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The CsA washout assay to detect HIV-1 uncoating in infected cells

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Summary

Uncoating is an early step of HIV-1 replication in which the viral capsid disassembles by p24 capsid ($p24^{CA}$) protein dissociating from the viral complex. Although uncoating is required for HIV-1 replication, many questions remain about the mechanism of this process as well as its impact on other steps in viral replication. Here we describe a recently developed assay to study the process of uncoating in HIV-1 infected cells. The CsA washout assay is a cell based assay that utilizes the HIV-1 restriction factor TRIM-CypA to detect and inhibit infection of coated viral complexes. Owl monkey kidney (OMK) cells are infected with a GFP reporter virus and TRIM-CypA restriction is switched on at various times post-infection allowing the kinetics of uncoating to be monitored in infected cells. This assay also can be used to examine the effect of different viral or cellular factors on the process of uncoating.

Keywords

HIV-1; capsid; uncoating; retrovirus; CsA washout assay; TRIM-CypA

1. Introduction

Background

In the early stages of HIV-1 replication the viral membrane fuses with the target cell membrane, resulting in the release of a viral complex into the cytoplasm of the cell. This viral complex consists of the genomic RNAs and associated proteins surrounded by a conical shell of $p24^{CA}$ protein called the capsid. For infection to progress, p24 capsid ($p24^{CA}$) protein must dissociate from the rest of the viral complex, a process referred to as uncoating. Uncoating is required for HIV-1 replication, but the mechanistic details of this process remain unclear. For example, it is not known if all $p24^{CA}$ proteins dissociate as the capsid structure disassembles or if a subset of molecules remain associated with the uncoated viral complex. Recent studies have largely focused on investigating the timing or cellular location of uncoating and viral determinants that mediate this process.

Initially uncoating was thought to occur immediately following viral fusion as viral complexes isolated from infected cells did not contain detectable levels of $p24^{CA}$ protein (1–

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3). However, poor capsid stability and detergent sensitivity likely caused premature $p24^{CA}$ disassembly in these experiments as $p24^{CA}$ has since been shown to localize with cytoplasmic viral complexes when using more careful isolation methods (4). A more recent model, based on biochemical data and fluorescence microscopy observations, suggests that the intact capsid docks at the nuclear pore after completion of reverse transcription (5, 6). It has also been proposed that uncoating takes place as the reverse transcribing viral genome is transported toward the nucleus largely due to fluorescence microscopy observations that both cytoplasmic reverse transcription complexes associated with and without $p24^{CA}$ protein can be found on microtubules (7, 8).

Many uncoating studies have focused on the role of $p24^{CA}$. Mutations in $p24^{CA}$ were identified that both altered capsid stability and decreased infectivity indicating that overall capsid stability is important for viral replication (9–13). The correct timing of uncoating after viral fusion also seems to be important for infectivity as $p24^{CA}$ mutants that uncoat more rapidly and mutants that uncoat more slowly than wildtype both decrease infectivity (9). Finally, experiments have suggested that there is a complex interplay between uncoating and reverse transcription of the viral genome. Some of the $p24^{CA}$ mutants with altered capsid stability also displayed defects in reverse transcription *in vivo* which initially suggested that uncoating occurs before reverse transcription (9). However, reverse transcription products can be detected in viral complexes that still contain $p24^{CA}$ protein using fluorescence microscopy (7). Furthermore, a recent study using two complementary assays to detect uncoating in HIV-1 infected cells has shown that inhibition of reverse transcription by nevirapine can prolong the process of uncoating (8).

Uncoating can be studied using three types of assays- in vitro, microscopy based, and cell based. For in vitro uncoating assays viral capsids are purified, incubated, and then the extent of uncoating is determined by comparing the relative amounts of particulate and soluble $p24^{CA}$ protein (9, 14). In microscopy based uncoating assays coated viral particles are directly detected in the cytoplasm of infected cells by staining with an antibody to $p24^{CA}$ (5, 8, 15). If this analysis is done at various times post-infection in conjunction with a marker for the virus to detect uncoated viral complexes then the kinetics of uncoating can be determined (8, 15). In the fate of the capsid assay, a cell based assay, infected cells are lysed several hours post-infection and uncoating is assayed by comparing the amounts of particulate and soluble forms of $p24^{CA}$ (16). Another cell based uncoating assay, the CsA washout assay, has recently been developed and is the subject of this chapter (8).

