

Involvement of the cytoskeleton in Junin virus multiplication

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The role of the cellular cytoskeleton in Junin virus (JV) infection was explored in two ways. Firstly, the action of inhibitors that affect individual cytoskeletal systems (microtubules or microfilaments) selectively was analysed. It was found that perturbations of microtubule or microfilament networks caused by colchicine, nocodazole, nifedipine, EGTA or DMSO strongly affected virion production and viral protein expression at non-cytotoxic concentrations. Secondly, the extent of association of viral proteins and infectious virus particles with the cytoskeletal fraction of monkey Vero cells was determined by using three non-ionic detergents, Triton X-100 (TX-100), NP-40 and octyl glucoside (OG). The cytoskeleton retained nearly 70% of the external JV envelope glycoprotein GP38 and about 40% of the JV nucleoprotein NP, according to TX-100 and OG insolubility results. Furthermore, 1% of the total cell-bound infectivity was detected in the detergent-insoluble fraction, suggesting that cytoskeletal components are involved in the initiation of the assembly and budding processes of JV particles at the plasma membrane.

Introduction

The cytoskeleton is a cellular network containing three classes of filaments (microfilaments, microtubules and intermediate filaments) and a set of accessory proteins (Hartwig, 1992). These components are assembled into a three-dimensional structure that defines the shape and structural organization of the cell and participates in cell motility, intracellular transport and chromosome movement during mitosis.

Several studies over the years have supported the hypothesis that the cytoskeletal filament organization or its associated proteins are involved at an early or late stage in the replication of animal viruses. Disruption of microtubule function can interfere with early phases of herpesvirus and simian virus 40 replication (Shimura *et al.*, 1987; Wittels & Spear, 1991). The genome replication and/or transcription of Sendai virus, human parainfluenza virus 3 and vesicular stomatitis virus appear to require the interaction of viral components with tubulin, actin or microtubule-associated proteins (Moyer *et al.*, 1986; Hill *et al.*, 1986; De *et al.*, 1991). The incorporation of a few specific cytoskeletal proteins, mainly actin, into purified virions has been described for several enveloped viruses, including retroviruses (Damsky *et*

al., 1977; Ott *et al.*, 1996), rhabdoviruses (Naito & Matsumoto, 1978) and paramyxoviruses (Örvell, 1978; Tyrrell & Norrby, 1978), and their interaction with viral components on the cell surface suggests a role for microfilaments in virus assembly and budding (Giuffrè *et al.*, 1982; Bohn *et al.*, 1986; Luftig & Lupo, 1994; Rey *et al.*, 1996). Furthermore, the cytoskeleton may also provide a structure for intracellular transport of infecting virus (Dales & Chardonnet, 1973) or for movement of virus macromolecules and particles to facilitate their spread between cells (Cudmore *et al.*, 1995).

Junin virus (JV), a member of the family *Arenaviridae*, is an enveloped virus containing two segments of ambisense single-stranded RNA. The most prominent virion proteins are the nucleocapsid-associated protein (NP, molecular mass 63 kDa) and the main envelope glycoprotein exposed on the virion surface (GP1 or GP38, molecular mass 38 kDa), derived by proteolytic cleavage from a cell-associated precursor (GPC, molecular mass 65 kDa). Although the identification of actin associated with purified JV led to the proposal that it plays an active role in JV maturation (Pasian *et al.*, 1983), a definitive requirement for the cellular cytoskeleton has not been fully demonstrated. Recently, it was reported that trifluoperazine and chlorpromazine, two phenothiazine derivatives known to produce actin microfilament disruption by inhibiting calmodulin-mediated mechanisms (Osborn & Weber, 1980*b*), are effective antiviral agents against multiplication *in vitro* of the

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arenaviruses Tacaribe, Junin and Pichinde (Candurra *et al.*, 1996). Furthermore, JV multiplication was inhibited by other microfilament-disrupting drugs such as local anaesthetics (Castilla *et al.*, 1994). However, the entry of the arenavirus lymphocytic choriomeningitis virus into BHK-21 cells was not blocked by cytochalasins B and D (Borrow & Oldstone, 1994), compounds that promote disassembly of microfilaments, arguing against a direct participation of actin, at least in the uptake of this virus.

To assess the involvement of the cellular cytoskeleton in the multiplication of JV, we have first examined the effect of several agents that affect microfilament and microtubule organization directly or indirectly. To obtain more convincing evidence, we have then extracted cytoskeletons from JV-infected Vero cells with non-ionic detergents and studied the association of virus particles and viral components with cytoskeletal extracts.

Methods

Cells, virus and antibodies. Vero cells were grown in Eagle's minimum essential medium (MEM) containing 5% inactivated calf serum and 50 µg/ml gentamycin. Maintenance medium (MM) consisted of MEM supplemented with 1.5% calf serum. The naturally attenuated IV4454 strain of JV was propagated in Vero cells and the titre of the stock suspension used in this study was 2×10^6 p.f.u./ml. Polyclonal anti-JV serum was obtained from rabbits inoculated with purified virus (Candurra *et al.*, 1989). A sample of this antiviral serum was adsorbed to Vero cell extracts by two cycles of incubation (1 h at 37 °C, overnight at 4 °C). Monoclonal antibody GB03-BE08, reactive against GP38 and its precursor GPC (Sanchez *et al.*, 1989), was generously provided by A. Sanchez (Center for Disease Control, Atlanta, GA, USA). Mouse monoclonal antibody to α -tubulin, polyclonal rabbit anti-actin antibodies and fluorescein isothiocyanate (FITC)-conjugated phalloidin, specific for filamentous actin, were purchased from Sigma.

