Mutational analysis of receptor interaction and membrane fusion activity of Sindbis virus

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Chapter 8

PE2 Cleavage Mutants of Sindbis Virus: Correlation Between Viral Infectivity and pH-Dependent Membrane Fusion Activation of the Spike Heterodimer

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Abstract

The spike glycoprotein E2 of Sindbis virus (SIN) is synthesized in the infected cell as a PE2 precursor protein, which matures through cleavage by a cellular furin-like protease. Previous work has shown that SIN mutants, impaired in PE2 cleavage, are non-infectious on BHK-21 cells, the block in infection being localized at a step after virus-receptor interaction but prior to RNA replication. Here, we studied the membrane fusion properties of SIN PE2 cleavage mutants and observed that these viruses are impaired in their ability to form an E1 homotrimer and to fuse with liposomes at a mildly acidic pH. The block in spike rearrangement and fusion could be overridden by exposure of the mutant viruses to very low pH values (pH < 4.5). Cleavage mutants with second-site resuscitating mutations in PE2 were highly infectious for BHK-21 cells. The ability of these viruses to form E1 homotrimers and to fuse at a mildly acidic pH was completely restored, despite a sustained lack of PE2 cleavage.
Introduction

Alphaviruses, such as Sindbis virus (SIN) and Semliki Forest virus (SFV), are enveloped viruses which contain three major structural proteins, the capsid protein, C, and two envelope glycoproteins, E2 and E1 (38). The glycoproteins are organized on the surface of the virion in 80 hetero-oligomeric spikes, which mediate the infectious entry of these viruses into cells. The E2 glycoprotein is primarily involved in the interaction of a virus particle with cell surface attachment receptors (37), whereas the E1 glycoprotein is necessary for the subsequent membrane fusion process (8, 40). Very recent crystallographic and cryoelectron microscopy studies have revealed that E1 in many respects resembles the flavivirus E glycoprotein. The alphavirus E1 protein appears to lie flat on the viral surface which drives lateral spike interactions, whereas E2 forms the spike protrusions (21, 30).

In the infected cell, the alphavirus structural proteins are translated as a large polyprotein. Once the C protein is cleaved off, the NH$_2$-terminus of PE2, the precursor protein of E2 (PE2 is called p62 in SFV), serves to direct the cotranslational translocation of the remaining polyprotein to the endoplasmic reticulum (ER) (38). This polyprotein is processed by signal peptidase within the ER, and the two envelope proteins associate to form PE2/E1 heterodimers (38). The PE2/E1 heterodimer matures further while passing through the Golgi and trans-Golgi network (TGN). In the TGN or in a post-Golgi compartment PE2 is cleaved close to its amino-terminus to form E2 and a residual peptide E3 (24). PE2 cleavage is mediated by a furin-like host protease at a consensus recognition sequence XBXBBXB close to the amino-terminus, in which B is a basic and X a hydrophobic amino acid (18). The peptide E3, which contains the XBXBBXB sequence, is retained on the SFV spike, but it is released in the case of SIN (24, 28).

Several investigators have shown that the efficiency of PE2 cleavage can be influenced by amino-acid changes within the cleavage site (5, 13, 14, 19, 22, 34, 39). Thus, a number of PE2 cleavage mutants of SIN, SFV, and Venezuelan Equine Encephalitis virus (VEE) have been identified, and these viruses appear to have defects in one or more biological functions due to the presence of uncleaved PE2. For example, in SFV, cleavage of p62 was prevented by replacement of Arg by Leu at position -1 of E2 (34). This p62 cleavage mutant virus (mL) was found to be non-infectious on BHK-21 cells. The block in infection appeared to involve both the virus-receptor interaction and fusion activity of the mutant virus. Viral infectivity on BHK-21 cells was restored by in vitro cleavage of p62 with trypsin or by exposure of the virus to very low pH values (22, 34, 40). Likewise, several SIN PE2 cleavage mutants have been shown to be non-infectious. For example, replacement of the Arg or Ser residues at position 1 of E2 (the last amino acid of the XBXBBXB cleavage site) by an Asn residue within the context of the infectious clones TRSB (corresponding to a laboratory-adapted strain of SIN) or TR339.
(containing the consensus sequence of wild type SIN) almost completely blocked viral infectivity (13, 14, 19, 25). SIN cleavage mutants in which part of the cleavage site was deleted were also found to be essentially non-infectious for BHK-21 cells (19).

Interestingly, it has been found that the lethality of PE2 cleavage mutation in TRSB-N (Arg-to-Asn substitution at position 1 of E2) could be reversed by resuscitating second-site mutations in PE2 (14). These investigations showed that after infection with TRSB-N virions occasionally small plaques were formed. Sequence analysis of the purified plaques revealed that some of these viruses carried second-site mutations in PE2, but yet remained cleavage-deficient. While these PE2 cleavage mutants with a resuscitating mutation in PE2 were found to be infectious on BHK-21 cells, the viruses appeared to have an attenuated virulence in CD-1 mice, as compared to the parental TRSB virus.

Recent data showed that the block in infection of PE2 cleavage-deficient SIN viruses without second-site resuscitating mutations, is not at the level of the initial virus-receptor interaction (19). By contrast, SIN PE2 cleavage mutants with an intact cleavage site, bind very efficiently to BHK-21 cells, even better than the parental TR339 virus. It has been shown that, the presence of the basic XBXB feature sequences mediates an efficient interaction with heparan sulfate (HS), which is abundantly expressed on BHK-21 cells and thus acts as a receptor for the virus (4, 20). Furthermore, it has been shown that the presence of uncleaved PE2 in virions does not influence RNA replication, or virus assembly and release (14, 19). Taken together, these data indicate that the block in infection of these SIN PE2 cleavage mutants lies downstream of the interaction of a virus particle with a cellular receptor but prior to RNA replication, suggesting that these mutant viruses are impaired in their membrane fusion properties.

