

Deacylation of the transmembrane domains of Sindbis virus envelope glycoproteins E1 and E2 does not affect low-pH-induced viral membrane fusion activity

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Abstract The envelope glycoproteins E1 and E2 of Sindbis virus are palmitoylated at cysteine residues within their transmembrane domains (E1 at position 430, and E2 at positions 388 and 390). Here, we investigated the *in vitro* membrane fusion activity of Sindbis virus variants (derived from the Toto 1101 infectious clone), in which the E1 C430 and/or E2 C388/390 residues had been substituted for alanines. Both the E1 and E2 mutant viruses, as well as a triple mutant virus, fused with liposomes in a strictly low-pH-dependent manner, the fusion characteristics being indistinguishable from those of the parent Toto 1101 virus. These results demonstrate that acylation of the transmembrane domain of Sindbis virus E1 and E2 is not required for expression of viral membrane fusion activity. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Acylation; Palmitoylation; Virus fusion; Membrane fusion liposome

1. Introduction

Sindbis virus (SIN), the prototype member of the genus alphavirus of the family Togaviridae, contains three major structural proteins: the capsid protein, C, and two envelope glycoproteins, E1 and E2 [1–3]. The E1 and E2 proteins, which mediate the infectious host cell entry of the virus, are exposed on the viral surface as 80 trimeric spikes each consisting of three E1/E2 heterodimers. E1 and E2 are type-I integral membrane proteins with a single transmembrane anchor sequence and a very small C-terminal domain located at the internal half of the viral membrane. The transmembrane sequences of E1 and E2 contain a number of cysteine residues, which are conserved among most of the members of the alphavirus genus and represent potential sites for covalent attachment of long-chain fatty acids. In SIN, these cysteine residues, at E1 position 430 and E2 positions 388 and 390, indeed appear to be palmitoylated [4].

Not only the spike proteins of alphaviruses, but in fact many transmembrane glycoproteins of animal viruses have been shown to be acylated [5–7]. These include the G protein of vesicular stomatitis virus [8], the hemagglutinin (HA) of

influenza virus [9–13] and the transmembrane subunit of the envelope glycoproteins of human and simian immunodeficiency viruses [14]. Although modification with long-chain fatty acids thus appears to be a common phenomenon among viral transmembrane proteins, the biological function of acylation remains elusive. Several investigators have used site-directed mutagenesis in order to selectively replace the cysteine residues that provide sites for acylation. Such modified proteins have been studied after expression in cultured cells or in systems generating recombinant viruses. However, conflicting results have been obtained with regard to the role that spike protein acylation plays in the life cycle of the viruses involved. Particularly, the potential function of fatty acids in the membrane fusion activity of viral spikes, such as the influenza virus HA, has remained controversial [9,10,13,15–19].

With regard to SIN, studies involving site-specific mutagenesis have demonstrated that deacylation of the transmembrane domains of the E1 and/or E2 spike glycoproteins slows down virus growth early in infection [4]. Furthermore, these deacylation mutant viruses are more sensitive to treatment with detergent compared with wild-type SIN [4]. Little is known about the potential effect of spike protein deacylation on the membrane fusion activity of SIN. However, the fact that the E1 and/or E2 deacylation SIN mutants infect a variety of cell types [4] suggests that the viral life cycle, including the membrane fusion step, is unlikely to be grossly affected by lipid modification.

Here, we studied the effect of deacylation of the transmembrane domains of SIN glycoproteins E1 and E2 on the viral membrane fusion capacity. Fusion of SIN virus derived from the infectious clone Toto 1101 and of several acylation mutants was evaluated in a liposomal model system on the basis of both lipid mixing and contents mixing. It is demonstrated that SIN fuses rapidly and efficiently with liposomes in a strictly low-pH-dependent manner. Moreover, deacylation of the transmembrane domains of E1 and/or E2 has no effect on the membrane fusion characteristics of the virus.

