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Reversible aggregation of HIV-1 Gag proteins mediated by nucleic acids

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ABSTRACT

HIV-1 Gag protein is the major structural protein for the assembly of virion particles. Although studies have been carried out using partially purified Gag proteins to investigate the mechanisms of viral particle assembly, the outcomes of an assembly reaction remain controversial. Here we have developed an improved procedure for purification of several untagged retroviral Gag proteins from *E. coli* to more than 95% purity and characterized Gag assembly in solution. We found that HIV-1 Gag proteins can undergo nucleic acid-dependent aggregation with several unexpected features: (1) they form spherical particles that are as large as microns in diameter; (2) the size of the aggregates vary with the molar ratio between nucleic acids and proteins, with the average size of these particles reaching maximal at a molar ratio of 1:2 between nucleic acids and proteins; and (3) these particles can be efficiently disassembled simply upon addition of excess nucleic acids into the solution, suggesting the presence of an ordered assembly. Single-stranded DNA oligos that are 10 nucleotides or shorter do not support the formation of these particles. Furthermore, the matrix domain of the Gag protein dramatically facilitates the formation of these aggregates. These studies uncover a previously uncharacterized pathway of HIV Gag assembly in vitro, and have implications for HIV-1 Gag assembly and pathogenesis in vivo.

1. Introduction

The Gag protein of the human immunodeficiency virus type-1 (HIV-1) is the major structural protein that is required for the assembly of HIV-1 virions [1]. The Gag protein, initially synthesized as a polyprotein precursor, is composed of four domains: the matrix (MA, or p17), the capsid (CA, or p24), the nucleocapsid (NC, or p7), and the C-terminal p6 domain. The overexpression of Gag protein alone in mammalian cells can produce viral-like particles (VLPs) that bud into the culture supernatant, even in the absence of cognate viral RNA genome, suggesting the promiscuity in the assembly process of HIV Gag proteins.

Several investigators have carried out studies of HIV assembly using purified Gag proteins [2–7]. However, the results remain controversial and the conditions under which these proteins undergo self-assembly have not been well defined. A major challenge is in the purification of Gag proteins to homogeneity. Although protocols have been developed to purify Gag proteins carrying hexahistidine tags to greater than 95% purity [6,7], the method to purify untagged Gag proteins to homogeneity remains lagging behind [2,3]. Because hexahistidine tag influences the activity of nucleic acid binding proteins [8], it is therefore essential to study Gag assembly in the absence of this affinity tag.

Here we report a much improved procedure to purify several untagged retroviral Gag proteins to greater than 95% purity. Using these proteins, we discovered reproducibly that HIV-1 Gag proteins can undergo reversible aggregation mediated by nucleic acids in solution. This property appears to be unique to HIV-1 Gag proteins and bears relevance to viral-induced cell death in vivo.

2. Materials and methods

2.1. DNA constructs

Gag_{BH10} full-length and $GagΔp6_{BH10}$ DNA were amplified using HIV-1 BH10 genome (pBKBH10S DNA, NIH AIDS Reagent Program) as template and inserted into the prokaryotic expression vector

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pET21a by using restriction enzymes NdeI and XhoI following standard cloning procedures. NL4-3 GagΔp6 gene was amplified using pGagMBPhis plasmid as template, a generous gift from Dr. James Hurley. Rous sarcoma virus (RSV) Gag ΔMBDΔPR DNA in pET3xc vector was generously provided by Dr. Volker Vogt. All the DNA constructs were sequenced and verified before protein expression and purification.

2.2. Gag protein expression and purification

All the retroviral Gag proteins used in current studies were purified to greater than 95% purity. The detailed purification protocol is given in Supplementary Methods.

2.3. In vitro assembly reaction

Protein stock solutions were thawed on ice and slowly diluted to 0.1 M NaCl with buffer F (20 mM Tris pH 8.0 at 21 °C, 10 mM β-mercaptoethanol). Nucleic acids were added right after the dilution and the solution mixed thoroughly by gentle pipetting. The nucleic acids included in the study were yeast tRNA (phenylalanine specific from brewer's yeast, Sigma), 20mer-Cy3, a 20-mer DNA (GTGGTGTCAATTACGGTAGC) with a Cy3 fluorophore conjugated to its 3′ end, oligo thymidylate of various lengths (IDT). The assembly reaction was incubated at 21 °C for 5 min before examination by fluorescence microscopy or applied to grids for electron microscopy.

