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# Semliki Forest virus mRNA capping enzyme requires association with anionic membrane phospholipids for activity

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**The replication complexes of all positive strand RNA viruses of eukaryotes are associated with membranes. In the case of Semliki Forest virus (SFV), the main determinant of membrane attachment seems to be the virus-encoded non-structural protein NSP1, the capping enzyme of the viral mRNAs, which has guanine-7-methyltransferase and guanylyltransferase activities. We show here that both enzymatic activities of SFV NSP1 are inactivated by detergents and reactivated by anionic phospholipids, especially phosphatidylserine. The region of NSP1 responsible for binding to membranes as well as to liposomes was mapped to a short segment, which is conserved in the large alphavirus-like superfamily of viruses. A synthetic peptide of 20 amino acids from the putative binding site competed with *in vitro* synthesized NSP1 for binding to liposomes containing phosphatidylserine. These findings suggest a molecular mechanism by which RNA virus replicases attach to intracellular membranes and why they depend on the membranous environment.**
*Keywords*: alphaviruses/membrane/RNA replication

## Introduction

The replication of all positive strand RNA viruses of eukaryotes seems to take place in membrane-associated complexes in the cytoplasm of infected cells. The membrane may be an essential part of the replication complex, needed for its organization and function (de Graaff and Jaspars, 1994; Buck, 1996). In addition, membrane attachment may act to concentrate the replicative enzymes and viral RNAs onto a two-dimensional membrane surface to promote the efficiency of replication and to compartmentalize the double-stranded viral replication intermediates which are sensed by antiviral systems of the host cell (Jacobs and Langland, 1996).

We are studying the RNA synthesis directed by an alphavirus, Semliki Forest virus (SFV). The alphaviruses belong to a large alphavirus-like superfamily, which additionally includes rubella virus, hepatitis E virus and several families of plant RNA viruses (Goldbach, 1987; Koonin and Dolja, 1993). The RNA replication of alphaviruses and rubella virus takes place in association with specific cytopathic vacuoles, which are modified endosomes and lysosomes (Froshauer *et al*., 1988; Peränen and Kääriäinen, 1991; Lee *et al*., 1994). The virus-encoded replicase of alphaviruses is produced as a large polyprotein, which is processed rapidly to form four non-structural proteins, NSP1–NSP4. Of these, NSP1 is an mRNA capping enzyme with guanine-7-methyltransferase and guanylyltransferase activities (Laakkonen *et al*., 1994; Ahola and Kääriäinen, 1995; Ahola *et al*., 1997). Point mutations abolishing these activities render the virus non-infectious, implying that RNA capping is an essential function (Wang *et al*., 1996). Based on studies of a temperature-sensitive mutant, NSP1 is also needed for the initiation or elongation of viral negative strand RNA (Sawicki *et al*., 1981; Wang *et al*., 1991). Thus, NSP1 is linked intimately to all steps of alphavirus RNA synthesis. NSP2 is a multifunctional protein with a papain-related protease domain responsible for the cleavage of the non-structural polyprotein (Hardy and Strauss, 1989), and a helicase-like domain possessing NTPase activity (Rikkonen *et al*., 1994). NSP3 is a phosphoprotein with essential but poorly defined functions in RNA synthesis (Peränen *et al*., 1988; LaStarza *et al*., 1994), while NSP4 is the catalytic subunit of the polymerase (reviewed in Strauss and Strauss, 1994).

In SFV-infected cells, NSP1, NSP3 and NSP4 are found almost quantitatively in the membrane fraction of cell lysates, whereas only ~25% of NSP2 is in this fraction (Peränen *et al*., 1988). As the amino acid sequences of the alphavirus NSPs are hydrophilic and devoid of predicted membrane-spanning segments (Takkinen, 1986), we have tried to approach the problem of membrane association and endolysosomal targeting by expressing the individual NSPs in different systems. Under those conditions, NSP4 is cytoplasmic but devoid of membrane affinity, NSP2 is almost quantitatively transported to the nucleus and NSP3 associates with poorly defined membrane vesicles (Peränen *et al*., 1990; Peränen and Kääriäinen, 1991). Only NSP1 displays a tight membrane binding and associates with plasma membrane, endosomes and lysosomes, features typical for the replication complexes (Froshauer *et al*., 1988; Peränen *et al*., 1995). We recently have shown that the high affinity membrane binding of NSP1 is due to covalent palmitoylation of cysteine residues 418–420. However, a non-palmitoylated mutant, C418–420A, retains weaker binding to membranes *in vivo* (Laakkonen *et al*., 1996). When the mutations, destroying the palmitoylation site, are introduced into the viral genome, the virus remains viable, replicates to high titers and the replication complexes retain their normal membrane-associated localization (T.Ahola, P.Kujala, T.Anttinen, P.Laakkonen, N.Ehsani, L.Kääriäinen and P.Auvinen, in preparation). Thus, the mechanism of membrane attachment of NSP1 in the absence of palmitoylation needs to be resolved.

We report here that the membrane binding of NSP1 is


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due to its affinity for negatively charged phospholipids. The binding site was localized to a short peptide in the middle of the NSP1 amino acid sequence. Lipid binding activated the enzymatic functions of NSP1, indicating that the protein is adapted to function only in a membranous environment.

