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Viral RNA Replication in Association with Cellular Membranes

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Abstract All plus-strand RNA viruses replicate in association with cytoplasmic membranes of infected cells. The RNA replication complex of many virus families is associated with the endoplasmic reticulum membranes, for example, picorna-, flavi-, arteri-, and bromoviruses. However, endosomes and lysosomes (togaviruses), peroxisomes and chloroplasts (tombusviruses), and mitochondria (nodaviruses) are also used as sites for RNA replication. Studies of individual nonstructural proteins, the virus-specific components of the RNA replicase, have revealed that the replication complexes are associated with the membranes and targeted to the respective organelle by the ns proteins rather than RNA. Many ns proteins have hydrophobic sequences and may transverse the membrane like polytopic integral membrane pro-

teins, whereas others interact with membranes monotopically. Hepatitis C virus ns proteins offer examples of polytopic transmembrane proteins (NS2, NS4B), a "tip-anchored" protein attached to the membrane by an amphipathic α-helix (NS5A) and a "tail-anchored" posttranslationally inserted protein (NS5B). Semliki Forest virus nsP1 is attached to the plasma membrane by a specific binding peptide in the middle of the protein, which forms an amphipathic α-helix. Interaction of nsP1 with membrane lipids is essential for its capping enzyme activities. The other soluble replicase proteins are directed to the endo-lysosomal membranes only as part of the initial polyprotein. Poliovirus ns proteins utilize endoplasmic reticulum membranes from which vesicles are released in COPII coats. However, these vesicles are not directed to the normal secretory pathway, but accumulate in the cytoplasm. In many cases the replicase proteins induce membrane invaginations or vesicles, which function as protective environments for RNA replication.

1 Introduction

The genome replication of all plus-strand RNA viruses infecting eukaryotic cells is associated with cellular membranes. The membranes can be derived from the endoplasmic reticulum (ER), or other organelles of the secretory pathway, mitochondria, chloroplasts, or from the endo-lysosomal compartment. The membrane association provides a structural framework for replication, it fixes the RNA replication process to a spatially confined place, increasing the local concentration of necessary components, and it offers protection for the alien RNA molecules against host defense mechanisms. The theme of immobilized polymerase and moving template may in fact be common also to most cellular DNA replication and transcription systems (Cook 1999) and might therefore reflect a primordial pathway in nucleic acid replication. The modes of membrane binding and targeting to specific intracellular organelles of the replication complexes of different viruses are so far poorly understood. However, this field at the interface of virology, cell biology, and biochemistry is attracting increased interest, as it represents an ancient feature shared by many virus groups. Some aspects have been nicely treated in earlier reviews (de Graaff and Jaspars 1994; Buck 1996). We will present in some detail relevant studies on alphaviruses, especially Semliki Forest virus (SFV), which has been the object of our own interest. We will then review recent work on other viruses based on their classification in three superfamilies (Koonin and Dolja 1993), except that we have treated nidoviruses as a separate group.

2
Alphaviruses as Models

2.1
RNA Replication in Cytoplasmic Vacuoles Derived from Endosomes

Association of SFV-specific RNA synthesis with membranes was demonstrated in several studies starting in the late 1960s (for reviews see Kääriäinen and Söderlund 1978; Kääriäinen and Ahola 2002). Simple fractionation of membranes derived from the postnuclear supernatant fraction of alphavirus-infected cells showed that essentially all RNA polymerase activity was associated with a "mitochondrial" pellet fraction sedimenting at 15,000 x *g*. On the other hand, early electron microscopic (EM) studies had revealed cytoplasmic structures typical for alphavirus-infected cells. These were designated as "cytopathic vacuoles type I" (CPV-I), hereafter referred as CPVs. Their size varied from 600 nm to 2,000 nm, and their surface consisted of small vesicular invaginations or spherules, of homogenous size, with a diameter of about 50 nm. EM autoradiography of SFV-infected cells pulse-labeled with tritiated uridine already suggested that CPVs, and possibly the spherules, were involved in virus-specific, actinomycin D-resistant RNA synthesis (Grimley et al. 1968).

However, the origin, nature, and function of CPVs remained unclear for about two decades until Froshauer et al. (1988) demonstrated that they were modified endosomes and lysosomes. Immunofluorescence and immuno-EM techniques showed that Sindbis virus-specific nonstructural proteins nsP3 and nsP4 were associated with CPVs. The authors proposed that CPVs were derived from endosomes, which were participating in the internalization of virus particles. This would have nicely explained the endosomal origin of CPVs as a direct consequence of fusion of the virus envelope with the endo/lysosomal membrane, which would bring the virus nucleocapsid and genome directly to the cytoplasmic surface of the organelle. Thus genome uncoating and subsequent synthesis of replicase components would result in the modification of endosomes to virus-specific CPVs. However, this hypothesis cannot explain why the amount of CPVs was not dependent on the amount of infecting virions. Moreover, typical CPVs were seen also in cells transfected with the genomic RNA of SFV, demonstrating that the endosomal targeting of the replication complexes must be a posttranslational event (Peränen and Kääriäinen 1991).

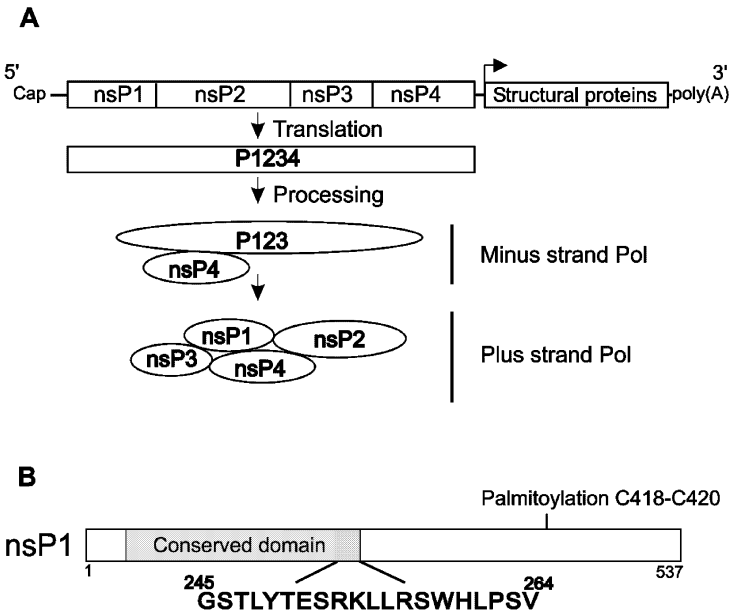


Fig. 1. **A** Genome organization of SFV. The translation and processing products relevant for SFV replication are shown. Physical interactions have been identified between nsP1 and nsP4, as well as nsP1 and nsP3 (Salonen et al. 2003). **B** Scheme of SFV nsP1 showing the two regions responsible for membrane binding. The amino acid sequence of the lipid binding peptide is given in *single-letter code*, and the position of palmitoylated cysteines is marked

Alphavirus nonstructural (=replicase) proteins are synthesized as a polyprotein precursor P1234, which is processed in a highly regulated manner into the individual components nsP1–nsP4 (Fig. 1A). Genetic and biochemical experiments have revealed many of the functions of the nsPs (reviewed in Strauss and Strauss 1994; Kääriäinen and Ahola 2002). Thus nsP4 is the catalytic RNA-dependent RNA polymerase subunit, nsP2 is involved in the regulation of the synthesis of the subgenomic 26S mRNA coding for structural proteins of the virion, whereas nsP1 is needed in the synthesis of the complementary (minus strand) RNA early in infection. nsP3 is essential for infection, but no specific function has been assigned for it as yet. Expression of the individual nsPs in *E. coli* and in insect cells revealed further functions of nsPs. nsP1 is an RNA capping enzyme with unique methyltransferase and guanylyltransferase activities (Mi and Stollar 1991; Ahola and Kääriäinen 1995), whereas nsP2 turned out to be a NTPase and RNA helicase (Gomez de Cedrón at

al. 1999), RNA triphosphatase (Vasiljeva et al. 2000), as well as the protease responsible for the processing of the nonstructural polyprotein precursor (Vasiljeva et al. 2001).

