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NELE TAMBERG

Studies on Semliki Forest virus replication and pathogenesis

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

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

I **Tamberg N**.*, Lulla V.*, Fragkoudis R., Lulla A., Fazakerley J. K. and Merits A. (2007). Insertion of EGFP into the replicase gene of Semliki Forest virus results in a novel, genetically stable marker virus. J Gen Virol 88:1225–1230.
* Authors contributed equally to this work

II Fragkoudis R., **Tamberg N**., Siu R., Kiiver K., Kohl A., Merits A. and Fazakerley J. K. (2009). Neurons and oligodendrocytes in the mouse brain differ in their ability to replicate Semliki Forest virus. J Neurovirol 15:57–70.

III Kiiver K.*, Tagen I.*, Zusinaite E., **Tamberg N**., Fazakerley J. K., and Merits A. (2008). Properties of non-structural protein 1 of Semliki Forest virus and its interference with virus replication. J Gen Virol 89:1457–1466.
* Authors contributed equally to this work

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In addition, some unpublished data are presented.

Author's contribution:

I I designed and performed all of the experiments together with V. Lulla or R. Fragkoudis, except the immunoprecipitation experiments to analyse polyprotein processing. I actively participated in the analysis of the data and the writing of the manuscript.
II I performed, together with F. Fragkoudis, the animal pathogenesis studies of recombinant viruses. I actively participated in the analysis of the data and the writing of the manuscript.
III I constructed and produced the recombinant viruses and participated in the growth curve experiments.

LIST OF ABBREVATIONS

aa	amino acid
C	capsid protein
cDNA	complementary DNA
CHIKV	Chikungunya virus
CPV	cytopathic vacuole
CSE	conserved sequence element
CNS	central nervous system
ds	double-stranded
EEEV	Eastern equine encephalitis virus
EGFP	enhanced green fluorescent protein
eIF	eukaryotic translation initiation factor
ER	endoplasmic reticulum
FMDV	Foot and mouth disease virus
GFP	green fluorescent protein
GMP	guanosine monophosphate
i.c.	intracerebral
icDNA	infectious cDNA
IFN	interferon
i.p.	intraperitoneal
IRES	internal ribosomal entry site
kb	kilobase
m^7GMP	7-methyl-GMP
MOI	multiplicity of infection
ns	nonstructural
nsP	nonstructural protein
NLS	nuclear localisation signal
NTR	nontranslated region
pfu	plaque forming unit
PKR	dsRNA-activated protein kinase
RdRP	RNA-dependent RNA polymerase
Rluc	Renilla luciferase
RRV	Ross River virus
SFV	Semliki Forest virus
SINV	Sindbis virus
ts	temperature-sensitive
VEEV	Venezuelan equine encephalitis virus
VLP	virus-like particle
WEEV	Western equine encephalitis virus

1. INTRODUCTION

Alphaviruses are small, enveloped, positive-strand RNA viruses that are spread worldwide. This genus includes many important human and animal pathogens that cause different diseases, including arthralgia and encephalitis. Semliki Forest virus (SFV) is one of the best-studied alphaviruses and serves as one of the prototypes. Studies of SFV have revealed many important aspects about alphavirus infection, including receptor-mediated endocytosis for virus entry, spatially and temporally regulated viral RNA replication, and mechanisms of membrane protein transport. In addition, SFV-infected rodents serve as one of the general model systems for studies of viral encephalitis.

Although alphaviruses have been extensively studied since their discovery more than 80 years ago, many questions concerning virus life cycle, virus-host interactions, and especially pathogenesis still remain unanswered. Several aspects of these processes are difficult to study, especially in living organisms, where infection is a complex process and involves different tissues and cell types. Recombinant viruses expressing marker proteins that are easy to visualise or quantify have facilitated infection studies of different viruses in both cultured cells and in animals. Unfortunately, most of designed alphavirus-based vectors tend to suffer from genetic instability, hampering their use in experiments where viral spread is needed.

The aim of the studies that form the basis of the current thesis was to develop suitable tools and then employ them to study different aspects of SFV infection and pathogenesis. This thesis describes the successful construction of a novel marker protein-expressing recombinant SFV with a highly stable genome. Pathogenesis studies in adult mice exploiting these tools revealed new insights into the infection process of SFV in the mouse central nervous system and have aided in the understanding of how the mouse brain responds to alphavirus infection. This thesis also describes new findings about the effects of non-structural protein 1 (nsP1) and its non-palmitoylated mutant on the host cell and SFV infection cycle, as studied by use of constructed recombinant viruses.

2. REVIEW OF LITERATURE

2.1. Alphaviruses

The family *Togaviridae* contains two genera, genus Alphavirus and genus Rubivirus. Rubella virus is the only member of the genus Rubivirus. The genus Alphavirus has more than 30 members including several important human and animal pathogens (Strauss and Strauss, 2008). Alphaviruses are distributed worldwide; their isolates have been reported on all continents except Antarctica. All members of the genus are closely related and even the most distantly related viruses have approximately 40% amino acid sequence identity (Strauss and Strauss, 2008).

Alphaviruses have classically been described as either Old World or New World viruses, based on the geographic location they were originally isolated. Old World viruses, including Chikungunya (CHIKV), O'nyong-nyong, Ross River (RRV), Sindbis (SINV) and Semliki Forest virus (SFV), are found in Europe, Africa, Asia and Australia and can cause fever, rash, and arthralgia. New World viruses, such as Western, Eastern and Venezuelan equine encephalitis viruses (WEEV, EEEV, VEEV, respectively), are found in North and South America and typically cause encephalitis (Strauss and Strauss, 1994).

The genus Alphavirus includes several important human and animal pathogens. EEEV and WEEV regularly cause fatal encephalitis in North and South America, although the number of people infected is generally small (Strauss and Strauss, 1994). VEEV is estimated to infect tens of thousands of people in Latin America every year. Typical symptoms of infection include headache, fever, myalgia and arthralgia; in some cases, neurological disease and death can occur (Aguilar et al., 2011). A recent CHIKV epidemic in Indian Ocean territories resulted in millions of infected people experiencing severe rash and joint pain and a mortality rate of 0.1% (Schwartz and Albert, 2010). Chikungunya fever has been identified in nearly 40 countries (Schwartz and Albert, 2010).

Most alphaviruses are spread by blood-sucking arthropods, and in nature, they alternate between replicating in insect vectors, mostly mosquitoes, and higher vertebrates. In insect vectors, alphavirus replication is asymptomatic, resulting in persistent lifelong infection with minimal effect on the biological functions of the host. In vertebrates, the duration of infection is usually short and ends with the death of the infected host or clearance of the virus by the immune system (Strauss and Strauss, 1994). Replication in cultured cells mirrors these properties of the alphavirus life cycle. In cells of vertebrate origin, the viruses cause highly productive, cytopathic infection, while in mosquito cells, the infection is apathogenic, persistent or chronic, resulting in the release of high titre progeny (Strauss and Strauss, 2008).

Most alphavirus infection knowledge comes from studies of the prototype viruses SFV and SINV. These are prototypes viruses due to their ability to grow to high titres in cell culture, ability to infect cells from a broad range of hosts and tissue types, and the fact that their laboratory strains are nonpathogenic to

humans. However, the natural strains of these viruses can cause severe human disease (Mathiot et al., 1990; Kurkela et al., 2008). Complete cDNA clones of SINV and SFV have made it possible to easily manipulate the virus genomic sequence and transcribe infectious viral RNA *in vitro* (Rice et al., 1987; Liljeström et al., 1991). Studies of SFV and SINV have shed light on many aspects of the alphavirus infection cycle and also have helped to elucidate the basics of several cellular processes. SFV was the first virus demonstrated to infect cells via endocytic uptake and low pH-triggered membrane fusion, and its capsid protein was one of the first proteins demonstrated to exhibit cotranslational folding (Helenius et al., 1980; Nicola et al., 1999). In addition, SFV and SINV have been valuable models for studies of togavirus uptake, structure, replication, and cellular pathways involved in such tasks as the cleavage and modification of glycoproteins and their transport to the plasma membrane (Kääriäinen and Ahola, 2002).

2.2. Virion and genome organisation

Alphavirus virions are uniform, highly organised icosahedral particles with a diameter of 65–70 nm and can be described as being composed of two interacting protein shells with a lipid bilayer sandwiched between (Figure 1) (Jose et al., 2009). Inside the virion, single copy of a single-stranded plus-sense RNA genome and 240 copies of capsid protein form the icosahedral nucleocapsid with diameter of 40 nm (Paredes et al., 1993; Cheng et al., 1995). The nucleocapsid is surrounded by a tight-fitting envelope, which consists of a host-derived lipid bilayer embedded with heterodimers of the viral glycoproteins E1 and E2. The glycoproteins penetrate the lipid bilayer and make contacts with the nucleocapsid by connecting each E1-E2 heterodimer to one monomer of capsid protein (Paredes et al., 1993; Cheng et al., 1995; Fuller et al., 1995). On the surface of the virion, glycoproteins form an icosahedral lattice with T=4 symmetry and three E1-E2 heterodimers arrange into 80 protruding structures called spikes (Vogel et al., 1986; Paredes et al., 1993). The receptor attachment sequence is found at the tip of the spike on the E2 molecule (Smith et al., 1995).

Alphavirus genomic 42S RNA (49S for SINV) is approximately 11.5 kb long. The 5’ two-thirds of the genome encodes the non-structural (ns) replicase proteins (nsP1, nsP2, nsP3 and nsP4) and the 3’ one-third codes the structural proteins (Figure 1). The ns proteins are translated directly from the 42S RNA. Structural proteins are translated from 4.1-kb long subgenomic 26S RNA, which is synthesised from an internal promoter located on the minus-strand of the replicative intermediate. Both the 42S and 26S RNA have 5’-terminal methylguanylate cap-structures and 3’ polyadenylate tails (Strauss and Strauss, 1994).

The alphavirus genome contains three nontranslated regions (NTR): one at each end of the RNA and one at the junction of the non-structural and structural regions. All NTRs contain important structural elements, so-called conserved

sequence elements (CSEs) necessary for virus replication. Approximately 40 of the first nucleotides of the 5' NTR form a conserved stem-loop structure (CSE1) and are believed to constitute the promoter for plus-strand RNA synthesis (Ou et al., 1983). CSE2 is located at the 5' end of the genome within the nsP1 coding sequence and contains two conserved stem-loop structures that function to enhance both minus- and plus-strand RNA replication (Ou et al., 1983; Frolov et al., 2001). The 24-nucleotide long basal subgenomic promoter (CSE3) lays at the junction of the ns and structural regions and partially overlaps with the sequence coding for C-terminus of nsP4 (Levis et al., 1990). The 3' NTR contains a conserved 19-nucleotide sequence just adjacent to the poly(A) tail (CSE4), which is necessary for efficient minus-strand synthesis (Pfeffer et al., 1998; Hardy and Rice, 2005).

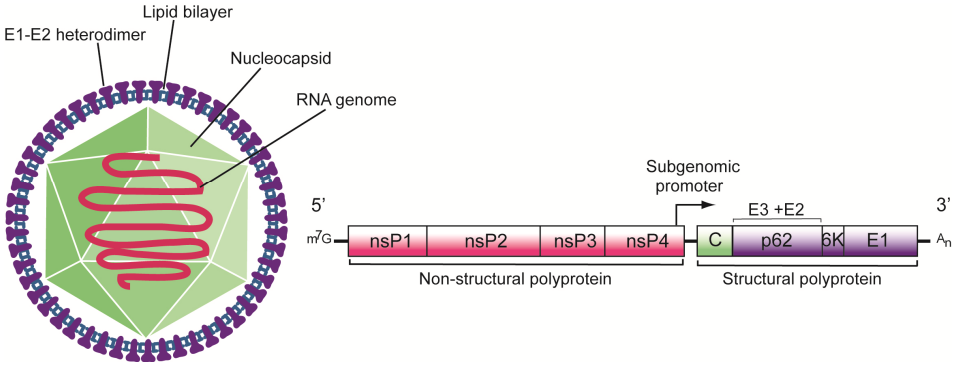

Figure 1. Alphavirus virion and genome organization. The alphavirus virion contains a nucleocapsid formed by plus-sense single-stranded 42S RNA genome (red) and capsid proteins (green). The nucleocapsid is surrounded by lipid bilayer (blue) embedded with heterodimers of viral glycoproteins E1 and E2 (purple). The alphavirus genome contains open reading frames encoding for non-structural and structural proteins. The non-structural proteins nsP1, nsP2, nsP3 and nsP4 are translated directly from the genomic RNA. The structural proteins C, p62, 6K and E1 are translated from the subgenomic RNA. p62 is the precursor of E2 and E3.

