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Visualization of retrovirus uptake and delivery into acidic endosomes

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Introduction

Diverse viruses exploit endocytosis to enter host cells and initiate infection. The acidic environment in endosomes activates viral surface proteins resulting in virus-endosome fusion (enveloped viruses) or in disruption/permeabilization of an endosomal membrane (non-enveloped viruses) [1, 2]. Viruses that do not rely on low pH to infect cells (many retro- and paramyxo-viruses) have long been thought to fuse directly with the plasma membrane. Accumulating evidence suggest, however, that these viruses can also enter and infect host cells *via* endocytosis [3-6]. We have recently reported that HIV-1 enters susceptible cell lines by receptor- and coreceptor-mediated endocytosis followed by pH-independent fusion with an endosomal membrane [7]. When HIV-1 did initiate fusion at the cell surface, the process was blocked at a step prior to the formation of a functional fusion pore, which is a prerequisite for the release of the nucleocapsid. The reliance of HIV-1 on endocytosis for entry is further supported by the demonstration that dynamin inhibitors suppress viral fusion and infection [7, 8]. These findings highlight the importance of measuring HIV-1 uptake and assessing the relationship between endocytosis and productive fusion in different cell types.

Virus endocytosis has traditionally been measured in acute experiments involving the removal of surface-bound viruses by proteases and assessment of the remaining viral proteins (for example, [9-11]). However, this approach is invasive and lacks the sensitivity to detect a small number of internalized virions. More recently, live cell imaging has been implemented to directly monitor single virus internalization and trafficking. Virus uptake has been deduced from the dispersal of fluorescently labeled clathrin-coat around the virus and/or from spikes in the virus-associated dynamin signal [12-14]. An alternative approach to detect single virus internalization has been introduced by Brandenburg and colleagues [15]. Polioviruses labeled with a pH-sensitive probe were judged to enter endosomal compartments when they became protected from changes in the extracellular pH. However, these measurements have not been implemented in a manner that would allow continuous

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monitoring of single virus internalization, since extracellular solutions were manually replaced at a selected time, once per experiment.

Here, we designed an imaging-based protocol to measure endocytosis and delivery of HIV-1 pseudoviruses (designated HIVpp) into acidic compartments. To achieve this goal, the viral surface was labeled with a pH-sensitive GFP variant that was quenched at mildly acidic pH. Similar to the strategy used to detect the formation of endocytic vesicles [13], virus internalization was monitored by assessing the changes in mean fluorescence of multiple viral particles in buffers of varied acidity. These measurements showed that the virus uptake and detachment from cells occurred concomitantly and that the latter process was faster than endocytosis. The fluorescence quenching assay also permitted time-resolved monitoring of single HIVpp endocytosis. We found that, shortly after being protected from changes in the external pH, HIVpp was delivered into acidic compartments. This result indicates that, similar to influenza virus [16], the internalized HIVpp is targeted to a quickly maturing population of endosomes. The methodology developed in this paper should be applicable to studies of uptake of both enveloped and non-enveloped viruses. Real-time imaging of single virus endocytosis and subsequent fusion with intracellular compartments would help delineate the steps of productive entry.

Experimental

Plasmids and virus production

To construct the vector expressing the ecliptic pHluorin-ICAM-1 chimera (designated EcpH-TM), the fragments of ecliptic pHluorin [17] and of the transmembrane domain of ICAM-1 (inter-cellular adhesion molecule 1) were produced by PCR using the following primers. EcpH: TAAGCTTCTC GAGAGTAAAG GAGAAGAACT TTTCCTGG and TGAATTCCTTG TATAGTTCAT CCATGCCATGTG; ICAM-1 transmembrane domain: AGAATTCACG GTATGAGATT GTCATCATC and TGGATCCTCA CCGCTGGCGG TTATAGAGGTA. The pCDM8 ICAM-1 vector (Addgene Inc., Cambridge, MA) was used as a template for the transmembrane domain of ICAM-1, and the MLV Gag-EcpH-expression vector [18], derived from the original ecliptic pHluorin construct, was used as a template for EcpH. PCR products of EcpH and ICAM-1 transmembrane fragment were cloned into p3xFLAG CMV9 vector (Sigma, St. Louis, MO) with *Hind*III and *Eco*RI, or *Eco*RI and *Bam*HI sites, respectively. To construct the HIV-1 Gag-mCherry expression vector, the Gag fragment of p96ZM651 gag-opt vector (NIH AIDS Research and Reference Reagent Program, ARRRP) was cloned into the pcDNA3.1zeo(+) plasmid (Invitrogen, Carlsbad, CA) using the *Hind*III and *Bam*HI sites. The mCherry fragment was produced by PCR with pRSET-BmCherry (from R. Tsien, University of California, San Francisco) as a template and following primers: AGGATCCAAG GGCGAGGAGG ATAACATGG and ACTCGAGTTA CTTGTACAGC TCGTCCATGC CGCCGGTGGA GTGGC. The fragment was cloned into the pcDNA3.1zeo(+) vector encoding the HIV-1 Gag using the *Bam*HI and *Xho*I sites.

Pseudoviruses were produced by transfecting 293T cells (ATCC, Manassas, VA) with plasmids encoding HIV R8ΔEnv (from D. Trono, University of Geneva, Switzerland), Gag-mCherry, EcpH-TM, and either HXB2 Env or JRFL Env, using the Ca-phosphate protocol, as described previously [7]. We found that HIV Gag-mCherry was not cleaved by viral proteases and was not released from the viral particles permeabilized by a brief treatment with saponin (data not shown). Therefore, even if a small fraction of virions do fuse with cells, these events should not result in the release in disappearance of the viral core marker.

