Labeling of Multiple HIV-1 Proteins with the Biarsenical-Tetracysteine System

Cândida F. Pereira¹,²,³, Paula C. Ellenberg¹,³, Kate L. Jones¹, Tara L. Fernandez⁴, Redmond P. Smyth¹,⁶, David J. Hawkes¹, Marcel Hijnen¹,⁴, Valérie Vivet-Boudou⁶, Roland Marquet⁶, Iain Johnson⁷, Johnson Mak¹,⁴,⁵ *

¹ Centre for Virology, Burnet Institute, Melbourne, Victoria, Australia, ² Monash Micro Imaging, Monash University, Clayton, Victoria, Australia, ³ Department of Medicine, Monash University, Clayton, Victoria, Australia, ⁴ Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australia, ⁵ Department of Microbiology, Monash University, Clayton, Victoria, Australia, ⁶ Architecture et Réactivité de l’ARN, Université de Strasbourg, CNRS, IBMC, Strasbourg, France, ⁷ Life Technologies Corporation, Eugene, Oregon, United States of America

Abstract

Due to its small size and versatility, the biarsenical-tetracysteine system is an attractive way to label viral proteins for live cell imaging. This study describes the genetic labeling of the human immunodeficiency virus type 1 (HIV-1) structural proteins (matrix, capsid and nucleocapsid), enzymes (protease, reverse transcriptase, RNAse H and integrase) and envelope glycoprotein 120 with a tetracysteine tag in the context of a full-length virus. We measure the impact of these modifications on the natural virus infection and, most importantly, present the first infectious HIV-1 construct containing a fluorescently-labeled nucleocapsid protein. Furthermore, due to the high background levels normally associated with the labeling of tetracysteine-tagged proteins we have also optimized a metabolic labeling system that produces infectious virus containing the natural envelope glycoproteins and specifically labeled tetracysteine-tagged proteins that can easily be detected after virus infection of T-lymphocytes. This approach can be adapted to other viral systems for the visualization of the interplay between virus and host cell during infection.

Introduction

Fluorescent viral fusion proteins have been instrumental in the visualization of the intracellular behavior of viruses during infection [1–3]. However, the incorporation of these 27 kDa fluorescent proteins (FPs) into most viral proteins has limited application due to its potential impact on viral protein function and infectivity [4–7]. This problem has been partially overcome with the development of the biarsenical-tetracysteine labeling system [8], in which the protein of interest is fused to a six to twelve amino acids tetracysteine motif (TC tag), which is only 0.5–1 kDa and therefore less likely to interfere with the structure or biological activity of the protein. The TC tag can be labeled with membrane-permeable biarsenal dyes such as FlAsH and ReAsH dye [9,10].

The TC tag has been inserted at the carboxy-terminus of the human immunodeficiency virus type 1 (HIV-1) matrix (MA) [11,12], HIV-1 integrase (IN) [13] and vesicular stomatitis virus (VSV) MA protein [5,14] without compromising virus infectivity. However, insertion of the TC tag into the cyclophilin-binding loop of HIV-1 capsid (CA) severely impaired infectivity to the extent that virus infectivity needed to be rescued by co-expression of TC tagged and wild-type (wt) proteins [15]. Another recent study has elegantly shown that the loop/linker regions, but not the α-helix regions of influenza A virus non-structural protein 1, can accommodate TC tags without affecting virus infectivity [16].