Here, we studied the fusogenic properties of PE2 cleavage mutants, based on the infectious clone TR339, using a liposomal model system. PE2 cleavage mutants in which either the Ser residue at position 1 of E2 was replaced by Asn (E2:N1) or from which the BXBB sequence within the PE2 cleavage site was deleted (FDF) were used. The results show that PE2 cleavage mutants were unable to fuse with liposomes at pH 5.0, indicating that the block in infection lies at the level of the fusion process. Incubation of PE2 cleavage mutants at low pH demonstrated that the viruses were impaired in their ability to form an E1 homotrimer, the fusion-active conformation of the viral spike protein. The block in rearrangement and fusion could be overridden by exposure of the virus to very low pH values (pH < 5.0). Furthermore, E2:N1 cleavage mutants with resuscitating mutations in PE2 were generated, analogous to those identified in TRSB-N, and found to be infectious in BHK-21 cells. These PE2 resuscitated mutant viruses do form E1 homotrimers at a physiologically relevant acidic pH, despite the lack of PE2 cleavage. Accordingly, their membrane fusion activity appeared to be completely restored.
Membrane fusion of Sindbis virus PE2 cleavage mutants

Materials and Methods

**Constructs.** The construction of the cDNA clones pTR339, pE2:N1 (called p39N1 in previous articles), and pFDF has been described previously (19, 20). The “p” prefix indicates the cDNA form of the virus clone. The cDNA pE2:N1/E3:R25 was constructed by substitution into pE2:N1 of an AatII-to-Stul fragment from pTRSB-E3R25 (14). The cDNA clones pE2:N1/T191 and pE2:N1/G216 were constructed by substitution into pE2:N1 of an Stul-to-BssHII fragment from pTRSB-NE2T191 resp. pTRSB-NE2G216 (14). The introduced mutations were confirmed by DNA sequence analysis at the University of North Carolina at Chapel Hill Automated Sequencing Facility with a 373A DNA sequencer with the Taq DyeDeoxy terminator cycle sequencing kits (Applied Biosystems).

**Production and characterization of pyrene- and [\(^{35}\)S]methionine-labeled virus particles.** Viruses were produced on BHK-21 cells. The cells were cultured in Glasgow’s modification of Eagle’s minimal essential medium (Gibco/BRL, Breda, The Netherlands), supplemented with 5% foetal calf serum (FCS), 10% tryptose phosphate broth, 200 mM glutamine, 25 mM Hepes, and 7.5% sodium bicarbonate. For the production of pyrene-labeled virus particles, BHK-21 cells, prior cultured for 48 h on medium containing 10 µg/ml 16-(1-pyrenyl)hexadecanoic acid (pyrene fatty acid; Molecular Probes, Eugene, OR, USA), were transfected, by electroporation, with in vitro transcripts of linearized cDNA clones. Pyrene-labeled SIN particles released from the cells at 20 h post-transfection were harvested and purified from the medium, as described before (3, 27, 36). For the production of [\(^{35}\)S]methionine-labeled virus particles, BHK-21 cells were transfected with in vitro transcribed viral RNA by electroporation, essentially as previously described (3, 27, 36). Briefly, at 3-4 h post-transfection, the culture medium was replaced by methionine-free DMEM (Gibco/BRL) supplemented with 5% FCS and 200 mM glutamine. After 2 h starvation, 200 µCi/5 ml [\(^{35}\)S]methionine was added to the medium and incubation was continued overnight. At 20 h post-transfection [\(^{35}\)S]methionine-labeled viral particles were harvested and purified from the medium, as described before (3, 27, 36, 43). The purity of the produced virus particles was analyzed with SDS-PAGE. Visualization of the protein bands and quantification of the PE2 content in virions were done by phosphorimaging analysis using Image Quant 3.3 software (Molecular Dynamics, Sunnyvale, CA, USA). The percentage PE2 in virions was determined, by relating the intensity of PE2 to the total intensity of E1, PE2 and E2, corrected for the contribution of E1 on the basis of the relative numbers of methionine residues in the E1, E2 and PE2 proteins (19, 32). The specific infectivity (PFU/cpm) was calculated for all batches [\(^{35}\)S]methionine-labeled viruses by plaque assay on BHK-21 cells (20).

**Binding assays.** Virus attachment to BHK-21 cells and heparin- and bovine serum albumin (BSA)-agarose beads (both from Sigma Chemical Co, St. Louis, MO, USA) was performed, essentially as described previously (19, 20). Briefly, BHK-21 cells (cultured in a 12-well plate; Costar, Cambridge, MA, USA) were washed two times with cold 5 mM Hepes, 150 mM NaCl, 0.1 mM EDTA, pH 7.4 (HNE) + 1% fetal calf serum (HNE+). Subsequently, 150 µl [\(^{35}\)S]methionine-labeled virus particles were added to the cells (ranging from \(10^6\) to \(10^7\) cpm ~ approx. \(10^9\) to \(10^{10}\) virus particles) and incubation was continued at 4 °C for 2 h with gentle agitation. The cells were then washed twice with HNE+ and trypsinized with 1*Tryptsin/EDTA (Gibco/BRL). For binding to heparin-agarose and
BSA-agarose beads, 1 ml of beads was washed with HNE+ three times, and resuspended in 1 ml HNE+. Fifty microliters of beads were added to 50 µl virus particles, similar to that of the cell binding assay, and incubated for 2 h at 4 °C with gentle agitation. Subsequently, the mixtures were washed three times with HNE+, and resuspended in 0.6% triton (Sigma) in HNE. Viral radioactivity was quantified by liquid scintillation analysis. Control reactions with no added virus were performed for each binding experiment, the cpm counted was subtracted from total cpm bound.

