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Cellular Membranes as a Playground for Semliki Forest Virus Replication Complex

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ACADEMIC DISSERTATION

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Cover graphics: 3D representation of BHK cells infected with Semliki Forest virus for 8 h. Replication complexes (yellow) were visualized by staining the viral replicase protein nsP1 (red) and dsRNA intermediates (green). NsP1-positive projections induced during virus infection connect neighbouring cells and contain replication complexes.

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To my dearest husband, Juan. You are my life, my love…

Abstract

All positive-strand RNA viruses utilize cellular membranes for the assembly of their replication complexes, which results in extensive membrane modification in infected host cells. These alterations act as structural and functional scaffolds for RNA replication, providing protection for the viral double-stranded RNA against host defences. It is known that different positive-strand RNA viruses alter different cellular membranes. However, the origin of the targeted membranes, the mechanisms that direct replication proteins to specific membranes and the steps in the formation of the membrane bound replication complex are not completely understood.

Alphaviruses (including *Semliki Forest virus*, SFV), members of family *Togaviridae*, replicate their RNA in association with membranes derived from the endosomal and lysosomal compartment, inducing membrane invaginations called spherules. Spherule structures have been shown to be the specific sites for RNA synthesis. Four replication proteins, nsP1-nsP4, are translated as a polyprotein (P1234) which is processed autocatalytically and gives rise to a membrane-bound replication complex. Membrane binding is mediated via nsP1 which possesses an amphipathic α-helix (binding peptide) in the central region of the protein.

The aim of this thesis was to characterize the association of the SFV replication complex with cellular membranes and the modification of the membranes during virus infection. Therefore, it was necessary to set up the system for determining which viral components are needed for inducing the spherules. In addition, the targeting of the replication complex, the formation site of the spherules and their intracellular trafficking were studied in detail.

The results of current work demonstrate that mutations in the binding peptide region of nsP1 are lethal for virus replication and change the localization of the polyprotein precursor P123. The replication complex is first targeted to the plasma membrane where membrane invaginations, spherules, are induced. Using a specific regulated endocytosis event the spherules are internalized from the plasma membrane in neutral carrier vesicles and transported via an actin-and microtubule-dependent manner to the pericentriolar area. Homotypic fusions and fusions with pre-existing acidic organelles lead to the maturation of previously described cytopathic vacuoles with hundreds of spherules on their limiting membranes.

This work provides new insights into the membrane binding mechanism of SFV replication complex and its role in the virus life cycle. Development of plasmid-driven system for studying the formation of the replication complex described in this thesis allows various applications to address different steps in SFV life cycle and virus-host interactions in the future. This *trans*-replication system could be applied for many different viruses. In addition, the current work brings up new aspects of membranes and cellular components involved in SFV replication leading to further understanding in the formation and dynamics of the membrane-associated replication complex.

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Pirjo Spuul
Helsinki, 2010

Contents

List of original publications

This thesis is based on the following publications, which are referred to in the text by their roman numerals:

I *Role of the amphipathic peptide of Semliki Forest virus replicase protein nsP1 in membrane association and virus replication* (2007); **Spuul P**., Salonen A., Merits A., Jokitalo E., Kääriäinen L., Ahola T.; J. Virol. 81(2):872-883

II *Assembly of alphavirus replication complexes from RNA and protein components in a novel trans-replicase system in mammalian cells*; **Spuul P**.*, Balistreri G.*, Hellström K., Golubtsov A., Jokitalo E., Ahola T.; manuscript.

III *Phosphatidylinositol 3-kinase, actin- and microtubule-dependent transport of alphavirus replication complexes from the plasma membrane to modified lysosomes* (2010); **Spuul P**.*, Balistreri G.*, Kääriäinen L. and Ahola T.; J. Virol. 84(15):7543-7557. Supplementary material is available at http://jvi.asm.org/cgi/content/full/84/15/7543/DC1

*equal contribution

In addition, some unpublished data is presented.

Abbreviations

aa	amino acid
BAR	Bin-amphiphysin-Rvs
BMV	Brome mosaic virus
BP	binding peptide
C	capsid
CHKV	Chikungunya virus
CL	cardiolipin
CLEM	correlative light and electron microscopy
CM	convoluted membranes
conA	concanavalin A
COP	coat protein
CPE	cytopathic effect
CPV	cytopathic vacuole
CSE	conserved sequence element
DMV	double-membrane vesicle
EEEV	Eastern equine encephalitis virus
EMCV	Encephalomyocarditis virus
ESCRT	endosomal sorting complex required for transport
FHV	Flock house virus
GFP	green fluorescent protein
GSL	glycosphingolipid
GT	guanylyltransferase
HCV	Hepatitis C virus
icDNA	infectious cDNA
LBPA	lyso-bis-phosphadic acid
MD	macro domain
MOI	multiplicity of infection
MT	methyltransferase
MVA	Modified Vaccinia Ankara
MVB	multivesicular body
NC	nucleocapsid
NMR	nuclear magnetic resonance
ns	non-structural
nt	nucleotide
NTR	nontranslated region
p.i.	post infection
p.t.	post transfection
Pa^-	non-palmitoylated
PA	phophatidic acid
PAK	p21-activated kinase
PC	phosphatidylcholine
PE	phosphatidylethanolamine

PFU	plaque forming unit
PG	phosphatidylglycerol
PI	phosphatidylinositol
PI3K	phosphatidylinositol 3-kinase
PM	plasma membrane
PS	phosphatidylserine
RC	replication complex
RdRp	RNA-dependent RNA polymerase
SFV	Semliki Forest virus
SINV	Sindbis virus
SM	sphingomyelin
TBSV	Tomato bushy stunt virus
TMV	Tobacco mosaic virus
ts	temperature-sensitive
VEEV	Venezuelan equine encephalitis virus
VP	vesicle packets
VRP	virus-like replicon particle
WEEV	Western equine encephalitis virus
WNV	West Nile virus
wt	wild type

1 Introduction

Membrane-associated replication has been shown to be a common feature for all positive-strand RNA viruses, although the nature and the functions of this association are still not completely understood. The current thesis is dedicated to dissecting the interplay between cellular membranes and the viral replication complex (RC) using Semliki Forest virus (SFV), an alphavirus, as an example. The literature review will first introduce the diverse world of positive-strand RNA viruses and then give a more detailed overview of alphaviruses, their virion structure, genome organization, life cycle and RC. Membrane association mechanism of the alphavirus RC and subsequent alteration of cellular membranes receives special attention. As alphaviruses induce many changes in infected cells, aspects of virus-host interactions relevant for this thesis will be introduced. A comparison of alphaviruses with other positive-strand RNA viruses will present the important issues related to membrane-associated replication.

1.1 Diversity of positive-strand RNA viruses

Positive-strand RNA viruses are the largest class of viruses, and include many important pathogens of humans and animals (Table 1). In addition, most plant viruses contain plus-strand RNA as their genome (Table 1) (Strauss and Strauss, 2008). According to RNA-dependent RNA polymerase (RdRp) sequence, positive-strand RNA viruses are divided into three major groups – picornavirus-like, flavivirus-like and alphavirus-like superfamilies (Koonin and Dolja, 1993). The hallmarks discriminating between each of these groups are the common features in their replicase proteins, as well as the arrangement of the genes encoding these proteins in the virus genome. However, there are significant differences within one superfamily concerning the virion components (icosahedral versus helical and enveloped versus nonenveloped), genome expression strategies and host range (bacteria, fungi, plants, insects and animals). Furthermore, the replicase proteins often show rearrangements and acquisition and deletion of domains. This is due to the high mutation rate and high frequency of recombination events typical for plus RNA viruses. These special features together with high yields and short replication times ensure RNA virus survival and adaptation. The error rate, caused by RdRp that lacks proofreading activity, is 10^{-3} to 10^{-5} substitutions per nucleotide site and per round of copying, leading to 0.1 to 10 mutations in average for a progeny of a 10 kb genome (Domingo et al., 1997). The low fidelity of the RdRp is directly linked to the relatively small genome size of plus-strand RNA viruses. There is variation in genome size and complexity ranging from segmented 4.5 kb in the case of Flock house virus (FHV) up to 32 kb in nonsegmented coronavirus genomes. However, the average genome size among plus-strand RNA viruses is around 10 kb. It has been speculated that increasing genome size above 15 kb is a challenge for RNA viruses and requires

additional mechanisms to maintain a balance between fast adaptation and genome stability (Gorbalenya et al., 2006).

Table 1. *Families of plus-strand RNA viruses containing important human pathogens (shown in bold) and well studied model viruses (Adapted from Strauss and Strauss, 2008).*

Family	Size of genome (kb)	Representative pathogens	Hosts for the family
Nodaviridae	~4,5	FHV	Insects
Tombusviridae	~4,8	TBSV	Plants
Picornaviridae	~7,5	**Poliovirus, Echovirus, Rhinoviruses, HAV, FMDV, EMCV**	**Humans**, cattle, primates, mice
Caliciviridae	~7,5	**Norwalk**	**Humans**, rabbits, swine, cats
Hepeviridae	7,2	**HEV**	**Humans**, primates, swine
Astroviridae	6,8-7,9	**Human astrovirus**	**Humans**, cattle, ducks, sheep, swine
Bromoviridae	~8,2	BMV	Plants
Togaviridae	~11,6	SFV, **CHKV, SINV, WEEV, VEEV, EEEV, rubella**	**Humans**, mammals, birds, horses, mosquitoes
Flaviviridae	9,5-12,5	**Dengue, YFV, WNV, BVDV, HCV**	**Humans**, swine, cattle, primaes, birds, mosquitoes
Coronaviridae	20-30	**SARS-CoV**, MHV	**Humans**, mice, birds, swine, cattle, bats

Abbreviations: FHV, Flock house virus; TBSV, Tomato bushy stunt virus, HAV, Hepatitis A virus; FMDV, Foot and mouth disease virus; EMCV, Encephalomyocarditis virus; HEV, Hepatitis E virus; BMV, Brome mosaic virus; SFV, Semliki Forest virus; CHKV, Chikungunya virus; SINV, Sindbis virus; WEEV, Western equine encephalitis virus; VEEV, Venezuelan equine encephalitis virus; EEEV, Eastern equine encephalitis virus; YFV, Yellow fever virus; WNV, West Nile virus; BVDV, Bovine Diarrhoea virus; HCV, Hepatitis C virus; SARS-Cov, Severe acute respiratory syndrome coronavirus; MHV, Mouse hepatitis virus.

Positive-strand RNA viruses have messenger RNAs as their genomes, which are directly translated into replicase polyproteins in the cytoplasm of the infected cell. These polyproteins are cleaved co-and post-translationally by viral and host cell proteases into replicase proteins. After the recruitment of viral genomic RNA, replicase proteins are targeted to cellular membranes giving rise to membrane-associated RCs. Subsequent alteration of cellular membranes seems to be one of the most striking common features among plus-strand RNA viruses (Salonen et al., 2005; Denison, 2008; Miller and Krijnse-Locker, 2008). RNA replication occurs via a negative-strand RNA template that can remain attached to the positive-strand thus creating a dsRNA replicative intermediate. The presence of dsRNA is a hallmark for positive-strand RNA virus replication (Weber et al., 2006).

1.2 Alphaviruses

Alphaviruses are members of family Togaviridae, which includes additionally the genus Rubivirus. This well-studied family of viruses belongs to the alphavirus-superfamily that also contains Hepatitis E virus as well as several plant and insect viruses (e.g. Brome mosaic virus, BMV and Tobacco mosaic virus, TMV) (Koonin and Dolja, 1993). A characteristic feature of this superfamily is the presence of three conserved domains, always in the order methyltransferase-helicase-polymerase. In addition, all these viruses have a cap0 structure (m^7GpppN) at the 5´end and a poly(A) tract or a tRNA-like structure at the 3´end of the plus-strand RNA (Koonin and Dolja, 1993).

The genus alphavirus has more than 40 members (Powers et al., 2001). Geographically, they are very widely spread, having been isolated from all the continents except Antarctica. Based on virus distribution, alphaviruses are divided into two groups: New World viruses (e.g. Eastern (EEEV), Western (WEEV), and Venezuelan Equine Encephalitis (VEEV) viruses) which can cause encephalitis in humans and domestic animals, whereas infections with Old World viruses (SFV and Sindbis virus, SINV as prototypes) mostly lead to fever, rash and arthralgia (Strauss and Strauss, 1994).

Most alphaviruses are arthropod-borne (arboviruses) being maintained in natural cycles by transmission between susceptible vectors and vertebrate hosts (Strauss and Strauss, 1994). In arthropod vectors (mainly mosquitoes) alphaviruses cause a persistent, lifelong infection with minimal cytopathic effects. However, in vertebrates, alphavirus infection is acute causing high-titre viremia and specific symptoms until the death of the infected host or clearance of the virus by the immune system. In addition, there are alphaviruses known to infect fish, Salmonid Alphaviruses, causing serious damage in salmon and rainbow trout stocks in Europe; no arthropod vector is known for these viruses. Despite the obvious importance of alphaviruses as pathogens, there are currently no effective antiviral drugs or vaccines available.

Two of the most extensively studied members of alphaviruses are SFV and SINV. These viruses have served as models to study different steps in the alphavirus life cycle and pathogenesis as they replicate efficiently and to high titres in a broad range of hosts and cultured cells. Recently, re-emerging Chikungunya virus (CHKV), a member of SF complex, has received a lot of attention because of its increasing global spread. CHKV has infected millions of people in areas around the Indian Ocean causing rash and severe joint pain and having a mortality rate of 0.5% (Schuffenecker et al., 2006).

The SFV prototype was isolated in 1942 from a pool of mosquitoes (*Aedes abnormalis*) in Semliki Forest in Uganda. The original isolate L10 is neurovirulent for mice and causes lethal encephalitis (Fazakerley, 2002). SFV can also infect humans causing fever, severe persistent headache, myalgia (muscle pain) and arthralgia (joint pain) (Mathiot et al., 1990). The original infectious clone of SFV (also used in the current work), derived from L10 after multiple passages, is designated pSP6-SFV4 and the virus produced by transcription of this infectious clone is labelled SFV4 (Liljeström and Garoff, 1991).

1.2.1 Structure and genome organization

The spherical virions of alphaviruses are approximately 70 nm in diameter and surrounded by a host-derived lipid envelope. Viral glycoproteins E1 and E2 form heterodimers that are assembled as trimers into spikes. Latter are embedded into the lipid bilayer forming an icosahedral lattice with triangulation number of four. Underneath the envelope, icosahedral nucleocapsid (NC) is composed of the capsid protein arranged as hexamers and pentamers in a T=4 lattice and encloses one copy of a single-stranded positive-sense genomic RNA of 11.5 kb (Jose et al., 2009).

The genomic 42S RNA contains two open reading frames which encode nine functional proteins via polyproteins precursors. The 5´ two-thirds of the genome encodes the non-structural polyprotein P1234, while the structural polyprotein is produced via an internal promoter and subgenomic 26S RNA corresponding to the last third of the genome. Both 42S and 26S RNAs are capped and polyadenylated (Strauss and Strauss, 1994) (Figure 1).

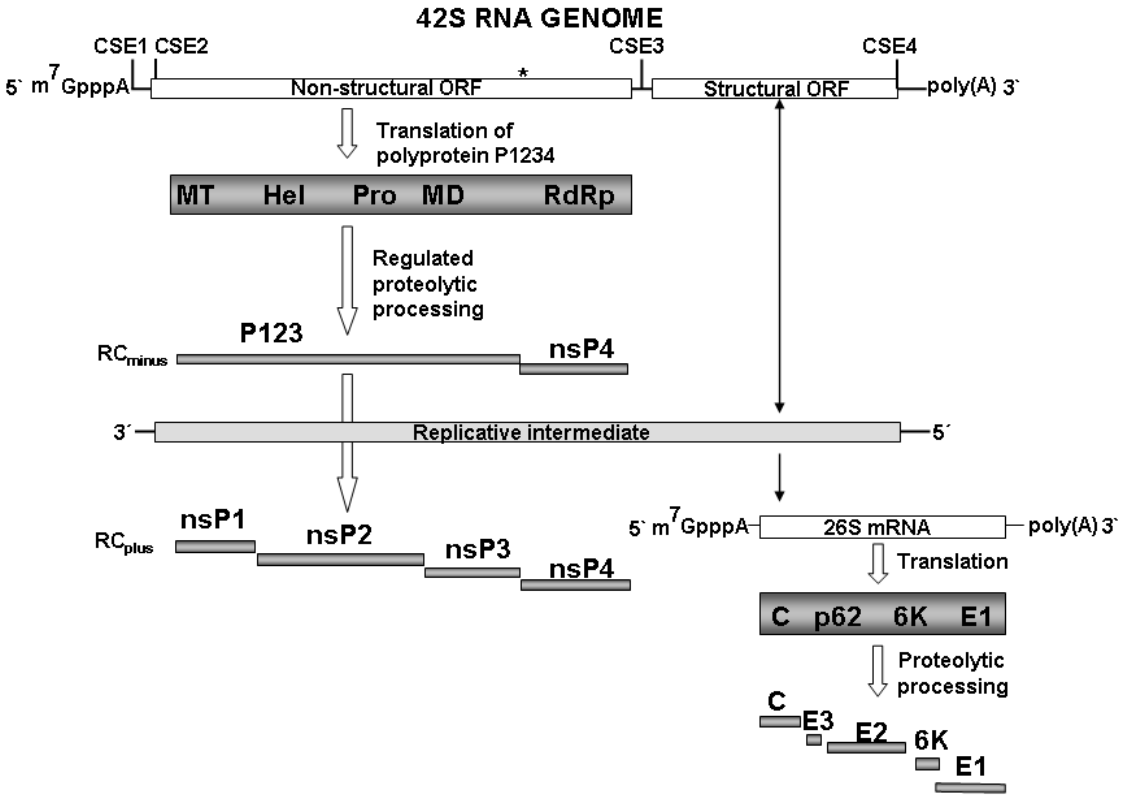


Figure 1 *Genome organization and expression strategy of alphaviruses (adapted from Strauss and Strauss, 1994). CSE, conserved sequence element; MT, methyltransferase;Hel, helicase; Pro, protease; MD, macro domain; RdRp, RNA-dependent RNA polymerase; C, capsid; E, envelope glycoprotein*

There are important structural elements including four conserved sequence elements (CSE) in the genomic RNA. These elements have been shown to facilitate

replication, transcription and packaging of viral RNAs by interacting with both viral and host proteins. The 5´ nontranslated region (NTR) regulates both minus- and plus-strand RNA synthesis. Approximately 44 first nucleotides (nt) at the 5´end the genome form a stem-loop structure (CSE 1) which has been implicated as a promoter for plus-strand RNA synthesis acting in the context of minus-strand (Ou et al., 1983). There is also a second element at the 5´end (CSE 2) located inside of the coding region of nsP1. This 51 nt element enhances both minus- and plus-strand synthesis and promotes the initiation of minus-strand synthesis (Frolov et al., 2001). The latter process is also assisted by the other element at the 3´ NTR of the genome (CSE 4). This 19 nt CSE is located just upstream of the poly(A) tract and is believed to interact with the 5´end of the genomic RNA (Kuhn et al., 1990; Hardy, 2006). In addition, there are 40-60 nt repeated sequence elements upstream of poly(A) believed to facilitate translation of viral RNAs by binding host factors (Ou et al., 1982). The 24 nt subgenomic promoter element (CSE 3), located in the junction between the regions coding for non-structural and structural proteins, is essential for transcription of the subgenomic RNA (Levis et al., 1990) (Figure 1). The packaging signal in alphavirus RNAs is located in the 5´ half of the genome but its location varies between species (Weiss et al., 1989; Weiss et al., 1994; Frolova et al., 1997).

1.2.2 Life cycle

Alphaviruses enter susceptible host cells by clathrin-mediated endocytosis (Helenius et al., 1980). As described above, alphaviruses are able to infect many cell types, including vertebrate and insect cells. Therefore, it is believed that alphaviruses use either conserved receptors expressed on many cells or multiple receptors. Laminin receptor and glycosaminoglycans have been shown to mediate the binding and entry of SINV (Lee et al., 2002; Wang et al., 1992). However, some cell-type-specific receptors have also been described; e.g C-type lectins (DS-SIGN, dendritic cell specific ICAM-3 grabbing non-integrin) seem to serve as a receptor for SINV entry into dendritic cells (Klimstra et al., 2003). Currently, no specific receptor for SFV has been identified.

After rapid internalization, mildly acidic pH (less than 6.2) in virus-containing endosomes triggers a series of conformational changes in the envelope proteins, which in turn result in the membrane fusion reaction between the viral envelope and the endosomal membranes (Gibbons et al., 2004; Kielian and Helenius, 1985). Fusion allows the release of the NC to the cytoplasm which is rapidly uncoated by the 60S ribosomal subunit –reviewed in (Wengler, 2009)–. Capped and polyadenylated genomic 42S plus-sense RNA is directly used as mRNA to translate the non-structural polyprotein P1234 (Kääriäinen et al., 1987). In the case of some alphaviruses (e.g. SINV), the primary polyprotein product is P123, as there is an opal termination codon after the nsP3 coding sequence. A translational read-through, occurring with 10-20% efficiency, results in the synthesis of P1234. The polyproteins form a membrane-bound RC and highly regulated processing of the polyprotein into four mature non-

structural proteins nsP1-4 (Figure 1) serves as a basic regulation mechanism for viral RNA synthesis (Kääriäinen et al., 1987; Merits et al., 2001; Vasiljeva et al., 2001) (see below). Therefore, both cleavage intermediates and fully cleaved ns proteins have important roles in virus replication. The processing is mediated exclusively by a specific protease activity located in nsP2 (Merits et al., 2001).

RNA synthesis is initiated by copying plus-sense 42S RNA into a genome length minus-strand. This process requires an early short-lived replicase complex composed of P123 and nsP4 (RC_{minus}) that is generated by *in cis* cleavage. RC_{minus} is effective in minus-strand synthesis but does not efficiently synthesise plus-strand RNAs (Lemm et al., 1994). Therefore, minus-strand synthesis occurs early in infection as the concentration of P123 is the highest and declines accordingly with the processing of P123. The latter processing converts the RC_{minus} into RC_{plus}, which is composed of individual ns proteins and is no longer capable of using 42S plus-strand RNA as a template. This stable late replicase uses minus-strand as a template and is responsible for the synthesis of 42S genomic RNA and 26S subgenomic RNA. 42S genomic RNA serves as a template for new rounds of RNA replication and is also recruited into the assembly of NCs, whereas 26S RNA is used to produce structural proteins –reviewed in (Strauss and Strauss, 1994; Kääriäinen and Ahola, 2002)–.

Ns proteins which fail to assemble properly and do not recruit the template are dissociated from each other and distributed to specific locations: nsP1 is targeted to the plasma membrane (PM) (Peränen et al., 1995), nsP2 is transported to the nucleus (Peränen et al., 1990), nsP3 forms aggregates in the cytoplasm (Salonen et al., 2003) and nsP4 is rapidly degraded by the ubiquitin pathway (Takkinen et al., 1991). The role of ns proteins not participating in the RCs is mostly associated with modifying host cell functions and is briefly described in a separate chapter (virus-host interactions).

Structural proteins are translated from 26S subgenomic RNA as a polyprotein (C-pE2-6K-E1) which is processed co-translationally (Figure 1). Capsid protein (C) is translated first and its autocatalytic cleavage releases the signal peptide at the N-terminus of pE2, which inserts the polyprotein into the ER. Further processing is carried out by host signal peptidases (Melancon and Garoff, 1987). In the lumen of ER, pE2 and E1 are processed, undergo post-translational modifications and their heterodimerization is needed for their transport to the cell surface via the Golgi complex. During the transport of heterodimers, pE2 is cleaved into E3 and E2 by furin which enables the induction of membrane fusion at low pH. Therefore, the cleavage of pE2 assures the maturation of infectious particles -reviewed in (Jose et al., 2009)-.

NC assembly is triggered by the recognition of an encapsidation signal in genomic RNA by 68 aa stretch of C followed by interactions of C and enclosure of the RNA (Weiss et al., 1994). NC interaction with glycoproteins at the PM initiates the budding of the virus (Suomalainen et al., 1992; Strauss et al., 1995) which occurs in cholesterol rich membrane rafts (Lu et al., 1999).

1.2.3 Replication complex

The RC of alphaviruses is composed of four viral ns proteins, replicating RNA and cellular membranes. Host factors may also be part of the complex but their identity and role remain poorly understood. Each ns protein is essential for virus replication (Strauss and Strauss, 1994). Their interactions with each other have been demonstrated by co-immunoprecipitation and they colocalize in the same vesicles as shown by immunostaining (Froshauer et al., 1988; Peränen and Kääriäinen, 1991; Barton et al., 1991; Salonen et al., 2003). The formation of the membrane-bound RC requires the polyprotein P123 stage as expression of individual nsPs does not result in the assembly of a functional complex (Salonen et al., 2003). It is believed that interactions between nsPs in the polyprotein facilitate formation of the membrane-associated replication complex.

