microscopy. Alternatively, mRNA from polysomes is directed to the RNA degradation machinery. Again, smaller complexes aggregate into larger complexes, and processing bodies are formed. Processing bodies and stress granules can dock with each other and exchange molecules (proteins and RNAs).

Processing bodies (PBs) represent another type of structure that is assembled on untranslated mRNAs (Anderson and Kedersha, 2009). SGs are connected closely to the PBs. However, whereas SGs are less prone to movement in the cytoplasm and are somewhat dynamic in shape, PBs move more and maintain a

spherical shape. The number of components shared by PBs and SGs, such as eIF4E, FAST, and RCK proteins, underlines their similarities. However, PBs also contain unique components, such as decapping enzymes (Dcp1 and Dcp2), decapping activators (Dhh1 and Pat1), heptameric Lsm1-7 complex, 5'-3' exonuclease Xrn1 and components of the mRNA deadenylation machinery. In addition, proteins involved in non-sense-mediated decay and the miRNA machinery are found in PBs, e.g., proteins such as GW182 and Ge-1 are considered the scaffolding factors of PBs (Balagopal and Parker, 2009; Moser and Fritzler, 2010). The fate of the mRNA included in PBs is often different from that of the mRNA included in SGs; mRNAs in PBs can be degraded or stored for further translation. Similar to SGs, PBs are formed via the aggregation of aggregates, which is initiated by the binding of decapping and deadenylation factors to the translationally repressed mRNAs (Fig. 6).

SGs and PBs are dynamic structures in both their composition and movement inside the cell. These properties make their isolation and biochemical characterization difficult, if not impossible. Furthermore, these distinct structures can dock with each other and exchange proteins and mRNA. According to the so-called triage model, a number of the mRNAs in the SGs are sorted for degradation, whereas others are bound by stabilizing proteins or sent for translation. Similarly, a number of the mRNAs in PBs are saved from degradation and are transported to SGs (Fig. 6).

RNA viruses, stress granules, and processing bodies. It is known that infection with alphaviruses, orthoreoviruses and poliovirus activates cellular eIF2α kinases. Therefore, it is not surprising that the formation of SGs is initiated at the beginning of infection and that their number increases (reviewed in Lloyd, 2012; White and Lloyd, 2012) In this respect, SGs can be viewed as part of the cellular antiviral response. However, in the later stages of infection by different viruses, the SGs disassemble. Furthermore, at this stage their generation cannot be reinitiated using the chemical compound sodium arsenite, typically used for this process. Sodium arsenite acts via the promotion of eIF2α phosphorylation and the consequent inhibition of translation initiation. As an extreme example, poliovirus 3C protease cleaves G3BP proteins and prevents the formation of SGs. West Nile and Dengue viruses sequester the SG nucleating proteins TIA-1 and TIAR to the RCs, and Dengue virus can sequester G3BP, caprin1, and USP10. Therefore, viruses can remove SGs as components not promoting infection, or alternatively, the virus can take advantage of the proteins included in these structures.

Stress granules are not the only components that appear and disappear in virus-infected cells. Infection with West Nile virus and hepatitis C virus (HCV) also causes the disappearance of PBs. In HCV infections, it has been shown that at least a small number of the components of PBs are needed for replication of the virus, e.g., Lsm1 and DDX3. Poliovirus is also known to disrupt components of the RNA decay pathway, such as Xrn1, Dcp1a and Pan3 proteins. Therefore, in simplified terms, SGs and PBs can be viewed by viruses as

obstacles to infection and/or as sources of cellular factors useful for virus replication.

The relationship between SGs and alphaviruses is understood poorly. A possible reason for this is the multiplicity of mechanisms that the viruses use for the inhibition of cellular translation and the maintenance of their own translation activity. Therefore, the complicated interactions between alphavirus and SGs are delicate and multilayered. Several studies have demonstrated direct and indirect associations between alphaviruses and SGs. First, it was demonstrated that SFV infection causes the formation of TIA-1- and TIA-containing SGs. Furthermore, the SGs and alphavirus RCs appear mutually exclusive; SGs are formed in the cell compartment most distant from the location of RC formation. This behavior indicates that alphaviruses somehow prevent SG formation near the RCs. The SGs disappear later in infection, when replicase organelles occupy the cells. Therefore, there is an obvious connection between the formation of replicase organelles and the disappearance of SGs. The virus mechanism causing the disassembly of SGs is unknown (McInerney et al., 2005). Furthermore, it is also interesting that the HuR protein, which binds to the U-rich 3' end of the SINV genome, can also be a component of SGs and PBs. The binding of HuR was shown to play a stabilizing role by reducing the decay rate of the SIN genome (Sokoloski et al., 2010); however, the CHIKV and Ross River virus apparently lack this structural element at the 3'UTR, although HuR is bound via alternative means (Dickson et al., 2012). Whether this phenomenon is associated with the disassembly of SGs is unknown. It is more likely that the connection between SGs and alphavirus replicase organelles is based on G3BP proteins. Without a doubt, G3BP proteins bind to replicase complexes in Old World alphaviruses because they have been identified in every pull-down study performed using tagged SINV replicase proteins. G3BPs are an important component of SGs. It has been demonstrated that the knockdown of G3BP enhances the translation of the SINV ns-polyprotein and has a mildly positive effect on SINV multiplication (Cristea et al., 2010).

3. RESULTS

Aims of the thesis:

I. To study the very end of SFV nsP3 protein as previously collected data indicated that C-terminus of nsP3 may not be as "useless" as previously figured.
II. The outbreak of Chikungunya virus, a close relative to SFV, brought up the need for simple and safe test system for screening virus inhibitors.
III. The study of nsP3 C-terminal end was further expanded. The role of SFV nsP3 in cellular stress granule formation and disassembly during alphavirus infection was investigated.
IV. Factors that bind SFV replicase were seeked to further expand the knowledge about alphavirus-host interactions.

3.1. Extreme C-terminal sequence of nsP3 contains several overlapping and functionally important motifs (I)

3.1.1. nsP3 is degraded rapidly in cells

Novel alphavirus vectors designed in our laboratory for the expression of marker proteins, such as EGFP, exhibited unexpected properties. First, the insertion of EGFP 30 aa residues upstream of the C-terminus of nsP3 resulted in a genetically unstable virus; the same insertion at 77 aa upstream of the C-terminus of nsP3 resulted in a highly stable recombinant virus. The observations indicated that sequence elements located at or near the first insertion site were important for SFV replication and were disrupted by the EGFP insertions. Second, when the virus was re-designed to release EGFP using nsP2-mediated processing, an unusual phenotype was observed; EGFP, which contained N- and C-terminal extensions derived from nsP2 protease recognition sequences, was located primarily in the nucleus of the infected cells, and despite containing a stabilizing glycine residue at the N-terminus (Varshavsky, 1996), the protein was surprisingly unstable. This observation was in stark contrast to the known high stability of EGFP and created additional benefits (and problems) in the constructed vector (Tamberg et al., 2007). The reasons for the instability remained unclear; however, it was assumed that the most likely reason for this phenomenon was the addition of the last 30 aa residues of nsP3 to the C-terminus of the EGFP molecule. Again, this observation highlighted the possible functional importance of this region.

The obvious problem with the idea that the C-terminus of nsP3 is a degradation signal is that nsP3 was thought to be stable protein in SFV-infected cells. Based on the considerations described, it was concluded that the functions of the nsP3 C-terminus warrant investigation. We selected a system for the inducible expression of nsP3. To generate the protein with its native N-terminal Ala-residue, a ubiquitin (ubi) fusion technique was used (Varshavsky, 1996). To

generate stably transfected cell lines, the ubi-nsP3 cassette was cloned into the expression vector pcDNA4/TO. T-REx cells are based on HEK293 cells and express a repressor that binds the promoter region in pcDNA-4/TO, inhibiting its expression; this effect can be reversed by the addition of the inducer tetracycline. Once the expression of the recombinant protein is induced, ubiquitin is cleaved precisely from nsP3 by cellular proteases. Subsequently, a similar approach was used for the construction of cell lines exhibiting the inducible expression of mutant forms of nsP3.

We used a radioactive pulse-chase method to analyze the half-life of nsP3 in the induced cells. This experiment demonstrated that nsP3 was relatively unstable, exhibiting a half-life of approximately 1 h; the use of proteasome inhibitors increased the half-life significantly (I, Fig. 1AB). In addition, in virus-infected cells, the nsP3 generated from the polyprotein was degraded rapidly (I, Fig. 1C); however, a fraction of the nsP3 incorporated into RCs remained relatively stable.