Virus-cell fusion assay

Virus fusion with TZM-bl cells was quantified using the β -lactamase assay, as described previously [7]. Briefly, viruses were added to cells (MOI 0.7-1) and centrifuged at 2095 $\times$ g, 4°C for 30 min. Cells were washed to remove unbound viruses, and virus uptake and fusion were initiated by raising the temperature to 37°C. Fusion was stopped at indicated times by adding 1 μ M of C52L peptide derived from the C-terminal heptad repeat domain of gp41 [19]. At the end of incubation, cells were loaded with the fluorescent β -lactamase substrate, CCF2-AM (Invitrogen). The intracellular β -lactamase activity (ratio of blue to green fluorescence) was measured after an overnight incubation at 14°C.

Protease protection assay for virus endocytosis

The virus uptake by TZM-bl cells was determined essentially as described in [20]. Briefly, unlabeled pseudoviruses bearing HXB2 or JRFL Env were collected and concentrated by centrifugation (100,000 $\times$ g, 2 hr, 4°C) onto a 20% of sucrose cushion. Virus binding to target TZM-bl cells (MOI 0.7) was aided by centrifugation at 2095 $\times$ g (30 min, 4°C). Following the shift to 37°C, external viruses were stripped by treating cells with 2 mg/ml pronase (10 min on ice), cells were then washed with DMEM with 10% serum and lysed. The amount of p24 in the lysate was determined using a p24 ELISA Kit (Cat# NEK050B001KT, Perkin Elmer Life Sciences, Boston, MA). The protease-resistant p24 signal at a given time point was normalized to the total amount of cell-bound p24 before raising the temperature.

HIV-1 immunostaining and immunoprecipitation

Double-labeled viruses ($1 \cdot 10^4$ IU) containing EcpH-TM and Gag-mCherry and HXB2 Env were allowed to adhere to poly-L-lysine coverglass slides (Lab-Tek, Rochester, NY) for 30 min at 4°C and blocked DMEM/10% serum. All subsequent incubation steps were performed at 4°C. Viruses were first incubated for 2 hr with 0.4 μ g/200 μ l/well of the IgG1b12 anti-gp120 human monoclonal antibody (from ARRRP, donated by Drs. Dennis Burton and Carlos Barbas) or with an isotype matched human IgG antibody (Sigma) diluted in DMEM/10% serum. Samples were washed and incubated for 1 hr with 2 μ g/ml CyTM5-conjugated goat anti-human antibody (KPL, Gaithersburg, MD), washed and imaged. Virus immunoprecipitation was carried out by incubating $\sim 1 \cdot 10^6$ IU of double-labeled pseudoviruses overnight at 4°C on a tube rotator with 10 μ g anti-FLAG M2 (Sigma) or mouse IgG isotype control antibodies covalently attached to Protein G Plus Protein A-agarose beads (Calbiochem). Beads were centrifuged, and supernatants containing unbound particles were removed and titrated on TZM-bl cells, as described in [7].

Calculation of virus detachment and endocytosis

As described in the text, we used the following equations to evaluate the fraction of virions residing on the cell surface (V_s), in neutral endosomes (V_{ne}), in acidic endosomes (V_{ae}), as well as those detached from target cells (V_d). The initial number of viruses attached to cells at time = 0 prior to raising the temperature was calculated as:

$$V_{ini} = F_{8.8}(0) - F_{5.4}(0) = F_{8.8*}(0) - F_{5.4}(0) = \Delta F_0, \quad (1)$$

where $F_{5.4}(t)$, $F_{8.8}(t)$ and $F_{8.8*}(t)$ are the EcpH signals from cell-associated virions in respective buffers. The fraction of cell surface-bound viruses was obtained from:

$$V_s = 100 \cdot [F_{8.8}(t) - F_{5.4}(t)] / \Delta F_0 \quad (2)$$

The fraction of virions residing in neutral and acidic compartments at any given time is:

$$V_{ne}=100 \cdot [F_{5.4}(t) - F_{5.4}(0)] / \Delta F_0 \quad (3)$$

$$V_{ae}=100 \cdot [F_{8.8*}(t) - F_{8.8}(t)] / \Delta F_0 \quad (4)$$

The fractions of detached virions and the total fraction of internalized particles ($V_e = V_{ne} + V_{ae}$) can be written as:

$$V_d=100 \cdot \{[F_{8.8*}(0) - F_{5.4}(0)] - [F_{8.8*}(t) - F_{5.4}(t)]\} / \Delta F_0 \quad (5)$$

$$V_e=V_{ae}+V_{ne}=100 \cdot \{[F_{8.8*}(t) - F_{8.8}(t)] + [F_{5.4}(t) - F_{5.4}(0)]\} / \Delta F_0 \quad (6)$$

Since we found that the number of viruses residing in non-acidic compartments (V_{ne}) was negligibly small, the equations 5 and 6 can be simplified.

$$V_d=100 \cdot [F_{8.8*}(0) - F_{8.8*}(t)] / \Delta F_0 \quad (7)$$

$$V_e=100 \cdot [F_{8.8*}(t) - F_{8.8}(t)] / \Delta F_0 \quad (8)$$

Single virus imaging

Double-labeled pseudoviruses containing EcpH-TM and Gag-mCherry were pre-bound to TZM-bl cells grown on a cover slip by spinoculating at 2095×g for 40 min at 4°C. Due to the better adherence of HXB2 Env-pseudoviruses to cells compared to JRFL Env-pseudoviruses, cover slips were inoculated with 6·10⁴ IU of HXB2 or with 4·10⁵ IU of JRFL virus. This protocol allowed each cell to bind between 12 and 35 double-labeled particles. Cover slips with cells were then transferred to the imaging chamber mounted on the microscope stage. Virus endocytosis (and fusion) was triggered by quickly shifting cells to 37°C. Solutions of different acidity were locally applied through a narrow tip positioned in close proximity to the imaged cells, using the image acquisition software-controlled 4-channel miniature perfusion system (Bioscience Tools, San Diego, CA). The following three isotonic buffers were sequentially applied to cells for 30 sec: pH 5.4 MES, pH 8.8 TAPS and with pH 8.8 TAPS supplemented with 30 mM NH₄Cl (pH 8.8*). To avoid applying excessive shear force, perfusion was carried out at a slow rate of ~0.2-0.3 ml/min.