2.4. Electron microscopic examination

For the negative staining of the Gag assembly product, 5 μL of the assembly reaction was applied to carbon-coated grid (200 mesh, copper, Electron Microscopy Sciences), and drained with a filter paper after 5 min. 5 μL of 5% uranyl acetate was then applied to the grid, and wiped off with filter paper after 5 min. After air-drying for 5 min, the grids containing assembly samples were ready to be imaged using a JEOL 1400-plus transmission electron microscope.

2.5. Flow cytometry

The flow cytometry experiments were conducted using a Miltenyi MACSQuant VYB digital benchtop flow cytometer. After the assembly or disassembly reaction was initiated, at designated time as indicated in the figure legends, the suspensions of particles were directly loaded onto the flow cytometer without any additional treatment. Both forward and side scattered light at 561 nm were monitored using 561/10 nm band-pass filters.

3. Results

3.1. Purification of untagged retroviral Gag proteins from E. coli

Previous studies have suggested that the presence of P6 domain in HIV-1 Gag causes significant degradation of this protein when overexpressed in *E. coli* [2]. The p6 domain of HIV-1 Gag protein is not required for particle assembly in cultured cells but is required for budding of the assembled particles from plasma membrane to extracellular milieu [1,9]. Consistent with this report [2], the full-length Gag proteins showed significant degradation in *E. coli* upon their overexpression (Lane 1, Fig. 1A). To improve the yield of protein for purification, we constructed overexpression plasmids for both BH10 and NL4-3 strains in which the p6 domain of Gag has been deleted (GagΔP6). Upon induction by addition of IPTG into the culture, the GagΔp6 proteins showed less degradation compared to

