Effects of Palmitoylation of Replicase Protein nsP1 on Alphavirus Infection

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The membrane-associated alphavirus RNA replication complex contains four virus-encoded subunits, the nonstructural proteins nsP1 to nsP4. Semliki Forest virus (SFV) nsP1 is hydrophobically modified by palmitoylation of cysteines 418 to 420. Here we show that Sindbis virus nsP1 is also palmitoylated on the same site (cysteine 420). When mutations preventing nsP1 palmitoylation were introduced into the genomes of these two alphaviruses, the mutant viruses remained viable and replicated to high titers, although their growth was slightly delayed. The subcellular distribution of palmitoylation-defective nsP1 was altered in the mutant: it no longer localized to filopodial extensions, and a fraction of it was soluble. The ultrastructure of the alphavirus replication sites appeared normal, and the localization of the other nonstructural proteins was unaltered in the mutants. In both wild-type- and mutant-virus-infected cells, SFV nsP3 and nsP4 could be extracted from membranes only by alkaline solutions whereas the nsP2-membrane association was looser. Thus, the membrane binding properties of the alphavirus RNA replication complex were not determined by the palmitoylation of nsP1. The nsP1 palmitoylation-defective alphaviruses produced normal plaques in several cell types, but failed to give rise to plaques in HeLa cells, although they induced normal apoptosis of these cells. The SFV mutant was apathogenic in mice: it caused blood viremia, but no infectious virus was detected in the brain.

The alphaviruses are a well-studied group of enveloped, plus-strand RNA viruses (36). Several members of the Alphavirus genus are capable of causing fatal encephalitis in rodents, domestic animals, and humans. Therefore, they can be used as a general model of virus-induced central nervous system disease (4). After virus entry, the genomic RNA (approximately 12 kb) is translated to yield the four essential virus-encoded proteins individually in animal cells has indicated that nsP4 membrane attachment of the replication complex cannot be predicted from the sequence. Expression of the nonstructural proteins individually in animal cells has indicated that nsP4 and nsP2 do not associate with membranes. In contrast, both nsP3 and nsP1 have some affinity for membranes; however, only nsP1 localizes to endosomes and lysosomes and exhibits tight binding to membranes, features typical for the replication complex (23–25). We have previously shown that Semliki Forest virus (SFV) nsP1 is palmitoylated on cysteine residues 418 to 420 (16, 24). Palmitoylation has a major effect on the membrane binding of SFV nsP1 when the protein is expressed alone (16, 17).

The amino acid sequences of the alphavirus nonstructural proteins are hydrophilic (35, 37), and thus the mechanism of membrane attachment of the replication complex cannot be predicted from the sequence. Expression of the nonstructural proteins individually in animal cells has indicated that nsP4 and nsP2 do not associate with membranes. In contrast, both nsP3 and nsP1 have some affinity for membranes; however, only nsP1 localizes to endosomes and lysosomes and exhibits tight binding to membranes, features typical for the replication complex (23–25). We have previously shown that Semliki Forest virus (SFV) nsP1 is palmitoylated on cysteine residues 418 to 420 (16, 24). Palmitoylation has a major effect on the membrane binding of SFV nsP1 when the protein is expressed alone in animal cells. The palmitoylated wild-type (wt) protein binds very tightly to membranes and can be released only by treatment with sodium carbonate at pH 12. In contrast, if the palmitoylation site is removed, nsP1 appears partially soluble and its membrane-associated fraction can be solubilized with 1 M NaCl (16). Based on these results, the hypothesis that nsP1 palmitoylation similarly affects the membrane binding of the entire alphavirus replication complex seems very attractive.

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The palmitoylation-independent mechanism of nsP1-membrane association appears to involve a direct interaction of the protein with anionic phospholipids. This interaction is at least partially mediated by a highly conserved nsP1 segment (amino acids 245 to 264), which is distant from the palmitoylation site in the primary sequence (3). Interaction with anionic lipids is required to activate the methyltransferase and guanylyltransferase activities of nsP1, needed in the capping of the viral RNAs (3). The capping activities (1, 5) are essential for viral RNA replication (38). The enzymatic activities and the palmitoylation site of nsP1 are independent of each other, since the nonpalmitoylated forms of the protein are enzymatically active and, conversely, enzymatically inactive proteins can become palmitoylated (2, 16, 18). Therefore, the palmitoylation site of nsP1 can be removed without affecting the essential enzymatic activities of the protein.