Inhibition of JV multiplication. Vero cell monolayers grown in 24-well microplates were infected with JV (m.o.i. approx. 0.1) for 1 h at 37 °C. Unadsorbed virus was removed and MM with and without nifedipine (20–100 µM), DMSO (0.5–10%), EGTA (0.5–5 mM), colchicine (5–60 µM) or nocodazole (5–50 µM) was added. All compounds were purchased from Sigma. Calcium-deficient MEM containing 0.017 mM Ca^{2+} was used in assays with nifedipine. After 24 h incubation at 37 °C, culture supernatants were harvested and extracellular virus yields were determined by plaque assay. The remaining cells were frozen and thawed twice, cellular debris was removed by centrifugation and supernatant fluids were assayed to determine cell-associated virus. Two replicate samples per dilution of each compound were tested.

Cell viability assay. Vero cells were seeded in 24-well microplates and after 24 h incubation, the cells were re-fed with MM containing twofold serial dilutions of the compounds. After 24 h incubation at 37 °C, the medium was removed, the cells were trypsinized and the number of viable cells was determined by the Trypan blue exclusion method.

Cell extraction. A protocol similar to that used by Morrison & McGinnes (1985) was employed to isolate cytoskeletal components from JV-infected cell cultures. Briefly, cell monolayers were washed twice with cold PBS and lysed with extraction buffer (EB) composed of 150 mM NaCl, 2.5 mM MgCl_2 , 10 mM HEPES-NaOH, pH 7.4, 0.4 mM PMSF

supplemented alternatively with 1% NP-40, 1% Triton X-100 (TX-100) or 60 mM octyl glucoside (OG). To perform TX-100 extraction at high salt concentration, EB containing 1 M NaCl was employed. After 5 min on ice, supernatant fluids containing the detergent-soluble material were removed and adjusted to 0.1% SDS and 1% sodium deoxycholate for immunoprecipitation. When high salt was included in the EB, supernatant fluids were diluted sixfold before immunoprecipitation. The insoluble material remaining on the culture dish was scraped off with a rubber policeman in EB without detergent and centrifuged for 5 min at 10 000 g. The pellets were solubilized in radioimmunoprecipitation assay (RIPA) buffer: 150 mM NaCl, 0.1% SDS, 1% TX-100, 0.4 mM PMSF and 1% sodium deoxycholate in 0.01 M Tris-HCl, pH 7.4.

For immunofluorescence staining of cytoskeletal fractions, Vero cells grown on coverslips and infected with JV (m.o.i. 0.1) were extracted with EB containing 1% NP-40 and 2 mM EGTA was added to stabilize microtubule structure. After washing once with EB without detergent, the material retained on the coverslips was used as the cytoskeletal fraction and processed for immunofluorescence.

Protein radiolabelling and immunoprecipitation. For analysis of virus protein distribution, JV-infected Vero cells at 42 h post-infection (p.i.) were incubated in methionine-free medium for 1.5 h and then labelled with 50 µCi/ml [^{35}S]methionine (sp. act. 1175 Ci/mmol, NEN) for 3.5 h. After labelling, the cells were washed three times in cold PBS and then extracted as described above to generate detergent-soluble and detergent-insoluble fractions. The soluble and insoluble samples from the different extraction procedures were mixed with hyperimmune rabbit anti-JV serum and incubated for 30 min at 37 °C and 90 min at 4 °C. Antibody-antigen complexes were collected with Protein A-Sepharose, washed three times in RIPA buffer and solubilized by boiling for 2 min in SDS-PAGE sample buffer. Proteins were electrophoresed on a 12% polyacrylamide slab gel and visualized by fluorography. The relative distribution of JV proteins between soluble and insoluble fractions was determined by densitometric scanning and quantification of the polypeptide bands detected by fluorography by using the ImageQuant software version 3.22 (Molecular Dynamics).

In another experiment, Vero cells were infected with JV or mock-infected and, at 42 h p.i., cells were incubated in methionine-free medium for 1.5 h and then labelled with 25 µCi/ml [^{35}S]methionine for 3.5 h. One set of cultures was supplemented with 2% DMSO in the methionine-free medium throughout the labelling period. The cells were next washed with PBS and lysed in RIPA buffer. Samples of clarified cell lysates were mixed with polyclonal anti-JV serum or anti-actin serum. Immunoprecipitation assay and gel electrophoresis of precipitated proteins were performed as described above.

Immunofluorescence staining. Vero cells grown on glass coverslips were infected with JV at an m.o.i. of 0.1 and either incubated for 24 h in the presence or absence of the cytoskeletal-disrupting compounds or incubated for 48 h and then detergent-extracted as described previously. Cells and cytoskeletal fractions remaining on coverslips were washed with PBS and fixed with methanol for 15 min at -20 °C for cytoplasmic staining or with 4% formaldehyde (freshly prepared from paraformaldehyde) for 15 min at room temperature for surface staining. Next, cells and cytoskeletons were washed with PBS and stained with anti-JV, anti-tubulin or anti-actin antibodies for 30 min at 37 °C, followed by incubation with an FITC-goat anti-mouse or anti-rabbit IgG (Sigma) for 30 min at 37 °C. In other experiments, actin was visualized directly by staining fixed cells with FITC-phalloidin for 1 h at 37 °C. After a final wash with PBS, the coverslips were mounted in a glycerol solution containing 1,4-diazabicyclo[2.2.2]octane (DABCO). The percentage of fluorescent cells in each preparation was calculated from 20 randomly selected microscope fields.

■ **Determination of succinate reductase activity.** This assay was carried out in soluble and insoluble fractions corresponding to the different extraction procedures and in non-extracted whole cells, as described by Pennington (1961).

