

Association of Sindbis Virus Proteins with Vero Cell Cytoskeleton

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Abstract: The involvement of Vero cell cytoskeleton in Sindbis virus replication was studied. Cytoskeleton disrupting drugs, i. e. Vinblastine sulphate (Vin) and Cytochalasin B (CB) were used. When the infected cells were exposed to Vin immediately after infection for 9 hours, all virus-specified proteins were still present. The core protein was observed albeit at lower quantity compared with the untreated cells. Treatment with CB did not affect 93K and core proteins but PE2 and E1 were absent in the virus-specified protein profiles. Triton X-100-extracted cells also revealed modification of the remaining cytoskeleton as infection progressed. Generally the filaments became thickened and clumpy from 6 hours post infection (p. i.). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis also revealed the core protein to be affiliated with the remaining cytoskeleton.

Key words: Cytoskeleton, Sindbis virus

INTRODUCTION

With improved electron microscopic techniques, studies on a variety of tissues and cells grown in culture revealed three major fiber systems. These are the microfilaments (5-6 nm in diameter), the intermediate filaments (7-10 nm) and the microtubules (20-25 nm). In general, this cytoskeleton gives cell the properties of shape and internal organization of the organelles. The components also have a role in intracellular movement, mitotic process and cytoplasmic traffic of all organelles and macromolecules within the cell (DeBrabander, 1982).

Various studies have shown that there were association of viruses or virus subparticles with one or more components of the cytoskeleton. Adenovirus and reovirus type 1 (Dales and Chardonnet, 1973) were observed to be associated with the microtubules. Other workers reported that in addition to the involvement of microtubules, intermediate filaments also played some roles in the replication of adenovirus (Belin and Boulanger, 1985) and reovirus (Sharpe *et al.*, 1982). Ng and Hong (1989) also found the presence of a flavivirus-specified protein, NS3, affiliated with the microtubules. Microfilaments were also observed to be involved in some ways in the replication of several viruses, for example vaccinia virus (Stokes, 1976; Hiller *et al.*, 1979), poliovirus (Lenk and Penman, 1979), paramyxovirus (Fragraeus *et al.*,

1978) and herpes simplex virus (Heeg *et al.*, 1981).

In this study we look into the possible involvement of the cell cytoskeleton during Sindbis virus replication using electron microscopy, SDS-PAGE and cytoskeleton disrupting drugs.

MATERIALS and METHODS

Cells and viruses: Vero cells were grown to confluency in Medium 199 containing 5% foetal calf serum and maintained after infection in Eagle's minimum essential medium (MEM) containing 0.1% bovine serum albumin. The Sindbis virus MRM 39 was a gift from Professor Westaway, Monash University, Australia. The source of the Sindbis virus was described by Doherty and co-workers (1963).

Extraction of cells with Triton X-100: The extraction procedure was adapted from Osborn and coworkers (1978). The cells were rinsed twice with cold phosphate buffered saline and once with stabilization buffer (0.1M PIPES, 1mM EGTA, 2.5 mM GTP, and 4% PEG 6000, pH 6.9). After these rinsings, the cells were exposed to the stabilization buffer containing 0.2% Triton X-100 for 3 to 4 minutes. The remaining cell cytoskeleton was rinsed twice again with stabilization buffer before processing for SDS-PAGE or electron microscopy.

Electron microscopy of the cytoskeleton: Cytoskeleton of uninfected and Sindbis virus-infected cells were prepared from Vero cells grown on glass coverslips and extracted as above. The remaining skeletons were dried at critical-point and shadowed with carbon at a 20° angle to the specimen plane, while rotating at 30 rpm. Further shadowing with carbon at 90° angle was performed for stabilization. The shadowed cytoskeleton sample were detached from the glass coverslips in 40% hydrofluoric acid and mounted on grids without supporting films. The specimens were viewed under Philips T400 or JEOL 1200EX electron microscope.