Two major limitations of the biarsenical-tetracysteine system are the high degree of background fluorescence [17,18], and the fact that the target cysteines in the TC tag must be in their reduced form prior to binding to the biarsenicals dyes [12,19]. Therefore, previous studies of TC tagged viruses have resorted to: (a) the use of a higher contrast twelve amino acids TC motif [12], (b) extracellular labeling of TC tagged proteins under acutely reducing conditions [12,13] and/or (c) increasing the number of available TC tagged proteins inside the target cell via pseudotyped viruses to improve the signal to noise ratio [11–13,15]. In the case of HIV-1, HIV-1 pseudotyped with VSV glycoprotein (VSV-G) instead of its natural envelope (Env) glycoproteins is commonly used to increase the number of HIV-1 proteins inside target cells. However, VSV-G pseudotyping does not represent the natural viral entry process for HIV-1. Furthermore, the extracellular labeling of viruses with biarsenal dyes under highly reducing conditions is not suitable for all virus systems [14].
The aim of the present study was to specifically label all of the structural proteins and enzymes of HIV-1 with a TC tag in the context of a full-length virus without affecting virus infectivity. We employed three different strategies in this study. Firstly, we inserted a six amino acids TC tag upstream and/or downstream of each of the protease cleavage sites in the Gag or Gag-Pol precursor protein. This resulted in specific labeling of MA, CA, nucleocapsid (NC), protease (PR), reverse transcriptase (RT), RNase-H (RN) and IN. Secondly, we inserted the TC tag into two highly flexible regions of the mature RT enzyme and the Env glycoprotein 120 (gp120). Thirdly, using a conditional complementation rescue approach, we generated an HIV-1 construct containing 80% TC-labeled NC proteins. We also show that virus production in the presence of biosynthetic dye followed by the removal of unbound dye is a highly specific method for the labeling of viral proteins containing a TC tag.

Results

Generation of mutant viruses containing tetracysteine-tagged proteins

The aim of this study was to specifically label all of the structural proteins and enzymes of HIV-1 with a TC tag in the context of a full-length virus without affecting virus infectivity. To this end, we created several full-length HIV-1 constructs containing TC tagged proteins (HIVTC). To minimize the potential impediment on virus infectivity, we used the six amino acids TC motif instead of the higher contrast twelve amino acids TC motif [10].

In the first labeling strategy we inserted the TC tag within the N-terminus or C-terminus of the major mature Gag and Pol proteins. The natural proteolytic cleavage sites were preserved by duplicating the first or last five amino acids of the viral protein coding sequences of the corresponding mature proteins (Figure 1A and Table 1). Using this strategy the following HIVTC were generated: HIVMA-C, HIVCA-N, HIVCA-C, HIVRN-N, HIVRN-C and HIVIN-N. The protein processing profiles of these HIVTC were very similar from HIVwt with a few exceptions. HIVRT-N, HIVRT-C, HIVRN-N, HIVRN-C and HIVIN-N showed different degrees of infectivity. HIVRT-N, HIVRN-C and HIVIN-N were approximately 50% less efficient. The capacity of HIVTC to infect the indicator cell line TZM-bl (Figure 1E) clearly showed that HIVMA-C replicates as efficiently as HIVwt, as reported previously [11,12]. The remaining viruses showed different degrees of infectivity, HIVNC-N, HIVNC-C, HIVPR-C and HIVEnv-V1 were 66%, 65%, 63% and 54% less infectious than HIVwt, respectively. HIVRT-N was 84% less infectious than HIVwt and the remaining viruses were approximately 95% less infectious than HIVwt.

Infectivity rescue of mutant viruses containing tetracysteine-tagged nucleocapsid proteins

Since the structural proteins of HIV-1 are more permissive to the insertion of the TC tag we attempted to rescue the infectivity of the HIVCA-N, HIVNC-N and HIVNC-C. A commonly used approach to rescue the infectivity of fluorescently-labeled viruses is to generate mixed virus particles through the co-transfection of virus producer cells with equal masses of plasmids encoding for virus containing wt and virus containing fluorescently-labeled proteins [4,15]. One major limitation of this approach is that it results in a heterogeneous population of virus particles containing: (a) only wt proteins, (b) only fluorescently-labeled proteins and (c) a mixture of wt and fluorescently-labeled proteins. We have therefore adapted our previously described conditional co-transfection system [21] to produce fluorescently-labeled HIV-1 containing a known ratio of wt/TC tagged proteins. The production of viral particles is limited to cells that simultaneously express both wt and TC tagged proteins. This system takes advantage of the fact that both Gag and Rev are required for the formation of infectious viral particles.

