

Sindbis Virus Glycoprotein E1 Is Divided into Two Discrete Domains at Amino Acid 129 by Disulfide Bridge Connections

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Received 12 May 2000/Accepted 10 July 2000

The E1 membrane glycoprotein of Sindbis virus contains structural and functional domains, which are conformationally dependent on the presence of intramolecular disulfide bridges (B. A. Abell and D. T. Brown, *J. Virol.* 67:5496–5501, 1993; R. P. Anthony, A. M. Paredes, and D. T. Brown, *Virology* 190:330–336, 1992). We have examined the disulfide bonds in E1 and have determined that the E1 membrane glycoprotein contains two separate sets of interconnecting disulfide linkages, which divide the protein into two domains at amino acid 129. These separate sets of disulfides may stabilize and define the structural and functional regions of the E1 protein.

Sindbis virus, the prototype of the *Alphavirus* subgroup of the family *Togaviridae*, contains three structural proteins. Two of these proteins (E1 and E2) form an icosahedral shell in association with the virus membrane. The third protein (C or capsid) combines with virus RNA to form an icosahedral structure that is nested within the virus membrane. The E1 glycoprotein contains 12 cysteine residues in its ectodomain, and it is likely that these cysteines are involved in disulfide bonds (5, 15). Previous studies have shown that these disulfide bonds are essential for the structural and functional properties of the virion (1, 5). Short exposures to the reducing agent dithiothreitol (DTT) can drastically decrease infectivity while the basic structure of the virus remains intact. If DTT treatment is extended to 5 h or more, the virus starts to break apart (5). This suggests that the functional domains, essential for virus infectivity, are relatively exposed on the surface of the virion, since they can be readily inactivated with DTT. By contrast, the structural domains, responsible for maintaining the integrity of the virus structure, are much more resistant to DTT, and the disulfides in this domain may be protected from the environment by the spike structure.

To better ascertain the position of the structural and functional domains in the spike structure, we performed a number of experiments in which the E1 protein was cleaved with its disulfides intact and the cleavage products were identified. If the disulfide bonds within E1 separate the structural and functional domains, the E1 cleavage products will indicate this, since multiple cleavage products from a particular domain would be linked by disulfide bridges. Additionally, several possible disulfide bonds were determined in the E1 glycoprotein region from amino acids 1 to 129.

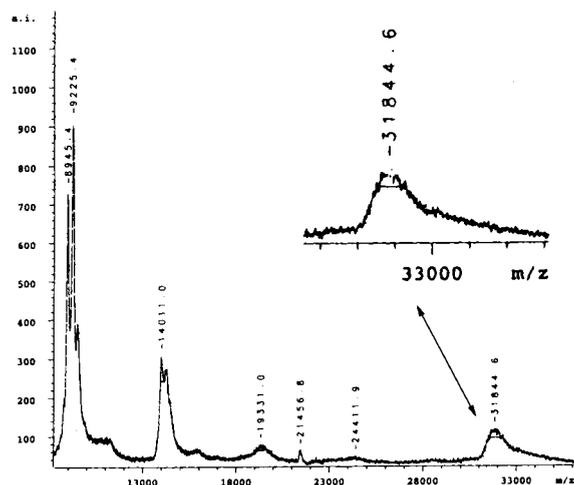
The E1 and E2 glycoproteins were separated from the capsid protein of intact virus as described previously (14), with the following modifications. Octylglucopyranoside (OGP) was substituted for Triton-X 100 detergent, and the bovine serum albumin carrier protein was excluded. The purity of E1 and E2 was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The E1 and E2 glycoprotein fraction was stored at -80°C until use.

The purified E1 and E2 glycoproteins were cleaved with cyanogen bromide (CNBr) by diluting the purified E1 and E2 glycoproteins in 6 M guanidine-HCl in 0.1 N HCl. A small CNBr crystal was then added. The solution was covered with argon gas and allowed to react in the dark at room temperature. The CNBr cleavages occur at low pH, and thus the scrambling of disulfides should not occur (3). The CNBr fragments from the E1 and E2 glycoproteins were separated on an HP 1100 high-pressure liquid chromatography apparatus at pH 2.0. Several of these fractions were analyzed by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS), and the masses were recorded. Edmond sequencing was performed on one prominent fraction, which indicated the sequence M/KVGLRIVYG matching E1 130 to 139.

