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Human Retroviruses: Methods and Protocols:

CryoEM Analysis of HIV-1 Capsid Assembly and Structural Consequences of TRIM5 α Binding

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Summary

After virus fusion with a target cell, the viral core is released into the host cell cytoplasm and undergoes a controlled disassembly process, termed uncoating, before or as reverse transcription takes place. The cellular protein TRIM5 α is a host cell restriction factor that blocks HIV-1 infection in rhesus macaque cells by targeting the viral capsid and inducing premature uncoating. The molecular mechanism of the interaction between capsid and TRIM5 α remains unclear. Here, we describe an approach that utilizes cryo-electron microscopy (cryoEM) to examine the structural changes exerted on HIV-1 capsid (CA) assembly by TRIM5 α binding. The TRIM5 α interaction sites on CA assembly were further dissected by combining cryoEM with pair-wise cysteine mutations that crosslink CA either within a CA hexamer or between CA hexamers. Based on the structural information from cryoEM and crosslinking results from in vitro CA assemblies and purified intact HIV-1 cores, we demonstrate that direct binding of TRIM5 α CC-SPRY domains to the viral capsid results in disruption and fragmentation of the surface lattice of HIV-1 capsid, specifically at inter-hexamer interfaces. The method described here can be easily adopted to study other important interactions in multi-protein complexes.

Keywords

CryoEM; uncoating; HIV-1 restriction factor; TRIM5 α ; HIV-1 Capsid

1. Introduction

The mature type 1 human immunodeficiency virus (HIV-1), the agent responsible for acquired immunodeficiency syndrome (AIDS), contains a conical capsid that encloses the RNA viral genome. The HIV-1 capsid comprises ~ 1,500 copies of capsid protein (CA) subunits which assemble into a fullerene cone-shaped structure (1). HIV-1 CA consists of an N-terminal domain (NTD) and a C-terminal domain (CTD) connected by a flexible linker. A number of atomic structures of individual CA domains as well as full-length protein have been solved (2–12). Due to its unprecedented polymorphism and asymmetric architecture, it is difficult to determine the structure of the native HIV-1 viral core. Several alternative approaches have been employed to shed light on the structure of HIV-1 capsid, including cryo-electron microscopy (cryoEM) of helical assemblies and two-dimensional arrays (13,

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14). Most recently, atomic models of the basic building blocks of the fullerene cone, CA pentamers and CA hexamers, were determined by the Yeager group using an elegant crosslinking strategy for X-ray crystallography (15–17). Recombinant full-length CA spontaneously assembles into helical tubes under high salt conditions (1), and the structure of these closely resembles authentic viral core. A pseudo-atomic model of helically assembled CA was recently constructed based on the cryoEM structure of in vitro assembled tubes (10). In addition to previously identified NTD-NTD, NTD-CTD and CTD dimer interactions (7, 13, 14) that are involved in capsid assembly, this CA assembly structure revealed a novel CTD-CTD trimer interface at the local three-fold axis of the CA lattice, which plays important roles in uncoating and capsid stability (10). Taken together, these structural findings have provided a firm foundation for the study of retroviral capsid function, including uncoating and interaction with host cell factors.

HIV-1 capsid uncoating is a very important, yet obscure, early post entry event. Recent studies have suggested that uncoating is a tightly controlled process: uncoating too early or too late impairs viral infectivity (18, 19). Because of its critical role during HIV-1 replication, capsid is a target of a host restriction factor, Trim5 (20–23). TRIM5 plays an important role in the innate immune defense against retroviruses, including HIV-1 (21, 24–27). The α splice variant of TRIM5 in rhesus macaque cells blocks HIV-1 after viral entry and before reverse transcription (21). Several studies suggest that TRIM5 α interacts with viral capsids and induces premature capsid uncoating (28–31). Very recently, TRIM5 was identified to have double duty in HIV-1 restriction. Other than inducing premature uncoating, TRIM5 is also involved in activating a cellular innate immune response by acting as a pattern recognition receptor for retrovirus capsid (32). TRIM5 α is a member of a tripartite motif (TRIM) family of proteins, which contain RING, B-box 2, and coiled-coil (RBCC) domains. TRIM5 α also has a C-terminal B30.2/SPRY domain (33–35). All four domains have distinctive functions and contribute collectively to the antiviral function of TRIM5 α . The RING domain is an E3 ubiquitin ligase (36, 37) and its self-ubiquitylation correlates to HIV-1 restriction (38). While the CC domain is necessary and sufficient for TRIM5 α homo-dimerization (25, 39–41), the B-box 2 domain is essential for higher-order association among TRIM5 α dimers and TRIM5 α binding avidity to capsid (42, 43). The C-terminal B30.2/SPRY domain binds to viral capsid and determines the specificity of restriction (25, 26, 31, 44–48). *In vitro*, specific recognition and binding to a hexagonal CA lattice requires both the CC and SPRY domains (40). Full-length chimera TRIM5–21R can spontaneously self-assemble into a hexagonal lattice that is complementary to the CA lattice (49). Our recent study also shows that, at high concentration, TRIM5 α CC-SPRY alone can bind the HIV-1 capsid assembly and disrupt the assembly lattices (50).