Results

Effect of cytoskeleton-disrupting compounds on JV multiplication

As a first approach to study the involvement of the cytoskeleton in arenavirus infection, the effect of several compounds affecting cytoskeletal integrity was determined. These compounds included three microfilament-disrupting agents that function by inhibiting divalent cation-mediated mechanisms: the Ca^{2+} chelator EGTA, which produces contraction of cytoplasmic actin (Britch & Allen, 1980); nifedipine, a Ca^{2+} -channel blocker preventing actin polymerization (Cavero & Spedding, 1983); and DMSO, which changes membrane permeability to divalent cations, inducing rearrangement of the microfilaments (Osborn & Weber, 1980*a*). Two antimicrotubular drugs, colchicine and nocodazole, which bind to tubulin and inhibit the assembly of microtubules (Hamel, 1996), were also assayed.

The lack of toxicity of the five compounds to Vero cells was investigated by assessing their effects on cell viability. In the range of concentrations assayed, a slight degree of cell rounding was observed after incubation with 5 mM EGTA, 10% DMSO and 60 μM colchicine, whereas no alterations were observed for any dose of nifedipine and nocodazole. Despite these morphological changes, no significant variations in the number of viable cells were observed after staining with Trypan blue in any case, since cell death did not exceed 10–15% of the control value for the maximal concentrations indicated (Fig. 1). Furthermore, the induced rounding of the cells was reversed on addition of normal medium to the treated cultures, emphasizing the lack of toxic effect of the compounds on Vero cells under these treatment conditions.

Dose-dependent inhibition of JV production was observed in the presence of all of the compounds in the range of non-cytotoxic concentrations tested (Fig. 1). The ability to reduce extracellular virus yields after 24 h infection was greatest for colchicine, DMSO and EGTA, attaining greater than 90% inhibition, whereas with nifedipine and nocodazole, the reduction was approximately 80%. Furthermore, the production of cell-associated virus was as sensitive to the inhibitors as was extracellular virus formation (data not shown).

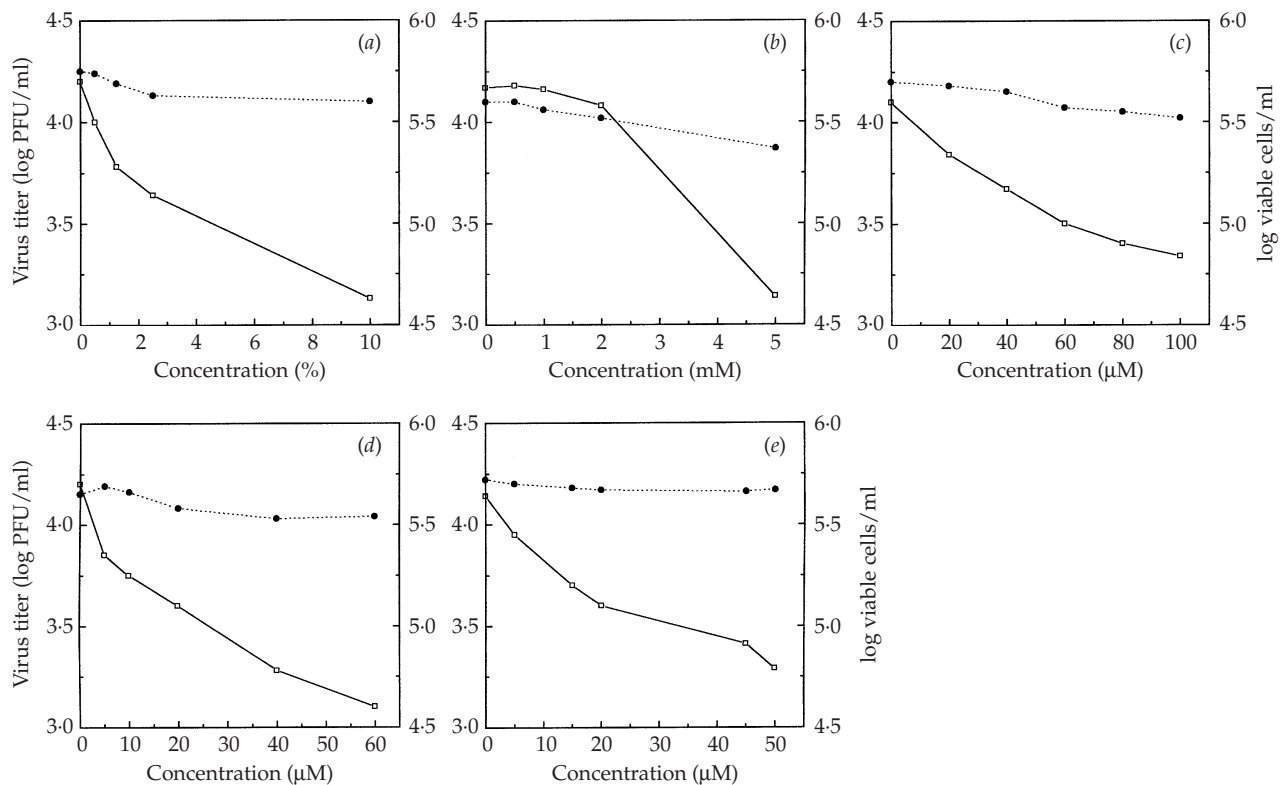


Fig. 1. Concentration-response curves of inhibition of virus production and cytotoxicity of microfilament- and microtubule-disrupting agents. Vero cells were incubated for 24 h in the presence of different concentrations of DMSO (a), EGTA (b), nifedipine (c), colchicine (d) or nocodazole (e). The number of viable cells was then determined by the Trypan blue exclusion method (dotted line). Another set of cultures was infected with JV and, after 24 h of infection in the presence of the compounds, extracellular virus yields were determined (solid line). Each point is the mean value of duplicate determinations.

