Mutational analysis of receptor interaction and membrane fusion activity of Sindbis virus
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Chapter 3

Adaptation of Alphaviruses to Heparan Sulfate: Interaction of Sindbis and Semliki Forest Virus with Liposomes Containing Lipid-Conjugated Heparin

Jolanda M. Smit, Barry-Lee Waarts, Koji Kimata, William B. Klimstra, Robert Bittman, and Jan Wilschut

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Abstract

Passage of Sindbis virus (SIN) in BHK-21 cells has been shown to select for virus mutants with high affinity for the glycoaminoglycan heparan sulfate (HS). Three loci in the viral spike protein E2 have been identified (E2:1; E2:70; E2:114), which mutate during adaptation and independently confer the ability to the virus to bind to cell-surface HS (W.B. Klimstra et al., J. Virol. 72:7357-7366, 1998). In this study, we used HS-adapted SIN mutants to evaluate a new model system, involving target liposomes containing heparin-conjugated phosphatidylethanolamine (HepPE) as an HS receptor analog for the virus. HS-adapted SIN viruses, but not non-adapted wild-type SIN TR339, interacted efficiently with HepPE-containing liposomes at neutral pH. Binding was competitively inhibited by soluble heparin. Despite the efficient binding of HS-adapted SIN viruses to HepPE-containing liposomes at neutral pH, there was no fusion under these conditions. Fusion did occur, however, at low pH, consistent with cellular entry of the virus via acidic endosomes. At low pH, wild-type or HS-adapted SIN viruses underwent fusion with liposomes with or without HepPE with similar kinetics, suggesting that interaction with the HS receptor analog at neutral pH has little influence on subsequent fusion of SIN at acidic pH. Finally, Semliki Forest virus (SFV), passaged frequently on BHK-21 cells, also interacted efficiently with HepPE-containing liposomes, indicating that SFV, like other alphaviruses, readily adapts to cell-surface HS. In conclusion, the liposomal model system presented in this paper may serve as a novel tool to study receptor interactions and membrane fusion properties of HS-interacting enveloped viruses.
Introduction

Alphaviruses, such as Ross River virus (RR), Semliki Forest virus (SFV), Sindbis virus (SIN), and Venezuelan equine encephalitis virus (VEE), are enveloped positive-strand RNA viruses belonging to the family of Togaviridae. The viral genome consists of a single-stranded RNA molecule, which is complexed with 240 copies of the capsid protein (50). The nucleocapsid is surrounded by a lipid bilayer in which the spike proteins are inserted. The viral surface contains 80 heterooligomeric spikes, a single spike consisting of a trimer of E2/E1 heterodimers. The E1 and E2 glycoproteins mediate the infectious entry of alphaviruses into cells. The E2 glycoprotein is primarily involved in the interaction of the virus particle with an attachment receptor on the cell surface (7, 28, 49), whereas E1 is required for the subsequent fusion process (19, 53).

The spike proteins of RNA viruses are capable of rapid adaptation to their growth environment. Recently, it has been shown that viruses from different families interact with glycoaminoglycans (GAGs), in most cases heparan sulfate (HS), as a cell-culture adaptation. Virus families or genera that exhibit such a GAG-adaptation include alphaviruses (2, 21, 28), flaviviruses (33), pestiviruses (25), picornaviruses (16, 43), and retroviruses (38, 41). GAGs are highly sulfated polymers of disaccharide repeats, and hence are negatively charged. They are ubiquitously expressed on cell surfaces, but vary with respect to their composition and quantity on different tissues and cell types (3, 52).

In the alphavirus genus, positively charged amino-acid substitutions have been identified in the viral spike protein E2 of SIN, RR, and VEE that are responsible for interaction with HS (2, 21, 28). With regard to SIN, three loci in E2 have been identified (E2:1; E2:70; E2:114) that mutate during the adaptation of SIN in BHK-21 cells and can independently confer the ability to bind to cell-surface HS (28). The sequence XBXBBBX or XBBXBX (where X is any residue and B is a basic residue) is a linear binding motif that allows proteins to attach to HS (9). The positive charge mutation at E2:1 results in the formation (although in opposite orientation) of a linear HS interaction sequence. The HS binding motifs are not present in the E2:70 and E2:114 regions, which suggest that these viruses interact with HS in a conformation-dependent manner. This phenomenon is known to occur in for foot-and-mouth disease virus type O, in which structural studies revealed that heparin makes contacts with all three major capsid proteins VP1, VP2, and VP3 (18). Despite the efficient interaction of the selected mutants of SIN, VEE, and RR with HS, the viruses were found to have an attenuated virulence in animals when compared to wild-type viruses. It has been proposed that HS-adapted mutants could bind to non-productive cellular structures, such as extracellular membranes and basal laminae, and therefore may be cleared from the blood more rapidly than wild-type viruses (2, 8, 21).
In previous studies, membrane fusion activity of SIN, SFV, and Tick-borne encephalitis virus (TBE) has been investigated using liposomes lacking a protein or carbohydrate receptor in the target membrane (5, 13, 46, 47). This suggests that receptor interaction is not a prerequisite for fusion. However, characteristics of virus-liposome fusion in the presence of an attachment receptor have not been studied. In this study, we used HS-adapted SIN mutants to evaluate a new model system, involving target liposomes supplemented with phosphatidylethanolamine-conjugated heparin (HepPE) as an attachment receptor analog for the virus. With HepPE in the target membrane, we were able to directly investigate the role of HS receptor interaction and its potential function in triggering or influencing membrane fusion of HS-adapted SIN with target membranes. It is demonstrated that HS-adapted SIN efficiently interacts with liposomes supplemented with remarkably low concentrations of HepPE in the membrane. Despite the efficient binding at neutral pH, there was no fusion under these conditions. Fusion was observed only at low pH, consistent with cell entry of SIN via acidic endosomes. Finally, it is shown that SFV, either passaged frequently in baby hamster kidney (BHK-21) cells or derived from the BHK-adapted infectious clone pSFV4, interacts efficiently with HepPE-containing liposomes, indicating that SFV like SIN readily adapts to cell-surface HS.