In this chapter, we describe, in detail, our protocols to examine the interaction between TRIM5 α and CA assembly by cryoEM. Based on the structural models derived from cryoEM and X-ray crystallography, we designed pair-wise cysteine mutations and chemical crosslinking that distinguish the inter- and the intra-hexamer interactions to probe which CA interface is affected by TRIM5 α interaction.

2. Materials

2.1. Equipment

1. FPLC system: AKTA Explorer with Unicorn software, 5mL Hi-Trap QP column and 5mL Hi-Trap SP column, and Hi-Load Superdex 75 26/60 column (GE Healthcare, Piscataway, NJ).
2. Vertical electrophoresis system: XCell SureLock Mini-Cell (Invitrogen, Carlsbad, CA).
3. Glow-discharge device model 100× (EMS, Hatfield, PA).
4. FEI transmission electron microscopes (FEI Corp., OR): Tecnai T12 with LaB₆ filament, a single-tilt room-temperature specimen holder and a Gatan 2K×2K CCD camera; Tecnai Polara F30 microscope with a Field Emission Gun, equipped with a Gatan 4K × 4K CCD camera.
5. Plunge-freezing devices: Vitrobot Mark III Automated Plunger (FEI Corp., OR) or home-made MRC-type gravity plunger.

2.2. Reagents

1. The cDNA encoding gag polyprotein, pr55^{gag} can be obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (51).
2. pET21 and Rosetta 2 (DE3) (EMD chemicals, Inc. San Diego, CA).
3. Electron microscope carbon-coated copper grids (SPI Supplies, West Chester, PA), Quantifoil R2/1 200 mesh holey-carbon copper grids (Quantifoil Micro Tools, Jena, Germany).
4. Filter paper type #1: 90 mm diameter (Whatman, Clifton, NJ).
5. DUMONT biology anti-capillary reverse tweezers (Ted Pella, Redding, CA).
6. All other reagents except those listed below are from Sigma (St. Louis, MO) and are of analytical grade or higher.
7. Pre-cast 4–12% SDS PAGE gel, 4× SDS sample buffer and 20× running buffer (Invitrogen, Carlsbad, CA), Amicon concentrators (Millipore, Billerica, MA). Luria-Bertani media (Bacto, NJ).
8. 5 nm Ni-NTA-Nanogold® gold beads from Nanoprobes (Yaphank, NY)
9. Polyethylenimine (Polysciences, PA)
10. pENT-TOPO vectors (Invitrogen)
11. Baculodirect C-term (Invitrogen)

2.3. Buffer systems

2.3.1 CA purification and assembly buffer

1. Lysis buffer: 25 mM sodium phosphate, pH 7.0, 0 mM NaCl

2. Hi-Trap QP column buffer: 25mM sodium phosphate, pH 7.0, 1mM DTT, and 0.02% sodium azide, 0 mM NaCl (Low Salt) - 1500 mM NaCl (High Salt).
3. Hi-Trap SP column buffer: 25 mM sodium phosphate, pH 5.8, 1 mM DTT, and 0.02% sodium azide, 0 mM NaCl (Low Salt) - 1000 mM NaCl (High Salt).
4. Hi-Load Superdex 75 26/60 column buffer: 25 mM sodium phosphate, pH 5.8, 100 mM NaCl, 1 mM DTT, and 0.02% sodium azide.
5. CA assembly buffer: 50 mM Tris-HCl, pH 8.0, 1 M NaCl.
6. Dilution buffer: 50 mM Tris-HCl, pH 8.0, 100 mM NaCl

