



REVIEW

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Retroviral matrix and lipids, the intimate interaction

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Abstract

Retroviruses are enveloped viruses that assemble on the inner leaflet of cellular membranes. Improving biophysical techniques has recently unveiled many molecular aspects of the interaction between the retroviral structural protein Gag and the cellular membrane lipids. This interaction is driven by the N-terminal matrix domain of the protein, which probably undergoes important structural modifications during this process, and could induce membrane lipid distribution changes as well. This review aims at describing the molecular events occurring during MA-membrane interaction, and pointing out their consequences in terms of viral assembly. The striking conservation of the matrix membrane binding mode among retroviruses indicates that this particular step is most probably a relevant target for antiviral research.

Introduction

Retroviruses are enveloped single-stranded RNA (+) viruses; they include some human pathogens such as human immunodeficiency virus (HIV), and oncoviruses such as the murine leukemia virus (MLV). Regardless of their diversity and the high divergence in their sequences, they share functional and viral protein structure similarities. Their genome contains the three retroviral genes: gag, pol, and env, and regulatory proteins in the case of complex retroviruses. One of the important steps in the process of retroviral infection is the formation of new infectious particles. It consists of the assembly of the viral core at the cellular membrane, budding, and maturation of the viral particles. In this review, we will focus especially on the events that occur at the molecular level during the interaction between Gag and membranes, more particularly between the Matrix domain of retroviral Gag proteins and the phospholipids, and we will place it in the context of the viral assembly process. Retroviral assembly relies on the viral Gag protein, and especially its ability to interact with the viral genomic RNA (gRNA) and cellular membranes. Gag is a polyprotein with three domains: the matrix domain, MA, that binds membranes, the capsid domain, CA, that contains Gag multimerization motifs and is responsible for the viral capsid formation (see [1] for

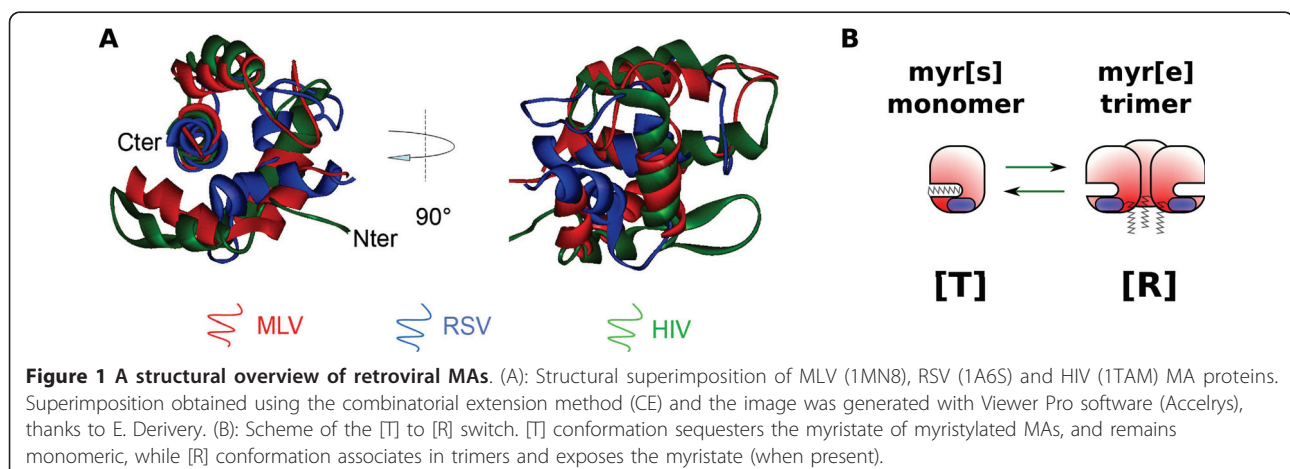
review), and the nucleocapsid domain, NC, that recruits the RNA genome and also promotes Gag multimerization [2,3]. The assembly process most probably initiates with the formation of a ribonucleoprotein complex composed of a few Gag molecules and the gRNA, which is going to interact with membranes [4,5]. Beta-retroviruses and spumaviruses are exceptions, that fully assemble in the cytosol before reaching membranes (see [6] for review on spumaviruses, and [7] for study on the role of MA in promoting cytosolic assembly of M-PMV). The formation of higher order Gag multimers leads to the formation of the viral particle at the plasma membrane, and subsequent budding and maturation, which consist of the proteolytic cleavage of Gag and structural rearrangement of the particle. The MA domain is not only carrying Gag trafficking and membrane binding determinants, but also dictating the specificity of the bound lipid. Many data have been recently published partially unveiling the molecular mechanism of MA lipid binding, enhancing the understanding of the role played by MA during Gag membrane targeting and assembly. In the light of the literature and our experiences, this review aims at proposing biochemical models for MA-lipid interactions for different retroviruses, and replacing the consequences of such interactions in the context of retroviral assembly. We will identify the elements conserved through retroviral evolution, and those that are specific to particular retroviral strains.

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Matrix proteins: a structural point of view

Despite low sequence similarity, MAs from different retroviruses share a conserved function in anchoring the viral Gag polyprotein to the plasma membrane. Indeed, most Gag chimeras with heterologous MA domains remain able to drive particle assembly [8-11]. One element allowing the interaction with the cellular membrane is N-terminal myristylation, a post-translational modification found in MAs from all retroviral families (myrMAs), including human immunodeficiency virus (HIV) [12], human T-lymphotropic virus (HTLV) [13], Mason-Pfizer monkey virus (M-PMV) [14] and exogenous murine leukemia virus (MLV) strains [15,16]. This myristate moiety is a common signal for membrane targeting of proteins, as it can insert into membrane bilayers. There are some exceptions, however, as Rous sarcoma virus (RSV), Visna virus, caprine arthritis-encephalitis virus (CAEV) and equine infectious anemia virus (EIAV) MAs are not myristylated [14]. Therefore, myristylation cannot be the only element involved in this targeting. Structural analysis of MA domains offers some clues for understanding its conserved biological role regarding membrane anchoring. Matrix structures from nine retroviruses have been resolved to date: HIV-1 [17-20] and 2 [21], SIV [22], HTLV-2 [23], bovine leukemia virus (BLV) [24], M-PMV [25], RSV [26], EIAV [27], and MLV [28]. They are all made of a globular core composed of four α -helices, whose overall organization is conserved among the retroviridae family [29,30] as shown by the superimposition Figure 1A. In the case of HIV-1, the unmyr-MA structure was resolved both by NMR [17,18] and crystallography [19], while the myr-MA structure was resolved by NMR only [20]. HIV-1 unmyr-MA (as well as SIV, but neither EIAV nor MLV MAs) crystallized as trimers, while it appeared mainly monomeric in classical NMR conditions. Overall structure was conserved between myr and

