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SEMLIKI FOREST VIRUS MEMBRANE PROTEINS. PREPARATION AND CHARACTERIZATION OF SPIKE COMPLEXES SOLUBLE IN DETERGENT-FREE MEDIUM

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Summary

After Triton X-100 delipidation and subsequent Triton X-100 removal in a sucrose gradient the membrane protein spikes of Semliki Forest virus remained soluble in aqueous buffers. It was shown they were present as octameric complexes with a molecular weight of $95 \cdot 10^4$ and that they contain less than 4% lipid and detergent by weight. In electron microscopy after negative staining they appeared as "rosette"-shaped particles. Part of the protein could also be found associated in ordered paracrystalline arrays.

The Semliki Forest virus (SF virus, a small animal virus of the toga virus group) consists of a nucleocapsid surrounded by a membrane. The membrane contains three polypeptides, E1, E2 and E3, with apparent molecular weights of $4.9 \cdot 10^4$, $5.2 \cdot 10^4$ and $1.0 \cdot 10^4$, respectively [1]. Spike structures, 7—8 nm long, are present on the membrane [2] and are necessary for infectivity. The molecular weight of a spike is $10 \pm 1 \cdot 10^5$, each spike probably consisting of one copy of each of the three glycopolypeptides [3]. Proteins E1 and E2 interact with the viral lipid bilayer membrane through hydrophobic peptide segments [4] and one or both span the bilayer [5]. The spikes can be isolated in lipid-free biologically active form using the nonionic detergent Triton X-100 (*p-t*-octylphenyl polyoxyethylene) [6]. The detergent binds to the hydrophobic part of the spikes, thereby keeping them in solution [4]. When the detergent is removed the spikes tend to precipitate because of their hydrophobic properties. This report describes a simple method to overcome the problem of precipitation and to obtain soluble spike proteins in high yields.

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The soluble spike protein was obtained from virus by a single preparative step using sucrose gradient centrifugation· radioactively labelled SF virus [7,8] was solubilized with Triton X-100 and the clear mixture was put onto a detergent-free sucrose gradient which had a small Triton X-100-containing zone on the top (for details see Fig. 1). The distribution of protein, phospholipid, RNA and Triton X-100 in the gradient after centrifugation was determined from the radioactivities [9]. The protein peak was pooled and the sucrose was removed by repeated concentration and addition of buffer (0.05 M Tris·HCl pH 7.4 containing 0.1 M NaCl), at 4°C in an Amicon ultrafiltration cell (XM 50 filter). The final concentrated protein solutions (2—4 mg/ml) were stored at 4 or −70°C.

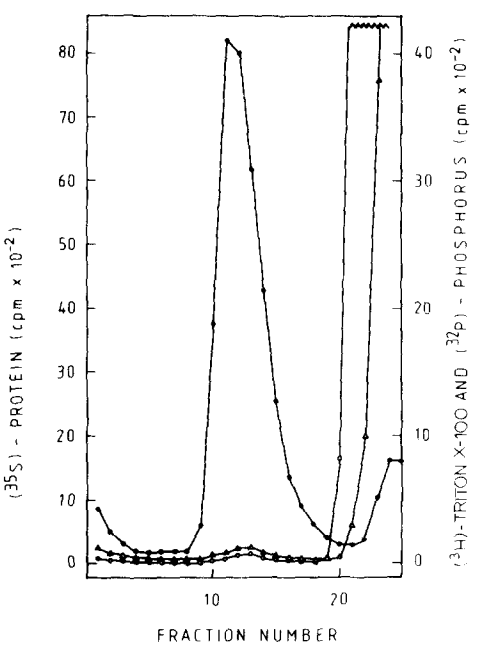

Fig.1. Preparation of soluble spike complexes by sucrose gradient centrifugation. The curves are compiled from two gradients which were identical except that in one [^{35}S]methionine-labelled virus and in the other ^{32}P-labelled virus and ^{3}H-ring-labelled Triton X-100 (a gift from Rohm and Haas, U.S.A.) were used. Sucrose gradients (13 ml, 20—50 % sucrose in 0.05 M Tris·HCl pH 7.4 containing 0.1 M NaCl) were overlaid with 0.3 ml of 1% Triton X-100 (w/v) in 15% sucrose. The samples (0.2 ml) layered on top contained a maximum of 2.5 mg SF virus solubilized in 2% Triton X-100 in 0.025 M Tris·HCl pH 7.4 containing 0.05 M NaCl. Gradients were centrifuged at 195 000 × *g* for 24 h at 20°C in an International B-60 ultracentrifuge using the titanium 488 swing-out rotor. Fractions (0.5 ml) were collected from below and aliquots measured for radioactivity. ● — ●, ^{35}S-labelled protein; △ — △, ^{32}P-labelled phospholipid; ○ — ○, ^{3}H-labelled Triton X-100. Sedimentation was to the left.