**Preparation of liposomes.** Liposomes (large unilamellar vesicles) were prepared by freeze/thaw extrusion as described before (3, 27, 36, 43). Briefly, lipid mixtures, dried from chloroform/ methanol, were hydrated in HNE and subjected to five cycles of freezing and thawing. Subsequently, the suspension was extruded 21 times through two 0.2 µM filters (Nucleopore, Inc., Pleasanton, CA, USA) in a LiposoFast mini-extruder (Avestin, Ottawa, Canada). Liposomes consisted of phosphatidylcholine (PC)/ phosphatidylethanolamine (PE)/ sphingomyelin (SPM)/ Cholesterol (Chol) with a molar ratio of 1:1:1:1.5. The phospholipids were obtained from Avanti Polar lipids (Alabaster, AL, USA) and cholesterol from Sigma. The phospholipid concentration of the liposomes was determined by phosphate analysis (2).

**Fusion assay.** Fusion of pyrene-labeled SIN with liposomes was monitored on-line in an AB2 fluorimeter (SLM/Aminco, Urbana, IL, USA), at 37 °C (3, 27, 36, 43). In the fusion reaction, pyrene-labeled SIN (approx. 10^10 particles) was continuously mixed with an 5-fold excess of liposomes in a final volume of 0.665 ml in HNE. At t=0 s, fusion was initiated by the addition of 35 µl 0.1 M MES (morpholinoethanesulfonic acid)-0.2 M acetic acid, pretitrated with NaOH to achieve the final desired pH. The fusion scale was calibrated such that 0% fusion corresponded to the initial pyrene-excimer fluorescence level and 100% fusion to the fluorescence value obtained after the addition of 35 µl 0.2 M octaethyleneglycol monododecyl ether (Fluka Chemie AG, Buchs, Switzerland) (3, 27, 36, 43). The extent of fusion was determined 60 s after acidification.

**Analysis of the conformational changes in the viral spike protein.** The conformational changes occurring in the viral spike protein were examined under the same conditions as in the fusion experiments (3, 36). After the indicated pH treatment, samples were neutralized by addition of a pretitrated volume NaOH, solubilized in SDS-PAGE sample buffer, and analyzed by SDS-PAGE. Running gels were further incubated for 30 min in 1 M sodium salicylate and dried. Visualization and quantification of the E1 trimer was done by phosphorimaging analysis, as mentioned above, by relating the intensity of the E1 trimer to the total intensity of E1, PE2, E2 and E1 trimer, corrected for the contribution of PE2 or E2 on the basis of the relative numbers of methionine residues in the E1, E2 and PE2 proteins.

**Results**

**Characterization of PE2 cleavage mutant SIN viruses.** In this study we investigated the membrane fusion activity of a number of SIN PE2 cleavage mutant viruses. PE2 cleavage mutations were introduced into the background of the infectious SIN clone TR339. Two mutants were chosen that have previously been shown to affect PE2 cleavage efficiency (Table 1; 19). In the mutant
construct, designated E2:N1, the Ser residue at position 1 of E2 is replaced by an Asn residue. This residue creates an N-linked glycosylation signal, and the glycosylation of the Asn residue at position 1 of E2 is proposed to interfere with furin activity (19). SDS-PAGE analysis of the E2:N1 mutant showed that indeed PE2 cleavage was completely inhibited. Furthermore, the virus was poorly infectious, as determined by plaque assay on BHK-21 cells. Likewise, the FDF mutant, in which the BXBB sequence was deleted, incorporated 100% uncleaved PE2 and also had a very low specific infectivity (Table 1).

<table>
<thead>
<tr>
<th>Virus</th>
<th>Amino acids at PE2 cleavage site</th>
<th>Furin cleavage site</th>
<th>% PE2</th>
<th>BHK specific infectivity(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR339</td>
<td>G R S K R S</td>
<td>Intact</td>
<td>4</td>
<td>18</td>
</tr>
<tr>
<td>FDF</td>
<td>G - - - S</td>
<td>Deleted</td>
<td>100</td>
<td>0.5</td>
</tr>
<tr>
<td>E2:N1</td>
<td>G R S K R N</td>
<td>Intact</td>
<td>100</td>
<td>1.5</td>
</tr>
</tbody>
</table>

\(^a\) (PFU/ cpm)

The first step in viral entry is the interaction of a virus particle with a cellular receptor. Consistent with previous reports (20), we found that TR339 virus does not bind very efficiently to BHK-21 cells (Figure 1A). The FDF mutant bound equally poorly to BHK-21 cells. On the other hand, the E2:N1 virus did bind efficiently to BHK-21 cells. Recent data have demonstrated that SIN viruses with an intact cleavage site interact with HS, a cellular receptor which is abundantly expressed on BHK-21 cells (19). Accordingly, we found that the E2:N1 mutant bound efficiently to heparin-agarose beads (Figure 1B). In a control, in which albumin-agarose beads were used, none of the viruses bound to the beads (data not shown). Since the two mutant viruses, as compared to TR339, bind similarly (FDF) or much better (E2:N1) to BHK-21 cells, it is clear that the block in infection for these mutants lies after binding of the virus particle to the cell surface, presumably at the level of the viral membrane fusion process.