unmyr-MA, but some differences arose, notably in the putative trimerization region and in the first alpha helix. As suggested earlier by Zhou and Resh [31], Tang and colleagues [20] showed that there is an equilibrium between two conformations of HIV-1 myrMA in solution. In the myr[s] conformation, the myristate moiety is sequestered inside the core of the protein (see scheme in Figure 1B). This is the conformation adopted by the majority of myr-MA at a concentration of 150-200 μ M. The other conformation, myr[e], promotes the exposure of the myristate and tends to assemble in trimers. This conformation is probably close to the conformation observed for unmyr-MA. The conversion from one state to the other is entropically regulated [20]. In particular, high concentration of MA (more than 400 μ M) promotes trimerization and stabilizes the myr[e] conformation. This will be extensively discussed in the next sections. Whether these myr[s] and myr[e] conformations exist for other retroviral MAs has never been demonstrated formally. However, a NMR study carried out on EIAV-MA (which is not myristylated) evidenced amino acid shifts at high MA concentration, and correlated with an increase of the trimeric versus monomeric state [32]. Even if no major conformation change was noticed, this may correspond to an entropic switch between two slightly different conformations, similar to HIV. We, therefore, propose a new nomenclature for the MA conformations, that can also apply for unmyristylated MAs. By analogy with the enzymology, the membrane-binding prone conformation will be denoted hereafter as relaxed [R], while the other conformation will be denoted as tensed [T] (Figure 1B). Another important element of MA necessary for membrane binding is most probably the highly basic region (HBR). Indeed, an exposed patch of basic amino acids has been observed or predicted on all retroviral MAs [30]. A comparison between structurally superimposed



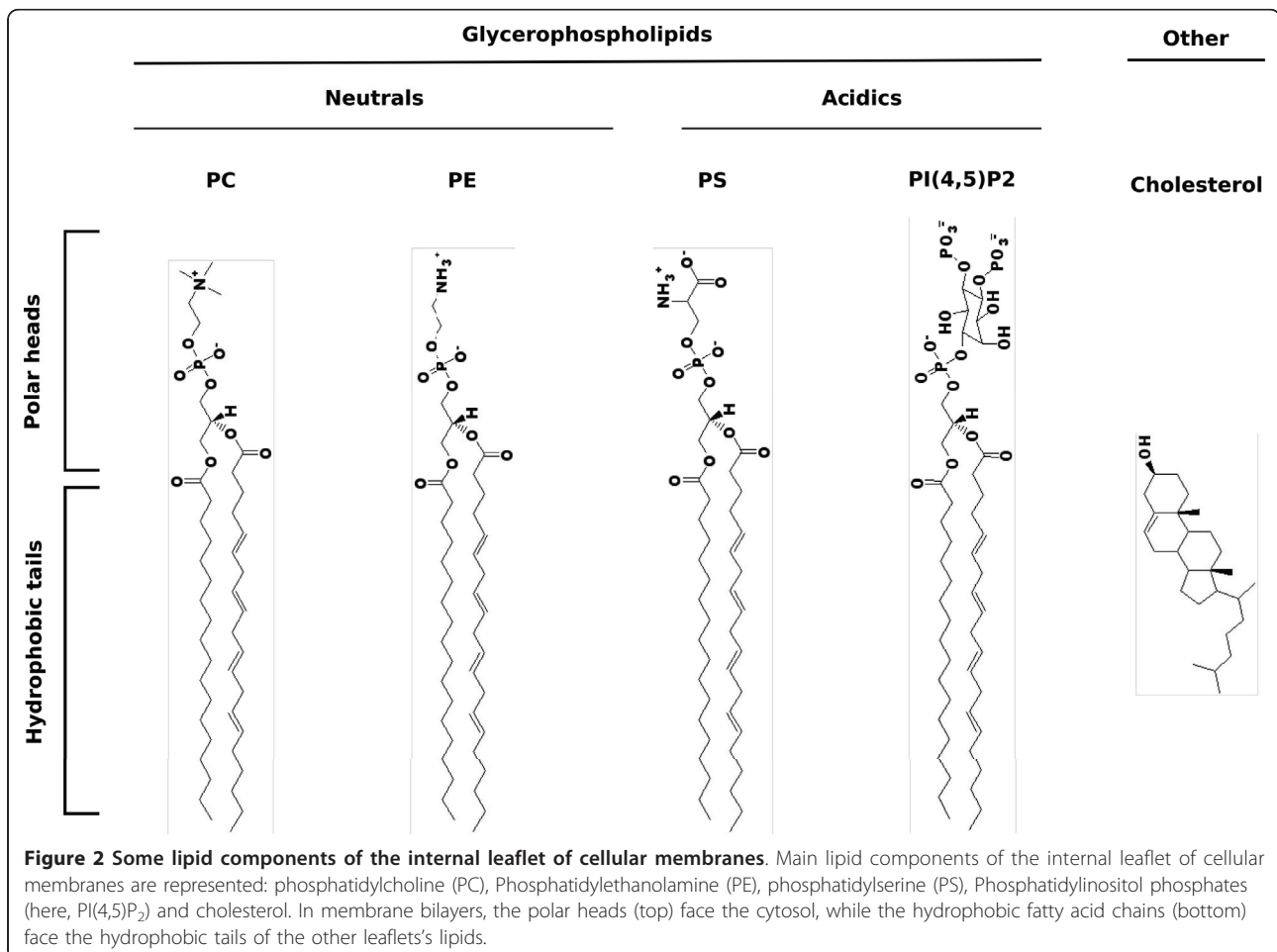
retroviral MAs shows that this domain “migrates” on the surface of the protein, but is always found in the proximity of the N-terminus [30]. This supports the idea that the N-terminus and the polybasic region of MA cooperate for efficient membrane binding, as HBR was hypothesized to promote interaction of MA with acidic phospholipid heads [30]. Moreover, other amino acids could be involved in Gag membrane anchoring, such as the N-terminal amino acids involved in [T] to [R] conversion in HIV-MA [33,34].