The image acquisition, local perfusion and autofocus function to compensate for the focal drift were implemented using the MultiTime macros available through the Zeiss LSM510 software. Cells and viruses were imaged using the C-Apo 40×/1.2 NA water immersion objective. Three Z-sections (bottom, middle and top) separated by 2.5 μm were acquired per each time point with the pinhole set to 340 μm in order to collect some out-of-focus light and thereby reduce the number of required Z-stacks. Image analysis was performed using the Volocity software (Improvision/Perkin Elmer). For every image frame, 3-dimensional objects positive for both EcpH and mCherry markers were identified and their total

fluorescence was calculated as a function of time. Single particle tracking was done using the red (mCherry) channel, as described previously [7].

Results

Production and characterization of pH-reporter virions

In order to continuously monitor virus uptake, the pH-sensitive GFP variant (referred to as ecliptic pHluorin (EcpH) [17]) was incorporated into the viral membrane by appending EcpH to the amino-terminus of the transmembrane domain of ICAM-1 (Figure 1A, EcpH-TM), an intercellular adhesion molecule known to selectively incorporate into HIV particles [21, 22]. Double-labeled pseudoviruses were obtained by co-transfecting 293T cells with plasmids encoding EcpH-TM, HIV Gag-mCherry and HIV Env (along with the HIVΔEnv backbone vector). Approximately 30-40% of Gag-mCherry labeled virions obtained by the above protocol contained detectable amounts of EcpH-TM in their membranes (Figure 1B). To eliminate the contribution to the fluorescence signal from the EcpH-TM labeled particles (likely membrane vesicles) lacking the Gag-mCherry, we analyzed only virions positive for both markers (see below). The choice of mCherry as a marker for the viral core was dictated by the relatively low sensitivity to a drop in pH ($pK_a < 4.5$ [23]), which should permit visualization of virions in acidic compartments where the EcpH fluorescence is fully quenched (Figure 1B). As expected, the EcpH- and mCherry-based markers attached to distant proteins did not exhibit FRET (Suppl. Fig. 1).

In order to assess the presence of Env in double-labeled virions, coverslip-immobilized particles were immunostained with the IgG1b12 anti-gp120 antibody and analyzed by fluorescence microscopy (Figure 1D, E). Nearly 55% of Gag-mCherry⁺/EcpH-TM⁺ particles exhibited detectable Env staining (Figure 1F). This relatively low colocalization of fluorescent markers and Env was more apparent than real due to the detection limit of fluorescence microscopy. In reality, EcpH-TM was incorporated into nearly all particles containing both Gag and Env, since >99% of infectious viruses could be precipitated by the M2 anti-FLAG antibody (Figure 1C). Because EcpH and Env signals tended to correlate (Figure 1G), incorporation of this membrane marker into virions did not appear to displace Env. These findings imply that monitoring delivery of double-labeled particles into acidic endosomes should faithfully reflect virus internalization.

Rationale for measuring virus uptake and dissociation

The high pK_a value (~ 7.0 [17]) of EcpH makes it useful for assessing early stages of virus uptake and endosomal acidification since its fluorescence is virtually fully quenched at pH 6.2 (Figure 1B). Thus, the drop in the EcpH fluorescence should, in principle, reflect the time-course of virus delivery into mildly acidic compartments. These measurements would not directly report the time of HIV envelopment by an endocytic vesicle if there is a significant lag before the interior of virus-carrying endosomes becomes acidic. Another confounding factor is that, in agreement with [7, 24], a large fraction of virions detach from cells during an imaging experiment. Virus detachment was manifested in the significant drop of the relatively pH-independent Gag-mCherry signal over time at 37°C (see Figure 3A), which was not due to sample photobleaching (Suppl. Figure 2A).

Our strategy for monitoring HIVpp detachment, uptake and delivery into acidic compartments was as follows. The initial number of virions adhered to cells at the beginning of incubation (V_{ini}) is redistributed into four populations of virions upon incubation at physiological temperature: surface-bound (V_s), detached (V_d), viruses in neutral endosomes (V_{ne}) and those in acidic endosomes (V_{ae}). Detached viruses no longer contribute to the fluorescence signal. To determine the fractions of virions in those populations, cells were

exposed to the following three buffers: pH 5.4 MES, pH 8.8 TAPS, and pH 8.8 TAPS supplemented with 30 mM NH_4Cl (referred to as the pH 8.8* buffer). The diagram in Figure 2A illustrates that only particles residing in neutral endosomes (V_{ne}) should contribute to the EcpH signal in a membrane-impermeant acidic buffer. Thus, virus accumulation in neutral endosomes would increase the EcpH fluorescence at external pH 5.4 (equation (3) of the Materials and Methods). Raising the external pH to 8.8 is expected to maximize the EcpH fluorescence of surface-bound viruses, but not those residing in acidic compartments, thus yielding $V_s + V_{\text{ne}}$. The inclusion of ammonium chloride in the pH 8.8* buffer would raise endosomal pH and reveal the signals from all cell-associated virions, $V_s + V_{\text{ne}} + V_{\text{ae}}$. Therefore, the signal difference in a pH 8.8 buffer with and without NH_4Cl is proportional to the number of particles in acidic compartments.

