# Envelope Lipids Regulate the In Vitro Assembly of the HIV-1 Capsid

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ABSTRACT During maturation of type 1 human immunodeficiency virus, a fraction of the capsid protein (CA) molecules in the budding virus particle form a conical capsid. However, the location and role of the remaining CA molecules are unknown. It has been recently reported that the C-terminal domain of CA is able to interact with lipid bilayers, suggesting that the CA molecules that do not form the capsid could be attached to the lipid envelope of the virus. Here, we have studied in vitro the effect of different envelope lipids on the CA polymerization process. Our results show that the negatively charged lipids phosphatidic acid and phosphatidylserine partially inhibit CA polymerization, whereas the nonbilayer forming lipid phosphatidylethanolamine facilitates CA assembly. These results suggest that specific lipids of the viral envelope could have a regulatory role in the maturation of type 1 human immunodeficiency virus.

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The human immunodeficiency virus type 1 (HIV-1), a member of the retrovirus family, is the agent that causes the acquired immune deficiency syndrome (AIDS). Each HIV-1 virion carries two copies of the single-stranded RNA viral genome, enclosed in a dynamic supramolecular assembly. The genome is first packed into a protein shell core known as capsid, formed by hundreds of units of the capsid protein (CA). This capsid is contained within a spherical space limited by a lipid bilayer, the so-called envelope, where different viral proteins are embedded.

After HIV-1 infection, the host cell machinery replicates the viral RNA and synthesizes three precursor polyproteins, namely Env, Gag, and Pol. The Gag polyprotein is formed by the matrix (MA), the capsid (CA), and the nucleocapsid (NC) proteins, as well as three small peptides. During the late phase of the virus cell cycle, the N-terminally myristoylated MA directs Gag toward the plasma membrane, whereas NC associates with the viral genome (1). Before virion budding, an immature particle is formed through the assembly of several thousands copies of Gag into higher-order oligomers. This Gag complex recruits lipid molecules from the host cell plasma membrane, to form the viral envelope. The assembled immature complex induces membrane curvature, leading to the formation of a bud. The budding process is finally completed as the nearly spherical immature virion pinches off from the plasma membrane.

During or shortly after budding, the HIV-1 protease cleaves Gag, in a process known as maturation. The resulting free MA molecules bind to the inner face of the lipid envelope, forming a seemingly discontinous layer where the cytoplasmic domain of the Env protein complex is anchored, whereas CA condenses to form the mature capsid, which encases the NC protein and the RNA. The mature capsid of HIV-1 has a conical shape and is basically formed by a net of connected hexameric rings, where the N-terminal domain of CA (CA-N) forms the hexamers and the C-terminal dimerization domain (CA-C) connects each ring to six neighbors (2). Until recently, it had been widely accepted that most CA molecules were employed to build the capsid. However, it has been recently demonstrated that between one-half and two-thirds of the total CA molecules do not assemble into the capsid (3–5). Therefore, the location within the mature virion and the function of most of the CA molecules remain to be elucidated. We have recently reported that CA-C is able to bind specifically to lipid bilayers (6). Briefly, our results showed that CA-C interacts strongly with the anionic lipids phosphatidic acid (PA) and phosphatidylserine (PS), whereas it displays a lower affinity for lipids without a net charge, such as phosphatidylcholine (PC), cholesterol (Cho), and sphingomyelin (SM). These results led us to propose that, after maturation of Gag, the CA molecules that are not employed to form the capsid might bind to the inner layer of the viral envelope.

To test this hypothesis, we have used a turbidity assay in vitro (7) to monitor the kinetics of CA polymerization and the effect of different lipids on such process. The CA assembly reaction is characterized by an initial lag phase, which is the time required to form a steady state of oligomeric intermediates; this stage is followed by an elongation phase (where large oligomers are formed), which ends when the equilibrium (a flat line) is reached (8). In Fig. 1 *A* it is shown that the presence of PC did not alter the time course of the reaction, and it only caused a minor increase in the turbidity at the equilibrium. However, the presence of anionic lipids or a lipid