## Results

### *Binding of NSP1 to membranes in Escherichia coli*

SFV NSP1 (537 amino acids) is devoid of hydrophobic sequences, yet it binds tightly to membranes in eukaryotic cells. The membrane affinity is due to covalent palmitoylation of cysteine residues 418–420, but a non-palmitoylated mutant protein, C418–420A, also binds weakly to membranes (Laakkonen *et al*., 1996). This peripheral type of membrane attachment might be mediated by cellular membrane proteins ('NSP1 receptors') or, alternatively, by direct interaction with membrane lipids. To examine this issue, we used NSP1 expressed in *Escherichia coli*, where the protein is not modified by covalent palmitoylation (Laakkonen *et al*., 1994). When bacterial extracts were floated in discontinuous sucrose gradients to separate membrane proteins from soluble components, more than half of NSP1 floated with the membranes. Some NSP1 was found throughout the gradient, and a fraction remained in the bottom fractions, which contained most of the bacterial proteins (Figure 1A and B). NSP1 in these preparations was susceptible to protease treatment, indicating that flotation was not due to trapping of the protein inside vesicles formed during cell lysis. Attachment of NSP1 to bacterial membranes was confirmed with immunoelectron microscopy, which showed that NSP1 was localized close to the plasma membrane of *E.coli* (Figure 1C).

### *Detergent inhibition and lipid reactivation of NSP1*

The unexpected finding of NSP1 in association with bacterial membranes prompted us to examine whether the protein could be solubilized with detergents. We previously had observed that two detergents, deoxycholate and Triton X-100 (TX-100), almost completely inactivated the guanine-7-methyltransferase activity of the bacterially expressed NSP1 (Laakkonen *et al*., 1994). We therefore tested a variety of other detergents (Hjelmeland and Chrambach, 1984): 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), *N*,*N*-bis-(3-D-gluconamidopropyl)-deoxycholamide (deoxy-BIGCHAP), *n*-octyl-β-D-glucopyranoside (octylglucoside), digitonin, *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propane sulfonate (zwittergent 3–14) and Thesit®. All of these, when used in concentrations theoretically able to solubilize membrane proteins, strongly inhibited the enzymatic functions of NSP1 (data not shown, but see below for examples). We therefore asked whether the activity of NSP1 was in some way dependent on lipids. Could it be maintained or regained by removal of detergents and addition of different phospholipids? The preparations of NSP1 used in these experiments were made by flotation, and correspond to fractions 2–3 (lanes 2–3) in Figure 1A. They contained, in addition to NSP1, bacterial membrane proteins and a small amount of lipids, as specified in Materials and methods.

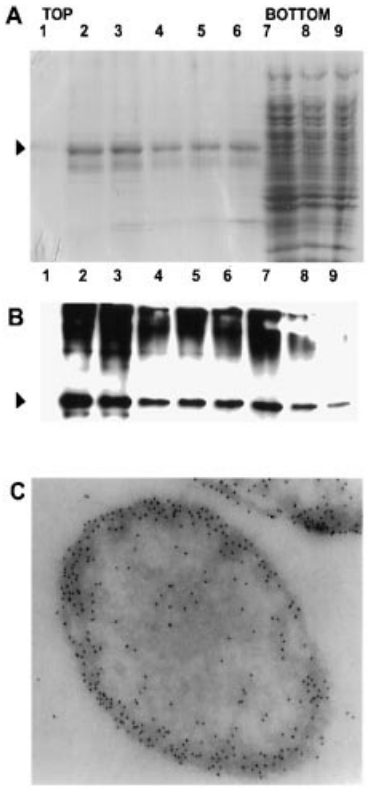


**Fig. 1.** Association of recombinant NSP1 with the plasma membrane of *E.coli*. (**A**) SFV NSP1 was expressed in *E.coli*, the bacteria were lysed and extracts subjected to flotation in a discontinuous sucrose gradient as described in Materials and methods. The numbered fractions were collected and analyzed by SDS–PAGE followed by Coomassie Blue staining. The sample originally was loaded in the 60% sucrose layer and, upon centrifugation, membrane-attached proteins floated to the 10–50% sucrose interface (fractions 2–3), whereas soluble proteins remained close to the sample loading layer (fractions 7–9). The position of NSP1 is indicated by an arrow. (**B**) The same fractions as in (A) analyzed by Western blotting with an antiserum against NSP1. The arrow points to the position of NSP1. (**C**) Cryoimmunoelectron micrograph of a bacterial cell expressing SFV NSP1, treated with anti-NSP1 antiserum followed by 10 nm gold particles conjugated to protein A.

When solubilized with octylglucoside, the methyltransferase activity of NSP1 was inhibited (Figure 2A, column 7). Removal of octylglucoside with the hydrophobic adsorbent Bio-Beads reduced the activity even further (column 5). Bio-Beads also reduced the enzymatic activity of the control preparation lacking detergent, possibly due to unspecific adsorption of the enzyme or other components of the reaction mixture (column 6). Selected phospholipids were mixed with the detergent to obtain mixed octylglucoside–phospholipid micelles, which were incubated with the NSP1 preparation before removal of the octylglucoside by Bio-Beads. Under these conditions, the removal of the detergent results in the creation of phospholipid vesicles (Rigaud *et al*., 1997). Of the phospholipids tested, phosphatidylserine (PS) preserved most of the methyltransferase activity (Figure 2A, column 3), whereas in the presence of phosphatidylcholine (PC, column 1) and phosphatidylethanolamine (PE, column 2) the activity was lost. A mixture of these three lipids had a small activating effect, probably due to PS (column 4). When NSP1 was treated with octylglucoside, PS and Bio-Beads, it could