To determine the sequences responsible for the reduced stability of nsP3, several constructs based on luciferase or EGFP markers were designed. Fragments of different lengths from the C-terminal end of nsP3 were added to the C-terminus of the reporter proteins. Using a transient expression system, it was determined that 6 aa residues from the very end of nsP3 were sufficient to reduce the stability of both markers (I, Figs. 2 and 3). Furthermore, using the last 6 aa residues from the full-length SINV nsP3 reduced the stability of the markers. In contrast, fusion of 6 aa residues from the C–terminal of the shorter form of SINV nsP3 did not result in significant degradation of the reporter proteins. These findings were supported further by analysis of the half-lives of nsP3del10 and nsP3del30 (nsP3 missing the last 10 or 30 C-terminal aa, respectively) using inducible T-REx-nsP3del10 and T-REx-nsP3del30 cell lines constructed for this purpose. The half-lives of the C-terminus-truncated nsP3 proteins were significantly higher (approximately 8 h) compared with those of the wild-type nsP3 (I, Fig. 4B and C).

3.1.2. C-terminal region of nsP3 determines its subcellular localization and is required for interaction with cellular protein(s)

Further analysis of the cell lines expressing nsP3 or the truncated nsP3 mutants revealed that both nsP3 and nsP3del10 exhibited a punctate or granular localization pattern (I, Fig. 5) similar to that observed in SFV-infected cells (Salonen et al., 2003). However, the localization of nsP3del30 was dispersed and/or formed filamentous stretches that did not colocalize with microtubules or actin fibers (data not shown). This observation was corroborated in the analysis of CHIKV nsP3 (Fros et al., 2012); deletion of the hypervariable domain in nsP3 led to the formation of filaments.

The significance of any finding for the individual ns-proteins in alphavirus is questionable unless it has also been demonstrated using polyprotein precursors. Therefore, plasmids expressing P123, P123del10 or P123del30 were generated,

and localization of the wt and mutant nsP3 released from these polyproteins was analyzed in HeLa cells transiently transfected with the constructs. Immunofluorescence analysis demonstrated the nsP3 and nsP3del10 exhibited punctate localization, whereas the localization of nsP3del30 was diffuse (I, Fig. 6A, B and C). Similarly, vectors that expressed the uncleavable polyproteins $P12^{CA}3$, $P12^{CA}3del10$ or $P12^{CA}3del30$ (CA represents the mutation of the nsP2 catalytic Cys 478 residue to Ala) were constructed and analyzed. Previous studies demonstrated that nsP3 is required for targeting the replicase complex to lysosomal membranes (Salonen et al., 2003). Indeed, targeting to the lysosomal membranes was observed for $P12^{CA}3$ and $P12^{CA}3del10$; $P12^{CA}3del30$ polyprotein behaved differently in, it was dispersed throughout the cells rather than exhibiting a discrete localization (I, Fig. 6D, E and F). Therefore, in addition to the degradation signal, nsP3 contains least one more important element in its C-terminus, and it was assumed that this element is involved in interacting with host proteins.

3.1.3. Effect of free nsP3 on virus infection

Whether the expression of the individual nsP3, nsP3del10 and nsP3del30 proteins affected SFV replication was investigated in the tetracycline-induced T-REx-nsP3, T-REx-nsP3del10 and T-REx-nsP3del30 cells. A comparison of the virus growth curves demonstrated that compared with the mock-induced cells, SFV4 replication was reduced in all the induced cells. The effect was mild in the induced nsP3-expressing cells, whereas expression of nsP3del10 and nsP3del30 resulted in a 10-fold and 5-fold drop in the virus titer, respectively (I, Fig. 7A, B and C). The more prominent effect might have been caused by the longer half-lives of the truncated proteins, which would result in higher levels of the mutant nsP3s compared with wt nsP3. In addition, SFV infection causes the shutdown of transcription and translation; therefore, because of its shorter half-life, any preexisting nsP3 would be degraded (I, Fig. 1A).

Several factors may have contributed to the inhibitory effect of nsP3 and its mutants on the SFV infection. The SFV replicon vector expressing EGFP under control of a subgenomic promoter was used to investigate whether the induction of the expression of wt or mutant nsP3 reduced the number of successfully infected cells. This replicon was used because it cannot spread in cell culture; accordingly, the number of successfully infected cells can be estimated easily. It was determined that the induction of nsP3 and nsP3del10 expression yielded a reduction of approximately 30% and 70%, respectively, in the number of infected cells. In contrast, the effect of nsP3del30 expression on the number of infected cells was negligible. Therefore, the expression of nsP3 inhibited entry and/or establishment of the SFV infection, indicating that nsP3 might play a role in the phenomenon of super-infection exclusion. However, the mechanism of action was unclear. Finally, how the induction of nsP3 expression affected viral RNA replication was investigated. Northern blot analysis demonstrated that over the course of the experiment, the induction of nsP3 or nsP3del10

expression reduced the numbers of SFV4 genomic and subgenomic strands but did not alter the ratio of these RNA strands. Again, the effect of expression of nsP3del30 was quite different in that it did not have a significant effect on the levels of genomic RNA; however, the amounts of subgenomic RNA were clearly reduced (I, Fig. 8).

3.1.4. Hypervariable C-terminus of nsP3 contains highly conserved sequence elements

The data demonstrated that the C-terminal domain of nsP3 mediates extremely important functions. Because the sequence is intrinsically disordered, the functionally important sequences in this region are likely represented by linear and relatively short conserved sequences. Indeed, multiple-sequence alignment of the C-termini of different alphavirus nsP3 proteins identified a number of conserved elements (I, Fig. 9A). In SFV, two conserved sequences, LTFGDFD and ITFGDFD, separated by a 10-aa sequence, were identified. In addition, the terminal 6 aa residues of nsP3, representing the P-side of the processing site between nsP3 and nsP4, were also conserved; this 6-aa sequence was also involved in the rapid degradation of nsP3 (see above). In contrast to the terminal 6 aa residues of nsP3, the functions of the conserved repeated sequences was investigated in the context of recombinant virus. A virus expressing nsP3 without aa residues 30-11 (aa count starting at the C-terminus of the protein) was designated SFVdel30-11. The deletion resulted in the complete removal of the ITFGFGD sequence, and only the LTFG portion of the LTFGDFD element remained. Compared to SFV4, the SFVdel30-11 exhibited a 50- to 100-fold lower titer (I, Fig. 9C). Based on the properties of this virus, additional deletion mutants were constructed based on the mapping of nsP3 interactions with the G3BP1 protein (see below).

3.2. Construction and use of stable Chikungunya virus replicon cell line (II)

Because of the massive outbreak of CHIKV in the Indian Ocean region in 2006 (Pialoux et al., 2007), a renewed interest in alphavirus infection was observed. The virus spread to Italy (Rezza et al., 2007), and it was recognized that the virus could, on principle, spread to any region suitable for the propagation of its insect vector. Currently there is no licensed vaccine or antiviral drug against CHIKV.

To select antiviral compounds against CHIKV, a safe and simple screening assay is required. Studies of hepatitis C virus have demonstrated that purified proteins and assays based on these proteins are inferior to assays based on cell lines supporting the constitutive replication of viral RNA. Unlike hepatitis C virus, CHIKV grows well in cell culture; however, this virus represents a dangerous pathogen and is handled as a biosafety level “3” pathogen in most

countries. Therefore, it was considered that cell lines expressing the CHIKV replicon might represent a simple and safe system for drug screening.

Infection using the wild type CHIKV replicon ends with the death of virus-infected cells. Based on SINV and SFV data published previously, it was assumed that the main cytoxicity determinant of CHIKV was likely nsP2. Following the establishment of cell lines stably expressing the SINV replicon, a cytotoxicity-attenuating mutation was identified in nsP2 at aa residue 726 (Frolov et al., 1999). The counterpart of this mutation in the SFV replicon (aa residue 718, Pro-to-Gly mutation) also resulted in a replicon with reduced toxic effects (Tamm et al., 2008). This replicon was not capable of establishing stable replication; however, following selection, the replicon acquired additional mutations in nsP2, resulting in a completely non-cytotoxic phenotype. Therefore, as a starting point, the CHIKV replicon (strain LR2006OPY1) was engineered to contain a similar Pro-to-Gly (PG) mutation at aa-residue 718 in nsP2. For selection, a cassette expressing puromycin acetyltransferase plus EGFP (the two proteins were separated by the foot-and-mouth 2A autoprotease) (II, Fig. 1A) was inserted under the control of a subgenomic promoter, and the resulting replicon was termed CHIKV-PG. CHIKV-PG RNA was electroporated into BHK-21 cells, and puromycin was added to the media to select for cells carrying replicons with reduced cytotoxicity (II, Fig. 1). The colonies formed by the cells containing these replicons were selected and expanded to cell lines, and the replicon RNAs were isolated and sequenced to identify potential changes. It was determined that mutations in addition to the P718G mutation were present in different regions of nsP2; the functional effects of these mutations were confirmed by reverse genetics. Based on this analysis, one mutant variant of the P718G nsP2, containing an insertion of five amino acids (GEEGS) between aa residues 647 and 648, was chosen for the construction of a replicon capable of stable replication in BHK-21 cells without exerting a cytotoxic effect. Using the data from the investigation of the C-terminal region of SFV nsP3, the CHIKV-NCT (NCT for noncytotoxic) replicon was engineered to also express Renilla luciferase (Rluc), which is a useful marker for screening purposes. The sequence encoding Rluc was inserted into the nsP3 sequence without disrupting a conserved motif. The insertion had no effect on the replication of CHIKV-NCT, which exhibited a reduced rate of RNA synthesis compared to the parental CHIKV replicon (II, Fig. 2A). Using this construct, a stable BHK-CHIKV-NCT cell line was created and was used for drug screening. The initial small-scale screening of 356 compounds led to the discovery of several inhibitors of CHIKV replication (II, Table 1). These compounds were also efficient against the full-length CHIKV and the related SFV, indicating that the stable BHK-CHIKV-NCT cell line was an effective and easy-to-use system for the selection of compounds inhibiting alphavirus replication.