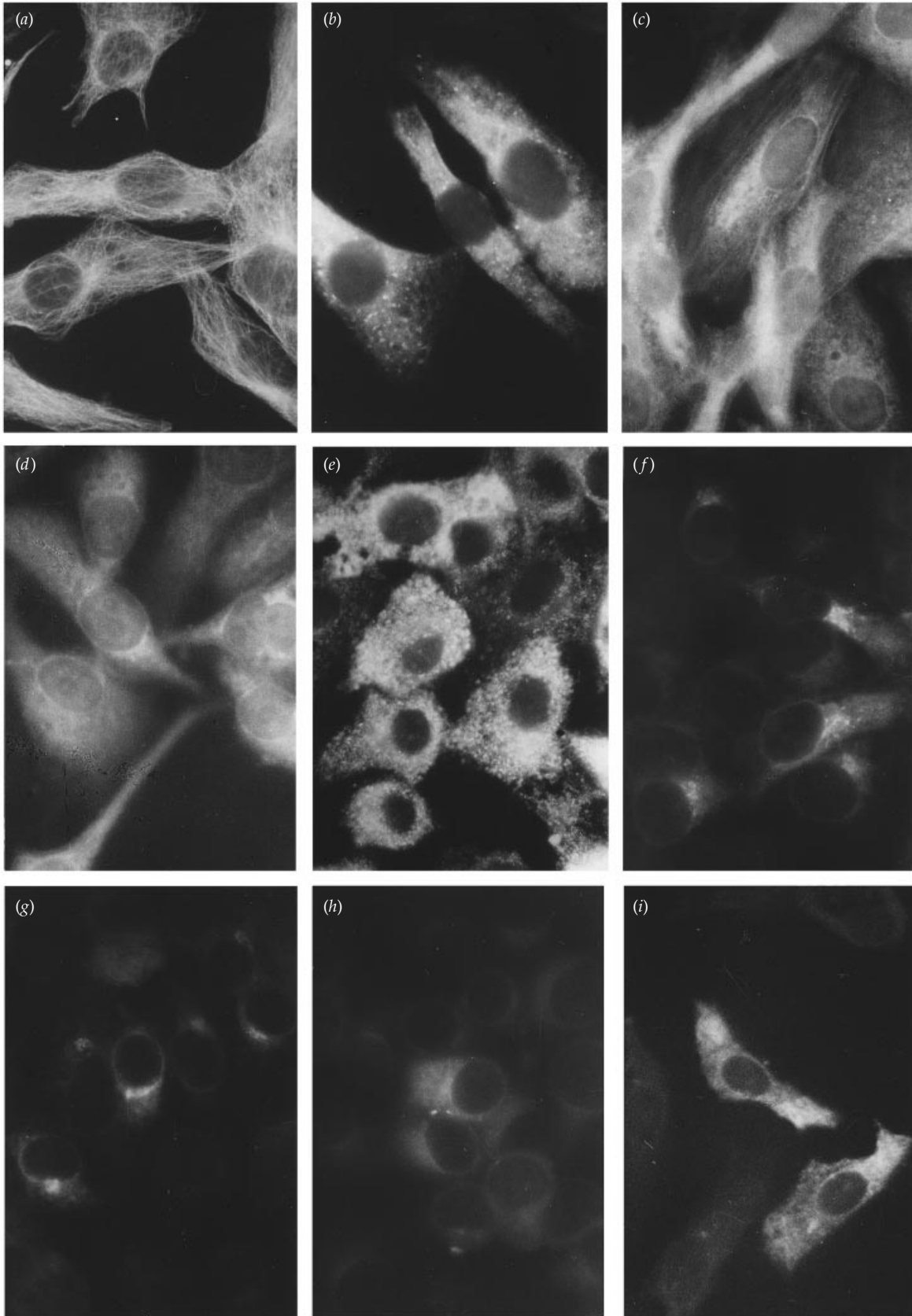


Fig. 2. For legend see facing page.

Action of inhibitors on the cytoskeleton and virus antigens

To confirm that the compounds affecting JV replication were effectively disrupting cytoskeletal organization under the assay conditions used, Vero cells were stained for actin filaments and microtubules in the absence or in the presence of the inhibitors.

The organization of microtubules in Vero cells, examined by using a monoclonal antibody to tubulin, was essentially the same as that described for other cell lines such as BHK-21 (Simon *et al.*, 1990) and CV-1 (Sharpe *et al.*, 1982). The staining pattern appeared as a network of thin, well-defined tubules radiating outwards from the microtubular organizing centre (MOC), located in the perinuclear region, to the cell periphery (Fig. 2*a*). After 24 h of treatment with 30 μ M colchicine (Fig. 2*b*) or 40 μ M nocodazole (not shown), the normal arrangement of microtubules was dramatically altered. The cytoplasm became stained with a punctate fluorescence as a result of tubulin depolymerization.

The distribution of actin-containing microfilaments was examined by using a monoclonal antibody to actin or FITC-phalloidin. Although microfilaments were not resolved efficiently under either conditions in Vero cells, the typical bundles of parallel stress fibres spanning the long axis of the cell were observed (Fig. 2*c*). Such filamentous structures were no longer visible after treatment with DMSO (Fig. 2*d*), EGTA or nifedipine (not shown). These morphological data support the notion that the compounds, under these treatment conditions, disrupt the cytoskeletal organization in Vero cells.

JV infection did not alter the morphological distribution of microfilaments or microtubules, except for the appearance of a less dense and straight network of microtubules observed at 24 h p.i. (data not shown).