Prior to shifting cells to 37°C all viruses are accessible to external solutions, hence, the initial number of cell-bound particles is proportional to the difference between the EcpH fluorescence in alkaline and acidic buffers at time $t = 0$ (ΔF_0 , see equation (1)). By normalizing the difference in the EcpH signal in buffers of different acidity at any given time to ΔF_0 , one can obtain the fraction of cell surface-bound, detached and internalized virions residing in different compartments (equations (2-6)). While we did not directly measure endosomal pH in the pH 8.8* buffer, this buffer raised the cytosolic pH (Suppl. Figure 3) and increased the EcpH fluorescence of internalized viruses (see Figures 2, 3 and 6). These results support the notion that the pH 8.8* buffer dissipates the pH gradient across both the plasma and endosomal membranes, thus raising the endosomal pH above neutrality. In our calculations, the differences in the V_{ne} fluorescence in membrane-impermeant buffers and in the pH 8.8* buffer are ignored. As we will show in the next section, this omission is inconsequential.

HIVpp endocytosis is restricted by the kinetically dominant detachment process

The amount of HIVpp input was adjusted so that a few hundred double-labeled particles were initially attached to TZM-bl cells in the image field. Virus uptake was triggered by quickly raising the temperature to 37°C. The virus dynamic on the cell surface was monitored by successive applications of pH 5.4, pH 8.8 and pH 8.8* buffers to cells in the image field. The local perfusion setup was synchronized with the image acquisition and was carried out every 8.5 min in brief sessions lasting about 1.5 min (30 sec for each buffer). Images were acquired during, but not between the perfusion sessions, and only three Z-sections were obtained for every time point. This protocol was designed to reduce adverse effects of shear force and non-physiological pH and to minimize sample photobleaching. A brief exposure to low pH minimizes the cytosol acidification ([13] and Suppl. Figure 3C), which could inhibit endocytosis. We also found that HIVpp-cell fusion was not affected by pre-treatment with acidic buffers (Suppl. Figure 2B).

A typical imaging experiment is illustrated in Figures 2 and 3. At the beginning of incubation at 37°C, the EcpH on all particles responded to the pH changes by switching between fluorescent and non-fluorescent states. Toward the end of the experiment, however, fewer red puncta were observed, and only a fraction of virions exhibited green fluorescence upon raising the external pH (Figure 2B, arrows vs. arrowheads). The dynamics of HIVpp detachment and entry were quantified by plotting the changes in mean EcpH and mCherry signals from double-labeled particles in solutions of different acidity (Figure 3). The application of the pH 5.4 buffer throughout the experiment abrogated the EcpH fluorescence, whereas the pH 8.8* buffer maximized the cell-associated signal (Figures 2 and 3A). Both EcpH and mCherry signals dropped over time even in the pH 8.8* buffer (Figure 3A), demonstrating that a large fraction of viruses detached from target cells. The lack of detectable EcpH fluorescence at acidic pH implies that, at any given time, the fraction of viruses residing in neutral endosomes (V_{ne}) was negligible. Indeed, if viruses

were to accumulate in neutral endosomes to a significant extent, the EcpH fluorescence at an external acidic pH would have increased relative to the initial level (Figure 2A). We therefore used simplified equations (7) and (8) to calculate V_d and V_e .

As expected, EcpH fluorescence was identical in pH 8.8 and pH 8.8* buffers before and immediately after raising the temperature. Over time, the difference between the EcpH signals in pH 8.8 and pH 8.8* buffers became more apparent (Figure 3, A and B), revealing the increased fraction of endosome-resident virions (V_e). To verify that the differences in the EcpH fluorescence with and without NH_4Cl were due to the virus uptake into acidic compartments, imaging experiments were performed at room temperature (25-27°C). As expected for conditions that slow down endocytosis, the pH 8.8* signal was only slightly greater than the pH 8.8 signal by the end of the experiment (Figure 3C). However, judging by the drop in the EcpH and mCherry signals over time at alkaline pH (Figure 3C and data not shown), viruses still effectively detached from cells at reduced temperature.

To obtain the plots of surface-associated (V_s), endocytosed (V_e) and detached (V_d) viruses vs. time of incubation, the EcpH quenching data shown in Figure 3 were analyzed using the equations 2, 7 and 8. This analysis showed that the majority of virions detached from cells, while a smaller fraction was internalized (Figure 4, A and B). In agreement with [7, 24], HIV-1 dissociation was virtually completed within ~40 min at 37°C. By that time, most of the cell-associated viruses were internalized with only a minor fraction remaining at the cell surface. The kinetics of virus detachment based on the decay in the overall mCherry signal was similar to that obtained from the EcpH quenching data (data not shown). We found that the virus uptake was marginal at room temperature, whereas its detachment was comparable to that at 37°C (Figure 4A, stars).

Interestingly, JRFL Env-pseudotyped particles appeared more prone to dissociate from cells and less capable of entering the cells than HXB2 Env pseudotypes. To verify whether this trend was real, we assessed the average values of virus shedding and endocytosis from several independent experiments (Figure 4, C and D). These results supported the initial observation that JRFL was more readily lost from the cell surface than HXB2. As determined by fitting the data with an exponential function (Figure 4, C and D, solid lines), JRFL and HXB2 uptake was respectively 4-fold and 2.5-fold slower than their dissociation from cells. As a result, only 13% of JRFL and 21% of HXB2 virions were endocytosed by the end of the experiment. HXB2 uptake tended to exceed that of JRFL, but these differences were not statistically significant ($P>0.29$) due to a limited sample size. Inverse correlation between HIVpp uptake and dissociation suggests that the kinetic competition between these processes determines the efficacy of virus entry and fusion with endosomes.

At 4°C, HIV-1 associates with HeLa-derived target cells in a CD4-independent manner [9, 20, 25]. We therefore asked whether the virus' propensity to associate with cells in a CD4-independent manner could affect its detachment at elevated temperature. Both single virus imaging (see Materials and Methods) and the p24 assay (Figure 4E) confirmed that HXB2 Env-pseudotyped particles attached to TZM-bl cells in the cold more efficiently than JRFL virions. This suggests that, in agreement with the inverse correlation between HIVpp dissociation and endocytosis (Figure 4, C and D), a tighter CD4-independent attachment to cells in the cold can slow down virus dissociation and thus favor its uptake.

