A role for actin in the polarized release of rotavirus

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ABSTRACT
Rotaviruses are characterized by a polarized release from the apical side of infected enterocytes and the rotavirus VP4 spike protein specifically binds to the actin network at the apical pole of differentiated enterocytic cells. To determine the functional consequences of this VP4-actin interaction, fluorescence recovery after photobleaching experiments were carried out to measure the diffusional mobility of VP4 associated with the microfilaments. Results show that VP4 binds to barbed ends of microfilaments by using actin treadmilling. Actin treadmilling inhibition results in the loss of rotavirus apical preferential release suggesting a major role for actin in polarized rotavirus release.
Rotaviruses belong to *Reoviridae* family and are a major cause of infantile viral diarrhoea worldwide. The non enveloped particles contain a segmented double stranded RNA genome and rotaviruses are characterized by their enteric tropism. In enterocytic differentiated cells, virions reach the apical pole through an atypical trafficking pathway where they are released, without cell lysis (6). Our previous data also shows that the spike protein VP4 specifically interact with and strongly remodel actin bundles of the brush border both in infected and transfected polarized Caco-2 cells (5). Since the structural VP4 protein is located at the most peripheral position of the virus, it could support molecular signals for virus targeting. In the present work we hypothesized that apical interaction between VP4 and actin may account for the apical release of progeny virions. We found that VP4 interacts with actin through a treadmilling process. Blocking treadmilling using Jasplakinolide resulted in random release of rotavirus in polarized intestinal cells.

**Analysis of the dynamic of the association of the protein EGFP-VP4 with actin microfilaments**

In order to determine the dynamics of VP4 association with the actin cytoskeleton, fluorescence recovery after photobleaching (FRAP) experiments were performed. Cos-7 cells were transiently transfected with a plasmid encoding EGFP-VP4 as previously described (5). Twenty four hours later, EGFP-VP4 expressing Cos-7 living cells were observed by confocal microscopy (5). Figure 1 illustrates how a subset of EGFP-VP4 labelled filaments may be photobleached by focusing high intensity blue laser light flash. The EGFP-VP4 fluorescence recovery was monitored with low power laser excitation and data show that EGFP-VP4 mobility on the actin filament or from the cytoplasm to the filament is a rather slow phenomenon, since the mobility is 0.4 µm/min as calculated from data recorded from the experiments displayed in figures 1 and 2.

Displacement of molecules along actin filaments frequently depends on myosin II motor activity. To establish if the mobility of EGFP-VP4 is dependent on such a motor activity, FRAP experiments were performed in EGFP-VP4 expressing Cos-7 living cells treated or not for 30 minutes with 20µM ML-7, an inhibitor of myosin II phosphorylation. As shown in figure 2, ML-7 did not influence EGFP-VP4 mobility on the actin cytoskeleton.

The efficiency of ML-7 treatment was checked by testing the phosphorylation state of myosin II by western blots shown in figure 2 B. Cells were again treated with ML-7 and with 10mM of diperoxovanadate, an inducer of myosin II phosphorylation. The efficiency of the inhibition of myosin II
phosphorylation with ML-7 confirmed that VP4 does not use myosin II motors to progress along the actin network. These experiments show that VP4 is specifically tethered to actin bundles with high recovery and reduced mobility a result in agreement with the low observed diffusional mobility compared to the rapid myosin II mobility (2 to 7µm/s) (7).

The mobility of EGFP-VP4 on the actin cytoskeleton is sensitive to jasplakinolide

Another possible mechanism supporting protein mobility on actin results from actin treadmilling, a process in which actin filament length remains approximately constant, but actin monomers preferentially add at the barbed ends and dissociate from the pointed ends of filaments. This oriented renewal of actin within microfilament causes a treadmilling concerning both actin monomers and actin binding proteins. Jasplakinolide is a cyclic peptide isolated from a marine sponge which binds to and stabilizes filamentous actin, inducing a blockade of actin treadmilling. The lateral mobility speed of EGFP-VP4 being compatible with an actin treadmilling process, FRAP experiments were performed on cells treated with jasplakinolide. In the meantime, cell treatment with 1µM jasplakinolide during 30 min did not modify the steady state appearance of actin bundles (not shown).

As shown in figure 2, the recovery of EGFP-VP4 fluorescence was reduced by jasplakinolide treatment of EGFP-VP4 expressing Cos-7 living cells, suggesting that EGFP-VP4 is an F-actin binding protein that undergoes the actin treadmilling to progress along the actin network.

Note that VP4 mobility is quite coherent with actin treadmilling velocity that is about 0.35 µm/min (4). It could be hypothesized that the inhibition of VP4 mobility would be linked to a competition of VP4 and jasplakinolide for actin microfilaments binding rather than to the jasplakinolide effect on actin treadmilling. However, jasplakinolide shares the same actin binding site that phalloidin, another actin-binding drug (2) and we previously revealed that in Cos-7 cells, VP4 and phalloidin are not competitive for F-actin binding even in presence of large amount of VP4 (5).

Apical release of rotavirus is sensitive to jasplakinolide

The above described effect of Jasplakinolide on actin-mediated VP4 mobility were studied in the context of rotavirus infection of intestinal Caco-2 cells. It was checked that neither rotavirus infection nor jasplakinolide altered the integrity of tight epithelial Caco-2 cells monolayers grown on membrane filters. In the presence of rotavirus and/or jasplakinolide throughout 18 hours, cell polarity and tight
junctions remained unaffected as attested by the stability of transepithelial electrical resistance (control cells: 210 ± 32 Ω.cm²; rotavirus infected cells: 206 ± 35 Ω.cm²; jasplakinolide treated rotavirus infected cells: 200 ± 19 Ω.cm²). Moreover, the lactate dehydrogenase enzymatic activity remained intracellular (96% +/- 1.5 of the total activity), indicating an absence of significant cell lysis.

