Nucleolar accumulation of Semliki Forest virus nucleocapsid C protein: influence of metabolic status, cytoskeleton and receptors

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Summary. The nucleolar accumulation of Semliki Forest Virus (SFV) C protein was examined as a function of intact microtubules, intact microfilaments and accessible intermediate filaments. The cytoskeletal components do not seem to play a role in directing C protein to the nucleolus but nucleolar accumulation is energy-dependent and saturable. This suggests the involvement of some receptor- (or chaperon-) interaction.

Introduction

Several viral capsid proteins have been shown to migrate to the cell nucleus,^{1–3} where they exert, as yet, poorly understood functions. Semliki Forest Virus (SFV) C protein plays a role in virus-host regulation.^{4,5} Its migration to the nucleolus³ might be responsible for this regulatory role. It has been shown recently that the nucleolar accumulation of C protein is a rapid event, and is complete before host cellular protein synthesis is shut off.⁶ The role of the cytoskeleton in intracellular transport^{7–12} has suggested a possible involvement of cytoskeletal components in C protein translocation. Furthermore, the speed of accumulation of C protein suggests the involvement of an energy-dependent process.

Material and methods

Preparation of subcellular fractions

Nucleolar fractions from SVF-infected semi-confluent Vero (African green monkey kidney) cells were prepared and separated by reducing SDS-PAGE, immunoblotted with an affinity-purified anti-C protein antiserum, and visualised by autoradiography. Briefly, cells were grown in 75-cm² plastic culture bottles (Corning, Stone) in DMEM (Biocrom; Berlin, Germany) supplemented with complement-depleted FCS (45 min at 56°C) 10%, penicillin 100 IU/ml, streptomycin 100 µg/ml and 2 mM glutamine (all Gibco, Paisley). Nucleolar fractions were prepared at 4°C by swelling the cells for 10 min in 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1.5 mM MgCl₂ (all from Merck, Darmstadt, Germany) supplemented with 50 mM iodoacetamide, aprotinin (Sigma, Munich, Germany) 20 µg/ml and 1 mM phenyl methyl sulphonyl fluoride

(PMSF; Boehringer Mannheim, Germany). Cells were disrupted in a Dounce homogeniser. The lysate was centrifuged for 8 s in an Eppendorf-Microfuge at 12000 *g*. The resultant post-nuclear supernate was termed cytoplasmic extract. The pellet was passed through a needle (0.45-mm gauge), layered on top of a 0.7-ml sucrose 45% w/v cushion in the hypotonic swelling buffer and pelleted for 30 min at 1700 *g*. After washing twice in the same buffer, the resultant nuclei were sonicated at 140 W for 1 min in 0.34 M sucrose, 0.05 mM MgCl₂, 10 mM triethanolamine (all Merck, Darmstadt, Germany), pH 7.4. The debris was centrifuged for 15 min at 12000 *g*, the nucleolar pellet was resuspended in swelling buffer and layered on top of a 0.2-ml sucrose cushion to be centrifuged as above. The pellet represented the purified nucleoli.

Quantitative Western blotting

Cells were infected with increasing concentrations of SFV (isolated from Vero cells according to the method of Kääriäinen and Gomatos¹³) and the nucleoli were isolated as described above, or nucleoli were incubated directly for 1 h with SFV C protein (isolated according to the method of Omar and Koblet¹⁴) and washed three times in swelling buffer before non-reducing PAGE on 12.5% gels¹⁵ and Western blot analysis according to the method of Towbin *et al.*¹⁶ After electroblotting the relevant C protein bands were revealed with affinity purified anti-C protein antibodies and ¹²⁵I-labelled second antibody.⁶ Autoradiographs were exposed for up to 3 weeks and the bands representing the C protein were scanned with a BioRad 620 densitometer with white light (BioRad, Richmond, CA, USA) to yield OD × mm as a measure of the amount of C protein. Serial dilutions of C protein or SFV were treated in an identical manner and served as an internal standard to correct for variations in electroblotting efficiency,

Fig. 1. a, Anti-C protein immunoblot of nuclear and cytoplasmic fractions of SFV-infected Vero cells infected for 3 h after addition of cycloheximide 500 $\mu\text{g}/\text{ml}$ in complete DMEM-medium with increasing concentrations of SFV. Lanes 1–4: standard dilutions of virus corresponding to 1, 180 pg; 2, 90 pg; 3, 45 pg; 4, 23 pg of C protein. Lanes 5–8: whole nucleolar extracts—5, control; 6–8 incubated with SFV corresponding to C protein 0.8, 1.7 and 3.9 pg/cell. Lanes 9–12: samples of cytoplasmic extracts (5%)—control (9), and incubated with SFV corresponding to C protein 0.8 (10) 1.7 (11) and 3.9 (12) pg/cell. **b,** Nucleolar association of SFV C protein as a function of cytoplasmic C protein. Densitometric evaluation of several immunoblots as shown in fig. 1a to give the correlation between cytoplasmic and nuclear C protein in response to SFV infection expressed in attograms(ag)/infected cell (1 pg = 1000 ag).

exposure time, specific radioactivity of the second and specific activity of the first antibody. By comparing $\text{OD} \times \text{mm}$ values measured with the standard curve of serial dilutions of SFV or C protein, absolute C protein concentration was quantified.