2. Materials and methods

2.1. Acylation mutant SIN viruses

A cDNA, containing the Toto 1101 infectious clone of SIN virus [20], as well as three acylation mutant cDNAs, were generously provided by Dr. Milton Schlesinger (Washington University, St. Louis, MO, USA). In the first acylation mutant, the transmembrane cysteine at E1 position 430 was replaced by an alanine (E1:C430A), which

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results in an almost complete lack of palmitoylation of E1 and a minor reduction in palmitoylation of E2 [4]. In the second mutant, the cysteines at E2 positions 388 and 390 were replaced by alanines (E2:C388A-C390A), resulting in a reduction of E1 and E2 palmitoylation by about 50% and 70%, respectively [4]. The third mutant, with all three transmembrane cysteine mutations in E1 and E2 (E1:C430A/E2:C388A-C390A), is completely devoid of E1 palmitoylation and has about 30% palmitoylation of E2 [4]. The residual fatty acid binding to E2 in the presence of the C388A and C390A mutations presumably arises from acylation at other cysteine residues in the cytoplasmic domain of the protein [21].

2.2. Production and characterization of virus particles

For the production of virus particles, RNA was synthesized and transfected into baby hamster kidney cells (BHK-21) by electroporation, as described previously [22]. Viruses released from cells at 20 h post-transfection were harvested, and these stocks were subsequently used directly for the production of pyrene- and [35 S]methionine-labeled viruses, essentially as described before for Semliki Forest virus (SFV) [23,24] or SIN [25]. The concentration of the virus preparations was determined by lipid phosphate [26] and protein [27] analysis. The purity of the virus particles was confirmed by SDS-PAGE. Viral infectivity was determined by titration on BHK-21 cells in 96-well plates.

2.3. Preparation of liposomes

Liposomes (large unilamellar vesicles) were prepared in 5 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.4 (HNE) by subjection of lipid mixtures, dried from chloroform solution, to five cycles of freezing and thawing and subsequent extrusion [28] through 0.2- μ m filters (Nuclepore Inc., Pleasanton, CA, USA) in a LiposoFast mini-extruder (Avestin, Ottawa, Canada). Liposomes consisted of phospholipids (Avanti Polar Lipids, Alabaster, AL, USA) and cholesterol (Chol, Sigma Chem. Co., St Louis, MO, USA). The phospholipids were phosphatidylcholine (PC) derived from egg yolk, phosphatidylethanolamine (PE) prepared by transphosphatidylation of egg-PC, and sphingomyelin (SPM) from egg yolk, mixed with Chol in a molar ratio of 1:1:1:1.5. Trypsin-containing liposomes were prepared likewise, only in this case lipids were dispersed in HNE containing 10 mg/ml trypsin (Fluka Chemie AG, Buchs, Switzerland). The trypsin-containing liposomes were separated from free trypsin by gel filtration on a Sephadex G-100 column in HNE. The phospholipid concentration of the liposome preparations was determined by phosphate analysis [26].

2.4. Fusion assays

Fusion of pyrene-labeled SIN with liposomes was monitored on-line in an AB2 fluorometer (SLM/Aminco, Urbana, IL, USA), as described previously [25]. Briefly, pyrene-labeled SIN (0.5 μ M viral phospholipid) and liposomes (200 μ M phospholipid) were mixed in a volume of 0.665 ml in HNE, in a quartz cuvette, magnetically stirred and maintained at 37°C. At $t=0$ s, fusion was initiated by the addition of 0.035 ml of 0.1 M MES, 0.2 M acetic acid, pre-titrated with NaOH to achieve the final desired pH. The fusion scale was adjusted such that the initial pyrene excimer fluorescence at 480 nm represented 0% fusion. The 100% fusion value was determined after addition of 0.035 ml of 0.2 M octaethylene glycol monododecyl ether (Fluka Chemie AG). The initial rate of fusion was determined from the tangent to the first part of the curve. The extent of fusion was determined 60 s after acidification.

Fusion of SIN with liposomes was also assessed by using a contents mixing assay based on degradation of the viral capsid protein by trypsin, initially encapsulated in the liposomes [25,29,30]. Briefly, [35 S]methionine-labeled virus (0.5 μ M phospholipid) was incubated with trypsin-containing PC/PE/SPM/Chol liposomes (200 μ M liposomal phospholipid) in the presence of 125 μ g/ml trypsin inhibitor (Boehringer, Mannheim, Germany) in the external medium, at 37°C. The mixture was acidified, under continuous stirring, to the desired pH with 0.1 M MES, 0.2 M acetic acid, as described above. After 30 s, samples were neutralized by the addition of a pre-titrated volume of 0.1 M NaOH, and further incubated for 1 h at 37°C. Subsequently, samples were analyzed by SDS-PAGE, the protein bands being visualized by autoradiography. Quantification of the viral proteins was done by phosphorimaging analysis using Image Quant 3.3 software (Molecular Dynamics, Sunnyvale, CA, USA).