Bulk uptake and productive endocytosis of HIVpp occur at the same rate

Our perfusion protocol was designed to minimize possible adverse effects of non-physiological pH [26, 27] and of the shear force produced by a local perfusion. To verify that these factors did not significantly affect virus endocytosis, we compared the above results with the kinetics of EcpH quenching at neutral pH in the absence of perfusion. Under

these conditions, the reduction in EcpH signals occurred as a result of both virus detachment and delivery into acidic compartments. Since HIVpp does not appear to accumulate in neutral compartments (Figure 3), the loss of EcpH signal should be proportional to $V_d + V_e$. The measured kinetics of the EcpH fluorescence decay in the absence of perfusion (Figure 4C, noisy gray line) and the combined fraction of endocytosed and detached viruses obtained from the perfusion experiments (black squares) were statistically indistinguishable ($P > 0.43$). These results imply that the perfusion protocol itself did not significantly perturb HIVpp uptake.

Next, we compared the rate of virus uptake obtained by the perfusion assay to the rate of the HIV-1 p24 accumulation in TZM-bl cells. The extent of virus internalization was determined from the fraction of protease-resistant p24, as described in [20]. For a given virus (JRFL or HXB2), the rates of EcpH protection from low pH and from a protease were close (Figure 5, $P > 0.40$). However, the fraction of the HIV-1 p24 resistant to proteolysis at any given time was greater than the fraction protected from an acidic environment. These differences are likely due to the limited access of proteases to partially internalized virions (e.g., those in clathrin-coated pits) which are still accessible to small molecules and ions [28]. To conclude, our imaging assay is suitable for the time-resolved measurements of the virus dynamics on the cell surface. This assay appears to more accurately report the point of virus sequestration within endosomal compartments compared to traditional biochemical assays employed to monitor the virus uptake.

The EcpH quenching assay reports the bulk virus uptake that may or may not lead to productive entry. We therefore sought to compare the above results with the functional measurements of productive HIVpp entry. The latter assay is based on the measurement of the time course of the virus escape from the C52L peptide [19], which competitively inhibits fusogenic conformational changes of Env. A fully inhibitory concentration of C52L was added at varied times of virus-cell incubation at 37°C, and the resulting HIV-cell fusion was measured by the β -lactamase assay [29]. Because HIVpp enters TZM-bl cells by endocytosis followed by fusion with endosomes [7], protection from the membrane-impermeant peptide should occur as a result of productive endocytosis which later culminates in virus fusion. The kinetics of HIVpp uptake (EcpH quenching) and of escape from C52L were indistinguishable (Figure 5B, $P > 0.90$). The finding that bulk virus uptake and its productive entry occur at similar rates supports the validity of the EcpH quenching assay.

Internalized HIVpp quickly enters mildly acidic compartments

Our initial analysis was concerned with the average fraction of internalized HIVpp obtained by integrating the pH-dependent EcpH signal from multiple particles. Next, we examined the exact time of single virus uptake and delivery into acidic endosomes. To improve the time resolution for detecting the virus uptake, extracellular pH was shifted between 5.4 and 8.8 every 24 sec (Figure 6A). Also, images (three Z-stacks) were acquired every 7-8 sec instead of 8.5 min (compare Figures 3 and 6). These experiments were carried out with HXB2 pseudoviruses that were more likely to get internalized than JRFL particles (Figure 4).

All cell-bound particles initially exhibited repetitive EcpH quenching-dequenching cycles (Figure 6 and Suppl. Videos 1-3). At later times, double-labeled virions permanently lost the EcpH fluorescence irrespective of the external pH. These events were often associated with the particle displacement towards the cell nucleus and thus marked the virus uptake and delivery into acidic compartments. Since the EcpH signal vanishes at pH 6.2 (Figure 1B), virions internalized during the alkaline perfusion cycle must experience a pH drop of more than 2 units (from 8.8 to ~6.2) in order to lose their green signal. We deduced the time of full virus engulfment by an endosomal membrane from the point when the EcpH

fluorescence ceased to respond to pH changes (Figure 6A). Ranking the internalization times of 26 randomly chosen particles yielded the rate of endocytosis which was not significantly different from that of the bulk virus uptake determined by ensemble analysis of EcpH quenching ($P>0.05$, Figure 4C, open triangles vs. black circles). The fact that raising the endosomal pH with the pH 8.8* buffer at the end of experiment fully recovered the EcpH fluorescence (Figure 6B and E, last panels) confirmed that the loss of this signal was due to the virus delivery into acidic endosomes.

Early endosomes undergo maturation whereupon their interior becomes increasingly acidic [30-32]. Viruses internalized during the alkaline perfusion cycle allowed the measurement of the time between uptake and delivery into mildly acidic compartments. If these virions spend considerable time in early non-acidic endosomes, they should appear as EcpH-positive particles during the next acid perfusion cycle(s) (Figure 6A). Surprisingly, the external pH-independent green fluorescence of virions that entered at alkaline pH was short-lived. The representative virus marked by an arrow in Figure 6B (see also Suppl. Video 1) was sealed off from external milieu at alkaline pH after ~14 min of incubation at 37°C, as evidenced by the brief retention of green fluorescence during the next low pH cycle (Figure 6C). The loss of the EcpH signal during the same low pH cycle (red block arrow) demonstrates that the virus-carrying endosome became acidic shortly after budding from the plasma membrane. Even assuming that the particle was internalized at the onset of the preceding alkaline cycle, the pH around the virus must have dropped in less than 48 sec (the duration of one full pH 8.8/5.4 perfusion cycle). In contrast, surface-associated virions (marked by arrowheads in Figure 6, B and E) continued to change their fluorescence in solutions of different acidity, thereby reporting the exact times of the local pH shift (Figure 6C, light green trace).