2.3. Infection cycle

2.3.1. Virus entry

Alphavirus infection of a susceptible cell starts with binding to its surface receptor. The viral glycoprotein E2 is primarily responsible for this interaction (Tucker and Griffin, 1991; Salminen et al., 1992). As alphaviruses have a very broad host range and replicate in cells from various tissues within their hosts, they must use different cell surface receptors for entry or use one conserved receptor that is expressed ubiquitously by all susceptible cells. To date, several

cell surface attachment molecules have been identified, and the data suggest that a single alphavirus can use different types of receptors and some of these receptors are also shared among different alphaviruses. It has been shown that the ubiquitously expressed NRAMP (Natural Resistance-Associated Macrophage Protein, a divalent metal ion transporter) mediates the entry of SINV, but not RRV, both into mammalian and insect cells (Rose et al., 2011). The laminin receptor has been shown to mediate the entry of SINV into mammalian cells and entry of VEEV into mosquito cells (Wang et al., 1992; Ludwig et al., 1996). SINV can also use cell-type specific receptors, such as c-type lectins, for entry (Klimstra et al., 2003). In addition to proteinaceous receptors, nonprotein attachment factors, including heparin sulphate, might be used by the alphaviruses to aid initial binding to the host cell surface (Jose et al., 2009). To date, no host cell receptor has been identified for SFV.

After binding to the receptor, virions enter host cells via clathrin-dependent endocytosis (Figure 2) (Helenius et al., 1980; DeTulleo and Kirchhausen, 1998). As infection proceeds, virus-containing endosomes mature, the intravesicular pH becomes mildly acidic, and this induces a series of conformational changes in virus glycoproteins. The E1-E2 heterodimers in the envelope are destabilised, and a previously hidden hydrophobic fusion loop in E1 is exposed (Kielian and Helenius, 1985; Wahlberg and Garoff, 1992; Ahn et al., 1999; Hammar et al., 2003). The fusion loop enters into the endosomal membrane and E1 homotrimers are formed (Wahlberg et al., 1992; Gibbons et al., 2000). This leads to the fusion of the viral envelope and endosomal membrane and the nucleocapsid is released into the cytoplasm. It has been shown that the presence of cholesterol and sphingolipids in the target membrane in mammalian cells is important for fusion (Kielian et al., 2010). The released nucleocapsid is disassembled by ribosomes, and viral RNA genome enters the cytoplasm (Singh and Helenius, 1992; Wengler et al., 1992).

2.3.2. Genome replication

The alphavirus RNA genome is capped, polyadenylated and immediately translated into ns polyprotein by host cell ribosomes after entry into the cytoplasm (Figure 2). The ns polyprotein is autocatalytically cleaved into four polypeptides in a precisely and temporally regulated manner to first yield the minus-strand synthesis complex and then transform it into stable plus-strand polymerase (Figure 2) (Lemm et al., 1994; Vasiljeva et al., 2003). The protease activity responsible for polyprotein processing resides in non-structural protein nsP2 (Hardy and Strauss, 1989; Merits et al., 2001). The polyprotein stage is essential for formation of functional replicase, as individual ns proteins cannot form a complex when expressed simultaneously in the same cell, whereas expression in a polyprotein form results in formation of complexes associated with cell membranes (Salonen et al., 2003).

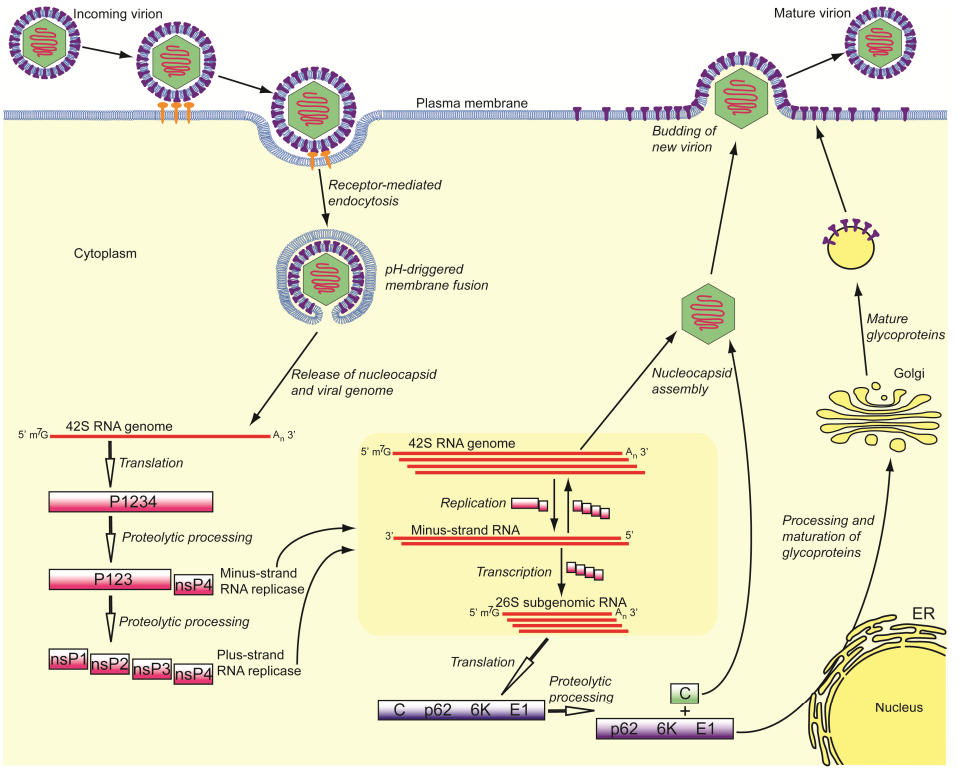

Figure 2. Alphavirus infection cycle. The alphavirus virion enters the host cell by receptor-mediated endocytosis. The fusion of endosome and viral membranes follows and the viral genomic 42S RNA is released into cytoplasm. The genomic RNA is immediately translated into ns-polyprotein P1234 (pink) that is proteolytically processed and forms first the minus-strand and then plus-strand replicase. The minus-strand replicase produces minus-strand RNA serving as a template for new positive-strand genomic 42S and subgenomic 26S RNAs. The subgenomic RNA drives the expression of structural polyprotein that is co- and post-translationally processed. First, the capsid protein (green) is released and it associates with newly synthesised genomic 42S RNA to form the nucleocapsid. Maturation of glycoproteins (purple) occurs in ER and Golgi. Mature glycoproteins are transported to the plasma membrane. The nucleocapsid associates with glycoproteins at the plasma membrane and virion budding occurs.

For most of the alphaviruses, the translation of the RNA genome yields two different polyproteins: P123 and P1234. The latter is synthesised as a result of translational read-through of an opal termination codon in the ns open reading frame with an efficiency of 10–20% (Li and Rice, 1993; Shirako and Strauss, 1994). In some alphaviruses, including most strains of SFV, the opal termination codon is not present and therefore only P1234 is produced (Takkinen, 1986). Shortly after translation, the full-length polyprotein P1234 likely cleaves itself *in cis* resulting in P123 and nsP4 to form the early replication complex (Figure 2). Using the genomic 42S RNA as a template, it

serves as a minus-strand RNA synthesis complex, which does not efficiently make plus-strands (Lemm et al., 1994; Shirako and Strauss, 1994; Vasiljeva et al., 2003). The synthesis of minus-strand RNA takes place only early in infection and is rapidly downregulated as the infection proceeds. Next, the cleavage of the polyprotein takes place between nsP1 and nsP2, and a short-lived complex is formed by nsP1, P23 and nsP4, which can produce both plus- and minus-strand RNA (Lemm et al., 1998; Gorchakov, Frolova, et al., 2008). Shortly after that, P23 is cleaved *in trans,* and a stable complex is formed by the fully processed individual ns proteins nsP1, nsP2, nsP3 and nsP4 that constitute the plus-strand replication complex responsible for synthesising subgenomic 26S and new genomic 42S RNA (Figure 2) (Lemm et al., 1994; Shirako and Strauss, 1994; Vasiljeva et al., 2003). The synthesis of plus-strand RNA continues at a maximal rate until the death of the infected cell, and over 200,000 copies of each RNA can be made in a single cell (Sawicki and Sawicki, 1980). The produced subgenomic 26S RNA serves as a template for virus structural protein synthesis, and 42S RNA is packaged into forming virions (Figure 2).

Among other ns polyprotein cleavage sites, the nsP2/3 site serves as an important temporal regulation step in the replication and infection cycle. First, it transforms the replicase into the plus-strand synthesis complex. Second, in later stages of infection, when enough nsP2 has accumulated in the cytoplasm for *in trans* cleavage, newly synthesised ns polyproteins are immediately cleaved into P12 and P34, and no additional functional replicases can form. Instead, ns polyproteins are efficiently cleaved into individual polypeptides and can fulfil their specific functions that are described in more detail below in chapter 2.4.

All ns proteins are essential for viral RNA replication. Analysis of temperature-sensitive (ts) mutant viruses has shown that nsP1 is specifically necessary for minus-strand synthesis (Hahn, Strauss, et al., 1989; Lulla et al., 2008), nsP2 regulates the synthesis of subgenomic 26S RNA (Suopanki et al., 1998), nsP3 plays a role in minus-strand and subgenomic RNA synthesis (Sang 1994, De 2003), and nsP4 is the catalytic subunit of the polymerase (Hahn, Grakoui, et al., 1989; Thal et al., 2007; Rubach et al., 2009).

The above described fine-tuned regulation of replication most likely evolved to make it efficient and also to regulate virulence. For virus propagation, different types of replication intermediates are needed in different amounts: plus-strand genomic and subgenomic RNAs are required in much greater quantities than minus-strands. Controlled non-excessive RNA production is especially important in insects, where production of avirulent infection and minimal effect on host cell viability is needed (Jose et al., 2009).

Alphavirus RNA replication is also a very dynamic process spatially and involves several different cellular compartments. This process takes place in association with modified intracellular membranes and the sites of replication are bulb-shaped membrane invaginations, called spherules (Froshauer et al., 1988; Kujala et al., 2001). The spherules are first formed at the host cell plasma membrane early in infection and as infection proceeds are internalised using endocytic processes (Frolova et al., 2010; Spuul et al., 2010). Later in infection,

spherules can also be found on modified endosomal and lysosomal membranes in large acidic cytoplasmic vacuoles, called type I cytopathic vacuoles (CPV-1) (Kujala et al., 2001; Spuul et al., 2010). It has been shown that double-stranded replication intermediates are located inside the spherules and virus ns proteins forming the replicase complex can be found at the cytoplasmic necks (Kujala et al., 2001; Frolova et al., 2010). In addition to viral proteins, host proteins are likely also in close association with spherule-forming viral replication complexes. It has been shown that both ns polyproteins and synthesis of dsRNA are necessary for spherule formation (Frolova et al., 2010). The viral ns protein nsP1 is responsible for membrane attachment of replication complexes, and nsP3 is necessary for directing plasma membrane-bound replication complexes into intracellular vesicles (Salonen et al., 2003; Varjak et al., 2010). Possible reasons for membrane-associated replication and spherule formation include enhancing replication efficiency by bringing all the necessary components together in close proximity and hiding dsRNA replication intermediates from host cell defence systems.

2.3.3. Virion assembly and budding

Alphavirus structural proteins are translated from the subgenomic 26S RNA as a polyprotein, C-p62-E2-6K-E1, and processed cotranslationally by a combination of viral and cellular enzymes (Figure 2) (Jose et al., 2009). Capsid protein (C) is a serine autoprotease and cleaves itself from the nascent structural polyprotein (Melancon and Garoff, 1987). This cleavage releases the signal peptide at the new amino terminus of the polyprotein and it is inserted into the endoplasmic reticulum (ER), where the translation is completed and the polyprotein is cotranslationally glycosylated, palmitoylated, and cleaved. The processing of the remaining structural polyprotein is mediated by a cellular enzyme signalase in the ER lumen and p62, 6K and E1 are produced. Glycoproteins p62 and E1 form heterodimers in the ER and p62 is cleaved by the cellular protease furin during transport from the Golgi to the plasma membrane to form E3 and E2 (Zhang et al., 2003). The p62-E1 heterodimer is more resistant to acidic conditions than the E1-E2 complex and survives the mildly acidic pH in transport vesicles. Once p62 is processed, the E1-E2 heterodimer is primed for the disassembly needed during virus entry into a new host cell (Wahlberg et al., 1989). For some alphaviruses, including SFV, VEEV and CHIKV, the released E3 protein is incorporated into virions (Wu et al., 2008; Voss et al., 2010; Zhang et al., 2011). For other alphaviruses, E3 is released.