Firstly, the HIVCA-N, HIVNC-N, HIVNC-C were modified to introduce an early termination codon and a frameshift into exon 2 of the Rev sequence (HIVTC-ARev) that inactivates Rev function and therefore prevents the nuclear export of the unspliced and single spliced HIV-1 mRNA. Thus, cells expressing only HIVTC-ARev cannot generate virus particles. Secondly, we generated a full-length HIV-1 construct containing modifications at the -1 frameshift slippery sequence and the immediate downstream RNA pseudoknot that regulates Gag-Pol expression (non-frameshift HIV-1 or HIVNFS). These codon modifications altered the coding sequence in the gag reading frame without affecting the protein sequence enabling the expression of Gag but not Gag-Pol. As HIVNFS lacks the capacity to produce HIV enzymatic proteins, cells expressing only HIVNFS are unable to produce mature and infectious viral particles. The built-in constraints of HIVTC-ARev and HIVNFS ensure that fluorescent infectious HIV-1 can only be produced when both proviral DNA constructs are simultaneously expressed in the same cell. These HIV-1 constructs are depicted in Figure 1A.

As the natural ratio of Gag to Gag-Pol synthesis is 20:1, the two plasmids were co-transfected at a ratio of 4 HIVTC-ARev to 1 HIVNFS or 8 HIVTC-ARev to 1 HIVNFS to generate a theoretical ratio of Gag to Gag-Pol of 25:1 and 22:3, respectively. These co-transfection ratios should also result in virus particles containing a ratio of TC tagged to wt Gag proteins of 80:20 or 88:9:11.1, respectively. Firstly, we compared the infectivity of these conditionally-produced fluorescent viruses with the original HIVTC viruses.
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A

| MA | CA | NC |
|----|----|----|
| HIV^{MA-C} | HIV^{CA-N} | HIV^{NC-N} |
| HIV^{CA-C} | HIV^{MA-N} | HIV^{NC-N} |
| HIV^{PR-C} | HIV^{RT1-N} | HIV^{RN-N} |
| HIV^{RT1-C} | HIV^{RT2-C} | HIV^{IN-C} |
| HIV^{RT2-C} | HIV^{RN-N} | HIV^{IN-N} |
| gp120 | gp41 |

B

- gp120-Env
- p66-RT
- p55-Gag
- p51-RT
- p32-IN
- p24-CA
- p17-MA

C

Reverse Transcriptase Activity (cpm)

Days

D

β-lactamase activity (% of HIV^{CA})

E

Luciferase activity (% of HIV^{CA})
peripheral blood mononuclear cells with HIVwt. Samples were collected at 3, 7, 11 and 14 days post infection, and the levels of virus replication were monitored using an in vitro reverse transcriptase assay that is specific for HIV-1 enzymatic activity. Data are representative of 2-3 independent experiments.

Table 1. Overview of the modifications introduced in the HIV-1 genome.

| Construct | Amino acid sequence |
|-----------|---------------------|
| HIV^NC-N | V50NYAGCCRECVSQYV |
| HIV^NC-C | KARVLAGCCPRGKAVL |
| HIV^RT-C | CTRNFAGCCPTTCLNFI |
| HIV^RT-C | GAETFAGCCPTTTGAEFT |
| HIV^RT-N | IRKVLAGCCPCRIVLK |
| HIV^PR-C | MVKQNCRECCAGPVQV |
| HIV^RT-N | PIKQNCRECCAGIKQNG |
| HIV^PR-N | PISPICCGPGCAIPI |
| HIV^RT | YVDGCPCGAGYVDGA |
| HIV^PR | FLGGICCCPGCGALDGI |

Discussion

The imaging of fluorescently-labeled viruses is limited to the visualization of a small number of viral proteins that can be fluorescently labeled without compromising virus infectivity. Due to its small size and versatility, the biosensory-tetracysteine system...
is an attractive generic approach to fluorescently label viral proteins. The TC tag has been successfully used to label the HIV-1 MA [11,12], HIV-1 IN [13] and VSV MA [5,14] proteins. This study describes the labeling of HIV-1 structural, enzymatic and envelope proteins with a TC tag in the context of a full-length HIV-1 and its effect on virus infectivity.

The insertion of the TC tag in the V1 and V2 flexible loops of the Env gp120 showed surprisingly different results. While the insertion in the V2 loop completely abolished virus infectivity, the insertion in the V1 loop was less detrimental to virus infectivity. This suggests that flexibility is not the only requirement for the insertion of the TC tag and that the V1 loop but not the V2 loop of gp120 is a good candidate for the insertion of a TC tag. Furthermore, due to the limited number of Env molecules in the virion [22], the new generation of brighter biarsenical dyes such as Alexa Fluor 568-FlAsH may be more suitable for the visualization of TC tagged Env [23].