Even though the specific functions of ns proteins have been described, the full understanding of the membrane-bound RC as a machinery with functional domains remains elusive. Here, I will summarize the major enzymatic activities and conserved domains associated with the RC and then describe more in detail the membrane binding mechanism of the RC and its interactions with cellular membranes.

The RdRp activity of the RC is located in the C-terminal part of nsP4 which possesses a conserved GDD motif characteristic for polymerases (Koonin and Dolja, 1993). Experiments with temperature-sensitive (ts) mutants have proven the role of nsP4 as the catalytic subunit of the complex (Keränen and Kääriäinen, 1979; Hahn et al., 1989). In vitro studies with the polymerase domain of nsP4 demonstrated the presence of terminal adenylyltransferase activity catalyzing the addition of adenine to the 3´end of RNA (Tomar et al., 2006). This might have a role in the maintenance of the viral poly(A) tract. In addition, nsP4 has been implicated to regulate the minus-strand synthesis shut-off (Sawicki et al., 1990). The N-terminus of nsP4 that is not conserved among other viral RdRps has been proposed to interact with the other ns proteins and host factors. Interaction with nsP1 is believed to be important in the formation of the RC (Shirako et al., 2000; Fata et al., 2002). NsP4 is short-lived in the cytoplasm of infected cells as it possesses an N-teminal tyrosine residue that leads to rapid degradation by the N-end rule pathway (De Groot et al., 1991). Replacement of that conserved residue results in inefficient RNA replication (Shirako and Strauss, 1998). Probably the only stable fraction of nsP4 is associated with RCs.

Macro domain (MD), a conserved ADP-ribose binding module (Karras et al., 2005), is the N-terminal part of nsP3. MD, named after its presence in the non-histone region of the histone macroH2A, is highly conserved among alphaviruses and shares sequence similarities with MDs present in other positive-strand RNA viruses, bacteria, archae and eukaryotes. (Pehrson and Fuji, 1998). The MDs of alphaviruses have been shown to bind poly(ADP-ribose) and poly(A) (Neuvonen and Ahola, 2009). Crystal structures of CHKV and VEEV macro domains revealed that these domains possess ADP-ribose binding modules and RNA-binding activity (Malet et al., 2009). In addition, mutations in the ADP-ribose binding site affect virus replication and age-dependent susceptibility to encephalomyelitis (Park and Griffin, 2009a). Studies with ts and insertion mutants have revealed that the nsP3 is involved

in minus-strand and subgenomic RNA synthesis (LaStarza et al., 1994; Wang et al., 1994).

The C-terminus of nsP3 is not conserved among alphaviruses and varies in length and sequence. However, this region is highly phosphorylated and seems to play a role in virus replication and pathogenecity (Vihinen and Saarinen, 2000; Vihinen et al., 2001). Apart from phosphorylation, the C-terminus of nsP3 has been shown to interact with poly(ADP-ribose) polymerase 1 that might play a role in virus replication in neuronal cells (Park and Griffin, 2009b).

The protease**,** which is located in the C-terminal portion of nsP2, is homologous to papain-like cysteine proteinases. The protease has a central role in the timing of minus- and plus-strand RNA synthesis, as already described in the previous section (Vasiljeva et al., 2003). It is the only protease needed for the processing of SFV polyprotein P1234 (Merits et al., 2001).

Helicase domain resides in the N-terminus of nsP2 (Koonin and Dolja, 1993) and possesses helicase activity needed for RNA duplex unwinding (Gomez et al., 1999). In addition, this domain mediates the NTP-binding activity and ability to hydrolyse ATP and GTP (Rikkonen et al., 1994).

As already mentioned above, the genomic and subgenomic RNAs of alphaviruses have a m^7GpppA (cap0) structure at their 5´ends. The RNA capping reaction has been shown to occur simultaneously with the synthesis of plus-strand RNAs in the cytoplasm of infected cells and by mechanism distinct from cellular nuclear capping (Cross and Gomatos, 1981; Cross, 1983). Already in these studies, it was shown that an ns polyprotein in a membrane fraction mediates the reaction (Cross, 1983). Later studies have revealed a fine-tuned interplay between two replicase proteins where nsP1 is a guanine-7-methyltransferase (MT) (Mi and Stollar, 1991; Laakkonen et al., 1994) and a guanylyltransferase (GT) (Ahola and Kääriäinen, 1995) whereas the N-terminus of nsP2 possesses RNA triphosphatase activity, catalysing the removal of the 5´γ-phosphate from the viral RNA before the addition of 7-methyl-GMP (m^7GMP) (Vasiljeva et al., 2000).

The MT activity of nsP1 was first demonstrated with nsP1 expressed in insect cells and E.coli (Mi and Stollar, 1991; Laakkonen et al., 1994). In these studies it was shown that nsP1 associated with membranes was able to transfer [^{3}H] methyl groups from labelled S-adenosylmethionine (SAM) to GTP and dGTP, substrates that cannot be used as acceptors by cellular capping enzymes. Another distinct feature appearing from these experiments was the finding that nsP1 cannot methylate capped RNAs or the cap analog GpppA. This was further characterized by Ahola and Kääriäinen (Ahola and Kääriäinen, 1995), showing that nsP1 possesses also a GT activity, by forming a covalent nsP1-m^7GMP complex. Methylation of guanine was an absolute requirement for the complex formation demonstrating a unique property of the alphavirus capping enzyme. Altogether these experiments showed a new cap formation mechanism in which methylation of the guanosine occurs before the transfer to the 5´end of the RNA.

Capping is an essential function for the virus, since defective mutants have been shown to be non-infectious. Work with SINV demonstrated that aa H39, R91, D94

and Y249 of nsP1 constitute a methyltransferase motif, as their mutation to Ala resulted in the loss of both MT-activity and viral infectivity (Wang et al., 1996). Corresponding residues in SFV are H38, D90, R93 and Y249 and moreover, these four aa are highly conserved among the entire alphavirus-like superfamily (Figure 2A) (Rozanov et al., 1992). Mutations in SFV showed that H38 did not affect MT activity but abolished GT activity, whereas the other three residues were crucial for MT activity (Ahola et al., 1997).

In addition, nsP1 is involved in negative-strand synthesis. Studies with SINV nsP1 indicate that a conserved C-terminal domain, containing residues A348 and T349, interacts with the N-terminus of nsP4. This interaction is a prerequisite for the recognition of the promoter for minus-strand synthesis (Shirako et al., 2000; Wang et al., 1991). In addition, N374 of nsP1, which is also located in the conserved C-terminal domain, interacts with nsP4 (Figure 2A) (Fata et al., 2002).

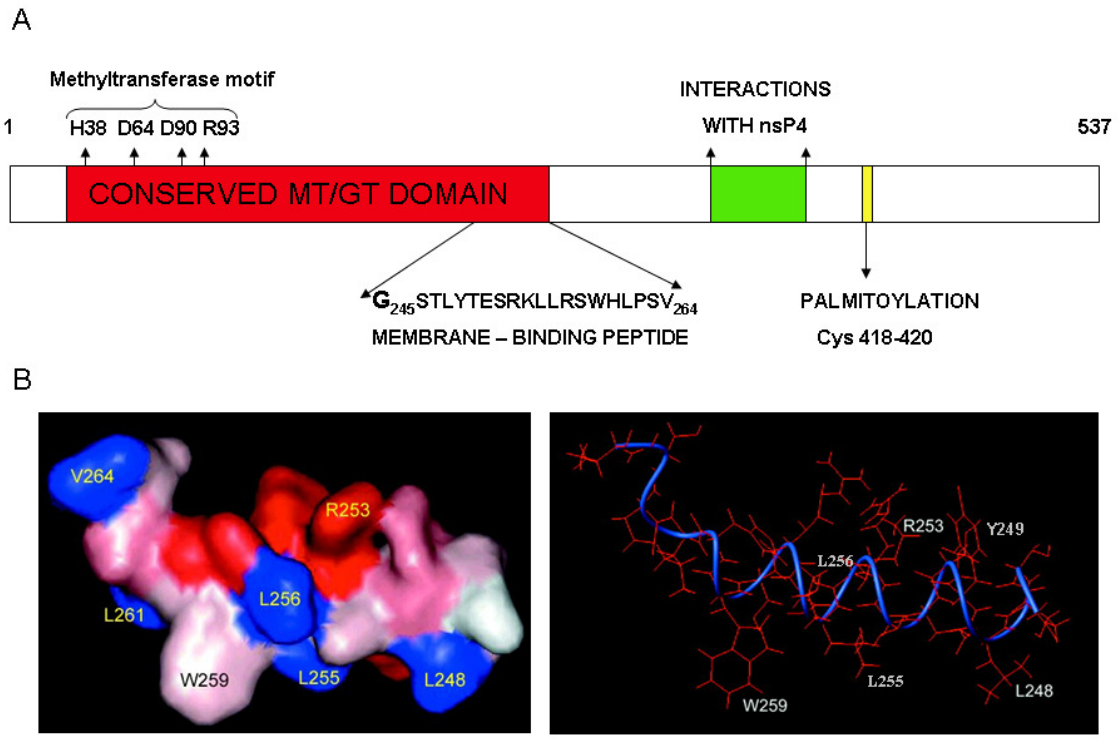


Figure 2 *A) Schematic view of SFV nsP1. Regions necessary for the membrane binding and enzymatic activities are shown. Numbers indicate the corresponding aa residues in SFV nsP1. b) Solution structure of the nsP1 binding peptide. In the left image, aa in the α-helix are color-coded according to their hydrophobicity (blue represents hydrophobic and red hydrophilic residues). Right image illustrates the energetically most preferred conformation of the peptide (adapted from Lampio, 2000).*

1.2.3.1 Association of RC with cellular membranes

As mentioned above, the alphavirus RC is membrane-associated and its activities are mainly located in a membrane fraction pelleting at 15,000 x g (P15-fraction) (Ranki and Kääriäinen, 1979; Peränen et al., 1988; Barton et al., 1991). In infected cells, all ns proteins are found in the membrane fraction but only nsP1, also if expressed alone, is exclusively associated with the membranes and therefore serves as the anchor for the whole complex (Peränen et al., 1988; Peränen et al., 1995). Association of nsP1 with smooth membrane fractions has been demonstrated with flotation experiments and it has been shown that nsP1 becomes membrane-associated very soon after its synthesis. NsP1 behaves like an integral membrane protein as its membrane binding is resistant to 1 M KCl, 2 M NaCl and up to 50 mM Na_2CO_3 (pH 12) but it can be disrupted with detergents like Triton-X100. However, a treatment with 15 mM NaOH disrupts the membrane association of nsP1 (Peränen et al., 1995). Immunofluorescence analysis of infected cells and Hela cells transfected with nsP1 demonstrated that nsP1 is located at the cytoplasmic surface of the PM and appears also on the membranes of large vacuoles in infected cells (Peränen et al., 1995; Salonen et al., 2003).

The membrane binding of nsP1 is a complex process and seems to comprise a two-step mechanism. First, nsP1 becomes S-acylated (palmitoylated) soon after its synthesis and this lipid modification occurs during the infection as well as when nsP1 is expressed in HeLa and insect cells (Laakkonen et al., 1996; Peränen et al., 1995). Palmitate is attached to nsP1 via thioester bond to cysteine residues: aa 418-420 in SFV nsP1 and aa 420 in SINV nsP1. Disruption of palmitoylation by mutating the cysteines to alanines changes the properties of the protein but does not abolish the membrane binding as 35-40 % of the protein still floats with membranes. However, non-palmitoylated (Pa^-) nsP1 behaves like a peripheral membrane protein being released from the membranes already with 1 M NaCl. In addition, Pa^- nsP1 possesses around 40 % of its enzymatic activities (Laakkonen et al., 1996). This suggests that palmitoylation tightens the membrane binding of the complex but is not the primary binding mediator. Nevertheless, mutations or deletions in palmitoylation sites are lethal for virus replication and compensatory mutations in nsP1 are needed to rescue the virus. Interestingly, these mutations are not restoring the palmitoylation demonstrating that palmitoylation itself is not needed for virus replication (Zusinaite et al., 2007). However, Pa^- viruses have been shown to be non-pathogenic in mice (Ahola et al., 2000). Therefore, the complete functional significance of the palmitoylation remains unknown.

nsP1 is very sensitive to deletions and only minor excisions are tolerated at the C-terminus without affecting the protein localization and enzymatic activities. However, with deletion analysis it was established that the first 270 aa are required for the protein to co-fractionate with membranes (Laakkonen et al., 1996). With more precise mutational analysis, the region mediating the association of nsP1 to membranes was mapped to a short binding peptide (BP, aa 245-264 in SFV nsP1) in the middle of the protein. This region was shown to be responsible for the binding of in vitro translated

nsP1 to liposomes as well as nsP1 expressed in *E.coli* to bacterial membranes. These two systems were utilized to study the binding mechanism in detail as in both cases nsP1 is not palmitoylated which allows to address the direct role of the BP (Ahola et al., 1999). These experiments demonstrated that nsP1 has affinity for negatively charged phospholipids, especially phosphatidylserine (PS), as in vitro-translated nsP1 bound preferentially to liposomes containing these lipids. In addition, the enzymatic activities of nsP1 were shown to be dependent on membrane binding. Detergent-abolished functions of nsP1 expressed in *E. coli* could be restored by providing phospholipids, especially PS as well as phosphatidylglycerol (PG) and cardiolipin (CL). However, they cannot be restored by neutral phospholipids like phosphatidylcholine (PC) and phosphatidylethanolamine (PE) or anionic glycolipids like gangliosides GD1a and GM1 (Ahola et al., 1999). These results support the idea that nsP1 and therefore the whole RC is adapted to function in a membranous environment. Moreover, it is interesting to note that PS is enriched in the cytoplasmic leaflet of eukaryotic PM and, as mentioned before, it is the compartment where nsP1 is targeted.

Synthetic peptides corresponding to the residues 245-264 in nsP1 (SFV) have been used to determine the structure of the BP and analyse critical residues involved in the binding process. These peptides have been shown to be able to compete with the binding of nsP1 to liposomes indicating that their structure and function is representative of the BP of nsP1 (Ahola et al., 1999). NMR experiments showed that in 30 % trifluoroethanol BP forms an amphipathic α-helix which has a hydrophobic surface composed of leucines, a valine and a tryptophane whereas polar residues, mostly positively charged lysine and arginines and negatively charged glutamate form the other face of the helix (Figure 2B) (Lampio et al., 2000). Positively charged R253 interacts with negatively charged membranes (Lampio et al., 2000) and its substitution by glutamate has a drastic effect to binding (Ahola et al., 1999). Mutations of the hydrophobic residues Y249, L255, L256 and W259 to alanines affect the binding as well. In addition, all these mutations have a severe effect on the MT activity of nsP1 (Ahola et al., 1999). W259 is particularly important in binding as it intercalates within the lipid bilayer to the depth of the ninth and tenth carbons of lipid acyl chains. W259A mutation also destroyed the ability of the synthetic peptide to compete with the binding of nsP1 to liposomes (Lampio et al., 2000). Importantly, the BP region is highly conserved among alphaviruses, especially the residues Y249, R253, L256 and W259 (numbering according to SFV nsP1) (Rozanov et al., 1992).

1.2.3.2 Alteration of cellular membranes – formation of spherules and CPV-Is

Cellular membranes involved in alphavirus replication were first described already in the late 60´s and early 70´s (Friedman and Berezesky, 1967; Friedman et al., 1972; Grimley et al., 1968). In these studies, it was demonstrated that the membranes of unique vacuolar structures termed cytopathic vacuoles type I (CPV-I) serve as the replication platform for alphaviruses and that virus replication induces bulb-shaped 50

nm membrane invaginations termed spherules on the limiting membranes of CPV-Is (Figure 3).

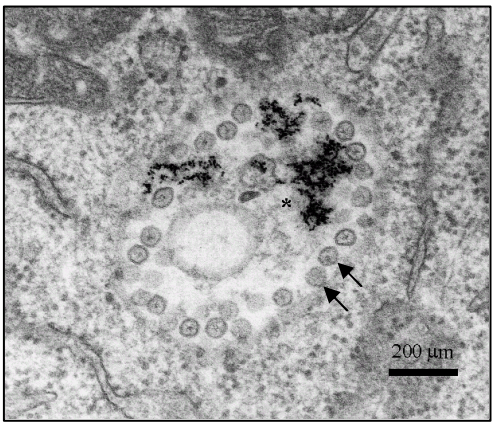


Figure 3 *Immuno-EM image of CPV-I and its invaginations, spherules (indicated with arrows), which are the sites for RNA synthesis. Labelled BSA-gold, used as a marker for endocytosis, is marked with an asterisk (Kujala et al., 2001).*

CPV-Is are modified endosomes and lysosomes (Froshauer et al., 1988; Kujala et al., 2001) with a diameter of 0.6 – 2 µm (Grimley et al., 1968) and they appear early in infection (2-3 h p.i) although this is dependent on virus multiplicity of infection (MOI) (Froshauer et al., 1988; Peränen and Kääriäinen, 1991). Their limiting membrane can be labelled with [^{3}H]-uridine, indicating that these membranes are the specific sites for viral RNA synthesis (Grimley et al., 1968). Regularly aligned spherules at the limiting membrane of these vacuoles usually possess a threadlike central density but they lack the core typical to virus particles making them evidently distinct from virions (Grimley et al., 1972). Spherules are connected to the cytoplasm through a membranous neck with an inner diameter of 8 nm, and electron-dense material extending into the cytoplasm can be seen in EM-images. Alphavirus-specific antibodies recognise the region around the pore suggesting that this is the site for RC attachment (Froshauer et al., 1988). Occasionally, spherules could be detected also as patches at the PM and viral replication has been shown to occur as well in these sites (Grimley et al., 1968; Froshauer et al., 1988). However, the relationship between the two sites of spherules and viral replication has been controversial. Froshauer et al. suggested that RCs are targeted to the late endosomes or lysosomes and this is determined by the mode of virus entry. The appearance of the spherules at the PM was attributed to recycling of the CPVs and fusing with the PM (Froshauer et al., 1988). However, Peränen and Kääriäinen showed that CPVs are also seen when the infection is started by transfection of viral genomic RNA into cells (Peränen and Kääriäinen, 1991). In addition, Kujala et al. proposed that the RCs are targeted to the PM by the nsP1 protein and the primary assembly of the RC might occur there. In addition, they suggested the possibility that the induction of the spherule structures takes place at the PM followed by endocytosis (Kujala et al., 2001). Continuation of that work was published by Salonen et al, where they demonstrated that uncleavable P12 polyprotein is exclusively localized at the PM but addition of nsP3 protein redirects the targeting to endosomes and lysosomes. The main suggestion was that the presence of nsP3 changes the properties of the polyprotein and exposes the endo-

lysosomal targeting signal, although P123 is not sufficient to induce the formation of the spherule structures (Salonen et al., 2003). Recently, another piece of work concerning the dilemma was published where it was noted that early in infection the RCs accumulate at the PM suggesting that they are first targeted there (Gorchakov et al., 2008). Therefore it is still not clear where and how the spherule structures are formed and how the membrane-bound RCs are assembled.

1.2.4 Virus – host interactions

In mammalian cells, alphavirus infection usually leads to severe cytopathic changes designated as cytopathic effect (CPE), and the host cell generally dies within 24 hours. As expression of viral proteins alone can modify cellular functions, it appears that many virus-host interactions are mediated by viral proteins excluded from the RCs. Already 3 h post infection host cell transcription and translation are severely inhibited. In Old World viruses, these effects seem to be mediated mainly by nsP2 whereas New World viruses use capsid protein for interfering with the host cell metabolism (Gorchakov et al., 2005; Garmashova et al., 2007; Aguilar et al., 2007). Phosphorylation of the host-cell translation initiation factor eIF2α by dsRNA-dependent protein kinase (PKR) seems to mediate the shut-off of host-cell protein synthesis (Ventoso et al., 2006). Translation of viral subgenomic RNA in infected cells does not require some of the host cell factors, like eIF2α, whereas translation of in vitro synthesised subgenomic RNAs followed canonical initiation (Ventoso et al., 2006; Sanz et al., 2009).

Expression of nsP1 alone induces filopodia-like extensions that are about 50 nm in diameter and many of them are branched. Filopodia are positive for nsP1 but not for F-actin distinguishing them from cellular filopodia. In addition, nsP1 causes disruption of the actin cytoskeleton, whereas the microtubule network remains intact. Similar changes are seen also during SFV infection. Pa^- nsP1 behaves differently as it does not induce filopodia-like extensions nor disrupt the actin cytoskeleton (Laakkonen et al., 1998).

1.3 Membrane-associated replication of other positive-strand RNA viruses

RNA replication associated with altered cellular membranes has been described for all positive-strand RNA viruses. Although extensively studied, the function of this interplay is still not completely understood. It is suggested that membranes would provide a structural framework for the RC, anchoring the process to a confined place as well as concentrating the essential components needed for replication. In addition, lipids seem to offer a functional platform for the replicase proteins as their enzymatic activities depend on membrane binding (as described above for nsP1) –reviewed in (Salonen et al., 2005; Miller and Krijnse-Locker, 2008)–. As also mentioned, RNA

replication produces dsRNA intermediates that are strong activators of the innate immune responses against viral infections. Therefore, the altered membranes may protect the replication intermediates from host defence mechanisms. It has been shown that interference with the correct assembly of membrane-bound complex/induction of membrane modifications makes the viral RNA more sensitive to ribonucleases (Barajas et al., 2009).

Membrane binding and targeting of viral RCs seems to be mediated via different mechanisms. Many viruses use amphipathic helices to anchor their RCs to the membranes similarly to alphaviruses. One well studied example is BMV, a plant virus belonging to the alphavirus-superfamily. In a recent study, it was shown that BMV anchors its RC to the membranes of ER via an amphipathic helix in the middle of the 1a protein (methyltransferase and helicase). Mutations in the helix abolished the membrane binding of the RC and were lethal to virus replication in plant host (Liu et al., 2009). Amphipathic helix fused with GFP was able to mediate the membrane association of the fusion protein (Liu et al., 2009). However, a longer region, termed helix E, was necessary to target the GFP to ER membranes (den Boon et al., 2001). HCV possesses several amphipathic helices in different replicase proteins. NS4B (a palmitoylated protein with functions in modulating host cell environment) has been shown to contain two amphipathic helices in the N-terminus and at least one membrane-associated helix in the C-terminus. In addition, NS4B has four transmembrane domains in the central part of the protein and two palmitoylation sites in the C-terminus. Therefore, NS4B represents a true integral membrane protein. Mutations in the amphipathic helices in the N- or C-terminus have been shown to affect the membrane binding and functionality of the RCs (Gouttenoire et al., 2010). Replicase component NS5A has an N-terminal amphipathic helix that is conserved throughout the HCV-related viruses and mediates the membrane binding of the protein (Brass et al., 2007). Fusion of the helix with GFP targets the protein to the ER indicating that the amphipathic helix contains the targeting signal to the ER (Brass et al., 2002). Exceptionally, HCV polymerase domain NS5B is also membrane-associated having a C-terminal transmembrane domain (Moradpour et al., 2004). Additionally, other replicase proteins are membrane-associated as well, leading to the situation where almost all the domains of the RC have individual membrane binding motifs. Therefore the membrane association of the HCV RC seems far more complicated than in some other cases. Poliovirus RCs are attached to the cellular membranes via several replicase proteins similar to HCV. $2C^{ATPase}$ protein possesses a predicted amphipathic α-helix in the N-terminus that mediates the membrane binding of the 2BC polyprotein (Echeverri and Dasgupta, 1995). Fusion of the first 72 aa form $2C^{ATPase}$ with chloramphenicol acetyltransferase (CAT) was able to target the recombinant protein to the membranes of ER (Echeverri et al., 1998). 3A mediates the membrane association of 3AB polyprotein through a hydrophobic sequence in the C-terminus of the 3A (Towner et al., 1996). 3AB is shown to anchor the RC to the membranes as it recruits the polymerase $3D^{Pol}$ through the interactions with 3B (Fujita et al., 2007). From these examples it is apparent that membrane binding mechanism of the RCs might be rather complex and differ between different virus groups.

