The Roles of Lipid and RNA in Regulating Retroviral Gag Membrane Binding and Targeting

by

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ABSTRACT

The matrix (MA) domain of HIV-1 mediates proper Gag localization and membrane binding by interacting with phosphatidylinositol-4,5-bisphosphate [PI(4,5)P$_2$], a phospholipid that is found predominantly at the plasma membrane (PM). HIV-1 MA also interacts with RNA, which prevents Gag from binding to membranes containing phosphatidylserine (PS), a prevalent negatively charged phospholipid. These results suggest that the MA-bound RNA promotes PM-specific localization of HIV-1 Gag by blocking non-specific interactions with cellular membranes that do not contain PI(4,5)P$_2$.

In this thesis, I examined whether PI(4,5)P$_2$ dependence and RNA-mediated inhibition collectively determine MA phenotypes across a broad range of retroviruses. By comparing a panel of Gag-leucine-zipper constructs (GagLZ) containing MA of different retroviruses, I found that membrane binding mediated by retroviral MA can be broadly divided into two categories: those that are PI(4,5)P$_2$-dependent and RNase responsive, and those that are neither. I also found that the PM-localization and virus-like particles (VLP) release of the former group is sensitive to the overexpression of a PI(4,5)P$_2$-depleting enzyme, polyphosphoinositide 5-phosphatase IV (5ptaseIV), while the latter group is much less sensitive to 5ptaseIV overexpression. Structural analyses further suggest that the basic patch size of the retroviral MA confer susceptibility to RNA-mediated membrane binding inhibition.

In my thesis, I also provided *in vitro* and cell-based evidence supporting that RNA-mediated suppression occurs in cells and that RNA can inhibit membrane binding of HIV-1 Gag at a concentration that is much lower than the estimated RNA concentration in the cell. Hence, RNA-mediated suppression is a physiologically relevant mechanism that prevents Gag from binding promiscuously to prevalent PS-containing membranes.

Finally, I examined the roles of $PI(4,5)P_2$ and RNA in regulating the targeting of HIV-1 Gag to the site of assembly, the virus-containing compartments (VCC), in primary macrophages. I found that the VCC localization and virus release of HIV-1 are severely impaired upon 5ptaseIV overexpression. However, HIV-1 MA only contributes to membrane binding but not in Gag targeting to the VCC. I also determined that HIV-1 nucleocapsid (NC) is important for VCC-specific localization of HIV-1 Gag. This suggests that targeting of HIV-1 Gag to the VCC adopts a different mechanism than Gag targeting to the PM in HeLa and T cells.

CHAPTER I

Introduction

Retroviruses are enveloped RNA viruses that can replicate in a host cell using a unique process known as reverse transcription (see **Life cycle of retroviruses**) ([1]). A number of retroviruses have been linked to diseases in humans and animals. These infections have killed millions of lives and resulted in severe financial losses. Human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS), was discovered in 1983. Since the beginning of the pandemic, there has been 39 million AIDS-related deaths with 35 million people presently living with HIV/AIDS worldwide ([2]). Retroviruses that infect livestock, such as Bovine leukemia virus (BLV) and Jaagsiekte sheep retrovirus (JSRV), have serious impact on the agricultural economy due to the death of cattle, increased veterinary costs, reduced reproductive efficiency and milk production ([3], [4]). To date, there is no cure or vaccine for these diseases. Thus, continued research efforts are needed for the development of effective treatments for and prevention against these viral infections.

Types of retroviruses

All retroviral genomes contain a highly conserved gene that encodes for an enzyme known as reverse transcriptase (RT) ([1], [5]). RT is essential for the virus to carry out reverse transcription, converting the viral RNA genome into complementary DNA (cDNA). Alignment of the RT sequence has unraveled the phylogeny relationship among

the different retroviruses ($\underline{6}$). Based on this, the family of retroviridae can be subdivided into six different genera, namely alpharetrovirus, betaretrovirus, gammaretrovirus, deltaretrovirus, epsilonretrovirus, lentivirus and spumavirus [for retrovirus phylogeny tree, see Weiss et al 2006 ($\underline{6}$)]. Aligning conserved amino acid residues within RT and other RT-like sequences have led to the discovery of retroelements including endogenous retroviruses (ERV) ($\underline{6}$, $\underline{7}$). These elements serve as footprints of ancient retroviral infections and can be used as molecular markers to study evolution. In human, ERV makes up a surprisingly large portion (8%) of the genome ($\underline{8}$, $\underline{9}$). While most human ERV (henceforth referred to as HERV) do not contain functional open reading frames (ORFs) and therefore are not able to replicate, some HERVs, such as HERV-K, contains ORFs for all retroviral genes. HERV-K belongs to the genus betaretrovirus. Upregulated HERV-K protein expression and/or RNA transcripts have been associated with various autoimmune diseases, cancer and HIV-1 infection ($\underline{10}$, $\underline{11}$). However, the direct role of HERV-K in disease pathogenesis remains controversial. Despite the intact retroviral genome, the expression level of HERV-K in general is very low, making it difficult to study *in vitro* ($\underline{12\text{-}14}$). Thus, to improve the protein expression of HERV-K, using bioinformatics approach, two groups reconstructed the infectious sequence of HERV-K ($\underline{14}$, $\underline{15}$). In one of the studies, the consensus sequence of HERV-K 113 and another closely related HERV-K was utilized to construct HERV-K$_{CON}$. These codon optimized HERV-K sequences are powerful tools for studying the biology of these ancient retroviruses and their relationships with diseases.

Retroviral genome

Retroviruses can also be broadly divided into simple and complex viruses based on their genomic organization (Fig. 1.1). All retroviruses contain four essential genes – *gag*, *env*, *pol* and *pro.* The gene product of *gag* directs the synthesis of virus core during assembly; *env* contains the information for the viral envelope protein, which is essential for the entry step of virus replication (see **Life cycle of retroviruses**); *pol* encodes the reverse transcriptase and integrase enzymes; and *pro* is necessary for virus maturation. Simple retroviruses, such as murine leukemia virus (MLV) and mouse mammary tumor virus (MMTV), only encode these four genes, while complex retroviruses, such as HIV and human T-lymphotropic virus (HTLV), contain additional non-structural genes (Fig. 1.1). HIV-1 encodes six other genes: *tat, rev, nef, vif, vpr* and *vpu*, each playing different roles during replication (**Table 1.1**). In addition to these genes, HIV-2 and some simian immunodeficiency viruses (SIV) also contain *vpx*, which is important in counteracting a restriction factor known as SAM domain and HD domain-containing protein 1 (SAMHD1) for successful infection in cells with myeloid lineage (see **Chapter IV**). Oncogenic retroviruses such as HTLV and Rous sarcoma virus (RSV) contain *tax* and *src* oncogene, respectively, which stimulate uncontrolled proliferation of host cells (1). Insertion of retroviral genome near the promoter region of a proto-oncogene in the host genome may also result in the onset of cancer.

Life cycle of retroviruses

A Retrovirus initiates infection by interacting its envelope proteins with host cell's surface-exposed receptor (Fig. 1.2) (16). Binding to the receptor leads to the internalization of the virus core into the cell. While most retroviruses enter the cell by

direct fusion with the plasma membrane, some retroviruses such as ecotropic (narrow host range) and amphotropic (wide host range) MLV have been shown to enter the cell through endocytosis ([17](#)). Following viral fusion with the host membrane, viral core is delivered to the cytoplasm where uncoating and reverse transcription take place. Most retroviral reverse transcription occur during early stages of virus life cycle, except for spumaviruses, where the process takes place during assembly ([16](#)). During reverse transcription, complementary DNA (cDNA) is generated from the viral genomic RNA. The cDNA is then transported into the nucleus as a pre-integration complex (PIC). In the nucleus, the cDNA is integrated into the host genome, a process mediated by viral integrase protein. The integrated viral genome is now known as a provirus.

During late stages of virus life cycle, viral genes are transcribed and translated to initiate the formation and release of new virions. For HIV-1, *tat* is one of the earliest genes to be expressed ([18](#)). Tat stands for trans-activator of transcription. As its name suggests, it increases transcription level of viral genes. Similar to *tat*, HTLV family encodes the *tax* gene, which, when expressed, also helps to increase expression level of other viral genes ([19](#)).

Viral genomic RNA is transcribed in the nucleus and transported out of the nucleus in its unspliced form ([20](#)). In HIV-1, the transport of singly spliced or unspliced RNA out of the nucleus is assisted by the viral-encoded Rev protein. Rev binds to the rev-response element (RRE) found on the RNA. Rev binding to RRE recruits Crm1/RanGTP complex, which facilitates nuclear export ([16](#)). Rev-like proteins are also found in other retroviruses, such as Rem (MMTV), Rex (HTLV-1) and Rec (HERV-K) ([21](#), [22](#)).

Following enhanced viral mRNA transcription and nuclear export, viral structural proteins such as Gag, Gagpol and Env are synthesized in the cytoplasm and are subsequently targeted to the site of virus assembly (see **Retroviral assembly)**. During assembly, viral genomic RNA is encapsidated into the forming virion. Specific incorporation of genomic RNA is mediated by the binding of Gag protein to the Psi packaging sequence found on the RNA (see **Gag polyprotein**) ([23]). In addition to viral RNA, many cellular RNA are also incorporated into the virus particle. For instance, tRNA^{Lys} and 7SL RNA are highly enriched in HIV-1 particles ([24-27]). tRNA^{Lys} is used by HIV-1 as the primer during reverse transcription, thus it is selectively packaged into the virus particle. The concentration of tRNA^{Lys} in HIV-1 virus particles is estimated to be ten times higher than in the cell cytoplasm ([28]), whereas 7SL RNA was estimated to be 250-fold enriched ([27]). Other RNA, such as mRNAs for ribosomal proteins, are also packaged non-specifically into the virions ([23], [29]).

At late stages of the virus assembly, newly formed virus particles are released from the cell surface. This pinching-off process is facilitated by the endosomal-sorting complex required for transport (ESCRT) proteins ([30]). The ESCRT proteins interact with the assembling Gag proteins via the late domain motifs, which are highly conserved among retroviruses (see **Gag polyprotein**). Finally, the viral protease packaged within the virus particles cleaves viral proteins into their individual forms, thereby causing a structural changes of the virus particle. This mature virus is then able to infect the next susceptible host cell.

Retroviral assembly

As mentioned above, retroviral assembly is driven by the Gag proteins. Viral core is made up of assembling Gag multimers, which appear as electron-dense sites under the electron microscope (EM). Based on the morphology and where these viral cores are found in the cell, the retroviral assembly can be further divided into four categories – Types A, B, C and D ([31]). Type A retroviruses, such as ERV-like elements, are intracellular-only viruses, where immature particles are seen to bud into the cytoplasm or the endoplasmic reticulum. Type B and D retroviruses form preassembled immature particles in the cytoplasm before being transported and released at the plasma membrane. B-type retroviruses usually display more prominent envelope spikes than D-type retroviruses. Examples of B- and D-type retroviruses include the MMTV and Mason-Pfizer monkey virus (MPMV), respectively. Finally, in type C retroviruses, the Gag proteins are transported as precursors to the plasma membrane such that virus budding occurs simultaneously with virus assembly. The C-type retroviruses includes equine infectious anemia virus (EIAV), MLV, HERV-K, RSV as well as human pathogens like HIV and HTLV. For the remaining sections of my dissertation, I will be focusing on the assembly of the C-type retroviruses.

The site of assembly of some retroviruses, such as that of HIV-1, is cell-type dependent. In HeLa cells and T cells, HIV-1 assembles at the plasma membrane ([32]). In a subset of T cells that adopts polarized morphology (such as those found in lymph nodes), HIV-1 is observed to assemble at one particular region of the plasma membrane, known as the uropod. Uropod is a region at the rear-end of a polarized T cell, and is found to contain numerous adhesion molecules for cell-to-cell contact ([32], [33]). In contrast to T

cells and HeLa cells, in primary macrophages, HIV-1 assembles and accumulates at seemingly intracellular compartments (32). These compartments, called the virus containing compartments (VCC), were thought to be endosomal compartments or multivesicular bodies (MVB) (34). While the exact nature of the VCC still remains unclear, some report that VCC is actually an invagination of the plasma membrane and contains unique structures and cellular markers that are distinct from the endosomal compartments/MVB.

In addition to HIV-1, HTLV-1 Gag also shows different localization in different cell types. In T cells, HTLV-1 Gag localize mainly to the plasma membrane, whereas in cell lines such as HeLa cells, HTLV-1 Gag is found to localize to both plasma membrane and intracellular compartment (35, 36). The varied Gag localization pattern in different cells may suggest that different trafficking machinery is being used for transporting Gag to its ultimate location for assembly, the plasma membrane.

Gag polyprotein

Gag is synthesized as a multi-domain polyprotein. All retroviral Gag contains matrix (MA), capsid (CA) and nucleocapsid (NC) structural domains (Fig. 1.3A) (37-41). Each domain plays critical roles during assembly. The MA domain is mainly responsible for membrane binding as well as targeting Gag to the site of assembly. In addition to these roles, MA also binds RNA (35, 39, 42-52). CA contains two subdomains – the N-terminal domain (NTD) and C-terminal domain (CTD). While the NTD is involved in viral core formation, the CTD is important in mediating Gag-Gag dimerization. The NC contains two zinc-finger domains, which bind to the Psi RNA packaging signal and encapsidate the genomic RNA during assembly. NC-RNA interaction also promotes

higher order Gag-Gag interaction by using RNA as a scaffold for Gag multimerization. Finally, B-, C- and D-type retroviruses also contain late domain (L) motifs to help in the release of virus particle from the plasma membrane. Since A-type retroviruses are considered as "intracisternal" particles and are not released from the cell surface, they are thought to not contain late domain motifs. There are three classes of L motif: PTAP, PPXY, YXXL (30, 38, 53, 54). For HIV-1, the main mechanism of release involves the interaction between the PTAP motif and Tsg101, a member of the ESCRT-1 complex, which recruits other ESCRT complexes to facilitate the pinching-off process (53).

In addition to these well-defined functional domains and motifs, some Gag proteins also contain spacer peptides, which vary in location within the Gag protein and play various roles during assembly. For instance, HIV-1 Gag contains spacer peptides 1 and 2 (SP1 and SP2) (37). While the role of SP2 is not well-understood, SP1 is important in proper particle formation. In RSV, the spacer peptide p10 contains nuclear export signal (NES), which, when mutated, prevents Gag from exiting the nucleus, resulting in inefficient assembly (55).

The focus of my dissertation is to understand the role of MA in mediating retroviral Gag membrane binding and targeting to the site of assembly. This research was carried out broadly, by examining the membrane binding behavior of various retroviral MA, and also highly focused, by looking at the targeting mechanism of HIV-1 Gag to the VCC in primary macrophages. As membrane binding is the first key step in retroviral assembly, this process serves as an attractive drug target for inhibiting virus replication. Therefore, an extensive understanding of the molecular mechanisms of Gag membrane binding and targeting by retroviral MA is necessary for identifying important drug targets.

In the remaining parts of this chapter, I will be focusing on discussing the roles played by retroviral MA during assembly.

The roles of retroviral MA in virus assembly

(i) Structure of retroviral MA

The retroviral MA domain makes up the N-terminus of the Gag protein (Fig. 1.3A). Despite a lack in sequence homology, structures of currently solved MA domains are remarkably similar to one another (39). To date, there are nine solved retroviral MA structures (39). These MA domains contain a globular core which is made up for four α-helices [for a comparison of retroviral MA structures, see Alfadlhi et al 2014 (39)]. Moreover, all retroviral MA contain basic residues (56). In some retroviruses, such as that of HIV-1, the basic residues are more clustered, forming a basic patch known as the highly basic region (HBR) (57-59). In other retroviruses, such as in HTLV-1, there is no obvious highly basic region; rather, the basic patches are found to be smaller and more distributed on the MA domain (36, 60, 61).

(ii) Myristate moiety of retroviral MA

The role of MA in membrane binding is brought about by two important signals: the N-terminal myristate and the basic residues (39, 57-59). Myristoylation is a co-translational modification process in which a saturated 14-carbon chain, the myristic acid, is covalently added to the surface-exposed glycine at the N-terminus of a forming protein. For HIV-1, myristate moiety is added to the first glycine residue (following methionine) of the Gag protein, following the cleavage of the first methionine amino acid from the forming polypeptide (57, 59). The myristate moiety allows the Gag protein to interact

with cellular membrane via hydrophobic interactions. Myristoylated Gag are found in most retroviruses such as HIV-1, HTLV-1, MLV, and HERV-K. Mutation of the N-terminus glycine residue, which prevents the addition of myristoyl group, renders the Gag protein unable to bind to cellular membrane ([59]), suggesting that myristate moiety is essential for membrane binding of Gag.

The presence of constitutively exposed myristate can be detrimental for the virus as random binding of Gag to different cellular membrane may occur. Therefore, it is important to sequester the myristate moiety until Gag reaches the site of assembly, where membrane binding should occur. In HIV-1, the "myristoyl switch" mechanism is proposed to explain how the myristate exposure is regulated ([62-69]). According to this hypothesis, the myristoyl moiety is buried inside the HIV-1 MA globular domain and is only exposed upon Gag conformational changes that are triggered by events happening at the site of assembly, such as Gag-Gag interaction and binding to specific lipid at the plasma membrane. Some myristoylated cellular proteins, such as ADP-ribosylation factor and recoverin, have also been described to use the "myristoyl switch" mechanism to regulate their membrane binding ([70], [71]). Interestingly, it was suggested that, despite being myristoylated, HTLV-1 Gag does not utilize this mechanism for its membrane binding regulation ([72], [73]). How HTLV-1 Gag regulates its myristoyl exposure is not well-understood. Furthermore, whether this mechanism is truly unique to HIV-1 Gag remains to be determined.

(iii) Basic region of retroviral MA contributes to electrostatic interaction with cellular membrane

Despite the importance of the myristate moiety in membrane binding, intriguingly, not all C-type retroviral Gag are myristoylated. Both RSV and EIAV Gag are not myristoylated, yet, they are able to bind to the plasma membrane (74, 75). This suggests that other forms of protein-membrane interaction are sufficient to provide the critical binding energy for stable membrane binding. One likely candidate is the electrostatic interaction between the basic patches on the retroviral MA and the negatively charged cellular membrane (56, 74, 76). Indeed, when all basic residues within the HBR is mutated to alanine, HIV-1 Gag failed to bind to the plasma membrane or negatively charged liposomes *in vitro*, suggesting that for HIV-1 Gag, both myristate and HBR are required for stable protein-membrane interaction (46, 59). Thus, in the case of RSV and EIAV Gag, it is possible that the electrostatic interaction is the main attractive force between the MA domain and the cellular membrane.

(iv) Cellular proteins that bind membrane also contain polybasic region

Not only retroviral Gag, but many cellular proteins also contain polybasic regions and are found to interact with cellular membrane via electrostatic interaction. These interactions can be specific or simply charge-based (77). Proteins containing pleckstrin homology (PH), epsin N-terminal homology (ENTH) domain, and FYVE domain, for example, bind membrane via specific interaction between the basic amino acid residues on the protein and the unique structure of the phospholipid headgroup (78). Other membrane proteins such as the effector domain of Src protein and Myristoylated Alanine-Rich C Kinase Substrate (MARCKS) bind to negatively charged membrane mainly by bulk charge interactions (78-80).

(v) HIV-1 MA and the PH domain of PLCδ1 bind PI(4,5)P$_2$

The PH domain of phospholipase C delta-1 (PLCδ1) binds specifically to phosphatidylinositol-4,5-bisphosphate (PI(4,5)P$_2$), a phospholipid that is predominantly found at the inner leaflet of the plasma membrane (Fig. 1.3B) ([77]). PI(4,5)P$_2$ is a type of phosphoinositide that makes up 1% of the total phospholipid at the plasma membrane with an overall charge of -3 or -4 at pH 7.0 ([78]). Upon binding, PLCδ1 cleaves PI(4,5)P$_2$ into diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP$_3$), where both serve as secondary messengers for the activation of signaling pathways in the cell. DAG remains on the cell membrane and activates the protein kinase C (PKC) cascade, whereas IP$_3$ enters the endoplasmic reticulum (ER), leading to an efflux of Ca^{2+}, which in turn activates other signaling cascades. In addition to activating cell signaling pathways, PI(4,5)P$_2$ is also involved in cytoskeleton rearrangement and in membrane trafficking, such as clathrin-mediated endocytosis and phagocytosis ([78], [81-83]).

The polybasic residues (Lys30 and Lys57) found in the PH domain of PLCδ1 is shown to interact with the phosphorylated inositol ring of PI(4,5)$_2$ by stereospecific interaction. Mutations at these sites of the PH domain results in severe reduction in PI(4,5)P$_2$ binding ([84]).

HIV-1 Gag has also been shown to interact specifically with PI(4,5)P$_2$ ([46], [66], [85-88]). Several *in vitro* studies further support that HIV-1 MA, in particular the HBR, interacts specifically with PI(4,5)P$_2$. In the first study, using NMR, Saad et al., demonstrated that HIV-1 MA interacts specifically with PI(4,5)P$_2$ and this interaction triggers myristate exposure ([66]). In another study, mass-spectrometric protein

footprinting was used to demonstrate that Lysines 29 and 31 of HIV-1 MA interact with PI(4,5)P$_2$ ([88]). However, there are major caveats to these structural studies. First of all, the HIV-1 Gag protein used for modeling was either non-myristylated or truncated. Secondly, soluble, short acyl-chain PI(4,5)P$_2$ was used, which did not fully represent the PI(4,5)P$_2$ found in the cells (Fig. 1.3B). Thus, in order to fully study the interaction of full-length myristylated Gag and PI(4,5)P$_2$ with long acyl chains, our lab reported the use of *in vitro* liposome binding assay. Using this assay, we showed that HIV-1 Gag binds specifically to PI(4,5)P$_2$-containing liposomes. Furthermore, mutating either the myristoylation site or the HBR of HIV-1 MA severely reduced HIV-1 Gag binding efficiency ([87]). These data provide *in vitro* evidence that HIV-1 Gag interacts specifically with PI(4,5)P$_2$ via its MA domain.

(vi) The role of PI(4,5)P$_2$ in retroviral assembly in cells

PI(4,5)P$_2$ present at the plasma membrane can be depleted or mistargeted to intracellular compartments by overexpressing either phosphatidylinositol polyphosphate 5 phosphatase (5ptaseIV) or the constitutively active Arf6 construct, respectively ([89]). PI(4,5)P$_2$ depletion by 5ptaseIV overexpression in HeLa cells was found to severely reduced plasma membrane localization of HIV-1 Gag and HIV-1 release efficiency ([87], [89]). Furthermore, expression of constitutively active Arf6 relocates HIV-1 Gag from the plasma membrane to the intracellular compartments. These results demonstrate that PI(4,5)P$_2$ is important for HIV-1 assembly and Gag targeting to the site of assembly.

The discovery of the role of PI(4,5)P$_2$ in HIV-1 assembly has prompted more studies to examine the role of phospholipids in other retroviral assembly. To date, MLV MA, HIV-2 MA, RSV MA and EIAV MA have been shown to bind to PI(4,5)P$_2$ ([90-93]),

although for EIAV, PI(3,5)P$_2$ seems to play a more important role in virus assembly (94).

Significant reduction in virus release efficiency of RSV, MLV, MPMV and HIV-2 are

also observed upon 5ptaseIV overexpression in cells (93, 95-97). Interestingly, HTLV-1

Gag as well as the HIV-1 Gag chimeric protein, which contains HTLV-1 MA in place of

HIV-1 MA (HTMA Gag), do not require PI(4,5)P$_2$ for efficient binding and that PI(4,5)P$_2$

depletion only modestly reduces their release from the cells (35).

(vii) Retroviral MA target Gag to the site of assembly

As mentioned previously, retroviral MA is also responsible in targeting Gag to the

site of assembly. For HIV-1, some mutations in the HBR, such as mutating Lysines 29

and 31 (29/31 KE), results in mislocalization of Gag to the intracellular compartments

and inefficient virus release (34, 87). This suggests that proper Gag targeting to the site of

assembly is important for efficient virus release. Similarly, mutations of some of the

basic residues in other retroviral MA, such as that of HTLV-1, RSV and MLV, also lead

to altered Gag localization and/or reduced virus release (36, 98, 99). Thus, the basic

residues of retroviral MA play dual roles during virus assembly: (i) facilitating membrane

binding and (ii) targeting Gag to the site of assembly.

However, while it is true that HIV-1 MA plays a major role in targeting Gag to

the plasma membrane in HeLa and T cells, the mechanism of Gag targeting to the uropod,

a specialized region of the plasma membrane, in polarized T cells seems to be dependent

more on Gag-Gag multimerization (100). Similarly, targeting of HIV-1 Gag to the VCC

in primary macrophages seems to require higher-order multimerization by HIV-1 NC.

The cell-type-dependent molecular mechanisms of HIV-1 Gag targeting to the site of

assembly will be further discussed in **Chapter IV** of my dissertation.

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(viii) The RNA-binding role of retroviral MA

Most retroviral MA contain an overall positive charge (56). This basic charge
allows retroviral MA to bind to the negatively charged cellular membrane as well as
nucleic acids (NA). Both BLV MA and HIV-1 MA have been shown to have NA-binding
abilities (101, 102). For BLV, it is thought that the MA domain plays a role in RNA
encapsidation (101). HIV-1 MA, in particular, Lysine residues 25 and 26 in the HBR, has
been implicated DNA/RNA binding (47, 48, 51). A recent RNA-seq study show that
HIV-1 MA binds RNA, in particular, specific types of tRNAs, in cells (103). *In vitro*
analysis of HIV-1MA further shows that some residues that binds RNA within the HBR
overlap with those binding $PI(4.5)P_2$ (104). Overall, these study support that HIV-1 MA,
particularly the HBR, can bind NA. However, it was not known whether RNA had any
regulatory effect on HIV-1 assembly, especially in membrane binding. Thus, to examine
the role of HIV-1 MA and RNA interaction, our lab utilized the *in vitro* liposome binding
assay and analyzed the effect of RNase treatment of HIV-1 Gag on membrane binding.
Surprisingly, we found that RNase treatment enables HIV-1 Gag to bind to negatively
charged liposomes (PC:PS) that does not contain $PI(4,5)P_2$ (46). The PC:PS liposomes
are made up of neutral lipid phosphatidylcholine (PC) and an acidic lipid
phosphatidylserine (PS) at 2:1 ratio. Importantly, deletion of NC, the major RNA-binding
domain, still renders HIV-1 Gag responsive to RNase treatment, suggesting that MA-
bound RNA is the one responsible for liposome binding inhibition (46). Based on these
results and previous studies looking at HIV-1 MA ability to bind RNA, we hypothesize
that the MA-bound RNA prevents HIV-1 Gag from binding non-specifically to the
abundant negatively charged cellular membranes and only allows binding to membranes

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that contain PI(4,5)P$_2$, such as the plasma membrane (Fig. 1.4). In other words, RNA regulates HIV-1 Gag membrane binding by HIV-1 Gag specific binding to the plasma membrane where assembly takes place.

In contrast to HIV-1 Gag, HTLV-1 Gag was found to be able to bind non-PI(4,5)P$_2$-containing (PC:PS) liposomes efficiently (35). Furthermore, unlike HIV-1 Gag, HTLV-1 Gag binding to PC:PS liposomes is not responsive to RNase treatment as efficient binding can already be achieved even in the absence of RNase treatment (35). HTLV-1 Gag provides the first evidence that PI(4,5)P$_2$ and RNA do not regulate the membrane binding of all retroviral Gag. However, while it is likely that the difference in membrane binding properties of HIV-1 Gag and HTLV-1 Gag is due to the MA domain, we were not able to specifically identify which retroviral domain in HTLV-1 Gag confers this lack of sensitivity to PI(4,5)P$_2$ and RNA. Moreover, it is unknown whether other retroviral MA can be similarly or differentially regulated by PI(4,5)P$_2$ and RNA. Thus, to address these issues, I conducted a broad analysis of different retroviral MA in their Gag membrane binding and targeting properties. By constructing protein chimeras (GagLZ), where each contain different retroviral MA, I was able to analyze specifically the role of MA-PI(4,5)P$_2$ and MA-RNA interactions during Gag assembly (60). The analyses and discussion of this part of my thesis can be found in **Chapter II** of my dissertation.

The data collected so far indicates that RNA-mediated membrane binding inhibition of HIV-1 Gag exists *in vitro*. While it is compelling to think that RNA can also regulate the interaction between HIV-1 MA and membrane in the cell, there is no direct evidence to show that this mechanism exists in the cell. Thus, to examine if RNA-mediated membrane binding inhibition of HIV-1 Gag can happen in cells, our lab

developed the cell-derived liposome binding assay (45). In these experiments, we harvested HIV-1 Gag from transfected HeLa cells and demonstrated that these HIV-1 Gag proteins are able to bind to PC:PS liposomes upon RNase treatment. Furthermore, to examine if RNA can indeed block HIV-1 Gag binding to PC:PS liposomes, we added back RNA to HIV-1 Gag that was previously RNase-treated and then RNase-inactivated. As expected, we found that RNA can re-establish the inhibition on Gag binding to these non-PI(4,5)P$_2$-containing liposomes (45). The results of the cell-derived liposome binding assays and the RNA add-back assays will be further discussed in **Chapter III** of the dissertation.

Overview of thesis

Retroviral assembly is driven by its structural Gag protein. Assembly is a key step in virus replication as it leads to the production of new virions in the cell. Since Gag is the main driver of this important process, it serves as an attractive drug target to inhibit viral replication. In this introduction, we have discussed some similarities between retroviral Gag and cellular proteins in their membrane binding properties. We have also considered the importance of cellular phospholipids and RNA in regulating Gag membrane binding. Membrane binding regulation by RNA is a new concept. Given that both retroviral MA and membrane-binding cellular proteins contain polybasic regions, it is likely cellular proteins can also bind RNA and that the RNA-mediated regulation is not only limited to retroviral proteins. Thus, a more in-depth analysis of how retroviral Gag bind to cellular membrane will potentially reveal important (cellular or viral) players in the assembly step and also further our understanding of how membrane binding of various cellular proteins are regulated.

My dissertation involves the study of roles of lipids and RNA in regulating retroviral assembly. The first aim of my thesis is to broadly examine the roles of lipid and RNA in regulating the membrane binding of retroviral Gag. This work is presented in **Chapter II**, which shows that membrane binding of retroviral Gag can be distinctly categorized into those that are $PI(4,5)P_2$-dependent, RNAse-responsive and those that are $PI(4,5)P_2$-independent and RNase non-responsive. Furthermore, towards better understanding of the role of RNA in regulating retroviral Gag membrane binding in the cell, in **Chapter III**, I have developed a cell-based liposome binding assay. I was able to validate our previous *in vitro* liposome binding results using cell-derived Gag in place od the *in vitro*-synthesized Gag. These results supports the idea that cellular RNA can interact with HIV-1 Gag and that RNA-mediated inhibition of HIV-1 Gag membrane binding can exist in the cell.

The second aim of my thesis is to elucidate the molecular mechanism of HIV-1 assembly in primary macrophages, a topic that remains poorly understood. In **Chapter IV** of my dissertation, I described the roles of $PI(4,5)P_2$ and RNA in determining the localization of HIV-1 Gag to VCC in macrophages. My results suggest that HIV-1 Gag localization to the VCC requires NC-mediated multimerization.

Finally, in **Chapter V**, I will summarize results of **Chapters II, III, IV**, propose future directions by providing some of the preliminary results pertaining to these projects, and discuss the future of the antiretroviral treatments based on the work of my dissertation.

Simple Retrovirus: MLV
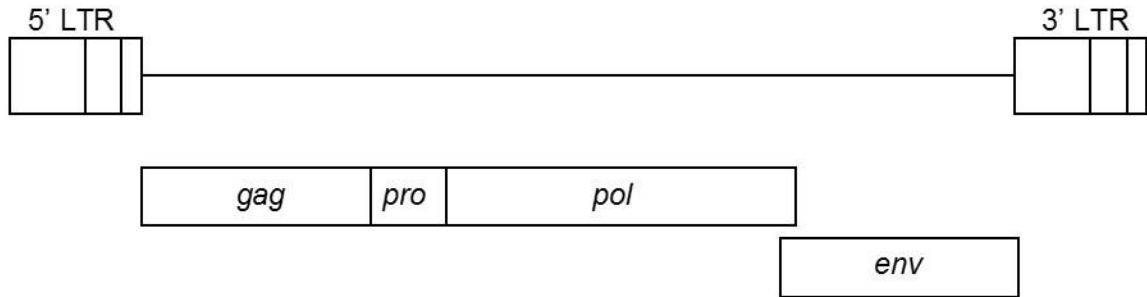
Complex Retrovirus: HIV
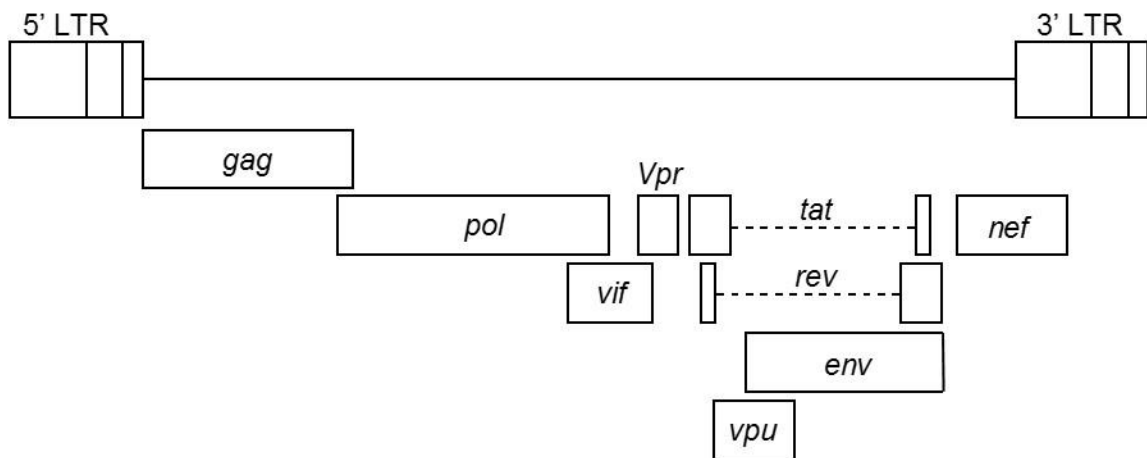
Fig. 1.1. Retroviral genome organization. A comparison of simple versus complex retroviral genome, as exemplified by MLV and HIV-1, respectively. Simple retroviruses only contain four essential genes – *gag, pro, pol and env*; whereas complex retroviruses contain additional accessory genes such as *tat, rev, vif, vpr, vpu and nef*.

