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Methods to Study Determinants for Membrane Targeting of HIV-1 Gag *In Vitro*

Gabrielle C. Todd¹ and Akira Ono¹

Akira Ono: akiraono@umich.edu

¹Department of Microbiology and Immunology, University of Michigan Medical School, 5736, Medical Science Building II, 1150 W. Medical Center Drive, Ann Arbor, MI, 48109 USA

Abstract

Assembly of HIV-1 viral particles is a critical step of the HIV-1 life cycle; yet many details of this complex process are unknown. The Gag polyprotein drives viral particle assembly at the plasma membrane via three different types of interactions: protein-protein, protein-RNA, and protein-membrane interactions. As an approach to tease apart the importance of these interactions during viral particle assembly, in particular at the step of Gag membrane binding, we have developed an *in vitro* liposome-binding assay. Below we describe how to prepare liposomes, which serve as model membranes, and how to assess their interaction with Gag by liposome flotation centrifugation. Additionally, we outline extensions of this basic assay that can be used to address the role of RNA in regulating Gag-membrane interactions.

Keywords

HIV-1 assembly; Gag; RNA; Membrane binding; Liposome flotation centrifugation

Introduction

Assembly of HIV-1 particles is choreographed by the Gag polyprotein, which uses each of its four major domains to carry out different roles in the assembly process [1, 2]. The matrix domain (MA) recruits Gag to the plasma membrane (PM), the capsid domain (CA) is involved in Gag multimerization, the nucleocapsid domain (NC) packages a dimer of genomic RNA, and the p6 domain recruits cellular factors to pinch off completed particles from the cell surface.

Of particular interest is the initial targeting of Gag to the PM, which is directed by MA [1–4]. MA has an N-terminal myristoyl group that is sequestered in a hydrophobic binding pocket in MA and exposed upon Gag-membrane binding [5]. A second important feature of MA is a concentrated region of basic amino acids between residues 17 and 31 known as the highly basic region (HBR). These residues enable Gag to specifically recognize the phospholipid, phosphatidylinositol (4,5) bisphosphate, or PI(4,5)P₂ [6–9]. PI(4,5)P₂ is enriched in the inner leaflet of the PM; thus specific recognition of this lipid by Gag

promotes productive viral particle assembly at the PM rather than unproductive assembly on other intracellular membranes [10].

MA also binds to RNA [11–13], presumably via the HBR [11, 13], and the presence of RNA inhibits interactions between Gag and membranes lacking PI(4,5)P₂ [8, 14–16]. Thus RNA-MA interactions appear to provide an additional layer of regulation for Gag-membrane association. To study the interplay among Gag, RNA, and membranes we have developed an *in vitro* liposome flotation assay to monitor relative membrane binding of Gag under various conditions [7, 8, 16, 17].

Materials

Liposome Preparation

1. Lipids from Avanti Polar Lipids, Inc.
 - a. POPC—[1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine].
MW 760.076 g/mol, can be stored for 6 months at –20 °C.
 - b. POPS—[1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (sodium salt)].
MW 783.988 g/mol, can be stored for 6 months at –20 °C.
 - c. Brain PI(4,5)P₂ —[L- α -phosphatidylinositol-4,5-bisphosphate (Brain, Porcine) (ammonium salt)].
MW_(ave) 1096.385 g/mol, can be stored for 3 months at –20 °C.
2. 250 μ l Hamilton syringe.
3. Glass vials (0.5–1 ml).
4. Chloroform, methanol, water for rinsing Hamilton syringes.
5. N₂ (g) tank or house nitrogen.
6. Vacuum source.
7. 20 mM Hepes pH 7.0.
8. Dry ice and acetone bath.
9. Mini Extruder, Avanti Polar Lipids, Inc.
10. Membranes, Avanti Polar Lipids, Inc., of the desired pore size (100 nm).
11. Filter Supports, Avanti Polar Lipids, Inc.
12. Forceps.
13. Sonicating water bath.

