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Semliki Forest virus based vectors and cell lines for studying the replication and interactions of alphaviruses and hepaciviruses

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II **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.
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Author's contribution:
I Growth curves, infectious center assay
II Experiments with recombinant viruses and VLPs
III Construction of HcRed-replicon vectors, all experiments except the survival test in presence of puromycin
IV Construction of expression constructs for epitope-tagged recombinant proteins, immunoprecipitation of epitope-tagged proteins, immunofluorescence analyses

LIST OF ABBREVATIONS

C	capsid protein
CPE	cytopathic effects
CPV	cytopathic vacuoles (or cytoplasmic vacuoles)
ds	double-stranded
EEE	Eastern equine encephalitis virus
eIF	eukaryotic translation initiation factor
EMCV	encephalomyocarditis virus
GT	guanylyltransferase
HCV	hepatitis C virus
IFN	interferon
IRES	internal ribosome entry site
MT	methyltransferase
NC	nucleocapsid
NLS	nuclear localization signal
nsP	non-structural protein
ORF	open reading frame
p.i.	post infection
PKR	double-stranded RNA-activated protein kinase
p.t.	post transfection
RC	replication complex
RdRp	RNA-dependent RNA polymerase
Rluc	*Renilla* luciferase
SFV	Semliki Forest virus
sg	subgenomic
SIN	Sindbis virus
ts	temperature sensitive
VEE	Venezuelan equine encephalitis virus
VLP	virus like particle
WEE	Western equine encephalitis virus

INTRODUCTION

Semliki Forest virus (SFV) was isolated from mosquitoes in Uganda in the area of Semliki Forest in 1942 (Smithburn and Haddow, 1944). Ten years later the Sindbis virus (SIN) was isolated from mosquitoes in Sindbis situated in the River Nile delta near Cairo (Taylor et al., 1955). Since then, both of these viruses have evoked the interest of many scientists and have become the prototypes of alphaviruses. Due to historical reasons, SFV has been more intensively studied in Europe, whereas most work on SIN has been carried out in the USA.

Besides providing basic knowledge on the molecular biology of alphaviruses, the studies of these viruses have also elucidated several general viral and cellular processes. For example, SFV was used as the first model system to study viral receptor-mediated endocytosis and acid-triggered membrane fusion, which takes place after the internalization of the virion (Helenius et al., 1980). Also, the SFV capsid protein was one of the first proteins shown to have co-translational folding (Nicola et al., 1999).

The simple genomic organization of alphaviruses and their efficient propagation in cell culture have made alphavirus-based vectors promising tools for both DNA and RNA vaccination as well as for anti-cancer therapy. For these same reasons, alphavirus-based vectors can be potent systems for biotechnological applications.

This current thesis focuses on the properties of the non-structural protein 1 (nsP1) of SFV and the development and utility of novel SFV-based vector systems. The studies described in this thesis reveal original concepts regarding the influence of nsP1 and its non-palmitoylated mutant forms on the host cell and the replication of SFV. As a part of these studies, novel SFV replicon vectors expressing anti-apoptotic bcl-2 were constructed and analyzed. Additionally, SFV-based vectors were successfully used to demonstrate an interaction between non-structural proteins of hepatitis C virus (HCV).

REVIEW OF LITERATURE

Alphaviruses

The genera Alphavirus and Rubivirus together form the family *Togaviridae*. These viruses have a positive-sense (+) single-stranded (ss) nonsegmented RNA genome. The genome is encapsulated by viral capsid (C) proteins and enveloped by a lipid bilayer derived from the host-cell plasma membrane.

Alphaviruses are relatively common in nature and are usually spread by mosquitoes to their mammalian or avian hosts (thus they are considered to belong amongst arboviruses – arthropod borne viruses). In mosquitoes, alphaviruses cause a persistent infection that has little or no effect on the viability of their vectors. In contrast, alphavirus infection of vertebrates causes severe illnesses such as encephalitis, hemorrhagic fever, rash, or arthritis and in some cases can be lethal for the host.

Alphaviruses are spread worldwide and can be divided into the Old World and New World subgroups. The New World alphaviruses, such as Eastern, Western, and Venezuelan equine encephalitis viruses (EEE, WEE, VEE, respectively) typically cause encephalitis with high mortality rates among horses and humans. Old World alphaviruses, such as SFV, usually cause subclinical infection. In some cases they cause rashes or arthritis – in Finland and other Nordic countries, a small SIN epidemic occurs every year in late summer-early autumn and a larger epidemic every seven years (Brummer-Korvenkontio et al., 2002). Therefore, Old World alphaviruses were considered medically less important – a view that has recently changed due to the Chikungunya virus outbreak that started in 2005 on the islands of the Indian Ocean and has resulted in at least 1.4 million cases in India alone (Enserink, 2007; Schuffenecker et al., 2006).

Virion

The virion of alphaviruses has T = 4 icosahedral symmetry and a 70 nm diameter. Inside the virion the viral genomic RNA is enclosed in the icosahedral nucleocapsid (NC). The latter comprises 240 copies of the capsid protein (Fuller et al., 1995; Soderlund et al., 1975; Strauss and Strauss, 1994). The NC is enveloped by a host-cell derived membrane containing 80 spikes formed by viral envelope glycoproteins. Each spike consists of three copies of each of three viral envelope proteins: transmembrane glycoproteins E1 and E2 and an extrinsic E3 protein (Cheng et al., 1995; Fuller et al., 1995; Strauss and Strauss, 1994). These spikes are embedded in a lipid bilayer and evenly cover the surface of the virion. Both the NC and outer surface of the envelope are highly ordered; each E2 protein interacts with one of the 240 copies of the C-protein via its cytoplasmic tail.

An interesting property of an alphavirus virion is that the lipid membrane bilayer is almost completely covered by a so-called skirt, made up of lateral

interactions among membrane glycoproteins that connect one spike to its neighbors (Cheng et al., 1995; Fuller et al., 1995). Due to these properties, the virions of alphaviruses have very regular structure and can be easily purified and concentrated.

Genomic organization

The genomic RNA of SFV, or 42S RNA, is approximately 11.5 kb in length and has a 5' cap and a 3' poly(A) tail (Strauss and Strauss, 1994; Takkinen, 1986) (Figure 1). It can be divided into two major regions: the 5' two-thirds encode non-structural (ns) proteins while the 3' one-third encodes structural proteins. The open reading frame (ORF) for ns-proteins starts with AUG at nucleotide 86 and ends with a UAA termination codon at nucleotides 7379–7381 (Takkinen, 1986). It encodes a large ns-polyprotein processed into mature ns-proteins involved in viral RNA synthesis. The region encoding the structural proteins is translated as a polyprotein from the subgenomic (sg) 26S mRNA, synthesized using the internal sg-promoter (Pettersson et al., 1980). The sgRNA is approximately 4.1 kb in length and is also capped and polyadenylated. It has a 51 base long 5' noncoding region, which partly overlaps with the terminal nucleotides of the ns-region and contains one common ORF for the virus capsid and envelope proteins (Garoff et al., 1974; Riedel et al., 1982).

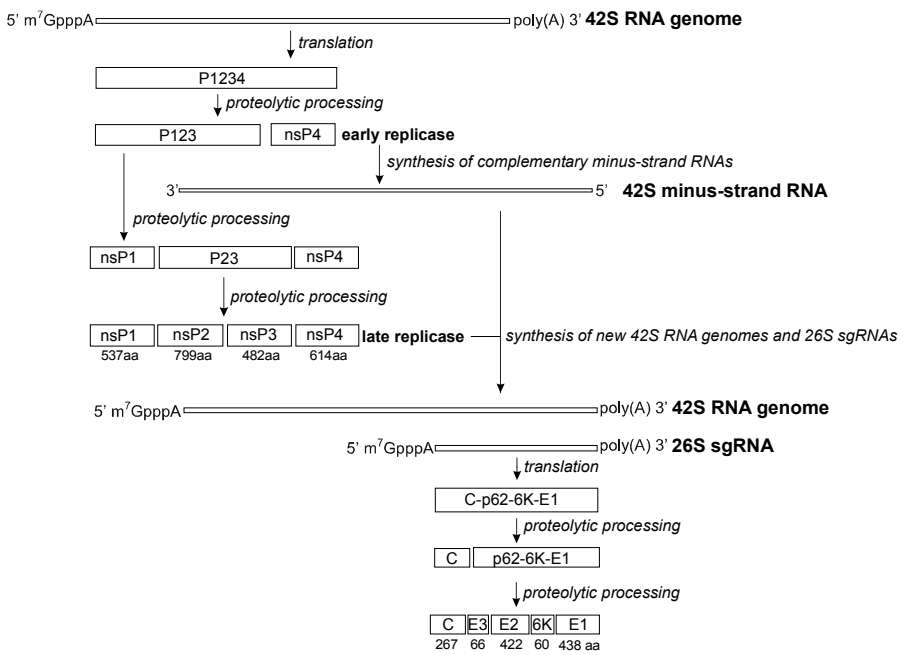

Figure 1. A schematic overview of the synthesis and translation of SFV RNAs and processing of polyproteins.