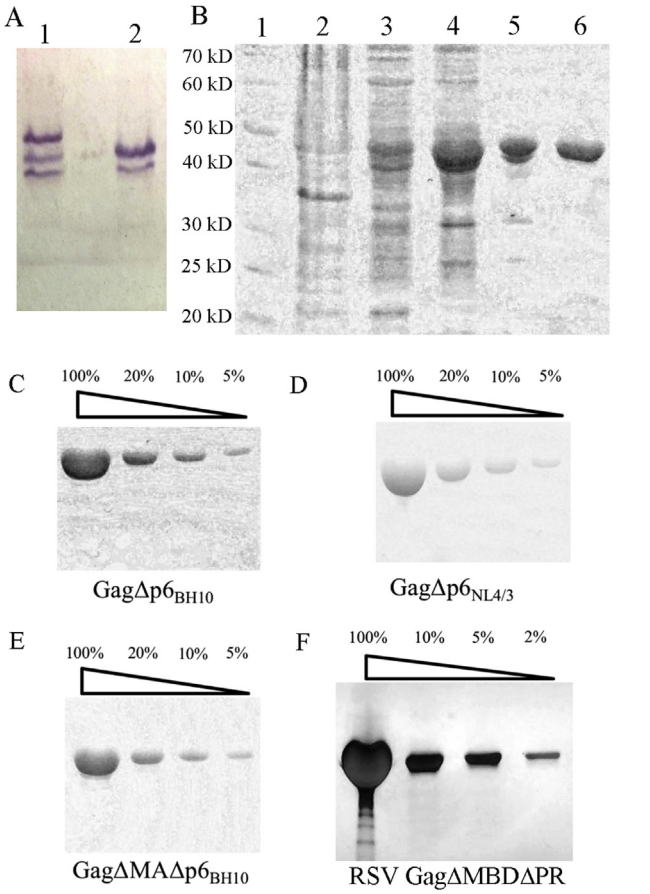

Fig. 1. SDS-polyacrylamide gel images from purification of recombinant retroviral Gag proteins. (A) Western blotting of the lysates from induced *E. coli* expressing full-length Gag_{BH10} (Lane 1) and $GagΔp6_{BH10}$ (Lane 2). (B) Products from major steps of protein purification for HIV-1 $GagΔp6_{BH10}$. Lanes: 1: protein marker; 2: total lysate of cell pellet after 5-h induction; 3: supernatant from cell lysate after 0.2% PEI treatment; 4: re-dissolution of protein pellet after 33% ammonium phosphate precipitation; 5: elution fraction after PC resin purification; 6: final product after heparin column chromatography. (C–F) Purity tests for various versions of recombinant retroviral Gag proteins: $GagΔp6_{BH10}$ (C); $GagΔp6_{NL4-3}$ (D); Gag ΔMA $Δp6_{BH10}$ (E); and RSV Gag ΔMBDΔPR (F). The amounts of protein loaded in each lane were 30 μg, 6 μg, 3 μg, and 1.5 μg for (C), (D) and (E), corresponding to 100%, 20%, 10% and 5% respectively. The amounts of protein loaded in each lane for (F) were 25 μg, 2.5 μg, 1.25 μg, and 0.5 μg, corresponding to 100%, 10%, 5% and 2% respectively.

that of the full length Gag protein (Lane 2, Fig. 1A), which provided good starting materials for protein purification. Rein and coworkers have developed procedures to partially purify untagged HIV-1 Gag proteins [2]. However, we have made two significant changes described as follows to achieve near homogeneity.

First, we added a polyethyleneimine (PEI) step before ammonium sulfate precipitation of the cleared cell lysate. PEI has been frequently used in the fractionation and purification of nucleic acids binding proteins [10], which can effectively remove nucleic acids that may present as a contaminant in the final purified proteins. Our initial attempt to purify Gag proteins without PEI addition suggest that this may indeed be the case, as indicated by the abnormal absorbance ratio at 280 and 260 nm displayed by the partially purified Gag proteins. This PEI step worked very well. As shown in Fig. 1B for the purification of GagΔp6 from HIV isolate BH10 ($GagΔp6_{BH10}$), majority of the GagΔp6 protein was retained in the supernatant after PEI precipitation (Lane 3). Moreover, majority of GagΔp6 protein was recovered back into the solution after precipitation using ammonium sulfate (Lane 4).

Second, we added a heparin column step after the phosphocellulose cation exchange column to further improve the purity of the protein. This step is necessary because we found that a major impurity was still present in the eluted protein from the cation exchanger using phosphocellulose. This impurity was a protein that was slightly smaller than the Gag protein. The Western blotting using anti-gp24 monoclonal antibody revealed that the impurity also reacted positively with this antibody, suggesting that the impurity was a degradation product from the Gag protein. We were able to separate the GagΔp6 protein from the degradation product by using concentration gradient of NaCl on the heparin column. As a result, the purity of the final GagΔp6$_{BH10}$ protein was over 95% through quantitative intensity comparison on a SDS-polyacrylamide gel (Fig. 1C). Using this new procedure, we were able to reliably purify several additional Gag proteins used in this work, all to greater than 95% purity. These include GagΔp6 protein from HIV isolate NL4-3, GagΔp6$_{NL4-3}$ (Fig. 1D), GagΔMAΔp6$_{BH10}$ (Gag protein lacking both p6 domain and amino acid 16-99 of the matrix domain [5]) (Fig. 1E), and a truncated version of the Rous sarcoma virus (RSV) Gag protein (AA85-577, RSV GagΔMBDΔPR) (Fig. 1F).

