Hexagonal Glycoprotein Arrays from Sindbis Virus Membranes

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Freeze-etch electron microscopy of Sindbis virus and of glycoprotein arrays derived from Sindbis membranes by nonionic detergent treatment shows that the local geometry of glycoprotein-glycoprotein interaction does not depend on the presence of the nucleocapsid.

Sindbis virus particles are composed of two species of glycoprotein (E_1 and E_2, both of molecular weights of approximately 50,000), a nonglycosylated core protein (C, molecular weight of approximately 30,000), a bilayer of lipid, and a molecule of RNA (8). The proteins are present in equimolar quantities, and from the observation that the glycoproteins form a T = 4 icosahedral lattice on the viral surface, it can be concluded that there are just 240 subunits of each (9). The glycoproteins are largely external to the lipid bilayer, but experiments on the closely related Semliki Forest virus show that both E_1 and E_2 have hydrophobic "tails" that penetrate the lipid bilayer and that can be crosslinked chemically to subunits of the core (K. Simons, H. Garoff, A. Helenius, and A. Ziemiecki, *In* B. Pullman, ed., *Frontiers of Physicochemical Biology,* in press). The crosslinking results suggest that transmembrane contacts are important for specificity in budding but leave open the question of bonding strength and specificity in lateral glycoprotein-glycoprotein association. We report here observations of hexagonal arrays of Sindbis glycoprotein, isomorphous in local packing to the viral surface lattice, demonstrating highly specific lateral interactions between glycoprotein units.

MATERIALS AND METHODS

Virus growth. Sindbis virus, HR strain (inoculum kindly supplied by B. Burge), was grown in roller bottle cultures of secondary chicken embryo fibroblasts as described earlier (9). Large batches of virus were grown at the Cell Culture Center, Massachusetts Institute of Technology, Cambridge, Mass. (with the kind assistance of D. Giard).

Virus purification. Small batches of virus (less than 15 mg) were concentrated by polyethylene glycol and purified by sucrose gradient centrifugation (7, 9). Larger batches, grown in 80 to 120 roller bottles (570-

cm² growth area) and up to 7,000 ml of growth medium were purified as follows. The virus-containing medium, clarified by centrifugation at 9,000 × *g* for 20 min, was banded on a 15 to 50% (wt/wt) sucrose gradient in the CF-32 continuous-flow rotor of a Beckman L5-50 ultracentrifuge. The rotor was loaded at 2,500 rpm with sucrose in Tris-buffered saline (TNE) (0.05 M Tris (pH 7.4), 0.10 M NaCl, 0.001 M EDTA), followed by loading of virus-containing medium at a flow rate of 2,500 ml/h, 30,000 rpm; the centrifugation was then continued for 90 min. The gradient was unloaded at 2,500 rpm, 1,600 ml/h, using 60% sucrose to force it out; 30 fractions of 15 ml were collected, and the virus-containing fractions were located by monitoring the optical density at 260 nm. The virus was recovered in 1 or 2 fractions, 38 to 40% sucrose. These fractions were diluted 10-fold with distilled water and stirred for 30 min on ice; the water-precipitated virus was pelleted by centrifugation at 8,000 × *g* for 10 min, resuspended in TNE, and purified finally on a discontinuous sucrose gradient, functioning as a combined velocity and density gradient (6).

Freeze-etching. Freeze-etching was performed by standard techniques (3, 5). The samples contained 0.25 to 0.5 mg of Sindbis virus in 50 µl of appropriate buffer, and 1-µl drops were frozen on 3-mm copper supports. Cryoprotective agents were not used. Etching temperatures of −100 to −105°C and etching times of up to 2 min were used before shadow casting with platinum/carbon. The replicas were cleaned with household bleach and rinsed in distilled water before mounting them on 400-mesh electron microscope grids.

Electron microscopy. For negative staining, we used 1% uranyl acetate or 2% potassium phosphotungstate, pH 7.2 (9). The negatively stained specimens, as well as the freeze-etch replicas, were examined in a Philips 301 electron microscope, and micrographs were taken either on glass plates or on film sheets.

