Mutational analysis of receptor interaction and membrane fusion activity of Sindbis virus
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Chapter 6

The Role of N-Linked Glycosylation of Sindbis Virus Glycoproteins E2 and E1 in Viral Infectivity and Membrane Fusion Activity

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Abstract

The ectodomains of Sindbis virus (SIN) glycoproteins E2 and E1 are glycosylated at asparagine residues in a consensus sequence Asn-X-Ser/Thr. Here, we studied the biological role of the N-linked oligosaccharides at positions E2:196 and E1:139, using SIN mutants with an asparagine-to-glutamine substitution at either one of these positions. It was observed that the removal of a single glycosylation site dramatically affects the infectivity of the virus on baby hamster kidney cells (BHK-21). Moreover, deglycosylated SIN mutants were impaired in their ability to fuse with liposomes under mildly acidic conditions, indicating that the reduced infectivity of the virus mutants on BHK-21 cells lies at the level of the fusion process. We suggest that the membrane fusion process of deglycosylated SIN mutants is affected by subtle changes in the conformation of the viral spike proteins, influencing the stability of the E2/E1 heterodimer.
Introduction

Sindbis virus, the prototype member of the alphavirus genus, has a highly structured envelope composed of a host-cell derived lipid bilayer in which 80 hetero-oligomeric spikes are assembled (42). A single spike consists of a trimer of E2/E1 heterodimers. The E2 and E1 glycoproteins, which mediate the infectious entry of the virus into its host cell, are type I transmembrane proteins with the amino-terminus facing the external surface of the virus particle. The carboxy-terminal domain of E2 interacts with the viral nucleocapsid, which contains the positive-strand genomic RNA associated with 240 copies of the capsid protein.

The structural proteins of alphaviruses are translated as a single polypeptide in the order NH$_2$-capsid-PE2-6K-E1-COOH from the 26S subgenomic RNA (42). Upon folding of the capsid protein, it cleaves itself off in an autoproteolytic fashion, and the remainder of the polypeptide is translocated to the rough endoplasmatic reticulum (ER). As soon as the nascent polypeptide enters the lumen of the ER, it is cleaved in three distinct polypeptides (PE2, 6K, and E1) by ER resident signal peptidases. The PE2 (PE2 is the precursor protein of E2) and E1 polypeptides start to fold immediately, disulfide bonds are formed and the proteins become glycosylated. The polypeptides contain oligosaccharide chains linked to the asparagine residues at positions E2:196, E2:318, E1:139, and E1:245 (40). It is believed that N-linked glycosylation, at this stage, increases the solubility of the folding intermediates and prevents the formation of protein aggregates (5, 8). In addition, molecular chaperones such as calnexin and calreticulin (16) bind to the oligosaccharide chains of PE2 and E1, and assist the folding of the proteins (31, 32). During the folding of the spike proteins in the ER, the PE2 and E1 subunits associate to form a PE2/ E1 heterodimer. Then, the PE2/ E1 heterodimer continues its folding and maturation while passing through the Golgi-apparatus. If the folding of the protein renders the oligosaccharide chains accessible to cellular processing enzymes, the mannosidic side chains are further modified to a complex-type oligosaccharide (48). Complex N-linked glycans were observed at position E2:318, and E1:245, an mannosidic side chain at position E2:196, and a mixture of both types at position E1:139. However, the modification of oligosaccharide chains to a complex-type was found to be dependent on the host cell (17, 18, 28).

Not only the spike proteins of SIN, but in fact many viral envelope proteins have been found to be glycosylated. These include the envelope glycoproteins of HIV-1, hepatitis C virus, dengue virus, influenza virus, and respiratory syncytial virus (14, 21, 24, 30, 50). A variety of functions have been ascribed to the N-glycans of viral glycoproteins, including the folding and transport of the proteins (5, 7, 12), the stability of the protein conformation (11, 14, 35), the activity or the immunological properties of the protein (9, 12, 36, 44), and host cell tropism (2, 3). For example, glycosylation of the asparagine residues in the stem region of
influenza hemagglutinin (HA) has been found to stabilize the metastable form of HA required for fusion activity (35). On the other hand, the N-linked oligosaccharide chains attached to the HA ectodomain in close proximity to the receptor-binding site appear to control the receptor binding specificity and affinity (13, 15, 36). Furthermore, glycosylation of HA at antigenic epitopes has been shown to interfere with the access of antibodies (33, 44), and may therefore contribute to the antigenic drift of influenza virus (43).

In contrast, little is known about the function of N-linked glycosylation of the spike proteins of alphaviruses. Moreover, the oligosaccharide chains show large variation in location, structure, and number among the alphavirus genus. Earlier studies showed that removal of a single glycosylation site in the ectodomain of SIN glycoproteins E2 or E1, resulted in virus mutants with reduced growth properties on baby hamster kidney cells (BHK-21), when compared to wild-type virus (38). A further decrease of the growth rate was observed with SIN mutants in which more than one glycosylation site were eliminated.