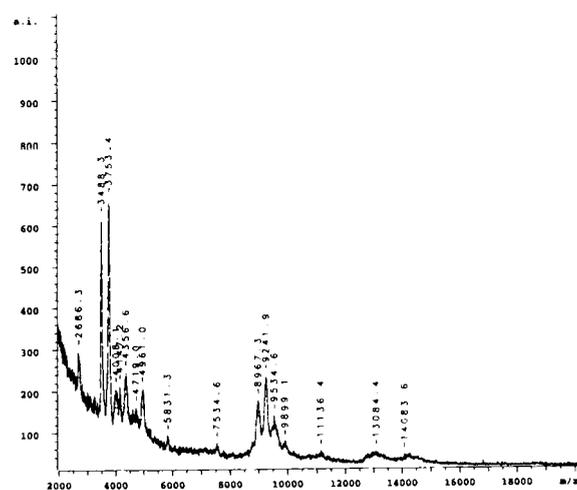
MALDI-TOF MS analysis was performed on fraction 19 as previously described (16). As seen in Fig. 1, the MALDI-TOF MS spectrum of fraction 19 contains an ion with a mass of 31,844.2 Da. This mass matches the E1 CNBr cleavage product from amino acids 130 to 426, which has a theoretical mass of 31,837.25 Da. Fraction 19 was then reduced with Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) by adding an equal volume of 20 mM TCEP in 0.2 M sodium citrate buffer (pH 3.0) and heating the mixture at 65°C for 45 min and was reanalyzed by MALDI-TOF MS. Upon reduction, the 31,844-Da mass disappeared, suggesting that it was composed of multiple CNBr fragments connected by disulfide bonds. The MALDI-TOF MS data, along with the partial Edman sequencing data, indicated that fraction 19 contains the E1 cleavage product from amino acids 130 to 426. If this were indeed this region of E1, it would indicate that this domain has become deglycosylated, since this mass does not take into account the carbohydrate binding sites at E1 positions 139 and 245 (17). No other mass combination can account for the mass seen in Fig. 1. The CNBr cleavage was done in 6 M guanidine-HCl in 0.1 N HCl for 18 h. Under these conditions, it is possible that some of the carbohydrates were hydrolyzed from the E1 protein by acid hydrolysis (10). It is possible that the vast majority of the E1 fragment from amino acids 130 to 426 in fraction 19 contained carbohydrates but was not seen by the mass spectrometer. The MALDI-TOF MS mass spectrometer is very sensitive, and it is possible that it was detecting only the deglycosylated E1 fragments. This may comprise only a small percentage of the total E1 fragments in that fraction. The partial sequence data ob-

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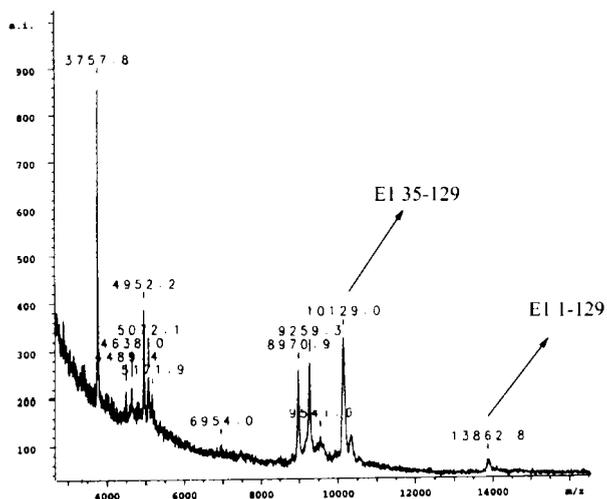
Fraction 19 Non-reduced



Fraction 19 Reduced with TCEP



E1 CnBr Cleavage Non-Reduced



E1 CnBr Cleavage Reduced

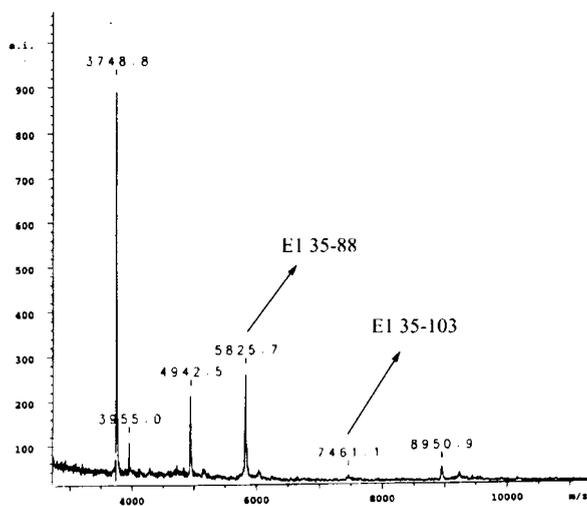


FIG. 2. Sindbis virus membrane glycoprotein E1 was purified from E2 and capsid protein (8) and cleaved with CNBr for 18 h in 6 M guanidine-HCl in 0.1 N HCl. The chemically cleaved peptides were desalted and analyzed on a Bruker Proflex MALDI-TOF MS instrument in both the reduced and nonreduced states. Protonated molecular ions ($M+H$) that are seen in the nonreduced fraction only include these of 10,129.0 Da (E1 35 to 129) and 13,862.8 Da (E1 1 to 129). Upon reduction, both of these ions disappear and ions of 5,825.7 Da (E1 35 to 88) and 7,461.1 Da (E1 35 to 103) appear, indicating that E1 1 to 129 is composed of at least two CNBr chemically cleaved domains disulfide bonded together.