Materials and Methods

Viruses. The viruses were generated from cDNA clones. The construction of the consensus Sindbis virus AR339 clone pTR339 and the HS-adapted SIN virus clones p3970 (called p39K70 in previous articles) and pTRSB has been described previously (28, 29, 36). The construction of the SFV clone pSFV4 has been described before (31). This clone was generated from a laboratory strain of SFV, adapted to growth on BHK-21 cells. A plaque-purified laboratory strain of SFV, also highly adapted to growth on BHK-21 cells, was a generous gift of Dr. Margaret Kielian (Albert Einstein College of Medicine, New York, NY).

The viruses were produced by high-efficiency electroporation of BHK-21 cells with in vitro transcripts of linearized cDNA clones as described before (31). Viruses released from the cells at 20 h post-transfection were harvested, and these stocks were subsequently used for the production of pyrene- or [35S]methionine-labeled SIN or SFV particles, as previously described (5, 46). The viruses were characterized by plaque assay on BHK-21 cells (28), phosphate analysis (4), and protein determination (42). The purity of the viruses was confirmed by SDS-PAGE.

Liposomes. Large unilamellar vesicles were prepared by n-octyl-β-D-glucopyranoside (OGP; Calbiochem, Darmstadt, Germany) dialysis followed by a freeze/thaw-extrusion protocol (5, 40, 46). This method is used, instead of the standard extrusion method, because HepPE is not completely soluble in a chloroform-methanol solution. Liposomes consisted of phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SPM), and cholesterol (Chol) or 6-photocholesterol (photoChol) in a molar ratio of 1:1:1:1.5, supplemented with HepPE as indicated. Briefly, PC/PE/SPM/Chol lipid
mixtures were dried from chloroform-methanol and hydrated in 100 mM OGP in 5 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.4 (HNE). Subsequently, HepPE, was added to the lipid-detergent mixed micelles and dialysis was initiated against HNE buffer to generate liposomes. The liposomes were subjected to five cycles of freezing/thawing and subsequent extrusion through 0.2-µm polycarbonate filters (Nuclepore Inc., Pleasanton, CA, USA) in a LipoFast mini-extruder (Avestin, Ottawa, Canada). PhotoChol-containing liposomes were prepared in subdued light. The size of the liposomes was determined by quasi-elastic light scattering analysis in a submicron particle sizer model 370 (Nicomp Particle Sizing systems, Santa Barbara, Calif.). The phospholipids were obtained from Avanti Polar Lipids (Alabaster, AL, USA), and cholesterol was from Sigma (St. Louis, Mo, USA). The photoactivatable analog of cholesterol, photoChol, was synthesized as described before (37). The HepPE conjugate, consisting of heparin (from porcine intestinal mucosa, MW\text{av} 10,000; Scientific Protein Laboratories, Wannakee, WI) coupled to dipalmitoyl-phosphatidylethanolamine was synthesized and purified as described previously (51). Trypsin-containing liposomes were prepared in a similar manner as outlined above, except that this case the lipids were dispersed in 100 mM OGP in HNE containing 10 mg/ml trypsin (Boehringer, Mannheim, Germany). 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 liposomes was determined by phosphate analysis (4).

Bindings assays. Virus binding to BHK-21 cells and heparin-, bovine serum albumin-agarose beads (both from Sigma) was performed essentially as previously described (28, 48). In the binding assay, 10^5 to 10^6 cpm of [35S]methionine-labeled SIN or SFV (approx. 10^6 to 10^7 virus particles) was allowed to attach to monolayers of BHK-21 cells or beads for 1 h at 4 °C. Subsequently, the cells or beads were washed with HNE + 1% FBS buffer. Virus binding was quantified by liquid-scintillation counting.

Binding of the virus to liposomes was assessed by a co-flotation assay, as described before (5, 39, 46). Briefly, [35S]methionine-labeled SIN or SFV particles (ranging from 10^5 to 10^6 cpm) were mixed with liposomes (100 µM phospholipid) and incubated for 1 h at 4 °C, unless indicated otherwise. Then, 0.1 ml of the mixture was added to 1.4 ml 50% sucrose (wt/vol) in HNE and loaded onto a discontinuous (60-50-35-20-5%, wt/vol) sucrose gradient. After ultracentrifugation at 4 °C, the gradient was fractionated into 10 samples, starting from the top. The radioactivity found in the top 4 fractions, relative to the total amount of radioactivity, was taken as a measure of virus-liposome binding. For the heparin binding competition experiments, heparin (MW\text{av} 6,000; Sigma) was added to the SIN particles 1 h prior to mixing with liposomes, and the mixture was incubated at 4 °C.