Here, we study the palmitoylation of nsP1 for the first time in the context of alphavirus infection and address two interconnected sets of questions: (i) whether the palmitoylation of nsP1 controls or contributes to the membrane association of alphavirus replication complexes, and (ii) in what other ways the palmitoylation of nsP1 affects alphavirus infection in cultured cells or in animals. Palmitoylation of nsP1 appears to be conserved during alphavirus evolution and induces morphological changes in infected cells.

MATERIALS AND METHODS

Cells and viruses. The recombinant vaccinia virus vTF7-3 was propagated in HeLa cells as described previously (11). The stocks of SFV and Sindbis virus (SIN) (used in this work) were derived from the infectious cDNA clones of these viruses, pSP6-SFV4 (22) and pTOTO1101 (29), respectively, which were kindly provided by Peter Liljestrom (Karolinska Institutet, Stockholm, Sweden) and Charles M. Rice (Washington University School of Medicine, St. Louis, Missouri), respectively. BHK cells were used for growing of the alphavirus stocks as described previously (14). Virus titers were determined by plaque assays on BHK cell or MBA-13 cell monolayers (14, 31).

MBA-13 is a transformed mouse brain cell line expressing an oligodendrocyte-specific marker, 2′,3′-cyclic nucleotide 3′-phosphodiesterase. These cells were maintained in Eagle’s minimal essential medium (MEM) supplemented with 5% fetal calf serum and 10 mg of gentamicin per ml. Subconfluent MBA-13 cells monolayers in 24-well tissue culture plates (Greiner) were infected with serial dilutions of blood and brain samples in medium, allowed to absorb for 1 h at 37°C, and overlaid with medium made viscous with 0.5% cellulose gum (CMC; Natrosol; Hercules Inc.) (1 part 4% CMC, 1 part 2% NaCO3 (pH 12). The membranes and resulting extraction supernatants were collected, clarified by centrifugation, and used to infect fresh BHK cells (50% confluent monolayers of BHK cells on 30-mm dishes). A 2-μg portion of plasmid DNA was incubated with 10 μl of Lipofectin (Gibco BRL) in OptiMEM (Gibco BRL) as specified by the manufacturer, and the mixture was incubated with cells for 1 h with gentle agitation. The transfection mixture was removed, and the cells were incubated with 1 ml of the normal serum-containing growth medium. The majority of the virus had detached from the dish (24 to 48 h) as a consequence of alphavirus infection. Control transfections were performed either with plasmid DNA or without any nucleic acid. In the controls, the cells showed no cytopathic changes. To generate virus stocks, the growth media were collected, purified by centrifugation as described previously (14), and stored at −80°C for up to 1 month. Equivalent virus titers were used for titer determination, and in all experiments described below.

DNA was isolated from the second-passage virus stocks with a High Pure viral RNA kit (Boehringer Mannheim) and subjected to reverse transcription with Superscript II (Gibco BRL). The nsP1 coding region was PCR amplified with appropriate primers, and several independent PCR products were directly sequenced to verify the maintenance of mutations or wt sequences. All clones sequenced contained the expected sequences at the palmitoylation site of nsP1.

RESULTS

DNA transfection and cell labeling. HeLa cells on 30-mm dishes were infected with the recombinant vaccinia virus vTF7-3 for 45 min, and then the cells were transfected with plasmids containing the gene of interest under the T7 polymerase promoter. A 2-μg portion of plasmid DNA was incubated with 8 μl of Lipofectin in 1 ml of OptiMEM on the cells for 1 h. Then the transfection mixture was replaced with MEM containing 0.2% bovine serum albumin (BSA). To detect palmitoylation of the proteins of interest, the transfected cells were labeled with 60 μCi of [3H]palmitate between 2 and 4 h posttransfection and treated as described above (16). Alphavirus-infected (multiplicity of infection [MOI] = 50) BHK cells on 60-mm dishes were labeled with 30 μl of [3H]palmitate between 2 and 4 h postinfection in MEM–0.2% BSA. The labeled cells were washed with ice-cold phosphate-buffered saline (PBS) and collected by scraping into 1% sodium dodecyl sulfate (SDS). Proteins were denatured by boiling the sample for 2 min. In some experiments, alphavirus-infected cells (MOI = 50) were labeled with [3H]S-methionine. In this case, the cells were preincubated in MEM–0.2% BSA but lacking methionine for 1 h prior to labeling, labeling was carried out in the same medium, and in some experiments the labeling was followed by a wash and a 2-h chase in MEM–0.2% BSA containing 20 times the normal medium concentration of methionine. Cells were similarly collected in SDS and proteins denatured by boiling.