3.2. In vitro assembly properties of HIV-1 GagΔp6 protein

To test the assembly of purified GagΔp6 $_{BH10}$ protein, we diluted the protein into Buffer F to reach 0.1 M NaCl at 21 °C. Upon addition of yeast tRNA into the diluted protein solution, the mixture immediately turned turbid. In contrast, control in the absence of nucleic acids remained clear (Fig. 2A inset). The species formed upon addition of nucleic acids was clearly observed under an optical microscope. Spherical particles of heterogeneous size, with diameters in the range of 1–10 μm were observed, as shown for GagΔp6$_{BH10}$ in Fig. 2A. To better evaluate this process, we used a short DNA oligomer of 20 nucleotides labeled with a single Cy3 fluorophore at its 3′ end (20mer-Cy3). When this DNA oligo was added to the diluted GagΔP6 protein solution to initiate the reaction, spherical particles of heterogeneous size, similar to those formed upon addition of yeast tRNA, were also observed under microscope (Fig. 2B). Moreover, these particles were fluorescent, as shown by a fluorescence image collected through Cy3 emission channel (Fig. 2C). The fluorescence image could be overlaid very well with its bright field image, indicating that all the particles formed under this condition contained 20mer-Cy3 inside (Fig. 2D). These giant spherical particles were also observed for GagΔp6$_{NL4-3}$, as shown in Supplementary Fig. 1A imaged using transmission electron microscopy (TEM). Interestingly, many particles showed some features on their surfaces. These surface features are highlighted in Supplementary Fig. 1B under a higher magnification. There were many needle-like thin structures about 100–200 nm in length that protruded from the surface of these particles. Throughout, the experiments were done in parallel for GagΔp6 from BH10 and NL4-3, and no differences in the outcomes were observed between the two GagΔp6 proteins.

To gain more mechanistic information about formation of these giant spherical particles, we next analyzed the role of nucleic acids by systematically varying the molar ratio between nucleic acids and protein, and then examine the size and quantity of particles formed using fluorescence microscopy. In these experiments, the 20mer-Cy3 DNA and GagΔp6$_{NL4-3}$ was used throughout. We noticed that when the molar ratio of nucleic acids to protein was below certain threshold (1/32), no particles were observed, clearly indicating the requirement of nucleic acids for this process to occur. As we further increased the amount of DNA included in the reaction, particles started to form. Both the average number of particles formed per field of view and the average particle size increased with increasing nucleic acids. The average particle size reached a maximum at a

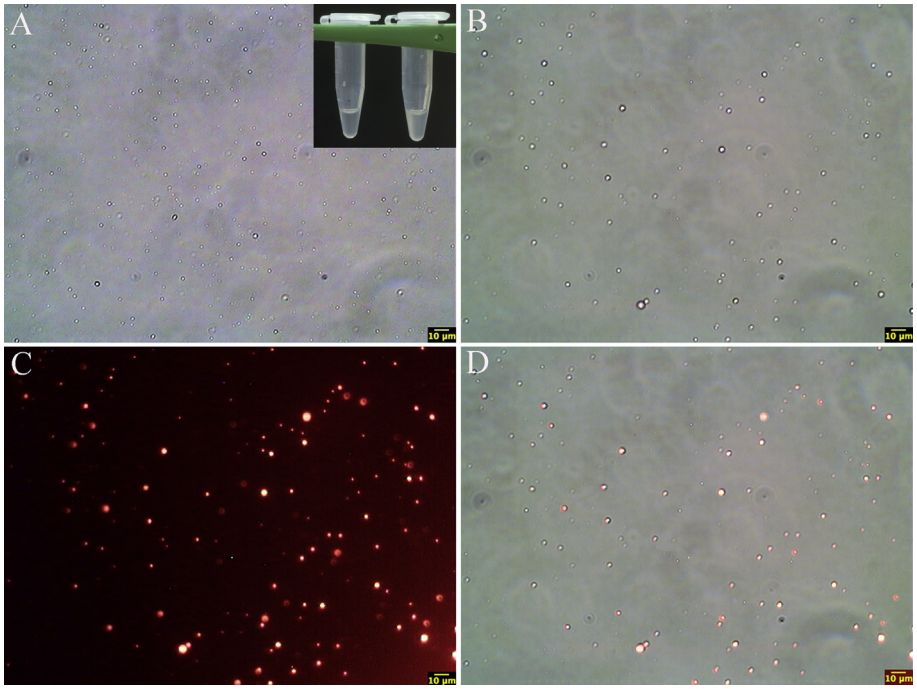

Fig. 2. In vitro assembly of HIV-1 GagΔp6 $_{BH10}$ protein. (A) GagΔp6$_{BH10}$ formed large heterogeneous particles of diameter around 1–10 μm, when 1 μg yeast tRNA was added to 5 μg GagΔp6$_{BH10}$ diluted with buffer F. Inset: GagΔp6$_{BH10}$ solution became turbid upon addition of yeast tRNA (right), while it remained clear in absence of yeast tRNA (left); (B–C) GagΔp6$_{BH10}$ formed similar particles with addition of 20mer-Cy3 compared to yeast tRNA, determined by microscope bright field figure (B) and fluorescence image (C). (D) Co-localization of the large particles in bright field with the red fluorescent particles in fluorescence image when overlapping two images of the same field from Panel B and C. All figures are taken under light microscope with a magnification of 400 times, and the scale bar indicates 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