Creation of potent monospecific antibodies allowed the identification and localization of the ns proteins in SFV-infected cells (Peränen et al. 1988; 1995). After crude cell fractionation most of nsP1, nsP3, and nsP4 were associated with the P15 membrane fraction, whereas about 50% of nsP2 was found in the nucleus (Peränen et al. 1990). Pairwise double staining with different anti-nsP antibodies revealed costaining of CPV-like structures in immunofluorescence microscopy, suggesting that all four nsPs were associated with CPVs. This was confirmed by double-labeling in cryo-immuno EM (Kujala et al. 2001). Moreover, bromouridine given in short pulses also localized to CPVs and spherules together with the nsPs, indicating that these structures were the sites of RNA replication. The CPVs costained with late endosomal (lamp-1, lamp-2, and rab7) and lysosomal markers (LBPA and LysoTracker). Interestingly, all nsPs had localization sites also outside of the CPVs. nsP2 was found in the nucleus, nsP1 at the plasma membrane, nsP3 in cytoplasmic spotlike structures, and nsP4 diffusely in the cytoplasm (Kujala et al. 2001). Therefore, only a fraction of nsPs are present in the actual replication complexes.

2.2 nsP1 as the Membrane Anchor of the Replication Complex

As none of the alphavirus nsPs has sequences typical for transmembrane proteins, we have studied their membrane binding by expressing them individually in BHK, HeLa, and insect cells. These studies revealed that only nsP1 had a specific association with membranes (Peränen et al. 1995), whereas nsP2 on its own was transported almost quantitatively to the nucleus and nsP3 was in cytoplasmic aggregates (Salonen et al. 2003), which in light microscopy gives an impression of vesicles of variable size (Vihinen et al. 2001). Finally, nsP4 was distributed diffusely in the cytoplasm.

Thus nsP1 was a promising candidate as the membrane anchor of the SFV replication complex. nsP1 turned out to be very tightly membrane bound, as the association was not sensitive to high salt, EDTA, or alkaline sodium carbonate treatments, which release peripheral membrane proteins (Peränen et al. 1995). The tight binding was due to palmitoylation of cysteine residues 418–420 (Fig. 1B). When these residues were mutated to alanines, nsP1 was still membrane associated, but less tightly,

as it could now be released by high-salt treatment. Thus elimination of palmitoylation altered nsP1 from an "integral" to a "peripheral" membrane protein (Laakkonen et al. 1996).

To study the significance of the palmitoylation of nsP1, the C418–420A mutation was introduced to the infectious cDNA of SFV, followed by transcription of genomic RNA, which was used for transfection of BHK cells. Infectious virus was released to the medium, indicating that palmitoylation of nsP1 was not essential for virus replication. However, there was some retardation in the kinetics of virus growth. Analysis of the membrane association of wild-type and palmitoylation-negative mutant (1pa−) replicase proteins of SFV showed that 1pa− nsP1 was bound less tightly to the membranes than the wild-type protein. Typical CPVs with spherules, indistinguishable from those in wild-type SFV-infected cells, were seen in EM. The same results were obtained when the single palmitoylated cysteine residue (C420) of Sindbis virus nsP1 was mutated to alanine. However, the SFV 1pa− mutant was apathogenic for mouse. After intraperitoneal infection blood viremia was detected, but no infectious virus was found in the brain (Ahola et al. 2000).

2.3 Membrane Binding Mechanism of nsP1

Because palmitoylation was not the decisive mechanism for membrane binding of nsP1, we studied the peripheral binding by producing the wild-type protein in *E. coli*, which cannot palmitoylate proteins. Enzymatically active nsP1 was associated with bacterial membranes as judged by flotation in sucrose gradients. In vitro translated nsP1 also associated with liposomes containing 20%–50% phosphatidylserine (PS) or other anionic phospholipids, but not with liposomes containing only phosphatidylcholine (PC). Solubilization of membranes containing nsP1 by detergents, such as Triton X-100 or octylglucoside, resulted in loss of the protein's methyltransferase and guanylyltransferase activities, which could be reactivated by reconstitution of nsP1 into vesicle membranes or into mixed detergent-lipid micelles containing anionic phospholipids (Fig. 2A). Detergents also inhibited the binding of the methyl donor *S*-adenosylmethionine to nsP1, and the binding was resumed under the same conditions as enzymatic activity. Thus binding to anionic phospholipids causes a conformational change, which activates the protein (Ahola et al. 1999).

The membrane-binding site in nsP1 was identified by site-directed mutagenesis and deletion mapping in both bacterial and in vitro expres-

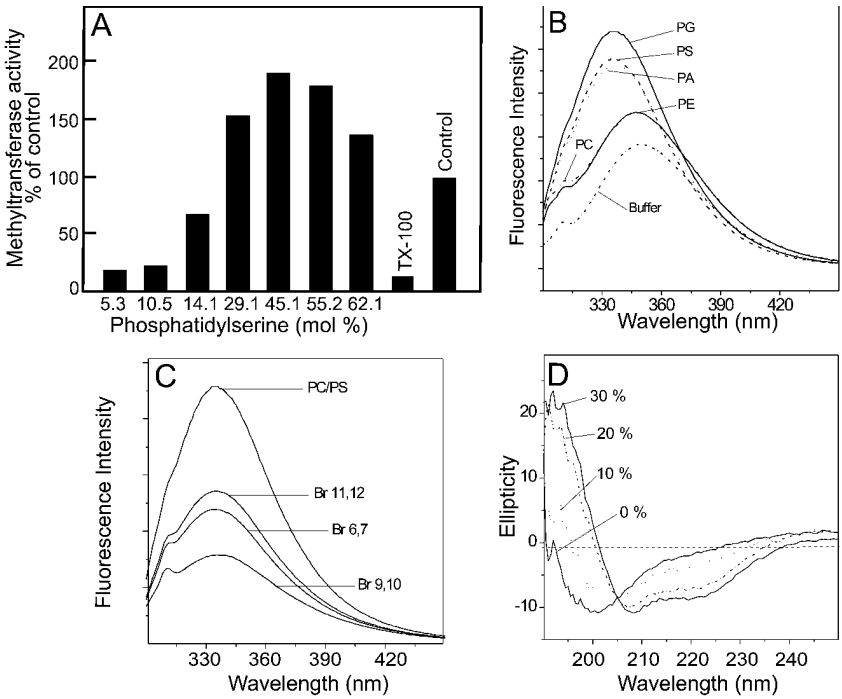


Fig. 2. Biochemical characterization of the lipid binding of SFV nsP1. **A** Inactivation and activation of nsP1 methyltransferase activity. Triton X-100 inactivates nsP1, compared with the control reaction in the absence of detergent (on the *right*). When TX-100 micelles containing increasing amounts of phosphatidylserine are added, nsP1 regains activity, even exceeding control levels at optimal concentration of the lipid. (Reproduced from Ahola et al. 1999, with permission). **B** Negatively charged phospholipids increase the intensity of tryptophan fluorescence of the lipid binding peptide. Tryptophan emission spectrum was recorded in the buffer or in the presence of small unilamellar vesicles consisting of phosphatidylcholine (*PC*) or PC with 30 mol % of phosphatidylglycerol (*PG*), phosphatidylserine (*PS*), phosphatidic acid (*PA*), or phosphatidylethanolamine (*PE*). **C** Depth of tryptophan W259 penetration to membrane measured by quenching of tryptophan fluorescence by brominated PCs. The position of bromine in the acyl chains is indicated. **D** The peptide adopts an α-helical conformation in the presence of PC-containing vesicles, as measured by circular dichroism spectroscopy. The numbers indicate the mol % of PS in the vesicles. (**B–D** reproduced from Lampio et al. 2000 with permission by the American Society of Biochemistry and Molecular Biology)

sion systems. Flotation of nsP1 with membranes or liposomes in discontinuous sucrose gradients was used as a criterion for membrane association. By this means a putative binding region of about 20 amino acid residues, starting from Gly245, was identified (Fig. 1B). The corresponding synthetic peptide consisting of Gly245–Val264 (GSTLYTESRKLLRSWHLPSV) was able to compete with the binding of in vitro synthesized nsP1 to liposomes containing PS, strongly suggesting that this region of nsP1 is responsible for its membrane association (Ahola et al. 1999).

