Investigating the role of F-actin in human immunodeficiency virus assembly by live-cell microscopy

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Running title: Live imaging of F-actin at HIV-1 budding sites
Abstract

Human immunodeficiency virus (HIV-1) particles assemble at the plasma membrane, which is lined by a dense network of filamentous actin (F-actin). High amounts of actin have been detected in HIV-1 virions, proposed to be incorporated by interaction with the nucleocapsid domain of the viral polyprotein Gag. Studies addressing the role of F-actin in HIV-1 particle formation using F-actin interfering drugs did not yield consistent results. Filamentous structures pointing towards nascent HIV-1 budding sites, detected by cryo-electron tomography and atomic force microscopy, prompted us to revisit the role of F-actin in HIV-1 assembly by live-cell microscopy.

HeLa cells co-expressing HIV-1 carrying fluorescently labeled Gag and a labeled F-actin binding peptide were imaged by live-cell total internal reflection microscopy (TIR-FM). Computational analysis of image series did not reveal characteristic patterns of F-actin in the vicinity of viral budding sites. Furthermore, no transient recruitment of F-actin during bud formation was detected by monitoring fluorescence intensity changes at nascent HIV-1 assembly sites. The chosen approach allowed us to measure the effect of F-actin interfering drugs on the assembly of individual virions in parallel to monitoring changes in the F-actin network of the respective cell. Treatment of cells with latrunculin did not affect efficiency and dynamics of Gag assembly under conditions resulting in disruption of F-actin filaments. Normal assembly rates were also observed upon transient stabilization of F-actin by short-term treatment with jasplakinolide. Taken together, these findings indicate that actin filament dynamics are dispensable for HIV-1 Gag assembly at the plasma membrane of HeLa cells.
Importance

HIV-1 particles assemble at the plasma membrane of virus producing cells. This membrane is lined by a dense network of actin filaments that might either present a physical obstacle for the formation of virus particles, or generate force promoting the assembly process. Drug-mediated interference with the actin cytoskeleton showed different results on formation of retroviral particles in different studies, likely due to general effects on the cell upon prolonged drug treatment. Here we characterized the effect of actin-interfering compounds on the HIV-1 assembly process by direct observation of virus formation in live cells, which allowed us to measure assembly rate constants directly upon drug addition. Virus assembly proceeded with normal rates when actin filaments were either disrupted or stabilized. Taken together with the absence of characteristic actin filament patterns at viral budding sites in our analyses, this indicates that the actin network is dispensable for HIV-1 assembly.
Introduction

Human immunodeficiency virus (HIV-1) particles are released from a virus producing cell through the formation of spherical, lipid enveloped virus buds bulging from the plasma membrane; constriction of the bud neck finally culminates in abscission of the virus envelope from the host cell membrane (1). Changes in membrane curvature accompanying bud formation are believed to be induced, at least in part, by cooperation between the viral polyprotein Gag, assembling into a curved protein lattice at the cytoplasmic face of the membrane, and the cellular endosomal sorting complex required for transport (ESCRT) machinery, which is involved in a number of cellular processes requiring membrane abscission. Additional host cell proteins may, however, be exploited by the virus to promote this process.

The plasma membrane is lined with a dense and dynamic network of filamentous actin (F-actin) which generates force for cellular processes involving deformation or reorganization of the membrane (e.g. filopodia formation, membrane ruffling or endocytosis, reviewed in (2, 3)). Subversion of the actin cytoskeleton for a variety of replication steps, including particle assembly and release, has been reported for numerous viruses (reviewed in(4)). It is thus conceivable that HIV-1 reorganizes and employs cortical F-actin to promote virus bud formation. In line with this, incorporation of high amounts of actin into HIV-1 (5-7) and a specific interaction of actin with the nucleocapsid (NC) domain of Gag (8, 9)have been reported. However, while the importance of actin cytoskeleton dynamics for the direct cell-to-cell transmission of HIV-1 through so-called virological synapses in tissue culture is undisputed((10, 11),
reviewed in (4, 12, 13)), the role of F-actin dynamics in the assembly of the viral structure itself is less clearly defined.

On theoretical grounds, both a negative influence of F-actin by obstructing membrane access of virion components and/or membrane deformation during bud formation, as well as a positive contribution of forces generated by actin polymerization to membrane bulging, could be envisioned. Analyses of the effect of F-actin interfering drugs on the efficiency of retroviral particle formation yielded different results. Depending on the drug and experimental condition used, complete abrogation of Gag membrane trafficking (14), partial inhibition of particle formation (10, 15), no effect of an F-actin disrupting drug on particle release (10), and even a moderate increase of particle formation in the case of short term cytochalasin D (cyto D) treatment of equine infectious anemia virus (EIAV) producing cells (16) was reported. This can be explained, at least in part, by a difficulty inherent to the ensemble measurements used to quantitate virus production: using bulk virus release as readout necessitates prolonged drug treatment periods, which may favor pleiotropic effects. Thus, potential direct effects on viral particle formation are not easily discriminated from indirect effects. In contrast, live cell imaging provides not only the opportunity to capture transient, non-synchronized interactions between virus and cellular components, but also allows focusing on single cells or individual events.