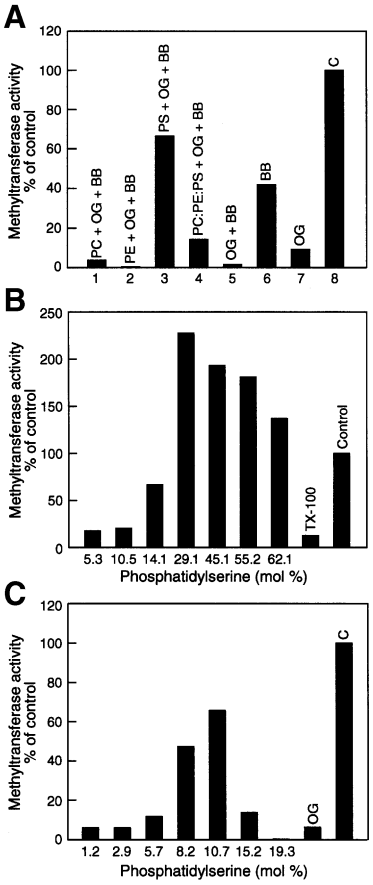


**Fig. 2.** Effects of detergents and lipids on the methyltransferase activity of NSP1. (**A**) NSP1 was first incubated briefly with mixed micelles of octylglucoside (OG) and the indicated phospholipids. The Bio-Beads (BB) were added and the incubation continued overnight, a treatment resulting in removal of the detergent and formation of phospholipid vesicles. In control experiments, lipid was omitted or NSP1 was incubated with Bio-Beads or octylglucoside only, or without additions (C). After removal of Bio-Beads, methyltransferase activity of the preparations was assayed, and is expressed as a percentage of the activity present in the control. (B and C) NSP1 was incubated for 30 min with micelles of TX-100 (**B**) or octylglucoside (**C**) containing the indicated amount (mol%) of phosphatidylserine. Methyltransferase activity of the preparations is expressed as a percentage of the control, which was not treated with detergent. Notice the different scales of activity in different panels. The results shown are the means of 2–3 experiments.

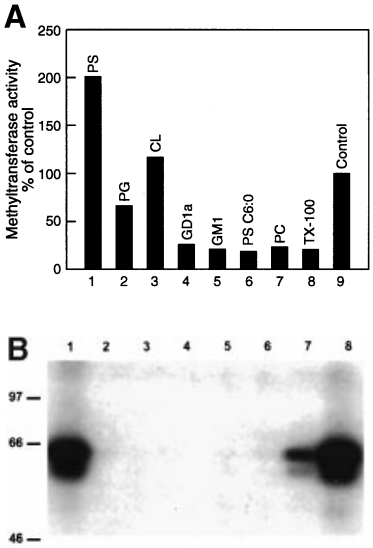


**Fig. 3.** Reversible inhibition of NSP1 by TX-100. The NSP1 preparation (total protein 44 μg/ml, total phospholipid 70 nmol/ml) was incubated either with or without (control) 5 mM TX-100. Portions of the detergent-containing mixture were aliquoted to tubes containing dried lipid (103 nmol), followed by bath sonication for 4 min. (**A**) Duplicate samples were assayed for guanine-7-methyltransferase activity. The results are expressed as a percentage of the control value. (**B**) Results of the guanylyltransferase assay. Lanes: 1, PS; 2, PG; 3, CL; 4, PS C6:0, 5, PC; 6, TX-100 only; 7, a sonicated control sample; 8, an untreated control sample. Molecular weight markers on the left are in kDa.

be floated quantitatively with the reconstituted PS vesicles, indicating that it had associated with them under conditions which also preserved the enzymatic activity (data not shown).

As PS maintained the activity of NSP1 when present in vesicles, we next studied the activity of NSP1 in the presence of defined mixtures of detergent and PS, under conditions, which lead to formation of mixed micelles of lipid and detergent. Two non-ionic detergents, TX-100 and octylglucoside, were chosen for these experiments. Treatments of NSP1 preparation with mixed micelles of TX-100 containing increasing amounts of PS resulted in hyperactivation of methyltransferase activity with a PS content within the range of 30–60 mol% (Figure 2B). Octylglucoside–PS mixed micelles had a less dramatic effect and an optimum around 10 mol% (Figure 2C).

To study whether the changes in the activity of NSP1 upon detergent solubilization and lipid rebinding were reversible, the following experiment was performed. NSP1 membrane preparation was treated first with TX-100 to inactivate the methyltransferase. Aliquots of the mixture were then transferred to tubes containing different dried lipids, and the enzymatic activity of mixed micelles formed after sonication was determined. A sample from which the detergent was omitted served as a control (Figure 3A, column 9). Reactivation of methyltransferase was obtained with phosphatidylglycerol (PG, column 2) and cardiolipin (CL, column 3), whereas neutral phospholipids such as PC (column 7) and anionic glycolipids, gangliosides GD1a and GM1 (columns 3 and 4) were inactive. The most effective lipid was PS (column 1). Interestingly, PS containing only short 6-carbon acyl chains could not activate NSP1 methyltransferase in the mixed micelles (column 6).

Guanylyltransferase activity as measured by covalent NSP1–$m^7$GMP complex formation (Ahola and Kääriäinen, 1995) was also tested under the same conditions. Reactivation was seen only in the presence of PS (Figure 3B, lane 1). The effect was remarkable since the sonication itself reduced the activity of guanylyltransferase by ~90% (lane 7) as compared with the untreated control (lane 8). This would suggest that the association with *E.coli* membranes was broken easily by sonication whereas association with PS-containing mixed TX-100 micelles was strong. Anyhow, resumption of guanylyltransferase activity suggests that NSP1 had regained its proper conformation in the presence of PS, which can be supplied as lipid vesicles or as a constituent of mixed micelles of detergent and lipid.