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Accessory protein	Major function
Nef	MHC-I and CD4 downregulation.
Rev	Binds rev response elements (RRE) found on viral RNA, transport of unspliced or incompletely spliced RNA out of nucleus.
Tat	Activation of HIV-1 gene transcription.
Vif	Counteracts the restriction factor APOBEC3G and prevents G-to-A hypermutation.
Vpr	Cell cycle arrest; rescues Env expression in macrophages by counteracting an unknown restriction factor.
Vpu	Downregulates tetherin to increase virus release from the cell surface.

Table 1.1. Functions of HIV-1 accessory proteins. HIV-1 genome contains genes that encode different accessory proteins that ensure its survival in the host. These proteins help in immune evasion, host restriction factor downregulation and also in increasing viral gene expression. For review, refer to ([105-107]).
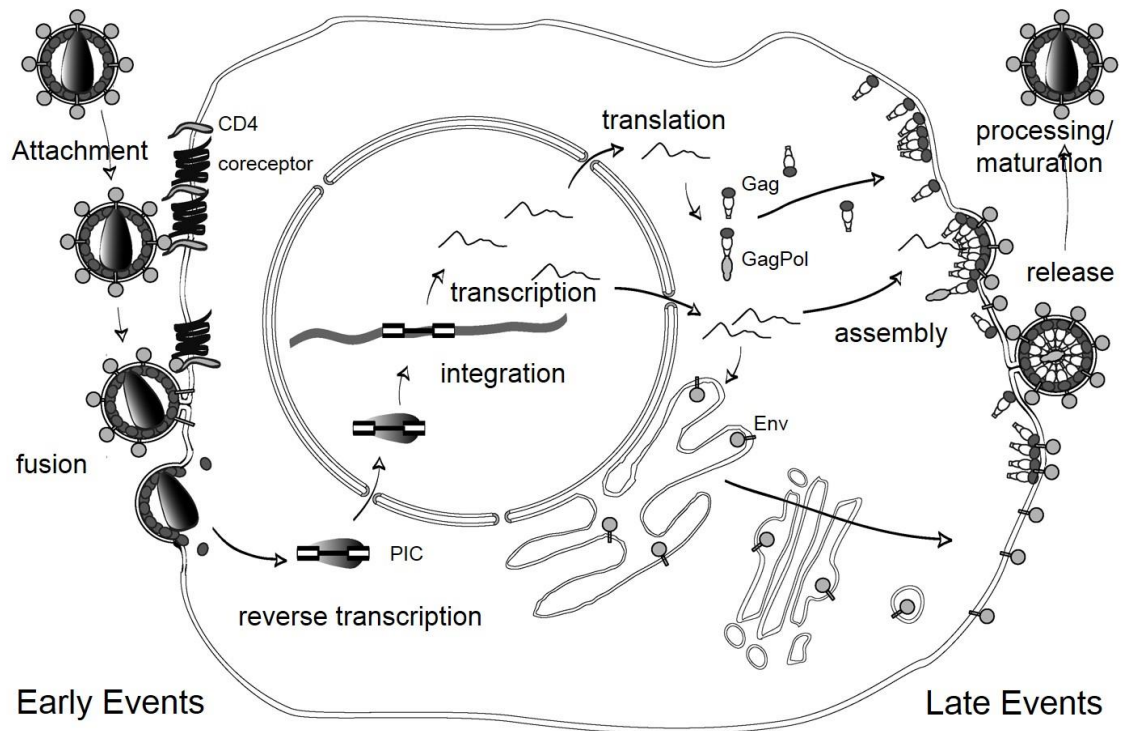
Fig. 1.2. An overview of retrovirus lifecycle. Retrovirus lifecycle, as exemplified by HIV-1 lifecycle, is broadly divided into early and late events. Early events include virus attachment, entry, membrane fusion, reverse transcription and integration. Late events of retrovirus replication include transcription and translation of viral genes, virus assembly, virus release and maturation.
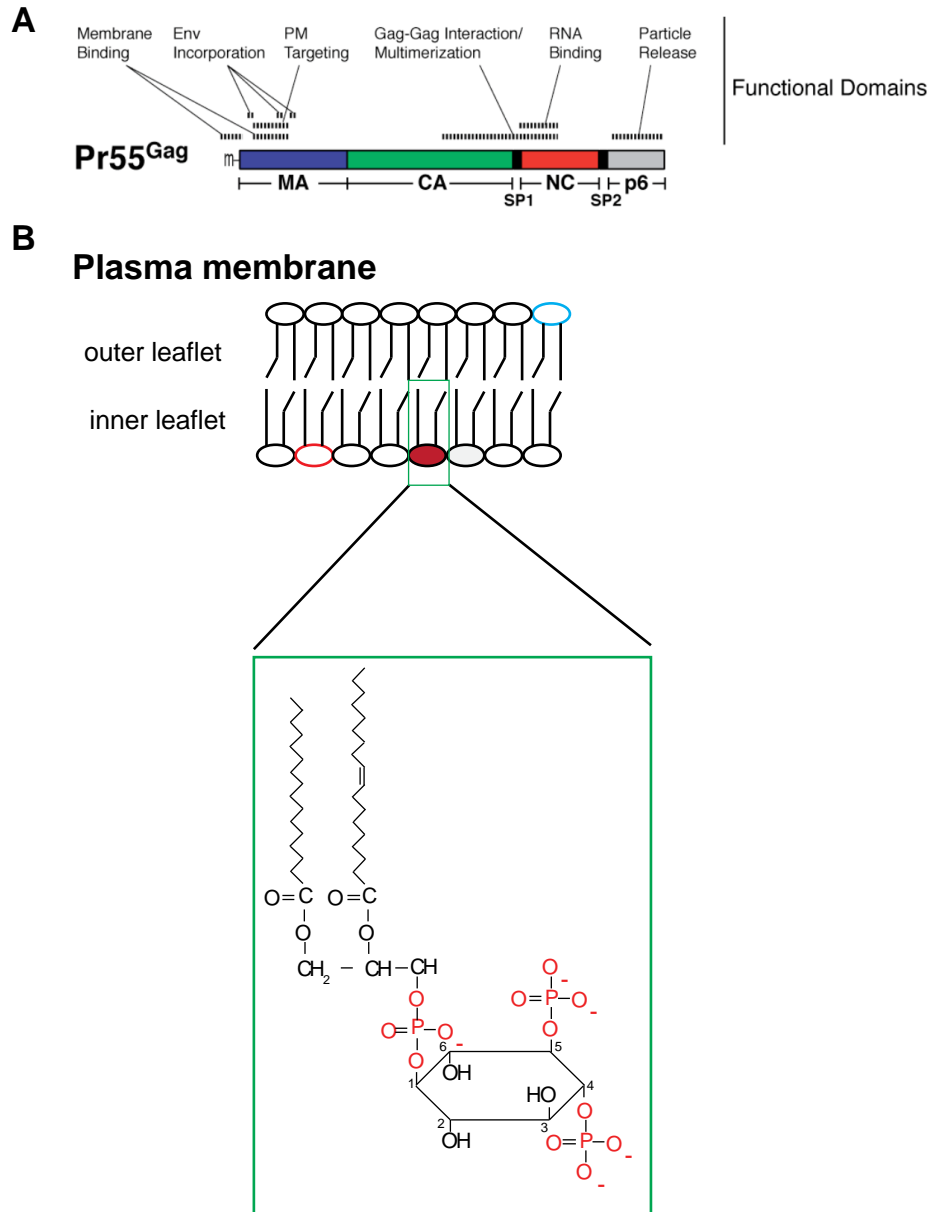
Fig.1.3. HIV-1 Gag structural and functional domains and PI(4,5)P$_2$ structure. (A) All retroviral Gag are synthesized as polyprotein containing matrix (MA), capsid (CA) and nucleocapsid (NC) structural domains. Each domain play essential roles during assembly. Retroviral Gag also contain late domain motif to help in virus release from cell surface. For HIV-1, late domain motif is found in the p6 region. Other retroviruses, such as HTLV-1 and MLV, the late domain motif is found in the MA region [for review, refer to (54)]. In addition to these structural domains, some retroviral Gag, such as HIV-1, also contain spacer peptides. (B) PI(4,5)P$_2$ is found in the inner leaflet of the plasma membrane. Its inositol ring is phosphorylated at the fourth and fifth position, making PI(4,5)P$_2$ a highly negatively charged phospholipid. Natural PI(4,5)P$_2$ contains long acyl chains (C16 to C20).
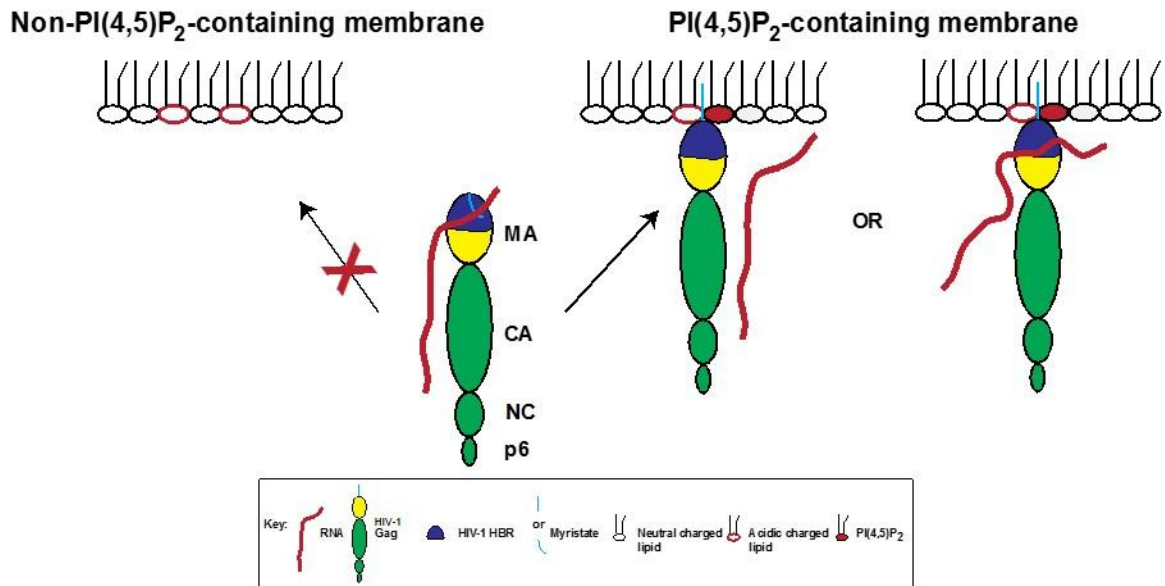
Fig. 1.4. A working model for RNA-mediated regulation of HIV-1 Gag membrane binding. The interaction between HIV-1 MA and RNA prevents HIV-1 Gag from binding to non-specific cellular membranes, i.e., membranes that do not contain $PI(4,5)P_2$. However, upon encountering $PI(4,5)P_2$-containing membrane, such as the plasma membrane, the HBR of HIV-1 MA interacts with $PI(4,5)P_2$, facilitating Gag membrane binding. It is not known at this point whether the RNA is displaced upon HIV-1 MA binding to $PI(4,5)P_2$ or remains bound to the MA domain.

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CHAPTER II

Membrane Binding and Subcellular Localization of Retroviral Gag Proteins are Differentially Regulated by MA Interactions with PI(4,5)P$_2$ and RNA

ABSTRACT

The matrix (MA) domain of HIV-1 mediates proper Gag localization and membrane binding via interaction with a plasma-membrane(PM)-specific acidic phospholipid, phosphatidylinositol-4,5-bisphosphate [PI(4,5)P$_2$]. HIV-1 MA also interacts with RNA, which prevents Gag from binding to membranes containing phosphatidylserine, a prevalent cellular acidic phospholipid. These results suggest that the MA-bound RNA promotes PM-specific localization of HIV-1 Gag by blocking non-specific interactions with cellular membranes that do not contain PI(4,5)P$_2$. To examine whether PI(4,5)P$_2$ dependence and RNA-mediated inhibition collectively determine MA phenotypes across a broad range of retroviruses and elucidate the significance of their interrelationships, we compared a panel of Gag-leucine-zipper constructs (GagLZ) containing MA of different retroviruses. We found that *in vitro* membrane binding of GagLZ via HIV-1 MA and RSV MA is both PI(4,5)P$_2$-dependent and susceptible to RNA-mediated inhibition. The PM-specific localization and virus-like particle (VLP) release of these GagLZ proteins are severely impaired by overexpression of a PI(4,5)P$_2$-depleting enzyme, polyphosphoinositide 5-phosphatase IV (5ptaseIV). In contrast, membrane binding of GagLZ containing HTLV-1, MLV, and HERV-K MA is PI(4,5)P$_2$-

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independent and not blocked by RNA. The PM localization and VLP release of these GagLZ chimeras were much less sensitive to 5ptaseIV expression. Notably, single amino acid substitutions that confer a large basic patch rendered HTLV-1 MA susceptible to the RNA-mediated block, suggesting that RNA readily blocks MA containing a large basic patch, such as HIV-1 and RSV MA. Further analyses of these MA mutants suggest a possibility that HIV-1 and RSV MA acquired $PI(4,5)P_2$ dependence to alleviate membrane binding block imposed by RNA.

INTRODUCTION

Assembly and release of retrovirus particles are mediated by the viral structural protein, Gag. Human immunodeficiency virus type-1 (HIV-1) Gag is synthesized as a precursor polyprotein, comprising four major structural domains: matrix (MA), capsid (CA), nucleocapsid (NC) and p6; and two spacer peptides: SP1 and SP2 (1-3). Each of these domains plays essential roles during assembly. MA is responsible for targeting and binding of HIV-1 Gag to the plasma membrane (PM), the site where virus assembly occurs. The N-terminal domain of CA is implicated in Gag lattice arrangement during virus particle formation, while the C-terminal domain contains CA dimer interface. Specific encapsidation of viral genomic RNA is determined by zinc finger motifs in NC, while NC binding to RNA also promotes Gag multimerization. The late domain motifs within NC and p6 recruit cellular ESCRT complexes that facilitate release of virus particles from the cell surface (4-6).

HIV-1 MA contains bipartite signals that mediate Gag binding to the PM: the N-terminal myristoyl moiety and the highly basic region (HBR), which spans residues 17 to

31 in the MA domain (7-9). The myristoyl moiety is sequestered within a hydrophobic pocket of HIV-1 MA. Structural changes caused by events such as Gag multimerization and Gag-PI(4,5)P$_2$ interactions trigger exposure of the myristoyl moiety, facilitating hydrophobic interactions between MA and lipid bilayer membranes (10-17). The HBR contributes to membrane binding via electrostatic interactions with the acidic phospholipids. Several studies based on a variety of approaches including protein footprinting, NMR and liposome binding showed that the HIV-1 MA HBR interacts with phosphatidylinositol-4,5-bisphosphate [PI(4,5)P$_2$], a phosphoinositide that is found predominantly at the cytoplasmic leaflet of the PM (14, 18-23). It has also been shown that mutations in the HBR result in alterations of HIV-1 Gag localization from the PM to either the intracellular compartments or the cytosol (21, 22, 24-28). These findings suggest that HBR also plays a role in targeting HIV-1 specifically to the PM. Notably, when cellular PI(4,5)P$_2$ is depleted by overexpression of polyphosphoinositide 5-phosphatase IV (5ptase IV), HIV-1 Gag fails to bind PM efficiently and either remains in the cytosol or localizes to intracellular compartments, resulting in a significant reduction in HIV-1 release (20, 21, 27, 29, 30). These results suggest that HIV-1 MA-PI(4,5)P$_2$ interactions are important for PM binding of HIV-1 Gag.

Using an *in vitro* liposome binding assay, we previously showed that HIV-1 Gag synthesized *in vitro* in rabbit reticulocyte lysates is unable to bind liposomes consisting of a neutral phospholipid phosphatidylcholine (PC) and an acidic phospholipid phosphatidylserine (PS) in a 2:1 ratio [PC:PS (2:1)], unless PI(4,5)P$_2$ is also present in the liposomes (21). These results suggest that bulk negative charge of liposomes is insufficient for efficient Gag-membrane interaction in the presence of mammalian cell

lysates and that Gag membrane binding under these conditions requires the presence of PI(4,5)P$_2$. In addition to PI(4,5)P$_2$, HIV-1 MA binds RNA (22, 30-41). Using the same liposome binding assay described above, we found that HIV-1 Gag can bind PC:PS (2:1) liposomes when it is first treated with RNase. These and other results suggest that the MA-RNA interaction negatively regulates HIV-1 Gag membrane binding in the absence of PI(4,5)P$_2$ by inhibiting HBR interaction with acidic lipids (22, 33, 42-44). The RNase responsiveness of Gag membrane binding is also observed for HIV-1 Gag derived from the cytosol of transfected HeLa cells, indicating that RNA present in human cells is capable of preventing Gag from binding to membrane in cells (33). Based on these results, we hypothesized that the interaction of MA HBR with RNA prevents premature or non-specific binding of HIV-1 Gag to membranes containing prevalent acidic lipids such as PS and thereby consequentially ensures its specific binding to the PM, which contains PI(4,5)P$_2$.

Basic surface patches are present not only on HIV-1 MA but on all retroviral MA domains for which structures have been determined. These surface patches are proposed or shown to interact electrostatically with phospholipid headgroups (45-49). Like HIV-1 MA, the MA domains of other retroviruses are also known to interact with RNA either in cells or *in vitro* (39, 50-53). Furthermore, in addition to HIV-1 Gag, Gag proteins of many retroviruses including HIV-2, equine infectious anemia virus (EIAV), murine leukemia virus (MLV), Rous sarcoma virus (RSV), and Mason-Pfizer monkey virus were shown to either interact with PI(4,5)P$_2$ via MA or produce virions in a manner that is susceptible to 5ptaseIV overexpression, suggesting that PI(4,5)P$_2$ is involved in efficient assembly and release of these viruses (20, 29, 54-58). However, the degree of

PI(4,5)P$_2$ dependence may vary among different retroviruses. For instance, a study showed that even though EIAV MA can bind both PI(3,5)P$_2$ and PI(4,5)P$_2$, EIAV assembly was sensitive to an inhibitor of PI(3,5)P$_2$ synthesis, but not to 5ptaseIV overexpression (59). As for RSV, while PI(4,5)P$_2$ was observed to promote Gag binding to liposome membranes (29), it remains unclear to what extent PI(4,5)P$_2$ plays a role in PM localization of Gag and virus assembly in cells, since previous studies yielded inconsistent results on RSV Gag sensitivity to 5ptaseIV overexpression (29, 56). In this regard, a comparative study of various retroviruses in the same experimental systems should allow us to determine the spectrum of PI(4,5)P$_2$ dependence for membrane binding, subcellular localization, and virus particle production.

Using the *in vitro* liposome binding assay, we previously found that, unlike HIV-1 Gag, HTLV-1 Gag does not require PI(4,5)P$_2$ for efficient membrane binding to PC:PS (2:1) liposomes (30). We also observed that RNase treatment of HTLV-1 Gag does not increase its binding to PC:PS (2:1) liposomes, unlike HIV-1 Gag, which is highly responsive to RNase treatment. These results suggest that HTLV-1 Gag membrane binding is not inhibited by RNA, unlike that of HIV-1 Gag. However, in that study, the presence of viral RNA and the downstream NC domain complicated the interpretation of the results as to the intrinsic properties of HTLV-1 MA relative to those of HIV-1 MA**.** As such, several major questions including the following remain to be answered. (i) Is the RNA susceptibility (or lack thereof) primarily determined by MA? If so, which feature of MA determines its susceptibility to RNA? (ii) What is the relationship between PI(4,5)P$_2$ interaction and RNA binding of HIV-1 MA? Are they inseparable or regulated differently? (iii) What is the significance of such relationships in assembly? For example,

was RNA recruited to enhance the specificity for PI(4,5)P$_2$ or did RNA-mediated block necessitate PI(4,5)P$_2$ interaction? Lastly, (iv) can PI(4,5)P$_2$ dependence and susceptibility to RNA (or lack thereof) serve as a general principle that can explain membrane binding and subcellular localization phenotypes of a broad range of retroviral MA domains?

In this study, in order to broadly analyze the specific roles of MA-PI(4,5)P$_2$ and MA-RNA interactions in membrane binding, we analyzed chimeric HIV-1 Gag derivatives where HIV-1 MA is replaced with MA domains of other retroviruses, each representing a retroviral genus, i.e., HTLV-1 (deltaretrovirus), MLV (gammaretrovirus), RSV (alpharetrovirus) and human endogenous retrovirus-K (HERV-K) (betaretrovirus). In this analyses, we focused on retroviruses that follow Type C assembly pathway, in which the most part of virus particle assembly process takes place at membrane (e.g., the PM) (60). To eliminate the effect of NC-RNA binding while allowing Gag multimerization, we replaced the HIV-1 NC, the major RNA-binding domain, with a leucine zipper motif in these constructs (GagLZ) (61). We examined their localization and VLP release efficiencies in cells as well as their membrane binding properties using both cell-based and *in vitro* methods. Our data demonstrate that PI(4,5)P$_2$ dependence and RNA-mediated inhibition are highly correlated properties among the five different retroviral MA domains. We found that there are two distinct groups among the retroviral MA domains that differ in their membrane binding phenotypes: those that are PI(4,5)P$_2$-dependent and RNase-responsive (MA of HIV-1 and RSV) and those that are neither PI(4,5)P$_2$-dependent nor RNase-responsive (MA of HTLV-1, MLV and HERV-K). Using structure-guided mutagenesis approach, we also elucidated an MA determinant for RNA susceptibility. We found that, strikingly, single point mutations that increase the size of

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an MA basic patch convert HTLV-1 MA to MA that is sensitive to RNA-mediated inhibition of membrane binding. However, unlike for HIV-1 MA and RSV MA, which also have a large basic patch, $PI(4,5)P_2$ failed to reverse the RNA-mediated inhibition for these HTLV-1 MA mutants, suggesting that $PI(4,5)P_2$ dependence and RNA susceptibility are genetically separable. These results support the model that that $PI(4,5)P_2$ dependence is an adaptation of retroviruses that contain a large basic patch on the MA surface, such as HIV-1 and RSV, to overcome the strong membrane binding block imposed by RNA. Other retroviruses such as HTLV-1 and MLV may have adopted smaller basic patches on their MA surface to avoid RNA-mediated membrane binding inhibition.

MATERIALS AND METHODS

Plasmids. pCMV-RRE-HIV-1 GagLZ was constructed from pCMV-RRE-HIV1.5-Gag which was described previously (30). To focus specifically on the role of MA-RNA interaction in Gag localization, the NC region of HIV-1 Gag, which also contain an RNA-binding domain, was replaced with leucine zipper dimerization motif (LZ) from yeast GCN4 activator (a kind gift from H. Gottlinger) (61). This construct, called HIV-1 GagLZ, is able to multimerize and form virus-like particles similar to wild-type HIV-1 Gag. pCMV-RRE-HTLV-1 MA GagLZ was constructed by replacing HIV-1 MA of pCMV-RRE-HIV-1 GagLZ with HTLV-1 MA spanning residues 1 to 116. pCMV-RRE-RSV MA GagLZ contains RSV MA-p2-p10 (RSV Gag residues 1-239) of the RHR construct (a kind gift from V. Vogt) (78) in place of HIV-1 MA. pCMV-RRE-MLV MA GagLZ was constructed by substituting HIV-1 MA with MLV MA (residues 1-131) from

pNCA (a kind gift from A. Telesnitsky) (79). Finally, pCMV-RRE-HERV-K MA GagLZ

contains HERV-K MA-SP1 (residues 1-148) of pCRVI/HERV-K/GagPro (a kind gift

from P. Bieniasz) (80, 81) in place of HIV-1 MA. Acidic-to-basic point mutants of

HTLV-1 MA GagLZ (D42K, E55K, D42K/E55K) were constructed using standard

molecular cloning techniques.

     pCMV-RRE-HIV-1 Gag-eCFP and pCMV-RRE-HTLV-1 Gag-eCFP were

described previously (30). pCMV-RRE-RSV Gag-eCFP, pCMV-RRE-MLV Gag-eCFP,

and pCMV-RRE-HERV-K Gag-eCFP were constructed by replacing the HIV-1 *gag*

reading frame in pCMV-RRE-HIV-1 Gag-eCFP with that of RSV *gag* in an expression

plasmid (78), MLV *gag* in pNCA (79) and HERV-K *gag* in pCRVI/HERV-K/GagPro

(80, 81), respectively, using standard molecular cloning techniques.

GagLZ proteins were C-terminally fused to a linker (Ala-Gly-Ser-Pro-Ala) and either an

eCFP or the Venus variant of YFP to yield fluorescently-tagged chimeric GagLZ

constructs. The first methionine residue of eCFP and YFP were deleted to prevent the

fluorescent protein from being translated by internal ribosomal entry. The 5ptaseIV

expression plasmid, pcDNA4TO/Myc5ptaseIV, and its derivative that lacks a functional

phosphatase domain (pCDNA4TO/5ptaseIV Δ1) were previously described (21, 27, 82).

pRS-HRevX plasmid (a gift from D. Derse) is derived from the pKS-Bluescript vector

encoding the HIV-1 *rev* gene driven by an RSV promoter. pCMV-Vphu, which encodes

the codon-optimized HIV-1 vpu gene, was kindly provided by K. Strebel (83). The Gag

expression plasmids used for *in vitro* transcription-translation-coupled reactions, pGEM-

HIV-1 GagLZ, pGEM-HTLV-1 MA GagLZ, pGEM-RSV MA GagLZ, pGEM-MLV MA

GagLZ, and pGEM-HERV-K MA GagLZ were constructed using pGEM-1 (Promega) as

a vector plasmid and Gag-encoding fragments derived from pCMV-RRE-HIV-1 GagLZ, pCMV-RRE-HTLV-1 MA GagLZ, pCMV-RRE-RSV MA GagLZ, pCMV-RRE-MLV MA GagLZ and pCMV-RRE-HERV-K MA GagLZ, respectively, using standard molecular cloning techniques. The resulting plasmids encode the Kozak sequence, followed by HIV-1 GagLZ, HTLV-1 MA GagLZ, RSV MA GagLZ, MLV MA GagLZ and HERV-K MA GagLZ.

Cells and transfection. HeLa cells were cultured as described previously (21, 22, 25, 84). For microscopy, 4.2×10^4 cells were plated into each well of eight-well chamber slides (Lab-Tek; Nalge Nunc International), grown for 24 h, and transfected with DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For VLP release, 5.6×10^5 cells were plated into each well of six-well plates (Corning), grown overnight, and transfected as described above. For microscopy, Gag expression plasmids were transfected along with pRS-HRevX. For VLP release assays, Gag expression plasmids were transfected along with pRS-HRevX and pCMV-Vphu. Co-expression of pCMV-Vphu does not lead to any change in chimeric GagLZ localization (unpublished data).

VLP release assay and immunoblotting. VLP release assay using immunoblotting was previously described (30). In some experiments, VLP release was examined using metabolic labeling and immunoprecipitation as previously described with modifications (21, 27, 85). Briefly, HeLa cells were transfected with CMV-driven plasmid encoding a chimeric GagLZ construct, along with pRS-HRevx and pCMV-Vphu. Sixteen hours post-transfection, culture medium was changed to RPMI-1640 lacking both methionine (Met) and cysteine (Cys) and supplemented with 2% FBS [RPMI-2 (−Met/−Cys)] and

incubated for 30 min. Subsequently, these cells were metabolically labeled with [^{35}S] Met/Cys (Perkin-Elmer) in fresh RPMI-2 (−Met/−Cys) for 4 h. Cell and virion lysates were prepared and subjected to immunoprecipitation with HIV-Ig antiserum (NIH AIDS Research and Preference Reagent Program). The virus release efficiency was calculated as the amount of virion-associated Gag as a fraction of the total amount of Gag synthesized during the labeling period.

Immunostaining and fluorescence microscopy. Fixation and immunostaining of transfected HeLa cells expressing Gag or Gag-fluorescent protein fusions were performed as described previously (27, 30). The presence of 5ptaseIV in cells was visualized by immunostaining with mouse anti-Myc antibody (clone 9E10; Santa Cruz Biotechnologies). For visualization of the plasma membrane, cells were incubated with Alexa Fluor 594-conjugated concanavalin A (ConA; Invitrogen) for 2 min after fixation. Cells were then imaged using Leica confocal fluorescence microscope. Pearson's correlation coefficients of ConA and Gag-YFP were calculated using the Coloc2 plugin in ImageJ. Twenty to fifty cells were analyzed for each condition. For determining the distribution of Gag localization patterns, images of about 20 fields were recorded by using an Olympus IX70 inverted fluorescence microscope at 100x magnification, and a range of 80 to 150 cells that were positive for both Gag and 5ptaseIV (either full-length [FL] or the Δ1 derivative) were evaluated for the Gag localization pattern in each condition in a blind manner. Classification of Gag localization patterns was validated using line profiles generated using ImageJ.

Liposome-binding assay. Preparation of liposomes, *in vitro* Gag translation, and sucrose gradient flotation centrifugation were performed as described previously (21, 30, 33). The RNase treatment experiments were also performed as previously described (30, 33).

Electrostatic potential calculations. The calculations of electrostatic potential of previously solved retroviral MA domains were performed using the DelPhi program (86). The electrostatic potentials were then mapped to molecular surface and visualized using the Chimera program (87). The retroviral MA structures used for comparison are as follow: HIV-1 MA (PDB: 2HMX), RSV MA (PDB: 1A6S), HTLV-2 (PDB: 1JVR) and MLV MA (PDB: 1MN8) (45, 63-65). The HTLV-1 MA structure was predicted based on the NMR structure of HTLV-2 MA (PDB: 1JVR) (45) using the SWISS-MODEL server (66, 67). The molecular surface of HIV-1 MA and HTLV-1 MA was visualized using Protean 3D.

Statistical analysis. Two-tailed Student t-tests were performed using Microsoft Excel. The paired t test was used for comparing data obtained from the same set of experiments. P values of <0.05 were considered statistically significant.

RESULTS

MA domains of different retroviruses determine subcellular localization patterns of Gag chimeras

As a first step to compare the properties of different retroviral MA domains, we tested the subcellular localization of eCFP-tagged full-length Gag proteins of different retroviruses in transfected HeLa cells. The cell surface was stained using Concanavalin A (ConA) labeled with AlexaFluor 594 to better distinguish Gag at the PM from those at

intracellular sites. As evident in the intensity profiles of ConA and Gag-eCFP, cells

expressing eCFP-tagged HIV-1 Gag, RSV Gag or HERV-K Gag displayed highest Gag-

eCFP signals at cell periphery (Fig. 2.1), indicating that these Gag-eCFP proteins localize

predominantly to the PM. In contrast, cells expressing eCFP-tagged HTLV-1 Gag, MLV

Gag and EIAV Gag showed eCFP intensity peaks at both cell periphery and cytoplasmic

regions, indicating that these Gag-eCFP proteins localize to both PM and intracellular

compartments (Fig. 2.1). To test whether the differential localization patterns can be

attributed primarily to MA domains and not downstream sequences, we constructed a

panel of Gag chimera by replacing the MA domain of HIV-1 Gag with that of RSV,

HTLV-1, MLV, HERV-K or EIAV (Fig. 2.2A). In these experiments, to eliminate the

effect of NC-RNA binding so as to focus on the direct effect of the MA-RNA interaction,

we replaced NC with a leucine zipper motif (GagLZ) in these constructs (Fig. 2.2A) (61).

HIV-1 Gag chimeras containing HIV-1 CA and this LZ sequence were previously shown

to support wild-type-level VLP formation in the absence of NC (61). Like their full-

length counterparts, eCFP-tagged HIV-1 GagLZ, RSV MA GagLZ and HERV-K MA

GagLZ localized predominantly at the PM in HeLa cells (Fig. 2.1). In contrast, eCFP-

tagged HTLV-1 MA GagLZ and MLV MA GagLZ localized to both the PM and

intracellular compartments, as observed with their full-length versions. Altogether, these

results suggest that the MA domains of HIV-1, HTLV-1, RSV, MLV, and HERV-K

determine the subcellular localization of Gag regardless of the downstream sequences.

Unlike other chimeric GagLZ constructs tested, however, EIAV MA GagLZ and its full-

length counterpart, EIAV Gag, showed different localization patterns. We found that

EIAV MA GagLZ localized mainly to PM, unlike wild type EIAV Gag, which localized

to both the PM and intracellular compartments (Fig. 2.1) (59). This suggests that the MA domain of EIAV Gag is not the sole determinant of Gag localization and that the downstream sequences of EIAV MA play a role as well. For this reason, we chose not to pursue EIAV MA GagLZ in subsequent comparative analyses of retroviral MA domains.