Acidic lipid binding: the biochemical characterization

In cells, analysis confirmed that Gag membrane binding depends on this bipartite signal for most retroviruses. On one hand, the myristate moiety is, as expected, necessary to ensure membrane binding for all myristylated MAs, as shown for MLV [16,35], HIV [36], or M-PMV [37]. On the other hand, mutations in the HBR disrupted Gag membrane-binding and assembly of HIV [38-41], MLV [42,43], feline immunodeficiency virus (FIV) [44], RSV

[45], HTLV-1 [46] and M-PMV [47], suggest that MA may interact with acidic membrane lipids.

To precisely identify the lipids that interact with retroviral Gag proteins, researchers focused on the lipids potentially present at the budding site. Phospholipids, including glycerophospholipids and sphingolipids, are the main components of cellular membranes, among which the most abundant are phosphatidylcholine (PC) and phosphatidylethanolamine (PE), both containing a neutral polar head. Some less abundant species, however, like phosphatidyl serine (PS), phosphatidyl glycerol (PG) or phosphatidylinositol phosphates (PIPs), contain acidic polar heads (cf. Figure 2). Apart from phospholipids, cellular membranes also contain other lipids, such as cholesterol, and an important proportion of transmembrane proteins. The composition of a membrane depends on its localization (internal/plasma membrane, inner/outer leaflets, etc.) and defines its functionality. Thus, retroviral assembly location restricts the panel of lipids potentially involved in the interaction with MA. Indeed, budding is mainly observed at the plasma



membrane for most retroviruses, including HIV [48], M-PMV, MLV [42,49], FIV, RSV, HTLV, but may also occur on internal membranes such as endosomes (see [50] and [51] for review). Moreover, the MA domain of Gag interacts with the inner leaflet of cellular membranes, whose main lipids are PC, PE, PS, PIP (here PI(4,5)P₂), and cholesterol [52], thereby susceptible to interact with MA (Figure 2).

Interaction between proteins and lipids can be studied *in vitro* using biomimetic membranes, and in particular large unilamellar vesicles (see [53] for review on using LUVs). The dissociation constant (K_d) can be measured, and corresponds to the lipid concentration at which half the protein is associated with the lipids: the lower the K_d, the higher the affinity. Most experiments were performed using recombinant MA proteins, because purification of the entire Gag protein is not easy. MA domain is separated from the rest of Gag by a flexible linker, thus isolated recombinant MA should recapitulate most functions of MA domain in Gag. It must be taken into account, however, that HIV-MA alone seems to have decreased affinity for membranes in comparison to the entire Gag [31]. Recombinant MA is also directly representative of the matured MA domain function in mature particles and during early stage of viral infection. As expected, purified recombinant MAs from RSV [54] and HIV-1 [55,56] can bind containing an acidic phospholipid, the phosphatidylserine (PS), which is an abundant species in the internal layer of cellular membranes. The order of magnitude of the K_d measurements made for recombinant RSV-MA and HIV-1 myristylated MA (myrMA) were of 10⁻³M, and about one order of magnitude lower upon forced dimerization of MA [54,55]. Nevertheless, the method used in these studies, i.e. LUV flotation, may underestimate the actual affinity, as the sucrose gradient may dilute the lipids. Indeed, we and others reported a value closer to 10⁻⁵M for unmyristylated HIV MA (HIV unmyrMA) by sedimentation assay [42] or by intrinsic fluorescence measurement [56,57]. Ehrlich and colleagues [56] showed that HIV-1 MA is also able to bind *in vitro* to another basic phospholipid, the phosphatidylglycerol (PG). These later studies were contested, however, because the authors also observed a binding of the CA domain of Gag to PG and PS that other authors questioned [54]. Recently, Barrera and colleagues [58] confirmed that CA has acidic lipid binding properties [58,59], rehabilitating the previous findings. It was also reported that EIAV MA can interact with PS (K_d < 10⁻⁶M at 0.1 M NaCl) and PC [60].

The binding of retroviral MAs to lipids was thus considered to be purely electrostatic, as the interaction with PS was inhibited at high ionic strength. The K_d values found would fit well with the computational models considering electrostatic interaction between acidic

lipids and basic MAs [30]. These reported K_d values would be rather low, though, to fully explain the binding of Gag to the plasma membrane in cells, and multimerization was invoked to explain MA binding to membranes [54,55].

Several retroviruses, however, show a dependency on a particular acidic phospholipid, the PI(4,5)P₂, for efficient particle production in cells. These include HIV [61,62], M-PMV [47] and MLV [42,62]. Phosphatidylinositol phosphates are a family of acidic glycerophospholipids, with a polar head made of an inositol ring that can be mono-, bi- or tri-phosphorylated (Figure 2 shows the example of PI(4,5)P₂). The sub-cellular localization of the different species is highly regulated by cellular kinases and phosphatases, such that they stand as major determinants of the identity of organelles' membranes (see [63], [64] and [65] for review).