Insertion of the TC tag at the C-terminus of the PR did not affect the proteolytic processing of HIV-1 proteins, indicating that it can potentially be used for the visualization of the production, assembly, budding and maturation of HIV-1. On the other hand, insertion of the TC tag at the N- and C-terminus as well as in the flexible “thumb” subdomain of the RT dramatically affected virus production, entry and infectivity. This is not surprising due to the fact that the RT undergoes highly precise and complex structural rearrangements during reverse transcription that might be affected by the smallest modifications [24,25]. A possible solution to this problem may be long-term culture of the HIVRT in lymphocytes to introduce compensatory mutations and therefore restore virus infectivity. Introduction of the TC tag at the N-terminus of the IN abolished HIV-1 infectivity, which indicates that as described previously [13], the C-terminus of IN is more permissive to the introduction of the TC tag.

We confirm that the TC tag can be inserted at the C-terminus of the MA protein without affecting virus infectivity [11,12]. Similarly to what has been described previously for the cyclophilin-binding loop of CA [15], insertion of the TC tag at both the N- and C-terminus of the CA protein completely abolished virus infectivity, suggesting that the stability of the highly ordered CA lattice might be compromised by the TC tag. The most encouraging result from this analysis is that the insertion of the TC tag at either the N- or C-terminus of the NC protein only partially affected virus infectivity. Using a conditional co-transfection system we were able to rescue the infectivity of both the HIV^{NC-N} and HIV^{NC-C} to 72% and 59% of the HIV^{wt} infectivity while retaining enough TC tagged NC proteins for visualization by fluorescence microscopy. These are the first infectious HIV-1 containing fluorescently-labeled NC proteins that will allow the visualization of the intracellular trafficking of the HIV-1 genome-NC complexes in live cells. Furthermore, the approaches described in this study can be applied to other virus systems. The genetic variances found within any group of viruses

Figure 2. Improve the infectivity of the \textit{gag}-TC viruses. (A) Schematic representation of proviral DNA constructs used in the study. The HIV^{TC-ΔRev} construct differs from HIV^{TC} by the introduction of an early termination codon and a frameshift into exon 2 of the Rev sequence, which was removed to inactivate Rev function. This mutation also affects Env expression. HIV^{NFS} is a full-length HIV-1 construct containing codon modifications in the -1 frameshift slippery sequences and the RNA pseudoknot in the \textit{gag} reading frame to enable the expression of Gag but not Gag-Pol. (B) The capacity of HIV^{TC-ΔRev/NFS} to infect target cells was assessed by measuring luciferase activity in the indicator TZM-bl cells that have been infected with the indicated viruses. Error bars, s.d. are based on the averages of 3-5 independent experiments.

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suggest that it is highly likely that a segment within the virus genome will be able to accommodate the six amino acids TC tag without compromising virus infectivity.

Using HIVMA as a model system, we have improved the biosensory-TC labeling system for the visualization of viral proteins during the early events of a natural virus infection. This generic protocol is built on three premises: firstly, the viral genome must contain segments that are sufficiently flexible for the insertion of a six amino acids TC tag without compromising the biological function of the protein and the infectivity of the virus; secondly, multiple copies of the TC tagged protein must be incorporated into the virion during assembly; thirdly, any non-specifically labeled cellular debris and non-incorporated free biosensory dye must be readily removed from the labeled viruses through virus purification procedures.

Furthermore, it is imperative to generate a wild-type virus in parallel with the TC tagged virus in the presence of the biosensory dye in order to evaluate the specificity of the labeling and the potential packaging of non-specifically labeled cellular proteins. By using a metabolic labeling approach that can easily be adapted to other viruses, we have bypassed the requirement for high concentrations of reducing agents during labeling. Using appropriate and specific virus purification procedures (such as sucrose density gradient centrifugation), we have found that non-specifically labeled cellular debris and excess dye can be efficiently removed, which leads to a substantial decrease in background fluorescence.

In conclusion, this study identifies novel tools for the imaging of HIV-1 proteins during its natural infection process. Furthermore, this study describes generic approaches to label viral proteins with a TC tag without compromising virus infectivity as well as a specific and generic method to improve the labeling of TC tagged viral proteins with biosensory dyes in the context of an infectious virus.