TNT Rabbit Reticulocyte Lysate

1. Plasmid containing the *gag* gene downstream of either a T7, T3, or SP6 phage transcription promoter. In our experiments, a fragment from the pNL4-3 strain of HIV-1 from nucleotides 639–5748 (*gag* is 790–2292) is oriented downstream of an SP6 promoter in a pGEM vector backbone.
2. Promega TnT[®] Coupled Reticulocyte Lysate Systems (we use the kit with SP6 RNA polymerase, L4600, but other variations are also available).
3. *Optional*: Homemade 1 mM amino acid mix (-Met) in 10 mM Hepes pH 7.0.
4. RNasin (Promega).
5. EXPRE³⁵ S³⁵ S Protein Labeling Mix (Perkin Elmer).
6. RNase A (Thermo Fisher).
7. 10× Rabbit reticulocyte lysate (RRL) buffer [18]: 200 mM Hepes-KOH pH 7.0, 1 M KCl, 5 mM MgCl₂.
8. Yeast total tRNA (Ambion).

Liposome Flotation

1. Prepare solutions of 85.5, 65, and 10 % (w/v) sucrose in 20 mM Hepes pH 7.0.
2. Centrifuge tubes (Beckman Coulter).
3. Rotor AH-650, or equivalent *swinging bucket rotor*.
4. Ultracentrifuge.

SDS-PAGE

1. 4× Resolving gel buffer: 1.5 M Tris-HCl pH 8.8, 0.4 % sodium dodecyl sulfate (SDS).
2. 4× Stacking gel buffer: 500 mM Tris-HCl pH 6.8, 0.4 % SDS.
3. 3× SDS loading buffer: 188 mM Tris-HCl pH 6.8, 30 % (v/v) glycerol, 15.2 % (v/v) β-mercaptoethanol, 9.4 % (w/v) SDS, 0.02 % (w/v) bromophenol blue.
4. 10× Running buffer: 248 mM Tris base, 1.92 M glycine, 35 mM SDS.
5. Gels (29:1 acrylamide:bis-acrylamide): 10 % resolving, 4 % stacking.
6. Rotator platform.
7. Fixing solution: 40 % methanol, 10 % acetic acid.
8. Soaking solution: 1 M salicylic acid, 2 % (v/v) glycerol.
9. Whatmann paper (3 mm).

10. Slab gel dryer.
11. Phosphor storage screen.
12. ImageQuant software.

Methods

Preparing Liposomes

To study Gag-membrane interactions, we prepare liposomes that have a 20 mM final lipid concentration with a 2:1 ratio of POPC:POPS. When PI(4,5)P₂ is incorporated, it typically comprises 7.25 mol %; the remaining mol % of lipid is divided in a 2:1 ratio of POPC:POPS [4, 8, 16]. We generally prepare 150 µl of each liposome solution using the volumes listed in Table 1 below for ease of extrusion. Detailed instructions and theory about liposome preparation can also be found on the website of Avanti Polar Lipid, Inc. (<http://avantilipids.com/>).

There are two types of liposomes that can be used to study Gag-membrane interactions. Unilamellar liposomes prepared by extrusion are composed of a single bilayer while multilamellar liposomes prepared by sonication consist of several concentric bilayers similar to the layers of an onion. The advantages of unilamellar liposomes are that they have well-defined diameters of varying sizes depending on the pore size of the membrane used during extrusion, and the amount of liposome surface available for protein binding is consistent between each liposome preparation. Multilamellar liposomes, however, are advantageous in that they are less dense than unilamellar liposomes and therefore flotation levels are somewhat higher. Thus different types of liposomes can be used depending on the desired application.

1. Before and after measuring out each type of lipid, rinse the Hamilton syringe five times using the same solvent in which the lipid is dissolved. Removing trace amounts of lipid with a good solvent is critical to prevent clogging of the syringe with precipitated lipid (*see Note 1*).
 - a. For POPC and POPS, rinse the syringe with chloroform.
 - b. For PI(4,5)P₂, rinse the syringe with a chloroform/methanol/water mixture (20:9:1).
2. Combine the desired amount of each lipid (Table 1) into a small glass vial. Vortex gently to mix the lipids.
3. Remove the solvent with a gentle stream of N₂(g) until the lipids form a milky film on the sides of the glass vial (*see Note 2*).

¹Avoid using plastic pipette tips or storage vessels when working with chloroform. Chloroform can dissolve some plastics leading to contamination of your liposomes with unknown quantities and types of hydrophobic compounds.