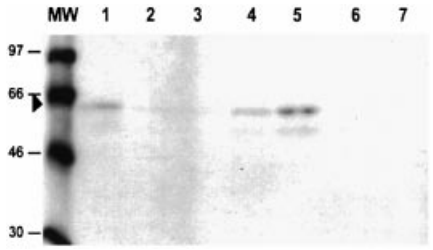


**Fig. 4.** Detergent and lipid effects on cross-linking of AdoMet with NSP1. A 10 µl aliquot of the NSP1 preparation (total protein 98 µg/ml, total phospholipid 130 nmol/ml) was incubated without detergent (control; lane 1) or in the presence of 2.5 mM TX-100 (lane 2) or 34 mM octylglucoside (lane 3). PS (low concentration, 0.9 mM, lane 4; high concentration, 5.2 mM, lane 5) or PC (low concentration, 0.9 mM, lane 6; high concentration, 5.2 mM, lane 7) was present in TX-100 micelles as indicated. Then, 2.5 µl of 500 mM Tris–HCl pH 7.5, 20 mM DTT, 20 mM EDTA and 2.5 µCi of *S*-adenosyl-L-[methyl-$^3$H]methionine were added, and the mixtures were subjected to UV cross-linking, followed by analysis with SDS–PAGE and autoradiography. Molecular weight markers (in kDa) are shown on the left, and the position of NSP1 is indicated by an arrow.

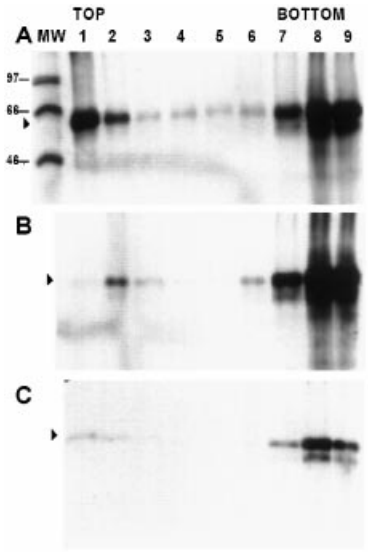


**Fig. 5.** Binding of NSP1 to liposomes. NSP1 or its derivatives were translated *in vitro* in the presence of multilamellar liposomes, and the translation mixtures were subjected to flotation in discontinuous sucrose gradients as detailed in Materials and methods. Fractions were collected and analyzed by SDS–PAGE and autoradiography to reveal the radiolabeled NSP1. (**A**) NSP1 translated with PS:PC (1:1) liposomes. Molecular weight markers (in kDa) are shown on the left. The lower band, just below full-length NSP1 (64 kDa), is due to initiation at a downstream site, as revealed by translation of the deletion constructs (not shown). (**B**) NSP1 translated with PC liposomes. (**C**) Mutant R253A, K254A, R257A translated with PS:PC (1:1) liposomes.

### *Association of AdoMet with NSP1 is inhibited by detergents*

To analyze NSP1 under conditions of detergent inactivation and lipid reactivation, we used UV light-induced cross-linking of the protein with one of its substrates, the methyl donor *S*-adenosyl-L-methionine (AdoMet) (Ahola *et al*., 1997). We found that the ability of the protein to cross-link with AdoMet was lost in the presence of TX-100 (Figure 4, lane 2) or octylglucoside (lane 3), and was reinstated by addition of PS (lanes 4 and 5). In lipid–TX-100 mixed micelles with the optimal concentration (5.2 mM) of PS (lane 5), cross-linking was even stronger than in the control (lane 1). Thus, it correlated with the loss and resumption of the methyltransferase activity (see Figure 3, lane 1, and Figure 2B), indicating that addition of detergent resulted in a conformational change which prevented the binding of the methyl donor to NSP1 and, therefore, the methyltransferase reaction. We also probed NSP1 with different concentrations of trypsin, in the absence of detergent or in the presence of octylglucoside micelles or mixed micelles of octylglucoside and PS, or octylglucoside and PC. Defined fragments were produced reproducibly as partial digestion products, but no differences in fragmentation patterns under these varied conditions were apparent (not shown).

### *Association of in vitro translated NSP1 with liposomes*

To examine the lipid association of NSP1 further, we translated it *in vitro* in the presence of multilamellar liposomes of different compositions. NSP1 became associated with liposomes containing negatively charged phospholipids (e.g. PS:PC 1:1) as assayed by flotation in discontinuous sucrose gradients (Figure 5A). Flotation was not quantitative, but a substantial fraction (~50%) of NSP1 floated with the liposomes. Liposomes containing only neutral phospholipids such as PC (Figure 5B) did not bind NSP1 (flotation $<$5%). PS alone formed liposomes only with difficulty. They bound NSP1, but they also inhibited translation and were not used in further experiments. Although PG could substitute for PS, or the molar concentration of PS could be reduced to 20 mol% (PS:PC 1:4), PS:PC (1:1) liposomes were used in all subsequent *in vitro* experiments.

NSP1 translated *in vitro* in the presence of liposomes was fully susceptible to proteolysis, indicating that it did not reside in the interior of the liposomes, but was attached to their surface. Flotation of NSP1 in this system was not dependent on its palmitoylation, since several different non-palmitoylated mutants (e.g. C418–420A) floated like the wild-type protein (not shown). Also, we were unable to demonstrate palmitoylation of wild-type NSP1 protein in this system using [$^3$H]palmitate either alone or in combination with coenzyme A and acyl-coenzyme A synthetase. Thus, enzymes capable of palmitoylating NSP1 may not be present in the reticulocyte lysate used for *in vitro* translation. In any case, we conclude that *in vitro* translated NSP1 could be used for studying the palmitoylation-independent mechanism of membrane association.