We also studied the expression of viral glycoproteins in the presence of the cytoskeleton-disrupting agents by indirect immunofluorescence with a monoclonal antibody reactive against GP38 and its precursor, GPC (Sanchez *et al.*, 1989). In control infected cells at 24 h p.i., JV glycoproteins were distributed throughout the cytoplasm with a very bright staining (Fig. 2*e*). When infected cells were treated with 30 μ M colchicine (Fig. 2*f*) or 40 μ M nocodazole (Fig. 2*g*), a large reduction in the expression of viral glycoproteins was observed and also the pattern of staining in positive cells changed: the immunofluorescence was not dispersed into the cytoplasm but accumulated as perinuclear spots. Altered viral glycoprotein distribution and reduced dissemination of JV infection were also observed after treatment with the microfilament-disrupting compounds. In the presence of 2% DMSO, viral proteins remained in a perinuclear location (Fig. 2*h*) and, after

Table 1. Quantification of the action of cytoskeleton-disrupting agents on JV glycoprotein expression

The number of glycoprotein-positive cells was counted in 20 microscope fields for each sample. Results are expressed as percent inhibition with respect to untreated control cells.

| Compound | Inhibition (%) | |
|------------|----------------|---------|
| | Cytoplasmic | Surface |
| DMSO | 74.2 | 79.4 |
| EGTA | 85.6 | 91.1 |
| Colchicine | 80.1 | 89.8 |
| Nocodazole | 65.5 | 72.0 |

5 mM EGTA treatment, fluorescence also accumulated as localized spots inside the cell (Fig. 2*i*), with a different pattern than that observed in untreated infected cells.

The effect of inhibitors on glycoprotein expression was quantified by counting the number of fluorescent cells after 24 h of treatment with the compounds. The inhibition of glycoprotein expression was in the range 60–90% for all the compounds, with similar levels of inhibition of both cytoplasmic and surface staining (Table 1).

Association of viral proteins with the cytoskeleton

The cytoskeleton of tissue culture cells has been defined classically as the protein network remaining insoluble after extraction of cells with non-ionic detergents (Osborn & Weber, 1977) and consequently, NP-40- or TX-100-insolubility has been considered as indicating cytoskeletal binding of a viral protein (Morrison & McGinnes, 1985; Bohn *et al.*, 1986). However, it is now known that resistance of proteins to TX-100 extraction can be due either to their association with cytoskeletal components via protein–protein interaction or to their association with TX-100-insoluble lipids. Two different experimental approaches can be used to determine the cause of TX-100-insolubility of a protein: (i) TX-100-insoluble lipids are soluble in OG, whereas this detergent does not disrupt the cytoskeleton; and (ii) protein–protein interactions with cytoskeletal proteins are likely to be disrupted by high salt concentrations and thus such proteins should be solubilized by TX-100 extraction in high salt, unlike proteins interacting with lipids (Brown & Rose, 1992). Thus, to determine whether JV proteins were associated with cytoskeletal elements during virus infection, we extracted infected cells under different detergent conditions. JV-infected Vero cells at 48 h p.i. were labelled with [³⁵S]methionine for 3.5 h before being extracted

Fig. 2. Immunofluorescence staining of tubulin, actin and JV glycoproteins GPC and GP38 in uninfected (*a–d*) or JV-infected (*e–j*) Vero cells. Cells were untreated (*a, c, e*) or treated with 30 μ M colchicine (*b, f*), 2% DMSO (*d, h*), 40 μ M nocodazole (*g*) or 5 mM EGTA (*i*). Cells were stained for cytoplasmic immunofluorescence with monoclonal antibody to α -tubulin (*a, b*) or FITC-phalloidin (*c, d*) or anti-JV monoclonal antibody GB03-BE08 (*e–j*), as described in Methods. Magnification \times 930.

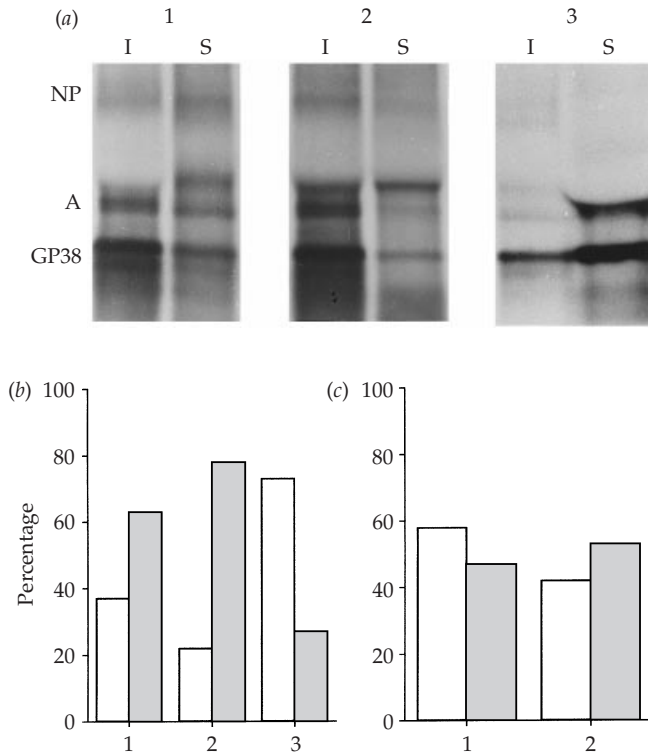


Fig. 3. TX-100- and OG-solubility of JV proteins in infected cells. (a) Vero cells infected with JV were labelled with 50 $\mu\text{Ci/ml}$ [^{35}S]methionine for 3.5 h at 48 h p.i. Cells were then extracted either with OG (1) or with TX-100 (2–3) in 150 mM NaCl (2) or 1 M NaCl (3). Both detergent-soluble (S) and -insoluble (I) materials were immunoprecipitated with anti-JV polyclonal antibodies and analysed by SDS-PAGE and fluorography. (b)–(c) The fluorograms shown in (a) were quantified and the results were expressed as percent soluble (empty bars) and percent insoluble (shaded bars) for GP38 (b) and NP (c).