Furthermore, drug effects can be directly visualized within short time windows. Consequently, parallel imaging of virus assembly and F-actin dynamics before, as well as directly after drug addition should allow discriminating between direct and indirect drug effects.
Two findings derived from microscopic analyses of HIV-1 assembly sites prompted us to revisit the question of potential F-actin involvement in HIV-1 Gag assembly by exploiting the strengths of a live-cell microscopy approach. First, Gladnikoff and coworkers (17) analyzed nascent retroviral budding sites at the surface of HIV-1 and murine leukemia virus (MLV) Gag expressing 3T3 and HeLa cells by atomic force microscopy (AFM). These authors observed prominent star-shaped structures with arm lengths of up to 4µm centered at a subset of budding sites. The large 'asters' were interpreted as structured F-actin assemblies recruited by retroviral Gag; this interpretation was supported by dependence of aster formation on the presence of the presumed actin-interacting NC domain of Gag (17). Second, a morphological connection between F-actin and nascent HIV-1 assembly sites was also conveyed by cryo-electron tomography (cET) of budding sites at the membrane of HIV-1 Gag or GagPol expressing glioblastoma cells (18). Filamentous actin structures were clearly detectable close to 34 of 39 budding sites analyzed and in some cases appeared to be connected to the rims of the bulging HIV-1 Gag layer (18).

Results from both studies suggested that a Gag mediated reorganization of F-actin structures at the plasma membrane might promote or facilitate the membrane deformations occurring during HIV-1 assembly. However, cET does not yield any dynamic information and AFM measurements were performed with a low temporal resolution (10 min/frame) compared to the time frame determined for completion of an HIV-1 bud (t_{1/2} ~ 3 min,(19)). In contrast, single virus tracing (SVT) provides the opportunity to directly observe both F-actin dynamics as well as HIV-1 particle assembly in live cells with high time resolution for a large number of individual budding sites, thereby allowing detection of rapid and transient changes occurring in an asynchronous
manner. Using previously characterized fluorescently labeled HIV-1 derivatives (20, 21) together with a fluorescently labeled F-actin binding peptide (Lifeact; (22)) we have analyzed potential interactions of F-actin and nascent viral budding sites in HeLa cells by SVT. The chosen approach allowed us to study F-actin dynamics at individual HIV-1 assembly sites and to investigate the influence of treatment with actin interfering drugs on the kinetics of HIV-1 assembly under defined conditions.

Materials and Methods.

Plasmids

Plasmids pCHIV and pCHIVeGFP have been described previously(21). Plasmid pLifeact.mCherry was kindly provided by Nikolas Herold. It was cloned by exchanging a BamHI/NotI fragment from pLifeact.GFP ((22); a kind gift of Michael Sixt) against the corresponding mCherry encoding fragment from pmCherry-N1 (Clontech).

Tissue culture and transfections

HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen), supplemented with 10% fetal calf serum (FCS; Biochrom), penicillin (100 IU/mL), streptomycin (100 μg/mL), 4 mM glutamine and 10 mM Heps (pH 7.4). For co-transfection, cells were seeded in 8-welled glass bottom chamber slides (LabTek, Nunc) at a density of 1.5x10^4 cells/well and transfected the following day with 400 ng of pCHIV derivatives and 100 ng of pLifeact.mCherry. Transfection was performed using either
Fugene6 (Roche) or ExtremeGene9 (Roche) according to the manufacturer's instructions. At 12-20 hours post transfection (h.p.t.), cells were transferred to imaging buffer (25 mM HEPES pH 7.4, 137 mM NaCl, 2.7 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂ and 30 mM glucose) and subjected to live cell imaging.

Cytotoxicity assay

Jasplakinolide solution (1 mM in DMSO) and latrunculin B (LAT-B) were purchased from Calbiochem; a 2.5 mM stock solution of LAT-B was prepared in 100% DMSO. The minimum non-toxic concentration for each drug was determined using a standard MTS cell proliferation assay. For this, HeLa cells were seeded in a 96-well plate at a density of 0.5x10⁴ cells per well and incubated overnight at 37°C, 5% CO₂. On the following day, the indicated concentrations of either LAT-B, jasplakinolide, or the corresponding DMSO concentration as a control, were added to the culture medium and incubation was continued for 2 h at 37°C. Cells were washed with pre-warmed phosphate buffered saline (PBS) and stained for dehydrogenase activity using reagents provided in the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions. Cells were incubated for 5 h at 37°C followed by measurement of absorbance at 495 nm.

Microscopy and single virus tracking

HeLa cells were transfected and prepared for live cell imaging as described above. Basic microscopy settings have been described elsewhere (21, 23). Briefly, live cell imaging
was performed using a highly sensitive TIR-FM setup (Objective type, Visitron Systems) based on a Zeiss Axiovert 200M fluorescence microscope equipped with an alpha plan FLUAR 100x/1.45 oil immersion TIRF objective. A 43 series ion laser (MellesGriot) with frequency range of 457 – 568nm was used for excitation at 488nm (eGFP) and 568nm (mCherry). For dual-color imaging the excitation wavelength was chosen by an acousto-optic tunable filter (VTiVisiTech International Vision Technology for Science assembled by Visitron Systems GmbH) coupled to an optic fibre connected with the laser condenser going through the TIRF objective. The TIRF angle was manually controlled. Image sequences were acquired using Metamorph (Visitron) using a sensitive EM-CCD camera (Cascade II, Roper Scientific, 512x512 pixel).