The formation of membrane-bound RC including the mechanisms as well as the components involved in inducing membrane alterations is the other large research topic concerning membrane-associated replication. Today, many aspects of the assembly of the membranous RCs are still not completely understood and seem to vary between different viruses. For several virus groups it has been shown that protein components can induce membrane alterations similar to rearrangements seen during virus infection. Expression of BMV 1a protein induces the formation of spherule-like structures on ER membranes. Mutations in the amphipathic α-helix are able to alter these structures, changing their size or appearance (Liu et al., 2009). Interestingly, co-expression of high levels of polymerase domain 2a changes the morphology of membrane alterations causing the induction of karmellae-like stack (double-membrane ER layers) around the nucleus (Schwartz et al., 2004). These results highlight a very delicate and important interplay between 1a and polymerase in order to form functional RCs. Similar observations have been made with Poliovirus replicase proteins. Expression of 2BC or $2C^{ATPase}$ results in the proliferation of ER membranes but co-expression of 3A is required in order to induce double-membrane vesicles (DMVs) that are associated with autophagy protein LC3 (Taylor and Kirkegaard, 2008). Only one HCV replicase protein NS4B is needed to induce the membranous web (Egger et al., 2002). C-terminal domain has been implicated to be involved in the process, although it is not sufficient to form the web (Aligo et al., 2009). Coronaviruses induce double-membrane vesicles (DMVs) that are thought to be the early RCs and later larger assemblies termed convoluted membranes (CMs) and vesicle packets (VPs) are associated with DMVs in the perinuclear area (Knoops et al., 2008; Hagemeijer et al., 2009). At least three nsPs (nsP3, nsP4 and nsP6) have been shown to have transmembrane domains. Expression of membrane-bound nsPs seems to induce the formation of DMVs. Additionally nsP3 and nsP6 contain hydrophobic domains that are shown to mediate membrane binding but their exact role is not known. It is suggested that they might resemble amphipathic helices that are shown to be involved in membrane remodelling (Oostra et al., 2008).

In addition to viral proteins, host components have been shown to be involved in the formation of viral RCs and subsequent membrane alterations. Tombusviruses (e.g. TBSV) induce spherule-like structures on the membranes of chloroplasts and peroxisomes. A recent study indicates that cellular endosomal sorting complex required for transport (ESCRT) proteins are necessary for spherule formation (Barajas et al., 2009). TMV replicates on the membranes of ER and host TOM1 and TOM2A are needed for membrane association and replication (Yamanaka et al., 2000; Tsujimoto et al., 2003). Poliovirus replication is dependent on coat protein (COP) I functions and RCs associate with COPI-coated membranes (Gazina et al., 2002).

1.4 Intracellular membrane organization

1.4.1 Lipid composition

Cells are synthesising thousands of different lipids that are organized in a very regulated manner in the cellular membranes. The lipid composition varies greatly between organelles ensuring the unique features of distinct membranes (van Meer et al., 2008) and references therein). The major structural lipids are glycerophospholipids such as PC, PE, PS, PI and PA and sphingolipids such as sphingomyelin (SM) and glycosphingolipids (GSLs). The major non-polar lipids are sterols, cholesterol being the most abundant (van Meer et al., 2008). In addition to the lipids, many proteins are present in cellular membranes resulting in mosaics of structural and functional domains, a concept that is known as a modular membrane organization (Gruenberg, 2001). The ways how the lipid organization and composition are being regulated by the cells include mainly the levels of free cholesterol and the degree of unsaturation of acyl chains in phospholipids (Maxfield and Tabas, 2005).

Membrane lipids have been shown to form ordered clusters that are referred to as lipid rafts. Lipid rafts are defined as small (10-200 nm), heterogeneous, highly dynamic, strerol-and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions (Pike, 2006). Signals that are shown to target the proteins to lipid rafts include glycosylphosphatidylinositol (GPI) anchor, acylation (palmitoylation, myristoylation) or certain transmembrane domains (Lucero and Robbins, 2004). Lipid rafts have elevated levels of cholesterol, SM and PS. Many signalling molecules and cytoskeletal components have been identified in lipid raft fractions. It is believed that rafts interact with the cytoskeleton –reviewed in (Pike, 2009)–.

PM is highly ordered and is enriched in cholesterol. PM has an asymmetric distribution of the lipids having mainly PS and PE in the inner leaflet whereas the outer membrane is enriched in SM and GSLs (Maxfield and Tabas, 2005). Early endosomes and recycling compartment are similar to plasma membrane as well as some membranes of trans-Golgi network (van Meer et al., 2008). In contrast, lysosomes do not contain high levels of cholesterol, PS and SM, instead a unique lipid, lyso-bis-phosphadic acid (LBPA) is enriched on these membranes (Kobayashi et al., 1998). ER membranes have low levels of cholesterol and the presence of a large fraction of unsaturated lipids renders the membranes more disordered (Maxfield and Tabas, 2005). Mitochondrial membranes are rather unique having PG and CL mainly in the inner membranes and to some extent on the outer membranes. PE and PC are enriched on the outer membranes of mitochondria (van Meer et al., 2008). Opposite to the PM, mitochondrial membranes have low levels of PS and cholesterol (van Meer, 1989).

Lipids on endosomal membranes are directly involved in protein sorting and membrane transport (Gruenberg, 2001). Late endosomes are important sorting stations of the endocytic pathway as they communicate with other organelles, like ER and Golgi. In addition, there is a very highly regulated and active transport between the intralumenal vesicles and the limiting membrane (van der Goot and Gruenberg, 2006).

Cellular cholesterol has a major role in organizing the cellular lipids. Therefore, its levels in membranes have to be highly controlled and maintained in a narrow optimal range -(Maxfield and Tabas, 2005) and references therein-. Intracellular cholesterol metabolism is mainly regulated by the ER. However, endocytic pathway is involved in controlling the trafficking and homeostasis of cholesterol. Cholesterol content in different endosomal compartment membranes is very variable and it is tightly regulated. Recycling endosomes are usually cholesterol rich whereas lysosomes show low levels of cholesterol (van Meer, 1989; Kobayashi et al., 1998) and references therein). Defects in cholesterol sorting and removal from late endosomes and lysosomes leads to dysfunction of the organelles and to many cholesterol storage diseases such as Niemann-Pick disease. It has been shown that cholesterol accumulation interferes with dynamic properties, such as motility and tubulation of late endocytic organelles (Lebrand et al., 2002). This is due to the perturbations in the ability of the organelles to switch microtubule motor proteins and therefore losing their bi-directional movement. Cholesterol-rich late endocytic organelles have been shown to retain the minus-end-directed dynein activity but cannot utilize the plus-end-directed kinesins. Apparently, Rab7 is involved in this process and its functions seem to be impaired by the extra loads of cholesterol. Rab GTPases are known to be among the key regulators of vesicular trafficking (Lebrand et al., 2002). In addition, cholesterol accumulation leads to the enlargement of late endocytic organelles up to 2-3 fold (Sobo et al., 2007).

1.4.2 Membrane curvature and remodelling

Membranes are shaped through complex interactions between proteins and lipids. Three major mechanisms are currently recognized that shape the cellular membranes. First, interactions with the cytoskeleton can lead to the pulling, pushing or stabilizing of the membranes. Motor proteins are involved as well as assembly and disassembly of the cytoskeleton. Second, heterogeneous lipid distribution within a lipid bilayer can affect membrane morphology. Accumulation of certain types of lipids (mainly with larger head-groups) to one leaflet can induce membrane curvature. Therefore, proteins that affect the lipid distribution can bend membranes. Third, many proteins have been shown to alter cellular membranes. Mainly, three different mechanisms have been described by which proteins can shape the membranes. First, scaffold-forming proteins (coat-forming proteins) form large rigid structures that deform the underlying membrane. These scaffolds can be used also to stabilize curvatures caused by other mechanisms. The most studied coat-forming protein complexes are COPI and COPII

and clathrin. Second, some proteins cluster lipids causing alterations in membrane shape. Finally, insertion of an amphipathic motif into the lipid bilayer increases the area of one leaflet and causes the membrane to bend –reviewed in (Shibata et al., 2009; Prinz and Hinshaw, 2009)–. It has been shown that most effective membrane bending domains do not penetrate deeply into the lipid bilayer (Campelo et al., 2008). In many cases, a combination of the above mentioned membrane shaping mechanisms is used.

It has been shown with COPII (contains Sar1p, Sec23/24 and Sec13/31) that in the absence of Sec13/31 tubule formation is induced, whereas spherical structures are formed in the presence of all the components (Lee et al., 2005). Sar1p, which is a member of Arf family GTPases, has an N-terminal amphipathic helix that is inserted into the lipid bilayer and results in tubulation of the membranes (Lee et al., 2005).

Proteins containing the Bin-amphiphysin-Rvs (BAR) domains, a six-helix bundle with a positively charged surface, are known to bend membranes –reviewed in (Prinz and Hinshaw, 2009)–. I-BAR domains have been shown to induce negative curvature including formation of PM protrusions such as filopodia (Mattila et al., 2007). These structures contain actin and other filopodia markers whereas I-BAR domains are lining their inner surface (Saarikangas et al., 2009). I-BAR proteins cluster PI(4,5)P2 lipids upon binding and induce membrane bending through electrostatic interactions. In addition, some I-BAR proteins contain an amphipathic helix that inserts to the membranes and enhances the formation of tubules (Saarikangas et al., 2009).

Negative membrane curvature is also induced by ESCRT proteins. ESCRT machinery consists of four large complexes (ESCRT-0,I,II,III) and many accessory components –reviewed in (Raiborg and Stenmark, 2009)–. These proteins mediate many processes in the cell including multivesicular body (MVB) biogenesis and the budding of some enveloped viruses. The membrane deformation is unique in the case of ESCRT proteins as they do not enter into the induced structure. Recently, it has been shown how coordinated actions of ESCRT complexes result in a MVB biogenesis (Wollert and Hurley, 2010). Current model is that ESCRT-0 clusters the ubiquitinated cargo into large domains whereas ESCRT-I and –II mediate the membrane budding into the lumen of the MVB. ESCRT-III localizes to the neck of the bud and performs the scission. ESCRT-III contains fours subunits in yeast: Vps20, Snf7, Vps24 and Vps2. It has been shown that Vps20 is recruited first to the neck of the bud followed by the recruitment of Snf7, the main player in scission process (Wollert and Hurley, 2010). Overexpression of Snf7 has been shown to tubulate liposome membranes and its human homolog induces the formation of spiral filaments on the plasma membrane (Hanson et al., 2008; Saksena et al., 2009). However, based on Wollert and Hurley recent study, these overexpression results do not reflect the processes in vivo.

2. Aims of the study

The main topic of this thesis is the interplay between the viral RC and cellular membranes. This interplay involves the membrane binding mechanism of the RC, origin of the membranes utilized, formation of membrane alterations and their dynamics in the infected cells. Therefore, the aims of this study were:

1) To describe the membrane binding mechanism of SFV RC in animal cells and test the role of nsP1 BP in RC localization and virus replication

2) To identify which cellular membranes are used to assemble the membranous RC spherules and to locate the targeting signals for specific membranes present in the replicase proteins

3) To set up a plasmid-derived system for studying the spherule formation process

4) To determine which viral components are required for the formation of the spherule structures

5) To study the intracellular dynamics of RCs and the maturation of the perinuclear viral compartment – CPV-Is.

3. Materials and Methods

Materials and methods used in the current study are described in the original publications as indicated. Table 2 gives an overview of the methods used. Materials and unpublished methods are summarized below.

Table 2. *Methods used in the present study.*

Methods	Publ.
Virological and cell culture methods	
Infections with viruses (SFV, MVA) and VRPs	I,III
Infectious center assay	I
Growth curve studies	I,III
Plaque cloning	I
Infections and drug treatments	III
Viral RNA labelling	III
DNA and RNA transfections	I,II,III
Luciferase assay	II
Cell fractionation	I
DNA and RNA methods	
PCR, cloning and DNA sequencing	I,II,III
viral RNA isolation and reverse transcription	I
RNA transcription	I,III
RT-PCR	II
Immunological methods	
Western blotting	I,II,III
Indirect immunofluorescence	I,II,III
Immuno-EM	I,II
Immunoprecipitation	I
Microscopy	
Light microscopy	I,II,III
TEM	I,II,III
SEM	III
CLEM	II,III
Data analysis	
ImageJ	I,II,III
ImagePro	III
TILLVisION	III
AutoQuant	II,III
Imaris Bitplane	II,III

Cells and viruses

BHK-21 cells were used for infections with viruses or virus-like replicon particles (VRPs) in combination with drug treatments (I, III), for viral RNA transfections (I, III), for infectious center assays (I), for virus growth curve studies (I,III), for plaque assays (I,III), for viral RNA labelling (III) and for DNA transfections (unpublished). The cultivation of BHK-21 cells has been described (I,III). HeLa cells were used for DNA transfections to express nsP1 and its derivatives (I) and for virus infections followed by drug treatments (III). HeLa cells were maintained as described (III). Primary mouse embryo fibroblasts (MEFs) were used for virus infections (III) and their cultivation is described in III. BSR T7/5 cell line expressing T7 polymerase was used in all the experiments in publication II to express constructs under T7 promoter and for virus infections (II). More detailed description of the BSR T7/5 cell line can be found in publication II.

SFV infectious clone pSP6-SFV4 (Liljeström and Garoff, 1991) was used as a backbone to obtain BP mutant viruses as well as the fluorescent SFV-ZsG virus (I,III). Propagation of wt SFV and its derivatives is described in detail (I). Modified recombinant vaccinia virus Ankara (MVA) was used to provide the T7 polymerase for the expression of nsP1 and its derivatives (I) as well as polyproteins under T7 promoter (unpublished). MVA was propagated in BHK cells (Carroll and Moss, 1997) and it was kindly provided by B. Moss (NIH, Bethesda, MD).

Plasmid constructs

Constructs used in the current thesis are listed in the Table 3. DNA cloning of these constructs is described in detail in the indicated publications.

NsP1 compensatory mutations D187G, M241I and D437G were derived from plaque purified virus stocks by reverse transcription-PCR (RT-PCR) and PCR fragments were transferred to pGEM-T (Promega). *Pst*I-*Dra*III fragments (containing the D187G or M241I mutations) and *Dra*III-*Stu*I fragment (containing the D437G mutation) were transferred to intermediate pSFV1Δ*Hind*III vector. The latter is a derivative of pSFV1 replicon-vector (Liljeström and Garoff, 1991), which has been modified with *Hind*III restriction, resulting in deletion of nsP4, nsP3 and part of nsP2 coding sequence. Unique *Sph*I and *Sac*I restriction sites in the pSP6-SFV4 were used to transfer the D202Y and M241I mutations from pSFV1Δ*Hind*III to R253A infectious cDNA (icDNA) and D437G mutation to wt icDNA. Plasmid pEGFP-N1 (Clontech) was used to obtain GFP fusion proteins with full-length nsP1 (aa 1-537) and the shorter versions of nsP1 (Fig. 11). The desired sequences were amplified from pTSF1 plasmid (Peränen et al., 1990). CCGCGG (*Sac*II site) was introduced with the forward primer and ACCGGT (*Age*I site) with the reverse primer. The resulting PCR products were cut with *Sac*II and *Age*I and fragments were transferred to pEGFP-N1. The constructs were verified by sequencing. P13 fusion construct was obtained by two-step PCR using pcP123 (Salonen et al., 2003) as a template. The first round was used to amplify the C-terminus of nsP1 using primers: 5´AACGGCGTATTGGATTGGGTT 3´ (F2) and 5´GGATGGTGCTGCACCTGCGTGA

TACTCTAG 3′ (Fus13_as). The N-terminus of nsP3 was amplified with primers 5′ GCAGGTGCAGCACCATCCTACAGAGTTAAG 3′ (Fus13_s) and 5′ GCTCTAGATT ACGTAGATGCGGCATACTTCCG 3′ (nsP3_as). Resulting PCR products were used as templates for the second round of PCR using primers F2 and nsP3_as and the product was transferred to pGEM-T vector. *Nhe*I-*Sac*II fragment was excised from pcP123 and replaced with the *Nhe*I-*Sac*II fragment from P13-pGEM-T. *Nhe*I-*Bsu*36I fragment was used to introduce the P13fusion into P123ZsG-pTM1 to obtain P13ZsG-pTM1.

Table 3. *Table 3. List of the plasmid constructs used in the current study.*

Plasmid	Publication			
	I	II	III	Unpublished
pTSF1 (T7), (Peränen et al., 1990)	wt Y249A R253A R253E K254E L255A+L256A L255A+L256V R257E W259A			
pSFV1 (SP6), (Liljeström and Garoff, 1991)			pSFV1-ZsG	
pSP6-SFV4 (SP6), (Liljeström and Garoff, 1991)	wt Y249A R253A R253E K254E L255A+L256A L255A+L256V R257E W259A D202Y+R253A D202Y+R253A+ +D437G D437G+R253A	wt	wt pSFV4-ZsG	D187G M241I D437G
pGEM (T7), Promega	$P12^{CA}3$ (Merits et al., 2001) $P12^{CA}3$ R253E $P12^{CA}3$ W259A			

Table 3 continues on the next page

Continuation of the Table 3

Plasmid	Publication			
	I	II	III	Unpublished
pTM1 (T7+EMCV IRES), (Moss et al., 1990)		P123 P1234 P1234^{GAA} P1^2^34 ubinsP4 ubinsP4^{GAA} P123Z4 P123Z4^{GAA}		P12^{CA}3 P13ZsG
pEGFP-N1 (CMV), Clontech	1xBP (aa 245-254) 2xBP 3xBP			aa 1-537 (nsP1) aa 1-247 (nsP1) aa 1-270 (nsP1) aa 1-324 (nsP1) aa 180-537(nsP1)
pcDNA4/TO (CMV), Invitrogen				nsP1 (Salonen et al., 2003) P13
pUC18 (added T7), **II**		TshortNSluc TshortSTluc TshortNSluc* TshortSTluc* Tmed Tlong		

* G→A mutation in the 5´UTR of the template constructs (**II**)

Antibodies, fluorescent conjugates and reagents

The primary antibodies used in this study are listed in the Table 4. Secondary antibodies with AF488/568/647 fluorophores were purchased from Invitrogen, Molecular probes (Eugene, OR).

Table 4. *Primary antibodies used in the present study.*

Antibodies	Host	Dilution	Source	Publ.
anti-nsP1	Rabbit	IF:1:1000, WB:1:10000	(Kujala et al., 2001)	**I,II**
anti-nsP2	Rabbit	WB:1:6000	(Kujala et al., 2001)	unpubl.
anti-nsP3	Rabbit	IF:1:1000, WB:1:10000	(Kujala et al., 2001)	**I,II,III**
anti-nsP4	Rabbit	IF:1:400, WB:1:6000	(Kujala et al., 2001)	**II**
anti-capsid	Rabbit	WB:1:3000	(Väänänen and Kääriäinen, 1980)	unpubl.
anti-E1	Rabbit	WB:1:3000	(Väänänen, 1982)	unpubl.
anti-dsRNA (J2)	Mouse	IF:1:300	Scicons	**II, III**
anti-α-tubulin	Mouse	IF:1:2000	Sigma Aldrich, Inc	**III**
anti-γ-tubulin	Mouse	IF:1:10	Sigma Aldrich, Inc	**III**
GM130	Mouse	IF:1:500	BD Transduction Lab.	**III**
anti-LAMP2	Mouse	IF:1:300	Abcam	unpubl.
anti-GFP	Mouse	WB:1:3000	BD Biosciences	**I**

IF, Immunofluorescence; WB, Western blotting

Fluorescent conjugates and reagents used in this work are listed in the Table 5.

Table 5. *Fluorescent conjugates and reagents used in this study.*

Fluorescent conjugates and reagents	Final conc. used	Source	Publ.
ConcanavalinA-AF488, AF568	10 μg/ml	Sigma Aldrich, Inc	**I**
Phalloidin-AF568	130 nM	Invitrogen; Molecular Probes	**III**
Lysotracker Red DND-99	100 nM	Invitrogen; Molecular Probes	**II,III**
(-)Blebbistatin	30 nM	Sigma Aldrich, Inc	**III**
Wortmannin	100 nM	Sigma Aldrich, Inc	**III**
LY294002	50 μM	Sigma Aldrich, Inc	**III**
PI-103	200 nM	Sigma Aldrich, Inc	**III**
Nocodazole	5 μM	Calbiochem	**III**
Rock inhibitor Y-27632	5 μM	Sigma Aldrich, Inc	unpubl.
IPA-3	10 μM	Sigma Aldrich, Inc	unpubl.
Filipin complex	50 ng/μl	Sigma Aldrich, Inc	unpubl.

Indirect immunofluorescence, microscopy and image analysis

Cells grown on glass coverslips were fixed at indicated times using 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 15 min at room temperature (RT). The fixed cells were washed three times with PBS and quenched with 50 mM NH_4Cl for 10 min. Samples were permeabilized using 0.1% Triton X-100 for 1 min at RT followed by washes with PBS. Dulbecco + 0.2% BSA was used as a blocking solution and to dilute the antibodies. After 20 min blocking at RT, samples were incubated with primary antibodies for 1 h at RT and washed three times with blocking solution. Incubation with secondary antibodies was performed in the dark for 1 h at RT followed by washes with blocking solution. Samples were post-fixed with 4% PFA and Mowiol® 4-88 (Calbiochem) containing 2.5% DABCO (Sigma) was used for mounting the coverslips. Cellular cholesterol was stained with filipin complex. PFA-fixed samples were washed three times with PBS and filipin diluted (50 ng/µl) in PBS containing 10% FBS was added to the samples. After 30 min incubation at +37°C samples were washed three times with PBS followed by quenching and permeabilization as described above. Cellular cholesterol visualized by filipin was imaged with Olympus Ax 70 Provis microscope with a 20x/0.50 Ph1air objective for quantification analysis. Pictures were collected by a Photometrics SenSys air-cooled CCD camera using AnalySIS program. Exposure was always set to 80 ms for filipin imaging. Quantification of cholesterol levels was done by using ImageJ (National Institutes of Health, Bethesda, MD). Mean intensities of filipin were measure from ~500 cells per sample group. Leica SP2 AOBS confocal microscope with HCX PL APO 63x/1,4-0,6 oil objective was used to analysed filipin stained infected samples and to obtain optical slices covering the whole cell for 3D analysis. Leica TCS SP5 confocal microscope with HCX APO 63x/1,30 Corr/CS 21 glycerol objective was used to analyse all the fixed samples except the ones stained with filipin (364 nm UV laser for exciting the filipin is present only in SP2 confocal). Deconvolution of confocal stacks was done with AutoQuant AutoDeblur 3D Blind Deconvolution (AutoQuant Imaging, Inc.). Imaris (Bitplane) program was used to make 3D reconstructions as maximum projections and distribution of filipin staining and viral RCs were analysed with the orthoslice mode.

4. Results

4.1 Membrane binding of the RC (I and unpublished)

4.1.1 Mutations in the BP region change the properties of nsP1 and the polyprotein P123 (I)

The primary membrane binding of nsP1 has been shown to be mediated via a BP in the middle of the protein. However, previous studies were carried out with synthetic peptides and nsP1 expressed in E.coli or synthesised in vitro (Ahola et al., 1999; Lampio et al., 2000). These studies suggested an important role for the predicted amphipathic helix in the membrane binding of nsP1 and highlighted the crucial residues in the BP region (Ahola et al., 1999; Lampio et al., 2000).

To study the role of the BP in the membrane binding of nsP1 expressed in mammalian cells, a set of nsP1 constructs carrying mutations in the BP region were made. These mutations change positively charged residues in the BP to neutral alanine or to negatively charged glutamate, or hydrophobic residues to alanine (**I, Fig. 1B**). The influence of these changes on nsP1 palmitoylation, subcellular fractionation and localization was observed.

Wt nsP1 was palmitoylated, pelleted exclusively in the P15 fraction and localized to the PM as well as to filopodia-like extensions as reported previously (Laakkonen et al., 1996; Laakkonen et al., 1998). ConcanavalinA (ConA) was used to visualize the PM. In contrast, mutations R253E and W259A abolished the membrane binding of the protein (**I**). These mutant nsP1 proteins were not palmitoylated (**I, Fig. 2A**), they were distributed between the P15 and S15 fractions like Pa^- nsP1 (**I, Fig. 2C**) and no PM localization was seen. Instead, they showed a diffuse pattern in the cytoplasm of the transfected Hela cells (**I, Fig. 3E,I**). With immuno-EM it was also confirmed that the mutation W259A disrupts the localization of nsP1 to the PM and the affinity to membranes, as the mutant protein appeared soluble (**I, Fig. 4**). Moreover, when the mutations R253E and W259A were transferred to the uncleaved polyprotein $P12^{CA}3$, no membrane binding was detected. These mutant polyproteins did not target the PM or the endo-lysosomal membranes as the wt $P12^{CA}3$ did; instead, they were diffuse in the cytoplasm (**I, Fig. 5**). These results suggest that the BP is important in the membrane binding of nsP1 as well as the entire RC.