We found that 36 out of 80 analyzed virions entered endosomes during the alkaline perfusion cycle. These particles were rendered non-fluorescent within one or two perfusion cycles (for another example of a relatively long-lived endosomal EcpH signal, see Suppl. Figure 4 and Video 2). Seven of these virions quickly lost their EcpH fluorescence while still at alkaline pH (data not shown) and were thus engulfed by endosomes and entered acidic compartments in a time shorter than a single perfusion cycle (21-24 sec). The early acidification of an endosomal lumen did not correlate with significant particle movement (Figure 6, C and D, dashed lines). The 44 virions that entered during the acid perfusion did not exhibit intracellular EcpH fluorescence (Figure 6A and [13]) and therefore did not permit the determination of the time required to deliver the virus into acidic compartments. An example of a virus entering at acidic external pH is shown in Figure 6 (panels D and E, see also Suppl. Video 3).

In order to determine the virus' residence time in neutral endosomes, we measured the interval from the time of retention of EcpH fluorescence at acidic pH and the subsequent loss of green signal (designated T_{EcpH} , Figure 7A). The exact time of the pH drop around the particle of interest was determined based on the EcpH fluorescence quenching of a neighboring surface-associated particle (Figure 7A, dashed line). Note that the above time interval is the lower limit of the actual time the virus spends in non-acidic endosomes, since its uptake occurred at some point during the preceding alkaline perfusion cycle. To account for this uncertainty, half the time of a perfusion cycle was added to the total time EcpH signal was observed after low pH application (T_{EcpH}), yielding an approximate residence time in neutral compartments. As shown in Figure 7B, nearly 80% of virions entered acidic endosomes within 25 sec after being internalized.

The practical implication of quick virus entry into acidic compartments is that the laborious perfusion assay could be potentially replaced by a less invasive protocol which simply

monitors the time course of EcpH quenching in the absence of perfusion. Indeed, the rate of EcpH quenching and the combined rate of HIV-1 detachment and internalization determined by our perfusion protocol were close (Figure 4C). After correcting the EcpH quenching data for the loss of signal due to virus detachment (measured independently), one can obtain the rate of virus uptake.

Discussion

The imaging-based method described in this work enables continuous monitoring of virus uptake and dissociation from cells. This assay revealed that HIVpp dissociated from HeLa cells expressing CD4 and coreceptors faster than they were internalized. Therefore, in agreement with [24], the kinetically dominant virus dissociation process limits its ability to infect these target cells. Interestingly, the JRFL Env-pseudotyped virions bound less efficiently to TZM-bl cells in the cold and detached somewhat more readily than isogenic particles bearing HXB2 Env. Hence, tighter receptor-independent HIVpp binding, likely occurring *via* interactions with heparans and other cellular factors [25, 33, 34], could slow down virus dissociation and favor endocytosis. Platt and colleagues [24] have convincingly demonstrated that HIV-cell binding mediated by polycations enhances the infectivity mainly by preventing virus detachment from cells.

It is worth pointing out that the current study examined HIV-1 uptake using pseudoviruses carrying a non-viral marker (EcpH-TM) and target cells engineered to express proper receptors. Thus, caution should be exercised when extrapolating these data to detachment and internalization of infectious HIV-1 grown in primary target cells. The PBMC-grown HIV-1 contains intercellular adhesion molecules, such as ICAM-1 [21, 22, 35]. Interactions between the viral ICAM-1 and LFA-1 (lymphocyte function-associated antigen 1) expressed on CD4⁺ T-cells and macrophages enhance infection through stabilizing the HIV-1 adhesion to natural target cells [36-38]. It is therefore expected that infectious HIV-1 would be less prone to detach from T-cells compared to pseudoviruses bound to HeLa-derived target cells. On the other hand, the HIVpp model has been extensively validated and employed for delineating the virus entry and nucleocapsid trafficking. Our control experiments (Figure 1) imply that the EcpH-TM is picked up by nearly all entry-competent pseudoviruses without noticeably affecting the incorporation or function of Env. These considerations and the necessity to monitor the virus uptake in real time appear to justify the usage of labeled pseudoviruses and adherent cell lines to gain insight into this process. The imaging-based method introduced in this work establishes proof-of-concept for the future studies of host cell-grown HIV-1 detachment and internalization by primary CD4⁺ T cells and macrophages.

We found that, once internalized, HIVpp are quickly sorted into acidic compartments of HeLa-derived target cells. Firstly, the lack of detectable EcpH signal from multiple virions at low external pH (Figure 3) implies that, at any given time, virtually all cell-associated viruses resided either at the cell surface or in acidic endosomes. Secondly, single virus tracking combined with continuous cycling between acidic and alkaline pH revealed that the residence time in neutral endosomes was usually less than 30 sec. By comparison, HeLa cells sorted a fluid-phase marker into mildly acidic endosomes within 10 min after internalization [39]. In different cell lines, Semliki Forest Virus and influenza virus were trafficked to acidic endosomes within 2-5 min, following their uptake [16, 40, 41]. These times are considerably longer than the brief HIVpp residence time in neutral compartments observed in this work. On the other hand, the interior of single endocytic vesicles carrying the EcpH-tagged transferrin receptor was acidified within ~30 sec, as determined by monitoring the EcpH fluorescence while changing the external pH [13]. This quick transferrin delivery into mildly acidic compartments agrees with the rate of HIVpp entry into

these compartments obtained in our experiments. Thus, although measurements of internalization of a conventional endocytic marker have not been performed in our experimental system, based on the work of others [13], we are confident that our results are valid. In addition, the similar kinetics of HIVpp entry into HeLa-derived cells in the absence of pH cycling and upon applying our perfusion protocol (Figure 4C) imply that transient exposure to acidic and alkaline buffers did not affect the virus uptake. We thus propose that, like influenza virus [16], HIVpp is preferentially sorted into a quickly maturing population of endosomes.