3.3. The role of nsP3 in inhibiting stress granule formation in SFV-infected cells (III)

Experiments with SINV mutants carrying the EGFP tag in nsP2 or nsP3 or the Flag-tag in nsP4 allowed the identification of host proteins that bind the targeted protein and, potentially, RCs. In these experiments, it was observed that regardless of the tagged ns-protein, immunoprecipitation always resulted in the co-precipitation of other nsPs. This observation may reflect the co-localization of the proteins in the virus replicase complexes but makes it difficult to identify which interaction partner is specific to which ns-protein. Therefore, the sets of host proteins identified in these experiments were similar (Cristea et al., 2006, 2010; Frolova et al., 2006; Atasheva et al., 2007; Gorchakov et al., 2008). Of the proteins binding to the SINV RCs, G3BP1 and G3BP2 were identified; however, it was uncertain if the proteins interacted with the RCs via nsP2, nsP3 or nsP4 (or even via nsP1).

3.3.1. G3BP is bound to SFV replicase via nsP3

Previously, G3BP1 and G3BP2 were shown to associate with the RCs in SINV; however, this also appears to be the case for the RCs in SFV (III, Fig. 1B–D) and CHIKV (Fros et al., 2012). Because the individually expressed nsP3 and G3BPs both localize in the granular structures, one could speculate that only nsP3s (and not the other ns-proteins) bind G3BPs. Therefore, the T-REx-nsP3 cells (see above) were used to determine whether nsP3 alone co-immunoprecipitated with G3BP, and both nsP3 and nsP3del10 co-immunoprecipitated with G3BPs. Interestingly, nsP3del30 failed to interact with G3BP1, indicating that the sequence motifs required for the interaction with G3BPs were located within the extreme C-terminal sequences of nsP3 (III, Fig. 2B). This observation was confirmed using a chimeric EGFP containing the 31 C-terminal aa residues of SFV nsP3 at its C-terminus. This protein bound G3BP1, whereas the normal EGFP did not bind G3BP1 (III, Fig. 2D).

As noted above, the two L/ITFGDFD repeat sequences at the C-terminus of nsP3 were identified as potential motifs for binding to unknown host proteins. Because nsP3del30 lacks intact copies of these sequences, it was proposed that these motifs, which are common to all Old World alphaviruses, are involved in interactions with G3BP1. To verify whether this interaction occurs in SFV infection, several mutant viruses were constructed as follows: SFVdel8 (contains both intact elements but lacks the 8 aa spacer between the elements), SFVdel78 (lacks the first repeat and the spacer sequence) and SFVdel789 (lacks both repeats and the spacer). The mutant viruses were viable and were expressed at levels comparable to the levels of wild type nsP3 in SFV4-infected cells (III, Fig. 3B). Further analysis confirmed that at least one repeat element was needed for binding to G3BP1; nsP3, nsP3del8 and nsP3del78 co-immunoprecipitated with G3BP1 although the mutant proteins bound less efficiently. In contrast, the nsP3del789 mutant was unable to bind G3BP1. Analyses

performed using confocal microscopy confirmed these results; the G3BP1 clearly co-localized with the RCs of SFV4 and SFVdel8 and, to a significantly lesser extent, with the RCs of SFVdel78 (III, Fig. 3C). However, as expected, the co-localization of G3BP1 and the RCs of SFVdel789 was not observed.

3.3.2. Recruitment of G3BP to RCs affects disassembly of stress granules

Many proteins co-localizing with the replicase organelles of RNA viruses play important roles in infection. G3BP proteins, however, have a relatively small effect on SINV infection (Cristea et al., 2010). It is possible that cellular proteins are bound by viral ns-proteins to prevent the normal functions of the proteins that are not beneficial to the virus. It has been known for some time that when stress granules in SFV-infected cells first appear, they are localized in the regions of cytoplasm not occupied by nsP3. Subsequently, as infection proceeds, the stress granules are disassembled. The mechanism behind these processes is unknown. Another feature of SFV infection is that once the cells reach the late phase of infection, stress granules can no longer be induced (McInerney et al., 2005). Interestingly, the cellular proteins TIA-1 and G3BP1, which are components of stress granules, re-localized in the cells infected with SFV4; G3BP1 exhibited a punctuated localization pattern, and TIA-1 relocated from the nucleus to the stress granules, resulting in the co-localization of these proteins. However, when the infection proceeded and the stress granules disappeared, the localization of TIA-1 was more dispersed, whereas the G3BP1 maintained a punctate pattern, co-localizing with viral replicase organelles (III, Fig. 4). This observation indicated that nsP3, because of its ability to interact with G3BP1, may be involved in the re-localization of G3BP1 and possibly in dissolving the stress granules.

When the MEF cells infected with SFVdel789 were compared with the SFV4-infected cells, it was evident that the majority of the cells were positive for stress granules; at 4.5 h post-infection, stress granules were detected in 71% of the SFVdel789-infected cells and in 63% of the SFV4-infected cells (this level of stress granules was observed slightly earlier, at 4 h p.i., likely because of the faster replication time of the wild type virus). Therefore, the differences in the formation of stress granules were small. However, in the SFVdel789-infected cells, the stress granules persisted longer than in the SFV4-infected cells (III, Fig. 5A). Surprisingly, in both sets of virus-infected cells, the stress granules eventually disassembled, indicating that the interaction between G3BP and nsP3 may affect this process but is not strictly required. Therefore, the formation and disassembly of stress granules in SFV-infected cells comprise a complicated process that appears to be regulated by several mechanisms.

Equilibrium exists between stress granule formation and polysomes that are active in translation. Because both SFV4 and SFVdel789 contain a translational enhancer at the beginning of their subgenomic RNA and translate large amounts of structural proteins in an eIF2α phosphorylation-independent manner, it was

hypothesized that the efficient recruitment of ribosomes to polysomes engaged in the translation of viral RNAs (mostly subgenomic RNAs) would shift the balance and facilitate the disassembly of formed stress granules. Replicon vectors, designated SFVdel789-ova and SFV-ova, were constructed to test this hypothesis. Both constructs express ovalbumin (ova) and lack a translational enhancer at the beginning of the subgenomic RNA. In this experiment, the percentage of stress granule-positive cells reached a maximum at 4 h p.i. and was 51% for SFV-ova and 80% for SFVdel789-ova. However, the stress granules still disassembled (although the rate was much slower than in the virus-infected cells), indicating that the enhancer had a relatively mild effect (III, Fig. 5B). However, even a replicon unable to synthesize subgenomic RNA caused the disassembly of stress granules, indicating that subgenomic RNA is not required for this process (unpublished data).

To explain this rather puzzling observation, the effects of different compounds able to induce the formation of *bona fide* stress granules were investigated. Sodium arsenite is typically used as a stressor for cells and functions via the induction of eIF2α phosphorylation. Another compound, pateamine A (Pat A), uses a different route to induce stress granules. SFV-ova- or SFVdel789-ova-infected MEF cells were stressed at 7 h p.i for 1 h using sodium arsenite, and an increase in the number of cells containing stress granules was not observed (the levels of stress granule-positive cells remained at 20% and 50%, respectively). This finding was not surprising because eIF2α is already phosphorylated in SFV-infected cells. In contrast, the addition of Pat A increased significantly the percentage of SG-positive cells, but only in the cells infected with SFVdel789-ova (the increase was from 50% to 70%) (III, Fig. 6A). Therefore, the eIF2α-independent translation of SFV RNAs and the ability of nsP3 to interact with G3BP1 are important for stress granule formation and disassembly. These data were confirmed in another set of experiments in which eIF2α-AA MEF cells expressing an eIF2α mutant that cannot be phosphorylated by PRK were used. When these cells were infected with either of the two replicon vectors, the percentage of stress granule-positive cells was close to zero. The data clearly confirmed that the activity of PKR, activated by SFV infection (Barry et al., 2009), and the consequent phosphorylation of eIF2α play an important role in the host response to alphavirus infection. As expected, when the infected eIF2alpha-AA MEF cells were stressed using sodium arsenite, an increase in the formation of stress granules was not observed. However, when the cells infected with SFVdel789-ova were treated with Pat A, the number of cells containing stress granules rose from 0% to 70%. Moreover, the Pat A treatment had only a small effect on the SFV-ova-infected eIF2alpha-AA MEF cells (III, Fig. 6A). This experiment confirmed the role of nsP3 in blocking the formation of stress granules in response to Pat A treatment.