Mutations K254E and R257E did not alter the properties of nsP1 as the mutant proteins behaved very similarly to wt nsP1 (**I, Fig. 2A,C and 3F,H**). Mutations Y249A, R253A and L255A+L256A influenced nsP1 in an intermediate manner. These mutant proteins were palmitoylated and pelleted abundantly in the P15 fraction (**I, Fig. 2A,C**). However, they seemed to be bound to the PM in a weaker manner as these mutant proteins were also found in the cytoplasm (**I, Fig. 3C,D,G**).

4.1.2 Mutations destroying the membrane binding of the RC are lethal for the virus (I)

As it was evident that the BP region is crucial for the membrane binding of nsP1 and the RC, we wanted to verify the significance of the changes in the BP region during virus replication. Therefore, all the mutations described above were transferred to the infectious cDNA clone of SFV (pSP6-SFV4) and their effects were monitored in infectious centre assay and growth curve studies.

The infectivity of the wt virus was 10^6 PFU/μg, which is very close to the value reported previously (Liljeström et al., 1991). Virus accumulated fast, both after transfection of 1 μg of infectious RNA or after infection with the primary virus stock (10 PFU/cell). CPE was observed within 24 h and the collected virus stocks had a high titre (10^9 PFU/ml). Opposite results were obtained with Y249A, R253E and W259A viruses. They did not show any CPE even after 72 hours and no plaques were observed in infectious centre assay (**I, Fig. 7, Table I).** This is in agreement with results obtained from nsP1 studies and shows that these mutations are lethal for SFV replication.

Mutant viruses carrying the changes K254E and R257E behaved very similarly to wt virus having even slightly higher infectivities (**I, Fig. 7, Table I**). These mutant viruses were also studied with EM for their ability to induce membrane alterations (spherules) in BHK cells (MOI=50). Similarly to wt, many CPV-Is with numerous spherules were detected at 5 h p.i. (not shown), demonstrating that these changes in the amphipathic peptide do not alter the membrane binding of the RC nor the ability to form spherules. Interestingly, mutations R253A and L255A+L256A altered virus growth and infectivity in an intermediate way. These mutant viruses accumulated extremely slowly after the transfection of RNA and showed mild CPE after 48 h. Strong CPE was observed after 72 h when also the virus stocks were collected. Infectivities of these viruses were up to 10^5 times lower than wt virus and resulted in very small or mixed size plaques in infectious centre assay. Remarkably, when these primary stocks were used to infect BHK cells, wt-like virus phenotypes were observed (**I, Fig. 7, Table I**).

4.1.3 Revertants arise for the BP mutants (I and unpublished)

As the viruses carrying the mutations R253A and L255A+L256A seemed to have an initial impairment in the growth that was overcome during the slow accumulation, this suggested that compensating changes had taken place. Sequencing was used to confirm the presence of the original mutations and to verify if any genetic changes had occurred during virus growth. Interestingly, the double mutant L255A+L256A virus consistently showed a pseudo-reversion in the position 256. Alanine in this position is encoded by GCG and in five independent experiments it reverted to GTG that encodes for valine. The GTG substitution was also transferred to icDNA and nsP1 sequence carrying the L255A mutation resulting in a double mutant L255A+L256V. This revertant virus behaved similarly to wt virus as it had comparable infectivity and growth rate, and it induced spherules undistinguishable from wt virus **(I, Fig. 8A,B)**. Respective revertant nsP1 was

targeted to the PM where it became palmitoylated (not shown) and induced filopodia-like extensions (**I, Fig. 8C**), confirming the beneficial effect of the valine.

Reversions were also detected with the R253A virus, although in this case changes had occurred outside of the BP region. Plaque cloning of the virus showed that mixed plaques seen in infectious center assay were caused by viruses with different additional mutations in nsP1. A fast growing revertant that was causing large plaques had two additional mutations, D202Y and D437G, in nsP1. Both of the changes were confirmed to be important to rescue the R253A virus, as separately they restored the growth of the virus only partially (**I, Fig. 7C**). Interestingly, when the mutation D437G was cloned into wt SFV4 backbone, it resulted in a faster accumulation of the virus. In addition, virus titres were higher compared to wt and the infectivity increased four-fold (Fig. 4, Table 6). Another revertant virus that yielded only very small plaques accumulated slower and had a lower infectivity (2 x 10^5 PFU/µg) than wt, had an additional mutation M241I (Fig. 4, Table 6). Therefore, this compensatory mutation did not restore the wt phenotype completely. Similarly, a very slow growing revertant (CPE appearing at 48 h p.i.) with a compensatory mutation D187G had low infectivity (8x10^2 PFU/µg) and yielded small plaques (Table 6).

Table 6. *Phenotypes of viruses containing compensatory mutations in nsP1.*

Virus	Plaque size (mm)	RNA infectivity (PFU/µg)	Stock collection time* (h)	Primary virus stock titre (PFU/ml)
wt	Large (3-4)	1 x 10^6	24	4.5 x 10^8
M241I + R253A	Small (1-1.5)	2 x 10^5	36	1 x 10^8
D187G + R253A	Small (0.5-1)	8 x 10^2	48	8.25 x 10^7
D437G	Large (4)	4 x 10^6	24	2.1 x 10^9

* The virus stock was collected after CPE became visible

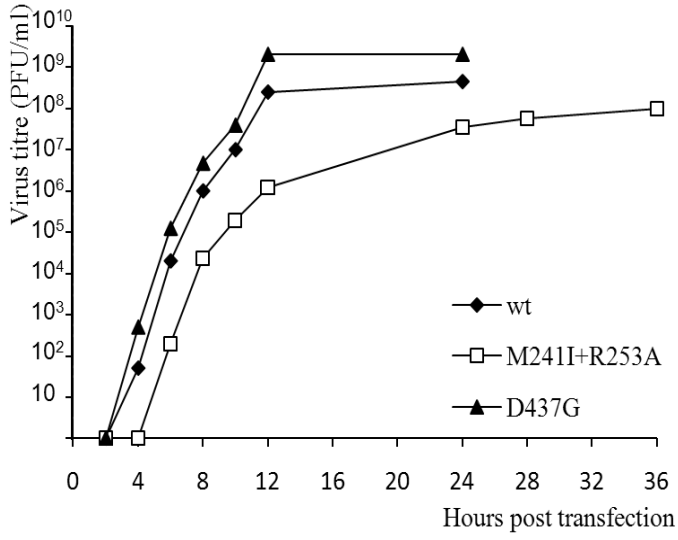


Figure 4 *Growth curves of viruses containing compensatory mutations in nsP1. Wt SFV is given as a reference. 1 µg of capped RNA transcript containing the indicated mutation was electroporated into BHK cells and aliquots of media were withdrawn at the indicated time points. Titers of the released viruses were measured by plaque assay.*

4.2 Spherule formation

The formation site of the spherule structures has been under debate for a long time, as described in the introduction. Therefore, we wanted to investigate the early steps of SFV replication and determine which membranes are used as the formation platform for the spherules. In addition, our aim was to set up a plasmid-driven reporter/replication system to study the formation process of the spherules in detail and to define the minimum set of viral components needed to induce the membrane alterations.

4.2.1 Spherule structures are formed at the PM (III)

To study the localization of SFV RCs during virus replication, we performed a time course experiment covering the whole replication cycle (**III, Fig. 1D**). BHK cells were infected with SFV (MOI=50), samples were fixed every 30 min and RCs were visualized by double-labelling with anti-nsP3 and anti-dsRNA antibodies. Early time points clearly indicated that RCs are first detected at the PM and they continue to accumulate there during the next couple of hours (**III, Fig. 1Db,c**). EM analysis of parallel samples demonstrated that the PM was filled with spherule structures whereas few spherules could be found in the cytoplasm (**III, Fig. 1Dd**). An interesting change was noticed after 2 h 30 min p.i., when most RCs were internalized and they seemed to be aligned in stripes (**III, Fig. 1De,f**). During the following hours, RCs were concentrated perinuclearly, and the double-stained organelles enlarged in size (**III, Fig. 1Dg,h**). Similar time course performed by using low and high virus loads (MOI 5 and 500, respectively) confirmed the results and showed that the localization of the RCs during the replication cycle follows a determined route starting from the PM followed by trafficking to the perinuclear area (Fig. 5).

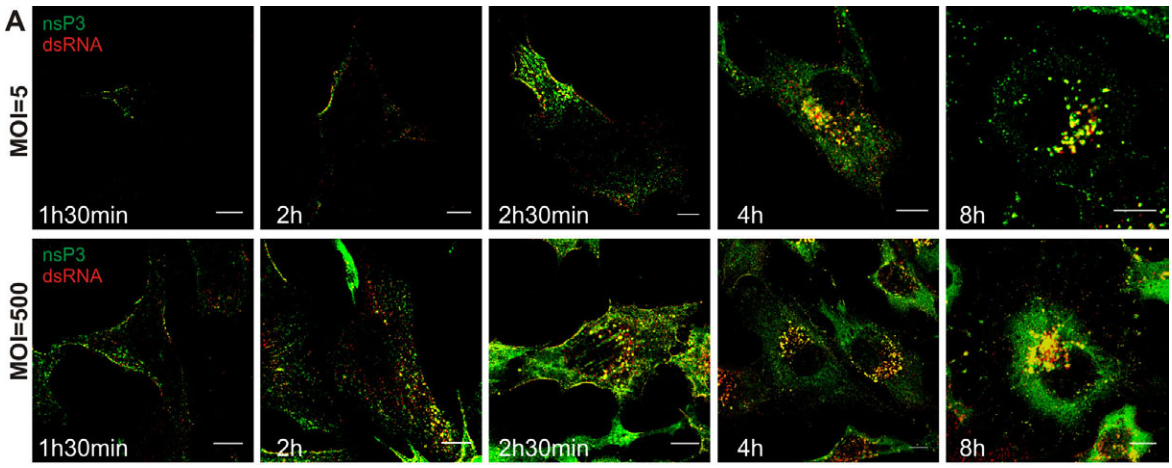


Figure 5 *Localization of the RCs during SFV infection using low (MOI=5) and high (MOI=500) virus loads. Samples were fixed at the indicated time points and RCs were double-labelled with anti-nsP3 and anti-dsRNA antibodies.*

The localization of the RCs was also confirmed by visual quantification performed during the time course with high virus loads. From the analysis of 500 cells in each time point, it was evident that early in infection, most RCs are localized at the PM – a phenotype that disappears during the course of replication and shifts towards the large RC-positive perinuclear vacuoles, the previously described CPV-Is (**III, Fig. 1E**).

The time course results were further confirmed with drug treatment experiments demonstrating that the RCs can be stopped at the PM by inhibitors of phosphoinositide 3-kinase (PI3K) (**III, Fig. 2**). Scanning electron microscopy revealed that wortmannin-treated infected cells had PMs covered with spherules, indicating that spherules are formed there -**III, Fig. 2** and described in more detail in the thesis of G. Balistreri (Balistreri, 2010)-. Similar results were obtained with blebbistatin, an inhibitor of myosin II. In the presence of this inhibitor, the internalization of the RCs was heavily delayed indicating a role for actin in the early stages of virus replication and the intake of spherules from the PM (**III, Fig. 5D,E**).

4.2.2 Plasmid-driven system to study spherule formation

The above described results concerning spherule formation were obtained by using infectious SFV where manipulation of the replication components is rather limited. Therefore, it was important to develop a plasmid-based system where production of replicase proteins as well as replication templates does not depend on virus replication.

Previously, it has been challenging to express large polyproteins and many different systems have been adopted. Nevertheless, the efficiency of transfection and the expression levels have not been satisfactory. We have constructed a set of SFV replicase derivatives under the T7 promoter, additionally containing the EMCV IRES element to promote translation. The template constructs contain the necessary cis acting elements needed for SFV replication (CSE 1-4, see introduction) and are as well under the T7 promoter (**II, Fig. 1**). In addition, marker genes were incorporated into the templates and also fused with the replicase protein nsP3 in polyprotein constructs. This set up was designed to rebuild a functional RC that could be visualized in cells and to follow replication based on luciferase gene expression.

4.2.2.1 Expression of polyproteins by the aid of MVA (unpublished)

MVA expressing the T7 polymerase has been used previously in our laboratory and it was utilized also for the current system. BHK cells were infected with MVA for 1 h followed by transfection with polyprotein constructs under the T7 promoter using Lipofectamine 2000. Samples were analysed at 6 h p.t. for Western blot and luciferase assay or fixed at 7 h p.t. for immunofluorescence analysis. All the polyproteins were expressed at high levels and at expected sizes as determined by Western blot analysis (Fig 6A). Cytoplasmic membrane fractions sedimenting at 15,000 x g (P15) from BHK cells transfected with uncleavable polyprotein constructs were analysed with SDS-PAGE

followed by Western blot. Immunostaining with anti-nsP3 antibody demonstrated that $P12^{CA}3$ and P1^2^34 polyproteins were exclusively associated with the P15 fraction whereas nothing was detected in the supernatant (S15) fractions (Fig. 6B). However, the localization of the replicase intermediates $P12^{CA}3$ (Fig. 6D) and P1^2^34 (not shown) in BHK cells gave confusing results. All the labelling of the RCs was at the PM and no cytoplasmic vesicles typical to uncleavable polyprotein expression were detected (Fig. 6D, upper panel). A similar phenotype was seen in SFV infected cells that were previously infected with MVA. Virtually all the RCs were blocked at the PM and no CPV-Is were seen even at 6 h p.i. (not shown). Interestingly, a different picture was seen in HeLa cells, where polyproteins localized mostly in vesicles that partially colocalized with the lamp2 marker (Fig. 6D, lower panel). As the late replication of MVA is restricted in HeLa but not in BHK cells this suggests that MVA inhibits certain cellular functions that are needed for the internalization of the RCs from the PM. Nevertheless, these results confirmed further that the RCs of SFV are first targeted to the PM and not to intracellular vesicles. Co-expression of polyproteins and templates containing the luciferase gene under ns or subgenomic promoter demonstrated that functional RCs are formed that are able to recruit the template provided *in trans* and perform replication (Fig. 6C). However, the background signals were very high, especially when expressing templates alone, suggesting that the MVA can recruit and utilize SFV templates very efficiently. Therefore, it was obvious that an MVA based expression system cannot be used to study the replication components of SFV and their delicate interplay. In addition, MVA is not suitable for morphological studies as MVA infection rearranges cellular membranes and organelles -(Gallego-Gomez et al., 2003; Schepis et al., 2006; Schramm et al., 2006) and our unpublished data-

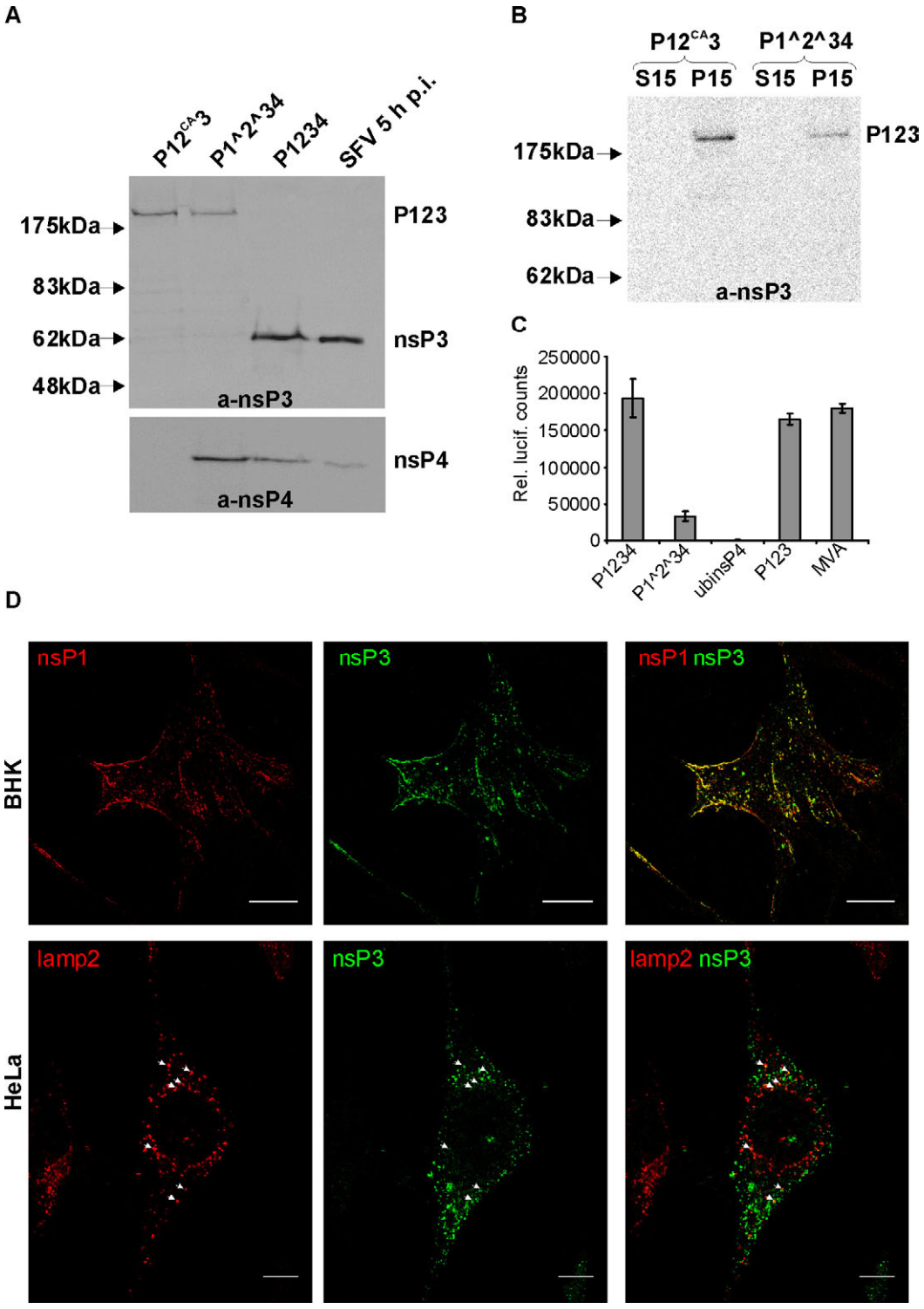

A
P12CA3
P1^2^34
P1234
SFV 5 h p.i.
175kDa
83kDa
62kDa
48kDa
P123
nsP3
a-nsP3
nsP4
a-nsP4
B
P12CA3
P1^2^34
S15 P15 S15 P15
175kDa
83kDa
62kDa
P123
a-nsP3
C
Rel. lucif. counts
250000
200000
150000
100000
50000
0
P1234
P1^2^34
ubinsP4
P123
MVA
D
BHK
HeLa
nsP1
nsP3
nsP1 nsP3
lamp2
lamp2 nsP3

Figure 6 *Expression of polyproteins by the aid of MVA. A) Western blot analysis of BHK cell extracts expressing the indicated polyproteins. Samples were lysed at 6 h p.t. and separated in SDS-PAGE followed by immunoblotting using anti-nsP3 and anti-nsP4 antibodies (indicated on the corresponding image). Lysate from SFV infected cells (5 h p.i.) was used as a reference for ns proteins. Molecular mass markers are shown on the left and expressed proteins on the right. B) Fractionation of BHK cell extracts expressing uncleavable polyproteins. Samples were lysed at 6 h p.t., nuclei removed and cytoplasmic membranes sedimenting at 15,000 x g (P15) and the remaining supernatant (S15) were analysed with SDS-PAGE and immunoblotting using anti-nsP3 antibody. Molecular mass markers are shown on the left and expressed proteins on the right. C) Luciferase assay was used to measure the specific replication activities of the polyproteins, TshortNsluc template was provided as capped RNA to all of the samples. MVA infection without polyprotein transfection served as a background control. Relative luciferase counts are indicated on the left; SD of duplicate samples is shown. D) Immunofluorescence analysis of $P12^{CA}3$ expression in BHK (upper panel) and in HeLa cells (lower panel). Anti-nsP1(red) and anti-nsP3 (green) antibodies were used to detect the localization of uncleavable polyprotein in BHK cells. An optical section from the bottom of the cell is shown to illustrate the PM staining of the polyprotein. Merged image is shown on the right. Colocalization of the lysosomal marker lamp2 (red) and anti-nsP3 (green) was tested in HeLa cells. Merged image on the right shows few colocalizing dots (indicated with arrowheads in all the images). Scale bars represent 10 μm.*

4.2.2.2 Expression of polyproteins in BSR T7/5 cells (II)

As MVA was interfering with SFV infection and cellular membranes, another T7 based system was needed to express the polyproteins and template constructs. BHK-derived cell line stably expressing T7 polymerase, BSR T7/5, has been used successfully with negative-strand RNA virus expression constructs (Freiberg et al., 2008). As our laboratory has been using BHK cells for SFV studies very extensively, we started to test the BSR T7/5 cell line. All the polyproteins were expressed at high levels comparable to SFV infection and with expected sizes (**II, Fig. 2**). Immunofluorescence showed that expression of the full length cleavable polyprotein P1234 together with the template construct resulted in PM staining with anti-nsP1 antibody, cytoplasmic aggregates with anti-nsP3 antibody and colocalization of these two antibodies in cytoplasmic vesicles (**II, Fig. 2**). Immuno-EM of parallel samples confirmed the results demonstrating that these vesicles were indeed endosomes and lysosomes (**II, Fig. 2**).

4.2.2.3 Specific replication activity of the polyproteins (II)

The functionality of these polyproteins and templates was assessed by luciferase assay (**II, Fig. 3**). Cotransfection of the full polyprotein P1234 plus the template with luciferase under the ns promoter (TshortNSluc) resulted in relatively high counts that were increasing up to 24 h. Negative control, where the polymerase active site was mutated to GAA, showed background level counts. The specific replication activity was ~75 fold

above the background. Another template, where luciferase was under the subgenomic promoter (TshortSTluc), yielded lower luciferase counts. However, the background signals were also lower increasing the specific replication activity ~150 fold above the background. In addition to the polymerase active site mutant, we used P1234 expression without the template or P123 together with the template as well as template alone as negative controls. Interestingly, template alone reproducibly gave higher counts than when expressed together with polyproteins suggesting that P123 might recruit the template and it cannot be used for translation. As the template constructs are under the T7 promoter, luciferase under ns promoter will always yield some background. That is also probably the reason why the TshortSTluc shows higher specific replication activity.

We aimed to increase the specific replication level and created a template with a single point mutation in the 5´ NTR. This G to A substitution disrupts a predicted hairpin structure in CSE1 that has been predicted to control the replication (see introduction). Surprisingly, the modified template Tshort increased the replication levels to ~200 fold in the case of TshortNSluc and to more than ~600 fold for the TshortSTluc as assessed by luciferase assay (**II, Fig. 3**). RT-PCR demonstrated that RNA levels increased up to 8 h when P1234 was cotransfected with modified TshortNSluc and the values were around 20% compare to SFV 4 h infection. Significantly lower RNA levels were detected when unmodified TshortNSluc was used (**II, Fig. 5**) Based on these results, we chose the modified TshortNSluc template for all the following experiments.

As our final aim was to study the formation of the spherules at the correlative light and electron microscopy (CLEM) level, we created P123Z and P123Z4 versions of the polyproteins where fluorescent protein from coral reefs, ZsG is fused with nsP3 (inserted into the unique *Xho*I site; **II, Fig. 1**) and therefore present in the RCs. These polyproteins localized similarly to the RCs seen during virus infection (**II, Fig. 2E,F**), expressed luciferase at the same level with the same timing compared to the versions without the fluorescent protein (**II, Fig. 4**) and were therefore used in further experiments.

Specific replication was tested also with immunofluorescence level by staining with anti-dsRNA antibody. Cotransfection of P123Z4 with Tshort showed that at least 90% of the cells are positive for dsRNA staining and dsRNA colocalized with ZsG positive structures (**II, Fig. 6**). When polymerase nsP4 was provided separately, the efficiency of dsRNA positive cells decreased to some extent, being around 80% (not shown). However these results together with luciferase assays (**II, Fig. 4**) indicate that providing P123, ubinsP4 and template as three separate plasmids reconstitutes the active RCs.

4.2.2.4 Template size determines the kinetics of replication activity (II)

The size of the templates used in the current studies was relatively small – 1500 bp, which is around 7 times smaller than the SFV genome. Therefore, it was decided to test whether the template size influences the kinetics of replication. In order to accomplish that, sequence for a fluorescent protein (tomato) was inserted under the subgenomic promoter of Tshort. This resulted in a Tmed template with 3000 bp and possibility to follow the replication based on tomato expression in immunofluorescence. In addition,

beta-galactosidase gene was inserted under the ns promoter after the luciferase gene, increasing the template size further up to 6000 bp (Tlong) (**II, Fig 1**). Therefore, three different sizes of templates were compared with luciferase assay and RT-PCR. Both methods demonstrated clearly that shorter templates were replicated faster and to higher levels. The longest template started to show specific replication only at 6 h p.t. compared to 4 h p.t. with the shortest template (**II, Fig. 4, 5** and data not shown).