The CsA washout assay is based on experiments conducted in the Bieniasz lab studying the HIV-1 restriction factor TRIM-CypA (17). TRIM-CypA inhibits HIV-1 replication by binding to the conical capsid and therefore should only inhibit infection of coated viral particles. (Figure 1; (18–20)). The drug cyclosporine A (CsA) blocks the interaction of TRIM-CypA with the viral capsid and thus acts a switch for turning off TRIM-CypA restriction (Figure 1; (18, 21, 22)). For this assay, owl monkey kidney (OMK) cells that endogenously express TRIM-CypA are synchronously infected with VSV-g pseudotyped HIV-GFP reporter virus in the presence of CsA (23). At various times post-infection CsA is washed out and any viral complex that has an intact capsid, or has not uncoated, becomes susceptible to TRIM-CypA restriction. Viral complexes that have uncoated, and therefore

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lack an intact capsid, are resistant to TRIM-CypA restriction and can infect the cell (Figure 2). Two days post-infection cells are harvested and subjected to flow cytometry for GFP to determine the percentage of infected cells. The percentage of GFP positive cells at each washout time point is representative of the percentage of uncoated virions at that time because only uncoated particles can infect the cell during TRIM-CypA restriction.

Representative data from the CsA washout assay with ethanol (EtOH) washout as the negative control is shown in Figure 3. The percentage of GFP positive cells is graphed for each washout time point. This percentage increases over time, leveling off four to five hours post-infection. This data can be normalized by setting the highest percentage of GFP positive cells (4 or 5 hours) to 100% and the time at which 50% of the virions have uncoated is calculated. In eight independent experiments uncoating initiated within the first hour after viral fusion with an average half-life of 40 minutes (8). Wildtype HIV displayed an average half-life of uncoating of 74 minutes in the CsA washout assay. However, the difference in the half-life of uncoating between VSV-g pseudotyped and wildtype virus can be accounted for by the differential rate of fusion of the two viruses (8). This result highlights the point that rates of uncoating as measured in the CsA washout assay can be influenced by the kinetics of viral fusion.

Compared to other uncoating assays the CsA washout assay has the benefit of being based on infectivity. In order for an uncoating event to be scored as positive the virus not only has to uncoat, but must be able to proceed through replication to reporter gene expression from the integrated provirus. However, because the assay is based on infectivity uncoating is detected through an indirect method using a restriction factor. As the mechanistic details of TRIM protein restriction of HIV-1 replication are unclear it is possible that resistance to TRIM-CypA restriction may occur before uncoating is completed. Therefore, given the differing strengths and weaknesses of the various uncoating assays, we believe it is important to use more than one assay to study uncoating when possible.

2. Materials

Cell lines: 293T cells, owl monkey kidney (OMK) cells

DNA plasmids: HIV-GFP, CMV-VSV-g

Cell culture media: Dulbecco’s Modified Eagle Media, 10% fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin, and 292 ug/ml L-glutamine

PEI: 1mg/ml in sterilized ddH_2O, MW 25,000 (Note 1)

Cyclosporine A: prepared in ethanol at 5 mM, used at a final concentration of 2.5 uM, store at −20°C

[1]At room temperature 1 mg/ml PEI is not completely soluble so when making the solution apply a low amount of heat until the PEI goes into solution. Let the solution cool to room temperature before aliquoting. The PEI solution will now be cloudy and some white precipitate may be visible. This is the optimal appearance and if the solution is not cloudy transfection will not be successful. Store 1 ml aliquots at −80°C. One tube may be used for multiple transfections provided it is stored at 4°C after thawing and its appearance remains cloudy.

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Polybrene: prepared in ddH_2O at 10mg/ml, used at a final concentration of 5 ug/ul, store at −20°C

CsA or EtOH media: Cell culture media, 1:2000 dilution of CsA or EtOH

Fix: 4 parts 1X phosphate buffered saline, 1 part 10% formaldehyde

Equipment: tissue culture incubator, tissue culture hood, centrifuge capable of spinning 96 well plates, 37°C water bath, flow cytometer capable of reading samples in a 96 well plate format, multichannel pipette

3. Methods

3.1 Virus generation

Virus can be generated using other methods, but we prefer to use PEI transfection of 293T cells. 293T cells are maintained in a tissue culture incubator under standard conditions at 37°C and in the presence of 5% CO_2.