Methods

Cells

293T cells were maintained in Dulbecco’s modified Eagle medium/high modified (with 4500 mg/L dextrose and 4 mM L-glutamine) medium (DMEM; Invitrogen, Mount Waverley, Victoria, Australia), supplemented with 10% (vol/vol) heat-inactivated cosmic calf serum (CCS; Hyclone, Tauranga, New Zealand), 100 U/mL of penicillin and 100 μg/mL of streptomycin (Invitrogen). MT-2 cells (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from D. Richman) [26,27] were cultured in Rosewell Park Memorial Institute (RPMI) 1640 medium (Invitrogen) supplemented with 10% vol/vol heat-inactivated fetal calf serum (FCS; Invitrogen) and penicillin/streptomycin. TZM-bl cells (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc.) are derived from the HeLa cell line and stably express high levels of CD4, CXCR4, and CCR5 on the cell surface and have integrated copies of the luciferase and β-galactosidase genes under control of the HIV-1 promoter. TZM-bl cells were cultured in DMEM supplemented with 10% CCS and penicillin/streptomycin. Peripheral Blood Mononuclear Cells (PBMCs) were isolated from buffy packs (supplied by the Red Cross Blood Bank, Melbourne) using Ficoll-Hypaque density gradient centrifugation (Amersham Pharmacia Biotech, Uppsala, Sweden). PBMCs were then stimulated with 10 μg/mL phytohemagglutinin (PHA; Remel, Lenexa, KS, USA) for 3 days and maintained in RPMI 1640 medium supplemented with 10% CCS, 2 mM L-glutamine (Invitrogen), 50 μg/mL gentamycin (Pfizer, Bentley, WA, Australia) and 10 U/mL of human interleukin-2 (IL-2; Roche, Mannheim, Germany).

Viruses

The pNL4-3 proviral DNA (NIH AIDS Research & Reference Reagent Program, Dr. Malcolm Martin [28]) contains the NL4-3 infectious molecular clone of HIV-1. The HIV-1 constructs with a tetracysteine (TC) tag were generated using stitch PCR mutagenesis on the full-length plasmid. Sequence-specific primers were used to introduce the TC motif at the sites indicated in Figure 1A and Table 1. The region of interest was then amplified using HIV-1 specific primers and subcloned back into the plasmid backbone using the appropriate restriction sites. The primers used in this study are described in Table 2. In the HIVMAC-G, HIVCA-N, HIVCA-C, HIVNC-N, HIVNC-C, HIVPR-C, HIVRT-N, HIVRT-C, HIVRN-N, HIVRN-C and HIVIN-N constructs, the TC tag was inserted five amino acids downstream and/or upstream of each of the protease cleavage sites in the Gag or Gag-Pol precursor protein and a NaeI restriction site was introduced downstream or upstream of the TC tag to improve screening. In addition, the first or last five amino acids of the viral protein coding sequence were repeated downstream or upstream of the TC tag to maintain the viral protease cleavage sites. The HIVMAC-Gplasmid differs from HIVTC by the introduction of an early termination codon and a frameshift into exon 2 of the Rev sequence, which inactivates Rev function. The Rev inactivation mutation was achieved by incorporation of a early...
The HIVNFS plasmid was constructed by replacing the wt sequence with 5'CTTCCTC-GGGGAAGATATGGCCATCACACAAAGGTAGACCT3' between DNA nucleotides 4240-4266 employing the ApaI and BclI sites of the HIV-1 molecular clone [29].

