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MIAZZA, Vincent Xavier, et al.

Abstract

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Reference


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Sendai virus induced cytoplasmic actin remodeling correlates with efficient virus production

Vincent Miazza a, Geneviève Mottet-Osmana, Sergei Startchick b, Christine Chaponnierc, Laurent Rouxa,⁎

a Department of Microbiology and Molecular Medicine, Faculty of Medicine University of Geneva, CMU, 1 rue Michel-Servet, 1211 Geneva 4, Switzerland
b Bioimaging Core Facility, Faculty of Medicine University of Geneva, CMU, 1 rue Michel-Servet, 1211 Geneva 4, Switzerland
c Department of Pathology and Immunology, Faculty of Medicine University of Geneva, CMU, 1 rue Michel-Servet, 1211 Geneva 4, Switzerland

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Cytoplasmic actins have been found interacting with viral proteins and identified in virus particles. We analyzed by confocal microscopy the cytoplasmic β- and γ-actin patterns during the course of Sendai virus infections in polarized cells. We observed a spectacular remodeling of the β-cytoplasmic actin which correlated with productive viral multiplication. Conversely, suppression of M during the course of a productive infection resulted in the decrease of particle production and the absence of β-actin remodeling. As concomitant suppression of β- and γ-actins resulted as well in reduction of virus particle production, we propose that Sendai virus specifically induces actin remodeling in order to promote efficient virion production. Beta- and γ-cytoplasmic actin recruitment could substitute for that of the endosomal sorting complex required for transport (ESCRT) mobilized by other enveloped viruses but apparently not used by Sendai virus.

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Introduction

Production of progeny virus is critical for virus survival since infection of new hosts generally depends on virus particle production. Virus particle production implies the gathering of all the components at the site of assembly where an orderly arrangement takes place so to create an entity separated from the host cell. For enveloped viruses, the assembly takes place at a cellular membrane, and involves a trafficking of the viral components which congregate at the membrane coming from different cellular compartments (e.g. cytosolic, exocytic or endocytic). Once assembled, the viral complex evaginates, forms a bud that pinches off and detaches from the cellular membrane. The assembly complex forms thanks to specific interactions built in the structure of the different viral components. These also temporarily interact with various cellular partners along the route to the place of assembly (trafficking pathways, cytoskeleton) and may recruit cellular machineries that help their trafficking and the pinching off of the bud that has formed, as for instance the endosomal sorting complex required for transport system (ESCRT) (Welsch et al., 2007).

Actins are found as a family of conserved cytoskeletal proteins that play fundamental roles in nearly all aspects of eukaryotic cell biology (Furukawa and Fechheimer, 1997). In vertebrates, six actin isoforms have been described encoded by distinct genes (Vandekerckhove and Weber, 1978). Muscle actins (α-skeletal and α-cardiac and α- and γ-smooth muscle) are organized in contractile units and are tissue specific, whereas cytoplasmic β- and γ-actins are ubiquitous and essential for cell survival (Karabinos et al., 2001). All actin isoforms exhibit similar primary sequences, which slightly differ at N-terminal amino acids. In particular, β- and γ-actins differ only by four residues at positions 1, 2, 3 and 9. Despite the high similarity, specific functions have been suggested, mainly for muscle actin isoforms (Lambrechts et al., 2004; Chaponnier and Gabbiani, 2004). For cytoplasmic actins, the definition of distinctive functions has been hampered by the inability to document their subcellular localization due to the unavailability of specific γ-actin antibodies.