with 125 mM NaCl containing 60 mM OG or 1% TX-100, as described in Methods. In preliminary experiments to optimize the extraction procedure, the detergent concentration, the extraction period and the temperature of incubation were varied. The effectiveness of the final extraction conditions was assessed by determining the succinate reductase activity as a mitochondrial membrane marker protein (Pennington, 1961). The purity of cytoskeleton samples was confirmed when succinate reductase activity in the soluble material was 80–90% of the total, whereas no activity was detected in the detergent-insoluble fraction (2% of the total activity is the detection limit of the enzyme assay). After each extraction procedure, viral polypeptides were immunoprecipitated from soluble and insoluble fractions and analysed by gel electrophoresis. The results showed that the two most abundant JV proteins, NP and GP38, were detected in both TX-100-soluble and TX-100-insoluble fractions (Fig. 3a panel 2, b, c), but with different distributions: GP38 was predominantly retained in the TX-100-insoluble portion (over 78% of the total), whereas NP was distributed roughly equally between the fractions (52 and 48% of the total in the insoluble and soluble portions, respectively). After OG extraction, NP became slightly more soluble (58% of

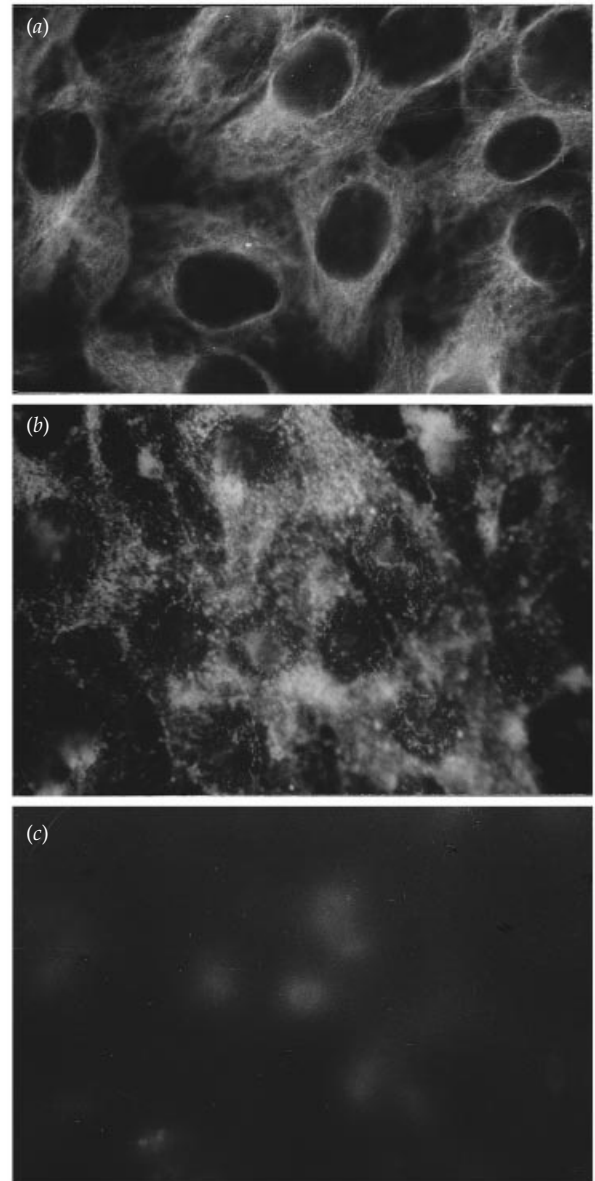


Fig. 4. Cytoskeletons extracted from JV-infected (a, b) or uninfected (c) Vero cells in NP-40-containing buffer immunostained with monoclonal antibody to α -tubulin (a) or anti-JV monoclonal antibody GB03-BE08 (b, c). Magnification $\times 900$.

NP present in the OG-soluble fraction) but still 42% was present in the insoluble fraction (Fig. 3a panel 1, c). By contrast, GP38 remained resistant to extraction and was located primarily in the OG-insoluble fraction of the cell (Fig. 3a panel 1, b). Although the JV polyclonal antiserum is equally reactive against NP and GP38 (as seen in Fig. 5), much more GP38 than NP appears to have been precipitated from extracted cells. It is likely that the detergent extraction conditions affected the interaction of NP with antibodies. As can be seen in the fluorograms, cellular actin was strongly immunoprecipitated by anti-JV antibodies from infected extracted cells and was also associated with the insoluble cytoskeletal fractions (Fig. 3a

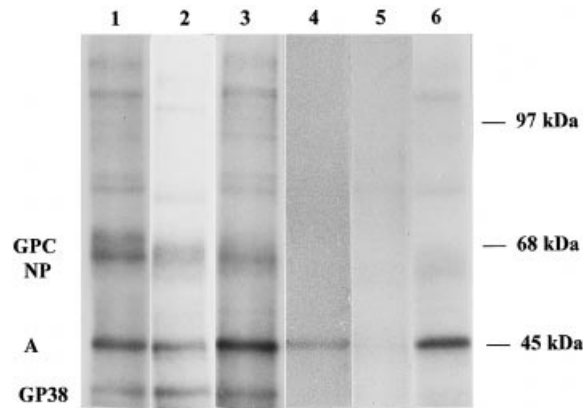


Fig. 5. Fluorogram of an SDS-PAGE gel showing proteins from JV-infected (lanes 1–3) and mock-infected (lanes 4–6) Vero cells immunoprecipitated with anti-JV antibodies (lanes 1 and 4), anti-JV antibodies extensively adsorbed to Vero cells (lanes 2 and 5) and anti-actin antibodies (lanes 3 and 6). A, actin. Positions of molecular mass markers are indicated to the right.

panels 1 and 2). Finally, to confirm that the detergent insolubility of JV glycoprotein was due to its interaction with the cytoskeleton, TX-100 extraction was performed in the presence of high salt (1 M NaCl). NP was not detected under these conditions, whereas GP38 and cellular actin became highly soluble (Fig. 3*a* panel 3, *b*), confirming the intimate association of both proteins with the cytoskeletal network.