The exact function of the 6K protein remains obscure, but it might function in glycoprotein trafficking, virion formation and budding. It is also incorporated into virions in small amounts (7–30 copies) (Jose et al., 2009). Recently it was discovered that during SFV structural polyprotein synthesis, ribosomal frameshifting occurs with an efficiency of 10–18% within the sequence encoding 6K,

and this results in production of TransFrame (TF) protein (Firth et al., 2008). It has been shown that TF is packaged into virions, but its exact functions have yet to be determined.

Nucleocapsids are formed in the cytoplasm of the infected cell (Figure 2). First, capsid protein recognises the encapsidation signal in the 42S RNA and then subsequent interactions between capsid proteins lead to encapsidation and nucleocapsid formation (Linger et al., 2004). The encapsidation signal is in the ns area, ensuring that only 42S, and not 26S RNA, is included into virions. In the SIN genome, the encapsidation signal is in the nsP1 sequence and in SFV RNA, the signal is in the region coding for nsP2 (Weiss et al., 1994; White et al., 1998). The newly formed nucleocapsids are transported or diffuse to the plasma membrane and are bound by cytoplasmic tails of E2 glycoprotein. Subsequently, nucleocapsids bud through the cell plasma membrane and acquire a lipoprotein envelope (Figure 2) (Jose et al., 2009).

2.4. Non-structural proteins

All four ns proteins are essential for alphavirus replication and, in addition, also have individual roles in the virus infection cycle and virus-host interactions.

The first of the ns proteins, **nsP1** (537 aa), is a membrane-binding protein possessing methyltransferase and guanylyltransferase activities involved in the capping of 42S and 26S RNAs (Mi and Stollar, 1991; Laakkonen et al., 1994; Ahola and Kääriäinen, 1995). In alphavirus capping reactions, nsP1 first transfers a methyl-group from S-adenosyl-methionine (AdoMet) to GTP, then a covalent intermediate complex between 7-methyl-GMP (m^7GMP) and nsP1 is formed and m^7GMP is transferred to virus RNA. The capping reaction of alphaviruses thus differs from the capping of cellular mRNAs, where guanylyltransferase first transfers GMP to RNA and then methylation of the GMP-RNA intermediate follows (Ahola and Kääriäinen, 1995). The enzymatic activities of nsP1 are essential for virus replication, as single point mutations abolishing enzymatic activity render the virus non-infectious (Wang et al., 1996).

NsP1 is tightly bound to the plasma membrane in both infected cells and also when expressed alone and serves as the sole membrane anchor for virus replication complexes (Peränen et al., 1995; Ahola et al., 2000; Salonen et al., 2003). The plasma membrane binding is mainly mediated by nsP1 amino acids 245–264 forming an amphipathic alpha-helix (Ahola et al., 1999; Spuul et al., 2007). Single point mutations in this area can affect nsP1 membrane binding activities and can be lethal to the virus (Spuul et al., 2007). The membrane binding is further tightened by post-translational palmitoylation at nsP1 cysteine residues 418–420 in SFV and cysteine 420 in SINV (Laakkonen et al., 1996; Ahola et al., 2000). Palmitoylation makes the nsP1 interaction with membranes very tight, resembling that of integral membrane proteins (Laakkonen et al., 1996). Palmitoylation, as such, is not needed for the enzymatic activities of

nsP1, for formation of functional replication complexes or for the viability of the virus (Ahola et al., 2000; Zusinaite et al., 2007). Nevertheless, deletion or substitution of cysteine residues 418–420 in nsP1 of SFV greatly hampers virus replication and causes accumulation of compensatory mutations (Zusinaite et al., 2007). Viruses expressing pamitoylation-deficient nsP1 are apathogenic in mice; these viruses are able to induce low levels of blood viraemia, but no infectious virus can be detected in brain tissue (Ahola et al., 2000).

In addition, nsP1 is also responsible for induction of filopodia-like structures on the cell surface that are characteristic of alphavirus-infected cells (Laakkonen et al., 1998; Zusinaite et al., 2007). The function of filopodia-like structures and also the exact functional significance of palmitoylation remain unknown.

The second ns protein, **nsP2 (**799 aa in SFV), has multiple enzymatic activities and important roles in virus infection. NsP2 is an RNA helicase and its N-terminal domain also contains nucleoside triphosphatase and RNA triphosphatase activities (Rikkonen et al., 1994; Gomez de Cedrón et al., 1999; Vasiljeva et al., 2000). RNA helicase activity has been suggested to be important for unwinding RNA duplexes or RNA secondary structures during replication. RNA triphosphatase activity is needed during capping reactions to remove the 5' γ-phosphate from viral RNA before the guanylyltransferase activity of nsP1 can attach the m^7GMP structure.

The C-terminal domain of nsP2 contains a papain-like protease responsible for processing of ns polyprotein (Hardy and Strauss, 1989; Merits et al., 2001). As described above, the protease participates in replication complex formation and the temporal regulation of replication. Mutations, which abolish proteolytic activity, are lethal to the virus (Strauss et al., 1992). The carboxy terminus of nsP2 also contains an enzymatically nonfunctional methyltransferase domain, which plays a role in regulation of minus-strand synthesis and development of cellular cytopathic effects (Mayuri et al., 2008).

Cell fractionation studies have shown that approximately 50% of nsP2 is in the nuclear fraction of infected cells (Peränen et al., 1990). The transport to the nucleus takes place early in infection and nuclear localisation is mediated in nsP2 of SFV by the pentapeptide PRRRV (aa 647–651) (Rikkonen et al., 1992). Mutations affecting nsP2 translocation to the nucleus yield attenuated phenotypes with reduced cytotoxicity, reduced transcriptional and translational shutdown of host cells, and renders virus apathogenic in adult mice (Rikkonen, 1996; Fazakerley et al., 2002; Gorchakov et al., 2005; Tamm et al., 2008). In addition, other sequences within nsP2, including the integrity of helicase and methyltransferase-like domains, are essential for mediating the virus-induced shutdown of cellular transcription (Gorchakov et al., 2005; Frolov et al., 2009; Akhrymuk et al., 2012) and suppression of antiviral responses (Frolova et al., 2002; Gorchakov, Frolova, et al., 2008). The exact mechanism for nsP2-mediated host cell transcription shutdown is described in chapter 2.5.1.

The functions of n**sP3** are not well understood. The nsP3 (482 aa in SFV) primary structure is composed of three discrete domains. The first 160 amino acids of the amino terminus of nsP3 form a small structurally conserved macrodomain, which is conserved among alphaviruses, rubella virus, hepatitis E virus, coronaviruses and also shares sequence similarity with the macrodomains displayed by proteins from eukaryotic organisms, eubacteria and archaebacteria (Koonin and Dolja, 1993; Pehrson and Fuji, 1998). The middle domain of nsP3 is only conserved among alphaviruses and the C-terminal domain (starting from Tyr324 in SFV) is hypervariable both in sequence and length (Strauss and Strauss, 1994).

In addition to localising to replication complexes, nsP3 can also be found in aggregates of variable size with irregular boundaries in the cytoplasm and a fraction of nsP3 localises to the nuclear envelope (Salonen et al., 2003; Cristea et al., 2006; Gorchakov, Garmashova, et al., 2008). This suggests that nsP3 has functions in addition to participating in RNA replication.

The only enzymatic activity identified for nsP3 is ADP-ribose 1''-phosphate phosphatase activity and it resides within the macrodomain of the protein (Malet et al., 2009; Neuvonen and Ahola, 2009). Alphavirus macrodomains can bind poly(ADP-ribose), RNA and, in some cases, ADP-ribose (Malet et al., 2009; Neuvonen and Ahola, 2009). Interestingly, poly(ADP-ribose) polymerase (PARP-1) has been shown to interact with SINV replication complexes through the C-terminal domain of nsP3 in neuronal cells (Park and Griffin, 2009a). The biological significance of these functions is poorly understood.

Based on current understanding, nsP3 is the only alphavirus ns protein modified by phosphorylation and gets phosphorylated at serine and threonine residues in the carboxy terminus (Peränen et al., 1988; Li et al., 1990; Vihinen and Saarinen, 2000; Vihinen et al., 2001). In SFV, all 16 identified phosphorylation sites are accumulated in a short 50-amino acid residue long area and complete elimination of these sites in nsP3 results in reduced levels of RNA synthesis in cultured cells and greatly reduced pathogenicity in mice (Vihinen et al., 2001). Studies of SINV ts mutants have shown that nsP3 phosphorylation is specifically important for minus-strand RNA synthesis (Dé et al., 2003).

Studies with both SFV and SINV have also suggested a role for nsP3 in the modulation of neuropathogenesis in mice (Tuittila and Hinkkanen, 2003; Park and Griffin, 2009b).

Studies have shown that nsP3 mediates the movement of alphavirus replication complexes from the plasma membrane into intracellular vesicles. This was elegantly demonstrated by a detailed analysis of subcellular localisation of different forms of ns polyprotein intermediates. While uncleavable P12 localised to the plasma membrane, addition of nsP3 targeted it to endosomes and lysosomes (Salonen et al., 2003).

The last of the ns proteins, **nsP4** (614 aa in SFV), is the catalytic subunit of alphavirus RNA-dependent RNA polymerase (RdRP) (Hahn, Grakoui, et al., 1989; Thal et al., 2007; Rubach et al., 2009). The C-terminus of alphavirus nsP4

shares sequence homology with RdRPs from other viruses and also contains the GDD motif observed in many viral RdRPs (Kamer and Argos, 1984). The N-terminus of nsP4 is conserved only among alphaviruses. While nsP4 is the catalytic core of the replication complex, it also needs other ns proteins for activity (Rubach et al., 2009). Mutational studies suggest that the amino terminus of nsP4 mediates functional interactions with other ns proteins, particularly with nsP1, and most likely also with host proteins (Shirako et al., 2000; Fata et al., 2002; Rupp et al., 2011).

The levels of nsP4 in infected cells are lower than the levels of other ns proteins. This is achieved by several processes. First, many alphaviruses contain the opal termination codon in their genome and the majority of translated ns polyproteins are in the form of P123, while translation of P1234 and thus also nsP4 happens only with 10–20% efficiency. Second, the N-terminal amino acid in nsP4 is a conserved tyrosine that directs nsP4 to rapid proteasomal degradation according to the N-end rule pathway (de Groot et al., 1991; Varshavsky, 1997). This destabilising tyrosine is essential, as replacing it with a nonaromatic residue results in poor RNA replication (Shirako and Strauss, 1998).

In addition, nsP4 has also demonstrated terminal adenylytransferase activity in *in vitro* experiments, suggesting a role in the maintenance and repair of the poly(A) tail of the viral genome (Tomar et al., 2006). The poly(A) tail is important for minus-strand synthesis and maintaining its integrity is essential for the virus.

2.5. Virus-host interactions

The outcome of any virus infection depends on both virus-encoded and host-specific properties. Efficient replication of viruses in infected hosts depends on proper functioning of the viral replicase and synthesis of viral proteins, along with disruption of the host antiviral response. Therefore, alphaviruses, similar to many other viruses, have developed mechanisms to hamper cellular antiviral processes while maintaining their own genome replication and virion production for spread in infected hosts.

2.5.1. Shutdown of host cell transcription and translation

One of the hallmarks of alphavirus infection in vertebrate cells is the shutdown of host cell transcription and translation without affecting the synthesis of viral RNAs and proteins. The transcriptional shutdown is essential to limit the production of antiviral proteins, mainly interferon-α/β (IFN-α/β), that play a role in early innate immune response and are the first defence mechanism against viral infection. The secreted IFNs both attenuate the ability of virus to replicate in cells already infected and also protect uninfected cells from new

rounds of infection until the development of an adaptive immune response (Jose et al., 2009).

The shutdown of cell transcription is mediated by nsP2 in Old World viruses and by capsid protein in New World viruses (Gorchakov et al., 2005; Garmashova et al., 2006, 2007; Aguilar et al., 2007). The nsP2-mediated transcriptional shutdown is achieved by ubiquitination and rapid degradation of Rpb1, a catalytic subunit of the RNA Polymerase II complex (Akhrymuk et al., 2012). Importantly, complete degradation of Rpb1 takes place before any other virus-induced change in infected cells occurs, suggesting that degradation of Rpb1 is the first and crucial step in the downregulation of the cellular antiviral response. The cellular transcription shutdown achieved by the New World virus VEEV is mediated through a different mechanism. The capsid protein of VEEV forms a complex with importin-α/β and the nuclear export receptor CRM1 and accumulation of these complexes in nuclear pores of infected cells rapidly inhibits the nucleocytoplasmic trafficking of proteins, eventually leading to transcriptional shutdown (Atasheva et al., 2008, 2010).