Single virus tracking and data analysis

Changes in Gag.eGFP fluorescence intensity over time were analyzed as a signature for the HIV-1 assembly progress. Individual assembly sites were detected and localized using a probabilistic approach for automated tracking of multiple virus particles introduced in (24). Mean fluorescence intensity at the position of identified particles was computed based on the standard deviation of the 2D Gaussian function ($\sigma_{xy}$). Signal intensity was measured within the radius of $\sigma_{xy}$. Local background intensity was determined based on an annulus with inner and outer radii of $\sigma_{xy}$ and $5\sigma_{xy}$, respectively and subtracted from the signal intensity measured within the radius of $\sigma_{xy}$. In our previous work, we showed that probabilistic tracking methods outperform deterministic ones and achieve accurate results (24). Our probabilistic approach for virus particle tracking was optimized and extended to improve the performance as well as to
automatically detect relevant events in the multi-channel microscopy image data. Briefly, with this approach multiple particles are tracked by solving a sequential estimation problem within a Bayesian framework using a Kalman filter for spatio-temporal filtering. For the dynamical model, we used Gaussian random walk dynamics and for the measurement model the intensity information was directly exploited. The tracking approach takes into account anisotropic uncertainty information based on the underlying probability distributions using the Mahalanobis distance. For preprocessing we used a background subtraction scheme based on the filtered image. In addition, we developed an automatic detection scheme for identifying budding events which exploits the steady increase of the intensity over time using a differential approach.

Since the temporal distribution of the occurrences of individual assembly sites was asynchronous, we aligned the intensities of different particles by synchronizing the time of initiation of individual assembly sites as described previously (19). Signals identified as typical HIV-1 assembly sites displayed a characteristic change of fluorescence intensity over time: an exponentially growing phase (phase I) representing the dynamic oligomerization of Gag, a plateau phase (phase II) indicating completion of Gag accumulation. A subsequent phase characterized by a rapid change in instantaneous velocity and fluorescence intensity (phase III) was observed only for a subset of traces. Here, the rate of assembly was calculated from phase I by using a saturating exponential function:

\[ y = A_i \left(1 - \exp\left[-k_i \left(t-t_0\right)\right] \right) \]

where \( A_i \) is maximum fluorescence intensity, \( k_i \) is the rate at which fluorescence intensity increases and \( t_0 \) is the initial time of assembly.
Semi-automatic quantitation of assembly sites in still images was performed using a previously described spot detection algorithm (25). Calculation of rate constants, statistical analyses and plotting of data for all experiments shown was performed using GraphPadPrism.

Analysis of LifeAct fluorescence density and coherency from still images

In order to check if virus budding has any statistically significant local effect on the underlying actin cytoskeleton, visible within the resolution of fluorescence microscopy, we analyzed actin patterns in close proximity around the budding sites using an automated image processing approach. We employed two parameters to quantify changes in actin structures, fluorescence density and coherency. Fluorescence density on F-actin stained images is a direct indicator for the local concentration of F-actin, while the coherency analysis is well suited to classify the quality of actin network structures in cells as previously described (26). Briefly, coherency is a side product of the structure tensor analysis, which extracts the local orientation of structures with respect to the squared gray value gradient within a small region of an image, constrained in this context by a Gaussian filter with a size of 9 pixels and a standard deviation of 3 pixels in each dimension (27). Coherency is defined as the squared relative difference of the two squared gray value gradients parallel and orthogonal to the local orientation, i.e. the two eigenvalues of the structure tensor. Therefore, sharp network structures like actin fibers yield maximum coherency values, while for a homogeneous gray value background or white noise the coherency parameter approaches zero.
This analysis has been employed before to extract the filament orientation distribution of actin networks from electron tomography data of fish keratocyte lamellipodia (28). Both fluorescence density and coherency in the red channel were measured within a small neighborhood around the individual budding sites and averaged over the time course of budding. The distributions of these temporal averages from all 215 tracked sites in 8 different cells are indicated as ‘virus’ in Figure 1 B and C. For comparison we performed identical analyses at the same number of random positions, uniformly scattered within the outline of the respective cells (Figure 1 B and C, random). Finally, we determined density and coherency averaging over all pixel positions inside the cell boundaries (Figure 1 B and C, 'total'), resulting in an average number of ~29,000 pixels analyzed per cell. This procedure corresponds to the limit of analyzing a large number (>> 29,000) of uniformly randomly located sites within each cell.

**Results**

In order to follow HIV-1 virion assembly by live-cell microscopy, we employed our previously described fluorescently labeled HIV-1 derivative pCHIVeGFP (21), which carries an eGFP moiety between the MA and CA domains of Gag. This derivative is non-replication competent due to the lack of viral long terminal repeat regions, but expresses all HIV-1 proteins except for Nef and produces particles with wild-type morphology and cell entry efficiency when complemented with an equimolar amount of its unlabeled counterpart pCHIV (21). Co-transfection of cells with pCHIV and pCHIVeGFP gives rise to punctate fluorescent assembly sites at the plasma membrane, which correspond mostly to individual virus buds and can be followed by live-cell TIR-FM with time resolution in...
the range of 1 s/frame (19). This experimental system was previously used to characterize the kinetics of the HIV-1 Gag assembly process (19) and the transient recruitment of a cellular protein associated with the endosomal sorting complex required for transport (ESCRT) to nascent budding sites (29).

For parallel visualization of F-actin in live cells we made use of an mCherry-tagged version of Lifeact, a 17 amino acid peptide that binds to filamentous actin structures within live eukaryotic cells without detectable interference with cellular processes (22). HeLa cells were co-transfected with Lifeact.mCherry and an equimolar mixture of pCHIV and pCHIVeGFP. Comparison of the dynamic alterations of Lifeact.mCherry in cells co-expressing HIV-1eGFP and Lifeact.mCherry with that in cells transfected with pLifeact.mCherry alone revealed no notable difference in F-actin dynamics observed at the ventral cell surface (supplemental movies S1, S2 and data not shown). At 12-20 h.p.t. individual cells co-expressing mCherry and eGFP, which displayed an early stage of budding site formation (evidenced by diffuse cytoplasmatic staining for Gag.eGFP and a very low number of detectable budding sites at the plasma membrane), were selected and subjected to microscopic observation over a period of 1-2h. Numerous individual Gag.eGFP punctae appeared at the plasma membrane and gradually increased in fluorescence intensity over a period of several minutes (supplemental movie S2), in accordance with previous findings (19, 30).