Infection cycle

Entry

Entry of SFV into host cells is initiated by the binding of the virion to a receptor on the cell surface. The identity of the receptor or receptors for SFV is still unknown, but it has been shown that the membrane glycoprotein E2 is responsible for binding the host (Smith et al., 1995). After binding, virus is rapidly internalized by clathrin-mediated endocytosis and transported to early endosomes (Helenius et al., 1980; Vonderheit and Helenius, 2005). Low pH in endosomes triggers a conformational change in the envelope proteins: the E1 and E2 dimers dissociate and E1 re-oligomerizes into an E1 homotrimer, which possesses fusogenic activity. Activated E1 is responsible for the fusion of the viral membrane with the endosomal membrane. The fusion results in the release of NC into the cytoplasm (Salminen et al., 1992; Wahlberg et al., 1992; Wahlberg and Garoff, 1992), where the viral genomic RNA is liberated by ribosomes (Singh and Helenius, 1992).

Replication

The three major types of viral RNAs detected in SFV infected cells are 42S(+) RNA, 42S(-) RNA, and 26S sgRNA. Their synthesis is finely regulated by the characteristic serial processing of the ns-polyprotein (Figure 1). First, the uncoated 42S RNA is translated into the large polyprotein P1234 (2432 amino acid residues), which serves as a precursor for individual ns-proteins nsP1, nsP2, nsP3, and nsP4. The polyprotein is processed via protease activity residing in nsP2 and subsequently forms the early and late replicase complexes (Merits et al., 2001; Sawicki and Sawicki, 1980; Vasiljeva et al., 2003). The short-lived early replicase, comprising processable intermediate P123 and cleaved nsP4, is responsible for the synthesis of complementary minus-strand RNA. Unlike the positive-strand 42S or 26S RNAs, the minus-strand does not accumulate in single-stranded form, but exists as double-stranded (ds) RNA. The synthesis of (-) RNAs occurs only at the early stages of infection (up to 4 h post-infection) while the synthesis of the positive-strand 42S and 26S RNAs continues at a maximal rate until the death of the infected cell (Sawicki and Sawicki, 1980). The late replicase of SFV is a stable complex, which is formed by cleavage of P123 into an intermediate nsP1+P23 species and finally to mature nsP1, nsP2, and nsP3. Replicase consisting from all four mature ns-proteins use (-) RNA as template for subsequent positive-strand RNA synthesis (Pettersson et al., 1980). This step of SFV replication takes place at the cytoplasmic membranes, more specifically on the surface of endosome- and lysosome-derived vesicles called cytopathic vacuoles type I (CPV I) (Froshauer et al., 1988; Grimley et al., 1968; Kujala et al., 2001). These vacuoles contain numerous small invaginations (spherules), which are the actual sites of viral RNA synthesis. The translation of structural proteins and the assembly of nucleocapsids occur also in the vicinity of CPVs (Froshauer et al., 1988).

All four ns-proteins are required for viral RNA synthesis. For the assembly of a functional replication complex (RC), the synthesis and processing of the polyprotein precursor is necessary – when co-expressed as individual proteins, nsP1, nsP2, nsP3, and nsP4 are not able to form active polymerase complex (Hahn et al., 1989b; Salonen et al., 2003). As described above, the timing of the synthesis of different viral RNAs is regulated by polyprotein processing. By using temperature sensitive (ts) mutants of SFV and SIN, the specific role each ns-protein plays in RNA synthesis has been demonstrated (Barton et al., 1988; Hahn et al., 1989b; Keranen and Kaariainen, 1979). That is, nsP1 is involved in the initiation of minus-strand RNA synthesis (Hahn et al., 1989b; Sawicki et al., 1981), nsP2 has been identified as a regulator of 26S RNA synthesis (Hahn et al., 1989b; Suopanki et al., 1998), nsP4 is the catalytic subunit of RNA polymerase, and nsP3 may be involved in the initiation of replication complex formation.

Virion assembly and budding

New SFV particles are released from infected cells by budding of the NC through plasma membrane regions that contain virus spike glycoproteins. NC itself is formed in the cytoplasm by incorporation of a single copy of genomic RNA into the capsid protein shell (Simons and Warren, 1984). The envelope proteins are transported through the *trans*-Golgi network to the cell surface where they generate spikes. The release of the virus from the cells is driven by NC-E2 interactions (Suomalainen et al., 1992). Unlike some other enveloped viruses, SFV does not appear to utilize lipid rafts during its virion formation (Briggs et al., 2003).

Non-structural proteins

nsP1 (537 aa) is a multifunctional protein with methyltransferase (MT) (Cross, 1983; Cross and Gomatos, 1981) and guanylyltransferase (GT) activities (Ahola and Kaariainen, 1995; Ahola et al., 1997) thereby catalyzing the capping reactions of the genomic and sgRNAs (Ahola and Kaariainen, 1995; Wang et al., 1996). The expression of nsP1 causes formation of characteristic extensions – filopodium-like structures on the cell surface. When expressed alone or in the context of properly processed P1234, nsP1 localizes to the inner surface of the plasma membrane (Laakkonen et al., 1996; Laakkonen et al., 1998). nsP1 facilitates the targeting of the replication complex to the cytoplasmic membranes (Peranen et al., 1995). The first and most important membrane binding site on nsP1 is a short amphipathic peptide (α-helix) with sequence G_{245}STLYTESRKLLRSWHLPSV$_{264}$, located in the middle of the nsP1 (Ahola et al., 1999). nsP1 functions only in a membranous environment, thus membrane binding via this amphipathic segment is essential for its enzymatic activities and, consequently, for SFV replication (Ahola et al., 1999; Spuul et al., 2007). In infected cells, as well as when expressed alone, nsP1 is palmitoylated at three adjoining cysteine residues (amino acid residues 418 to 420 of nsP1 of SFV). This modification takes place after the association of the

protein with the membrane (Laakkonen et al., 1996) rendering nsP1 highly hydrophobic, thus tightening its interaction with the membrane. Palmitoylation itself is not essential for membrane attachment or enzymatic activities of nsP1 – non-palmitoylated forms of nsP1 still associate with the membrane and form membranous replication complexes with normal appearance. However, such mutant proteins were reported not to induce formation of the filopodial extensions of the plasma membrane (Laakkonen et al., 1996). *In vivo* experiments conducted in mice show that loss of nsP1 palmitoylation reduces the pathogenicity of SFV (Ahola et al., 2000).

nsP2 (799 aa) contains many enzymatic activities required for viral replication and represents the main viral factor involved in virus-host cell interactions for Old World alphaviruses. Its C-terminal half is a papain-like cysteine protease (Merits et al., 2001) responsible for the processing of P1234. RNA triphosphatase, NTPase, and RNA helicase (Gomez de Cedron et al., 1999; Rikkonen, 1996; Rikkonen et al., 1994a) activities required for RNA synthesis and modification are localized in the N-terminal half of nsP2. Mutations in the C-terminal region of nsP2 decrease the level of 26S RNA synthesis, suggesting a role for this protein in the initiation of sgRNA synthesis (Suopanki et al., 1998).

nsP2 of SFV or SIN is responsible for the rapid suppression of cellular transcription and translation (Garmashova et al., 2006; Garmashova et al., 2007). It seems that these cytotoxic activities depend on the presence of the free form of nsP2 in infected cells since inhibition of P123 processing abolishes nsP2-dependent cytopathic effects (CPE) (Garmashova et al., 2006; Gorchakov et al., 2008). Mutations in the C-terminal portion of nsP2 render alphaviruses and their replicon vectors (alphavirus genomes in which the structural region is substituted with a gene of interest; see below for details) less cytotoxic to vertebrate cells (Lundstrom et al., 2003). A nuclear localization signal (NLS; $P_{647}RRRV_{651}$) has been identified in the C-terminal part of nsP2. Due to this element, approximately half of the synthesized nsP2 is found in the nucleus of SFV-infected cells (Peranen et al., 1990; Rikkonen, 1996; Rikkonen et al., 1992; Rikkonen et al., 1994b). The nuclear function or functions of nsP2 are likely connected with both viral pathogenesis and cytotoxicity, although their exact nature is unknown (Fazakerley et al., 2002; Frolova et al., 2002; Gorchakov et al., 2005; Tamm et al., 2008). It is very likely that interactions with nuclear structures and components have multiple effects. For example, an SFV mutant with the single amino acid change (R_{649}-D) in nsP2, which prohibits the transport of nsP2 into the nucleus, is unable to suppress interferon expression (see below for details) and is less virulent in mice (Fazakerley et al., 2002). It has also been observed that non-cytopathic alphavirus vectors that do not induce complete translation shutoff of the vertebrate host often have one or more mutations in the coding region of nsP2 (Agapov et al., 1998; Frolov et al., 1999; Frolova et al., 2002; Perri et al., 2000).