Cytoplasmic actins are part of the various cellular components that have been found involved in virus life cycle, at early steps, during transport of the viral genome to the site of genetic expression and genome replication, or, at later steps, during formation of new virus particles (for a general textbook about viral multiplication cycle, see Flint et al., 2004). Virus infections are generally altering infected cell shape. They can induce a transient increase in actin polymerization as in the case of human respiratory syncytial virus [HRSV; (Ulloa et al., 1998)], and also remodeling of actin filament pattern. Growing actin filaments have been observed in budding virus particles (Bohn et al., 1986), and many Paramyxovirus particles have been shown to contain actin, such as measles virus, mumps virus, Sendai virus (SeV),
Newcastle disease virus (NDV) and HRSV (Fagraeus et al., 1978; Ulloa et al., 1998; also reviewed in Takimoto and Portner, 2004). Disruption of actin polymerization by the drug cytochalasin-D has generally a deleterious effect on virus particle shedding (Burke et al., 1998; Stallcup et al., 1983), alluding to the fact that polymerized actin plays a positive role in virus production. Despite this body of descriptive work, little is known about specific functions of the cytoplasmic actins, and in particular about a possible differential role for β- and γ-actins.

SeV is a prototype for the Paramyxovirus family. Newly formed virus particles bud from the plasma membrane and in polarized MDCK cells, budding occurs exclusively from the apical side (Boulan and Sabatini, 1978 and Fouillot-Coriou and Roux, unpublished). Early on, it was recognized to include actin in its particle (Lamb et al., 1976; Orvell, 1978; Fagraeus et al., 1978), and later, SeV matrix protein (SeV-M) was shown to directly interact with actin (Giuffre et al., 1982). More recently, it has been suggested that the SeV-M C-terminus contains an actin binding domain and, since the deletion of this motif prevents M shedding into virus-like particles, it was postulated that actin is actively involved in M shedding (Takimoto et al., 2001).

By taking advantage of newly prepared monoclonal antibodies against the cytoplasmic β- and γ-actin isoforms (Dugina et al., 2009), we investigated by confocal microscopy analysis the effect of SeV infection on the pattern of cytoplasmic actins in MDCK cells.

**Results**

Cytoplasmic actin remodeling in SeV infected cells

The observation of the cytoplasmic actin cytoskeleton in polarized MDCK cells revealed a pattern for the two isoforms, β- and γ-actins, previously described for polarized epithelial cells (Dugina et al., 2009). β-actin is mainly distributed into bundles at cell–cell contacts (Fig. 1Aa). This preferential accumulation at the cell junctions is clearly shown by the Z sections. Bundles of γ-actin at the cell to cell junctions are very thin, as γ-actin appeared more evenly distributed in projection images (Fig. 1Ab) and, was rather seen under the apical plasma membrane, in Z sections. Little co-localization of the two isoforms was evident, especially at the apical pole of the cell, due to this different segregation (Figs. 1Ac, Z section and Ad, XY apical single section).

SeV infection resulted in spectacular morphological changes of the actin cytoskeleton. In projection images, bundles at the cell–cell junctions were no longer visible. Both β- and γ-actins, appeared in a punctuated pattern with tiny spots of higher concentration (Fig. 1Ae and f). The β-actin was found to re-localize at the apical cellular membrane (Fig. 1Ae, Z section). The γ-actin appeared less homogenously distributed at the apical membrane and Z section showed streaks of higher concentration (Fig. 1Af, Z section). A clear co-localization of β- and γ-actins was evidenced at the apical plasma membrane by the yellow streaks in the Z section in Fig. 1Ag and by the yellow staining in the single apical XY section (Fig. 1Ah).

The observations in Fig. 1A were made at 18 h post infection when viral macro molecules exponentially accumulate and when virus particles are actively produced. To investigate whether the changes in actin cytoskeleton pattern could simply result from the virus entry step, infected cells were observed much earlier (Fig. 1B). At 1, or even 4 h post infection, the β-actin was still mainly concentrated at the basal cell to cell junctions, as evidenced by the projection images and the Z sections (Fig. 1B, images b and c, β-CYA). The γ-actin even distribution at the apical cell membrane was not perturbed (Fig. 1B, images b and c, γ-CYA). In conclusion, SeV infection is accompanied by a drastic cytoplasmic actin remodeling that appeared not due to the infecting process, but required active viral protein synthesis.