Differential effects of PI(4,5)P$_2$ depletion on subcellular localization of GagLZ chimeras in HeLa cells

We and others previously reported that 5ptaseIV overexpression abolishes localization of HIV-1 Gag to the PM and instead increases the hazy cytosolic localization in HeLa cells (21, 27, 29, 30). To assess the effect of PI(4,5)P$_2$ depletion on subcellular localization of chimeric GagLZ proteins described above, HeLa cells were transfected with a plasmid encoding YFP-tagged chimeric GagLZ constructs (GagLZ-YFP), along with a plasmid encoding either Myc-tagged full-length (FL) 5ptaseIV or its Δ1 derivative, and were examined by fluorescence microscopy (Fig. 2.2B and 2.4). The 5ptaseIV Δ1 derivative lacks the functional phosphatase domain and therefore does not deplete cellular PI(4,5)P$_2$ (21, 27, 29, 30). We used YFP-tagged chimeric GagLZ constructs in this experiment because they display higher signal-to-background ratios than eCFP-tagged ones. Substitution of FP in these chimeric GagLZ constructs did not alter their subcellular localizations (compare Fig. 2.4, 5ptaseIV Δ1, with Fig. 2.1). To measure the effect of 5ptaseIV expression on localization of the chimeric GagLZ constructs quantitatively, we analyzed 80-150 cells expressing both Myc-tagged and YFP-tagged proteins per condition and categorized them in a blind manner into 3 different groups based on GagLZ-YFP localization patterns: (i) predominant localization at the PM (black bar), (ii) localization to both PM and intracellular compartments (white bar), and (iii)

hazy cytosolic localization (grey bar) (Fig. 2.4A). These three localization patterns were also validated via comparison of the GagLZ-YFP signal intensity profiles with the signal intensity profiles of ConA-AlexaFluor 594, the PM marker (Fig. 2.4B). In 5ptaseIV Δ1-expressing cells, a majority of HIV-1 GagLZ, RSV MA GagLZ, and HERV-K MA GagLZ showed punctate PM localizing pattern. However, when 5ptaseIV FL was expressed, most cells expressing HIV-1 GagLZ and RSV MA GagLZ displayed hazy cytosolic signals, indicating a defect in Gag membrane binding. In contrast, HERV-K MA GagLZ still localized to the PM with a modest increase in the population of cells showing dual localization to the PM and intracellular compartments in 5ptaseIV FL-expressing cells. On the other hand, 5ptaseIV overexpression did not drastically alter localization of HTLV-1 MA GagLZ and MLV MA GagLZ; most cells showed GagLZ localized to both the PM and intracellular compartments regardless of whether they expressed 5ptaseIV Δ1 or FL. While localization of these GagLZ proteins to intracellular compartments appeared to be increased in some of the cells expressing 5ptaseIV FL, PM localization was still observed in these cells.

In addition to the analysis of cell populations described above, we also sought to measure quantitatively the effect of 5ptaseIV expression on the chimeric GagLZ localization on single cell bases. To this end, we acquire images using confocal microscopy and calculated the Pearson's correlation coefficient (PCC) between YFP-tagged GagLZ constructs and ConA- Alexa Fluor 594. Confocal imaging (Fig. 2.2B) showed qualitatively same localization patterns for GagLZ chimeras as observed by epifluorescence microscopy (Fig. 2.4) but eliminated out-of-focus signals. In the PCC analysis (Fig. 2.3A), we used the distributions of wild type HIV-1 Gag-YFP, a chimeric

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HIV-1 Gag-YFP which contains HTLV-1 MA (HTMA Gag-YFP) (30), and membrane-binding-defective HIV-1 mutant (1GA Gag-YFP) as controls (Fig. 2.3A). These Gag constructs were previously determined to display predominantly PM, PM+intracellular and hazy cytosolic localizations, respectively. The PCC of ConA with HIV-1 Gag-YFP was found to be above 0.6; ConA with HTMA Gag-YFP was around 0.5; and ConA with 1GA Gag-YFP was below 0.1 (Fig. 2.3B). In this analysis, we found that in 5ptaseIV Δ1-expressing cells, HIV-1 GagLZ and RSV MA GagLZ showed high PCC values with ConA (above 0.6) (Fig. 2.3B). When 5ptaseIV FL was expressed, HIV-1 GagLZ and RSV MA GagLZ showed great reductions in PCC with ConA (below or near 0). In contrast, only modest (while statistically significant) changes were observed in PCC values for HTLV-1 MA GagLZ, MLV MA GagLZ, or HERV-K MA GagLZ with ConA between 5ptaseIV Δ1- and FL-expressing cells. These data (Fig. 2.3B) quantitatively support our observation shown in Fig. 2.4, which revealed that GagLZ chimeras containing HTLV-1 MA, MLV MA, and HERV-K MA are able to localize at the PM regardless of the 5ptaseIV expression.

Altogether, these results suggest that while $PI(4,5)P_2$ is required for localization of HIV-1 GagLZ and RSV MA GagLZ to the PM, it is not essential for PM localization of chimeric GagLZ constructs containing HERV-K MA, HTLV-1 MA or MLV MA in HeLa cells.

Virus-like particle production of HIV-1 GagLZ and RSV MA GagLZ, but not that of HTLV-1 MA GagLZ, MLV MA GagLZ and HERVK MA GagLZ, is severely inhibited upon cellular $PI(4,5)P_2$ depletion in HeLa cells

In parallel with the microscopy analysis, we also sought to determine the effect of PI(4,5)P$_2$ depletion on the virus-like particle (VLP) release of the GagLZ chimeras with different retroviral MA domains. To this end, we examined the effect of 5ptaseIV expression on VLP production of the untagged GagLZ constructs. We transfected HeLa cells with a plasmid encoding one of the GagLZ chimeras, along with expression plasmids for Rev, humanized Vpu (Vphu), and either 5ptaseIV FL or the Δ1 derivative. Sixteen hours post-transfection, cell and viral lysates were collected. GagLZ proteins in lysates were then detected by immunoblotting using HIV-immunoglobulin, and the virus release efficiency was calculated. Consistent with the diminished PM localization, we observed that VLP release efficiency of HIV-1 GagLZ and RSV MA GagLZ was greatly reduced (~3-10 fold) upon coexpression of 5ptaseIV FL relative to when co-expressed with 5ptaseIV Δ1 (Fig. 2.5). In contrast, the VLP production of HTLV-1 MA GagLZ, MLV MA GagLZ and HERV-K MA GagLZ was only modestly reduced (less than 2 fold) upon 5ptaseIV FL co-expression, consistent with the PM localization of these GagLZ proteins, which was not visibly altered upon 5ptaseIV FL expression (Fig. 2.5). We noticed that the expression levels of RSV MA GagLZ in cells (and hence in VLPs) were much higher than other GagLZ tested (such as MLV MA GagLZ), potentially affecting quantification of RSV MA GagLZ (Fig. 2.5A). However, when reduced amounts of lysates were loaded, VLP release of RSV MA GagLZ still showed significant sensitivity to 5ptaseIV FL, whereas MLV MA GagLZ VLP release did not (Fig. 2.6A, 2.6B). Consistent with the immunoblotting experiments described above, analyses of VLP release efficiency using metabolic labeling followed by radioimmunoprecipitation showed that VLP release efficiency of RSV MA GagLZ was reduced 5 fold in cells

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expressing 5ptaseIV FL relative to cells expressing 5ptaseIV $\Delta 1$, whereas that of HTLV-1 MA GagLZ was reduced only 2 fold (unpublished data). Of note, the lower molecular weight band of RSV MA GagLZ, which has been also observed with its full-length counterpart (62), is likely due to internal initiation from methionine residue 139. Deletion of this site led to the elimination of this band but did not affect sensitivity of VLP release to 5ptaseIV FL expression (Fig. 2.6C). Overall, these results indicate that cellular PI(4,5)P$_2$ is essential for efficient VLP production in HeLa cells for GagLZ chimeras containing HIV-1 MA or RSV MA but not for those containing HTLV-1, MLV, or HERV-K MA.

MA domains of HIV-1 and RSV, but not those of HTLV-1, MLV and HERV-K, mediate membrane binding of GagLZ in a PI(4,5)P$_2$-dependent manner

The results described above demonstrate that there are two groups of retroviral MA domains that differ from each other in terms of PI(4,5)P$_2$ dependence: while PI(4,5)P$_2$ is essential for the PM localization of GagLZ and VLP production mediated by some retroviral MA domains (HIV-1 and RSV), it is dispensable for those facilitated by other retroviral MA domains (HTLV-1, MLV and HERV-K). To further examine the role of PI(4,5)P$_2$ in mediating membrane binding via retroviral MA domains, we performed an *in vitro* liposome binding assay. We observed that similar to full-length HIV-1 Gag, HIV-1 GagLZ bound poorly to control liposomes containing PC and PS in a 2:1 ratio [hereafter PC:PS (2:1) liposomes], but its binding efficiency increased significantly when 7.25 mol% of PI(4,5)P$_2$ was included in PC:PS (2:1) liposomes (Fig. 2.7). Similarly, RSV MA GagLZ bound poorly to PC:PS (2:1) liposomes, but inclusion of PI(4,5)P$_2$ significantly enhanced its binding (Fig. 2.7). In contrast, HTLV-1 MA GagLZ bound

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readily to PC:PS (2:1) liposomes, and the presence of $PI(4,5)P_2$ did not significantly

increase its binding efficiency (Fig. 2.7), as was the case with full-length HTLV-1 Gag

(30). Interestingly, GagLZ chimeras containing MLV MA or HERV-K MA also bound

readily to liposomes in the absence of $PI(4,5)P_2$ (Fig. 2.7). Overall, these results indicate

that membrane binding mediated by HTLV-1 MA, MLV MA and HERV-K MA does not

require the presence of $PI(4,5)P_2$, whereas $PI(4,5)P_2$ is important for efficient membrane

binding of HIV-1 MA and RSV MA.

PC:PS (2:1) liposome binding of GagLZ chimera via HIV-1 MA or RSV MA, but not via HTLV-1 MA, MLV MA or HERV-K MA, is susceptible to RNA-mediated block

We recently showed that binding of full-length HTLV-1 Gag to PC:PS (2:1)

liposomes is not inhibited by RNA, unlike that of full-length HIV-1 Gag (30). To

determine whether HTLV-1 MA is responsible for this lack of sensitivity to RNA-

mediated inhibition and whether other retroviral MA domains are susceptible to such

RNA-mediated inhibition as well, we compared GagLZ chimeras for RNase

responsiveness in their binding to PC:PS (2:1) liposomes. We observed that, like full-

length HIV-1 Gag, HIV-1 GagLZ is responsive to RNase treatment, indicating that MA-

bound RNA rather than NC-bound RNA is likely responsible for the membrane binding

block to PC:PS (2:1) liposomes (Fig. 2.8). Similar to HIV-1 GagLZ, binding of RSV

MA GagLZ to PC:PS (2:1) liposomes was poor, and its binding efficiency increased

greatly upon RNase treatment (Fig. 2.8). In contrast, chimeric GagLZ containing either

HTLV-1 MA, MLV MA, or HERV-K MA, which bound to PC:PS (2:1) liposomes

efficiently in the absence of $PI(4,5)P_2$, did not respond to RNase treatment (Fig. 2.8).

These results indicate that while RNA suppresses membrane binding via HIV-1 MA and RSV MA, the membrane binding mediated by HTLV-1 MA, MLV MA, and HERV-K MA are insensitive to RNA-mediated suppression.

Altogether, we observed a striking correlation between $PI(4,5)P_2$ dependence and susceptibility to RNA-mediated block of membrane binding across MA domains of different retroviruses. A retroviral MA that requires $PI(4,5)P_2$ for efficient membrane binding is responsive to RNase treatment. Conversely, if a retroviral MA binds membrane in a $PI(4,5)P_2$-independent manner, its membrane binding does not change upon RNase treatment. This correlation also extends to the GagLZ behaviors in cells. While PM localization and VLP release efficiency of $PI(4,5)P_2$-dependent, RNase-responsive GagLZ (containing HIV-1 MA or RSV MA) are highly sensitive to 5ptaseIV overexpression, the $PI(4,5)P_2$-independent and RNase-non-responsive GagLZ (containing HTLV-1 MA, MLV MA or HERV-K MA) are minimally sensitive to 5ptaseIV overexpression.

RNA serves as an inhibitor for the membrane binding of retroviral MA with a large basic surface patch

Our results thus far suggest that there is a correlation between $PI(4,5)P_2$ dependence and sensitivity to RNA-mediated inhibition in the membrane binding of retroviral MA domains. To examine whether these properties could be attributed to a feature in the retroviral MA structures, we compared the basic patch distribution on surfaces of previously solved retroviral MA structures, namely, HIV-1 MA, RSV MA, MLV MA and HTLV-2 MA (63-65), as done previously (49) (Fig. 2.9). The comparison of electrostatic potential maps suggests that both HIV-1 MA and RSV MA contain a

large basic patch on their surfaces, whereas HTLV-2 MA and MLV MA contain several smaller basic patches. We also modeled the structure of HTLV-1 MA based on the HTLV-2 MA structure (PDB: 1JVR) (45) using SWISS-MODEL, a protein structure homology-modeling server (66, 67). When we compared distribution of basic amino acids between the predicted model of HTLV-1 MA and an NMR structure of HIV-1 MA (2HMX), we found that HIV-1 MA contains a larger basic surface patch than those found on HTLV-1 MA (Fig. 2.10). To further examine whether the size of a surface basic patch on retroviral MA plays a role in regulating membrane binding, we introduced acidic-to-basic substitutions for two amino acid residues close to a small basic patch (D42 and E55) in HTLV-1 MA. These changes were made in the HTLV-1 MA GagLZ context and tested for their effects on binding to PC:PS (2:1) liposomes with or without RNase treatment. Despite the increased positive charge, which could enhance electrostatic interaction with negatively charged liposomes, we found that all mutants show reduced binding to PC:PS (2:1) liposomes relative to wild-type HTLV-1 MA GagLZ (Fig. 2.11). While the E55K mutation reduced liposome binding only slightly, the reduction in liposome binding was prominent with mutants with the D42K change. Upon RNase treatment, however, all mutants bound to PC:PS (2:1) liposomes as efficiently as wild-type HTLV-1 MA GagLZ (Fig. 2.11). These results indicate that the mutant MA domains retain the ability to bind membrane as revealed by RNase treatment and hence are unlikely to be grossly misfolded. More importantly, these results demonstrate that single or double amino acid substitutions, which increase the size of a basic patch, can readily convert an otherwise RNA-insensitive MA to one that is susceptible to RNA-mediated inhibition. Altogether, these results suggest that the size of MA basic patches is a

determinant for susceptibility of retroviral-MA-mediated membrane binding to the RNA-mediated inhibition.

PI(4,5)P$_2$ fails to facilitate membrane binding of HTLV-1 MA GagLZ containing acidic-to-basic point mutations

Unstructured polybasic peptides can display a specificity for PI(4,5)P$_2$ over PS due to the higher charge density (68). Therefore, we further tested whether increasing the size of basic surface patch of HTLV-1 MA GagLZ also increases its PI(4,5)P$_2$ dependence in membrane binding *in vitro.* We observed that, unlike HIV-1 GagLZ, which significantly increased liposome binding in the presence of PI(4,5)P$_2$, the HTLV-1 MA GagLZ mutants did not show a significant enhancement of membrane binding in a PI(4,5)P$_2$-dependent manner (Fig. 2.11). This was especially clear with D42K and D42K/E55K, which failed to bind efficiently to both non-PI(4,5)P$_2$-containing and PI(4,5)P$_2$-containing liposomes (Fig. 2.11). We further tested by microscopy whether such membrane binding defect is reflected in the localization of HTLV-1 MA GagLZ mutants in cells. While YFP-tagged wild-type HTLV-1 MA GagLZ displayed PM localization in addition to intracellular localization, the D42K/E55K mutant showed no PM signal in HeLa cells, indicating that this mutant fails to bind the PM even in the presence of PI(4,5)P$_2$ (Fig. 2.12). Consistent with this observation, the PCC analysis revealed a stark difference in the extent of colocalization with ConA between WT HTLV-1 GagLZ (PCC > 0.3) and the mutant (PCC < - 0.1) (Fig 2.12). Overall, our data suggest that an increase in size of a basic surface patch of a retroviral MA results in a stronger block by RNA in membrane binding, but such change alone is insufficient to confer PI(4,5)P$_2$-dependent membrane binding ability to the retroviral MA.

DISCUSSION

In this work, we have revealed a clear correlation between $PI(4,5)P_2$ dependence and RNA-mediated inhibition of the membrane binding mediated by various retroviral MA domains. Using chimeric GagLZ constructs that differ only in MA domains allowed us to compare intrinsic properties of different MA domains without the potentially differential effects of the downstream regions, while allowing the Gag chimeras to multimerize and form VLPs (61, 69, 70). Using this approach, we demonstrated that GagLZ chimeras that are $PI(4,5)P_2$-dependent (those containing HIV-1 MA and RSV MA) in their membrane binding are susceptible to RNA-mediated inhibition. In contrast, GagLZ chimeras that do not require $PI(4,5)P_2$ for efficient membrane binding (those containing HTLV-1 MA, MLV MA and HERV-K MA) are not inhibited by RNA. Since the chimeric GagLZ constructs displayed subcellular localization patterns that were indistinguishable from their full-length counterparts, membrane binding properties of the different retroviral MA in the GagLZ backbone examined in this study are likely to reflect those in the native contexts. Consistent with our results, a recent fluorescence fluctuation spectroscopy study showed that unlike HIV-1 MA, which is known to be inefficient in membrane binding, HTLV-1 MA can readily bind membrane in cells (71). It should be noted that our assays are designed to analyze $PI(4,5)P_2$ dependence of Gag proteins in the presence of RNA and mammalian cell components. Therefore, the lack of observed $PI(4,5)P_2$ dependence for GagLZ membrane binding via MLV MA does not necessarily contradict with previous analyses of the interaction between $PI(4,5)P_2$ and purified unmyristylated MLV MA, which were focused on the affinity between the purified components (55).

A comparison of the electrostatic potential of previously solved retroviral MA structures showed that HIV-1 MA and RSV MA contain a large basic surface patch compared to HTLV-2 MA and MLV MA. Furthermore, point mutations in HTLV-1 MA, which increase the size of a small basic patch on the MA surface, rendered the protein defective in binding to PC:PS (2:1) liposomes and RNase-responsive, the membrane binding phenotypes reminiscent of those of HIV-1 GagLZ and RSV MA GagLZ. Conversely, we previously observed that multiple single point mutations that reduce the size of the basic surface patch of HIV-1 MA enhanced binding of HIV-1 MA mutants to PC:PS (2:1) liposomes (22). The susceptibility of Gag membrane binding to RNA-mediated inhibition does not appear to be a mere consequence of the increased total charge of MA. The net charges of HIV-1 MA and HTLV-1 MA (residues 1-116, which are present in our GagLZ chimeras) are similar (+4.16 and +4.39, respectively, at pH7) even though RNA suppresses membrane binding of only the former but not the latter, highlighting the importance of how basic residues are distributed over the MA surface. Altogether, these results suggest that an increase in the size of a basic surface patch on retroviral MA results in an increase in stable RNA binding, which in turn imposes a stronger block on retroviral MA membrane binding.

Interestingly, HTLV-1 MA GagLZ mutants (D42K and D42K/E55K), which have larger basic patches and are susceptible to RNA-mediated inhibition, were unable to bind efficiently to PI(4,5)P$_2$-containing liposomes unlike HIV-1 MA GagLZ and RSV MA GagLZ. The inability to bind PI(4,5)P$_2$ and the susceptibility to RNA block were also observed with an HIV-1 Gag mutant, HBR/RKswitch, where all lysine and arginine residues within HIV-1 HBR are substituted with each other (72). The failure of the

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HTLV-1 MA GagLZ mutants and HBR/RKswitch in utilizing $PI(4,5)P_2$ for membrane binding indicates that the presence of a large basic surface patch is insufficient for $PI(4,5)P_2$ interaction, yet is sufficient for MA-RNA interaction. Therefore, it appears likely that HIV-1 MA and RSV MA, which contain a large basic surface patch, have evolved to counteract the RNA-mediated inhibition by interacting specifically with $PI(4,5)P_2$. On the other hand, HTLV-1 MA and MLV MA avoid strong RNA-mediated suppression of membrane binding possibly due to their smaller basic patches. In light of the observation that both $PI(4,5)P_2$-dependent and -independent MA domains mediate efficient VLP production, the significance of the $PI(4,5)P_2$ dependence for HIV-1 and RSV may be to counteract the RNA-mediated suppression rather than to direct PM-specific localization.

The model described above also suggests an alternative mechanism for localization of Gag to intracellular vesicles observed for several retroviruses. Such intracellular localization has been explained as the consequence of nonspecific endocytosis of virions formed at the PM (73, 74). However, in our comparison, while HIV-1 GagLZ and RSV MA GagLZ were found almost exclusively at the PM where $PI(4,5)P_2$ localizes in cells, HTLV-1 MA GagLZ and MLV MA GagLZ localized both at the PM and intracellular compartments despite the fact that they all share the same multimerization domains. This suggests that intracellular localization via VLP endocytosis for these GagLZ would have to be an MA-specific process rather than non-specific internalization. Alternatively, according to our model, due to the weaker RNA-mediated block of membrane binding, HTLV-1 MA GagLZ and MLV MA GagLZ are able to bind to different cellular membranes via prevalent acidic lipids, thus localize to

both PM and intracellular compartments in cells. We observed that HTLV-1 MA GagLZ and MLV MA GagLZ bind liposomes containing PS or other acidic lipids (phosphatidylglycerol or phosphatidic acids) with similar efficiencies (unpublished data). Nevertheless, because of the relative abundance and broad subcellular distribution (75, 76), it is still quite possible that PS mediates Gag membrane binding via HTLV-1 MA or MLV MA in cells and thus leads to their intracellular localization.

Subcellular localization patterns and the severity of the effect of 5ptaseIV expression on VLP production generally correlated well with the membrane binding phenotypes of GagLZ chimeras in this study. However, subcellular localization of HERV-K MA GagLZ was inconsistent with membrane binding and VLP production phenotypes of this GagLZ protein. HERV-K MA GagLZ localized predominantly to the PM, and upon 5ptaseIV expression, a substantial fraction localized to intracellular compartments, even though in vitro membrane binding of this GagLZ derivative and its VLP release in cells are independent of the presence of $PI(4,5)P_2$. One can speculate that in cells HERV-K MA GagLZ may be able to bind both $PI(4,5)P_2$ and another PM-specific molecule, only the latter of which promotes productive assembly.

The direct comparison of 5 different retroviral MA domains in this study highlighted the difference in severity of VLP release inhibition by 5ptaseIV expression. In particular, it is of note that 5ptaseIV overexpression inhibits GagLZ particle production mediated by RSV MA but not as severely as that by HIV-1 MA. While the cause of the difference between the two GagLZ chimeras remains unknown, these results potentially reconcile two apparently contradicting previous studies; while Chan et al. reported that 5ptaseIV has minimal effect on full length RSV Gag VLP production when

compared to HIV-1 Gag (29), Nadaraia-Hoke et al. reported that PM localization and virus release are reduced upon 5ptaseIV overexpression (56). Thus, as was the case with RSV MA GagLZ in this study, it is possible that the effect of 5ptaseIV overexpression on full-length RSV Gag may be less severe than that on HIV-1 Gag but still detectable in a sensitive assay (as speculated in a recent review (77)).

Our current study demonstrated that VLP release efficiency of MLV MA GagLZ is only modestly reduced upon 5ptaseIV overexpression (less than 2 fold), whereas previous studies showed that full-length MLV Gag VLP production is greatly reduced by 5ptaseIV overexpression (3-8 fold) (20, 55). Such discrepancies exist possibly due to the difference in downstream sequences and/or the difference in the type of cell lines in which the experiments were carried out. In particular, the native NC domain may modulate membrane binding phenotypes possibly by facilitating capture of RNA that can in turn bind to MA and necessitate $PI(4,5)P_2$ interaction for efficient membrane binding. While this study focused on intrinsic properties of various retroviral MA domains when compared in the same context, ongoing studies are aimed at the modulatory role of NC-mediated RNA interactions in lipid-RNA competition over MA domains.

In summary, this study has demonstrated that membrane binding of retroviral MA can be either $PI(4,5)P_2$-dependent and sensitive to RNA-mediated inhibition or $PI(4,5)P_2$-independent and RNA-insensitive. We also showed that RNA-insensitive MA can be readily converted to an RNA-sensitive one by expanding an MA surface basic patch, but such expansion is insufficient for the ability to utilize $PI(4,5)P_2$ for efficient membrane binding in the presence of the RNA block. Based on our study, we propose that the block imposed by RNA on membrane binding has driven retroviruses with a large basic patch

on the MA surface to acquire the ability to use $PI(4,5)P_2$, whereas other retroviruses may have maintained smaller basic patches on their MA domains to evade this inhibition. Our study also highlights a potential role for RNA as a broad inhibitor that negatively regulates membrane binding of cytoplasmic proteins that have basic patches.
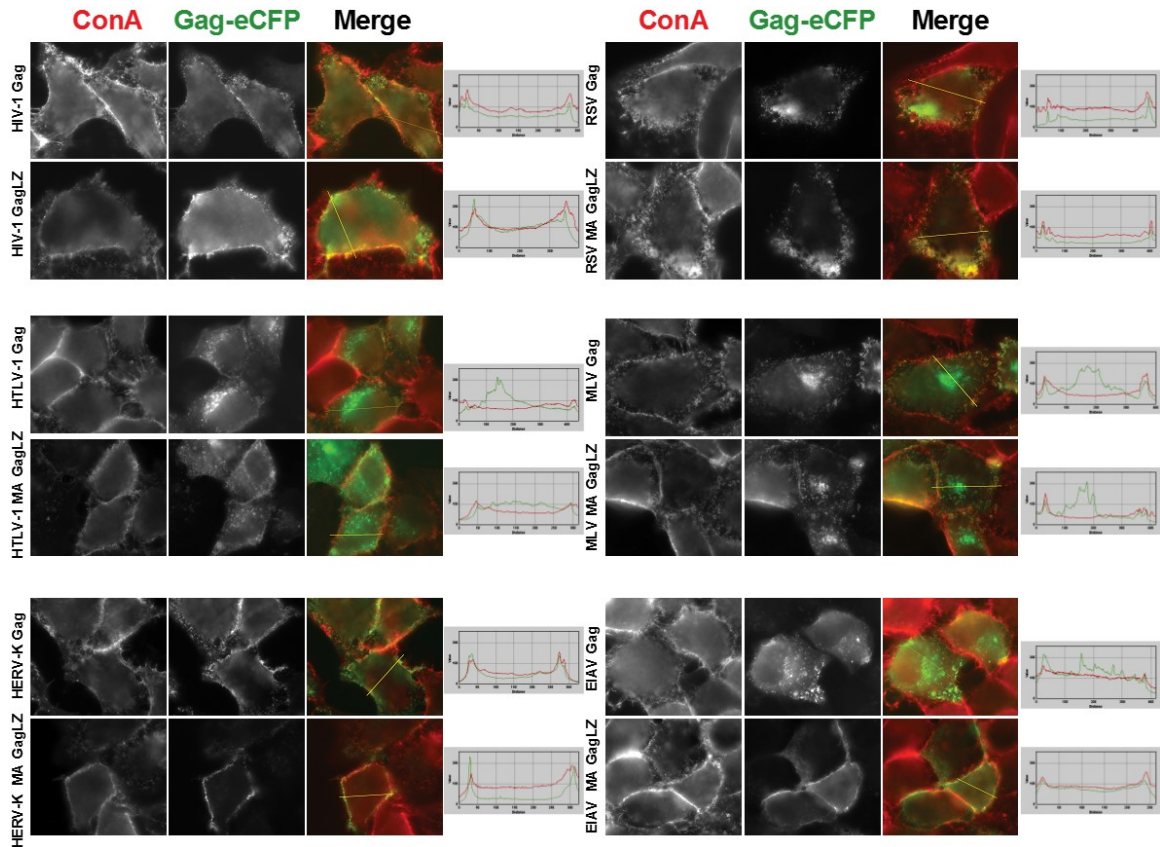
Fig. 2.1. Retroviral MA determines localization of Gag chimeras in HeLa cells. HeLa cells expressing Gag-eCFP or GagLZ-eCFP constructs were fixed and analyzed using an epi-fluorescence microscope. PM was detected by staining with Concanavalin A (ConA) conjugated with Alexa Fluor 594. Fluorescence intensity profiles along the lines drawn in merged images are shown on the right. Gag-eCFP, green line; ConA-Alexa Fluor 594, red line. Gag-eCFP constructs that localize only at the PM have fluorescence intensity peaks only at the same locations as ConA-Alexa Fluor 594 (i.e., at cell periphery). In contrast, Gag-eCFP constructs that localize both at the PM and intracellular compartments have intensity peaks at inner locations of the cell in addition to the peaks matching those of ConA-Alexa Fluor 594 at cell periphery. Note that HIV-1 Gag-eCFP, HIV-1 GagLZ-eCFP, RSV Gag-eCFP, RSV MA GagLZ-eCFP, HERV-K Gag-eCFP, HERV-K MA GagLZ-eCFP and EIAV MA GagLZ-eCFP localized predominantly on the PM, whereas HTLV-1 Gag-eCFP, HTLV-1 MA GagLZ-eCFP, MLV Gag-eCFP, MLV MA GagLZ-eCFP and EIAV Gag-eCFP localized to both PM and intracellular compartments.
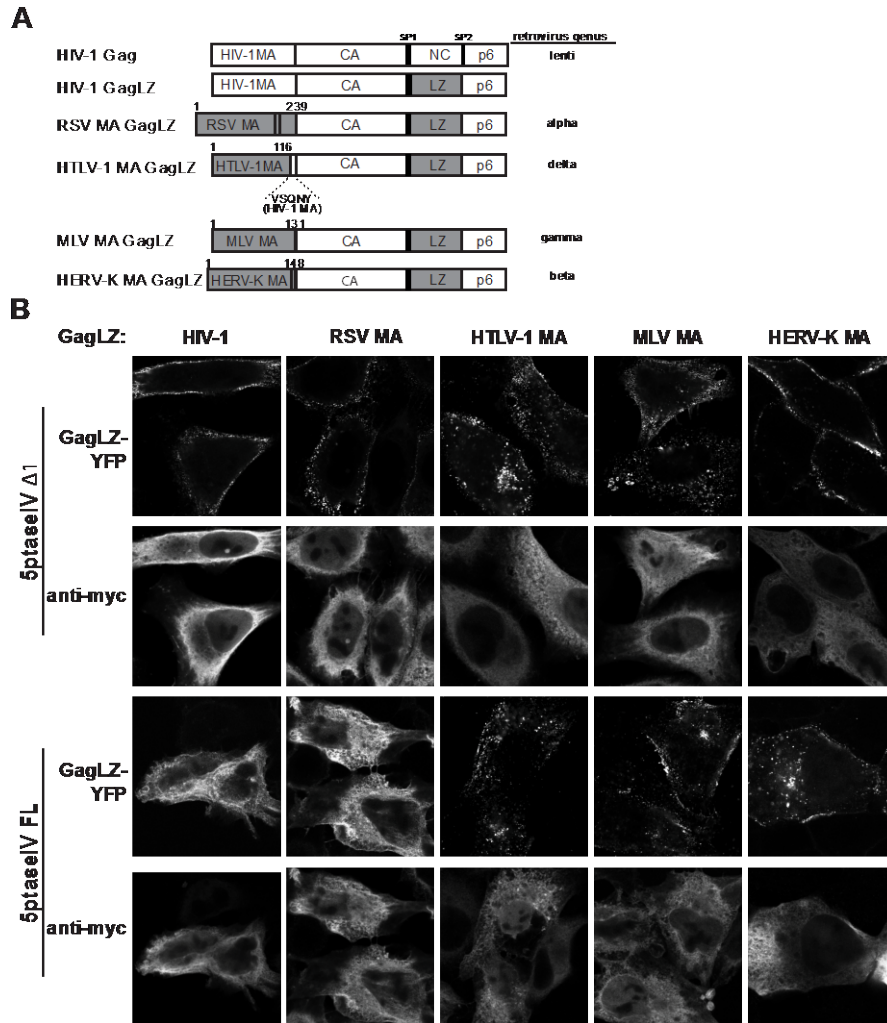
Fig. 2.2. PM localization of HTLV-1 MA GagLZ, MLV MA GagLZ, and HERV-K MA GagLZ persists upon 5ptaseIV overexpression, unlike that of HIV-1 GagLZ and RSV MA GagLZ. (A) Schematic illustrations of HIV-1 Gag, HIV-1 GagLZ, RSV MA GagLZ, HTLV-1 MA GagLZ, MLV MA GagLZ, and HERV-K MA GagLZ are shown. The first amino acid, methionine, is included in the numbering of MA residues although the methionine is removed upon N-terminal myristylation. All Gag proteins were expressed from the same CMV-promoter-driven vector backbone. (B) HeLa cells expressing YFP-tagged HIV-1 GagLZ, RSV MA GagLZ, HTLV-1 MA GagLZ, MLV MA GagLZ or HERV-K MA GagLZ, along with Myc-tagged full-length (FL) 5ptaseIV or the Δ1 derivative, were stained with ConA labeled with Alexa Fluor 594 (not shown), immunostained with mouse monoclonal anti-Myc antibody and anti-mouse IgG conjugated with Alexa Fluor 647 (anti-Myc), and analyzed using a confocal fluorescence microscope. Note that overexpression of 5ptaseIV FL induced mislocalization of HIV-1 GagLZ and RSV MA GagLZ to the cytosol and abolished PM localization. In contrast, 5ptaseIV FL overexpression did not drastically alter localization of HTLV-1 MA GagLZ and MLV MA GagLZ to the PM and intracellular compartments. Overexpression of 5ptaseIV increased the localization of HERV-K MA GagLZ to intracellular compartments, but PM localization persisted regardless of the intracellular localization.
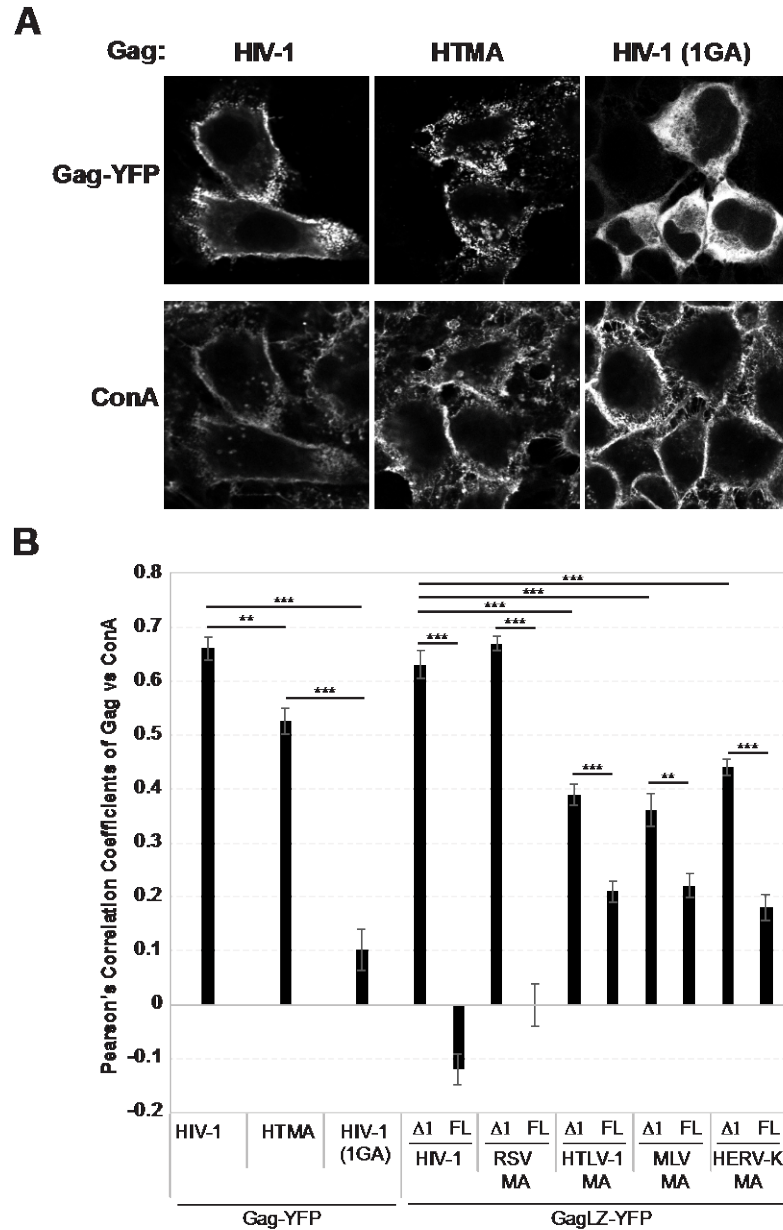
Fig. 2.3. Pearson's Correlation Coefficient for colocalization of chimeric GagLZ with ConA in cells expressing 5ptaseIV Δ1 or full-length 5ptaseIV. (A) HeLa cells expressing YFP-tagged HIV-1 Gag, HTMA Gag and HIV-1 (1GA) Gag were stained with ConA labeled with Alexa Fluor 594. Cells were then fixed and analyzed using a confocal fluorescence microscope. (B) Pearson's correlations coefficients (PCC) for colocalization of Gag-YFP or GagLZ-YFP with ConA were calculated and are shown as means ± SEM. Twenty to fifty cells were analyzed per condition. **, P<0.005; ***, P<0.001.
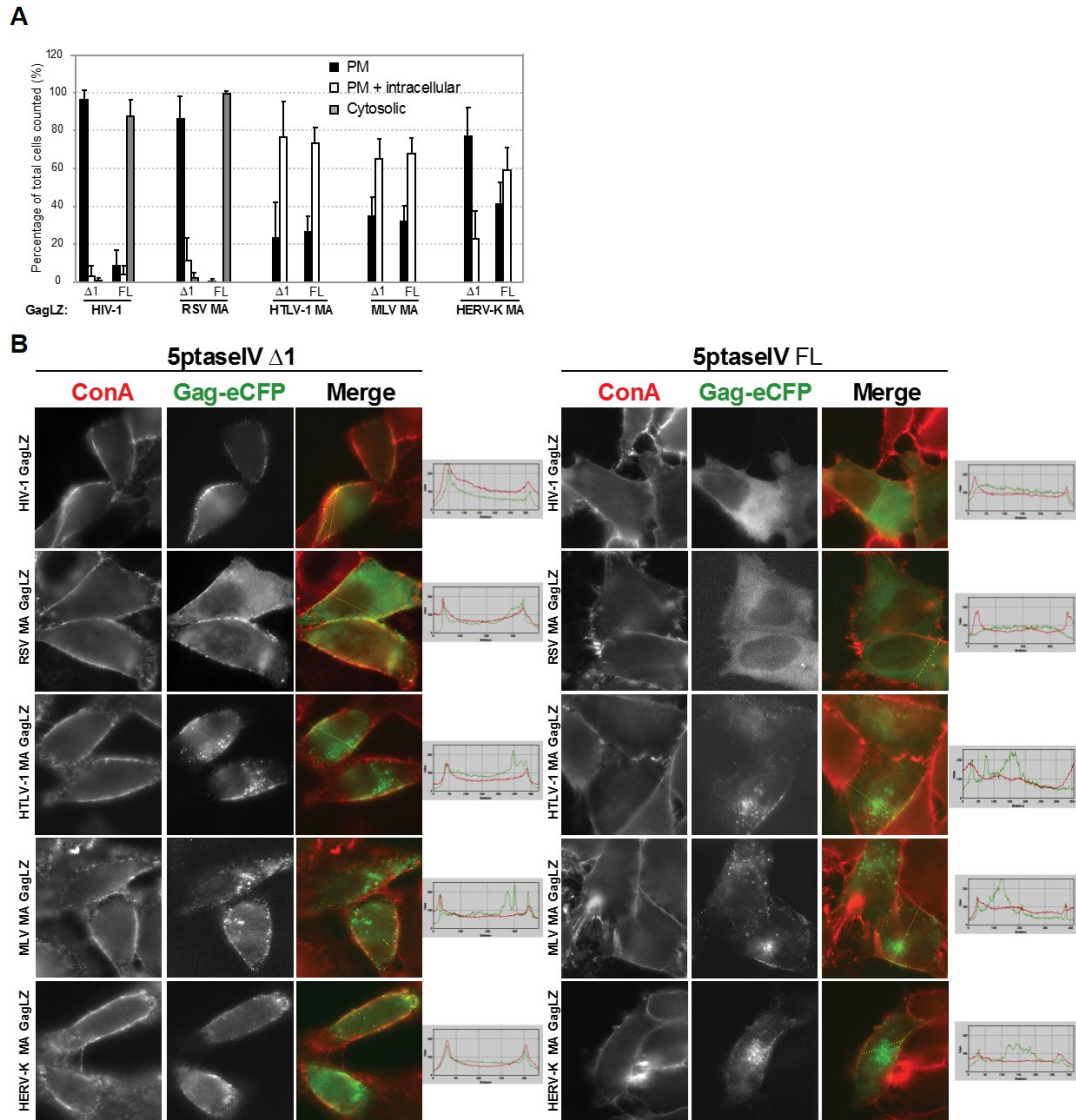
Fig. 2.4. The effect of 5ptaseIV overexpression on subcellular localization of chimeric retroviral MA GagLZ in HeLa cells. (A) The number of cells with (i) Gag localized predominantly at the PM (black), (ii) Gag localized at both PM and intracellular compartments (white), and (iii) Gag localized in the cytosol (grey) were counted. A range of 80 to 150 cells, which were positive for both 5ptaseIV and Gag, were examined for each condition. (B) HeLa cells expressing YFP-tagged HIV-1 GagLZ, RSV MA GagLZ, HTLV-1 MA GagLZ, MLV MA GagLZ or HERV-K MA GagLZ, along with Myc-tagged full-length (FL) 5ptaseIV or the Δ1 derivative, were stained with ConA labeled with Alexa Fluor 594, immunostained with mouse monoclonal anti-Myc antibody and anti-mouse IgG conjugated with Alexa Fluor 350 (anti-Myc) (not shown), and analyzed using an epi-fluorescence microscope. Intensity profiles of ConA and Gag-YFP were plotted. Note that overexpression of 5ptaseIV FL but not Δ1 induced mislocalization of HIV-1 GagLZ and RSV MA GagLZ to the cytosol and abolished PM localization. In contrast, 5ptaseIV FL overexpression did not substantially alter localization of HTLV-1 MA GagLZ and MLV MA GagLZ to the PM and intracellular compartments.