nsP3 (482 aa) is the only phosphoprotein encoded by SFV (Peranen et al., 1988). nsP3 does not have known enzymatic activities and its functions and

biological role are not completely understood. The nsP3 of SFV can be divided into three domains. The first domain is conserved among alphaviruses, coronaviruses, hepatitis E virus, and Rubella virus. In addition, the homologues of this domain can be found in bacteria, archaea, and eukaryotes. In this last group, the motif is attached to histone H2A (Koonin and Dolja, 1993; Pehrson and Fuji, 1998). The middle domain of nsP3 is conserved only among alphaviruses. The C-terminal domain (downstream of Tyr_{324} in the case of SFV nsP3) is hypervariable (Strauss and Strauss, 1994). SFV mutants with deletions in the hypervariable region or knocked-out phosphorylation sites within nsP3 are capable of replication but have reduced virulence in infected mice (Galbraith et al., 2006; Vihinen et al., 2001). nsP3 tolerates insertion of different foreign marker genes in its C-terminal part (Frolova et al., 2006; Tamberg et al., 2007) allowing for the construction of reporter viruses that enable the monitoring of virus infection in cell culture or in infected animals.

nsP3 is the only ns-protein with similar subcellular localization in both infected cells or when expressed alone. In both cases it localizes to the cytoplasm and forms vesicle-like structures characteristic of the whole RC (Froshauer et al., 1988; Peranen and Kaariainen, 1991; Salonen et al., 2003; Vihinen et al., 2001). When expressed as a part of an uncleavable polyprotein P123 or P23, the nsP3 directs these polyproteins to intracellular vesicles which resemble CPV structures seen in SFV-infected cells (Salonen et al., 2003). Analysis of non-phosphorylated or deletion variants of nsP3 revealed that neither phosphorylation nor the presence of the C-terminal domain of nsP3 are needed for its ability to connect with or participate in formation these structures (Vihinen et al., 2001). In the case of SIN, it has been shown that reduced phosphorylation of nsP3 correlates with reduced minus-strand RNA synthesis (De et al., 2003).

nsP4 (614 aa) is an RNA-dependent RNA polymerase (RdRp), the catalytic subunit of the replicase complex (Barton et al., 1988; Hahn et al., 1989a; Keranen and Kaariainen, 1979). During the early years of alphavirus studies, experiments with SIN mutant ts6 revealed that nsP4 is essential for the synthesis of both positive- and minus-strand RNAs and has a role in the elongation of the RNA chain (Barton et al., 1988; Hahn et al., 1989a; Sawicki et al., 1981). The presence of an X-Gly-Asp-Asp (A-GDD) motif, common to many RdRps, in the C-terminal part of nsP4 was suggestive of polymerase activity (Kamer and Argos, 1984; Koonin, 1991; Poch et al., 1989). However, only very recently were the enzymatic activities of purified recombinant nsP4 experimentally demonstrated (Rubach et al., 2008; Thal et al., 2007; Tomar et al., 2006). Aside from its RdRp activity, nsP4 has divalent cation-dependent terminal adenylyltransferase (TATase) activity likely responsible for adding adenosine residues to the 3' end of a positive-strand RNA substrate – an activity required to build up the poly(A) tail of the genome. During replication of the alphavirus genome, the poly(A) tail serves as part of the promoter for minus-strand synthesis, thus maintaining its integrity is absolutely essential for the viability of the virus (Hardy and Rice, 2005; Kuhn et al., 1990; Levis et al.,

1986). The first N-terminal amino acid residue of nsP4 is Tyr for all known alphaviruses. According to the N-end rule, Tyr is a destabilizing residue; therefore, the fraction of nsP4 not incorporated into replicase complexes is rapidly degraded in infected cells (Bachmair et al., 1986; de Groot et al., 1991). The enzymatic functions of nsP4 in the replication complex are dependent upon the properties of its N-terminal residue, which must be an aromatic amino acid (Tyr, Trp, or Phe) or His (Shirako and Strauss, 1998). The presence of other aa-residues inhibits viral replication. However, it has been shown that such defects can be rescued by the presence of compensatory changes in nsP4 and, surprisingly, also nsP1 or at the 5'-end of the viral genome.

Structural proteins

Structural proteins of alphaviruses are translated from the sgRNA as a poly-protein, which is processed co- and post-translationally (Figure 1) (Garoff et al., 1978; Strauss and Strauss, 1994). When synthesis of the structural polyprotein is initiated, the **capsid** protein (267 aa) acts as a serine autoprotease. It folds during its synthesis bringing together three catalytic residues so that the C-terminal protease domain of C is activated and releases itself from the rest of the polyprotein by cleaving the peptide bond between Trp_{267} and Ser_{268} (Choi et al., 1997; Melancon and Garoff, 1987; Nicola et al., 1999). Then, the C-terminal Trp_{267} is buried in a hydrophobic pocket, which inhibits the proteolytic activities of C (Choi et al., 1997). In the cytoplasm, the positively charged N-terminal segment of mature C is responsible for binding to the packaging signal in the viral genomic RNA leading to the formation of the nucleocapsid (Geigenmuller-Gnirke et al., 1993; Weiss et al., 1989).

After the cleavage of C from the polyprotein, a new N-terminus of a p62-6K-E1 polyprotein is exposed. It contains a signal for the co-translational insertion of p62 into the ER membrane. Translocation of the polyprotein terminates when the transmembrane region of p62 reaches the membrane. When the cytoplasmic tail of p62 and the 6K peptide is synthesized, the translocation of E1 into the ER membrane is reinitiated. Peptide 6K carries the signal sequence required for insertion of E1 into the ER membrane. In the lumen of ER, the polyprotein is processed by host cell signal peptidase to p62, 6K, and E1 (Garoff et al., 1978; Melancon and Garoff, 1986; Melancon and Garoff, 1987).

p62 is a precursor for **E3 and E2 glycoproteins.** Via its E3 domain, the p62 forms a heterodimer with E1. This interaction occurs prior to the exit of structural proteins from the ER, after which the formed E1-p62 complex is transported to the cell surface through the secretory pathway (Golgi apparatus). **E1** (438 aa) is a fusion protein (Klimjack et al., 1994). A p62 acts as a chaperone and holds E1 in an inactive form, thereby prohibiting the fusion of cellular membranes of infected cells. The p62 protein is cleaved into mature E2 (422 aa) and E3 (66 aa) by the cellular furin protease during transport from *trans*-Golgi to the cell surface (de Curtis and Simons, 1988; Zhang et al., 2003). This cleavage is necessary for the infectivity of virions since association of E1

with E2 in mature virus particles is more labile and acid-sensitive than the interaction of p62 with E1 (Wahlberg et al., 1989). In contrast to virions of most alphaviruses that lose the peripheral E3 protein, this protein is retained by SFV virions and forms heterotrimeric complexes ($[E1E2E3]_3$) with the other SFV spike glycoproteins (Garoff et al., 1974).

The 6K is a short transmembrane peptide (60 aa) that separates p62 from E1 and carries the signal sequence for the insertion of E1 into the ER membrane (Hashimoto et al., 1981; Liljestrom and Garoff, 1991a; Lusa et al., 1991; Melancon and Garoff, 1986; Welch et al., 1981). A recent study revealed that the coding sequence of 6K contains a frame shift signal, which is used to express a previously unknown structural protein designated **TF** (TransFrame protein; ~8 kDa). This new protein has been found in SFV virions, but its functions are not yet understood (Firth et al., 2008).

Virus-host interactions

The pathogenesis of alphavirus infection is highly dependent on both viral as well as host cell factors. The host factors affecting SFV pathogenesis include well-known antiviral factors such as type I interferons (IFN) and the double-stranded RNA-activated protein kinase (PKR). In addition, anti-alphavirus effects of numerous IFN-induced proteins have also been described.

Viral factors depend on virus strain and possible mutations that could lead to a weaker cytotoxic phenotype. The original SFV isolate, strain L10, and the SFV4 strain, derived from infectious clone pSP6-SFV4 (Liljestrom et al., 1991), are neurovirulent in mice, causing lethal encephalitis in mice of all ages. In contrast, pathogenicity of avirulent strains A7 (McIntosh et al., 1961) and its derivative A7[74] is age-dependent, being lethal for newborn but not adult mice (Atkins et al., 1999; Taylor et al., 1955). Normally, vertebrate cells are killed by wt SFV infection, but several mutations in nsP2 enable the establishment of persistent infection (Frolov et al., 1999; Perri et al., 2000).

Infection of the cell with alphavirus or alphavirus-based replicon vectors typically has a severe inhibitory effect on the host cell transcription and translation. Shutdown of host cell protein synthesis occurs at a very early stage of infection when viral structural proteins are not yet synthesized and the level of the viral sgRNA is too low to be detected. These findings indicate that cytotoxic effects of SFV (and other Old World alphaviruses) are caused by ns-proteins and/or by RNA replication *per se* (Frolov and Schlesinger, 1994a; Gorchakov et al., 2005). In many cell lines, virus induced cytopathic effects lead to apoptosis of infected cells. These effects, as stated below, are also dependent on the host. Importantly, in cells of invertebrate vectors, alphavirus infection is not cytotoxic and only moderately effects host gene expression.

