JOURNAL OF VIROLOGY, July 1975, p. 141–145
Vol. 16, No. 1

Printed in U.S.A.

Sindbis Virus Glycoproteins Form a Regular Icosahedral Surface Lattice

C.-H. VON BONSDORFF*[1] AND S. C. HARRISON
Gibbs Laboratory, Harvard University, Cambridge, Massachusetts 02138

Received for publication 7 February 1975

Electron micrographs of negatively stained Sindbis virus particles show that the glycoproteins are organized with trimer clustering in a T = 4 icosahedral surface lattice.

Sindbis virus is structurally among the simplest of the lipid-containing animal viruses that mature by budding out through the plasma membrane of an infected cell (1, 4, 15). It has a spherical nucleocapsid, which assembles in the cytoplasm from subunits of a single protein species (C, mol wt 30,000) and viral RNA (21). During budding, this core acquires a membrane composed of a lipid bilayer coated on its outer surface with subunits of two distinct glycoprotein species (E_1 and E_2, both of mol wt ~50,000) (17). In addition, a much smaller glycoprotein, E_3, has been found in the closely-related Semliki Forest virus (8). The lipids are derived from the host-cell plasma membrane (11). X-ray scattering and electron microscopy have shown that in the virus particle, the inner polar groups of the bilayer lie at a radius of about 210 Å, where they appear to contact the tips of the core subunits, and the outer polar groups lie at about 260 Å (10). The glycoproteins project about 80 Å outward, and studies of Semliki Forest virus indicate that both E_1 and E_2 also cast hydrophobic "tails" into the bilayer (22).

The surface organization of Sindbis glycoprotein has not hitherto been determined. Electron micrographs of negatively stained particles have generally shown a halo of fringe-like glycoprotein "spikes," though a number of images showing surface contrast have been published (3, 9, 20). We present here electron micrographs, demonstrating that Sindbis glycoprotein forms a regular icosahedral lattice on the particle surface. This result has important implications for the mechanism of budding.

MATERIALS AND METHODS

Virus growth. Secondary cultures of chicken embryo fibroblasts were used for the growth of Sindbis virus (inoculum kindly supplied by B. Burge, Massachusetts Institute of Technology, Cambridge, Mass.). The cells were grown at 37 C in glass roller bottles (1,200-cm^2 growth surface) in Eagle minimum essential medium supplemented with 2% tryptose phosphate broth, 1% calf serum, and 1% chicken serum. The cells were infected at a multiplicity of infection of 0.1 to 0.01 PFU/cell in 10 ml of minimal essential medium per bottle. After absorption for 1 h at 37 C, 80 ml of minimal essential medium supplemented with 2% tryptose phosphate broth and 1% calf serum was added to each bottle, and the virus was grown for 18 to 20 h at 37 C. Alternatively, minimal essential medium supplemented with 0.2% bovine serum albumin (fraction V, Armour Pharmaceutical Co., Chicago, Ill.) was used during virus growth.

Virus purification. After an initial centrifugation at 10,000 × *g* for 20 min at 4 C, the virus-containing supernatant fluid was concentrated either by polyethylene glycol (Matheson, Coleman and Bell, Norwood, Ohio) precipitation as described by Sefton and Keegstra (19) or by pelleting the virus by centrifugation for 2 h at 100,000 × *g* in the SW27 rotor of a Beckman Spinco model L-50. The virus was suspended in TNE buffer (0.05 M Tris, pH 7.4, 0.15 M NaCl, 0.001 M EDTA). Purification of the virus was achieved in a discontinuous sucrose gradient, which functions as a combined velocity and density gradient (13, 16). The gradient consisted of a 1.5-ml cushion of 50% (wt/wt) gradient was layered. Onto this first gradient, an 18-ml, 10 to 20% (wt/wt) gradients was layered. A sample of about 5 ml was centrifuged at 25,000 rpm for 5 h at 4 C in an SW27 rotor. The visible virus band was collected (2 to 2.5 ml), and the purity of the virus was checked by electron microscopy and polyacrylamide gel electrophoresis. The modifications in virus growth and purification used were not found to cause any differences in virus yields or structure.