Actin remodeling correlates with a productive infection

To verify whether active viral macromolecules synthesis was sufficient to promote actin remodeling, we took advantage of the features of mixed virus infections. These are established by a mixture of defective interfering particles (DI) and infectious virions [SeVwt helper virus, called in the context standard virus (St)]. Under conditions of mixed virus (St + DI) infections performed at high multiplicity ( moi=100), all infected cells survive, in contrast to the destruction of the cell monolayer following infection with helper SeVwt only (Roux and Holland, 1979). The SeV St + DI infected cells contain quantitatively as many viral nucleocapsids as SeVwt infected cells. They, however, exhibit a rapid M protein turnover associated with a drastic decrease of virion production (Fig. 2B and Roux and Waldvogel, 1981; Roux and Waldvogel, 1982; Tuffereau and Roux, 1988). These St + DI infected cells, which readily establish a persistent infection, represent therefore a situation where the viral macromolecules are normally synthesized in conjunction with almost no virus particle production.

Actin cytoskeleton observation of such mixed virus infected cells showed no significant change in its pattern relative to the mock infection conditions (compare in Fig. 2A, images b,c,d to f,g,h). This pattern, however, was quite different from that of the productive infection (Fig. 2A, images j,k,l, which corresponds to Fig. 1A, images e, f,g). This indicates that SeV multiplication cycle promotes actin remodeling in conjunction with virus particle production.

**Statistical analysis of actin remodeling**

A statistical validation of the cytoplasmic actin pattern was performed with Metamorph/MetaXpress software (Molecular Devices, Sunnyvale, CA) to measure the cellular staining intensity along the Z axis of cellular stacks. The image analysis was performed as follows. The stack of images taken for Z positions from –3 to +3 was processed to obtain a “best focus” image of actins that reflects the best cell structure for basic definition of borders. On the resulting image, outlines of cells were provided manually, following the actin pattern. Each outline corresponds to a border between what was called a cell interior and its border (corresponding in fact to the actin bundle) as shown on Fig. 3A. When border between adjacent cells is rather wide, the interior border should still follow the frontier between dark interior and bright border between cells. In the next step, based on the indicated border, analysis masks were built for cell interior and border. The interior mask was built on the indicated border itself (Fig. 3B). To build the outer border mask, the image in Fig. 3b was dilated by 16 pixels. The resulting dilated image was combined with original interior mask by XOR operation to obtain the exterior mask as shown in the Fig. 3C. In the next step, scans of the whole stack of images were obtained and the measure of total intensity in the areas delimited by interior and exterior masks was performed. In Figs. 3D and E, the measured areas of, respectively, the inner intensity and the intensity of the borders of the cells are shown. The total intensity for both measured areas was reported in spreadsheet for each plane of the stack. The resulting values were than plotted against the plane number. With such an analysis, one would predict that the ratios of the inner surface/outer surface β-actin staining intensity increase after SeV infection over the mock control at the apical cell pole. Fig. 4A shows that it is indeed the case. For the mock infected cells (blue markers and line), the inner/outer ratios remained below 1 over the entire cell stack (2 section axis, 0 to 1, bottom to top), in contrast to the SeV infected cells where the ratios clearly exhibit a trend raising to 3 (SeV infected, markers and red line). Note that during a mixed virus infection (St+DI infected, markers and green line), the analysis confirmed the lack of movement of the β-actin toward the apical cell pole, this in conditions where the virus particle production is impaired.
The γ-actin analysis (Fig. 4B) leads to less conclusive results, where the trend line of the ratios derived from SeV infected cells differed less from the mock infected cell values. This was not unexpected, since the observed remodeling of γ-actin was less spatially different (see Fig. 1A and f). The Metamorph analysis was not meant to discriminate between a diffuse apical staining and streaks of more intense staining at the apical pole as shown in Fig. 1A (b–f). Because our statistical analysis could not discriminate γ-actin remodeling, we concentrated our further study on β-actin only.