Fusion assays. Fusion of pyrene-labeled SIN or SFV with liposomes was measured on-line at 37 °C in an AB2 fluorometer (SLM/Aminco, Urbana, USA) at excitation and emission wavelengths of 345 and 480 nm, respectively (5, 46, 48). Briefly, pyrene-labeled SIN or SFV (1 µM phospholipid) and liposomes (100 µM phospholipid) were mixed in 0.665 ml of HNE buffer and stirred magnetically in a quartz cuvette. At t=0 s, fusion was triggered by injection of 35 µl 0.1 M MES (morpholinoethanesulfonic acid) and 0.2 M acetic acid buffer, pre-titrated with NaOH to achieve the final desired pH. Fusion was calibrated such that 0% fusion corresponded with to the initial pyrene-excimer fluorescence and 100 % fusion was obtained after the addition of 35 µl 0.2 M octaethylene glycol monododecyl ether (Fluka, Buchs, Switzerland), to achieve an infinite dilution of the
pyrene probe. The initial rate of fusion was determined from the tangent to the initial phase of the curve. The extent of fusion was determined 60 s after acidification.

The mixing of the aqueous contents of interior of virus and the liposomal lumen was determined as the degradation of the viral capsid protein by trypsin, initially encapsulated in the liposomal lumen (54, 46, 47). Briefly, [\(^{35}\)S]methionine-labeled SIN particles (ranging from \(10^5\) to \(10^6\) cpm) were mixed with trypsin-containing liposomes (100 µM phospholipid) in presence of 125 µg of trypsin-inhibitor (Boehringer, Mannheim, Germany) per ml in HNE, at 37 ºC. The mixture was acidified to the desired pH, as described above. After 60 s the reaction mixture was neutralized by the addition of a pre-titrated volume of NaOH and further incubated for 1 h at 37 ºC. Control incubations were carried out with empty liposomes, or in the presence of Triton X-100 and in absence of trypsin inhibitor. All samples were analyzed by SDS-PAGE, the protein bands being visualized and quantified by phosphorimaging analysis using Image Quant 3.3 software (Molecular Dynamics, Sunnyvale, CA, USA). Capsid degradation was determined by relating the intensity of the capsid protein to the intensity of E1 and E2 in a control experiment in which empty liposomes were used. This ratio was used to calculate the expected intensity of the capsid protein from the reaction in which trypsin-containing liposomes were used. The difference between the expected and the found intensity was taken as a measure of the capsid degradation.

Results

Characterization of HS-adapted SIN mutants. In this study we investigated HS interaction and membrane fusion activity of alphaviruses in a liposomal model system using lipid-conjugated heparin. Heparin is a sulfated polysaccharide, which is commonly used as an analog for HS in receptor-ligand assays since ligand interaction with heparin and analogs of HS have little qualitative difference (27).

<table>
<thead>
<tr>
<th>Virus</th>
<th>nsP3:528</th>
<th>E2:1</th>
<th>E2:70</th>
<th>E1:72</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR339</td>
<td>Arg</td>
<td>Ser</td>
<td>Glu</td>
<td>Ala</td>
</tr>
<tr>
<td>3970</td>
<td>Arg</td>
<td>Ser</td>
<td>Lys</td>
<td>Ala</td>
</tr>
<tr>
<td>TRSB</td>
<td>Gln</td>
<td>Arg</td>
<td>Glu</td>
<td>Val</td>
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</table>

To evaluate the new liposomal model system a comparison was made between the HS-adapted SIN mutants 3970 and TRSB, and the non-adapted SIN TR339 (carrying the consensus sequence of SIN) (28, 29, 36). The HS-adapted SIN
mutant designated 3970 differs from the TR339 clone at position E2:70 (Table 1). The other HS-adapted SIN strain, TRSB, differs from TR339 by a positive charge amino-acid substitution at position E2:1 and a conserved valine for alanine substitution at position E1:72 (Table 1).

First, the specific infectivity of each of the viruses was determined by plaque assay on BHK-21 cells (Table 2). In agreement with earlier data, the specific infectivity of TR339 was much lower than the infectivities of the HS-adapted mutants (28, 29). Next, we determined whether the high specific infectivity of 3970 and TRSB was a result of more efficient binding to BHK-21 cells (Table 2). The results show that the HS-adapted mutants 3970 and TRSB bind very efficiently to monolayers of BHK-21 cells, whereas the TR339 virus binds very poorly to these cells (Table 2). Accordingly, TRSB and 3970 virus bound efficiently to heparin-agarose beads (Table 2). In a control, in which albumin-agarose beads were used, none of the viruses bound to the beads (data not shown).

