Published in final edited form as:

Methods Mol Biol. 2014 ; 1087: 29–36. doi:10.1007/978-1-62703-670-2_3.

The Fate of HIV-1 Capsid: A Biochemical Assay for HIV-1 Uncoating

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Abstract

The uncoating process of HIV-1 is a poorly understood process, so the development of a reliable assay to study uncoating is critical for moving the field forward. Here we describe an uncoating assay that currently represents the state-of-the-art biochemical procedure for monitoring uncoating and core stability during infection. This assay is based on the biochemical separation of soluble capsid protein from particulate capsid cores and provides information about the fate of the capsid during infection.

Keywords

HIV-1 core; Uncoating; Capsid; TRIM5 α ; Sucrose cushion

1 Introduction

Soon after human immunodeficiency virus (HIV-1) enters the host cell, a poorly understood process known as “uncoating” takes place. The viral RNA (vRNA) genome is converted to vDNA within the viral nucleoprotein core, sometimes called the reverse transcription complex (RTC). The RTC is composed of capsid protein in addition to matrix protein, Vpr, reverse transcriptase, and integrase [1]. Synthesis of full-length vDNA within the RTC produces the pre-integration complex (PIC) [1]. For HIV-1, biochemical isolation of the cytosolic PIC reveals little or no HIV-1 capsid protein [2–5], and recent studies suggest that reverse transcription and separation of CA from the complex are linked [6, 7]. Subsequently, the PIC is actively transported to the nucleus, where vDNA is integrated into cellular chromatin.

Here we describe an assay to follow HIV-1 uncoating within cells by measuring stability of the HIV-1 core acutely after infection [6, 8–15]. The HIV-1 core is a cone-shaped shell composed of ~1,500 monomers of capsid protein [16, 17]. Upon release into the cytosol, this cone-shaped capsid protein lattice comes apart (uncoating). In its simplest definition, uncoating is the shedding of soluble capsid monomers or small lattice subunits from the retroviral core or ribonucleoprotein complex. Since only about 40 % of the total capsid protein within the virion appears to be incorporated within the capsid protein lattice [18, 19], it is believed that monomeric capsid is in dynamic equilibrium with the assembled capsid (viral core). This implies that the core exists in a meta-stable state only when the soluble

capsid is in high concentration, keeping the equilibrium shifted towards the core formation by mass action; however, the fact that complexes containing capsid have been detected in the cytoplasm of cells early during infection implies that cellular factors might be involved in stabilization of the core [4, 20, 21]. Here we describe a procedure that can be applied any time during infection to determine the relative amount of capsid that is within cores (pelletable) versus the amount that is soluble.

2 Materials

Prepare all solutions using Millipore water and analytical grade reagents. All solutions might be stored at 4 °C unless otherwise indicated.

1. Phosphate-buffered saline (PBS) 1×: To prepare 1,000 ml dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄, and 0.24 g of KH₂PO₄ in 800 ml of Millipore water. Adjust pH to 7.4 and the total volume to 1,000 ml using Millipore water.
2. Pronase solution: Prepare pronase at 7 mg/ml in complete DMEM medium containing 10 % serum.
3. Hypotonic lysis buffer is composed of 10 mM Tris-HCl, pH 8.0; 10 mM KCl and 1 mM EDTA and tablet protease inhibitors (Roche).
4. Sucrose cushion is a solution of 50 % sucrose weight/volume in 1× PBS.
5. 7.5 ml Dounce homogenizer with pestle B was obtained from Kontess glass CO.
6. DMEM Invitrogen.
7. Cf2Th and HeLa cells are available from ATCC.
8. Polyallomer centrifuge tubes (14×89 mm from Beckman Coulter) catalog #358649.
9. Beckman ultracentrifuge Optima L-100 K.
10. Beckman rotor SW41.
11. Fluorescent Western blotting system.
12. Li-Cor scanning.
13. Lipofectamine 2000 (Invitrogen).
14. Polybrene(Sigma).