Labelling of proteins in drug-treated infected cultures: Vero cells were infected with Sindbis virus at a multiplicity of infection of 10 PFU/cell. Cytoskeletal disrupting drugs were added at 0 hour p. i. Two cytoskeletal drugs used were Vinblastine sulphate (Vin-10 µg/ml) and cytochalasin B (CB-10 µg/ml). The drugs were exposed onto the cells for 9 and 12 hours. Three hours before harvesting, the MEM was replaced with medium deprived of methionine but supplemented with the drugs. After two hours of starvation, the cultures were incubated for one hour with 2µCi/ml of ³⁵S-methionine, in the presence of the drugs. At the end of the labelling period, the uninfected and Sindbis virus-infected cells were harvested with 2% SDS and electrophoresed in either 8% or 10% SDS-PAGE (Laemmli, 1970).

The cytoskeleton samples for SDS-PAGE were labelled as above but were extracted as described above before harvesting with 2% SDS.

RESULTS

Electron microscopy of the cytoskeleton: Electron microscopy was used to study Sindbis virus-induced morphological changes in the Vero cell cytoskeleton during infections. Uninfected and Sindbis virus-infected cells were extracted with Triton X-100 (Osborn *et al.*, 1978). The extraction procedure was carried out at 25°C on monolayers grown on coverslips. The remaining cytoskeletons were dried at critical point and shadowed as described in the Materials and Methods section.

At low magnification, the progressive changes to the cytoskeleton could be correlated with advancing cytopathic effects [(CPE)–(Fig. 1)]. Fig. 1 a gave an overall view of the intricate cytoskeletal arrangement on a Vero cell. The nuclei of the cells were held in place by fine filaments. This was more clearly illustrated at higher magnification of the boxed area (Fig. 1 b—arrows). With increased infection time (6 hours p. i.—Fig. 1 c), the cell periphery seemed to have shrunk towards the nucleus (arrow). A thicker mesh-work radiating from the perinuclear region was observed (arrowheads). By 9 hours p. i. (Fig. 1 d), the cytoskeleton had lost its organised structure and had shrunk further towards the nucleus.

To observe the gross modification of the cytoskeleton arrangement in greater detail, higher magnification were used (Fig. 2). At 3 hours p. i. (Fig. 2b), the change in morphology of the cytoskeleton was not very obvious when compared with the uninfected cells (Fig. 2a). There were some filaments which were broken (arrows). After 6 hours of infection (Fig. 2d & e), the arrangement of the cytoskeleton was completely different compared with the uninfected cells (Fig. 2c). The filaments seemed to have merged together into large clumps (arrowheads). After 6 hours p. i. the number and the size of these clumps increase and at late infection (between 12 to 15 hours p. i.) the connecting filaments became fragmented (not shown).

Viral proteins remaining on the extracted cells: As there were obvious changes in the morphology of the cytoskeleton network during infection, it is highly probable that they were virus-induced. The extracted cells (i. e. the remaining cytoskeleton) was then electrophoresed by SDS-PAGE (Fig. 3) to see if any virus proteins were associated with the skeleton. The core protein was seen affiliated with the remaining cytoskeletal structure; more obviously at 12 hours p. i. (Lane 6) although its presence was seen at 9 hours p. i. (lane 4). Probably at earlier stages of infection (3 to 6 hours p. i.), the concentration of the protein was not high enough for detection (Lane 2).

Labelling of proteins in drug-treated cultures: Skeleton-disrupting drugs, Vin and CB were added to uninfected and Sindbis virus-infected cells for 9 and 12 hours (immediately after infection). It was interesting to investigate to which component of the cytoskeleton the core protein was attached. Vin caused the precipitation of tubulin into paracrystals (Olmstead and Borisy, 1973; Bensch *et al.*, 1969 and Marantz *et al.*, 1969). The CB treatment would result in the disintegration of actin containing microfilaments (Wessels *et al.*, 1971 and

Spooner *et al.*, 1971). Only whole cell lysate were analysed. Very low amount of material was left when extraction was done on drug-treated cells. This was probably due to the more stressful condition of the cells caused by the additive effects of the drugs and virus.

After 9 hours of Vin treatment (Fig. 4a—lane 5) there was not much difference in the virus protein profiles when compared with the untreated sample (Fig. 4a—lane 6). When the incubation time was extended to 12 hours p. i., three viral proteins were no longer visible (Fig. 4a—lane 4). They were the 93K, PE2 and E1 proteins. The core protein was present but at lower quantity. In the CB-treated cells, the presence of 93K and core proteins were not affected by this drug. However PE2 and E1 proteins were no longer or only faintly detectable compared to the untreated infected cells (Fig. 4b—lanes 3& 4, compare with lane 5). This observation was also made even when the drugs were present for only 3 to 6 hours (not shown).