F-actin distribution patterns with respect to HIV-1 assembly sites.
Formation of prominent µm-sized F-actin structures at the plasma membrane of virus producing cells and in the vicinity of HIV-1 buds as described in (17) should be detectable by TIR-FM, although the spatial resolution of this technique would not allow capturing of structural details. Computational image analysis was performed to obtain information on a statistically significant number of individual sites (Figure 1).

We first analyzed the average spatial distribution of F-actin with respect to HIV-1 budding sites using an automated image analysis approach (26, 27). The parameters fluorescence density and fluorescence coherency, respectively, were employed in order to detect characteristic F-actin patterns at all individual HIV-1 assembly sites tracked from 8 individual cells (n=215). The density of Lifeact.mCherry fluorescence is considered as a direct indicator for the concentration of F-actin at the position of measurement. In contrast, coherency is a parameter chosen to classify the quality of actin network structures in cells. By definition, it extracts the relative strength of the edges of structures compared to their surroundings; thus, sharp and aligned network structures like actin filaments yield maximum coherency values, while coherency values approach zero for a homogeneous gray background or for random noise.

We had earlier tested the sensitivity of this parameter to structural changes using simulated microscopy images based on stochastic realizations of a biophysical filament model as a benchmark (26), demonstrating that coherency measurements are well suited to detect changes in the density, intensity and average fiber length of filamentous networks. In the case of star shaped structures, coherency would detect the peripheral aster region, whereas values at the center point may not be significant due to averaging of gradient orientations. However, based on a published fluorescence microscopy image...
of typical actin aster shapes(17) we expect that density as our second parameter complements this lack of sensitivity by yielding significantly higher values at the central point of asters compared to their surroundings. Therefore we averaged coherency over an annulus with boundaries at 3 and 7 pixels, respectively, around the tracked virus position, while actin density was measured within a circle with a diameter of 6 pixels around the individual budding sites. Both measures were averaged over the time course of assembly in order to detect correlations in the positions of budding sites and the location of ordered F-actin structures.

Figure 1 B and C show the distributions of coherency and density measurements, respectively, for 215 individual assembly sites (‘virus’). These data were compared to corresponding data sets obtained for either the same number of random positions, uniformly scattered within the boundaries of the respective cell (Figure 1B and C, ‘random’), or from averaging over the whole area (~29,000 pixel) of the individual cell (Figure 1B and C, ‘total’). This analysis did not reveal significant differences in either F-actin coherency or density for HIV-1 budding sites compared to other sites in the same cell.

*Investigation of actin dynamics at nascent budding sites.*

The image analyses summarized in Figure 1 were generated by averaging over the time course of Gag.eGFP assembly and would thus not detect transiently occurring patterns of co-localization that are only visible for part of the observation period. We therefore inspected image series for transient accumulation of F-actin at nascent viral budding
sites. Previous characterization of Gag assembly dynamics had revealed that the initial phase of this process follows a saturating exponential at prototypic budding sites (19, 30) and that gradual or transient recruitment of ESCRT-components for time periods of less than one minute during this process can be detected (29, 31). Here, we first measured Gag assembly kinetics during the initial exponential phase as described previously (19) in order to control for a possible influence of Lifeact.mCherry expression on HIV-1 assembly. Analysis of >200 tracks from 12 individual cells yielded an average rate constant of $k = 0.005 \pm 0.0026$ s$^{-1}$ in very good agreement with the rate of $k = 0.0043 \pm 0.0005$ s$^{-1}$ obtained for HeLa cells expressing HIV-1-eGFP alone (19). This result indicates that co-expression of Lifeact.mCherry did not alter the dynamics of the HIV-1 assembly process.

We then analyzed the Lifeact.mCherry signal recorded over time at the positions where HIV-1 budding site formation was detected in TIR-FM (Figure 2 and supplemental movie S3). Only budding sites which were recorded from the onset of Gag.eGFP detection for at least 250 frames were selected for analysis. Figure 2A shows the averaged HIV-1 assembly traces of 58 individual assembly sites from 5 different cells recorded in the eGFP channel. Averaged signals recorded in parallel at the identical positions in the mCherry channel are shown in Figure 2B, while Figure 2C displays averaged traces recorded in the red channel at 58 randomly selected non-assembly sites in the same cells. No gradual increase in the Lifeact.mCherry signal over the course of Gag assembly was detected. However, transient recruitment of F-actin at varying time points with respect to the assembly process would be masked by averaging intensities from numerous individual sites. Thus, we also performed manual frame-by-frame inspection of mCherry signal intensities at computationally tracked individual assembly sites (Figure
2D-F). In order to account for potential F-actin bursts occurring at early assembly nucleation sites with a number of Gag.eGFP molecules below the detection limit of our approach (32) we included 150 frames (300s) preceding detectable Gag.eGFP accumulation at the respective site in these analyses. Figure 2D and E show examples for a prototypic assembly site (yellow circles) and a non-assembly site (red circles), recorded in the green channel and red channel, respectively. Figure 2F shows the intensity values recorded at the individual assembly site indicated by the yellow circle in Figure 2D for Gag.eGFP (green line) and Lifeact.mCherry (yellow line). The red line shows the Lifeact.mCherry signal recorded during the same time period at the non-assembly site indicated by the red circle in Figure 2D. Inspecting 200 individual tracks for assembly and non-assembly sites did not reveal any characteristic intensity changes associated with Gag assembly: neither a gradual change of mCherry intensity, nor transient bursts of mCherry differing notably from transient fluctuations of Lifeact.mCherry intensity at random membrane positions were detected at nascent Gag assembly sites. In summary, neither characteristic F-actin distribution patterns in close vicinity of nascent budding sites nor typical transient changes in F-actin distribution over the course of HIV Gag assembly were detected by live-cell imaging under our experimental conditions.