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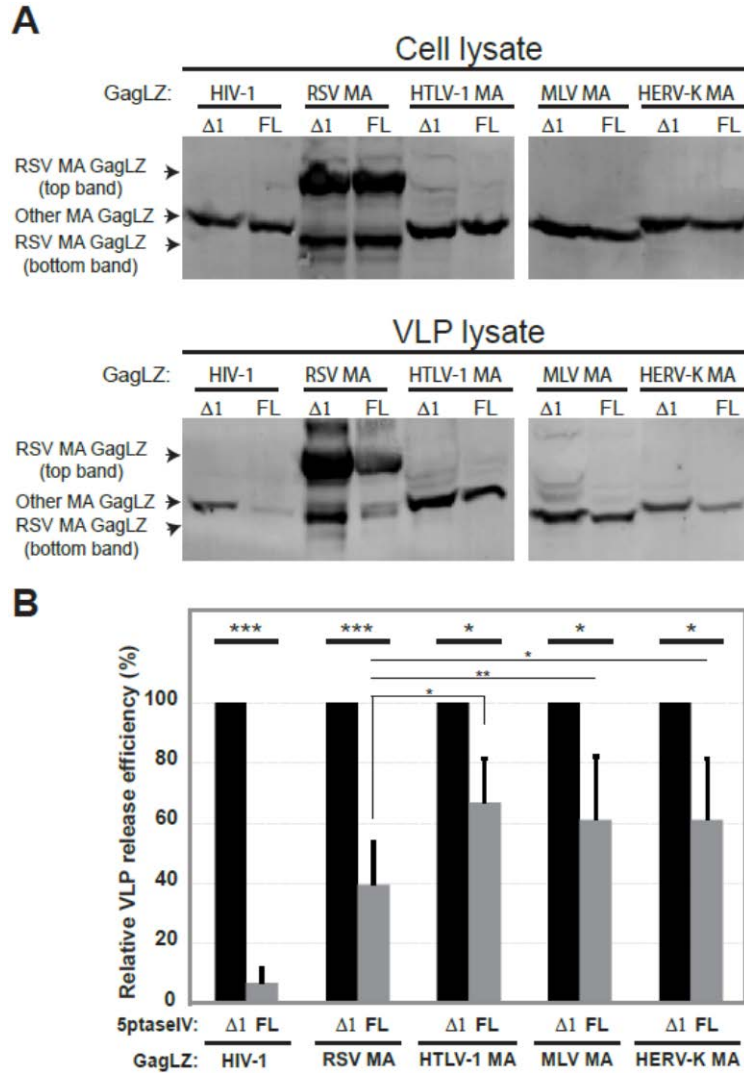
Fig. 2.5. VLP release of HTLV-1 GagLZ, MLV MA GagLZ and HERV-K MA GagLZ is not as sensitive to full-length 5ptaseIV overexpression as that of HIV-1 GagLZ and RSV MA GagLZ. (A) Cell and VLP lysates of HeLa cells expressing HIV-1 GagLZ, RSV MA GagLZ, HTLV-1 MA GagLZ, MLV MA GagLZ or HERV-K MA GagLZ, along with 5ptaseIV FL or its Δ1 mutant, were subjected to SDS-PAGE and analyzed by immunoblotting using HIV-immunoglobulin. (B) Relative virus release efficiency was calculated as the amount of VLP-associated GagLZ as a fraction of total GagLZ present in cell and VLP lysates and normalized to the virus release efficiency in 5ptaseIV Δ1-expressing cultures. The average VLP release efficiencies by cells expressing HIV-1 GagLZ, RSV MA GagLZ, HTLV-1 MA GagLZ, MLV MA GagLZ and HERV-K MA GagLZ along with 5ptaseIV Δ1 were 11.6%, 41.0%, 21.2%, 28.9%, 14.4%, respectively. Note that the average VLP release efficiencies in the control condition do not correlate with the extents of sensitivity to full-length 5ptaseIV. Data from at least 7 different experiments are shown as means ± standard deviations. P values were determined using Student's t-test using raw data. ***, P<0.001; **. P<0.005; *, P<0.05.
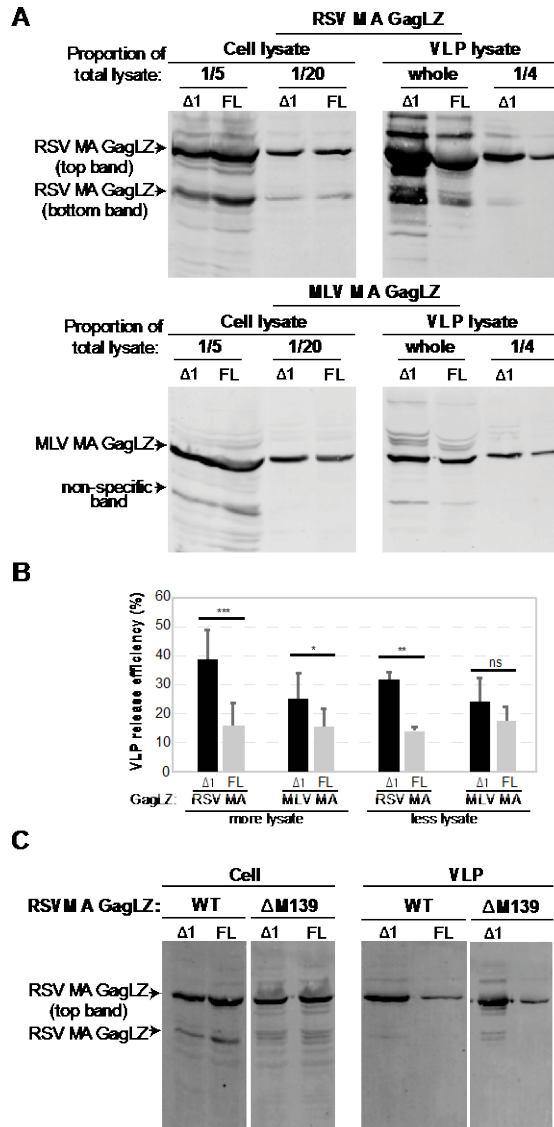
Fig. 2.6. VLP release of MLV MA GagLZ is not as sensitive to 5ptaseIV overexpression as that of RSV MA GagLZ. (A) Cell and VLP lysates of HeLa cells expressing RSV MA GagLZ or MLV MA GagLZ, along with 5ptaseIV FL or its Δ1 mutant, were subjected to SDS-PAGE and analyzed by immunoblotting using HIV-immunoglobulin. In Figure 2.5, 1/5 of total cell lysates and the whole VLP lysates derived from one well of 6-well plates were loaded per lane (standard condition). Here, smaller fractions of lysates (1/20 of cell and 1/4 of VLP lysates) are loaded for comparison. (B) Virus release efficiencies of GagLZ were calculated as in Fig. 2.5B. Data from at least 3 different experiments are shown as means ± standard deviations. P values were determined using Student's t-test using raw data. ***, P<0.001; **, P<0.005; *, P<0.05; ns, not significant. (C) Cell and VLP lysates of HeLa cells expressing WT RSV MA GagLZ or the ΔM139 mutant, along with full-length (FL) 5ptaseIV or the Δ1 derivative, were subjected to SDS-PAGE and analyzed by immunoblotting using HIV-immunoglobulin. Note that the prominent lower molecular weight band was absent in the lanes of the ΔM139 mutant.
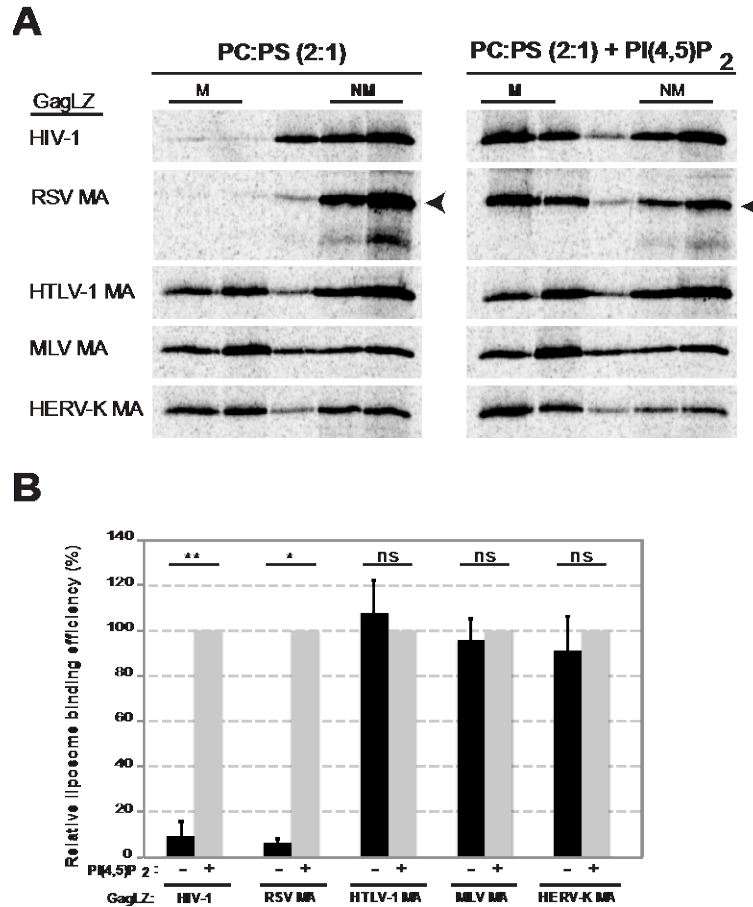
Fig. 2.7. HTLV-1 MA GagLZ, MLV MA GagLZ and HERV-K MA GagLZ proteins, unlike HIV-1 Gag LZ and RSV MA GagLZ, bind efficiently to liposomes in the absence of PI(4,5)P$_2$. (A) [^{35}S]-labeled HIV-1 GagLZ, RSV MA GagLZ, HTLV-1 MA GagLZ, MLV MA GagLZ and HERV-K MA GagLZ were synthesized in vitro using rabbit reticulocyte lysates and incubated with control liposomes [PC:PS (2:1)] or liposomes containing 7.25 mol% PI(4,5)P$_2$ [PC:PS (2:1) + PI(4,5)P$_2$]. The reaction mixtures were then subjected to membrane flotation centrifugation, and a total of five 1-ml fractions were collected from each sample. M, membrane-bound Gag; NM, non-membrane-bound Gag. Note that RSV MA GagLZ is synthesized as two predominant bands, but only the top band corresponding to the size of full-length RSV MA GagLZ is quantified. (B) The liposome binding efficiency is presented as the percentage of membrane-bound Gag versus the total Gag synthesized in the reaction. Each reaction is normalized to the binding efficiency to PI(4,5)P$_2$-containing liposomes. The average liposome binding efficiencies of HIV-1 GagLZ, RSV MA GagLZ, HTLV-1 MA GagLZ, MLV MA GagLZ and HERV-K MA GagLZ to PI(4,5)P$_2$-containing liposomes were 53.0%, 31.7%, 33.1%, 73.6%, 51.1%, respectively. Data from at least three experiments are shown as means ± standard deviation. P values were determined by Student's t-test. **, P<0.005; *, P<0.05; ns, not significant.

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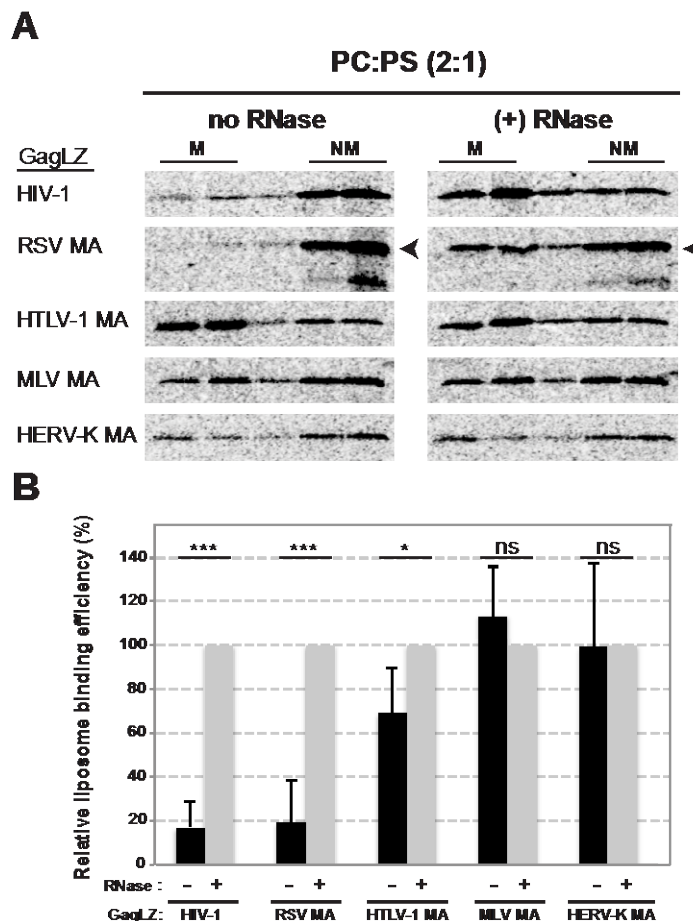
Fig. 2.8. Membrane binding of HTLV-1 MA GagLZ, MLV MA GagLZ and HERV-K MA GagLZ proteins are not susceptible to RNA-mediated inhibition, unlike that of HIV-1 Gag LZ and RSV MA GagLZ. (A) HIV-1 GagLZ, RSV MA GagLZ, HTLV-1 MA GagLZ, MLV MA GagLZ and HERV-K MA GagLZ proteins were synthesized using rabbit reticulocyte lysates and either treated or not treated with RNase A. The reaction mixtures were incubated with the PC:PS [2:1] liposomes and were subsequently subjected to membrane flotation centrifugation. Five 1-ml fractions were collected from each sample. M, membrane-bound Gag; NM, non-membrane bound Gag. Note that RSV MA GagLZ is synthesized as two predominant bands, but only the top band corresponding to the size of full-length RSV MA GagLZ is quantified. (B) The relative liposome binding efficiency was calculated as the percentage of membrane-bound versus the total Gag synthesized in the reaction and normalized to binding efficiencies in RNase-treated samples. The average liposome binding efficiencies of HIV-1 GagLZ, RSV MA GagLZ, HTLV-1 MA GagLZ, MLV MA GagLZ and HERV-K MA GagLZ to RNase-treated conditions were 56.7%, 22.9%, 44.5%, 56.5%, 51.4%, respectively. Data from at least six different experiments are shown as means ± standard deviations. P values were determined using Student's t-test. ***, P<0.001; *, P<0.05, ns, not significant.

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HIV-1 MA (PDB: 2HMX)   **RSV MA (PDB: 1A6S)**   **HTLV-2 MA (PDB: 1JVR)**   **MLV MA (PDB: 1MN8)**

Front: Front: Front: Front:

Back: Back: Back: Back:
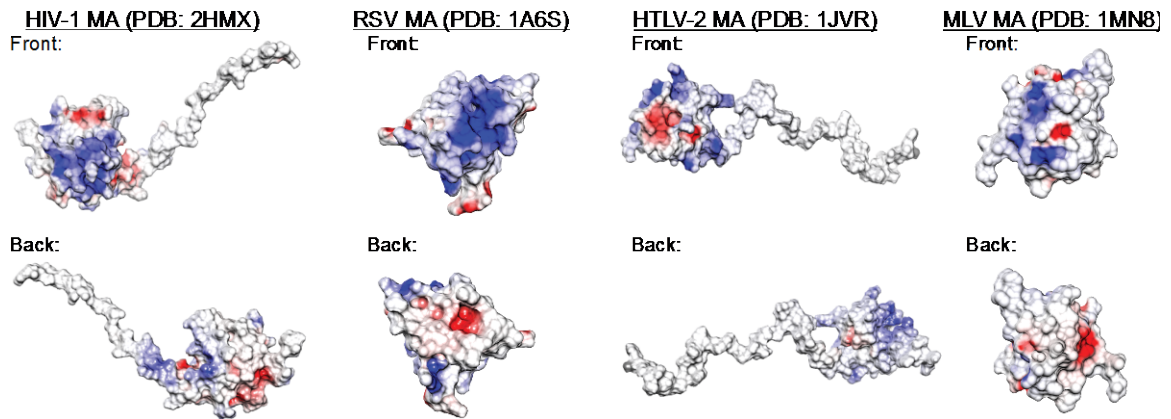
Fig. 2.9. Distribution of basic surface patches on retroviral MA structures. Previously solved retroviral MA domains are represented by their molecular surfaces. The electrostatic potentials were calculated using DelPhi, mapped to the molecular surfaces, and are visualized using Chimera. Blue represents basic patches, whereas red represents acidic patches on the MA domains. Note that HIV-1 MA and RSV MA contain large basic surface patches than HTLV-2 MA and MLV MA, whose basic patches are smaller and distributed all over the MA domain.
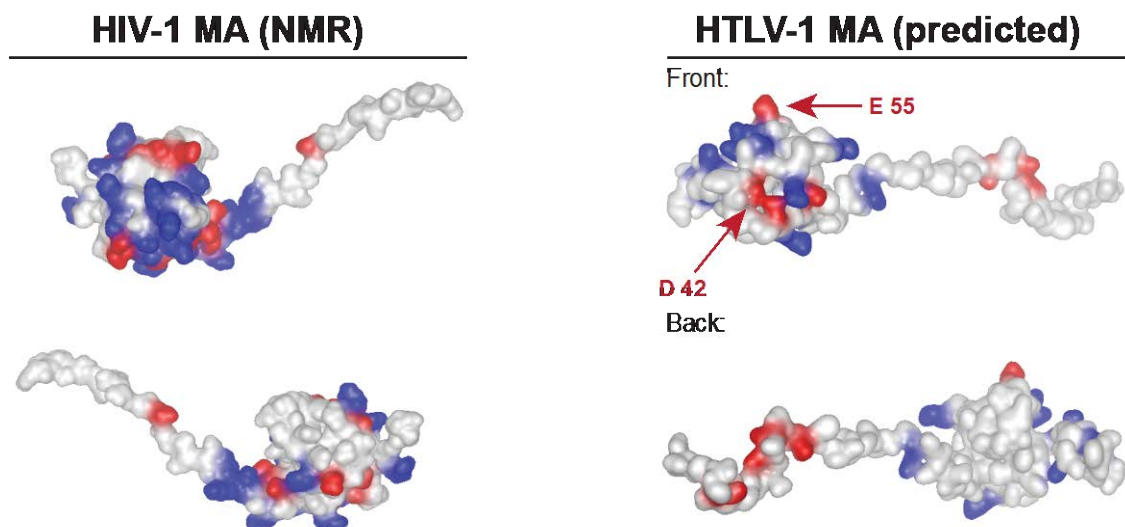
Fig. 2.10. A comparison of HIV-1 MA and HTLV-1 MA as represented by their molecular surfaces. HIV-1 MA was obtained from a previously solved structure (PDB: 2HMX). The structure of HTLV-1 MA was modeled based on the NMR structure of HTLV-2 matrix (PDB: 1JVR) using the Swiss-Model server. Basic residues are represented in blue, while acidic residues are represented in red. Note that HIV-1 MA contains a larger basic patch than those present in HTLV-1 MA. The red arrows identify residues D42 and E55 of HTLV-1 MA, which are mutated to lysine in the experiments shown in the following panels.

69
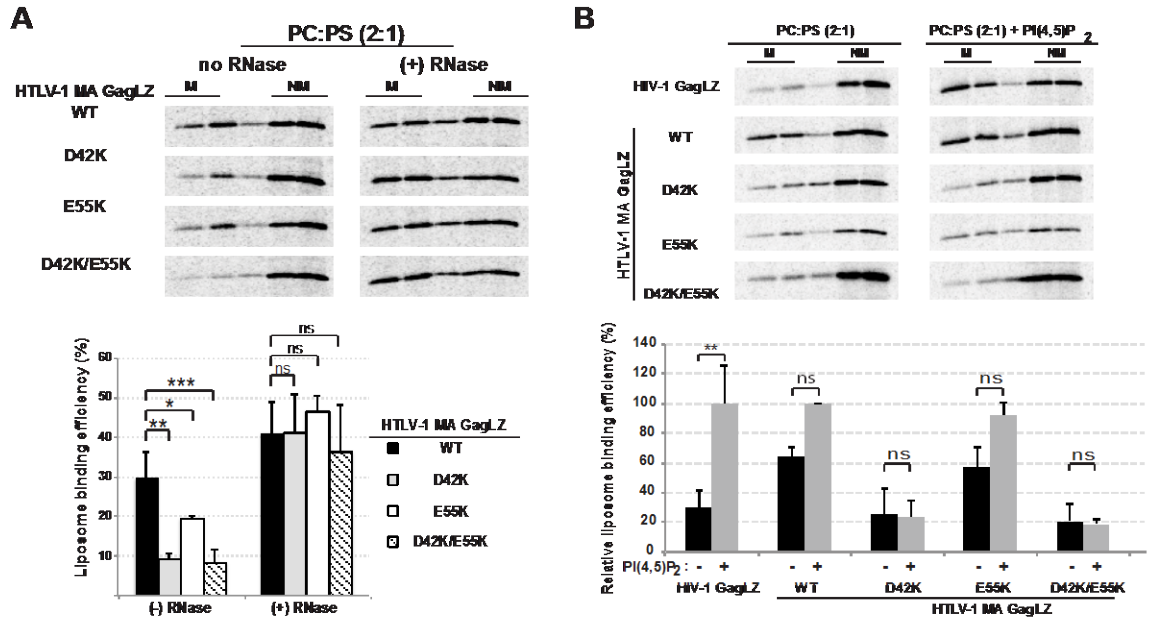
Fig. 2.11. RNA inhibits membrane binding of HTLV-1 MA GagLZ mutants that contain an expanded basic patch in the MA domain. (A) [^{35}S]-labeled WT and mutant HTLV-1 MA GagLZ proteins were synthesized using rabbit reticulocyte lysates and either treated or not treated with RNase A. The reaction mixtures were incubated with the control liposomes [PC:PS (2:1)] and were subsequently subjected to membrane flotation centrifugation. Five 1-ml fractions were collected from each sample. M, membrane-bound Gag; NM, non-membrane-bound Gag. The liposome binding efficiency is presented as the percentage of membrane-bound Gag versus the total Gag synthesized in the reaction. Data from at least three experiments are shown as means ± standard deviation. P values were determined by Student's t-test. ***, P<0.001; **, P<0.005; *, P<0.05; ns, not significant. (B) [^{35}S]-labeled WT and mutant HTLV-1 MA GagLZ were synthesized in vitro using reticulocyte lysates and incubated with control liposomes [PC:PS (2:1)] or liposomes containing 7.25 mol% PI(4,5)P$_2$ [PC:PS (2:1) + PI(4,5)P$_2$]. The reaction mixtures were then subjected to membrane flotation centrifugation, and a total of five 1-ml fractions were collected from each sample. M, membrane-bound Gag; NM, non-membrane-bound Gag. Each reaction is normalized to the binding efficiency of WT HTLV-1 MA GagLZ to PI(4,5)P$_2$-containing liposomes. The average liposome binding efficiencies of WT HTLV-1 MA GagLZ is 43.0%. Data from at least three experiments are shown as means ± standard deviation. P values were determined by Student's t-test. **, P<0.005; *, P<0.05; ns, not significant.

70
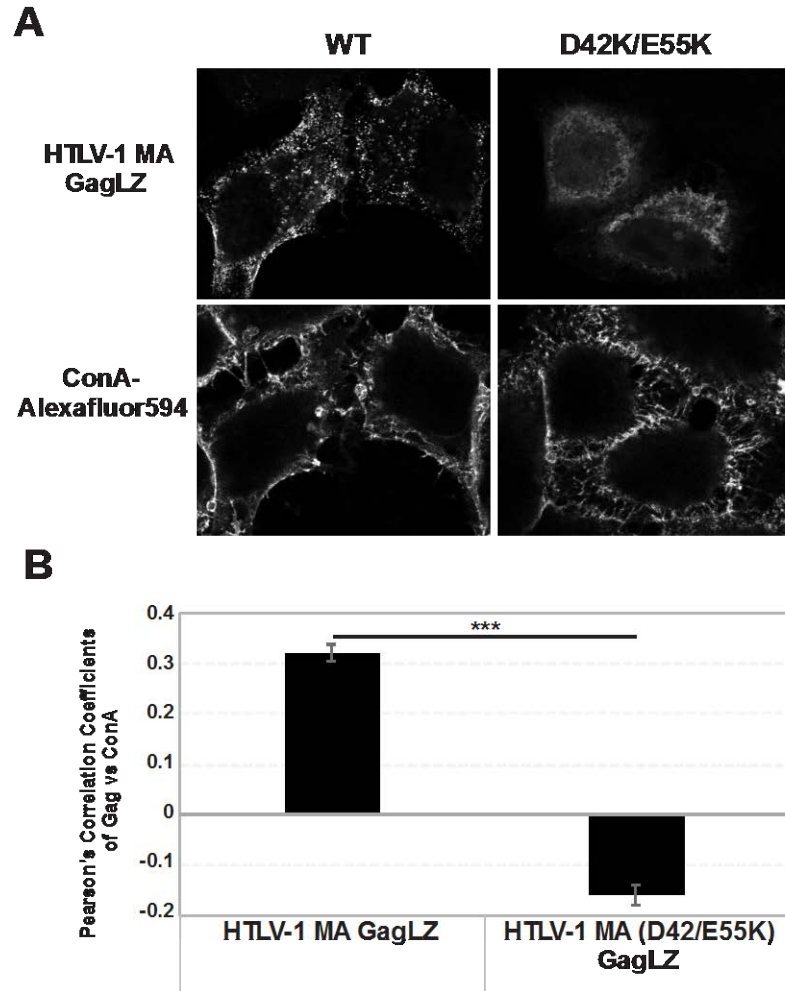
Fig. 2.12. HTLV-1 MA GagLZ mutant that contains an expanded basic patch in the MA domain fails to localize to the plasma membrane or intracellular compartments. (A) HeLa cells expressing YFP-tagged HTLV-1 MA GagLZ constructs with either WT or D42K/E55K mutant MA sequences were fixed and stained with ConA conjugated with Alexa Fluor 594. Note that punctate signals of WT HTLV-1 MA GagLZ localized to both PM and intracellular compartments, whereas D42K/E55K HTLV-1 MA GagLZ displayed no PM signal. (B) Pearson's correlation coefficients (PCC) for colocalization between Gag-YFP and ConA were calculated and shown as means ± SEM. At least twenty cells per condition were analyzed. ***, P<0.001.