²For solutions containing PI(4,5)P₂, the dried film may form large white chunks as the water component of the solvent from this lipid will be the slowest to evaporate. Once all of the water is removed by N₂(g), resuspend the lipids in ~100 µl of chloroform with gentle vortexing and dry once more with N₂(g). The lipids should now dry in a smooth film.

4. Once all of the bulk solvent is removed, place the uncapped vials under vacuum for 4 h or overnight to remove any trace amount of solvent.

Unilamellar Liposomes: Extrusion

1. Resuspend the lipid film in 150 μ l of 20 mM Hepes pH 7.0 and vortex for 1 min. The solution should be cloudy (*see* Note 3).
2. Let the liposome suspension stand for 1 h at room temperature (RT) to hydrate.
3. Disrupt the nonuniform, multilamellar liposomes by ten cycles of freeze-thaw between an acetone-dry ice bath and a water bath set above the transition temperature (for rapid thawing we use a water bath set to 50 °C).
4. Assemble the extruder according to the instructions on the website of Avanti Polar Lipids, Inc. Briefly:
 - a. In the retainer nut (side B), place the Teflon bearing followed by the internal membrane support, O-ring side up.
 - b. Inside of the O-ring, place a filter support.
 - c. Place a polycarbonate membrane with the desired pore size over the filter support such that it covers the internal membrane support O-ring completely. We use membranes with a pore size of 100 nm for our experiments.
 - d. Place a second filter support in the center of the polycarbonate membrane (*see* Note 4).
 - e. Place the second internal membrane support on top of the stack, O-ring side down.
 - f. Slide the extruder outer casing (side A) over the top internal membrane support and screw it into the retainer nut. Tighten by hand, making sure that the apexes of the outer casing and retainer nut line up.
5. Rinse the extruder syringes three times with water and three times with 20 mM Hepes pH 7.0.
6. To reduce the dead volume in the extruder, pass one to three syringe volumes worth of 20 mM Hepes pH 7.0 through the extruder and discard.

³Care must be taken to ensure that liposomes are resuspended and extruded at temperatures above the gel-liquid crystal temperature of the lipid with the highest transition temperature. The liposomes described in this chapter all have transition temperatures well below room temperature; thus we perform all subsequent extrusion steps at room temperature. More information about the transition temperatures of lipids can be found on the Avanti Polar Lipids, Inc. website.

⁴If having difficulty placing the filter supports or membrane due to static electricity, place a droplet of water between each layer.

7. Take up the liposome solution into syringe A and insert it into side A of the extruder set up. Remove any excess buffer that is pushed out of side B with a Kim wipe.
8. Insert an empty syringe B into side B of the extruder setup.
9. Place the assembly in the holder (*see* Note 5).
10. Gently push the liposome solution from syringe A to syringe B and back again, repeating 10–60 times.
11. Push the liposome solution into syringe B and remove the assembly from the holder. Holding the assembly vertically with syringe B on the bottom, remove syringe A, and pull down on the plunger in syringe B to remove all of the liposome solution from the chamber. Transfer the extruded liposome solution from syringe B into an Eppendorf tube and store at RT (or above the transition temperature) until use (*see* Note 6).
12. Clean the extruder syringes three times with 20 mM Hepes pH 7.0, three times with water, and once with 100 % ethanol (*see* Note 7).
13. Disassemble the extruder and discard the membrane and filter supports. Rinse all of the components with milli-Q water.

Multilamellar Liposomes: Sonication

1. Resuspend the lipid film in 150 μ l (or adjusted volume) of 20 mM Hepes pH 7.0 and vortex for 1 min to mix. The solution should be cloudy.
2. Let the liposome suspension stand for 1 h at room temperature (RT) to hydrate.
3. Transfer the liposomes to a plastic Eppendorf tube and sonicate the liposomes for 30 min.
4. Shake the liposome solution overnight at 4 °C.

In Vitro Transcription /Translation of Gag Polypeptide

Preparation of recombinant full-length, myristoylated Gag using bacterial expression systems is often challenging due to poor solubility and proteolytic degradation. Therefore, to obtain full-length myristoylated Gag, we generate it by *in vitro* transcription/translation (TNT) in either rabbit reticulocyte lysates or wheat germ extracts, both of which can be purchased as kits from Promega. Below, we give a brief description of a typical experiment using rabbit reticulocyte lysate which can be altered as desired to use wheat germ extract

⁵If the transition temperature of the lipid mixture is above room temperature, the extruder holder can be placed on a heat block set to the desired temperature so that extrusion can be carried out at elevated temperatures.

