Molecular Determinants That Regulate Plasma Membrane-Specific Binding of HIV-1 Structural Protein Gag

by

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Abstract

Human Immunodeficiency Virus type 1 (HIV-1) assembly is a multistep process mediated by the viral precursor polyprotein Gag ($Pr55^{Gag}$). Matrix (MA), which constitutes the N-terminal domain of $Pr55^{Gag}$, is essential for membrane binding and targeting of Gag to the plasma membrane (PM). MA has a bipartite signal for membrane binding: a myristate moiety at the N-terminus and amino acid residues 17-31 that form a highly basic region (HBR) on the surface of MA. The N-terminal myristate is normally sequestered into the MA globular domain, and a structural change exposes myristate, thereby enhancing membrane binding. The HBR on the other hand is thought to bind acidic lipids. Previous results from the lab suggest that a PM-specific acidic lipid, phosphatidylinositol-(4,5)-bisphosphate [$PI(4,5)P_2$], is important for Gag localization to the PM.

In this thesis, I have shown that Gag interacts specifically with $PI(4,5)P_2$ and that this interaction is important for efficient membrane binding of Gag. To elucidate the molecular mechanisms by which Gag-$PI(4,5)P_2$ interaction is regulated, site-directed mutagenesis was performed on the MA HBR. Using this approach, we identified three lysines that facilitate membrane binding by interacting with $PI(4,5)P_2$. Strikingly, mutations in two other lysines in the MA HBR enhance $PI(4,5)P_2$-independent membrane binding by exposing myristate. Thus, MA HBR has opposing roles in membrane binding. Notably, another major finding of this thesis is that RNA also negatively regulates membrane binding of Gag. In the absence but not in the presence of $PI(4,5)P_2$, RNA

bound to the MA HBR abolishes membrane binding of Gag. Overall, the results from this thesis suggest that the MA HBR regulates membrane binding both positively by binding to PI(4,5)P_2 and negatively through myristate sequestration and RNA binding. This regulation ensures that Gag is targeted specifically to the PM, where it likely interacts with other viral and cellular molecules for efficient virus assembly and release.

CHAPTER I

Introduction

Human Immunodeficiency Virus (HIV) is the causative agent of Acquired Immune Deficiency Syndrome (AIDS). There are currently 33.3 million people living with HIV around the world. Even though researchers have focused on understanding various aspects of HIV since its discovery 29 years ago, there is still no vaccine available to prevent the spread of infection. The existing anti-retroviral regimen is effective in slowing the progression of the disease but the adverse effects and the emergence of drug-resistant strains still pose a problem. In addition, the current drugs are expensive and are not readily accessible to people in developing countries. In 2009 alone, there were 2.6 million new infections and 1.8 million deaths due to AIDS [1]. Thus, there is a continuous need for newer and inexpensive drugs while the quest for an effective vaccine continues.

HIV types, groups and subtypes

There are two types of HIV: HIV-1 and HIV-2. HIV-2 is relatively uncommon and less efficient at transmission and disease progression. HIV-1 is the major type that is responsible for the worldwide pandemic and can be divided into three groups: M (major), O (Outlier), N (non-M/O) [2]. In 2009, another new group P was proposed that seem to be derived from gorillas and is distinct from the three groups mentioned above [3, 4].

Within group M, there are nine distinct subtypes (A, B, C, D, F, G, H, J, K) and several major circulating recombinant forms (CRFs). Half of the global infections are caused by subtype C. The second most common is subtype A. Subtype B dominates the western world and is the most studied subtype in the research labs. However, it accounts for only 12% of global infections. This prevalence should be considered while developing drugs as most drugs are tested for efficacy against HIV-1 subtype B and may or may not work the same for other subtypes or groups.

HIV Genome

HIV is an enveloped virus and belongs to the family Retroviridae. The genome is single- stranded RNA about 9 kb in length and encodes three major polyproteins, Gag, Pol and Env. In addition, HIV has six accessory proteins: Tat, Rev, Nef, Vpr, Vif and Vpu (Fig.1.1). Gag and Pol are made from unspliced RNA, singly spliced RNAs ~4kb encode Env, Vpu (Vpx in the case of HIV-2), Vif and Vpr and doubly spliced RNAs ~2kb encode Tat, Rev and Nef [5]. Gag is the major structural polyprotein that is sufficient to form virus-like particle. Pol is made as a fusion with Gag protein through ribosomal frameshift and is proteolytically cleaved to form viral enzymes: protease, reverse transcriptase and integrase. Env (gp160) is the viral glycoprotein that is cleaved to form a surface subunit (gp120) and a transmembrane subunit (gp41). These subunits form trimers of heterodimers and decorate the viral lipid bilayer [6, 7]. Tat binds nascent viral RNA and transactivates HIV promoter by enhancing transcription initiation and stabilizing elongation of full-length viral transcripts [8]. Rev interacts with nuclear export machinery to allow unspliced and singly spliced RNA to be transported to the cytosol where they can be translated [9]. Unspliced RNA is also packaged as genomic RNA. Nef

has several functions, including downregulation of CD4 and MHC-1 molecules from the surface of infected cells[10-12]. Vpr is important for the transport of preintegration complex to the nucleus and also participates in cell-cycle arrest [13, 14]. Vif reduces hypermutation of viral genome by preventing the incorporation of Apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G (APOBEC3G), a cytidine deaminase, into the virions [15]. Vpu degrades CD4 molecules [16]and in addition has been recently shown to counteract the cellular restriction factor tetherin [17, 18](For review on accessory genes, see [19]). The viral genome itself is structured at several levels. It has Trans-Activating Response (TAR) hairpin that interacts with Tat. Primer binding site interacts with $tRNA^{Lys}$ that primes reverse transcription. The packaging signal (Ψ) (and kissing loop region within Ψ) interacts with nucleocapsid (NC) domain of Gag and is crucial for specific encapsidation of two genomic RNAs into the virion. The Rev-response element interacts with Rev allowing export of unspliced or singly spliced RNA into the cytosol. In addition, the viral RNA has one major splice donor, several splice acceptors and a ribosomal frameshift sequence that allows accurate translation of viral proteins. The whole genome structure has been recently analyzed and several additional unrecognized regulatory structures were also found [20].

HIV life cycle

HIV life cycle is depicted in Fig. 1.2.

Attachment and entry

HIV begins its life cycle by attaching to the cellular receptor CD4 via gp120 [21]. This causes structural change within gp120 that facilitates its interaction with one of the

coreceptors, CCR5 or CXCR4, depending on the tropism determined by the V3 loop of gp120 [22, 23]. Once attached, the structural change in the transmembrane domain, gp41, allows the insertion of the fusion peptide in the target cell membrane [6]. This leads to the formation of a six-helix bundle that mediates fusion of the viral membrane with the cellular membrane [24, 25]. This process is receptor mediated and occurs on the plasma membrane of the cell. Recently, however, endocytosis has also been shown as a viable pathway for the entry of the virus and both endocytosis of the virus and fusion of viral and endocytic membranes seem to be dependent on the cellular protein dynamin [26]. Currently, there are two drugs that block entry process by either preventing the formation of the six-helix bundle or by blocking CCR5 interaction with gp120 [27].

Uncoating, reverse transcription and integration

Once the viral membrane fuses with the cellular membrane either at the plasma membrane or endosomes, the viral core is released into the cytosol. The transport of the genome towards the nucleus is dependent on microtubules [28]. How and when uncoating of the core occurs is still unclear. Integrity and timely disassembly of the capsid (CA) protein, a cleavage product of Gag, is required for efficient transport, reverse transcription and nuclear import[29], for review see [30]. Species-specific cellular restriction factor, Tripartite motif-containing protein 5-α (TRIM5-α) interferes with the uncoating process preventing successful reverse transcription and nuclear transport [31]. Reverse transcription, where the single stranded RNA is converted to double-stranded DNA by the reverse transcriptase is completed during the uncoating process. It is during this time, HIV is vulnerable to another cellular restriction factor APOBEC3G, which can be incorporated into the virion. APOBEC3G is a cytidine deaminase and causes lethal

hypermutation of the viral genome [32, 33]. Viral protein Vif interacts with APOBEC3G and facilitates its degradation, thus preventing it from being incorporated into the virion [15, 34], for review see [35]. The complex that contains reverse-transcribed DNA and several viral and host proteins that allow it to be actively transported into the nucleus is called pre-integration complex (PIC). Active transport of PIC is important for infection of terminally differentiated cells like macrophages by HIV. Even though viral proteins like Vpr, matrix (MA) and integrase have been suggested to mediate entry of PIC into the nucleus, their involvement is still controversial [36-38] [39]. tRNAs have also been implicated in nuclear import of PIC [40]. Once inside the nucleus, the viral DNA integrates into the cellular DNA with the help of integrase. Association of cellular lens epithelium derived growth factor (LEDGF) with integrase and chromosomal DNA may allow specific targeting of integration near active genes [41] [42]. Rev, on the other hand, has been recently shown to interact with LEDGF and limit integration events [43]. There are currently several drugs that inhibit reverse transcriptase and a single drug that inhibits integrase. Detailed understanding of uncoating and nuclear import is necessary for the development of newer drugs that can block HIV replication prior to the integration of the viral genome into chromosomal DNA.

Transcription, RNA export and translation

Once integrated, the viral DNA is called a provirus. HIV promoter is in the 5' long terminal repeat (LTR) sequence and has regulatory elements, including sites for NF-κB, TATA-box binding protein. The transcription mediated by the cellular RNA polymerase II and viral protein Tat is important for both enhancing the rate of transcription initiation and for efficient elongation[44], for review see [45]. In addition to

interacting with several cellular cofactors involved in initiation and elongation of transcription, Tat also recruits chromatin-remodeling proteins to the 5'LTR before the assembly of RNA Pol II complex [46] [19]. When viral mRNAs are first produced, most viral RNAs that reach the cytoplasm are completely spliced and encode Tat, Rev and Nef. Once Rev is made in sufficient amounts, unspliced and singly spliced RNAs are transported to the cytoplasm, where they encode the remaining viral proteins. Rev interacts with the viral RNA at the RRE and recruits CRM/exportin 1[47]. In addition, it interacts with several other nuclear export proteins for the transport of viral RNA to the cytosol. Oligomerization of Rev on RRE is important for its function as it may increase the concentration of nuclear export signal (NES) sites of Rev on a single mRNA and promote efficient export [19, 48]. There are currently no drugs that inhibit either transcription or nuclear export of the viral RNAs.

Assembly and Release

Once viral structural protein is synthesized as the precursor polyprotein $Pr55^{Gag}$, it mediates the virus assembly process. As defined by sites of the viral protease-dependent cleavage that occurs during or after virus release, this polyprotein has four major structural domains: MA, CA, NC and p6 [49, 50]. In addition, it also contains spacer peptides 1 and 2 (SP1 and 2). Each of these structural domains performs a crucial role during the assembly process (Fig. 1.3). MA mediates the targeting of Gag to the site of assembly and facilitates membrane binding. The C-terminal domain of CA forms the Gag dimerization interface, whereas NC promotes higher order multimerization of Gag through binding to RNA, which is thought to serve as a scaffold. In addition, NC contains two zinc finger/knuckle structures that are required for specific encapsidation of viral

genomic RNA. The late domain motifs in p6 recruit cellular protein complexes called endosomal-sorting complex required for transport (ESCRT) that aid in the release of the virus from the cell membrane. Approximately 2000 Gag molecules are present within the virion and Gag alone is sufficient to form virus-like particles. However, for the virus to be infectious, several components need to be at the assembly site for their packaging into the virion, including Gag, GagPol, which contains all the viral enzymes, Env, viral RNA, and host factors that are required for virus infectivity, such as $tRNA^{Lys3}$ and cyclophilin. Once the virus assembles and is ready for release, it encounters another cellular restriction factor, tetherin, that acts as a physical leash between viral membrane and cell membrane. This leash-like structure keeps the virus tethered to the cell surface. However, viral protein Vpu counteracts tetherin by removing it from the assembly site and by facilitating its degradation. This counteraction by viral protein is important for efficient release of the virus into the extracellular space [17, 18]. Once the virus is released, viral protease cleaves the polyprotein Gag and Gag-Pol causing a huge conformational change within the virus. This structural change makes the virus infectious and the virus is now ready to infect another cell. There are several drugs that inhibit the protease activity. However, there are currently no drugs that inhibit the assembly process.