1. Seed out 293T cells in a 10 cm tissue culture dish so that they will be 80–90% confluent on the day of transfection.
2. Next day, make transfection mixture by adding 6 ug HIV-GFP proviral plasmid, 4 ug CMV-VSV-g plasmid, and 40ul PEI to 1 ml Dulbecco's Modified Eagle Media. Flick tube gently to mix. Incubate at room temperature for 15 minutes.
3. Add transfection mixture dropwise to the 10cm dish. Gently rock the plate to mix. Incubate cells for 24 hours.
4. Aspirate media from the 10cm dish. Add 10 ml of new warmed cell culture media. Incubate cells for 16–18 hours.
5. Remove media from the 10cm dish by pipetting. Filter this viral supernatant using a 0.45 um syringe filter. (Note 2)
6. Aliquot filtered virus into cryovials and store at −80°C until use.

3.2 Virus titration

Virus is titrated under the same conditions as the CsA washout assay using a ½ dilution series in the presence of CsA or EtOH. OMK cells are maintained in a tissue culture incubator under standard conditions at 37°C and in the presence of 5% CO_2. (Note 3)

1. Seed out OMK cells in a 96 well tissue culture plate at a concentration of 7500 cells/well. This will yield about 90% confluency on the next day. For each virus tested at least 12 wells of a 96 well plate will be used with an additional 3 wells of uninfected cells in cell culture media as a negative control for flow cytometry.

[2]293T cells are adherent but may dislodge from the plate during the collection of viral supernatant. If this happens pipette the viral supernatant into a 15ml conical tube and centrifuge at 1,500 x g for 5 min to pellet the cells. Then carefully remove the media without disturbing the pellet and filter through a 0.45 um syringe filter.

[3]OMK cells are larger in size and grow slower than HeLa or 293T cells. OMK cells are used for this assay because they endogenously express TRIM-CypA. Other cells lines could be used for the assay if TRIM-CypA is stably expressed.

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2. Next day, prepare 2X CsA media by adding 1ul CsA and 1 ul polybrene to 1ml cell culture media. Prepare 1X CsA media by adding 0.5 ul CsA and 0.5ul polybrene to 1 ml cell culture media. Prepare 2X and 1X EtOH media the same way.
3. Aspirate media from one row of the 96 well plate (12 wells across). Add 100ul 2X CsA media to the first well of a row. Add 100ul 1X CsA media to the next 5 wells. Add 100ul 2X EtOH media to the next well. Add 100ul 1X EtOH media to the next 5 wells.
4. Add 100ul of virus to the first well of the row and pipette several times to mix with the media (1/2 dilution). Transfer 100ul to the next well of the row and pipette to mix (1/4 dilution). Continue serial dilution across the next 4 wells and discard the last 100ul in a bleach solution.
5. Perform the same serial dilution with the EtOH test wells (second half of the row).
6. Place the plate in the centrifuge and spinoculate at 1200 x g at 16°C for 1.5 hours.
7. Make CsA and EtOH media. Store at 4°C until the plate is removed from the centrifuge.
8. Remove the plate from the centrifuge and incubate at 37°C for 30 minutes. Place CsA and EtOH media in the 37°C water bath.
9. Aspirate inoculation media from all wells and replace with 200ul warmed CsA or EtOH media. Incubate cells for 2 days.
10. Aspirate media and add 100ul trypsin to each well. Incubate at 37°C until cells detach with gentle tapping.
11. Add 100ul Fix to each well of a new 96 well plate.
12. Resuspend the trypsinized cells by pipeting. Add resuspended cells to corresponding wells in the new 96 well plate that contain fix.
13. Wrap the edges of the plate in parafilm and then wrap the entire plate in foil. Store at 4°C. Incubate fixed cells at 4°C for at least 4 hours to allow any residual virus to fix. We typically fix the cells overnight and run flow the next day.
14. Run the fixed samples on a flow cytometer for GFP to determine the percentage of infected cells.
15. Determine the correct viral dilution to use by comparing the CsA and EtOH reactions. Choose a dilution where the percentage of infected cells in the CsA reaction is below 50% so that only one virus is infecting a cell. The percentage of GFP positive cells in the corresponding EtOH reaction should be 0–0.1% indicating that TRIM-CypA restriction is not saturated at that dilution. (Note 4)

[4]We prefer viral dilutions that yield 30–40% GFP positive cells in order to observe changes in infectivity over a broad range. When comparing two or more viruses we choose dilutions for each virus that yield similar infectivities.

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3.3 CsA washout assay

The assay takes place over 4 days from seeding out the OMK cells to harvesting the infected cells. For the assay OMK cells are maintained in a tissue culture incubator under standard conditions at 37°C and in the presence of 5% CO_2.

