

Identification of the pore forming element of Semliki Forest virus spikes

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Abstract Pore formation at mildly acidic pH by SFV spike proteins was investigated using isolated and modified virions. Modification of the virions was performed by limited proteolysis in presence of octylglucoside and resulted in the formation of E1 particles and spikeless particles, respectively. Pore formation was detected by measuring the influx of propidium iodide into the viral particles. The results obtained clearly showed that the presence of E1 alone is sufficient to promote pore formation at mildly acidic pH. Thus E1 represents the pore forming element of the viral spike proteins.

Key words: Semliki Forest virus; Pore formation; E1 protein

1. Introduction

One of the major events in the life cycle of Semliki Forest virus (SFV), the entry of the host cell, is known to be mediated by the viral spikes [1–3]. The composition of these spikes was determined to be a trimer of spike proteins, which in turn consist of two transmembrane glycoproteins, E₁ (MW 51,000) and E₂ (MW 52,000), and a peripheral glycoprotein E₃ (MW 11,000) being attached to E₂ [4]. Thus, each spike is made up of (E₁E₂E₃)₃.

Several studies have revealed the ability of SFV spikes to alter the permeability of membranes upon exposure to mildly acidic conditions. Such treatment induces an irreversible conformational change of the spike proteins E₁ and E₂ [5,6]. Exposure of purified SFV to mildly acidic conditions (pH 5.8) led to shrinking of the nucleocapsid, as revealed by sedimentation analysis and electron microscopy [7]. The shrinking of the nucleocapsid is an indication of exposure of the capsid to low pH-conditions [8]. This effect was shown to be dependent on the presence of the spikes, providing evidence for the ability of the spikes to enable ion fluxes across the viral membrane. Furthermore, experiments with SFV-infected insect cells suggested that the spikes triggered the formation of unspecific pores in the plasma membrane, allowing the passage of ions and small molecules up to 900 Da [9]. This was further supported by experiments using the whole-cell patch clamp technique [10]. Recent research revealed the influx of the fluorescence marker propidium iodide into SFV-infected insect cells as well as into SFV itself upon exposure to mildly acidic conditions. In case of the virions, this influx, too, was dependent of the presence of the spikes (Käsermann and Kempf, unpublished observation). These permeability changes proved to be sensitive to millimolar concentrations of Zn²⁺, a phenomenon known to be

characteristic for pore forming agents such as bacterial toxins and viruses [9,11].

The question remained open which of the envelope proteins was responsible for pore formation. One candidate was a small integral membrane protein, the 6k protein, of which a small amount is present in the membrane of the virus (approximately 3% compared to the spike proteins; [12]). However, several lines of evidence strongly suggested that this protein is not involved in the pore formation described above. One line of evidence is the dependency of permeability changes of the viral membrane in the presence of the ectodomain of the spikes as outlined above. Additionally, investigations of a deletion mutant of SFV lacking the 6k protein showed unaltered behaviour with respect to low pH induced pore formation in infected cells (Dick and Kempf, to be published elsewhere). Furthermore, results presented by Lanzrein and coworkers [13] suggested that the E₁ might be responsible for the low-pH induced pore formation. Increasing permeability of the membrane of infected cells by lowering the pH was found to be impeded strongly by preincubation with a monoclonal antibody raised against E₁, but not by antibodies against E₂.

2. Experimental

SFV was propagated on *Aedes albopictus* cells, clone C6/36 (Igarashi, 1978). These cells were grown in Mitsuhashi-Maramorosh (MM-Medium, Amimed) containing 16% heat-inactivated fetal calf serum (FCS, Sera-Lab), 100 µg streptomycin and 100 U penicillin/ml. The cells were passaged weekly by 1:20 dilution's. For virus preparation, *Aedes* cells were infected with SFV at a multiplicity of infection (MOI) of 10 plaque forming units (PFU) per cell. Six hours post infection (hpi) the supernatant was replaced by MM-medium that was diluted 1:10 with phosphate buffered saline (PBS) containing 0.2% bovine serum albumin (BSA, Fluka). After 24 h the supernatant was harvested and cellular debris were removed by centrifugation at 3,000 × g. Virus was then concentrated by centrifugation through a cushion of 12% (w/w) sucrose in PBS at 130,000 × g for 2 h and 40 min. The pellet was resuspended in morpholinoethanesulfonic acid-buffered saline (MBS; pH 7.3). Virus protein concentrations were determined according to the method of Lowry et al. [14] and the purity of the preparations was checked by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