The interaction of the synthetic membrane binding peptide with liposomes was assayed by utilizing the fluorescence of tryptophan residue W259 (Lampio et al. 2000). Tryptophan emission spectrum changes when it is embedded into an apolar environment. There was a marked increase in the fluorescence intensity and a blue shift of the emission in the presence of monolamellar liposomes, which consisted of PC and negatively charged phospholipids (PS, phosphatidylglycerol, or phosphatidic acid) (Fig. 2B). By using phospholipids with bromide substitution in different carbon atoms of the acyl chain, for quenching of the tryptophan fluorescence, it was possible to estimate that W259 penetrated to the level of carbon atoms 9 and 10 of the PC acyl chains in the outer leaflet of the liposomes (Figs. 2C and 3A). The circular dichroism spectrum of the binding peptide was dependent on the content of the apolar constituents (liposomes or trifluoroethanol). In a buffer solution the binding peptide was mostly in a random coil, whereas in the presence of liposomes with 20%–30% PS or in 30%–50% trifluoroethanol the peptide attained an α-helical conformation (Fig. 2D). The solution structure of the binding peptide, determined by NMR spectroscopy in 30% trifluoroethanol, revealed an amphipathic α-helix (Fig. 3A). One face consisted of hydrophobic residues, leucines 248, 255, 256, and 261, valine 264, and residues S252 and W259 interacting with the apolar fatty acid chains on the cytoplasmic leaflet of the membrane (Fig. 3). The other face contained positively charged residues R253, K254, and R257 lying parallel to the polar head groups of the bilayer surface. The hydrophobic surface of the peptide is rather stable, whereas the polar residues show considerable mobility in trifluoroethanol.

Point mutations R253E and W259A in the nsP1 protein, when expressed in *E. coli*, resulted in the loss of enzymatic activity and the lack of ability to float with membranes in sucrose gradients (Ahola et al. 1999). Thus these two residues were considered to be essential in the interaction of nsP1 with membranes. This view was supported by competition experiments done with synthetic mutant peptides. Both were unable to inhibit the binding of in vitro synthesized nsP1 to liposomes, in

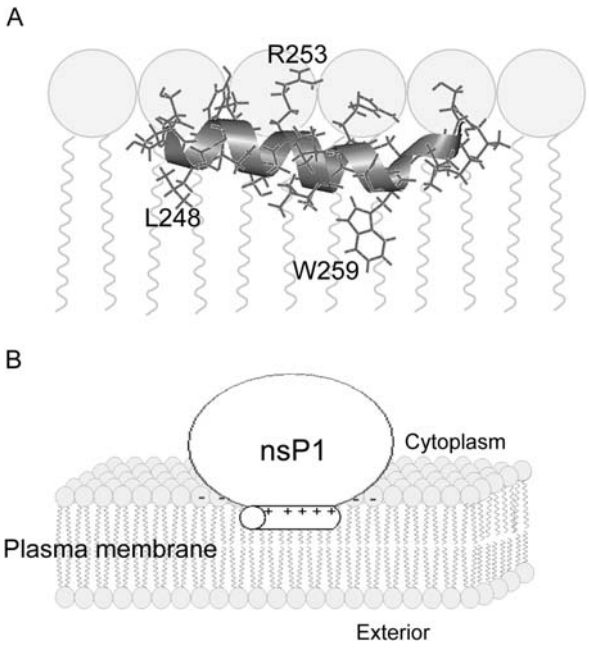


Fig. 3A, B. Monotopic binding of nsP1 to membrane via amphipathic α-helical peptide. **A** The NMR structure of the peptide is shown in interaction with the cytoplasmic leaflet of the lipid bilayer. **B** Highly schematic overview of interaction of nsP1 with a lipid bilayer

contrast to the synthetic wild-type binding peptide (Lampio et al. 2000). When the corresponding mutations were introduced to the SFV genome, neither W259A nor R253E was able to produce infectious virus after transfection (Salonen et al., unpublished data). Altogether, these results indicate that the interaction of nsP1 binding peptide with membranes is an essential and structurally finely tuned process, dependent on interaction with anionic phospholipids.

2.4 Polyprotein Conducts the Assembly and Targeting of the Replication Complex

The nsPs are derived from a common precursor P1234, the initial cleavage products of which (P123 plus nsP4) are necessary for the first step in the RNA replication, the synthesis of complementary RNA (Lemm et al. 1994, 1998) (Fig. 1A). To understand the role of this and other cleav-

age intermediates, we have produced them both in wild-type form and as noncleavable polyprotein variants, in which the autoprotease of nsP2 was inactivated by a mutation of the active site cysteine to alanine (superscript "CA"). The constructs were expressed in insect and mammalian cells, and the localization of individual proteins was followed by confocal microscopy and the complex formation by immunoprecipitation (Salonen et al. 2003).

The cleavable polyproteins (P12, P23, P123, P1234) containing an active nsP2 protease were processed to their constituents, most of which were distributed in the cell as though they were expressed alone. For instance, P12 yielded nsP1 and nsP2, which were targeted to the plasma membrane and nucleus, respectively. However, flotation analysis and immunoprecipitation recapture experiments showed that expression of P123 and P1234 resulted in membrane-bound complexes, containing all the individual proteins. This was different from the coexpression of all four nsPs individually, allowing us to conclude that the membrane association of the complex is guided by the polyprotein intermediate.

The uncleavable polyproteins were palmitoylated and had enzymatic activities typical for nsP1 and nsP2, and those containing nsP3 were phosphorylated, suggesting that the individual domains had folded properly in the context of the polyprotein. When $P12^{CA}$ was expressed in HeLa cells, it was localized exclusively at the cytoplasmic side of the plasma membrane and in long filopodia-like extensions (Fig. 4A), indistinguishable from those previously described for cells expressing nsP1 alone (Laakkonen et al. 1998). This indicated that the affinity to plasma membrane of nsP1 in the polyprotein overruled the attraction of nsP2 for nuclear transport. However, an interesting change in the localization was seen when $P12^{CA}3$ was expressed (Fig. 4B). No filopodia-like extensions were seen, and instead intracellular vesicular staining was observed, which in immuno-EM resembled CPVs (Fig. 4C and D). Double immunofluorescence with antisera against nsPs and lamp-2 suggested that at least a fraction of the vesicular structures were late endosomes or lysosomes (Salonen et al. 2003). Thus it seems that endosomal targeting is a joint action of nsP1 and nsP3 domains in the nonstructural polyprotein. The polyprotein is attached to the membrane first by the nsP1 binding peptide, which adopts α-helical structure. Concomitantly the nsP1 domain undergoes a conformational change, which activates the methyltransferase and guanylyltransferase. Palmitoylation of cysteine residues 418–420 thereafter anchors the protein irreversibly to the membrane. We propose that the targeting of nsP1 and polyproteins with this

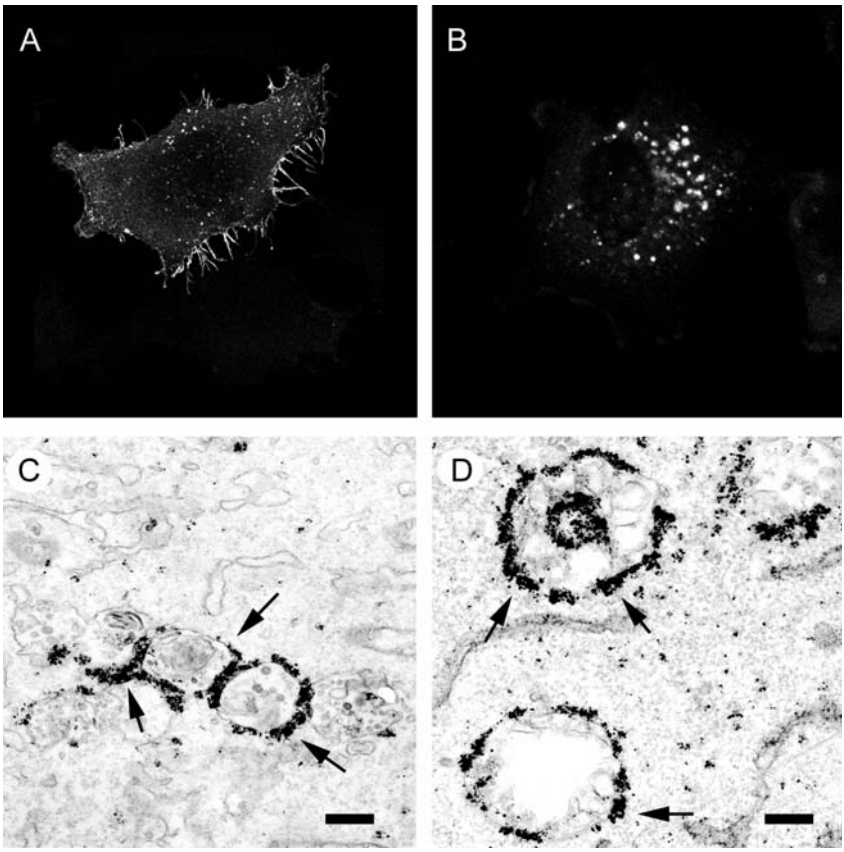


Fig. 4. Immunolocalization of SFV nonstructural polyproteins expressed by the aid of recombinant adenovirus vectors (**A–C**) and nsP3 during SFV infection (**D**) in HeLa cells. Cleavage-deficient $P12^{CA}$ (A) localizes to the plasma membrane and filopodia, whereas $P12^{CA}3$ (**B**) displays vesicular staining. At the ultrastructural level $P12^{CA}3$ (**C**) localizes to the outer membrane of cytoplasmic vesicles (*arrows*), which resemble the characteristic CPV structures carrying the viral replication complex in SFV-infected cells (**D**). *Bars* 200 nm

domain to the plasma membrane may simply be dictated by the optimal PS concentration in its cytoplasmic leaflet.