Cell fractionation and extraction experiments. After swelling in hypotonic buffer (10 mM Tris [pH 7.5], 10 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride), cells were disrupted by Dounce homogenization and subsequently fractionated to yield nuclear (5 min at 500 × g), P15 (20 min at 15,000 × g), and S15 fractions as described previously (1). Portions of the P15 fraction were subjected to the following treatments for 30 min on ice: (i) 50 mM Tris (pH 7.5)–100 mM NaCl (ii) 50 mM Tris–1 M NaCl, (iii) 75 mM NaClO4 (pH 11.5), and (iv) 75 mM NaClO4 (pH 12). The membranes and resulting extraction supernatants were isolated by recentrifugation at 15,000 × g for 20 min.

Studies of mouse pathogenesis. Specific-pathogen-free BALB/c mice (4 to 6 weeks old) were infected intraperitoneally with defined doses of virus in PBS. Duplicate samples of blood and brain tissue were collected daily for 8 days postinfection. Mice were anesthetized with CO2, and blood was collected from the right ventricle, after which the blood was perfused with PBS and the brains were collected in 10 ml of cold PBS. The blood samples were subjected to low-speed centrifugation, and the supernatant was diluted 1:10 in PBS, while the brain samples were homogenized in PBS and centrifuged. Diluted sera and supernatants of brain homogenates were stored at −70°C until used for titer determination.

Other methods. Immunoprecipitation of alphavirus nonstructural proteins, after SDS denaturation, and Western blotting were carried out as described previously (18, 27). Indirect immunofluorescence analysis was performed as described previously (17). Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assays (Boehringer Mannheim) for fixed cells were done as specified by the kit manufacturer. Cells were treated for transmission electron microscopy as described previously (15).

Palmitoylation of SIN nsP1. We have previously shown that SFV nsP1 is covalently modified by palmitoylation of one or more of the three consecutive cysteines at positions 418 to 420 (16). In all alphaviruses sequenced, this region of nsP1 contains 1 to 3 cysteine residues (Fig. 1A). To examine whether palmitoylation of these cysteines is a general feature of alphaviruses, we chose to study SIN nsP1, since SFV and SIN are quite divergent members of the alphavirus genus: the overall amino acid sequence identity of SFV and SIN nsP1 is 64%.
The sequences around the putative palmitoylation site also show considerable variation between different alphaviruses (Fig. 1A). It is especially noteworthy that in some viruses, exemplified by SIN, nsP1 contains only one cysteine (at position 420 in SIN) at the putative palmitoylation site. Although there is no known consensus sequence for protein palmitoylation, in several cases the site of modification comprises multiple palmitoylatable cysteine residues (28).

Initially, SIN nsP1 was expressed in HeLa cells by transient transfection with the recombinant vaccinia virus-T7 system. The cells were labeled with tritiated palmitic acid to reveal the covalent modification of proteins by palmitoylation. Cell lysates were immunoprecipitated with antisera against nsP1 and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography to visualize radioactive labeling of nsP1. The engineered mutants were analyzed by labeling of cells expressing nsP1 alone (Transfected) or in the context of alphavirus infection (Infected) with [3H]palmitate. Mutant or wt nsP1 was analyzed by labeling of cells expressing nsP1 alone (Transfected) or in the context of alphavirus infection (Infected) with [3H]palmitate. Mutant or wt forms of SFV or SIN nsP1 were used as indicated at the top. The cell extracts were immunoprecipitated with antisera against nsP1 and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography to visualize radioactive labeling of nsP1. Equal amounts of each wt-mutant pair of samples were loaded on the gel. Approximately 10 times more infected cell extracts than transfected cell extracts were used in this analysis due to the higher level of expression of the proteins in transfected cells. (C) The labeled cell extracts used in panel B were analyzed by Western blotting with antisera against nsP1. Equal amounts of each wt-mutant pair were loaded on the gel.