DISCUSSION

The present study by electron microscopy indicated that Vero cells cytoskeletons were interactive with Sindbis virus during infection. This was further supported by the affiliation of the core (C) protein with that Triton X-100 extracted cell's skeleton (Fig. 3-Lanes 4 and 6).

With Vin treatment, most of the viral proteins were present in the whole cell extracts. Thus all the virus proteins were synthesized despite the disruption of microtubules by Vin (Fig. 4a—Lane 5). Although after 12 hours of exposure, only low quantity of C protein was present. The loss of 93K, PE2 and E1 proteins (Fig. 4a—Lane 4) were most likely due to the toxicity on the cells caused by long exposure to Vin, therefore, not directly to the lack of intact microtubules.

After CB treatment for 3 to 12 hours p. i., only PE2 and E1 proteins were affected (Fig. 4b—Lanes 3 and 4). These results might reflect that the disruption of the microfilament affected the normal cleavage of the 93K to PE2 and E1. This would require further investigation.

This preliminary work indicated that the C protein would most likely be associated with the third component of the cytoskeleton i. e. the intermediate filament, and that both Vin and CB would not disrupt this component.

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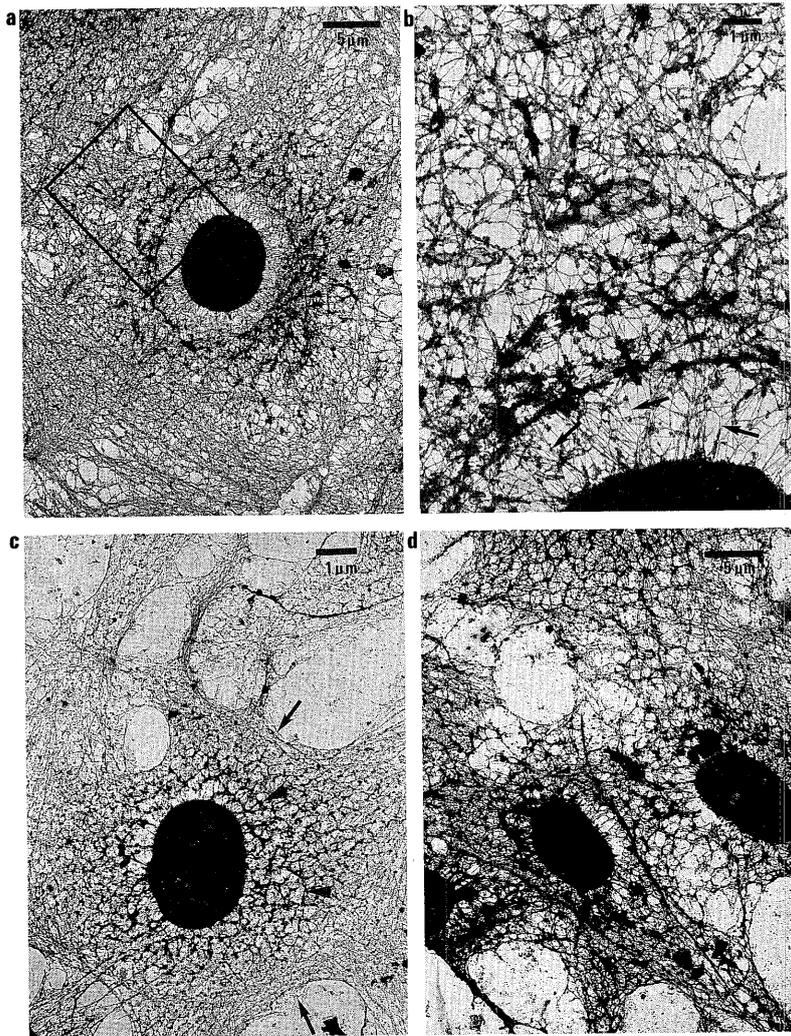


Fig. 1. Electron micrographs of Triton X-100 extracted uninfected and Sindbis virus-infected Vero cells. (a) Uninfected Vero cells. After Triton X-100 extraction procedure, an intricate filamentous array of the Vero cell cytoskeleton is seen. (b) Higher magnification of the boxed area shown in (a). Arrows indicate the very fine, delicate filaments attaching the nucleus to the cytoplasmic area. (c) At 6 hours p. i. with Sindbis virus, the cell periphery seemed to have shrunk towards the perinuclear region (arrows). Arrowheads indicate thickened clumps of the cytoskeletal filaments. (d) After 9 hours of infection, the cytoskeletal network is totally disrupted and disorganised. The cell volume has been reduced considerably when compared with the uninfected cell [compare with (a)].