### Table 2. Characterization of HS adapted Sindbis viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>BHK specific infectivity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>BHK cell binding&lt;sup&gt;b&lt;/sup&gt;</th>
<th>HS-agarose beads binding&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR339</td>
<td>5</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>3970</td>
<td>256</td>
<td>42</td>
<td>50</td>
</tr>
<tr>
<td>TRSB</td>
<td>186</td>
<td>51</td>
<td>56</td>
</tr>
</tbody>
</table>

<sup>a</sup> (PFU/ cpm)  
<sup>b</sup> % cpm bound  
<sup>c</sup> % cpm bound

**Binding of HS-adapted SIN to HepPE-containing liposomes.** To study the receptor interaction of HS-adapted SIN mutants, we used target liposomes in which HepPE was incorporated in the membrane. Liposomes consisted of PC/ PE/ SPM/ Chol (molar ratio 1:1:1:1.5) with various concentrations of HepPE. In the binding experiment, <sup>35</sup>S)methionine-labeled SIN (approx. 10<sup>8</sup>-10<sup>9</sup> virus particles) was incubated with the liposomes (100 µM phospholipid) at neutral pH, for 1 h at 4 ºC. Subsequently, the liposome-bound virus was separated from non-bound virus by flotation on a sucrose-density gradient. Figure 1A shows the gradient profiles. In the presence of liposomes supplemented with 0.02 mol% HepPE, essentially all SIN 3970 particles floated to the top of the gradient (circles), demonstrating that the virus bound quantitatively to the liposomes. Half-
maximal binding was observed with liposomes supplemented with 0.01 mol% HepPE (diamonds). The virus did not bind to liposomes without HepPE in the membrane (squares).

Figure 1B shows the final extent of binding of SIN 3970 to HepPE-containing liposomes, plotted as a function of the molar ratio of HepPE to total phospholipid in the liposomal membrane. Clearly, the binding increased steeply at a ratio of 1 HepPE molecule per 10,000 phospholipid molecules, while binding was maximal at a ratio of 1:5000. A liposome of 200 nm diameter, consisting of phospholipid and cholesterol in a molar ratio of 2:1, has approximately 150,000 phospholipid molecules in its outer membrane leaflet (55). Thus, maximal binding of SIN 3970 to HepPE-containing liposomes was obtained with on average only 30 HepPE molecules exposed on the outer surface of a target liposome, while half-maximal binding occurred with approx. 15 surface-exposed HepPE molecules per liposome. It should be noted that in all likelihood the liposomes used in the present study were, on average, smaller than 200 nm, as discussed in more detail below. This implies that the number of HepPE molecules per liposome was proportionately smaller as well.

![Figure 1](image1.png)

**Figure 1. Binding of HS-adapted SIN 3970 to HepPE-containing liposomes.**

\[^{35}S\]Methionine-labeled SIN (approx. $10^8$-$10^9$ virus particles) was incubated with PC/PE/SPM/Chol liposomes (100 μM liposomal phospholipid) at pH 7.4 for 1 h at 4 °C. Binding was determined by co-flotation analysis on sucrose density gradients, as described in Materials and Methods. (A) Gradient profiles obtained after incubation of virus with control liposomes lacking HepPE (squares), liposomes supplemented with 0.01 mol% (diamonds) or liposomes supplemented with 0.02 mol% HepPE (circles). (B) Extents of binding of SIN to HepPE-containing liposomes as a function of the molar ratio HepPE to total lipid in the liposomes. Bars represent the average of triplicate binding assays.

Next, a comparison was made between the liposome binding capacity of HS-adapted SIN 3970 and TRSB and non-adapted SIN TR339, using liposomes with and without 0.01 mol% HepPE in the membrane. Figure 2 shows the results. For
SIN 3970 and TRSB more than half-maximal binding to the HepPE-containing liposomes was observed, whereas SIN TR339 bound very poorly to these liposomes (shaded bars). There was no binding of any of the viruses to liposomes lacking HepPE in the membrane (solid bars).

Figure 2B presents the binding of SIN to liposomes supplemented with 0.01 mol% HepPE after incubation during various periods of time either in the cold or at 37 °C. Binding of the HS-adapted SIN viruses was fast and efficient at various temperatures. Complete binding of the viruses to HepPE-containing liposomes was observed even after 1 min incubation at 37 °C. Again, non-adapted SIN TR339 bound poorly to the liposomes, although both at 4 °C and at 37 °C there was a detectable degree of binding presumably due to a low affinity of the TR339 virus for HS (confer Table 2).

Competition of SIN binding to HepPE-containing liposomes by soluble heparin. To determine whether the HS-adapted SIN viruses interact specifically with the heparin moiety on the liposomal membrane, binding competition experiments were carried out with soluble heparin. SIN was incubated with soluble heparin for 1 h at 4 °C. Subsequently, HepPE-containing liposomes were added to the reaction mixture and incubation was continued for 1 h at 4 °C in the presence of the soluble heparin. Liposome-bound virus was separated from non-bound virus by flotation on a sucrose-density gradient. Figure 3 shows the results. In the presence of 5 mg/ml heparin, SIN 3970 virus failed to float with the liposomes to the top of the gradient (squares), while in the absence of soluble heparin efficient
binding of the virus to the liposomes was observed (diamonds). Clearly, soluble heparin blocks binding of the virus to HepPE-containing liposomes, indicating that HS-adapted SIN specifically interacts with the heparin moiety on the liposomal membrane. It is of interest that maximum competition of SIN 3970 binding to HepPE-containing liposomes was achieved only at relatively high concentrations of soluble heparin (5 mg/ml). At lower soluble heparin concentrations, partial competition was observed (results not shown). This indicates that the interaction between HS-adapted SIN and HepPE-containing liposomes is very tight, suggesting that multiple interactions between a single virus and several HepPE molecules on the liposomal membrane are involved.