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CHAPTER III

Evidence in support of RNA-mediated inhibition of phosphatidylserine-dependent HIV-1 Gag membrane binding in cells

ABSTRACT

The matrix domain promotes plasma-membrane-specific binding of HIV-1 Gag through interaction with an acidic lipid phosphatidylinositol-(4,5)-bisphosphate. In *in vitro* systems, matrix-bound RNA suppresses Gag interaction with phosphatidylserine, an acidic lipid prevalent in various cytoplasmic membranes, thereby enhancing the lipid specificity of matrix. Here we provide *in vitro* and cell-based evidence supporting that this RNA-mediated suppression occurs in cells and hence is a physiologically relevant mechanism that prevents Gag from binding promiscuously to phosphatidylserine-containing membranes.

INTRODUCTION

Membrane binding of HIV-1 Gag is one of the essential steps in virus assembly, which takes place primarily at the plasma membrane (PM) (1). The matrix (MA) domain of Gag is essential for directing virus assembly specifically to the PM. MA has an N-terminal myristoyl moiety that facilitates hydrophobic interaction of Gag with membranes (2, 3). The second signal required for efficient association of Gag with

membranes is the highly basic region (HBR) in MA, which spans residues 17 to 31 of MA. The HBR mediates the electrostatic interaction with cellular acidic lipids (4-10), in particular, a PM-specific phospholipid, phosphatidylinositol-(4,5)-bisphosphate [$PI(4,5)P_2$] (9, 11-13). Notably, *in vitro* studies have shown that MA or its HBR binds RNA as well (13-17). Furthermore, we and others have shown that RNA bound to HBR also regulates membrane binding of Gag *in vitro*. RNA bound to MA abolishes Gag binding to negatively charged membrane composed of a neutral lipid phosphatidylcholine (PC) and an acidic lipid phosphatidylserine (PS) at the 2:1 ratio; however, addition of $PI(4,5)P_2$ allows Gag to alleviate the block imposed by RNA (9, 16, 18). These results collectively support an attractive model in which RNA binding to MA HBR prevents Gag from binding non-$PI(4,5)P_2$ acidic lipids, of which PS is the most abundant in the cytoplasmic leaflet of cellular membranes. This RNA-mediated inhibition can thus enhance the specificity of Gag binding to membranes that contain $PI(4,5)P_2$, which in cells is the PM. However, because the available data supporting this model were all obtained by *in vitro* studies, it remains unknown whether RNA-dependent regulation of membrane binding actually takes place in cells. In this study, we show that the cellular level of RNA is sufficient for blocking Gag binding to PS. In addition, we observed that Gag present in the cytosol is bound to RNA partially via MA HBR and that MA-HBR-dependent PS binding of cytosolic Gag is indeed suppressed by RNA.

RESULTS

Inhibition of Gag binding to PC- and PS-containing liposomes by RNA takes place at RNA concentrations lower than that in cells

Myristoylated full-length Gag synthesized in reticulocyte lysates does not bind negatively charged liposomes containing PS at the physiological concentration of this acidic lipid (~30%)(12, 19, 20). Even at higher concentrations (~50%), palmitoyl-oleoyl-PS (POPS), the most abundant form of PS in viral and plasma membranes (21), does not support efficient liposome binding although di-oleoyl-PS does (20). In previous studies (9, 22), we observed that RNase A treatment drastically increases binding of Gag to liposomes with a 2:1 ratio of POPC and POPS (here termed PC+PS liposome). To determine the minimal RNA concentration that is sufficient to inhibit membrane binding of Gag, we added back different amounts of RNA to RNase-treated Gag. Gag synthesized using rabbit reticulocyte lysates as described previously (9, 12), was treated with 0.028 Units (400 ng) of RNase A (Qiagen) and incubated in a 30-µl reaction at 37°C for 20 min. RNase A was then inactivated by 10 µl of RNasin (40 U/µl; Promega). The reaction containing RNase-treated Gag was then incubated with different concentrations of yeast tRNA (Ambion) for 30 min at 30°C. After another 15-min incubation of Gag with PC+PS liposomes (final volume 50 µl), the mixture was subjected to equilibrium flotation centrifugation using sucrose gradient as described previously (12). We found that tRNA in the range of 0.01-0.1 µg/µl was sufficient in inhibiting Gag binding to PC+PS liposomes (Fig. 3.1A). Considering that a typical HeLa cell volume is at most 5×10^{-6} µl (ranging 0.5-5×10^{-6} µl) (23) and that a cell has around 10-30 pg of RNA (based on the typical RNA yield from a known number of cells) (24, 25), there is at least 2-6 µg/µl of RNA in a HeLa cell (data not shown). Consistent with this, total RNA concentration measured for a mammalian cell line is ~4 µg/µl (26). Under normal physiological conditions, eighty percent of cellular RNA is rRNA, 15-20 % are tRNA or other small

RNAs and the rest being mRNA (27, 28). Thus, the concentration of tRNA that inhibited

Gag binding to PC+PS liposomes is lower than the concentrations of both total RNA and

tRNA in HeLa and other cell lines (26), suggesting that RNA-mediated inhibition of Gag

binding to PS-containing membranes can occur in cells. Notably, Gag binding to PC+PS

liposomes containing 7.25 mol% $PI(4,5)P_2$ was less sensitive to tRNA (Fig. 3.1B), a

finding consistent with the model in which $PI(4,5)P_2$ is capable of interacting with Gag

even in the presence of MA-bound RNA.

RNA binding in cells is mediated partly by MA

While both total RNA and tRNA concentrations in cells would be sufficient for

the inhibition of Gag binding to PS-containing, but not $PI(4,5)P_2$-containing, membranes,

it is likely that the majority of cellular RNA species are not accessible to the MA HBR in

cells due to their localization or interactions with cellular proteins. To determine whether

MA HBR is able to interact with RNA in cells, we quantified the amount of RNA bound

to Gag immunoprecipitated from cells. In these experiments, we examined non-

myristoylated Gag derivatives encoded in molecular clones that also lack active protease.

We used non-myristoylated Gag constructs to eliminate the variation in

immunoprecipitation efficiencies caused by differences in Gag epitope exposure between

non-membrane-bound Gag and membrane-bound Gag that forms higher-order multimers

(29, 30). We assessed the effect of the 6A2T substitutions in the MA HBR, in which all

the basic amino acid residues were replaced with neutral ones (9). As a control, we also

examined the RNA binding when NC, the main RNA binding domain of Gag, is replaced

with a dimerization motif, leucine zipper (LZ), of an yeast transcription factor GCN4

(31). This Gag derivative (GagLZ) lacks NC-mediated RNA binding, yet is capable of

forming virus-like particles in the context of intact MA (31-33). HeLa cells were transfected using Lipofectamine 2000 according to the manufacturer's instruction with pNL4-3/1GA/PR- (34) and derivatives containing LZ, 6A2T, 6A2T/LZ changes [constructed using previously reported molecular clones (34-36) by standard molecular cloning techniques]. Six hours post-transfection, cells were labeled with [^{35}S]-methionine/cysteine. Twenty-four hours post-transfection, cells were lysed using the polysome lysis buffer (23), and Gag was immunoprecipitated using HIV-1 immunoglobulin as described previously (9). A fraction of the immunoprecipitated materials was treated with proteinase K, and co-immunoprecipitated RNA was isolated using Qiazol and Qiagen miRNAeasy kit as recommended by the manufacturer. In parallel, the remaining immunoprecipitated materials were analyzed on SDS-PAGE, and the amount of Gag was quantified using phosphorimager analysis. The RNA was then quantified using Quant-iT Ribogreen reagent and normalized to the amount of immunoprecipitated Gag. As expected, there was significantly less RNA co-immunoprecipitated with GagLZ than with full-length Gag (Fig. 3.2). This result is consistent with previous reports showing diminished RNA contents in extracellularly released NC-deficient particles (32, 37, 38). Importantly, the amount of RNA bound to Gag was also reduced when MA HBR was mutated in the full-length Gag context. While introduction of the same MA mutations in the GagLZ context (1GA/6A2T GagLZ) did not cause a statistically significant reduction in RNA binding compared with GagLZ, the amount of RNA bound to 1GA/6A2T GagLZ was most severely reduced relative to full-length Gag among the Gag derivatives. Altogether, these results suggest that not only NC but also MA HBR participates in RNA binding of full-length cytosolic Gag. Consistent

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with this observation, *in vitro* studies have shown that MA has stronger affinities for some RNA aptamers compared to NC, suggesting a potential specific interaction between MA and RNA(13-15, 18, 39). However, these data do not rule out the possibility that the RNA bound to NC is the same RNA that binds MA in cells. If this is the case, due to its inherent high affinity for RNA, NC may facilitate MA-RNA interaction by bringing the RNA in close proximity to the MA HBR.

Gag in the cytosol binds negatively charged liposomes upon removal of RNA

RNA present in *in vitro* translation reactions prevents PS-dependent liposome membrane binding of non-myristoylated Gag synthesized in these reactions (9). To address whether the RNA bound to Gag in cells inhibits PS-dependent membrane binding, HeLa cells (2×10^6 cells) were transfected with HIV-1 molecular clones encoding non-myristoylated Gag as in Fig. 3.2. Twenty hours post-transfection, the cells were treated with 100 μl of 0.04% digitonin (in 20 mM HEPES, 150 mM NaCl with Complete™ protease inhibitor) for 10 min on ice in a microcentrifuge tube. The permeabilized cells were then centrifuged at 13200 rpm for 10 min in a microfuge to separate the cytosol from perforated cells. The supernatant was then treated or not treated with RNase A (0.7 U or 10 μg) at 37° C for 20 min and further incubated with PC+PS liposomes at 37° C for 15 min. The liposome-bound proteins were then separated on equilibrium flotation centrifugation as previously described (9, 12). Gag present in each fraction was detected using Typhoon Trio imager (GE) after immunoblotting using HIV-Ig as the primary antibody and anti-human Alexa Fluor 488 as the secondary antibody. As observed previously for *in vitro* synthesized Gag (9), RNase treatment significantly enhanced binding of cytosolic Gag to PC+PS liposomes (Fig. 3.3). These results indicate

that RNAs in cells can block binding of cytosolic Gag to negatively charged membranes that do not contain PI(4,5)P$_2$. This RNA-mediated inhibition was also observed when NC was replaced with LZ (Fig. 3.3A and B) or deleted (data not shown), suggesting that neither RNA binding to NC nor NC-driven Gag multimerization is essential for RNA-mediated inhibition of MA-dependent Gag membrane binding. When MA HBR was mutated in the GagLZ context (Fig. 3.3A and B), there was negligible membrane binding regardless of RNase treatment. This observation indicates that the MA HBR mediates the PC+PS liposome binding of RNase-treated cytosolic Gag examined in these experiments. Experiments described above were performed using cells expressing non-myristoylated Gag derivative to maximize the yield of cytosolic Gag. To determine whether RNA regulates wild-type (WT) Gag in the cytosol in a similar manner, the cytosol of HeLa cells transfected with a molecular clone encoding WT Gag were harvested 8 hours post-transfection. At this time point, fluorescently tagged WT Gag shows a cytosolic localization pattern in a larger fraction of cells than at 24 hours post-transfection (data not shown). We found that PC+PS liposome binding of WT Gag derived from the cytosol of transfected HeLa cells was enhanced upon RNase treatment (Fig. 3.3C and D) as observed for non-myristoylated Gag. These results support the conclusion that RNA prevents cytosolic Gag from binding to prevalent acidic lipids and further suggest that a substantial population of non-membrane-bound Gag that has been observed in cells is maintained due to the block mediated by RNA.

DISCUSSIONS AND CONCLUSIONS

The data presented in this study show that RNA-mediated inhibition of Gag binding to PS-containing membranes can occur in cells and thus is a physiologically relevant mechanism for the regulation of Gag-membrane binding and HIV-1 assembly. While the observed MA-HBR-dependent RNA binding (Fig. 3.2) supports a direct mechanism whereby RNA outcompetes PS or other non-PI(4,5)P_2 acidic lipids for electrostatic interactions with HBR, we do not rule out an indirect mechanism in which a cellular RNA-dependent factor is involved in regulation of MA-acidic lipid interactions . Interestingly, however, a recent report showed that nucleotides facilitate selective binding of synaptotagmin C2 domains to PI(4,5)P_2-containing membranes by blocking electrostatic interactions with PS in untargeted membranes (40). Therefore, the competition between nucleotides and acidic lipids may be a widely used mechanism to promote specific targeting of proteins to PI(4,5)P_2-containing membranes.

In addition to the regulation of Gag-membrane binding, previous *in vitro* studies suggested that the competition between RNA and acidic lipids explains the structural change of full-length HIV-1 Gag between bended and extended shapes (41) and regulation of the NC-dependent RNA chaperone activity (42). These findings collectively highlight the switch function of MA HBR during the late phase of HIV-1 life cycle. Nevertheless, molecular details of the balance between MA-PI(4,5)P_2 and MA-RNA interactions remain to be determined. In particular, the potential roles for simultaneous binding of MA and NC domains to the same RNA molecule in coordinating Gag membrane binding with Gag multimerization or other steps in the assembly process warrant further investigation. RNA aptamers have been emerging as a new class of

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potential therapeutics (43). With the current evidence supporting that the MA-RNA interaction regulates membrane binding of Gag in cells, RNA aptamers that specifically interact with MA with a higher affinity than $PI(4,5)P_2$ might be useful to block HIV-1 assembly in HIV-1-infected individuals.
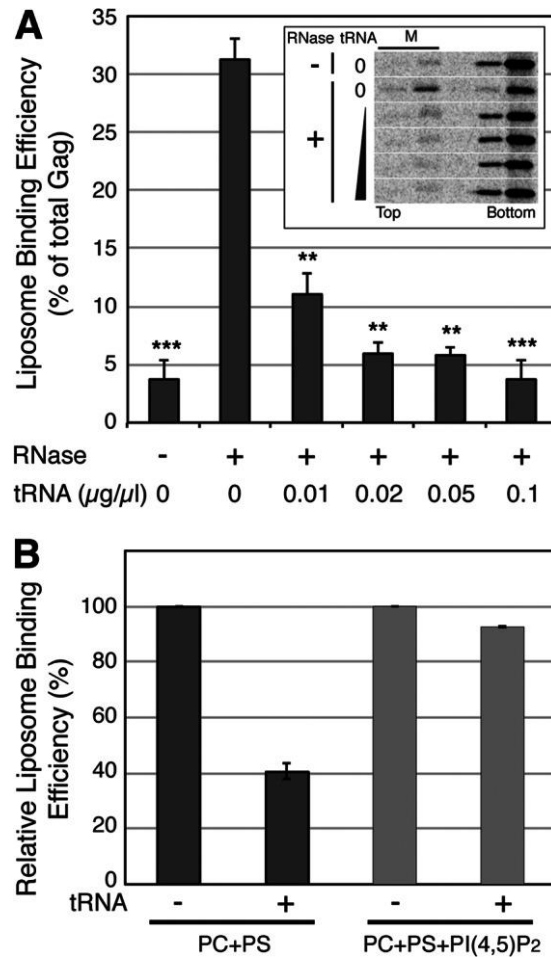
Fig. 3.1. tRNA below intracellular levels inhibits Gag binding to liposomes containing PS but not PI(4,5)P$_2$. (A). [^{35}S]-labeled Gag synthesized using rabbit reticulocyte lysates was treated with 400 ng of RNase A at 37°C for 20 minutes. RNase A was blocked using RNasin, and the mixtures were further incubated with indicated concentrations of tRNA at 30°C for 30 minutes. PC+PS liposomes were then added and incubated further for 15 minutes before performing equilibrium flotation centrifugation. Five 1-ml fractions were collected, and 25 µl of each fraction was loaded and analyzed on SDS-PAGE (inset). The liposome binding efficiency was calculated as the amount of membrane-bound Gag (M in inset) as a fraction of total Gag. Average of 3 different experiments is shown. Error bars represent standard deviation. P values were determined by using Student's t test. **, $P < 0.01$; ***, $P < 0.001$. (B). [^{35}S]-labeled Gag incubated sequentially with RNase A, RNasin, and 0.1 µg/µl tRNA as in A was examined for binding to PC+PS liposomes that contain 7.25 mol% PI(4,5)P$_2$ (PC:PS:PI(4,5)P$_2$ = 62:31:7.25) in comparison with binding to PC+PS liposomes. The liposome binding efficiency was calculated as in panel A, and the relative liposome binding efficiency was calculated in comparison with the binding efficiency of RNase-treated Gag not incubated with tRNA for each experiment. Average of 2 different experiments is shown. The average liposome binding efficiencies of RNase-treated Gag not incubated with tRNA were 33% for PC+PS liposomes and 59% for PI(4,5)P$_2$-containing liposomes in these experiments.
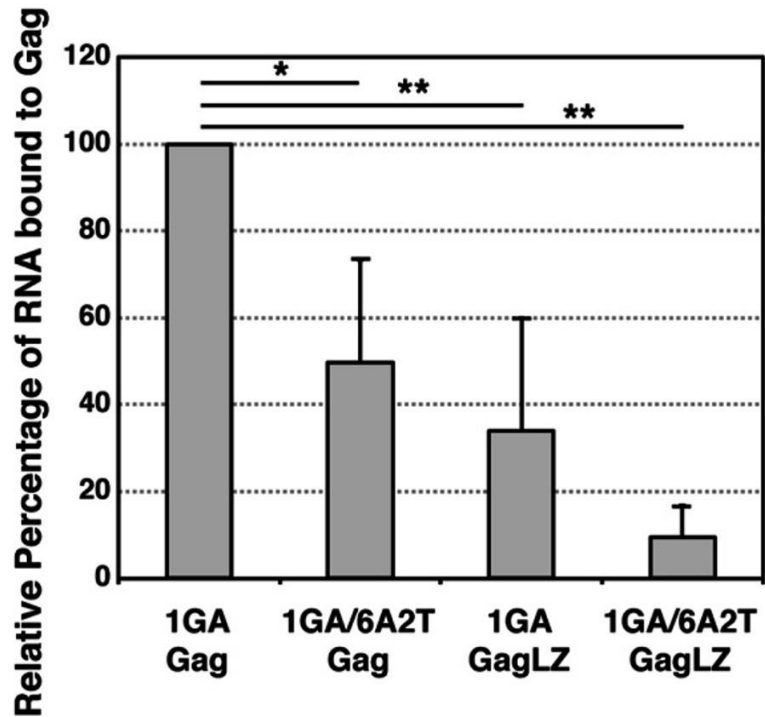
Fig. 3.2. MA HBR interacts with RNA in cells. HeLa cells were transfected with pNL4-3/1GA/PR-, pNL4-3/1GA/6A2T/PR-, pNL4-3/1GA/LZ/PR- or pNL4-3/1GA/6A2T/LZ/PR- and metabolically labeled with [^{35}S] Met/Cys overnight. Cells were lysed and immunoprecipitated with anti-HIV Ig antibody. A fraction of immunoprecipitated materials were examined for the amounts of bound RNA. The amount of co-immunoprecipitated RNA was quantified by fluorometry using Ribogreen reagent. The amount of RNA was normalized to the Gag levels, and the RNA bound to pNL4-3/1GA/PR- was set to 100%. Average of 3 different experiments is shown. Error bars represent standard deviation. *, $P < 0.05$; **, $P < 0.01$. The amounts of RNAs bound to 1GA/6A2T Gag and 1GA GagLZ are not significantly higher than that of 1GA/6A2T GagLZ.
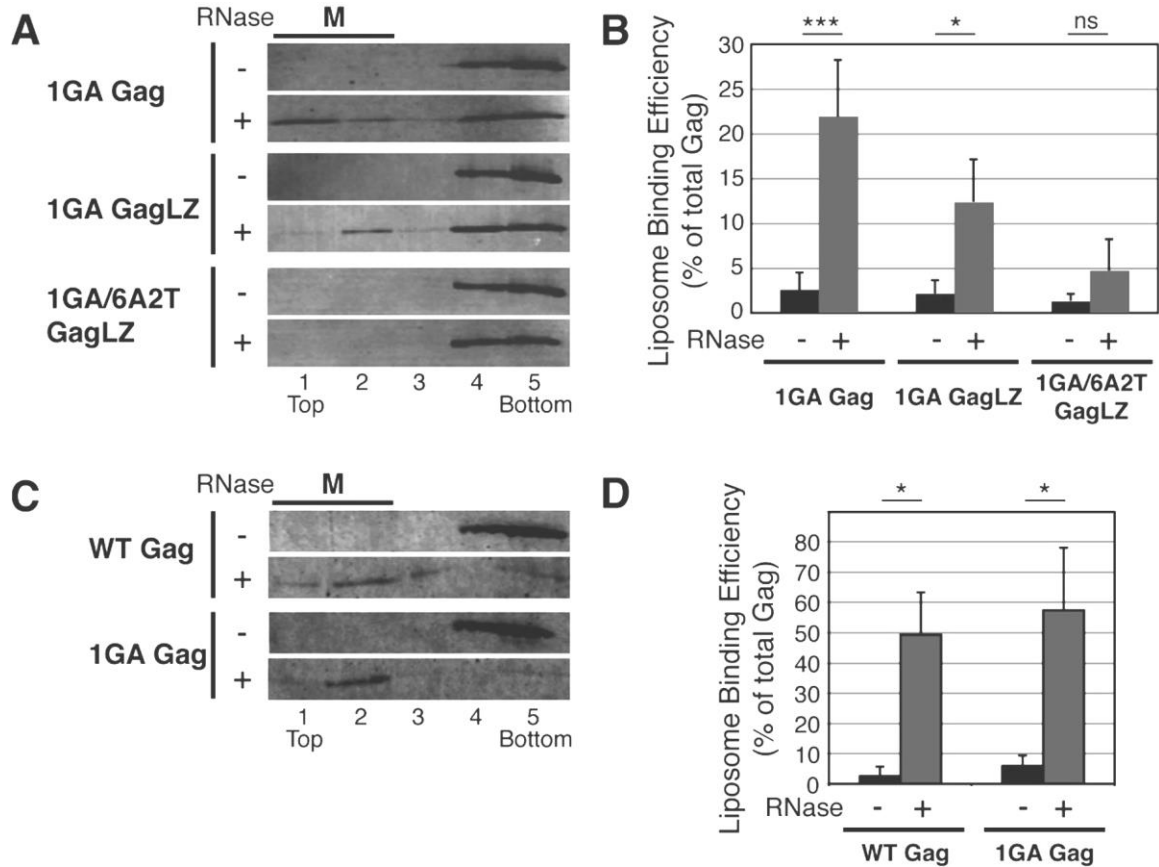
Fig. 3.3. RNA inhibits membrane binding of Gag present in the cytosol. (A) and (B), HeLa cells (2×10^6) were transfected with pNL4-3/1GA/PR-, pNL4-3/1GA/LZ/PR- or pNL4-3/1GA/6A2T/LZ/PR-. Twenty hours post-transfection, cells were treated with digitonin, and supernatants containing cytosolic Gag (100 µl) were separated from perforated cells by centrifugation. The supernatants were divided in two aliquots and treated with RNase A or left untreated. Five microliter of PC+PS liposomes (14.6 µg lipid/µl) were then added, and the reaction was further incubated for 15 minutes before performing sucrose gradient centrifugation. Five fractions were collected and analyzed by western blotting using anti-HIV Ig as the primary antibody and anti-human Alexa Fluor488 as the secondary antibody. M: membrane-bound Gag. (C) and (D), HeLa cells (6×10^6) were transfected with pNL4-3/PR-, which encodes WT Gag, or pNL4-3/1GA/PR-. Eight hours post-transfection, cells were treated with digitonin, and supernatants containing cytosolic Gag (200 µl) were separated from perforated cells by centrifugation. The supernatants were divided in two aliquots and treated with RNase A or left untreated. Ten microliter of PC+PS liposomes (14.6 µg lipid/µl) were then added, and the reaction was analyzed as in A and B. The amount of membrane-bound Gag versus the total amount of Gag was calculated and is shown as liposome-binding efficiency (B and D). Data from at least three independent experiments are shown as means ± SD. ns, not significant; *, P < 0.05; ***, P<0.001.

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Chapter IV

Molecular mechanism of HIV-1 assembly in primary macrophages

ABSTRACT

HIV-1 Gag is necessary and sufficient for the assembly process. The site of assembly of HIV-1 is cell-type dependent. In HeLa and T cells, HIV-1 assembles at the plasma membrane (PM). The PM binding and localization of HIV-1 Gag is mediated by the matrix (MA) domain, specifically the highly basic region (HBR), via interaction with a PM-specific acidic phospholipid, phosphatidylinositiol-4,5-bisphosphate [$PI(4,5)P_2$]. HIV-1 MA also interacts with RNA, which prevents Gag from binding to membranes containing phosphatidylserine, a prevalent cellular acidic phospholipid. These results suggest that the MA-bound RNA promotes PM-specific localization of HIV-1 Gag by blocking non-specific interactions with cellular membranes that do not contain $PI(4,5)P_2$. Interestingly, in polarized T cells, nucleocapsid(NC)-dependent multimerization was found to be important in Gag targeting to the uropod, the rear-end PM protrusion of the cell. The site of assembly of HIV-1 in primary macrophages is the virus-containing compartments (VCC). Whether targeting of HIV-1 Gag to the VCC is MA-dependent or NC-dependent is not known. Thus, to study the molecular mechanism of HIV-1 Gag targeting to the VCC, we first examined the impact of $PI(4,5)P_2$ depletion by the overexpression of polyphosphoinositide 5-phosphatase IV (5ptaseIV) in primary macrophages. We found that the VCC localization and virus release of HIV-1 are

severely impaired. Furthermore, we determined that myristoyl moiety, but not the intact HBR is important in VCC localization. Our analysis of HIV-1 Gag with MA-replacement mutations suggest that HIV-1 MA only contributes to membrane binding but not in Gag targeting to the VCC. We further determined that HIV-1 NC is important for VCC-specific localization of HIV-1 Gag. HIV-1 Gag is still able to localize to the VCC efficiently when the NC domain is replaced with a leucine zipper dimerization motif (GagLZ), which is capable of promoting Gag-Gag multimerization. Altogether, our data suggest that targeting of HIV-1 Gag to the VCC requires NC-dependent multimerization, a mechanism that is reminiscent of HIV-1 Gag targeting to the uropod in polarized T cell.

INTRODUCTION

Macrophages represent one of the primary cell targets of HIV-1 and play important roles in HIV-1 pathogenesis (1-3). They are terminally differentiated phagocytic cells of myeloid lineage and are found at various tissues in the body. Similar to T cells, macrophages express HIV-1 receptor, CD4, albeit at lower level, and co-receptors CCR5 and CXCR4. CCR5 is expressed in higher levels than CXCR4 in macrophages. HIV-1 enters the host through the mucosal and gastrointestinal surfaces (4). With macrophages residing at these sites, it is thought that they represent one of the earliest immune cells that encounters HIV-1 at mucosal surfaces.

Macrophages are long-lived and are seen to accumulate HIV-1 particles for long period of time (5-7). They are more resistant to cytopathic effects of virus replication than CD4 T cells, and are not efficiently depleted during acute infection (3, 8). Thus, they

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are thought to represent one of the latent reservoirs for viruses that evade or are resistant to HAART (1, 4, 9, 10). Furthermore, because macrophages are found circulating the body and are capable of passing the blood-brain barrier, they are important as a means of HIV-1 dissemination and establishment of infection at the brain, causing HIV encephalitis (HIVE) (9, 11).

The site of assembly of HIV-1 is defined by the presence of immature particles as well as budding virus structures from the limiting cellular membrane as visualized by electron microscope (EM) [see (12, 13) for EM images]. In addition, the presence of Env protein and Gag can also be used to identify the site of assembly. In T cells and other cell lines, such as 293T cells, COS cells and HeLa cells, HIV-1 assembly occurs at the plasma membrane (14-16). Ultrastructural study of macrophages reveal that the HIV-1 particles accumulates intracellularly and contain not only mature particles but immature ones as well (5, 17-19). These compartments where HIV-1 accumulates in primary macrophages are called the virus containing compartments (VCC).

Composition of VCC

The identity of VCC is currently unknown. Previously, VCC was categorized as an endosomal compartment or multivesicular bodies (MVB) as Gag was found to colocalize with CD63, an endosomal/MVB marker (20). In another study, HIV-1 particles were seen to bud from major histocompatibility complex class II (MHCII) compartments, where CD63 are also present (17). By assembling and budding into the MHCII compartments, HIV-1 was also thought to have used exocytosis pathway to release the particles into extracellular milieu (17). In support of this hypothesis, immuno–EM studies showed that numerous internal vesicles containing HIV-1 was found to be in

close proximity with the plasma membrane, resembling the exosomal secretion pathway in B cells (17, 18, 21, 22). Other than serving as the site of assembly, VCC also seems to be involved in cell-to-cell transmission of HIV-1. In particular, HIV-1 Gag is observed to traffic from intracellular compartments to macrophage-T cell contact sites (23).

Despite accumulating evidence suggesting that HIV-1 bud into endosomal/MVB compartments, recent studies seem to content these findings. Firstly, Jouve et al. found that VCC was not as efficiently stained by BSA-Gold, an endocytic tracer, as the endocytic/MVB compartments (19). Furthermore, using a membrane-permeable acidic organelle probe, DAMP (D1552), Jouve et al. also estimated the pH of VCC to be 6.8 (19). This finding is inconsistent with the pH found in the endocytic compartments, which has acidic environment. More importantly, VCC seems to be accessible from the cellular surface. This surface connection was visualized by EM after staining the infected macrophages with membrane-impermeable dye, ruthenium red (RR) (21, 24, 25). In these cells, HIV-1 particles are found in the RR-positive intracellular compartments that has narrow channels (<20nm) connecting to the cell surface (24, 25). Thus, the current consensus is that VCC are deep invaginations of the plasma membrane (21).

However, not all VCC are cell-surface accessible as some but not all VCC can be stained with cell-impermeable dye or small molecules (26, 27). Furthermore, sequential feeding of HIV-1 infected macrophages with two different fluorescently-labeled 10kDa dextran revealed that not all VCC remain accessible to both compounds. This indicates that VCC exposure to the extracellular milieu is transient (26). Overall, current data suggest that VCC in macrophages are heterogeneous and dynamic in nature.

Recent studies have focused on characterizing the membrane composition of the VCC. VCC is shown to contain tetraspanin markers such as CD9, CD53, CD81 and CD82 (18, 25, 28, 29). In addition, CD18, a leukocyte-specific β2 integrin, CD44, cell adhesion protein/hyaluronic acid receptor and CD36, an LDL-receptor, are also present (29-31). Moreover, PH-GFP, a $PI(4,5)P_2$-specific marker, is shown to overlap with CD81 and Gag puncta at intracellular sites in macrophages, suggesting that $PI(4,5)P_2$ is also present at the VCC (28). $PI(4,5)P_2$ is a negatively charged phospholipid that is found predominantly at the plasma membrane (32, 33) and was previously shown to be important for HIV-1 release and Gag localization to the plasma membrane (13, 34). In addition to these membrane protein markers, interestingly, CD63 segregates from the two VCC markers, CD81 and CD9, in uninfected cells (25). The VCC only become CD63-positive upon HIV-1 infection. It is unknown at the moment whether this is because HIV-1 actively organizes the membrane at the VCC, or because the cycling of CD63-positive compartments to the cell surface is trapped due to the expanding VCC in the cell (25, 35). Nonetheless, it seems that CD63 is not required for HIV-1 localization to the VCC nor is it required for productive virus release (25, 35).

3D morphological analyses of RR-stained membranes have shown that VCCs are made up of a complex meshwork of convoluted, membranous structures (24). Furthermore, comparison between RR-stained uninfected versus infected macrophages reveal that VCC exist prior to virus infection. Consistent with this, intracellular staining of CD81, CD9 and CD53 are observed in uninfected macrophages. However, upon HIV-1 infection, the volume of VCC expands, possibly through the accumulation of virus

particles in the compartments (24). Thus, current studies suggest that VCC formation is not a result of *de novo* membrane synthesis upon HIV-1 infection.

VCC staining by tetraspanin markers also overlap with actin staining and are shown to contain focal adhesions proteins such as vinculin, paxillin and talin (29). VCC are also reported to be in close association with KIF3A microtubules (36). Actin depolymerization by latrunculin A or cytochalasin treatments disperse Gag localization from the VCC (14, 28) and increase virus release from infected macrophages (28). Altogether, these results suggest that the integrity of VCC seems to be dependent upon actin assembly.