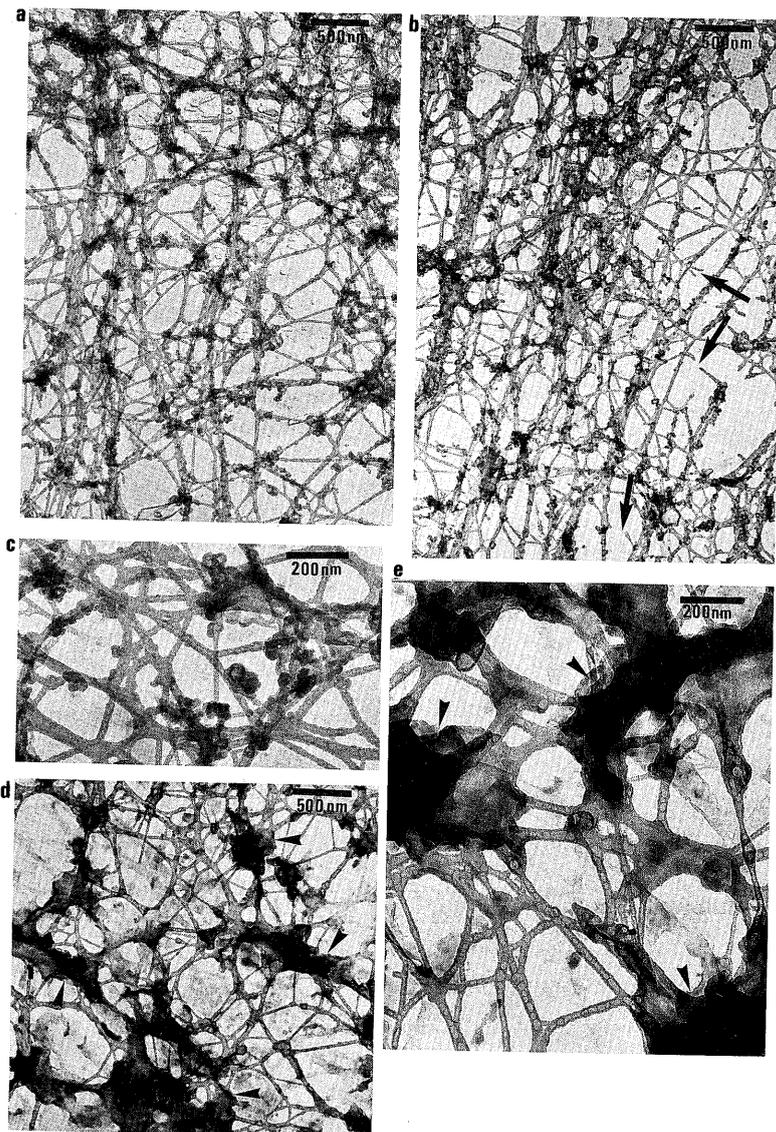


Fig. 2. Electron micrographs of Triton X-100 extracted uninfected and Sindbis virus-infected Vero cells. (a) Uninfected Vero cells. Higher magnification shows the cell cytoskeletal filaments to be composed of long parallel filaments with shorter interconnected filaments between them. (b) At 3 hours p. i., the gross arrangement of the filaments did not differ very much from the uninfected cells. However the shorter interconnecting filaments from the parallel ones could be seen to be detached (arrows). (c) Uninfected cells cytoskeleton at similar magnification to (e). (d, e) With increased infection time (6 hours p. i.), numerous large clumps of the filaments (arrowheads) are very obvious [compare with uninfected cells (c) at similar magnification with (e)].