**Figure 3.** Effect of soluble heparin on binding of HS-adapted SIN 3970 to PC/PE/SPM/Chol liposomes supplemented with 0.01 mol% HepPE at pH 7.4, 1 h at 4 °C. Binding was determined as described in the legend to Fig.1. Curves: squares, binding in the presence 5 mg/ml soluble heparin; diamonds, without soluble heparin.

**Fusion activity of pyrene-labeled SIN with liposomes with or without HepPE in the target membrane.** It has been suggested that receptor interaction, rather than low pH, triggers conformational changes in the viral spike protein of SIN which would subsequently lead to fusion of the viral envelope with the plasma membrane of the cell (1, 17, 24). To study the membrane fusion activity of SIN upon interaction with the HepPE attachment receptor, fusion was measured in a direct manner using pyrene-labeled virus, as described previously (46, 47). Pyrene-labeled SIN 3970 or TRSB (1 µM phospholipid) and PC/PE/SPM/Chol liposomes supplemented with 0.01 mol% HepPE (100 µM phospholipid) were mixed, with continuous stirring, and incubated for 1 min at 37 °C, pH 7.4, to achieve binding of the virus to the liposomes (see Figs. 1 and 2). While under these conditions with 50-60% of the viruses bound to the HepPE receptor on the liposomal target membrane, there was no detectable fusion (Figure 4, curves b, c).
In a control experiment, in which non-adapted SIN TR339 was used, there was no fusion at neutral pH either (curve a). However, all three viruses fused rapidly and efficiently with HepPE-containing liposomes at pH 5.0. Under these conditions, a decrease of pyrene-excimer fluorescence intensity of 50% was observed. This indicates that SIN bound to HepPE-containing liposomes at neutral pH becomes fusion-active only upon exposure to acidic pH.

**Figure 4. Low-pH-dependent fusion of HS-adapted SIN mutants with liposomes.** On-line fusion experiments were performed at 37 °C, as described in Materials and Methods. The final virus and liposome concentrations were 1.0 and 100 µM (membrane phospholipid), respectively. (A) shows fusion curves SIN with PC/PE/SPM/Chol liposomes supplemented with 0.01 mol% HepPE; curves a, TR339; curves b, 3970; curves c, TRSB. (B) and (C) show the initial rate of fusion, as determined from the tangent to the first part of the curve, of SIN TR339 (diamonds) and HS-adapted 3970 (squares) and TRSB (circles). (B) fusion with PC/PE/SPM/Chol liposomes supplemented with 0.01 mol% HepPE; (C) fusion with control PC/PE/SPM/Chol liposomes without HepPE. All fusion measurements were repeated at least three times.
To further establish that SIN, bound to HepPE in target liposomes, retains the capacity to fuse at low pH, fusion was measured on isolated virus-liposome complexes. Pyrene-labeled SIN 3970 was incubated with HepPE-containing liposomes for 1 h at 4 °C. Then, liposome-bound virus was separated from non-bound virus by flotation on a sucrose-density gradient. Subsequently, the membrane fusion activity of the virus-liposome complex was measured at pH 5.0. Under these conditions, the liposome-bound virus fused rapidly and efficiently, indicating that virus, pre-bound to target liposomes through the interaction with HepPE at neutral pH, remains fully fusion-active when exposed to acidic pH (data not shown).

Figure 4B shows the initial rates of fusion of SIN TR339, 3970, and TRSB with liposomes supplemented with 0.01 mol% HepPE as a function of the pH of the medium. Similar fusion kinetics were observed for the HS-adapted SIN 3970 and TRSB versus the non-adapted SIN TR339. Furthermore, using target liposomes without HepPE, we observed indistinguishable fusion kinetics for HS-adapted SIN viruses and non-adapted SIN TR339 (Figure 4C). Clearly, all of the SIN viruses used fuse with liposomes in a strictly low-pH-dependent manner, exhibiting similar fusion kinetics irrespective of the presence of HepPE in the target membrane.

The decrease of pyrene excimer fluorescence intensity by approx. 50%, seen in the above measurements, corresponds to 50% fusion under the assumption that when a virus particle fuses with a liposome the pyrene probe is diluted infinitely. However, upon fusion of a virus particle with a comparatively small liposome, a residual excimer fluorescence intensity will remain, implying that in this case the actual extent of fusion may be underestimated in the pyrene assay. In this respect it is important to note that the liposomes produced by the OGP dialysis method, as used in our present experiments, tend to be smaller than the liposomes we use routinely (5, 46, 48). Moreover, inclusion of increasing concentrations of HepPE (> 0.02 mol%) in the membrane results in a further reduction of the size of the liposomes, as judged by a decreasing opalescence of the preparation. As a consequence, it is likely that, using the pyrene excimer fusion assay under these conditions, one in fact underestimates the extent of fusion due to incomplete dilution of the fluorophore.