1. Seed out OMK cells in a 96 well tissue culture plate at a concentration of 7500 cells/well to yield about 90% confluency on the day of the washout assay. For the assay each experimental time point is performed in triplicate with a corresponding EtOH control, also in triplicate. For each virus three wells are needed for the CsA continuous treatment (positive control), and three wells are needed for continuous EtOH treatment (negative control). For flow cytometry three wells of uninfected OMK cells continuously incubated in cell culture media are used as a negative control. (Note 5)
2. Next day, make spinoculation mastermix for each experimental condition in a 15 ml conical tube. For each well 100ul of spinoculation mix will be used. For one reaction, add 0.05ul polybrene, 0.05ul CsA or EtOH, and the appropriate dilution of GFP reporter virus to cell culture media to a total volume of 100ul. (Notes 6 and [7])
3. Aspirate media and add 100ul spinoculation media to each well using a multichannel pipette.
4. Place the 96 well plate in a centrifuge cooled to 16°C. Spinoculate at 1200 x g at 16°C for 1.5 hours. (Note 8)
5. Make CsA and EtOH media. Store at 4°C until the plate is removed from the centrifuge.
6. Remove the plate from the centrifuge and incubate at 37°C for 30 minutes. Place CsA and EtOH media in 37°C water bath. (Note 9)
7. Aspirate spinoculation media from all wells. Replace with 100ul warmed CsA or EtOH media. Perform washout for the zero hour time point by replacing the spinoculation media with 200ul warmed cell culture media. Return the plate to the tissue culture incubator until the 15 min time point.
8. Remove the plate from the incubator and perform washout for the 15 min time point by aspirating the CsA or EtOH media and adding 200ul warm cell culture media to each well. (Note 10)

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[5]For the assay presented in Figure 3 cells were seeded in 63 wells of a 96 well plate.

[6]To synchronize infection the temperature of the media needs to be at or below 16°C (see note 8). Therefore, when preparing to make the inoculation media only incubate the cell culture media in a 37°C water bath for 10–15 minutes, long enough to bring the temperature up from 4°C but not to 37°C.

[7]Order of addition is media, polybrene, CsA or EtOH, and then virus.

[8]For VSV-g pseudotyped virus, viral fusion is synchronized by the combination of spinoculation at 16°C, a temperature at which the virus can only bind and endocytose, and then temperature shift to 37°C, by replacing the inoculation media with warmed media, to allow completion of viral fusion. For virus with the wildtype HIV envelope spinoculation can be done at 23°C to achieve the temperature arrested state of fusion, followed the same temperature shift to 37°C through media change.

[9]The 30 minute incubation at 37°C is to allow the cells to recover from spinoculation. The media will warm a little, but the following media change will provide the temperature change to allow viral fusion.

[10]When performing the assay on more than one plate change the media on the plates in the same order so that the time lags between the different washout time points is consistent from plate to plate. This is especially important for the early time points where there is only a 15min interval.

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9. Continue washout in the same manner for the 30 min, 45 min, 1 hr, 2 hr, 3 hr, 4 hr, and 5hr time points. Incubate cells for 2 days.
10. Aspirate media and add 100ul trypsin to each well. Incubate the plate at 37°C until cells detach with gentle tapping.
11. Add 100ul Fix to each well of a new 96 well plate.
12. Resuspend the trypsinized cells by pipeting. Add resuspended cells to corresponding wells in the new 96 well plate that contain fix.
13. Wrap the edges of the plate in parafilm and then wrap the entire plate in foil. Store at 4°C. Incubate fixed cells at 4°C for at least 4 hours to allow any residual virus to fix. We typically fix the cells overnight and run flow the next day.
14. Run the fixed samples on a flow cytometer for GFP to determine the percentage of infected cells.

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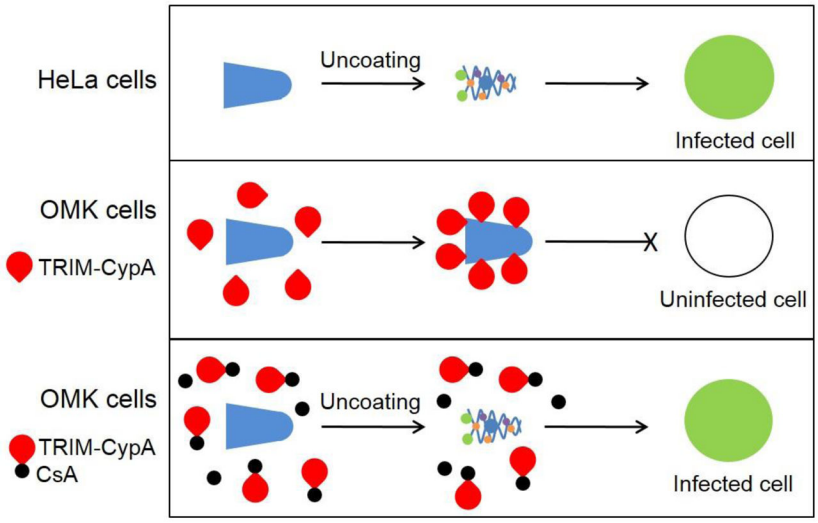