Polyacrylamide gel electrophoresis in sodium dodecyl sulphate showed that the protein preparation contained the three membrane glycopolypeptides E1, E2 and E3 (Fig. 2). The haemagglutinating activity of the spikes was intact [10]. The nucleocapsid protein and viral RNA were recovered in the pellet fraction of the sucrose gradient, and almost all the phospholipid and Triton X-100 were found in the top fractions (Fig. 1). Use of ^{3}H-labelled Triton X-100 and ^{32}P-labelled SF virus showed that the protein preparation

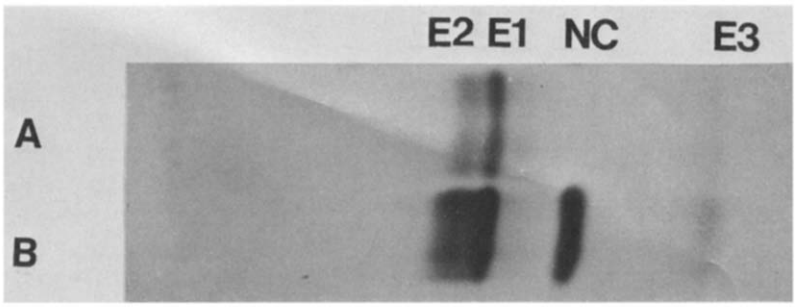

Fig.2. Polyacrylamide slab gel electrophoresis of [^{35}S]methionine-labelled spike octamers (A) and SF virus (B) in the presence of sodium dodecyl sulphate. The gels, the buffer system and the sample preparation were as described by Neville [20]. The bands were developed by the fluorographic film detection method of Bonner and Laskey [21]. NC, nucleocapsid protein. Anode to the right.

contained 0.03 mg residual Triton X-100 and 0.01 mg phospholipid per mg protein (two determinations). The actual phospholipid content may be somewhat lower since the SF virus proteins are known to be phosphorylated [11]. Gel filtration of the protein in a Sepharose 4B column (1.0 × 80 cm) equilibrated with 0.05 M Tris·HCl pH 7.4 containing 0.1 M NaCl yielded a Stoke's radius (a) of 8.0 nm. Thyroglobulin, glutamate dehydrogenase, urease and gammaglobulin were used as standards. Analytical ultracentrifugation in a Beckman Spinco Model E centrifuge equipped with photoelectric scanner at 20°C in the above buffer yielded a sedimentation coefficient ($S^0_{20,w}$) of 29.1 ± 0.2. The AnD rotor was run at 42 040 rev./min. No concentration dependence was detected in the concentration range (0.1—0.5 mg protein per ml) used. Both in gel filtration and ultracentrifugation the protein behaved as a homogeneous particle. The partial specific volume ($\bar{\nu}$ = 0.724) was calculated from the aminoacid, carbohydrate, Triton X-100 and phospholipid content [6,12]. A molecular weight (M_r) of 95 ± 3·10^4 was calculated from the equation:

$$M_r = \frac{6\pi\eta_{20,w}\, N\, a\, S^0_{20,w}}{1 - \bar{\nu}\,\rho_{20,w}}$$

(in which $\eta_{20,w}$ and $\rho_{20,w}$ are the viscosity and density of water at 20°C respectively, and N is Avogadro's number). This molecular weight indicated that the complexes were made up of eight spike units. Electron microscopy of negatively stained samples showed "rosette"-shaped particles with an average diameter of 19.0 ± 2.0 nm (Fig. 3).

Elongated tactoid arrays of spike protein (Fig. 4 A—C) were also frequently observed in negatively stained preparations. The repeat distance in the most ordered parts of the paracrystalline aggregate was 17.5 ± 1.0 nm. As seen in Fig. 4B, the repeat structure seems to arise feom parallely oriented spikes joined together as rows (or sheets). It is most likely that the well-defined, stain-resistant regions contain the hydrophobic ends of the spike proteins. The weaker stain-resistant bands (best seen in Fig. 4A) probably indicate the position of the hydrophilic ends.