Recently, we have shown that a photoactivatable analog of cholesterol, 6-photocholesterol (photoChol), has the capacity to reversibly quench pyrene excimer and monomer fluorescence intensity (37). With this compound we were able to investigate directly whether the pyrene assay indeed does underestimate the actual extent of fusion. Fusion of pyrene-labeled SIN with photoChol-containing liposomes is not only monitored on the basis of dilution but also on the basis of quenching of the pyrene probe in the target membrane. Figure 5 shows the results. Clearly, at pH 5.0, fusion of SIN 3970 with liposomes consisting of PC/PE/SPM/photoChol with or without 0.01 mol% HepPE (curves a,b)
appeared more rapid and more efficient than fusion with corresponding liposomes containing regular cholesterol (curves c,d). With photoChol-containing liposomes, the initial rate of fusion was extremely fast: 35-40% of the virus particles underwent fusion with the liposomes within the first second after acidification. Furthermore, the apparent extent of fusion was over 70%. Taken together, these results indicate that the extent of fusion of SIN with comparatively small liposomes, as assessed by the regular pyrene assay, represents an underestimation of the actual extent of fusion.

Figure 5. Fusion of pyrene-labeled SIN 3970 with liposomes, containing photocholesterol, at pH 5.0. Fusion was measured on-line at 37 °C as described in the legend to Fig. 4. Curves a, PC/PE/SPM/photoChol liposomes supplemented with 0.01 mol% HepPE; curves b, PC/PE/SPM/photoChol liposomes; curves c, PC/PE/SPM/Chol liposomes supplemented with 0.01 mol% HepPE; curves d, PC/PE/SPM/Chol liposomes. All fusion measurements were repeated at least two times.

Contents mixing during fusion of SIN with trypsin-containing liposomes. To further quantify the extent of SIN-liposome fusion under the conditions of our experiments, we also applied an entirely different fusion assay based on mixing of the interior of the virus with the liposomal lumen. Contents mixing was measured as the degradation of the viral capsid protein by trypsin, encapsulated in target liposomes, in the presence of trypsin inhibitor in the medium (46, 47, 54). [35S]Methionine-labeled SIN, either HS-adapted SIN 3970 or non-adapted SIN TR339, and trypsin-containing supplemented with 0.01 mol% HepPE liposomes (100 µM phospholipid) were incubated for 1 min at 37 °C, pH 7.4, to allow the virus to bind to the liposomes. Figure 6 shows that there was very little capsid degradation under these conditions (lanes a). This, again, demonstrates that virus-receptor interaction at neutral pH does not induce fusion of the virus.
Figure 6. Transfer of the viral capsid into the liposomal lumen assayed as the degradation of the viral capsid protein. [35S]methionine-labeled SIN (approx. $10^8$–$10^9$ virus particles) was incubated with trypsin-containing PC/PE/SPM/Chol liposomes supplemented with 0.01 mol% HepPE (100 µM liposomal phospholipid) at 37 °C, and viral capsid protein degradation was determined, as described in Materials and Methods. (A) and (B) show the results for HS-adapted SIN 3970 and non-adapted SIN TR339, respectively. Lanes a and d, trypsin-containing liposomes; lanes b and e, empty liposomes; lanes c and f, trypsin-containing liposomes in presence of TX-100 and absence of trypsin inhibitor in the medium. Lanes a-c, pH 7.4; lanes d-f, pH 5.0. (C) and (D) show the quantification of the extent of capsid protein degradation. Solid bars, TR339; shaded bars, 3970; open bars, TRSB. (C), target liposomes supplemented with 0.01 mol% HepPE; (D), target liposomes without HepPE. All capsid degradation experiments were repeated at least two times.
with target liposomes. On the other hand, when SIN 3970 or SIN TR339 were incubated with the liposomes at pH 5.0, almost all of the capsid protein was degraded (lanes d). In control experiments, in which SIN was incubated with empty HepPE-supplemented liposomes at pH 7.4 or pH 5.0, no capsid degradation was observed (lanes b,e). The ratio of the radioactivity of the capsid band relative to the total amount of radioactivity was close to 0.4, as expected on the basis of the number of methionine residues in the structural proteins of SIN. Complete capsid degradation was observed when Triton X-100 was added to the reaction mixture, in absence of trypsin inhibitor in the medium (lanes c,f). The extent of capsid degradation, as function of the pH, for HS-adapted SIN 3970 and TRSB or non-adapted SIN TR339 after fusion with HepPE-containing liposomes at different pHs is shown in panel 6C. For SIN TR339 (solid bars), little to no capsid degradation was observed at pH 7.4, whereas at pH 5.0 82% of the capsid protein was degraded. For HS-adapted SIN 3970 (shaded bars) and TRSB (open bars), similar results were obtained. Furthermore, in all cases the extent of capsid degradation after fusion of the viruses with liposomes without HepPE was similar to that observed upon fusion with HepPE-containing liposomes (Figure 6D). Interestingly, the extents of fusion measured with the trypsin assay closely correspond to those observed with the pyrene assay in the presence of photoChol in the target membrane (confer Fig. 4). This underlines the above conclusion that the regular pyrene assay underestimates the actual extent of fusion due to incomplete dilution of the probe into the comparatively small dialysis liposomes. Clearly, upon exposure to low pH, under the conditions of our experiments the large majority of the viruses fuse with the liposomes whether or not these liposomes contain the HepPE attachment receptor.