Tetherin and VCC

Tetherin is an interferon-inducible cellular restriction factor that restricts virus particle release from the cell surface (37). HIV-1-encoded Vpu protein is able to overcome this restriction by downregulating tetherin surface expression and targeting tetherin for degradation (37, 38). In T cells, tetherin accumulates at virus budding sites and restricts the release of HIV-1 particles that lack the *vpu* gene (37). These Vpu-deleted HIV-1 strain are internalized and accumulate in intracellular vacuoles (39, 40). Similar to T cells and other cell lines, HIV-1 infection in macrophages upregulates tetherin expression (27, 41). However, whether tetherin is involved in VCC formation remains controversial. Tetherin is present at the VCC in uninfected and HIV-1 infected macrophages (27, 41). In one report, tetherin knockdown was found to shrink the size of Gag-positive VCC in infected macrophages, suggesting that tetherin also plays a role in VCC formation (27). The authors also proposed that HIV-1 infection in MDM results in high levels of tetherin induction and that Vpu is insufficient to counteract the tetherin

effectively in MDM. This consequently leads to tetherin-mediated trapping of HIV-1

particles at the VCCs. In contrast, Giese et al. reported that HIV-1 Gag is detected at

VCC regardless of the presence of Vpu, although mature Gag by p17 staining at VCC

was increased in macrophages infected with Vpu-deficient viruses (41). This suggests

that tetherin restricts and accumulates Vpu-deficient HIV-1 particles at VCC. They

further analyzed the intracellular CD81 and CD9 staining in untreated or tetherin-

knockdown macrophages and saw no difference in their localization regardless of tetherin

level. Altogether, Giese et al. concluded that tetherin is not important for VCC

morphology (41).

SAMHD1 and Vpx

One of the reasons why it remains challenging to study HIV-1 infection in

primary macrophages is because macrophages resist HIV-1 infection much better than

CD4 T cells (1). In addition to the presence of tetherin, HIV-1 faces additional replication

restrictions in macrophages. A macrophage-specific interferon-inducible restriction factor

seems to reduce HIV-1 Env expression and virus release (42). Although the restriction

factor is yet to be determined, it was found that HIV-1 Vpr is able to counteract this

restriction pathway. Moreover, cells of myeloid lineage, including dendritic cells,

monocytes and macrophages, express a restriction factor known as SAM domain and HD

domain-containing protein 1 (SAMHD1) at high levels (43). Interestingly, HIV-1 does

not encode any protein to counteract this inhibition (see **Chapter I**). SAMHD1 restricts

HIV-1 replication by depleting the intracellular pool of deoxynucleoside triphosphates

(dNTPs), an essential component for synthesizing viral cDNA during reverse

transcription (43). As a result of lower dNTPs concentration, incomplete or inefficient reverse transcription occur, leading to abortive infection.

In contrast to HIV-1, viruses of SIV_{sm}/HIV-2 lineage encode the *vpx* gene (44, 45). Vpx is an accessory protein that counteracts SAMHD1 restriction by targeting it to proteasome degradation, thereby allowing infection to proceed. Vpx is packaged into the virion by interacting with the p6 domain of Gag (44, 46). Upon virus entry, Vpx is released into the cytosol of the target cell where it interacts with E3 ubiquitin ligase complex to target SAMHD1 for degradation.

HIV-1 infection is highly sensitive to the presence of SAMHD1. However, when Vpx is exogenously introduced, such as in the form of pseudotyped virus-like particles (VLP) to the target cell prior or during infection, HIV-1 infection can occur efficiently (44, 46). More importantly, introduction of Vpx into the cell does not alter macrophage maturation (upon LPS stimulation) or affect their survival (47). This method of using Vpx-VLP for increasing HIV-1 infection rate in macrophages was previously used to examine the role CD36 at the VCC and its effect on HIV-1 release (31).

Unanswered questions

Even though much effort has been carried out to characterize the structure and composition of VCC, very few have examined the molecular determinants that targets Gag to the VCC. HIV-1 assembly is driven by its structural protein, Gag. Gag is synthesized as a polyprotein (Pr55^{Gag}) containing multiple structural domains, where each domain plays critical role during the assembly process (48-50). The Matrix (MA) domain makes up the N terminus of the Gag protein and is responsible for Gag targeting to and

membrane binding at the site of assembly. CA and NC are involved in Gag-Gag interaction, resulting in the formation of Gag multimers. The p6 domain contains the late domain motif, which is responsible for the recruitment of ESCRT proteins, to aid in the release of virus particles from the cell surface.

In cells where the site of HIV-1 assembly takes place at the plasma membrane, it was hypothesized that the MA domain of HIV-1 Gag mediates membrane binding and Gag targeting. HIV-1 MA contains two key signals: the N-terminus myristoyl moiety, which helps in establishing hydrophobic interactions with the membrane, and the highly basic region (HBR) (51-54). The HBR binds membrane via specific electrostatic interactions with $PI(4,5)P_2$, a plasma membrane-specific phospholipid. Mutations in HBR have resulted in membrane binding defect *in vitro* or mislocalization of HIV-1 Gag in HeLa and T cells (13, 34, 55-59). Moreover, depletion of cellular $PI(4,5)P_2$ by overexpressing polyphosphoinositide-5-phosphatase IV (5ptaseIV) has resulted in a defect of Gag binding to the plasma membrane and a reduction in virus release from the cell (13, 34). Altogether, these results suggest that myristoyl moiety, HBR and $PI(4,5)P_2$ interaction are required for Gag localization to the plasma membrane and efficient virus assembly.

In addition to $PI(4,5)P_2$, HIV-1 MA, in particular the HBR, also binds RNA (60-66). Removal of MA-bound RNA by RNase treatment reduces HIV-1 Gag membrane binding specificity to $PI(4,5)P_2$ *in vitro* (55). Based on these results and other studies, we hypothesize that MA-bound RNA prevents HIV-1 Gag from binding cellular membranes that do not contain $PI(4,5)P_2$. This way, MA-RNA interaction ensures specific binding of HIV-1 Gag to the plasma membrane, where $PI(4,5)P_2$ is found (see **Chapter I**). Recent

studies have found that not only *in vitro* synthesized HIV-1 Gag, but the membrane binding of cell-derived HIV-1 Gag are also responsive to RNase treatment, suggesting that RNA-mediated membrane binding inhibition can exist in cellular context as well (67). Interestingly, a recent RNA-seq study reported that HIV-1 MA-bound RNA are in the form tRNA, specifically GluCTC, GluTTC, GlyGCC, GlyCCC, LysCTT, LysTTT, ValAAC, and ValCAC tRNAs (68). Whether these tRNA has specific regulatory role in HIV-1 Gag membrane binding is not yet known.

In polarized T cells, HIV-1 Gag is targeted to a specific site of the plasma membrane known as the uropod (69). The uropod is a plasma membrane protrusion at the rear end of a polarized T cell. This region is enriched in membrane proteins that are also found in the VCC, such as CD9, CD44, CD81 and CD82. Uropod also contain important adhesion molecules such as P-Selectin Glycoprotein Ligand 1 (PSGL-1) and Intracellular adhesion molecules (ICAMs) such as ICAM-1 and ICAM-3 (69-71). Uropod serves as a contact site between cells and participates in virological synapse (VS) formation, where cell-to-cell transmission of HIV-1 occur (71). Interestingly, Gag targeting to the uropod is not MA-dependent; rather, NC-mediated multimerization is required (71). Such NC-dependent multimerization is thought to cluster Gag at the plasma membrane and mediates interactions with uropod-specific microdomains (UDMs), which eventually co-migrate with Gag to the uropod.

The information above describes that HIV-1 Gag uses different mechanisms for targeting to specialized sites in the cell. While HIV-1 MA interactions with $PI(4,5)P_2$ and RNA seem to regulate Gag binding to the plasma membrane in HeLa and T cells, NC-dependent Gag multimerization plays a major role in Gag targeting to the uropod in

polarized T cells. It is not known which of these factors are important in HIV-1 Gag targeting to the VCC in macrophages. Since $PI(4,5)P_2$ is present at the VCC, we hypothesize that HIV-1 Gag localization to VCC is $PI(4,5)P_2$-dependent. Alternatively, Gag-Gag multimerization can play a role in VCC-targeting as well.

To test our hypotheses, we first examined the role of $PI(4,5)P_2$ in VCC targeting of HIV-1 Gag and in HIV-1 assembly. We analyzed the localization of HIV-1 Gag in infected macrophages that are overexpressing 5ptaseIV and quantified HIV-1 release efficiency from these cells. We found that $PI(4,5)P_2$ depletion results in a reduction in HIV-1 Gag targeting to the VCC and that virus release efficiency is reduced in 5ptaseIV-expressing macrophages. Next, we compared a panel of HIV-1 Gag with MA-mutations or multimerization-defective mutations on their abilities to localize to the VCC. Our results indicate that Gag-Gag multimerization is important for specific VCC localization. Altogether, HIV-1 Gag localization to the VCC is NC-dependent, and that $PI(4,5)P_2$ serves as an important membrane anchor, but plays a less important role in targeting Gag to the VCC than in other cells such as HeLa cells and T cells.

MATERIALS AND METHODS

Cells and plasmids. Monocyte derived macrophages (MDM) were obtained from peripheral blood mononuclear cells and isolated from buffy coats from healthy donors (New York Blood Center, NY) as previously described (20). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) for 7 days before they

are used for experiments. 293T cells was cultured and maintained in DMEM (Lonza) supplemented with 10%FBS (HyClone) as described previously (71).

The HIV-1 molecular clones encoding Gag-Venus (pNL4-3/GagVenus) was described previously (34). pNL4-3/KFS/398/IRES-Myc-5ptaseIV and pNL4-3/KFS/398/IRES-Myc-5ptaseIVΔ1 encode Myc-5ptaseIV and an inactive deletion mutant, Myc-5ptaseIVΔ1, respectively, following an internal ribosome entry site (IRES) sequence in place of the *nef* gene as previously described (72). These plasmids were derived from a parental plasmid, pNL4-3/KFS/398, which has the *nef* gene sequence replaced with a sequence containing multiple restriction sites derived from a plasmid, p398-6 (a kind gift from K. T. Jeang) and contain a frameshift mutation (KFS), which disrupts Env expression (73). pNL4-3/GagVenus/KFS/398/IRES-Myc-5ptaseIV and pNL4-3/GagVenus/398/IRES-Myc-5ptaseIVΔ1 were constructed using standard molecular cloning techniques using pNL4-3/GagVenus. pNL4-3/1GA GagVenus, pNL4-3/29KT/31KT GagVenus, pNL4-3/Fyn10-ΔMA GagVenus, pNL4-3/Kmyr-ΔMA GagVenus, pNL4-3/PH-ΔMA GagVenus, pNL4-3/HTMA GagVenus, pNL4-3/delNC GagVenus, pNL4-3/EE75,76AA GagVenus, pNL4-3/GagLZ-Venus, pNL4-3/Gag-LZ4-Venus were also constructed using standard molecular cloning techniques using pNL4-3/GagVenus as described previously (70, 74-76).

Virus stock and infection. Vesicular stomatitis virus G glycoprotein (VSV-G) pseudotyped HIV-1 stocks were prepared as previously described with modification (71). Briefly, 3.4×10^6 293T cells were transfected with 9μg of pNL4-3-derived molecular clones, 9μg of pCMVNLGagPol-RRE and 3μg of pHCMV-G. Two days post-transfection, virus-containing supernatants were filtered through a 0.45-μm filter and

virus particles were pelleted using ultracentrifugation (35,000 rpm, 4C, 45 minutes). Virus pellets were resuspended in 900ul of RPMI-10. VSV-G pseudotyped SIV virus-like particles containing Vpx were prepared by transfecting 9μg of SIV3+ [a kind gift from Dr. Andrea Cimarelli (46)], and 3μg of pHCMV-G into 3.4×10^6 293T cells. Virus particles were then pelleted in the same way as described above.

MDM were first transduced with Vpx-containing VLPs (SIV-Vpx) for 2 hours and then incubated with pseudotyped HIV-1 particles with high titer virus stocks for 6 more hours. Virus-containing supernatants were then removed and cells were washed and cultured for additional 48 hours (Fig. 4.1A).

Virus release assay. Virus release assay was performed as previously described with modifications ((13). Briefly, MDM were treated with SIV-Vpx followed by infection using pseudotyped HIV-1 molecular clones. At 48 h post-infection, culture medium was changed to RPMI-1640 lacking both methionine (Met) and cysteine (Cys) and supplemented with 2% FBS [RPMI-2 (−Met/−Cys)] and incubated for 30 min. Subsequently, these cells were metabolically labeled with [^{35}S]Met/Cys (Perkin-Elmer) in fresh RPMI-2 (−Met/−Cys) for 4 h. Cell and virion lysates were prepared and subjected to immunoprecipitation with HIV-Ig antiserum (NIH AIDS Research and Preference Reagent Program). The virus release efficiency was calculated as the amount of virion-associated Gag as a fraction of the total amount of Gag synthesized during the labeling period.

Immunostaining and Confocal fluorescence microscopy. For visualization of the plasma membrane, MDM infected with VSVG-pseudotyped HIV-1 particles were incubated with Alexa Fluor 594-conjugated concanavalin A (ConA; Invitrogen) for 5 min

at room temperature before fixation. Cells were then washed and fixed with 4%

paraformaldehyde (Electron Microscopy Sciences) in phosphate-buffered saline (PBS)

for 30 min at room temperature, permeabilized in PBS containing 0.1% Triton-X for 2

min, and washed thoroughly with PBS. The cells were immunostained with anti-CD81

antibody (#555675, BD Biosciences Pharmingen. San Diego, CA) for 1 h at room

temperature, washed twice with PBS, and stained with goat anti-mouse IgG conjugated to

Alexa Fluor 647 (Invitrogen). The immunostained cells were subsequently washed with

PBS and mounted. Cells were visualized using Leica confocal fluorescence microscope.

Pearson's correlation coefficients of ConA and GagVenus as well as CD81 and

GagVenus were calculated using the Coloc2 plugin in the ImageJ software program. Ten

cells per donor were analyzed for each condition. MDM from at least 2 donors were used

in the experiments.

RESULTS

SIV-Vpx transduction increases HIV-1 infectivity in macrophages but does not change CD81 staining pattern

HIV-1 is known to replicate poorly in myeloid cells such as monocytes,

macrophages and dendritic cells (1, 4, 6). This is mainly due to the presence of a

restriction factor known as SAM domain and HD domain-containing protein 1

(SAMHD1), which depletes cellular dNTPs, thereby reducing HIV-1 reverse

transcription efficiency (43). On the other hand, SIV_{sm}/HIV-2 are able to replicate

efficiently in myeloid cells. Recent studies reveal that this phenomenon is attributed to

the presence of Vpx protein, which targets SAMHD1 for degradation, resulting in successful reverse transcription and infection (44). HIV-1 does not contain *vpx* gene; however, transduction of Vpx-containing virus like particle (SIV-Vpx) prior or during infection is able to relieve restriction and enhance HIV-1 infection (46, 47).

We first compared the effect of SIV-Vpx transduction on GagVenus expression level in monocyte-derived macrophages (MDM). The general scheme of SIV-Vpx transduction followed by HIV-1 infection of MDM is shown in Fig. 4.1A**.** As expected, we found that more cells express GagVenus in MDM that were transduced with SIV-Vpx (Fig. 4.1B). We next compared the localization of CD81, a VCC marker, in uninfected and HIV-1-infected cells with or without SIV-Vpx transduction. HIV-1 infection in MDM without SIV-Vpx transduction was allowed to progress up to 6 days post-infection. We found that CD81 localize to both the plasma membrane and intracellular compartments regardless of SIV-Vpx transduction (Fig. 4.1C). Moreover, GagVenus co-localize with CD81 in all conditions, suggesting that SIV-Vpx does not alter GagVenus localization in MDM.

PI(4,5)P$_2$ depletion reduces HIV-1 release efficiency from MDM

We previously reported that 5ptaseIV overexpression, which depletes cellular PI(4,5)P$_2$, in HeLa cells and T cells significantly reduced HIV-1 release efficiency (20, 34, 72, 74). To assess the role of PI(4,5)P$_2$ in HIV-1 release in MDM, we infected SIV-Vpx-transduced MDM with HIV-1 molecular clone encoding full-length 5ptaseIV or the catalytically inactive 5ptaseIV-Δ1. At 48 hours post-infection, cells were metabolically labeled with [^{35}S]Met/Cys for 4 hours and ^{35}S-labeled HIV-1 Gag in cell and virus lysate were immunoprecipated using HIV-Ig. We found that HIV-1 release efficiency is reduced

by more than 3-fold in 5ptaseIV-expressing cells than in 5ptaseIV-Δ1-expressing cells (Fig. 4.2A, 4.2B). This indicates that PI(4,5)P$_2$ is important for efficient release of HIV-1 from MDM.

PI(4,5)P$_2$ is required for HIV-1 Gag localization to the VCC in MDM

In addition to virus release, we previously also demonstrated that PI(4,5)P$_2$ is required for HIV-1 Gag localization to the site of assembly, the plasma membrane, in HeLa and T cells (20, 34, 72, 74). To assess whether PI(4,5)P$_2$ depletion can influence HIV-1 Gag localization to the VCC in MDM, we transduced MDM with SIV-Vpx followed by infection using pseudotyped HIV-1 particles containing GagVenus and either full-length 5ptaseIV or 5ptaseIV-Δ1. In 5ptaseIV-Δ1-expressing cells, we found that GagVenus colocalized with CD81 (Fig. 4.3A). In contrast, in full-length 5ptaseIV-expressing cells, HIV-1 GagVenus failed to localized to the VCC. Instead, GagVenus displayed hazy cytosolic signal or is mislocalized to CD81-negative compartments (Fig. 4.3A).

To quantitatively measure the effect of PI(4,5)P$_2$ depletion on GagVenus localization to the VCC on a single-cell basis, we measured the Pearson's correlation coefficient (PCC) between GagVenus and CD81 signals from confocal images (Fig. 4.3B). In this analysis, we found that GagVenus and CD81 showed higher PCC ($\approx$0.50) in 5ptaseIV-Δ1-expressing cells than in 5ptaseIV-expressing cells (PCC $\approx$ 0.19). Altogether, these data support that PI(4,5)P$_2$ is required for HIV-1 Gag localization to the VCC in MDM.

Gag targeting to the VCC requires myristate moiety but not intact HBR

Previous studies in HeLa cells demonstrate that the interaction between HIV-1 MA and PI(4,5)P$_2$ is important for HIV-1 Gag localization to the plasma membrane (13, 34). In particular, the two signals present in HIV-1 MA, the myristoyl moiety and the highly basic region (HBR), are essential for proper targeting and binding of Gag to the plasma membrane. To test whether membrane binding is necessary for HIV-1 Gag localization to the VCC, we transduced MDM with SIV-Vpx and infected them with HIV-1 molecular clone containing GagVenus that has myristoylation-defective mutation (1GA GagVenus) (Fig. 4.4A, 4.4B). We found that 1GA GagVenus displayed hazy cytosolic signal in MDM, indicative of membrane binding defect, and does not colocalize with CD81 (Fig. 4.4B, Fig. 4.4C). Consistent with this, PCC of 1GA GagVenus and CD81 (≈ 0.2) is lower than PCC of WT GagVenus and CD81 (Fig. 4.4C). This indicates that myristoyl moiety of HIV-1 Gag is required for membrane binding in MDM and that membrane binding is required for VCC localization.

We next tested the role of HBR in Gag targeting to the VCC. Previous studies have shown that mutations in the HBR results in HIV-1 Gag relocalization to perinuclear region in HeLa cells and a reduced binding efficiency to PI(4,5)P$_2$-containing membrane *in vitro* (34, 77). To see if HBR is important in Gag targeting to the VCC, we infected MDM with pseudotyped HIV-1 particles encoding GagVenus that contains basic-to-neutral mutations in the HBR (Fig. 4.4A) (34, 77). Surprisingly, we saw that 29KT/31 KT GagVenus colocalized with CD81 with similar PCC as WT GagVenus and CD81 (Fig. 4.4B, 4.4C). Basic-to-acidic mutation of the same amino acid residues (29KE/31KE) in HIV-1 Gag was previously reported to also show colocalization with

CD81 (23). Thus, this suggests that an intact HBR is not required for Gag targeting to the VCC.

MA-RNA interaction in not required for Gag localization to the VCC

In addition to PI(4,5)P$_2$, HIV-1 MA also interacts with RNA (55, 60-67). Our recent study showed that removal of the MA-bound RNA by RNase treatment enables HIV-1 Gag to bind negatively charged liposomes containing phosphatidylserine (PS) even in the absence of PI(4,5)P$_2$ *in vitro* (55). In contrast to HIV-1 Gag, we found that membrane binding of HTLV-1 Gag or the chimeric HIV-1 Gag protein, containing HTLV-1 MA in place of HIV-1 MA (HTMA Gag or HTLV-1 MA GagLZ) do not require PI(4,5)P$_2$. In addition, membrane binding of HTLV-1 Gag and HTLV-1 MA GagLZ are not responsive to RNase treatment (74, 78). We found that HTLV-1 Gag and the chimeric Gag proteins also localize to both the plasma membrane and intracellular compartment in HeLa cells, consistent with the lack of PI(4,5)P$_2$-dependence and RNA-mediated membrane binding inhibition (74, 78).

To test whether Gag localization to VCC requires MA-RNA interaction, we constructed an HIV-1 molecular clone encoding Gag chimera in which the HIV-1 MA is replaced with HTLV-1 MA [HTMA Gag (74)] (Fig. 4.4A). When MDM are infected with this construct, we found that HTMA Gag colocalized with CD81 with similar efficiency as WT GagVenus (Fig. 4.4B, 4.4C). This suggests that RNA-mediated membrane binding inhibition is not required for HIV-1 Gag localization to the VCC.

HIV-1 MA is not required for VCC localization in MDM

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To further study the role of membrane binding in Gag localization to the VCC, we replaced the HIV-1 MA with various membrane binding motifs, namely: (i) PH domain, which binds to PI(4,5)P$_2$ (32, 33); (ii)K-myr, which contains a myristoylation signal and the K-Ras4B polybasic sequence and binds to membrane through myristoylation and basic residues (79, 80); (iii) Fyn(10), which contains N-terminal 10 residues of Fyn kinase encoding myristoylation and dual-palmitoylation signals and binds membrane via hydrophobic interactions (34) (Fig. 4.5A). These MA-replacement mutants were introduced into HIV-1 molecular clones encoding GagVenus, and the pseudotyped virus particles were generated and used to infect MDM. Interestingly, we found that, similar to WT GagVenus, PH-ΔMA-GagVenus, Kmyr-ΔMA-GagVenus and Fyn(10)- ΔMA-GagVenus are able to colocalize with CD81 (Fig. 4.5B, 4.5C), indicating that HIV-1 MA is not required for VCC localization in MDM. Altogether, our data suggest that HIV-1 MA's role in membrane binding is necessary for Gag localization to the VCC, but its role in Gag targeting is dispensable from VCC localization. We also found that PI(4,5)P$_2$ mainly serves as an important membrane anchor that facilitate HIV-1 Gag membrane binding.

Higher-order multimerization is required for HIV-1 Gag localization to the VCC in MDM

The lack of requirement of HIV-1 MA for VCC localization suggests that downstream region of HIV-1 Gag may be important for targeting to the VCC. Our previous work looking at HIV-1 Gag targeting to uropod in polarized T cells demonstrated that NC-dependent multimerization is necessary for this specific localization (70, 71). To test whether multimerization is similarly important for specific

targeting to the VCC in MDM, we tested a panel of HIV-1 Gag containing various multimerization mutations (Fig. 4.6A).

We first tested EE75,76AA Gag, an HIV-1 Gag mutant with mutation in the CA domain. This mutant was previously shown to form electron-dense patches at the plasma membrane, suggesting their abilities to multimerize, but are not able to cause membrane curvature or form virus particles (70, 76, 81). We found that GagVenus containing EE75,76AA mutation was able to colocalize with CD81 as efficiently as WT Gag (Fig. 4.6B, 4.7A). These results are analogous with our previous findings in that uropod localization of HIV-1 Gag does not require membrane curvation or full particle formation (70).

We next examined if NC is required for VCC targeting. We first compared the ability of HIV-1 delNC Gag, an NC-deleted HIV-1 Gag mutant, to localize to the VCC. delNC Gag was previously shown to bind membrane but display lower level Gag-Gag interaction than WT Gag in HeLa and T cells (71, 82). We found that deletion in NC results in Gag failing to localize specifically to CD81 (Fig. 4.6B, 4.7A). Interestingly, delNC Gag are found at the plasma membrane and binding to tubular-like intracellular compartments (Fig. 4.6B). The PCC of delNC GagVenus and ConA, a plasma membrane marker, show that they colocalize with relatively high efficiency (Fig. 4.7B). In contrast to this, WT Gag showed lower colocalization with ConA as most Gag puncta are found in the intracellular compartments in MDM (Fig. 4.6B, 4.7B). These results suggest that NC is required for specific targeting of HIV-1 Gag to the VCC, which is reminiscent of the NC-dependent Gag targeting to uropod in T cells.

We next examined how NC contributes to VCC targeting. Specifically, we wanted to know if NC contributes to the targeting by promoting Gag-Gag multimerization, as was previously observed in polarized T cells. To test this, we replaced HIV-1 NC with a leucine zipper dimerization motif (GagLZ) (Fig. 4.6A). GagLZ was previously shown to multimerize efficiently and are able to form virus-like particles that are indistinguishable from WT-HIV-1 Gag (83). We found that GagLZ restored colocalization with CD81 to similar extent as WT Gag and that less Gag is found on the plasma membrane (Fig. 4.6B, 4.7A, 4.7B). This indicates that the key role of NC in targeting to the VCC is to serve as a multimerization domain for promoting higher order interactions between Gag proteins.

Finally, we examined the minimal level of multimerization that is needed for Gag targeting to the VCC. Our previous studies demonstrated that an LZ domain mutant that forms a tetramer (LZ4) is able to restore Gag localization to the uropod in T cells (70, 84) (Fig. 4.6A). Thus, it is possible that similar level of multimerization is sufficient to target Gag to VCC in MDM. Interestingly, we observed that Gag-LZ4 showed an intermediate phenotype between delNC Gag and GagLZ (Fig. 4.6B). Although Gag-LZ4 was able to colocalize with CD81 at higher PCC than delNC Gag, a large portion of Gag-LZ4 remained at the plasma membrane and colocalize with ConA (Fig. 4.6B, 4.7). This suggests that tetrameric Gag is insufficient to drive Gag targeting to the VCC in MDM, unlike in polarized T cells, where Gag-LZ4 was able to restore specific targeting to the uropod.

Altogether, our data demonstrate that Gag targeting to VCC in MDM share similar mechanism as Gag targeting to the uropod in T cells. We found that MA only

serves as a membrane binder and is not required for targeting. We also found that NC-dependent multimerization plays a key role this process. Interestingly, a higher order of multimerization may be needed for VCC targeting MDM than for uropod targeting in T cells, despite that a whole virus particle formation is not required for these processes.

DISCUSSION

Human primary macrophages represent one of the natural target cells of HIV-1. Despite its importance, very little is known about HIV-1 replication in these cells. In particular, how HIV-1 Gag is targeted to the site of assembly, known as the VCC, in macrophages is not well understood. In this study, we found that the targeting mechanism of HIV-1 Gag to the VCC is driven by the NC domain, resembling the mechanism of Gag targeting to the uropod in polarized T cells. We observed that deletion of NC results in a failure of Gag to specifically localize to with CD81, a VCC marker, and are found to localize at the plasma membrane instead (Fig. 4.6 and 4.7). In contrast, WT Gag or Gag mutants that are capable of higher-order of multimerization (such as EE75,76AA Gag and GagLZ) are found mostly in the intracellular compartments and colocalizing with CD81. Based on these results, we propose a working model in which NC-mediated multimerization facilitates Gag movement from the plasma membrane to the VCC (Fig. 4.8). In this model, we propose that VCC are connected to the plasma membrane, and that HIV-1 Gag (i) is initially targeted to the plasma membrane, (ii) binds to the membrane, (iii) begins to multimerize and (iv) is subsequently transported to the VCC. Gag multimerization may result in reorganization of microdomains and/or promote the association with VCC-specific microdomains. While the presence and identity of VCC-

specific microdomain is beyond the scope of this study, we speculate that it is likely to comprise of membrane proteins that are enriched in the VCC, such as CD9, CD18, CD36, CD53 , CD81 and CD82 (18, 25, 29, 31). Interestingly, delNC Gag does not only localize to the PM but it localizes to a seemingly tubular membranenous webs as well. Therefore, it is tempting to speculate that VCC are not only connected to the cell surface but are interconnected intracellularly. Consistent with this idea, PH-GFP, a PI(4,5)P$_2$ marker, does not fully colocalize with CD81 in the intracellular compartments in MDM (28). Instead, PH-GFP was found to label the plasma membrane as well as CD81-negative intracellular compartments. This suggests that other seemingly intracellular compartments may contain PI(4,5)P$_2$, possibly enabling Gag to bind to these membranous compartments. While we propose that HIV-1 Gag multimerization drives VCC localization, what determine VCC as the site of assembly and how HIV-1 Gag distinguishes VCC from any other intracellular compartments is not known.

The role of tetherin in retaining virus particles at VCC remains controversial. In one study, Chu et al. reported that knockdown of tetherin by RNAi redistributes and reduces the size of Gag-positive VCC, hence implicating tetherin in the formation of VCC (27). On the other hand, Giese et al. argued that tetherin does not influence the integrity of VCC [or Intracellular plasma membrane-connected compartments (IPMC)], and that the presence or absence of Vpu -- the virus-encoded tetherin antagonist -- does not influence Gag localization pattern in macrophages (41). Consistent with this finding, we find that Gag localization to the VCC is unlikely to be a consequence of tetherin effect. EE75,76AA Gag was previously shown to be unable to recruit tetherin to the site of assembly in HeLa cells (76). However, in this study, we found that both WT Gag and

EE75,76AA Gag are able to localize to VCC in MDM, suggesting that Gag targeting to VCC is independent of tetherin recruitment (Fig. 4.6). Nevertheless, one major caveat of this experiment is that cell-type differences may influence the phenotypes of this EE75,76AA Gag mutant (i.e., the ability to recruit tetherin), and more in-depth studies on Gag-tetherin interaction in macrophages needs to be carried out.

Our study showed that HIV-1 MA can be replaced with any membrane binding motifs, such as PH domain, Kmyr or Fyn(10), suggesting that the HIV-1 MA contributes to VCC localization by serving as a membrane binder. We also found that $PI(4,5)P_2$ remains important for efficient virus release and Gag localization to the VCC (Fig. 4.2 and 4.3). Thus, these data suggest that MA-$PI(4,5)P_2$ interaction is required for Gag binding to the membrane, thereby allowing Gag to multimerize and be targeted to the site of assembly.

Our previous study looking at the role of HTLV-1 MA in Gag targeting and membrane binding showed that, unlike HIV-1 MA, HTLV-1 MA membrane binding does not require $PI(4,5)P_2$ and is not inhibited by RNA (74, 78). Consistent the lack of $PI(4,5)P_2$ requirement and RNA-mediated inhibition, we observed that Gag containing HTLV-1 MA (HTMA Gag) localizes to not only the plasma membrane but intracellular compartments as well in HeLa cells. In contrast the observations in HeLa cells, in MDM, we found that HTMA Gag is able to localize specifically to the VCC (Fig. 4.2). This further emphasizes that HIV-1 MA domain does not play significant role in Gag targeting to the VCC. However, we do not eliminate the possibility that HTMA Gag, due to its lack of $PI(4,5)P_2$-dependence and RNA-mediated membrane binding inhibition, may initially be targeted to both the plasma membrane and intracellular CD81-negative compartments,

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such as endosomal compartments, and subsequently fuse with the expanding VCC during infection.

In conclusion, our study provides crucial insights into the molecular mechanism of Gag targeting during HIV-1 assembly in macrophages. We showed that HIV-1 Gag targeting to the site of assembly in macrophages is a two-step process: successful membrane binding via $PI(4,5)P_2$-MA interaction, followed by NC-dependent higher-order multimerization. This multimerization-driven targeting mechanism is reminiscent of Gag targeting to the uropod in polarized T cells. However, unlike in T cells, where tetramers are sufficient to restore uropod-specific Gag localization, multimers of higher than a tetramer may be required for VCC localization.