SeV-M protein involvement in the actin remodeling

SeV-M protein is recognized as the central organizer of virus particle formation. When M is unstable (as in St + DI infections, Roux and Waldvogel, 1982) or suppressed (Mottet-Osman et al., 2007), virus particle production is significantly decreased. In MDCK cells, M protein is found at the cell apical pole, where virus production takes place exclusively, in co-localization with β-actin (Fig. 5A) and γ-actin (not shown). Moreover, ectopic expression of M results in drastic morphological cellular changes as exemplified by the images in Fig. 5B, alluding to its ability to perturb the cytoplasmic actin network, mainly responsible for the cell shape. In fact, in these cells the actin pattern is simply no longer visible (not shown). Note that, expression of a mutant M protein (M30) known to be incompetent for virus particle production (Mottet et al., 1996 and unpublished), has lost this disrupting property. Based on this observation, we hypothesized that M could be directly involved in the actin remodeling observed in infected cells.

To verify this hypothesis, MDCK cell lines were derived that constitutively express a siRNA targeted to a green fluorescent protein (GFP) gene sequence (MDCK-siRNA), as was already described for a BHK-siRNA cell line (Mottet-Osman et al., 2007). These cells, and a control cell line, were further infected with a recombinant SeV (SeVMgfpt) harboring the GFP target sequence (gfpt) in the 3′ untranslated region of the M mRNA (Mottet-Osman et al., 2007). SeV-Mgfpt developed, in the MDCK cells, an infection comparable to that of SeVwt, evidenced by similar levels of viral proteins (Fig. 6A, cellular extracts, compare lane 3 and 4 to lanes 1 and 2, N, HN and F0 proteins). Moreover, in MDCK control cells (siRNA-, lanes 1 and 3), comparable virus particle production was observed as well (35S-viral particle). In MDCK cells expressing the siRNA (siRNA+ lanes), the M protein was suppressed by more than 98% (cellular extracts, lane 4).

Fig. 1. Actin cytoskeleton remodeling following productive Sendai virus infection. MDCK cells, grown on glass coverslips, were mock infected or infected with SeVwt (moi = 3). A) Eighteen hours post infection, cells were fixed, permeabilized and processed for indirect immuno-fluorescence staining using anti-β-actin (a,e) or anti-γ-actin (b,f) coupled, respectively, to FITC and TRITC secondary antibodies. a,b,c,e,f,g: Projection confocal microscopy images. c,g: Merges of the corresponding two stainings. Below each image, corresponding Z sections. d,h: Single XY sections (merge images) with dotted white lines in c,g indicating the level of the sections. B) As in A), except that the cells, infected at moi=100, were fixed at 1 h or 4 h post infection, and that anti-N antibodies were also used (coupled to Cyan-5). The squared panels represent projection confocal microscopy images, with below corresponding Z sections. Bars = 10 μm. N: Z section visualized with anti-N antibodies, showing the incoming nucleocapsid protein.
resulting in a corresponding virus particle production decrease. With this approach, it became possible to analyze, in the context of a regular infection, the effect of M suppression on the \( \beta \)-actin pattern. Fig. 6B presents images of MDCK cells illustrative of the \( \beta \)-actin pattern in cell middle XY sections (5/9) or in sections closer to the apical pole (top section, 8/9). Fig. 6Bd shows that the \( \beta \)-actin remodeling is reproduced in conditions of normal infection with the top section showing a marked accumulation of \( \beta \)-actin. In conditions of M protein suppression, however (Fig. 6Bh), the concentration of \( \beta \)-actin at the apical pole is not observed. These observations are confirmed by the Metamorph analysis program, which indeed shows that in MDBK-siRNA cells the ratios of average internal/external \( \beta \)-actin staining intensities remain close to that of the mock infected cells (Fig. 6C, Mgpt-siRNA, red dashed curve). Fig. 6D shows that siRNA expressing MDCK cells allows \( \beta \)-actin remodeling in conditions where M protein is present, promoting a normal virion production (SeV-siRNA, blue dashed line). These results provide the demonstration that the M protein is involved in the remodeling of the \( \beta \)-actin pattern in the infected cells in conjunction with virion production.