Interaction of SFV with HepPE-containing liposomes. Next, we addressed the question whether SFV, another member of the genus Alphaviruses, has the capacity to adapt to HS during passage in cell culture. To this end, we used SFV derived from the infectious clone pSFV4 as well as a strain of virus passaged many times on BHK-21 cells. The pSFV4 clone was generated from a laboratory strain of SFV, which had also been passaged frequently on BHK-21 cells (31). HS adaptation of SFV derived from the infectious clone pSFV4 was evaluated in binding assays. Figure 7A shows that SFV from pSFV4 bound efficiently to monolayers of BHK-21 cells. To assess the specificity of this interaction, we used heparin- versus albumin-agarose beads as cell surrogates in suspension binding assays (28). The results show that SFV bound efficiently to heparin-agarose beads, whereas the virus did not bound to albumin-agarose beads (Figure 7A).

Figure 7B shows that pSFV4-derived virus bound efficiently to HepPE-containing liposomes at neutral pH. The extents of binding were very similar to those obtained with HS-adapted SIN (confer Fig. 1A). With liposomes supplemented with 0.02 mol% HepPE, more than 90% of the virus bound, while with 0.01 mol% HepPE in the target liposomes half-maximal binding was
observed. There was no binding to liposomes lacking HepPE in the membrane. Very similar results were obtained with a plaque-purified laboratory-adapted strain of SFV (results not shown). Taken together, these results demonstrate that SFV passaged frequently on BHK-21 cells, is strongly adapted to interaction with HS.

**Figure 7. Interaction of SFV with BHK-21 cells, heparin-agarose beads and HepPE-containing liposomes.** (A) \([^{35}\text{S}]\)methionine-labeled SFV particles (approx. 10⁸-10⁹ virus particles) were added to BHK-21 cell monolayers or heparin- or albumin-agarose beads, and binding was measured after incubation for 1 h at 4 °C, as described in Materials and Methods. Bar a, binding to BHK-21 cells; bar b, binding to heparin-agarose beads; bar c, binding to albumin-agarose beads. (B) Binding of \([^{35}\text{S}]\)methionine-labeled SFV (approx. 10⁸-10⁹ virus particles) to PC/PE/SPM/Chol liposomes supplemented with various concentrations HepPE (100 µM liposomal phospholipid) at pH 7.4, for 1 h at 4 °C. Binding of SFV to liposomes was assessed as described in the legend to Fig. 1. Each bar represents the average of triplicate binding assays.

Finally, we investigated the membrane fusion activity of SFV upon interaction with HepPE-containing liposomes. Figure 8 shows the results. Interaction of pyrene-labeled SFV with HepPE in the liposomal membrane did not result in fusion at neutral pH (curve c). However, the virus fused rapidly and efficiently with HepPE-containing liposomes at pH 5.5 (curve a). Moreover, similar fusion kinetics were observed with or without HepPE in the target liposomes 5.5 (curve a versus b). There was no fusion of SFV with liposomes lacking the HepPE receptor analog at neutral pH (curve d).
Figure 8. Low-pH-dependent fusion of pyrene-labeled SFV with liposomes. Fusion of pyrene-labeled SFV with PC/PE/SPM/Chol liposomes with or without 0.01 mol% HepPE (100 µM liposomal phospholipid) at 37 °C was determined as described in the legend to Fig. 4. Curves a, PC/PE/SPM/Chol liposomes supplemented with 0.01 mol% HepPE; curves b, PC/PE/SPM/Chol liposomes; curves c, PC/PE/SPM/Chol liposomes supplemented with 0.01 mol% HepPE; curves d, PC/PE/SPM/Chol liposomes. All fusion measurements were repeated at least three times.

Discussion

The study presented in this paper evaluates a new liposomal model system in which a lipid-conjugated heparin (HepPE) is incorporated in the target membrane as an attachment receptor for HS-adapted alphaviruses. The sulfated polysaccharide heparin is commonly used as an analog for HS in receptor-ligand assays, since ligand interaction with heparin and analogous HS have little qualitative difference (27). It is demonstrated here that HS-adapted SIN 3970 and TRSB, at neutral pH, interact efficiently with liposomes supplemented with remarkably low levels of HepPE in the membrane (Figures 1 and 2). Without HepPE in the target membrane the HS-adapted SIN viruses were unable to bind to the liposomes, indicating that the viruses bind specifically to the heparin molecule. Furthermore, SIN strain TR339, which is not adapted to HS, was unable to bind to HepPE-containing liposomes under the same conditions. Moreover, binding competition experiments showed that soluble heparin blocked binding of HS-adapted SIN to HepPE liposomes, further underlining the notion these viruses specifically interact with the lipid-conjugated heparin moiety on the liposomal membrane. Despite the efficient interaction of SIN with HepPE-containing liposomes at neutral pH, there was no fusion under these conditions, as measured with the pyrene- and the trypsin-assay (Figures 4 and 6), as discussed in more detail below.