The interaction between MAs and PI(4,5)P₂ has been observed *in vitro* by NMR (EIAV [32], HIV-1 [66] and HIV-2 [21]), using LUVs (HIV-1 [67-69] and MLV [42]), by mass spectrometric footprinting (HIV-1 [70]) and by surface plasmon resonance (SPR)(HIV-1, [71]). The K_d values measured by NMR were rather high for all tested lentiviruses (EIAV, HIV-1, and HIV-2), ranging from 125 to 185 μM, and cannot account for membrane binding in cells. It is noteworthy though that these interactions were observed with short chain PIPs (Di-C4-PI(4,5)P₂). In contrast, SPR analysis was performed both with Di-C4- and Di-C8-PI(4,5)P₂ (longer acyl chains), and K_d values decreased significantly in the case of Di-C8-PI(4,5)P₂, suggesting that acyl chains are involved in the interaction between MA and PI(4,5)P₂ [71]. The K_d of this interaction could not be calculated in the LUV systems, however, neither for the recombinant HIV MA domain [42,55], nor for the recombinant RSV MA domain [54]. This suggests that unlike PS binding, the mechanism of PIP/HIV-MA interaction could be more complex than a simple electrostatic interaction. The region of HIV-MA involved in the interaction with PI(4,5)P₂ differs slightly depending on the method used (NMR [66] or footprinting [70]), but mapped to the HBR in both cases. New NMR techniques, using reverse micelle encapsidation instead of soluble lipids could settle it, but only preliminary results have been published to date [72].

We recently reported a definite different behavior in the case of MLV-MA [42]. UnmyrMLV-MA was able to bind PIPs-containing LUVs in a dose-dependant manner. An interaction is observed not only with PI(4,5)P₂, but also with all the PIPs species, with K_d values ranging from 20 to 50 μM. To the contrary, unmyrMLV-MA does not bind PS containing LUVs, even if the residues involved in the interaction with PIPs map to the HBR. However, adding PI(4,5)P₂ and PS together in the same LUV dramatically increased the affinity of MLV-MA for PI(4,5)P₂, but not

for the other PIPs. Therefore, as for HIV, interaction with PIPs appears to result from a specific interaction, rather than a purely electrostatic mechanism [42].

Specificity and regulation of the interaction with acidic phospholipids

In the light of the data presented above, we can question the specificity and the biological relevance of the interaction of retroviral Gag with the different acidic phospholipid species, as MA can interact *in vitro* with different acidic phospholipids, with important differences in Kd and interaction mode.

The lipidomics data emerging from the analysis of viral particles, however, seems to confirm the specificity for both PI(4,5)P₂ and PS, as they are highly enriched in MLV particles [73]. This is consistent with the *in vitro* data obtained with MLV-MA, showing that there is in fact a cooperation between PI(4,5)P₂ and PS which allows strong MA anchoring to the membrane. Indeed, even if MLV-MA can bind any PIPs but not PS-containing LUVs, the protein actually displayed a strong stereospecificity for PI(4,5)P₂, but exclusively when PS is added to the same LUV (resulting in a fourfold decrease in Kd, [42]). Thus, MA probably interacts with both PI(4,5)P₂ and PS, but we hypothesize that PS binding may occur only after initial docking of MA on the PI(4,5)P₂. In HIV particles, PI(4,5)P₂ is enriched, while PS is present at high concentrations. Together with data emerging from MLV study, these results indicate that *in vitro* binding of HIV-MA to both PI(4,5)P₂ and PS may be biologically relevant. Other families of lipids may also regulate MA association with membranes. In particular, HIV myrMA show more affinity for cholesterol-containing biomimetic membranes [57], and cholesterol enhances the binding specificity of HIV-MA to PI(4,5)P₂ [67], in accordance with the finding that retroviruses can bud in cholesterol-enriched membrane domains such as lipid rafts [74-76].

Surprisingly enough, another element, the RNA, was recently found to be involved in the regulation and the specificity of HIV-MA membrane binding [69]. Indeed, HIV-MA has long been known to bind RNA efficiently *in vitro* [67,70,77-79], as does BLV-MA [80] and RSV-MA [81]. Moreover, HIV-MA specifically interacts with RNA, bearing a high degree of homology to a region within the Pol open reading frame of the HIV-1 genome, suggesting that the RNA molecule interacting with MA in cells might be the viral gRNA [79]. Interestingly, the basic residues of HIV-1 MA involved in the interaction with RNA are also necessary for PI(4,5)P₂ binding [66,70,77,79]. Thus, RNA might be a competitive inhibitor of the interaction with PI(4,5)P₂. As a matter of fact, Chukkapalli and colleagues observed that RNase treatment increased binding of Gag to both neutral and

acidic LUVs (PC, +/- PS, +/- PI(4,5)P₂) [69]. The hypothesis is that RNA would inhibit the entropic switch, stabilizing the [T] conformation (Figure 3Ab), thus preventing membrane-binding in general. On the other hand, Alfadhli and colleagues [67] simultaneously found that PI(4,5)P₂ is the only lipid that can remove nucleic acids bound to HIV-1 myrMA recombinant protein. This favors the idea that RNA would ensure the specificity of the interaction of MA with the PI(4,5)P₂, which therefore appears as a relevant cellular partner of Gag during the assembly process, allowing MA to switch from a “transport” [T] conformation to a “membrane binding” [R] conformation. RNA-mediated regulation of HIV-MA binding to PI(4,5)P₂ seems to be supported by the data emerging from *in cellulo* studies. A functional link between the genomic RNA exporting pathway and the HIV-1 MA-driven assembly has been established recently, even if the precise mechanism has not been elucidated [82-85]. Whether gRNA plays a role in MA/lipid interaction for other retroviruses is not known as yet. EIAV or MLV does not seem to have the same dependency on gRNA export pathway for proper assembly [84,85] as compared to HIV-1. In contrast, RSV-MA is able to interact with both PS [54] and RNA [81]. The measured affinity for PI(4,5)P₂ was found to be low in the case of RSV MA alone [54], but given the results obtained with HIV-MA, further investigation could prove useful. Thus, from an evolutionary point of