To complement the cell fractionation data, we examined the effect of detergent extraction on the distribution of viral proteins by immunofluorescence staining. Vero cells grown on coverslips and infected with JV were exposed to NP-40-containing EB. After removing the materials solubilized into the buffer, the cytoskeletal structures that remained on the coverslips were washed with the same buffer lacking NP-40 and processed for immunofluorescence staining with monoclonal antibodies to tubulin and to GP38. Extracted cells showed the remnants of fragmented microtubules and the straight filaments were converted to shorter and curlier structures (Fig. 4*a*, compare with Fig. 2*a*). In agreement with the results obtained by fractionation of pulse-labelled cells, GP38 antigens detected by immunohistochemistry appeared to be retained after extraction. Cytoplasmic staining of infected extracted cells revealed a regular, finely dotted distribution of viral glycoproteins (Fig. 4*b*). No JV proteins were detected in control uninfected cells subjected to detergent extraction (Fig. 4*c*).

It has been reported previously that cellular actin copurifies with virus particles and is incorporated into extracellular virions (Pasian *et al.*, 1983). Thus, in an alternative attempt to study the association of viral components with the cytoskeletal network, RIPA was performed with anti-JV and anti-actin antibodies. In JV-infected cells, the main viral proteins co-precipitated with actin upon addition of anti-JV serum (Fig. 5, lane 1) and the band of immunoprecipitated actin

Table 2. Association of infectious virus particles with the cytoskeletal fraction

Vero cells were infected with JV and, at 48 h p.i., cells from one set of cultures (intact cells) were washed with PBS after collecting the supernatants and the cells were frozen (cell homogenate). Cells from another set of cultures (extracted cells) were lysed in EB containing 1% NP-40 and the cytoskeletal fraction was prepared and frozen. All samples were titrated on Vero cells. Results of two independent experiments are shown.

| Sample | Virus titre (p.f.u./ml) | |
|-------------------------|-------------------------|-------------------|
| | Experiment I | Experiment II |
| Intact cells | | |
| Supernatant | 1.1×10^5 | 8.1×10^4 |
| Cell homogenate | 1.6×10^4 | 1.9×10^4 |
| Extracted cells | | |
| Cytoskeleton homogenate | 1.4×10^2 | 2.6×10^2 |

was more intense than the weak band corresponding to actin precipitated from uninfected cells with anti-JV antibodies (Fig. 5, lane 4). To confirm the co-precipitation of actin with viral proteins, anti-JV serum was extensively adsorbed to Vero cell extracts before performing the immunoprecipitation. Under these conditions, actin-binding antibodies were removed (Fig. 5, lane 5) but actin still co-precipitated with viral proteins from JV-infected cells (Fig. 5, lane 2). Addition of anti-actin serum to JV-infected cells immunoprecipitated a significant amount of GP38, whereas only a small amount of NP was detected (Fig. 5, lane 3), confirming the results obtained in the detergent-extraction experiments shown in Fig. 3. Proteins were also precipitated from control uninfected cells with anti-actin serum (Fig. 5, lane 6). When RIPA was carried out on JV-infected cells treated with cytoskeleton-disrupting agents, viral proteins were almost undetectable, even with anti-JV antibodies (data not shown), as previously shown by immunofluorescence staining.

Infectivity linked to the cytoskeleton

Since both NP and GP38 were detected in the cytoskeletal fraction, we tested whether infective virus particles were also associated with the cytoskeleton. Vero cells were infected with JV (m.o.i. 0.1) and at 48 h p.i. one set of cultures was washed with PBS after collecting the supernatants, following which the cells were frozen in PBS and used to determine cell-associated virus. Another set of cultures was lysed in EB containing 1% NP-40, washed once in this buffer without detergent and the cytoskeletal fraction was prepared and frozen in the same buffer. Titres of all samples were determined on Vero cells. As shown in Table 2, approximately 1% of the total cell-bound infectivity was detected in the cytoskeletal fraction after

detergent extraction at 48 h p.i. No infectivity was detected in the soluble fraction after cell treatment with EB, at least at dilutions of 10^{-2} , under which conditions the viability of the cell monolayer used for the plaque assay was not affected by the detergent.

Discussion

We have shown in this report that cytoskeletal components may be involved in the assembly of JV particles in infected Vero cells. A variety of experimental approaches were required to confirm the interaction of JV components with the cytoskeleton. This interaction was first suggested by the significant decrease in virus protein expression and infectious virus production in the presence of compounds interfering specifically with the cytoskeletal network. Perturbation of the microtubule system, induced by nocodazole or colchicine, as well as nifedipine-, EGTA- or DMSO-induced alterations of microfilaments, had an inhibitory effect on JV production (Fig. 1). In previous studies, phenothiazines, which also affect microfilaments, exhibited an antiviral effect on attenuated and pathogenic strains of JV and other arenaviruses (Candurra *et al.*, 1996). It is uncertain whether these inhibitors of cytoskeleton assembly reduce virus yields by a direct effect on the cytoskeletal network or by indirectly inhibiting another cellular function. Even though the chemicals act through different mechanisms, all the compounds share the ability to produce microfilament or microtubule disruption in cells. In the present study, treatment conditions that blocked the formation of JV infective units were always associated with a drastic perturbation of the cytoskeletal network, as detected by specific immunofluorescence staining (Fig. 2). This supports the idea that this disruption was responsible for the inhibition.