2.3.2 TRIM5 α CC-SPRY expression and purification buffer

1. Lysis buffer: 25 mM sodium phosphate, pH 7.5, 250 mM NaCl, 10 mM beta-mercaptoethanol, 0.1 mM PMSF, inhibitor cocktail (Roche), and 0.02% sodium azide.
2. Hi-Load Superdex 75 26/60 column buffer: 25 mM sodium phosphate, pH 7.5, 150 mM NaCl, 2 mM DTT, 10% glycerol, and 0.02% sodium azide.
3. Hi-Trap QP column buffer: 25 mM Tris-HCl, pH 7.5, 2 mM DTT, 10% glycerol, and 0.02% sodium azide, a gradient of 0 – 1000 mM NaCl.

2.3.3 TRIM5 α CC-SPRY binding buffer (also stock buffer)

1. Binding buffer: 10 mM Tris-HCl, pH 7.5, 330 mM NaCl, 1 mM TCEP, 0.02% azide, 5% glycerol

2.3.4 Nano-gold labeling buffer

1. 0.05 μ M 5 nm Ni-NTA-Nanogold gold beads, 10 mM imidazole, 866 mM NaCl, 42 mM Tris, pH 8.0, 0.2 mM TCEP, 0.004% sodium azide, 1.25% glycerol

2.3.5 Native core isolation buffer

1. STE buffer: 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA

2.3.6 Crosslinking buffer

1. Oxider buffer: 60 μ M CuSO₄, dissolved in water, and 267 μ M 1,10-phenanthroline dissolved in 100% ethanol in a 1:1 ratio
2. Quench buffer: 20 mM iodoacetamide and 3.7 mM neocuproine.

3. Methods

3.1 Expression and purification of HIV-1 CA proteins

1. Amplify and subclone wildtype full-length CA (Pro1-Leu231) into pET21 using NdeI and XhoI sites.
2. Express full-length CA in *E. coli*, Rosetta 2 (DE3), cultured in Luria-Bertani media and induce with 0.4mM IPTG. Culture at 23°C for 16 hrs.

3. Lyse cells by sonication in the lysis buffer and clear the cell lysate by ultracentrifugation at 100,000×g for 1hr at 4°C
4. Let the supernatant flow through a Hi-Trap QP column equilibrated with the lysis buffer.
5. Collect flow through fractions.
6. Reduce the pH of the sample to 5.8 with diluted acetic acid and reduce conductivity to below 2.5ms/cm by adding Milli-Q water. Centrifuge the sample at 13,000 rpm with a SLA-1500 rotor for 1hr at 4°C.
7. Pass the resulting supernatant through a Hi-Trap SP column and collect peak fractions. CA should elute at ~10 ms/cm.
8. Concentrate the sample to 15–18 ml and pass it through a Hi-Load Superdex 75 26/60 gel-filtration column.
9. Collect peak fractions and concentrate with Amicon concentrators to 10 mg/ml, based on the extinction coefficient at UV 280.
10. Analyze protein samples by SDS-PAGE and stain with Coomassie-Blue to assess protein purity.
11. For storage, add 5% glycerol to the sample. Flash freeze 25µl aliquots and store them at –80°C.

3.2 Expression and purification TRIM5α CC-SPRY

1. Amplify and clone the coiled-coil and SPRY domains of human and rhesus TRIM5α (TRIM5α CC-SPRY; residues 132–493 and 134–497, respectively) into the pENT-TOPO vector (Invitrogen), modified to encode a Strep-tag at the N-terminus and a His₆-tag at the C-terminus of the proteins. The Strep-tag is cleavable with TEV protease.
2. Prepare baculoviruses expressing human and rhesus TRIM5α CC-SPRY using the Baculodirect C-term (Invitrogen) according to the manufacturer's protocols.
3. Infect SF21 insect cells with baculoviruses at a MOI of 2 for 40 h.
4. Harvest cells by centrifugation at 4000×g for 30 min. Lyse cells by sonication in the lysis buffer containing 25 mM sodium phosphate, pH 7.5, 250 mM NaCl, 10 mM beta-mercaptoethanol, 0.1 mM PMSF, inhibitor cocktail (Roche) and 0.02% sodium azide.
5. Clear cell lysate by ultracentrifugation at 100,000×g for 1hr at 4°C.
6. Let the supernatant flow through 5 mL Ni-NTA column. The protein is eluted with the lysis buffer containing 500 mM imidazole. Aggregates should be removed by passage over a Hi-Load Superdex 200 16/60 column (GE Healthcare) in a buffer containing 25 mM sodium phosphate, pH 7.5, 150 mM NaCl, 2 mM DTT, 10% glycerol, and 0.02% sodium azide.