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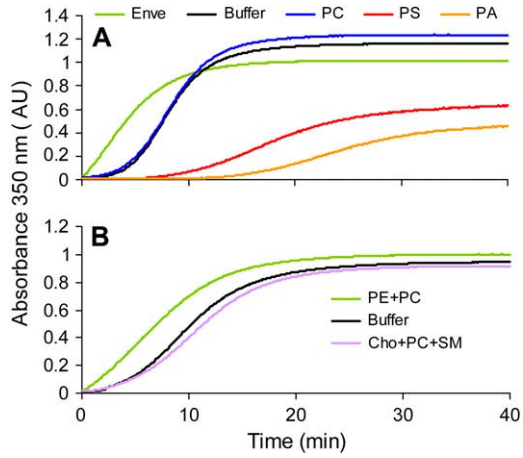


**FIGURE 1 Polymerization of CA in the presence of lipids. (*A*) Polymerization of CA in the absence of lipids (*black*), or in the presence of PC (*blue*), PS (*red*), PA (*orange*), and a lipid mixture mimicking the lipid composition of the HIV-1 envelope (*green*) (9). (*B*) Polymerization of CA in the absence of lipids (*black*), or in the presence of a mixture of 33% Cho, 33% PC, and 33% SM (*magenta*) or 25% PE and 75% PC (*green*). CA concentration was 22 $\mu$M in all cases. Phosphate buffer 50 mM, NaCl 2.25 M, pH 8, was used. Lipid small unilamellar vesicles, 0.3 mM, were employed, and the blanks subtracted. Different CA stocks were employed in panels *A* and *B*, resulting in the small differences observed for polymerization in buffer. Each curve is the average of at least three experiments. Errors were smaller than 5%.**

mixture mimicking the composition of the HIV-1 envelope, had a marked influence on CA polymerization.

The presence of PA or PS led to a clearly longer lag phase and the increase of the half-time of the polymerization reaction, accompanied by a marked reduction in the slope of the elongation phase (polymerization rate) and the turbidity at the equilibrium. These changes are very similar to those observed after a reduction in the CA concentration or the addition of the isolated CA-C domain, which may sequester CA molecules in polymerization-incompetent complexes (7). This suggests that, in the presence of anionic lipids, a population of the CA molecules might remain attached to the lipid bilayer, and then the number of solubilized, polymerization-competent CA molecules were reduced. As a negative control of the polymerization reaction, the assay was performed with a CA mutant with two specific mutations (E180D-V181D), which severely hamper the polymerization process (7) but that do not predictably alter the affinity for lipid membranes (6). Using this mutant either in the absence or presence of PA, no increase of turbidity was detected, indicating that the turbidity changes observed in the experiments with wild-type CA under the same conditions were caused by the ordered polymerization of the protein (data not shown). The lipid composition of the HIV-1 envelope seems to be finely tuned, as it is selectively enriched in certain lipid types with respect to the plasma membrane of the host cell (see below) (9,10). Hence, we studied the CA polymerization in the presence of a mixture of lipids mimicking those present in the purified virus envelope (50% Cho, 16% PC, 12% PE, 12% SM, 8% PS, and 2% PA, according to Aloia et al. (9)). Interestingly, in the presence of this lipid mixture, the CA polymerization process was clearly facilitated, the lag phase being virtually absent. As in the case of PA and PS, a decrease in the absorbance at the equilibrium was also observed (Fig. 1).

Intrigued by the ability of lipids present in the viral envelope to enhance the CA polymerization, we decided to investigate whether this feature could be attributed to a single lipid type or, on the other hand, it was due to the combined effect of the mixture. Accordingly, we assayed the influence on the polymerization of CA of the lipids composing the envelope that had not yet been tested. Firstly, we studied the combined effect of a equimolar mixture of Cho, SM, and PC, as this lipid mixture is usually employed to reproduce the composition of the physiologically relevant lipid rafts. As in the case of the isolated PC, only a minor influence on the assembly was detected (Fig. 1 *B*). The study of the effect of phosphatidylethanolamine (PE) poised an additional challenge, since PE tends to form nonbilayer structures, which are prone to cause scattering artifacts. Then, a mixture of 25% PE and 75% PC, which can form stable small unilamellar vesicles, was used. The obtained results (Fig. 1 *B*) were similar to those observed with the mixture of the envelope lipids, since the lag phase was again greatly reduced. This indicates that PE can facilitate the polymerization of CA. However, in contrast to the results obtained with the envelope lipid mixture, the turbidity at equilibrium was slightly higher, compared to those in the absence of lipids. This result suggests that the decrease in the turbidity at the equilibrium observed in experiments performed with envelope lipids (Fig. 1 *A*), can be attributed to the effect of the anionic lipids present in this mixture. The influence of membrane lipids in protein polymerization is not a novel mechanism, as it has been reported that specific lipids catalyze the formation of fibers of the human islet amyloid polypeptide (11). However, further investigation will be required to elucidate the mechanism by which PE accelerates CA assembly.