molar ratio of 1:2 between the nucleic acid and the protein. Further increase in nucleic acids leads to fewer particles formed and also smaller size of spherical particles. The details of these results are summarized in Supplementary Table 1.

The decrease in particle assembly with increasing DNA upon a defined molar ratio between the DNA and the protein suggests that the self-assembly process may be reversible. To further examine this dependence quantitatively, we have used an oligo thymidylate, $(dT)_{30}$, to repeat the experiment. For this set of experiments, we were able to titrate nucleic acids well above the moles of proteins so to examine a wider range of nucleic acids to protein ratios. As summarized in Supplementary Table 2, the threshold behavior of self-assembly was again observed. The particles started to form only when the molar ratio of nucleic acids to protein was at 1/45 or higher. Average size of the particles reached a maximum at a ratio of 1:2. As we further increased concentration of $(dT)_{30}$, to more than two fold of GagΔp6 protein, particle disappeared. No visible particles were formed at even higher concentrations of $(dT)_{30}$ tested as examined under microscope. This quantitative dependence of self-assembly on nucleic acids, where a peak in the assembly reaction occurs at a certain ratio of nucleic acids to protein, indicates that this process is reversible. To further test this phenomenon, we first added 20mer-Cy3 to diluted $Gag\Delta p6_{BH10}$ at a molar ratio of 1:2, where the peak of assembly occurs. As expected, the sample quickly turned turbid after the addition of 20mer-Cy3, and the spherical particles were clearly visible under fluorescence microscope (Fig. 3A). After 5 min, we then added additional $(dT)_{30}$ to reach a molar ratio of 8:1, and interestingly, the turbidity quickly disappeared after the addition of excess $(dT)_{30}$. As shown by fluorescence microscopy, the majority of large particles formed by GagΔp6 and 20mer-Cy3 20mer disappeared after addition of $(dT)_{30}$ (Fig. 3B). This process can also be monitored using flow cytometry. As shown in Fig. 3C, the addition of 20mer-Cy3 to the diluted $Gag\Delta p6_{BH10}$ at a molar ratio of 1:2 yielded a large number of heterogeneous particles, indicated by the total forward scattered light. However, upon further addition of excess $(dT)_{30}$, majority of the large particles disappeared (Fig. 3D). As a control, $Gag\Delta p6_{BH10}$ diluted in buffer F in the absence of nucleic acids did not produce any significant particles. The fact that the particle formation can be reversed by addition of excess nucleic acids suggests that the formation of these giant particles is in a dynamic equilibrium with particle disassembly. The peak assembly occurs at a molar ratio of 1:2 between nucleic acids and proteins, which further suggests an ordered assembly of Gag proteins mediated by nucleic acids. The threshold dependence of this self-assembly was also observed for yeast tRNA (Supplementary Table 3). Furthermore, the addition of dNTP instead of nucleic acids did not result in any assembly of the particles, despite the various concentrations we have tested. This result suggests that a nucleic acid molecule of a minimum length is required to mediate this reversible aggregation.