Effect of pharmacological interference with actin dynamics on HIV-1 Gag assembly kinetics.

Although live-cell imaging did not reveal any apparent differences in spatial or temporal F-actin distribution in the presence of Gag assemblies, these findings did not rule out an
influence of F-actin dynamics on assembly kinetics. To address this issue, we made use of the fact that live-cell microscopy offers the unique opportunity to analyze virus assembly upon short-term treatment with F-actin interacting drugs while directly monitoring the effect of the drug on F-actin dynamics. Latrunculin B (LAT-B), which binds and sequesters G-actin monomers and thereby prevents actin polymerization (33, 34), and jasplakinolide, whose binding to actin oligomers and F-actin filaments stabilizes multimeric actin structures(33, 35), were employed.

In order to define effective drug concentrations with minimal adverse effects, cytotoxicity of LAT-B and jasplakinolide under the conditions used was assessed by a colorimetric cell proliferation assay (Celltiter96AQeous, Promega; data not shown).

Based on these control experiments, final concentrations of 300 nM LAT-B and 200 nM jasplakinolide, respectively, were chosen for all experiments. HeLa cells were co-transfected with plasmids expressing Lifeact.mCherry and HIV^GFP and grown in the absence of actin interfering drugs. At 20h.p.t., cells were transferred to imaging buffer and microscopically screened for individual cells representing an early stage of assembly site accumulation. Image acquisition was initiated at a rate of 0.5 frames/s and LAT-B, jasplakinolide or DMSO as control, respectively, was added under continuous microscopic observation.

Figure 3A, B and supplemental movie S4 summarize the results obtained for LAT-B. Typically, within 100 frames (200 s) after drug addition filamentous actin structures were disrupted and converted into mobile actin clusters moving along the cell membrane with a velocity of ∼2μm/min, closely resembling the globular structures detected in Dictyostelium cells following short-term LAT-B treatment (36). These structures
gradually disappeared over time (Figure 3A, top row and Supplemental movie S4). Parallel observation of the Gag.eGFP signal in the same cell revealed that assembly sites continued to form after the addition of drug over the whole observation period of 1 h, although filamentous actin structures were efficiently disrupted (Figure 3A lower row and supplemental movie S4). Furthermore, we analyzed rates of assembly after drug addition. A value of $k = 0.0047 \pm 0.0023$ s$^{-1}$, not significantly different from the value determined in control cells ($0.005 \pm 0.0026$ s$^{-1}$; data not shown), was obtained for 71 averaged exponential assembly phases from 5 individual LAT-B treated cells (Figure 3B).

Analogous live-cell imaging experiments were performed in the presence of 200 nM jasplakinolide. In this case, live imaging of Lifeact.mCherry at high time resolution allowed us to distinguish different stages of the jasplakinolide effect, dependent on the time after drug treatment. Addition of jasplakinolide to the imaging buffer initially resulted in apparent stabilization and thickening of stress fibers (Figure 3C, top row and supplemental movie S5). Approximately 5 min after drug addition, formation of amorphous actin aggregates was observed in the cytoplasm, accompanied by filament disintegration. Within ~15-25 min of treatment, all visible F-actin had collapsed into a large aggregate close to the nucleus and cells started to detach from the chamber slide. This is in accordance with findings reported by Bubb et al. (35). Jasplakinolide stabilizes F-actin filaments in vitro (37) and in live cells, but it also promotes enhanced actin filament nucleation in cells due to stabilization of actin oligomers. The latter effect ultimately results in sequestration of actin in large amorphous polymers, depleting G-actin from the pool available for filament assembly(35).
Based on this observation, we divided our analysis of Gag assembly kinetics and budding site formation into the two phases defined by the parallel analysis of Lifeact.mCherry, revealing differential effects on assembly that correlated with the phenotype of the virus producing cell (Figure 3C and supplemental movie S5). During the phase of apparent actin filament thickening, assembly sites continued to form with an average assembly rate $k = 0.0041 \pm 0.0025 \text{ s}^{-1}$ (Figure 3D), again similar to the rate determined for control cells. However, whereas new budding sites displaying exponential assembly kinetics appeared throughout the observation period for control and LAT-B treated cells, respectively (Figure 4A, top and middle panel), almost no new assembly sites were observed after the first 30 min of jasplakinolide treatment (Figure 4A, bottom panel) when most cells had reached the actin aggregation state. Visual analysis of image series form jasplakinolide treated cells confirmed that new assembly sites were not detected in cells in which the actin cytoskeleton had collapsed.

In order to unmask potential more subtle effects on assembly rates, we analyzed the distribution of assembly rate constants observed dependent on the time points after jasplakinolide or LAT-B addition. In agreement with our earlier observations (19) a rather broad range of rate constants was determined when comparing individual sites, but this range did not vary depending on the time point after drug addition (Figure 4A). The distribution of rate constants determined for individual exponential assembly phases in jasplakinolide treated cells was similar to that observed in control or LAT-B treated cells, respectively (Figure 4B). We conclude that HIV-1 assembly in jasplakinolide treated cells proceeded with normal rates during the phase of F-actin filament thickening and then ceased completely upon actin network collapse. This was supported by the analysis of cells treated with cucurbitacin E, which has been reported to inhibit F-actin
depolymerisation by a different mechanism of action (38). Again, Gag assembly in the early phase of cucurbitacin E treatment proceeded with a similar rate ($k = 0.0054 \pm 0.0028 \text{ s}^{-1}$) as in control cells (Figure 3E, F and supplemental movie S6).