Translational shutdown

Inhibition of translation in infected cells is a host defense mechanism designed to put a stop to virus multiplication. However, many viruses have evolved to circumvent this mechanism and/or to use it for their own benefits. It has been demonstrated that multiple mechanisms are involved in a SIN-induced shutdown of translation, but a full set of mechanisms has not been elucidated for any alphavirus. The complex nature of alphavirus-induced shutdown of host translation and the mechanisms used by the virus is illustrated by the following example. In host cells, the appearance of viral RNA replication intermediates – dsRNA molecules – activates type I IFNs, which act by inducing transcription of several antiviral genes including PKR. Activated PKR phosphorylates the eukaryotic translation initiation factor 2α subunit (eIF2α). Normally, eIF2 (an oligomer composed of three subunits α, β, γ) interacts with GTP and Met-tRNAi. This ternary complex associates with 40S ribosomal subunits and binds to the 5' end of mRNA where it scans the RNA sequence until the suitable initiation codon is found. The 60S ribosomal subunit then joins the complex and translation starts. These events include GTP hydrolysis and release of eIF2-GDP, which is recycled by eIF2B. Phospo-eIF2α, generated by the activity of PKR, restricts the activity of eIF2B and thus blocks the formation of the GTP-eIF2-Met-tRNAi ternary complex. Since the translation of most cellular and viral mRNAs proceeds via ternary complex-dependent mechanisms, the defense mechanism described above leads to a global decrease in protein synthesis in alphavirus infected cells. Despite the global inhibition of cellular and viral genome translation (Ventoso et al., 2006), there persists a high level of alphavirus sgRNA translation. This results in accumulation of large amounts of structural proteins during the late stage of alphavirus infection. A unique genetic element known as the translational enhancer, located at the 5' end of the coding region of the C protein of SFV and SIN, has been found to be responsible for this effect (Frolov and Schlesinger, 1994b; Sjoberg et al., 1994). The translational enhancer functions only in infected cells in the presence of high levels of phospho-eIF2α (Frolov and Schlesinger, 1994b; McInerney et al., 2005; Sjoberg and Garoff, 1996). In the case of SIN it has been shown that the enhancer, which forms a stable hairpin structure in sgRNA, allows the usage of an alternative initiating factor 2A (eIF2A) that does not require GTP for delivering Met-tRNAi to the initiation complex (Ventoso et al., 2006).

Transcriptional shut-down and suppression of type I interferon response

In addition to the inhibition of cellular translation, alphaviruses also suppress cellular transcription. Interestingly, the Old and New World alphaviruses downregulate cellular transcription via different viral factors. In the case of Old World alphaviruses – such as SFV and SIN – the nsP2 is responsible for shutting down host transcription whereas this task is relegated to the capsid protein in the New World alphaviruses VEE and EEE (Garmashova et al., 2007). The alphavirus-induced shutdown of transcription can be divided into two parts. The general transcriptional shutdown results in almost complete

inhibition of host mRNA production. A targeted shutdown of transcription of antiviral genes occurs during the earlier phases of infection. For example, it was shown that the highly specific inhibition of transcription is responsible for downregulating expression of interferon genes leading to suppression of an IFN-induced antiviral response (Gorchakov et al., 2005).

Type I IFNs are the main factors in the initiation of an innate immune defense against different virus infections. Induction of type I IFNs in most cell types is triggered by the recognition of viral replication intermediates, such as dsRNA, by the cytoplasmic RNA helicases RIG-I and MDA-5 (Yoneyama et al., 2005; Yoneyama et al., 2004). Expression and secretion of type I IFNs leads to autocrine and paracrine stimulation of cells, which establishes an antiviral state and blocks the spread of the virus. Both SIN and SFV cause the induction of type I IFNs and are very sensitive to the effects of IFN expression. For example, they cannot efficiently replicate in cells pre-treated with IFNs (Frolova et al., 2002). These viruses overcome this block by preventing the IFN response at its start. Different mutations in nsP2 result in phenotypes that induce significantly more α/β IFNs than wt virus, suggesting the main role of nsP2 in host-virus interaction is to counteract induction of IFN. As an example, a mutation (RDR) in the NLS of SFV nsP2 disturbs its nuclear localization and leads to a mutant virus that efficiently inhibits general cellular transcription and translation, nevertheless, it still induces significantly more type I IFNs than wt virus (Breakwell et al., 2007). In the case of the mutant virus SIN/2V (G_{806}-V, blocks nsP2/3 cleavage and results in accumulation of P23, which localizes exclusively to the cytoplasm), it was shown that the virus efficiently inhibits cellular translation but not transcription, leading to an increased α/β IFN response (Gorchakov et al., 2005). Another mutant virus known as SIN/G (P_{726}-G) produces nsP2 that localizes mainly to the cell nucleus and does not inhibit either cellular translation nor transcription, therefore efficiently inducing type I IFNs (Frolova et al., 2002; Gorchakov et al., 2005). Thus, multiple changes in nsP2 result in mutant viruses incapable of suppressing IFN expression. This effect has a complex nature – nuclear localization of nsP2 is required but is not sufficient to suppress transcription of IFN genes.

Apoptosis

Multiple studies have shown that SIN and SFV induce apoptosis in both cultured vertebrate cells (BHK-21, AT-3, N18, RIN, oligodendrocytes in glial cell culture) and cells of the central nervous system (CNS) of mice (Glasgow et al., 1997; Levine et al., 1993; Lundstrom et al., 1997; Scallan et al., 1997). The entry process of viral particles is not necessary for the induction of apoptosis since transfection of the cells with viral genomic RNA or RNA of alphavirus-based replicon vectors also results in apoptosis. It is known that SFV causes p53-independent apoptosis, thus DNA damage as a triggering event is probably not the cause (Glasgow et al., 1998). The exact mechanisms involved in apoptosis induction by alphaviruses are still disputed.

There are many examples indicating the involvement of an intrinsic (mitochondrial) pathway in the induction of apoptosis by alphavirus infection. It has been shown that over-expression of the anti-apoptotic gene *bcl-2*, a potent inhibitor of the mitochondrial apoptosis pathway, inhibits or slows down SFV- and SIN-induced apoptosis (Levine et al., 1993; Lundstrom et al., 1997; Scallan et al., 1997). At the same time, protection of cells by expressing bcl-2 *in trans* strongly depends on the particular viral strain (virulent *versus* avirulent) (Ubol et al., 1994). In cells, bcl-2 is anchored to the outer mitochondrial membrane, the ER, and the nuclear membrane and acts to block the release of cytochrome c from mitochondria into the cytosol (Kluck et al., 1997; Krajewski et al., 1993). Cytochrome c localizes to the mitochondrial intermembrane space where it functions as a part of the electron transport chain. After apoptotic stimuli, cytochrome c is released and triggers the formation of the apoptosome that activates caspases (Li et al., 1997). Thus, the protective effect of bcl-2 expression against alphavirus-induced apoptosis indicates a role for the mitochondrial pathway in apoptosis induction. However, there is also evidence supporting involvement of other apoptotic pathways. It has been shown that in rat 6 (R6) embryo fibroblasts, SFV or SIN infection activates caspase-3, which proteolytically inactivates bcl-2. The activation of caspase-3 occurs only in response to infection with alphaviruses, while in cells treated with other apoptotic stimuli, such as NH_4Cl, bcl-2 prevents the activation of the apoptotic response (Grandgirard et al., 1998). Recently, a variant of the mitochondrial pathway of alphavirus-induced apoptosis was characterized. In this case, apoptosis was initiated via the activity of pro-apoptotic Bak and subsequently amplified via the activity of Bid (Urban et al., 2008). Importantly, it was shown that replication of SFV, which resulted in Bak-mediated release of cytochrome c and activation of caspases 3 and 7, was required for apoptosis induction. Subsequent activation of caspase 8 amplified virus-induced apoptosis (Urban et al., 2008). Thus, the mitochondrial pathway of alphavirus-induced apoptosis consists of multiple steps.

Alphavirus-based vectors

Virus-based expression vectors have been developed from viruses belonging to different systemic groups including both DNA and RNA genomic viruses. The main advantage of virus-based vectors over alternative expression systems is their inherent ability to enter the cell. At the same time, basic properties of such vectors reflect those of their parental viruses. As such, all vectors have properties which can represent advantages in some applications and disadvantages in other cases. The basic properties alphavirus-based vectors inherit from their parental viruses are cytoplasmic replication, strong but transient expression of transgenes, and cytotoxicity for most vertebrate cells. Therefore, alternatively designed alphavirus-based vectors are being developed for three applications: vaccine development, gene therapy of central nervous system diseases, and anti-

cancer therapy (Atkins et al., 2008). At least one alphavirus-vector based vaccine has reached preclinical evaluation (Hubby et al., 2007).

Most alphavirus-based vectors in use today were developed from the genomes of SFV, SIN, and VEE. These vectors, like corresponding viruses, are able to multiply very effectively in many different cell types including neuronal cells. During replication, competent vectors initiate high levels of expression of the inserted gene of interest (Atkins et al., 2008).