Electron microscopy. Samples from the purified virus preparations were diluted in TNE buffer 10- to 20-fold to obtain a suitable concentration for electron microscopy. A drop of the virus was applied to a copper grid covered with carbon-reinforced Formvar. The drop was removed with filter paper and immediately replaced by the negative stain. The stain was allowed to settle for only a few seconds before draining with filter paper. A 1% aqueous solution of uranyl acetate or a 2% solution of neutral potassium phos-

[1] Permanent address: Department of Virology, University of Helsinki, Helsinki, Finland.

photungstate was used as a negative stain. The specimens were examined in a Philips 300 electron microscope, and micrographs were taken at original magnifications of 30,000 to 60,000×.

RESULTS

A field of Sindbis virus, negatively stained with potassium phosphotungstate, appears in Fig. 1. A number of particles show a lattice of stain-filled lines, intersecting at sixfold or fivefold nodes. Figure 2 shows selected images from this and similar fields, and Fig. 3 shows that uranyl acetate can also contrast the same surface features. Such images are much rarer in uranyl acetate, however, suggesting considerable variability in stain penetration.

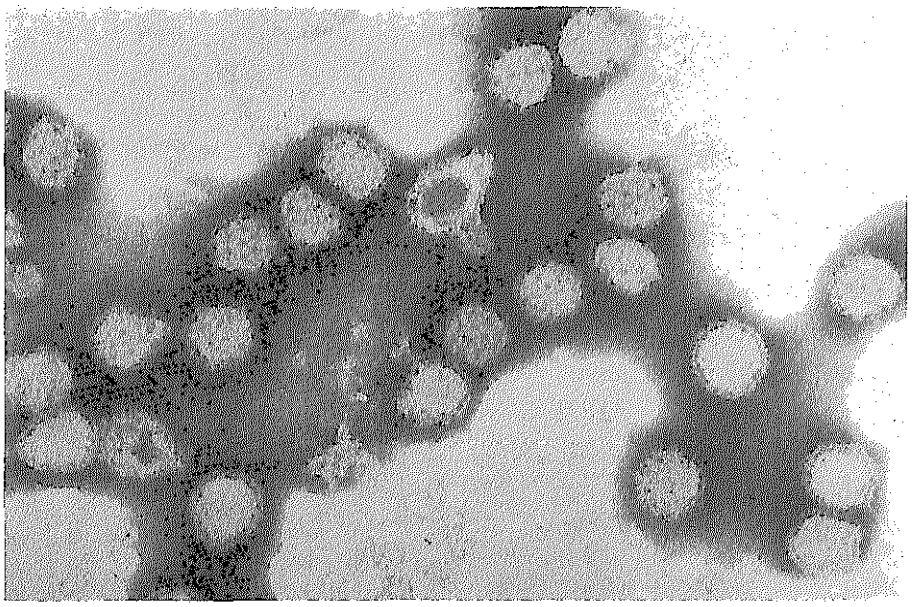


FIG. 1. *Field of Sindbis virus particles negatively stained with potassium phosphotungstate. Several particles show a triangular network of surface features. The glycoprotein layer of particles showing this surface contrast appears to be more deeply penetrated by stain, since the peripheral halo is less pronounced than in particles not showing surface features. Magnification 140,000×.*

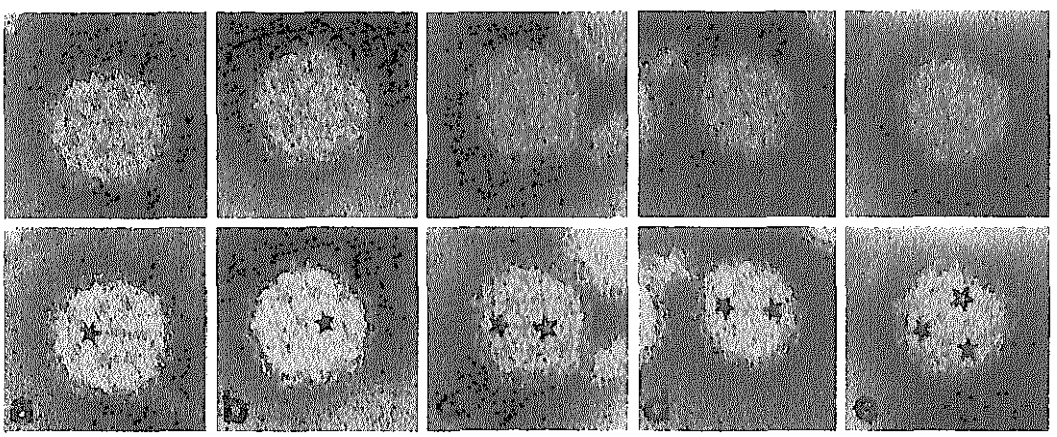


FIG. 2. *Selected KPT-stained particles. Each particle is shown twice, with fivefold nodes marked on the lower image. Filled stars indicate confident assignments, open stars, less certain identification. Note that images (c), (d), and (e) show two or more fivefold positions, in a relationship corresponding to T = 4. The constrasted lattice extends almost to the edge of the particle, where it appears to cut into the fringe (b and c). The clarity of contrast of the lattice indicates that these images are largely one-sided. Magnification 250,000×.*