These experiments showed that drug-induced interference with the F-actin network did not result in decreased Gag assembly rates. On the other hand, cortical actin could be envisioned as an obstacle for nascent bud formation. Resolution of dense cortical actin networks by latrunculin might then facilitate Gag access to the plasma membrane, leading to increased budding frequency. Visual inspection of image series had indeed suggested an increase in the appearance of new assembly sites upon LAT-B treatment in some cases (see supplemental movie S4). To address this question quantitatively, we analyzed the number of assembly sites detected per cell and the rate of new assembly site formation in individual cells (Figure 5). While the total number of assembly sites detected at the plasma membrane varied considerably between individual cells, neither the average value (Figure 5A) nor the variation between individual cells (Figure 5B) differed significantly between LAT-B treated and control cells, respectively.

Similar results were obtained when comparing the rate of assembly site initiation measured in live-cell experiments. This rate was assessed either through accumulation of assembly sites over time (Figure 5C) or through calculating the average interval between consecutive detections of individual assembly sites (Figure 5D). In both cases we observed variation between individual cells under the same experimental conditions, but the evaluations did not reveal any clear difference between control and LAT-B treated cells. In accordance with the data shown in Figure 4, accumulation of new assembly sites in jasplakinolide treated cells ceased at ~30 min after drug addition.
Discussion

Based on previous reports implicating F-actin in HIV-1 morphogenesis, we have analyzed actin density, actin recruitment and the presence of specific F-actin structures at viral budding sites using live cell TIR-FM. No specific features were detected in the vicinity of nascent assembly sites. Disruption of the F-actin network by treatment with LAT-B affected neither the rate of budding site initiation nor Gag assembly kinetics at individual sites, while the F-actin network was completely disrupted. Furthermore, addition of the F-actin stabilizing compound jasplakinolide also had no significant effect on assembly kinetics and rate of budding site appearance during the early phase when F-actin filament thickening was apparent. Cessation of budding site formation at time points later than 30 min post drug addition in the case of jasplakinolide treatment cannot be attributed to the observed disruption of F-actin filaments at this stage, since particles continued to form with normal efficiency upon filament disruption through LAT-B. We thus consider it likely that termination of assembly in the case of jasplakinolide is due to indirect pleiotropic effects upon complete collapse of the cytoskeleton. Based on these results, we conclude that cortical F-actin is not specifically recruited to HIV-1 budding sites and does not influence the nucleation of assembly sites or the kinetics of assembly in HeLa cells. Our data thus clearly do not support a role of F-actin in HIV-1 Gag membrane transport and assembly, which had been inferred from previous studies.
Sasaki and coworkers had reported complete abrogation of membrane localization and particle production upon treatment of T-cells with mycadelide B (14) and a partial block of HIV-1 particle release upon long-term treatment with cytoD (15), respectively. Employment of inhibitory drugs for 3 h (the shortest possible time permitting ensemble measurements) performed by Jolly et al. (10) revealed no effect of cytoD and a modest effect of LAT-A on bulk Gag release from T-cells. Whereas these data were taken as evidence for a role of actin dynamics in Gag membrane transport and particle formation, in our view the findings taken together rather argue for indirect effects of long term drug treatment. Although no overt cytotoxicity was observed in these studies, more subtle effects on cell metabolism, protein expression, energy status and/or transport pathways may offer an alternative explanation for the observed moderate reductions in particle formation. Consistent with this interpretation, differential effects depending on the time of drug treatment were observed in the case of infectious equine anemia virus (EIAV): whereas prolonged incubation with cytoD reduced the amount of particles released by ~50%, short-term treatment with either cytoD or LAT-B resulted in enhancement of bulk particle release by 1.5 to 2-fold (16).

While live-cell imaging methods are also not without caveats due to the necessity of heterologous labels, specific microscope setups and extensive image analysis, we believe that our study illustrates an important advantage of a live imaging approach. By directly correlating the effect of drug treatment on F-actin to that on particle assembly in the same individual cell, it was possible to dissect differential effects that would be obscured in ensemble measurements. This was particularly apparent in the case of jasplakinolide treatment, where two distinct stages of drug action could be clearly correlated to different effects on Gag assembly. Of note, our bulk toxicity measurements
had not shown pronounced cytotoxic effects under conditions at which a large proportion of cells was severely compromised as judged by F-actin live-cell imaging. These observations underline the point that bulk experiments involving the use of chemical compounds with pleiotropic effects have to be interpreted with caution and indicate live-cell imaging methods as an alternative approach.