**Fusion activity of pyrene-labeled PE2 cleavage mutant SIN viruses.** To study the fusogenic properties of the PE2 cleavage mutants, we used SIN variants biosynthetically labeled with the fluorescent probe pyrene. Because the mutant viruses are almost non-infectious, RNA transcripts derived from the cDNA were transfected directly into BHK-21 cells, cultured beforehand in the presence of pyrene fatty acid. At 20 h post-transfection the pyrene-labeled virus particles were
Figure 1. Binding of PE2 cleavage-deficient SIN viruses to BHK-21 cells or heparin-agarose beads. Approx. $10^{10}$ [$^{35}$S]methionine-labeled virus particles were added to the cells or beads and binding was measured after 2 h incubation at 4 °C, as described in Materials and Methods. Each bar represent the mean of triplicate binding assays. Each set of triplicates was repeated three times. The error bars represent standard deviations. (A) Binding to BHK-21 cells. (B) Binding to heparin-agarose beads.

harvested and purified from the medium (3, 27, 36). With this procedure no additional passage of the virus is involved, thereby minimizing the possibility of generating revertant viruses. Subsequently, fusion of pyrene-labeled SIN was measured in a liposomal model system (3, 27, 36). Upon fusion of pyrene-labeled virus particles with target liposomes, the pyrene phospholipids are diluted into the liposomal membrane, resulting in a decrease of pyrene excimer fluorescence intensity. For this assay, liposomes were prepared with an average diameter of 200 nm. Figure 2 presents the results. SIN derived from the infectious clone TR339 fused rapidly and efficiently with the liposomes at pH 5.0 (curve a), 60% of the virus fusing within 60 s after acidification. No fusion was seen at neutral pH (data not shown). In contrast to TR339, PE2 cleavage mutant E2:N1 hardly fused with the liposomes at pH 5.0 (curve b). At 60 s after acidification to pH 5.0, an extent of fusion of only 7% was observed. Like E2:N1, PE2 cleavage mutant FDF, was unable to fuse at pH 5.0 (curve c).

**Construction and characteristics of viable E2:N1 revertants.** Using SIN PE2 cleavage mutant TRSB-N, Heidner et al. (14) showed that after infection of cells with TRSB-N virions occasionally small plaques were formed. These viruses were found to be infectious revertants either with a restored PE2 cleavage phenotype or with second-site mutations in PE2 along with retention of the PE2 cleavage defect.

Some of the second-site resuscitating mutations, identified in TRSB-N, were now introduced into the E2:N1 PE2 cleavage mutant virus, i.e. in the TR339 background. In the first mutant, E2:N1/T191, the Pro residue (CCG) at position 191 of E2 was replaced by a Thr (ACG). In the second mutant, E2:N1/G216, the
Figure 2. Fusion of PE2 cleavage-deficient SIN viruses with liposomes at pH 5.0. Online fusion experiments, with pyrene-labeled viruses, were performed at 37 °C, as described in Materials and Methods. All fusion measurements were repeated at least three times. Approx. $10^{10}$ virus particles were mixed with a 5-fold excess of liposomes, consisting of PC/PE/SPM/Chol (molar ratio 1:1:1:1.5). Curve a, TR339; curve b, E2:N1; curve c, FDF.

Glu residue (GAA) at position 216 of E2 was replaced by a Gly (GGA). In the third mutant, E2:N1/E3:R25, the Cys residue (TGT) at a position corresponding to position 25 of E3 was replaced by an Arg (CGT). We were interested in whether the introduced mutations promoted viral viability and membrane fusion capacity, with retention of the PE2 cleavage-defective phenotype.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Substitution mutation</th>
<th>% PE2</th>
<th>BHK specific infectivity$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2:N1</td>
<td></td>
<td>100</td>
<td>1.5</td>
</tr>
<tr>
<td>E2:N1/T191</td>
<td>E2 position 191 Pro → Thr</td>
<td>100</td>
<td>230</td>
</tr>
<tr>
<td>E2:N1/G216</td>
<td>E2 position 216 Glu → Gly</td>
<td>100</td>
<td>136</td>
</tr>
<tr>
<td>E2:N1/E3:R25</td>
<td>E3 position 25 Cys → Arg</td>
<td>100</td>
<td>160</td>
</tr>
</tbody>
</table>

$^a$ (PFU/ cpm)

First, PE2 cleavage of each of the mutants was determined by SDS-PAGE analysis of $^{[35]}$S methionine-labeled protein from purified particles (Table 2). In agreement with earlier observations on the corresponding TRSB-N revertants (14), all of the second-site mutant viruses maintained the defect in PE2 cleavage. On the other hand, the specific infectivities of the mutants were increased by a factor of
approx. 150-200, compared to the parental E2:N1 virus (Table 2). Thus, all three resuscitating mutations restored virus viability while retaining the PE2 cleavage-deficient phenotype. It is noteworthy, that the specific infectivities of the resuscitated mutants (Table 2) were significantly higher than the specific infectivity of the TR339 virus (Table 1).