Gag-RNA interaction is important for assembly and specific RNA encapsidation

Viral genomic RNA and $tRNA^{Lys}$ that acts as the primer for HIV reverse transcription are specifically packaged into the virus particle. In addition, there are various cellular RNAs that are either specifically enriched in virus particles like 7SL [51] or randomly packaged [52], but their function in virus lifecycle is unknown. Genomic RNA is specifically packaged into the virion through the interaction of Ψ with the zinc

fingers of NC[53, 54]. However, where and when this interaction occurs is still unclear. In another retrovirus Rous Sarcoma Virus (RSV), there is evidence that some Gag proteins shuttle into the nucleus to pick up unspliced RNA that can act as genomic RNA [55]. Even though it is known that HIV-1 MA domain has a nuclear localization signal [36], the physiological relevance is debated [56]. On the other hand, $tRNA^{Lys}$ incorporation is independent of genomic RNA packaging. C-terminal domain of CA is thought to bind Lysyl-tRNA synthetase (LysRS) and this interaction is necessary for selecting $tRNA^{Lys}$. In addition, GagPol, especially reverse transcriptase (RT) is important for stabilizing the interaction leading to efficient incorporation of the $tRNA^{Lys}$ into the virion [57]. Once packaged, NC domain of Gag acts as a chaperone and facilitates the $tRNA^{Lys}$ annealing to the primer-binding site on the viral RNA [58]. Gag-RNA binding also has a major role in assembly. It is thought that NC- bound RNA acts as a scaffold, concentrating Gag molecules in a single place, where they can multimerize efficiently. Even though genomic RNA is thought to provide this scaffolding function, cellular RNAs can also behave similarly and aid in higher-order multimerization of Gag [59]. MA can also interact with RNA [60-65] and this interaction may play a redundant role with NC-RNA interaction in virus assembly and particle production [66]. It is, however, unknown which RNA is bound to MA, as there is no consensus sequence agreement between groups that have isolated RNAs bound to MA with higher affinity. In addition, the physiological role of this MA-RNA interaction is unclear.

Site of HIV-1 assembly

HIV-1 assembly occurs at the plasma membrane (PM) [67] or, in the case of macrophages, in deep invaginations of the PM called virus-containing compartments

(VCC). These compartments are connected to the cell surface via membrane-lined tubular conduits [68-70]. Endosomes were also proposed previously to serve as native sites for assembly of HIV-1 particles, as Gag was frequently observed in these compartments. However, most of the WT virus particles localized in these endosomal compartments are now thought to originate from endocytosis of particles already assembled at the PM [71](for reviews, see [72, 73]). Consistent with the notion that endosomes do not constitute a native virus release pathway, redirecting Gag to endosomal compartments severely inhibits release of HIV-1 in HeLa, COS, and 293T cells perhaps because assembled particles are trapped in these compartments [71, 74-77]. It is therefore possible that the PM serves as the preferred site of HIV-1 assembly because it allows nascent particles to readily exit to the extracellular space. However, recent evidence suggests that at least in T cells, mutant Gag proteins targeted to the intracellular compartments can still be released as extracellular particles and are not necessarily trapped in these compartments [78]. Therefore, easy access to the extracellular space is unlikely to be the only reason that necessitates specific localization of HIV-1 Gag to the PM. Additional advantages that might be associated with virus assembly at the PM include lipid raft association and cell-cell transmission of the virus, both of which facilitate efficient replication of the virus. The focus of my thesis is to determine the molecular mechanisms by which Gag is specifically targeted to the PM.

Gag membrane binding is driven by bipartite signal in MA

MA is the membrane proximal domain of $Pr55^{Gag}$ (Fig.1.4A). The first 104 amino acids of the MA form a compact globular domain consisting of five major α-helices that are capped by a mixed three-stranded β-sheet [79, 80]. MA is myristoylated at the amino-

terminus and facilitates membrane binding of Gag [81]. In addition, a highly basic region (HBR) spanning residues 17-31 (Fig.1.4A), which is exposed and clustered around the mixed β-sheet on the surface of MA, is important for efficient membrane binding and proper targeting of Gag to the PM [74-76, 82-84]. Mutagenesis studies suggest that the region spanning residues 84-88 is also important for PM localization of Gag [76, 85]. However, this region is buried within MA and hence it is likely that mutations in this region indirectly alter the HBR and affect Gag localization. Consistent with this hypothesis, a revertant of an MA mutant with a substitution in this region had a compensatory second site mutation in the MA HBR, suggesting that these regions are functionally linked [86]. Besides having determinants for membrane binding and proper targeting, MA, especially the HBR region, is also important for other processes in viral life cycle, such as envelope glycoprotein incorporation and several post-entry processes [for review see [61]]. MA HBR also interacts with RNA, but the physiological relevance of this interaction is unknown. Chapter III of this thesis focuses on this interaction as a potential regulator of Gag-membrane binding.

The N-terminal myristoyl moiety is sequestered in the globular domain of MA

Myristoylation is a process that occurs co-translationally, where a 14-carbon saturated fatty acid, myristic acid, is attached to the N-terminal glycine that is exposed after the first methionine is removed. The enzyme that catalyzes this reaction is the myristoyl-CoA: protein N-myristoyltransferase (NMT) [for detailed review see [87]]. In addition to the absolute requirement for Glycine at position 1 (numbering of residues in this thesis is based on the fact that glycine will be the first residue after the first methionine is removed), human NMT prefers polar residues at positions 2 and 3, large

hydrophobic at position 4, small polar residues such as serine at position 5 and lysine at position 6. About 0.5 to 3% of eukaryotic proteins are thought to be myristoylated. These myristoylated cellular proteins are involved in many cell-signaling cascades and include protein kinases and phosphatases, cytoskeleton-associated proteins, calcium binding proteins, small G-proteins etc. In addition to the eukaryotic proteins, certain viral and bacterial proteins have also been shown to be myristoylated. As mentioned above, the MA domain of Gag is myristoylated at the N-terminus. Mutating the N-terminal glycine to alanine severely reduces membrane binding of Gag and inhibits virus particle release [74, 81, 85, 88, 89]. In addition, mutating MA residues 2-5 also reduces membrane binding and virus particle production, which is consistent with the idea that these residues are required for NMT recognition [86, 89].

Differential membrane binding of the MA domain alone and of full-length Gag led to the hypothesis that a structural change in MA alters its myristate-mediated membrane binding [90-93]. According to the original hypothesis of myristoyl switch, the myristate moiety is sequestered in the MA globular domain, and upon Gag multimerization, a structural change exposes the acyl chain facilitating efficient membrane binding. Consistent with this model, mutations of MA residues 6 and 7 were shown to inhibit myristate exposure [89, 94, 95], and certain other mutations or deletions in the MA globular domain were observed to enhance Gag membrane binding, presumably through inhibition of myristate sequestration [84, 89, 90, 92, 95, 96]. The presence of myristoyl switch was eventually demonstrated by NMR analysis [97]. In this study, MA was shown to exist as an equilibrium mixture of myristate-sequestered monomer and myristate-exposed trimer. Notably, this study observed that the myristoyl

moiety moves into or out of a hydrophobic cavity of MA in a manner coupled to MA trimerization without involving a drastic structural change of MA. Interestingly, C-terminal addition of the CA sequence, which contains a dimerization domain, shifted the equilibrium toward the myristate-exposed trimeric state by increasing the self-association of otherwise low-affinity molecules. These results suggest a model in which Gag multimerization stabilizes MA trimerization thereby facilitating myristate exposure and membrane binding.

Consistent with the multimerization-coupled myristoyl switch model, liposome or lipid monolayer binding studies showed that addition of the CA-derived dimerization domain to HIV-1 MA enhances its affinity for membranes [98, 99]. However, membrane binding of these Gag constructs was dependent on the presence of acidic phospholipids. Furthermore, similar observation was also obtained for RSV MA that lacks myristate [100]. Therefore, these studies suggest that multimerization-dependent enhancement of membrane binding is explained at least partly by the increased avidity of the whole Gag complex to the membranes via clustering of multiple HBRs and myristate moieties on MA. In addition to the avidity, virus particle formation could also partly account for enhanced membrane binding of full-length Gag in cells. Gag molecules within virus particles that remain on the cell surface are in effect irreversibly membrane-bound in fractionation experiments. Altogether, it is likely that the myristate exposure is not the sole reason for the difference between membrane binding of MA and that of full-length Gag [90-93]. Further in vivo and in vitro studies are required to sort out mechanisms contributing to the difference between membrane affinities of MA and full-length Gag.

Gag multimerization is not the sole trigger for exposure of the myristoyl moiety. As detailed later, the interaction of the HBR with a PM-specific acidic lipid, phosphatidylinositol-(4,5)-bisphosphate [$PI(4,5)P_2$] was also shown to trigger the myristate exposure for efficient membrane binding [101]. Interestingly, non-myristoylated Gag that exists in a monomer-dimer equilibrium shifts to a monomer-trimer equilibrium when inositol hexakisphosphate (IP6) is added to the solution [102]. As IP6 is structurally similar to the head group of $PI(4,5)P_2$, it is conceivable that $PI(4,5)P_2$ may also modulate trimerization of Gag, which is coupled to myristate exposure. In addition to $PI(4,5)P_2$, binding of calmodulin to MA and pH changes were shown to cause structural changes that modulate the status of myristate exposure [103, 104]. The role of calmodulin and pH changes in Gag membrane binding in cells has yet to be elucidated.

The MA HBR binds acidic lipids

Even though myristoylation is necessary, it is thought to be insufficient for efficient membrane binding of proteins [105, 106]. Myristoylation only provides reversible membrane binding, and a second signal is thought to be required for strong localization of the myristoylated protein to the membrane [106]. This second signal may be a polybasic cluster, a second acylation such as palmitoylation, or a protein-protein interaction that increases avidity of the protein complex. In the case of HIV-1 Gag, the interaction of the HBR with acidic lipids can provide such second signal[74].

The PM is one of the cellular membranes that are rich in anionic lipids such as phosphatidylserine (PS) and phosphoinositides that preferentially localize on the

cytoplasmic leaflet [107]. This distinctive localization makes the cytoplasmic surface of the PM acidic. Proteins or protein domains with polybasic clusters on the surface, such as pleckstrin homology (PH) domains, myristoylated alanine-rich protein kinase C substrate (MARCKS), and k-ras, are targeted to this negatively charged surface where they interact with acidic lipids either in a lipid-head-group-specific manner or simply via electrostatic interactions [108-110]. Mutations within the polybasic cluster of these proteins significantly alter their PM binding and localization [111, 112]. Similarly, mutations in the MA HBR mislocalize Gag to intracellular compartments and reduce membrane binding of Gag [74-76, 82-84]. Structural and mutagenesis studies suggest that the HBR is exposed on the surface of the MA domain and interacts with acidic lipids [74, 79, 80, 84, 98, 113]. Notably, however, Gag mutants lacking the MA globular domain except the N-terminal myristoylation signal can still bind membranes and release virus, suggesting that the HBR is important for efficient membrane binding only in the context of full-length MA [96, 114-117].