Optical diffraction. Optical diffraction patterns were recorded on a folded-optical-bench diffractometer.

RESULTS

As shown previously (9), negative staining reveals a regular arrangement of Sindbis virus

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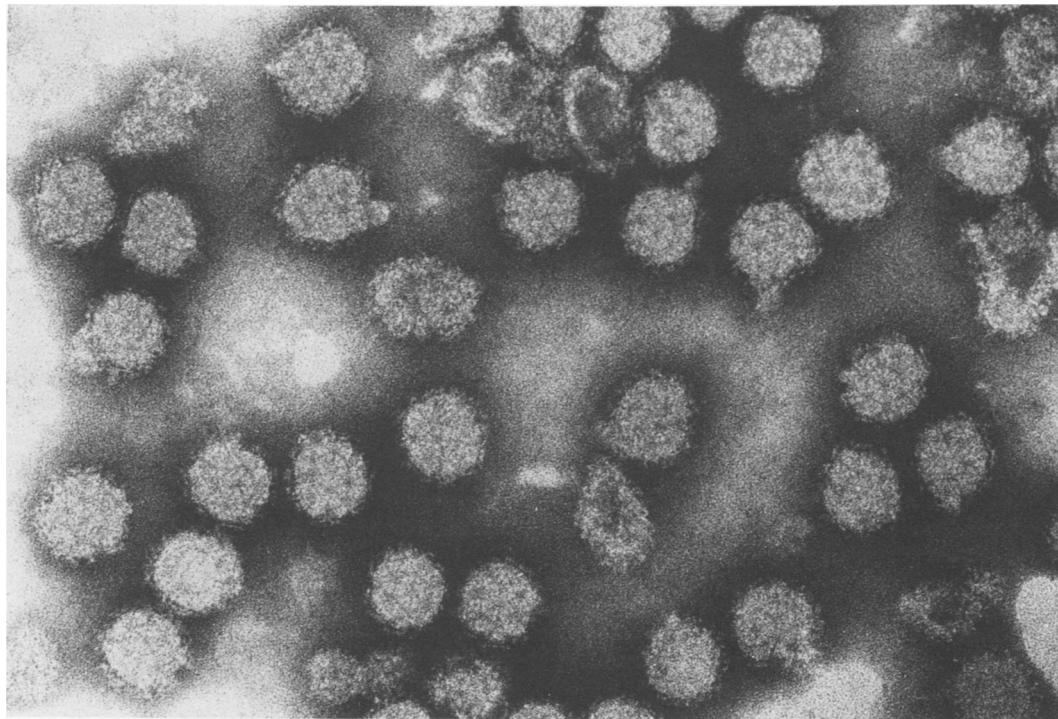
FIG. 1. *Sindbis virus, negatively stained with potassium phosphotungstate, pH 7.2. ×180,000.*

glycoprotein, described as trimer clustering in a $T = 4$ icosahedral surface lattice (Fig. 1). Visualization of the virus particle surface by freeze-etching yielded images consistent with this structure. Figure 2A shows an aggregate of particles that emerged from the ice during etching. The aggregation, commonly observed, is apparently due to concentration of virus in the eutectic phase during freezing (2). The pattern of lines and nodes on the particle surface is seen clearly over a relatively restricted area because of the geometry of shadow casting on a sphere. Nonetheless, in favorably oriented particles, the lattice may be analyzed by assuming a triangular network of grooves, meeting in five- and sixfold nodes. A fivefold node is particularly clearly seen in Fig. 2B, and Fig. 2C through F shows particles with two well-contrasted fivefold positions. In all cases, there is a sixfold node between two fivefolds, as required by a $T = 4$ surface lattice. Assuming regular bonding, each "triangle" contains three subunits each of glycoproteins E_1 and E_2, arranged with local threefold symmetry.