The shutdown of translation in alphavirus-infected cells is partially mediated by a cellular defence mechanism, the dsRNA-activated protein kinase (PKR) (Gorchakov et al., 2004, 2005). The presence of viral dsRNA activates host PKR, which phosphorylates and transiently inactivates the eIF2α subunit of cellular translation initiation factor eIF2. eIF2 is responsible for delivering initiator methionyl-tRNA to ribosomes and is essential for translation initiation. The phosphorylation of eIF2α results in a significant decrease in cellular translation, but the translation of alphavirus subgenomic RNA does not depend on eIF2α (Ventoso et al., 2006). Several alphaviruses, including SINV and SFV, have evolved a unique mechanism to bypass the requirement for eIF2 through the presence of a highly stable RNA hairpin loop downstream of the AUG initiator codon in 26S RNA, also called an enhancer sequence, which likely functions by stalling the ribosomes on the correct site to initiate translation (Sjöberg et al., 1994; Frolov and Schlesinger, 1996; Ventoso et al., 2006).

Another pathway leading to eIF2α phosphorylation and translation shutdown in alphavirus-infected cells is the unfolded protein response in the ER (Nivitchanyong et al., 2009; Barry et al., 2010). Studies have shown that one of the major ER stress sensors, eIF2α kinase 3 (PERK), is activated upon SINV infection leading to eIF2α phosphorylation (Nivitchanyong et al., 2009). The alphavirus glycoproteins E1 and E2 are translated and processed in the ER and Golgi and likely exceed the ER folding capacity, resulting in induction of the unfolded protein response.

2.6. Infection of the nervous system

SFV and SINV are Old World alphaviruses, but in rodents they cause encephalitis and therefore have been extensively used as models for alphavirus-induced neurological disease and acute viral encephalitis in general. The pathogenesis of alphavirus infection in the central nervous system (CNS) depends both on host age and virus strain, which together determine if the infection is restricted or widespread, and whether cells die, clear the virus, or become persistently infected (Fazakerley, 2002; Griffin, 2005).

Virus inoculation into the abdominal cavity (intraperitoneal inoculation, i.p.) is the most commonly used route to study the neuroinvasivness of alphaviruses. SFV and SINV inoculated i.p. first replicate in a number of tissues, resulting in high titre plasma viraemia that can be detected by 24–48 hours post-infection and is generally undetectable by day 4 (Fazakerley, 2002). High titre plasma viraemia is a prerequisite to infect the CNS and, in nature, also crucial for efficient transmission of virus to insect vectors during a blood meal. After entry into the CNS, alphaviruses first establish small perivascular foci of infection and in cases of permissive hosts, widespread replication in the CNS follows (Fazakerley et al., 1993). The cell types infected in the brain are mainly neurons and in case of SFV, also oligodendrocytes (Fazakerley, 2002).

All strains of SFV and SINV productively infect neonatal and young suckling mice (up to 11 days old). They replicate efficiently in the CNS, induce apoptotic death of infected cells, and animals die rapidly within couple of days (Fazakerley, 2002; Griffin, 2005). However, the outcome of infection in older mice can range from avirulent infection to lethal encephalitis depending on the virus strain used. The L10 strain of SFV efficiently causes lethal encephalitis in adult mice by any route of inoculation (Gates et al., 1985; Fazakerley et al., 1993; Oliver et al., 1997). The prototype strain SFV4 is neurovirulent and efficiently causes lethal encephalitis after intracerebral (i.c.) inoculation. An i.p. course of infection with SFV4 is productive only when high doses of virus are used; low doses fail to establish high titre plasma viraemia and the virus does not efficiently enter the CNS (Tarbatt et al., 1997; Tuittila et al., 2000; Fragkoudis et al., 2007). The SFV A7 and A7(74) strains are apathogenic in mice older than 2 weeks (Gates et al., 1985; Fazakerley et al., 1993; Oliver et al., 1997). SFV A7(74) is neuroinvasive in adult mice and efficiently enters the CNS when inoculated i.p. even at low doses, but infection is apathogenic and spread in the CNS remains limited and concentrated to perivascular foci. Replication is severely restricted in CNS neurons, and the virus is cleared from the brain (Fazakerley et al., 1993; Oliver et al., 1997). Interestingly, when SFV A7(74) is administered i.c. it still remains avirulent and restricted in mature neurons, but widespread replication in oligodendrocytes in the major white matter tracts can be observed (Fazakerley et al., 2006). Age-dependent susceptibility to fatal disease has also been described for SINV (Labrada et al., 2002; Griffin, 2005). It should also be noted that many other neurotrophic viruses cause more severe disease and replicate to higher titres in neonatal mice

than in older animals, and often the sharp decrease in susceptibility to fatal infection is during the first two weeks of life (Griffin, 2005).

The molecular determinants of increased susceptibility of younger hosts to severe alphavirus-induced encephalitis remain poorly understood. Studies with mice having selective immune dysfunctions suggest that it is not dependent on maturation of innate or adaptive immune responses, but rather on the maturity of neurons and neuronal circuits (Fazakerley et al., 1993; Oliver et al., 1997). For example, infection of SFV A7(74) remains restricted in mature neurons in athymic *nu/nu* mice lacking functional T-lymphocytes and even in mice with severe combined immunodeficiency (SCID) lacking both T- and B-lymphocytes (Fazakerley et al., 1993; Oliver et al., 1997). Restriction of replication in mature neurons is not mediated by the type-I IFN system (IFN-α/β) either (Fragkoudis et al., 2007). These results indicate that intrinsic properties of neurons and neuronal networks are important in determining the neuropathogenicity of alphavirus infection.

During the first postnatal weeks, when the susceptibility to fatal encephalitis declines, several changes take place in the mouse CNS. It is a time when detailed connections in neuronal network are finalised and axogenesis, synaptogenesis, gliogenesis and myelination are being completed (Fazakerley, 2002; Griffin, 2005). While apoptosis of immature neurons is part of normal development of the nervous system and immature neurons depend on trophic factors for survival, as neurons mature they become less dependent on growth factors and survival signalling pathways dominate (Fazakerley, 2002; Griffin, 2005). Mature neurons are irreplaceable and apoptosis or death of these cells can have severe consequences to the host. Upregulation of antiapoptotic pathways might restrict alphavirus infection in mature neurons. In mature neurons, the metabolic processes are also downregulated, and the production and transport of smooth membrane vesicles needed for alphavirus replication is decreased. In agreement with this theory, when gold compounds, which induce smooth membrane production in neurons, are administered to adult mice before infection, SFV A7(74) spreads efficiently in the mature CNS and neurons become permissive to replication (Scallan and Fazakerley, 1999).

2.6.1. Immune response in the mouse CNS

The immune response in the CNS is tightly regulated and depends on interactions between neurons and glial cells, blood brain barrier (BBB) function and constitutive production of regulatory molecules. Microglial cells represent the macrophage lineage in the CNS and their functions are inhibited by specific neurons under normal conditions. As part of normal immunologic surveillance, activated T cells cross the BBB and if antigen is not found they leave or die (Griffin and Metcalf, 2011).

When the CNS is infected, a rapid response follows. The exact mechanism for sensing alphavirus infection is not yet known, but likely toll-like receptors

and intracellular RNA helicases sensing the viral dsRNA are involved (Griffin and Metcalf, 2011). The type-I interferon system is important for initial control of alphavirus infection and both humoral and cellular arms of adaptive immune response participate in elimination of the virus. Following alphavirus infection type-I interferons are rapidly induced and the levels correlate with the virus titre in blood and viral RNA in the brain (Bradish et al., 1972; Fragkoudis et al., 2007). Virus strains apathogenic in wild-type mice are virulent and induce widespread infection in IFNAR-/- animals lacking functional IFN-α/β receptors (Hwang et al., 1995; Ryman et al., 2000; Fragkoudis et al., 2007).

Recovery of adult mice from infection with avirulent alphavirus strains requires immune-mediated clearance of the virus. Infection with avirulent strains is cleared from the brain in 7 to 10 days post-inoculation and it is a nonlytic process requiring both, functional T- and B-lymphocytes (Fazakerley, 2002; Griffin, 2005). In mice, which lack functional B-cells (μMT mice), clearance of SFV A7(74) is impaired both in blood and brain, while in mice, which lack functional T-cells (*nu/nu* mice), the viraemia is transient but CNS infection is persistent (Amor et al., 1996; Fragkoudis et al., 2008). Interestingly, avirulent strains of both SFV and SINV can establish persistent CNS infection in immunocompromised *nu/nu* or SCID mice, while no evidence of pathology can be detected (Amor et al., 1996).

The infectious virus is cleared very rapidly from CNS while the levels of viral RNA decrease gradually during several weeks in infected mature neurons (Metcalf and Griffin, 2011). As infected mature neurons are irreplaceable and cannot be eliminated without possible severe consequences, viral RNA cannot be completely cleared and long-term persistence of low levels of viral RNA in surviving cells has been described (Levine and Griffin, 1992; Fragkoudis et al., 2008; Metcalf and Griffin, 2011). Both antibody secreting cells and IFN-γ are essential for prevention of viral reactivation after viral clearance (Burdeinick-Kerr et al., 2007).

In adult mice, SFV A7(74) infection occasionally results in transient inflammatory demyelination of axons and the most likely mechanism generating these lesions is $CD8^+$ T-cell-mediated elimination of infected oligodendrocytes (Subak-Sharpe et al., 1993; Amor et al., 1996).

2.7. Alphavirus-based vectors

Alphavirus-based vectors expressing marker proteins, which are easy to visualise or quantify, have been successfully used to study virus infection both in cell culture and in animal models. Recombinant viruses expressing various host proteins have helped to analyse the effect of these proteins on the viral infection cycle. Although most viral vectors used for medical or biotechnology purposes are based on viruses with DNA genomes or retroviruses, vectors based on RNA viruses, including alphaviruses, have shown promising results. The main advantages of alphavirus-based vectors over DNA or retrovirus-based

systems are the high-level expression of foreign gene, cytoplasmic replication and no danger of integration of virus genetic material into host cell genome. Other characteristics of alphavirus-based vectors are the ability to replicate in a broad host cell range, high cytotoxicity and apoptotic rate of infected cells, very high but short-term foreign gene expression, and very high induction of the type-I interferon response. As alphavirus-based vectors mediate transient expression, the main applications that have been investigated are recombinant vaccine construction, treatment of CNS diseases and anti-cancer therapy (Atkins et al., 2008).

Infectious cDNA clones have been developed for many alphaviruses, including SFV, SINV, EEEV, VEEV, CHIKV and RRV (Atkins et al., 2008). Alphavirus-based vectors contain a cDNA copy of the entire viral genome inserted downstream of a bacteriophage RNA polymerase promoter, usually from bacteriophage SP6 or T7. *In vitro* transcribed RNA introduced into mammalian cells initiates the infectious cycle and infectious virus is produced. Alternatively, strong promoters working in eukaryotic cells can be successfully used to drive the expression of infectious virus RNA from a DNA plasmid transfected into mammalian cells (Atkins et al., 2008).

The first alphavirus-based vector constructed was the recombinant virus-like particle (VLP), also called a "suicide" particle vector (Figure 3A) (Liljeström and Garoff, 1991). In this design, the virus structural genes are deleted or substituted with foreign genes, but all the sequences needed for efficient replication remain. The structural proteins are provided *in trans* by helper RNAs that encode the capsid or envelope proteins but lack the packaging signal (Frolov et al., 1996; Smerdou and Liljeström, 1999). Introduction of *in vitro*-transcribed helper RNAs along with vector RNA into packaging cells will result in efficient replication of both and formation of VLPs from structural proteins translated from subgenomic RNA made from the helper RNA templates. The formed suicide particles are able to infect susceptible cells but can only go through one round of efficient replication. However, these vectors are mostly valuable for gene and biotechnology purposes and generally not suitable for studies of alphavirus infection where spreading and propagation of virus is important.