3. Results

3.1. Low-pH-dependent fusion of pyrene-labeled SIN with liposomes

Fusion of SIN was measured on-line in a liposomal model system, using a lipid mixing assay based on pyrene excimer fluorescence [23–25]. In order to exclude any potential effects of the fluorescence labeling procedure on the biological properties of the virus, we first evaluated the specific infectivities of pyrene-labeled Toto 1101 and acylation mutant SIN virus preparations. Viral infectivity was determined by titration on BHK-21 cells and related to the number of virus particles present based on biochemical analyses [25]. In all cases a particle to infectious unit ratio of 4–5 was found, similar to that seen in unlabeled virus (data not shown). These results demonstrate that neither the fluorescence labeling procedure nor the presence of the acylation mutations in E1 and/or E2 had any significant effect on the specific infectivity of the viruses.

The pyrene fusion assay relies on a decrease of pyrene excimer fluorescence owing to dilution of pyrene-labeled phospholipids from the viral into the liposomal membrane. This decrease can be translated directly to the extent of fusion, since each individual fusion event results in a large dilution of the probe and, thus, in an essentially complete disappearance of the excimer fluorescence intensity of the virus particle involved. Fig. 1 presents the fusion kinetics of pyrene-labeled Toto 1101 SIN virus with PC/PE/SPM/Chol liposomes. At pH 4.6, the virus fused rapidly and efficiently with the liposomes, the extent of fusion being approximately 55% at 10 s after acidification of the virus-liposome mixture (curve a). With increasing pH, fusion became slower and less extensive (curves b, c). At pH 7.4 there was no detectable fusion (curve d). We also measured fusion of pyrene-labeled Toto 1101 SIN virus with liposomes lacking either SPM or Chol or both, and observed that fusion was absent (data not shown). These results indicate that the membrane fusion activity of SIN derived from the Toto 1101 infectious clone is triggered by a mildly acidic pH and exhibits a similar overall lipid dependence as

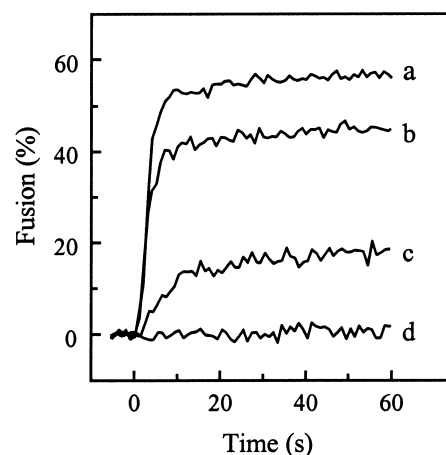


Fig. 1. Low-pH-dependent fusion of pyrene-labeled SIN derived from cDNA clone Toto 1101 with liposomes. Fusion of pyrene-labeled SIN Toto 1101 with PC/PE/SPM/Chol liposomes was measured on-line at 37°C as described in Section 2. Final concentrations of virus and liposomes corresponded to 0.5 μ M and 200 μ M phospholipid, respectively. Curve a, pH 4.6; curve b, pH 5.0; curve c, pH 5.75; curve d, pH 7.4.