The specificity of the tomato expression was tested with immunofluorescence using anti-dsRNA antibody. Cotransfection of P123Z + ubinsP4 + Tlong resulted in a strong colocalization between ZsG and dsRNA signal in tomato positive cells. Polymerase mutant ubinsP4GAA expressed together with P123Z + Tlong did not show any tomato expression or dsRNA staining in ZsG positive cells (**II, Fig. 7**).

4.2.2.5 Formation of the spherules as determined by CLEM (II)

As the luciferase assay, RT-PCR and immunofluorescence indicated that functional RCs are formed during plasmid transfections in which the template is recruited *in trans*, CLEM was performed to assess the formation of spherules. For the correlation, P1234Z or P123Z + ubinsP4 were cotransfected with Tshort and Tlong and samples were fixed at 24 h p.t. As the Tlong had the tomato gene under the subgenomic promoter, double correlation was performed to assure image acquisition from positive cells with active replication (**II, Fig. 7**). Correlation was done with confocal microscope where an image stack was taken in average of three cells per sample group and a reflection image visualized the cells and the grid. The latter was used as a map to relocate the cells in EM and analyse the cellular structures (**II, Fig. 7**). Coexpression of P1234Z or P123Z + ubinsP4 together with the long template resulted in induction of numerous spherule structures very similar compared to the ones seen during SFV infection (**II, Fig. 7**). Large clusters of spherules were found at the bottom of the cells (**II, Fig. 7Aa-c**), at the rim of the cells (**II, Fig. 7Ad**) as well as on the limiting membranes of cytoplasmic vesicles (**II, Fig. 7Bd**). Interestingly, the expression of the polyprotein together with Tshort resulted in very small spherules (20-25 nm in diameter) lining the membranes of cytoplasmic vacuoles (Fig. 7). Reproducibly, dark material outside of the vacuoles accompanied the spherules (Fig. 7, black arrows). This suggests that template size is at least one factor that determines the morphology of the induced membrane alterations. These results demonstrate that current plasmid-derived system is able to reconstitute SFV replication and membrane-bound RCs are assembled in a similar manner compared to virus infection. Preliminary experiments with polyprotein expression without the template indicate that replication proteins themselves are not sufficient to induce the spherule structures seen during virus infection (not shown). However more experiments are needed to verify which viral components and possibly enzymatic activities are needed to induce the spherule structures.

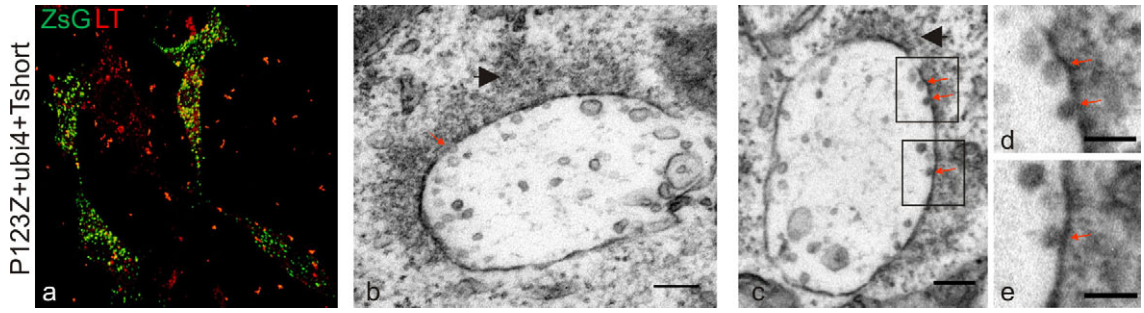


Figure 7. *Template length affects the morphology of induced spherule structures as analyzed by CLEM. BSR cells were transfected with P123Z+ubi4+Tshort and samples were fixed at 24 h p.t. CLEM was performed to verify the formation of spherules. Lysotracker (LT) was added to the samples before fixation in order to stain the acidic organelles. 3D reconstructed ZsG-positive cells (a) were analyzed with EM (b, c). Note that many of the ZsG-positive structures are acidic (a). Two representative organelles from analyzed cells are shown (b, c). Scale bar in EM images b and c is 100 nm. Small spherule-like structures induced by the P123Z+ubi4+Tshort can be detected on the limiting membranes of late endosomes; red arrows indicate the connection between spherules and cytoplasm. Dark electron-dense material (black arrowheads) is found in the close proximity of the small spherules. The boxed areas of image c are shown as images d and e. Scale bar in zoomed images is 50 nm.*

4.3 Intracellular dynamics of RCs (III)

As many results indicated that the RCs are first targeted to the PM and then transferred to the perinuclear area where they are found on the membranes of large vacuoles, we wanted to investigate further, which cellular pathways and components are utilized and what are the dynamics of RCs in live cells. Therefore, we constructed a recombinant virus SFV-ZsG carrying a fluorescent protein in the nsP3 region. ZsG protein was fused with nsP3 as described above (see 4.2.2.3) and was therefore a potential marker for RCs. As insertion of a fluorescent protein into this specific site has not been tested for virus replication, we analysed in detail the properties of the recombinant virus. In addition, as it is known that a fraction of nsP3 is not present in the RCs but forms aggregates in the cytoplasm, it was important to verify the value of ZsG signal as marker for the RCs. SFV-ZsG virus behaved very similarly to wt SFV and immuno-blot analysis confirmed the stability of nsP3-ZsG throughout the replication cycle (**III, Fig. 2A**). Staining with anti-dsRNA antibody illustrated that most ZsG-positive dots were also positive for dsRNA (**III. Fig. 2B**) and live cell imaging in the presence of lysotracker showed that ZsG signal was surrounding the acidic organelles (**III, Fig. 2D**). Notably, it seemed that SFV RCs were covering the majority of the acidic compartment that was concentrated in the perinuclear area. We observed also some ZsG-positive structures that were not acidic at 4 h p.i. indicating that these were likely to be nsP3 aggregates distinct from the RCs. However,

these results confirmed that ZsG protein is tolerated in the active complex and does not interfere with virus replication and correct localization of the RC. Furthermore, a CLEM experiment was performed to verify whether the ZsG signal can be correlated with the presence of spherules. The fact that RCs (and therefore ZsG-positive structures) are very abundant in the bottom PM around 2 h 30 min p.i. (**III, Fig 1Df**) was taken as the basis to follow the ZsG signal with confocal microscope and to analyse the bottom sections with TEM. Interestingly, the bottom PM that was positive for ZsG signal was filled with hundreds of spherules forming clusters of 6-10 spherules (**III, Fig 2C**). Taking these results together, we concluded that ZsG fused with nsP3 is a reliable marker to follow the RCs in live cells and SFV-ZsG was used in all live cell imaging experiments.

4.3.1 Spherules are internalized from the PM and transported by actin and microtubule dependent manner to the pericentriolar area

Live cell imaging was performed in parallel with a wide field microscope to gain an overall picture of the dynamics of RCs, and with a confocal microscope to dissect different steps during the trafficking of the RCs and to describe these steps in detail. Initial live cell imaging experiments with wide field microscopy indicated that the RC spherules were internalized from the PM in small neutral vesicles that are very dynamic and show many homotypic fusions which in turn gives rise to larger ZsG-positive vesicles (**III, suppl. videos S1,S2**). These neutral RC-carriers fused with acidic organelles that were then transported to the perinuclear area (**III, Fig. 4E, suppl. video S4**). Further fusion events between acidic ZsG-positive organelles in the perinuclear area resulted in the formation of very large static vacuoles, the previously described CPV-Is (**III, Fig. 4B-E, suppl. videos S3,S4**).

Particle tracking revealed that early in infection (around 2-2.5 h p.i), RCs in the periphery of the cell undergo very dynamic multidirectional movements. Speed measurements indicated that these small neutral RC-carriers use actin-dependent transport (**III, Fig 4A,C,D**) (Kaksonen et al., 2000). As blebbistatin experiments in fixed samples demonstrated that internalization of the RCs is severely delayed in the presence of this inhibitor, we used also blebbistatin during live cell imaging. Accordingly, addition of the drug early in infection almost instantly stopped all the movements of the ZsG-positive vesicles (**III, Fig. 5B,C**).

Another type of movement of the RC-vesicles was becoming more prevalent around 2.5-4 h p.i., when many neutral RC-vesicles were fusing with acidic organelles, followed by a fast directional long distance movement to the perinuclear area. Tracking of these vesicles suggested that microtubules are utilized for their transport (**III, Fig. 4**) (Matteoni and Kreis, 1987). Disruption of the microtubules with nocodazole supported this idea and illustrated clearly that no long distance directed movements occurred in the presence of the drug (**III, Fig 6**). Interestingly, addition of the drug early infection did not stop the multidirectional short distance movements of the RCs (not shown).

3D reconstruction of the ZsG-positive structures after live cell imaging at 4 h p.i. illustrated that nocodazole inhibited the accumulation of the RCs in the perinuclear area

and RCs no longer covered the surface of the acidic organelles. Instead, they stayed scattered throughout the cytoplasm and many did not manage to fuse with acidic organelles. Some patches of the ZsG signal were observed on the surface of the acidic organelles that had increased in size due to the presence of nocodazole (**III, Fig 6**).

The role of the microtubules was also studied in fixed samples. DsRNA staining of treated infected cells demonstrated that scattered RCs were active for replication. Time course experiment in the presence of nocodazole demonstrated that early in infection RCs still targeted the PM and formed the stripes in the bottom of the cell that are aligned with actin (not shown). However, the transport to the perinuclear area was blocked as RC-positive structures stayed scattered in the cytoplasm even late in infection (**III, Fig. 6**).

4.3.2 CPV maturation and stable perinuclear compartment (III and unpublished)

The described CPV-Is were shown to be modified endosomes and lysosomes with numerous spherules on their limiting membranes and having a diameter of up to 1-2 μm (Grimley et al., 1968; Froshauer et al., 1988; Kujala et al., 2001). We noticed that these large modified vacuoles are the end result of the complicated delivery of the RCs starting from the PM and reaching the perinuclear area 3-4 h later. As described in the previous chapter, microtubule-dependent transport is utilized to concentrate the RCs in the pericentriolar area and that always results in the formation of a half ring-shaped compartment around the Golgi complex (Fig. 8A). During live cell imaging recordings we noticed that ZsG-positive large acidic vacuoles keep fusing with each other and are gradually losing their motility (**III, Fig. 4B-E**). Around 4-5 h p.i. the majority of the acidic compartment of the cell was covered with RCs and transformed into a stable viral compartment (**III, Fig. 2D and 4B**).

By staining the infected cells with filipin, we noticed that CPV-Is were very rich in cholesterol unlike normal late endosomes and lysosomes. When quantifying the cellular cholesterol levels during SFV infection, slight increase was observed compared to mock-infected BHK cells (Fig. 8B). Confocal microscopy images demonstrated that all CPV-Is have accumulated cholesterol as they colocalize strongly with filipin staining (Fig. 8C). It is known previously that cholesterol accumulation in late endosomes and lysosomes leads to enlargement of the organelles and their motility is affected (Sobo et al., 2007). A very similar picture can be seen also with CPV-Is.

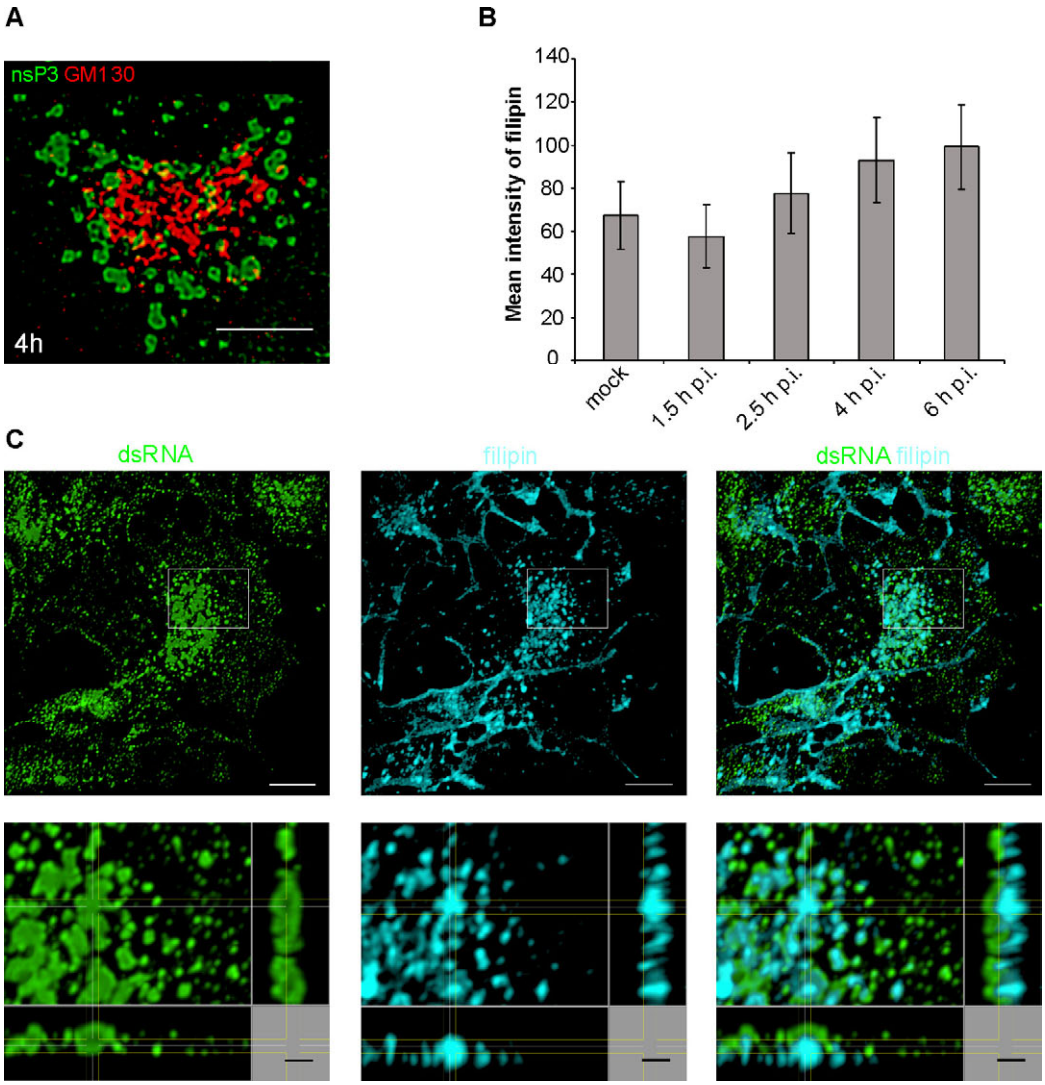


Figure 8 *Perinuclear viral compartment and accumulation of cholesterol in CPV-Is. A) Later in infection (4 h p.i.), CPV-Is (stained with anti-nsP3, green) are accumulating in the perinuclear area surrounding the Golgi complex (GM130 staining, red). Cells were infected with SFV (MOI=50), fixed at 4 h p.i. and processed for immunofluorescence imaging. The scale bar represents 10 µm. B) Cellular cholesterol levels visualized by filipin staining were measured in uninfected (mock) and SFV-infected BHK cells. Approximately 500 cells per sample group were analyzed and the mean intensities of filipin measured. SD was calculated for each sample group. C) Confocal microscopy images of SFV- infected cells. Samples were fixed at 6 h p.i and stained with filipin (cyan) and anti-dsRNA (green) antibodies. Maximum projection of the whole cell is presented in the upper panel, and an orthoslice view of the zoomed area (indicated with the white box) is shown in the lower panel. Merged images are illustrated on the right. Scale bar in the upper panel represents 10 µm and in the orthoslice image 2 µm.*

4.3.3 Role of RC trafficking in SFV replication and virus release (III and unpublished)

DsRNA staining of drug-treated infected cells indicated that blocking of the RCs at the PM or in the scattered vesicles does not visibly affect the viral replication. Therefore we labelled the viral RNA with uridine during the replication cycle in the presence of wortmannin or nocodazole. Non-treated sample served as the control. In concordance with the results described above, there was only a minor difference between the RNA levels in the presence of the drugs compared to the control (**III, Fig. 7, inset**). In addition, virus release in the presence of the drugs was tested and growth curves of the respective viruses were analysed. Interestingly, wortmannin enhanced virus release at 6 h p.i. and the titers reached a plateau after that. Surprisingly, nocodazole reduced the release of SFV compared to the non-treated sample (**III, Fig. 7**). However, this could be related to the later stages in virus life cycle as it was reported previously that nocodazole inhibits the transport of the envelope protein to the PM (Cid-Arregui et al., 1995). To test whether nocodazole interferes with the synthesis of the viral proteins, we performed an immunoblot analysis of SFV infected cells at 2 h and 4 h p.i. Staining with antibodies against viral non-structural and structural proteins demonstrated that there is no difference between a nocodazole-treated and non-treated sample (Fig. 9).

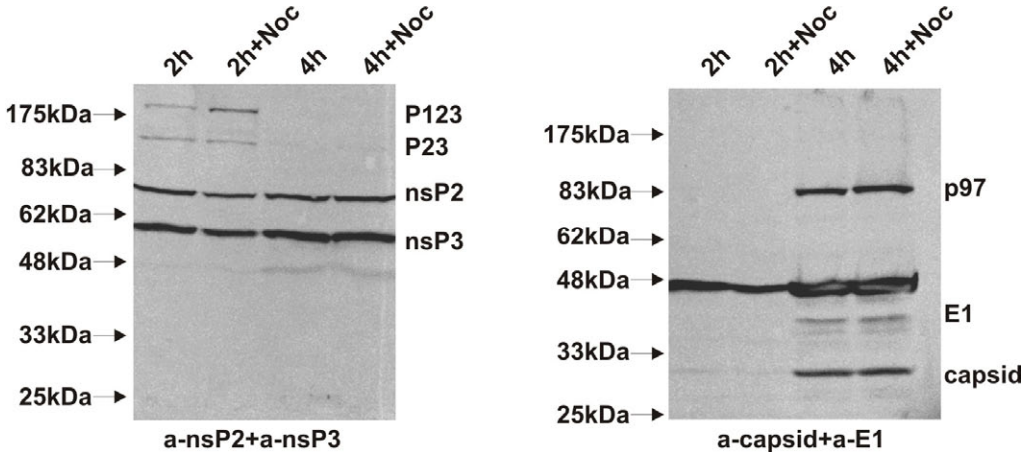


Figure 9 *Nocodazole treatment does not affect viral protein synthesis during SFV infection. Western blot analysis of SFV infected cells in the presence of nocodazole. Untreated samples served as positive controls. BHK cells were infected with SFV (MOI=500) and nocodazole was added from the beginning of infection. Samples were lysed at 2 h and 4 h p.i and cell extracts were subjected to immunoblot analysis. Anti-nsP2 and anti-nsP3 antibodies were used to verify the synthesis of non-structural proteins and anti-capsid and anti-E1 demonstrated the accumulation of structural proteins. Molecular mass markers are given on the left and the proteins expressed are indicated on the right. p97 is the first cleavage product of the structural polyprotein after releasing the capsid.*

4.3.4 Role of actin during the replication cycle (III and unpublished)

We noticed that during the movement of RCs from the PM to the perinuclear area, cellular actin became gradually disrupted and this correlated with the stage of RC trafficking (**III, Fig. 5A,F**). Interestingly, as long as the RCs were still aligned in stripes, there were actin fibres left. Concentration of the RCs to the perinuclear area seemed to be coordinated with actin disruption and at least a portion of the disrupted actin was localized together with CPV-Is in the perinuclear area (not shown). It should be noted that not all cellular actin became disrupted at 6 h p.i, as it was present in the PM projections (**III, Fig. 5F**). Palmitoylated nsP1 alone has been shown to rearrange the actin cytoskeleton, induce the filopodia-like extensions (Laakkonen et al., 1998) and nsP1 is localized at the PM. Therefore, we tested whether nsP1 could be involved in some signalling pathway leading to the rearrangement of the actin cytoskeleton. NsP1-transfected and SFV-infected cells were treated with several inhibitors interfering with actin dynamics and IPA-3, an inhibitor of p21(Cdc42/RacI)-activated kinase I (PakI) as well as Rho kinase (ROCK) inhibitor Y-27632 were selected for further studies.

In nsP1-transfected cells, PakI inhibition did not stop the formation of filopodia-like extensions that were negative for actin (Fig. 10, lower panel). However, the actin cytoskeleton looked less disrupted compared to the non-treated sample (Fig. 10). A more striking effect was seen with infected cells. IPA-3 treated sample had a much less disrupted actin cytoskeleton even at 8 h p.i. (Fig. 11B, central panel) whereas non-treated sample had practically no actin fibres left at this point (Fig. 11B, upper panel). It seemed that RC trafficking to the perinuclear area was delayed in IPA-3-treated samples, indicating a positive effect of actin disruption to the transport of RCs. However, at 8 h p.i. many cells had perinuclear CPVs in the presence of IPA-3 having more intact actin cytoskeleton than in untreated sample. In addition, actin-rich projections seen in infected cells (at 4-6 h p.i.) were practically not formed in treated samples even at 8 h p.i. (Fig. 11B, central panel).

Interestingly, we saw the opposite effect with the ROCK inhibitor Y-27632. Infected cells treated with this pharmacological inhibitor showed very severe actin disruption already at 4 h p.i. and actin-rich projections reaching out to the neighbouring cells were more abundant, more branched and longer (not shown). In addition, RCs seemed to be transported even faster to the perinuclear area and CPV-Is reached extremely large sizes by 4 h p.i. (Balistreri et al., 2010). At 8 h p.i., untreated sample showed even more abundant projections that were positive for nsP1 as well as for dsRNA and they formed a connecting network between neighbouring cells (Fig. 11B, upper panel). Y-27632 inhibitor enhanced this phenotype even further (Fig. 11B, lower panel). Therefore it seems that actin is needed in the early steps of RC internalization but actin disruption facilitates the transport of the RCs to the perinuclear area and the maturation of the CPV-Is. Nevertheless, CPV-Is are formed also without disrupting actin, indicating an additional role for the actin rearrangement.