Double-subgenomic alphavirus-based vectors are also very commonly used. In this design, sequences encoding virus proteins are left intact and a duplicated subgenomic promoter is introduced to guide the expression of the foreign gene (Figure 3B) (Frolov et al., 1996; Atkins et al., 2008). To mimic the expression of virus structural proteins and also increase the levels of foreign protein, a translation enhancer element from the capsid protein can be used upstream of the foreign gene (Sjöberg et al., 1994; Smerdou and Liljeström, 1999; Ventoso et al., 2006). Double-subgenomic vectors enable direct observation of infection and have been successfully used to study alphavirus pathogenesis in animals, the entry and spread of the virus in the CNS, virus cell tropism and virus-host interactions (Levine et al., 1996; Cook and Griffin, 2003; Vähä-Koskela et al., 2003; Toribio and Ventoso, 2010). However, these vectors tend to suffer from

genomic instability, most likely due to recombination events between duplicated sequences and because the foreign gene is introduced as an independent transcription unit and has no selective value for the virus (Pugachev et al., 1995, 2000; Thomas et al., 2003). In addition, introducing a second subgenomic promoter slows down virus replication and reduces the levels of structural proteins by competition with the native subgenomic promoter for viral transcriptase complexes (Raju and Huang, 1991; Thomas et al., 2003). One solution to alleviate the genomic instability is the use of an internal ribosomal entry site (IRES) instead of a duplicated subgenomic promoter. This strategy was successfully utilised in Rubella virus and VEEV vectors, but for unknown reasons works poorly in the case of SFV (Pugachev et al., 2000; Volkova et al., 2008; Rausalu et al., 2009).

Another possibility is to insert foreign sequences into virus ns or structural polyprotein coding sequences so that the foreign sequence becomes a part of the viral gene expression unit (Figure 3C, D). These vectors should have higher genomic stability and marker expression mimics that of viral proteins, which is not always achieved when independent transcriptional units are used. Design of these vectors is more difficult as correct expression, processing and final conformation to ensure functionality of virus ns and structural proteins is essential for virus viability.

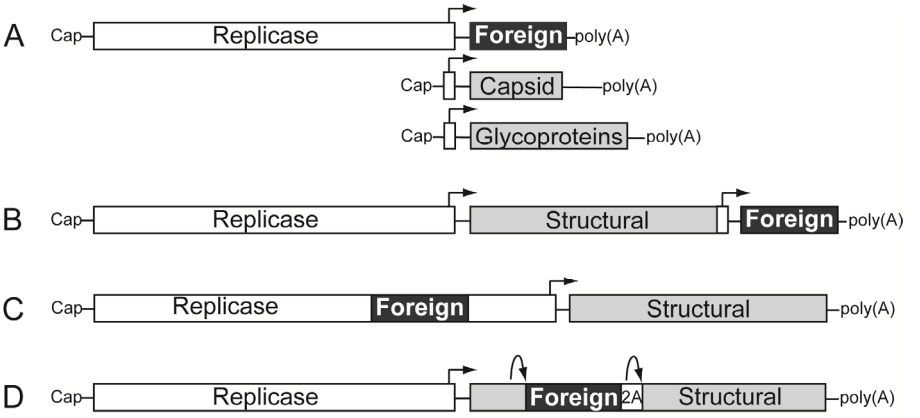

Figure 3. Alphavirus-based expression vectors. (A) Virus-like particle vectors. In the replication-competent vector, the structural genes are substituted with foreign genes. Virus structural proteins needed for particle formation are provided by helper vectors. (B) Double-subgenomic vectors contain a duplicated subgenomic promoter for foreign gene expression. (C) Foreign protein is expressed in fusion with virus ns proteins. (D) Foreign protein is expressed as a cleavable component of the viral structural polyprotein. Capsid mediates the C-terminal and inserted 2A peptide the N-terminal cleavage of foreign protein from the polyprotein.

SINV genomes with markers inserted into ns polyprotein to produce nsP2- or nsP3-fusion proteins have been described (Figure 3C) (Bick et al., 2003; Frolova et al., 2006; Atasheva et al., 2007). Luciferase or green fluorescent protein (GFP) coding sequences have been successfully inserted into the nsP3 hypervariable area without affecting the infectivity or replication of the virus. Luciferase enabled quantification of the production of viral ns proteins in infected cells and GFP was used to purify nsP3-containing complexes and visualise their time-dependent dynamics in infected cells (Bick et al., 2003; Frolova et al., 2006). To find suitable areas in nsP2 for marker insertion, a transposon-based approach was used to generate a cDNA library of SINV genomes with GFP randomly inserted into nsP2. Several sites within nsP2 were identified that tolerated insertions and resulted in viable virus presenting only slightly slower growth kinetics than wild-type virus (Atasheva et al., 2007). These insertions generated important information about functional domains in nsP2 and recombinant viruses expressing GFP in fusion with nsP2 have been successfully used to purify nsP2-containing complexes from infected cells. Recently, several studies have also used SFV-based vectors expressing nsP3-marker fusion proteins to monitor the detailed dynamics of replication complexes in living mammalian cells or to study the antiviral response in mosquito cells (Spuul et al., 2010; Siu et al., 2011).

The production of marker proteins as part of the viral ns polyprotein has also been successful for other plus-strand RNA viruses. For example, recombinant Hepatitis C virus expressing EGFP has been used to visualise virus replication complexes and recombinant Equine arteritis virus has been used to facilitate testing of antiviral compounds in infected cultured cells (Moradpour et al., 2004; van den Born et al., 2005).

A marker gene has been successfully introduced as a cleavable component also into SINV structural polyprotein between sequences coding for capsid and E3 proteins (Figure 3D) (Thomas et al., 2003). The carboxy terminal release of foreign protein from the virus structural polyprotein was mediated by an introduced 2A peptide from foot and mouth disease virus (FMDV). N-terminal cleavage was mediated by capsid protein. The resulting viruses replicated efficiently in cultured cells, had high genomic stability and presented mortality rates comparable to their parental strain in new-born mice (Thomas et al., 2003).

3. RESULTS AND DISCUSSION

Objectives of the present study

Recombinant viruses expressing marker proteins easy to visualise, trace, or quantify have facilitated virus infection studies in both cultured cells and in animals. The aim of the present study was to design novel recombinant SFV-based viruses stably expressing marker proteins and to use these viruses to study different aspects of SFV infection in cell culture and animal models.

1. Design recombinant viruses expressing marker proteins with stable genomes suitable for infection studies in cell cultures and animal models.
2. Determine the genetic stability and infection properties of recombinant viruses in cultured cells.
3. Use suitable marker-expressing viruses to study the course of infection and neuropathogenesis of SFV in adult mouse brain and cultured neurons
4. Study the effects of nsP1 and its palmitoylation-deficient form on host cells and the SFV infection cycle.

3.1. Design and characterisation of novel marker viruses (I, II)

3.1.1. Design of novel marker viruses

We investigated the possibilities of expressing the marker protein as part of both SFV ns and structural polyproteins. We hypothesised this would result in stable marker expression accurately reflecting the activities of viral genomic and subgenomic promoters during infection. All these properties are crucial for a good recombinant virus suitable for both *in vitro* studies with cell cultures and *in vivo* mouse pathogenesis experiments. As marker insertion into a virus transcriptional unit could have possible negative effects on virus viability, replication, polyprotein processing and virion formation, we analysed the infection properties of recombinant marker viruses in detail in cultured cells before pathogenesis studies.

In our first recombinant virus design, we investigated the possibility of inserting the coding sequence of EGFP into non-structural area of virus genome, specifically into the nsP3 coding region, to produce an nsP3-EGFP fusion protein (I, Fig 1a). The resulting recombinant virus SFV4(3F)-EGFP was viable, expressed the nsP3-EGFP fusion protein at the expected size (I, Fig. 2a) and produced punctuate cytoplasmic EGFP fluorescence that colocalised with nsP3 in infected cells (I, Fig. 1c). However, both plaque purification and Western blot analysis revealed that SFV4(3F)-EGFP was genetically highly unstable.

In SFV4(3F)-EGFP, the marker was inserted into the hypervariable domain of nsP3, before the last 30 amino acid residues of the carboxy terminus. In addition, two recombinant SINVs with marker insertion into two different locations within the hypervariable domain of nsP3, distinct from the position used in SFV4(3F)-EGFP, have been described (Bick et al., 2003; Frolova et al., 2006). While one resulting recombinant SINV was successfully used and harboured a stable genome, the second virus was unstable (Bick et al., 2003; Frolova et al., 2006). Both our and previously published results indicate that the reportedly unstructured carboxy terminus of nsP3 is sensitive to insertions into certain positions and most likely contains linear sequence elements important for the viral life cycle. In agreement with this hypothesis, data published after completion of our studies indicated that the area we used for marker insertion lies within a conserved element playing a critical role in virus replication cycle (Varjak et al., 2010). Marker insertion in SFV4(3F)-EGFP into this conserved element most likely disrupted its functionality causing defects in formation and functioning of replication complexes or affecting other functions of nsP3.

We concluded that, similar to SINV nsP3-fusion constructs (Bick et al., 2003; Ryman et al., 2005; Frolova et al., 2006; Ventoso et al., 2006) SFV4(3F)-EGFP could be useful for studying replicase gene expression, replicase protein localisation and interactions in cell cultures, but its instability hampers the use of the construct for *in vivo* pathogenesis studies.

To eliminate the possible negative effect of the nsP3-EGFP fusion protein on virus life cycle, new recombinant viruses were designed that produce marker protein that is cleaved from nsP3 and nsP4 by a viral protease. Starting with SFV4(3F)-EGFP, we restored the native C-terminus of nsP3 and inserted an optimised artificial nsP2 protease cleavage site between nsP3 and EGFP (I, Fig. 1a). The introduced nsP2 cleavage site was based on an original nsP3/4 site that is the most efficiently cleaved site in the SFV ns polyprotein (Lulla et al., 2006). The resulting virus, designated SFV4(3H)-EGFP, was viable and had similar infectivity to parental SFV4, as determined by infectious centre assay. Importantly, in the SFV4(3H)-EGFP-infected cells, EGFP had diffuse cytoplasmic and nuclear staining, and no colocalisation with nsP3 could be seen (I, Fig. 1d). These results indicate that EGFP was efficiently released from the viral ns polyprotein and not incorporated into virus replication complexes. In addition, Western blot analysis demonstrated the presence of individual nsP1, nsP2, nsP3, nsP4 and EGFP proteins in infected cells (I, Fig. 2a).

To enable easy monitoring of structural protein synthesis, we also investigated the possibility of expressing marker protein as part of virus structural polyprotein. We constructed a recombinant SFV with the EGFP inserted between sequences coding for capsid and glycoproteins, similar to previously described recombinant SINV (Thomas et al., 2003). In this design, the EGFP should be released from the viral structural polyprotein cotranslationally by N-terminal cleavage by the capsid protein and the C-terminus is released by an inserted 2A peptide from FMDV (II, Fig. 1a). The resulting recombinant virus, designated SFV4-stEGFP, was viable and efficiently expressed marker protein.

Structural polyprotein of the new virus was efficiently processed to release both free capsid and free EGFP. Low levels of high-molecular mass proteins containing EGFP could also be detected, indicating that some EGFP remained attached to glycoproteins in an EGFP-2A-p62 form (II, Fig. 1c). The explanation for this could be that the 2A peptide function was not 100% efficient.

3.1.2. Characterisation of novel marker viruses in cultured cells (I, II)

One-step growth curves demonstrated that both SFV4(3H)-EGFP and SFV4-stEGFP replicated to high titres in BHK-21 cells (I, Fig. 1b; II, Fig. 1e). Relative to parental SFV4, accumulation of recombinant viruses was slightly delayed and SFV4-stEGFP was more attenuated in growth than SFV4(3H)-EGFP. However, as both marker viruses were able to replicate to high titres within 10 h (10^9 pfu/ml), we concluded that insertion of EGFP into virus polyproteins had only a mild effect on viral life cycle in cultured cells.

The slower growth of SFV4(3H)-EGFP is most likely mainly caused by increased time needed for replication of the longer genome. In agreement with this, the viral RNA synthesis in SFV4(3H)-EGFP-infected cells had a similar 1 h delay as seen in growth curves when compared to parental SFV4 (I, Fig. 3a). The more attenuated phenotype observed for SFV4-stEGFP could, in addition to the longer genome, be due to reduced efficiency in structural polyprotein processing as evidenced by the presence of small amounts of unprocessed EGFP-2A-p62 (II, Fig. 1c). This could have affected both glycoprotein trafficking and virion assembly. In addition, proteins encoded before the 2A sequence in the polyprotein can be found in molar excess over proteins C-terminal to the 2A (Donnelly et al., 2001). If this also happens in SFV4-stEGFP-infected cells, then the virus glycoproteins are produced in lower amounts than capsid protein, most likely affecting efficient virion formation.