To produce virions containing only the E₁-glycoprotein in their membranes (E₁-particles), the protocol described by Omar and Koblet [6] was used with minor modifications: briefly, purified SFV was incubated in MBS in the presence of trypsin (sequencing grade, 69 U/mg; Boehringer). The ratio of trypsin to viral protein was optimized and finally 1.3 U/mg protein were used. After incubation for 24 h at 37°C, 20 µg of soybean trypsin inhibitor (STI; type I-S, Sigma) per U trypsin were added. Spikeless particles were obtained by the same treatment of SFV as described above when 27 U trypsin/mg virus protein instead of 1.3 U/mg were used. To obtain mock-treated particles, SFV was subjected to the treatment used for preparation of spikeless particles. However, STI was added at the beginning of the incubation instead of adding it at the end. To avoid damage to the envelope, untreated virions as well as the digested virions were never frozen, but were kept on ice and were used freshly for flux experiments. All particles obtained in the above described procedures were subjected to SDS-PAGE analysis to control the final product. Initial experiments showed the necessity of removing

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the octyl- β -D-glucopyranoside (OG; Fluka) from the virion suspension to avoid artificial membrane permeability (data not shown). Therefore, all suspensions of particles to be used in flux experiments were dialyzed over night in a microdialysator (system 500, Pierce) against MBS containing SM-2 Biobeads (biotechnology grade, Bio-Rad).

The influx experiments consisted in monitoring the influx of propidium iodide (PI: 3,8-diamino-5-[3-[diethylmethylammonio]propyl]-6-phenylphenanthridinium, molecular weight 668) into the respective particles. PI is a fluorescence marker for RNA, i.e. its fluorescence is strongly enhanced upon binding to RNA. Thus, influx can be detected through the increased fluorescence emitted by PI. Measurements were performed with an amount of virions equivalent to approximately 50 μ g viral proteins in MBS containing 24 nmol/ml PI. After having established a constant baseline of fluorescence, the pH was lowered to 5.9 by adding 1 M MES to start the flux experiment. At the end of each experiment, the viral membranes were solubilized by adding Triton X-100 to a concentration of 0.1%. Addition of Triton X-100 resulted in an increase of fluorescence, indicating that upon solubilization of the membrane the viral RNA became even more accessible to PI. Furthermore this additional increase is also indicative that the lipid envelope was intact during the flux measurements. Fluorescence was recorded in a luminescence spectrometer LS-5B (Perkin-Elmer). The excitation wavelength was set to 528 nm, while emission was measured as the integral over the wavelength range above 570 nm.

3. Results and discussion

SDS-PAGE analysis of the particles prepared according to the methods outlined above revealed the feasibility of selective removal of the proteins E₂ and E₃ by using a low trypsin to protein ratio (1.3 U/mg) in the presence of OG (Fig. 1). Complete removal of all spike proteins, i.e. preparation of spikeless particles was accomplished by using a significantly higher concentration of trypsin (27 U/mg viral protein), whereas addition of STI to such a batch at the beginning of the incubation resulted in the preparation of particles with unchanged protein patterns with respect to untreated SFV (mock treated particles). The C-protein was found to be unaffected by all these treatments, as revealed by SDS-PAGE analysis (Fig. 1), indicating that the envelope remained intact.