We need to point out, however, that whereas imaging the ventral surface in TIR-FM allows quantitative analyses of new budding site formation and the assembly kinetics of the immature Gag lattice, our experimental system is not well suited for analyses of the subsequent release step. Trapping of viruses in the confined space between cell membrane and cover slip prevents a clear assignment of the actual membrane scission event. Accordingly, our data do not exclude a role of actin in the actual HIV-1 particle release. A contribution of F-actin to virion abscission could explain the modest effects of cytoD and/or latrunculin observed by others on retroviral particle formation in short-time incubation experiments; however, data on HIV-1 suggested a negative effect of F-actin disassembly on release (10), while it was reported to have a positive effect in the case of EIAV (16). Again, ensemble measurements have to be interpreted with caution, since particle formation between individual cells in the same culture is asynchronous; bulk material collected may thus represent a mixture of events initiated before and during the treatment phase. A role of actin in virus release and spread, but not for intracellular transport or assembly of the particle is not without precedent, as actin dynamics is essential for vaccinia virus transmission, while not being relevant for the virus formation process (reviewed in (39)). In the case of measles virus, jasplakinolide treatment was recently reported to specifically affect the step of particle abscission at the plasma membrane (40). Investigating the role of actin dynamics for the HIV-1 membrane
abscission event by live-cell imaging, however, is currently prevented by the lack of a suitable microscopic readout to monitor release events. Obviously, our data do not argue against the previously reported role of F-actin dynamics in retroviral cell-cell transmission. Work from several labs has clearly shown that actin plays a role in the formation of virological synapses and for viral transmission through cell surface surfing, nanotubes or cytonemes (reviewed in(13, 41, 42)), while the current study focused on the assembly of individual HIV-1 particles.

Previous cET studies had indicated the presence of dense F-actin networks in the vicinity of HIV-1 budding sites and sometimes apparently associated with the budding site (18), and this was confirmed in the accompanying paper of Stauffer et al. (43). Our live-cell TIR-FM studies do not argue against such structures, but suggest that the F-actin density does not detectably differ between budding sites and other regions of the plasma membrane. This is in agreement with the observation made by Stauffer et al., that actin was not enriched in HIV-1 particles as compared to the average actin content of the corresponding host cell (43). Furthermore, although we cannot exclude the presence of short, dynamic and possibly also more latrunculin resistant, actin oligomers (44, 45), our studies did not reveal large actin-derived asters at HIV-1 budding sites as had been reported in a previous AFM study (17). This difference cannot be explained at present, but our parallel cETstudy (43) did not detect a difference in actin association when the NC-domain was replaced by a dimerizing leucin zipper, while AFM detection of asters was completely lost in this case (17). Thus the relation between the aster structures uncovered by AFM and the actin networks detected by cET and TIR-FM is currently unclear.
In summary, this work, together with the accompanying manuscript by Stauffer et al. (43), indicate that F-actin and F-actin dynamics as well as the putative actin-binding NC domain of Gag are dispensable for HIV-1 assembly. Neither actin filaments (disrupted by LAT-B) nor actin treadmilling (disrupted by jasplakinolide) are required for HIV-1 Gag transport, for formation of new budding sites or for assembly of the immature Gag shell.

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References


Figure legends

Figure 1: Analysis of F-actin density and coherency at nascent HIV-1 budding sites. (A) HeLa cells were co-transfected with pCHIV/pCHIVeGFP and pLifeact.mCherry and analyzed by live-cell TIR-FM at 20 h.p.t. as described in materials and methods. The figure shows TIR-FM still images from supplemental movie S2 recorded in the green (left panel) and red (right panel) channel, respectively, at 20 min after initiation of image acquisition. Squares indicate a representative HIV-1 budding site, circles a representative non-budding site. Scale bar represents 10 µm. (B, C) Distribution of F-actin coherency (B) and density (C) at 215 HIV-1 budding sites (‘virus’), 215 randomly selected non-budding sites (‘random’), or all pixels within the boundaries of the respective cells (‘total’), respectively. HIV budding sites and non-budding sites were identified from recordings in the GFP channel and density and coherency of the
corresponding signal in the red channel were determined as described in materials and methods. The box plots illustrate the distribution of data obtained from eight individual cells. Each box is divided at the median value and lower and upper boundaries of the box indicate the 25th and 75th percentiles of the distribution. Whiskers extend to the most extreme data points within 1.5 times the interquartile range (i.e. the range between 25th and 75th percentile).

**Figure 2: Live-cell analysis of Lifeact.mCherry intensity changes at HIV-1 assembly sites.** HeLa cells were co-transfected with pCHIV/pCHIV\textsuperscript{eGFP} and pLifeact.mCherry. At 20 h.p.t., TIR-FM live cell imaging was performed with a resolution of 0.5 frames/s. Individual HIV-1 assembly sites were tracked as described under materials and methods. (A-C) Analysis of mean signal intensity changes over time; mean values (lines), SD (gray bars) and exponential fit (white) are shown. (A) Mean intensities from exponential assembly phases (58 individual HIV-1 assembly sites from 5 cells, recorded in 5 independent experiments) are depicted. (B) Mean of the corresponding traces recorded in the red channel (Lifeact.mCherry) for all positions analyzed in (A). (C) Mean of traces recorded in the red channel at 58 non-assembly sites from the cells analyzed in (A). (D) Image from the live-cell analysis of a representative cell (shown in supplemental movie S3). Examples for an individual assembly site (yellow) and a non-assembly site (red), respectively, are indicated by circles. Scale bar represents 10\textmu m. (E) Images from supplemental movie S3 recorded at the indicated time points (min:sec) in the green and red channels at the assembly site or non-assembly site encircled in (D), respectively. (F) Signal intensity changes over time.
recorded at the individual assembly and non-assembly sites site shown in (E) (green, Gag.eGFP at assembly site; yellow, Lifeact.mCherry at assembly site; red, Lifeact.mCherry at non-assembly site).