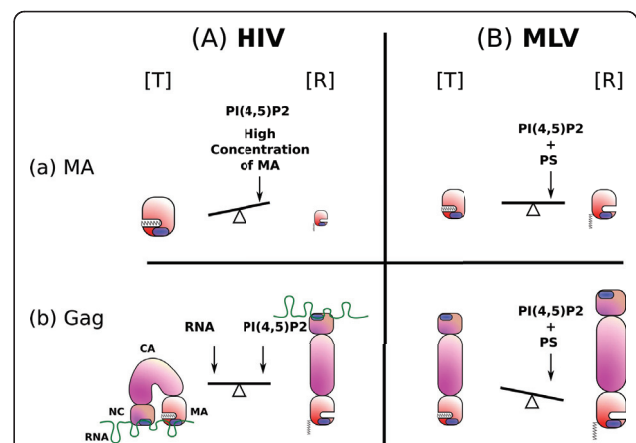


Figure 3 A model for [T] to [R] equilibrium in different conditions. Some elements are susceptible to influence the MA [T] vs [R] equilibrium, in the context of MA alone (in the mature particle, during the early step of infection, or *in vitro*), or as a domain of the Gag polyprotein. The “initial” equilibrium (in solution, purified protein, concentration around 1 μM) between the [T] and [R] conformations of HIV (A) and MLV (B) MAs (a) or Gag (b) are depicted, the size of the protein representing the relative amount of each form. The factors susceptible to induce a majority of a given conformation are written in bold characters. Others, such as PI(4,5)P₂ in the case of HIV-MA, are only able to (slightly) displace the equilibrium, even at a saturating concentration (Aa).

view, it would be interesting to determine if these regulation modes involving PI(4,5)P₂ and RNA are conserved among retroviruses, including those lacking MA myristylation.

In summary, we have proposed a model in which two different retroviral MAs use alternative mechanisms to bind membrane lipids, but end up with the same lipid specificity. MLV-MA is able to interact initially with PI(4,5)P₂, and this interaction triggers a conformational modification that allows PS binding. In contrast, HIV-MA would have initially low affinity for PI(4,5)P₂, especially in the presence of gRNA [69]. However, PI(4,5)P₂ seems to be the only compound able to compete with RNA for HIV-MA binding [67], and once RNA is removed, HIV-MA would be able to interact both with PI(4,5)P₂ and PS, and this interaction may be stabilized by other elements, as discussed in the next section. Therefore, in spite of different lipid binding modes, the specificity of binding could be highly conserved among retroviruses.

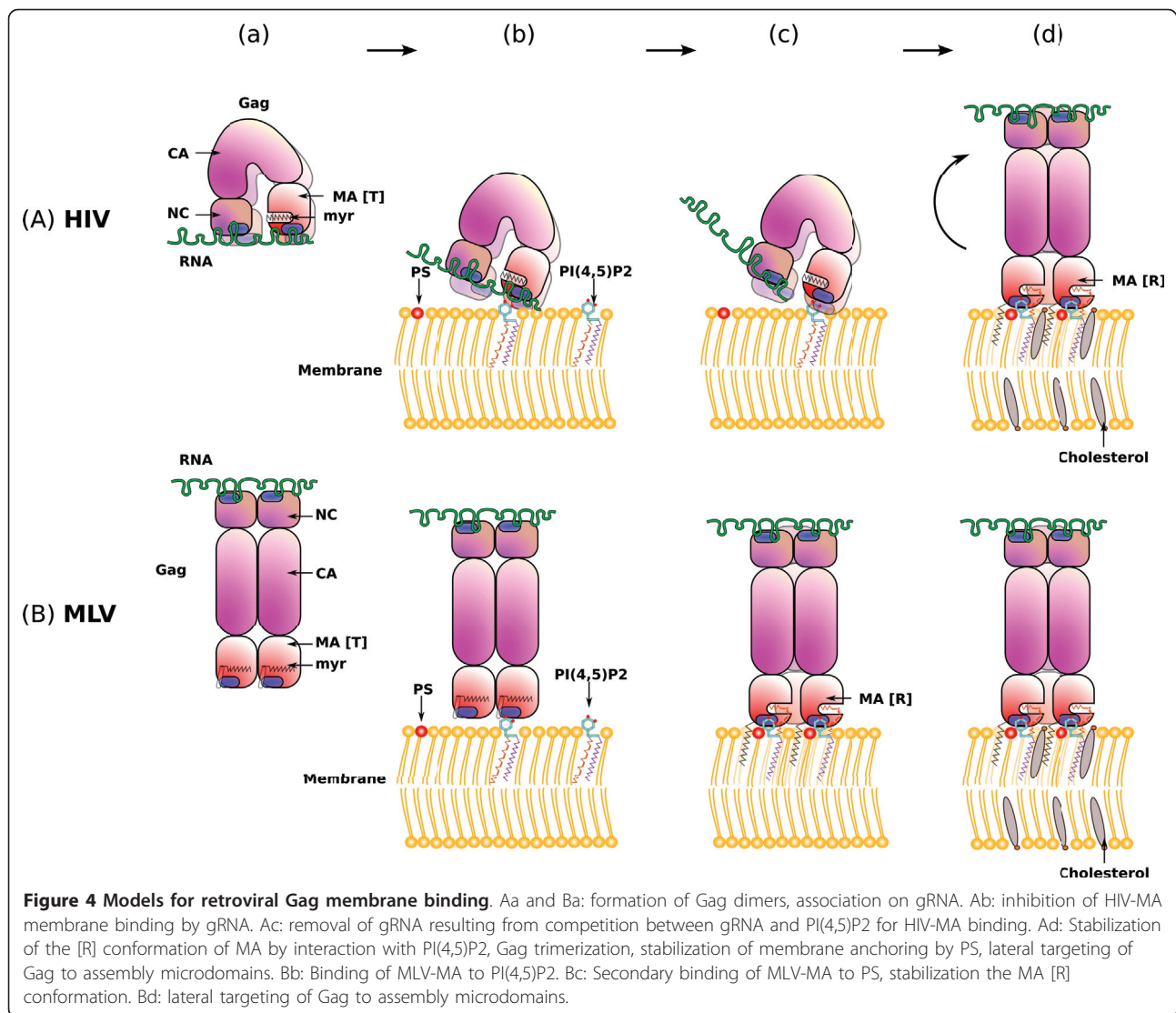
Let's switch again! Stabilization of the [R] conformation