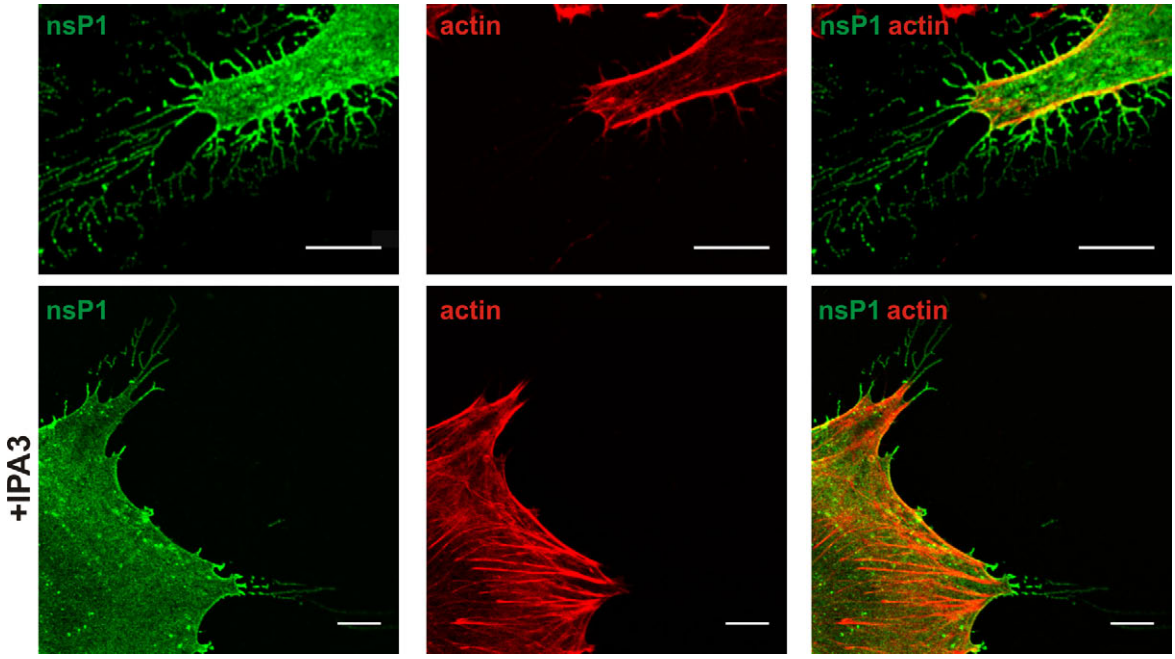
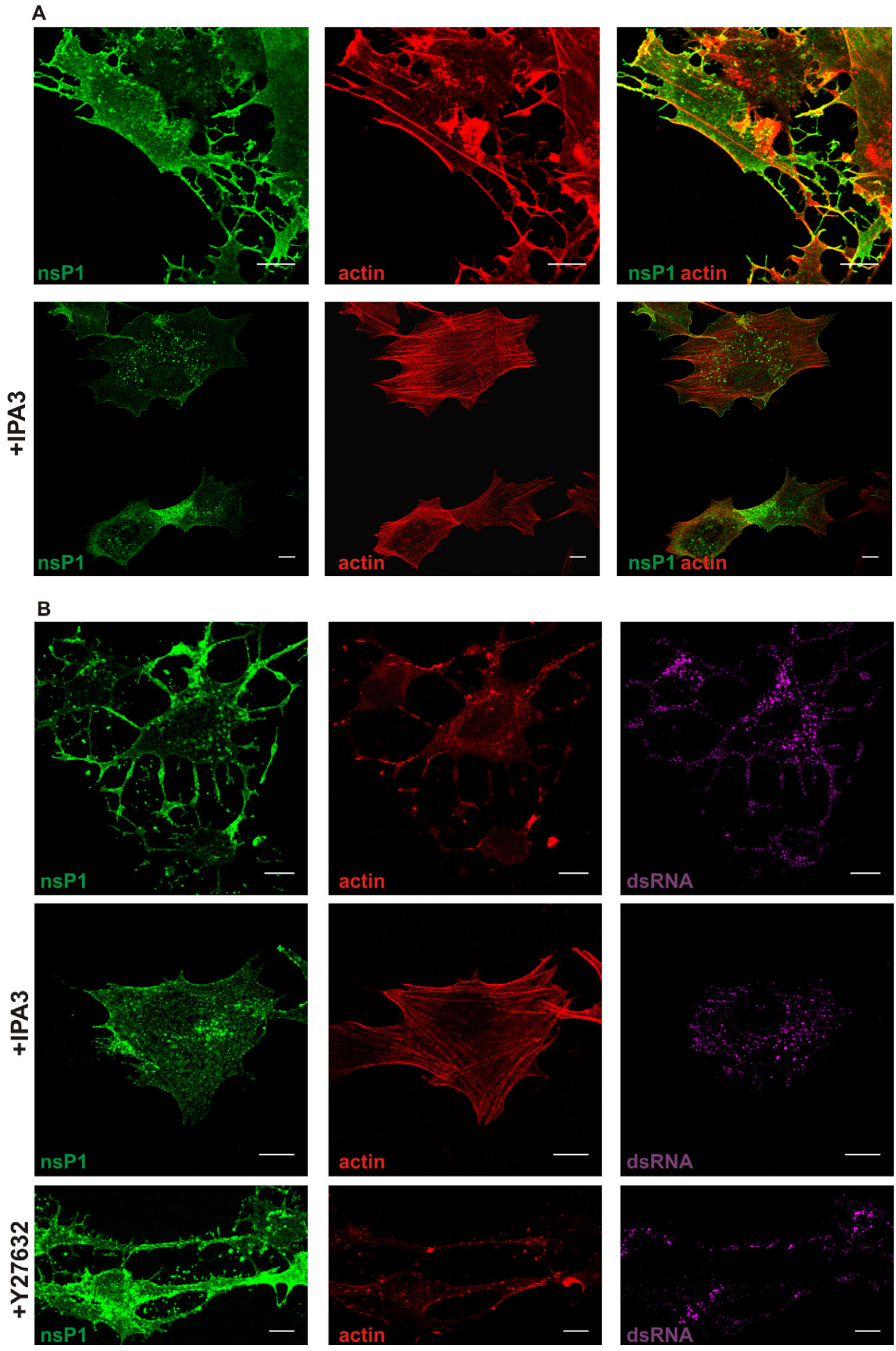


Figure 10 *Actin reassembly in nsP1 transfected cells. BHK cells were transfected with nsP1 4/TO and treated with IPA-3 (added 3 h p.t.) (lower panel). Untreated sample served as a control (upper panel). Cells were fixed at 8 h p.i., permeabilized and stained with anti-nsP1 (green) and phalloidin-A568 to visualize the actin (red). Merged images are shown on the right.). Confocal optical sections from the bottom of the cells are shown to illustrate the filopodia. Scale bar represent 10 μm.*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nsP1
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actin
dsRNA
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nsP1
actin
dsRNA

Figure 11 *Actin reassembly and formation of PM projections in SFV-infected BHK cells. A) BHK cells were infected with SFV (MOI=500). IPA-3 was added to the cells at 1 h p.i. and samples were fixed at 4 h p.i. Untreated sample served as a control. Cells were stained with anti-nsP1 (green) and phalloidin-A568 (red). Merged images are shown on the right. B) BHK cells were infected with SFV (MOI=500). IPA-3 or Y-27632 was added to the cells at 1 h p.i. and samples were fixed at 8 h p.i. Untreated sample served as a control. Cells were stained with anti-nsP1 (green), anti-dsRNA (magneta) and phalloidin-A568 (red). A,B) Confocal optical sections from the bottom of the cells are shown to illustrate the filopodia. Scale bar represent 10 μm.*

4.4 Targeting of the RC

As described in the previous section, the RCs of the alphaviruses are first targeted to the PM where the spherule structures are formed. Subsequently, RC spherules are internalized by an endocytic event leading to transport to the perinuclear area where they are found on the limiting membranes of large cytopathic vacuoles. Therefore, we were interested in determining the signals that drive the RCs first to the PM and then trigger the endocytic event.

4.4.1 Tandem BP mediates affinity for membranes but not specificity (I)

NsP1 alone is located on the cytoplasmic side of the PM (Peränen et al., 1995; Laakkonen et al., 1998) and is the membrane anchor for the whole RC. Previous work and experiments in the current thesis have shown that the BP mediates the primary binding of the RC to membranes. Therefore, it was interesting to see whether the BP itself contains a sufficient targeting signal for the PM. For that purpose, a set of GFP fusion proteins were made in which the BP was located in the N-terminus of the GFP as one, two or three copies. In addition, a spacer region GGSGSAG was added between the BP and GFP and the first methionine in GFP was changed to leucine to avoid additional initiation (**I, Fig. 6A**). These recombinant proteins were expressed in HeLa cells for 18-24 h and assessed by western blot, fractionation and flotation assays using anti-GFP antibody and with immunofluorescence following the epifluorescence of GFP (**I, Fig. 6**). PM was visualized with ConA. All the fusion proteins were expressed and no additional products were detected with western blot analysis (**I, Fig. 6B**). Simple fractionation analysis and flotation in sucrose gradient demonstrated that at least two copies of the BP are needed to change the properties of the GFP. 3xBP-GFP fusion protein was located exclusively in P15 fraction and floated with membranes demonstrating that this protein had acquired membrane binding features (**I, Fig. 6C,D**). Immunofluorescence analysis of GFP distribution in HeLa cells confirmed the results as two copies of BP were needed to change the localization of the recombinant protein (**I, Fig. 6F**). Nevertheless, even three copies of BP did not target the GFP to the PM; instead it was accumulating in the cytoplasm in a punctate pattern (**I, Fig. 6G**). The identity of the 3xBP-GFP-positive organelles was observed with immuno-EM using anti-GFP antibody. Interestingly, most

of the fusion protein was located on the mitochondrial membranes but also endo-lysosomal membranes were stained (Fig. 12). However, no signal was seen at the PM.

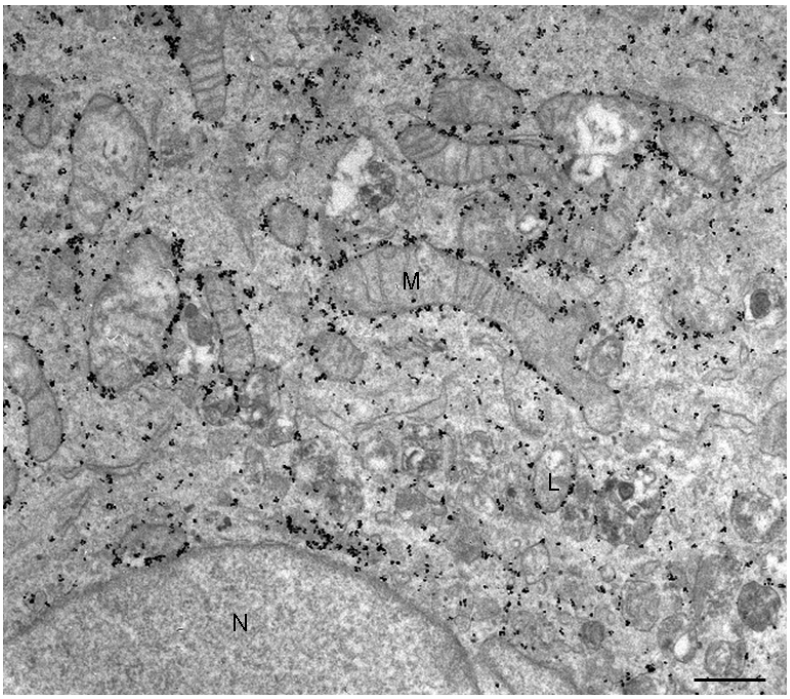


Figure 12 *Localization of 3xBP-GFP fusion protein expressed in HeLa cells analyzed with immuno-EM. The sample was fixed at 15 h p.t. and recombinant protein was stained with anti-GFP antibody. Scale bar represents 500 nm. N – nucleus, M – mitochondria, L – lysosome.*

4.4.2 Full-length nsP1 is needed for PM targeting (unpublished)

BP mediated the membrane binding of a fusion protein but did not target the protein to the PM suggesting that other parts of nsP1 are involved in determining the PM localization. Therefore, a set of nsP1 deletion constructs fused with the N-terminus of EGFP were made (Fig. 13A). All the fusion proteins were expressed at expected sizes and no additional products were detected with western blot analysis except for the 1-324-EGFP where a minor product with a smaller size was detected (Fig. 13B) Expression of recombinant proteins in HeLa cells showed that full-length nsP1 and nsP1-EGFP (aa 1-537) were localized mainly at the PM where filopodia-like extensions were induced (Fig. 123). The N-terminal portion of nsP1 without the BP sequence (aa 1-247) was diffuse in the cytoplasm as expected (Fig. 13C). Addition of the BP to the latter construct (aa 1-270) changed the localization of the protein and seemed to yield similar properties as for the 3xBP-GFP protein (Fig. 13C). A longer fusion protein (aa 1-324) showed a similar picture with some PM staining but no filopodia were induced (Fig. 13C). Fusion protein 180-537-

EGFP showed a completely different picture being equally distributed in the nucleus and in a reticular network of unknown identity (Fig. 13C).

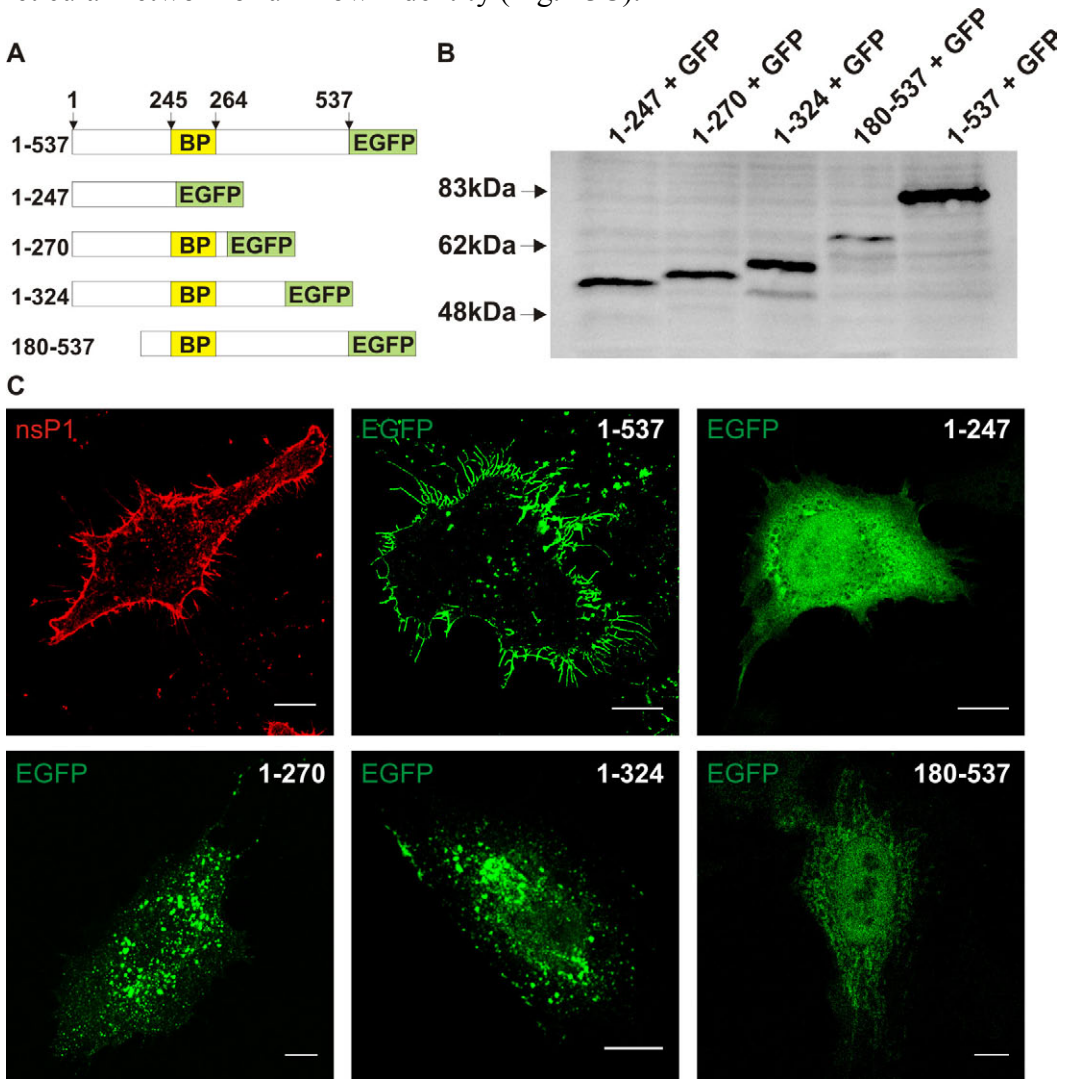
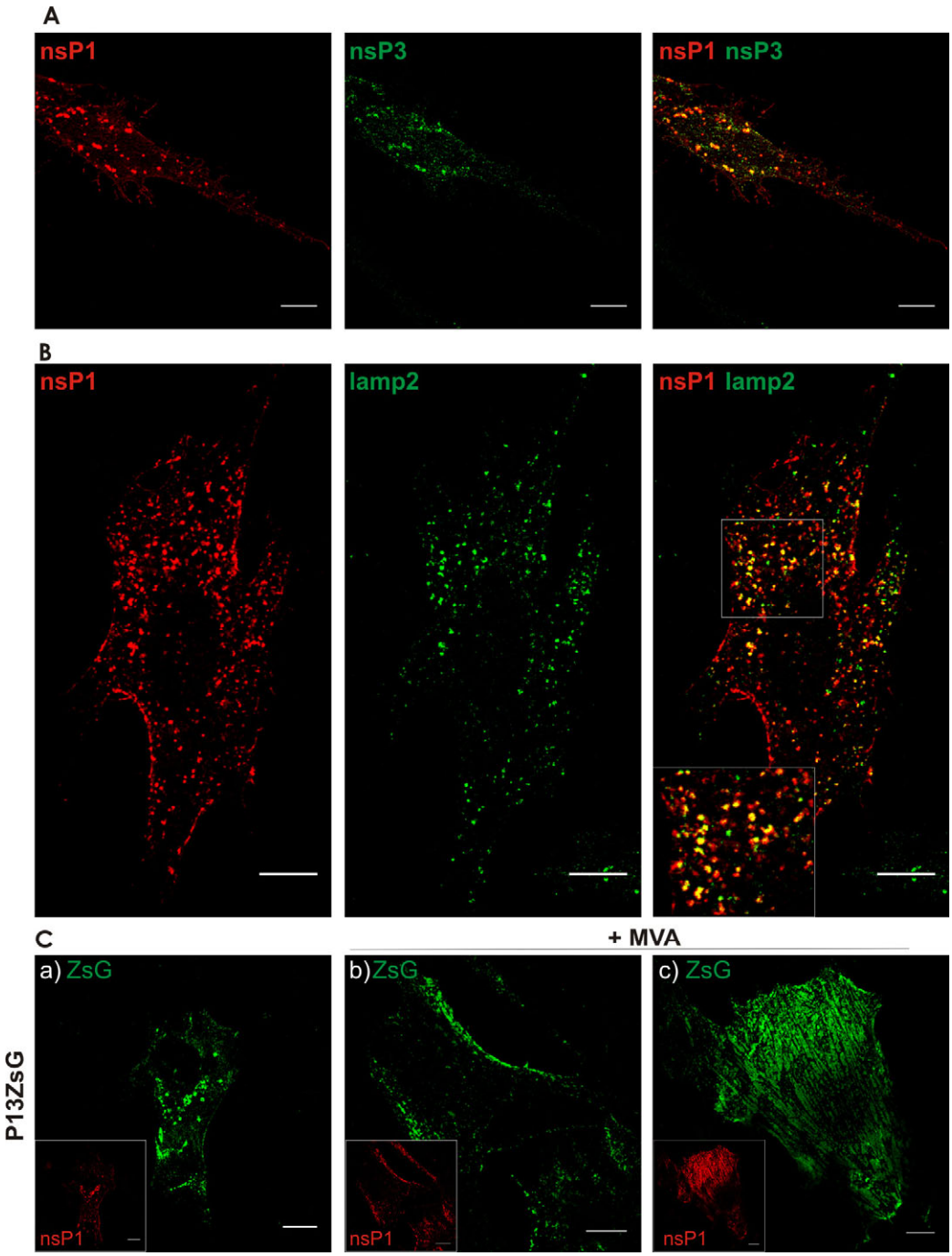


Figure 13 *Expression of nsP1 deletion constructs fused with EGFP in HeLa cells. A) Schematic representation of the full-length nsP1 (aa 1-537) and its deletion mutants fused with EGFP (indicated with a green box). BP region (aa 245-264) is highlighted with a yellow box. B) Western blot analysis of nsP1-EGFP and its deletion mutants expressed in HeLa cells. Samples were lysed at 24 h p.i and expression of the correct fusion proteins was verified with anti- GFP antibody. Molecular mass markers are given on the left. C) Localization of wt nsP1 and EGFP fusion proteins (shown in panel A) in HeLa cells. Samples were fixed at 24 h p.t. nsP1-pCDNA4/TO was used as a control for nsP1 expression under CMV promoter and localization of nsP1 was visualized with anti-nsP1 antibody (red). EGFP fusion proteins were analyzed based on EGFP epifluorescence. Scale bar in all the images represents 10 µm.*

4.4.3 Targeting signals of RC are located in nsP1 and nsP3 (unpublished)

Previous work has suggested that nsP3 contains signals to shift the RC from the PM to endo-lysosomal membranes (Salonen et al., 2003). The authors showed that $P12^{CA}$ localizes exclusively to the PM whereas $P12^{CA}3$ is targeted to endo-lysosomes. To confirm whether the targeting signals are located in nsP1 and nsP3, a fusion protein P13 lacking the nsP2 moiety was made. Western blot analysis showed that majority of P13 protein is expressed as a fusion protein. A minor product was detected with anti-nsP1 antibody migrating at the size of nsP1 however anti-nsP3 antibody recognized only the fusion protein (data not shown). Localization studies in HeLa and BHK cells demonstrated that P13 fusion protein was targeted to the PM. In addition, a large fraction of the protein was detected in vesicular structures in the cytoplasm (Fig. 14A). Colocalization with the lysosomal marker lamp2 indicated that most of these vesicles are of endo-lysosomal origin (Fig. 14B). For live cell imaging studies, P13ZsG fusion under T7 promoter was made. Expression of this recombinant protein showed that it behaves similarly to P13 as it is found on the PM and on the membranes of perinuclear vesicles (Fig. 14Ca). Anti-nsP1 staining was used to verify that ZsG signal represents the P13 (Fig. 14a, inset image). Interestingly, when this protein was expressed by the aid of MVA, the fusion protein was blocked at the PM, very similarly to $P12^{CA}3$ (Fig. 14Cb, compare with Fig. 6D). Cells expressing P13ZsG at high levels showed strong accumulation of the fusion protein at the bottom PM in aligned stripes (Fig. 14Cc, compare with III, Fig. 1Df). These results indicate that P13 contains the targeting signals of the RC.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nsP1
nsP3
nsP1 nsP3
B
nsP1
lamp2
nsP1 lamp2
C
+ MVA
P13ZsG
a) ZsG
b) ZsG
c) ZsG
nsP1
nsP1
nsP1

Figure 14 *P13 contains the targeting signals to PM and to endo-lysosomes. A) P13 under CMV promoter was expressed in BHK cells and the sample was fixed at 18 h p.t. Anti-nsP1 (red) staining shows the PM staining and intracellular vesicles typical to P123 expression. Anti-nsP3 (green) stains the same intracellular vesicles and some staining could be seen at the PM. Merged image is shown on the right. B) Colocalization of P13 positive structures (in red) in HeLa cells with lamp-2 marker (green). The sample was fixed at 18 h p.t. Colocalization is shown on the right, zoomed area is indicated with the white box. C) P13ZsG under T7 promoter was expressed in BSR cells expressing T7 polymerase (a) and BHK cells by the aid of MVA expressing T7 polymerase (b,c). Samples were fixed at 6 h p.i. and stained for anti-nsP1 (red). In image c, the optical slice from the bottom of the cell is shown. Scale bar in all the images represents 10 µm.*

5. Discussion

Similarly to other +RNA viruses, alphaviruses replicate their RNA in association with altered cellular membranes –reviewed in (Salonen et al., 2005)–. The current thesis was dedicated to dissecting the close interplay between the SFV RC and cellular membranes. The results presented here show that the membranes of the PM and the endocytic pathway serve as a playground for SFV RCs to gradually build up a stable perinuclear viral compartment. Early in infection the PM of the infected cell becomes enriched in viral replication proteins that induce the formation of the membranous RC-spherules. Through a specific endocytic event, spherules are internalized and subsequently transported to the perinuclear area. By 4 h p.i. viral RCs have taken over almost all of the cellular acidic organelles, caused them to cluster around the Golgi complex and turned them into productive stable viral factories. In addition, drastic changes in the actin cytoskeleton during the replication cycle result in the induction of abundant actin-rich PM protrusions that seem to connect neighbouring cells.

5.1 Membrane binding mechanism of SFV RC

The membrane-associated replication of SFV was demonstrated already in the late 1960s (Grimley et al., 1968) and subsequently the endo-lysosomal origin of the utilized membranes was shown (Froshauer et al., 1988; Kujala et al., 2001). The membrane binding properties of the RC have been studied for 15 years by our group (Peränen et al., 1995; Laakkonen et al., 1996; Laakkonen et al., 1998; Ahola et al., 1999; Ahola et al., 2000; Lampio et al., 2000; Salonen et al., 2003). SFV RC is anchored to membranes via nsP1, as other ns proteins do not show membrane binding properties (Peränen et al., 1995; Salonen et al., 2003). In infected or transfected cells, nsP1 is found at the inner surface of the PM (Laakkonen et al., 1996; Kujala et al., 2001), suggesting that it is specifically targeted there. The primary binding of nsP1 is thought to be mediated via an amphipathic helix (binding peptide, BP) in the middle of the protein (Ahola et al., 1999; Lampio et al., 2000). In addition, palmitoylation of cysteine residues in the C-terminal part of the protein tightens the binding and renders nsP1 similar to integral membrane proteins (Laakkonen et al., 1996). Nevertheless, palmitoylation is not needed for the enzymatic activities of nsP1 but mutations preventing palmitoylation render the virus non-pathogenic (Ahola et al., 2000).