In this study, we examined the role of the oligosaccharide chains, linked to asparagine residues E2:196 and E1:139 of the spike proteins of SIN, in the infectious cell entry and membrane fusion activity of the virus. To this end, we used the infectious SIN clone TE12, and two single deglycosylated mutants, in which the asparagine residues at position E2:196 or at position E1:139 were substituted by glutamines. It is demonstrated that the removal of either one of these glycosylation sites dramatically affects the infectivity of the virus on BHK-21 cells. Furthermore, the fusogenic properties of the mutant viruses were evaluated in a liposomal model system, as described previously (45, 46). There was no detectable fusion of the single deglycosylated SIN mutants in a pH range from 4.5 to 7.4, indicating that the reduced infectivity of these viruses lies at the level of the fusion process.

**Materials and Methods**

**Viruses.** All viruses were generated from cDNA clones. The construction of SIN clone TE12, and the single deglycosylated SIN virus clones E2:196 and E1:139 has been described previously (27, 38).

The viruses were produced by high-efficiency electroporation of BHK-21 cells with in vitro transcripts of linearized cDNA clones, as described before (26). Viruses released from the cells at 20 h post-transfection were harvested, and these stocks were used directly for the production of pyrene-labeled SIN particles, as previously described (6, 45, 46). Labeling of SIN with the pyrene-probe does not affect the infectivity of the virus (45, 46). The viruses were characterized by plaque assay on BHK-21 cells (22), phosphate analysis (4), and protein determination (37). The purity of the viruses was confirmed by SDS-PAGE. The pyrene concentration of the labeled virus preparations was determined by measuring the emission spectrum of the pyrene probe in an AB2 fluorometer (SLM/ Aminco, Urbana, IL) at an excitation wavelength of 345 nm.
Liposomes. Large unilamellar vesicles (LUV) were prepared by a freeze/thaw-extrusion procedure, as previously described (6, 45). Liposomes were prepared with an average size of 200 nm. Liposomes consisted of phosphatidylcholine (PC) from egg yolk, phosphatidylethanolamine (PE) prepared by transphosphatidylation of egg PC, sphingomyelin (SPM) from egg yolk, and cholesterol (Chol) in a molar ratio of 1:1:1:1.5. The lipids were obtained from Avanti Polar Lipids (Alabaster, AL). The phospholipid concentration of liposome preparations was determined by phosphate analysis (4).

Fusion assay. Fusion of pyrene-labeled SIN with liposomes was monitored continuously in an AB2 fluorometer, at excitation and emission wavelengths of resp. 345 and 480 nm (6, 45, 49). Briefly, pyrene-labeled SIN (1.0 µM viral phospholipid) and liposomes (100 µM phospholipid) were mixed in a magnetically stirred and thermostatted (37 °C) quartz cuvette of the fluorometer in a final volume of 0.665 ml in HNE. At t= 0 sec, fusion was initiated through the addition of 35 µl 0.1 M MES, 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 excimer fluorescence value and 100% fusion to infinite dilution of the probe (6, 45, 49). 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.

Results

Characterization of pyrene-labeled deglycosylated SIN viruses. To directly evaluate the potential role of spike protein glycosylation in the infectivity and cell entry of SIN, virus mutants derived from the clone TE12 were used in which the asparagine residue at E2:196 or E1:139 had been substituted for a glutamine (Table 1). Pyrene-labeled stocks of the parental virus and both mutants were generated to allow fusion measurements, as described below. However, first, the purified pyrene-labeled virus preparations were characterized by plaque assay on BHK-21 cells. It was observed that the plaque titer of the single deglycosylated SIN viruses was at least 3 logs reduced, compared to the parental TE12 virus.