As depicted in Fig. 2, PI-influx experiments with E₁-particles showed a rapidly increasing fluorescence after lowering the pH to 5.9, reaching constant values within a period of approximately 0.5 min. The data of this time period could be fitted using a hyperbolic Michaelis-Menten algorithm (Fig. 3). Solubilization of the particle membrane with Triton X-100 at the end of the experiment led to a further manifold increase of the

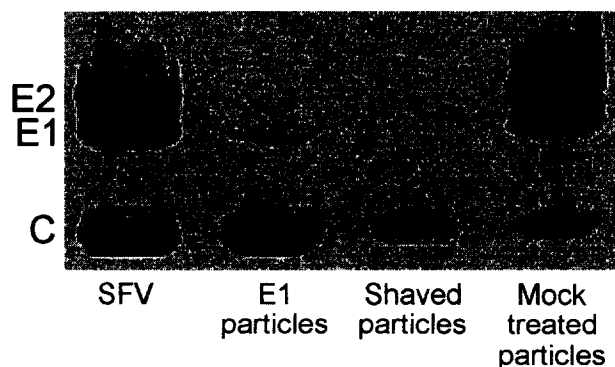


Fig. 1. Analysis of SFV spike proteins. Identical quantities of SFV were treated with various amounts of trypsin in presence of octylglucoside and the integrity of the spike proteins was subsequently analyzed by SDS-PAGE on a 10–15% gradient gel. The figure shows a scanned image of the Coomassie blue-stained gel.

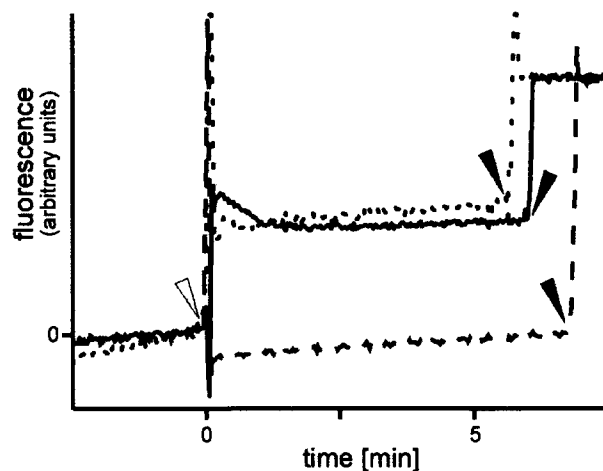


Fig. 2. Influx of propidium iodide (PI) into viral particles. The pH of the suspension containing the viral particles and PI was adjusted to pH 5.9 by addition of 1 M MES at time = 0 min (open arrow). Influx of propidium iodide into the viral particles is depicted by the fluorescence increase. At the end of the experiment the viral particles were disrupted by addition of TX-100 (solid arrows) to make all RNA accessible to propidium iodide. Solid line = E₁ particles; dashed line = 'shaved particles'; and dotted line = mock treated particles.

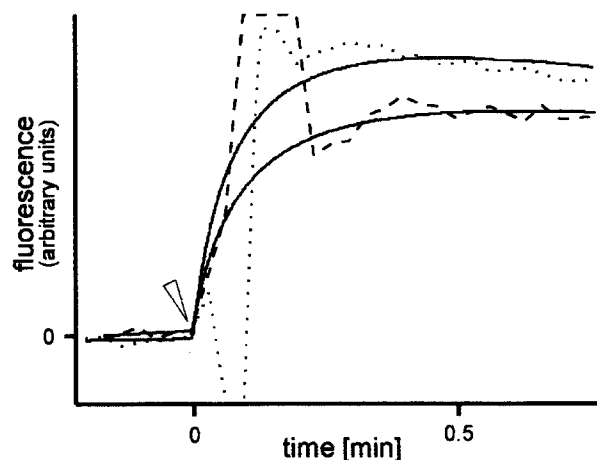


Fig. 3. Kinetics of propidium iodide influx. The data corresponding to the first 45 s after lowering the pH in Fig. 2 were fitted using a hyperbolic Michaelis-Menten algorithm. The peak maxima and minima at time = 0, due to the experimental handling, were omitted. The dotted line shows the data for E₁ particles, the dashed line for mock treated particles, and the corresponding curve fits are depicted as solid lines.

fluorescence, confirming that the particles had not been destroyed in the course of the experiment. Furthermore, it also shows that upon solubilization of the viral membrane the RNA becomes even more accessible for interactions with PI.