3.3.3. nsP3 alone does not block stress granule formation

The data presented above suggested that at least two viral processes, the interaction of nsP3 with G3BP and the eIF2α-independent translation of viral RNAs, are involved in the dynamics of stress granule formation and disappearance. It was clear that viral RNA translation is needed for stress granule disassembly. However, it was not clear whether nsP3 alone could prevent stress granule formation. Therefore, T-REx-nsP3 and T-REx-nsPdel30 cells, induced for the expression of viral proteins, were treated with Pat A. In both cell lines, the formation of stress granules was observed. The nsP3 (but not nsP3del30) was co-localized with G3BP1 prior to the addition of the stressor; however, Pat A also caused the co-localization of TIA-1 with both nsP3 and G3BP1 (III, Fig. 7A). This phenomenon was not observed in SFV4-infected cells, indicating that nsP3 was included in the stress granules. When the T-REx-nsP3del30 cells were stressed, TIA-1 and G3BP co-localized, and the nsP3del30 remained diffuse.

In another set of experiments, MEF cells were transfected with plasmids expressing P123 or P123del30. The co-localization of G3BP1 and eIF3 was used to confirm the presence or absence of stress granules in the transfected cells. When Pat A was added to the cells expressing P123, nsP3 remained in contact with G3BP1, and IF3 (used as marker of SGs) was diffuse in the cytoplasm, indicating that unlike the control cells, these cells were unable to form SGs (III, Fig. 7B). However, in the cells expressing P123del30, nsP3del30 did not co-localize with G3BP1, and when the cells were stressed, the percentage of stress granule-positive cells (co-localization of G3BP1 and eIF3 observed by immunofluorescence) reached 80%. Therefore, the interaction of nsP3 and G3BP prevents stress granule formation when nsP3 is bound to the virus replication organelles or, at the very least, is interacting with membrane-bound structures formed with nsP1 and nsP2 (Salonen et al., 2003). In T-Rex-nsP3 cells, the interaction of nsP3 and G3BP was maintained; however, instead of preventing G3BP from participating in stress granule formation, nsP3 was dragged into these structures.

3.4. Replicase organelles in SFV are enriched in RNA-binding proteins, which can affect virus replication (IV)

Based on our data and data from other studies, it is clear that G3BP1 and G3BP2 bind to the RCs of SINV, CHIKV and SFV. However, because replicase proteins of positive strand RNA viruses usually interact with hundreds of host proteins, this observation is only a small part of the whole story. Indeed, several studies performed using SINV have led to the identification of numerous factors that bind ns-proteins (and therefore RCs), and a number of proteins interacting with nsP2 in CHIKV have been reported. However, these studies have not been performed in SFV. Importantly, immunoprecipitation analyses have produced similar results; however, to approach the complete picture of alphavirus-host interactions, alternatives to immunoprecipitation methods are needed.

3.4.1. Purification of alphavirus replication organelle membranes carrying functional RCs

Alphavirus RCs are small and fragile structures. As one can imagine, the purification of these structures from infected cells represents a considerable challenge, and the purification of RCs has not been achieved. In infected cells, RCs are not present as isolated structures but rather are clustered with the virus replication organelles. In the late phase of infection, SFV RCs are bound to membranes of endosomal and lysosomal origin, vesicles referred as cytopathic (or cytoplasmic) vesicles type I (CPV-I). This localization of RCs is unique to SFV. RCs in other positive-strand RNA viruses also form virus replication organelles, but on different types of membranes, e.g., mitochondrial, ER, Golgi, and plasma membranes (reviewed in Miller and Krijnse-Locker, 2008). The formation of virus replication organelles represents a modification of cellular organelles, and therefore, one would expect to see numerous virus-induced changes in the protein composition of these organelles compared with the non-modified organelles. However, similar to RCs, the purification of replicase organelles, especially in a functional form, is challenging, and functional RC purification has not been successful for many positive strand viruses. We hypothesized that because alphavirus RCs form on the plasma membrane and the formation of replicase organelles is associated with their endocytosis, it should be possible to purify CPV-I vesicles from infected cells and to compare their content with endo/lysosomal vesicles obtained from mock-infected cells. Ultracentrifugation is the classical method used for the isolation of membranes; however, this process is time-consuming, and most importantly, it has been shown that the RCs in vesicles purified in this manner tend to lose their functionality (Clewley and Kennedy, 1976). The reasons for this loss of functionality are unknown; however, the situation may be similar to that of arteri- and coronaviruses, in which host factors essential for replication are not co-purified with RC-containing membranes under these conditions (van Hemert et al., 2008). Therefore, the more traditional method used for studying endocytosis represented a possible alternative. In these studies, the use of dextrans, typically carrying various fluorescent probes, has been the method of choice. In a continuation of this approach, methods based on the use of dextran-covered magnetic beads have been developed (Glebov et al., 2006; Wittrup et al., 2010). Such methods lead to the accumulation of magnetic beads in endosomes and lysosomes, and when these cells are disrupted, the organelles can be collected via magnetic separation and used for investigation of the proteome. SFV-infected cells have been shown to endocytose immuno-gold, which were localized in CPV-I structures (Kujala et al., 2001); therefore, it was assumed that the magnetic beads would also end up in these structures.

For this method, SFV cells infected at a relatively low MOI and at a late phase of infection were chosen. These parameters were chosen because in nature, a high MOI is unlikely, and studies of stress granule formation have demonstrated these structures are not observed at a high MOI. In addition, a

high MOI leads to the presence of viral structural proteins in cellular endo-lysosomes because SFV entry is also mediated by endocytosis. Human HeLa cells were used for these experiments because alphaviruses infect human cells and are often considered potential tools for gene vaccination and anti-cancer therapy approaches. After infection of the HeLa cells with SFV4 at a MOI of 1, dextran-covered magnetic beads were added to the growth media; the control cells were mock-infected but treated similarly. At 12 h post-infection, the cells were lysed, and the magnetic fractions were obtained both from the mock-infected and infected HeLa cells; these fractions were rich in endolysosomes but largely devoid of plasma membrane and ER markers (IV, Fig. 2C). Furthermore, the magnetic fraction obtained from the HeLa cells contained all four ns-proteins from SFV4 (IV, Fig. 2C). To test the activity of the SFV replicase in the magnetic fraction, this fraction was compared with the activity present in a crude P15 fraction. The P15 fraction is rich in various cellular membranous structures (ER, Golgi, mitochondria, etc.) and has been used previously in various alphavirus studies. The magnetic fraction from the infected cells demonstrated approximately the same level of activity as the P15 fraction, indicating that the fraction contained membranes with active RCs (IV, Fig. 3).

3.4.2. Quantitative proteomics approach to characterize the contents of magnetic fractions

The analysis of the magnetic fraction from the cells infected with an SFV mutant carrying the ZsGreen marker in nsP3 demonstrated that this fraction contained a significant amount (approximately 40–50%) of vesicles lacking ZsGreen and accordingly, the viral replicase (IV, Fig. 2D–F). To accurately compare the protein contents of the magnetic fractions from infected and mock-infected cells, a SILAC-based (stable isotope labeling with amino acids in cell culture) quantitative proteomics approach (Ong et al., 2002) was used. This method is based on the non-radioactive isotopic labeling of cells during which all the proteins in the cells labeled with amino acids (usually with lysine and arginine) have a large mass because of the presence of heavy nitrogen, carbon or oxygen isotopes. Accordingly, the peptides obtained after protease treatment also exhibit higher molecular masses and, compared with the same peptides from non-labeled cells, exhibit a slightly higher mass/charge (m/z) ratio. This property is extremely useful because a mass spectrometer detects the m/z ratio for any peptide at a very high accuracy but cannot detect absolute quantities. In addition to the m/z ratio, the peptides from heavy and light samples have similar properties; therefore, it is possible to detect changes in their abundance by comparing the amounts of the light and heavy peptides. Therefore, SILAC allows the detection of relative changes in protein amounts (Ong et al., 2002).

In this study, the HeLa cells grown in SILAC media and labeled with heavy arginine and lysine were termed H-HeLa, whereas the HeLa cells grown in normal (light) media were termed L-HeLa. Subsequently, the H-HeLa cells were infected with SFV4, and the non-infected L-HeLa cells were used for

comparisons; to reduce the false identification of proteins, the opposite combination was also used. After isolating the magnetic fraction from the infected and mock-infected cells, the protein compositions were compared, producing a list of proteins that were reproducibly increased in abundance in the magnetic fraction from the infected cells (IV, Table 1). This list included many proteins previously known as interaction partners of alphavirus replicase proteins (with G3BP proteins at top of the list), supporting that the method allowed the detection of proteins truly localized in SFV replicase organelles. In addition, nearly 50 proteins not previously connected to alphavirus infection were identified (IV, Table 1). These proteins belonged to different functionally connected clusters (IV, Fig. 4), among which the cluster of RNA-interacting proteins was most prominent; this cluster was therefore chosen for further investigation.