<table>
<thead>
<tr>
<th>Virus</th>
<th>E2:196</th>
<th>E1:139</th>
</tr>
</thead>
<tbody>
<tr>
<td>TE12</td>
<td>Asn</td>
<td>Asn</td>
</tr>
<tr>
<td>E2:196</td>
<td>Gln</td>
<td>Asn</td>
</tr>
<tr>
<td>E1:139</td>
<td>Asn</td>
<td>Gln</td>
</tr>
</tbody>
</table>
(Table 2). On the other hand, analysis of the protein and phosphate contents of the virus preparations demonstrated that the total amounts of virus produced were similar for the parental TE12 virus, and the E2:196 and E1:139 mutants (data not shown). Calculation of the number of virus particles showed that about $1.0 \times 10^{12}$ particles per ml were present in all three purified virus preparations (data not shown). For the calculation, a theoretical amount of $5.45 \times 10^{-17}$ g of protein or $4.6 \times 10^{-20}$ mol of phosphate per viral particle was used (see also corresponding numbers for SFV; 23). Determination of the PFU-to-particle ratio revealed that almost all of the produced SIN TE12 particles were infectious on BHK-21 cells, the PFU-to-particle ratio being less than 1:10 under the conditions of the experiment (Table 2). By contrast, the PFU-to-particle ratios of both single deglycosylated SIN mutants were found to be greater than 1 to 40,000 (Table 2). Thus, it appears that elimination of a glycosylation site in the ectodomain of SIN glycoprotein E2 or E1 dramatically affects the infectivity of these viruses. Next, we investigated whether the high PFU-to-particle ratio of the single deglycosylated SIN mutants was related to the membrane fusion activity of these viruses.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Viral infectivity on BHK-21 cells (PFU/ml)</th>
<th>PFU to particle ratio based on protein</th>
<th>PFU to particle ratio based on phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>TE12</td>
<td>$6 \times 10^{11}$</td>
<td>1:8</td>
<td>1:4</td>
</tr>
<tr>
<td>sM139</td>
<td>$1 \times 10^{8}$</td>
<td>1:82.000</td>
<td>1:41.000</td>
</tr>
<tr>
<td>sM196</td>
<td>$5 \times 10^{7}$</td>
<td>1:164.000</td>
<td>1:64.000</td>
</tr>
</tbody>
</table>

**TABLE 2. Characterization of deglycosylated SIN viruses**

**Low-pH-dependent fusion of pyrene-labeled deglycosylated SIN viruses with liposomes.** Fusion was evaluated in a liposomal model system on the basis of lipid mixing, using pyrene-labeled SIN, as described previously (45, 46). Pyrene-labeled SIN (1 µM phospholipid) and PC/PE/SPM/Chol liposomes (100 µM phospholipid) were mixed, with continuous stirring, and incubated for 1 min at 37 °C. Then, fusion was triggered by acidification of the medium to pH 5.0. Figure 1 shows the results. At pH 5.0, SIN TE12 fused rapidly and efficiently with liposomes (curve a). At 60 s after acidification, an extent of fusion of approximately 52% was observed. However, under the same conditions, there was no detectable fusion observed with SIN mutants E2:196 and E1:139 (curve b, c).
Membrane fusion of Sindbis virus glycosylation mutants

To investigate whether deglycosylation of SIN influences the detailed pH-dependent fusion properties, fusion was evaluated in a pH range from pH 4.5 to 7.4. Figure 2A presents the initial rate of fusion of SIN TE12 (circles), E2:196 (triangles), and E1:139 (squares) as function of the pH. At pH 5.0, 22% of the SIN TE12 virus particles fused with the liposomes within the first second after acidification. Higher or lower pH values than pH 5.0, resulted in a slower rate of fusion. The pH threshold of fusion was 6.5, similar to that of other SIN strains (45, 46). The initial rates of fusion for the single glycosylated SIN mutants were undetectably low, in the entire pH range from 4.5 to 7.4. Figure 2B shows the extent of fusion, determined 60 sec after acidification. Again, throughout the entire pH range no fusion was observed for the single deglycosylated SIN mutants E2:196 (triangles) and E1:139 (squares), whereas the parental SIN TE12 virus (circles) showed high extents of fusion with an optimum at pH 5.0.

Discussion

The present analysis of the SIN clone TE12 and two SIN glycosylation mutants revealed that oligosaccharide chains coupled to the E2 and E1 viral envelope proteins strongly facilitate the membrane fusion activity of the virus. It is demonstrated that the removal of a glycosylation site within the ectodomain of either E2 or E1 dramatically affects both infectivity and low-pH-dependent membrane fusion activity of the virus, indicating that the reduced infectivity of the
mutant viruses lies at the level of the membrane fusion process occurring in acidic endosomes.

How can N-linked glycans influence the membrane fusion process of SIN? One possibility is that the oligosaccharide chains are directly involved in the fusion reaction. However, it seems unlikely that an oligosaccharide chain in E2 plays a direct role in the fusion process of SIN, since alphavirus fusion is mediated by the E1 glycoprotein (6, 20, 45, 49). Moreover, preliminary characterization of a double deglycosylated SIN mutant carrying a second-site resuscitating mutation in E2 revealed that membrane fusion is partially restored, despite a sustained lack of glycosylation (unpublished results). Another possibility is that the N-linked glycans are required for the proper folding of the viral glycoproteins. Elimination of a glycosylation site could induce a conformational change within the viral spike that may not be tolerated in the stringent lattice of the icosahedral SIN particle. However, detailed cryo-EM analysis demonstrated no detectable conformational differences between the parental SIN TE12 virus and the SIN glycosylation mutants, other than those directly associated with the lack of oligosaccharide chains (38). This indicates that deglycosylation of E2 or E1 does not result in major conformational alterations of the viral spike. Therefore, we suggest that the elimination of a single glycosylation site in either E2 of E1 induces an only subtle change in the conformation of the viral spike protein, as discussed in more detail below. This conformational change could affect the stability of the E2/E1 heterodimer, and therefore influence the structural rearrangements of the viral glycoproteins during the membrane fusion reaction.