It has previously been shown that permeability changes induced by viruses or bacterial toxins can be hampered by divalent cations such as Zn²⁺ [11]. The above described experiments with E₁ particles were, therefore, performed in presence of ZnCl₂. As shown in Fig. 4 addition of 2 mM Zn²⁺ completely inhibited the flux of PI across the membrane after lowering of pH. This is in full agreement with the findings presented in reference [9], which reported the ability to inhibit fluxes of small

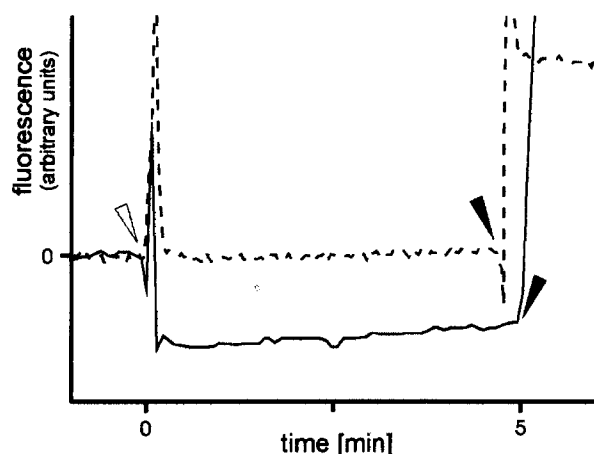


Fig. 4. Inhibition of propidium iodide influx into viral particles by Zn^{2+} . Suspensions containing the viral particles and propidium iodide were supplemented with 2 mM $ZnCl_2$. The pH was adjusted to pH 5.9 at time = 0 min (open arrow). Absence of fluorescence increase indicates inhibition of propidium iodide flux into the particles. At the end of the experiment the viral particles were disrupted by addition of TX-100 (filled arrows) to render all RNA accessible to propidium iodide. Solid line = E_1 particles; dashed line = mock treated particles.

molecules (<900 Da) through the pores formed by SFV spike proteins induced at low pH.

To confirm the influx being dependent solely on the presence of E_1 , the influx experiments were carried out with 'shaved' particles, showing a non-increasing fluorescence after pH lowering (Fig. 2). Experiments with mock treated particles yielded data similar to those of E_1 particles or untreated SFV (Fig. 2).

Comparison of the results of the PI-influx-experiments using E_1 -particles with those using 'shaved' particles shows the pore forming ability of protein E_1 . The increase of fluorescence after addition of acid in suspensions containing E_1 -particles indicates the influx of PI molecules into these virions. This influx is not due to damage of the particle membrane, as is revealed by the absence of influx into shaved particles. Further confirmation for this conclusion was found in experiments with suspensions containing 2 mM Zn^{2+} , which were found to inhibit the flux of PI into E_1 -particles. Therefore, the change of permeability detected by the PI-influx-experiments is comparable to the one described by Lanzrein and collaborators [9], which was identified to be a consequence of pore formation.

The examination of mock treated particles in influx experi-

ments showed that the long exposure of the virions to increased temperature (37°C) and detergent does not impair the function of the viral spikes.

In conclusion, the results presented strongly suggest that E_1 is the pore forming element of SFV spikes.

Thus, the pre-uncoating steps in the entry pathway of SFV show some similarity with those of influenza A virus, as proposed by Helenius [15]. SFV binds to the host cell membrane and is taken up by endocytosis. Within the endosome the spikes are exposed to a mildly acidic pH which in turn leads to the conformational change of the spike proteins and the formation of a pore across the viral membrane. Therefore, the E_1 protein might play a similar role in uncoating of SFV as was proposed that the M_2 protein does in influenza A virus. Whether the uncoating of SFV nucleocapsid follows a mechanism similar also to the one of influenza A remains to be elucidated and is the subject of current work.

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