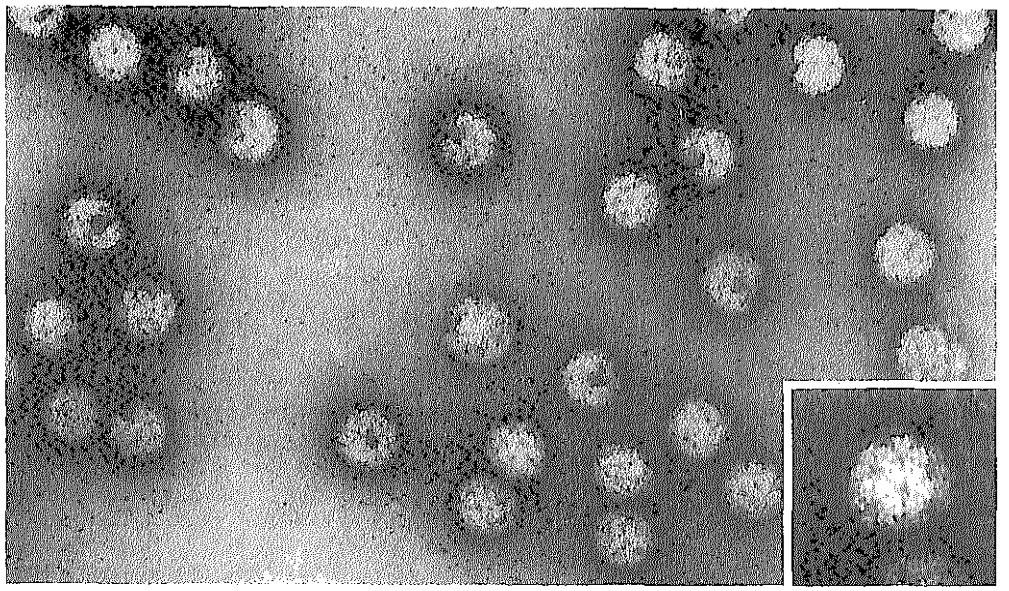
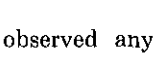


FIG. 3. *Field of Sindbis virus particles negatively stained with uranyl acetate. The nodes of the contrasted lattice are more pronounced than the interconnecting lines. The particles appear less deformed than in potassium phosphotungstate, and the fringe is less pronounced. The inset shows a particle with a particularly well contrasted surface network. Magnification 140,000×; inset 250,000×.*

The radius of the potassium phosphotungstate-stained particles that appear least flattened is 350 Å, with the peripheral "fringe" extending outward from about 260 Å. These dimensions agree well with the radial density profile determined by small-angle X-ray scattering: the outer polar groups of the lipid bilayer lie at about 260 Å. Uranyl acetate-stained particles have a less prominent halo and a correspondingly smaller apparent outer radius.

In a number of images, the lattice is coherent across the entire particle. This regularity suggests that the contrast comes not from a fortuitous local clustering of glycoprotein subunits, but from the organization of these molecules into a regular surface lattice. The presence of fivefold as well as sixfold nodes indicates icosahedral symmetry, and our analysis of the images proceeds on this assumption. The distribution of fivefold nodes can be used to determine the triangulation number (12). Chemical analysis of the closely related Semliki Forest virus is most consistent with T = 4 and certainly limits consideration to T = 3, T = 4, and T = 7 (Laine et al. [14] estimate 290 subunits each of E_1 and E_2). Unfortunately, it is difficult to discern more than one unambiguous fivefold position on any one particle, since contrast near the perimeter is reduced by the edge-on view of the spike-like surface protein. Superposition contrast from the upper and lower surfaces may also complicate many images. Those images that do appear to contain two 5's all correspond to T = 4 (Fig. 2). We have certainly not observed any clear cases of  , the distribution characteristic of T = 3. Moreover, the presence in a number of images of three sixfold positions at the corners of a lattice triangle is inconsistent with T = 3. We can also rule out T = 7 by observing that the distance between sixfold nodes is about 160 Å (Fig. 2). In a T = 7 lattice, these nodes would then correspond to stain-penetrated features at a radius of 350 Å. This is just at the particle boundary in potassium phosphotungstate and outside the apparent boundary in uranyl acetate. It seems unreasonable that such clearly defined features could arise from contrast only at the very outer tips of the glycoprotein. In images where the lattice extends toward the edge of the particle, it appears to be "cut" into the fringe, and the expected mean radius of contrast should be between 260 and 300 Å. In a T = 4 lattice, the only possibility consistent with all the images, the inter-sixfold distance implies a radius of 260 Å for the contrasted features. (The angle subtended at the center of the particle by two neighboring sixfold positions in a T = 7 surface lattice was calculated from the average of the inter-sixfold distances obtained from the three-