In the most commonly used alphavirus-based vectors, the structural part of the viral genome is replaced with a gene of interest. This replacement results in a self-replicative vector, commonly called a replicon vector. To compensate for the lack of structural proteins and to produce virus-like particles (VLP), a helper system has been developed (Liljestrom and Garoff, 1991b; Smerdou and Liljestrom, 1999). The helper vector(s) contain *cis*-signals for replication/ transcription and encode structural proteins, but typically lack a packaging signal; thus, cells co-transfected with a replicon and helper RNAs produce VLPs that are limited to a single cycle of infection.

To enhance the expression of a foreign gene, the translational enhancer from the viral capsid gene (Sjoberg et al., 1994) is often fused to the 5' end of corresponding coding sequences. Alternatively, mutations leading to less cytotoxic phenotypes can be introduced into the SFV vectors in order to prolong the time of recombinant gene expression (Lundstrom et al., 2003).

RESULTS AND DISCUSSION

Objectives of the present study

The present dissertation is based on the studies of the biological properties of nsP1 of SFV and on the construction and use of SFV-based vectors. The main objectives of this work are:

1. To analyze the properties of non-palmitoylated mutant forms of nsP1 and their influence on virus replication; to estimate the possibility of utilizing corresponding mutations in the context of SFV replicon vectors.
2. To determine the effect of transiently expressed nsP1 and its non-palmitoylated form on cell metabolism by using inducible cell lines as models; to analyze the effects of their expression on the replication of SFV.
3. To investigate whether inclusion of the anti-apoptotic gene *bcl-2*, which inhibits the induction of apoptosis via a mitochondrial pathway, can protect cells against SFV-induced apoptosis and, thus, be used for construction of SFV-based expression vectors with reduced cytotoxicity.
4. To demonstrate the use of SFV-based vectors in other basic research studies, e.g., characterizing the interactions of replicase proteins of hepatitis C virus.

Mutations at the palmitoylation site of SFV nsP1 attenuate virus replication and cause accumulation of compensatory mutations (I)

During RNA synthesis, nsP1 participates in the capping of SFV genomic and sgRNAs (Ahola and Kaariainen, 1995; Wang et al., 1996). The association of nsP1 with the cell membrane is absolutely essential for its enzymatic activity and, therefore, effective viral replication. A short amino acid stretch in the middle of nsP1 is responsible for binding the cell membrane (Ahola et al., 1999). Additionally, binding is enhanced by palmitoylation of three contiguous cysteines (C_{418}-C_{420}) (Laakkonen et al., 1996).

Since it has been reported that substitution of C_{418}-C_{420} with alanine residues has no (or very little) effect on SFV replication (Ahola et al., 2000) but reduces pathogenicity of the virus, this mutation was considered as a basis for construction of an SFV-based vector with reduced cytotoxicity. When SFV replicon vectors bearing the mutation C_{418}-C_{420} to AAA were introduced to cells, revealed that the mutation almost completely abolished infectivity of the SFV replicon. This result was in sharp contrast to the previously reported data for the full-length virus. In order to explain this discrepancy and to further analyze effects of nsP1 palmitoylation on SFV replication, three pSFV1-based vectors were constructed carrying mutated palmitoylation sites in nsP1 and expressing a *d1EGFP* gene as a marker. In these vectors, three cysteine residues (C_{418}-C_{420}) were either replaced with alanines (SFV1mut3A/d1EGFP), deleted entirely

(SFV1Δ3/d1EGFP), or deleted in combination with the adjacent $R_{414}SLT_{417}$ sequence (SFV1Δ7/d1EGFP) (I, Fig. 1). Analysis of cells transfected with *in vitro* transcripts of these vectors gave an unexpected result: in accordance with previous observations only a few EGFP positive cells were detected among cells transfected with SFV1mut3A/d1EGFP and SFV1Δ3/d1EGFP RNAs, whereas approximately 40% of the cells transfected with SFV1Δ7/d1EGFP RNA were EGFP positive. For a comparison, in these experiments >90% of cells transfected with transcripts for a control vector (wt SFV1/d1EGFP) were EGFP positive. Thus, mutations in the nsP1 palmitoylation site have a severe effect on the replication of SFV RNA and on the expression of the marker gene. The fact that SFV1Δ7/d1EGFP was considerably more infectious than the other two mutant replicons suggested that some other defect(s) was responsible for the extremely low infectivity of the SFV1mut3A/d1EGFP and SFV1Δ3/ d1EGFP replicons and not the absence of palmitoylation. To analyze the effects of these mutations in the context of the full-length genome of SFV, all three mutations were introduced into the infectious cDNA clone pSP6-SFV4 and the infectivity of corresponding RNA transcripts was analyzed by an infectious center assay. Again, it was found that RNAs transcribed from SFV4mut3A and SFV4Δ3 exhibited extremely low infectivity (at least 10,000 times lower than those of SFV4), whereas infectivity of SFV4Δ7 RNA was reduced only about 40-fold in comparison with wt SFV4. Thus, the defect was not specific for replicon vectors of SFV.

The most likely explanation for the contradictory observations of SFV4mut3A and the virus carrying essentially the same mutation (Ahola et al., 2000) is the accumulation of mutations with a positive impact on virus replication during its propagation. To test this hypothesis, the release of viruses from transfected and infected BHK-21 cells was compared (I, Fig. 2). The release of infectious virions from cells transfected with SFV4mut3A and SFV4Δ3 RNAs occurred significantly later – 6-8 h post infection (p.i.) – than in the case of cells transfected with wt SFV4 RNA (4 h p.i.) indicating the possibility of genetic rearrangement or adaptation of mutant genomes. Consistent with previous findings, the release of SFV4Δ7 virions was delayed to a lesser extent. In contrast, when the BHK-21 cells were infected with the primary stocks (collected from transfected cell cultures) of wt and mutant viruses, no considerable difference between the growth curves of these viruses was detected. These results support the hypothesis that changes had taken place during the propagation of the mutant viruses, and these changes restored the ability of SFV4mut3A and SFV4Δ3 to replicate at high levels in BHK-21 cells.

The mutations in the palmitoylation sequence of nsP1 (multiple point mutations or deletions) were designed in a way to make their reversion highly unlikely. Indeed, it was found that the original mutations were preserved in the collected virus stocks indicating that genetic rearrangements/adaptations must have occurred in other regions of the genomes of mutant viruses. To identify the possible second-site compensatory mutations, four isolates for each mutant were plaque-purified and their ns-regions were sequenced. The analysis of

sequences revealed three single mutations (P181Q, L234F, and Q357L) in SFV4mut3A isolates and two double mutations (M124V+A197D and ΔG224+T352S) in SFV4Δ3 isolates (I, Table I). All these mutations localized to nsP1. Interestingly, no mutations were found in the ns-region of any plaque-purified isolates originating from SFV4Δ7.

Introduction of the identified mutations into the genomes of SFV4mut3A and SFV4Δ3 increased the infectivity of the *in vitro* synthesized RNAs to a level comparable to wt SFV4 RNA transcripts (I, Table 1). Thus, the identified mutations rescued the infectivity of SFV harboring mut3A or Δ3 mutations. The effect of compensatory mutations to the growth of mutated viruses was further analyzed using two recombinants, SFV4mut3A-P181Q and SFV4Δ3-ΔG224+T352S. It was found that, unlike SFV4mut3A and SFV4Δ3, the growth of SFV4mut3A-P181Q and SFV4Δ3-ΔG224+T352S in transfected BHK-21 cells was not delayed, although virion accumulation was slightly reduced when compared with cells infected with SFV4 (I, Fig. 3a). When BHK-21 cells were infected with the respective primary viral stocks, no differences between mutant viruses and SFV4 were detected (I, Fig. 3b).

The compensation of the original defect by second-site mutations is a rather common phenomenon for alphaviruses (and for RNA viruses in general) since many random mutations are generated during RNA replication. However, it is interesting that all compensatory changes for SFV4mut3A and SFV4Δ3 were found in the nsP1 region. It either reflects the fact that the original defect cannot be compensated with changes elsewhere, since multiple functions of nsP1 were affected by the mut3A and Δ3 changes or, alternatively, too few mutant genomes were plaque-purified and sequenced. The data presented below favor the former hypothesis, since at least two different defects were described for nsP1 with mut3A and Δ3 mutations.

Although the reversion of the original mutations was not observed in this study, the analysis of palmitoylation of mutant forms of nsP1 expressed in HeLa cells was carried out to exclude the possibility of palmitoylation at an alternative site. The experiment clearly revealed that only wt nsP1 was palmitoylated (I, Fig. 4b) confirming that mut3A, Δ3, and Δ7 indeed abolish palmitoylation and compensatory mutations did not restore that particular property.

The original mutation of C_{418}-C_{420} to alanine residues had a minor effect on the enzymatic properties of recombinant nsP1 (Laakkonen et al., 1996). To find whether this is also the case for nsP1s harboring mut3A, Δ3, and Δ7 mutations with or without compensatory changes, these proteins were expressed in *E. coli* and their GT activities were assayed. The GT activities of all these recombinant proteins were found to be similar to those of wt nsP1 (I, Fig. 4a). Thus, it was concluded that defects (if any) in enzymatic activity of mutant nsP1s were not the driving force behind the very low infectivity of the mut3A and Δ3 constructs.