7. Collect the fraction containing TRIM5 α CC-SPRY and dilute it 3-fold with a buffer containing 25 mM Tris, pH 7.5, 2 mM DTT, 10% glycerol, and 0.02% sodium azide.
8. Pass the fraction through a 5 mL Hi-Trap QP column (GE Healthcare) and elute the proteins using a gradient of 0 – 1 M NaCl. Alternatively, load the fraction onto a 5 mL StrepTrap-HP column (GE-Healthcare) and elute with 2.5 mM desbiotin
9. Flash freeze 20 μ l aliquots and store them at -80°C

3.3 In vitro HIV-1 CA assembly

1. Add 2 $\times$ assembly buffer to freshly-thawed stock protein to a final CA concentration of 2 mg/ml (*see* Note 1).
2. Incubate the mixture at 37°C for 1hr and mix frequently. At the end of incubation, transfer the sample to ice for further analysis.

3.4 TRIM5 α CC-SPRY and CA assembly binding assay

We use a binding assay with SDS-PAGE to quantify the binding ratio of TRIM5 α CC-SPRY/CA.

1. Add TRIM5 α_{hu} CC-SPRY or TRIM5 α_{rh} CC-SPRY aliquots from 4 mg/ml stock solutions to the preassembled CA tubes (*see* Note 2).
2. Incubate the reaction mixture on a rocking platform at room temperature for 1hr with gentle mixing at 10 min intervals.
3. At the end of incubation, withdraw 5 μ l samples from the reaction mixtures and immediately use these for cryoEM analysis.
4. Collect 6 μ l samples from the same reaction mixtures and mix these with 4 $\times$ LDS loading buffer supplemented with 10 mM DTT for SDS-PAGE analysis (t).
5. Pellet the remaining sample at 20,000 $\times$ g with an Eppendorf centrifuge 5417R for 15 min.
6. Collect the supernatants (s) carefully without disturbing the pellets (p)
7. Resuspend the pellet in 1/3 of volume.
8. Mix the supernatant and the resuspended pellet solution with 4 $\times$ LDS loading buffer.
9. Load the total (t), supernatant (s), and pellet (p) samples, without boiling, onto 10% SDS-PAGE and stain with Coomassie Blue (Fig. 1).

¹For the CA assembly assay, the sequence of mixing reaction components has an impact on the final number of assembled tubes. The most efficient assembly is achieved by adding 2 $\times$ assembly buffer to the CA protein stock solution followed by addition of Milli-Q water.

²Before carrying out binding experiments, freshly thawed TRIM5 α CC-SPRY solution should be spun at 20,000 $\times$ g for 1 min at 4°C to remove aggregates, thus minimizing the errors in the binding ratio measurements.

10. Scan the SDS-PAGE gels and calculate the integrated intensities of CA and TRIM5 α_{rh} protein bands in pellet fractions measured using ImageJ 1.40g program.
11. Calculate the binding molar ratio according to the formula (TRIM5 α_{rh} band intensity/TRIM5 α_{rh} molecular weight)/(CA band intensity/CA molecular weight).

3.5 CryoEM specimen preparation

1. Glow discharge several 200 mesh perforated Quantifoil grids at 25 mA for 25 second.
2. Prepare liquid ethane and wait until it has cooled to near solidifying temperature (*see* Note 3).
3. Mount a grid onto an anti-capillary forceps and apply 5 μ l sample to the carbon side of the grid (*see* Note 4).
4. Mount the forceps onto the plunge-freezing device and make the carbon side face away from you for manual plunger.
5. Quickly blot the excessive solution by pressing filter paper against the bar side for about 6 second.
6. Remove the filter paper and release the plunger at the same time.
7. Transfer the frozen EM grids to storage for later cryoEM analysis.

3.6 CryoEM study of TRIM5 α CC-SPRY interaction with CA assemblies

1. Set up a TRIM5 α CC-SPRY and CA assembly binding experiment by following the method described in **Subheading 3.4**.
2. Prepare a cryoEM specimen by following the method described in **Subheading 3.5**.
3. Load the frozen grid into a Tecnai TF20 microscope.
4. Screen the grid at 200 $\times$ magnification and find the suitable area with thin ice (*see* Note 5).
5. Record all the thin ice areas suitable for imaging in a stage position file.
6. Change the magnification to 59,000 $\times$ and move to the first position
7. Switch to diffraction mode; insert and center an objective aperture (*see* Note 6).