As correct ns polyprotein processing is essential for functional replication complex formation and marker insertion within the replicase region could significantly affect this process, we studied the dynamics of ns polyprotein processing in detail in SFV4(3H)-EGFP-infected cells. We could not detect any apparent disturbance of processing besides the presence of very small amounts of EGFP-nsP4 fusion protein (I, Fig. 2a). The lack of nsP3-EGFP indicates the introduced optimized cleavage site between nsP3 and EGFP is very efficiently processed. As released EGFP-nsP4 can be only cleaved *in trans*, its accumulation indicates that the 3/4 site is more efficiently cleaved *in cis* and most likely less so *in trans*. Although it is generally believed that in SFV-infected cells, the first cleavage in ns polyprotein processing between nsP3 and nsP4 takes place *in cis*, in cell-free reaction conditions used to study polyprotein processing, the requirements only for *in trans* cleavage have been successfully elucidated (Vasiljeva et al., 2003; Lulla et al., 2006). Our results indirectly

support the hypothesis of *in cis* cleavage of the 3/4 site in the ns polyprotein during replication complex formation in SFV-infected cells and represent, to our knowledge, the best available proof for it.

Interestingly, our data also indicate that addition of a fourth cleavage site in the ns polyprotein did not dramatically change the dynamics of processing nor affect functioning of the replicase. The full significance of this fact is not clear, but it does highlight the specific role of 3/4 site processing in the alphavirus life cycle. Although we did not attempt to use a similar approach to place marker genes between nsP1/nsP2 or nsP2/nsP3, it is rather likely that these manipulations would have had a major impact on replicase maturation and resulted in defective, possibly non-infectious genomes.

SFV is an arbovirus and its infection cycle in nature involves apathogenic replication in blood-sucking insects. We demonstrated that in addition to mammalian BHK-21 cells, both SFV4(3H)-EGFP and SFV4-stEGFP efficiently infected and replicated in a mosquito cell line C6/36 (II, Fig. 2). Cells infected with SFV4(3H)-EGFP and SFV4-stEGFP efficiently expressed EGFP that did not colocalise with viral replication complexes, indicating proper polyprotein cleavage. Similar to the parental SFV4, no cytopathic effect was induced in infected C6/36 cells.

3.1.3. Genetic stability of recombinant viruses *in vitro* and *in vivo* (II)

The genetic stability of SFV4(3H)-EGFP and SFV4-stEGFP was determined by passaging viruses five times through BHK-21 cells or adult mouse brains (II, Table 1). Usually the genetic stability of a recombinant viruses is evaluated by methods that are not very sensitive and do not enable quantitative measurements (Pugachev et al., 1995; Thomas et al., 2003). We wanted to also detect small changes in the virus population and employed a more sensitive technique to quantify the genetic stability of recombinant viruses - plaque purification followed by monitoring of EGFP fluorescence produced by purified virus isolates. Using this approach, we demonstrated that upon passage in BHK-21 cells, SFV4(3H)-EGFP had significantly increased genomic stability relative to the above-described SFV4(3F)-EGFP. After five consecutive passages of SFV4(3H)-EGFP, more than 90% of plaques analysed were EGFP-positive (II, Table 1). However, SFV4-stEGFP demonstrated increasing instability upon passage in BHK-21 cells (II, Table 1).

The *in vitro* propagated and plaque-purified SFV4(3H)-EGFP isolates presenting the EGFP-negative phenotype were analysed. We identified large in-frame deletions or elimination of the entire EGFP marker gene with all duplicated sequences in their genomes. This indicates that mutations shortening the virus genome give a slight growth advantage and thus accumulate in the virus population. Our attempts to further increase the genomic stability of SFV4(3H)-EGFP by removing 6 duplicated amino acids from the N-terminus

EGFP or by decreasing the length of duplicated nsP3 sequence added to the C-terminus of EGFP resulted in viable virus, but genetic stability was not increased.

Importantly, both SFV4(3H)-EGFP and SFV4-stEGFP presented very high genetic stability upon *in vivo* passage through adult mouse brains (II, Table 1). In the case of both recombinant viruses, after the fifth consecutive passage, more than 90% of analysed plaques were expressing functional EGFP. From this data we concluded that both recombinant viruses were suitable for further mouse pathogenesis experiments.

The significant differences between *in vitro* and *in vivo* stability could be due to the different cellular environments and differences in virus amounts used. While mice received 1000 pfus of virus for passaging, about 100-fold more virus was used to infect cultured cells for each passage. Due to the use of a higher amount of infectious virions for each passage, it is more likely that mutant viruses with deletions in marker-encoding region are included into inoculums. It is also possible that the growth advantage of viruses with marker deletions in mouse brains were less pronounced than in cultured cells.

3.1.4. NsP3 carboxy terminus contains a hypothetical degradation signal (I)

In BHK-21 cells infected with SFV4(3H)-EGFP, the EGFP signal monitored by fluorescence microscopy could be detected as early as 2 h post-infection. The signal increased until 6 h and then decreased rapidly. In general, EGFP is a stable protein with an estimated half-life of 24 h and once expressed should be detectable for longer than 24 h. Thus, the rapid decrease of EGFP signal in infected cells suggested possible low stability of the virus-encoded marker protein. To assess EGFP stability, infected cells were metabolically labelled, chased and subsequently EGFP, nsP1 and nsP3 were immunoprecipitated. While only a small decrease in nsP1 and nsP3 signal could be detected over a 24 h period, the levels of EGFP had decreased significantly already during 3 h chase-period and the estimated half-life of the marker protein was less than 2 h (I, Fig. 2b). These results demonstrated that the EGFP encoded by SFV4(3H)-EGFP is significantly destabilised and we suggested that additional amino acid residues in the amino or carboxy terminus of virus-encoded EGFP must be responsible for its rapid degradation. The N-terminal amino acid residue of processed EGFP expressed by SFV4(3H)-EGFP is a Gly, a stabilising residue according to the N-end rule (Varshavsky, 1997). This suggests that the destabilising sequence must be at the C-terminus. In the processed EGFP, 30 amino acid residues from the carboxy terminus of nsP3 are fused to the EGFP C-terminus and we hypothesised that this must contain destabilising sequences. Indeed, further detailed analysis performed by another member of our lab identified strongly destabilising sequences within the C-terminal ends of nsP3 proteins from both SFV and SINV (Varjak et al., 2010).

We also studied the half-life of EGFP expressed in SFV4-stEGFP-infected BHK-21 cells and determined it to be a stable protein (II, Fig. 1d). The unexpected differences in the stability of EGFP expressed by these recombinant viruses makes them excellent tools in infection studies. The short half-life of EGFP expressed by SFV4(3H)-EGFP is very useful because fluorescence intensity closely reports ns polyprotein synthesis. EGFP can be monitored early in infection and its levels rapidly decrease when synthesis of ns polyprotein is downregulated. EGFP expressed in SFV4-stEGFP-infected cells can be detected later in infection, as virus structural polyprotein synthesis starts and the fluorescence levels remain high until the death of the cells. Thus, the protein is a reliable marker for detection of all infected cells in the later stages of infection.

3.2. Course of infection of recombinant viruses in adult mice (II)

In adult mice, the two most studied SFV strains, SFV L10 and SFV A7(74), are virulent and avirulent, respectively. The prototype strain SFV4 used in this study to design recombinant viruses is neurovirulent by intranasal or i.c. inoculation but does not efficiently reach the CNS by i.p. inoculation (Fazakerley, 2002; Fragkoudis et al., 2007). To assess the virulence and neuroinvasion of EGFP-expressing marker viruses, both i.p. and i.c. routes of inoculation were used.

SFV4, SFV4(3H)-EGFP and SFV4-stEGFP were all avirulent in adult Balb/c mice following i.p. inoculation. Infected mice were monitored for 2 weeks and remained healthy even when high doses of viruses ($5*10^5$ pfu) were used. The extraneural replication of SFV4(3H)-EGFP was identical to parental SFV4, while SFV4-stEGFP replication was significantly attenuated. By 24 h post-inoculation, SFV4(3H)-EGFP had induced plasma viraemia at similar levels to SFV4, but no infectious virus could be detected at this time in the blood of mice infected with SFV-stEGFP (II, Fig. 3a). Neither the parental SFV4 nor recombinant viruses were neuroinvasive, and no infectious virus or any EGFP fluorescence signal could be detected in brains at 4 days post-infection (II, Fig. 3b).

To determine if recombinant viruses were neurovirulent and could replicate in the brain as efficiently as the parental SFV4, an i.c. route of inoculation was used. SFV4(3H)-EGFP presented a strong neurovirulent phenotype, similar to parental SFV4, but SFV4-stEGFP was slightly, but clearly, attenuated. Animals infected with SFV4 or SFV4(3H)-EGFP reached clinically determined end points, indicative of substantial disease by 2 days post-inoculation. The course of infection in SFV4-stEGFP-infected mice was slower and mice reached clinically defined end points by 3 days post-inoculation. The recombinant SFV4(3H)-EGFP replicated efficiently in the CNS and induced high levels of infectious virus in the brain, similar to SFV4 (II, Fig. 3c). The SFV4-stEGFP

was slightly attenuated and the induced CNS titres were approximately 10-fold lower than in case of SFV4 or SFV4(3H)-EGFP (II, Fig. 3c).

We concluded that the course of infection with SFV4(3H)-EGFP was indistinguishable from parental SFV4. The SFV4-stEGFP had a slightly attenuated phenotype but nevertheless remained neurovirulent and the course of infection was in general similar to parental SFV4 and distinct from other strains of SFV, SFV L10 and SFV A7(74) (II, Fig. 3).

The slightly attenuated phenotype of SFV4-stEGFP in adult mice could be caused by accumulation of small amounts of unprocessed EGFP-2A-p62 or non-equimolar synthesis of glycoproteins and capsid, as described above in chapter 3.1.2. Previous studies have indicated that recombinant SINV expressing foreign proteins as part of the virus structural polyprotein presented slightly attenuated phenotypes. In these experiments, subcutaneous inoculation of newborn mice with recombinant SINV resulted in 100% mortality, similar to parental strain, but the average survival time was increased by 2–3 days (Thomas et al., 2003). In the case of recombinant SINV, the structural polyprotein was reported to be properly processed.

During this study, we also inoculated mice with the SFV L10 and SFV A7(74) strains in parallel with SFV4 and SFV4-based marker viruses. The obtained data provided some general insights into the pathogenesis of SFV. We observed that SFV4 induces approximately 1000-fold lower plasma viraemia than SFV L10 or SFV A7(74) following i.p. inoculation (II, Fig. 3a). In agreement with previously published data, we also observed that SFV4 is not efficiently neuroinvasive following low dose i.p. inoculation, while SFV L10 and SFV A7(74) are (II, Fig. 3b) (Fragkoudis et al., 2007). Our data strongly support the hypothesis that induction of low-level blood viraemia is the reason why SFV4 is not able to invade the CNS after i.p. inoculation. It is not clear why SFV4 does not replicate to high titres in peripheral tissues, but it is likely mediated by the type-I interferon response because in IFNAR$^{-/-}$ mice, SFV4 and SFV A7(74) induce similar very high virus titres in blood (Fragkoudis et al., 2007).

3.2.1. Neurons and oligodendrocytes differ in their ability to permit replication of SFV (II)

We used immunohistochemistry to determine the cell tropism of recombinant viruses in adult mouse brains. We observed that both SFV4(3H)-EGFP and SFV4-stEGFP efficiently infected neurons and oligodendrocytes in the CNS but never astrocytes (II, Fig. 5). This is in agreement with general cell tropism results published for SFV (Gates et al., 1985; Fazakerley et al., 2006).

Histopathological analysis of brains from mice inoculated i.c. with SFV4(3H)-EGFP demonstrated only a few EGFP-positive cells, which were generally dull green and were most frequently in white matter tracts (II, Fig. 4a,b). Immunostaining with antibodies specific to nsP3 or virus structural

proteins demonstrated that many neurons and oligodendrocytes were infected, but a strong EGFP signal could be observed only in oligodendrocytes and the EGFP signal was very low or undetectable in majority of infected neurons (II, Fig. 4c,d, Fig. 5c,d,g). As EGFP encoded by SFV4(3H)-EGFP is a good indicator of on-going ns protein synthesis due to its short half-life, these results suggest that synthesis of virus replicase proteins is rapidly downregulated in neurons while it remains active for longer periods in oligodendrocytes. As nsP3-specific immunostaining signal could be seen in many neurons and oligodendrocytes, we conclude that the replicase complexes were long-lived and present in both neurons and oligodendrocytes.