Involvement of \( \beta \)- and \( \gamma \)-actins in SeV particle production

In general, virus evolution leads to sequestration of cellular functions in the most appropriate way, such that what is not needed for virus multiplication and transmission is generally not mobilized by the viral functions. In the present case, the mobilization of the
cytoplasmic actins (by likely the viral M protein) to the site of virus particle formation strongly suggests a participation of these factors in virus particle formation and/or production. To investigate this putative role of the cytoplasmic actins, we used once more the siRNA silencing technology to suppress the \( \beta \)- and \( \gamma \)-actins individually or in concert (Fig. 7). A549 cells were used rather than MDCK cells, since these latter’s were not susceptible enough to siRNA transfection. Although A549 responded better, they were still responding poorly, such that individual suppression never reached more than 50% (Figs. 7A, lanes 2 and 6 for \( \beta \)-actin, and 6B, lanes 3 and 7 for \( \gamma \)-actin). It appeared, then, that suppression of one isoform often created a compensatory increase in the other (see for instance in Fig. 7A, upon suppression of \( \beta \)-actin, increase in \( \gamma \)-actin, lanes 3 and 7, and conversely, upon suppression of \( \gamma \)-actin, Fig. 7B, lanes 3 and 7, increase in \( \beta \)-actin, lanes 2 and 6). Finally, this compensatory effect presumably prevented apparent suppression of \( \beta \)-actin when both isoforms were suppressed in concert (see lanes 4 and 8, in Fig. 7A). In spite of these drawbacks, suppression had an unexpected effect on virus particle production as shown in Figs. 7D and F. Particularly upon concomitant \( \beta \)-\( \gamma \) actin suppression, an 80% decrease in virus production was consistently observed. This clear effect appeared surprising in view of the poor \( \beta \)-\( \gamma \) actin suppression shown in Figs. 7A and B. A similar paradox was reported in Dugina et al. (2009) in which suppression of \( \gamma \)-actin was barely observed by Western blotting, while abolition of cell motility (driven by \( \gamma \)-actin) was spectacular. This, plus the compensatory effect mentioned earlier (and observed as well by Dugina et al., 2009), suggests that suppression may disturb some dynamic process that cannot be recorded by Western blotting. Note that suppression of cytoplasmic actins had no deleterious effect on the virus multiplication efficiency as evidenced by a comparable level of \( {^{35}}S \)-radiolabeled N proteins (rising above the cellular protein background) in the cellular extracts of the different infections (Fig. 7C). In conclusion, these data support the role of cytoplasmic actins in SeV particle production. This conclusion received further support through sensitivity of virus production to cytochalasin-D (Fig. 7E), a drug known to disrupt actin filaments (Cooper, 1987).

Discussion

Upon SeV acute infection of polarized cells, \( \beta \)-actin and \( \gamma \)-actin patterns are significantly altered. The most spectacular remodeling concerns \( \beta \)-actin which is transferred from the baso-lateral membrane to the apical side of the plasma membrane. The change in \( \gamma \)-actin is more subtle, but not less pronounced. From a smooth distribution at the apical membrane, it exhibits a more heterogeneous pattern with streaks of higher concentration which co-localize in Z
sections with the β-actin. This remodeling requires the development of the viral multiplication cycle as opposed to the only process of infection, since it is not observed early after infection, at a time when the virus load is mainly composed of the infecting virus. Furthermore, this actin cytoskeleton remodeling is dependent on a productive infection, as it was not observed in mixed virus (St+DI) infected cells where the viral particle production is significantly impaired. As suppression of the viral matrix M protein in the context of an infection reduces β-actin remodeling and in view of the negative effect on virus production that takes place upon β- and γ-actin suppression, we propose that the remodeling participates in the active virus particle formation and production.