³Ice contamination is a normal problem during the cryoEM grid preparation. One common source of contamination is contaminated liquid ethane. Ethane should be completely cooled to near solidifying temperature and kept minimally exposed to air to avoid frost. Also, the remaining ethane gas at the bottom of the tank is not suitable for sample preparation.

⁴The CA tubes are assembled at 1 M NaCl. The salt increases the background noise of cryoEM images considerably. For imaging, a drop (3–5 μ l) of dilution buffer can be added to the bar side of the grid, and, then, a filter paper can be used to quickly blot away excess solution from the bar side. Due to the quick exchange between sample solution and dilution buffer, structural effects on the sample can be kept to a minimum.

⁵Before collecting cryoEM micrographs, it is important to select the areas with the most suitable ice thickness. The data collected at thinner ice areas will normally give a better contrast. It is convenient and time-saving to screen the whole grid first at 200 $\times$ magnification to find thin ice areas. The thin ice area should show uniform contrast indicating the ice thickness is uniform. Furthermore, the holes should be clearly visible as thicker ice will reduce the contrast from holes on carbon film.

⁶Due to the low contrast of frozen-hydrated biological specimens, an objective aperture can be used to enhance the contrast, albeit at the cost of resolution. The objective aperture can also reduce charging problems. Insert and carefully center the objective aperture. Due to potential residual contamination and slight misalignment, the objective stigmatism needs to be readjusted.

8. Switch back to imaging mode and lower the magnification to 3900 $\times$.
9. Record a CCD image using a long exposure time of $\sim 1 - 2$ seconds and a beam spot size of 9 or 10.
10. Identify a thin ice-covered hole with a feature and move the stage to a position in which the feature is at the center of the CCD.
11. Switch to exposure mode and collect a CCD image under low dose condition ($10 - 15 \text{ e}^-/\text{\AA}^2$).
12. Examine the morphology of the CA assembly after incubating with binding buffer, human and rhesus TRIM5 α CC-SPRY (Fig. 2A, 2B & 2C).
13. CryoEM has shown that the structure of tubular CA assembly has been disrupted by rhesus TRIM5 α CC-SPRY, resulting in release of linear fragments of CA (Fig. 2C & 2D).

3.7 Nano-gold labeling of TRIM5 α CC-SPRY on capsid assemblies

It is difficult to visualize the TRIM5 α CC-SPRY density on CA tubes in cryoEM micrographs due to low contrast. Since the C-terminus of TRIM5 α CC-SPRY is tagged with His₆, we used Ni-NTA nano-gold labeling to confirm its binding to CA assemblies (52). The method is described in detail here.

1. Assemble the CA tubes as described in **Subheading 3.3**.
2. Set up both human and rhesus TRIM5 α CC-SPRY/ CA assembly binding assays, as described in **Subheading 3.4**.
3. Add 2.7 μl of 5 nm Ni-NTA-Nanogold $\text{\textcircled{R}}$ gold to the assemblies and incubate the mixture at room temperature for 20 minutes.
4. Centrifuge the mixture at 3,000 $\times g$ and resuspend the pellet in assembly buffer.
5. Apply the samples immediately to glow-discharged EM grids for negative staining with 1% uranyl acetate solution.

In rhesus TRIM5 α CC-SPRY/ CA samples, EM micrographs showed the nano-gold beads clearly decorating the CA tube surfaces (Fig. 2F). In contrast, in human TRIM5 α CC-SPRY/ CA samples, few gold beads were found to bind to CA tubes (Fig. 2E). These results compliment the previous binding results from SDS-PAGE analysis.

3.8 Inter-molecular crosslinking of CA assemblies

The CA assembly is built with NTD hexameric rings connected by CTD dimers. The hexamers are stabilized by intra-hexamer NTD-NTD and NTD-CTD interactions (15). The inter-hexamer linkage can be strengthened through a CTD-CTD trimer interface at the local three-fold axis (10). To probe which interface is disrupted by TRIM5 α , we used two pair-wise cysteine mutations A14C/E45C, which crosslinks CA into hexamers (Fig. 3A) (15), and P207C/T216C, which crosslinks three CA molecules from neighboring hexamers (Fig. 3B), to stabilize the intra- and inter-hexamer interfaces, respectively. Incubation of TRIM5 α_{rh} CC-SPRY with crosslinked CA assemblies and examining the structural effect

using cryoEM provided insight into which interface is more susceptible to TRIM5 α disruption (50).