Phosphatidylinositol-(4,5)-bisphosphate is important for PM targeting of Gag

As alluded to earlier, recent studies have shown that the basic residues in the HBR interact with the acidic phospholipid, $PI(4,5)P_2$ [84, 101, 118, 119]. $PI(4,5)P_2$ belongs to a family of phospholipids called phosphoinositides. These lipids are derivatives of phosphatidylinositol and have a hydrophobic diacylglycerol backbone esterified to a polar inositol head group that can be phosphorylated at three of the five hydroxyl residues (Fig. 1.4b). Thus, seven different phosphatidylinositol phosphates (PIPs) are formed, which vary in the number and position of phosphates on the inositol ring [120]. Various cellular proteins with basic residues on the molecule surface have different affinities and

specificities to the head groups on phosphoinositides [109]. Through the recruitment of these proteins in either a head-group-specific or simply a charge-dependent manner, PIPs control several important cellular processes including membrane fusion, budding, and actin dynamics [121]. Notably, PIPs themselves are spatially and temporally regulated by the action of kinases and phosphatases that add or remove phosphate on the inositol ring, respectively. The predominant localization of various phosphoinositides is shown in Fig.1.5. Among the PIPs, $PI(4,5)P_2$ is the most abundant and is specifically localized on the cytoplasmic leaflet of the PM.

The first clue for the role of PIPs in HIV-1 assembly came from an in vitro assembly study where both RNA and inositol pentakisphosphate (IP5) were shown to be required for Gag to form a proper virus-like structure [122]. IP5 is an inositol derivative structurally related to the head group of $PI(4,5)P_2$. The requirement for $PI(4,5)P_2$ in HIV-1 assembly became evident from the study in which cellular $PI(4,5)P_2$ was depleted or mislocalized in HeLa cells, either using phosphatidylinositol polyphosphate 5 phosphatase type IV (5ptaseIV), which removes D-5 phosphate from the inositol head group [123] or constitutively active Arf 6, which induces $PI(4,5)P_2$-enriched intracellular vesicles, respectively [124, 125]. Both methods of $PI(4,5)P_2$ perturbation mislocalized Gag to internal compartments and significantly reduced virus particle release [77]. Subsequently, several in vitro structural and biochemical studies, including the work in Chapter II of this thesis showed that Gag interacts with $PI(4,5)P_2$ via basic residues in the HBR [101, 118, 119, 126]. Shkriabai, et al. examined the accessibility of lysines in non-myristoylated full-length Gag proteins after Gag was mixed with a water-soluble analogue of $PI(4,5)P_2$ that has short acyl chains and found two lysines in MA HBR as

potential PI(4,5)P_2 binding site [119]. Saad, et al. determined the structure of the complex between myristoylated MA and a water-soluble PI(4,5)P_2 analogue using NMR and identified binding of PI(4,5)P_2 to MA as a trigger for myristate exposure [101]. Notably, this study not only showed interactions between the acidic head group of PI(4,5)P_2 and basic amino acids in the HBR but also revealed the interaction between 2' acyl chain and a hydrophobic pocket that is normally occupied by the myristoyl moiety . While these studies utilized the water-soluble analogues of PI(4,5)P_2, the work in Chapter II of this thesis demonstrated that full-length myristoylated Gag binds to membrane-associated PI(4,5)P_2 that contains native-length acyl chains and that this interaction is important for efficient membrane binding of Gag in cells[118].

Gag and host vesicular trafficking pathways

It is still unclear how Gag reaches the PM where it can interact with PI(4,5)P_2. Several endosomal trafficking proteins have been implicated in the transport of Gag to the PM. Inhibiting interaction of Gag with adaptor proteins AP-1 or AP-3 blocks virus particle production and relocalizes Gag [127, 128]. Similarly siRNA knockdown of Rab9 [108] and a kinesin motor KIF4 also causes accumulation of Gag in intracellular compartments and inhibits virus particle production [129]. Several other proteins like Golgi-localizing, gamma-adaptin ear homology domains (GGAs), ADP-ribosylation factors (ARFs) and AP-2 have also been shown to modulate the assembly process [130, 131]. Thus, it is possible that Gag uses existing host vesicular trafficking pathway or multiple pathways to get to the PM. Further research is needed to pinpoint which pathway is predominantly used.

Overview of thesis

The work presented in this thesis illustrates the molecular determinants for PM association of Gag. Chapter II determines how $PI(4,5)P_2$ is important for efficient membrane binding of Gag in addition to PM targeting of Gag. Chapter III demonstrates how MA HBR utilizes opposing regulatory mechanisms for facilitating membrane binding of Gag. The basic residues in the HBR not only facilitate membrane binding by interacting with $PI(4,5)P_2$ but also inhibit non-specific membrane binding via sequestration of myristate and binding to RNA. Chapter IV discusses the results of chapter II and III along with the future directions for the project and describes some of the preliminary data related to the future directions.

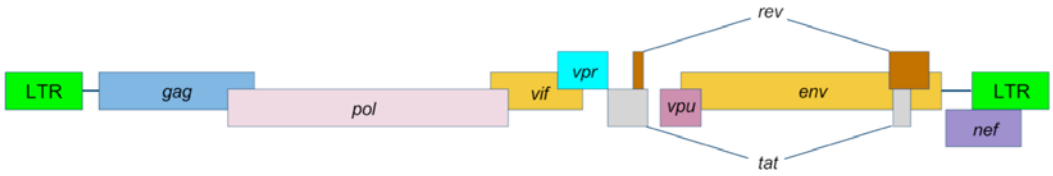

Fig.1.1. HIV Genome Organization. HIV has 3 major genes, *gag*, *pol* and *env* that are common to all retroviruses. In addition HIV also has 6 accessory genes: *tat*, *rev*, *vpu*, *vpr*, *vif* and *nef*. Completely spliced RNAs around 2kb encode for Tat, Rev and Nef. Once Rev is synthesized to sufficient amounts, it assists the transport of unspliced and singly spiced RNAs into the cytosol. Singly spliced RNAs around 4kb encode Env, Vpu, Vif and Vpr. Unspliced RNA encode for Gag and Pol.

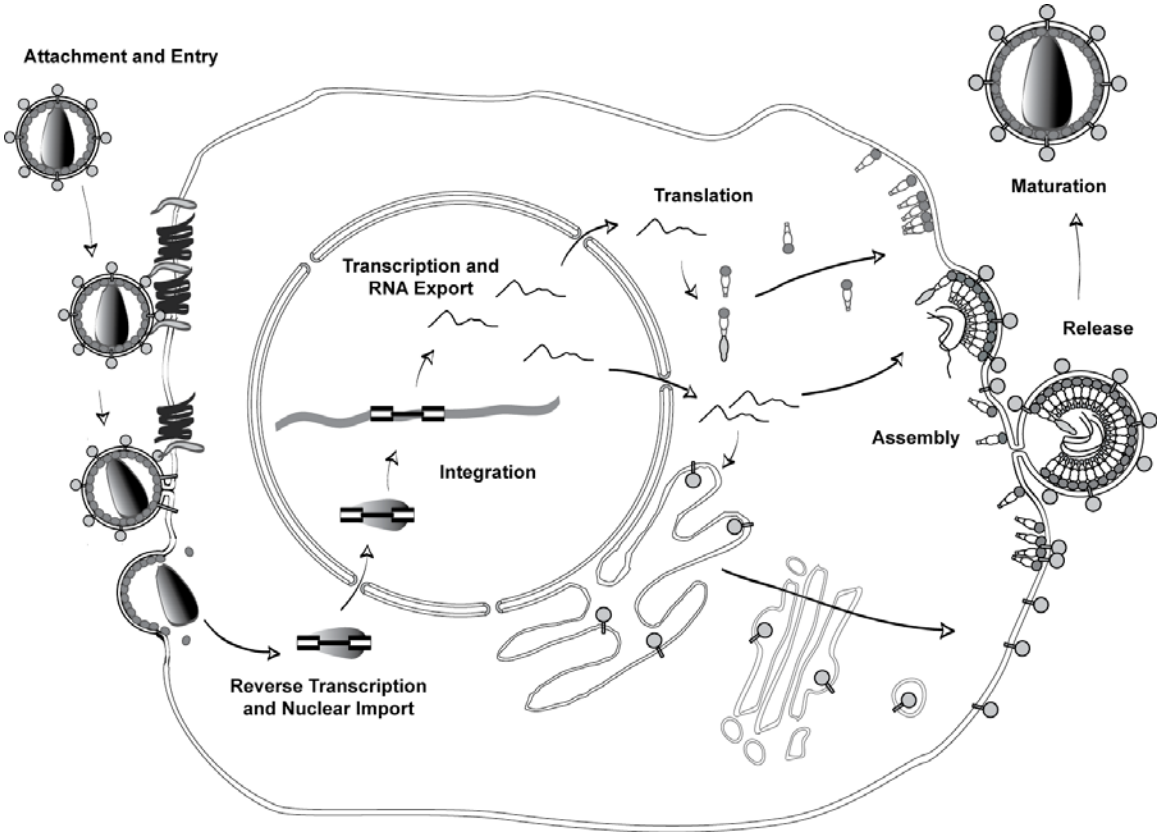

Fig. 1.2. HIV Life Cycle. HIV Env binds the cellular receptor CD4 and coreceptors CCR5 or CXCR4 and mediates fusion between cell membrane and viral membrane. This release the core into the cytosol, where the viral RNA is reverse transcribed into DNA. The DNA is then transported into the nucleus where it integrates with the cellular chromosome. Once integrated, the DNA is called provirus and behaves like a cellular gene and is transcribed and translated. Once the structural protein Gag is made, it initiates viral assembly on the plasma membrane. Other viral proteins depicted at the assembly site are GagPol, which has all the viral enzymes and Env, which uses secretory pathway to reach the assembly site. RNA transcribed from the provirus can also act as genomic RNA and a dimer of this genomic RNA is picked up by Gag during assembly. Once released from the cell, the viral protease becomes active and cleaves all the viral polyproteins leading to a structural change in the virus. The virus then becomes infectious and is ready to infect new cells.