E1 35 to 103 may have been disulfide bonded to cysteines in E1 104 to 129, although masses for E1 104 to 129 were not seen.

The peptides resulting from the E1 CNBr cleavage were further digested with trypsin at pH 6.0 by adding 1.2 μ l of Promega modified trypsin (0.52 mg/ml) per 50 μ l of the E1 and E2 CNBr cleavage. At this pH, disulfide bond scrambling should still be diminished. The masses found after trypsin subdigestion are shown in Table 1. The mass of 3,098.3 Da

matched E1 51 to 79, and the mass of 4,493.9 Da matched E1 62 to 103. E1 51 to 79 contains four cysteines, suggesting that the cysteines in the fragments are disulfide bonded to the other cysteines in this fragment. E1 62 to 103 contains these four cysteines plus two additional cysteines. E1 62 to 103 is most probably due to an incomplete digestion between E1 62 to 79 and E1 80 to 103 by trypsin, since E1 51 to 79 contains an even number of cysteines and since fragments containing only these four cysteines can be seen under both nonreduced and reduced conditions (see below).

The CNBr chemically cleaved peptides which were digested with trypsin were then reduced with TCEP and analyzed again by MALDI-TOF MS. Upon reduction, a mass of 1,900.1 Da appeared (Table 1), which matches E1 62 to 79, further supporting the contention that the cysteines between amino acids 62 to 79 are disulfide linked to each other. No other masses from the reduced sample could be correctly assigned to E1 cleavage or digestion products.

Figure 3 shows a diagram of the proposed disulfide-bonded regions of E1 1 to 129. Although no direct disulfide bonds were determined, E1 1 to 129 is divided into at least three disulfide bonded domains. At least one disulfide in E1 35 to 103 is disulfide bonded to the cysteine at position 114 in E1 104 to 129, since E1 1 to 129 disappeared upon reduction and E1 35 to 103 also appeared upon reduction. The four cysteines in the region from 62 to 79 are probably all disulfide bonded together, since trypsin-digested subfragments containing only these four cysteines could be found in both the reduced and nonreduced fragments. Cysteines 94 and 95 are probably not disulfide bonded together, since they are only one residue apart. Disulfide bonds between adjacent cysteines and cysteines one residue apart have been reported but are considered rare (6, 13). It is possible that both of them are free.

The data presented above suggest that the E1 membrane glycoprotein is divided into two domains at amino acid 129. The disulfides in E1 1 to 129 are included within this region and are separate from the disulfides in E1 130 to 426. The E1 region from 1 to 129 contains eight cysteines, and possible disulfide locations within this region were determined as shown in Fig. 3. This observation has implications for understanding the process by which alphaviruses infect cells and suggest that the influenza virus model for virus cell membrane fusion may not apply in this case. Influenza virus has hydrophobic "fusion" domains located at the NH_2 terminus of the hemagglutinin (7). These hydrophobic domains are exposed as the virion is exposed to the acidic pH of an endosome. Integration of these domains into the target cell membrane initiates the process of virus-cell membrane fusion. By contrast, the putative fusion domain of the alphaviruses (11, 12) is located 79 amino acids downstream of the amino terminus. The 79 amino acids upstream of the fusogenic domain are involved in disulfide bridges, creating a complex three-dimensional structure. This structure must be disassembled for the fusogenic domain to attack a target membrane. The disassembly of the disulfides requires disulfide reshuffling, a process that cannot occur at acidic pH. Thus, the disulfide-bonded conformation of the amino-terminal domain of E1 may explain the need to return virus cell complexes to neutral pH after exposure to acid to allow membrane fusion (9). The structure of the alphavirus membrane is much more complicated than that of the influenza virus membrane. The alphavirus membrane is an icosahedral shell whose structure is determined by protein-protein interactions (4). These interactions are also dependent on disulfide bonds, and these bonds may also have to be reduced before membrane fusion can occur. Even if a reductive event breaks the disulfide bridges in the amino-terminal region, it is