An active role of cytoplasmic actins during a virus multiplication cycle has already been reported for the human respiratory syncytial virus (HRSV), another member of the Paramyxovirus family (Ulloa et al., 1998). In this case, actin was equally found in virus particles (what is the case also for SeV, not shown) and increased actin polymerization (mainly β-actin) was observed upon infection. This increased polymerization could be related to formation of cytoplasmic extension containing β-actin and virus particles. These observations were interpreted as an indication that HRSV has developed an actin-based motility system participating in virus cell to cell transfer. The focus of the two papers was clearly different as were the experimental approaches, but in the end the two studies point to an involvement of the cytoplasmic actins in the viral multiplication cycle.

The fact that there appears to be a strong correlation between β-actin remodeling and virus particle production, and the fact that the level of virus particle production is adjusted by that of the viral M protein, constitute a strong argument to point to M as an effector of actin remodeling. This goes in line with the past report of physical interaction between M and actin (Giuffre et al., 1982). In our hand, however, it was not possible to confirm this direct interaction by co-

![Fig. 5. SeV-M cellular localization in relation with the actin cytoskeleton and effect upon ectopic expression. MDCK cells, grown on glass coverslips, were mock infected or infected with rSeV-HA-M (moi = 3), or transfected with EBS plasmids expressing HA-Mwt or HA-M30. A) Eighteen hours post infection, the cells were fixed and processed for confocal microscopy as in Fig. 1. β-actin was visualized with an Alexa488-conjugated goat anti-mouse IgG while HA-M was visualized using a TRITC-conjugated donkey anti-rat IgG. a to f: Projection images of mock or SeV infected, with below a Z section of each image. g: Top plane section of f. B) Forty hours post transfection cells were prepared for confocal microscopy and in A. HA-Mwt and HA-M30, three examples of cells expressing HA-Mwt or HA-M30, respectively.](image-url)
immunoprecipitation (not shown) alluding to the fact that M may act indirectly. A faint interaction was detected between β-actin and the viral glycoprotein HN (not shown). An indirect action of M through HN (known to interact with M), however, seems unlikely, since normal virus production takes place when HN is suppressed to more than 95% (Gosselin-Grenet et al., 2010). Whether direct or indirect, the action of M on the cytoskeleton is further evidenced by its ability to totally perturb the cell morphology upon ectopic expression. This activity, now, is independent of any other viral protein, and this may explain its erratic effect.

The ability to specifically visualize the β- and γ-actins led to propose that the two isoforms perform different functions, based, in part, on their different subcellular localization (Dugina et al., 2009). In mock infected MDCK cells, the spatial distribution of the two isoforms differs, as does the remodeling of the two isoforms after infection. Is this the sign that the β- and γ-actins play different roles in the SeV production? The fact that both isoforms congregate at the apical pole should not exclude a different task. Recruitment of actin could represent a mean for SeV to promote vesicle formation, independent of the ESCRT machinery (Gosselin-Grenet et al., 2007). In the same way that HN and F participate in pulling and M in pushing the bud (Chen and Lamb, 2008), β- and γ-actins could participate in the former and latter task, respectively. This would correspond to the protrusion and contraction actions of, respectively the γ- and β-actin during cell migration and division (Dugina et al., 2009). Alternatively, Chen and Lamb (2008) in their review about the mechanism of enveloped virus budding, mention abscission during cytokinesis as a process that can be assimilated to the detachment of the viral bud. Similarly, Dugina et al. (2009) assign to the β-actin a role in cytokinesis where it forms the contractile ring during telophase. Applied to virus particle formation, β-actin could help the pinching off of the bud and γ-actin could act in the pushing of the bud. These proposed mechanisms of action should not obliterate a more straightforward role of the actin in the transport of the cytoplasmic viral constituents to the site of viral assembly. This would imply M interacting with other viral partners as well (i.e. nucleocapsids, cytoplasmic portion of a glycoprotein), since M by itself is clearly having a “wild” effect.