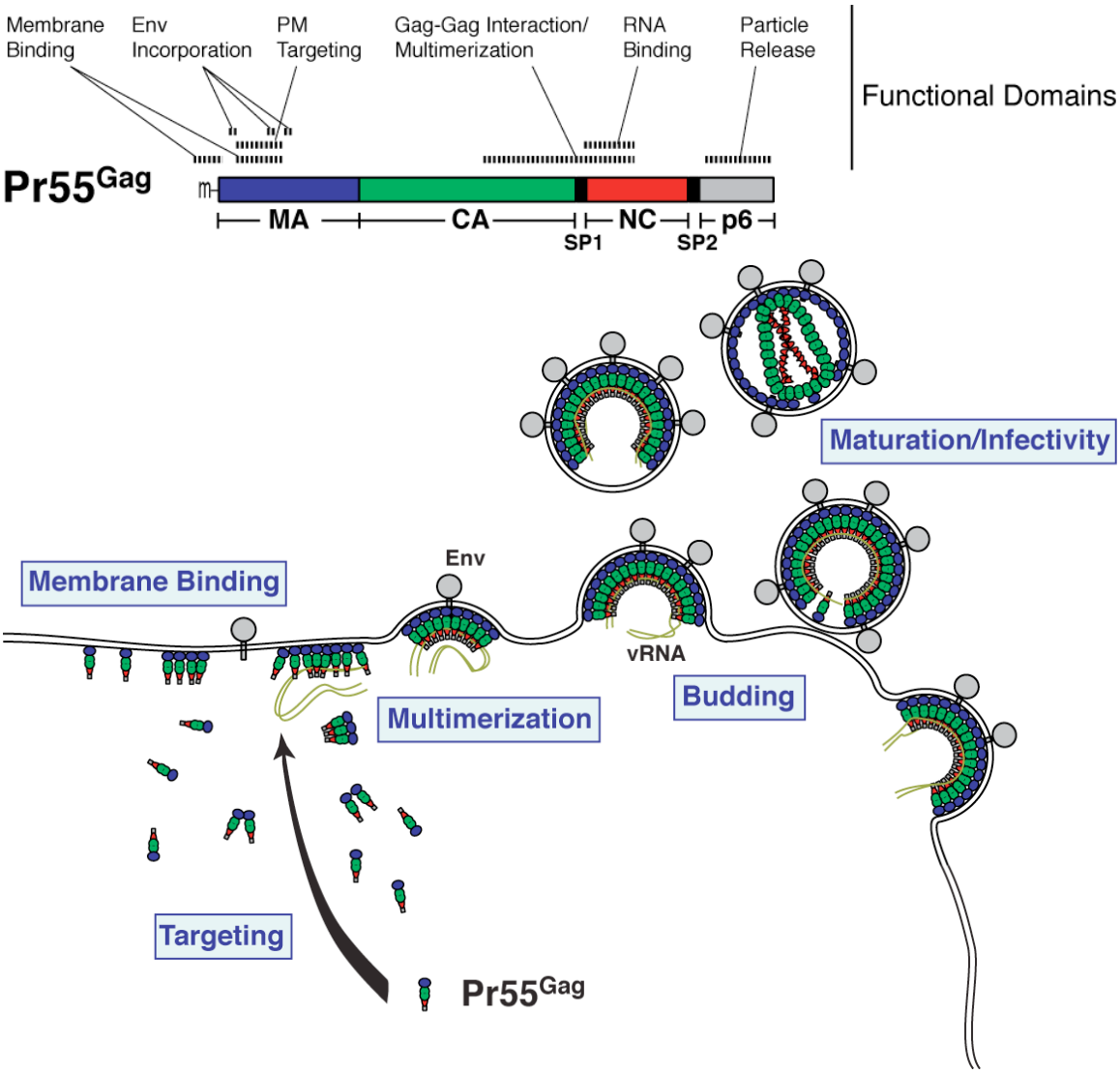

Fig. 1.3. HIV-1 Gag domain structure and the assembly process. Gag synthesized as a polyprotein $Pr55^{Gag}$ is the structural protein of HIV-1. As defined by the viral protease cleavage, it has 4 major proteins: Matrix (MA), Capsid (CA), Nucleocapsid (NC) and p6. In adidition it has two spacer peptides (SP1 and SP2). HIV-1 assembly is a multi-step process that includes targeting to the site of assembly, membrane binding and multimerization and eventual release into the extracellular space where virions matures into a fully infectious particle. Gag polyprotein is sufficient for assembly as there are functional domains that mediate each step of the assembly process. MA is important for targeting, membrane binding and Env incorporation. CA and NC are important for multimerization. NC also mediates viral RNA incorporation. p6 recruits cellular proteins that mediate release of the virus.

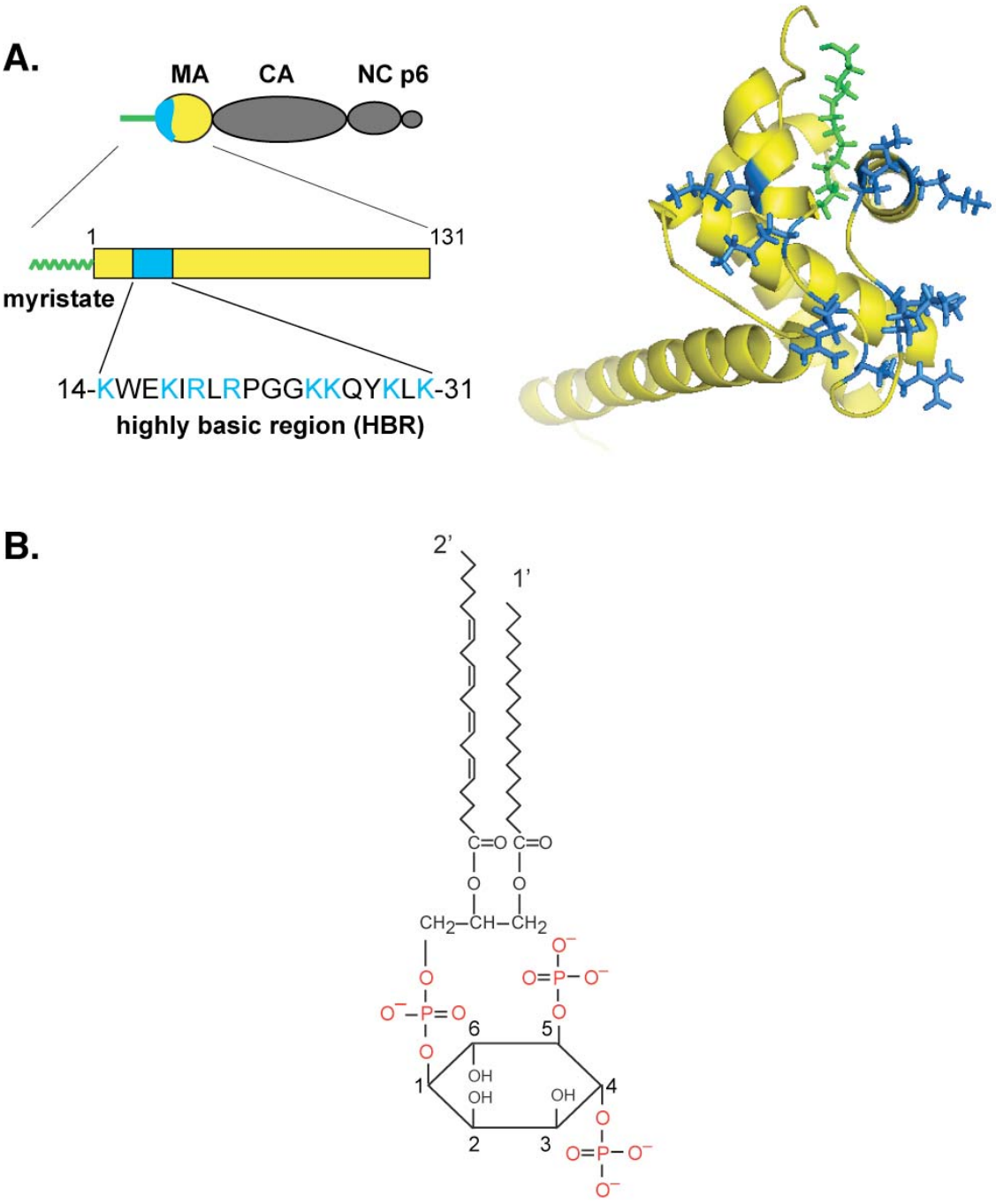

Fig. 1.4. Bipartite signal in MA domain of Gag and PI(4,5)P_2 structure. A). Schematic representation and NMR structure showing the bipartite membrane-binding signal within matrix domain (yellow) of Gag. Both the N-terminal myristoyl moiety (green) and the highly basic region (blue) are important for membrane binding of Gag. Structural information in the right panel is obtained from PDB database ID: 1UPH (Ref 97) B). PI(4,5)P_2 structure. PI(4,5)P_2 has a polar inositol head group, which is phosphorylated at D-4 and D-5 on the inositol ring. The acyl chains of PI(4,5)P_2 found commonly in cells are saturated stearic acid at position 1' and unsaturated arachidonic acid at position 2'.

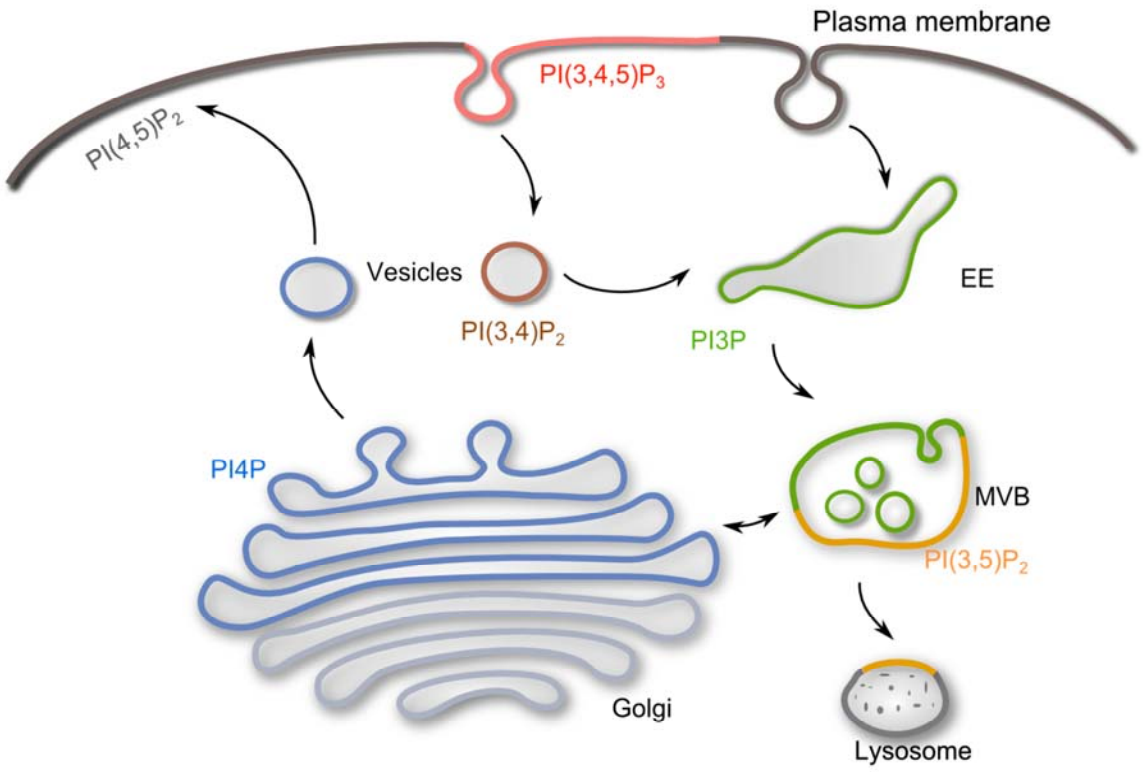

Fig.1.5. Sub-cellular localization of phosphoinositides. Different phosphoinositides have different sub-cellular localization within the cell due to the action of kinases and phophatases that add or remove phosphates on the inositol headgroup. Predominant localization of 5 different phosphoinositides is shown. $PI(4,5)P_2$ and $PI(3,4,5)P_3$ are on the plasma membrane, PI4P is in Golgi, PI3P is in the early endosomes (EE), $PI(3,4)P_2$ is in the vesicles and $PI(3,5)P_2$ is in the multi-vesicular bodies (MVB).