The first important difference between wt nsP1 and the mut3A or Δ3 mutants was revealed by the analysis of their subcellular localization in transfected cells. As expected, wt nsP1 localized almost exclusively to the

plasma membrane and caused extensive induction of filopodium-like extensions (I, Fig. 5a). While nsP1-mut3A and nsP1-Δ3 also localized to the plasma membrane, they were also found in the cytoplasm and did not cause the formation of filopodium-like extensions (I, Fig. 5b, c). Neither did expression of nsP1-Δ7 result in formation of filopodium-like extensions, but in contrast to the other two mutant nsP1s, the nsP1-Δ7 localized mainly at the plasma membrane of transfected cells (I, Fig. 5d). The addition of the compensatory mutations to nsP1-mut3A and nsP1-Δ3 resulted in more extensive plasma membrane localization of the recombinant proteins (I, Fig. 5e-i). In most cases, compensatory mutations restored the ability to induce the formation of filopodium-like extensions (except nsP1-mut3A-L234F), although these structures were always less prominent than those induced by wt nsP1. Thus, the subcellular localization of nsP1 was affected by mutations in its palmitoylation site and was, at least in part, restored by identified compensatory changes.

The second property of nsP1 affected by mutations in its palmitoylation site was its ability to interact with other proteins of the SFV replicase complex. It has been shown that nsP1 interacts directly with nsP3 and nsP4 and that these interactions can be detected even if the proteins are expressed pair-wise using a transient expression system (Salonen et al., 2003). To determine the effects of mut3A, Δ3, and Δ7 on association of nsP1 with nsP4, wt nsP1 and corresponding mutants were co-expressed with nsP4 in Cos-7 cells. Complexes were immunoprecipitated under native conditions. It was found that wt nsP1 and nsP1-Δ7, but not nsP1-mut3A and nsP1-Δ3, were able to form a complex with nsP4 (I, Fig. 6). Importantly, the introduction of the compensatory mutations P181Q, L234F, or Q357L into nsP1-mut3A and M124V+A197D or ΔG224+T352S into nsP1-Δ3 restored their interaction with nsP4. These findings suggest that an impaired interaction between nsP1 and nsP4 is another reason for the low infectivity of SFV4mut3A and SFV4Δ3 and that the compensation occurred through its restoration.

Taken together, these findings demonstrate a pleiotropic effect of mutations in the palmitoylation site of nsP1. In addition, these results demonstrate the extreme plasticity of the virus, since defects caused by mutations in the palmitoylation site could be compensated by numerous single second-site changes. This analysis also indicates that palmitoylation-deficient replicon vectors cannot be used as non-cytotoxic expression systems, since their infectivity is significantly attenuated, and when combined with compensatory mutations, their properties resemble too closely those of the cytotoxic wt replicons. Indeed, this assumption was later confirmed by direct analysis of cytotoxicity of such vectors (Liis Karo-Astover, MSc thesis, 2007).

Properties of SFV nsP1 and its interference with virus replication (II)

As described above, alphavirus infection causes CPE in host cells. In the case of SFV or SIN the free form of nsP2 is required for induction of CPE (Garmashova et al., 2006; Gorchakov et al., 2008). However, it has not been demonstrated whether the expression of other ns-proteins can cause similar effects. Expression of nsP1 in infected or transfected cells results in characteristic changes, such as induction of filopodium-like structures at the plasma membrane and rearrangements of actin filaments (Laakkonen et al., 1998). These effects implicated nsP1 as a likely candidate to induce cytotoxic effects.

To study the properties of nsP1 and its effect on host cells, tetracycline-inducible stable cell lines (based on HEK293 T-REx cells) were constructed for wt nsP1 and its palmitoylation-negative form (mutation C_{418}-C_{420} to AAA, designated hereafter as nsP1^{6D}). The half-life of both proteins expressed in these cells was determined; the results obtained from this system were compared with data on the half-life of nsP1 measured in infected HEK293 T-REx cells (II, Fig. 1b). It was found that the half-lives of nsP1 and nsP1^{6D} in induced cells were approximately 5 h, which was in good agreement with the half-life of nsP1 measured in SFV4-infected HEK293 T-REx cells (approximately 4 h). The analysis of subcellular localization of recombinant proteins in induced cells revealed that nsP1 and nsP1^{6D} both localized to the plasma membrane, where wt nsP1, but not nsP1^{6D}, induced formation of typical filopodium-like structures similar to those observed for SFV infected cells (II, Fig. 2). However, in contrast to SFV4-infected cells, the recombinant nsP1 did not localize to intracellular vesicles, a finding which is in agreement with previous data (Salonen et al., 2003). Various assays carried out with constructed cell lines revealed that inducing expression of nsP1 or nsP1^{6D} did not affect cellular transcription, translation, or cell viability (II, Fig. 3).

It is a well known fact that many viruses create intracellular conditions causing an infected cell to develop resistance to further superinfection by the same or closely related viruses. This phenomenon is called superinfection exclusion or homologous interference and is also characteristic of alphaviruses (Johnston et al., 1974). The localization of nsP1 to the cell membrane suggested that it might have a role in this phenomenon. To examine that possibility, cell lines in an induced or uninduced state were infected with VLPs containing a packaged SFV1-d1EGFP replicon (II, Fig. 4b). This construct is incapable of spreading in infected cell culture, thus the efficiency of infection could easily be analyzed by counting the percentage of EGFP positive cells. The experiment revealed that induction of nsP1 or nsP1^{6D} expression did not affect the ability of SFV VLPs to enter the cells and replicate. However, since the test system did not fully reproduce the conditions of superinfection it cannot be excluded that nsP1, in combination with other components of SFV, has some role in this phenomenon.

To determine whether expression of nsP1 or nsP1^{6D} interferes with other steps of SFV multiplication, induced or mock-induced cell lines were infected with SFV4 and titers of released virus at 8 and 12 h p.i. were compared (II, Fig. 4c). It was found that titers of SFV4 released from induced HEK293 T-REx control cells did not differ from those released from non-induced cells at either selected time point indicating that the presence of an inducer alone has no effect on SFV multiplication. In contrast, induction of expression of either nsP1 or nsP1^{6D} severely decreased SFV multiplication. At 8 h p.i., the decrease was more apparent in induced T-REx-nsP1 cells than in T-REx-nsP1^{6D} cells. At 12 h p.i., the situation was reversed: the titer from induced T-REx-nsP1 cells was decreased 2.5-fold compared to an approximately 5-fold decrease observed for induced T-REx-nsP1^{6D} cells. Thus, expression *in trans* of wt nsP1 and its palmitoylation-deficient variant inhibited extracellular accumulation of the virus.

Since the expression of nsP1 or its mutant form did not affect SFV entry, the reduced extracellular virus titers should reflect defects in production of viral transcripts, virus structural proteins, and/or assembly and release of virions. To monitor whether the expression of nsP1 or nsP1^{6D} had any effect on the translation of viral genomic and sgRNAs, the SFV4-based vectors carrying *Renilla* luciferase (Rluc) as a marker gene in their non-structural (SFV4(3H)-Rluc) or structural regions (SFV4-StRluc) were used (Tamberg et al., 2007) to infect constructed cell lines. It should be taken into consideration that the translation of SFV genomic RNA is proportional to its copy number only at early stages of infection; in later phases of infection the translation of genomic RNA is inhibited, but its synthesis remains active. Therefore, the expression of Rluc in cells infected with SFV4(3H)-Rluc was measured during the first 6 h of infection. In contrast, Rluc activity from SFV4-StRluc should be proportional to the amount of expressed viral structural proteins throughout the course of infection, therefore, it was monitored up to 12 h p.i. Treatment of HEK293 T-REx control cells with tetracycline prior to infection resulted in a small increase of translation of the replicase ORF (II, Fig. 5a) but had no effect on the translation of the structural protein ORF (II, Fig. 5b). In contrast, tetracycline induction of the T-REx-nsP1 cell line resulted in a four-fold reduction of replicase ORF-derived Rluc activity (II, Fig. 5c). Marker expression, mediated by the sgRNA, was strongly suppressed only at early stages of infection. By 12 h p.i., no difference between induced and uninduced cells was detectable (II, Fig. 5d). Induction of the T-REx-nsP1^{6D} cell line resulted in less than a two-fold reduction of replicase ORF-derived Rluc activity (II, Fig. 5e) but caused strong and persistent inhibition of structural protein ORF-derived Rluc activity (II, Fig 5f) indicating that the mechanism of action of the mutant protein differs from that of wt nsP1.