The interaction of retroviral MAs with PI(4,5)P₂ seems to be a conserved, highly specific, and regulated feature among retroviruses. As previously mentioned, PI(4,5)P₂ binding seems to be associated with conformational changes, as shown by NMR for HIV-1 MA [66] and EIAV-MA [86]. For HIV, it corresponds to the myr[s] and myr[e] conformations ([T] and [R] respectively) evidenced by structural studies [20], and it is probably also the case for EIAV except that it is not myristylated. This supports a pre-existing hypothesis first proposed by Zhou *et al* [31]: the existence of a “myristyl switch”, that is actually an entropic equilibrium between the [T] conformation that sequesters the myristate inside the protein, and the [R] conformation that promotes trimerization and exposure of the myristate moiety allowing its insertion in the cellular membranes. A refinement of this model was proposed by Saad and colleagues, as the NMR data on HIV-MA suggested that the insertion of the myristate into the lipidic bilayer may be compensated by the extraction of the 2' fatty acid chain of the PI(4,5)P₂ out of the membrane, that would then be sequestered into the hydrophobic core of the MA domain (Figure 4Ad) [66]. Anraku and colleagues compared the affinity of HIV-1 MA and Gag for phosphorylated inositol ring alone and for medium length fatty acid chain lipids (Di-C8-PI(4,5)P₂), in order to compare the relative contribution of electrostatic interactions (with inositol phosphate ring) and hydrophobic interactions (with acyl chains) [71]. In accordance with the data from Saad *et al.* [66], acyl chains were found to have a major contribution in the interaction. This

model, however, is built on data obtained with short chain fatty acids, and needs further confirmation in lipid bilayer conditions.

As a model for HIV-MA/PI(4,5)P₂ interaction, we propose that the [T] conformation has a high affinity for RNA, and a low affinity for PI(4,5)P₂. On the contrary, the [R] conformation has a high affinity for PI(4,5)P₂. PI(4,5)P₂ would compete with RNA for HIV-MA binding as recently proposed [69,87] and its interaction with MA would in turn stabilize the [R] conformation as shown by Saad and colleagues [66] (Figure 3Aa). In this model, PI(4,5)P₂ has two roles: in addition to being the “substrate” (i.e. the bound molecule), it is also an effector, stabilizing the binding prone conformation, [R] (Figure 3Aa). In other words, PI(4,5)P₂ is able to displace a pre-existing equilibrium toward the [R] conformation, as suggested by Tang *et al* [20]. Symmetrically, RNA would have an “allosteric inhibitor” effect in stabilizing the [T] conformation (Figure 3Aa). This property may prevent a specific binding to membranes lacking PI(4,5)P₂. This model could explain why many authors were unable to measure the affinity of HIV-1 MA for PI(4,5)P₂ in the LUV system [42,55]. At low HIV-MA concentrations (from 1 μM to 20 μM), the equilibrium would be only slightly displaced toward the [R] conformation, even at a saturating PI(4,5)P₂ concentration (Figure 3Aa[42,54]). The [T] conformation had very low affinity for the lipid; we and others concluded that the affinity of MA for PI(4,5)P₂ was negligible in these conditions [42,55]. Many other elements could also influence the [T] to [R] equilibrium *in vivo*, to allow specific interaction of Gag with membranes. As mentioned earlier, a high concentration of MA promotes trimerization, and at the same time stabilizes the [R] conformation [20] (cf. Figure 3Aa). In addition, multimerization of Gag seems to correlate with the appearance of the [R] state, as multimerizing regions in CA promote myristate exposure [20] and increase lipid binding of MA-CA constructs [55] *in vitro*. In cells, it has been shown that proteolytic cleavage of Gag induces partial dissociation of p17MA from the membrane, confirming that uncleaved Gag stabilizes the [R] conformation of MA [31,88,89]. Another parameter that seems to influence the [T] to [R] transition is pH, as shown recently by Fledderman *et al.* [90]. High pH stabilizes the [T] form, while acidification favors myristate exposure. In addition, the same laboratory also reported that Calmodulin (CaN), a Ca²⁺ sensor protein determinant that interacts with HIV MA, promotes the myristyl switch [91].

The equilibrium constant between the [T] and [R] conformations also seems to vary greatly from one MA to another. As a matter of fact, in NMR conditions (high MA concentration, around 0.5 mM), HIV-1 and HIV-2 MAs behave differently in the presence of PI(4,5)P₂, the



[R] conformation remains undetectable for HIV-2 MA [21], unlike HIV-1 [66]. As far as other viruses are concerned, less data are available. It is possible that PI(4,5)P₂ also stabilizes an [R] conformation of EIAV-MA as suggested by 2-D NMR data obtained by Chen *et al* [86], showing a slight amino acid shift upon PI(4,5)P₂ binding. In contrast, MLV MA may display a more complex behavior. We were able to calculate two K_d values for MA/PI(4,5)P₂ interaction, either in the presence or absence of PS. The [T] conformation might be able to bind PI(4,5)P₂ with a K_d of 25 μM, while the [R] conformation might be stabilized by the presence of PS, allowing PI(4,5)P₂ to switch to the extended lipid conformation, with a resulting K_d value approaching 5 μM (Figure 3Ba) [42]. Another hypothesis is that the majority of MA is already in the [R] conformation, and that PS modulates the affinity of the interaction with PI(4,5)P₂.