Figure 1. Rationale of the CsA washout assay

HIV uncoats and infects HeLa cells which can be assayed using flow cytometry when a GFP reporter virus is used (top panel). In OMK cells TRIM-CypA binds to the capsid and restricts HIV infection (middle panel). HIV can infect OMK cells in the presence of cyclosporine A (CsA) which binds TRIM-CypA and prevents its binding to the HIV capsid (bottom panel).

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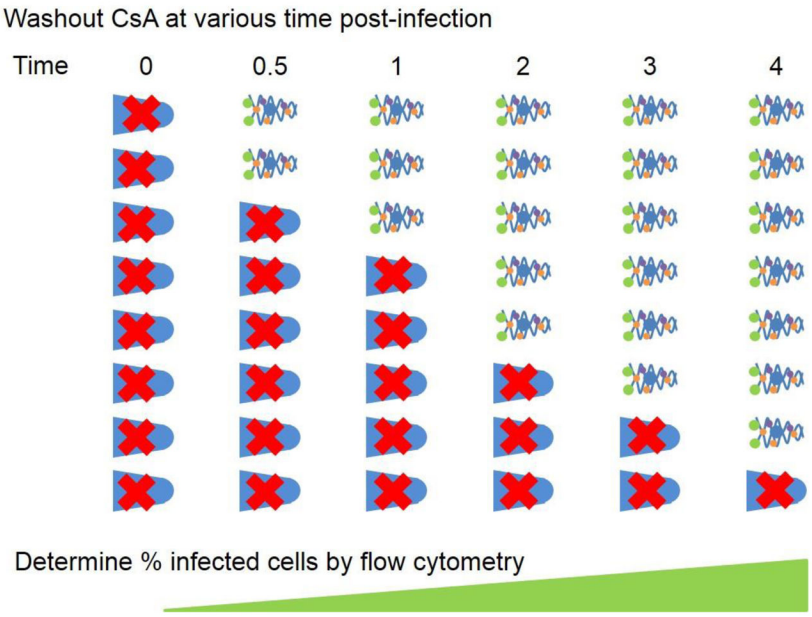

Figure 2. Schematic of the CsA washout assay

HIV GFP reporter virus infects OMK cells in the presence of CsA. When CsA is washed out at various times post-infection coated viral complexes are restricted by TRIM-CypA, while uncoated particles can continue through the replication process to infect cells. The percentage of infected cells, and thus the percentage of uncoated viral complexes, at each time of CsA washout is determined by flow cytometry.

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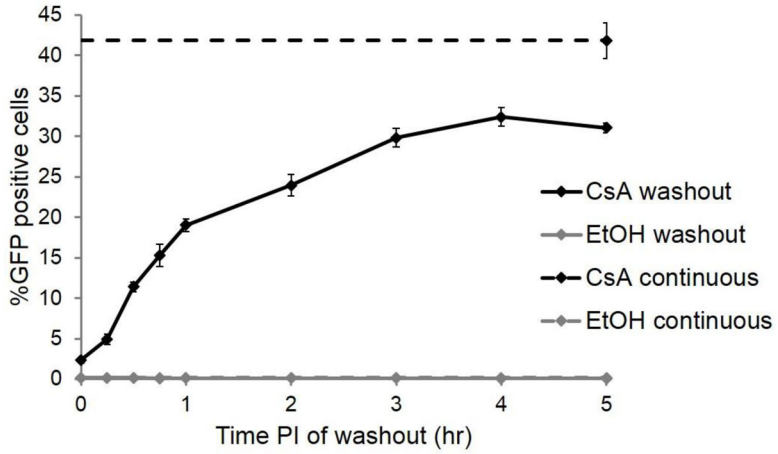

Figure 3. Representative results from the CsA washout assay

The CsA washout assay was conducted using an HIV GFP reporter virus (HIV-GFP). Graphed is % of GFP positive cells at each time point of CsA washout, with each data point representing an average of three wells. The presence of CsA in the media continuously is a positive control. Ethanol (EtOH) washout and continuous treatment are negative controls. Error bars denoted standard error among triplicate wells.