To determine the extent to which this regular arrangement of glycoprotein subunits depends on the structural integrity of the virus particle, we prepared viral membranes by treatment with nonionic detergent. Helenius and Söderlund (4) have shown that with Semliki Forest virus, 0.2

mg of Triton X-100 per mg of viral protein will release the nucleocapsid while leaving the membrane more or less intact. A negatively stained specimen of a concentrated Sindbis virus preparation, to which 0.2 mg of Triton X-100 per mg of virus has been added, is shown in Fig. 3A. Membranes appear as flattened sacs, with spike-like glycoproteins contrasted along their margins. The surfaces of these vesicles show no regular features in negative stain, but freeze-etched specimens of similar Triton-containing preparations reveal quite striking hexagonal arrays (Fig. 3B and 4). Except for the absence of fivefolds, the patterns of grooves and nodes in these arrays are identical, both in dimension and in appearance, to the pattern on the surface of intact Sindbis virus (Fig. 5). Optical diffraction from suitable micrographs shows a high degree of order: diffraction maxima extend to a resolution of about 3.5 nm. We interpret these structures as two-dimensionally crystalline arrays of Sindbis glycoprotein, hexagonal (p6) variants of the normal surface lattice. Caspar and Klug (1) have shown the close relationship between icosahedral surface lattices and p6 planar arrays.

These arrays, and the corresponding flattened vesicles seen in negative stain, were present only in specimens containing between 0.2 and 0.4 mg of Triton X-100 per mg of virus. Micrographs
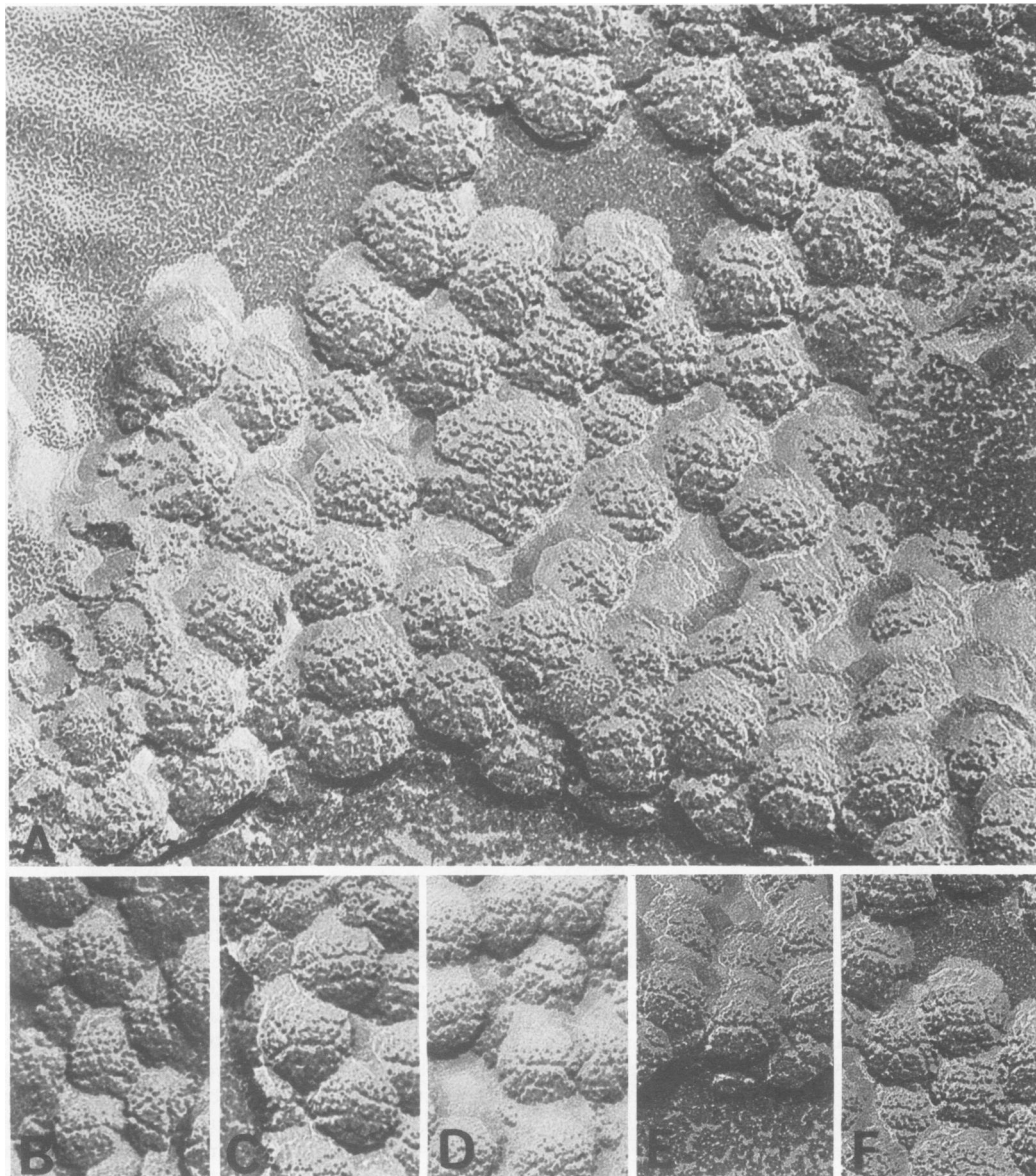
FIG. 2. *(A) Freeze-etched replica of Sindbis virus in TNE. The surface of uncleaved particles emerging from the ice shows a pattern of grooves resembling that seen in negative staining. The distance between parallel grooves is 15.0 to 17.0 nm. ×220,000. (B through F) Individual particles selected for their structure details. In the upper part of B is a particle in which five grooves radiate from one node. In C through F, the central particles all show two fivefold nodes with a sixfold node between them. In most cases, only three of the five radiating grooves can be seen, due to unidirectional shadowing, and the particles show a "double fork" pattern: ✕●●✕. ×180,000.*