### *Mapping of the lipid-binding site in NSP1*

We have shown previously that constructs containing residues 1–269 and 1–429 of NSP1 displayed membrane binding in eukaryotic cells which was very similar to that of the non-palmitoylated full-length protein (Laakkonen *et al*., 1996). Thus, in mapping the lipid-binding site, we concentrated on the N-terminal half of this 537 amino acid protein. The above-mentioned N-terminal fragments bound to liposomes *in vitro*, albeit less efficiently than the full-length protein (data not shown), whereas the C-terminal half (residues 264–537, Δ2–263) showed no

**Table I.** Membrane binding and methyltransferase activity of NSP1 derivatives

| Construct | MT[a] | Flotation[b] | |
|---|---|---|---|
| | | *E.coli* | *In vitro* |
| Wild-type | 100 | +++ | +++ |
| Δ2–263 | 0 | +/– | – |
| Δ248–537 | 0 | – | – |
| Δ3–24 | 0 | – | – |
| Δ25–57 | 0 | ++ | +/– |
| Δ58–75 | 0 | +++ | +/– |
| Δ76–107 | 0 | ++ | + |
| Δ108–127 | 0 | +++ | +/– |
| Δ140–158 | 0 | ++ | +/– |
| Δ171–195 | 0 | + | +/– |
| Δ196–222 | 0 | + | +/– |
| Δ223–244 | 0 | + | +/– |
| Δ245–268 | 0 | – | – |
| K17A, K21A | 42 | +++ | +++ |
| R71A, R72A | 0 | +++ | + |
| K99A, K100A | 10 | +++ | +++ |
| R222A, K225A | 27 | +++ | +++ |
| R230A K231A K232A | 56 | +++ | ++ |
| R253A K254A R257A | 0 | – | +/– |
| R253A | 20 | +++ | ++ |
| R253E | 0 | – | + |
| K254A | 30 | +++ | +++ |
| K254E | 7.3 | +++ | ++ |
| R257A | 39 | +++ | ++ |
| R257E | 6.7 | ++ | ++ |
| Y249A | 0 | +++ | +/– |
| L255A, L256A | 4.8 | +++ | +/– |
| R253E, L256E | 0 | – | +/– |
| W259A | 3.2 | – | +/– |

[a]Methyltransferase activity as a percentage of wild-type.
[b]Flotation as compared with wild-type = 100%. Scale: – = 0–10%; +/– = 10–20%; + = 20–40%; ++ = 40–60%; +++ = 60–100%.

binding (Table I). We constructed a set of 10 small deletions covering residues 1–268 (see Figure 6 and Table I). The deletions were planned to take into account the predicted secondary structure of the N-terminal half of NSP1 (Ahola *et al*., 1997; Figure 6). In addition to the deletions, we constructed a set of point mutations altering clusters of lysines and arginines to alanine (Figure 6 and Table I), as positively charged residues might have a role in the selective binding of the protein to negatively charged phospholipids.

The constructs were tested for flotation in the *in vitro* translation system with PS:PC 1:1 liposomes, as well as after production in *E.coli* with the bacterial membranes (Table I). The flotation experiments were repeated 2–5 times, and they gave reproducible results. Bacterial extracts were also tested for methyltransferase activity, which was normalized by the expression level of each protein variant, as quantitated by Western blotting (Table I).

All deletion variants were enzymatically inactive, as expected, since they were made in the part of the protein implicated in methyltransferase activity (Ahola *et al*., 1997). None of them bound to liposomes and/or bacterial membranes as efficiently as the wild-type protein according to the flotation criterium. However, some exhibited behavior intermediate between that of the wild-type and the negative control (Table I), whereas Δ3–24 and Δ245–268 consistently did not float in either system. Further

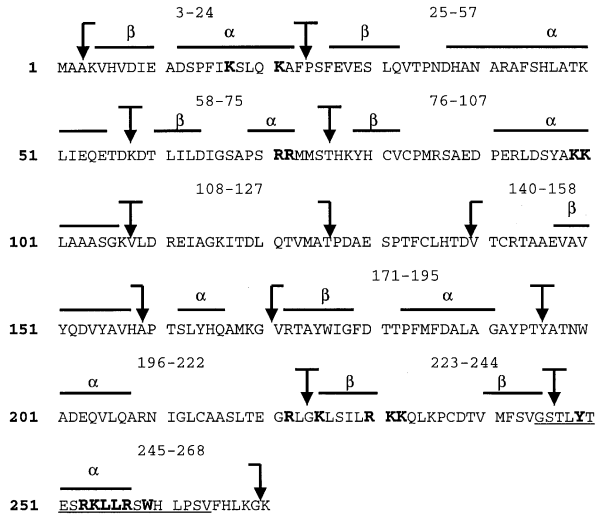


**Fig. 6.** A schematic diagram of the NSP1 constructs used. The amino acid sequence of the N-terminal half of SFV NSP1 is presented together with the predicted secondary structure of the protein (α-helices and β-strands, as indicated). Mutated residues are shown in bold, and deletions are indicated above the sequence by numbers and arrows. The sequence of the synthetic peptide is underlined.

information was obtained from the results with the point mutations. All of them except for one, the triple mutant R253A, K254A, R257A, showed lipid affinity approaching that of the wild-type protein. With the *in vitro* system, the double mutant R71A, R72A also showed poor flotation. This may be due to heavy aggregation of the protein as >90% of R71A, R72A was found in inclusion bodies, which were excluded before the flotation experiment in the *E.coli* system. In the methyltransferase assay, R71A, R72A was inactive, consistent with its aggregation tendency, as was R253A, K254A, R257A, and other constructs showed intermediate levels of activity. Mutation K17A, K21A did not support a role for the extreme N-terminus of NSP1 in membrane binding. Thus, the effect of Δ3–24 may be due to a disturbance of protein folding.

