The Effects of Palmitoylation on Membrane Association of Semliki Forest Virus RNA Capping Enzyme*

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The nonstructural protein Nsp1 of Semliki Forest virus has guanine-7-methyltransferase and guanylyltransferase-like activities, required in the capping of viral mRNAs. It is palmitoylated and tightly associated with the cytoplasmic surface of the plasma membrane, endosomes, and lysosomes. To localize the acylation site(s) and the putative membrane-targeting domain, a number of deletions were made in the nsp1 gene. Most deletions resulted in the expression of nonpalmitoylated, enzymatically inactive, cytoplasmic protein. Palmitate could be released from Nsp1 with neutral hydroxylamine, indicating a thioester linkage to a cysteine residue. Therefore we mutated the conserved cysteine residues of Nsp1 to alanine. Triple mutation of Cys418, Cys419, and Cys420 resulted in nonpalmitoylated Nsp1, which was enzymatically active and still associated with membranes. However, it could be released from the membranes with 1 M NaCl, whereas 50 mM sodium carbonate (pH 12) was required to release wild type Nsp1, suggesting a conversion from an integral to a peripheral membrane protein. Indirect confocal immunofluorescence microscopy showed that the nonpalmitoylated Nsp1 colocalized with the plasma membrane marker, concanavalin A. However, it was not detected in filopodia, which were heavily stained in cells expressing wild type Nsp1. These results indicate that the acylation of Nsp1 was not needed for its targeting to the plasma membrane, but it was necessary for the migration to the filopodial extensions of the plasma membrane.

Semliki Forest virus (SFV), 1 a member of alphaviruses, is an enveloped animal virus with a single-stranded RNA genome of positive polarity (1). Replication of alphaviruses occurs in association with modified endosomes and lysosomes (2–4). The viral RNA genome is translated to a large polyprotein, which is autocatalytically processed into four nonstructural proteins, Nsp1–4. Each nsp protein is essential for the RNA replication of the virus (1). Some of their functions have been elucidated by genetic criteria and by expressing them individually in different host cells. Recent studies have shown that Nsp1 is a guanine-7-methyltransferase and a putative guanylyltransferase (5–7). Nsp1 is specifically associated with the cytoplasmic surface of the plasma membrane, endosomes, and lysosomes both in transfected and in infected cells. It has been suggested that Nsp1 is responsible for targeting of the replication complex to membranes (8). In addition, Nsp1 is palmitoylated and tightly bound to membranes (8), although it does not contain any stretches of hydrophobic amino acids that might constitute a membrane-spanning domain, suggesting that palmitate might be responsible for its membrane association.

Protein palmitoylation in eukaryotic cells has been widely studied, because a number of cellular and viral proteins are fatty acylated by the addition of palmitate (see Refs. 9–12 and references therein). This C16 saturated fatty acid is added post-translationally to proteins through an ester bond. In contrast to other lipid modifications, such as myristoylation and prenylation, palmitoylation is often reversible and the palmitate moiety turns over with a half-time considerably shorter than that of the protein to which it is attached. Palmitoylation is responsible for the membrane association of Ras (13, 14), a neuronal growth-associated 43-kDa protein (GAP-43; Ref. 15), α-subunits of heterotrimeric G-proteins (16), and a cellular 64-kDa protein (17).

In the present work we have localized the palmitate attachment site of SFV Nsp1 to cysteine residues 418–420 and studied the effects of palmitoylation on Nsp1 using a nonpalmitoylated derivative. The absence of palmitate reduced the hydrophobicity of Nsp1 and converted it to a loosely attached peripheral membrane protein. Acylation significantly affected the subcellular distribution of Nsp1, because only the palmitoylated Nsp1 was detected in the filopodial extensions of the plasma membrane.

MATERIALS AND METHODS

Cells and Viruses—HeLa cells were maintained in Dulbecco’s modified minimal essential medium supplemented with 10% fetal calf serum (Life Technologies, Inc.) and 100 units/ml of both penicillin and streptomycin (Life Technologies, Inc.). The vTF7–3 recombinant vaccinia virus stock was prepared in HeLa cells as described previously (18).