Note, however, that the quick maturation of influenza-carrying endosomes was defined based on the accumulation of the late endosomal marker, Rab7, whereas the lumen of Rab7-positive endosomes became acidic after a considerable delay [16, 40]. The exceptionally quick acidification of HIVpp-carrying endosomes indicates that this virus enters through a distinct endocytic pathway, which may ensure the quick transport to a perinuclear space where the virus appears to fuse [7]. Sorting into quickly maturing endosomes may therefore be beneficial for enhancing the efficiency of nuclear entry and integration. It is not clear, however, what prevents HIVpp from fusing with the plasma membrane and/or what cellular factors facilitate its fusion with endosomes. Future studies of the HIVpp trafficking and dynamic colocalization with endosomal markers at the time of fusion should further our understanding of its productive entry pathway.

The experimental approach described in this paper can be readily modified for monitoring the uptake of viruses that fuse at low pH. To avoid prematurely activating viral fusion proteins, an acidic buffer used in the triple-perfusion protocol should be substituted by a neutral buffer. Even though EcpH would not be fully quenched at neutral pH, the increase in its signal upon shifting to alkaline pH should still be sufficient to monitor virus endocytosis and detachment. The usage of amine-reactive dyes like CypHer5 [15], which fluoresce brighter at acidic pH, would provide a better and perhaps more universal labeling strategy for studies of virus uptake. The ability to detect both virus delivery into acidic compartments and its subsequent fusion in the same experiment is essential for elucidating the key steps of viral entry. This could be accomplished by incorporating EcpH-TM into viruses containing a lipophilic dye and a diffusible content marker [7].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BlaM	beta-lactamase
EcpH	elciptic pHluorin
Env	envelope glycoprotein
FRET	fluorescence resonance energy transfer
HIVpp	Human Immunodeficiency Virus-based pseudoviruses
ICAM-1	Inter-Cellular Adhesion Molecule 1

LFA-1 lymphocyte function-associated antigen 1

Synopsis

Diverse enveloped viruses enter cells by endocytosis and fusion with intracellular compartments. Recent evidence imply that HIV also infects permissive cell lines by fusing with endosomes in a pH-independent manner. This finding highlights the importance of time-resolved monitoring of viral uptake. Here, we designed an imaging-based assay to measure endocytosis in real time through probing the virus' accessibility to external solutions. Exposure of viruses bearing a pH-sensitive GFP variant on their surface to solutions of different acidity altered the fluorescence of surface-accessible particles, but not internalized viruses. By sequentially applying acidic and alkaline buffers with or without ammonium chloride, we were able to quantify the fractions of internalized and non-internalized virions, as well as the fraction of detached particles, over time. The exact time of single virus internalization was assessed from the point when a particle ceased to respond to a perfusion with alternating acidic and alkaline buffers. We found that, surprisingly, HIV pseudoparticles entered acidic compartments shortly after internalization. These results suggest that the virus might be sorted to a quickly maturing pool of endocytic vesicles and thus be trafficked to fusion-permissive sites near the cell nucleus.

Figure 1.

Construction and characterization of pH-reporter pseudoviruses. (A) A diagram of the ecliptic pHluorin (EcpH) attached to the transmembrane domain of ICAM-1 (designated EcpH-TM). (B) Diagrams and images of pseudoviruses labeled with EcpH-TM and the HIV-1 Gag-mCherry at the alkaline and acidic pH. (C) Double-labeled HXB2 Env-pseudotyped viruses can be quantitatively pulled down with the anti-FLAG M2 antibody, but not with an isotype control antibody. (D) A representative image of HXB2 Env-carrying pseudoviruses co-labeled with Gag-mCherry (red), EcpH-TM (green) and immunostained for Env, using the IgG1b12 antibody (blue). Particles positive for all three markers appear white. (E) A contour plot of Gag-mCherry, EcpH-TM and Env co-localization obtained by measuring the total intensities of red, green and blue signals, respectively, for 1,027 individual particles. A "hot spot" of high Env-expressing particles is observed for viruses with larger amounts of Gag-mCherry and moderate amounts of EcpH-TM. (F) Env co-localization with Gag-mCherry⁺ particles (left bar) and with Gag-mCherry⁺/EcpH-TM⁺ particles (right bar). (G) Incorporation of EcpH-TM does not compete with incorporation of Env, as evidenced by a weak positive correlation between the EcpH and Env signals from Gag-mCherry⁺ particles.

Figure 2. Rationale for HIVpp endocytosis measurements and snapshots of viruses on cells subjected to the triple-perfusion protocol. (A) Expected changes in the EcpH-TM fluorescence of virion entering target cells upon exposure to solutions of different acidity. The Gag-mCherry fluorescence of double-labeled viruses is not shown for simplicity. Viruses residing on the cell surface (V_s), in neutral and acidic endosomes (V_{ne} and V_{ae}), as well as detached viruses (V_d) are illustrated. Viruses in acidic endosomes are colored gray, while those in neutral environment are green. An alkaline buffer supplemented with NH_4Cl raises the endosomal pH and reveals the EcpH signal from all three populations of virions (right panel). (B) Representative images of TZM-bl cells with bound EcpH-TM/Gag-mCherry labeled viruses were acquired during successive perfusion with pH 5.4, pH 8.8 and pH 8.8/ NH_4Cl buffers prior to (upper panel) and 43 min after (lower panel) shifting to 37°C. Arrows mark surface-accessible virions that respond to changes in extracellular pH while arrowheads indicate internalized particles that respond only to perfusion with the pH 8.8/ NH_4Cl buffer that raises the endosomal pH. Scale bar 10 μm .

Figure 3.