Effects of nsP1 palmitoylation on alphavirus viability and growth. To study the effects of palmitoylation on alphavirus replication, the sequences encoding palmitoylation-defective nsP1 proteins were transferred to bT10 cells. Both mutant viruses were grown in BHK cells after RNA transfection. To confirm that no reversion had occurred, RNA was isolated from the virus stocks and subjected to reverse transcription and PCR amplification of the nsP1 encoding region. Sequencing of several independent PCR clones indicated that the mutations had been maintained. Thus, the viruses SFV1pa- and SIN1pa- do not contain cysteine residues at the palmitoylation site of nsP1.

BHK cells infected with the wt and mutant viruses were also subjected to labeling with tritiated palmitic acid (Fig. 1B and C). Localization of the replicase proteins in SFV1pa- and SIN1pa- infected cells was determined by indirect immunofluorescence microscopy using antibodies against the replicase proteins, to reveal possible differences in their subcellular distribution. There were two features distinguishing wt- and mutant-virus-infected cells. First, the cell morphology was altered during wt virus infection by the induction of filopodium-like extensions (Fig. 3A and C). These were not found in SFV1pa- or SIN1pa- infected cells (Fig. 3B and D) or in...
noninfected control cells (data not shown). These filopodium-like structures are also known to be induced by the wt nsP1 protein, but not by its nonpalmitoylated mutant derivative, when nsP1 is expressed alone in various cell types by transfection, but their significance and mechanism of formation are not understood (17). Thus, the same phenomenon of filopodium induction and its dependence on the palmitoylation of nsP1 can be observed in alphavirus-infected cells. Second, the distribution of nsP1 was different in the wt- and mutant-virus-infected cells. wt nsP1 was seen exclusively in association with membranes, on the plasma membrane and in its filopodial extensions, as well as on intracellular vesicles. In contrast, the nonpalmitoylated nsP1 of SFV1pa- or SIN1pa- showed a diffuse staining throughout the cytoplasm in addition to being present on intracellular vesicles and as patches on the plasma membrane (Fig. 3B and D). The distribution of the other nonstructural proteins was not altered in SFV1pa- and SIN1pa-infected cells compared to those infected with the wt virus; the cytoplasmic replication sites known as CPVIs were prominently observed, as described previously (10, 23, 24).

The ultrastructure of SFV- and SIN-induced CPVI structures was studied by electron microscopy. The internal morphologies of wt- and mutant-virus-infected cells appeared very similar. Characteristic CPVI structures with spherule invaginations were present in both SFV1pa- and SIN1pa-infected cells (Fig. 4), indicating that their formation is not dependent on nsP1 palmitoylation. However, it seemed that the time course of the appearance of CPVIs and spherules was delayed in SFV1pa-infected cells compared with the wt virus infection, consistent with the above result on virus growth and nonstructural-protein production.

Membrane attachment of nonstructural proteins. To biochemically characterize the membrane association of SFV nonstructural proteins, we isolated cytoplasmic membranes pelletting at 15,000 x g and the remaining supernatant fractions from BHK cells infected with either SFVwt or SFV1pa-. The distribution of each of the four nonstructural proteins between the membrane-attached and soluble fractions is shown in Fig. 5A. The nonstructural proteins were present in smaller quantities in mutant-virus-infected cells in accordance with the results of the pulse-chase experiment. In addition, the distribution of nsP1 between the pellet and supernatant fractions was altered in the mutant. wt nsP1 was exclusively membrane bound, while a small fraction of the mutant protein was soluble (Fig. 5A, lanes nsP1 in the wt and 1pa- panels). However, this change in the distribution of nsP1 did not affect the distribution of the other nonstructural proteins. In both cases, nsP4 was exclusively membrane bound and almost half of nsP3 was soluble (Fig. 5A). The anti-nsP4 antiserum cross-reacted with some cellular proteins (which were also present in uninfected cells), giving rise to additional bands in nsP4 blots (Fig. 5). nsP2 appeared predominantly in the soluble fraction in this particular experiment (Fig. 5A), but in repeated tests it more commonly partitioned equally between the two fractions.