The RNA-interacting proteins PCBP1, hnRNP M, hnRNP K and hnRNP C were selected for analysis of their effect on alphavirus replication and for confirmation of the validity of the analysis method. In the SFV4-infected HeLa cells (at 8 h and 12 h p.i.), all four host proteins clearly co-localized with dsRNA and nsP3 and therefore with the SFV RCs (IV, Fig. 5). For this to occur, a fraction of these proteins must have re-localized from the nucleus to the lysosomes (this conclusion was based on the detection of the lysosomal marker Lamp2) positive for nsP3 (IV, Fig. 6). Furthermore, confocal microscopy analysis confirmed the SILAC data; the selected proteins were re-localized to the site of SFV replication.

3.4.3. PCBP1, hnRNP M, hnRNP C and hnRNP K affect SFV4 infection

Co-localization with replicase organelles may indicate that the selected proteins have an effect on the SFV infection. However, PCBP1, hnRNP M, hnRNP C and hnRNP K knockdown (kd) had no effect on SFV4 entry and the establishment of infection. To demonstrate the effects of kd on the expression of viral ns- and structural proteins, the replicon vector SFV-nsP3-Rluc-SG-Ffluc was designed. The replicon expresses Renilla luciferase (Rluc) fused to nsP3 and firefly luciferase (Ffluc) under the control of a subgenomic promoter; the activity of the protein was proportional to the expression of the corresponding part of the viral genome. At 48 h prior to infection, HeLa cells were transfected with control siRNA or with a set of siRNAs targeting the host protein mRNA.

The cells infected with the replicon-containing VRPs were lysed at 4, 6 and 8 h p.i., and the activities of both luciferases were measured. When PCBP1 was targeted, the SF, replicon did not perform well, and the activities of both luciferases were approximately 50% less compared with the control cells at all time points (IV, Fig. 7B), confirming that PCBP1 contributed to the expression of SFV proteins. This effect may be at the level of translation of viral RNAs but more likely reflects decreased replication of the SFV replicon. In contrast, the kd of hnRNP M and hnRNP C resulted in increased amounts of both

luciferases, approximately 3- and 5-fold, respectively, at all measured time points. When hnRNP K was targeted, only the synthesis of Rluc and Ffluc was increased by 8 h p.i. compared with the control transfection (IV, Fig. 7B). Therefore, these proteins suppressed SFV gene expression. Again, it was unclear at which stage the SFV infection was affected by the kd of cellular factors. To investigate the release of new infectious virions, the cells transfected with siRNAs were assayed using a one-step growth curve. The experiment demonstrated that the kd of PCBP1 and hnRNP K did not alter the number of new virions released (IV, Fig. 8A). In contrast, when hnRNP M and hnRNP C were knocked down, the SFV replicated at 3- and 5-fold higher titers, respectively (IV, Fig. 8A). In this case, there was a good correlation between the observed increase in the expression of proteins from the viral RNA and the effect on the titer of the released virions. Because the most obvious link (though not the only link) between these effects was the activation of viral RNA replication, the levels of viral RNAs in the cells at 4, 6 and 8 h p.i. were analyzed using northern blots. The assay demonstrated that in the hnRNP M kd, increased levels of viral RNAs were detected at 8 h p.i. The effect was more prominent in the hnRNP C kd cells, in which the same levels of viral RNAs were detected at all the time points (IV, Fig. 8B). For the hnRNP M and hnRNP C kd cells, the increased expression of both genomic and subgenomic viral RNA strands was detected. Taken together, the data indicated that RNA replication and transcription were the major stages of viral infection affected by these cellular proteins. The same is most likely true for PCBP1 and hnRNP K; the kd of either of these proteins did not have a long-lasting effect, which explains the lack of effect in the growth curve experiment. However, consistent with the data from the replicon vector experiment, the kd of PCBP1 caused a decrease in viral RNA levels at 4 h p.i., whereas the kd of hnRNP K led to an increase in viral RNA levels.

The host factors for positive-strand RNA viruses are usually conserved host proteins; however, this does not mean that the proteins perform the same function in the infection process of different viruses. The alphavirus genus is somewhat heterogeneous; therefore, the effects of kd of these proteins on infection with CHIKV and SINV were investigated in a replicon experiment. Replicons similar to those in SFV were constructed, packed into VRPs and used to infect cells in which the levels of PCBP1, hnRNP M, hnRNP C or hnRNP K were knocked down using appropriate siRNAs. It was determined that the kd of PCBP1, hnRNP M, and hnRNP C affected these replicons in a manner similar to the SFV replicon; the expression of the marker was reduced in the PCBP1-kd cells but increased in the hnRNP M- and hnRNP C-kd cells (IV, Fig. 9). In contrast, when hnRNP K was targeted with siRNAs, the levels of both luciferases expressed by the SINV and CHIKV replicon did not increase. Instead, a decrease in marker expression was observed, which is consistent with previously published results (Burnham et al., 2007; Bouraï et al., 2012). Therefore, the effect of one host protein on different alphaviruses can differ.

4. DISCUSSION

Macromolecular assemblies are highly complex, and viral replicases are no exception. Although alphaviruses contain only four ns-proteins, several important questions regarding the organization of the proteins within the RC have remained unanswered, including where the proteins are located, their stoichiometry, how the domains are organized, and what their dynamics are. The real situation is even more complex; because no virus exists alone- cellular factors are also involved, which raises even more questions.

Currently, the functions of nsP3 are somewhat enigmatic. The enzymatic functions of nsP3 in the infection process are not well-defined. The C-terminal one third of the protein is yet another puzzle because the region is extremely hypervariable among the alphaviruses (Strauss and Strauss, 1994). Based on a sequence analysis using the IUPred web server (Dosztányi et al., 2005), the C-terminal portion of the SFV nsP3 is predicted to be intrinsically unstructured (Fig. 7), similar results were obtained for nsP3s from other alphaviruses, regardless of used prediction programs (data not shown).

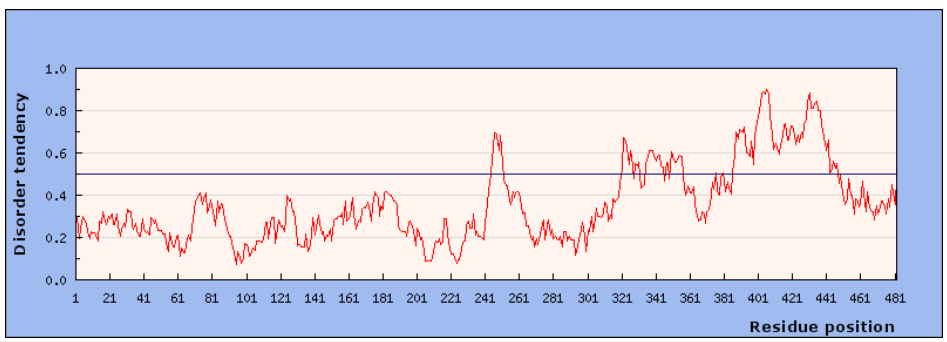

Figure 7. Analysis of the SFV nsP3 protein sequence was performed using the IUPred web server. The analysis indicated that one third of the C-terminal of nsP3 is largely unstructured.

Intrinsically disordered domains are not rare and can be found in many proteins (Dyson and Wright, 2005). Notably, approximately half of all human proteins contain long unstructured regions. Unlike the globular domains of proteins, disordered segments of proteins are relatively more exposed to the environment; therefore, the domains have the potential to interact with a larger number of partners and can be efficiently modified (e.g., phosphorylated). These domains are also present in positive strand RNA viruses. The best studied example is the C-terminal region of NS5A from hepatitis C virus, in which it has been demonstrated that despite the lack of a globular structure, the region is very important for viral infection (He et al., 2006; Liang et al., 2007).

A closer look at the nsP3 C-terminal sequence demonstrated that despite the hypervariability of this region, a number of short sequence elements are conserved among a number of alphaviruses. Recently, it was reported that one or two proline-rich motifs are conserved among alphaviruses. These motifs were shown to be a target for proteins containing Src-homology 3 (SH3) domains. Further investigation indicated that nsP3 interacts with amphiphysin-1 and amphiphysin-2 via these motifs and that these cellular proteins likely facilitate CPV formation (Neuvonen et al., 2011) but are not absolutely necessary to establish infection.

In study I, we discovered one short linear motif that was present in the nsP3s of Old World alphaviruses in two copies (I, Fig. 9); however, the motif was lacking in the nsP3 of New World alphaviruses. Study III demonstrated that this motif is responsible for binding G3BP1 and G3BP2 and sequestering these proteins to SFV RCs. This property of the motif was corroborated indirectly in another study comparing the hypervariable domains of VEEV and SINV nsP3s. It was determined that the C-terminus of the VEEV nsP3 cannot bind G3BP (Foy et al., 2012), which is consistent with the lack of an interaction motif. However, this observation does not mean that the C-terminus of the nsP3 in these viruses is less important. Indeed, a recent publication reported a different short sequence element located at the end of the C-terminus in New World alphavirus nsP3. This motif is present in either one or two copies and is important for efficient replication. Furthermore, there is a likely correlation of these motifs with pathogenicity because more pathogenic VEEV strains contain two of these motifs (Foy et al., 2013). These observations demonstrated that the C-terminal of nsP3s in both Old and New World alphavirus are similar in their structural arrangement (presence of different important motifs) and dissimilar in their primary sequences and therefore in their binding partners.