The switch from the [T] to the [R] conformation may have further implications at the level of the entire Gag protein, thus influencing the assembly process. Indeed, Datta *et al.* recently proposed a model in which HIV-Gag would be in a bent conformation in solution, with MA and NC in close proximity [92,93] (Figure 3Ab). This model is supported by the fact that both NC and MA can bind IP6 (an inositol ring containing six phosphorylations, thus somewhat homologous to PI(4,5)P₂) *in vitro*, and is consistent with hydrodynamic and small-angle neutron scattering data. This is also in agreement with the idea that RNA can bind both NC and MA [67,70,77-79]. This is not compatible, however, with the immature particle organization, in which Gag is in an extended rod-shaped conformation [94]. Consequently, the authors propose that viral assembly is coupled with major conformational modifications of Gag (Figure

3Ab). The same group showed that correct *in vitro* assembly of viral like particles necessitates both RNA and IP6 (that can be considered as an analog of PI(4,5)P₂). It is still the case when the NC domain is replaced by a multimerization domain such as a leucine zipper, suggesting that RNA not only plays a role in assembly via its interaction with the NC domain, but probably also at the level of the MA domain [95].

The ability of HIV-Gag to auto-assemble into viral-like particles *in vitro* seems to be linked with a switch from Gag dimers to Gag trimers that can be mediated by IP6 [93,95]. As it has been shown that PI(4,5)P₂ promotes HIV-MA trimeric association [87], the effect of IP6 addition could mimic the effect of PI(4,5)P₂ binding in cells, in stabilizing the [R] conformation and promoting the formation of MA trimers. This could further trigger Gag structural reorganization via dimer to trimer transition (Figure 4Ad). A similar mechanism could drive the assembly of all retroviruses, as other retroviral MAs have multimerization properties upon PI(4,5)P₂ binding. For example, MLV-MA multimerizes in the presence of PI(4,5)P₂ under certain conditions (unpublished personal data), and EIAV-MA forms trimers [32].

MLV-Gag, however, seems to differ in some points from lentiviral Gag proteins. Datta et al. showed that *in vitro* recombinant MLV-Gag is readily in a rod-shaped conformation in solution, with a much more rigid structure (Datta, Zuo, Campbell, Wang, Rein: Personal communication) (Figure 3Bb). This property might argue for an absence of an RNA mediated maintenance of the [T] conformation for MLV-MA. This correlates with the fact that the [R] conformation of MLV-MA appears more stable, as 100% of MLV-Gag is associated with membranes in cells [42], in contrast with HIV-Gag which is no more than 60% membrane bound [96]. However, we cannot exclude the possibility that RNA could regulate the interaction of MLV-MA with lipids.

The mechanisms of interaction between retroviral MAs and lipids are quite original, and whether some particularities of these binding modes can also apply to other viral or cellular proteins is not known. For instance, other retroviral proteins could interact with lipids using a similar mechanism. For example, Nef and Tat, two regulatory proteins of HIV, also bind membranes. In fact, Nef is a myristylated protein able to bind acidic phospholipids, but the curvature of the membrane induced upon Nef binding is not consistent with the extraction of a fatty acid out of the membrane [97] as in the model proposed for HIV-MA [66]. A myristyl switch mechanism is still possible, however, as the binding of Nef to biomimetic membranes is a biphasic process, with a first phase of electrostatic interaction with acidic phospholipids, and a second phase of structural modifications (in particular,

the formation of an amphipathic helix) [97]. As for Tat, it was recently shown that it also interacts with PI(4,5)P₂ before crossing the plasma membrane and being secreted into the extracellular environment [98-100].

Conclusion: Cellular consequence of Gag binding to PI(4,5)P₂ and PS

Taking all the previously discussed data together allowed us to propose a model for the role played by MA during HIV and MLV assembly initiation, at the molecular level (Figure 4). In this model, Gag first polymerizes on gRNA (Aa and Ba), but adopts a bent conformation in the case of HIV (Aa), with both MA and NC interacting with gRNA, while MLV-Gag is readily in a rod-shaped conformation (Ba). For both viruses, the [T] conformation of MA is initially dominant, with myristate trapped in the protein core. When HIV-MA reaches PM (Ab), PI(4,5)P₂ is able to compete with gRNA for MA binding (Ac). Removal of gRNA and interaction with PI(4,5)P₂ stabilize the [R] conformation of MA (exposed myristate), which in turn promotes the trimerization and the reorganization of Gag into its rod-shaped conformation (Ad). The presence of PS could stabilize the interaction between MA and PI(4,5)P₂ (Ad). Gag would then be laterally targeted to membrane microdomains containing high levels of saturated lipids, such as lipid rafts (Ad). In the case of MLV, initial binding to PI(4,5)P₂ (Bb) is followed by a secondary binding to PS (Bc) that would further stabilize the [R] conformation of MA, exposing the myristate. Like HIV, lateral targeting of Gag to rafts or other microdomains is likely to occur afterwards (Bd).

These mechanistic observations are useful to re-evaluate the data available regarding assembly and budding localization in cells. Analysis of the retroviral particle envelope content evidenced that budding membranes resemble the plasma membrane in terms of lipid composition [73,75,101-105]. The ratio between lipids, however, differs from the average plasma membrane composition. In particular, viral particles of HIV and MLV are enriched not only in PI(4,5)P₂ and PS, but also in cholesterol, ceramides, GM3 and sphingolipids [73]. This can reflect the fact that viral particles are produced in specific membrane microdomains. Moreover, HIV virions are also enriched in lipid raft markers such as GPI-anchored proteins [106], actin and actin-associated proteins, such as Ezrin-Radixin-Moesin proteins (ERMs) [107,108], and in tetraspanins [108-116]. ERM and tetraspanins are also found in particles of MLV [107,117,118]. In consequence, retroviral budding has been proposed to occur preferentially in two types of membrane microdomains associated with actin cytoskeleton: lipid rafts and tetraspanin enriched microdomains (TEMs). There is a spatial and functional distinction, however, between these two kind

of domains [119-121], even if they are adjacent and may interact [122-124].