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CHAPTER II

Interaction between the HIV-1 Gag matrix domain and phosphatidylinositol-(4,5)-bisphosphate is essential for efficient Gag-membrane binding

ABSTRACT

HIV-1 particle assembly mediated by the viral structural protein Gag occurs predominantly on the plasma membrane (PM). Although it is known that the matrix (MA) domain of Gag plays a major role in PM localization, molecular mechanisms that determine the location of assembly remain to be elucidated. We observed previously that overexpression of polyphosphoinositide 5-phosphatase IV (5ptaseIV) that depletes PM phosphatidylinositol-(4,5)-bisphosphate [$PI(4,5)P_2$] impairs virus particle production, and redirects processed Gag to intracellular compartments. In this study, we examined the impact of $PI(4,5)P_2$ depletion on the subcellular localization of the entire Gag population using Gag-fluorescent protein chimeras. Upon 5ptaseIV overexpression, in addition to perinuclear localization, Gag also showed a hazy cytosolic signal, suggesting that $PI(4,5)P_2$ depletion impairs Gag-membrane binding. Indeed, Gag was less membrane-bound in $PI(4,5)P_2$-depleted cells, as assessed by biochemical analysis. These observations are consistent with the hypothesis that Gag interacts with $PI(4,5)P_2$. To examine a putative Gag interaction with $PI(4,5)P_2$, we developed an *in vitro* binding assay using full-length myristoylated Gag and liposome-associated $PI(4,5)P_2$. Using this

assay, we observed that PI(4,5)P_2 significantly enhances liposome binding of wild type Gag. In contrast, a Gag derivative lacking MA did not require PI(4,5)P_2 for efficient liposome binding. To analyze the involvement of MA in PI(4,5)P_2 binding further, we examined MA basic amino acid substitution mutants. These mutants, previously shown to localize in perinuclear compartments, bound PI(4,5)P_2-containing liposomes weakly. Altogether, these results indicate that HIV-1 Gag binds PI(4,5)P_2 on the membrane and that the MA basic domain mediates this interaction.

INTRODUCTION

Retroviral particle production is a complex multistep process mediated by a viral structural protein, Gag. HIV-1 Gag is synthesized as a precursor polyprotein, $Pr55^{Gag}$. This precursor polyprotein consists of four major domains which, upon virus release, are cleaved by the viral protease (PR) to generate the mature Gag proteins: p17 matrix (MA), p24 capsid (CA), p7 nucleocapsid (NC), and p6. In addition, $Pr55^{Gag}$ contains two spacer peptides SP1 and SP2 [1-3]. MA, which constitutes the N-terminal domain of $Pr55^{Gag}$, is important for Gag-membrane binding and targeting of Gag to the plasma membrane (PM). CA and NC promote Gag multimerization, and p6 is essential for the release of virus particles [1-3].

Membrane binding of HIV-1 Gag is mediated by two signals in MA: The N-terminal myristic acid and conserved basic region between MA amino acids 17 and 31 [4-6]. The myristate moiety is considered to be regulated by a mechanism termed a myristoyl switch [7-15]. In this model, the N-terminal myristate is normally sequestered in the MA globular domain, but a structural change exposes myristate and enhances Gag-

membrane binding. The patch of conserved basic residues likely contributes to membrane binding by interacting with acidic phospholipids in the inner leaflet of the PM [5, 16, 17]. In addition, the MA basic domain is involved in specific localization of Gag to the PM. In HeLa and T cells, mutations in the basic domain of MA shift the Gag localization from PM to intracellular vesicles containing late endosomal marker proteins [18-22]. In apparent contrast to HeLa and T cells, in macrophages, even when wild type (WT) Gag is expressed, the virus particles localize primarily at compartments positive for late endosomal markers [22-25]. However, these compartments, which first appeared to be internal, were later shown to be invaginations of the PM [26, 27]. Moreover, even in macrophages, a population of Gag was clearly observed at the cell surface, especially at early time points [28]. This specific localization of Gag to the PM or PM-related compartments suggests that host cellular factors play a role in determining the site of virus assembly.

Many cellular proteins that bind the PM have membrane targeting sequences, such as pleckstrin homology (PH) domains. These domains are known to have clusters of basic amino acids that interact with negatively charged lipids collectively known as phosphoinositides [29-31]. Different phosphoinositides localize in different subcellular membranes, thereby influencing the location of proteins to which they bind [32, 33]. Phosphatidylinositol (4,5) bisphosphate [$PI(4,5)P_2$] is concentrated primarily on the cytoplasmic leaflet of the PM, which is also the site where Gag assembles in most cell types. As mentioned above, Gag also contains a patch of basic amino acids that are important for PM localization. Furthermore, IP5 and IP6, which are structurally similar to $PI(4,5)P_2$, were shown to interact with Gag in *in vitro* Gag assembly studies [34, 35].

Altogether, this information led to the hypothesis that $PI(4,5)P_2$ plays a role in PM localization of Gag through interaction with the MA basic amino acids.

Previously, we showed that perturbing PM $PI(4,5)P_2$ in HeLa cells markedly reduces virus production. This was demonstrated using two different approaches: overexpression of polyphosphoinositide 5-phosphatase IV (5ptaseIV) and a constitutively active ADP-ribosylation factor 6 (Arf6) mutant, Arf6/Q67L [36]. Overexpression of 5ptaseIV reduces cellular $PI(4,5)P_2$ levels by hydrolyzing the phosphate group at the D5 position of the inositol ring [37], whereas expression of Arf6/Q67L induces $PI(4,5)P_2$-enriched vesicles within the cytoplasm [38, 39]. Altering $PI(4,5)P_2$ levels by 5ptaseIV overexpression drastically reduced virus release efficiency when compared to cells expressing 5ptaseIV Δ1 mutant that lacks the functional phosphatase domain. In addition, in 5ptaseIV-overexpressing cells, mature Gag relocalized from the PM to CD63-positive compartments. Expression of Arf6/Q67L also reduced virus release efficiency. In this case, Gag was retargeted to newly induced $PI(4,5)P_2$-enriched vesicles. These results support the model that $PI(4,5)P_2$ promotes or stabilizes the binding of Gag to the PM by interacting with Gag.

Recently, two studies addressed the question of whether Gag interacts with $PI(4,5)P_2$[7, 40]. One study examined the accessibility of lysines after Gag is mixed with $PI(4,5)P_2$. They identified two lysines at MA residues 29 and 31 as potential $PI(4,5)P_2$-interacting amino acids. Interestingly, we have observed previously that the mutations at these MA residues relocalize Gag from the PM to compartments positive for late endosome markers in HeLa cells [20, 22]. The other group determined the structure of a complex between MA and $PI(4,5)P_2$ using nuclear magnetic resonance (NMR). As is

often observed for interactions between $PI(4,5)P_2$ and other proteins, in this structure, the inositol head group of $PI(4,5)P_2$ was in direct contact with several basic residues in MA, although Lys 29 and 31 were not found to be involved. Strikingly, in addition to the head group, the 2' fatty acid chain of $PI(4,5)P_2$ also binds to MA at a hydrophobic cleft. Furthermore, binding of $PI(4,5)P_2$ to MA increased the exposure of the myristate moiety. Based on these findings, the authors proposed that $PI(4,5)P_2$ not only acts as a membrane anchor for Gag, but also as a trigger for myristate exposure. Altogether, results from these studies are consistent with the model that Gag binds $PI(4,5)P_2$ directly during virus assembly.

However, two major caveats apply to these studies. First, because all the components need to be soluble in the approaches used, both studies used water-soluble $PI(4,5)P_2$ with short acyl chains, which does not accurately represent the natural $PI(4,5)P_2$ found in the cells. Secondly, both studies used Gag derivatives that are unable to drive virus assembly in cells: isolated MA and MACA were used in the NMR study, and non-myristoylated Gag was analyzed in the lysine accessibility experiments. Thus, it remains to be determined whether the Gag-$PI(4,5)P_2$ interaction occurs in a physiologically relevant environment.

In this study, we analyzed the overall membrane binding ability of Gag in control and $PI(4,5)P_2$-depleted cells using microscopy and an equilibrium flotation assay. The data indicate that $PI(4,5)P_2$ is important for general membrane binding as well as PM localization, supporting the hypothesis that Gag and $PI(4,5)P_2$ interact. To determine whether Gag interacts with $PI(4,5)P_2$ in a more defined system, we developed an *in vitro* liposome-binding assay in which full-length myristoylated Gag and $PI(4,5)P_2$ with

natural-length acyl chains were used. Using this assay, we analyzed both WT and MA domain mutants for their ability to bind liposomes with varied amounts of $PI(4,5)P_2$. Our results demonstrate that full-length myristoylated Gag binds membrane-associated $PI(4,5)P_2$ and that this interaction mediated by the MA basic amino acids is important for Gag-membrane binding. We also demonstrate that efficient Gag membrane binding and Gag localization to the PM are separable processes that each require cellular $PI(4,5)P_2$.

MATERIALS AND METHODS

Cells and Plasmids

HeLa cells were cultured and maintained as described previously [41]. The HIV-1 molecular clone pNL4-3 was described previously [42]. Molecular clones encoding Gag-Venus (pNL4-3/GagVenus) and Gag-monomeric red fluorescent protein (mRFP1) (pNL4-3/GagmRFP) were constructed by replacing the SphI-SalI region of pNL4-3 with the corresponding regions of pHIV-1-GagvYFP3M and pHIV-1-GagmRFP, respectively. Both pHIV-1-GagvYFP3M and pHIV-1-GagmRFP were derived from pON-HIG [43] and express the full-length Gag fused to a glycine-rich hinge (PGISGGGGGILD) and a fluorescent protein. Additionally, these two constructs encode *tat*, *rev*, and *nef*, and contain a large deletion in *pol* and silent mutations that destroy the *gag-pol* frameshift site without changing the protein sequence of Gag. The fluorescent protein gene vYFP3M harbors mutations that enhance the monomeric property of the encoded protein (A206K, L221K, F223R) [44]. In this manuscript, this Venus YFP derivative is referred to as Venus for simplicity. The 5ptase expression plasmid pcDNA4TO/Myc5ptaseIV [37] and the Δ1 mutant [36] were previously described. The plasmids expressing 5ptaseIV under

the control of HIV-1 long terminal repeat (LTR) promoter in the presence of Tat (pHIV-Myc5ptaseIV and pHIV-Myc5ptaseIV Δ1) were made by replacing the Lck gene within pHIVLck+ (a kind gift from Dr. K.Strebel) with sequences encoding either Myc-tagged full-length 5patseIV or the Δ1 mutant, respectively, from corresponding pcDNA plasmids. Molecular clones encoding Gag derivatives in which the initiation codon or the entire MA sequence was substituted with the sequence of the N-terminal 10 amino acids from Fyn Kinase [pNL4-3/Fyn(10)fullMA and pNL4-3/Fyn(10)ΔMA, respectively] were constructed using PCR mutagenesis. pGEMNLNR, the *in vitro* expression plasmid for Gag, was made by inserting the Nar I-EcoR I fragment from pNL4-3 into the multiple cloning site of pGEM®-1 (Promega Corporation). The derivatives of pGEMNLNR encoding MA mutants, pGEMNLNR/1GA, pGEMNLNR/29KE/31KE, pGEMNLNR/29KT/31KT, pGEMNLNR/Fyn(10)fullMA, and pGEMNLNR/Fyn(10)ΔMA, were made by replacing the Gag sequence of pGEMNLNR with those from pNL4-3 derivatives, pNL4-3/1GA [18], pNL4-3/29KE/31KE [20], pNL4-3/29KT/31KT [45], pNL4-3/Fyn(10)fullMA, and pNL4-3/Fyn(10)ΔMA, respectively. pNL4-3/Fyn(10)fullMA/GagVenus and pNL4-3/Fyn(10)ΔMA/GagVenus were constructed by replacing the Gag sequence of pNL4-3/GagVenus with those from pNL4-3/Fyn(10)fullMA and pNL4-3/Fyn(10)ΔMA, respectively. A molecular clone encoding non-functional viral protease, pNL4-3/PR$^-$, was described previously [46].

Transfection, Virus Release Assay, and Immunoblottinng

Transfection, metabolic ^{35}S labeling of transfected cells, and immunoprecipitation of viral proteins using HIV immunoglobulin (HIV-Ig; AIDS Reagent and Reference Reagent Program) were performed as described elsewhere [20, 22, 41]. Virus release

efficiency was calculated as previously described [36]. Immunoblotting was performed as described previously[47] using HIV-Ig, rabbit polyclonal anti-GFP (Clontech, Mountain View, CA), or rabbit polyclonal anti-DsRed (Clontech) as primary antibodies. Detection of specific signals was performed using Super Signal West Pico chemiluminescence detection kit (Pierce, Rockford, IL).