Construction of nsp1 Mutants—DNA manipulations were done according to standard methods (19). Deletions were made to plasmids pTSF1 (20) and pBAT-Nsp1 (6), which contain the nsp1 gene under the T7 promoter using appropriate restriction enzymes. Thereafter T4 DNA polymerase/mung bean nuclease was used to chew away or fill in nucleotides to maintain the reading frame. Deleting amino acids 2–64 was done with NcoI and EcoRV, amino acids 121–148 with PstI and BglII, amino acids 141–187 with PmaCI and PfluI, amino acids 189–286 with NheI and SnaBI, amino acids 238–319 with Th/HIN1, and amino acids 461–488 with Stul and MluI. C-terminal deletions 220–537 and 430–537 were generated by polymerase chain reaction with a common upstream primer 5′-TATACCATGGCCGCCAAGTGCGATGTGCT-3′ and two downstream primers (i) 5′-ACTAAGCTTACCTTTCTACGTTGAAATACGG-3′ corresponding to nucleotides 788–807 of nsp1 codon region and (ii) 5′-CTCAAGCTTACTCTCTTTTTAATCGCC-3′ corresponding to nucleotides 1265–1284, both with 10 additional nucleotides containing a HindIII recognition site. The conserved cysteine residues in nsp1 gene (see Fig. 1) were changed to alanines in plasmid pTSF1 using the unique site elimination (U.S.E.) mutagenesis kit (Pharmacia Biotech Inc.) according to manufacturer’s instructions. The sequence of mutated regions was verified by the dideoxy chain termination method using the T7 sequencing kit (Pharmacia).

Expression in Escherichia coli—To determine the enzymatic activities, the mutated proteins were expressed in E. coli as described for wild
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RESULTS

Deletion Analysis of Nsp1—We have previously shown that SFV Nsp1 is palmitoylated and specifically associated with the cytoplasmic surface of the plasma membrane, endosomes, and lysosomes both in SFV-infected and in nsp1-transfected cells (6, 8). A deletion series was prepared using suitable restriction enzymes with the aim to characterize the domain(s) responsible for palmitate attachment and membrane targeting of Nsp1 (Fig. 1). These truncated Nsp1 derivatives were synthesized in HeLa cells using the recombinant vaccinia virus (vTF7-3) expression system (18). Proteins were labeled with [3H]palmitate for 3 h starting at 2 h post-transfection, cell lysates were immunoprecipitated with a polyclonal anti-Nsp1 antiserum, and the proteins were analyzed by SDS-PAGE (data not shown). The expression levels of the truncated proteins were verified by Western blotting, and they were similar to those of the wt Nsp1 (data not shown). Only one of the mutant proteins, with deletion of amino acid residues 461–488, was labeled with [3H]palmitate to about the same extent as wt Nsp1. Another protein with a C-terminal truncation (Δ430–537) was labeled to a barely detectable extent, whereas no labeling was observed with the rest of the truncated proteins (Fig. 1).

The association of the [35S]methionine/cysteine-labeled truncated proteins with membranes was studied by discontinuous sucrose gradient centrifugation (8). None of the proteins with deletions within the N-terminal half of Nsp1 floated with membranes to the top of the gradient. A protein with a minor C-terminal deletion (Δ461–488) floated with the membranes like wt Nsp1 (see Fig. 5A). Larger deletions (Δ430–537 and Δ270–537) resulted in about a 50% decrease in the membrane association (Fig. 1). The intracellular localization of the truncated proteins and wt Nsp1 was studied using indirect immunofluorescence. Cells expressing wt Nsp1 showed bright staining of the plasma membrane with extensive decoration of filopodia-like extensions (Fig. 2A). Identical fluorescence staining was observed with the deletion mutant Δ461–488 (data not shown). All deletion mutants, which had lost their membrane association, showed cytoplasmic fluorescence similar to that of the deletion mutant Δ270–537 (Fig. 2B).

To assay for the enzymatic activities, Nsp1 derivatives were expressed in E. coli, and the methyltransferase activity and the ability to form a covalent complex with 7-methyl-GMP were determined from the total cell lysate (6, 7). The mutant Δ461–488 showed reduced activity in both assays. All the other deletion mutants were completely inactive (Fig. 1). The loss of enzymatic activities and loss of the ability to become palmitoylated was not due to the instability of the proteins because they were detected in Western blots using anti-Nsp1 antibody in amounts similar to the wild type Nsp1.