It is not surprising that nsP3 contains several short motifs in its hypervariable region because viral proteins use unstructured segments to increase the number of interactions with the host cells. Generally, the unstructured regions are involved in molecular interactions via short linear interaction motifs (SLiM) (Diella et al., 2008), and the functional importance of the sequence has been demonstrated (I, II, Neuvonen et al., 2011; Foy et al., 2013). The large general databases, ELM and MnM, are used for storing and investigating this type of information (Rajasekaran et al., 2009; Gould et al., 2010). Having a hypervariable region implies that nsP3 exhibits scaffolding rather than enzymatic functions. Intriguingly, although HCV and alphaviruses are not closely related, the intrinsically disordered regions in HCV NS5A and alphavirus nsP3 contain short fragments rich in proline, an SH3-binding element, which have been shown to bind amphiphysin (Zech et al., 2003; Neuvonen et al., 2011).

In addition to the presence of important conserved interaction motifs at the end of nsP3, we determined that SFV nsP3 and full-length SINV nsP3 were relatively stable and were degraded rapidly. The sequence responsible for degradation was mapped to the end of the protein, within the last 6 aa-residues. Unfortunately, this sequence matched the sequence element needed for the

recognition of the protease site between nsP3 and nsP4; therefore, we could not mutate the element to investigate its effects on virus infection directly. Our results indicated that when nsP3 was bound to replicase, it was stable, similar to nsP4. The true significance of the rapid degradation of nsP3 is unknown. However, recent evidence suggests that the increased amount of SFV nsP3 expressed in infected cells leads to the drastic stabilization of nsP4, presumably by including excessive amounts of the protein into RCs (Sirle Saul, unpublished data). Therefore, it is possible that the instability of nsP3 is required to regulate the formation of RCs. The observation that the shorter form of SINV nsP3 lacks the destabilization motifs appears to support this possibility.

It was determined that when the expression of nsP3 in the T-Rex-nsP3 cell line was induced, SFV infection was affected in these cells in multiple ways. First, a lower number of cells were infected, and a similar phenomenon was observed, although to a greater extent, when a more stable nsP3del10 was expressed. Based on the results from study II, it was concluded that both nsP3 and nsP3del10 were bound to cellular G3BP. This observation suggested that the change in the intercellular environment was the nsP3-mediated mechanism that reduced the number of infected cells because in the T-REx-nsP3 and T-REx -nsP3del10 cells, the expression of nsP3 or nsP3del10 led to the formation of G3BP-positive aggregates. It is possible that the aggregates sequester other cellular factors that are needed for the successful initiation of infection. However, this does not exclude the possibility that nsP3 influenced directly the beginning of viral infection through interfering with polyprotein processing or affecting the stability of nsP4 because the studies using cells expressing nsP3del30 (did not bind G3BP) supported multimodal action. When nsP3del30 was induced, it did not affect the number of cells infected successfully. Second, the expression of nsP3 also reduced viral RNA replication and transcription. These effects were most prominent for the more stable nsP3del10. Interestingly, expression of nsP3del30 reduced virus multiplication and genomic RNA synthesis.

As demonstrated previously by McInerney et al. in 2005 and in study III, eIF2α was phosphorylated in infected cells, which led to the formation of stress granules. However, this effect was temporary, and the stress granules disappeared later in infection. Furthermore, the formation of stress granules cannot be induced using chemicals (McInerney et al., 2005), such as sodium arsenite and Pat A. A different phenomenon was observed when the cells were infected with the mutant SFVdel789 nsP3, which does not bind G3BP. Although eIF2α was phosphorylated in response to virus infection and sodium arsenite did not induce the formation of stress granules, the PatA treatment allowed the production of stress granules using an eIF2α phosphorylation-independent pathway. This phenomenon led us to ask the following question: what mechanism (other than the interaction of nsP3 and G3BP) was behind the disappearance of stress granules? It was determined that one factor that reduced the number of stress granule-positive cells was the presence of the enhancer element at the beginning of the subgenomic RNA, which allows the active translation of

subgenomic RNAs in cells in which eIF2α is phosphorylated. This translation activity could lead to changes in the intracellular balance, causing the stress granules to disassemble. Another factor that could indirectly affect the number of stress granules is the ability of Old World alphaviruses to shut down cellular transcription by designating the RNA polymerase II subunit for degradation (Akhrymuk et al., 2012); and mRNAs are required for the formation of stress granules.

Unfortunately (or fortunately), there are no published reports on processing bodies in alphavirus-infected cells, a topic that requires further study. Interestingly, study IV demonstrated (IV, Fig. 5) that similar to G3BP1, one of the well-known proteins in processing bodies, PCBP1 (Fujimura et al., 2009), was localized to the SFV replication organelles. Both PCBP1 and its close relative PCBP2 are found in processing bodies. Whether this interaction and relocalization of PCBP1 had the same effect on processing bodies as that of the G3BP interaction on stress granules is unknown because quantitative measurements were not performed. However, it was not difficult to identify PCBP1, which did not co-localize with virus replication organelles, located in granules in the SFV4-infected cells (IV, Fig. 5). Therefore, it appears that unlike stress granules, processing bodies are not disassembled in SFV-infected cells.

Many positive-strand RNA viruses play tricks with components of stress granules to prevent stress granule formation. The 3C protease in poliovirus has been shown to cleave G3BP but not the other nucleating proteins TIA-1 and TIAR (White et al., 2007). In contrast, the West Nile and Dengue flaviviruses reduce the number of stress granules via a stem-loop structure at the 3' end of the negative strand, which allows sequestration of TIA-1 and TIAR into the replication complexes (Emara and Brinton, 2007). Similarly, viruses could target processing bodies, which also represent obstacles for virus infection. It has been demonstrated that flavivirus infection leads to a reduction in processing body numbers, likely through the recruitment of components of processing bodies (e.g., Lsm proteins) to the sites of replication. Again, poliovirus, which appears to favor more drastic measures, uses its protease to disrupt processing bodies (reviewed in Lloyd, 2012).

The common "interest" of different positive strand RNA viruses in stress granules indicates that the interactions demonstrated in study II are not accidental. However, the disassembly of stress granules in SFV-infected cells may be an indirect consequence resulting from the need for G3BP proteins in the RCs. However, this option is unlikely because the effect of G3BP knockdown appeared to have only a mild effect on Sindbis virus infection (Cristea et al., 2010). A similar conclusion can be drawn from the experiments using SFVdel789, which did not interact with G3BP1 and G3BP2. This raises another question: what are the functions of the proteins recruited to the sites of replication? In experiments using Rubella virus (another togavirus), G3BP1 did not co-localize with dsRNA but localized with single-stranded RNA, suggesting that the protein plays a role in encapsidation of the virus and not in replication (Matthews and Frey, 2012). Furthermore, it has been shown that that replicase

components of HCV can bind G3BP1 and that knockdown of G3BP1 reduces virus replication (Yi et al., 2011). Therefore, it is likely that the interaction of alphavirus nsP3s with G3BP proteins has multiple functions.

Because the ns-proteins encoded by alphaviruses are not sufficient to perform all of the functions essential for successful infection, numerous host components are engaged during the infection process. Large-scale studies to identify binding partners of SFV replicase have not been performed partly because several studies of proteins interacting with the SINV replicase have been published previously. However, to affect virus replication, a cellular protein does not necessarily need to interact with the virus replicase RNA; alternatively, this interaction may be transient and difficult to detect. Furthermore, the co-localization of viral replicase complexes and cellular proteins do not necessarily indicate the functional significance of these interactions for virus infection. Nevertheless, it is logical to assume that co-localization may have a purpose for the virus or host. For the majority of viruses, the proteins co-localizing but not necessarily interacting with viral replicase are relatively difficult to detect. However, the unique pathway of alphavirus replication organelle formation provided an opportunity to use a different approach and to monitor changes in the proteome of cellular organelles associated with their conversion into virus replicase organelles. Unlike the studies dedicated to analyzing the changes in the total proteome of the cell following virus infection, this approach allowed us to concentrate on factors associated with virus replication rather than on the detection of the changes caused by suppression of cellular translation or activation of the antiviral innate immune response.

Previously, the isolation of cellular membranes corresponding approximately to viral replication organelles was demonstrated only in a small number of positive-strand RNA viruses. For example, lipid raft domains, which are used as sites of replication for hepatitis C virus, were investigated using two-dimensional electrophoresis and mass spectrometry as well as SILAC combined with mass spectrometry (Mannová et al., 2006). Similarly, Golgi-enriched fractions, which are associated with coronavirus RNA replication, were analyzed using SILAC-based quantitative proteomics (Vogels et al., 2011). In both cases, more than one hundred host proteins that displayed significant increases or decreases in abundance following virus infection were identified. Therefore, the quantitative proteomic analysis of virus replication organelles has proven valuable for studying proteins that may be associated with the replication of RNA viruses. However, in contrast to study IV, none of the membranes contained functional replicase complexes; therefore, it is possible that crucial cellular factors were lost during the purification procedures. To our knowledge, our study is the first to investigate the proteome of positive-strand RNA virus replication organelles; study IV successfully analyzed the proteome of functionally active viral replicase organelles and from 300 identified proteins, approximately 80 were enriched 2.5-fold or more compared with the organelles from the non-infected cells. It is likely that a number of these proteins were not bound directly to alphaviral RNAs and/or proteins but rather were associated with cellular factors

that bound viral components. For the selected proteins, the co-localization of SFV and replicase organelles was confirmed using confocal microscopy. In addition, the siRNA-mediated silencing of host protein expression confirmed that these factors indeed affected SFV, SINV and CHIKV infection in cultured HeLa cells, each in different ways.