Immunostaining and Fluorescence Microscopy

Fixation and immunostaining of HIV- expressing HeLa cells was performed as described previously [20, 36]. For quantitative analysis of Gag localization phenotypes, cells transfected with pNL4-3/GagVenus or its derivatives, or pNL4-3/GagmRFP, along with pHIV-Myc5ptaseIV or its Δ1 derivative, were fixed and immunostained for the presence of 5ptase expression with rabbit anti-Myc antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Images of 10 to 15 fields were recorded using a Nikon TE2000 microscope, and cells positive for full-length 5ptaseIV or the Δ1 mutant were evaluated for Gag localization pattern. At least 100 5ptaseIV-positive, Gag-positive cells were examined for each condition.

Cell-Based Membrane Binding Analysis

HeLa cells expressing WT or Fyn(10)fullMA Gag, along with full-length 5ptaseIV or the Δ1 mutant, were pulse-labeled with [^{35}S] Met/Cys for 5 minutes and chased for 20 minutes. After sonication of labeled cells, the cell homogenates were subjected to a low-speed centrifugation to remove unbroken cells and the nucleus-associated materials as pellets. These pellets contain approximately 10% of total labeled Gag (data not shown) regardless of which Gag or 5ptaseIV constructs were expressed in the labeled cells. The post-nuclear supernatants were collected and subjected to

membrane flotation centrifugation as previously described [14]. The top fractions containing membrane-bound materials, and also the bottom fractions containing non-membrane-bound materials, were pooled and subjected to immunoprecipitation using HIV-Ig. Labeled Gag in fractions was quantified by phosphorimager analyses as described previously [48, 49].

Liposome Binding Assay

Chloroform solutions of 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine (POPC), 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-[Phospho-L-serine] (POPS), and L-α-Phosphatidylinositol-4,5-bisphosphate(Porcine brain) (PI(4,5)P_2), and powder forms of 1-Stearoyl-2-Arachidonoyl-*sn*-Glycero-3-[Phosphoinositol-4,5-Bisphosphate] (Tri-ammonium Salt), 1-Stearoyl-2-Arachidonoyl-*sn*-Glycero-3-[Phosphoinositol-3,5-Bisphosphate] (Tri-ammonium Salt), 1-Stearoyl-2-Arachidonoyl-*sn*-Glycero-3-Phosphoinositol-3,4,5-trisphosphate (Tetra-ammonium Salt) were purchased from Avanti Polar lipids Inc., Alabaster, AL. Either POPC and POPS, or POPC, POPS and one of the phosphoinositides, were mixed in a tube at ratios described in figure legends and the chloroform was evaporated using a nitrogen stream. The dried lipids (total 730 μg) were resuspended in 50 μl of cold 20 mM HEPES buffer (pH7.0) (HB), sonicated for 30 minutes in a water bath sonicator at 4°C, and further incubated in a shaker at 4°C overnight [50-52].

In vitro translation of Gag was performed using TNT® SP6 Coupled Reticulocyte Lysate System (Promega Corporation, Madison, WI). Twenty five μl of a reaction mixture that contains 12.5 μl of reticulocyte lysate was prepared with [^{35}S] methionine and cysteine as instructed by the manufacturer, incubated for 30 minutes at 30°C, 5 μl of the liposome

suspension (total 73 μg of lipids) prepared as above were added, and further incubated for 90 minutes in the presence of liposomes.

Subsequently, the reaction was diluted to 200 μl using HB and mixed with 1 ml of HB containing 85.5% sucrose in an ultracentrifuge tube. This mixture was then layered with 2.8 ml HB containing 65% sucrose and 1 ml HB containing10% sucrose, and centrifuged at 115,000 × g at 4°C for 16 hours (Sorvall rotor AH-650, 35000 rpm). Five 1 ml fractions were collected from the top of each tube. The top two fractions represent liposome-bound Gag, and the bottom fractions represent non-liposome-bound Gag. The fractions were analyzed by SDS-PAGE, and the band intensity of Gag in each fraction was quantified using a phosphorimager. The amount of labeled Gag in the top two fractions versus the total amount of labeled Gag was calculated and is shown as liposome binding efficiency. For Figs. 2.5, 2.6, and 2.9, the relative liposome binding efficiency for each condition was calculated in comparison to the binding efficiency of WT Gag with PI(4,5)P_2-containing liposomes.

Statistical Analysis

The two-tailed Student's *t*-test was performed using GraphPad Prism version 3.0cx for Macintosh, GraphPad software, San Diego, California (www.graphpad.com). The paired *t*-test was used for comparing the data within the same set of experiments. *p* values less than 0.05 were considered statistically significant.

RESULTS

Overexpression of 5ptaseIV relocalizes HIV-1 Gag to the perinuclear compartments and cytosol.

In a previous study, we used a monoclonal antibody against p17MA to show that overexpression of 5ptaseIV relocalizes Gag to perinuclear compartments [36]. However, this antibody recognizes only mature MA, but not $Pr55^{Gag}$ [10]. To determine the localization of the whole population of Gag, in this study we used Gag tagged with Venus yellow fluorescent protein [53](GagVenus) or monomeric red fluorescent protein [54](GagmRFP). To examine the impact of 5ptaseIV overexpression on Gag localization, HeLa cells were transfected with either GagVenus or GagmRFP, along with 5ptaseIV or its derivative lacking a functional phosphatase domain (the Δ1 mutant), and analyzed with a fluorescence microscope (Fig. 2.1A, and data not shown). In cells expressing the 5ptaseIV Δ1 mutant, a majority of GagVenus and GagmRFP showed punctate PM localization as expected for WT Gag. When full-length 5ptaseIV was expressed, however, most Gag signals were detected either in perinuclear compartments or in the cytosol. To assess the effect of 5ptaseIV more quantitatively, cells expressing GagVenus or GagmRFP were counted and divided into three groups based on Gag localization: Gag localized to the PM, Gag predominantly in the intracellular compartments, or Gag only in the cytosol without PM or intracellular localization (Fig. 2.1B). The cells expressing the Δ1 mutant of 5ptaseIV had Gag localized to the PM in more than half of the cells. Very few cells showed perinuclear localization. The remaining cells displayed only hazy cytosolic localization. These Venus and mRFP signals likely represent full-length Gag tagged with the fluorescent proteins, as virtually no other protein species containing the fluorescent tags were detected by immunoblotting of cell lysates (Fig. 2.1C). In contrast to cells expressing 5ptaseIVΔ1, cells expressing full-length 5ptaseIV had drastically increased hazy cytosolic localization and intracellular localization, whereas cells that had

PM localization were reduced to only 5% of total Gag-positive cells. These results indicate that, when cellular PI(4,5)P_2 is depleted, Gag relocalizes not only to intracellular compartments as we have shown previously [36], but also to the cytosol. These data confirm the importance of PI(4,5)P_2 in PM localization of Gag. In addition, the increase in the hazy cytosolic signal in 5ptaseIV-expressing cells suggests that PI(4,5)P_2 might also be essential for total membrane binding of Gag.

Overexpression of 5ptaseIV causes a Gag-membrane binding defect.

To analyze more quantitatively whether PI(4,5)P_2 depletion impairs membrane binding of Gag, we examined the membrane binding of WT Gag in HeLa cells expressing either 5patseIV or the Δ1 mutant, by equilibrium flotation centrifugation. The cells were pulse-labeled with [^{35}S] Methionine-Cysteine for 5 minutes, and chased for 20 minutes, and post-nuclear supernatants of cell homogenates were subjected to equilibrium flotation centrifugation. The labeled Gag in both top fractions (membrane-bound) and bottom fractions (non-membrane-bound) was immunoprecipitated with HIV-Ig and quantified by phosphorimager. We found that the membrane binding of Gag was significantly decreased when full-length 5ptaseIV was expressed compared to the Δ1 mutant of 5ptaseIV (Fig. 2.2A & B). These results indicate that PI(4,5)P_2 is not only important for plasma membrane localization but also contributes to general membrane binding of Gag. A large part of membrane-bound Gag still detected in cells overexpressing 5ptaseIV may be a population associated with the intracellular membranes observed in Fig. 2.1. Notably, we did not observe a significant reduction in the membrane binding of Fyn(10)fullMA Gag (Fig. 2.2A & B), a Gag derivative in which

the N-terminal myristoylation site is replaced by Fyn membrane binding signal containing one myristoylation and two palmitoylation sites. These data indicate that addition of a strong membrane binding signal to MA can reverse the membrane-binding defect imposed by $PI(4,5)P_2$ depletion.

The Presence of $PI(4,5)P_2$ increases Gag binding to liposomes.

As described above, cellular $PI(4,5)P_2$ depletion not only relocalized Gag from the PM to intracellular compartments, but also reduced the total membrane-binding ability of Gag. These results are consistent with the hypothesis that Gag interacts with $PI(4,5)P_2$ directly. To test this hypothesis, we developed an *in vitro* system using liposomes that consist of phosphatidylcholine(PC), phosphatidylserine (PS) and varied amounts of $PI(4,5)P_2$. An *in vitro* transcription and translation coupled reaction with rabbit reticulocyte lysate was used to synthesize full-length myristoylated Gag in the presence of [^{35}S] methionine-cysteine. This *in vitro* Gag synthesis has been used successfully for studying Gag-membrane binding and assembly [5, 10, 11, 55-59]. After 30 minutes of Gag synthesis, the reaction was mixed with liposomes and further incubated for 90 minutes. The liposome-bound Gag and non-liposome-bound Gag were separated by equilibrium flotation centrifugation. The amount of labeled Gag in each fraction was quantified using a phosphorimager, and the percentage of Gag bound to the liposomes versus total Gag was calculated. When the $PI(4,5)P_2$ concentration in the liposomes was increased gradually, the liposome-bound Gag also increased (Fig. 2.3A & B), suggesting that the Gag-$PI(4,5)P_2$ interaction mediates Gag binding to liposomes.

In the experiments described above, the ratio between PC and PS was kept

constant (2:1) while the amounts of $PI(4,5)P_2$ were gradually increased. Therefore, it is possible that the enhanced Gag binding to liposomes in the presence of $PI(4,5)P_2$ may be due to the increase in the overall negative charge of liposomes, not the increase in the $PI(4,5)P_2$ concentration *per se*. To address this possibility, we analyzed Gag binding to PC-PS liposomes containing elevated levels of PS. As $PI(4,5)P_2$ is likely trivalent or tetravalent in the negative charge under the conditions tested, whereas PS is monovalent [60], we examined liposomes containing 52 or 60 mol % PS that would bear equivalent levels of the negative charge as liposomes containing 30 mol% PS and 7.25 mol% $PI(4,5)P_2$ used in Fig. 2.3A and B. Liposomes with 52 or 60 mol % PS showed increased Gag binding compared to liposomes containing 30 mol % PS (Fig. 2.4A and B), suggesting that electrostatic interaction between Gag and lipids does play a role in Gag-liposome binding. Nevertheless, these high concentrations of PS were insufficient to mediate the high level of Gag binding observed with liposomes containing 30 mol% PS and 7.25 mol% $PI(4,5)P_2$ (Fig. 2.4Aand B). These results suggest that enhanced Gag binding to $PI(4,5)P_2$-containing liposomes does not simply result from the increase in the overall negative charge of liposomes.