FIG. 1. Deletion analysis of Nsp1. Deleted amino acids are marked on the left and visualized in the cartoon by thin lines. The positions of conserved cysteine residues in the alphavirus proteins are shown in wt Nsp1. FL, the membrane association of [35S]methionine/cysteine-labeled proteins from transfected HeLa cells was studied by flotation analysis in discontinuous sucrose gradients. After centrifugation the fractions were collected from the bottom. Each fraction was subjected to immunoprecipitation with anti-Nsp1 antiserum and analyzed by SDS-PAGE. Quantitation of [35S]radioactivity in the immunoprecipitated bands was carried out using Ultrascan XL Enhanced Laser Densitometer (Pharmacia). The numbers denote the percentage of Nsp1 floated with membranes to the top of the gradient (mean of two experiments). Minus (−) means <5% of the material floated. PA, [3H]palmitate incorporation (mean of two experiments). Transfected HeLa cells were labeled with [3H]palmitate for 3 h starting at 2 h post-transfection, and cell lysates were immunoprecipitated using anti-Nsp1 antiserum, analyzed by SDS-PAGE, and visualized by fluorography. Minus (−) stands for an undetectable level. For the determination of the enzymatic activities, the proteins were expressed in E. coli. MT, methyltransferase activity; MB, enzymatic activities and loss of the ability to become palmitoylated.
Characterization of the Palmitoylation Site—Because our recent work indicated that Nsp1 contains at least one palmitoyl group sensitive to alkaline methanolysis (8), we further investigated the properties of palmitate linkage in Nsp1. Incubation with hydroxylamine at neutral pH leads to hydrolysis of the thioester bond between the palmitate and cysteine, whereas alkaline pH is needed to hydrolyze oxyester bonds between palmitate and serine or threonine (22). As demonstrated in Fig. 3, the [3H]palmitate label was released from Nsp1 by hydroxylamine treatment at neutral pH. Coomassie Blue staining of a duplicate gel confirmed that this treatment did not remove Nsp1 from the gel (data not shown). These results indicate that palmitate was linked to Nsp1 via a thioester bond to a cysteine residue.

Because deletion analysis did not reveal the region for palmitate attachment, we mutated the cysteine residues of Nsp1 conserved in alphaviruses (Fig. 1) to alanines. Mutant proteins were expressed in HeLa cells to assay for [3H]palmitic acid incorporation. Because the expression levels differed significantly, [3H]palmitate incorporation (Fig. 4 A) was normalized by estimating the amount of the respective proteins by laser densitometry of Western blots (Fig. 4 B). The mutants C135A and C142A (Fig. 4, lanes 3 and 4) were always expressed poorly. All mutants, except C418A,C419A,C420A (Fig. 4, lane 11), were labeled with palmitate almost as well as the wt Nsp1 (70–100% of wt level). No labeling of C418A,C419A,C420A could be observed even in 15-fold longer exposures of the gel (data not shown). C418A,C419A,C420A retained 40% of the enzymatic activities of wt Nsp1 when expressed in E. coli (data not shown). Thus, the mutation appeared not to destroy the protein conformation. We conclude that palmitate addition occurred at least on one of the cysteine residues 418–420. To determine to which cysteine palmitate was added, we prepared two more point mutants, C418A,C419A and C420A. Both of these mutant proteins were enzymatically active and could be labeled with [3H]palmitic acid (Fig. 4, lanes 12 and 13), suggesting either that any of the cysteines 418–420 may be palmitoylated or that palmitate is added to several of these cysteines in wild type Nsp1.

Membrane Association of Nonpalmitoylated Nsp1—To assess the role of palmitoylation in the membrane association of Nsp1, the nonpalmitoylated form of Nsp1 (C418A,C419A,C420A) was expressed in HeLa cells, and the post-nuclear supernatant was subjected to flotation analysis in a discontinuous sucrose gradient. About 35–40% of C418A,C419A,C420A and 95–100% of wt Nsp1 floated with membranes (Fig. 5, A and B). To characterize the nature of membrane association, the floated membrane fraction was subjected to various treatments followed by sedimentation of the membranes. The supernatant and pellet fractions were subjected to immunoprecipitation with anti-Nsp1 antibody followed by SDS-PAGE and quantitation of the radioactivity of the Nsp1 bands. About 75% of wt Nsp1 was still membrane-associated after treatment with 50 mM NaCO₃, pH 11.5. Only when the pH was raised to 12, most of the wt Nsp1 was released from the membranes (Fig. 5 C). About two-thirds of the C418A,C419A,C420A protein could be released from the membrane fraction already by treatment with 1 M NaCl (Fig. 5 D). The floated membranes were also subjected to Triton X-114 phase separation. Wild type Nsp1 was heavily aggregated (Fig. 6, lane 1), and at least 50% of the nonaggregating Nsp1 partitioned into the detergent phase (Fig. 6, lane 2). In contrast, only a minor fraction of C418A,C419A,C420A protein was detected in rapidly sedimenting aggregates (Fig. 6, lane 4), and the major part partitioned into the aqueous phase (Fig. 6, lane 6). These results show that palmitoylation of wt Nsp1 is responsible for its tight membrane association.