The mechanisms for the membrane binding of the SFV RC and the role of the BP in virus replication were studied in the current thesis (**I**). As all previous studies concerning the role of the BP were carried out with synthetic peptides and nsP1 translated *in vitro*, the effect of the BP for the membrane affinity and localization of nsP1 in mammalian cells were studied as well. Based on the previous NMR studies with the BP, it was assumed that the hydrophobic residues which are concentrated on one side of the amphipathic α-helix will be in contact with the acyl chains of the phospholipids upon binding to membranes,

whereas the positively charged polar residues would interact with the head groups of the anionic phospholipids (Fig. 2B). This is supported also by a helical wheel projection of the BP (aa 245-262) clearly showing that the helix posses a hydrophobic face and a polar face (Fig. 15A,C). The polar face consists of basic (positively charged) residues as well as polar residues such as serine and threonine. Analysis of amphipathic helices has indicated that the presence of basic residues on the polar face favours the binding of the peptide to negatively charged lipids through electrostatic interactions. In contrast, lack of basic residues and enrichment in polar residues (serine and threonine) changes the sensitivity of the peptide for lipids (the charge of the lipids is not crucial any more due to the lack of electrostatic interactions between the helix and lipids) but renders the peptide to sense membrane curvature (Drin and Antonny, 2010). It is thought that lack of basic residues makes the curvature a prerequisite for membrane adsorption, as increasing curvature spreads the lipids apart and favours the intercalation of hydrophobic residues. It has been experimentally proven that addition of basic residues to the polar face of the helix drives the peptide to negatively charged lipids but these peptides have lost their sensitivity for membrane curvature (Drin et al., 2007). Therefore it is believed that the presence of basic residues in the polar face promotes the binding to flat membranes rich in negatively charged lipids and often induces membrane curvature (Drin and Antonny, 2010). Based on the helical wheel projection (Fig. 15A) it could be interpreted that BP of nsP1 preferably binds to flat membranes containing negatively charged lipids. The latter are enriched on the cytoplasmic side of the PM. Therefore, the membrane binding of the BP seems to be mediated through electrostatic and hydrophobic interactions.

A set of mutations was inserted into the BP region, including changes in basic residues to more neutral alanine or to negatively charged glutamate, and hydrophobic residues to alanine (Fig. 15). Some of these mutations showed drastic effects on the enzymatic activities of nsP1 expressed in E.coli and for liposome binding of synthetic peptides and in vitro synthesised nsP1 (Ahola et al., 1999; Lampio et al., 2000). Therefore, these mutations were tested in the context of virus infection and for the properties of nsP1 expressed in animal cells.

The effects of the mutations could be divided into three groups: 1) neutral/positive (K254E and R257E), 2) lethal (Y249A, R253E and W259A) and 3) strongly deleterious, leading to the acquisition of compensatory mutations (R253A and L255A+L256A). Interestingly, the residues that were crucial in the membrane binding concentrate on one side of the helical wheel (Fig. 15A, red ovals) whereas more flexible residues are on the other side (Fig. 15A, black ovals). When marking the important residues on the predicted α-helix (Fig. 15C, red circles), it seems that residues R253, L256 and W259 form a central patch extending from the polar to the hydrophobic face.

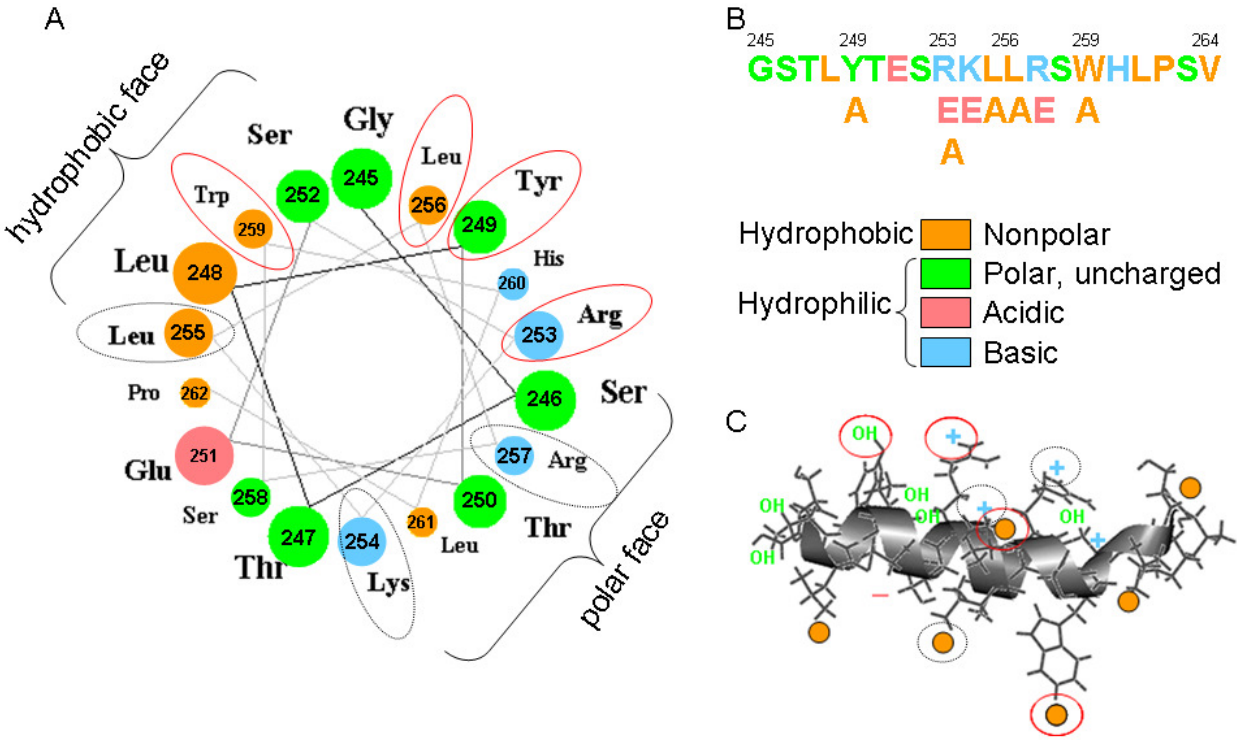

Figure 15 *A) Helical wheel projection of the amphipathic a-helix (BP) of SFV nsP1 (aa 245-262 are projected). Publicly available software was used to draw the helical wheel: http://cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html. The size of the aa circles is decreasing according to the position in the helix. The colour code of the circles is explained in panel B. Crucial residues for the membrane binding of RC and SFV replication are highlighted with red ovals and residues that allowed changes are highlighted with black ovals. B) The linear aa sequence of the BP. Mutations used in this study are indicated under the sequence. The colour code is explained in the figure. C) Solution structure of the BP (determined by NMR) shows amphipathic nature as hydrophobic residues (marked with orange circles) are concentrated on one side whereas basic (blue +) and hydroxylated polar residues (green OH) form the polar face of the helix. Crucial and flexible residues in the membrane binding of RC and SFV replication are marked with red and black circles, respectively.*

Experimental data demonstrated that the mutations K254E and R257E did not alter the properties of nsP1 nor the infectivity of the virus. Comparison of the BP sequences between alphaviruses shows that these two residues are rather variable. K254 is D254 in the case of EEE and R257 is Q257 in SINV, Aura and some others. Therefore it is not surprising that acidic glutamate was tolerated in these positions. Furthermore, it has been shown that the presence of negatively charged residues in the middle of the polar face might enhance membrane binding (Mishra and Palgunachari, 1996). Interestingly, these mutations heavily reduced the methyltransferase activity of nsP1 in *E. coli* (Ahola et al., 1999). It is possible that in animal cells where nsP1 is palmitoylated and the membrane binding is a more complex process, nsP1 behaves differently than when expressed in *E. coli*. In addition, nsP1 as a part of the RC might act differently than nsP1 alone. It is also

possible that even a low level of methyltransferase activity may be sufficient for virus viability. Discrepancies between nsP1 expressed in E. *coli* and in animal cells could be also noticed with the mutant R253A. The methyltransferase activity of mutant nsP1 was 20% and in vitro synthesised R253A nsP1 bound to liposomes. However, this mutation weakened the membrane binding of nsP1 expressed in HeLa cells and was lethal to virus replication. This suggests further that the membrane binding of nsP1 and the entire RC in animal cells is more complex than in *in vitro* systems and that it was necessary to test the effect of the mutations pinpointed previously in the context of infectious virus. This was especially useful in the case of R253A and L255A+L256A mutants, which acquired compensatory mutations that probably restored the membrane binding and allowed replication. These additional mutations highlighted important regions in nsP1 for the membrane binding process and for the RC to be functional.

R253A virus acquired several different compensatory mutations elsewhere in nsP1 while maintaining the original mutation. Interestingly, these additional mutations compensated the initial impairment with different efficiencies and resulted in viruses with diverse phenotypes. In many cases, an aspartate was changed to glycine indicating that removal of the charge might be an important factor. Compensatory mutations D202G and D437G were both needed to restore the R235A virus replication to wt level highlighting an interplay between different regions of nsP1. Interestingly, in the position 437 SIN originally has a glycine. When this mutation was cloned into wt SFV4 backbone, it increased virus infectivity around four fold. Thus this supports the previously proven concept that the wt ns protein sequences represent evolutionary compromises and have been adapted to a certain environment.

In contrast to R253A, L255A+L256A virus always yielded the same pseudorevertant, L255A+L256V, demonstrating that only this combination was viable. The Ala-Val dipeptide was encoded by the sequence GCTG**T**G, whereas the original mutation was GCTG**C**G (Ala-Ala). In the wt sequence, there is a leucine in the position 256 and the corresponding codon is CTG. As can be seen, pseudoreversion to the wt amino acid residue would have required a two nucleotide change, so it is logical that valine as the closest amino acid to leucine and requiring only one nucleotide change was chosen. Other possible amino acid residues that could be achieved with changing only one nucleotide in the alanine codon beside valine are threonine, proline, serine, glutamate or glycine. Therefore solely valine, as the only hydrophobic residue in this list, can be allowed. When the L255A+L256V mutation was transferred to icDNA, it proved its ability to compensate the low initial infectivity and yielded high titres with time similar to wt. Thus, alanine in the position 256 is not tolerable and certain degree of hydrophobicity is needed in this position of the peptide.

Very low infectivity of R253A and L255A+L256A in the infectious centre assay demonstrates that only in very few cells mutant viruses appear and are capable of starting replication, and Figure 7A (**I**) clearly illustrates the situation of slow accumulation. Actually, the infectivity of these viruses can be used to calculate the frequency of these mutations, 1.2×10^{-5} being the likelihood for the L255A+L256V mutation and 2.5×10^{-4} for the compensatory mutations in R253A virus. This is in agreement with the error rate caused by RdRp polymerase reported previously (Domingo et al., 1997). Therefore these

results confirm an outstanding potential of alphaviruses, and actually all plus-strand RNA viruses for adaptation and survival due to high mutation rate. Even within one passage, replication can be adapted and compensatory mutations arise. Nevertheless, the functional importance of these additional mutations remains unknown until more thorough studies will be carried out.

Mutations Y249A, R253E and W259A turned out to be totally lethal as no replicating viruses were observed. Y249A mutant protein was membrane-bound to some extent but weaker binding was evident as the protein was found also in the cytoplasm. However, it has been shown previously that Y249A is absolutely deficient in the methyltransferase activity (Ahola et al., 1999), and therefore it may be possible that the loss of infectivity of the corresponding virus is a consequence of the absence of enzymatic activity rather than membrane association. Although mutants R253E and W259A possessed severely reduced methyltransferase activity, it is more plausible that in these cases the loss of enzymatic activity was due to the deficiency in membrane binding, as these mutant proteins remained soluble in the cytoplasm. Therefore we can conclude that the residues R253 and W259 are directly linked to the ability of the RC to bind membranes. The exact role of other critical residues such as Y249 in the binding process remains uncertain as the infectivity is directly dependent on the enzymatic activities, which are altered by the membrane association and therefore it is very difficult to say what is the cause and what is the consequence. Alternatively, it is possible that due to an altered conformation of nsP1, its interactions with other ns proteins are disrupted and no functional RC is formed. Further experiments could be carried out in the plasmid-derived system where the production of the replicase components does not depend on replication (see below).

Taken together, the results in this thesis confirm the essential role of the BP in the anchoring of the RC and point out important residues in the binding process. Altogether, these four residues Y249, R253, L256 and W259 are the most conserved residues in the BP region of alphaviruses indicating that membrane binding of other alphaviruses might be mediated by similar mechanisms.

The membrane binding mechanism of amphipathic helices has been shown to include three main steps. First, electrostatic interactions attract the unfolded peptide to the vicinity of negatively charged lipids. Then, the hydrophobic residues are inserted into the lipid bilayer. These two steps are considered as fast processes. The third slow step coverts the unfolded peptide into an amphipathic helix that completes the binding process –reviewed in (Drin and Antonny, 2010)–. As palmitoylation of nsP1 seems to change the properties of the protein from the peripheral type to the integral type it could be imagined that palmitoylation has to occur before the slow step (formation of the amphipathic helix). From our results it was clear that palmitoylation is dependent on membrane binding (soluble R253A and W259A mutant nsP1 were not palmitoylated) indicating that palmitoylation should take place after the electrostatic interactions. The enzymatic activities of nsP1 are not dependent of palmitoylation suggesting that the first binding step, electrostatic interactions are sufficient to change the properties of nsP1. However, Pa^- virus is replication deficient indicating that proper membrane binding and assembly of the RC requires all the steps in the binding process. Based on the principles described above

for the membrane binding of amphipathic helices and our experimental data I would propose a model, how the binding and the assembly of SFV RC might occur.

First, the polyprotein is attracted to the PM through the electrostatic interactions between nsP1 (mainly the BP region) and negatively charged phospholipids (peripheral type of binding). Basic residues in the BP play an important role. This step is followed by palmitoylation that triggers a possible conformational change in nsP1 allowing the insertion of hydrophobic residues to the lipid bilayer. Finally, the formation of amphipathic helix completes the binding and attaches the RC firmly to the membranes. The change triggered by palmitoylation seems to be needed for the proper interactions between nsP1 and nsP4 (Zusinaite et al., 2007) and for the functionality of the RC. It is possible that induction of membrane bending requires the palmitoylation as Pa^- nsP1 does not form the filopodia. Although as discussed in the next chapter, conformational change in nsP1 might be needed to bind host factors that assist in induction of membrane alterations. Anyhow, it has been shown that the change in nsP1 is important not the palmitoylation itself as the compensatory mutations can overcome the absence of palmitoylation (Zusinaite et al., 2007). It would be interesting to test whether these Pa^- nsP1 variants with compensatory mutations would have acquired properties similar to integral type of proteins. However, it remains to be elucidated what is the role of palmitoylation as it seems to be involved in pathogenesis of the virus (Ahola et al., 2000).

+RNA virus RCs are designed to assemble on intracellular membranes and their function is strictly dependent on lipid composition. Continued lipid synthesis seems to be crucial for the replication of many viruses. Cerulenin, an inhibitor of lipid biosynthesis, severely inhibits the replication of alphaviruses, as well as poliovirus and HCV (Perez et al., 1991; Guinea and Carrasco, 1990; Sagan et al., 2006). However, it is apparent that different viruses have adapted to function in association with different lipids. Membrane binding and enzymatic activities of SFV RC are dependent on negatively charged phospholipids, such as PS (Ahola et al., 1999; Lampio et al., 2000). Similarly, SIN infection was defective in cells devoid of PS (Kuge et al., 1989) implying a preference for negatively charged phospholipids for the entire family. This is not surprising as the binding module, amphipathic peptide, is very conserved throughout the whole alphavirus family as discussed above. Replication of BMV is very sensitive to biosynthesis of unsaturated fatty acids and defects in that pathway severely affect specific early steps in virus replication (Lee et al., 2001). The authors speculated that the assembly and functioning of the BMV RC might need highly fluid membranes. Similar observations were made with Japanese encephalitis virus, where an unsaturated fatty acid (oleic acid) enhanced virus replication (Makino and Jenkin, 1975). In contrast, poliovirus replication is inhibited by the addition of oleic acid (Guinea and Carrasco, 1991). However, this virus seems to increase the cellular phophatidylcholine levels (Vance et al., 1980). Geranylgeranyl lipids are required for the replication of HCV and flaviviruses. In addition, cholesterol seems to play an important role in the replication of these viruses (Ye, 2007; Mackenzie et al., 2007). All these examples illustrate that the interplay between RCs and cellular membranes is very complex and further studies are needed to explain the functional mechanisms of the membrane association of the viral RCs. However, it is clear

that the assembly and activity of the RCs require intact membranes and specific properties/composition of the cellular lipids.

Membrane binding of the RC is absolutely needed as even a single point mutation in the membrane binding region can be lethal for virus replication. Similar observations have been made with other +RNA viruses. In the case of BMV, all the alterations made in the 1a amphipathic helix region were lethal showing a very delicate conformation of the RC. Interestingly, even mutations that did not destroy spherule formation rendered the virus non-infectious (Liu et al., 2009). In the case of flaviviruses, hepaciviruses and picornaviruses, the membrane binding of the RCs is more complicated as more than one replication protein is membrane-associated and contributes to the formation of the membrane-bound complex. However, it is intriguing to see that alterations even in one membrane binding motif are sufficient to abrogate membrane binding and viral replication. In HCV, NS4B has several membrane binding domains, including amphipathic helices in the N-and C-termini. RC formation and virus replication was completely destroyed when replacing the hydrophobic or aromatic residues with alanines in either of the helices (Gouttenoire et al., 2009a; Gouttenoire et al., 2009b). The same effect was seen with poliovirus, where aa changes in the hydrophobic domain of 3A destroyed membrane association, resulted in impaired replication and were lethal to virus (Giachetti et al., 1992; Towner et al., 1996). Interestingly, all these motifs described to be crucial for the membrane binding of viral RCs show amphipathic peptide features. Some viruses have additional binding mediators such as transmembrane motifs, but all +RNA RCs described to date have amphipathic peptides in their RCs mediating membrane binding. Therefore it is likely that this motif is used by many viruses in order to fulfil specific tasks such as altering cellular membranes. And as all the +RNA viruses described rearrange cellular membranes, it is tempting to believe that amphipathic peptides are involved in this process. As alphaviruses have only one replicase protein that mediates the binding through one amphipathic peptide, they serve as a good model for studying the membrane binding mechanism. Nevertheless, care should be taken as alphaviruses together with rubiviruses are the only group known to utilize endo-lysosomal membranes to build up their membranous RCs, and alphavirus superfamily seems to be unique in that their membrane alterations resemble spherules. All the other virus groups induce more extensive membrane alterations such as the membranous web (HCV), DMVs associated with autophagy (poliovirus) or DMVs, CMs and VPs (coronaviruses) (Gouttenoire et al., 2010; Taylor and Kirkegaard, 2008; Knoops et al., 2008). This might reflect the fact that all these other virus groups use more than one replicase protein and many motifs to attach their RCs into the membranes, resulting in more complex alterations.

5.2 Spherule formation

As mentioned in the previous chapter, all positive-strand RNA viruses are known to induce membrane alterations to facilitate their replication. However, the mechanisms by which viral RCs are able to create these complicated membranous assemblies remain still incompletely understood.

Amphipathic helices have been shown to be involved in either curvature sensing, both positive and negative (described above) or remodelling of membranes. Analysis of SFV BP suggested that the presence of basic residues in the polar face would exclude curvature sensing properties, perhaps favouring involvement in membrane shaping. However, the cases reported up to date show that insertion of an amphipathic helix induces positive curvature –reviewed in (Prinz and Hinshaw, 2009; Drin and Antonny, 2010)–. SFV nsP1 and RC lead to the formation of filopodia and spherules, respectively. These structures represent negative curvature. In many cases the formation of membrane alterations involves the assembly of several proteins forming large complexes. In addition, the preservation of defined shapes might require additional factors. Therefore it is likely that nsP1 and RC while localized to the PM recruit cellular proteins that assist in membrane bending and formation of the membranous RC.

It cannot be said whether the formation of filopodia-like extensions induced by palmitoylated nsP1 is mechanistically similar to the formation of spherules. As nsP1 is the only membrane anchor of the complex it could be imagined that the same membrane bending principles mediated by the BP are used. However, nsP1 in the context of RC might be folded in a unique way or act differently and the outcome is rather distinct, namely a very uniformly sized spherule. Alternatively, nsP1 alone might bind different host factors mediating the formation of tubule-like structures. As I-BAR proteins have been shown to induce PM protrusions (Saarikangas et al., 2009) it is possible that similar host proteins are assisting nsP1. In the case of the RC, nsP1 or some other replicase protein might recruit distinct cellular proteins that regulate the induction of spherules. Evidently, a more complex and controlled process must underlie spherule formation as these structures are very uniform in size and their induction seems to require all the replicase components including the template. Furthermore, template size affected the size of the spherules indicating a distinct mechanism from membrane alterations caused by nsP1 alone. Among host proteins, the most suitable candidates for mediating spherule formation would be the ESCRT proteins as they induce negative curvature and assist in the formation of structures similar to spherules. The biogenesis of MVBs and the induction of intralumenal vesicles seem rather similar, especially the steps of vesicle formation (Prinz and Hinshaw, 2009). Therefore it is possible that some specific ESCRT proteins are recruited to the RCs. As ESCRT components are also involved in enveloped virus budding e.g. HIV, (Raiborg and Stenmark, 2009) it indicates that PM structures similar to spherules can be induced by ESCRT proteins.

In the case of Tombusviruses (TBSV) it has been shown that cellular ESCRT proteins are recruited to the viral RC assembly sites on the membranes of peroxisomes and they assist in the formation of the membranous RCs (Barajas et al., 2009). In the same paper it was shown that in the absence of certain ESCRT proteins the activity of viral RC was greatly reduced and viral RNA templates became more sensitive to ribonuclease treatment, suggesting that RCs were not properly assembled and membranous spherules could not protect the template anymore. In the plant host, viral replication was inhibited when several dominant negative ESCRT protein mutants were expressed. In addition, it was observed that dominant negative ESCRT proteins Vps4p and Snf7-1p interfered with

spherule induction and resulted in membrane alterations with irregular size and shape (Barajas et al., 2009).

Changes in the membrane binding domains have been shown to affect the membrane bending properties of the proteins. Our EM studies showed that K243E and R257E mutations in the BP region did not change the morphology of induced spherules, as they looked indistinguishable from the wt ones. Also the pseudorevertant L256V caused spherules very similar to wt. In the case of BMV, it was shown that changes in the 1a amphipathic helix had severe effects on virus replication. Interestingly, even mutations that allowed the formation of spherules (although smaller in size) were intolerable for the virus (Liu et al., 2009). That might reflect the fact that spherule formation (membrane alteration) is a tightly regulated process and only mutations that do not change the properties of these alterations are tolerated, such as K254E, R257E and L256V. It would be interesting to test whether mutations L256A or R253A that still allowed the membrane association of nsP1 induced membrane spherules different from the wt as in the case of BMV (Liu et al., 2009), but these changes are too drastic for the virus and lethal for replication. It was seen with the plasmid-derived system that template size has a positive correlation with the spherule size, indicating that viral RNA is one factor that determines the size of the membrane alterations for SFV. Therefore if mutations in the amphipathic peptide do not allow the formation of spherules needed to accommodate the viral RNA, it might be lethal for the virus. That might be the case also for BMV, where they saw that smaller spherules do not support viral replication. It would be interesting to test whether these smaller spherules caused by the mutations in the amphipathic peptide would support the replication of smaller templates.

It is interesting to speculate, whether the mutations L255A+L256A and R253A allow the formation of early minus-strand RC and minus-strand synthesis to some extent. This would be required in order to produce minus-strands or plus-strands with the compensatory mutations. In the case that a membrane-associated complex is formed, this could be followed in the plasmid-driven system. Especially interesting would be to see, whether the compensatory mutations would change the properties of spherule formation. It could be hypothesised that in the case of a low infectivity, the membrane alterations would be different from the wt case or that they are less in number.

5.3 Targeting of the viral RC to cellular membranes

All the viral RCs described are targeted to specific membranes. SFV RC is targeted to the PM and this seems to be common to all alphaviruses. It has been reported that SIN RCs are first accumulating at the PM (Gorchakov et al., 2008). Based on our studies, in addition to SIN, also CHKV RCs appear at the PM at the initial stages of replication (our unpublished data). BMV replicates on the membranes of ER (Restrepo-Hartwig and Ahlquist, 1996). ER is also used by many other viruses, such as HCV and flaviviruses (Welsch et al., 2009). Picornaviruses seem to use autophagosomal membranes for their replication (Jackson et al., 2005). Nodavirus RCs are targeted to mitochondrial membranes (Miller et al., 2001), and TBSV to the membranes of peroxisomes (McCartney et al.,

2005). However, in many cases the details of the targeting mechanism and the origin of the targeted membranes remain incompletely understood.