⁶Liposomes can be extruded starting from either side of the assembly. However, it is critical that liposomes are removed from the apparatus in the syringe opposite the one from which they entered the extruder to ensure that all liposomes have passed through the membrane. Thus we find it easiest to label the sides A and B and always input from side A and extract from side B.

⁷It is important that organic solvents other than alcohols are not used with the extruder syringes because organic solvents can dissolve the glue holding on the Teflon plunger tip.

[19], purified full-length Gag [20], purified Gag domains [5], or cell-derived Gag [16] (see Note 8).

Rabbit Reticulocyte Lysate Transcription/Translation Reaction

1. Combine the components listed in Table 2, adding the DNA template last.
2. Incubate the reaction for 90 min at 30 °C for optimal synthesis of Gag protein.
3. Follow the steps for the liposome flotation assay (see below).

Monitoring the Gag-Membrane Interactions in the Absence of RNA

1. Prepare a master mix of the TNT reaction with enough for two 25 µl reactions.
2. Incubate the master mix pool for 90 min at 30 °C for optimal synthesis of Gag protein.
3. Dilute RNase A (10 µg/µl) 1:10 in 20 mM Hepes pH 7.0.
4. For a negative control, remove 25 µl of the Gag TNT reaction from the pool and add 1.5 µl of 20 mM Hepes pH 7.0.
5. For the RNase-treated condition, remove 25 µl of the Gag TNT reaction from the pool and add 1.5 µl of the diluted RNase A (1.5 µg RNase A per reaction).
6. Incubate the negative control and RNase-treated reaction at 37 °C for 20 min.
7. Follow the steps for the liposome flotation assay (see below).

Testing the Effect of Specific RNAs on Gag-Membrane Interactions: RNA Add-Back Assay

1. Prepare a master mix of the TNT reaction with enough for three 25 µl reactions.
2. Incubate the master mix pool for 90 min at 30 °C for optimal synthesis of Gag protein.
3. Dilute RNase A (10 µg/µl) 1:10 in 20 mM Hepes pH 7.0.
4. For a negative control, remove 25 µl of the Gag TNT reaction from the pool and add 1.5 µl of 20 mM Hepes pH 7.0.
5. For the rest of the pool, add the diluted RNase A (1.5 µl per reaction).
6. Incubate the negative control and RNase-treated reaction at 37 °C for 20 min.

⁸These protocols involve radiolabeling of the Gag protein. Specific protocols for handling radioactive samples and for collecting radioactive waste vary among institutions. *Be sure to follow the standard procedures outlined by your institution.*

7. Inactivate the RNase A by addition of RNasin, 8 μ l to the negative control reaction and 8 μ l per reaction to the RNase A-treated pool. Incubate for 10 min at 37 °C.
8. Add 8 μ l of 1 $\times$ RRL buffer to the negative control reaction.
9. Remove 34.5 μ l from the RNase A-treated pool as the RNase control and add 8 μ l of 1 $\times$ RRL buffer.
10. Remove 34.5 μ l from the RNase A-treated pool and add 8 μ l of RNA, refolded in RRL buffer (*see the Refolding RNA section*).
11. Incubate negative control, RNase control, and RNA add-back reaction(s) at 37 °C for 30 min.
12. Follow the steps for the liposome flotation assay (*see below*) adding 7.5 μ l of liposomes (the final reaction volume should be 50 μ l).

Refolding RNA

To test the impact of various RNAs on Gag-membrane interactions, RNAs can be chemically synthesized or prepared by *in vitro* transcription and purified using standard techniques [21]. Prior to adding these RNAs to our Gag protein, they should be refolded. If refolding conditions for a particular RNA species are known, they can be used. If no specific conditions have been established, we recommend refolding in a buffer similar to that of the rabbit reticulocyte lysate. Although the exact composition of Promega's rabbit reticulocyte reaction buffer is proprietary information, the literature from which Promega's protocol is derived is known [18]. Thus we refold under these buffer conditions.