HIV-1 particles were produced by poly(ethylenimine) (PEI; Polysciences Inc., Warrington, PA, USA) transfection of 293T cells cultured in 10-cm plates with: 3.5 mg pDRNL or HIVTC plasmids to generate unlabeled viruses; 2.5 mg pDRNL or HIVTC plasmids and 1 mg pMM310 β-lactamase-Vpr plasmid (kindly donated by...
M. Miller, Merck Research Laboratories) to generate β-lactamase-
Vpr-labeled viruses; 5 µg HIV

plasmids and 2.5, 1.25 or 0.63 µg HIV

plasmid to generate HIV

viruses. Forty hours post-transfection supernatant from 293T cells was harvested, purified, filtered and virus production quantified by micro-electronic transistors (RT) assay. Briefly, a sample of the culture supernatant was mixed with an equal volume of 3% vol-
vol Nonidet P-40, followed by addition of RT reaction cocktail containing the template primer poly(rA)-(dT)15 (GE Healthcare, Rydalmer, Australia) and [γ-32P]-dTTP (PerkinElmer, Waldham, MA, USA). Following incubation for 3 h at 37°C, RT activity was determined by the level of [γ-32P]-dTTP using a TopCount NXT™ Microplate Scintillation and Luminescence Counter (Perkin Elmer, Glen Waverley, Australia). Viral particles were concentrated by ultracentrifugation through a 20% sucrose cushion at 100,000 g for 1 h at 4°C using an L-90 ultracentrifuge (SW 41 rotor; Beckman, Fullerton, CA, USA) and virus pellets were resuspended in 1x phosphate buffered saline (PBS; Invitrogen) and quantified using a HIV-1 antigen (p24 CA) Micro enzyme-linked immunosorbent assay (ELISA) (Vironostika: Biomerieux, Buxtel, The Netherlands).

Metabolic labeling of virus with biosynthetic dyes

Eight hours post-transfection the virus producer cells were incubated with 1 µM FlAsH-EDT2 (Invitrogen) pre-mixed with 12.5 µM 1,2-Ethanedithiol (EDT; Sigma-Aldrich, Sydney, Australia) and virus production was allowed to proceed for 40 h. Viral particles were isolated, purified, filtrated and concentrated as described above. Non-incorporated free biosynthetic dye was removed from the virions by sucrose density gradient centrifugation. Briefly, biosynthetic dye-labeled viral particles were layered on top of sucrose density gradients prepared in PBS in 2.5% increments ranging from 30 to 55% sucrose and centrifuged for 16 h at 100,000 × g (SW41 rotor; L-90 Ultracentrifuge). Afterwards, gradient fractions were collected, a micro-RT assay was used to determine the location of the virion particles in the gradient fractions, the virus-containing fractions were pooled together and the virus was re-concentrated by ultracentrifugation and quantified by (p24 CA) Micro ELISA.

Protein isolation and western blot analysis

Intracellular viral protein was isolated from transfected 293T cells by washing cells twice with PBS then lysing with Triton-buffered saline (TBS) lysis buffer containing 1% Nonidet P-40, 20 mM phenylmethylsulfonylfluoride (PMSF), 1 µM pepstatin and 1 µM leupeptin. Cell lysates were freeze-thawed in liquid nitrogen and then clarified by centrifugation. Concentrated virions were resuspended in 1x phosphate buffered saline (PBS; Invitrogen) and quantified using a HIV-1 antigen (p24 CA) Micro enzyme-linked immunosorbent assay (ELISA) (Vironostika: Biomerieux, Buxtel, The Netherlands).

β-lactamase assay

One million MT-2 cells were infected with HIV

and HIV

viruses (normalized to 100 ng of p24) for 1 h at 37°C, 5% CO2, washed and loaded with the β-lactamase substrate CCF2-AM (GeneBLAzer in vivo Detection Kit, Invitrogen) for 7 h at RT. Afterwards, the cells were washed and fixed with 4% formaldehyde (Polysciences, Warrington, PA) in 0.1 M Pipes buffer, pH 6.8. The extent of virus-cell fusion was determined by measuring the production of viral RT activity by using a micro-RT assay.

Infection of lymphoid cells

Synchronized infections were performed as described previously [30]. MT-2 cells were spinoculated with virus at 17°C for 2 h at 1,200 × g. Afterwards, the cells were washed twice with PBS to remove unbound virus and incubated with warm media at 37°C, 5% CO2 for 20 min to initiate infection. Afterwards, the cells were washed, treated with 2 mg/ml of protease from Streptomyces griseus (pronase E, Sigma) for 10 min on ice and washed extensively with PBS containing 20% FCS. The cells were then fixed with 4% formaldehyde in 0.1 M Pipes buffer, pH 6.8 or incubated at 37°C, 5% CO2 for 4 h before fixation and cytospined into glass slides. Cell-free viruses (same batch as used for the infection of lymphoid cells) were fixed with 4% formaldehyde (Polysciences) in 0.1 M Pipes buffer, pH 6.8, spread on glass slides, incubated at 4°C for 16 h and washed twice with PBS.