To determine whether expression of nsP1 or nsP1^{6D} affects the level of viral RNA accumulation, the total RNA extracted from induced and uninduced HEK293 T-REx, T-REx-nsP1, and T-REx-nsP1^{6D} cells at 12 h p.i. with SFV4(3H)-Rluc was analyzed by northern blotting (II, Fig. 6). Expectedly, the

treatment of control HEK293 T-REx cells with tetracycline had no effect on viral RNA levels. The amount of viral RNAs in uninduced HEK293 T-REx cells was similar to that of T-REx-nsP1 cells while the viral RNA level in uninduced T-REx-nsP1^{6D} cells was slightly lower. Induction of nsP1 expression decreased the amount of genomic RNA and, to a lesser extent, of sgRNA. In contrast, induction of nsP1^{6D} expression resulted in a strong reduction of the amounts of both genomic and sgRNAs.

In conclusion, these results showed that expression of nsP1 did not affect cell metabolism and viability, thus they indirectly support the current opinion that nsP2 plays the lead role in SFV cytotoxicity. Highly concordant results obtained by northern blot and analysis of marker protein expression from recombinant viruses revealed that expression of nsP1 and nsP1^{6D} exert different effects on SFV replication. In general, the expression of both of these proteins inhibited the synthesis of viral RNAs and their translation resulting in a significant decrease of released virus titers.

Novel vectors expressing the anti-apoptotic protein Bcl-2 (III)

Current SFV replicon vectors provide high-level but transient expression of a gene of interest. The reason behind this phenomenon is the ability of both SFV and SFV-based vectors to cause rapid and almost complete shutdown of cellular macromolecule synthesis and induce apoptotic cell death precluding long-term expression of a foreign gene. The possibility of reducing the cytotoxicity of alphavirus vectors by introducing selected point mutations to their ns-region was described above. An alternative option toward avoiding cell death and prolonging expression of a gene of interest is to use vectors co-expressing anti-apoptotic genes. Since the expression of *bcl-2* by stable cell lines prevents alphavirus-induced apoptosis (Levine et al., 1993; Lundstrom et al., 1997; Scallan et al., 1997), it was considered to be a suitable candidate for a suppressor of vector-induced cytotoxicity. However, it should be noted that the use of stable cell lines has an intrinsic problem – the over-expression of oncogenic *bcl-2* can cause numerous changes in the phenotypes of infected cells and alter cellular gene expression. Thus, stable cell lines permanently expressing *bcl-2* are not adequate models for the analysis of the direct effect of the bcl-2 protein on SFV infection. Instead, the most effective alternative option is drive expression of bcl-2 from promoters within SFV vectors themselves. To achieve this, the SFV1 vector system (Liljestrom and Garoff, 1991b) was used to construct novel bicistronic replicons by adding a second expression cassette to the initial monocistronic vector (SFV-PL-d1EGFP) (III, Fig, 1). This manipulation resulted in replicons with the marker gene *d1EGFP* placed under the native 26S sg promoter while the expression of bcl-2 was achieved via the minimal (-20 to +6 with respect to sgRNA start site) sg-promoter of SFV or by insertion of internal ribosomal entry site (IRES) of encephalomyocarditis virus

(EMCV IRES) or crucifer-infecting tobamovirus (CR IRES). In control vectors the coding sequence of *bcl-2* was replaced with that of *HcRed* (the respective protein has no effect on cell metabolism). For the survival test, the *d1EGFP* was replaced with a gene conferring resistance to puromycin (*Pac*).

The analysis of the expression of d1EGFP, bcl-2, HcRed, and nsP1 in BHK-21 cells infected with VLPs revealed that the expression patterns of nsP1 and d1EGFP were similar for all analyzed constructs. In contrast, significant differences were observed for the expression of bcl-2 or HcRed. Their expression was strongest and earliest when directed by EMCV IRES, whereas CR IRES-mediated expression was only slightly stronger than that achieved by the use of the second sg promoter (III, Fig. 2). Regardless of the level or time of bcl-2 expression, all replicons led to a shutdown of cellular protein synthesis of similar potency (III, Fig. 3). No protective effect by expressed bcl-2 was observed in survival tests using BHK-21 cells and puromycin selection. In this assay, almost all mock-transfected cells died within 24 h after puromycin was added to the growth medium. Cells transfected with SFV replicon RNAs expressing the puromycin resistance gene survived longer but died 72 h post transfection (p.t.) (III, Fig. 4a), despite the expression of an anti-apoptotic protein. Furthermore, the greatest percentage of surviving cells 48 h p.t. was observed for a construct void of bcl-2 expression. Analysis of the viability of infected cells provided a similar result: cells infected with bcl-2 expressing VLPs were less viable than cells infected with VLPs carrying the monocistronic vector (III, Fig. 4b). Thus, expression of bcl-2 neither increased the viability of the infected cells nor delayed cell death. If anything, bcl-2 expression accelerated SFV-induced toxicity in BHK-21 cells.

The anti-apoptotic effect of bcl-2 is associated with its ability to prevent the release of cytochrome c from mitochondria (Kluck et al., 1997; Yang et al., 1997). Results showing that high levels of bcl-2 do not protect cells against SFV-induced death suggest that SFV-induced apoptosis in BHK-21 cells may not depend on the mitochondrial pathway. To verify this hypothesis, the release of cytochrome c from mitochondria was analyzed by immunofluorescence microscopy. This approach allows detection of the characteristic punctate pattern of cytochrome c (elongated mitochondrial structures) in the cytosol of mock-infected cells (III, Fig. 5A, 7A). In agreement with previous data, it was found that infection of BHK-21 cells with SFV-PL-d1EGFP VLPs or SFV4 did not affect cytochrome c localization in mitochondria (III, Fig. 5–8). In contrast, it quickly translocated to the cytoplasm in response to treatment with staurosporine, a non-selective protein kinase inhibitor (III, Fig. 5B, 7B). At later time points (24 h p.i.), cytochrome c condensed into the perinuclear area of the cells (III, Fig. 6F, 7E), but the punctated pattern did not diffuse. Thus, there was no release of cytochrome c into the cytosol in response to SFV as measured by immunofluorescence microscopy, and it can be presumed that SFV-induced apoptosis did not occur via the mitochondrial (cytochrome c dependent) pathway.

It is well known that cell death caused by alphavirus infection depends on cell type. For example, mature neurons or cells of invertebrate origin do not

undergo apoptosis upon infection. Similarly, it is possible that the pathway(s) leading to apoptosis might be different in various cell types. As AT3-Neo and AT3-bcl2 cells were the first cell lines in which the protective effect of bcl-2 against alphavirus-induced apoptosis was demonstrated (Levine et al., 1993), they were also used to estimate the effects of bcl-2 expression on SFV replication in stably transformed cells. The corresponding experiments revealed that after infection of AT3-Neo cells with VLPs containing bicistronic replicon vectors, almost all the infected cells died within 48 h (III, Fig. 9a). Thus, bcl-2 expressed by replicons via a sg-promoter or IRES elements did not provide a protective effect. In contrast, if these experiments were performed with AT3-bcl2 cells, the number of viable cells remained constant for 24 h. Furthermore, the numbers of viable cells increased considerably by 48 h p.i. (III, Fig. 9b). At the same time, the d1EGFP signal was lost, indicating that AT3-bcl2 cells overcome SFV infection. However, taken together, these data indicate that suppression of SFV infection in AT3-bcl2 cells was probably not caused by bcl-2 expression. Instead, the permanent expression of bcl-2 most likely resulted in the expression of anti-viral proteins (such as zinc-finger antiviral proteins) that protected cells against alphavirus infection. In conclusion, these findings demonstrate the very complex nature of alphavirus-induced apoptosis.

Complex formation between hepatitis C virus NS2 and NS3 proteins (IV)

A common feature of the majority of positive-strand RNA viruses of vertebrates is the synthesis of polyprotein(s) that are subsequently cleaved by viral and/or host proteases to produce enzymes for RNA synthesis and structural proteins for virion assembly. In the case of hepatitis C virus (HCV), the structural proteins (core, E1, E2, and p7) are released from the N-terminal part of a single viral polyprotein by host signal peptidases, while the viral NS2 and NS3 proteins are responsible for the processing of the non-structural part of the polyprotein (Figure 2).

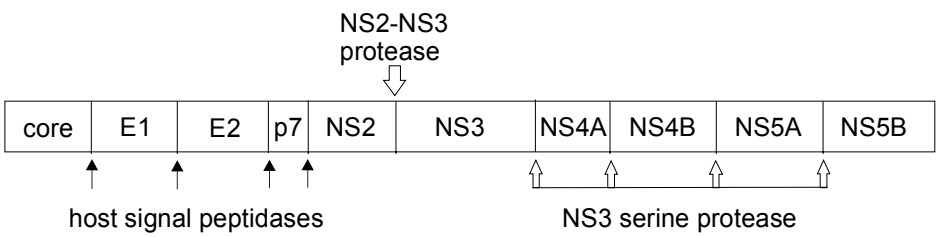

Figure 2. A schematic overview of proteolytic processing of the HCV polyprotein. The black arrows indicate the cleavage-sites for host signal peptidases, while open arrows show junctions processed by HCV proteases.