Figure 3: Effect of pharmacological interference with actin dynamics on HIV-1 assembly kinetics. HeLa cells were co-transfected with pCHIV/pCHIVeGFP and pLifeact.mCherry. At 20 h.p.t., medium was changed to imaging buffer and TIR-FM imaging in the green channel and red channels was initiated. (A, B) Disruption of actin filaments by LAT-B: At t=0, a final concentration of 300 nM LAT-B was added to the imaging buffer and image series were recorded. (A) Images from supplemental movie S4 are displayed for t=0 and for the indicated times after drug addition in the red (3A top row) or green channel (3A bottom row), respectively. The scale bar represents 10 µm. (B) Average assembly rate determined after LAT-B treatment. 71 individual assembly sites from 5 cells, recorded in 4 independent experiments, were tracked as described in materials and methods. Mean values (black line), SD (gray bars) and a single exponential fit to the average data (white line) are shown. (C, D) Transient stabilization of actin filaments by jasplakinolide. At t=0, a final concentration of 200 nM jasplakinolide was added to the imaging buffer and TIR-FM observation was continued. (C) Images from supplemental movie S5 recorded directly before, and at the indicated times after drug addition in the red (top row) or green channel (bottom row), respectively. Scale bar represents 10 µm. (D) Average assembly rate determined after jasplakinolide treatment. 55 individual assembly sites from 5 cells, recorded in 5 independent experiments, were tracked as described in materials and methods. Mean values (black line), SD (gray bars)
and a single exponential fit to the average data (white line) are shown. (E, F) Transient stabilization of actin filaments by cucurbitacin E. At t=0, a final concentration of 200 nM cucurbitacin E (Calbiochem) was added to the imaging buffer and live imaging was continued (supplemental movie S6). (E) Frames from supplemental movie S6 recorded at the indicated times after drug addition in the red (top row) or green channel (bottom row), respectively. The scale bar represents 10 µm. (F) Average Gag assembly rate in the presence of cucurbitacin E. A total of 63 individual assembly sites from 3 cells, recorded in 3 independent experiments, were tracked as described in materials and methods. Mean values (black line), SD (gray bars) and a single exponential fit to the average (white line) data are shown.

Figure 4: Variation of Gag assembly rates upon F-actin interference. (A) Variation of assembly rates over time. Assembly rate constants from 5 different cells treated with DMSO (top panel; n=92 sites recorded in 5 independent experiments), LAT-B (middle panel; n=106 sites recorded in 4 independent experiments) or jasplakinolide (bottom panel; n=66 sites recorded in 5 independent experiments), respectively were determined from the exponential assembly phase of individual traces and plotted as a function of time of initial detection of the site. t = 0 corresponds to the time point of drug addition. (B) The histogram illustrates the distribution of number of particles assembling with the respective rates based on the data sets shown in (A).

Figure 5: Effect of drug treatment on the appearance of assembly sites at the plasma membrane. (A, B) HeLa cells were co-transfected with pCHIV/pCHIVeGFP and
pLifeact.mCherry as described in materials and methods. At 12 h.p.t., tissue culture medium was replaced by medium containing 0.1% DMSO (grey bars) or 300 nM LAT-B (black bars), respectively, and incubated for 2 h at 37°C in 5% CO₂. Cells were fixed with 3% PFA and total numbers of budding sites detected per cell were quantitated for n >100 Gag expressing cells per condition by semi-automated analysis of TIR-FM images as described in materials and methods. (A) Mean values and SD from three independent experiments. (B) Individual cells were stratified according to the numbers of budding sites detected at the membrane. The histogram shows a comparison between DMSO control cells (gray bars) and LAT-B treated cells (black bars). (C, D) Formation of new budding sites over time. HeLa cells were co-transfected with pLifeact.mCherry and pCHIV/pCHIVeGFP. At 20 h.p.t., TIR-FM imaging was initiated and DMSO, LAT-B or jasplakinolide, respectively, were added during live cell recording. Automated detection of individual assembly sites appearing after the time point of drug addition (t=0) was performed as described in materials and methods. (C) Number of budding sites per cell detected on images recorded at different time points after drug addition. Mean values and standard deviations from five individual cells per condition are shown (green, control; blue, LAT-B; orange, jasplakinolide). (D) Time intervals between consecutive initiations of individual assembly events. Average intervals were calculated for five cells (1-5) per condition by linear regression analysis from the time points of appearance for all assembly sites traced for a given cell (n >330 per condition). The plot shows average values and 95% confidence intervals for each individual cell.
A Gag.eGFP Lifeact.mCherry

density [a.u.]
coherency [a.u.]
virus random total

1.0
0.8
0.6
0.4
0.2
0.0

B C

A Gag.eGFP Lifeact.mCherry
density [a.u.]
coherency [a.u.]
virus random total

1.0
0.8
0.6
0.4
0.2
0.0

B

C

coherency [a.u.]
density [a.u.]
virus random total

0.0
0.2
0.4
0.6
0.8
1.0
A B C

D E F

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A

B

control

LAT-B

jasplakinolide

number of traces

k-value [s\textsuperscript{-1}]

k-value [s\textsuperscript{-1}]

number of traces

k-value [s\textsuperscript{-1}]

k-value [s\textsuperscript{-1}]

number of traces

k-value [s\textsuperscript{-1}]

k-value [s\textsuperscript{-1}]

number of traces

k-value [s\textsuperscript{-1}]

k-value [s\textsuperscript{-1}]

number of traces

k-value [s\textsuperscript{-1}]

k-value [s\textsuperscript{-1}]

number of traces

k-value [s\textsuperscript{-1}]

k-value [s\textsuperscript{-1}]

number of traces
Figure A: Bar graph showing the average number of assembly sites for DMSO and LAT-B conditions.

Figure B: Histogram showing the number of assembly sites per cell (binned) for different time intervals.

Figure C: Line graph showing the number of assembly sites over time for control, LAT-B, and jasplakinolide conditions.

Figure D: Scatter plot showing the time interval for control, LAT-B, and jasplakinolide conditions.