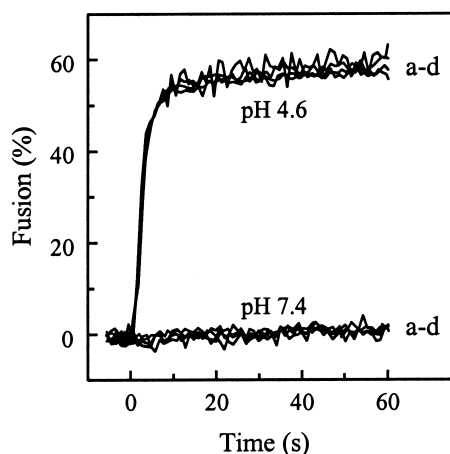


Fig. 2. Low-pH-dependent fusion of pyrene-labeled acylation mutant SIN viruses with liposomes. Fusion of pyrene-labeled acylation mutant viruses with liposomes was measured at pH 4.6 and 7.4, as described in the legend to Fig. 1. Curves a, SIN Toto 1101; curves b, E1:C430A; curves c, E2:C388A-C390A; curves d, E1:C430A/E2:C388A-C390A.

fusion of wild-type SIN (laboratory-adapted strain AR339) [25] or SFV [24,31].

3.2. Fusion characteristics of SIN acylation mutants

Fig. 2 presents the fusion kinetics of the three acylation mutant SIN viruses, E1:C430A, E2:388A/C390A, and E1:C430A/E2:C388A-C390A, in comparison with the original Toto 1101 virus. Clearly, the fusion kinetics of the acylation mutants were indistinguishable from those of the unmodified virus. Importantly, none of the viruses had any significant fusion activity at neutral pH.

Fig. 3 compares the detailed pH dependence of Toto 1101 (open triangles) and the acylation mutant virus E1:C430A/E2:C388A-C390A (closed triangles). Panel A shows the initial rate of fusion as a function of the pH, determined from the tangent to the first part of the fusion curves. Rates were very similar for unmodified and acylation mutant viruses ranging from pH 4.0 to pH 7.4. Fig. 3B presents the extent of fusion as a function of pH measured 60 s after acidification. Again, throughout the pH range from 4.0 to 7.4, no differences were observed between the unmodified and acylation mutant SIN. Under optimal conditions, 18–20% of the virus particles fused within the first second after acidification, with an extent of fusion of 60% at 60 s post-acidification. The pH threshold for fusion was 6.0 for both viruses. The acylation mutant viruses E1:C430A and E2:C388A-C390A gave essentially identical results in terms of the initial rate and extent of fusion (data not shown).

3.3. Contents mixing of [35 S]methionine-labeled SIN with trypsin-containing liposomes

In the above experiments fusion was evaluated on the basis of lipid mixing. Another, very stringent, criterion for fusion involves the coalescence of the interior of the virus with the liposomal lumen. Contents mixing was assayed as the degradation of the viral capsid protein by trypsin, initially encapsulated in the liposomes. Fig. 4A shows the capsid degradation of SIN Toto 1101 and the acylation mutant virus E1:C430A/E2:C388A-C390A. In both cases, incubation of the virus with trypsin-containing liposomes at pH 4.6 resulted in the degra-

dation of a substantial fraction of the capsid protein (lanes a,d). At pH 7.4 no capsid degradation was observed (lanes b,e). The controls, in which the viruses were incubated with empty liposomes at pH 4.6, did not show degradation of the capsid protein either (lanes c,f). The ratio of the radioactivity of the capsid band relative to the total radioactivity ($C/[C+E1+E2]$) of the samples incubated at pH 7.4 and the control samples with empty liposomes was close to 0.4, as expected on the basis of the number of methionine residues in the SIN structural proteins [2]. Complete degradation of the capsid protein was observed when Triton X-100 was added to the reaction mixture, in the absence of trypsin inhibitor (results not shown). Fig. 4B shows the extent of capsid degradation as function of the pH, quantified by phosphorimaging. For SIN Toto 1101, incubated at pH 4.6, approximately 70% of the capsid protein was degraded, whereas pH 5.0 and pH 5.5 the corresponding values were 41% and 19%, respectively. With the triple acylation mutant virus, E1:C430A/E2:C388A-C390A, we detected an extent of capsid degradation of 72% at pH 4.6, 45% at pH 5.0, and 25% at pH 5.5. Similar results were obtained with the separate E1 or E2 acylation mutant