It could be argued that the morphology of the assembled CA polymers could be altered in the presence of lipids, which would complicate the interpretation of the results. To rule out this possibility, the morphology of the assembled CA polymers in the presence of lipids was studied by transmission electron microscopy. The results shown in Fig. 2 revealed that CA, in the presence of 25% PE and 75% PC, assembled predominantly as hollow tubes, with an external diameter of ~40 nm, similar to those observed when CA was assembled in the absence of lipids (7). These results indicate that the presence of lipids does not substantially modify the final polymeric state of CA.

The affinity of CA for the different lipids was determined as described (6). The obtained partition coefficients, $K_p$, indicate that CA binds with high affinity to all the lipids

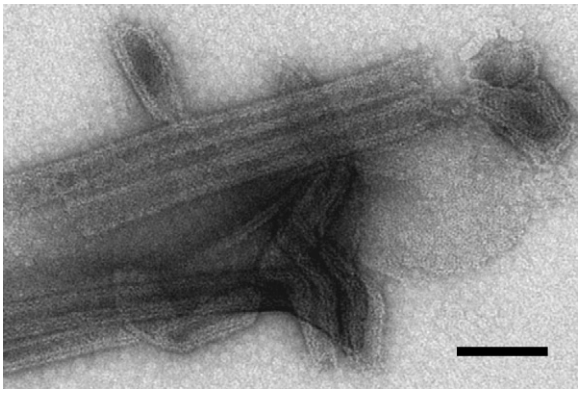

**FIGURE 2 Transmission electron microscopy of CA polymers assembled in the presence of 25% PE-75% PC. Samples were treated with glutaraldehyde 0.1% for 30 min., deposited on ionized Formvar/carbon-coated copper grids, negatively stained with uranyl acetate 2%, and visualized in a JEM-1010 microscope. CA concentration was 60 $\mu$M. Lipid prepared as small unilamellar vesicles, and at 0.1 mM, were employed. The scale bar represents 100 nm.**

assayed, although the value for the anionic lipids (for PA, $K_p = 47{,}600 \pm 6000$) was significantly higher than that for zwitterionic lipids (for PC, $K_p = 13{,}000 \pm 6700$), or for the lipid mixture 25% PE-75% PC ($K_p = 6000 \pm 4000$) (data not shown).

The mechanism by which the HIV-1 capsid is assembled during virion maturation remains poorly understood. However, recent cryoelectron tomography results argue strongly for the presence in vivo of local and/or templating interactions that promote HIV-1 capsid assembly (5). It has been observed that capsid growth initiates at the narrow end of the core, which is very close to the lipid envelope. This has led Briggs and co-workers to propose that the viral membrane could act as a template for capsid assembly (12). This hypothesis, which implies that only a subset of the CA molecules would drive the formation of the capsid, seems reasonable, since after proteolytical processing of Gag in the immature virion, several hundred copies of CA per virion are released. If all these molecules had the same ability to associate, it would be reasonable to assume that many independent polymerization seeds would be formed, resulting in multiple assembly loci (13). In this study, we have observed that CA displays a high affinity for anionic lipids, and that these are able to sequester a large population of the total amount of protein resulting in the partial inhibition of its assembly in vitro. On the other hand, we observed that polymerization of CA is facilitated in the presence of PE, a lipid for which CA shows lower affinity. The inner layer of the viral envelope, the only surface directly accessible to CA after maturation, is enriched in certain types of lipids when compared to the plasma membrane of the host cell. It has been suggested that these lipid types are essentially PE, PS, and PA (10), precisely those able to modulate CA assembly according to our experiments. In view of these results, we suggest that after Gag proteolysis in the virion, an important fraction of the released CA molecules could bind to the inner face of the lipid envelope. A significant number of these CA molecules would remain attached to anionic lipids, becoming polymerization-incompetent, whereas a smaller number of CA subunits would bind to PE-rich membrane patches. As the nucleation process is facilitated when CA is attached to PE, these anchored proteins would constitute seeding loci from where capsid polymerization might be triggered.

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