In SFV4-stEGFP-infected brains, widespread EGFP signal was seen in the corpus callosum and other white matter tracts, and large EGFP-positive foci could be detected throughout the brain (II, Fig. 4g). In contrast to SFV4(3H)-EGFP, EGFP signal was detected both in many neurons and oligodendrocytes (II, Fig. 5b,h). Immunostaining revealed that the majority of EGFP-positive cells were nsP3-positive, but also nsP3-positive and EGFP-negative cells could be found. The latter were most common around the expanding edge of foci of infection indicative of cells at early stages of infection (II, Fig. 4i-k). These results demonstrate that both neurons and oligodendrocytes switch to virus structural protein synthesis in adult mouse brains.

3.3. Mature but not immature neurons rapidly downregulate virus protein synthesis (II, unpublished)

We wanted to confirm the rapid downregulation of virus replicase synthesis observed in mature neurons in adult mouse brains and also analysed the infection in cultured neurons. To enable easy quantification of virus polyprotein synthesis, we constructed recombinant viruses expressing Renilla luciferase (Rluc) as part of virus ns or structural polyproteins, designated SFV4(3H)-Rluc and SFV4-stRluc, respectively. We infected immature and mature primary rat hippocampal neuronal cultures with SFV4(3H)-EGFP or SFV4(3H)-Rluc and observed the rapid suppression of replicase synthesis of both viruses in mature neurons but not in immature neurons (II, Fig. 6a, 7a). Interestingly, we also observed the rapid downregulation of virus structural protein synthesis specifically in mature neurons and not in immature neurons infected with SFV4-stRluc (II, Fig. 7b). Rapid downregulation of virus structural protein synthesis likely also takes place *in vivo*, but remained undetected due to the very long half-life of EGFP produced by SFV4-stEGFP.

We also studied SFV replicase synthesis in undifferentiated and differentiated cells from the neuronal cell line CSM14.1, which is a stable rat embryonic neuronal cell line immortalised with a temperature-sensitive SV40 Large T-antigen (Durand 1990). At permissive temperature, these cells proliferate and maintain undifferentiated status, but undergo differentiation at non-permissive culture conditions, and thus represent a cell line easy to

maintain and differentiate. In fact, this cell line has been successfully used as a model system to study SINV replication in immature and mature neurons (Vernon 2005, Park 2009). We infected undifferentiated and differentiated CSM14.1 neurons with SFV4(3H)-Rluc and again saw rapid downregulation of virus ns-polyprotein synthesis specifically in mature cells (Figure 4). The levels of virus replicase proteins gradually increased until 24 h post-infection in undifferentiated cells, but reached a peak by 8 h post-infection in differentiated neurons. Taken together, with the use of two different cell lines, we demonstrated that only mature neurons rapidly downregulated both virus ns and structural protein synthesis. In immature neurons, synthesis of virus proteins remained high even in later stages of infection.

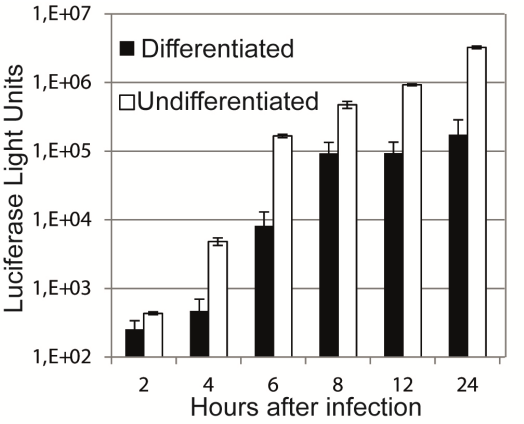

Figure 4. Mature but not immature neurons downregulate virus ns polyprotein synthesis. Undifferentiated and differentiated CSM14.1 neuronal cultures were infected with SFV4(3H)-Rluc and at selected time points, cells were lysed and Rluc activity was measured. The means of triplicate cultures with standard deviations are shown.

3.4. Properties of SFV4 and SFV A7(74) infection in immature and mature neurons (unpublished)

Age-dependent neurovirulence has been described for many alphaviruses and obtained data suggest that the maturity of neurons and not the immune system plays the major role in this phenomenon (Fazakerley et al., 1993; Oliver et al., 1997; Griffin, 2005; Fragkoudis et al., 2007). Nevertheless, the exact mechanism behind it has not been revealed. Our data obtained from adult mouse brains and cultured neurons was very similar and suggested that CSM14.1 cells might be a good model system to study SFV age-dependent neurovirulence. We compared the infection cycle of two SFV strains differing in their neurovirulence, SFV4 and SFV A7(74), in CSM14.1 cells. Both of these strains replicate efficiently in the CNS of neonatal mice, but only SFV4 is strongly

neurovirulent in adult mice and the replication of SFV A7(74) remains restricted specifically in neurons in the mature CNS (Fazakerley et al., 1993).

To enable the visualisation of on-going infection, we used recombinant marker viruses expressing a fluorescent protein ZsGreen in fusion with nsP3, designated SFV4-Xho-ZsGreen, and SFVA7(74)-Xho-ZsGreen. In these recombinant viruses, the marker gene is also inserted into the nsP3 hyper-variable domain as in the above-described SFV4(3F)-EGFP, but into a slightly different location, resulting in genetically stable viruses (A. Merits. unpublished data). First, we compared the growth kinetics of SFV4 and SFVA7(74)-based recombinant viruses in undifferentiated and differentiated CSM14.1 cells (Figure 5A). Both strains replicated efficiently in immature neurons, and to our surprise, they also replicated relatively well in differentiated cells. In the case of both strains, the release of infectious virions was considerably delayed in differentiated neurons compared to undifferentiated cells. This difference can be explained, at least to some extent, by limited synthesis of virus ns proteins in mature neurons (Figure 4). Compared to SFV4-Xho-ZsGreen, SFVA7(74)-Xho-ZsGreen presented delayed growth in neuronal cultures and the difference was more pronounced in mature neurons (Figure 5). However, both strains were able to efficiently spread in and kill both immature and mature neuronal cultures. These results are different from previous mouse studies where SFV A7(74) replication did not spread and remained confined to small foci in adult mouse brains (Fazakerley et al., 1993).

We also analysed the synthesis of virus replicase proteins in undifferentiated CSM14.1 cells infected by SFV4 and SFVA7(74)-based reporter viruses expressing Rluc (Figure 5B). The obtained data indicated that both virus strains efficiently synthesised the ns polyproteins, but SFV A7(74) had a slight delay compared to SFV4, similar to infectious virion accumulation (Figure 5). However, by 12 h post-infection, the SFV A7(74) expressed equal or slightly higher amounts of ns polyproteins compared to SFV4 (Figure 5B). The delay in replicase synthesis in SFV A7(74)-infected cells can contribute to the delay in virion formation but also additional defects in other virus life cycle steps should be considered and we plan to analyse them in near future.

Taken together, compared to SFV4, SFV A7(74) had slightly delayed growth in immature and, especially, in mature CSM14.1 neurons. Although, the replication of SFV A7(74) in differentiated CSM14.1 cells was not as severely affected as in an adult CNS, this cell line is a valuable model for analysis of the aspects of SFV neuropathology that cannot be studied in animal models. Many studies have shown that it is rather common that small changes in virus replication in tissue culture conditions translate into more severe defects in animal models. There are several possibilities how the *in vitro* differentiated neurons could differ from mature neurons in the mouse brain, including the metabolic activity and cellular membrane availability for virus replication, antiviral gene expression, formed cellular connections between both neurons and neuron-glial cells aiding the antiviral response.

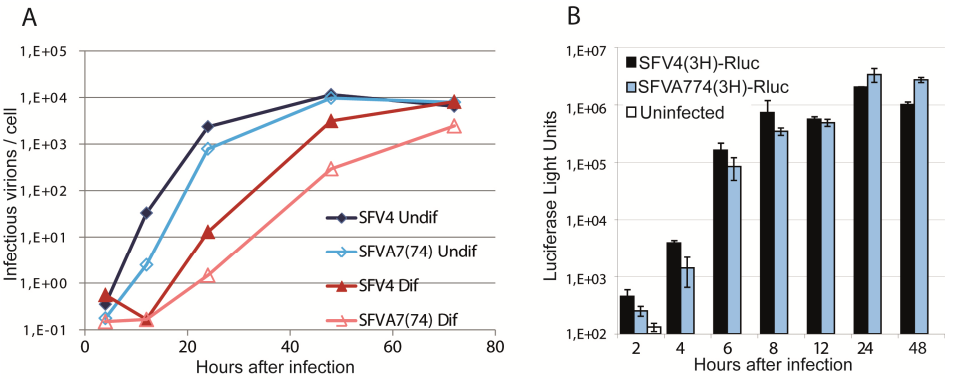

Figure 5. (A) The growth kinetics of SFV4 and SFV A7(74) in undifferentiated and differentiated neuronal cells. CSM14.1 cells were infected with SFV4-Xho-ZsGreen or SFVA774-Xho-ZsGreen at an MOI of 0.1 at 31°C and at selected time points post-infection, the amount of infectious virus released into media was measured. (B) The synthesis of virus ns polyproteins in undifferentiated cells. Undifferentiated CSM14.1 cells were infected with SFV4(3H)-Rluc or SFVA774(3H)-Rluc at an MOI of 3 at 31°C and at selected time points the Rluc activity, indicative of virus ns polyprotein synthesis, was measured. The means of triplicate cultures with standard deviations are shown.

3.5. Properties of nsP1 and its interference with virus infection (III)

Alphavirus nsP1 is an important player in virus infection. It is involved in the capping of virus RNAs and is the sole membrane anchor for the replication complexes (Kääriäinen and Ahola, 2002; Salonen et al., 2003). nsP1 is modified by palmitoylation but the exact biological significance of this modification is unknown. nsP1 expression in transfected or infected cells results in clearly detectable changes such as induction of filopodia-like structures and rearrangements of actin filaments, suggesting that this protein might contribute to induction of cytotoxic effects characteristic to alphavirus-infected cells (Laakkonen et al., 1998).

We used tetracycline-inducible stable HEK293 T-Rex cell lines expressing SFV nsP1 or its palmitoylation-negative mutant form nsP1^{6D} (aa 418–420 CCC→AAA) to study the effect of nsP1 on the host cell and SFV infection cycle. The induced expression levels of nsP1 and nsP1^{6D} in stable cell lines were comparable to nsP1 levels in virus-infected cells (III, Fig. 1a). nsP1 expressed in both stable cell lines and in SFV-infected cells was stable with an approximate half-life of 5 h (III, Fig. 1b). Similar to previously published data, both nsP1 and nsP1^{6D} expressed by stable cell lines localised to the plasma membrane, while only nsP1 induced a large number of filopodia-like structures (III, Fig. 2) (Laakkonen et al., 1998; Zusinaite et al., 2007).

As nsP1 induces filopodia-like structures and mediates rearrangements of actin filaments (Laakkonen et al., 1998), we hypothesised that this ns protein

might contribute to the cytopathic effect seen in SFV-infected cells. Surprisingly, expression of nsP1 or its palmitoylation-deficient form had no detectable effect on any cellular process tested, including transcription, translation and cell viability (III, Fig. 3). These findings support the hypothesis that nsP2 alone is the main factor for inducing cellular transcription/translation shutdown and the death of infected cells (Garmashova et al., 2007; Akhrymuk et al., 2012).

Infection by many different viruses, including alphaviruses, induces an intracellular state blocking further infection by homologous virus – a phenomenon called superinfection exclusion or homologous interference. Several studies have proposed, but not directly demonstrated, that in alphavirus infection, the cleaved form of nsP2 is likely mediating this process (Karpf et al., 1997; Sawicki et al., 2006; Lulla et al., 2012). Possible participation of other ns proteins in alphavirus superinfection exclusion has not been properly addressed. We determined that the induction of nsP1 or nsP1^{6D} expression prior to infection did not affect the numbers of successfully infected cells (III, Fig. 4b), indicating that individual nsP1 does not play a role in superinfection exclusion. After completion of our study, a similar stable cell line approach was used to demonstrate involvement of nsP3 in slightly reducing the infection efficiency of incoming virus (Varjak et al., 2010).