To determine whether the high specific infectivity of the resuscitated mutants was due to an improved binding of these viruses to BHK-21 cells, cell-binding assays were performed. Figure 3A shows the results. As indicated above (Figure 1), the E2:N1 virus binds efficiently to BHK-21 cells. Likewise, the resuscitated mutants E2:N1/T191, E2:N1/G216, and E2:N1/E3:R25 also bound very efficiently to BHK-21 cells. Clearly, all PE2 cleavage-deficient mutants, retaining the XBXBBX sequence, bind better to BHK-21 cells than TR339, explaining the higher specific infectivity of the resuscitated mutants. Since the resuscitated mutants are still PE2 cleavage-deficient, retaining the XBXBBX furin cleavage site, it is likely that these viruses bind to HS on BHK-21 cells (19). To investigate this directly, we determined the binding capacity of the resuscitated mutants for heparin- versus albumin-agarose beads. All viruses appeared to bind efficiently to the heparin-beads (Figure 3B). None of the viruses bound to albumin-beads (data not shown). Taken together, PE2 cleavage-deficient resuscitated viruses are infectious, and have a high specific infectivity on BHK-21 cells, presumably due to an interaction of the uncleaved PE2 with HS on the cell surface. The high infectivity of the resuscitated mutant viruses, as compared to the low infectivity of the parent E2:N1 virus, suggests that the second-site mutations somehow restore the fusion capacity of these viruses. This question was addressed subsequently.

Figure 3. Binding of PE2 cleavage-deficient second-site resuscitated SIN viruses to BHK-21 cells or heparin-agarose beads. Binding was measured after 2 h incubation at 4 °C, as described in the legend to Figure 1. Bars: a, E2:N1; bars b, E2:N1/T191; bars c, E2:N1/G216; bars d, E2:N1/E3:R25. (A) Binding to BHK-21 cells. (B) Binding to heparin-agarose beads.
Fusion activity of pyrene-labeled PE2 cleavage mutant SIN viruses carrying a resuscitating mutation in PE2. Figure 4 presents the fusion kinetics of pyrene-labeled PE2 resuscitated cleavage mutant SIN viruses with liposomes at pH 5.0. The E2:N1/T191 mutant fused efficiently with liposomes, the extent of fusion being about 55% within 60 s (curve a). The E2:N1/G216 and E2:N1/E3R25 mutants also fused efficiently with liposomes at pH 5.0 (curves b, c). Again, the E2:N1 virus was unable to fuse at pH 5.0 (curve d). Thus, it appears that, as a result of a single amino-acid substitution, the non-infectious PE2 cleavage-deficient E2:N1 virus regains infectivity due to restoration of its membrane fusion competence, despite retention of the uncleaved PE2 phenotype.

Figure 4. Fusion of PE2 cleavage-deficient SIN viruses with liposomes. On-line fusion experiments were performed at 37 °C, as described in the legend to Figure 2. Curve a, E2:N1/T191; curve b, E2:N1/G216; curve c, E2:N1/E3R25; curve d, E2:N1.

PE2 cleavage mutant SIN viruses show an acidic pH-shift in the activation of membrane fusion. Previous work, based on SFV, showed that the block in infection of the p62 cleavage mutant mL (Arg-to-Leu substitution at position -1 of E2) could be overridden by exposure of the virus to a very low pH (34). Therefore, we were interested in whether incubation at a very low pH could activate fusion activity of PE2 cleavage mutant SIN.

Figure 5A presents the fusion kinetics of several pyrene-labeled PE2 cleavage mutants at pH 4.0 in the liposomal model system. As expected, the resuscitated mutant E2:N1/T191 (curve a) fused efficiently, with fusion kinetics identical to those of the TR339 virus (curve b). Importantly, however, under these extreme pH conditions the non-infectious E2:N1 and FDF mutants were able to fuse as well (curve c, d). As a control, the different pyrene-labeled SIN viruses were incubated at pH 4.0 in the absence of liposomes. In all cases, the fluorescence intensity remained constant, demonstrating that, also under these extreme pH conditions,
the decrease in pyrene excimer fluorescence intensity observed in the presence of liposomes was due to dilution of the fluorescent probe from the viral membrane into the liposomal membrane (data not shown). These results demonstrate that the PE2 cleavage mutants FDF and E2:N1 are fusion-competent, but only when the viruses are exposed to an unphysiologically low pH.

Figure 5B shows the detailed pH dependence of fusion of SIN PE2 cleavage mutants with liposomes. For TR339, optimal fusion was observed at pH 5.0 (squares). The threshold for fusion was pH 6.25, similar to that of the laboratory-adapted SIN strain AR339 (36). The PE2 cleavage mutants FDF (diamonds) and E2:N1 (circles) showed a clear-cut downward shift in the pH dependence of fusion, with a threshold at pH 5.0. Only at very low pH values fusion of these viruses was fast and extensive. Interestingly, an intermediate pH dependence was observed for the resuscitated mutant viruses E2:N1/T191 (upward triangles) and E2:N1/G216 (downward triangles). The other resuscitated mutant virus, E2:N1/E3:R25, showed a comparable pH dependence to that of the E2:N1/T191 and E2:N1/G216 mutants (data not shown). The threshold for fusion was very close to pH 5.75 for all cleavage-deficient SIN viruses with a resuscitizing mutation in PE2. Taken together, SIN PE2 cleavage mutants exhibit an acidic pH-shift in their ability to fuse with liposomes, and resuscitating mutations in PE2 partly reverse this pH-shift.