Many of the identified RNA-binding proteins have the potential to associate with alphavirus replicase, and several of the proteins could be components of stress granules and processing bodies, suggesting that alphaviruses could interact with RNA granules in addition to interacting with G3BP. Apparently, stress granules and processing bodies are formed on microtubules (Aizer et al., 2008; Loschi et al., 2009), and it has been demonstrated that processing bodies use microtubules for movement inside the cell. This suggests the hypothesis that perhaps SFV and a number of other alphaviruses (ab)use hnRNPs for the movement of replication complexes from the PM to the vicinity of the nucleus, where static CPV-I structures are formed. Consistent with this hypothesis, several RNA-binding proteins and components of the cytoskeleton were identified among the proteins that were more abundant in the magnetic fraction obtained from the infected cells compared with the un-infected cells. Surprisingly, however, no protein that could potentially affect membrane curvature was over-represented in this fraction, possibly because proteins important for alphavirus replication may simply not increase in abundance in modified endo/lysosomes; therefore, the proteins remained undetected as important partners. Alternatively, as demonstrated by Cristea et al. (2010) in SINV, alphavirus replicases could change partners over the course of infection. It is logical to assume that proteins affecting membrane curvature are actively involved at the stage of infection when spherules are formed at the PM; this event takes place early in infection (Spuul et al., 2010). If this is true, then the method cannot detect the increased abundance of these proteins because magnetic purification cannot be used in the analysis of early RCs, which are not yet internalized by endocytosis. Therefore, the method is more suitable for the detection of proteins affecting virus replication, especially at late stages of infection. Interestingly, however, the knockdown of the chosen proteins resulted in immediate effects on virus replication, observed as early as 4 h p.i., resulting in increased or decreased levels of viral RNAs. A possible explanation for this is that host proteins, which are required for (or act against) RNA replication, are bound to RCs at the early stage of infection and remain there for the remainder of the infection process. It is even possible that a number of these proteins actually function during the early stages of replication and that their effects subsequently diminish (even though the proteins themselves remain bound to replicase organelles). Detailed studies will be required to determine whether this is the case. It should also be noted that further studies will be complicated by genetic redundancy; one hnRNP can substitute for another, and a simple knockdown experiment will not necessarily be sufficient for functional analysis. Indeed, this phenomenon was evident in an investigation of the roles of G3BPs in alphavirus infection; when only one G3BP protein was knocked down, the silencing effect was absent, whereas

reducing the levels of both proteins had a positive effect on SINV virus replication (Cristea et al., 2010).

According to current knowledge, all positive-strand RNA viruses in eukaryotes re-organize intracellular membranes to create specific virus replication organelles and tend to separate the viral replicase activities to distinct compartments, isolating them from the rest of the cytoplasm. Compared with several other positive-strand RNA viruses, the formation of replication organelles of alphaviruses is more straightforward because the process begins on the PM, where small invaginations connected to the cytosol are formed. RCs formed step-by-step bind to modified endolysosomes, and large CPV-Is are generated (Frolova et al., 2010; Spuul et al., 2010). In contrast, poliovirus, coronaviruses and HCV use complex mechanisms to create convoluted membranes and double-membrane structures to support RNA synthesis. For example, at the beginning of infection, poliovirus uses Golgi membranes, and ER and lysosome membranes are recruited at a later stage. Furthermore, single-membrane structures detected at the beginning of infection are later converted into double-membrane structures (Belov et al., 2012). However, no viruses (with the exception of togaviruses) use the outer surface of the cell as the site for RC formation and the extensive movement of formed RC structures. This observation raises open questions, including the following: what drives the movement of alphavirus RCs from the PM towards the nucleus, and which of the viral and host components are responsible for this behavior? As indicated in studies performed by Salonen *et al.* (Salonen et al., 2003), nsP3 directs uncleavable polyprotein $P12^{CA}3$ (and likely RCs) from the PM to lysosomes. Our data (I, Fig. 6) demonstrated that if the last 30 amino acids are removed, then $P12^{CA}3$del30 is still removed from the PM but is dispersed in the cytosol; therefore, there must be additional re-location determinants in other regions of nsP3, and their interaction partners in cells will be the subject of future studies.

Other open questions include the following: how do alphaviruses modify cellular membranes to form spherules, what is the mechanism that causes membrane curvature, are only viral proteins needed, are cellular proteins recruited, and does the lipid content of membranes change? It seems likely that the formation of spherules requires newly synthesized lipids because inhibiting fatty acid synthesis with cerulenin diminishes SFV replication (Perez et al., 1991). If so, then alphaviruses are similar to many other positive strand RNA viruses, such as HCV or Dengue virus, in which ongoing lipid synthesis is necessary for the formation of membranous structures that support virus RNA synthesis (Yang et al., 2008; Heaton et al., 2010). The proteomics data (study IV) identified two components of fatty acid synthesis pathways that were over-represented in the SFV replication organelles, fatty acid synthase (FASN) and ATP citrate lyase (ACLY) (IV, Fig. 4). The identification of these molecules raises the possibility of investigating this pathway in more detail.

In conclusion, there are many gaps in this story, and any novel data, even technically solid negative data, will be useful to move the field forward.

SUMMARY

This study focused on the replication complex components of alphaviruses using mostly Semliki Forest virus (SFV) as a model. It was determined that the non-structural protein nsP3, a component of the replication complex, exhibits a number of previously uncharacterized properties. Initially, in virus-infected cells, nP3 is produced as a part of the non-structural polyprotein P1234, which is cleaved into subunits. The functions of nsP3 have remained elusive because the protein lacks meaningful enzymatic activities. Furthermore, the last one third of the protein is intrinsically unstructured and is not conserved among alphaviruses.

At the beginning of the investigation, it was determined that the stretch of six amino acids at the end of the protein was sufficient for the rapid degradation of the protein. This element is homologous to the sequence required for recognition by nsP2 for polyprotein processing and the release nsP4 from nsP3. Before the degradation signal are two almost identical sequence elements that are conserved among Old World alphaviruses but not New World alphaviruses. Through further investigation, it was determined that the removal of these elements changed the localization pattern of the nsP3 inside the cells from an aggregate-like distribution to a diffuse distribution and to perinuclear fibers. The expression of nsP3, nsP3del10 (degradation signal removed) and nsP3del30 (degradation signal and two conserved elements removed) before infection reduced the efficiency of virus multiplication by affecting different steps of the infection cycle. The expression of nsP3 and nsP3del10 reduced the number of infected cells, and the expression of nsP3del30 led to reduced synthesis of subgenomic RNA.

In many studies, G3BP proteins have been demonstrated to bind to the components of alphavirus replicases. Further investigation of the two conserved elements led to the discovery that both elements bind G3BP1 and G3BP2, which are important components of cellular stress granules (SG). Therefore, we identified the viral elements that are needed for this interaction. Removing the elements from the virus disrupted the co-localization of G3BP proteins with SFV replicase. In alphavirus-infected cells, SGs appear, and later in the infection process, the SGs disappear. It was determined that nsP3 proteins facilitate the sequestering of G3BP1 and 2 from SGs; this is one of the mechanisms used by alphaviruses to disrupt SGs. Furthermore, the translational enhancer at the beginning of the subgenomic RNA was demonstrated to contribute to this process. Unfortunately, the removal of the G3BP-binding elements and translational enhancer from the virus increased the number of stress granules in infected cells and delayed the disappearance of SGs, indicating that other viral mechanisms are also involved in this process.

Although we demonstrated that G3BP1 and G3BP2 are bound to the SFV replicase, we did not identify other cellular factors. This study is the first to identify cellular proteins that bind the replicase of SFV. In alphaviruses, the replication complexes are bound to modified endosomes and lysosomes. We

obtained functionally intact replication complexes from the infected cells by feeding the cells with dextran-covered magnetic nanoparticles. The nanoparticles were translocated to the endosomes and lysosomes, and using magnetic fractionation, we collected the replication complexes. Stable isotope labeling of amino acids in cell culture, combined with quantitative proteomics, was used to identify eighty distinct cellular proteins that were more abundant in the replicase complex-carrying vesicles than in the vesicles collected from non-infected cells. In total, four proteins were chosen to validate our approach, PCBP1, hnRNP M, hnRNP C, and hnRNP K. All four proteins co-localized with SFV replicase, and silencing hnRNP M and hnRNP C enhanced the replication of SFV, Chikungunya virus (CHIKV) and Sindbis viruses (SINV). The knockdown of PCBP1 decreased SFV-mediated translation, whereas hnRNP K silencing increased RNA synthesis. However, the effect of hnRNP K silencing on CHIKV- and SINV-mediated translation was opposite of that observed for SFV.