3 Methods

Brief description of the assay. To perform this biochemical assay, cells must be infected with a large quantity of retroviral particles (step 1 of Fig. 1). Infection is allowed to proceed for a specific amount of time. Cells are harvested and treated with Pronase and washed three times (step 2 of Fig. 1). Cells are then lysed by using a Dounce homogenizer (step 3 of Fig. 1). Subsequently, a post-nuclear supernatant (PNS) is prepared by spinning the lysates at 1,000 × g and collecting the supernatant (step 4 of Fig. 1). A fraction of the PNS is stored as “input.” The PNS is then spun through a 50 % sucrose cushion, and “soluble” and “pellet” fractions are collected (steps 5 and 6 of Fig. 1). Input, soluble and pellet fractions are

analyzed by fluorescent Western blot to quantify the soluble and particulate capsid (step 7 of Fig. 1).

1. Count human HeLa or canine Cf2Th cells transduced with the empty vector LPCX or with vector expressing TRIM5_{rh} and seed 2×10^6 cells on 10 cm plates (*see Notes 1 and 2*).
2. The next day, add 10 ml of freshly produced virus in the presence of polybrene (4–8 $\mu\text{g/ml}$) (*see Note 3*). Incubate cells for 30 min on ice. Change media and allow infection to occur for a specific amount of time (*see Note 4*).
3. Harvest the cells by adding 1 ml of complete DMEM medium with 10 % serum containing pronase (7 mg/ml) and incubate on ice for 5 min (*see Note 5*).
4. Use $1 \times$ PBS (ice-cold) to wash the cells three times (centrifuge at 4°C for 7 min at $500 \times g$ in a eppendorf table top centrifuge).
5. Remove PBS and add 2.5 ml of hypotonic lysis buffer and incubate on ice for 15 min.
6. Use pestel B of a Dounce homogenizer and perform 15 strokes (*see Note 6*).
7. Centrifuge the lysate at $1,000 \times g$ for 4 min at 4°C to separate the nuclear pellet from the cytosolic (supernatant) fraction.
8. From the supernatant fraction, take 100 μl as the “Input.”
9. Prepare a polyallomer centrifuge tube (14×89 mm from Beckman Coulter) containing 7 ml of 50 % sucrose in PBS (4°C).
10. Layer 2.3 ml of the supernatant on top of the sucrose cushion.

¹Many different vectors can be used to stably express TRIM5 or other restriction factors within target cells (Fig. 3). pLPCX (Clontech) uses the strong CMV promoter to drive expression [22]. In some cases, restriction factors such as TRIM5 can be quite toxic to transduced cells, and this may necessitate using vectors with lower levels of expression. One possibility is to use pMIG, which drives transgene expression from the LTR of the retrovirus vector [23, 24]. Alternatively, one can replace the CMV promoter with a weaker promoter such as the HSV TK promoter, or place the transgene downstream of an internal ribosome entry site (IRES) [25].

²The effect of restriction factors such as TRIM5 can be studied in most cell lines, though it has been reported that restriction activity is not as strong in some lines as in others [15]. This might be due to low levels or absence of host factors essential for restriction activity [26, 27]. Restriction activity from heterologous TRIM5 transgenes is fine in most human cell lines such as HeLa, though there can be significant competition from endogenous TRIM5, with interference in restriction activity [28]. Because of concerns about interference with endogenous TRIM5, two of the preferred lines for TRIM5 restriction studies are canine Cf2Th cells and feline CRFK cells. TRIM5 was disrupted in the canine genome and the capsid-interacting PRYSPRY domain is missing from the feline orthologue [29]. Both of these cell lines have been used successfully for fate-of- capsid assays.

³The titer of the virus is extremely important for the success of this assay. To produce a high titer virus we cotransfect a 10 cm dish of 293 T cells using a 5 μg of codon-optimized HIV-1 gag- pol, 10 μg of a GFP-reporter construct, and 3 μg of the VSV-G envelope by using Lipofectamine. Virus collection is carried out 48 h post-transfection. Polybrene could be used in a range 4–8 $\mu\text{g/ml}$ or higher. It is recommended to test the toxicity on the cell line that will be used to perform the experiment. HeLa and Cf2Th cells can tolerate as much as 8 $\mu\text{g/ml}$ for 16 h without sign of obvious toxicity. To increase the effective titer of a given retrovirus stock, an alternative to polybrene is so-called “spinoculation” [30].