Figure 5. Low-pH dependent fusion of PE2 cleavage-deficient SIN viruses with liposomes. On-line fusion experiments were performed, as described in the legend to Figure 2. (A) Curve a, E2:N1/T191; curve b, TR339; curve c, E2:N1; curve d, FDF. (B) The extent of fusion was determined 60 s after acidification. Line (■) TR339; line (▲) E2:N1/T191; line (▼) E2:N1/G216; line (●) E2:N1; line (♦) FDF.
Rearrangement of the spike heterodimer is impaired in PE2 cleavage mutants. Earlier studies on SFV and SIN have demonstrated that, under low-pH conditions, the E2/E1 heterodimer dissociates and a trypsin-resistant E1 homotrimer is formed. (3, 16, 29, 36, 40, 41). It is believed that the E1 homotrimer is the fusion-active conformation of the viral spike protein (16, 40). To determine whether the PE2 cleavage mutants were blocked in their ability to rearrange to the fusion-active conformation, the conformational changes of the viral spike proteins were studied. [35S]Methionine-labeled virus was incubated at low pH in the presence of liposomes. At 60 s after acidification the mixture was neutralized and the appearance of the E1 homotrimer was analyzed on SDS-PAGE. Figure 6 shows the results. The spike protein of TR339 already rearranged to form an E1 homotrimer at pH 5.75. Under optimal conditions for fusion (pH 5.0), 76% of the TR339 E1 protein was converted to the trimeric configuration. The PE2 cleavage mutants FDF and E2:N1 showed a large pH shift in the formation of the E1

![Figure 6](image_url)

**Figure 6.** E1 trimerization of PE2 cleavage-deficient SIN viruses at 37 °C and the indicated pH values. Approx. \(10^{10}\) [35S]methionine-labeled SIN particles were incubated with an 5-fold excess of liposomes consisting of PC/PE/SPM/Chol (molar ratio 1:1:1:1.5). After 60 s, samples were neutralized and analyzed for the appearance of E1 trimers on SDS-PAGE, as described in Materials and Methods.
trimer. Clearly, with these viruses, at pH values of 5.0 or above no significant trimer formation occurred. Only when these mutants were incubated at a very low pH, the E1 spike protein rearranged to its trimeric configuration. By contrast, the resuscitated cleavage mutant E2:N1/T191 did convert to an E1 homotrimer at pH 5.0. The extent of E1 trimerization increased by incubation at pH values lower than pH 5.0. Similar results were obtained with the other resuscitated mutants, E2:N1/G216 and E2:N1/E3:R25 (data not shown). These results suggest that the PE2/E1 heterodimer of the PE2 cleavage mutants FDF and E2:N1 is stable at pH 5.0, rearranging to the fusion-active state only at very low, non-physiological, pH values. On the other hand, the PE2/E1 heterodimer of the resuscitated viruses is less stable than that of the parental E2:N1 virus and rearranges to a fusion-active conformation at a physiological mildly acidic pH (pH 5.0).

Figure 7 presents a comparison of E1 trimerization and fusion for the PE2 cleavage mutant SIN viruses at different pH values. Clearly, there is a distinct correlation between the appearance of the E1 homotrimer and the ability of the virus to fuse with liposomes. Under optimal conditions for fusion, the extent of E1 trimerization and fusion of TR339 at 60 s after acidification were 76 and 59%, respectively. At pH 5.75, already 64% of the E1 glycoprotein had rearranged to the fusion-active conformation and half-maximal fusion was observed. On the other hand, the PE2 cleavage mutant FDF, at pH 5.0, showed extents of E1 trimerization and fusion of only 5% and 3%, respectively. At pH 4.0, the FDF mutant did become fusogenic, 70% of the E1 protein converting to a homotrimer, and 40% of the virus particles fusing with liposomes, under these conditions. Likewise, the PE2 cleavage mutant E2:N1 showed little E1 trimerization and fusion at pH 5.0 (6 and 8%, respectively), whereas at pH 4.0 the extents of E1 homotrimer formation and fusion for this virus were 70% and 43%, respectively.

At pH 5.0, the resuscitated mutant E2:N1/T191 showed extents of E1 trimerization and fusion of 38% and 49%, respectively. At a pH lower than 5.0, the extent of E1 trimerization and fusion further increased. At pH 5.75, only a very small amount (2%) of the E1 glycoprotein was converted to a homotrimer, and accordingly fusion was negligible. Similar results were obtained for the other resuscitated mutants, E2:N1/G216 and E2:N1/E3:R25 (data not shown). The pH-threshold of fusion for TR339 is 6.2 (Figure 5B), which is 0.5 pH unit higher than that of the resuscitated viruses. The resuscitated mutants clearly showed a pH shift in the formation of an E1 homotrimer and fusion as compared to TR339. Taken together, the lower stability of the PE2/E1 heterodimer of the resuscitated mutants, as compared to the parental E2:N1 mutant, enables the virus to become fusion-active under physiological pH conditions, consistent with the restored infectivity of these viruses in BHK-21 cells.
Membrane fusion of Sindbis virus PE2 cleavage mutants

Figure 7. Relative pH-dependence of E1 trimerization and fusion for several PE2 cleavage-deficient SIN viruses. The extents of E1 trimerization (shaded bars) and fusion (open bars) are shown at 60 s post-acidification to the indicated pH values. To compare E1 trimerization and fusion, the final extents of these processes at pH 4.0, were set to 100%. (A) TR339 (absolute extents: 76% E1 trimerization, 55% fusion). (B) FDF. (absolute extents: 70% E1 trimerization, 40% fusion). (C) E2:N1. (absolute extents: 70% E1 trimerization, 43% fusion). (D) E2:N1/T191. (absolute extents: 70% E1 trimerization, 56% fusion). E1 trimerization was determined as described in the legend to Figure 6. Fusion was measured as described in the legend to Figure 2.