Lipid rafts are membrane domains enriched in cholesterol and sphingolipids, but can also be enriched in PI(4,5)P₂ and PS under specific conditions [125-128]. Rafts were initially identified as detergent-resistant membranes, and this property was widely utilized to characterize raft-associated lipids and proteins, including HIV-Gag [74,129-137], MLV-Gag [76,136] and HTLV-1-Gag [136,138]. The existence in living cell, the exact nature, and the actual size of lipid rafts has, however, been intensely debated over the past decades. The current consensus is that lipid rafts are nanoscale concentrations of specific lipids, notably cholesterol and sphingolipids, and proteins (reviewed in [128,139]). Their size is around 10 to 20 nm but they can coalesce and organize membrane bioactivity in many ways.

The association of HIV-Gag with lipid rafts depends on both membrane association signals of MA, the myristate and the HBR (reviewed in [140,141]). Lower order multimerization is also necessary because the association of CA mutants with lipid rafts is delayed [74], however, higher order association appears to be dispensable as demonstrated by NC mutants [142]. Lipid raft targeting is a slower process than membrane association, giving the idea that initial docking of Gag at the plasma membrane is followed by lateral transport to assembly microdomains as proposed by Ono and Freed [74].

Saad and colleagues [66] proposed a very elegant model in agreement with a preferential budding of HIV in raft microdomains. Their NMR data suggests that the 2'-fatty acid of the PI(4,5)P₂ is extracted from the membrane bilayer upon MA binding, and sequestered inside the protein, in the same hydrophobic pocket the myristate occupied. Unlike the 2'-chain, the 1'-chain is usually saturated, as is the myristate (cf. Figure 4). If this model proves to be correct, Gag would then be anchored to the membrane via two saturated chains (myristate and 1'-chain) and this could result in a lateral targeting of Gag to lipid rafts, where saturated lipids are enriched (Figure 4d, Bd).

The trapping of PI(4,5)P₂ into lipid rafts by Gag may have important consequences in terms of cellular responses. Indeed, in non-infected cells, it seems that the ratio of raft-associated PI(4,5)P₂ versus raft-excluded PI(4,5)P₂ is finely regulated. Any modification of one pool seems to have profound consequences, in particular on cytoskeleton remodelling, cell morphology and modulation of signaling pathways, such as the PI3K-Akt pathway [143].

Whether Gag, and in particular the MA domain, is able to aggregate lipid raft microdomains (directly or indirectly) or bind to pre-formed platforms is not as yet known, even if recent findings argue for dynamic

aggregation of raft components by Gag [116]. Annexin 2 could potentially play a role, as this protein interacts with Gag [108,144] and is able to aggregate lipids, in particular cholesterol, PS, and PI(4,5)P₂ [145,146]. Other viral proteins may be involved too. It was recently shown that gPr80^{gag}, a long glycosylated form of MLV-Gag, increases the release of MLV and HIV particles via lipid rafts [76]. A similar role has been observed for HIV-Nef [147], which also increases the "raft-like" properties of HIV particles [105] and modifies the cholesterol metabolism of producer cells [148]. However, it is not known how these two proteins act to relocate assembly in these microdomains.

On the other hand, several authors have reported that retroviral assembly occurs in association with tetraspanins [108-116,149-151]. Some tetraspanins can modulate viral infectivity and regulate cell to cell transmission [115], while the role of others, such as CD63, is currently debated [152]. The tetraspanins are a family of small transmembrane proteins that operate as major lateral organizers of membrane domains. They form tetraspanin-enriched microdomains (TEMs) or tetraspanin webs, in close relation with the cytoskeleton (reviewed in [153]). TEMs are enriched in cholesterol, GM1 and sphingolipids, but only a small fraction of the tetraspanins are found in the detergent resistant membrane (DRM) fractions, unlike raft proteins. Some tetraspanins, including CD9, CD63, CD81, and CD51 are associated with PI4K, a kinase that allows the synthesis of PI(4)P, the main precursor of PI(4,5)P₂. In particular, HIV-Gag seems to associate specifically with CD63 and CD81 and less with CD82 [108,109,113-115] while HTLV-1 Gag associates preferentially with CD82 at the plasma membrane [149-151]. It is noteworthy that CD82 does not associate with PI4K and that this may be related to the unusual particle production mode of HTLV, with preferential budding at the cell-to-cell contact areas and low production of cell-free virions. One unresolved question is whether there is a collaboration between rafts and TEMs during particle assembly or whether distinct budding microdomains exist in the cell. In support of the first hypothesis, it was observed that some tetraspanins are able to address protein complexes toward lipid rafts, inducing the activation of specific signalization pathways. In particular, CD81 is necessary to partition the B cell receptor (BCR) and the CD19/CD21/CD81 complex into rafts [122,123], while CD82 links the actin cytoskeleton, T cell receptors and raft domains [124]. This suggests that tetraspanins may help to target Gag to lipid rafts, or, the other way around, that Gag could recruit tetraspanins and lipid raft components in order to activate particular signalization pathways necessary for sustaining HIV infection. This later model is supported by recent work by Kremontsov *et al.* showing the strong trapping of

CD9 and the transient trapping of cholesterol, GM1 and CD55 into the HIV-1 assembly microdomains [116]. Interaction between TEMs and lipid rafts could result in the activation of TCR signalization pathway from which HIV could benefit. This pathway comprises, for example, the protein Lck, a Src-kinase participating in T-cell activation [154], that interacts with HIV-Gag and increases particle production [155]. Moreover, the activation of TCR not only causes the accumulation of raft lipids in the membrane areas involved in the TCR signaling pathway but also recruits PS, which is probably necessary for Gag stabilization in PM microdomains during particle formation [127].

The enriched literature on retroviral assembly has allowed us to postulate a quite precise model of the molecular events that drive the anchoring of Gag to cellular membranes preceding particle formation, but these models remain to be tested experimentally. The high conservation of the overall process is striking, especially concerning the specificity of the interaction between Matrix domain of Gag and cellular lipids (PI(4,5)P₂, PS, cholesterol), and suggests that targeting retroviral assembly by therapeutical approaches may be a good strategy to combat HIV infection.

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Authors' contributions

EH wrote the manuscript and made the figures. DM contributed to the manuscript writing and editing. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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