Yeast total tRNA can be used as a positive control. At 1 μ g/ μ l final concentration in our 50 μ l add-back reaction, yeast tRNA can completely inhibit Gag binding to PC + PS liposomes, but has no impact on Gag binding to PC + PS + PI(4,5)P₂ liposomes. At 100 ng/ μ l yeast tRNA, Gag binding to PC + PS liposome is inhibited by ~50 % relative to the RNase-treated condition. Thus the desired final concentration of your RNA of interest may need to be titrated to optimally discern its impact on Gag-membrane binding.

1. To maintain a 50 μ l final reaction volume, 8 μ l of RNA stock can be added.
2. For refolding, mix the desired amount of RNA with water to a final volume of 7.2 μ l.
3. Heat the RNA for 1 min at 95 °C to denature and snap cool on ice.
4. Add 0.8 μ l of 10 $\times$ RRL buffer, mix well, and incubate at 37 °C for 15 min (*see Note 10*).
5. Keep the refolded RNA on ice until use.

¹⁰Do not heat RNA in the presence of divalent ions! This will result in degradation of your RNA. Therefore it is important to add the stock buffer containing Mg²⁺ *after* the RNA has cooled.

Liposome Flotation Assay

1. Following Gag synthesis (and possible RNase treatment and/or RNA add-back), add 7.5 μ l of liposomes and incubate at 37 °C for 15 min (*see* Note 11).
2. Bring the final reaction volume up to 200 μ l with 20 mM Hepes pH 7.0.
3. In a 5 ml ultracentrifuge tube add 1 ml of 85.5 % sucrose.
4. Add the 200 μ l reaction to the 85.5 % sucrose solution and vortex *gently* for 5 s.
5. Carefully layer on 2.8 ml of the 65 % sucrose solution.
6. Carefully layer on 1 ml of the 10 % sucrose solution.
7. Balance pairs of tubes with additional 10 % sucrose solution if necessary.
8. Centrifuge for 16 h at 35,000 rpm (100,000 xg) at 4 °C.
9. Collect five 1 ml fractions from the top of the gradient by pipetting.
10. Vortex each fraction thoroughly. Then mix 100 μ l of each fraction with 50 μ l of 3 $\times$ SDS loading buffer.
11. Boil samples for 5 min and resolve on a 10 % gel by standard SDS-PAGE techniques (*see* Subheading 2 for our buffer compositions).
12. Remove the stacking gel and incubate the resolving gel in fixing solution for 30–60 min on a rotating platform.
13. Rinse the gel twice briefly with water.
14. Incubate the gel in soaking solution for 30–60 min.
15. Dry the gel onto a piece of Whatman paper at 80 °C for 2 h.
16. Expose the gel to a Phosphor Storage screen overnight, scan, and quantify Gag bands (~55 kDa) using ImageQuant software. We consider the top two fractions of each gradient to contain all floated liposomes and therefore membrane-bound Gag. Unbound Gag will remain at the bottom of the gradient. The proportion of liposome-bound Gag can be quantified as the sum of Gag signal in fractions 1 and 2 over the total amount of Gag present in fractions 1–5. For representative data, please see previously published results [7, 8, 16, 17].

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¹¹The amount of liposomes needed to obtain good levels of flotation may need to be titrated for specific applications.

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Table 1

Required lipid quantities for liposome preparation

	Stock concentration	2:1 POPC +: POPS	2:1 POPC +: POPS + 7.25 % PI(4,5)P ₂
Lipid	(μg/μl)	(μl)	(μl)
POPC	10	152	141
POPS	10	78	73
PI(4,5)P ₂	1	–	239

Quantities of each lipid needed for 20 mM final total lipid concentration in 150 μl of liposomes

Table 2

Recipe for rabbit reticulocyte lysate transcription/translation assay

Component	Volume (μl)
Milli-Q H ₂ O	8
25× TNT reaction buffer	1
TNT rabbit reticulocyte lysate	12.5
RNasin	0.5
SP6 RNA polymerase	0.5
1 mM amino acid mixture (-Met)*	0.5
³⁵ S Met/Cys (10 μCi/μl)	1
DNA template (1 μg/μl)	1
	25

Composition of a standard TNT reaction (*see Note 9)