$PI(4,5)P_2$ may facilitate Gag binding due to the highly concentrated negative charge of its head group. Alternatively, the configuration of phosphate residues may play a key role in the specificity of the Gag-$PI(4,5)P_2$ interaction. To evaluate these possibilities, we compared $PI(3,5)P_2$ and $PI(3,4,5)P_3$ with $PI(4,5)P_2$ at 7.25 mol% for the ability to recruit Gag to liposomes containing 30 mol % PS. We observed that $PI(3,5)P_2$ and $PI(3,4,5)P_3$ substantially enhanced Gag binding to liposomes compared to the control liposomes. However, Gag binding to $PI(3,5)P_2$-containing liposomes was consistently

less than that to PI(4,5)P_2-containing liposomes. We did not observe significant difference between PI(4,5)P_2 and PI(3,4,5)P_3 (Fig. 2.4C and D). It is of note that, using water-soluble phosphoinositides, Saad et al. observed that the isolated MA domain also binds PI(4,5)P_2 and PI(3,4,5)P_3 but not PI(3,5)P_2[7]. These data suggest that not only the number but also the position of phosphates on the inositol head group affects the Gag-phosphoinositide interaction. Altogether, these results demonstrate that there is an interaction between the head group of PI(4,5)P_2 and full-length Gag and that this interaction facilitates membrane binding of Gag.

To confirm that the observed Gag binding to liposomes reflects the Gag-membrane interaction in cells, we also examined, using liposome-binding assay, a non-myristoylated Gag (1GA) that was previously shown to be defective in membrane binding in cell-based assays [4-6, 14, 15]. As shown in Fig. 2.5A, WT but not 1GA Gag was myristoylated in the *in vitro* translation system we used. The 1GA mutant binds liposomes inefficiently even in the presence of 7.25 mol % PI(4,5)P_2 (Fig. 2.5B and C). These results indicate that myristoylation is required for efficient Gag-liposome interaction. These results further demonstrate that, unlike assays using water-soluble PI(4,5)P_2, the liposome-binding assay developed here is suited for examining the interaction between Gag and PI(4,5)P_2 in the context of membrane binding.

MA mediates Gag interaction with PI(4,5)P_2.

MA was shown previously to be important for both membrane binding and PM localization [1-3]. To assess the importance of MA in the interaction with PI(4,5)P_2 in liposome-binding assay, we sought to delete the entire MA sequence. Since such MA

deletion removes the site for myristoylation that is indispensable for membrane binding, we instead used a Gag derivative that lacks the entire MA sequence but contains the N-terminal 10 amino acids of the Fyn kinase, Fyn(10)ΔMA Gag (Fig. 2.6A). This Fyn-derived sequence is myristoylated and dually palmitoylated, thereby providing this Gag derivative with a high membrane affinity. As a control, we used Fyn(10)fullMA Gag (Fig. 2.6A), which retains the entire MA sequence following the Fyn-derived peptide. As shown in Fig. 2.6B and C, liposome binding of Fyn(10)fullMA Gag was still augmented by $PI(4,5)P_2$ as observed for WT Gag. However, when MA was deleted as in Fyn(10)ΔMA Gag, Gag bound both control and $PI(4,5)P_2$-containing liposomes with similar efficiences. These results demonstrate that MA confers dependence on $PI(4,5)P_2$ for liposome binding.

PM localization and virus release efficiency of Fyn(10)fullMA Gag are still sensitive to 5ptaseIV overexpression.

In contrast to the data obtained from the liposome-binding assay, in the cell-based membrane binding analysis, Fyn(10)fullMA Gag did not show any significant membrane-binding defect upon $PI(4,5)P_2$ depletion (Fig. 2.2). In the case of WT Gag, 5ptaseIV alters Gag localization to the PM in addition to reducing membrane binding. To investigate whether the $PI(4,5)P_2$ dependence of Fyn(10)fullMA Gag for liposome binding is manifested as a defect in the PM localization, we analyzed the effect of 5ptaseIV overexpression on localization of Fyn(10)fullMA GagVenus as in Fig. 2.1. In 5ptaseIVΔ1-expressing cells, Fyn(10)fullMA GagVenus was predominantly localized on the PM. In cells overexpressing full-length 5ptaseIV, however, we observed that a

majority of Fyn(10)fullMA GagVenus was localized to intracellular compartments (Fig. 2.7A and B). These results indicate that, even though general membrane binding of Fyn(10)fullMA Gag is not affected in cells (Fig. 2.2), PM localization of this Gag derivative still requires cellular PI(4,5)P_2. To examine whether the altered Gag localization affects virus particle production, we examined the virus release efficiency of Fyn(10)fullMA Gag in 5ptaseIV-overexpressing cells. HeLa cells were transfected with a molecular clone encoding WT Gag or Fyn(10)fullMA Gag, along with an expression vector for either 5ptaseIV or the Δ1 mutant. The cell and viral lysates were immunoprecipitated with HIV-Ig after metabolic labeling, and the virus release efficiency was calculated. Consistent with the altered localization of Gag, the virus release efficiency of Fyn(10)fullMA Gag as well as WT Gag was reduced when the cells express 5ptaseIV(Fig. 2.8A and B). These results indicate that cellular PI(4,5)P_2 is required separately both for efficient membrane binding and PM localization of WT Gag.

The liposome-binding assay showed no difference in binding of Fyn(10)ΔMA Gag to control and PI(4,5)P_2-containing liposomes (Fig. 2.6). Therefore, we predicted that Gag localization and virus particle production of Fyn(10)ΔMA Gag would be insensitive to 5ptaseIV overexpression in contrast to Fyn(10)fullMA. Venus-tagged Fyn(10)ΔMA Gag localized not only to the PM but also to intracellular vesicles in a majority of cells expressing 5ptaseIV Δ1 (Fig. 2.7A and B). This promiscuous localization of Fyn(10)ΔMA Gag, which was also observed previously for a similar Gag derivative lacking MA[61], underscores the importance of MA in specific Gag localization to the PM. Notably, overexpression of full-length 5ptaseIV did not cause a readily detectable qualitative difference in Gag localization (Fig. 2.7A and B).

Nevertheless, 5ptaseIV overexpression significantly reduced Fyn(10)ΔMA Gag virus release efficiency (Fig. 2.8A and B). We are currently investigating the possibility that cellular PI(4,5)P_2 might play an additional role in HIV-1 particle production.

Matrix basic domain mutants display a PI(4,5)P_2 binding defect *in vitro*.

As described in the introduction, MA contains a basic amino acid cluster that was previously shown to play an important role in plasma membrane localization of Gag [18-21]. To see if the basic residues in this cluster facilitate the MA-PI(4,5)P_2 interaction, we tested the mutants in which lysines were replaced with either glutamic acid (29KE/31KE) or threonine (29KT/31KT) at MA residues 29 and 31, using *in vitro* liposome binding assay. Full-length Gag with WT MA or with MA mutations 29KE/31KE or 29KT/31KT were synthesized using *in vitro* translation system, and were allowed to interact with the control liposomes or liposomes containing PI(4,5)P_2. There was a 2.5-fold decrease in binding of both mutants to PI(4,5)P_2-containing liposomes when compared to WT Gag (Fig. 2.9A & B). Both of these Gag mutants were previously shown to localize in intracellular vesicles using immunoflorescence microscopy [20, 22]. These results indicate that the efficient interaction of Gag with PI(4,5)P_2 requires basic amino acids in the MA domain, especially residues at positions 29 and 31, and suggest that this interaction is important for the plasma membrane localization of Gag.

DISCUSSION

We have shown previously that PI(4,5)P_2 is essential for plasma membrane localization of HIV-1 Gag. In this study, using microscopy (Fig. 2.1) and biochemical methods (Fig. 2.2), we observed that PI(4,5)P_2 is not only important for PM localization

but also contributes to overall membrane binding of HIV-1 Gag. Using a liposome binding assay, we further demonstrated that the MA domain of Gag and $PI(4,5)P_2$ interact with each other. Recently, using different approaches, two groups [7, 40] reported evidence supporting a possibility that MA and $PI(4,5)P_2$ interact directly. By solving the structure of the MA-$PI(4,5)P_2$ complex by NMR, one of these studies provided detailed information on the molecular contacts between MA and $PI(4,5)P_2$ at a high resolution [7]. As described in the Introduction, however, whether the interaction observed in these studies is physiological was unknown. In the liposome binding assay described in this report, we used membrane-associated $PI(4,5)P_2$ with natural-length acyl chains. Furthermore, the Gag synthesized by the *in vitro* translation system is full-length, myristoylated Gag precursor that is capable of driving HIV assembly in cells. Thus, this assay allowed us to examine the interaction between Gag and $PI(4,5)P_2$ using components relevant to virus assembly in cells. Using this assay, we observed that only when $PI(4,5)P_2$ is present does Gag bind efficiently to the liposomes. Altogether, these results demonstrate that the full-length HIV-1 Gag indeed interacts with membrane-associated $PI(4,5)P_2$, and that this interaction contributes to efficient Gag-membrane binding in cells.

Using the liposome-binding assay, we observed an increase in the level of Gag binding when more $PI(4,5)P_2$ was present in the liposomes. Though there was negligible binding to liposomes containing up to 2 mol % $PI(4,5)P_2$, increasing the $PI(4,5)P_2$ level to 3.5 mol % or more enhanced Gag binding significantly. It has been shown that 5 mol % $PI(4,5)P_2$ was required for liposome binding of cellular $PI(4,5)P_2$-binding proteins including ezrin, myosin VI and gelsolin [62-64]. As $PI(4,5)P_2$ is thought to be present at

1-1.5 mol % of total PM lipid in cells [65-67], one might pose a question that the binding seen in the presence of higher levels of this lipid may not be physiologically relevant. However, several points bear consideration. First, the $PI(4,5)P_2$ concentration in the PM has been determined using erythrocytes [65-67]. These cells have a unique cytoskeleton structure [68-70] and lack the regular endocytosis function [71]. As actin cytoskeleton rearrangement and endocytosis are two major $PI(4,5)P_2$-regulated cellular functions in other cell types [60, 72-74], $PI(4,5)P_2$ levels in erythrocytes may differ from those in other cell types. Second, it is important to point out that liposomes used in this assay are not completely representative of the PM, especially with regard to membrane microdomain structures. They lack cholesterol and other lipids that may form lipid rafts, which are shown to be important for HIV-1 assembly in cells [48, 75-80], particularly at the step of Gag-membrane binding [48]. Furthermore, it has been proposed that $PI(4,5)P_2$ may be sequestered to form microdomains and induce raft-like structures [81-83], which would lead to higher local $PI(4,5)P_2$ concentrations. Finally, in cells, $PI(3,4,5)P_3$ might also support the PM binding of Gag. A recent paper has shown that proteins with polybasic clusters dissociated from the PM only when both $PI(4,5)P_2$ and $PI(3,4,5)P_3$, but not $PI(4,5)P_2$ alone, were depleted [84]. This may also be the case for HIV-1 Gag, which is sensitive to 5ptaseIV overexpression. Since 5ptaseIV removes the D5-phosphate not only from $PI(4,5)P_2$, but also from $PI(3,4,5)P_3$ [37], the defect observed in 5ptaseIV-expressing cells could be due to dephosphorylation of both phosphoinositides. Consistent with this possibility, the NMR study [7] showed that HIV-1 MA bound soluble $PI(4,5)P_2$ and $PI(3,4,5)P_3$ with equal affinity. A substantial interaction was also observed between full-length Gag and membrane-associated $PI(3,4,5)P_3$ in the current study. Thus,

$PI(3,4,5)P_3$ might also contribute to the membrane binding of Gag along with $PI(4,5)P_2$ in cells.