Next we studied if induction of nsP1 or nsP1^{6D} expression prior to infection with SFV interfered with the virus infection cycle. We demonstrated that expression of nsP1 and its mutant significantly inhibited the extracellular accumulation of virions (III, Fig. 4c). As nsP1 did not affect virus entry, the reduced extracellular titres could be due to the defects in synthesis of virus RNA or polyproteins, assembly and release of virions. To monitor the accumulation of virus polyproteins, we used recombinant viruses SFV4(3H)-Rluc and SFV4-stRluc and produced Rluc activity was used to estimate the accumulation of virus polyproteins in infected cells (II, Fig. 1a). We found that expression of nsP1 and nsP1^{6D} significantly reduced synthesis of virus polyproteins. The ns polyproteins levels were approximately 2–3-fold lower in both nsP1- and nsP1^{6D}-expressing cells compared to non-induced cells (III, Fig. 5). The accumulation of structural polyprotein was even more reduced in both nsP1- and nsP1^{6D}-expressing cells, however, the structural polyprotein levels were restored later in infection in nsP1-expressing cells but remained low in the presence of nsP1^{6D} (III, Fig. 5). To verify these findings, we also analysed synthesis of virus-specific RNA in nsP1-expressing and in non-induced cells (III, Fig. 6). The accumulation of virus RNAs correlated well with virus polyprotein synthesis.

Taken together, expression of nsP1 interfered with the virus infectious cycle and resulted in reduced levels of virus genomic RNA and replicase proteins, delayed synthesis of structural proteins and reduced levels of released infectious virus. The expression of nsP1^{6D} had similar effects, except the synthesis of subgenomic RNA and accumulation of structural proteins remained low also later in infection.

Previously published data show that palmitoylation of nsP1 is important for efficient SFV replication and pathogenesis in mice. Nevertheless, the exact biological function of this modification remains unknown. In our experiments, the effects of nsP1 and its palmitoylation-deficient mutant were very similar on virus infection cycle. Thus, we could not reveal novel biological functions of nsP1 palmitoylation or the exact steps of infection cycle where this modification might be essential. These questions remain to be answered by further studies.

There are several possible mechanisms that would explain how nsP1 and nsP1^{6D} expressed *in trans* could interfere with virus replication, some of which were not known at the time this study was performed. SFV replication takes place in association with modified cellular membranes and starts initially at the plasma membrane where the sites of replication, membrane invaginations called spherules, are initially formed (Frolova et al., 2010; Spuul et al., 2010). Both nsP1 and nsP1^{6D} expressed by stable cell lines were localised to plasma membrane and could affect membrane interactions of virus ns polyproteins and reduce efficiency of virus replication complex formation. Plasma membrane-bound nsP1 and nsP1^{6D} could also be brought into spherules as part of the invaginating membrane and affect the proper functioning of replication complexes. Reduced numbers of functional replicase would result in decreased levels of virus RNA and polyproteins, as observed in our study.

It is unlikely that nsP1 or nsP1^{6D} expressed by stable cell lines would be efficiently incorporated into virus RNA synthesis machinery and result in aberrant activity of replicase. Ns proteins expressed in a polyprotein efficiently form a strong complex while individually expressed ns proteins do not interact with each other very well, indicating that *in trans* expressed nsP1 cannot most likely efficiently interact with the active enzymatic complex (Salonen et al., 2003). However, this does not exclude many subtle ways that nsP1 or nsP1^{6D} might interfere with replicase functioning.

Expression of nsP1 and nsP1^{6D} might also reduce the availability of nsP1-interacting host factors important for replication. Studies have shown that interactions between nsP1 and nsP4, which are crucial for minus-strand synthesis, are also host-dependent, suggesting a possible role for host factors mediating this interaction and virus replication (Shirako et al., 2000; Fata et al., 2002; Rupp et al., 2011).

Both nsP1 and nsP1^{6D} can compete with replicase complexes for the substrates for cap synthesis, and this has also been suggested for truncated forms of SINV nsP1 (Li et al., 1997). In HeLa cells, stable expression of nsP1 did not have a significant effect on SINV replication while expression of the truncated forms of nsP1 strongly interferes with SINV multiplication (Li et al., 1997). The main mechanism suggested by authors was competition for substrates for cap synthesis reactions between cellular and viral nsP1 proteins.

4. SUMMARY

We designed and characterised novel SFV-based reporter viruses expressing marker proteins easy to visualise or quantify, facilitating infection studies both in cell cultures and animals. Novel recombinant virus SFV4(3H)-EGFP expressing EGFP as cleavable component of the virus non-structural polyprotein demonstrated excellent genomic stability during passaging both in cultured cells and in mouse brains. The growth kinetics, accumulation of viral proteins and RNA, and shutdown of host cell translation were slightly reduced in infected cell cultures, but the pathology and course of infection in adult mice was indistinguishable from parental SFV4. Thus, SFV4(3H)-EGFP represents an excellent tool for infection studies, both in cell cultures and animal models. This design has subsequently served as a prototype for several recombinant viruses successfully used by our group and others.

The recombinant virus SFV4-stEGFP expressing EGFP as a cleavable component of the structural polyprotein demonstrated excellent genetic stability after passages in adult mouse brain, but showed delayed growth and increased instability upon passage in BHK-21 cells. SFV4-stEGFP was also slightly attenuated in adult mice. Nevertheless, it remained neurovirulent, efficiently replicated in the CNS, and produced high levels of EGFP easy to visualise, and thus represents an excellent tool for monitoring infection.

While EGFP is in general a stable protein, we determined that the half-life of SFV4(3H)-EGFP-encoded marker protein was less than 2 h, indicative of the presence of highly destabilising sequences. We hypothesised that the additional amino acids in its carboxy terminus, originating from the duplication of the C-terminus of nsP3, must contain an efficient degradation signal. This assumption was later experimentally confirmed (Varjak et al., 2010).

Experiments described in this thesis reveal novel properties of the adult mouse CNS in response to SFV infection. We demonstrated that the synthesis of virus replicase proteins is rapidly downregulated in neurons, but it remains active for longer periods in oligodendrocytes in adult mouse brains. Nevertheless, the replication complexes themselves were long-lived and were present in both neurons and oligodendrocytes. We also observed that both neurons and oligodendrocytes switch from replicase to structural protein synthesis. We also demonstrated that the response to infection depends on the differentiation state of neurons. Only mature neurons rapidly downregulated both virus non-structural and structural protein synthesis. In immature neurons, synthesis of virus proteins remained high even in later stages of infection.

We used a combination of inducible stable cell lines expressing SFV nsP1 or its palmitoylation-negative mutant nsP1^{6D} and constructed reporter viruses to study the effect of nsP1 on the host cell and also on SFV infection cycle. We demonstrated that expression of nsP1 or its palmitoylation-deficient form had no detectable effect on any cellular processes tested, including transcription, translation or cell viability, indicating that nsP1 does not play a direct role in the induction of CPE, a hallmark of alphavirus infection. We also demonstrated that

nsP1 does not play a role in superinfection exclusion. *In trans* expression of nsP1 or its palmitoylation-deficient mutant interfered with virus infectious cycle and reduced the accumulation of virus RNA, non-structural and structural polyproteins, and ultimately release of infectious virions.

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SUMMARY IN ESTONIAN

Semliki Forest viiruse replikatsiooni ja patogeensuse uurimine

Alfaviirused on laialt levinud selgroogsete patogeenid, mis põhjustavad erinevate sümptomitega haigusi, sealhulgas artriiti ja entsefaliiti. Alfaviiruste genoomiks on üheahelaline positiivse polaarsusega RNA molekul, mille üks avatud lugemisraam kodeerib mittestruktuurseid, replikatsioonikompleksi moodustavaid valke ja teine struktuurseid, virioni moodustamiseks vajalikke valke. Alfaviiruste genoomi replikatsiooni toimumist ja erinevate viirusvalkude osalust selles protsessis on detailselt kirjeldanud paljud uuringud, kuid viirus-peremees suheteid ja patogeensust mõjutavad protsesse ei mõisteta veel nii hästi. Neid protsesse on sageli raske adekvaatselt koekultuuri tingimutes modelleerida, samuti on infektsiooni jälgmine elusorganismis väga keeruline, sest viirusnakkus on kompleksne ja hõlmab erinevaid kudesid ning rakutüüpe.

Üheks kõige põhjalikumalt uuritud alfaviiruseks on Semliki Forest viirus (SFV). SFV-alased uuringud on andnud väärtuslikke teadmisi ka teiste alfaviiruste infektsioonitsüklite ja erinevate nakkusega seotud rakuliste protsesside kohta. Käesoleva uurimistöö peaeesmärgiks oli välja töötada uudsed SFV nakkuse uurimist hõlbustavaid markervalke ekspresseerivad rekombinantsed viirused ja kasutada neid viirusnakkuse protsessi ja dünaamika analüüsimiseks nii koekultuuri tingimustes kui ka hiirtes. Konstrueeritud rekombinantsed viirused ekspresseerisid markervalke osana kas viiruse mittestruktuursest või struktuursest polüproteiinist. Rekombinantne viirus, mis sisaldas roheliselt fluorestseeruvat valku (EGFP) osana viiruse mittestruktuursest polüproteiinist, säilitas margergeeni oma genoomis nii koekultuuri tingimustes kui ka täiskasvanud hiire ajus mitme järjestikuse viiruspõlvkonna jooksul. Nii täiskasvanud hiires toimuvale infektsioonile kui ka patogeensusele ei omanud lisatud marker mingit mõju, hoolimata sellest, et koekultuuri rakkudes oli konstrueeritud viiruse replikatsioon mõnevõrra aeglasem kui ilma võõrgeenita viirusel. Ka need rekombinantsed viirused, kus EGFP oli sisestatud viiruse struktuursesse polüproteiini, olid täiskasvanud hiire ajus paljunedes geneetiliselt stabiilsed. Koekultuuri tingimustes oli antud viiruse paljunemine aga aeglustunud ja viirusgenoom ebastabiilsem, ka viiruse paljunemine täiskasvanud hiirtes oli märgatavalt ebaefektiivsem kui markergeenita viirusel. Samas ei olnud sellise rekombinantse viiruse patogeensus markergeeni lisamise tõttu täiskasvanud hiirtes muutunud. Kokkuvõtlikult võib öelda, et vaatamata täheldatud effektidele viiruse replikatsioonile, ei muutnud markeri lisamine viiruse genoomi mittestruktuursesse või struktuursesse alasse viirusnakkuse üldist kulgu ega iseloomu täiskasvanud hiirtes. Seega sobivad meie poolt disainitud rekombinantsed viirused SFV infektsiooni uurimiseks hiirtes.

Jälgides rekombinantsete viiruste nakkust täiskasvanud hiire kesknärvisüsteemis leidsime, et erinevat tüüpi rakkudes oli SFV replikatsioon erineva dünaamikaga. Neuronites suruti SFV replikaasivalkude süntees kiiresti maha,

kuid oligodendrotsüütides toodeti viiruse mittestruktuurseid valke ka infektsiooni lõppstaadiumis. Samas olid juba formeerunud replikatsioonikompleksid mõlemat tüüpi rakkudes stabiilsed ning detekteeritavad ka hilistes infektsioonistaadiumites. Lisaks leidsime, et neuronite vastus SFV infektsioonile sõltub antud rakutüübi diferentseerituse astmest – efektiivne viirusvalkude sünteesi mahasurumine toimus ainult diferentseeritud neuronites ja noortes diferentseerumata neuronites sünteesiti viirusvalke kõrgel tasemel ka hilistes infektsioonietappides. Sellest tulenevalt oli SFV paljunemine noortes neuronites palju efektiivsem ja infektsiooniliste virionide vabanemine toimus antud rakkudes tunduvalt varem kui diferentseeritud neuronites.

Käesolev uurimistöö keskendus ka mittestruktuurse viirusvalgu nsP1 omaduste kirjeldamisele. Katsed rekombinantsete viiruste ja nsP1 valku indutseeritavalt ekspresseerivate rakuliinidega näitasid, et nsP1 ei mõjuta rakkude elulemust või metabolismi ning seega tõenäoliselt ei osale antud valk alfaviirustele omase tsütopaatilise efekti tekitamises. Eelnev nsP1 ekspressioon ei mõjutanud ka viiruse sisenemist rakkudesse, mistõttu võib oletada, et nsP1 ei osale SFV superinfektsiooni peatamises. Samas mõjutas *in trans* ekspresseeritud nsP1 SFV nakkuse kulgu, vähendades viiruse RNA ning valkude taset rakus ning seeläbi ka vabanenud infektsiooniliste virionide hulka.

Saadud tulemused aitavad paremini mõista nii SFV kui teiste alfaviiruste infektsiooni ja patogeneesi selgroogsete, kaasa arvatud inimese, organismis.

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List of publications

1. **Tamberg N**., Lulla V., Fragkoudis R., Lulla A., Fazakerley J. K., and Merits A. (2007). Insertion of EGFP into the replicase gene of Semliki Forest virus results in a novel, genetically stable marker virus. J Gen Virol 88:1225–1230.
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