dimensional reconstruction of human wart virus [5]. The ratio of this average distance to the mean radius of contrast of the surface features in human wart virus is about 0.45. In T = 4, this ratio is 0.62. It may be determined directly from the angle between two icosahedral twofold axes, since local sixfold positions are strict diads in a T = 4 lattice [6].) Since negative stain penetrates along lines joining the sixfold and fivefold nodes, we conclude that the outer portions of the glycoprotein subunits form trimer clusters in this T = 4 icosahedral surface lattice.

Similar but less distinct images of this kind have been obtained before from Sindbis and Semliki Forest viruses (3, 10), and an interpretation in terms of a T = 4 surface lattice was suggested (10). It appears that the events during negative staining are critical; too little penetration between surface projections produces no contrast, except for the characteristic halo of glycoprotein viewed edge-on, and too extensive penetration leads to particle disruption (see Fig. 1). We have observed these images on a number of grids from several preparations of Sindbis virus. They are much less frequent in fields of uranyl acetate-stained particles, although the general degree of specimen preservation seems better than with potassium phosphotungstate.

DISCUSSION

These observations represent the first clear demonstration of a regular surface lattice on a lipid-containing virus. They have important implications for the nature of the contacts between protein subunits in the Sindbis particle and for their role in the budding process. A T = 4 structure for Sindbis core has not yet been demonstrated directly, but the regularity of its structure argues strongly for an icosahedral design. Chemical analysis of Semliki Forest virus yields 240 ± 10 protein subunits per core, and there is good evidence in both Sindbis and Semliki Forest virus for an equimolar ratio of E_1, E_2, and C (6, 16). A T = 4 structure for the core and regular match of surface lattices on the inside and outside of the membrane are therefore implied. Note that the chemical data mean that each of the 240 structure units of the surface lattice comprises one chain each of E_1 and E_2 (and of E_3, if present). Uterman and Simons (22) have shown that in Semliki Forest virus both E_1 and E_2 have hydrophobic tails that penetrate the lipid bilayer, and Garoff and Simons (7) have recently used cross-linking and labeling experiments to show that these tails extend completely across the bilayer. They propose that interactions between these tails and the core subunits determine the specificity of assembly at the cell surface. In particular they suggest that glycoproteins are substituted into the outer surface of the cell membrane, where they can diffuse laterally (compare reference 2). Cores, apposed to the inner surface, recruit new viral glycoprotein by the formation of specific molecular contacts: one E_1 and one E_2 tail per core subunit. The regularity of the structure we observe lends support to this model. A similar organization of inside and outside protein is necessary if regular interactions between them occur, e.g., if core protein has specific binding site(s) for some sequence in the glycoprotein tail(s). Moreover, the trimer clustering of glycoprotein subunits indicates significant noncovalent lateral bonding between them. The structure thus suggests that budding can be described as a straightforward self-assembly process. The core, a spherical structure, wraps itself in a membrane by virtue of specific interactions with a viral glycoprotein-substituted bilayer. The interactions probably involve contacts with both the glycoprotein tails and the inner lipid polar groups. The formation of these contacts and of lateral glycoprotein-glycoprotein bonds can drive the extrusion of the Sindbis virus particle. The development of a curved, budding particle is the only way in which all possible contacts between a core and a glycoprotein-substituted bilayer can occur and the only way in which contacts in an outer, icosahedral surface lattice can be made. The spherical shape of the core and the regularity of the surface lattice imply that completion of all such contacts will end in release of the particle from the cell surface.