NS3 of HCV is a serine protease that interacts with its cofactor, viral protein NS4A, to gain proteolytic activity (Lin et al., 1995). In turn, the autoprotease activity of NS2 requires the presence of the first 180 N-terminal residues of NS3. Thus, the processing of the HCV polyprotein and replication of the virus depend on the complexes formed by virus-encoded proteases. The existence of numerous interactions between HCV non-structural proteins has been demonstrated using adenovirus vectors expressing HCV NS2-NS5B polyprotein (Dimitrova et al., 2003). At the same time, a pair-wise interaction of NS2 with NS3 has not been demonstrated. To demonstrate this bimolecular interaction, an epitope-tag technique was used. To confirm the suitability of the designed approach, a well characterized interaction between NS3 and NS4A was analyzed using the same system.

The expression of epitope-tagged NS2, NS3, and NS4A proteins from conventional plasmid-based vectors in the human hepatic cell line, Huh7, was deemed ineffective (IV, Fig. 1B). To overcome this problem, SFV replicon vectors expressing HCV NS2, NS3, or NS23 polyprotein were constructed and tested (IV, Fig. 1C).

Localization patterns of individually expressed HCV proteins in recombinant SFV infected Huh7 cells were found to be similar to those in transiently transfected Cos7 cells (IV, Fig. 2). NS2 was perinuclear and displayed a network-like localization pattern. NS3 was diffused throughout the cytoplasm and was, to some extent, also detected in the nucleus. NS4A exhibited a dotted distribution throughout the cytoplasm, but was most intensively localized to perinuclear regions. In Huh7 cells, either co-infected with SFV-3F12NS2 and SFV-NS3 or infected with SFV-3F12NS23, both proteins co-localized to the perinuclear area (IV, Fig. 4) and a similar localization was observed in transiently transfected cells (IV, Fig. 3).

An interaction between NS2 and NS3 was studied by co-immunoprecipitation (IP) under native conditions. The interaction between NS2 and NS3 in Huh7 cells co-infected with SFV-3F12-NS2 and SFV-NS3 or infected with SFV-3F12NS23 was confirmed by a reciprocal IP procedure: NS3 was found to co-precipitate with 3F12-tagged NS2 when using a 3F12 antibody (IV, Fig. 7A and B), and NS2 co-precipitated with NS3 when using an anti-NS3 antibody (IV, Fig. 7C and D).

Thus, it was demonstrated that NS2 and NS3 of HCV interact and that complex formation did not depend on whether NS2 and NS3 were expressed as individual proteins or in the form of the NS23 polyprotein precursor. SFV-based vectors were suitable to guarantee an expression of a target protein or proteins in different cell types and may be utilized as tools to overcome technical difficulties due to low levels of expression often observed for alternative systems.

SUMMARY

Studies of the properties of SFV nsP1 and its non-palmitoylated variants revealed that mutations in the nsP1 palmitoylation site significantly reduce the infectivity of SFV genomes or SFV-based replicons. During infection of cell cultures, positively selected viruses with compensatory mutations emerged. When introduced into the genomes of the original mutant viruses, these compensatory mutations rescued the infectivity of their carriers. The GT activities of recombinant nsP1s carrying mutations in their palmitoylation sites and the proteins harboring compensatory changes did not differ from that of wt nsP1. Therefore, possible defects in enzymatic functions of nsP1 were not the reason behind the attenuated phenotypes of genomes with the initial mutations. Instead, it was found that nsP1, carrying the initial mutation, had an altered subcellular localization and was not able to interact with the polymerase subunit of the alphavirus replicase complex. The introduction of respective compensatory mutations restored, at least in part, these properties.

Experiments described in this thesis revealed new information on the functions of nsP1 in virus replication. Expression of nsP1 or the expression of its non-palmitoylated version alone did not repress the metabolism of host cells or reduce their viability. Individual expression of nsP1 proteins did not interfere with SFV entry into the cells either. Nevertheless, *in trans* expression of nsP1 or its mutant form had negative effects on the translation of viral genomic and subgenomic RNAs, inhibited the viral RNA synthesis, and resulted in significant reduction in the titer of released virus.

In order to analyze the mechanism behind SFV induced apoptosis, a novel SFV-based bicistronic vector system co-expressing anti-apoptotic bcl-2 was developed. Although bcl-2 was expressed at high levels, its presence did not prolong the expression of a marker gene nor rescue the infected cells from virus-induced death. At the same time, these bicistronic vectors were found to be suitable for synchronic and transient expression of two genes of interest.

The subcellular localization of hepatitis C virus proteins NS2, NS3, and NS4A was analyzed using the SFV-based vectors. Co-expression of these proteins resulted in altered localization patterns. This effect was caused by a previously undescribed interaction between NS3 and NS2. In addition, the reliability of the system was demonstrated by detecting a well-known interaction between NS3 and NS4A of HCV.

SUMMARY IN ESTONIAN

Semliki Forest viirusel põhinevad vektorid ja rakuliinid alfa- ja hepatsiviiruste replikatsiooni ja interaktsioonide uurimisel

Semliki Forest viirus (SFV) kuulub perekonda *Alphavirus* sugukonnas *Togaviridae*. Alfaviirused on üheahelalise positiivse polaarsusega RNA genoomiga viirused. Genoomne RNA jaguneb kaheks piirkonnaks: 5'-poolne kaks kolmandikku kodeerib mittestruktuurseid valke, 3'-poolne kolmandik kodeerib struktuurseid valke. Kuna struktuursed valgud replikatsioonis ei osale, on vastav regioon alfaviirustel põhinevates ekspressioonivektorites asendatud uuritava geeniga. Lihtsa ülesehituse ja efektiivse paljunemise tõttu on alfaviirused olnud oluliseks töövahendiks erinevate viiruslike ning rakuliste protsesside kirjeldamisel; samas on alfaviirustel-põhinevad vektorid paljutõotavad geeni- või kasvajavastases teraapias ning biotehnoloogias.

Käesoleva töö eesmärkideks oli uurida SFV mittestruktuurse valgu nsP1 ning tema erinevate mittepalmitüleeritud vormide mõju viirusnakkusele ning peremeesrakule. Samuti uuriti võimalust takistada anti-apoptootilise valgu bcl-2 ekspressiooni abil SFV poolt põhjustatud apoptoosi ning kasutati SFV-l põhinevaid vektoreid C-hepatiidi viiruse proteaaside interaktsioonide kirjeldamiseks.

Uurimine näitas, et palmitüleerimist takistavate mutatsioonide sisseviimine nsP1 kodeerivasse alasse vähendas järsult viiruse paljunemise efektiivsust. Samas taastasid replikatsiooni käigus tekkinud ja selekteerunud kompenseeriva toimega mutatsioonid RNA-de nakkuslikkuse ja viiruse paljunemise efektiivsuse. Leiti, et mitte-palmitüleeritud mutantide funktsionaalset defekti ei põhjustanud mitte palmitüleerimise kui sellise puudumine, vaid hoopis nsP1 mutantsete vormide võimetus seonduda SFV polümeraasi katalüütilise subühikuga ning ka mutantsete valkude ebakorrektne rakusisene paiknemine. Kompensatoorsete mutatsioonide sisestamine taastas, vähemalt osaliselt, nsP1 esialgsed omadused.

Indutseeritavate rakuliinide abil uuriti nsP1 ja tema mitte-palmitüleeritud vormi ($nsP1^{6D}$) mõju peremeesrakule ja viirusnakkusele. Leiti, et eraldi ekspresseerituna ei avalda nsP1 ega tema mutantne vorm peremeesraku metabolismile ega elulemusele pärssivat mõju, samuti ei takistanud selliste valkude ekspressioon SFV sisenemist vastavatesse rakkudesse. Küll aga takistas nsP1 ja $nsP1^{6D}$ ekspressioon oluliselt SFV genoomse ja subgenoomse RNA translatsiooni, inhibeeris viiruse RNA sünteesi ning seega ka uute viirusosakeste moodustamist ja vabanemist rakust.

Alfaviirusnakkus ning isegi alfaviirusel põhineva vektori replikatsioon põhjustab peremeesraku apoptootilist surma. Mitmete uurimuste tulemused viitavad sellele, et SFV põhjustatud apoptoos algatatakse mitokondriaalse raja kaudu ning et seda on võimalik vältida või edasi lükata anti-apoptootilise valgu bcl-2 ekspresseerimise abil. Selle võimaluse uurimiseks konstrueeriti uudsed bitsistroonsed SFV replikon-vektorid, mille abil leiti, et vähemalt BHK-21

rakkudes ei takista bcl-2 ekspressioon SFV indutseeritud apoptoosi. Samas on konstrueeritud vektorid efektiivseteks töövahenditeks juhtudel kui on vaja transientselt ja sama-aegselt ekspresseerida kahte uuritavat valku.

SFV-vektorite abil näidati, et C-hepatiidi viiruse proteaasid NS2 ja NS3 moodustavad omavahel kompleksi. Samuti leiti, et kompleksi moodustumine ei sõltu peremeesraku tüübist ega sellest, kas vastavad valgud on ekspresseeritud paarikaupa või ühise eelvalgu kujul.

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