Early in alphavirus infection the minus-strand RNA synthesis is regulated by the processing of the nonstructural polyprotein. The first cleavage releases nsP4 from P1234 giving rise to the minus-strand polymerase (Fig. 1A). The further processing of P123 is regulated by the slow

in cis cleavage of the nsP1/2 site, which is essential for the next cleavage at the P2/3 site (Vasilieva et al. 2003). Thus the polyprotein has time to fold properly and bind to membranes by the aid of the nsP1 domain. The proper folding of the complex enables protein-protein interactions, which cannot be achieved when the components are expressed individually (Salonen et al. 2003). The polyprotein has a half-life of about 15 min before it is processed into the final components. During this time a replication complex synthesizes possibly only one minus-strand RNA before it is transformed into a stable plus-strand polymerase, which operates as the unit of replication within the spherule (Kujala et al. 2001).

3 Alphavirus-Like Superfamily

Rubella virus belongs to the *Togaviridae* family together with alphaviruses, and rubella virus replication complexes resemble in many ways those of SFV. Spherule-lined endo-lysosomal vacuoles are also found in rubella virus-infected cells (Magliano et al. 1998). Rubella virus replicase protein and newly synthesized RNA are located on the vacuoles, and specifically in spherule structures (Kujala et al. 1999). The role of spherules as sites of RNA replication is supported by localization of double-stranded RNA to them by antibodies against dsRNA (Lee et al. 1994).

Plant viruses belonging to the alphavirus-like superfamily replicate on various intracellular membranes, for instance, brome mosaic virus (BMV) and tobacco mosaic virus (TMV) on the ER, alfalfa mosaic virus on the vacuolar (tonoplast) membrane, and turnip yellow mosaic virus on the chloroplast envelope (Restrepo-Hartwig and Ahlquist 1996; Más and Beachy 1999; Prod'homme et al. 2001; van der Heijden et al. 2001). For BMV (Fig. 5A; Table 1), the targeting determinant of the replication complex has been mapped to the 1a protein, and more precisely to its N-terminal domain, part of which is distantly related to alphavirus nsP1. 1a is peripherally but tightly bound to membranes and exposed to the cytoplasm. Relatively large regions of 1a are needed for membrane association and ER targeting, but the exact molecular basis for membrane binding is not yet known (den Boon et al. 2001). Further comparative studies are needed to determine whether replicase proteins in the alphavirus-like superfamily share similar mechanisms of membrane association and targeting, but in the case of several viruses the capping enzyme domain binds to membranes (Magden et al. 2001). Interestingly,

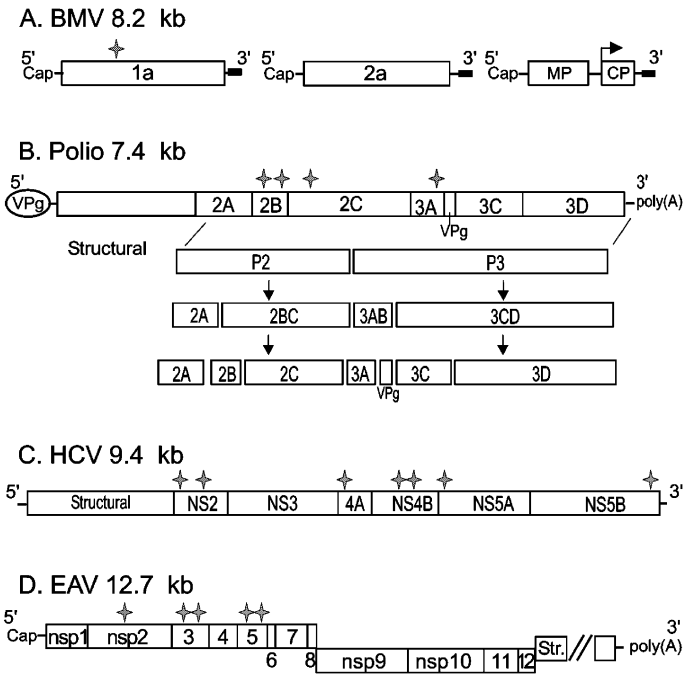


Fig. 5A–D. Genome organization of model viruses described in the text, representing the major groups of plus-strand RNA viruses. The different genomes are not in the same scale, and the structural region of the EAV genome is not represented. Regions attaching proteins to membranes are marked with *star symbols* (see also Table 1)

TMV replication in *A. thaliana* specifically and absolutely requires host genes TOM1 or TOM3. They encode related multipass transmembrane proteins, which seem to interact with the TMV replicase and are speculated to participate in its membrane anchoring (Yamanaka et al. 2002).

In cells infected with these plant viruses structures closely resembling the spherules, described above for alphaviruses, have been detected by EM-techniques (Prod'homme et al. 2001; Schwartz et al. 2002 and references therein). The spherules produced by BMV in yeast cells have recently been characterized in exquisite detail (Schwartz et al. 2002). BMV 1a protein alone, in the absence of other viral components, can induce spherule formation. When viral RNA is coexpressed with 1a, it is apparently protected inside the spherule in a membrane-associated, nuclease-resistant state. When the viral polymerase protein 2a is coexpressed, it associates with the spherules through interaction with 1a, and viral minus- and plus-sense RNA synthesis takes place in close association with

Table 1. Properties of replicase proteins

Virus	Replicase Proteins	Size aas	Function	Mode of membrane binding	Localization alone	Remarks	References
Togaviridae	nsP1	537	MT, GT	α-Helix; monotopic[1,2]	PM[3]	RC is in spherules	[1]Ahola et al. 1999
SFV	nsP2	799	NTPase, TP, Hel, Pro	–	Nucleus	In CPVs and at PM[5]	[2]Lampio et al. 2000
	nsP3	482	Unknown	–	CP; aggregates[4]		[3]Laakkonen et al. 1996
	nsP4	614	RdRp	–	CP	P123 is required for	[4]Salonen et al. 2003
	P123	1818	Early RC	Via nsP1	PM, endosomes[4]	targeting the RC[4]	[5]Kujala et al. 2001
Bromoviridae	1a	961	MT,GT,(Hel?)	Monotopic?[1]	ER	RC is in spherules,	[1]den Boon et al. 2001
BMV	2a	822	RdRp	–	CP	which can be induced by 1a alone[2]	[2]Schwartz et al. 2002
Picornaviridae	2A	149	Protease	–	CP	RC is in DMVs[4], which	[1]de Jong et al. 2003
Poliovirus	2B	97	Unknown	α-Helix[1] + TM[2]	ER, Golgi[1]	can be induced by the	[2]Agirre et al. 2002
	2C	329	NTPase	α-Helix; monotopic[3,4]	ER	coexpression of 2BC	[3]Paul et al. 1994
	3A	87	Unknown	HP C-term.[5]	ER	and 3A[5]	[4]Echeverri&Dasgupta 1995
	3B	22	VPg	–	CP		[5]Towner et al. 1996
	3C	182	Protease	–	CP		[6]Cho et al. 1996
	3D	461	RdRp	–	CP		[7]Lyle et al. 2002
	2BC	426	Unknown	Via 2B and 2C[6]	ER, Golgi, PM?		
	3AB	109	RNA priming	Via 3A[5,7]	ER, Golgi, PM?		
	3CD	643	Protease (main)	–	CP		
Flaviridae	NS2	149	Protease	TM; polytopic[1]	ER	Membranous web is the	[1]Yamaga et al. 2002
HCV	NS3	631	Pro, NTPase,Hel	–	CP, Nucleus	potential site of RC	[2]Wölk et al. 2000
	NS4A	54	Pro cofactor	HP N-term.[2]	ER	NS4B alone is able to	[3]Hügle et al 2001
	NS4B	261	Unknown	TM; polytopic[3]	ER	induce the web[6]	[4]Brass et al. 2002
	NS5A	447	Unknown	α-Helix; monotopic[4]	ER		[5]Ivashkina et al. 2002
	NS5B	591	RdRp	TM, tail-anchored[5]	ER		[6]Egger et al. 2002