To further probe the dependence of this aggregation on the lengths of the nucleic acids molecules, we have tested oligo thymidylate of various lengths, ranging from $(dT)_5$ to $(dT)_{20}$. The results are summarized in Supplementary Table 4, for $(dT)_5$ and $(dT)_{10}$, no particles were formed regardless of their concentrations used, tested at three different ratios of nucleic acids to protein (1:25, 1:5, and 1:1). In contrast, particles of similar morphology could be observed for oligo thymidylates that are 12 nucleotides or

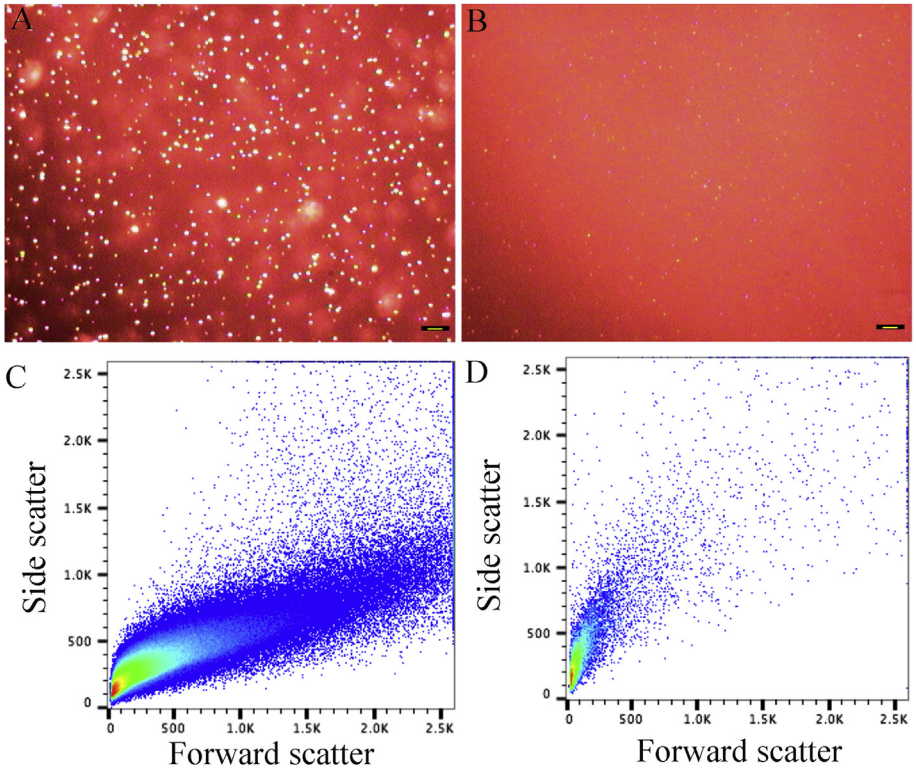

Fig. 3. Microscopy photos and flow cytometry of in vitro assembly and disassembly of $Gag\Delta p6_{BH10}$ protein. (A) 5 μg $Gag\Delta p6_{BH10}$ was diluted with buffer F, and then mixed with 20mer-Cy3 at a molar ratio of nucleic acids to protein as 1 to 2. (B) After the assembly of $Gag\Delta p6_{BH10}$ with 20mer-Cy3 under the same conditions as in panel A, $(dT)_{30}$ was added to the sample to reach a molar ratio of nucleic acids to protein as 8:1. After incubation at 21 °C for 10 min, it was sampled and observed under microscope in fluorescence mode. The yellow scale bars indicate 10 μm for both panels. (C) Flow cytometry of the assembly formed by $Gag\Delta p6_{BH10}$ incubated with 20mer-Cy3. The sample was prepared under same assembly conditions as in (A), and analyzed 5 min after the addition of nucleic acids. (D) Flow cytometry of the assembly formed by $Gag\Delta p6_{BH10}$ first incubated with 20mer-Cy3 and then mixed with excessive $(dT)_{30}$. The sample was prepared under same assembly conditions as in (B). After addition of excess $(dT)_{30}$, the sample was incubated at 21 °C for 10 min before flow cytometry analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

longer. Early studies demonstrated that the minimum length of nucleic acids required to stably bind to the NC domain of HIV-1 Gag protein was 5 nucleotides [11]. The fact that $(dT)_5$ is inadequate to promote the self-assembly of GagΔp6 indicates that binding to nucleic acids by the NC domain is not sufficient to induce the formation of these giant particles, additional nucleotides in the nucleic acid molecules are required.