from samples with higher concentrations of detergent (1 mg/mg of protein or greater) showed small aggregates and nucleocapsids.

DISCUSSION

The Triton-induced hexagonal arrays are identical in lattice dimensions and local organization to the viral surface. They are found only after detergent treatment under the stage II dissociation conditions of Helenius and Söderlund (4), when essentially all particles have been disrupted and cores may be separated from membranes. We have observed a number of

arrays containing many more triangular cells than the 80 that cover the surface of a single virus particle. The arrays must therefore be formed by fusion of membranes from several particles. The arrays are probably patches of glycoprotein in somewhat larger detergent-lipid bilayers or vesicles, a conclusion consistent with the size of many vesicles seen in negative stain (Fig. 4A). It is clear that spontaneous formation of such structures requires both a high degree of specificity in lateral contacts and sufficient mobility of glycoproteins in the lipid bilayer that fivefold vertexes anneal to sixfolds. If we think of the intact viral surface as a two-dimensionally crystalline array of glycoprotein, then incorporation of Triton into the lipid bilayer corresponds to addition of solvent. The result is that the surface crystal partially dissolves, membranes from different particles fuse, and glycoprotein recrystallizes with a hexagonal lattice. If more Triton is added (stage III or IV of Helenius and Söderlund [4]), the hexagonal array also dissolves, and small protein-lipid-detergent or protein-detergent micelles result.

Formation of extensive arrays of this sort on the surface of an infected cell might be expected to inhibit budding, since formation of fivefold vertexes would require removal of a large wedge of protein. We therefore consider it unlikely that such arrays represent an intermediate state in viral assembly. Rather, we take their spontaneous formation in vitro as an indication of precise and reasonably strong glycoprotein-glycoprotein bonding. These noncovalent contacts are presumably part of the driving force that favors budding.