The interaction of HS-adapted SIN with HepPE-containing liposomes is extremely efficient. Half-maximal binding was observed with liposomes containing as little as 0.01 mol% HepPE in the membrane, demonstrating that about 15
HepPE molecules on the outer surface of a liposome are sufficient for efficient binding of the virus to the liposomal membrane. Almost no binding was observed when, on average, 1-2 HepPE molecules were incorporated in a single liposome. Therefore, we hypothesize that a SIN virus particle, after initial binding to a single HepPE molecule in the liposomal membrane, subsequently recruits more HepPE molecules to the site of interaction, resulting in multiple interactions between the virion and most if not all of the HepPE molecules at the liposome surface. This hypothesis is substantiated by the observation that a high concentration of soluble heparin was required for competition. This concentration was at least an order of a magnitude greater than that required for complete competition of binding of HS-adapted SIN to BHK-21 cells (28). An explanation for this difference could be that binding of HS-adapted SIN to cells involves fewer interactions with HS per virus particle than binding to HepPE-supplemented liposomes. This may be related to a limited mobility of HS-carrying core proteins on the cell surface, possibly restricting recruitment of multiple HS moieties to the site of interaction (27).

There is convincing evidence to indicate that SIN, like SFV (22, 23, 34, 35), infects its host cell by receptor-mediated endocytosis and subsequent fusion from within acidic endosomes (15, 20, 46, 47). In this regard, it is intriguing to note that recently Hernandez and coworkers (24) published a paper, supporting earlier data (1, 6, 17), suggesting that exposure to an acidic compartment within cells may not be an obligatory step in alphavirus infection. Accordingly, it has been proposed that virus-receptor interaction triggers conformational changes in the viral spike protein, inducing fusion of the viral membrane with the plasma membrane of the cell. The results presented in this study clearly demonstrate that, despite the efficient interaction of SIN with the HepPE receptor in target liposomes at neutral pH, there is no fusion under these conditions. This indicates that the presence in the liposomal membrane of HepPE, an analog of the HS attachment receptor used by cell culture-adapted strains of SIN, has no functional role in triggering membrane fusion activity of SIN at neutral pH. Therefore, it appears that the sole requirement for SIN-liposome fusion, even after interaction with the HS receptor analog, is exposure of the virus to low pH. This is in agreement with earlier data demonstrating that not only SIN, but also SFV, and TBE, fuse efficiently at low pH with liposomes lacking a protein or carbohydrate receptor (5, 13, 46, 47). Formally, it cannot be excluded that initial receptor interaction could influence the detailed characteristics of the pH-dependent membrane fusion process of SIN. However, similar fusion kinetics were observed for HS-adapted SIN versus non-adapted SIN with or without HepPE in the target membrane, indicating that receptor interaction has no influence on the detailed pH dependence of virus fusion in the liposomal model system. Taken together, our present results demonstrate that, even after attachment of SIN to HepPE, as an HS receptor analog, fusion remains strictly dependent on exposure of the virus to a mildly acidic pH. This finding strengthens the notion that SIN infects its host cells via
receptor-mediated endocytosis and low-pH-dependent fusion from within acidic endosomes. It is however, important to note that our present study does not address HS-independent virus-receptor interactions. It is likely that some, if not all alphaviruses have cellular receptors in addition to or instead of HS (7, 8, 28).

Newly isolated or unpassaged strains of SIN, RRV and VEE viruses, all members of the alphavirus genus, do not bind to heparin and attach poorly to cells in culture relative to laboratory-adapted strains (2, 21, 28). Passage of non-HS-adapted SIN TR339 on BHK-21 cells resulted in virus mutants which bind with high affinity to BHK-21 cells and interact with HS (28). In vivo, these HS-adapted viruses typically exhibit an attenuated phenotype (8, 28). Similar results were obtained in passaging of RRV and VEE on cells. In the present paper, we demonstrate that SFV, derived from the infectious clone pSFV4 (31), efficiently interacts with cell-surface HS. It was found that this virus efficiently binds to BHK-21 cells. Evidence that the efficient binding of SFV to BHK-21 cells involves cell-surface HS, was obtained from heparin binding experiments. We observed that SFV interacts with immobilized heparin-agarose beads. Furthermore, it is shown that SFV binds to HepPE in liposomal membranes. These results suggest that SFV utilizes HS for infection of cells. Thus, adaptation to HS attachment receptors appears to represent a common cell culture-adaptive mechanism among the alphavirus genus.

There is extensive evidence that viruses from different families and genera have the capacity to interact with GAGs, in most cases with HS (2, 7, 10, 11, 12, 14, 26, 28, 30, 32, 33, 44, 56). In several instances, high-affinity binding to HS, as with alphaviruses, has been found to be a cell culture adaptation for a number of viruses, including flaviviruses, pestiviruses, picornaviruses, and retroviruses. In some cases, however, interaction with HS was not found to be a cell culture adaptation. For example, herpes simplex virus type 1 interacts with HS carrying a specific sulfation pattern, which serves as functional receptor or co-receptor for the virus (45). Since numerous viruses interact with HS, the liposomal model system presented here may serve as a novel tool to study basic virus-receptor interactions and membrane fusion properties of viruses from different families or genera.

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