Table 1. (continued)

Virus	Replicase Proteins	Size aas	Function	Mode of membrane binding	Localization alone	Remarks	References
Arteriviridae	nsp1	260	Protease	–	Nucleus, CP	RC is in perinuclear DMVs[2], which can also be formed by expression of self-cleaving nsp2-nsp3[3]	[1]van der Meer et al. 1998
EAV	nsp2	570	Protease	HP[1]	ER		[2]Pedersen et al. 1999
	nsp3	232	Unknown	HP[1]	ER		[3]Snijder et al. 2001
	nsp4	204	Protease (main)	–	CP		
	nsp5	163	Unknown	HP[1]	ER?		
	nsp6–8	302	Unknown	–	CP		
	nsp9	643	RdRp	–	CP		
	nsp10	467	Hel, NTPase	–	CP		
	nsp11–12	338	Endonuclease?	–	CP		

Abbreviations: MT, methyltransferase; GT, guanylyltransferase; TP, triphosphatase; Hel, helicase; Pro, protease; RdRp, RNA-dependent RNA polymerase; RC, replication complex; PM, plasma membrane; CP, cytoplasm; CPV, cytopathic vacuole; ER, endoplasmic reticulum; HP, hydrophobic; DMV, double membrane vesicle; TM, transmembrane.

the spherules, possibly in their interior, from where plus-sense RNA is released to the cytoplasm. Calculations based on immunolabeling suggest that many (maximally a few hundred) 1a proteins may be present in a spherule; leading to a hypothesis that 1a may form a shell-like structure coating the inside of the spherule (Schwartz et al. 2002). BMV replication in yeast requires a certain concentration of unsaturated fatty acids, as demonstrated through a mutation in the host fatty acid desaturase gene and its complementation by addition of unsaturated fatty acids. Under restrictive conditions, 1a can still normally recruit viral RNA and 2a to membranes, but minus-strand synthesis is strongly inhibited. Unsaturated fatty acids, present in membrane lipids, generally increase membrane fluidity and plasticity. Therefore, proper assembly or function of the BMV replication complex seems to require a relatively fluid membrane (Lee et al. 2001).

Uniquely among positive-sense RNA viruses, a highly purified detergent-solubilized replication complex of cucumber mosaic virus can catalyze a complete cycle of minus-strand and plus-strand synthesis on an exogenously provided specific template (Hayes and Buck 1990). However, this preparation seems to be relatively unstable and it has not been characterized further. In contrast, partially purified template-dependent preparations, such as that isolated from TMV-infected cells (Osman and Buck 1996), will be useful in further analyzing the role of membranes in RNA replication.

4
Picornavirus-Like Superfamily

4.1
Poliovirus as a Model

Poliovirus is one of the best-characterized viruses. Its structure and replication have been described in recent reviews (Pfister et al. 1999; Racaniello 2001; Semler and Wimmer 2002). Even though poliovirus does not have an envelope, the synthesis of the structural and nonstructural proteins takes place in association with cytoplasmic membranes in close vicinity to the RNA replication site (Caliguiri and Tamm 1970). The entire positive-strand RNA genome is translated to a single polyprotein, which is nascently cleaved into three polyproteins, P1, P2, and P3. P1 is the precursor of virion structural proteins, whereas P2 and P3 represent nonstructural proteins participating in the replication of viral

RNA (Fig. 5B; Table 1). Protease 2A cleaves P1 from the nascent polyprotein, while cleavages between nonstructural and structural proteins are carried out by protease $3C^{pro}$ (or rather $3CD^{pro}$). Nonstructural protein P2 yields protease $2A^{pro}$ and 2BC, which in turn is cleaved to 2B and the NTPase 2C. P3 yields 3AB and $3CD^{pro}$, which are processed to 3A and 3B (=VPg) and to 3C and $3D^{pol}$, respectively (Fig. 5B). VPg, a terminal protein of 22 aa, is linked to the 5′ end of the genome. The 5′ nontranslated region consists of a cloverleaf structure and an internal ribosome entry site.

The incoming poliovirus genomes seem to migrate to specific perinuclear sites, where replication starts. RNA recombination, which occurs during the synthesis of the complementary (minus-strand) RNA, takes place in these perinuclear sites (Egger and Bienz 2002). Throughout infection plus- and minus-strand RNAs are synthesized in the same replication complexes approximately in a ratio of 100 to 1 (Bolten et al. 1998). Poliovirus replication complexes consist of clusters of vesicles of 70–400 nm in diameter, which after isolation are associated as large "rosettelike structures" of numerous vesicles interconnected with tubular extensions. The rosettes can dissociate reversibly into tubular vesicles, which carry poliovirus nonstructural proteins on their surface and synthesize poliovirus RNA in vitro (Semler and Wimmer 2002). Immunoisolated poliovirus-specific vesicles contain cellular markers for the ER, lysosomes, and trans-Golgi network, suggesting complex biogenesis of the RNA replication complexes (Schlegel et al. 1996).

Involvement of the secretory route in the biogenesis of poliovirus replication complexes was suggested by the finding that brefeldin A, which inhibits the transport of secretory proteins from the ER to the Golgi complex, also inhibits poliovirus replication both in vivo and in vitro (Racaniello 2001). The importance of ER as the primary source for poliovirus replication complexes was confirmed recently by experiments in which COPII coat components were shown to colocalize with poliovirus nonstructural proteins on budding vesicles upon their exit from ER. Resident ER proteins were excluded from the released vesicles, which were not destined to the Golgi complex, but accumulated in the cytoplasm (Rust et al. 2001). These results are in conformity with previous findings, which showed that poliovirus infection inhibits the transport of secretory and plasma membrane proteins (Doedens and Kirkegaard 1995). Thus it seems that in poliovirus-infected cells a continuous proliferation and loss of ER membranes takes place. This process does not supply the Golgi complex with its normal lipids. The sensitivity of poliovirus replication to lipid synthesis inhibitors such as cerulenin could be

explained by this scenario (Racaniello 2001; Pfister et al. 1999). Overexpression of viral or cellular ER-associated proteins also inhibits poliovirus replication, possibly by competing for the capacity of ER to generate new membrane material (Egger et al. 2000).

Expression of P2 and P3 without structural proteins results in membrane alterations similar to those seen in infected cells (Teterina et al. 2001). However, vesicles formed from nonreplicating poliovirus RNA could not be recruited to support the replication of superinfecting poliovirus RNA, suggesting that functional replication complexes are formed only *in cis* by the direction of the incoming RNA. This must be first translated to yield the replicase proteins for its own replication in situ (Egger et al. 2000). Assuming that the newly synthesized plus-strands create in turn new replication complexes by a similar *in cis* process, the "rosette structures" might well consist of closely packed assemblies of a parent replication complex and its numerous daughters, which are loosely bound to each other (Semler and Wimmer 2002).

Numerous studies, in which poliovirus nonstructural proteins were expressed individually or in combinations, in the absence of RNA synthesis, have helped to understand the biochemical functions of nonstructural proteins and their role in membrane association during the biogenesis of the replication complexes (Racaniello 2001; Semler and Wimmer 2002). The 2B protein is targeted to ER membranes and to the Golgi complex. It interferes with the secretory pathway in mammalian and yeast cells (Barco and Carrasco 1995; Doedens and Kirkegaard 1995; de Jong et al. 2003). It has been reported to disassemble the Golgi complex (Sandoval and Carrasco 1997). 2B has a predicted cationic amphipathic α-helix within the N-terminal 34–49 aa and a potential transmembrane domain (aa 61–81), which according to modeling form tetrameric aqueous pores, which could be responsible, for example, for the observed hygromycin sensitivity and increased permeability of poliovirus-infected cells. To cause these effects 2B has to be transported to the plasma membrane, evidently on the cytoplasmic surface of the transport elements (Agirre et al. 2002; de Jong et al. 2003).