Discussion

In this paper, we provide evidence that the block in viral infectivity of SIN PE2 cleavage mutant viruses on BHK-21 cells lies at the level of the membrane fusion activity of the viral envelope glycoprotein (Figures 2, 6-7). Earlier studies on similar SIN PE2 cleavage mutants had shown that viral infection in vertebrate cells was not blocked at the level of virus-receptor interaction, RNA replication or virus assembly (14, 19). Our present results show that SIN PE2 cleavage mutants are impaired in their fusion activity because of the inability of the immature PE2/E1 heterodimer to rearrange to an E1 homotrimer, at a physiological acidic pH. The E1 trimer has been shown to represent the fusion-active conformation of the alphavirus spike (3, 11, 16, 36, 40). The lethality of SIN PE2 cleavage defect could be overridden by second-site resuscitating mutations in PE2 (Table 2), which destabilize the PE2/E1 heterodimer interaction. Thus, the spike regains the ability
to form a fusion-competent E1 trimer at a physiologically relevant acidic pH (Figures 4-7).

Among members of the alphavirus genus, PE2 cleavage has been associated with different biological functions of the viruses involved. Recent work has shown that SIN PE2 cleavage mutants with an intact cleavage site (XBXBBX) interact electrostatically with HS, a glycosaminoglycan which is abundantly expressed on BHK-21 cells (19). These findings are in agreement with our results, showing that the E2:N1 mutant (with an intact cleavage site) binds efficiently to BHK-21 cells and heparin-beads, as compared to the PE2 cleavage mutant FDF (with a deleted cleavage site) (Figure 1). PE2 cleavage mutant E2:N1 binds much more efficiently to BHK-21 cells than the parental SIN TR339, confirming that the TR339 virus does not interact directly with HS (20). For SFV, p62 cleavage mutants have been generated by mutation of the cleavage site from RHRR to RHRL (mL mutant) (22, 34) or SHQL (1, 39). In contrast to our PE2 cleavage-deficient SIN viruses, these SFV mutants show a large reduction in binding capacity to BHK-21 cells, as compared to wild-type SFV. However, in the case of SFV the cleavage site was disrupted to create the p62 cleavage-deficient viruses. Therefore it is difficult to interpret these results in the context of our present data on SIN mutants which are known to bind to cells through the furin cleavage site. Despite the efficient binding of the E2:N1 PE2 cleavage mutant to BHK-21 cells, the virus was still found to be non-infectious, due to its inability to fuse (Table 1; Figure 2). However, after introduction of resuscitating mutations in PE2 E2:N1 regained fusion competence and infectivity on BHK-21 cells, as discussed in more detail below. Interestingly, the specific infectivity of these viruses on BHK-21 cells was found to be very high, even 10-fold higher than that of the parental TR339 (Table 2). Our results demonstrate that the resuscitated PE2 cleavage mutants are more infectious than TR339, very likely as a result of the more efficient interaction of these viruses with HS (Figure 3).

The inability of cleavage mutants E2:N1 and FDF to fuse at a physiologically relevant acidic pH appears to be caused by a downward shift in the pH dependence of the viral membrane fusion reaction (Figure 5B). Within the overall process of low-pH-dependent alphavirus membrane fusion, the E2/E1 heterodimer first dissociates, followed by formation of a trypsin-resistant E1 homotrimer (3, 11, 16, 36, 40). In the present study, it became clear that the PE2/E1 heterodimer of the cleavage mutant viruses is too stable to rearrange to the fusion-active E1 homotrimeric conformation, at a physiologically relevant acidic pH (Figures 6-7). However, when the viruses were incubated at pH 4.0, the spike did form an E1 homotrimer, resulting in expression of full membrane fusion activity (Figures 6-7). In conclusion, the low specific infectivity of SIN PE2 cleavage mutants is the result of an impairment of the viral spike to rearrange to the fusion-active conformation at a mildly acidic pH, due to the high stability of the immature PE2/E1 heterodimer. This is in agreement with observations on the SFV p62 cleavage-
deficient mL mutant, showing that viral infectivity on BHK-21 cells was restored by exposure of the virus to pH 4.5 (34).

Several investigators have identified second-site resuscitating mutations in the E3, E2 or E1 glycoproteins which promote infectivity of PE2 cleavage-deficient alphaviruses (5, 14, 39). Some of these resuscitating mutations, found with the TRSB-N SIN clone (14), were now introduced in the E2:N1 virus to investigate the role of these mutations in viral infectivity versus the stability of the spike heterodimer. These E2:N1 mutants with resuscitating mutations in PE2 were indeed infectious on BHK-21 cells (Table 2), and at the same time exhibited a restored membrane fusion capacity at a physiologically relevant acidic pH (Figures 4-5). In this respect it is important to note that viruses which do cleave PE2 and interact efficiently with the cell attachment receptor HS are more infectious on BHK-21 cells than the resuscitated PE2 cleavage-deficient viruses (20). This indicates that SIN cleavage mutants with resuscitating mutations in PE2 have an intermediate infectivity on BHK-21 cells, which in fact correlates precisely with the intermediate pH dependence of their membrane fusion activity with liposomes (Figure 5B). For example, the TR339 virus with a PE2 cleavage-deficient phenotype, is already fully fusion-competent at pH 5.5, whereas the resuscitated PE2 cleavage mutant viruses express full membrane fusion activity only at pH values of 5.0 or below. Analysis of the conformational changes occurring in the viral spike protein showed that the PE2/E1 spikes of the resuscitated mutants were able to form an E1 homotrimer at a physiological acidic pH, in contrast to the heterodimer of the E2:N1 virus (Figure 6). In conclusion, the presence of the resuscitating mutations in PE2 destabilizes the PE2/E1 heterodimer such that the viral spike protein regains the ability to undergo conformational changes with formation of a fusion-active E1 trimer at a physiological relevant pH, despite retention of the uncleaved PE2 phenotype.