The tightness of the membrane association of the nonstructural proteins was then analyzed by extracting the P15 membranes with either physiological or 1 M NaCl or with NaCO3 at pH 11.5 or 12. Treatments with 25 mM EDTA, 25 μg of RNase A per ml, or 1 mM ATP on ice or at room temperature had no effect on the membrane binding of the nonstructural proteins (data not shown). As expected, upon salt or high-pH treatment, nsP1 derived from SFVwt-infected cells displayed the highest membrane affinity of all of the nonstructural proteins (Fig. 5B, panel nsP1 wt). It could be released from the membranes only by alkaline extractions, and even at pH 12 a large fraction of the protein remained membrane bound. In contrast, half of the mutant protein (panel nsP1 1pa-), could be extracted with 1 M salt, and alkaline extraction was highly effective in solubilizing the protein. These results are comparable to those obtained when the palmitoylated and nonpalmitoylated forms of nsP1 were expressed alone in animal cells (16). The only difference may be that initially a larger fraction of nonpalmitoylated nsP1 remained membrane bound in the infected cells (Fig. 5A, panel 1pa-) (16). This could be due to a limited number of membrane binding sites being available for nonpalmitoylated nsP1, whose capacity could be exceeded by the high level of overexpression achieved with the vaccinia virus-T7-mediated transfection system used for expression.

**FIG. 2.** One-step growth curves of SIN and SFV. The wt (solid diamond) or nsP1 palmitoylation-defective (1pa-) (open square) SFV (A and C) and SIN (B) were used to infect BHK or HeLa cells, as indicated (MOI = 5). Duplicate samples were collected at the indicated time points, and the amount of infectious virus was measured by plaque formation in BHK cells. This experiment was done three times with closely similar results; results from a representative experiment are shown.
studies of individual nonstructural proteins; alternatively, the complex formation of nonstructural proteins in infected cells may influence their membrane association (see Discussion).

The clear-cut difference in the membrane affinity of wt and nonpalmitoylated mutant forms of nsP1 had essentially no effect on the extractability of the other nonstructural proteins (Fig. 5B, panels nsP2, nsP3, and nsP4). nsP4 displayed the second tightest binding to membranes, with the majority of the protein being released only by alkaline extractions. In contrast, the membrane association of nsP2 was highly sensitive to salt and only a small fraction of the protein remained membrane bound in 1 M NaCl or at pH 11.5. It is interesting that the behavior of nsP4 and nsP3 resembled that of the nonpalmitoylated form of nsP1 in these experiments carried out with membrane fractions derived from infected cells.

Plaque formation in different cell types. In the virus growth experiments described above, all the virus plaque titer measurements were carried on in BHK cells, since it was observed early on that determination of the SFV<sub>1pa</sub>- titer in HeLa cells was impossible. Both SFV<sub>wt</sub> and SFV<sub>1pa</sub>- formed visible plaques in BHK cells (Fig. 6) and in chicken embryo fibroblasts (CEF) (data not shown) after overnight incubation. After 2 days, the plaques were large and easily visualized (Fig. 6A and B). Similar plaque morphology was observed also with SIN<sub>wt</sub> and SIN<sub>1pa</sub>- in BHK cells and in CEF, as well as with SFV<sub>wt</sub> and SFV<sub>1pa</sub>- in MBA-13 cells (data not shown). In contrast, only the wt viruses could form plaques in HeLa cells (SFV<sub>wt</sub> in Fig. 6C and E). Even after 6 days in culture, only individual, very small plaques could be observed in HeLa cells infected with the mutant virus; Fig. 6D shows a typical field with no plaques, and Fig. 6G shows an individual small plaque.

We then tested whether the difference in plaque formation could be due to the lack of apoptotic cell killing and lysis of HeLa cells by SFV<sub>1pa</sub>- . HeLa cell plaques at 1, 2, 3, or 4 days after infection were stained with anti-nsP1 and anti-E2 antibodies, preceded by the TUNEL staining used to detect apoptotic cells. SFV<sub>wt</sub> plaques contained nsP1 (Fig. 6E) and E2 (data not shown) positive cells throughout. TUNEL-positive dying cells were detected in the center of plaques, but there was a TUNEL-negative, apoptosis-free zone on the edge of the