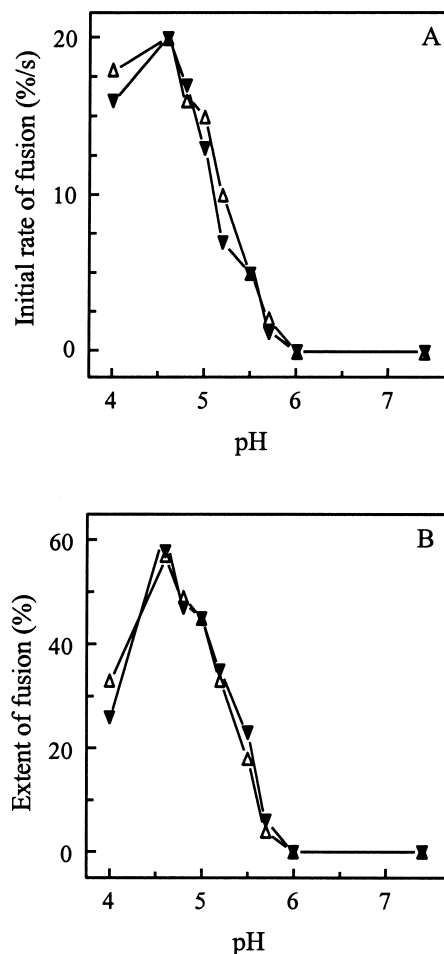


Fig. 3. Comparison of the pH dependence of fusion of pyrene-labeled acylation mutant and SIN Toto 1101 viruses. Fusion of SIN Toto 1101 (open triangles) and the acylation mutant virus E1:C430A/E2:C388A-C390A (closed triangles) was determined at different pH values, as described in the legend to Fig. 1. A: The initial rates of fusion were determined from the tangents to the first parts of the fusion curves. B: The extents of fusion were determined 60 s after acidification.

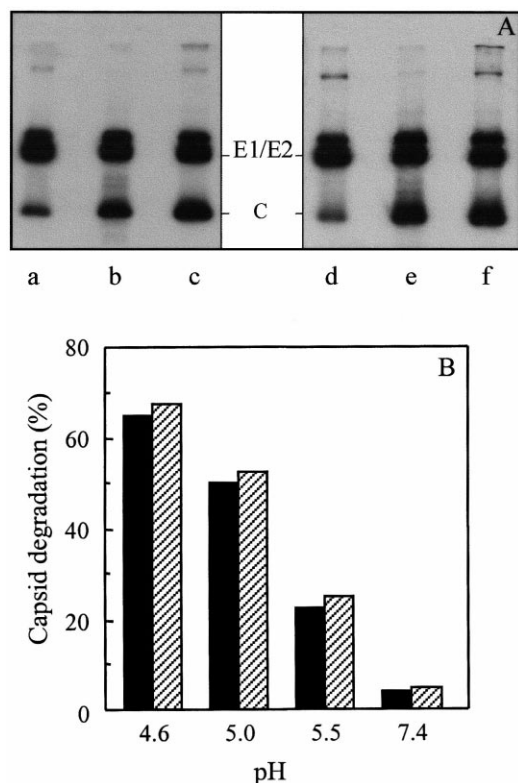


Fig. 4. Degradation of the viral capsid protein by trypsin, initially encapsulated in the liposomes. The trypsin fusion assay was carried out as described in Section 2. Final concentrations of [35 S]methionine-labeled virus and trypsin-containing PC/PE/SPM/Chol liposomes corresponded to 0.5 μ M and 200 μ M phospholipid, respectively. A: Viral structural proteins of SIN Toto 1101 (lanes a–c) and acylation mutant E1:C430A/E2:C388A-C390A (lanes d–f), visualized by autoradiography. Lanes a and d, trypsin-containing liposomes at pH 4.6; lanes b and e, trypsin-containing liposomes at pH 7.4; lanes c and f, empty liposomes at pH 4.6. B: Quantification of the extent of capsid protein degradation on the basis of the ratio C/[C+E1+E2] by phosphorimaging analysis. The ratios C/[C+E1+E2] of the controls, in which empty liposomes were incubated with the viruses under otherwise identical conditions, were taken as the 100% values. Solid bars, SIN Toto 1101; hatched bars, E1:C430A/E2:C388A-C390A.

viruses (results not shown). The extents of capsid protein degradation seen in the pH range from 4.6 to 5.5 (Fig. 4B) are very similar to the corresponding extents of lipid mixing observed in the pyrene fluorescence assay (Fig. 3B).