When 2C is expressed alone in mammalian cells it is localized to the ER, causing its expansion into tubular structures. As opposed to 2B, it does not prevent the transport of VSV G-protein to the plasma membrane (Suhy et al. 2000). The fragment responsible for the membrane binding of 2C has been mapped to the N-terminal part of 2C within aa 18–54 (Pfister et al. 1999). It has been predicted that this region has an amphipathic α-helix, which starts either from residue 10 (Paul et al. 1994) or 21 (Echeverri and Dasgupta 1995). 2C has NTPase activity,

which can be inhibited by guanidine, the well-known specific inhibitor of poliovirus replication (Pfister and Wimmer 1999). The ATPase activity of 2C is needed specifically in the initiation of minus-strand RNA synthesis, the only step inhibited by guanidine (Barton and Flanegan 1997). Because 2C binds specifically to the 3′ end of the minus-strand RNA, it may also have a function in the synthesis of the plus-strand RNAs, which takes place even in the presence of guanidine, that is, without ATPase activity. Anyhow, the tight membrane association of 2C and its intimate participation in minus-strand RNA synthesis mean that this process must also take place in association with membranes, although it has been difficult to prove (Egger and Bienz 2002). 2BC, like 2B, is a membrane protein, which interferes with the vesicular transport in both animal and yeast cells. Thus the 2B moiety in 2BC is responsible for the transport inhibition (Doedens and Kirkegaard 1995). 2BC induces vesicles similar to those seen in poliovirus-infected cells and causes permeability increase of the plasma membrane, like 2B (Teterina et al. 1997).

3A expressed alone efficiently inhibits the vesicular transport of secretory proteins from the ER to the Golgi. It remains associated with ER membranes but can be mobilized into secretory vesicles, similar to those in poliovirus-infected cells, by coexpression with 2BC (Dodd et al. 2001). In poliovirus-infected cells 3AB delivers the 22-aa-long VPg peptide to the 5′ end of both minus- and plus-strand RNA molecules. Only membrane-associated 3AB can be cleaved by the viral proteases ($3C^{pro}$ and $3CD^{pro}$), and thus serve as the source of VPg (Pfister et al. 1999). 3AB associates tightly with cellular membranes, resembling the binding of integral membrane proteins. The binding region has been mapped to the C-terminal amino acids 59–80 of 3A, specifically to a hydrophobic region consisting of aa 73–80. However, the exact binding mechanism is not known (Towner et al. 1996). The 3B (=VPg) portion of 3AB has affinity to the catalytic subunit $3D^{pol}$, and its precursor 3CD, which in turn recruits the template RNA into the membrane-associated replication complex by interaction with 3C and 3D (Egger et al. 2000; Pfister et al. 1999).

In summary, the assembly of the poliovirus replication apparatus is a complex process of specific membrane recognition, followed by protein-protein and RNA-protein interactions. At the same time the vesicles develop by a poorly understood autophagocytosis-like process to double-membrane vesicles (DMVs) and large rosettes containing proteins from ER, Golgi, and lysosomes (Schlegel et al. 1996; Suhy et al. 2000). Because of the extreme proliferation of the ER, the secretory apparatus becomes exhausted. The Golgi complex disappears, probably through retrograde

transport that is not compensated by normal lipid flow from the ER. Development of poliovirus-induced vesicles must be associated with multiple fusion events directed either by viral or cellular proteins. Another possibility would be that the membrane proteins from the Golgi complex, and perhaps beyond it, would be enclosed to the poliovirus-specific vesicles through retrograde transport via ER. In any case, the result is that the membranes of the secretory and endocytotic apparatuses become mixed.

4.2 Other Members of the Picornavirus Superfamily

Many plant viruses in the picornavirus superfamily appear to replicate in association with membranes derived from the ER. In comovirus- and nepovirus-infected cells the ER is proliferated and vesiculized, but in contrast to poliovirus-infected cells, the Golgi complex remains normal. Replicase proteins and newly synthesized RNA are associated with the ER-derived structures. Sensitivity to cerulenin, as an inhibitor of RNA synthesis, seems to be a common property in the picornavirus superfamily (Carette et al. 2000; Ritzenthaler et al. 2002). Cowpea mosaic comovirus 32-kDa and 60-kDa replicase proteins are both targeted to subregions of the ER when individually expressed, and they also cause morphological alterations of the membrane system. The 32-kDa protein is a hydrophobic component specific for comoviruses, whereas the 60-kDa protein may contain membrane binding regions analogous to poliovirus 2C and 3A (Carette et al. 2002). For tobacco etch potyvirus, the 6-kDa protein (analogous to poliovirus 3A) appears to be decisive in directing the replicase to the ER. The 6-kDa protein associates tightly with ER membranes by a single central hydrophobic domain (Schaad et al. 1997). The protein may be inserted to the membrane posttranslationally, but its exact binding mechanism and topology are not known.

Although the polymerase of the insect nodaviruses appears to be distantly related to the picornavirus-like superfamily (Koonin and Dolja 1993), these viruses have capped mRNAs and the ultrastructure of the replication complex resembles that of the alphaviruses. The outer mitochondrial membranes of flock house virus (FHV)-infected *Drosophila* cells contain numerous spherulelike invaginations, connected to the cytoplasm by narrow necks. The number of spherules increases during infection, leading finally to disruption of mitochondrial structure. The single virus-encoded replicase component, 112-kDa protein A, localizes to the outer mitochondrial membrane, which is also the site of viral

RNA synthesis (Miller et al. 2001). The N-terminal 46 aa of protein A contain a mitochondrial targeting signal and a transmembrane helix, such that the N-terminus is embedded in the mitochondrial matrix while most of protein remains on the cytoplasmic side (Miller and Ahlquist 2002). This transmembrane topology distinguishes nodavirus replicase from the replicase proteins of alphaviruses.

An interesting result has been obtained with the crude membrane-bound replication complex isolated from FHV-infected cells. When treated with micrococcal nuclease and supplied with an exogenous template, the FHV replicase synthesizes a complementary minus-strand resulting in a dsRNA product. However, when glycerophospholipids are added to the mixture, relatively large quantities of plus-strand RNAs are also produced, that is, a complete RNA replication cycle takes place. Several phospholipid species can stimulate this reaction, for instance, phosphatidylcholine bearing acyl chains of 14–18 carbons. It has been speculated that glycerophospholipid might directly interact with a component of the crude membrane preparation, perhaps activating an enzymatic function, or alternatively, that the lipid might facilitate a membrane modification or assembly process required specifically for plus-strand synthesis (Wu et al. 1992). Because of these advances and the simplicity of the nodavirus replicase, this virus group is promising for further analysis of membrane-associated replication.

5
Flavivirus-Like Superfamily

Of the members of the *Flaviviridae*, hepatitis C virus (HCV) and Kunjin virus are the best studied in the context of membrane-associated replication. Here they will be discussed only briefly, because these aspects of HCV and Kunjin virus have been reviewed recently (Dubuisson et al. 2002; Westaway et al. 2002).

Analogous to picornaviruses, the whole positive-strand RNA genome of flaviviruses is translated to a large polyprotein. The structural proteins consist of a capsid protein and envelope glycoproteins followed by nonstructural proteins (Fig. 5C; Table 1). Because the envelope proteins are translocated to and glycosylated in the ER, it would be expected that the nonstructural proteins would associate directly to the ER membrane. Nevertheless, in heterologous expression systems HCV nonstructural proteins NS2, NS4A, NS4B, NS5A, and NS5B each alone bind to the ER, whereas the soluble protease/helicase (NS3) associates with the mem-

brane by interaction with NS4A, a cofactor for the protease domain of NS3 (Dubuisson et al. 2002; Wölk et al. 2000).

NS2 is a polytopic integral membrane protein introduced to the ER membrane by internal signal sequences. It is a protease responsible for the cleavage between NS2 and NS3, but it is not essential for RNA replication (Yamaga et al. 2002). NS4B is also a polytopic membrane protein cotranslationally inserted into the ER membrane with its own internal signal sequences (Hügle et al. 2001). Although the exact function of NS4B is not known, its interaction with NS3 and NS5B modulates the RNA polymerase activity (Piccininni et al. 2002). NS5A phosphoprotein is tightly associated with membranes through an N-terminal amphipathic helix of about 30 residues. When these residues are joined to GFP, the fusion protein is associated with ER membranes, suggesting that the N-terminus of NS5A has also an address for the ER, in addition to membrane binding (Brass et al. 2002; Dubuisson et al. 2002). The monotopic binding of NS5A to membranes resembles the situation in alphaviruses, except that the binding peptide of NS5A is "tip-anchored," rather than residing in the middle of the protein like in nsP1 (Ahola et al. 1999). NS5B, the catalytic subunit of the HCV RNA polymerase, has a C-terminal membrane insertion sequence of 21 aa, which is targeted to the ER membrane posttranslationally, like typical tail-anchored membrane proteins (Ivashkina et al. 2002).