FIG. 3. Localization of nsP1 in infected cells. BHK cells infected with SFV<sub>wt</sub> (A), SFV<sub>1pa</sub>- (B), SIN<sub>wt</sub> (C), or SIN<sub>1pa</sub>- (D) were fixed at 5 h (A and B) or 6 h (C and D) postinfection and stained by indirect immunofluorescence using anti-nsP1 antibodies. Magnification, ×875.
expanding plaques, containing only nsP1- and E2-positive cells (Fig. 6E and F). We could find only few plaques in SFV wt-infected HeLa cells. However, these plaques always contained apoptotic cells (Fig. 6G and H). The SFV wt plaques were much smaller on day 4 (Fig. 6G and H) than were the SFV wt plaques on day 2 (Fig. 6E and F). In contrast, there was no difference in the size of the plaques caused by SFV wt and SFV 1pa2 in BHK cells, and these plaques also contained similar amounts of apoptotic cells. Furthermore, when HeLa cells were infected with the wt and mutant SFV under normal medium conditions, without CMC to inhibit virus diffusion through solution, the rate of apoptosis measured by DNA laddering was similar in SFV wt- and SFV 1pa2-infected HeLa cell cultures. Thus, the difference in plaque formation in HeLa cells cannot be explained by the inability of SFV 1pa2 to cause apoptosis.

Pathogenesis in mice. wt type SFV4 causes fatal encephalitis in mice (13). To study the effect of nsP1 palmitoylation in an animal system, mice were injected intraperitoneally with different doses of the wt and nsP1 palmitoylation-defective viruses. In our assay, the death of mice inoculated with SFV wt occurred in a dose-dependent fashion. Animals infected with 10^7 PFU died in 5 days, those infected with 10^6 PFU died by day 7, and those infected with 10^5 PFU died by day 11 (Fig. 7). Strikingly, none of the SFV 1pa2-infected mice died, even those infected with the highest dose (10^7 PFU) (Fig. 7).

The course of infection in mice inoculated with 10^6 PFU was monitored by measuring the amount of infectious virus present in blood and in brain tissue every day. SFV wt caused detectable viremia from days 1 to 3 (Fig. 8A), whereas SFV 1pa2 was present in the blood only on day 1 (Fig. 8B). Nevertheless, this indicates a clearly detectable general infection. In the brain tissue, SFV wt was measurably present from day 2 until death, approximately day 6 postinfection (Fig. 8C). In contrast, infectious SFV 1pa2 was never observed in the brain tissue (Fig. 8D).

**DISCUSSION**

We have analyzed the effects of the prevention of the covalent palmitoylation of nsP1, the mRNA-capping subunit of the replicase complex (1, 18, 33), on alphavirus RNA replication. In addition to capping, nsP1 proved to be palmitoylated (Fig. 1), which indicates the conservation of this modification during alphavirus evolution and points to its likely functional significance. We constructed mutant derivatives of both SFV and SIN encoding nonpalmitoylated forms of nsP1 (Fig. 1). These mutant viruses remained viable and grew to high titers, showing only slightly delayed replication (Fig. 2).

**Plaque formation and pathogenesis.** The nsP1 palmitoylation-defective alphaviruses, SFV 1pa2 and SIN 1pa2, gave rise to plaques similar to those produced by the wt viruses in BHK and MBA-13 cells and CEF. However, in HeLa cells, SFV 1pa2 and SIN 1pa2 produced only individual, very small plaques (Fig. 6). The death and lysis of most alphavirus-infected cells is caused by apoptosis, or programmed cell death (12, 21). Therefore, we verified that the capacity of SFV 1pa2 and SIN 1pa2 to induce apoptosis in HeLa cells appeared to be normal. The capability of SFV 1pa2 to replicate and produce infectious virions in HeLa cells was also close to that of the wt virus (Fig. 2); the restriction in replication in HeLa cells appeared only when the cells were grown under a viscous CMC layer. Thus, a possible cause of the lack of plaque formation is a failure of SFV 1pa2 and SIN 1pa2 to spread between HeLa cells under these conditions. It is intriguing that the restriction was found
only in HeLa cells, which are normally quite permissive to alphavirus infection.

wt SFV killed mice in a dose-dependent manner, whereas all the mice infected with SFV_{1pa-} survived (Fig. 7), indicating that nsP1 palmitoylation contributes to SFV pathogenesis and efficient infection of animals. SFV_{1pa-} caused a transient viremia, but no infectious virus was detected in brain homogenates, in contrast to the wt virus, which was prominent in the brain (Fig. 8). In future studies, it will be important to determine whether SFV_{1pa-} can penetrate the blood-brain barrier and replicate or spread in neurons to any extent. It is an exciting possibility that the palmitoylation of nsP1, perhaps in association with the ability of the palmitoylated protein to induce cell surface projections (Fig. 3) (17), contributes to the cell-to-cell spread of alphaviruses. An attenuated strain of

SFV, A7(74), is restricted in spreading in neurons (8), due to mutations in the structural and 5' noncoding regions of the virus genome (4). More generally, mutations in the structural and noncoding regions of the genome have been shown to
attenuate several alphavirus strains (36). Examples of attenuation caused by mutations in the nonstructural region are rare; in addition to the prevention of nsP1 palmitoylation, mutation of the nuclear localization signal of nsP2 and perhaps some mutations in the C-terminal region of nsP3 reduce SFV pathogenicity (4, 30).