1. Assemble 30 μ l P207C/T216C or A14C/E45C CA in the presence of 50 μ M DTT under the conditions described in **Subheading 3.3**.
2. Incubate assembled tubes with binding buffer and TRIM5 α_{th} CC-SPRY (18 μ M), as described in **Subheading 3.4**.
3. Pellet both samples by centrifugation at 20,000 $\times$ g, at room temperature, in an Eppendorf centrifuge 5417R for 15 minutes.
4. Resuspend the pellet in 30 μ l assembling buffer and oxidize it with 1 μ l of 30 $\times$ oxidizer mix for 5 seconds.
5. Quench the resuspended pellet solution immediately with 20 mM iodoacetamide and 3.7 mM neocuproine.
6. Mixed quenched pellet solution with 4 $\times$ LDS loading buffer.
7. Load the sample, without boiling, on 10% SDS-PAGE and stain with Coomassie Blue.
8. Our SDS-PAGE analysis confirmed that CA A14C/E45C is crosslinked into hexamers and that CA P207C/T216C is crosslinked into trimers (Fig. 3A lane 1, 3B lane 1). When incubated with TRIM5 α_{th} CC-SPRY followed by oxidation, the trimer band (Fig. 3B lane 2) was reduced dramatically, whereas the hexamer band (Fig. 3A lane 2) exhibited only a minor reduction, if anything; instead, the dimer of hexamer band was almost completely diminished. These results suggest that TRIM5 α disrupts the CA lattice at inter-hexamer interfaces.
9. Incubate TRIM5 α_{th} CC-SPRY with both non-crosslinked (Fig. 4A lane 2) and crosslinked (Fig. 4A lane 4) P207C/T216C mutant CA assembly as described in **Subheading 3.4**.
10. Analyze the interaction using method described in **Subheading 3.6**.
11. Previous CryoEM studies have shown that non-crosslinked P207C/T216 mutant CA tubes are susceptible to TRIM5 α disruption (Fig. 4B), while the crosslinked assembly is resistant to such structural damage (Fig. 4C).

3.9 Isolation of crosslinked HIV-1 cores

We also examined the structural effect of TRIM5 α CC-SPRY on native cores. HIV-1 cores with cysteine mutations A14C/E45C and P207C/T216C were isolated using a modified “spin-thru” method previous reported (53).

1. Transiently transfect sixty dishes of 6 $\times$ 10⁶ 293T cells with 10 μ g plasmid DNA (using 10 μ g of HIV-1 construct R9, R9.Env-, or R9.A14C/E45C, or R9.P207C/T216C) using polyethylenimine (3.6 μ g/ml, Polysciences) in each 10 cm dish.
2. Collect virus-containing supernatants after 48 hrs and clarify supernatant by filtration (0.45 μ m pore-size).

3. Pellet clarified supernatant (600 ml) through 3ml cushions of 20% sucrose ($120,000 \times g$, 2.5 h) in a Beckman SW32Ti rotor then gently suspended in a total of 1.2 ml STE buffer for 2 hr at 4°C.
4. Subject the concentrated virus suspension to equilibrium ultracentrifugation ($120,000 \times g$, 16 h, 4°C, Beckman SW-32Ti rotor) through a layer of 1% Triton X-100 into a linear gradient of 30%–70% sucrose in STE buffer. Collect 1-ml fractions.
5. Determine the CA concentrations using p24 ELISA.
6. Collect the peak p24 fractions near the bottom of the gradient and concentrate to ~100 μ l by diafiltration with an Ultracel-10K protein concentrator
7. Use non-reducing SDS-PAGE analysis to confirm that both A14C/E45C cores and P207C/T216C cores are spontaneously crosslinked into hexamers and trimers, respectively.