FIG. 3. *Sindbis virus after treatment with 0.2 mg of Triton X-100 per mg of purified virus. (A) Negatively stained in uranyl acetate. Empty-looking membrane sacs are seen, some more than 500 nm in diameter. Glycoprotein spikes are seen at the margins of these vesicles, and released nucleocapsids are visible in the lower part of the picture. (B) Freeze-etched replica from the same sample. In two separate areas, regular arrays of lines (grooves) are discerned. One of them is clearly associated with the cleaved vesicle. In the center are seen structures interpreted as released nucleocapsids. ×180,000.*
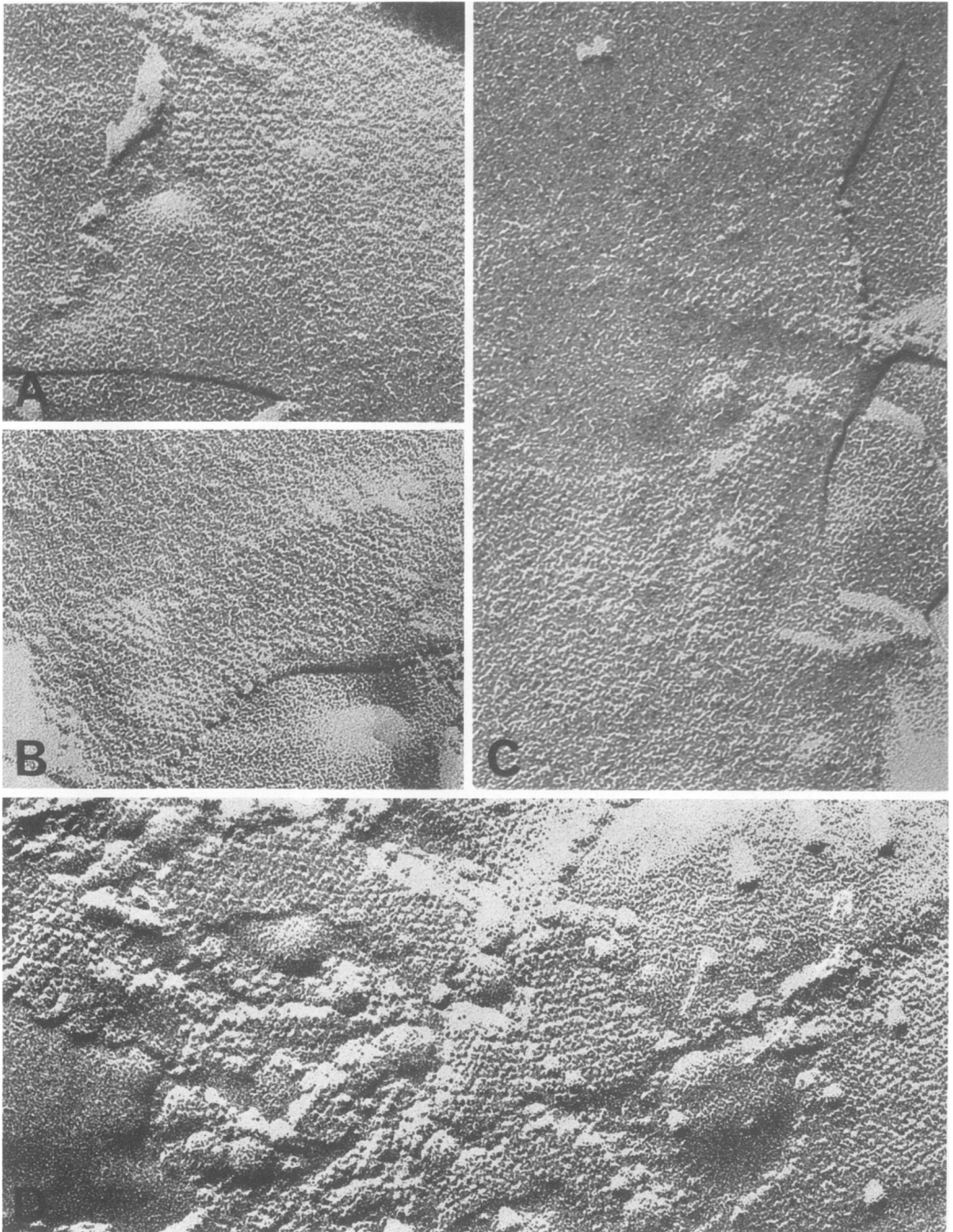