Expression of the entire HCV polyprotein induced a special ER-derived membranous web, where a cluster of tiny vesicles was embedded in a membranous matrix, often accompanied by tightly associated vesicles surrounding the web. According to immuno-EM analysis, all HCV proteins were associated with the web, but not with the vesicles. When the ns proteins were expressed individually or in combinations, NS4B alone induced the web, whereas NS3-NS4A complex induced a multitude of single vesicles having no direct analog in polyprotein-expressing cells (Egger et al. 2002). NS5A and NS5B did not modify the ER. In cells expressing functional HCV subgenomic replicons all ns proteins associated with ER membranes according to light and electron microscopic analysis (Mottola et al. 2002).

Even though the genome organization of HCV and flaviviruses is similar, there are some differences as well. Flaviviruses have a nonstructural glycoprotein NS1 (46 kDa), which is translocated into the lumen of ER and transported through the secretory route to the exterior of the cell. NS1 also plays an essential role in RNA replication, evidently by recognizing portions of the other replicase proteins penetrating the ER membrane (Westaway et al. 2002). Another difference is that there are

two small membrane-associated proteins, NS2A (25 kDa) and NS2B (14 kDa), preceding the soluble NS3 protein (60 kDa), which has protease-helicase-RNA triphosphatase activities. NS2B acts as a cofactor for the NS3 protease. NS4A (16 kDa) and NS4B (27 kDa) are poorly conserved membrane proteins. Finally, NS5 (104 kDa) protein is the catalytic subunit of the RNA polymerase complex. It is a soluble protein, unlike its homologous counterpart NS5B of HCV.

Extensive immunofluorescence microscopy studies of Kunjin virus-infected cells have established that NS1, NS2A, NS3, NS4A, and NS5 are regularly associated with dsRNA, which has served as marker for genuine replication complexes. These markers also colocalize with cellular markers of trans-Golgi membranes (Mackenzie et al. 1999), even in cells that do not express the viral glycoproteins (Mackenzie et al. 2001).

Electron microscopy of Kunjin virus-infected cells has revealed dramatic changes in the organization of the ER membrane. Proliferation of the ER leads to convoluted membranes (CM) and paracrystalline structures (PC) and to vesicle packets of smooth membranes (VP) (Westaway et al. 1997, 2002). The majority of NS proteins, dsRNA, as well as nascent labeled viral RNA have been immunolocalized to VPs, which are derived from trans-Golgi membranes late in infection. VPs are "vesicle sacs" consisting of a cluster of individual vesicles (diameter about 50–100 nm) surrounded by a membrane. Interestingly, VPs were not detected on expression of Kunjin replicons, whereas CMs and PCs were formed. In these cells the dsRNA was scattered throughout the cytoplasm in small isolated foci, suggesting that all membrane structures induced by the replicase proteins are not necessarily sites of RNA replication (Mackenzie et al. 2001; Westaway et al. 1999). Comparison between replicon cell lines producing RNA and NS proteins with different efficiencies suggests that the induction of virus-specific membranes is dose dependent and requires a certain level or concentration of viral products to manifest (Mackenzie et al. 2001).

Tombusviruses, plant viruses classified in the same supergroup with flaviviruses, replicate on peroxisomal or chloroplast membranes, or on the mitochondrial outer membrane depending on the virus species. Tombusvirus infection induces multivesicular bodies, where the limiting membrane of the organelle is transformed into numerous spherules (Rochon 1999). A putative polymerase of carnation Italian ringspot virus is a 92-kDa protein translated by read-through of an amber termination codon at the end of a 36-kDa protein. Both the 36-kDa and 92-kDa proteins are targeted to the mitochondrial membranes and anchored there via two hydrophobic domains located close to the N-terminus

(Weber-Lotfi et al. 2002). However, the expression of the 36-kDa protein alone was not sufficient to induce the vesiculation of mitochondria and hence the formation of spherules (Rubino et al. 2000).

6 Nidoviruses

Coronaviruses and arteriviruses, which are grouped in the order *Nidovirales,* express their replicase genes from two large open reading frames through complex proteolytic processing, leading to 12 or more end products, depending on the virus (Snijder and Meulenberg 2001; Lai and Holmes 2001). The replicase proteins of equine arteritis virus (EAV) (Fig. 5D; Table 1), as well as newly synthesized RNA, accumulate in perinuclear granules and vesicles, which are of ER origin (van der Meer et al. 1998). An electron microscopic study of EAV-infected cells revealed DMVs of approximately 80-nm diameter, carrying the replication complex (Pedersen et al. 1999). Usually the inner and outer membranes of the DMVs were tightly apposed but clearly separate. The mechanism for DMV formation appears to be a protrusion of paired ER-membranes, because DMVs having the outer membrane continuous with ER could be seen. These profiles sometimes contained a neck between the paired ER-membrane and a forming DMV, which had not yet pinched off. The formation of DMVs is not dependent on RNA synthesis, because DMVs strikingly resembling those seen in EAV-infected cells can be induced by heterologous expression of the nsp2-nsp3 region of the polyprotein (Snijder et al. 2001). On individual expression of these proteins, DMVs were not observed. The large nsp2 has a long central hydrophobic sequence, which may represent its membrane anchor, whereas nsp3 and nsp5 have several hydrophobic sequences, suggesting that they are polytopic membrane proteins. They all, and their precursors, also behave biochemically as integral membrane proteins (van der Meer et al. 1998).

DMVs are also the sites of coronavirus RNA replication (Gosert et al. 2002). Coronavirus-induced DMVs are larger than those induced by EAV, over 200 nm in diameter, and they are surrounded by tightly apposed membranes that have fused into a lipid trilayer. Viral RNA and replicase proteins are found on the surface of DMVs, where RNA synthesis also takes place. The coronavirus replicase proteins are membrane associated and include the integrally bound components p210 and p44, the coronavirus counterparts of EAV nsp2 and nsp3 (Gosert et al. 2002). At the moment, the origin of the coronavirus replication complexes (DMVs) is

not clear; involvement of membranes from secretory and endosomal compartments has been suggested. It should be noted that the majority of the viral replicase proteins may be located elsewhere than in the active replication complexes, as in SFV-infected cells (Kujala et al. 2001).

7 Concluding Remarks

7.1 Targeting of the Replicase Complex

Many viruses replicate on the cytoplasmic side of the ER membrane. For instance, picornavirus nonstructural proteins are targeted to the ER through 2B, 2C, 3A, and their precursors, bromovirus replication complex by the 1a protein, and arterivirus replicase by the nsp2-nsp3 complex (Table 1). However, the targeting mechanisms remain to be solved, as no specific receptors for viral components on the ER have been identified. Because all HCV and flavivirus proteins are translated from the same polyprotein as their envelope glycoproteins, it might be expected that their ns proteins also remain at the ER membrane. However, their location is guaranteed by their own independent affinity for ER membranes. In the case of Kunjin virus the NS1 glycoprotein, which is translocated to the cisternal side of the ER, may be responsible for the transport of the replication complex to the trans-Golgi region by transmembrane contact.

For some viral replicase proteins, classic targeting sequences directing them to a specific compartment have been identified. FHV virus protein A is directed to the mitochondrial outer membrane by a specific targeting sequence. A similar mechanism seems to operate in the targeting of the tombusvirus replication complex to either mitochondria or chloroplasts, depending on the virus species. The RNA replication of alphaviruses on the membranes of the endosomal apparatus (plasma membrane, endosomes, and lysosomes) might be explained by direct interaction of the amphipathic α-helix of nsP1 with these PS-rich membranes. It is an interesting question whether specific lipid components, or the lipid composition of the target membranes, might attract replicase components of other viruses as well.

The genomes of animal viruses discussed in this review are expressed as polyproteins. Picornaviruses and flaviviruses express both structural and nonstructural proteins in the same polyprotein, whereas togaviruses

and nidoviruses express their nonstructural proteins as a separate polyprotein. The polyprotein strategy evidently guarantees the proper targeting and assembly of the membrane-associated RNA replication complex. For instance, in SFV-infected cells the delayed proteolysis of the ns polyprotein enables the folding and assembly of "soluble" components (nsP2-nsP4) with the nsP1 membrane anchor. In the case of picornaviruses and flaviviruses several membrane anchors are present.