3.10 CryoEM study of TRIM5 α CC-SPRY interaction with native HIV-1 cores

1. Add rhesus or human TRIM5 α CC-SPRY proteins to a solution of isolated HIV-1 A14C/E45C or P207C/T216C cores (~11 μ g/ml) so that the final CC-SPRY concentration is about 18 μ M.
2. Incubate the mixture at room temperature for 1 hr on a rocking bed.
3. Prepare cryoEM specimen using the method described in **Subheading 3.5**.
4. Load the grid into a Tecnai TF20 microscope.
5. Randomly collect 80 low dose projection images of each sample at 19,000 $\times$ magnification, under which the field of view covers about 5 μ m² surface areas (Fig. 4A, 4B, 4D & 4E).
6. Quantify the number of cores in each sample using average number of cores per image frame.
7. Plot mean value of cores per image with one standard deviation for error bar (Fig. 4C & 4F).

Our results showed that TRIM5 α_{rh} CC-SPRY disrupts A14C/E45C crosslinked cores. On the other hand, inter-hexamer crosslinking (P207C/T216C mutations) rendered the cores resistant to disruptive effect of TRIM5 α_{rh} CC-SPRY (50).

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Figure 1.

SDS-PAGE analysis of TRIM5α CC-SPRY binding to CA assemblies. CA tubes were incubated in binding buffer with TRIM5α_{hu} CC-SPRY (10 mM), TRIM5α_{rh} CC-SPRY (18 mM). Samples of the reaction mix before centrifugation (t), of supernatant (s), and of pellet (p) are shown (reproduced from (50), licensed under the Creative Commons Attribution 2.5 Generic License).

Figure 2.

CryoEM analysis of the TRIM5 α CC-SPRY interaction with wild-type CA tubes. (A–C) Low-dose projection images of CA assemblies (64 μ M), incubated with binding buffer (A), human (B), or rhesus (C) TRIM5 α CC-SPRY (18 μ M). (D), Representative assembly fragments, observed after TRIM5 α_{rh} CC-SPRY binding. Arrows indicate the TRIM5 α_{rh} CC-SPRY density. Nano-gold labeling of human (E) and rhesus (F) TRIM5 α CC-SPRY on CA tubes. Scale bars are 100 nm in (A–C) and (E&F) and 50 nm in (D) (reproduced from (50), licensed under the Creative Commons Attribution 2.5 Generic License).

Figure 3. SDS-PAGE analysis of TRIM5α CC-SPRY interaction with CA assemblies using intra- and inter- hexamer crosslinking. Non-reducing and reducing SDS-PAGE analysis of A14C/E45C CA tubes (A) and P207C/T216C CA tubes (B) incubated with binding buffer and TRIM5α_{th} CC-SPRY (18 μM), followed by oxidation. The gel is subjected to Coomassie Blue staining. At the top of panel (A) and (B), pseudoatomic model of hexameric NTD ring (pdb code: 1gwp) and CTD trimer (pdb code: 2kod) are shown, respectively. Residues A14/E45 and P207/T216 are highlighted by red spheres (reproduced from (50), licensed under the Creative Commons Attribution 2.5 Generic License).

Figure 4.

Inter-hexamer crosslinking prevents CA structural disruption by TRIM5 α_{rh} CC-SPRY. (A) Non-reducing SDS-PAGE analysis of TRIM5 α_{rh} CC-SPRY (18 μ M) binding to cross-linked P207C/T216C CA tubes, visualized by Coomassie Blue staining. (B&C) CryoEM analysis of the structural effect of TRIM5 α_{rh} CC-SPRY binding to P207C/T216C CA tubes without (B, corresponding sample in panel A, lane 2) and with (C, corresponding sample in panel A, lane 4) crosslinking. Scale bar, 100 nm (reproduced from (50), licensed under the Creative Commons Attribution 2.5 Generic License).

Figure 5.

Structural effects of TRIM5α CC-SPRY_{rh} on isolated HIV-1 cores. (A&B) Low-dose projection images of purified mutant A14C/E45C cores (11 μg/ml) incubated with human (A) or rhesus (B) TRIM5α CC-SPRY (18 μM). Scale bar, 100 nm. (C) Quantification of the number of cores on the cryoEM grids. The mean values of the average number of cores per image are plotted, with the error bars representing one standard deviation. (D&E) Representative low-dose projection images of purified P207C/T216C cores, incubated with rhesus TRIM5α CC-SPRY (18 μM), without (D) and with (E) oxidation for crosslinking. Scale bars, 100 nm. (F) Quantification of the number of cores on the cryoEM micrographs (reproduced from (50), licensed under the Creative Commons Attribution 2.5 Generic License).