3.3. *In vitro assembly of HIV-1 GagΔMAΔp6 protein*

Previously, the MA domain was also reported to recognize viral genome RNA although the affinity of the MA domain towards nucleic acids may be weaker than that of the NC domain [12] and this MA-RNA interaction could be abolished by the substitution of more than two basic residues [13]. To determine the role of MA domain in the formation of these giant spherical particles, we next constructed a mutant for BH10 Gag protein. In addition to the deletion of the p6 domain, a large portion of the MA domain, which corresponds to amino acids 16 to 99 in Gag, was also deleted, resulting in GagΔMAΔp6$_{BH10}$. Previously, it was reported that this large truncation in the MA domain changed the site of virus budding from plasma membrane to endoplasmic reticulum, but the morphology of resulting virions remains largely unchanged [14].

As shown in Supplementary Fig. 2A–B, purified GagΔMAΔp6$_{BH10}$ assembled into particles that were visible under an optical microscope. However, these particles were visually two to three times smaller in size compared to those particles formed with GagΔp6 under the same conditions. After centrifugation at 17,000 g for 5 min, majorities of the particles were centrifuged to form the pellet, but leaving submicron particles in the supernatant. These submicron particles left in the supernatant could be clearly visualized under fluorescence microscope, although not by the bright field microscope (Supplementary Fig. 2C–D). In contrast, all the particles formed by GagΔp6 protein were pelleted after centrifugation at 17,000 g for 5 min. The smaller particles formed by GagΔMAΔp6$_{BH10}$, compared to GagΔp6$_{BH10}$, can also be shown using agarose gel and flow cytometry, as shown in Supplementary Fig. 3. These results suggest that the MA domain facilitates the formation of these giant particles. Deletion of amino acids 16 to 99 of the MA domain does not prevent protein aggregation. However, the kinetics of protein aggregation is likely to be slowed down significantly.

Are these reversible aggregations unique to HIV-1 Gag proteins? To test this, we have purified RSV GagΔMBDΔPR using the similar procedure for HIV Gag and studied its aggregation properties in solution. The RSV GagΔMBDΔPR shares similarities to HIV-1 GagΔMAΔp6 in domain structures but only 23% primary sequence identity. This protein was purified to greater than 95% purity (Fig. 1F), and we initiated the assembly reaction in the same manner as various HIV-1 Gag proteins. As shown in Fig. 4A, RSV GagΔMBDΔPR formed relatively homogenous virus-like particles as seen under TEM. The diameters of these particles ranged from 70