In previous studies we have isolated octameric Triton X-100·spike

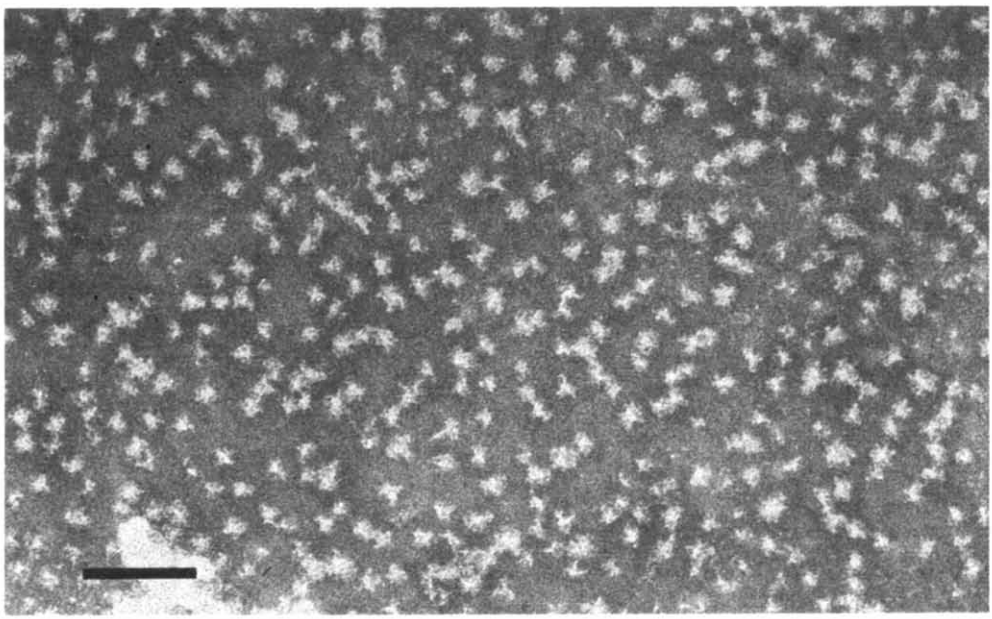

Fig.3. Negative staining of spike protein complexes. Staining with 2% neutral potassium phosphotungstate on carbon-reinforced Formwar grids as previously described [7]. A Siemens Elmiskop 1A with initial magnification of × 40 000 was used. The bar is 100 nm.

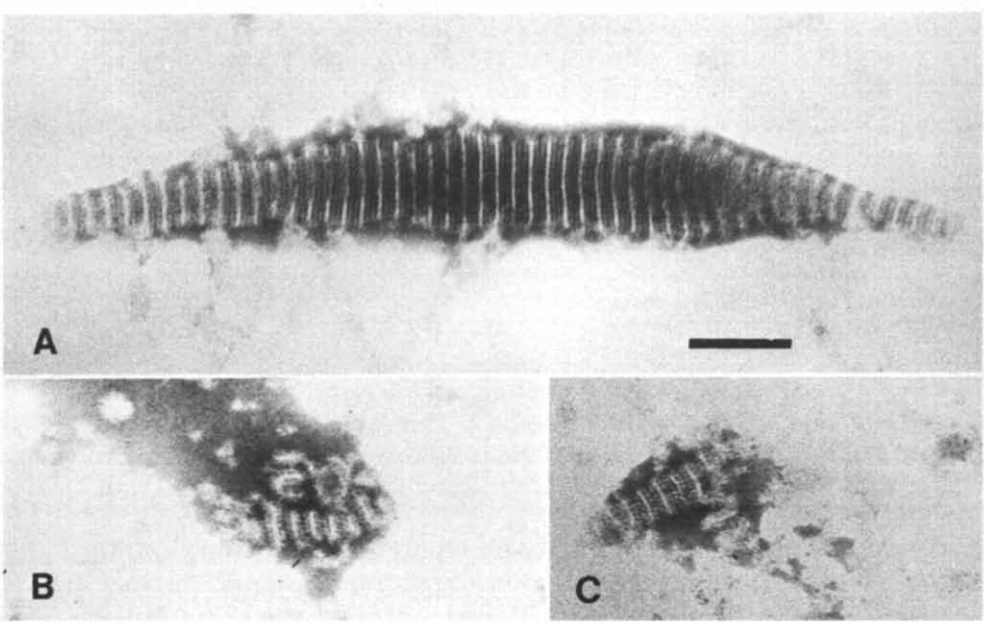

Fig.4. Spike tactoids observed in negatively stained specimen of spike protein. Staining as in Fig. 2. The bar is 100 nm.

protein complexes that are similar to those described here [6]. They are formed when Triton X-100 is used to solubilize the membrane in the absence of sucrose. The complexes contain 0.20 g Triton X-100 per mg protein and

the molecular weight is $1.1 \cdot 10^6$. Due to the bound Triton X-100 they have a larger Stoke's radius (9.4 nm) and a lower sedimentation coefficient (23.5 S^0_{20w}). These complexes depend on Triton X-100 for solubility; our attempts to remove the detergent from the isolated Triton X-100·protein complexes by gel filtration, ion-exchange chromatography, dialysis and sucrose gradient centrifugation have resulted in protein precipitation. The conditions for the formation of soluble complexes such as described here therefore appear to be critical.

Once formed the soluble spike complexes remain stable and they can be handled as ordinary water-soluble proteins without difficulty. They can be added to cells, membranes and liposomes without the detrimental effects of detergents. We have so far used them to obtain reconstituted membranes [13], to study the interaction of the proteins with sodium dodecyl sulfate [14] and work is in progress to evaluate their efficiency as subunit vaccines. The interesting tendency of the spikes to form ordered paracrystalline arrays may also allow some detailed structural studies.

Formation of water-soluble aggregates of integral membrane proteins after detergent solubilization and subsequent detergent removal has been reported previously for cytochrome b_5; cytochrome b_5 reductase, cytochrome *f* and the glycoproteins from influenza and parainfluenza virus [15—19]. These complexes have not yet been characterized in detail. It may be possible to obtain soluble complexes with other membrane proteins as well. The sucrose gradient centrifugation procedure described here has been recently used successfully in our laboratory to prepare soluble complexes of membrane penicillase from *Bacillus licheniformis* (Simons, K. and Sarvas, M., unpublished results).

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