Fig. 6. Analysis of β-actin pattern in conditions of M protein suppression. MDCK cell samples constitutively expressing a siRNA directed against the GFP target sequence (gfpt, see Materials and methods) and control MDCK cell samples were infected with SeVwt or SeVMgfpt, a virus recombinant whose M gene carries the siRNA target sequence. A. Infected cells were radiolabeled from 15 to 24 h post infection. Cell extracts and virus particles in the supernatants were processed as described in Materials and methods. Cell extracts were analyzed by Western blots using antibodies to HN, F0, N and M. 35S-virions were directly analyzed by PAGE and revealed by enhanced fluorography. B. MDCK control and -siRNA, infected or not with SeVMgfpt, were grown in cover slips and processed for confocal microscopy analysis as in Fig. 1, except that Alexa568 coupled anti-mouse β-actin antibody was used. The confocal images were all taken with 9 plane sections. Examples of middle (plane 5) or top (plane 8) sections are shown. C. Metamorph analysis of the confocal images is performed as described in Fig. 4, except that, since all the images have been taken with the same number of planes, the graph can be presented with mean values and deviations from the mean. Mock: 3 image fields, 60 cells analyzed. Mock-siRNA: 2, 61 cells analyzed. Mgfpt: MDCK cell control infected with SeVMgfpt, 3 image fields, 53 cells. Mgfpt-siRNA: MDCK-siRNA cells infected with SeVMgfpt, 5 field images, 78 cells. D. As in C, but infections were made with SeVwt. Mock: 5 image fields, 55 cells analyzed. Mock-siRNA: 4, 82 cells analyzed. SeV: 4 image fields, 44 cells. SeV-siRNA: 3 field images, 47 cells.
In conclusion, with the aid of specific antibodies that allow the distinction between $\beta$- and $\gamma$-cytoplasmic actins, we describe here profound modifications in the actin cytoskeleton that follow SeV infection. If the presence of actin in virus particles has been described for quite some time, if cytoskeleton pattern modification has been recognized before and if actin was already shown to interact with Paramyxovirus M proteins, to our knowledge, this represents the first published evidence for specific participation of M in actin remodeling with an effect on virion production.

Materials and methods

Cells and treatment

All cells were grown at 37 °C under 5% CO2 atmosphere. MDCK and A549 cells were grown in Dulbecco modified Eagle’s medium (DMEM) supplemented with 5% fetal calf serum (FCS). For siRNA suppression experiments, A549 cells were grown in Dulbecco modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). MDCK cells were either DMSO treated or cytochalasin-D (Sigma) treated at 2 h post infection (pi). MDCK-siRNA and -NGFR (control) were prepared exactly as described by Mottet-Osman et al. (2007) for BHK-siRNA and -NGFR.

Antibodies

Mouse monoclonal antibodies against the cytoplasmic $\beta$- and $\gamma$-actin isoforms, mAb 4C2 (IgG1) and mAb 2A3 (IgG2b) respectively, were obtained and characterized as described in Dugina et al. (2009). Antibodies used in this study include also an anti-SeV-N (a rabbit serum raised against SDS-denatured N protein, $\alpha$-NSDS), anti-SeV-M (MAb 383 obtained from Claes Örvell, Laboratory of Clinical Virology, Huddinge Hospital, Huddinge, Sweden), and anti-influenza HA epitope (anti-HA) rat IgG1 monoclonal antibody (clone 3F10, Roche).

Plasmids

The pEBS-H-AM30 was prepared by the replacement of the HA-M gene of pEBS-HA-M (Gosselin-Genet et al., 2007) with HA-M30 (Mottet et al., 1999), using SacI and KpnI restriction sites.

Viruses

Sendai virus H strain (SeVwt) was prepared and characterized as before (Roux and Holland, 1979). Recombinant Sendai virus GP42 was a kind gift of Daniel Kolakofsky and was published (Garcin et al., 1998). The recombinant SeV-HA-M was recovered as described...
previously (Fouillot-Coriou and Roux, 2000) from pFL4-HA-M plasmid. In order to obtain persistently infected cells, confluent MDCK cells were infected at a high multiplicity of infection (moi of 100) with DII4 stocks (Calain et al., 1992) for 48 h. The surviving cells were then split and grown as described for normal uninfected MDCK cells. Recombinant rSeV-Mgfpt rescue was previously described (Mottet-Osman et al., 2007).