On the basis of the above results, we concentrated further efforts on the amino acid residues 245–268 region of NSP1, which had shown interesting behavior in both the deletion and point mutation analyses. Residues 249–259 are predicted to form a short α-helix (Figure 6) which has some amphipathic character and contains three positively charged residues, R253, K254 and R257. First, NSP1 was truncated further to contain only residues 1–247 (Δ248–537). This derivative did not float with membranes (Table I), in contrast to the construct containing residues 1–269. Secondly, the three positively charged residues were changed individually to alanine or glutamate, which produced a set of proteins showing behavior intermediate between that of the wild-type and the triple mutant. Mutations in residue R253 had the strongest effects. Glutamate substitution had more drastic effects than alanine (Table I). Thirdly, mutations of the nearby hydrophobic residues (Y249, L255, L256 and W259, Table I) reduced the methyltransferase activity considerably and affected the binding to liposomes and membranes.

### *Inhibition of lipid binding of NSP1 by an amphipathic peptide (amino acids 245–264)*

We also employed a synthetic peptide spanning residues 245–264 of SFV NSP1, and a control peptide of the same

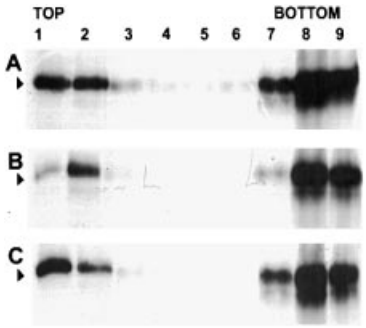


**Fig. 7.** Competition for binding of *in vitro* synthesized NSP1 to liposomes in the presence of synthetic peptide representing amino acids 245–264 of NSP1 (B) or a randomized sequence with identical amino acid composition (C). NSP1 was translated *in vitro* in the presence of multilamellar liposomes consisting of PS:PC (1:1). The translation mixtures were subjected to flotation in discontinuous sucrose gradients as in Figure 5. (**A**) Control translation in the absence of peptides. (**B**) Translation in the presence of 0.5 mM peptide GSTLYTESRKLLRSWHLPSV. (**C**) Translation in the presence of 0.5 mM RLSEYKLGHTSLPWRSTVLS. In both cases, the respective peptide was included in the 67, 60 and 50% sucrose layers at 0.05 mM concentration. The amount of floating NSP1 in (B) was 45% of that in (A). In (C), the percentage was 110%. The arrow indicates the position of NSP1.

amino acid composition but with the order of the residues scrambled, in the *in vitro* translation. Flotation of the translational products revealed that compared with the untreated control (Figure 7A), peptide 245–264 inhibited the binding of NSP1 to liposomes by 55% (Figure 7B), whereas the control peptide had no inhibitory effect (Figure 7C).

## Discussion

In this report, we have studied the mechanism by which SFV NSP1, and thereby probably the entire alphavirus RNA replication complex, binds to intracellular membranes. Wild-type NSP1 is a palmitoylated protein (Peränen *et al*., 1995). However, our previous studies have shown that palmitoylation is not essential for the membrane attachment of NSP1 (Laakkonen *et al*., 1996). More recently, we have shown that SFV in which the palmitoylation sites of NSP1 have been eliminated is viable and replicates effectively in cell cultures (T.Ahola, P.Kujala, T.Anttinen, P.Laakkonen, N.Ehsani, L.Kääriäinen and P.Auvinen, in preparation). Thus, here we have studied the membrane attachment of non-palmitoylated NSP1 synthesized in *E.coli* and *in vitro*.

Non-palmitoylated NSP1 had affinity for both bacterial plasma membranes (Figure 1) and phospholipid liposomes (Figure 5). In the liposome system, the membrane binding of NSP1 required the presence of negatively charged phospholipids such as PS, which is a major lipid in the cytoplasmic leaflet of eukaryotic membranes where NSP1 normally is localized (Peränen *et al*., 1995; Laakkonen *et al*., 1996). However, in the membranes of *E.coli*, the most abundant negatively charged phospholipids are PG and CL, whereas PS is present only in trace amounts (Ames, 1968). PG was also effective in mediating the binding of NSP1 to liposomes, so it may have the same activity in attaching NSP1 to the bacterial plasma membrane. Interestingly, the same types of negatively charged phospholipids that were needed for binding of NSP1 to liposomes were also found to be activators of the methyltransferase and guanylyltransferase reactions catalyzed by NSP1 (Figure 3). Solubilization of NSP1 with a variety of detergents strongly inhibited the enzymatic reactions, but reconstitution either in vesicles or in mixed micelles of detergent and lipid reactivated them (Figures 2 and 3). The activation of SFV NSP1 by mixed TX-100–PS micelles took place over a large molar range of the lipid, whereas less lipid was needed in mixed micelles containing octylglucoside (Figure 2). This might reflect the ability of the detergent to dissolve phospholipid: in octylglucoside–egg PC micelles, the number of octylglucoside molecules changed from ~84 molecules when $n_{PC}$ was 2–3 to close to 350 molecules when $n_{PC}$ was 30. When the amount of PC was increased further, mixed micelles changed to lamellar structures (Eidelman *et al*., 1988). We assume that the binding between NSP1 and mixed micelles needs an optimal density of polar head groups of PS, which depends on the properties of the detergent.