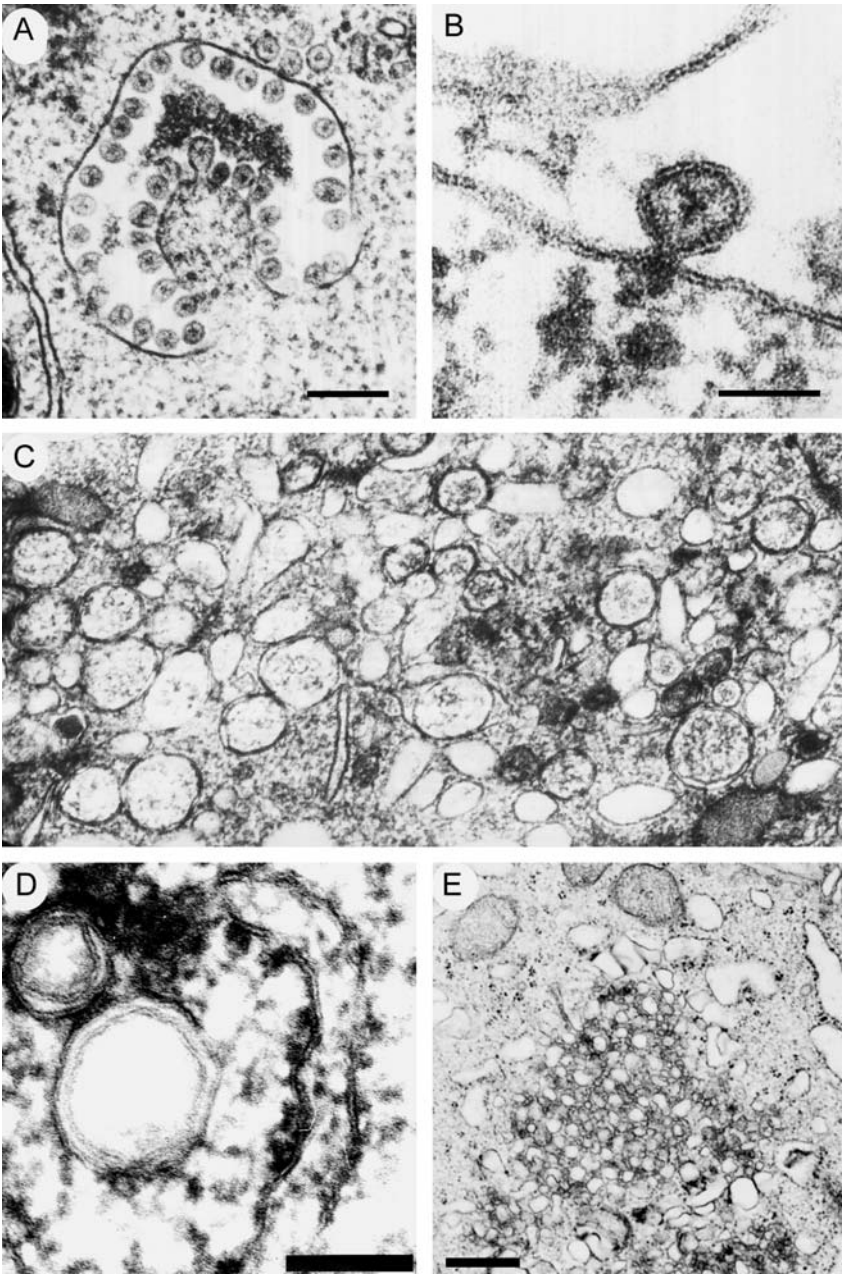
7.2 Mechanisms of Membrane Binding

The modes of membrane attachment are also variable. Monotopic binding by amphipathic α-helix has been suggested for several replicase proteins, which lack continuous hydrophobic anchor sequences (Table 1). Except for SFV nsP1 (Lampio et al. 2000) and HCV 5A (Brass et al. 2002), these conclusions have been based only on sequence-based predictions and mutagenesis studies. The amphipathic α-helix strategy has been proposed for components of picornavirus and for several plant virus replication complexes. Monotopic hydrophobic anchors have been proposed for poliovirus 3A and related plant virus proteins, and polytopic membrane anchors have been demonstrated for HCV proteins NS2 and NS4B. Recent results have shown that the catalytic subunit of HCV RNA polymerase is a typical tail-anchored ER protein, whereas the polymerases of other viruses are soluble proteins (Table 1).

7.3 Membrane Modification by Replicase Proteins

Alphaviruses and rubella virus give rise to specific cytoplasmic vesicles with regular membrane invaginations, spherules (Fig. 6A and B), that

Fig. 6A–E. Ultrastructure of membranes involved in virus replication. **A** Typical cytopathic vesicles (CPVs) in SFV-infected BHK cell (4 h p.i.) with characteristic spherules inside. **B** A single spherule showing a neck opening to the cytoplasm with filled electron-dense material (courtesy of Dr. Ari Helenius and Dr. Jürgen Kartenbeck). **C** Poliovirus type 1-infected COS-1 cell (4 h p.i.) showing typical cytoplasmic vesicles ranging from 70 to 400 nm (courtesy of Dr. Karla Kirkegaard and Dr. Thomas Giddings). **D** EAV-infected BHK cell (4 h p.i) showing double membrane vesicles (DMVs) in close vicinity to RER (courtesy of Dr. Eric Snijder and Dr. Ketil Pedersen). **E** Membraneous web in UHCV-57.3 cell expressing the entire HCV open reading frame, 48 h after tetracycline removal (courtesy of Dr. Kurt Bienz). *Bars* 200 nm (**A**), 60 nm (**B**), 100 nm (**D**), 500 nm (**E**)


A
B
C
D
E

seem to be the actual units of RNA replication (Kujala et al. 2001). So far, it is not known how these structures arise. Similar spherules have been described in BMV-infected plant and in yeast cells expressing nonstructural proteins of the virus. It has been proposed that the internal surface of the spherules is covered by the replicase protein 1a of BMV (Schwartz et al. 2002). As the spherules seem to be a general feature for the members of the alphavirus superfamily, it will be interesting to see whether the suggested protein coating of the inner surface of spherules is a general mechanism within the whole superfamily. Involvement of host cell proteins cannot be excluded, as membrane bending and vesiculation in cells is a complex process requiring several factors (Hurley and Wendland 2002).

Membrane vesicles and multivesicular bodies induced by poliovirus (Fig. 6C) and other picornaviruses contain proteins from the Golgi complex, endosomes, and lysosomes, suggesting multiple fusion events during their development. Isolated membrane structures, "rosettes," consist of clusters of vesicles, which can be separated from each other at low ionic strength and low temperature. The individual vesicles represent units of replication (Egger et al. 1996). They have a tubular extension, which resembles the neck of a spherule through which the nascent RNA is proposed to be "secreted" from the site of synthesis (Froshauer et al. 1988). The DMVs (Fig. 6D) seem to the sites of arterivirus and coronavirus RNA synthesis as well. Evidently the vesicles wrap the template RNA, which in the case of poliovirus, alphaviruses, and flaviviruses is probably double-stranded, shielding it from host nucleases. During virus infection, translation and assembly of virus particles take place in close association with the replication complexes, utilizing nascent RNAs immediately after their synthesis.

A common feature for the viruses discussed in this review is that the nonstructural proteins, in the absence of RNA template, seem to be sufficient to induce the membrane modifications seen in the infected cells. Such is also the case for the membranous web seen during HCV polyprotein expression (Fig. 6E). Future studies at the molecular level should reveal how the different viruses and proteins can cause these fundamental structural changes in the membranes. A suitable lipid composition of the membrane may also be required for these dynamic membrane assembly and modification processes (Lee et al. 2001).

7.4
Functional Implications of Membrane Attachment

As the polymerase complex itself is firmly attached to the membrane, the template apparently has to move through the complex, which often also contains helicase, capping enzyme, and other subunits. In most instances, this would also mean that the same template would be repeatedly utilized by circling through the same replication complex. The dimensions of the membrane vesicles seen in EM images are such that the RNA would be relatively tightly packed within them, analogous to dsRNA virus cores. For picornavirus-like viruses there are special challenges, as for each round of RNA synthesis a protein component is consumed as a primer. It should also be emphasized that membrane lipids provide active components for the replication complex. They may directly bind to replicase proteins, thereby changing their conformation and activating them (Ahola et al. 1999).

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