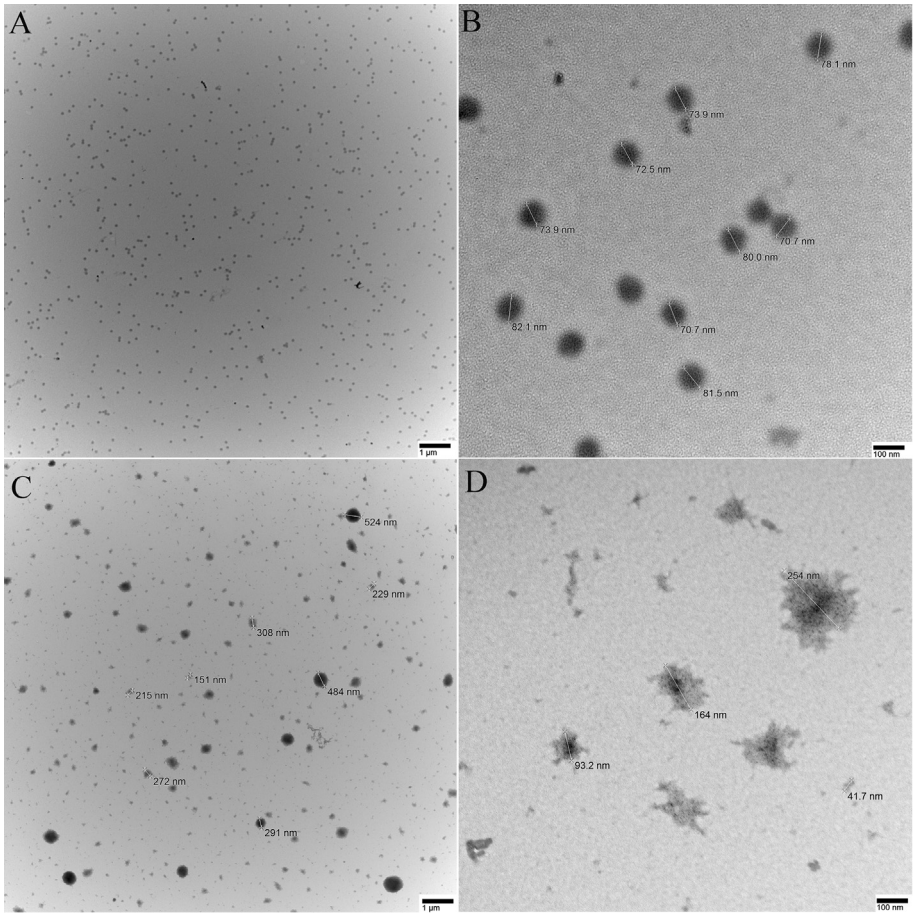

Fig. 4. Particles assembled by RSV GagΔMAΔp6 and HIV-1 GagΔMAΔp6$_{BH10}$ in presence of yeast tRNA. (A–B) 5.2 μg RSV GagΔMAΔp6 was mixed with 1 μg yeast tRNA after dilution with buffer F, corresponding to a molar ratio of 1:2.5 between tRNA and protein. EM photos were taken at magnification of 13,000 (A) and 100,000 (B), respectively. (C–D) 5.4 μg HIV-1 GagΔMAΔp6$_{BH10}$ was mixed with 1 μg yeast tRNA after dilution with buffer F, corresponding to a molar ratio of 1:3.3 between tRNA and protein. EM photos were taken at magnification of 13,000 (C) and 100,000 (D), respectively. The scale bars indicate 1 μm, 100 nm, 1 μm, and 100 nm for Panels A, B, C and D respectively.

to 80 nm in general (Fig. 4B), consistent with previous literature report [15]. In contrast, GagΔMAΔp6$_{BH10}$ formed much bigger and heterogeneous particles, with size ranging from 50 nm to 1 μm in diameter (Fig. 4C and D). These results thus suggest that the reversible aggregation mediated by nucleic acids appears to be unique to HIV-1 Gag proteins.

4. Discussion

Nucleic acid-binding proteins overexpressed in *E. coli* are typically bound with nucleic acids in the cell lysate due to their intrinsic affinities toward nucleic acids. These nucleic acids need to be separated from the proteins of interest for the purpose of purification. This step is especially important when downstream assays will be conducted with nucleic acids for the purified proteins, because the presence of nucleic acids contaminant will interfere the outcomes of these assays. PEI is a polycation that can efficiently compete with many nucleic acids binding proteins for sequestration and precipitation of nucleic acids, which has been broadly used in the purification of DNA-binding proteins to separate proteins from cellular nucleic acids [10]. However, it has not been used previously for purification of HIV Gag proteins. Here we have developed a much improved protocol for purification of untagged retroviral Gag proteins from *E. coli* extract. The use of PEI allowed us to remove nucleic acids contaminants and the addition of heparin affinity column further purified Gag proteins of interest away from the degradation products, both of which are critical for this success. The resulting proteins reached a purity of >95% in general (Fig. 1) and the homogeneities of these purified proteins were also confirmed by electrospray mass spectrometry.

Our results indicate that HIV-1 Gag proteins can undergo reversible aggregation mediated by nucleic acids. The MA domain facilitates the assembly of these giant particles. None of these results have been reported previously. Our results thus uncover a novel pathway of HIV Gag protein assembly in vitro. Because the formation of these aggregates does not require specific nucleic acid sequence, these results raise the possibility that these aggregates may form intracellularly depending on the molar ratio of Gag proteins to nucleic acids in the cytosol. The mechanisms of T cell death upon HIV-1 infection remain incompletely understood [16]. Because intracellular protein aggregates can cause cell death [17,18], our current study thus suggests a new possibility for future investigation. Whether these aggregates may form in T cells and play a role in HIV pathogenesis needs to be investigated in the future.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.12.054.

Transparency document

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