A

2 hours   6 hours   48 hours

Infect MDM with pseudotyped SIV-Vpx VLPs | Remove virus, wash cells, infect MDM with pseudotyped HIV particles | Remove virus, wash cells, add fresh media | Analysis

B

	Brightfiled (40x)	GagVenus	ConA-Alexafluor594
(-) SIV-Vpx			
(+) SIV-Vpx			

C

	ConA-Alexafluor594	GagVenus	CD81
(-) SIV-Vpx, uninfected			
(+) SIV-Vpx, 2 days post-infection			
(-) SIV-Vpx, 6 days post-infection			
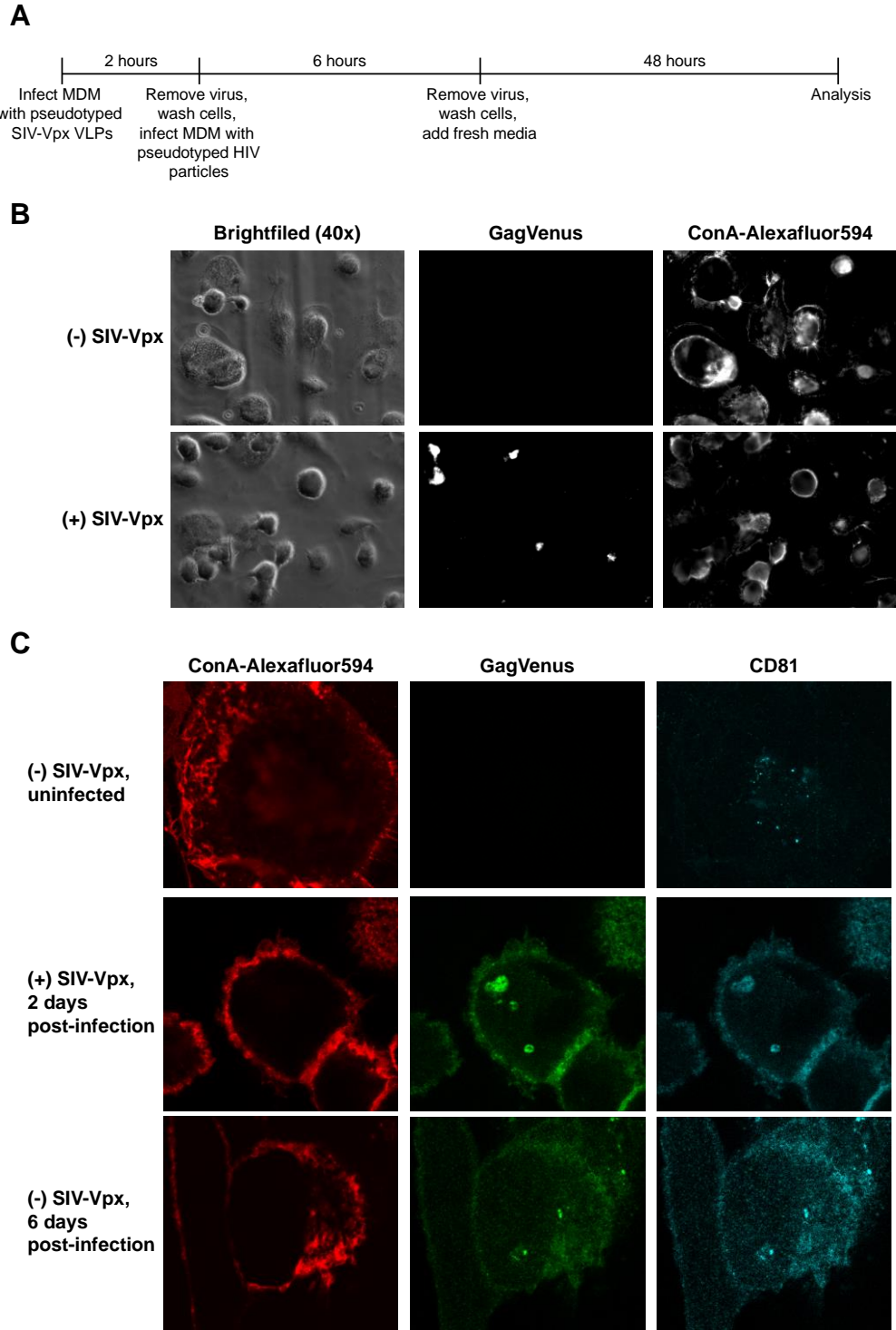
Fig. 4.1. SIV-Vpx VLP transduction does not affect HIV-1 Gag and CD81 localization in MDM. (A) MDM were transduced with SIV-Vpx for 2 hours followed by infection with pseudotyped HIV-1 particles. Cell are then washed and infection is allowed to progress up to 48 hours post-infection. (B) SIV-Vpx transduction improved HIV-1 infection in MDM. (C) SIV-Vpx does not alter HIV-1 Gag and CD81 localizations in MDM.
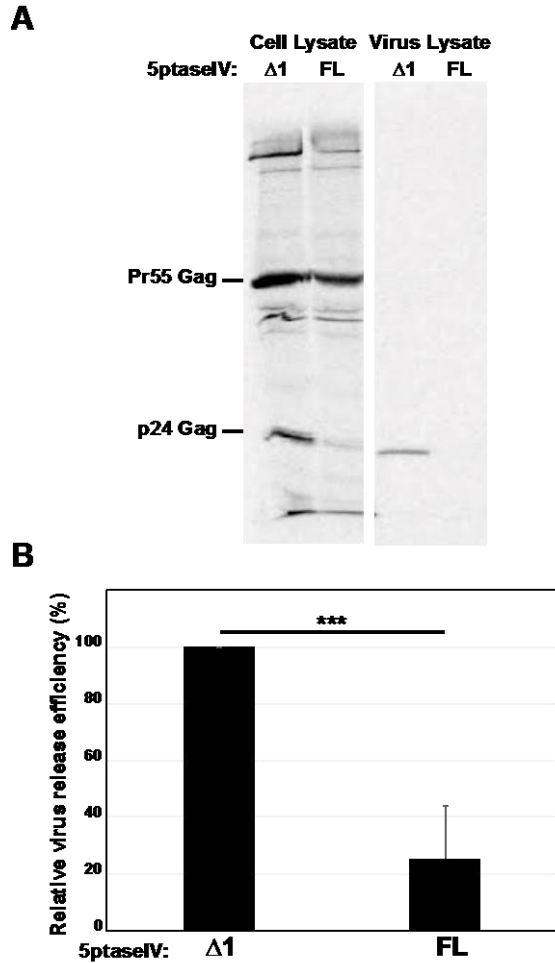
Fig. 4.2. HIV-1 release from MDM is sensitive to 5ptaseIV overexpression. (A) MDM infected with pseudotyped HIV-1 particles along with 5ptaseIV FL or its Δ1 mutant, were metabolically labeled for 4 hours. Cell- and virus-associated Gag were recovered by immunoprecipitation and analyzed by SDS-PAGE. (B) Relative virus release efficiency of HIV-1 were calculated. Data from 7 donors are shown as means ± standard deviations. The average virus release efficiencies by HIV-1 infected MDM along with 5ptaseIVΔ1 were 18.4%. P values were determined using Student's t-test using raw data. ***, P<0.001.

A

B
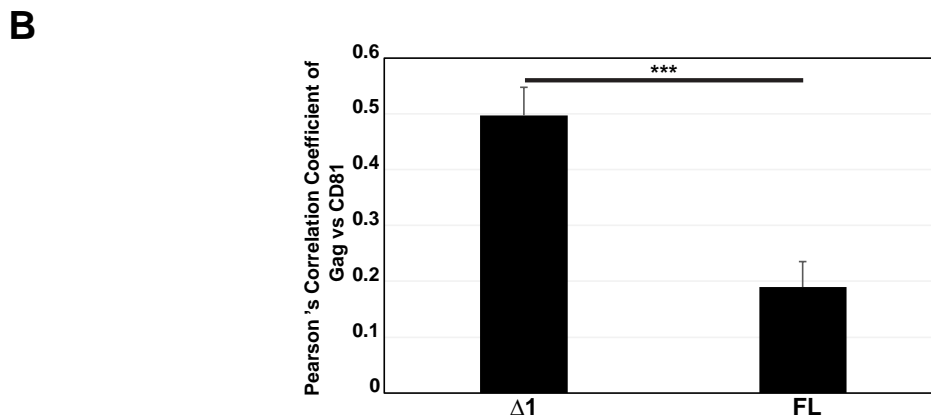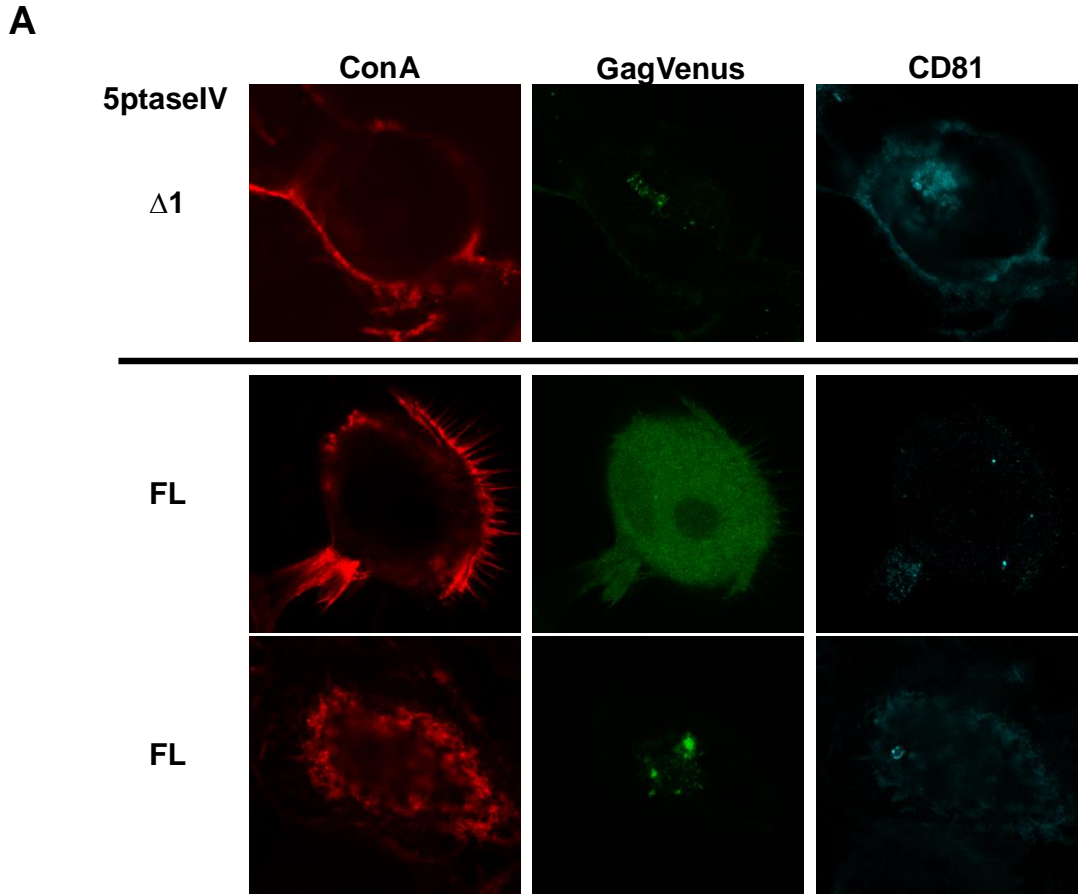
Fig. 4.3. HIV-1 Gag failed to localize to VCC upon 5ptaseIV overexpression. (A) MDM were infected with pseudotyped HIV-1 GagVenus particles along with 5ptaseIV FL or its Δ1 and were stained with ConA labeled with Alexa Fluor 594, fixed and immunostained with mouse monoclonal anti-CD81 antibody and anti-mouse IgG conjugated with Alexa Fluor 647, and analyzed using a confocal fluorescence microscope. Note that HIV-1 GagVenus in 5ptaseIV-expressing cells were unable to localize to CD81-positive sites. (B) Pearson's correlations coefficients (PCC) for colocalization of GagVenus with CD81 were calculated and are shown as means ± SEM. Ten cells were analyzed per condition. ***, P<0.001.
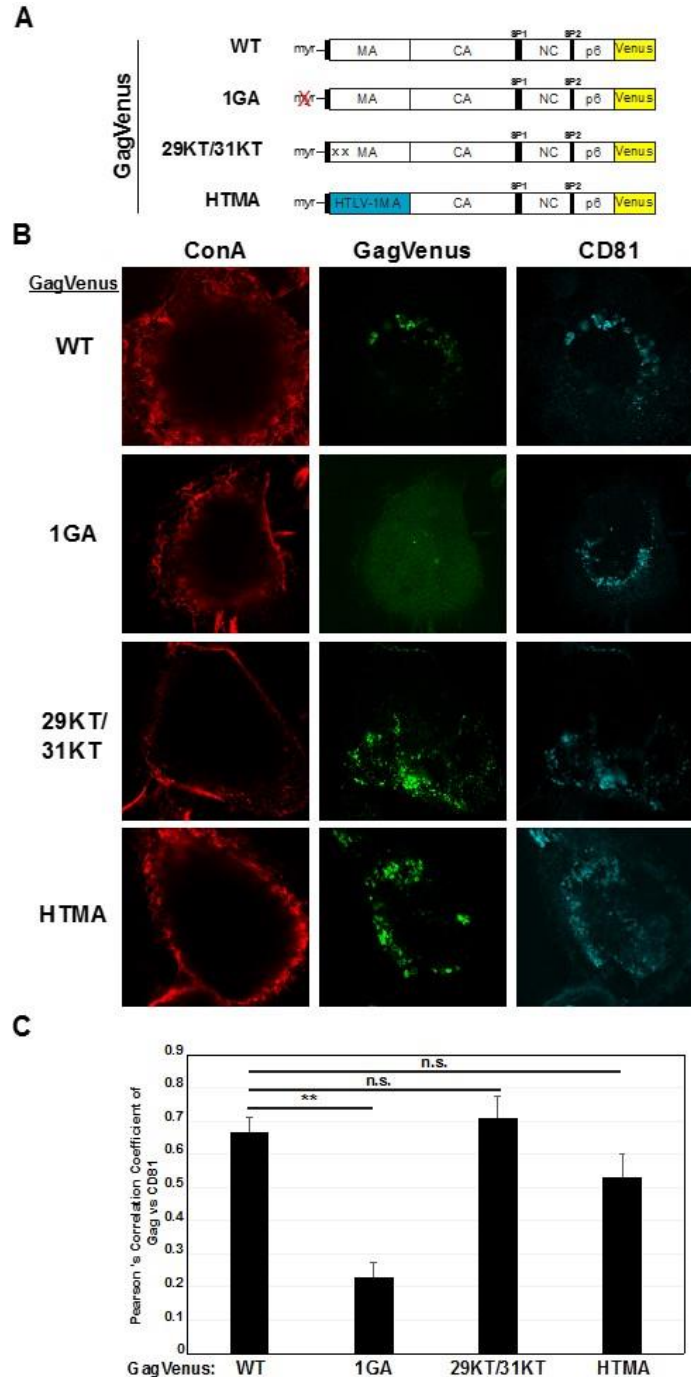
Fig. 4.4. Myristoyl moiety but not intact HIV-1 MA is required for VCC localization. (A) Schematic illustrations of WT, 1GA, 29KT/31KT and HTMA GagVenus are shown. (B) MDM were infected with pseudotyped HIV-1 GagVenus containing MA mutations and were stained with ConA labeled with Alexa Fluor 594, fixed and immunostained with mouse monoclonal anti-CD81 antibody and anti-mouse IgG conjugated with Alexa Fluor 647, and analyzed using a confocal fluorescence microscope. (C) Pearson's correlations coefficients (PCC) for colocalization of GagVenus with CD81 were calculated and are shown as means ± SEM. Ten cells were analyzed per condition. **, $P<0.005$; n.s., not significant.
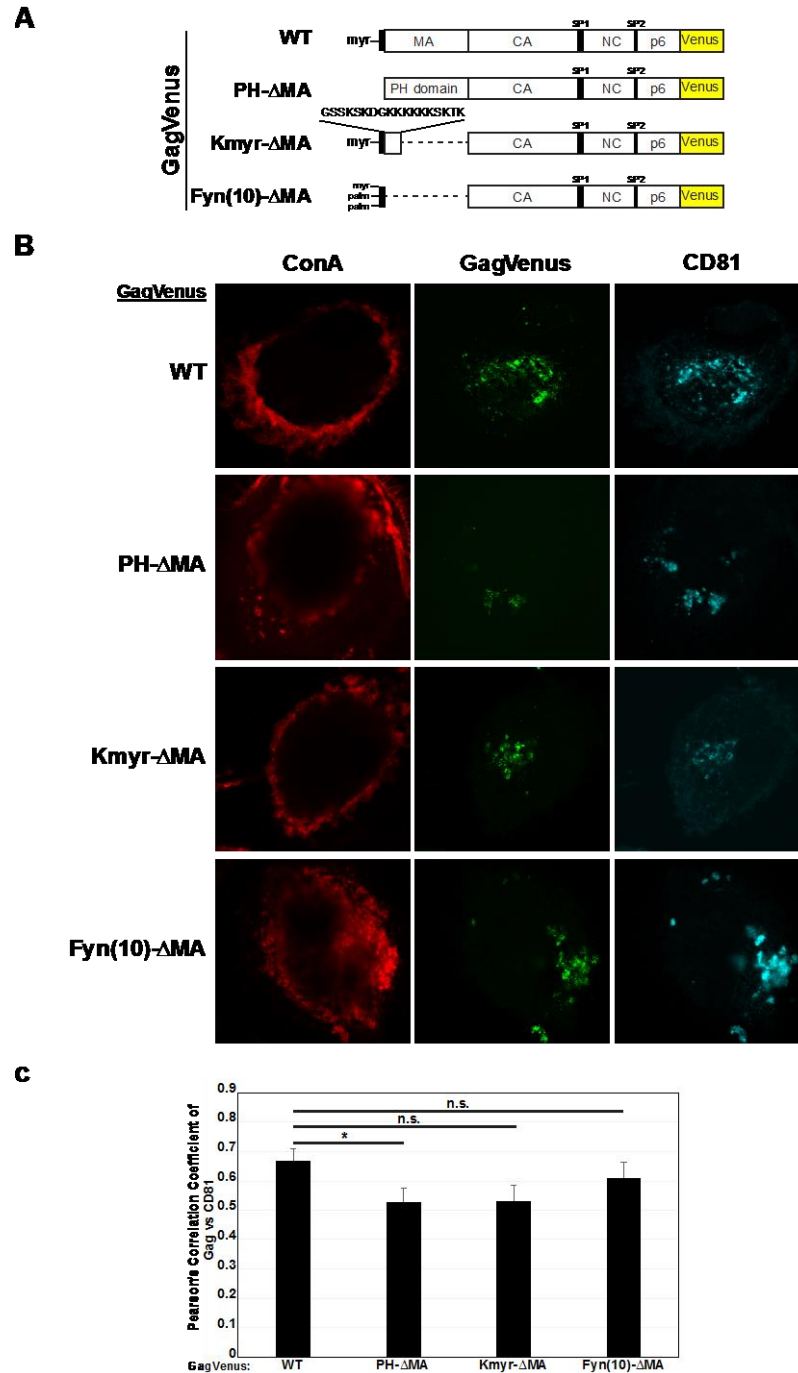
Fig. 4.5. Only membrane binding role of HIV-1 MA is required for VCC localization. (A) Schematic illustrations of WT, PH-ΔMA, Kmyr-ΔMA and Fyn(10)-ΔMA GagVenus are shown. (B) MDM were infected with pseudotyped HIV-1 GagVenus containing MA mutations and were stained with ConA labeled with Alexa Fluor 594, fixed and immunostained with mouse monoclonal anti-CD81 antibody and anti-mouse IgG conjugated with Alexa Fluor 647, and analyzed using a confocal fluorescence microscope. (C) Pearson's correlations coefficients (PCC) for colocalization of GagVenus with CD81 were calculated and are shown as means ± SEM. Ten cells were analyzed per condition. *, P<0.05; n.s., not significant.

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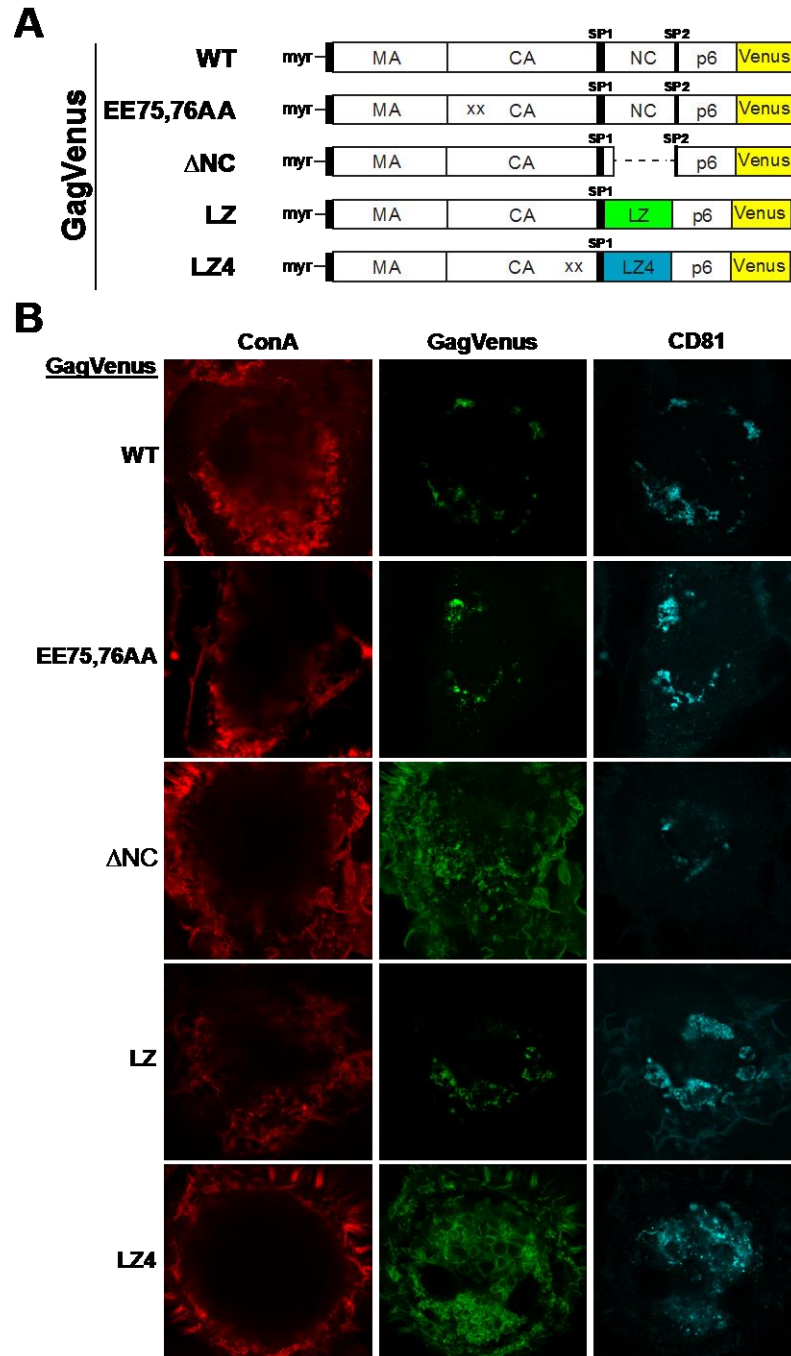
Fig. 4.6. HIV-1 Gag localization to VCC requires higher order multimerization. (A) Schematic illustrations of WT, EE75,76AA, delNC, LZ and LZ4 GagVenus are shown. LZ4 GagVenus also contains WM184,185AA CA mutation to abolish Gag-Gag dimerization. (B) MDM were infected with pseudotyped HIV-1 GagVenus containing NC mutations and were stained with ConA labeled with Alexa Fluor 594, fixed and immunostained with mouse monoclonal anti-CD81 antibody and anti-mouse IgG conjugated with Alexa Fluor 647, and analyzed using a confocal fluorescence microscope. Ten cells were analyzed per condition.
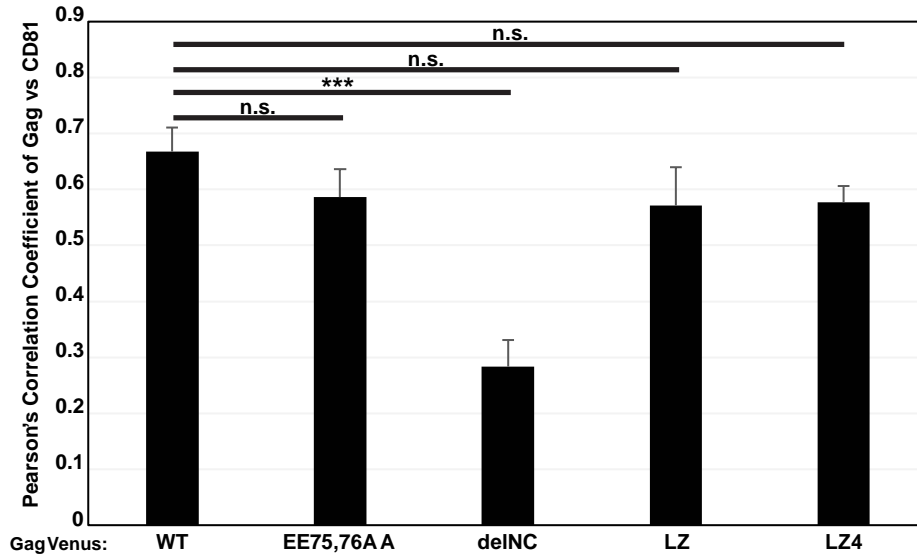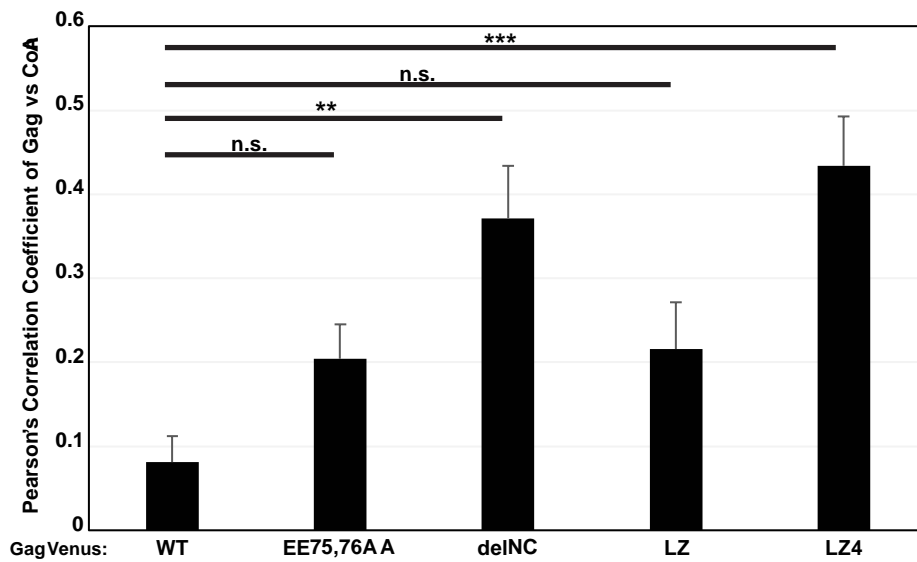
Fig. 4.7. PCC of HIV-1 GagVenus mutants with CD81 or ConA. (A) Pearson's correlations coefficients (PCC) for colocalization of GagVenus containing NC mutations with CD81 were calculated and are shown as means ± SEM. Ten cells were analyzed per condition. ***, P<0.001; n.s., not significant. (B) Pearson's correlations coefficients (PCC) for colocalization of GagVenus containing NC mutations with ConA were calculated and are shown as means ± SEM. Ten cells were analyzed per condition. **, P < 0.005; ***, P<0.001; n.s., not significant.
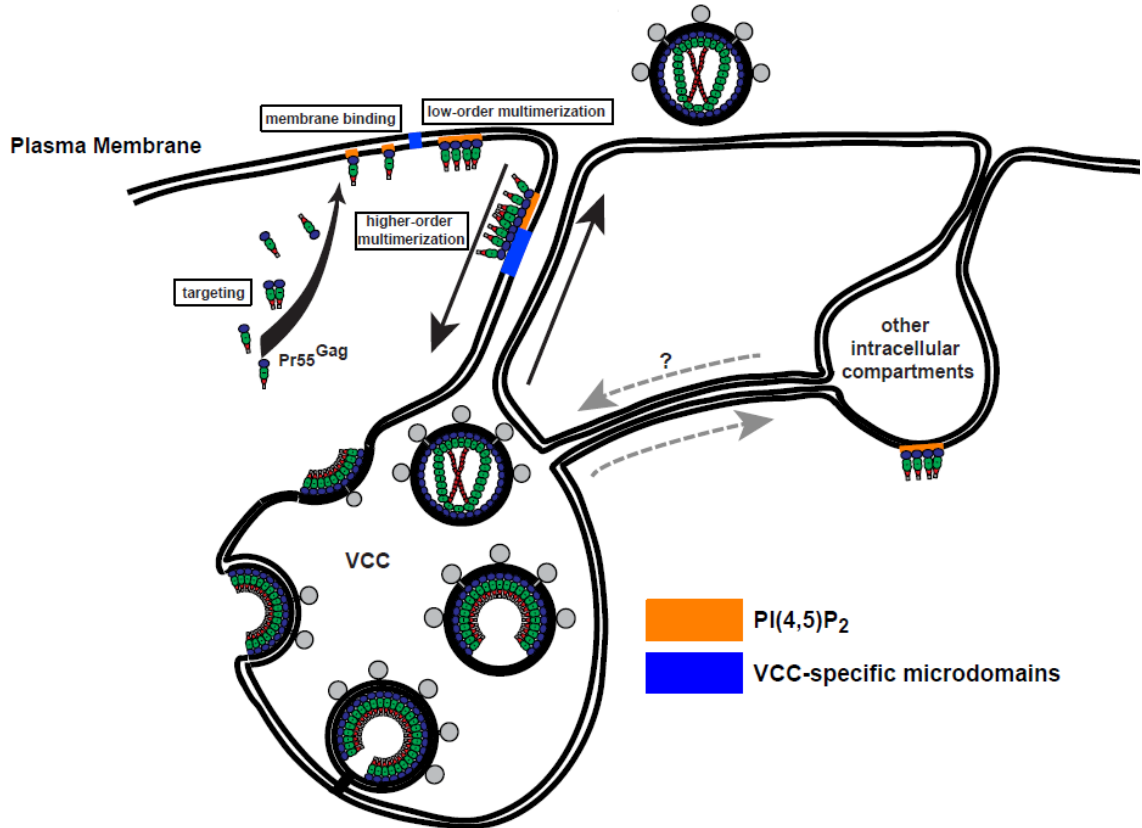
Fig. 4.8. A working model for HIV-1 Gag targeting to the VCC. HIV-1 Gag are synthesized in the cytosol and are first targeted to the plasma membrane. At the plasma membrane, HIV-1 Gag bind to PI(4,5)P$_2$ begin to multimerize in NC-dependent manner. As higher-order multimers begin to form, HIV-1 Gag are transported intracellularly to the VCC where assembly takes place. In addition to being connected with the plasma membrane, VCC may also be connected intracellularly.

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CHAPTER V

DISCUSSIONS AND FUTURE DIRECTIONS

The focus of my thesis is to examine how retroviral Gag drives the assembly process for successful virus production. In Chapter II, I performed a broad analysis of the different retroviral MA in targeting Gag to the site of assembly. Further focusing on HIV-1 assembly, in Chapter III, I examined whether membrane binding inhibition by RNA on HIV-1 Gag can occur in cells and provided evidence that RNA can inhibit HIV-1 Gag membrane binding in a concentration-dependent manner. Finally in Chapter IV, I investigated the molecular mechanism of HIV-1 Gag targeting to the site of assembly in primary macrophages, the VCC. In this chapter, I will summarize the findings from previous chapters, discuss the implications and future directions of these findings and comment on the future of medicine against HIV-1 infection.

SUMMARY OF DATA

(i) Membrane binding mediated by retroviral MA can be divided into two broad categories: those that are PI(4,5)P$_2$-dependent and RNase-responsive, and those that are neither

Previously, we proposed a working model for the regulation of HIV-1 Gag membrane binding in which MA-bound RNA prevents HIV-1 Gag from binding to non-PI(4,5)P$_2$-containing cellular membrane [see Fig.1.4] (1). This RNA-mediated membrane

binding inhibition can be relieved upon binding to PI(4,5)P$_2$, which is predominantly found at the plasma membrane. Therefore, MA-RNA interaction ensures HIV-1 Gag specific binding to the plasma membrane. However, whether PI(4,5)P$_2$ or RNA can regulate membrane binding of other retroviral Gag is not known. In **Chapter II**, in order to broadly study the specific roles of retroviral MA in Gag membrane binding, we constructed HIV-1 GagLZ chimeric proteins, where the HIV-1 MA is replaced with different retroviral MA, each coming from different retroviral genus (2). We observed a correlation between PI(4,5)P$_2$ dependency and RNase responsiveness in the membrane binding of different retroviral MA GagLZ. That is, if the membrane binding of the chimeric GagLZ is PI(4,5)P$_2$-dependent, it is also RNase-responsive. On the other hand, if the chimeric GagLZ does not require PI(4,5)P$_2$ for membrane binding, it is also not RNase-responsive. This correlation seems to extend to the subcellular localization of chimeric GagLZ in cells and their sensitivity to 5ptaseIV overexpression. We found that chimeric GagLZ that require PI(4,5)P$_2$ for membrane binding *in vitro*, localize mainly to the plasma membrane in HeLa cells. Upon 5ptaseIV overexpression, these GagLZ fail to localize to the plasma membrane and their VLP release efficiency is significantly reduced. In contrast, chimeric GagLZ that do not require PI(4,5)P$_2$ for membrane binding *in vitro* are usually found to localize at the plasma membrane and intracellular compartments (with the exception of HERV-K MA GagLZ). The localization and VLP release of these chimeric GagLZ are less sensitive to 5ptaseIV overexpression than the chimeric GagLZ that are PI(4,5)P$_2$-dependent. Moreover, a comparison of previously solved retroviral MA domains showed that all MA contain basic patches. Specifically, GagLZ that are observed to be PI(4,5)P$_2$-dependent and RNase-responsive (HIV-1 MA

and RSV MA) show bigger surface exposed basic patches than those whose membrane binding do not require $PI(4,5)P_2$ and are not inhibited by RNA [HTLV-2 MA (HTLV-2 is a close relative of HTLV-1), MLV MA]. Further mutational analyses of HTLV-1 MA showed that size of basic patches correlates with susceptibility to RNA-mediated membrane binding inhibition. Altogether, these results suggest that RNA serves as a general membrane binding block for retroviral MA with large basic patches and that some retroviruses overcome this block either by binding to $PI(4,5)P_2$ or by having smaller basic patches to subvert the inhibition.

(ii) RNA-mediated membrane binding inhibition can occur in cells

Many studies have demonstrated the RNA binding ability of HIV-1 MA; however, it was only recently that RNA was found to have regulatory role in membrane binding of HIV-1 Gag during assembly. Even though cellular-based assays are in support of the results from the *in vitro* liposome binding assays (1-3), whether RNA-mediated membrane binding inhibition actually occurs in cells has not been demonstrated. In **Chapter III**, we modified our *in vitro* liposome binding assay, by using HIV-1 Gag derived from the cytosolic fraction of transfected HeLa cells, to show that cell-derived HIV-1 Gag also require $PI(4,5)P_2$ for efficient liposome binding (4). Furthermore, the membrane binding of these cell-derived HIV-1 Gag are responsive to RNase treatment. Consistent with *in vitro* studies, the HBR of cell-derived HIV-1 Gag is important for membrane binding and RNA-binding. We also determined that RNA can inhibit HIV-1 Gag membrane binding to negatively-charged liposomes (PC:PS liposomes) in a concentration-dependent manner. Since the inhibition can take place at much lower RNA

concentration than the estimated total RNA concentration in HeLa cells, it is likely that RNA-mediated membrane binding inhibition can occur in cells as well.