Analysis of pH-dependent changes in fluorescence of cell-associated HIVpp. (A) EcpH-TM/ Gag-mCherry labeled virions on TZM-bl cells were sequentially perfused with pH 5.4, pH 8.8 and pH 8.8/NH₄Cl buffers. The first perfusion cycle was carried out at room temperature, after which time the cells were shifted to 37°C. The duration of each perfusion session is marked by gray boxes. The pH 5.4 perfusion was initiated ~15 sec before the image acquisition. The open and black circles show the changes in the EcpH and mCherry signals, respectively. Each datum point is the mean fluorescence of a 3D region of interest after subtracting the background signal. The gray dashed lines trace the changes in fluorescence signals in pH 8.8 and pH 8.8* buffers. (B) The blow up view of one perfusion session. (C) Control imaging experiment performed at 26°C. Fluorescence measurements were carried out as described in panel (A).

Figure 4.

Calculated fractions of cell surface-adhered (V_s), endocytosed ($V_e = V_{ne} + V_{ae}$), and dissociated (V_d) viruses as a function of time at 37°C. (A) Changes in the fractions of cell-adhered (open triangles), internalized (black circles) and dissociated (open circles) HXB2 Env-bearing virions were calculated as per cent of the initially pre-bound particles from the representative experiment shown in Figure 3, using the equations 2, 7, 8. Virus detachment and endocytosis at room temperature (RT) are shown by open and black stars, respectively. (B) Same as in A, but for JRFL Env-bearing virions. (C and D) Kinetics of HXB2 (C) and JRFL (D) dissociation (open circles) and internalization (black circles) obtained by averaging the data from 3-5 independent experiments. Solid lines are single exponential fits to data points. The rate of endocytosis based on single particle tracking is shown by open triangles in (C) after normalizing the final extent of virus uptake to that determined by multi-particle analysis. Also shown are the rate of EcpH quenching upon HXB2 endocytosis in the absence of perfusion (gray noisy line) and the sum of endocytosis and detachment ($V_e + V_d$, black squares). (E) JRFL and HXB2 pseudovirus binding to TZM-bl cells expressed as a fraction of HIV-1 p24 input that remained associated with cells after virus spinoculation at 4°C and washing ($n \geq 6$). There was a statistically significant difference in pseudovirus binding ($P < 0.043$). Error bars are SEM.

Figure 5.

The rates of virus endocytosis measured by different techniques. (A) Pseudovirus uptake by TZM-bl cells determined based on the EcpH quenching assay and based on the p24 assay. The HIV-1 p24 internalization assay measured the fraction of viruses that remain associated with cells following the protease treatment. Endocytosis of JRFL and HXB2 cells is shown by open and filled symbols, respectively. Virus uptake based on the EcpH quenching data (re-plotted from Figure 4, C and D) is shown by circles, while time course of p24 internalization is shown by triangles. (B) Comparison of the HXB2 uptake (EcpH quenching data, filled circles) and the virus escape from the inhibitory peptide C52L (open diamonds), as measured by the beta-lactamase assay. Error bars are SEM of at least three independent experiments.

Figure 6.

Imaging single HIVpp endocytosis. (A) Perfusion protocol and particle internalization at alkaline and acidic pH. PM, plasma membrane, CCP, clathrin-coated pits, NE, neutral endosomes, AE, acidic endosomes. EcpH-labeled viruses residing in alkaline and acidic compartments are shown in green and gray colors, respectively. (B and C) Representative images and single particle tracking for an HXB2 Env-pseudotyped virion internalized by a TZM-bl cell at alkaline pH (see also Suppl. Video 1). Internalization of pre-bound viruses was monitored by continuously perfusing cells with alternating pH 5.4 and pH 8.8 buffers, each applied for 24 sec (gray and white areas on the graphs, respectively). The external pH and the time after raising the temperature are shown on top of images. The graph illustrates the mCherry (dark red) and EcpH (dark green triangles) signals from the selected particle (arrow) as a function of time. The pH 5.4 perfusion session applied after ~14 min (second image panel) did not initially quench the EcpH fluorescence, which dropped to the background level shortly thereafter (red block arrow) and did not recover upon subsequent exposure to pH 8.8 (third image panel). A reference particle (arrowhead), which remained on the cell surface throughout the experiment, went through repetitive EcpH quenching-dequenching cycles (light green trace), while its mCherry fluorescence remained steady (light red). (D and E) Single particle tracking and representative images of a virus (arrow) endocytosed during the low pH cycle (see also Suppl. Video 3). The EcpH signal that was lost at low pH (second image panel) did not reappear after shifting to a basic solution (third image panel). Arrowhead marks a surface-accessible virus. The blue numbers above the traces in panels C and D correspond to sequential images in panels B and E, respectively. Perfusion with the pH 8.8/NH₄Cl buffer recovered the EcpH fluorescence of the endocytosed particle (last image panels in B and E). The particle's velocity is shown by a dashed line.

Figure 7. HIVpp residence time in non-acidic endosomes. (A) TZM-bl cells with bound HXB2 Env-pseudotyped viruses were perfused with alternating pH 8.8 TAPS and pH 5.4 MES (gray-shaded areas) buffers every 21 sec. The EcpH fluorescence of surface bound particles (crosses and dashed line) marks the time of pH drop near the virion of interest (black triangles and solid line). The virus residence time in neutral endosomes was obtained from the interval between the first time point when the EcpH signal failed to drop at acidic pH and the time when this signal disappeared due to entry into acidic compartments (T_{EcpH}). To account for the uncertainty arising from the virus internalization at some time during the preceding alkaline cycle, the cycle half time ($T_{1/2} = 10.5$ sec) was added to the measured interval, thus yielding the residence time in non-acidic endosomes ($T_{ne} = T_{EcpH} + T_{1/2}$). (B) Distribution of virus residence times in non-acidic endosomes measured as described above.