Subcellular Localization of Nonpalmitoylated Nsp1—To investigate the effect of the loss of palmitate on the subcellular distribution of Nsp1, we used indirect immunofluorescence microscopy. Recombinant vaccinia virus-infected HeLa cells were transfected with the plasmid encoding wt Nsp1 or C418A, C419A,C420A. The cells were fixed with paraformaldehyde at 3 h post-transfection and stained with rhodamine-conjugated concanavalin A to visualize the plasma membrane. Thereafter, the cells were permeabilized to reveal Nsp1 by indirect immunofluorescence (fluorescein isothiocyanate label). In wt nsp1-transfected cells colocalization of Nsp1 with the plasma membrane marker was observed (Fig. 7, A and B). Again, the filopodia-like extensions were decorated with both stains. The filopodia of C418A,C419A,C420A-transfected cells were not visible by anti-Nsp1 staining (Fig. 7, C and D). The colocalization of
A and gradient, 0.5-ml fractions were collected from the bottom (on the transfected cells was subjected to flotation in a discontinuous sucrose, all proteins with N-terminal deletions were cytoplasmic, of Nsp1 is evidently needed for membrane association, because

permitted, and the membrane association of these mutant proteins was studied. Our results suggest that the N-terminal part of Nsp1 is evidently needed for membrane association, because all proteins with N-terminal deletions were cytoplasmic.

the plasma membrane staining with C418A, C419A, C420A was confirmed using laser scanning confocal microscope to overlay the stains of C418A, C419A, C420A and concanavalin A. A clear colocalization was detected at the cell surface, but not in the filopodia (data not shown).

**DISCUSSION**

Semliki Forest virus capping enzyme Nsp1 is a palmitoylated membrane protein, tightly associated with the cytoplasmic surface of plasma membrane, endosomes, and lysosomes. To define the regions responsible for palmitoylation and membrane association, a series of deletions in the nsp1 gene was prepared, and the membrane association of these mutant proteins was studied. Our results suggest that the N-terminal part of Nsp1 is evidently needed for membrane association, because all proteins with N-terminal deletions were cytoplasmic, whereas the N-terminal fragment (Δ270–537) and proteins with C-terminal deletions retained some membrane affinity even in the absence of palmitoylation. However, the results of this analysis have to be taken with caution for the following reasons. All truncated proteins, expect the one with a minor deletion at the C terminus of Nsp1 (Δ461–488), lost their enzymatic activities and were unable to incorporate radioactively labeled palmitate, suggesting that the deletions may have affected the proper folding of Nsp1. These results also give the impression that Nsp1 cannot be divided into separable functional domains. Similar results were recently obtained when the methyltransferase activity of truncated derivatives of Sindbis virus (a related alphavirus) Nsp1 protein was assayed (23).

Our present results show that palmitate is attached to Nsp1 via a thioester linkage to a cysteine residue, which is the most common way to link palmitate to proteins (24). To determine the palmitoylation site, we mutated the cysteine residues conserved in the Nsp1 of the alphavirus family to alanines. The incorporation of radioactively labeled palmitate into the mutant proteins was studied. On the basis of these results we conclude that palmitoyl group was attached to cysteine residues 418–420. To define which of the cysteines were palmitoylated, two more mutants, C418A, C419A and C420A, were generated. Both of these mutant proteins were palmitoylated, suggesting that at least two of the three cysteine residues are accessible to the palmitoyl transferase in the wt Nsp1. At least one cysteine residue is found at that position in all alphaviruses from which sequence data are available. No amino acid sequence motifs have been identified for the prediction of protein palmitoylation, except N-terminal Met-Gly-Cys, found in myristoylated and palmitoylated members of the heterotrimeric G-protein subfamily (12). In the case of Nsp1, the palmitate is attached to cysteines in the central region of the protein with no obvious sequence homology to the palmitoylation sites of other proteins.
Protein palmitoylation seems to have several different functions. It has been shown to be responsible for membrane association of several cytoplasmic proteins: the α-subunits of G-proteins (16), Ras proteins (13, 14), the neuronal protein, GAP-43 (15), and endothelial nitric oxide synthase (25). Because many integral membrane proteins, for example, transmembrane and the envelope proteins of alpha- and rhabdoviruses (1, 26, 27), also contain palmitate, a more complex role for palmitoylation was suggested. To date experimental evidence exists to suggest that palmitoylation mediates protein–protein interactions (28, 29), regulates protein activity (30) and stability (31), and is involved also in protein targeting to different cellular organelles like the plasma membrane (17, 32) and caveolae (34).

The subcellular site for palmitoylation of Nsp1 is not known, but because Nsp1 is rapidly associated with membranes (8) and targeted to the plasma membrane also in the absence of palmitate, it seems reasonable to assume that palmitoylation of Nsp1 occurs at the plasma membrane. Recently, a membrane-associated palmitoyltransferase was identified at the plasma membrane, which is able to add palmitate to G-protein α-subunits in vitro (35).

Because the absence of palmitate did not abolish the enzymatic activities of Nsp1, we assume that the conformation of the nonpalmitoylated protein was not changed significantly. Our results show that the absence of palmitate reduced the tightness of membrane association and converted Nsp1 from an associated form of Nsp1 was detected in filopodial extensions. The results are in agreement with recent studies, which suggest additional roles for palmitoylation besides sole membrane association of proteins.

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