(iii) NC-dependent multimerization promotes HIV-1 targeting to the VCC in macrophages

Our previous results showed that $PI(4,5)P_2$ is important for HIV-1 Gag targeting and membrane binding to the plasma membrane during virus assembly in HeLa and T cells. Additionally, HIV-1 MA, in particular the HBR, is important for correct Gag targeting in cells and interaction with $PI(4,5)P_2$. Interestingly, we found that HIV-1 Gag targeting to the uropod, the rear-end protrusion of the plasma membrane, was dependent on NC-mediated multimerization in polarized T cells (5). In **Chapter IV**, we sought to understand the targeting mechanism of HIV-1 Gag to the site of assembly in primary macrophages, one of the natural target cells of HIV-1. HIV-1 assembles at the virus-containing compartments (VCC) in primary macrophages. We found that HIV-1 Gag targeting to the VCC also depends on NC-mediated multimerization. Furthermore, $PI(4,5)P_2$ depletion of HIV-1 infected macrophages leads to membrane binding defect or mislocalization of HIV-1 Gag and reduction in virus release. Our analysis of HIV-1 Gag mutants containing different membrane binding motifs in place of MA suggested that HIV-1 MA only contributes to membrane binding and plays a less significant role in Gag targeting to the VCC. Moreover, HIV-1 Gag with deletion in the NC domain failed to localize specifically to the VCC. This defect is rescued when dimerization motif (LZ), which enables Gag to form higher-order multimer, is added back in place of NC. Based on our results, we propose a two-step process of HIV-1 Gag targeting to the VCC: HIV-1

Gag binding to $PI(4,5)P_2$, followed by NC-driven Gag-Gag multimerization, leading to Gag transport to the VCC.

IMPLICATIONS AND FUTURE DIRECTIONS

The role of other phosphoinositides and negatively charged phospholipids in retroviral Gag assembly

The work discussed thus far mainly involves the study of $PI(4,5)P_2$ and an abundant negatively charged cellular phospholipid, phosphatidylserine (PS), in retroviral Gag membrane binding. However, the cell membrane clearly comprises of more than just these lipids (6, 7). Thus, it may be important to understand the roles of other phospholipids in regulating the membrane binding of these retroviral Gag. Our previous *in vitro* studies showed that liposomes containing either $PI(4,5)P_2$ or $PI(3,4,5)P_3$ are able to support efficient binding of HIV-1 Gag (8). It remains to be determined whether RSV MA GagLZ, which also showed $PI(4,5)P_2$ dependency in membrane binding, is able to bind $PI(3,4,5)P3$ efficiently as well. Additionally, our working model propose that $PI(4,5)P_2$ is able to alleviate RNA-mediated membrane binding block. It will also be interesting to further examine the efficiency of other phosphoinositides in helping HIV-1 Gag overcome this membrane binding block by RNA.

In our study, 5ptaseIV overexpression was used to deplete cellular $PI(4,5)P_2$. This enzyme converts $PI(4,5)P_2$ to PI4P (7, 9). In addition, 5ptaseIV is able to hydrolyze $PI(3,4,5)P_3$, which is also present at the plasma membrane albeit at much lower abundance than $PI(4,5)P_2$, yielding $PI(3,4)P_2$. Whether PI4P or $PI(3,4)P_2$ can support

efficient HIV-1 Gag binding has not been tested yet. Interestingly, 5ptaseIV overexpression in HeLa and macrophages not only results in membrane binding defect of HIV-1 Gag, but also causes mislocalization of HIV-1 Gag to perinuclear region or CD63-positive compartments (in HeLa cells) and non-CD81-containing compartments (in macrophages). While the identity of these compartments are not fully known, it is tempting to speculate that these mistargeted HIV-1 Gag can possibly be interacting with other phospholipids in cells, such as PI4P (in Golgi) or $PI(3,4)P_2$ (in endosomal compartments), which may be increased in abundance upon 5ptaseIV perturbations.

Our studies also demonstrate that GagLZ containing the MA domain of HTLV-1, MLV or HERV-K are able to bind PS-containing liposomes efficiently (2). When RNase-treated, HIV-1 Gag, HIV-1 GagLZ and RSV MA GagLZ derivative are also able to bind PS-containing liposomes in the absence of $PI(4,5)P_2$ (1-3). While it was inferred that these Gag proteins bind negatively charged liposomes via non-specific electrostatic interactions, further studies need to be performed to rule out the possibility that PS can play a specific role in retroviral Gag membrane binding. As a preliminary study, we compared the membrane binding efficiencies of chimeric GagLZ to other negatively charged phospholipids, namely, phosphatidylglycerol (PG) and phosphatidic acid (PA) (Figure 5.1). We found that HTLV-1 MA GagLZ, MLV MA GagLZ and HERV-K MA GagLZ are able to bind liposomes that contain either PS, PG or PA (PC:PS, PC:PG and PC:PA, respectively) with similar efficiencies. Importantly, HIV-1 GagLZ and RSV MA GagLZ are unable to bind to these liposomes prior to RNase treatment, further demonstrating that $PI(4,5)P_2$ is required for their efficient membrane binding. However, upon RNase treatment, both HIV-1 GagLZ and RSV MA GagLZ are able to bind PC:PS,

PC:PG and PC:PA liposomes with similar efficiencies. Overall, our data support that *in vitro*, GagLZ containing MA domains of HTLV-1, MLV or HERV-K are able to bind to membrane via electrostatic interactions. Additionally, removal of RNA from HIV-1 MA and RSV MA also allows these chimeric GagLZ to bind to negatively charged liposomes via electrostatic interactions.

PS is the most abundant negatively charged phospholipid (5-10%) in the cell, whereas PG and PA are found in lower abundance (roughly 1%) (10, 11). Given the large quantity of PS in the cell, retroviral MA may still prefer to bind to PS over other negatively charged phospholipids. Thus, further studies examining the effect of PS pertubrations in cells, either by PS-decarboxylase overexpression or knockdown of phosphatidylserine synthases (11), on Gag localization and virus release efficiency will provide a better understanding of the role of PS in retrovirus assembly.

Identifying cellular proteins that have similar membrane binding properties as HIV-1 Gag

As mentioned in **Chapter I**, HIV-1 MA share some structural similarities with $PI(4,5)P_2$-binding cellular proteins, such as the PH domain of PLCδ1. Both HIV-1 Gag and the PH-domain contain basic cluster that interacts specifically with the phospholipid heagroup of $PI(4,5)P_2$ (7, 12). HIV-1 MA also interacts with RNA and that RNA is able to regulate HIV-1 Gag membrane binding. However, whether RNA can regulate membrane binding of cellular proteins, such as PLCδ1, is not known. To begin identifying cellular proteins that are both $PI(4,5)P_2$-dependent and RNase responsive, we collected cellular proteins that are found in the cytosolic fraction of HeLa cells as

previously mentioned in **Chapter III**. The proteins are treated with or without RNase, and are allowed to bind to either PS-containing liposomes (PC:PS) or $PI(4,5)P_2$-containing liposomes [PC:PS + $PI(4,5)P_2$]. The mixtures were subjected to membrane floatation centrifugation and the cellular proteins that are found in the membrane-bound fractions are visualized by SDS-PAGE followed by silver staining (Fig. 5.2). Surprisingly, we found that many cellular proteins show increased binding to PC:PS liposomes upon RNase treatment. We also found that some cellular proteins show more efficient membrane binding to $PI(4,5)P_2$-containing liposomes than PC:PS liposomes (Fig. 5.2). Further analysis of these proteins by mass-spectrometry will help identify cellular proteins whose membrane binding are both $PI(4,5)P_2$-dependent and RNase responsive.

Despite the ease of using cytosolic fractions to identify cellular proteins that have similar membrane binding properties as HIV-1 Gag, one caveat for using such method is that $PI(4,5)P_2$-specific proteins may be found in low abundance in the cytosol as these proteins are already binding to the plasma membrane. Thus, methods to increase the amount of $PI(4,5)P_2$-binding proteins in the cytosol, such as by 5ptaseIV overexpression, may be necessary. 5ptaseIV overexpression will reduce the plasma membrane binding of $PI(4,5)P_2$-specific proteins, hence increasing their concentrations in the cytosolic fraction.

Identifying MA-bound RNAs that have regulatory role in HIV-1 Gag membrane binding

In **Chapter III**, we found that yeast tRNA is able to inhibit HIV-1 Gag binding to PS-containing liposomes (PC:PS) in a concentration dependent manner *in vitro*. The membrane binding inhibition can occur at a much lower RNA concentration than the

estimated RNA concentration in the HeLa cell. In the same study, we found that the membrane binding of cell-derived HIV-1 Gag are also RNase responsive, suggesting that RNA-mediated membrane binding block can occur in the cell. Thus, it is likely that cellular RNA can regulate HIV-1 Gag membrane binding. Consistent with this finding, a recent study reported that MA-bound RNA in cells almost exclusively comprises of tRNAs, in particular, Glu, Gly, Lys, Val tRNAs (13). However, it is also likely that other RNA, such as viral RNA, can regulate Gag membrane binding. Purohit et al. previously identified an RNA consensus sequence that specifically interact with HIV-1 MA (14). This RNA sequence shares high sequence homology as a region in the Pol open reading frame. This suggests that HIV-1 MA can also interact with genomic RNA (gRNA) at least *in vitro*. HIV-1 NC recognizes gRNA via the psi packaging signal (see **Chapter I**). Thus, it is likely that HIV-1 NC "pre-selects" gRNA and brings it to close proximity with HIV-1 MA. Whether MA selects specific RNA for binding or whether these RNA are pre-selected by HIV-1 NC is currently unknown. Furthermore, it is also not known whether these specific MA-bound tRNA or gRNA are able to regulate HIV-1 Gag membrane binding. Thus, as an initial study, we compared ability of different RNA species in inhibiting HIV-1 Gag membrane binding to PS-containing liposomes. Using the same RNA concentration (100ng/ul), we found that the psi region of the gRNA can inhibit liposome binding as efficiently as yeast tRNA (Fig. 5.3). Interestingly, Lys tRNA, which is used as a primer for reverse transcription and is packaged specifically into the virus particle (15) (see **Chapter I)**, has no inhibitory effect on HIV-1 Gag membrane binding. This result suggests a possibility that not all Gag-interacting RNA have a regulatory effect on HIV-1 Gag membrane binding. In addition, given that both HIV-1

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MA and NC are able to bind RNA, it is not known if both domains have to be engaged to the same strand of RNA for the RNA to have a regulatory effect. If this is the case, longer RNA may have higher inhibitory effect in membrane binding than shorter RNA. Further experiments looking at the roles of HBR and NC, as well as the physical properties of RNA, will shed light on how specific RNA confer inhibitory effect on HIV-1 Gag membrane binding.

Virus assembly at cell surface versus "intracellular" compartments

HIV-1 has proven time and again that it is a highly successful virus. Selecting plasma membrane as a site of assembly certainly benefit HIV-1 with ease of egress and efficient virus transfer to adjacent cells. Similarly, directing HIV-1 Gag to the uropod in polarized T cells is beneficial as uropod contain numerous adhesion molecules and possibly allowing easier establishment of virological synapse. Thus, it seems unlikely that HIV-1 Gag localization to the seemingly intracellular compartments (the VCC) in primary macrophages is a mere coincidence. Macrophages have extremely low dNTP (16) that discourages efficient virus replication. Moreover, given that macrophages are long-lived cells, they may serve as a virus reservoir. Macrophages are also found to be involved in cell-to-cell virus transmission. Indeed, numerous studies have demonstrated that formation of virological synapse (VS) are observed in co-culture of infected macrophages and T cells (17-19). A recent finding show that VCC-localizing HIV-1 Gag is trafficked to macrophage-T cell contact site, and that an HIV-1 MA mutant (29KE/31KE) failed to be recruited to the contact sites (18). This suggests that HIV-1 Gag transport to cell-to-cell contact sites is MA-dependent. However, exactly how Gag is transported to the VS is not yet understood. Further analysis of HIV-1 Gag MA mutants

and its efficiency in transporting to cell contact site may reveal the molecular mechanism of this process.

In **Chapter IV**, we found that deletion of HIV-1 NC results in a failure of Gag to be targeted to the VCC specifically. Furthermore, HIV-1 Gag mutants with low multimerization ability (delNC Gag, Gag-LZ4) are found to localize to the plasma membrane, suggesting that HIV-1 Gag is first targeted to the plasma membrane and is subsequently transported to the VCC (see Fig. 4.8). In polarized T cells, NC-dependent multimerization is also found to drive Gag localization to the uropod. HIV-1 Gag is found to associate with the uropod-directed microdomains (UDM). The UDM comprises of P-selectin glycoprotein ligand 1(PSGL-1), CD43 and CD44 (5, 20). Likewise, HIV-1 Gag in macrophages may also interacts with specific microdomains prior or during its transport to the VCC. Whether this form of Gag-microdomain association is required for VCC localization is not known.

Similar to the uropod, VCC is also enriched in tetraspanins (such as CD81, CD9 and CD53) (21). In addition, Chan et al. previously reported that HIV-1 particles derived from macrophages or T cell line showed similar lipid composition (22). In particular, $PI(4,5)P_2$, cholesterol and GM3 are enriched in virus particles when compared to the plasma membrane lipid composition, consistent with the observation that Gag associates with and causes reorganization of microdomains on the cellular membrane. Unfortunately, the comparison of lipid composition of virus particle versus the VCC was not carried out and may in fact prove to be a challenging task. Thus, it remains to be determined whether the membrane organization of the VCC is similar or different from the plasma membrane or the virus particle. Future studies looking at the association of

membrane markers with HIV-1 Gag at the plasma membrane versus the VCC may reveal the presence (or absence) of VCC-directed microdomains and whether HIV-1 Gag organizes the microdomains at the VCC during infection. Further experiments of siRNA knockdown of some VCC-localized tetraspanin markers may also give clue to the importance of tetraspanin in HIV-1 Gag transport to the VCC.

VCC localization may require higher order multimerization than uropod localization

In **Chapter IV**, we also observed that Gag-LZ4 was unable to fully restore VCC-specific localization. This observation is different in polarized T cells, where Gag-LZ4 was targeted specifically to the uropod (23). LZ4 was previously reported to be able to form tetramer (24). Thus, these results suggest that Gag-tetramer is sufficient for uropod localization but is insufficient for VCC localization. Certainly, one major caveat is that whether Gag-LZ4 truly form tetramers in these cells is not known. Nevertheless, it is tempting to speculate that there could be intrinsic differences in protein transport mechanisms between polarized T cell and MDM. Previous studies reported that Gag multimerization defects can reduce steady-state membrane binding (25-27). For these reasons, Fyn(10) sequence was added to HIV-1 NC mutants (including Gag-LZ4) in the study of Gag localization to the uropod to compensate for the reduced membrane binding (5). In contrast to these observations, multimerization defects did not seem to influence membrane binding of HIV-1 Gag in MDM as Gag was still observed to be localizing to either CD81-containing compartments and/or the plasma membrane efficiently (Fig 4.6B). These observations may suggest that HIV-1 Gag is able to bind membrane more efficiently in MDM than in other cells such as HeLa and T cells. However, transporting

all membrane-bound HIV-1 Gag to the VCC may result in overcrowding at the VCC. Thus, selecting and transporting only HIV-1 Gag with higher order multimer (more than tetramer) to the VCC may serve as additional checkpoints to help prevent overcrowding at the VCC and ensure proper virus assembly.

VCC localization – an HIV-1 specific phenomenon?

Many pathogens other than HIV-1 are found to infect macrophages. Bacteria such as *Salmonella enterica* has been found to replicate in modified phagosomes [known as Salmonella containing vacuole (SCV)] in macrophages (28). *Mycobacterium tuberculosis* is also found to replicate efficiently in macrophages by preventing fusion of phagosomes with lysosomes (29). *Leishmania major* utilize granulocytes as "Trojan horse" to enter and establish infection in macrophages silently (30). Phagosomes containing pathogens such as *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus fumigatus* actively recruits CD82 (31). Moreover, influenza virus is able to infect alveolar macrophages (32) and are found to incorporate CD9 and CD81 into its viral envelope (33). Given that tetraspanin-enriched compartments are found even in the absence of HIV-1 infection in macrophages and that many viruses are reported to associate with tetraspanins (34-36), it seems unlikely that VCC localization could be a HIV-1-specific phenomenon. The difference in experimental procedures and the difficulty in infecting macrophages may be some of the reasons why no other pathogens have yet been found to localize at VCC in macrophages.

Perhaps the lack of motivation in studying virus replication (or specifically, assembly) in macrophages is due to the difficulty in infecting these cells and the high variability from donor to donor. Furthermore, the distribution of macrophages in the body

is widespread and isolation of tissue macrophages is usually inefficient. Thus far, the most relevant *in vitro* HIV-1 infection relies upon monocyte derived macrophages (MDM). Over the years, MDM have served as the standard model for studying HIV-1 infection in macrophages and established protocols in isolation and differentiation of MDM have been reported. The use MDM to study virus-macrophage interaction and along with the advent of SIV-Vpx transduction system, which down-regulates the SAMHD1 restriction factor, will hopefully provide an easier and more standardized way to study retroviral replication in macrophages.

Understanding how HIV-1 Gag operates as one entity during assembly is important

Retroviral assembly is a multi-step process leading to the formation of nascent virions. Despite being stepwise, each structural domain of Gag actually influences one another during assembly. For example, HIV-1 MA is mainly responsible for membrane binding. However, the exposure of myristoyl moiety is dependent on Gag-Gag interaction (that is, CA and NC domains) and binding to PI(4,5)P$_2$ (37-45). Similarly, membrane binding efficiency of HIV-1 Gag has been shown to be influenced by Gag-Gag interaction (25-27). This suggests that downstream sequence of HIV-1 MA can influence membrane binding. Pertaining MA-RNA interaction, it is not known whether MA binds RNA that is pre-selected by the NC or that MA and NC can bind two different RNA strands at one time. Importantly, how NC-RNA interaction influence MA-RNA interaction is not known. Thus, a deeper analysis into how Gag functions as one entity will help in identifying key steps during assembly for the development of more efficient inhibitors to abolish HIV's ability to produce new virions in infected cells.

FUTURE OF ANTIRETROVIRAL MEDICINE

Targeting the membrane binding step of HIV-1 assembly

Our results show that different RNA species can inhibit HIV-1 Gag binding to negatively charged (PS-containing) liposomes to various degrees (Fig. 5.3). Based on our working model, we find that $PI(4,5)P_2$ can successfully outcompete RNA for HIV-1 MA. By harnessing RNA's ability to regulate HIV-1 Gag membrane binding, a therapeutic RNA aptamers can be developed to inhibit HIV-1 replication. One potential direction towards developing this RNA aptamer is by selecting RNA sequence that have higher binding affinity to the HIV-1 MA than $PI(4,5)P_2$. HIV-1 MA association with C4 $PI(4,5)P_2$ was estimated to be 150 ± 30 uM (42). Thus, effective RNA aptamers should be able to bind HIV-1 with Kd values that are lower than of MA-$PI(4,5)P_2$. To date, numerous studies have been performed to identify short-length RNA that can inhibit HIV-1 replication. These studies usually use the *in vitro* selection method known as Systematic evolution of ligands by exponential enrichment (SELEX) to isolate RNA or DNA that bind specifically to HIV-1 proteins or gRNA (14, 46-49). Previously reported RNA ligands that was bound to HIV-1 MA specifically showed Kd values ranging from ~1 to 10nM (49). It remains to be determined if these RNA are able to regulate HIV-1 Gag membrane binding. Further consideration regarding RNA folding structure and whether it is aminoacylated (for tRNA) may be important in designing effective RNA aptamers. As an initial study, our RNA add-back liposome binding assay as mentioned in **Chapter III** can be used to screen for important RNA species that can inhibit HIV-1 Gag

membrane binding. Thus, our study lays groundwork for potential development of therapeutic RNA aptamers that will inhibit HIV-1 infection.

In **Chapter II**, we discovered that RNA can serve as a general inhibitor for retroviral membrane binding. We also found that retroviral MA containing bigger basic patch on its surface is more susceptible to RNA-mediated membrane binding inhibition and that retroviral MA with smaller basic surface patch is less inhibited by RNA. With HIV-1 ability to mutate, revertants are likely to arise upon the introduction of antiretroviral treatments to the cells. These revertants may become resistant to the given antiviral treatments. Thus, when designing inhibitors against HIV-1 infection, it is important to anticipate what type of HIV-1 mutants may appear when such selective pressure is introduced to the virus. Based on my results from **Chapter II**, I speculate that the likely HIV-1 revertants upon the treatment of RNA aptamers will be those containing mutations within the HIV-1 HBR that reduces the overall basic patch size of HIV-1 MA, such that RNA block on membrane binding is subverted.

In addition to developing RNA aptamers that can block HIV-1 membrane binding, one can also identify small molecule inhibitors that block essential component that is involved in HIV-1 Gag membrane binding. Recently, Compound 7 was identified to be effective in blocking HIV-1 MA interaction with PI(4,5)P$_2$ (50, 51). While effective, this drug is still at its infancy and more study is needed to identify the mechanism of this inhibition. Our previous study looking at HIV-1 replication in PI(4,5)P$_2$-minimal T cells have identified a mutation in HIV-1 MA (74LR) that adapts to this selection pressure by increasing its infectivity in T cells (52). Whether compound 7 will result in similar HIV-1 revertant has not yet been tested.

Targeting the Gag multimerization step of HIV-1 assembly

Our study looking at HIV-1 assembly in polarized T cell and macrophages have suggested the importance of NC-mediated multimerization in targeting Gag specifically to the site of assembly. In HeLa and T cells, high order HIV-1 Gag multimerization usually occurs after the arrival of HIV-1 Gag to the site of assembly, that is, the plasma membrane. However, it seems that Gag-Gag multimerization happens earlier during the assembly in polarized T cells and macrophages, as this step is required prior to the arrival to the site of assembly. Since multimerization is likely occur earlier, the use of inhibitors that disrupt Gag-Gag multimerization may be more effective in polarized T cells and macrophages than in other cells. As mentioned in **Chapter I**, NC promote Gag multimerization by binding to RNA and using it as a scaffold for higher order Gag interactions. NC binds RNA via its zinc finger motif, which is highly conserved among all retroviruses. To date, there are many compounds that target HIV-1 NC by ways of disrupting the zinc finger motifs or ejecting the zinc cations (50). For example, zinc ejector compound such as cystamine and Pyridinioalkanoyl thioesters (PATEs) were shown to have low cytotoxicity and are effective in reducing HIV-1 replication in MDMs or monocytic U1, a cell line that is chronically infected with HIV-1 (53-55). The mode of action of these zinc ejectors in inhibiting virus replication is not known, although it was proposed that it could potentially disrupt NC-RNA binding and reverse transcription. Whether PATEs and similar compounds inhibits HIV-1 replication by mistargeting Gag to non-VCC or non-uropod sites in macrophages or polarized T cells, respectively, is not known.

CONCLUSION

Membrane binding and targeting of retroviral Gag proteins to subcellular sites are important steps during retroviral assembly. The basic patch found on the retroviral MA are important regions that facilitates MA-lipid interaction, MA-RNA interaction and Gag targeting to the site of assembly. Additionally, our cell-derived liposome binding assay has provided evidence that RNA-mediated inhibition of HIV-1 Gag membrane binding can occur in cells. Such study will lay groundwork for the discovery of relevant cellular/viral RNA that have regulatory effects on HIV-1 Gag membrane binding in cells. Extending our cell-derived liposome binding assay, cellular proteins that share similar membrane binding properties as HIV-1 Gag (that its, $PI(4,5)P_2$-dependent and RNase responsive) can be identified. Such analysis will further our understanding in the role of RNA in regulating the membrane binding of cellular proteins. Finally, our study on HIV-1 assembly in macrophages has revealed an important mechanism of HIV-1 Gag targeting to the VCC. Further examination of Gag movement from VCC to cell contact sites will shed light on important factors necessary for virus dissemination via cell-to-cell transmission and provide new strategies for inhibiting virus spread.
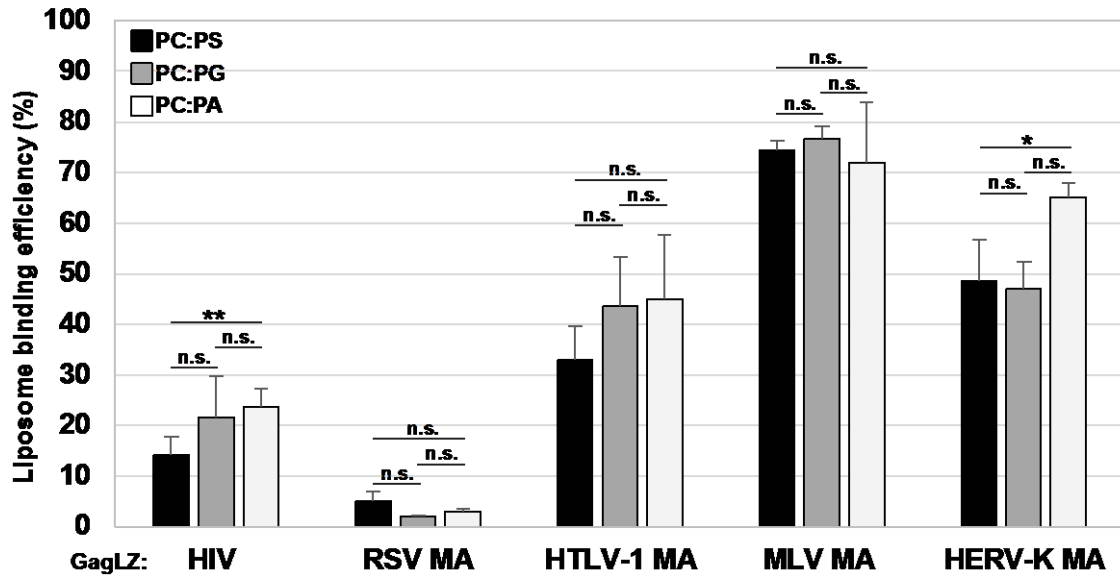
Fig. 5.1. Binding efficiency of chimeric GagLZ to various negatively charged liposomes.
HIV-1 GagLZ, RSV MA GagLZ, HTLV-1 MA GagLZ, MLV MA GagLZ and HERV-K MA
GagLZ proteins were synthesized using rabbit reticulocyte lysates and were incubated with
PC:PS [2:1], PC:PG [2:1] or PC:PA [2:1] liposomes. The samples were subsequently subjected to
membrane flotation centrifugation. Five 1-ml fractions were collected from each sample. The
liposome binding efficiency was calculated as the percentage of membrane-bound versus the total
Gag synthesized in the reaction. Data from at least three different experiments are shown as
means ± standard deviations. P values were determined using Student's t-test. **, P<0.005; *,
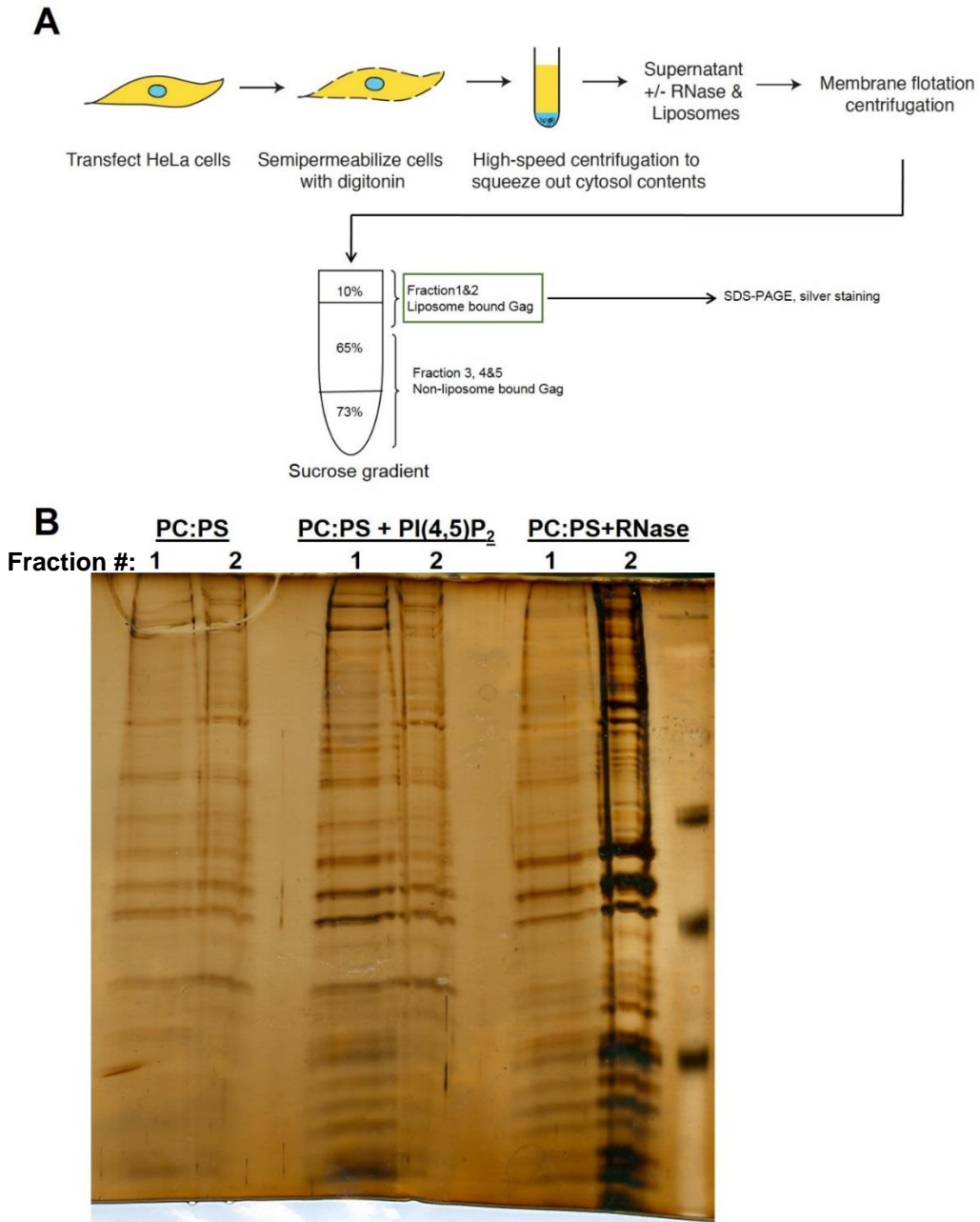P<0.05, n.s., not significant.

Fig. 5.2. Many cellular proteins bind PI(4,5)P$_2$-containing liposomes and are RNase-responsive. (A) Schematic diagram of cell-derived liposome binding assay. This method was adapted from (56) with modifications. Briefly, HeLa cells that are transfected with HIV-1 molecular clone are harvested and subjected to low amount of digitonin treatment. Cells are then centrifuged at high speed and cytosolic fractions are collected and treated or not treated with RNase A, followed by incubation with liposomes. Samples are subsequently subjected to membrane floatation centrifugation. Five 1-ml fractions were collected from each sample. The top 2 fractions are subjected to SDS-PAGE followed by silver staining to visualize the membrane-bound cellular proteins. (B) A representative image of silver-stained gel. Note that more proteins are detected upon binding to PI(4,5)P$_2$-containing liposomes and in RNase-treated condition.
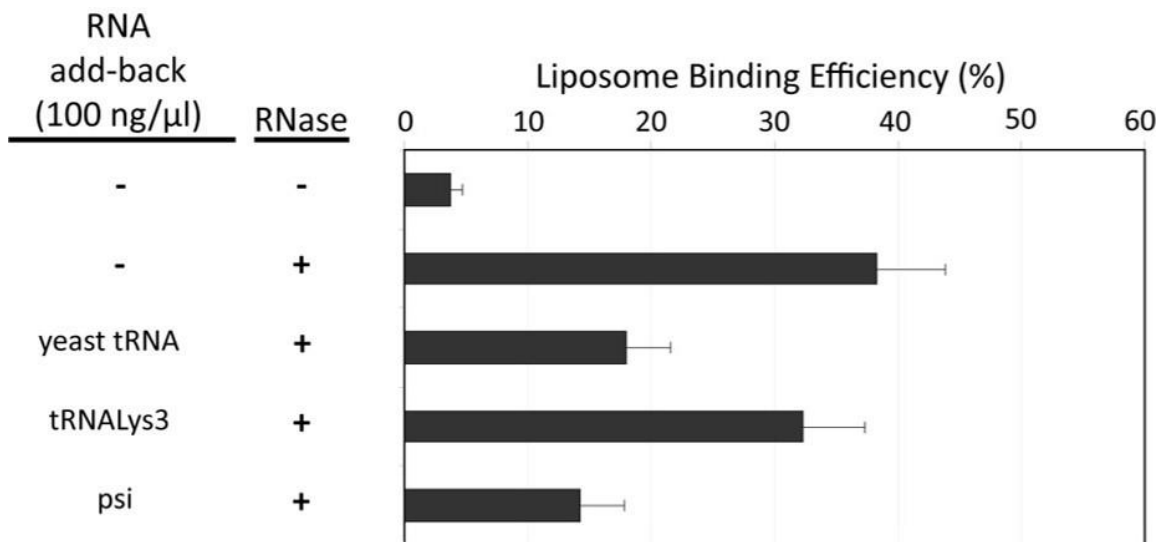
Fig. 5.3. Different RNA species can inhibit HIV-1 Gag PC:PS liposome binding to varying degrees. [^{35}S]-labeled HIV-1 Gag synthesized using rabbit reticulocyte lysates was untreated or treated with RNase A. RNase A was blocked using RNasin and the mixtures were further incubated with indicated RNA species at 100ng/ul final concentration. PC:PS liposomes were then added and incubated further before performing equilibrium flotation centrifugation. Five 1-ml fractions were collected and samples each fraction was loaded and analyzed on SDS-PAGE. The liposome binding efficiency was calculated as the amount of membrane-bound Gag as a fraction of total Gag.

[The data from this figure was collected by Dr. Gabrielle Todd.]

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