The role of the cytoskeletal framework in JV replication may involve association of viral components with this cell structure. Until now this has not been explored in arenavirus-infected cells. From the high resistance of surface glycoprotein GP38 to both TX-100 and OG extraction (more than 70% insolubility) and its sensitivity to TX-100 extraction in high salt, we conclude that GP38 associates effectively with cytoskeletal components through an ionic interaction. The viral nucleocapsid protein, NP, was distributed nearly equally in the soluble and insoluble fractions, suggesting at least a temporary or dynamic interaction between this protein and cytoskeletal components that may depend on other factors or protein modifications. Morphological studies using immunofluorescent staining with monoclonal antibodies against GP38 confirmed the presence of JV glycoprotein predominantly in the insoluble fraction of extracted cells.

In an earlier report, the presence of actin in purified JV virions was described (Pasian *et al.*, 1983) and in this study, GP38 was shown to be precipitated from JV-infected cells by anti-actin antibodies. These data point to actin as the cytoskeletal component interacting with JV. However, we

cannot rule out the possibility that other cellular polypeptides may also play a role in this interaction because, as shown in Figs 1 and 2, perturbation of the microfilament and microtubule networks were equally inhibitory for JV multiplication. Further work is needed to characterize precisely the components of the cytoskeleton associated with JV proteins.

The association of viral components and the cytoskeletal network has already been demonstrated for various virus species and cellular systems. In fractionation studies, the NP and matrix (M) proteins of Sendai virus (Sanderson *et al.*, 1995), the HA protein of measles virus (Bohn *et al.*, 1986), the NP and M1 proteins of influenza A virus (Avalos *et al.*, 1997) and the Gag protein of human immunodeficiency virus type 1 (HIV-1) (Rey *et al.*, 1996) have been shown to interact with cytoskeletal elements in virus-infected cells. A possible co-localization of viral NP and cytokeratin was also observed for avian influenza virus (Arcangeletti *et al.*, 1997).

The function of the virus protein–cytoskeleton interaction in the JV infectious cycle remains unclear. In particular, cytoskeletal components appear to be involved in virus assembly and budding and release of enveloped viruses, processes that require the interaction of viral components and their transport to the cell membrane. For example, retroviruses (including HIV-1) (Hunter, 1994; Perotti *et al.*, 1996) and measles virus (Bohn *et al.*, 1986) bud from actin-enriched sites in the infected cell. For most enveloped viruses, the association of external glycoproteins with the internal nucleocapsids at the plasma membrane or in internal membranes is mediated by the M protein (Sanderson *et al.*, 1993). The critical role of this protein in the virion assembly process seems to be facilitated by the strong interaction of M protein with host cytoskeletal components (Avalos *et al.*, 1997). In contrast to most other enveloped viruses, arenaviruses do not possess an internal protein that corresponds to the M protein. The arenavirus glycoproteins are synthesized in the endoplasmic reticulum and migrate via the Golgi complex to the plasma membrane. Inhibitors of the intracellular exocytic pathway, such as brefeldin A, monensin or carbonyl cyanide *m*-chlorophenylhydrazine, block glycoprotein transport to the cell membrane and the formation of infectious virions (Damonte *et al.*, 1994; Candurra & Damonte, 1997). The only stage of arenavirus maturation that has been visualized by electron microscopy is the final process of budding at the plasma membrane (Compans, 1993) and there are no published data regarding the interactions of glycoproteins and nucleocapsids in the budding process.

Different mechanisms have been proposed for arenavirus assembly (Compans, 1993): (i) a direct interaction in the nucleocapsids between NP and the cytoplasmic tail of the glycoproteins; (ii) lateral interactions of glycoproteins to form a domain on the cytoplasmic surface of the viral envelope, which would play a role analogous to the M proteins of other viruses in binding nucleocapsids; or (iii) other virion proteins may participate in the assembly, for example the 10–14 kDa

polypeptide containing a zinc-finger motif, reported in association with NP (Iapalucci *et al.*, 1989; Salvato *et al.*, 1992), that has no known function in the virus life cycle. In any case, the lack of an M protein may lead to unusual protein interactions during virus assembly and thus a closer interaction between viral glycoproteins and cytoskeletal components may be required to target viral proteins to a common location and to attain virus maturation. To support this idea, the involvement of the cytoskeleton in JV maturation was reassessed when infectious particles were detected in detergent-extracted and washed cell fractions. This finding indicates that part of the cell-bound infectivity that remained associated with the cytoskeleton was not sensitive to detergent treatment. By contrast, when a JV suspension collected from cell supernatants was treated with extraction buffer and assayed by plaque titration, no infectivity was recovered. Thus, the interaction of the viral glycoprotein with the cytoskeleton may trigger virus assembly and exocytosis, and the consequent association of virus particles with the structural network seems to confer distinct stability properties on the budding particles. Although a similar protective effect of actin filaments towards infective virus retained on the cytoskeleton after detergent extraction has been proposed for measles virus (Bohn *et al.*, 1986), at present we cannot totally dismiss the suggestion that the plaque-forming units detected could represent infectious ribonucleoproteins.

Taken together, the biological, biochemical and morphological data reported in this paper support the notion of an active role of the host cytoskeleton in arenavirus maturation. Further work will be necessary in order to elucidate the molecular interactions between viral proteins and cytoskeletal components.

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