4. Discussion

The results presented in this paper demonstrate that SIN derived from the infectious clone Toto 1101 fuses rapidly and efficiently with receptor-free Chol- and sphingolipid-containing liposomes in a strictly low-pH-dependent manner. Fusion was evaluated on the basis of both membrane lipid mixing and internal contents mixing between the virus and the liposomes. The pH dependence of fusion of the Toto 1101 virus is very similar to that of the wild-type, laboratory-adapted, SIN strain AR339 [25] and argues strongly for a cell entry mechanism of SIN involving receptor-mediated endocytosis and acid-induced fusion of the viral envelope with the endosomal membrane. This is entirely consistent with recent observations of Glomb-Reinmund and Kielian [32]. These investigators

showed that infection of BHK-21 cells by SIN is inhibited by agents that interfere with endosomal acidification, such as NH_4Cl , bafilomycin or concanamycin. Further support for a cell entry mechanism of SIN involving receptor-mediated endocytosis was provided by DeTulleo and Kirchhausen [33], who observed that BHK-21 cell infection by SIN is affected by a mutated form of dynamin which inhibits the budding of clathrin-coated vesicles.

In the light of this fairly convincing evidence, it is intriguing that quite recently [34] Brown and coworkers have presented data which support their previous suggestion [35,36] that exposure to an acidic pH may not be an obligatory step in the infection of cells by alphaviruses. These investigators observed that the infection of mosquito cells by SIN was not blocked by chloroquine under conditions such that the drug did raise the pH of the endosomal compartment of the cells [34]. Although it is possible that alphaviruses use different routes of infection in vertebrate and insect cells, our previous [23–25,37] and present studies in model systems, designed to specifically address the issue of alphavirus fusion activation, strongly argue for exposure to a mildly acidic pH being an essential step in the triggering of the fusion process.

The principal result of this study demonstrates that acylation of the transmembrane domains of the SIN glycoproteins E1 and E2 is not required for expression of viral membrane fusion activity. Indeed, deacylation of E1 and/or E2 has no effect on the kinetics or the detailed pH dependence of the fusion process. This conclusion is in agreement with similar results obtained for other enveloped viruses, such as vesicular stomatitis virus [8] and human and simian immunodeficiency viruses [14]. The potential role of acylation in the membrane fusion activity of influenza HA, however, remains controversial. HA-mediated fusion has been studied extensively in cultured cells expressing the isolated HA, with erythrocytes or erythrocyte ghosts serving as target membranes. In such systems, deacylation of HA does not affect membrane lipid mixing, as measured by fluorescence dequenching of the fluorophore R18 [17,19]. Likewise, deacylation has been observed to have little effect on syncytia formation mediated by HA expressed on the cell surface [10,11]. By contrast, other investigators have reported distinct effects of deacylation of HA on various stages of the fusion process, including initial fusion pore flickering [15] and late fusion pore dilation as assessed by syncytia formation [9,19]. It is possible that this apparent discrepancy is, at least in part, a consequence of the fusion process being primarily studied in HA-expressing cells rather than with virus. In these cell systems the surface density of HA may vary considerably and also its biological activity may be affected when it is expressed in the absence of the M2 protein [38]. It is a major advantage of our present study that it involves the use of whole virus, derived from a cDNA clone, rather than SIN envelope glycoproteins expressed on the surface of cultured cells. Similar approaches followed for influenza, involving reverse genetics and the generation of mutant virions, however, have again resulted in conflicting conclusions. Zurcher et al. [16] reported that deacylation of HA affects virus assembly, whereas little effect on either assembly or infectivity was observed by others [18,39,40].

With regard to alphaviruses, SIN in particular, the lack of effect of E1 and/or E2 deacylation on viral membrane fusion activity, reported here, and the limited effects on virus assem-

bly and release [6] explain the observation made by us in the present study and by others before [6] that deacylation mutant viruses are fully infectious on cultured cells, exhibiting normal specific infectivity values. On the other hand, the conserved nature of the cysteine residues in the transmembrane domains of the E1 and E2 envelope glycoproteins suggests that fatty acid modification of these proteins has an important function in the life cycle of alphaviruses. Our present results suggest that this biological function is not at the level of the low-pH-induced virus membrane fusion process.

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