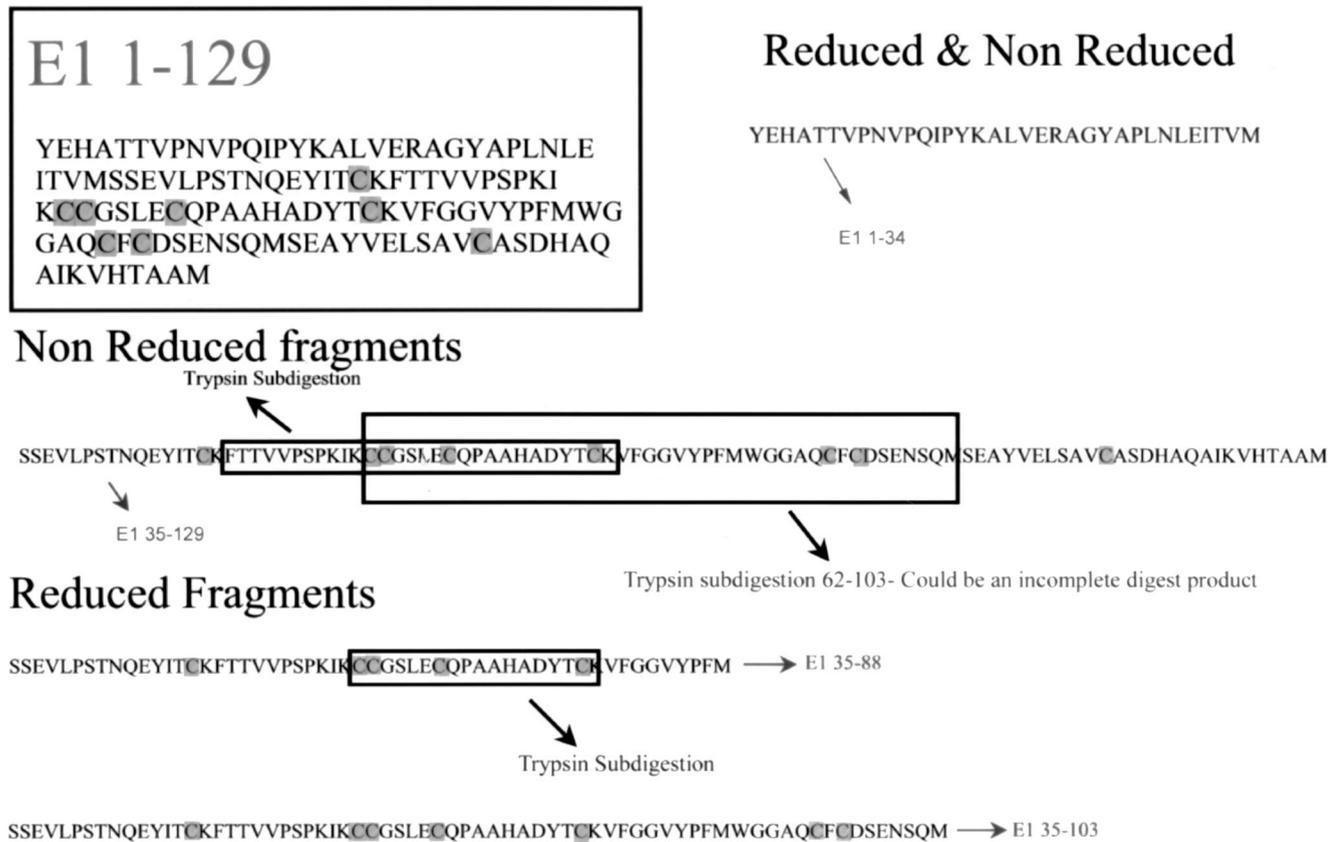


FIG. 3. Sindbis virus E1 domain 1 to 129 is divided into at least three disulfide-bonded domains. Although no direct disulfide bonds were determined, E1 1 to 129 can be cleaved chemically and digested into at least three peptides under conditions where the disulfides in this region remain intact.

difficult to envision how the putative fusogenic domain at E1 79 to 98 can engage the target membrane with a run of 79 nonhydrophobic amino acids between the fusogenic domain and the target membrane. It may be that the fusogenic domain attacks the target membrane by folding upon itself to produce a fusogenic protein hairpin. This hypothesis is unlikely, since folding of the fusogenic domain in half would render it too short to integrate into the membrane bilayer of the target cell (2). These observations suggest that alphaviruses may infect cells by a mechanism that is unique and very different from the influenza virus paradigm. This mechanism is under investigation in our laboratory.

This research was supported in part by grants from the Foundation for Research, Carson City, Nev., and the National Institutes of Health (grant AI42775 to D.T.B.).

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