⁴In the case of TRIM5_{rh} acceleration of HIV-1 uncoating is observed best at 16 h postinfection; however, it could be seen as early as 8 h postinfection [8, 12]. In the case of MLV uncoating, effects of TRIM5-mediated restriction could be detected as early as 1 h postinfection [9].

⁵Pronase treatment is intended to eliminate the virus associated nonspecifically with cellular membrane and not in the infectious pathway [31].

⁶The numbers of strokes used for preparation of the extracts depends upon the cell line being use. The number of strokes should be sufficient to disrupt the integrity of 80–90 % of the cells, as demonstrated by trypan blue staining, with minimal disruption of the nucleus and other organelles.

11. Centrifuge in an ultracentrifuge using a Beckman SW41 rotor for 2 h at 4 °C at $100,000 \times g$.
12. After centrifugation, collect 1 ml from the top of the centrifuge tube as the “Supernatant” (*see Note 7*).
13. Use vacuum to carefully remove the sucrose and resuspend the pellet in 40 μ l of 2 $\times$ loading buffer as the “pellet” (*see Note 8*).
14. Analyze the Input, supernatant and pellet fractions by fluorescent Western blotting using antibodies against p24 (HIV-1 capsid) or p30 (MLV capsid) as shown in Fig. 2 (*see Note 9*).

Acknowledgments

This work was funded by an R01 AI087390 to F.D.-G, NIH grant RO1AI59159 to J.L., Swiss National Science Foundation grant 3100A0-128655 to J.L., and a K99/R00 Pathway to Independence Award to F.D.-G. from the National Institutes of Health 4R00MH086162-02.

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⁷Collect at least 1 ml of the “Input” supernatant being careful not to take the endosomal fraction that will be a white band in between the lysate and the sucrose cushion. In order to be analyzed by Western blot, the supernatant should be concentrated by alcoholic precipitation (SHOULD BE MORE SPECIFIC: ethanol? What about TCA?).

⁸The pellet fraction containing the virion cores or particulate capsid is not visible, so the sucrose must be removed without perturbing the bottom of the tube.

⁹The preferred antibody for p24 (HIV-1 capsid) is from Immunodiagnostics (Cat# 1103). For MLV capsid, we purified the antibody from supernatant of the R187 hybridoma (ATCC). Fluorescent western blot will permit reasonable quantification of the virion proteins in the different fractions.

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Fig. 1.
Schematic diagram showing the steps of the fate of the capsid assay

Fig. 2.

Rhesus TRIM5 α (TRIM5 α_{rh}) decreases the amount of HIV-1 retroviral cores during infection. HeLa cells containing the empty vector LPCX or stably expressing TRIM5 α_{rh} were incubated with equivalent amounts of HIV-1-GFP at 4 °C for 30 min. The cells were washed and returned to 37 °C, and infection was allowed to proceed for 16 h. Cell extracts were fractionated on sucrose gradients. Input, soluble capsid and particulate capsid/retroviral cores fractions were analyzed by Western blotting using antibodies against the HIV-1 capsid protein (p24). **(b)** Similar results were obtained in three independent experiments and the standard deviation is shown. Statistical differences were given as $p < 0.001$; twoway ANOVA followed by the Bonferroni post test

Fig. 3.

Retroviral constructs used to express TRIM5 proteins. Schematic diagram of three different vectors that have been used to transduce TRIM5 into target cells. pLPCX and pMIG are derived from murine leukemia viruses. pFUPI is derived from HIV-1. TRIM5 expression from pLPCX is driven by the strong CMV promoter. pMIG and pFUPI offer lower levels of expression. *LTR* long-terminal repeat; Δ U3 a deletion of the main transcriptional enhancer elements within the LTR, ψ psi, the *cis*-acting RNA packaging signal, *CMV* cytomegalovirus immediate early promoter, *UBI* UbC gene promoter, *PuroR* puromycin acetyltransferase coding sequence