The mechanism by which non-palmitoylated NSP1 bound to negatively charged lipids and was activated by them was addressed indirectly. Detergent-treated, inactive NSP1 failed to bind its substrate AdoMet, suggesting that NSP1 had undergone a conformational change. However, this change may be subtle since partial proteolysis with trypsin did not produce an altered cleavage pattern. Furthermore, the conversion between inactive and active states of the protein was reversible (Figure 3). In Triton X-114 phase separation, non-palmitoylated NSP1 behaves as a soluble protein, suggesting that ionic interactions with the negatively charged lipids might play an important role in the membrane attachment of this protein (Laakkonen *et al*., 1996). Our previous finding that non-palmitoylated NSP1 is partly released from its membrane association by treatment with 1 M NaCl supports this view (Laakkonen *et al*., 1996). With this in mind, we tentatively mapped the lipid-binding site of NSP1 with deletion and point mutation analysis (Table I and Figure 6). The results suggested that a small segment in the central region of the protein (amino acids 245–264) was important for the membrane binding. Significantly, mutations in this region, which interfered with membrane binding, also inactivated the enzymatic activities of NSP1 (Table I), supporting the idea that membrane association and enzymatic activities were tightly linked. To confirm the importance of amino acids 245–264 in the membrane binding of NSP1, we employed a synthetic peptide of the corresponding amino acid sequence. The peptide was able, although in relatively high concentrations, to compete with NSP1 for binding to liposomes (Figure 7). Competition was not observed with a peptide of the same composition scrambled in the sequence of amino acids.

Membrane binding of proteins through amphipathic α-helices has been well established for several cellular proteins. Structural data at a peptide level have been obtained for blood coagulation factor VIII (Gilbert and Baleja, 1995) and the enzyme CTP:phosphocholine cytidylyltransferase (Dunne *et al*., 1996), whereas the crystal structure strongly suggests this mechanism for prostaglandin $H_2$ synthase-1 (Picot *et al*., 1994) and apolipoprotein A-I (Borhani *et al*., 1997). Interestingly,

both CTP:phosphocholine cytidylyltransferase and factor VIII show specific activation in the presence of negatively charged lipids or PS, respectively (Gilbert *et al*., 1990; Cornell, 1991), indicating that short peptides may mediate selective binding and lead to conformational changes in the presence of lipids. Competition for membrane binding against the full-length protein by peptides derived from it has been demonstrated in the case of factor VIII (Foster *et al*., 1990; Gilbert and Baleja, 1995).

In the above cases of protein–membrane association, the interaction between charged amino acids and the polar head groups of phospholipids is supported by hydrophobic interactions, often involving aromatic amino acids. For the suggested membrane-binding peptide of NSP1, both positively charged and hydrophobic residues were important for function (Table I). Interestingly, the proposed lipid-binding site is one of the most conserved segments within NSP1-like proteins (Rozanov *et al*., 1992). Especially intriguing is the pattern of conservation preserving hydrophobic, aromatic or positively charged residues at positions 249, 253, 256 and 259 (SFV NSP1 numbering). Thus, this amphipathic helix could mediate membrane binding and lipid-dependent activation as a common feature of all related viral replicases. To establish this, other proteins from this superfamily need to be examined.

Adaptation to function in the context of membranes seems to be common to replicase enzymes of positive strand RNA viruses. In studies of whole replication complexes, it is usually observed that when membrane preparations of viral replicases are solubilized with detergents, at least some forms of viral RNA synthesizing activity are lost (de Graaff and Jaspars, 1994; Buck, 1996). In the case of flock house virus, liposomes consisting of glycerophospholipids were able to induce the synthesis of positive RNA strands *in vitro*, leading to complete replication (Wu *et al*., 1992). However, the components or interactions affected by detergents or lipids are mostly unknown. For poliovirus, the best studied of all RNA viruses (Rueckert, 1996), the situation seems complex, since at least two of the virus-encoded replicase components are able to interact tightly with membranes on their own. Both proteins 2C and 3AB contain a predicted amphipathic α-helix important for membrane binding (Echeverri and Dasgupta, 1995; Towner *et al*., 1996; Teterina *et al*., 1997), suggesting at least some analogy with alphavirus NSP1. Interestingly, detergent-solubilized 3AB is active in some *in vitro* assays, but inactive in others, possibly due to a conformational change (Lama *et al*., 1994).

In conclusion, the results of this study represent one step towards a molecular definition of the mechanism whereby a viral RNA replication complex is attached onto intracellular membranes and made dependent on them. Analytical understanding of this mechanism should allow perturbation of the membrane attachment in a controlled manner, which should in turn clarify its functional significance in the virus life cycle.

## Materials and methods

### Materials

Bovine brain-derived phospholipids were from Sigma, and they contained mixtures of fatty acid moieties, except for PS 6:0 which was from Avanti Polar Lipids. Gangliosides were isolated from pig brain (Lampio *et al*., 1988). Detergents were from Boehringer Mannheim, and radiochemicals from Amersham. Synthetic peptides were made at the Division of Biochemistry, Department of Biosciences, University of Helsinki with an ABI 433 (Applied Biosystems) synthesizer using 9-fluorenylmethoxycarbonyl chemistry, and purified with a C18 reverse phase column. They were of correct composition and devoid of contaminating peptides as assessed by matrix-assisted laser desorption–ionization time-of-flight mass spectrometry.