In conclusion, despite not exhibiting catalytic activity, nsP3 likely plays a role as a scaffolding protein, and because its tail is intrinsically unstructured, one of the roles of nsP3 is to interact with several cellular partners and (based on our study) with G3BP1 and G3BP2, which are other components of stress granules. In addition, based on the proteomics data, the number of cellular partners that can associate with alphavirus replication complexes is high, and their roles need further investigation.

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SUMMARY IN ESTONIAN

Alfaviiruste replikatsioonikompleksiga seotud viiruslike ning peremeesraku komponentide funktsionaalne analüüs

Alfaviirused kuuluvad Togaviiruste sugukonda ning nende seas leidub mitmeid inimeste ja loomade patogeene, haiguste sümptomiteks võivad olla artriit ja/või entsefaliit. Fülogeneetiline alfaviiruste jaotamine kattub geograafilise paiknemisega ning seetõttu on alfaviirused omakorda jaotatud Uue Maailma ja Vana Maailma alfaviirusteks. Üheks enimkasutavaks mudeluurimisobjektiks on Vana Maailma alfaviiruste hulka kuuluv Semliki Forest viirus (SFV), mis ka antud töös vaatluse all.

Alfaviiruste genoom on üheahelaline positiivse polaarsusega RNA molekul, virion ikosaheedriline ja ümbritsetud membraaniga. Kui on toimunud rakkude edukas nakatumine viirusega käitub genoom kui tavaline rakuline mRNA ja sellelt transleeritakse mitte-struktuurne polüproteiin, mis protsessitakse iseseisvateks valkudeks. Mitte-struktuursed valgud osalevad replikatsioonikompleksi moodustamises, mis esmalt sünteesivad genoomsele ahelale juurde negatiivse ning moodustub kaheahelaline intermediaat, sellelt omakorda sünteesitake uued genoomsed ahelad ning subgenoomsed RNAd, mille järestus kattub genoomi viimase kolmandikuga ja mis on vajalik struktuursete valkude transleerimiseks.

Alfaviirustel on neli mittestruktuurset valku: nsP1, nsP2, nsP3 ja nsP4, neist on jäänud kõige mõstatuslikumaks nsP3, millel pole kirjeldatud, erinevalt teistest, replikatsiooniks vajalikku ensümaatilist funktsiooni. nsP3 saab jagada tinglikult kolmeks regiooniks: makro-domään, ainult alfaviirustel esinev domään ning kolmandana mitte-konserveerunud regioon.

Antud doktoritöös "Alfaviiruste replikatsioonikompleksiga seotud viiruslike ning peremeesraku komponentide funktsionaalne analüüs" kirjeldati, et nsP3 omab lühikest karboksüterminaalset degradatsioonisignaali, mis kattub piirkonnaga, mis on vajalik äratundmisjärjestus viiruse mitte-struktuurse polüproteiini protsessingus, et vabastada nsP3 küljest nsP4. Lisaks leiti, et eespool degradatsioonisignaali on Vana Maailma alfaviirustel konserveerunud kahes koopias motiiv. Nende motiivide eemaldamine muutis nsP3 paiknemist rakus, mitte enam tsütoplasmaatilistes graanulites, vaid difuussena ning esines ka fibrille perinukleaarses alas, viidates, et on kadunud interaktsioonid mingite rakuliste faktoritega. Kui ekspresseerida enne SFV-ga nakatamist rakkudes nsP3, nsP3del10 (eemaldatud degradatsioonisignaal) või nsP3del30 (eemaldatud nii degradatsioonisignaal kui konserveerunud motiivid), siis vähendab see rakkudest vabastatud viiruspartiklite arvu. Selle fenomeni edasine uurimine näitas, et nsP3 ja nsP3del10 ekspresseerimine mõjutab viiruse võimet saavutada rakkus edukas infektsioon, kuid nsP3del30 vähendab subgenoomse RNA sünteesi.

Töö nende kahe nimetatud motiiviga jätkus, ilmnes, et vähemalt üks neist on vajalik, et tagada edukas nsP3-poolne G3BP1 ja G3BP2 sidumine, antud valgud

on rakus stress graanulite moodustamiseks vajalikud komponendid. Kui eemaldada nood elemendid viiruselt, siis G3BP1 ja G3BP2 ei ole enam võimelised kolokaliseeruma replikatsioonikompleksiga. Alfaviiruste infektsiooniga rakus kaasneb stressi graanulite teke, mis hiljem kaovad. Samuti kasutades keemilisi induktoreid, ei ole võimalik hilises infektsioonifaasis stressi graanuleid enam esile kutsuda. Üks mehhanisme, millega alfaviirused seda soodustavad ongi G3BP1 ja G3BP2 seondamine replikatsioonikompleksiga- kui antud kaks nsP3 motiivi eemaldada, siis stressi graanulite kadumine on aeglustunud ning kasutades induktorit Pateamine A-d, taasilmuvad nood rakulised granulaarsed struktuurid.

Hoolimata, et teame, et G3BP1 ja G3BP2 seonduvad SFV replikatsioonikompleksile, on vähe teadmisi teistest valkudest, mis seda samuti teevad. Viisime läbi esimesena uuringu SFV-ga, et teha kindlaks tema replikatsioonikompleksiga seotud valke. Kuna SFV replikatsioonikompleksid on seondunud lüsosoomidele, siis õnnestus meil eraldada funktsionaalselt terved replikaatsioonikompleksid, söötes rakkudesse dekstraaniga kaetud magneetilised nanopartiklid. Nanopartiklid jõuavad endo- ja lüsosoomidesse, misjärel oli vajalik rakkude plasmamembraani lõhkumine ja vesiikulite magneetiline püüdmine. Kasutades kvantitatiivse proteoomika võimalusi, õnnestus tuvasta 80 valku, mis olid üleesindatud fraktsioonis, mis pärit nakatunud rakkudest (võrrelduna fraktsiooniga mittenakatatud rakkudest). Neli valku (PCBP1, hnRNP M, hnRNP C, hnRNP K) valiti, et kinnitada metoodikat töötamist. Kõik neli kolokaliseerusid SFV replikatsioonikompleksiga. Kasutades siRNA vahendatud ekspressiooni allasurumist, ilmnes, et hnRNP M ja hnRNP C allasurumine võimendas viiruse paljunemist, kuid PCBP1 allasurumine vähendas viirusvalkude translatsiooni ning hnRNP K suurendas viiruse RNA sünteesi.

Kokkuvõtvalt, nii meie kui teiste uurimisgruppide tulemused näitavad, et tõenäoliselt omab nsP3 valk SFV replikatsioonikompleksis pigem struktuurset rolli ning vahendab olulisi interaktsioone peremeesraku valkudega. Samuti on need uuringud, mis uurivad peremeesraku valkude rolli infektsioonis, alles algusjärgus, mistõttu on vajalikud edasised pürgimised.

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PUBLICATIONS

CURRICULUM VITAE

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List of Publications:

1. **Varjak M**, Zusinaite E, Merits A. 2010. Novel functions of the alphavirus nonstructural protein nsP3 C-terminal region. Journal of Virology, 84: 2352–2364
2. Pohjala L, Utt A, **Varjak M**, Lulla A, Merits A, Ahola T, Tammela P. 2011. Inhibitors of alphavirus entry and replication identified with a stable Chikungunya replicon cell line and virus-based assays. PLoS One, 6(12): e28923
3. Panas MD, **Varjak M**, Lulla A, Eng KE, Merits A, Karlsson Hedestam GB, McInerney GM. 2012. Sequestration of G3BP coupled with efficient translation inhibits stress granules in Semliki Forest virus infection. Molecular Biology of the Cell, 23:4701–4712
4. Rodriguez-Andres J, Rani S, **Varjak M**, Chase-Topping ME, Beck MH, Ferguson MC, Schnettler E, Fragkoudis R, Barry G, Merits A, Fazakerley JK, Strand MR, Kohl A. 2012. Phenoloxidase activity acts as a mosquito innate immune response against infection with Semliki Forest virus. PLoS Pathogens, 8(11):e1002977
5. **Varjak M**, Saul S, Arike L, Lulla A, Peil L, Merits A. 2013. Magnetic fractionation and proteomic dissection of cellular organelles occupied by the late replication complexes of Semliki Forest virus. Journal of Virology, 87: 10295–10312

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Publikatsioonide nimekiri:

1. **Varjak M**, Zusinaite E, Merits A. 2010. Novel functions of the alphavirus nonstructural protein nsP3 C-terminal region. Journal of Virology, 84: 2352–2364
2. Pohjala L, Utt A, **Varjak M**, Lulla A, Merits A, Ahola T, Tammela P. 2011. Inhibitors of alphavirus entry and replication identified with a stable Chikungunya replicon cell line and virus-based assays. PLoS One, 6(12): e28923
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