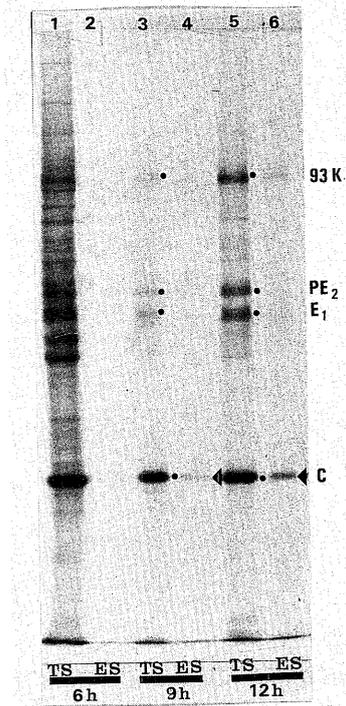


Fig. 3. SDS-PAGE of Sindbis virus-infected and Triton X-100 extracted Vero cells. Lanes 1 & 2 show the protein profiles of unextracted (TS) and Triton X-100 extracted (ES) cells at 6 hours p. i. No viral proteins were seen affiliated with the remaining cytoskeleton (Lane 2). Lanes 3 & 4 show the protein profiles of cells infected with Sindbis virus for 9 hours. In the extracted lysate (Lane 4) only the core (C) protein is faintly visible. Lanes 5 & 6 show the protein profiles of cells infected with Sindbis virus for 12 hours. Again only the C protein is visible after Triton X-100 extraction (Lane 6). Close circles denote positions of the virus-specified proteins and arrowheads indicate the position of C protein in the extracted samples. TS denotes total cell substrate and ES represents the remaining substrate after Triton X-100 extraction.

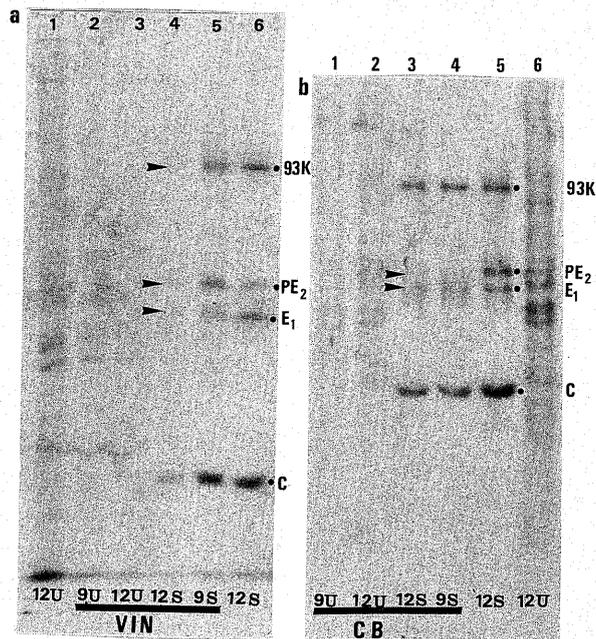


Fig. 4. Polyacrylamide gel electrophoresis (PAGE) of uninfected and Sindbis virus-infected Vero cell lysates. (a). Vin-treated and untreated cells (8% SDS-PAGE). Lane 1 shows the protein profile of mock-infected Vero cells exposed to maintenance medium for 12 hours. Lanes 2 & 3 show protein profiles of mock-infected and Vin-treated cells for 9 & 12 hours respectively. Lanes 4 & 5 show the protein profiles of Sindbis virus-infected cells treated with Vin for 12 & 9 hours after infection respectively. Lane 6 shows the protein profile of Sindbis virus-infected cells 12 hours p. i. without Vin treatment. (b). CB-treated and untreated cells (10% SDS-PAGE). Lanes 1 & 2 show the protein profiles of uninfected Vero cells after 9 or 12 hours of treatment with CB. Lanes 3 & 4 show the protein profiles of Sindbis virus-infected Vero cells upon exposure to CB for 12 or 9 hours. Lanes 5 & 6 show the protein profiles of Sindbis virus-infected (12 hours p. i.) and uninfected Vero cells respectively (without CB treatment). Close circles denote virus-specified proteins 93K, PE₂, E₁ & C. U and S represent uninfected and Sindbis virus-infected cells respectively. Arrowheads indicate the missing viral proteins after drug treatment.