DNAase and RNAase treatment of nucleoli

Nucleolar fractions were incubated for 30 min at 37°C with a mixture of RNAase A 30 $\mu\text{g}/\text{ml}$ and DNAase 50 $\mu\text{g}/\text{ml}$ (Boehringer) in 50 mM Tris-HCl, pH 7.8, 9 mM MgCl_2 , 10 mM 2-mercaptoethanol.

Drug treatment of Vero cells before infection

To assess the role of the actin microfilaments, cells were pre-incubated for 2 h with 1 μM cytochalasin D (Fluka, Buchs, Switzerland) before infection (1 h at 4°C, 5 pfu/cell). Nucleoli were harvested 3 h after infection (hpi) and the relative C protein migration was determined by comparison with the positive control.

Nocodazole (33 μM ; Aldrich, Milwaukee, USA), a microtubule disrupting agent,^{17,18} was applied in the same manner. Pre-treatment of Vero cells with lower

Fig. 2. Nucleolar association of C protein as a function of C protein applied directly to isolated nucleoli. One Vero cell nucleus contains an average of 1.3 nucleoli as counted microscopically; 1 ag = 1000 fg (femtogram).

concentrations of nocodazole ($0.13 \mu\text{M}$) arrests the cells before mitosis.¹⁹ Since the effect is reversible,^{20, 21} cells proceed spontaneously and simultaneously through mitosis when fresh medium is replaced. Therefore, cells were treated overnight with $0.13 \mu\text{M}$ nocodazole; the medium was then changed and the Vero cells were infected as above to determine the effect of mitosis on nucleolar accumulation of C protein. Nuclei were again harvested at 3 hpi.

ATP-depleted cells were produced by incubation with medium containing 10 mM sodium azide (Merck) and 6 mM deoxyglucose (Aldrich).²² The pre-incubation time was again 2 h and nucleoli were harvested at 3 hpi.

The influence of intermediate filaments on the trafficking of C protein was revealed by electroporating²³ $10 \mu\text{l}$ of anti-vimentin antibody (Amersham) into 10^6 Vero cells in $100 \mu\text{l}$ of DMEM and $90 \mu\text{l}$ of electroporation buffer²³ at 0.4 kV and 25 μF . Cells were plated out for 3 h and infected overnight. Controls were electroporated without antibody.

Results

C protein accumulated in the nucleoli of SFV-infected Vero cells reached a saturation plateau with increasing concentrations of cytoplasmic C protein (fig. 1a and b). However, if nucleolar extracts were incubated directly with increasing amounts of C protein, saturation was achieved with about thousand-fold lower concentrations of C protein and at about hundred-fold lower nucleolar concentrations of C protein (fig. 2). The increased binding of C protein molecules to purified nucleoli *in vivo* compared to that to nucleoli isolated after simple incubation with

Table. Effect of chemical treatment on the relative incorporation of SFV C protein into nucleoli

Treatment	Nucleolar associated C protein (percentage of untreated control)*
Cytochalasin	100
Nocodazol	99
Anti-vimentin antibody	94.5
Mitosis stimulation	92
ATP-depletion	7

*OD of C-protein bands after Western blot of naturally infected cells was set to 100 % and the OD of treated cells was expressed as a percentage of these untreated controls (n = 2).

protein C could be reversed by DNAase+RNAase treatment of the nucleolar fraction of naturally infected Vero cells (data not shown).

The effect of different drugs that act on the organisation of the cytoskeleton was determined by scanning the OD of the relevant C protein bands on Western blots of nucleolar extracts and comparing them with untreated controls. The table summarises the results of these experiments. Interference with cytoskeletal components did not influence markedly migration of the C protein. In contrast, ATP depletion had a great effect on nucleolar migration. This corresponds with the observation that starving cells fail to incorporate C protein into their nucleoli (data not shown).

Discussion

The results of this study show that the cytoskeleton does not play a role in nucleolar accumulation of SFV C protein. Interestingly, the breakdown of the nuclear

membranes during mitosis did not lead to increased accumulation of C protein. Increased accumulation would have been expected because dividing 3T3-L1 cells take up gold particles coupled to the karyophilic protein nucleoplamin at a rate seven times that of growth-arrested cells.²⁴ However, ATP depletion impedes nucleolar migration of SFV C protein dramatically. This finding corresponds to results of an *in vitro* system for the study of C protein karyophilicity.³ Although the cytoskeleton does not seem to promote C protein migration, an association of C protein with the cytoskeleton during uncoating of the viral nucleocapsid, as found for vesicular stomatitis virus (VSV) N protein,²⁵ cannot be excluded. Recently, Singh and Helenius²⁶ showed that ribosomes are implicated in

the uncoating of SFV nucleocapsids and that this process is energy-independent until there is sequestration of the C protein molecules.

The finding that, *in vivo*, thousand-fold more C protein concentrations were needed to saturate the nucleoli with about 100-fold more C-protein than *in vitro* reflects the fact that additional substrates for C protein binding are present inside the infected cell both in the cytoplasmic and nucleolar fractions. The finding that nucleolar accumulation *in vivo* and *in vitro* is energy-dependent and saturable argues in favour of a receptor or chaperon being responsible for the migration process.

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