**Immune-fluorescence and confocal Laser Scanning Microscopy**

For immune-fluorescence staining, cells grown on glass coverslips were rinsed with DMEM containing 20 mM HEPES at 37 °C, fixed in 1% PFA in pre-warmed DMEM for 30 min, followed by 3 min treatment with methanol at −20 °C. Cells were subsequently incubated with the different primary antibodies, followed by incubation with appropriate secondary antibodies: FITC-conjugated goat anti-mouse IgG1 and TRITC-conjugated goat anti-mouse IgG2b (Southern Biotechnology Associates Inc., Birmingham, AL), for respectively the anti-actin isoforms antibodies; Alexa488-conjugated goat anti-mouse IgG and Alexa568-conjugated goat anti-mouse IgG (Molecular Probes); Cy3-conjugated goat anti-rabbit, FITC-conjugated donkey anti-rat IgG and TRITC-conjugated donkey anti-rat IgG (Jackson); DAPI was used for nuclear staining. After several washes in PBS, cells were mounted in Mowiol 4–88 (Calbiochem, 475904). Images were acquired using a confocal microscope (LSM510, Carl Zeiss, Oberko-chen, Germany) equipped with oil immersion objectives (Plan-Neofluar 40x/1.3 and Plan-Apochromat 63x/1.4, Zeiss). A sequential scanning for different channels (multitrack function) was selected to avoid crosstalk between fluorescent dyes. For serial optical section stacks with Z-step of 0.3–0.5 μm were collected. Stacks and Z sections were collected and processed using LSM 510 3D software for 3D reconstruction of the cells. Images were processed using Adobe Photoshop software.

**Transfections**

A549 cells in 35 mm-diameter dishes were transfected with 100 nM of human β-actin siRNA (target sequence: AATGAAGATCAA-GATCATTGC, from Qiagen) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions, 48 h before SeV infection. Transfection efficiency ≥90% was estimated using BLOCK-IT™ (Invitrogen). MDCK cells on coverslips in 24-well dishes were transfected using ESCORT™ Transfection Reagent (Sigma-Aldrich) according to the manufacturer’s instructions with 1 μg of the different pEBs plasmids.

**Virus infections and radiolabeling**

Infections with Sendai virus (SeV) and its various recombinants were performed at 33 °C. Virus stocks were adequately diluted (moi) and laid over monolayers of MDCK cells. Recombinant rSeV-Mgfpt rescue was previously described (Mottet-Osman et al., 2007).

**Virus particles and cellular extracts**

The virus particles were isolated from the clarified cell supernatants by centrifugation through a 25% glycerol cushion (Beckman SW55 rotor, 2 h, 50,000 rpm, 4 °C) and directly resuspended in 1% β-

mercaptoethanol, 2% sodium dodecyl sulfate, 80 mM Tris–HCl pH 6.8, 10% glycerol and 0.005% bromphenol blue (SDS sample buffer). Infected cells were directly resuspended in 150 μl of SDS sample buffer and sonicated for 10 s (Branson Sonic Sonifer B-12, lowest speed).

**SDS-PAGE analyses, Western blotting, autoradiography and quantification**

The total cellular extracts and the virus were analyzed by SDS-PAGE. After electrophoresis, the proteins were transferred using a semi-dry system onto polyvinylidene difluoride membranes (Millipore). Blots were then incubated with specific antibodies, followed by the appropriate horseradish peroxidase (HRP)-coupled secondary antibodies. Protein detection was performed by using the enhanced chemiluminescence system (Amersham Biosciences). The radiola-

beled virus particle samples were analyzed by SDS-PAGE and the gels, treated for enhanced fluorography (DMSO plus 5% 2,5-diphenylox-

azol, PPO), were exposed to Hyperfilm MP (Amersham Biosciences). The autoradiographs were scanned and the intensity of the replication signal was measured using ONE-Dscan version 1.0 (Scananalytics; CSP).

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**References**