ACKNOWLEDGMENTS

Electron micrographs were taken in the laboratory of Daniel Branton, Biological Laboratories, Harvard University.

We acknowledge support from the National Science Foundation Program in Human Cell Biology, grant GB-43573X. S. C. H. is the recipient of Public Health Service Research Career Development award CA-70169 from the National Cancer Institute.

LITERATURE CITED

1. Acheson, N. H., and I. Tamm. 1967. Replication of Semliki Forest virus: an electron microscopic study. Virology 32:128–143.
2. Birdwell, C. R., and J. H. Strauss. 1974. Replication of Sindbis virus. IV. Electron microscope study of the insertion of viral glycoproteins into the surface of infected chick cells. J. Virol. 14:366–374.
3. von Bonsdorff, C.-H. 1973. The structure of Semliki Forest virus. Commentat. Biol. Soc. Sci. Fenn. 74:1–53.
4. Brown, D. T., M. R. F. Waite, and E. R. Pfefferkorn.

1972. Morphology and morphogenesis of Sindbis virus as seen with freeze-etching techniques. J. Virol. **10**:524–536.

5. Crowther, R. A., and L. A. Amos. 1971. Three-dimensional image reconstructions of some small spherical viruses. Cold Spring Harbor Symp. Quant. Biol. **36**:489–494.
6. Caspar, D. L. D., and A. Klug. 1962. Physical principles in the construction of regular viruses. Cold Spring Harbor Symp. Quant. Biol. **27**:1–24.
7. Garoff, H., and K. Simons. 1974. Location of the spike glycoproteins in the Semliki Forest virus membrane. Proc. Natl. Acad. Sci. U.S.A. **71**:3988–3992.
8. Garoff, H., K. Simons, and O. Renkonen. 1974. Isolation and characterization of the membrane polypeptides of Semliki Forest virus. Virology **61**:493–504.
9. Harrison, S. C., A. Jack, D. Goodenough, and B. M. Sefton. 1974. Structural studies of spherical viruses. J. Supramol. Struct. **2**:486–495.
10. Harrison, S. C., A. David, J. Jumblatt, and J. E. Darnell. 1971. Lipid and protein organization in Sindbis virus. J. Mol. Biol. **60**:523–528.
11. Hirschberg, C. B., and P. W. Robbins. 1974. The glycolipids and phospholipids of Sindbis virus and their relation to the lipids of the host cell plasma membrane. Virology **61**:602–608.
12. Klug, A. 1965. Structure of viruses of the papilloma-polyoma type. II. Comments on other work. J. Mol. Biol. **11**:424–431.
13. Kääriäinen, L., and H. Söderlund. 1971. Properties of Semliki Forest virus nucleocapsid. I. Sensitivity to pancreatic ribonuclease. Virology **43**:291–299.
14. Laine, R., H. Söderlund, and O. Renkonen. 1973. The chemical composition of Semliki Forest virus. Intervirology **1**:110–118.
15. Lenard, J., and R. W. Compans. 1974. The membrane structure of lipid-containing viruses. Biochim. Biophys. Acta **344**:51–94.
16. Scheele, C. M., and E. R. Pfefferkorn. 1969. Kinetics of incorporation of structural proteins into Sindbis virions. J. Virol. **3**:369–375.
17. Schlesinger, M. J., S. Schlesinger, and B. W. Burge. 1972. Identification of a second glycoprotein in Sindbis virus. Virology **47**:539–541.
18. Schlesinger, S., and M. J. Schlesinger. 1972. Formation of Sindbis virus proteins: identification of a precursor for one of the envelope proteins. J. Virol. **10**:925–932.
19. Sefton, B. M., and K. Keegstra. 1974. Glycoproteins of Sindbis virus: preliminary characterization of the oligosaccharides. J. Virol. **14**:522–530.
20. Simpson, R. W., and R. E. Hauser. 1968. Basic structure of group A arbovirus strains Middelburg, Sindbis and Semliki Forest examined by negative staining. Virology **34**:358–361.
21. Strauss, J. H., Jr., B. W. Burge, and J. E. Darnell. 1969. Sindbis virus infection of chick and hamster cells: synthesis of virus-specific proteins. Virology **37**:367–376.
22. Uterman, G., and K. Simons. 1974. Studies on the amphipatic nature of the membrane proteins in Semliki Forest virus. J. Mol. Biol. **85**:569–587.