Fig. 4. *Selection of hexagonal arrays found in Sindbis virus specimens treated with Triton X-100. The line spacing is 15.0 to 17.0 nm; a weaker line can often be seen in the middle of the stronger features. In larger arrays (e.g., C), a slight bending of the line pattern frequently occurs, but fivefold nodes never appear. ×180,000.*
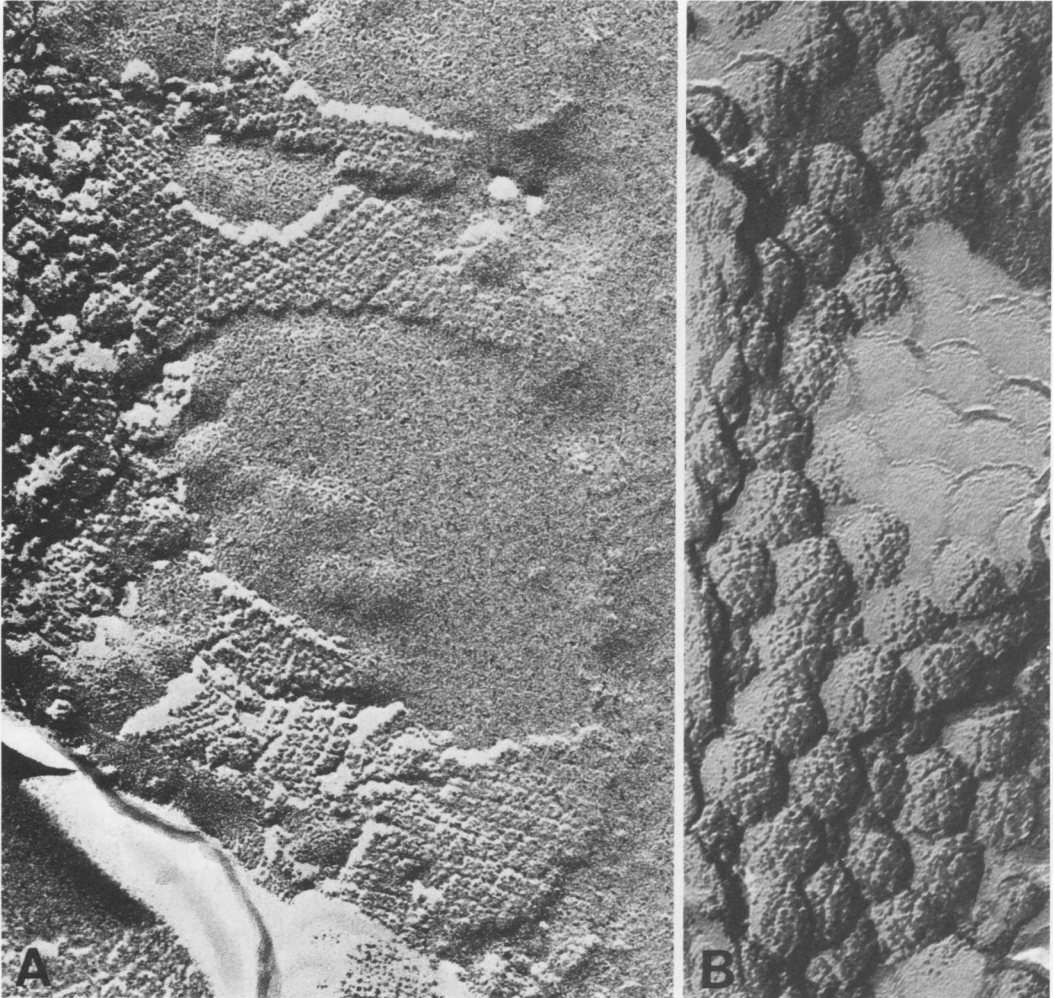
FIG. 5. *(A) Hexagonal arrays observed in a Sindbis specimen containing 0.4 mg of Triton X-100 per mg of virus. (B) For comparison, an etched virus sample, showing surface grooves with spacing and appearance similar to those of the glycoprotein array in A. ×180,000.*

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