Immunofluorescence staining

Cells and virus were permeabilized and stained with mouse anti-
matrix (SVM-33) antibody (MH-SVM33C9, ATGC, Manassas, VA (Akzo Nobel N.V.) and/or mouse anti-capsid (AG3.0) antibody (NIH AIDS Research & Reference Reagent Program, Dr. Jonathan Allan) [31] and goat Cy5-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch, USA). After staining, the cells were counterstained with Hoechst 33258 (Invitrogen). Samples were mounted in Fluormount-G (Electron Microscopy Sciences, Hatfield, PA) and images were captured in a z series on a charge-coupled device (CCD) camera (CoolSnap HQ, Photometrics, Tucson, AZ) through a 100x 1.4 numerical aperture (NA) oil immersion lens on a DeltaVision microscope (Applied Precision, Issaquah, WA) and deconvolved using soft-WoRx deconvolution software (Applied Precision).

Quantitative PCR for HIV-1 reverse transcription products

Quantification of HIV-1 reverse transcription products and standardization of cell numbers was performed using quantitative PCR. Concentrated virus stocks were treated with 90 U/ml of Benzonase (Sigma) for 15 min at 37°C before infection to remove any contaminating plasmid DNA from the transcription procedure.
A heat-inactivated virus control (2 h at 56°C) was used to confirm efficient removal of plasmid DNA for each sample. MT-2 cells were infected with equivalent amounts of virus as determined by a HIV-1 antigen (p24-CA) Micro ELISA by spinoculation at 17°C for 2 h at 1,200 x g [30]. Cells were then washed with warm PBS and maintained thereafter in fresh medium at 37°C. Cells were harvested at various time points post-infection and lysed in PCR lysis buffer containing 10 mM Tris [pH 8.0], 30 mM KCl with 0.5% vol/vol Triton-X100, 0.5% vol/vol NP-40 and 75 mg/ml proteinase K (Roche). Samples were incubated at 56°C for 2 h before the proteinase K was inactivated at 95°C for 10 min, samples were then stored at −20°C. Quantitative PCR was performed on an MX3000P QPCR machine (Stratagene). Each PCR reaction contained 1X Brilliant II SYBR Green Master mix (Stratagene), 400 nM each primer and 5 μl of cell lysates (1:10 dilution) in a 15 μl reaction volume. The HIV-1 specific primers M667 (5'-GGCTAATTAGGGAACCCACTG-3') and M661 (5'-CTTGAATTTTCCCCACTG-3') were used to detect early HIV-1 cDNA (template- or strong-stop DNA). The HIV-1 specific primers M667 (5'-GGCTAATTAGGGAACCCACTG-3') and M661 (5'-CTTGAATTTTCCCCACTG-3') were used to detect late HIV-1 reverse transcriptase products 2nd strand transfer. HIV-1 PCR conditions were an initial denaturation at 95°C for 15 min followed by 40 rounds of cycling at 95°C for 10 s, then 60°C for 30 s. Cell numbers were standardized for the human CCR5 gene using the primers LK46 (sense: 5'-GCTGTTGTGGCTCTCCTCCAGGA-3') and LK47 (anti-sense: 5'-CTCAGAGCCGTGTGCCCTCCTCTC-3'). CCR5 PCR conditions were an initial denaturation at 95°C for 10 min followed by 40 rounds of cycling at 95°C for 20 s, 30°C for 40 s and 72°C for 40 s.

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Author Contributions

Conceived and designed the experiments: CFP JM. Performed the experiments: CFP PCE KLJ TF RPS. Analyzed the data: CFP PCE KLJ TF RPS. Contributed reagents/materials/analysis tools: DJH MH VVB RM JJ. Wrote the paper: CFP JM.

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Author/s:
Pereira, CF; Ellenberg, PC; Jones, KL; Fernandez, TL; Smyth, RP; Hawkes, DJ; Hijnen, M; Vivet-Boudou, V; Marquet, R; Johnson, I; Mak, J

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