### Protein expression and flotation

The optimal conditions for expression of SFV NSP1 and its derivatives in *E.coli* have been described (Laakkonen *et al*., 1994; Ahola *et al*., 1997). The bacteria were lysed with a French press (Laakkonen *et al*., 1994), but the lysis buffer was modified to omit the detergent and contained 50 mM Tris pH 8.0, 50 mM NaCl, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride and 10% glycerol. The lysate was centrifuged at 15 000 *g* for 20 min, and the supernatant fraction was used for further experiments.

Combined *in vitro* transcription–translation was performed with the TnT T7 kit (Promega) using appropriate circular plasmids containing the desired genes under control of the T7 promoter. Reactions were performed in a final volume of 25 µl. In most experiments, 5 µl of multilamellar liposomes were included in the reaction mixture. These were prepared by vortexing the desired lipids [dried from stocks in chloroform/methanol (2:1) solutions by nitrogen evaporation] with a buffer containing 50 mM Tris pH 7.5, 50 mM NaCl, to give a total lipid concentration of 8.3 mM.

Membrane association of NSP1 in *in vitro* translation mixtures and in bacterial lysates was analyzed in discontinuous sucrose gradients as follows. The samples were mixed with 67% (w/w) sucrose, to give a final concentration of 60% (w/w). Discontinuous gradients were prepared in SW50.1 (Beckman) ultracentrifuge tubes by layering first 0.5 ml of 67% (w/w) sucrose, then 0.5 ml of the sample in 60% sucrose followed by 3 ml of 50% (w/w) sucrose and 1 ml of 10% sucrose. The sucrose solutions were prepared in a buffer containing 100 mM NaCl and 50 mM Tris pH 7.5. The gradients were centrifuged overnight (>16 h) at 35 000 r.p.m. in a SW50.1 rotor at 4°C. During the centrifugation, membranes and membrane-associated proteins floated to the top of 50% sucrose layer, while soluble proteins remained in the 60 and 67% layers of the gradient. Fractions were collected from the top.

### Enzyme assays and cross-linking

Guanine-7-methyltransferase was assayed in a 25 µl volume containing 100 mM HEPES, pH 6.95, 2 mM $MgCl_2$, 2 mM DTT, 20 µM AdoMet, 0.75 µCi of *S*-adenosyl-L-[methyl-$^3$H]methionine and 10 mM GTP for 30 min at 30°C, and analyzed as before (Laakkonen *et al*., 1994). Cross-linking of tritiated AdoMet with NSP1 by UV light and formation of the covalent guanylate complex were assayed as described previously (Ahola *et al*., 1997).

### Preparation of lipid vesicles by detergent removal with hydrophobic beads

The desired phospholipids (total of 500 µg, ~0.7 µmol) were dried thoroughly in Eppendorf tubes, and 110 µl of 68 mM octylglucoside (7.5 mmol) in 50 mM HEPES pH 7.0, was added. After vigorous mixing, the tubes were incubated for 2 h at room temperature in a nitrogen atmosphere to allow the formation of mixed micelles. Then, 90 µl of the same buffer and 50 µl of a flotated NSP1 preparation (total protein 44 µg/ml, total phospholipid 70 nmol/ml) were added followed by vortexing. The micelles were transferred into microcentrifuge tubes each containing 26 mg of washed and pre-wetted Bio-Beads (Bio-Rad) as described (Rigaud *et al*., 1997). The tubes were incubated overnight at 6°C with end-over-end mixing. Two 15 µl samples of each preparation were assayed for methyltransferase activity. In control experiments, Bio-Beads, lipids or detergent were omitted from the reaction mixtures.

### Preparation of mixed micelles

Phospholipids (41–820 nmol) were pipetted into Eppendorf tubes, and the solvent evaporated under a stream of nitrogen. The dried lipids were resuspended in 50 µl of 50 mM HEPES pH 7.0, containing either octylglucoside (68 mM) or TX-100 (5 mM) in a nitrogen atmosphere. The micelles were incubated at room temperature for 2–3 h before use, with occasional vortexing. A 10 µl aliquot of the NSP1 preparation (total protein 44 µg/ml, total phospholipid 70 nmol/ml) and a further 10 µl of buffer were added. After 30 min incubation at room temperature, duplicate samples were assayed for methyltransferase activity.

### DNA constructs

Point mutations were constructed in plasmid pTSF1 (Peränen *et al*., 1995), a derivative of pGEM3 (Promega), where the SFV NSP1-coding region is under the control of the T7 promoter. The unique site elimination method (U.S.E. mutagenesis kit, Pharmacia Biotech) was applied according to the manufacturer's instructions. The presence of the desired mutations was verified by sequencing. Deletion constructs were made by PCR as described (Ho *et al*., 1989). The sequences of the oligonucleotides used are available upon request. The reaction products were cloned with the TA cloning kit (Invitrogen) and sequenced. An appropriate fragment of these constructs was transferred to pTSF1 using suitable restriction sites. For production in *E.coli*, the NSP1 derivatives were cloned into the tightly regulated expression plasmid pBAT4 (Peränen *et al*., 1996) as *Nco*I–*Hin*dIII fragments.

### Other methods

For cryoelectron microscopy, ultrathin frozen sections were prepared as described by Tokuyasu (1988) from *E.coli* expressing NSP1. Protein was determined by the method of Bradford (1976) using bovine serum albumin as a standard, and total phospholipid (lipid-bound phosphorus) according to Rouser *et al*. (1970). Western blotting to detect SFV NSP1 has been described (Laakkonen *et al*., 1994). Autoradiograms were quantitated with a Fuji Bas 1500 Bioimaging Analyzer instrument.

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