In alphaviruses, nsP1 is the sole membrane anchor of the complex and as shown in this thesis, directs the other replicase proteins to the PM. Time course experiments demonstrated clearly that early in infection active RCs first appear at the PM and continue to accumulate there. Treatment with pharmacological inhibitors supported the idea further, as the RCs were blocked at the PM by different inhibitors for P13K and by interfering with the actin cytoskeleton. Blebbistatin, an inhibitor of non-muscle myosin II delayed the internalization. A similar effect was also seen with dynasore, genistein and AktI inhibitor (Balistreri et al., 2010). However, these inhibitors affect the actin cytoskeleton and therefore it is not clear whether the effect is direct as we saw that actin is crucial for internalization. Use of pharmacological inhibitors should be interpreted with care and alternative experiments have to be performed to support the effect. We have started to test siRNAs against clathrin, caveolin, dynaminII and PI3K, but more experiments are needed to make conclusions from these studies.

The internalization of the RCs was also blocked by MVA, a DNA virus that replicates in the cytoplasm of infected cells and creates membranous miniorganelles derived from ER membranes to support its replication (Tolonen et al., 2001). MVA is a modified version of vaccinia virus, generated by repeated passing in chicken embryo fibroblasts, resulting in a virus with 65 genes deleted or inactive compare to wt virus (Antoine et al., 1998). These changes allow MVA to produce infectious virus in BHK cells but not in HeLa cells where the virus assembly is blocked (Carroll and Moss, 1997; Drexler et al., 1998). Although it has been shown that MVA does not induce cellular changes and rearrangement of actin cytoskeleton similar to wt vaccinia, nearly all of these studies have been carried out in HeLa cells (Schepis et al., 2006; Schramm et al., 2006). A comprehensive microscopy study of MVA influence to BHK cells and their cytoskeleton/membranes has not been carried out. Therefore, it cannot be said why MVA blocks the SFV RCs at the PM, but it is clear that in BHK cells MVA induces changes that restrict the internalization of spherules.

An interesting example of the targeting of viral RCs was demonstrated with FHV. Its protein A is targeted to the outer membranes of mitochondria through an N-terminal sequence that contains a transmembrane domain (Miller and Ahlquist, 2002). Replacing this sequence with an ER-targeting motif redirected the RC to the membranes of ER and even enhanced the replication of FHV (Miller et al., 2003). This intriguing result raises a question whether the specific membranes are an absolute requirement for plus RNA virus replication. Based on this example it could be said that there must be some additional reasons why different viruses have evolved to target specific membranes. Another important observation was made in the Miller et al. (2003) study concerning the morphology of the membrane alterations induced. Interestingly, change of the membranes where RCs were assembled also altered the outcome of induced structures. Instead of clustered mitochondria with spherule-like invaginations seen during FHV replication, perinuclear layers appeared due to the ER targeting (Miller et al., 2003). Therefore these results suggest that targeting motifs and membrane binding domains determine the

membrane shaping properties. Alternatively, the origin of the membranes used for RC assembly might determine at least to some extent the morphology of the alterations.

5.4 Targeting signals in replicase proteins nsP1 and nsP3

Expression of the replicase proteins as a polyprotein P123 is an absolute pre-requisite for the assembly and correct localization of the RCs. Only nsP4 can be provided separately (Salonen et al., 2003). As mentioned already before, nsP1 is targeted to the PM when expressed alone whereas the polyprotein is internalized from the PM and transported to the membranes of endosomes and lysosomes. The specific signals needed for the shift in the localization were assumed to be in nsP3, as P12 still localizes to the PM but P123 is found also on the membranes of the cytoplasmic vesicles (Salonen et al., 2003). In this study, we wanted to investigate further and map more precisely the targeting signals.

Amphipathic helices have been shown to direct the proteins to the PM or other specific locations. For example, HCV NS5A contains an N-terminal amphipathic helix that mediates the membrane binding and targeting of the protein. The helix itself is able to direct GFP to ER membranes indicating that it contains the necessary targeting information (Brass et al., 2002). Many cellular signalling molecules have been shown to contain amphipathic helices that target them to the PM and mediate membrane binding. G protein-coupled receptor kinase 5 (GRK5) contains a C-terminal predicted α-helix that is able to target GFP to the PM (although only partially) (Thiyagarajan et al., 2004). The authors suggest that other regions of the protein might be needed for stronger PM localization.

Mutational analysis of the BP region highlighted that residues W259 and R253 are crucially involved in the targeting of SFV nsP1, and the entire RC to its preferred destination, the inner surface of the PM. However, the BP itself was not sufficient to target EGFP to the PM suggesting that the amphipathic helix outside of the nsP1 context does not contain the targeting signals to the PM. Nevertheless, a tandem copy of the BP rendered EGFP to a membrane binding protein, localizing mainly to the membranes of mitochondria. This is rather surprising as mitochondrial lipid composition is very different from that of the PM. PS levels are extremely low as well as cholesterol (van Meer, 1989). On the other hand, PG and CL (lipids that were able to restore the enzymatic activities of nsP1 in addition to PS), are present at low levels at the outer membranes of mitochondria (van Meer, 1989; van Meer et al., 2008) possibly providing a preferred targeting platform for the tandem-BP-GFP protein. Interestingly, PG and CL are lipids unique to mitochondria (van Meer et al., 2008). The fact that they activated the enzymatic activities of nsP1 expressed in E. coli would suggest that mitochondrial membranes might serve as an alternative targeting/binding platform for nsP1. It is likely that BP outside of the context of nsP1 in which it is located in the middle of the protein, has acquired altered properties being in a tandem copy in the N-terminus of the GFP. As the major lipids at the outer membranes of mitochondria are PC and PE (van Meer et al., 2008), it cannot be ruled out that tandem-BP-GFP would preferentially target these lipids.

Therefore it seems that other signals in nsP1 contribute in the targeting process and might add e.g. necessary charge to specifically bind lipids at the PM. Compensatory mutations arising during mutant virus replication might highlight the important regions in nsP1 that can contribute to the targeting and binding process. Attempts to make deletion mutants of nsP1 confirmed that nsP1 is a very delicate protein and its fold seems to be very sensitive to deletions. Fusion protein 1-324-EGFP (nsP1 aa 1-324 fused with EGFP) was found to localize to the PM to some extent showing that this portion might have most of the signals needed for targeting. Therefore longer deletion constructs could be made to test, how large a portion of nsP1 is needed for preferred PM localization. A similar observation was made with BMV where the BP itself rendered the GFP to be a membrane binding protein (Liu et al., 2009), but a longer region from 1a was needed for ER targeting (den Boon et al., 2001).

Since based on previous studies it was thought that nsP3 contains the signals for endo-lysosomal targeting, we wanted to test whether P13 would have all the necessary elements for correct targeting. Indeed, this fusion protein was located at the PM and intracellular vesicles positive for lamp2. Initial live cell imaging studies have shown that these intracellular vesicles are acidic and gather perinuclearly similarly to CPVs (with permission of Maarit Neuvonen). Moreover, when P13 under T7 promoter was expressed by the aid of MVA, it was blocked at the PM and accumulated at the bottom of the cell in aligned stripes very similarly to $P12^{CA}3$ expressed by the MVA and also to the RCs early in infection. Therefore these experiments indicate that P13 contains all the targeting signals of the RC and behaves very similarly to $P12^{CA}3$ polyprotein. Further experiments are needed to map the regions inside of nsP3 needed for the targeting. Initial results suggest that a 50 aa stretch in the C-terminus of nsP3 that is phosphorylated might be involved in the internalization of the RCs from the PM. Removal of that region blocks the RCs at the PM (Balistreri et al., 2010). Therefore it would be interesting to test whether this region fused with nsP1 would target the protein to endo-lysosomes or whether some other signals in nsP3 are needed.

Our results suggest that RCs are localized in lipid rafts at the PM (Balistreri et al., 2010). We have shown that cholesterol depletion did not affect the targeting of the RCs to the PM, or virus replication. However, in methyl-β-cyclodextrin treated cells RCs did not segregate properly any more, regular cluster-like distribution was lost and it seemed that they covered the PM in large patches. Most importantly, the internalization of the RCs was completely blocked indicating that signalling occurring in the raft-like domains was disrupted (Balistreri et al., 2010). It has been proposed that lipid microdomains such as lipid rafts are important for segregating proteins into specific ordered regions/domains, and this serves as one basis for regulated signalling (Maxfield and Tabas, 2005) and references therein). As lipid rafts have been shown to be enriched in G-protein palmitoyltransferases (Dunphy et al., 2001), it is tempting to speculate that palmitoylation of nsP1 could occur in these membrane domains. In addition, lipid rafts contain high levels of PS making it a suitable platform for the binding and functioning of the RCs (Pike, 2009).

5.5 Intracellular dynamics

Following the viral RCs in live cells has enabled a new exciting line of studies concerning the dynamic properties of the RCs, the maturation process of the virally induced membrane alterations, and most importantly the interplay between viral RCs and cellular components. Initial studies have been carried out describing the dynamic nature of the RCs of HCV, MHV and poliovirus (Wölk et al., 2008; Hagemeijer et al., 2009; Egger and Bienz, 2005). Based on these studies it is apparent that RCs are very dynamic and they utilize cellular cytoskeleton (especially microtubules) to traffic in infected cells. Interestingly, RCs seem to assemble in smaller membrane-associated entities that are very dynamic and concentrate gradually in the perinuclear area resulting in a stable viral compartment. These features are strikingly similar in our studies.

SFV RCs are first targeted to the PM and through signals in nsP3, the RCs are internalized and transported to the perinuclear region. Live cell imaging showed that RCs are internalized in neutral vesicles that utilize actin cytoskeleton for their transport. After fusing with acidic organelles, a switch occurs and microtubules are used to concentrate the RCs around the Golgi complex. Nocodazole treatment confirmed the role of microtubules in the later stages of RC trafficking. Interestingly, these long range microtubule-dependent movements were also blocked by blebbistatin (added after the internalization of the RCs), suggesting an interplay between actin and microtubule networks. Indeed, coordinated behaviour, both structural and regulatory, of these two networks has been described (Rodriguez et al., 2003; Petrasek and Schwarzerova, 2009) and references therein). However, treatment with nocodazole did not stop the movements of the small neutral RC-carriers; they were internalized from the PM and matured to some extent. They performed fusions with each other and their size increased up to 600 nm. This suggests that early in infection the dynamics of the RCs do not depend on microtubules. On the other hand, fusions with acidic organelles and further maturation until the formation of the CPV-Is is strictly dependent on microtubules, as in nocodazole-treated samples RCs did not reach the perinuclear area nor manage to saturate the surface of the acidic organelles. These results highlight a very delicate and regulated interplay between the actin and microtubule networks during RC trafficking.

Late in infection, a very stable perinuclear compartment was formed. Large acidic CPV-Is surround the Golgi complex and show very immobile dynamics. Notably, almost all cellular acidic vesicles are recruited into this virus-formed compartment indicating an extremely efficient mechanism of the virus to exploit the host cell. In addition, filipin staining demonstrated that CPV-Is are very rich in cholesterol. It has been shown previously that cholesterol accumulation in late endosomes and lysosomes leads to enlargement of the organelles and their motility is affected (Lebrand et al., 2002; Sobo et al., 2007). A similar picture can be seen also with CPV-Is, which can increase to up to 2 μm in size and are rather static.

As RCs seem to localize in lipid rafts at the PM and initiate internalization from these domains (Balistreri et al., 2010), it is possible that this induced large scale endocytosis event alters the PM enough to trigger the cellular response to reduced cholesterol levels at the PM. Staining with filipin indicated that from 2-2.5 h (the peak time for internalization)

cholesterol levels started to increase and kept the trend until late infection. In the case that cholesterol remains trapped in the CPV-I induced late endocytic compartment and is not transported further, the cellular cholesterol biosynthesis pathway would still remain active as negative feedback mechanism is inhibited (Maxfield and Tabas, 2005). This could be seen also in Figure 8B. The mechanisms by which RCs manage to interfere with normal cholesterol trafficking remain to be elucidated.

There might be several reasons why CPV-Is are accumulating cholesterol. One of the most probable interpretations is that viruses have evolved to create this very stable compartment for their replication, and accumulation of cholesterol would allow stabilizing the dynamic organelles. As the late endocytic compartment is a very active sorting and membrane trafficking station, it would not be an ideal place for viral replication. Late endosomes have a very active communication even at the intra-organelle level and induced spherule structures would be subjected immediately to fusions with internal vesicles (van der Goot and Gruenberg, 2006). In addition, late endocytic organelles are very dynamic and would not allow the virus to bring CPV-Is in close contact to ER where structural proteins are synthesised. It has been proposed that cholesterol-rich membranes are important for RC functioning. WNV has been shown to redistribute cellular cholesterol to its replication sites on ER membranes and induce the synthesis of cholesterol. Depletion of cellular cholesterol has a drastic effect on WNV replication. Additionally, increased membrane platforms created by loaded cholesterol are thought to provide a larger surface area for viral protein production and virion formation (Mackenzie et al., 2007). In the case of SFV, we have seen that cholesterol depletion does not stop replication (Balistreri et al., 2010) implying that cholesterol is not functionally needed for RC assembly and replication. However, it is needed to create the viral perinuclear compartment. We saw in cell culture that virus replication can occur at the PM without affecting RNA synthesis or virus release. However, in the organismal level, the situation might be different. Our initial results indicate that the virus with altered nsP3 and subsequent defect in RC internalization is apathogenic in mice (Vihinen et al., 2001; Balistreri et al., 2010). Therefore it is possible that at least one of the roles of RC trafficking, besides the creation of a stable viral compartment, is to avoid or modulate the immune response. It has been proposed for vaccinia virus that perturbations in intracellular membrane trafficking might affect the secretion of molecules eliciting immune response. Interestingly, vaccinia virus also clusters the late endosomes although no cholesterol accumulation has been shown (Schepis et al., 2006). In addition, WNV has been shown to downregulate the interferon stimulated antiviral response due to the redistribution of cellular cholesterol (Mackenzie et al., 2007).

In addition to these hypotheses for the role of concentration of viral RCs to perinuclear region, some others have been proposed. Egger and Bienz (Egger and Bienz, 2005) suggested that accumulation of poliovirus RCs perinuclearly and fusing to large structures might be evolutionaryly advantageous. It could promote virus recombination as the migration of RCs might enhance encounter of viral genomes. However, in the case of MHV and HCV it was demonstrated that large perinuclear RC-assemblies are very static and do not exchange material between each other. This would suggest that complementation of different viral genomes has to occur during RC assembly in the small

structures (Wölk et al., 2008; Hagemeijer et al., 2009). Wölk et al proposed that it might be beneficial to spread the small RCs throughout the cytoplasm to start productive replication in many foci thereby increasing the possibility for the emergence of adaptive genomes. Unlike the other studied plus RNA viruses (MHV, poliovirus, SFV), HCV seems to maintain the pool of small RCs throughout the replication cycle. The authors propose that this might be due to the fact that HCV is a noncytolytic virus and establishes a latent infection. Therefore scattering RCs in the cytoplasm might preserve physiological cell structure and function, an important feature for noncytolytic viruses (Wölk et al., 2008). HCV studies have been carried out with a cell line stably expressing HCV replicon. Therefore, it is not so clear whether the existence of small RCs throughout the replication cycle is due to the continuous expression of the replicon or whether it really is a unique feature for viruses establishing latent infections. It will be interesting to see future work carried out in this direction. Nevertheless, in all the above mentioned cases (HCV, MHV, poliovirus and SFV) microtubule-dependent transport served as means to gather the RCs to the perinuclear area. Disruption of the microtubules with nocodazole resulted in scattered small RCs in the cytoplasm that were active in replication. Therefore it remains to be seen why plus-strand RNA viruses have evolved the microtubule-dependent concentration of RCs in the perinuclear area.

5.6 Rearrangement of actin cytoskeleton

During SFV infection, actin cytoskeleton undergoes several rearrangements that correlate with the state of infection and RC localization. Expression of palmitoylated nsP1 alone seems to lead to F-actin disruption and formation of filopodia-like extensions that are devoid of F-actin (Laakkonen et al., 1998). As seen in this thesis, the disruption of actin seems to follow the trafficking of the RCs, and together with the inward movement of the RCs actin becomes disassembled. In addition, nsP1 mediates the induction of another type of PM projections that are rich in actin and become especially prominent during later stages of virus infection. Induction of the latter actin-rich projections is accompanied by the disruption of intracellular F-actin and seems to involve a signalling pathway mediated by nsP1. IPA-3, an inhibitor of group A p21-activated kinases (PAKs), mainly PakI, interfered with the action of nsP1 and clearly decreased the amount of induced projections. In addition, the disruption of actin cytoskeleton was less prominent even at 8 h p.i. (Fig. 11B). PakI is known to be involved in the signalling pathway leading to disassembly of actin stress fibres and the formation of actin-rich projections (Fig. 16) (Daniels and Bokoch, 1999; Bokoch, 2003). Therefore it is tempting to speculate that nsP1 might activate directly or indirectly PakI kinase. Interestingly, PAKs and their substrates are shown to mainly localize at the PM and in neuronal cells activated PakI at the PM induces neurite outgrowth (Daniels and Bokoch, 1999; Bokoch, 2003). In addition, PakI was reported to induce hepatocyte growth factor-induced projections in epithelial cells (Miao et al., 2003).

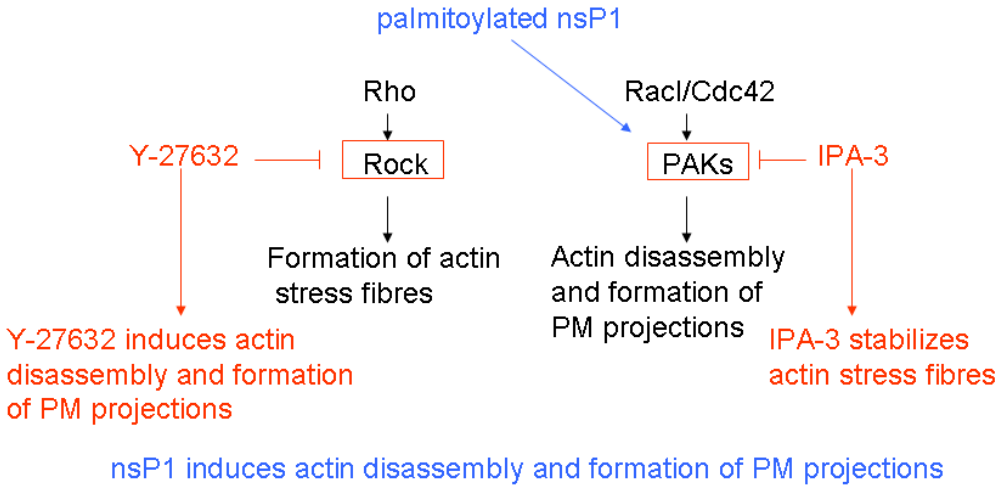

Figure 16 *Schematic view of the possible actions of nsP1(shown in blue) through PAKs (PakI). Effects of the inhibitors IPA-3 and Y-27632 are highlighted in red.*

The same phenomenon was seen with Rho kinase (Rock) inhibitor Y-27632. In our study, Y-27632 enhanced the actions of nsP1 and induced even more prominent actin-rich projections, in concordance with previous results (Miao et al., 2003; Favoreel et al., 2005). As seen in Figure 16, Rock is involved in actin polymerization process and induces the formation of actin stress fibres. Therefore its inhibition promotes the disassembly of actin fibres and formation of protrusions as seen also in our study.

The role of the actin rich projections during virus infection remains a matter for hypothesis. For several viruses, it has been shown that induction of similar actin-rich structures facilitates intercellular virus spread and allows the virus to avoid neutralizing antibodies. Therefore it is proposed that connecting neighbouring cells is used by viruses as an important strategy to spread in the presence of active antiviral immunity (Favoreel et al., 2000). In the case of alphaherpesvirus PRV (Pseudorabies virus), actin- and microtubule containing projections are induced along which virus particles move from one cell to another without actually budding out and infecting a new cell (Favoreel et al., 2005). It was demonstrated that these projections could be enhanced by Y-27632 inhibitor resulting in increased virus spread. They showed also that the formation of actin-rich projections was accompanied by the disassembly of actin stress fibres. Interestingly, in the case of PRV, a viral protein kinase US3 is responsible for inducing the projections and deletion of that gene results in the block of intercellular virus spread. US3 from HSV-2 has been shown to display homology with PAKs and therefore this viral protein is considered as viral analogue to activated PakI (Murata et al., 2000). Similar intercellular virus spread has been reported also for vaccinia virus, for which virus particles are seen to move inside long protrusions reaching neighbouring uninfected cells (Schepis et al., 2006; Cordeiro et al., 2009). In the case of TMV, a plant virus belonging to the alphavirus superfamily, a membrane-bound RC together with movement protein has been shown to traffic through tubular structures traversing plasmodesmata (Kawakami et al., 2004).

Movement proteins alone can induce the formation of the tubules that extend between cells through plasmodesmata (Gallagher and Benfey, 2005). In plant cells, traffic through plasmodesmata is a regular process to transport proteins, organelles or other components from one cell to another. In animal cells, these processes seem to be less well understood. However, recent reports suggest a concept of cytoplasmic continuity that is achieved by tunnelling nanotubes between neighbouring cells (Gerdes et al., 2007). Transported organelles have been shown to be of endo-lysosomal origin and acto-myosin-dependent transport is needed for their trafficking (Gerdes et al., 2007). Therefore actin-rich projections seen in SFV infected cells might facilitate intercellular virus spread during infection. Phalloidin and anti-nsP1 staining clearly shows that these structures connect neighbouring cells and anti-dsRNA staining demonstrated that many RCs are present in these structures. Therefore it is possible that membranous RCs move along the projections aiming to reach uninfected cells and start replication there. Initial live cell imaging observations indicate that RCs are able to move along these structures and enter the neighbouring cell (our unpublished data). However, more experiments are needed to test whether the projections induced during virus infection could serve as a means to transport the viral RCs or alternatively virus particles between the cells. This would also provide a possibility to enter cells lacking suitable receptors for the virus.

Induction of the filopodia-like extensions in nsP1-transfected cells and to lesser extent also in infected cells seems to be mediated via a different mechanism than the actin-rich projections. Filopodia-like extensions do not contain F-actin and their formation was IPA-3 independent suggesting that PakI signalling pathway is not used to induce these structures (Fig. 10). Interestingly, in infected cells, these filopodia-like extensions are not as abundant as in transfected cells and seem to appear later, when free nsP1 starts to accumulate at the PM. In a recent study, it has been shown that nsP1-induced filopodia are used as targets by IL-2 activated NK cells and nsP1-transfected cells are effectively killed (Helander, 2010). Therefore, it is difficult to imagine that virus would have evolved the mechanism to induce filopodia that are used as targets by the immune system. It is more likely that these structures are mainly caused by the overexpression of nsP1, and during infection they represent a minor portion compared to nsP1-induced actin-rich projections.

6. Conclusions

The interplay between cellular membranes and viral RCs has become an increasingly important field of research during the past few years. It is well accepted that all positive-strand RNA viruses assemble their RCs on cellular membranes resulting in extensive membrane alterations. The current thesis dissected different aspects of membrane-associated replication using a model alphavirus, SFV, as an example. The main findings of this study can be summarized as follows:

1) PM serves as the targeted membrane for alphavirus replicase proteins and as the assembly platform for the membranous RC spherules.
2) The BP in the middle of nsP1 mediates the membrane binding of nsP1 and the RC.
3) Mutational analysis of the BP supported the amphipathic nature of the peptide and highlighted the conserved R253, L256 and W259 as crucial residues in the membrane binding of the RC and for the replication of the virus. In the position 256 only a hydrophobic residue is tolerated. In addition, Y249 is essential for virus replication.
4) By the aid of the plasmid-derived system it was shown that template size seems to affect the morphology of the membrane structures induced and the replicase proteins themselves might not be sufficient to induce the spherules
5) Internalization of the membranous RCs requires the activity of PI3K as well as intact actin cytoskeleton.
6) RCs are internalized in neutral carrier vesicles that utilize actin cytoskeleton and display homotypic fusion.
7) Microtubule-dependent transport is responsible for directing the RCs into the perinuclear area where they surround the Golgi complex and mature into CPV-Is.
8) Late in infection, viral RCs have usurped most of the cellular acidic organelles creating a stable cholesterol-rich viral compartment. The role of the formation of perinuclear static membrane assemblies, induced also by other plus RNA viruses, remains to be elucidated.
9) Targeting of the SFV RC to the PM is mediated by nsP1; mutations in residues R253 and W259 destroy the PM localization of the RC. However, the BP itself is not sufficient to retarget a fluorescent protein to the PM. Signals for the internalization of the RCs from the PM are found in nsP3. Therefore P13 contains all the targeting signals of the RC.
10) During RC trafficking, the cellular actin cytoskeleton is rearranged and long branched actin-rich projections connecting neighbouring cells are induced. These processes seem to need active PakI. Later in infection, actin is destroyed in these tubular structures but they remain positive for nsP1 and also for viral RCs.

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