The maturation of the alphavirus spike heterodimer through cleavage of the PE2 precursor of E2 has a distinct function in the viral life cycle. During transport of the heterodimer from the ER to the surface of the infected cell, the uncleaved PE2 protein is presumed to function as a chaperone, protecting the spike from premature destabilization within the acidic TGN (9). Subsequently, after cleavage of PE2 in a post-TGN compartment by a furin-like protease, the mature viral spike protein is primed for expression of membrane fusion activity when exposed to a mildly acidic pH within the endosomal compartment of a new target cell. The results of this study indicate that, while cleavage maturation is a common phenomenon in viral assembly, it is not absolutely required for viral infection. SIN PE2 cleavage mutants with resuscitating mutations in PE2 were found to be infectious on BHK-21 cells, despite retention of the uncleaved PE2 phenotype. Apparently, the resuscitating mutations in PE2 destabilize the PE2/E1 heterodimer to such an extent that the spike has the capacity to rearrange to the fusion-active conformation at the luminal pH of endosomes, while at the same
time the heterodimer is stable enough to survive the mildly acidic lumen of the TGN (pH ~6.0 (35)) during transport to the cell surface.

Interestingly, another alphavirus PE2 cleavage mutant called S12 (Ser-to-Asn substitution at position 1 of E2), derived from SAAR86, was found to be fully infectious on BHK-21 cells (31, 33). In the light of our present results, we would argue that the PE2/E1 heterodimer of the S12 mutant is less stable than that of the E2:N1 virus, despite the fact that both viruses carry the same amino-acid substitution at E2 position 1. Differences in the genetic background of S12 and E2:N1, outside the PE2 cleavage region, could account for the different spike stability, in agreement with our present observation that distant mutations in the E2:N1 mutant have the capacity to restore viral infectivity by destabilization of the spike heterodimer. We postulate that the S12 spike heterodimer has a very precise pH dependence, so that it survives the TGN during maturation, but that it does become fusion competent at acidic pH in endosomes. The stability of the spike heterodimer and its fusion competence at low pH values of SAAR86 or S12 remain to be determined.

The results in Figures 6 and 7 show that there is a clear correlation between expression of low-pH-dependent membrane fusion activity of SIN and formation of the E1 homotrimeric conformation. However, close scrutiny of the data reveals that mutant SIN viruses with a relatively low pH threshold for fusion require a lower extent of trimer formation to reach a certain level of fusion than the TR339 virus with a high pH threshold for fusion. This suggests that there are different levels of pH control of viral fusion activation and that, besides E1 trimerization, other pH-dependent conformational alterations are involved. In previous studies on SFV (3) and SIN (36), we have shown that, kinetically, fusion in the liposomal model system occurs with a distinct delay after virus-liposome binding and E1 trimerization. This lag phase prior to the onset of fusion may well involve the rearrangement of several E1 trimers into a fusion complex. Interestingly, the duration of the lag phase decreases with decreasing pH (3, 36). Thus, our present observation suggesting that, at a relatively high pH, a relatively high extent of E1 trimerization is required for fusion, may well reflect the comparatively long lag phase between E1 trimerization and the onset of fusion under these conditions.

The present results suggest that there is a direct correlation between the infectivity of SIN on BHK-21 cells and low-pH-dependent membrane fusion activity of the virus with liposomes. Not only is SIN-liposome fusion clearly dependent on low pH, it also appears that subtle shifts in the pH dependence of fusion have a profound effect on viral infectivity. This result provides additional support for the notion that exposure to a mildly acidic pH is an obligatory step in the infectious entry of SIN into its host cell. This conclusion is in agreement with recent observations of Glomb-Reinmund and Kielian (12) and DeTulleo and Kirchhausen (6), indicating that SIN enters cells by receptor-mediated endocytosis and fusion from within acidic endosomes. On the other hand, very recently
Hernandez et al. (15) provided evidence, based on investigation of mosquito cell infection by SIN, suggesting that exposure to an acidic pH may not be an obligatory step in alphavirus cell entry. Clearly, it can not be excluded that alphaviruses use different routes of cell entry in mosquito and vertebrate cells. Also, our observations do not rule out the possibility that interaction of SIN with cell-surface components induces conformational alterations priming the spike to subsequently rearrange to its fusion-active structure (7, 26). Nevertheless, from our present results it would appear that low pH is a very important factor in alphavirus spike heterodimer destabilization and thus in the activation of viral membrane fusion activity. This conclusion is completely consisted with recent structural data on the SIN spike (30). Furthermore, SIN closely resembles SFV in terms of both spike organization (21, 30) and membrane fusion characteristics (3, 27, 36). For SFV it has been clearly demonstrated that it infects cells via receptor-mediated endocytosis and fusion from within acidic endosomes (6, 10, 12, 17, 23, 42).

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**References**

Membrane fusion of Sindbis virus PE2 cleavage mutants