Membrane association of the replication complex. The binding of the alphavirus RNA replication complexes to the cytoplasmic surface of endosomes and lysosomes should be mediated by one or more of the virus-encoded nonstructural proteins nsP1 to nsP4. Here we have performed, for the first time, a comprehensive biochemical characterization of the membrane binding properties of each of the SFV nonstructural proteins in infected cells. These experiments were carried out both for infections where nsP1 was wt (palmitoylation positive) and for infections where nsP1 was palmitoylation defective (Fig. 5). The nonstructural proteins were analyzed at an early time point in infection, when a large fraction of the nonstructural proteins so far synthesized is expected to be localized in active replication complexes. At least a fraction of each nonstructural protein was bound to the cytoplasmic membranes tightly, in a salt-resistant and partially alkali-resistant manner (Fig. 5B). It is especially noteworthy that all of the nsP4, the core RNA-dependent RNA polymerase subunit, displayed tight membrane association. nsP4 is present in smaller quantities than the other nonstructural proteins, due to its instability (7) and, in many alphaviruses, also due to its mode of production, involving a low-frequency read-through of a stop codon (36).

Nonpalmitoylated nsP1, nsP3, and nsP4 all showed very similar sensitivities to various extraction procedures (Fig. 5B), while the bulk of nsP2 was more easily detached from the membranes. However, a fraction of nsP2 also displayed tighter binding to membranes. nsP1 itself showed an increased affinity for membranes upon palmitoylation, similar to that previously observed in cells expressing this protein in the absence of other replicase components (16), but this clear-cut change had no effect on the membrane affinity of the other nonstructural proteins. In particular, in the wt situation, other nonstructural proteins could be extracted from membranes while most of nsP1 remained bound (Fig. 5B). Inspection of infected cells by immunofluorescence microscopy was in agreement with the biochemical analysis in that there was essentially no difference in the distribution of other nonstructural proteins beyond nsP1 in cells infected with SFVwt or SFV1pa2. Thus, it can be concluded that palmitoylation of nsP1 is not an important factor in contributing to the membrane binding of alphavirus RNA replication complexes.

How, then, do the alphavirus replicase proteins attain and maintain their relatively tight interaction with membranes? Nonpalmitoylated nsP1 can interact with membranes containing anionic phospholipids (3), and nsP3 could also contribute to the membrane attachment of the replicase (23), although on its own its membrane binding appears to be too weak to account for the properties shown in Fig. 5B (H. Vihinen, T. Ahola, and L. Kääriäinen, unpublished results). It is possible that multiple copies of either or both of these proteins are present in the replication complexes, which would enhance their membrane affinity. Furthermore, the characteristic spherule structures found in connection with the replication complexes, most probably formed through some activities of the nonstructural proteins, may act to protect the replication com-

FIG. 7. Pathogenicity of SFVwt and SFV1pa2 in mice. Five BALB/c mice were inoculated intraperitoneally with each indicated dose (PFU) of the two viruses. Mouse survival is plotted against the time after inoculation. The experiment was carried out so that the identity of the virus (wt or mutant) used in the infections was not known to the persons performing the experiment. This experiment was carried out twice with closely similar results; data from one experiment are shown.

FIG. 8. SFVwt or SFV1pa2 titers in infected mice. Samples were collected on each day after inoculation of mice with 10⁶ PFU of the respective viruses, as described in Materials and Methods. Two mice were sacrificed each day for each virus. The amount of infectious virus in blood (A and B) and in brain tissue samples (C and D) was measured by a plaque assay in MBA-13 cells. The asterisks indicate living mice without detectable virus titer, and crosses indicate mice killed by the virus.
plexes or tighten their membrane binding. It will be important to experimentally address these possibilities in order to more fully understand the membrane association of alphavirus replication complexes, which may serve as models for other plus-strand RNA viruses of eukaryotes.

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