The efficiency of jasplakinolide treatment in Caco-2 cells was controlled by measuring high density actin polymers. Caco-2 cells treated or not with 1µM jasplakinolide for 16 hours were subjected to Triton X-100 ice cold detergent extraction, then cell lysates were analysed by sucrose gradient sedimentation as previously described (3). Sucrose gradients were recovered in 12 fractions from the top to the bottom, the thirteenth fraction corresponding to the resuspended pellet. The actin content of each fraction was analysed by western blot. As expected, jasplakinolide treatment induced a stabilization of high density actin polymers as shown by the high actin content of the pellet (figure 3).

In order to test if the actin-based VP4 mobility was essential for virus production, infectious particles released in the presence of jasplakinolide were quantified. Filter grown differentiated Caco-2 cells were infected with RF strain rotavirus (5). Virus release was quantified at 18 hours post infection, from cells treated or not for 16 hours with 1µM jasplakinolide (drug treatment was started 2 hours post infection). As shown in figure 4, jasplakinolide strongly perturbed rotavirus release from Caco-2 cells, since the drug was able to cause a loss of rotavirus polarized release.

The present experiments are the first to explore the functional consequences of VP4 interaction with actin. The results indeed show that association of VP4 with microfilaments is sensitive to the renewal of actin and that VP4 binding to microfilaments is initiated from barbed ends upon renewal of actin monomers. This means that the actin cytoskeleton likely plays an important role in the exocytosis process of progeny virions. Since the total virus production from jasplakinolide treated or untreated Caco-2 cells remained unchanged, actin-based VP4 mobility does not appear to directly control assembly of VP4 with immature virions. In the meantime, actin treadmilling seems to be essential to ensure polarized release of rotavirus.
FIGURE 1. EGFP-VP4 FRAP experiment on the actin cytoskeleton. Cos-7 cells were transiently transfected with a plasmid encoding EGFP-VP4. At 24h post-transfection, living Cos-7 cells expressing EGFP-VP4 were observed with a confocal microscope. A part of a EGFP-VP4 labelled filament was photobleached with a high power laser excitation (photobleached region is indicated with a white arrow). The EGPF-VP4 fluorescence recovery was observed for 20 minutes after photobleaching. Image gallery displays projections of all 0.3µm xy focal sections taken throughout the height of a VP4-EGFP expressing cell. EGFP-VP4 fluorescence images were acquired 2 minutes before photobleaching (Tb-2min), and then after the photobleaching: 1 minute (Tb+1min), 6 minutes (Tb+6min), 12 minutes (Tb+12min), 15 minutes (Tb+15min) et 20 minutes (Tb+20min). Scale bar = 10µm. This figure is representative of three independent experiments.

FIGURE 2. Panel A: quantification of EGFP-VP4 FRAP experiments on the actin cytoskeleton. Quantification was obtained from the measurement of fluorescence intensity from fluorescence image data using METAMORPH software. The analyzed data were from experiments similar to the one presented in figure 1. Graphic displays the EGFP-VP4 fluorescence recovery in the photobleached region expressed as a percentage compared to the fluorescence before photobleaching. Fluorescence intensities were standardized by comparison to fluorescence intensities from an unphotobleached part of the cell in order to correct the spontaneous photobleaching, which occurs even with low power laser excitation during image acquisition. Diplayed are: untreated cells (black line); 30 minutes 20µM ML-7 treated cells (dotted line); 30 minutes 1µM jasplakinolide treated cells (discontinuous dotted line). Data are means +/- sem from three independent experiments.

Panel B : To control the efficiency of the ML-7 treatments, Cos-7 cell lysates were analyzed by Western blots using anti-diphosphorylated myosin light chain (ppmlc) from Cell Signaling or anti-myosin light chain (mlc) from Sigma Aldrich. lane a: control cell lysate, lane b: 30 min 10µM Diperoxovanadate (DPV) treated cell lysate, lane c: lysate from cells treated 30 min with 10µM ML-7 and 20µM of ML-7. DPV was prepared as described in (1).
FIGURE 3. Jasplakinolide effect on actin polymerization in infected Caco-2 cells. Caco-2 cells were grown until differentiation and infected with RF strain bovine rotavirus at a multiplicity of infection of 10 PFU/cell. From 2 hours p.i., cells were treated or not with 1µM Jasplakinolide. Infection was carried out for 18 hours. Caco-2 cells were subjected to 1% ice cold Triton X-100 extraction and ultracentrifugation in sucrose gradient as previously described (3). Actin content of sucrose gradient fractions and pellet were analyzed by western blot. High density actin polymers were recovered in the pellet fraction C.

FIGURE 4. Apical secretion of rotavirus from infected differentiated Caco-2 cells is affected by Jasplakinolide. Caco-2 cells were filter-grown, infected and treated with 1µM Jasplakinolide as described above (figure 3). Infectious virus release was quantified in apical and basolateral media (AP, BL). Collected virus were frozen, thawed, and then assayed for virus titration by plaque assay on MA104 cells. Histogram displays the mean values of infectious virus +/- standard deviations from 3 independent experiments.

REFERENCES