Figure 2. Detailed pH-dependent fusion of pyrene-labeled deglycosylated SIN viruses with liposomes. Fusion of SIN TE12 (circles), E2:196 (triangles), and E1:139 (squares) virus with liposomes was determined at different pH values, as described in the legend to Figure 1. All fusion measurements were repeated at least two times. (A) The initial rates of fusion were determined from the tangents to the first part of the curve. (B) The extents of fusion were determined 60 s after acidification.
Inhibition of glycosylation by glucosidase inhibitors or mutagenesis of the glycosylation sites has been shown to cause misfolding and aggregation of several viral glycoproteins, resulting in the retention of these proteins within the ER (7, 29). Although, it has been observed that individual N-linked oligosaccharides differ in terms of their importance for folding of the glycoprotein in the ER, some N-glycans can be eliminated with little or no consequence while others appear to be essential (1, 19, 34, 35, 41). For SFV, the glucosidase inhibitor castanospermine blocked binding of p62 (corresponding to PE2 in SIN) and E1 to calnexin and calreticulin, and as a result, 80% of the folding intermediates ended up in protein aggregates and were retained within ER (32). Apparently, the removal of a single glycosylation site in E2 or E1 does not dramatically affect the folding of the protein. This may suggest that calnexin and/or calreticulin still assist the folding of the protein, albeit perhaps to lesser extents. On the other hand, the reduced binding capacity of the molecular chaperones might allow subtle changes in the folding intermediate of the protein, which could result in a slightly different conformation of the spike heterodimer.

A role for N-glycans in the stability of the viral glycoproteins has also been proposed for a number of other enveloped viruses, including influenza virus, dengue virus, HIV-1, and human respiratory syncytial virus (9, 10, 11, 14, 35, 50). For example, treatment of HIV-1 infected cells with N-butyldexnojirimycin (NB-DNJ), a α-glucosidase inhibitor, blocks syncytium formation and the formation of infectious virus (9). Although binding to CD4 occurs, the conformational shift and cleavage of gp120 that results in the exposure of gp41 does not (11). Thus, viral entry is blocked by the increased stability of gp120 in the viral envelope. Moreover, it has been observed that in the presence of NB-DNJ there is a regional misfolding in the V1/V2 loops of gp120 (10). On the other hand, deglycosylation of the stem region of influenza HA resulted in a higher pH threshold for fusion, indicating that the oligosaccharides preserve the metastable form of HA required for triggering fusion (35). Also, the removal of the glycosylation site in the dengue virus E protein resulted in virus mutants with a higher pH threshold for fusion (14). In a later study, it was shown that this oligosaccharide chain protects the fusion peptide of the virus, which suggests that the N-linked glycan stabilizes the E dimer contacts within the viral envelope (39). Although the overall fold of alphavirus E1 is similar to that of flavivirus E, the alphavirus E1 protein is not glycosylated at this position (25, 38, 39).

Recently, the three-dimensional localization of the oligosaccharide moieties on SIN have been revealed by cryo-EM image difference map analysis of a panel of deglycosylated SIN mutants (38). It was shown that the asparagine residue at position E2:196 lies at the external side of the spike trimer that forms the receptor binding motif of alphaviruses (47). The glycosylation site at E2:318 lies immediately downstream of the conserved Trp-Ile-Val region, a region that is assumed to interact with the fusion peptide of E1. Residues E1:139 and E1:245 lie
within the viral “skirt” around the base of each spike. A crystallographic dimer of the ectodomain of SFV E1 shows that the E1:139 residue lies within the central domain of the protein, whereas the E1:245 residue lies within the dimerization domain of the protein (25). The location of the glycosylation sites would suggest that the individual sites have different effects on the stability of the E2/E1 heterodimer. For example, one could argue that the oligosaccharide chain at E2:318 is involved in E2-E1 dimer interactions. Deglycosylation of the protein at this position could thus destabilize the E2/ E1 heterodimer, which might result in a higher pH threshold for fusion. On the other hand, the removal of the glycosylation sites in E1 could induce a subtle change in the protein conformation, such that it cannot rearrange to a fusion competent complex at mildly acidic pH.

We are currently investigating the influence of N-linked glycans on the stability of the E2/ E1 heterodimer.

References