Even if $PI(4,5)P_2$ is not available for Gag binding, addition of the efficient membrane binding signal derived from Fyn kinase to the N-terminus of Gag would be expected to allow Gag to bind any liposomes with equal efficiency. However, when Fyn(10)fullMA Gag was analyzed using the liposome-binding assay, binding of this Gag derivative to control liposomes was weak, and the presence of $PI(4,5)P_2$ augmented the binding several fold (Fig. 2.6). Notably, binding to control liposomes was enhanced when MA was removed as observed with Fyn(10)ΔMA Gag that bound control and $PI(4,5)P_2$-containing liposomes with a similar efficiency (Fig. 2.6). Thus, MA renders Gag-liposome binding dependent on $PI(4,5)P_2$. We speculate based on these data, and a recent report [85], that MA in the context of full-length Gag might have a negative effect on liposome binding, which may be eliminated by the MA-$PI(4,5)P_2$ interaction. In the absence of MA, this negative effect no longer exists, hence Fyn(10)ΔMA Gag binds both control and $PI(4,5)P_2$-containing liposomes efficiently. In contrast to the liposome binding assay, where there is only a limited number of membrane components, the cell-based assay showed that Fyn(10)fullMA Gag binds membranes well, even in 5ptaseIV-expressing cells (Fig. 2.2). This difference may be due to cellular components that are absent from liposomes. These factors might reduce the suppressive effect imposed by MA to a level low enough for the Fyn sequence to overcome. This difference illustrates that the defined nature of lipid membrane used in the liposome-binding assay allows the contribution of $PI(4,5)P_2$ to be evaluated in the absence of other membrane factors that, in the cell, can contribute to Gag-membrane binding.

In the liposome-binding assay described in this report, very little Gag associated with liposomes in the absence of $PI(4,5)P_2$, even though more than 30 mol % of the liposomes consist of another acidic lipid, PS. In contrast, a number of groups have observed that PS can support liposome binding of Gag in the absence of $PI(4,5)P_2$ [5, 86-90]. Some studies used a very high level of PS (e.g., PC:PS at a 1:2 ratio), which may account for the efficient PS-mediated membrane binding (discussed below). Dalton *et al*. showed recently, however, that myristoylated HIV-1 MA efficiently binds to PC:PS (2:1) liposomes [87]. This discrepancy can be accounted for by differences in experimental systems. One such difference is that Dalton et al. observed efficient binding of myristoylated MA or its derivative fused with CA C-terminal domain (MA-CACTD) to PS using much higher concentrations of the proteins than obtained in our assay. In this way, they were able to measure a weak interaction between MA and PS. In addition, a high concentration of Gag likely facilitates Gag multimerization, thereby increasing its avidity for membrane. Indeed, Dalton et al. found that dimerization of MA-CACTD significantly enhances binding to PS-containing liposomes. Whether Gag forms multimers under the conditions we used in our assay is currently unknown. Second, the use of MA (or MA-CACTD) versus full-length Gag may also contribute to the difference in the PS interaction. It is possible that the structure of isolated MA and the MA domain of full-length Gag may be not identical and that such conformational differences may affect the affinity for PS. It was recently proposed that full-length Gag adopts a folded conformation in which MA and NC are in close proximity [91]. In the context of full-length Gag, such a folded conformation may restrict the access of PS to the MA basic domain, which is freely accessible for PS in isolated MA or MA-CACTD. Lastly, yet

another important difference is that, in our experiments, components of rabbit reticulocyte lysate used for synthesizing Gag were present during incubation of Gag with liposomes, whereas Dalton *et al* analyzed liposome binding of purified proteins in the absence of other cellular components. Thus, in our system, negatively charged molecules such as IP6 and RNA, or positively charged molecules such as polyamines, both of which are present in mammalian cells, may interfere with the weak electrostatic interaction between Gag and PS. Indeed, both IP6 and RNA were previously shown to bind MA [35, 92]. Altogether, we consider our *in vitro* assay as an approach complementary to existing ones in that it focuses on relatively strong interactions between Gag and membrane lipids, which overcome the interference by charged molecules present in mammalian cells.

Even in our liposome binding assay, elevating the fraction of PS from 30 mol % to 52 or 60 mol % augmented Gag binding (Fig. 2.4A and B). However, liposomes containing 7.25 mol % $PI(4,5)P_2$ along with 30 mol % PS allowed more Gag binding than those containing 60 mol % PS (Fig. 2.4A and B), suggesting that the high charge density of $PI(4,5)P_2$ plays a key role in Gag binding. These data are largely consistent with the computational work of Murray *et al* [93], in which an electrostatic interaction was shown to determine the MA-membrane interaction. In this modeling study, when $PI(4,5)P_2$ was present in the lipid bilayer, it significantly increased the electrostatic potential due to its high negative charge density, thus enhancing membrane binding of Gag [93]. Compared with $PI(4,5)P_2$, however, $PI(3,5)P_2$ was consistently less efficient in facilitating Gag-liposome binding (Fig. 2.4C and D). These findings, in agreement with the NMR study [7], suggest that, in addition to a high density of negative charge, a specific configuration

of phosphates on the inositol head group may play a role in the preferential interaction of Gag with PI(4,5)P_2.

Previously, we have observed that mutations at MA residues 29 and 31 within the highly basic sequence relocalized Gag from the PM to compartments positive for late endosome markers in HeLa and T cells. Using the liposome-binding assay, we observed that the 29KE/31KE Gag interacts less efficiently with PI(4,5)P_2-containing liposomes than WT Gag. This effect is not simply due to a drastic change of charge from positive to negative, since the 29KT/31KT Gag, in which the same lysine residues are substituted with neutral amino acids, showed a similar level of reduction in binding to PI(4,5)P_2-containing liposomes. It is also unlikely that these mutations caused a major conformational change that in turn resulted in the reduced binding to PI(4,5)P2-containing liposomes, as Gag derivatives containing these amino acid changes are capable of forming virus particles with apparently a normal morphology at intracellular vesicles [18, 20]. Interestingly, Shkriabai *et al.* observed that the same lysine residues were masked from solvent when water-soluble PI(4,5)P_2 was added to non-myristoylated, but full-length Gag [40]. In contrast, the NMR study performed using isolated MA in complex with PI(4,5)P_2 did not show an interaction of MA residues 29 and 31 with PI(4,5)P_2 [7]. This difference in the residues involved in PI(4,5)P_2 interaction may be due to differences in the conformation of the MA domain of Gag versus mature MA. We are currently examining the role of CA and NC domains in PI(4,5)P_2-dependent liposome binding. These experiments will also likely provide insights into the ability of PS to support the MA-liposome binding discussed above. It is of note that the binding of the mutants 29KE/31KE and 29KT/31KT to PI(4,5)P_2-containing liposomes was not totally

abolished, suggesting that there might be other basic amino acids playing a role in the Gag-PI(4,5)P_2 interaction. The analysis of other basic MA amino acid mutants for their ability in binding PI(4,5)P_2 is described in chapter III.

One possible caveat of the liposome binding assay developed and used in this report might be the non-uniformity of liposomes. PI(4,5)P_2 is not as soluble in chloroform as other lipids [94]. Therefore, in the procedure we used to prepare liposomes, this differential solubility may lead to non-uniform mixing of lipids, which may in turn result in variable percentages of PI(4,5)P_2 in each individual liposome. In addition, as discussed above, reticulocyte lysates present in the assay likely contain molecules that modulate the Gag-PI(4,5)P_2 interaction. Therefore, this assay should not be used to determine the affinity of Gag for PI(4,5)P_2. Nonetheless, the assay described here will be valuable in investigating requirements for the Gag-phosphoinositide interaction.

In summary, we have shown that full-length Gag binds to PI(4,5)P_2 associated with membranes under conditions relevant for virus assembly in cells, and this MA-mediated interaction is important for efficient membrane binding of Gag. We also confirmed that, apart from membrane binding, cellular PI(4,5)P_2 is also important for PM localization of Gag. Due to the continuous need for new drugs against HIV-1, it is important to look at interactions between viral proteins and cellular factors like PI(4,5)P_2 as potential drug targets. Although manipulation of cellular PI(4,5)P_2 may not be a viable anti-retroviral strategy because of the physiological importance of this lipid in cells, inhibitors that block Gag binding to PI(4,5)P_2 could perhaps be developed. Further

characterization of the Gag-PI(4,5)P_2 interaction using the liposome-binding assay established in this study will likely provide groundwork for this goal.

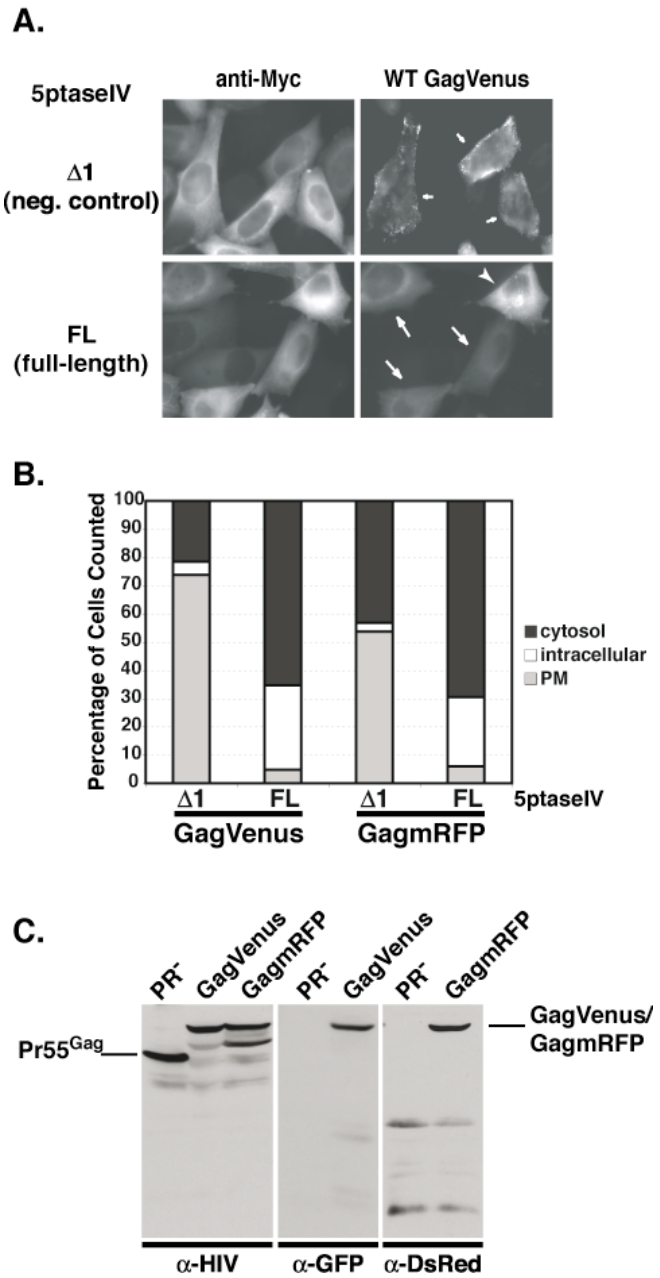

Fig. 2.1. Overexpression of 5ptaseIV relocalizes Gag from the PM to perinuclear compartments and cytosol

HeLa cells expressing GagVenus (A and B) or GagmRFP (B) along with full-length 5ptaseIV (FL) or the Δ1 derivative (Δ1) were immunostained with anti-Myc (for 5ptaseIV) and analyzed by a Nikon TE2000 microscope. The number of cells with 1) Gag localized at the PM (small arrows/grey); 2) Gag localized predominantly at intracellular compartments (arrow heads/white); and 3) Gag localized only in the cytosol (large arrows/black), were counted. At least 100 5ptaseIV-positive, Gag-positive cells were examined for each condition. In C, lysates of HeLa cells transfected with pNL4-3/PR$^-$, pNL4-3/GagVenus, or pNL4-3/GagmRFP, were subjected to SDS-PAGE and analyzed by immunoblotting using HIV-Ig, anti-GFP, or anti-DsRed. Note that predominantly single bands were detected using antibodies against fluorescent proteins.

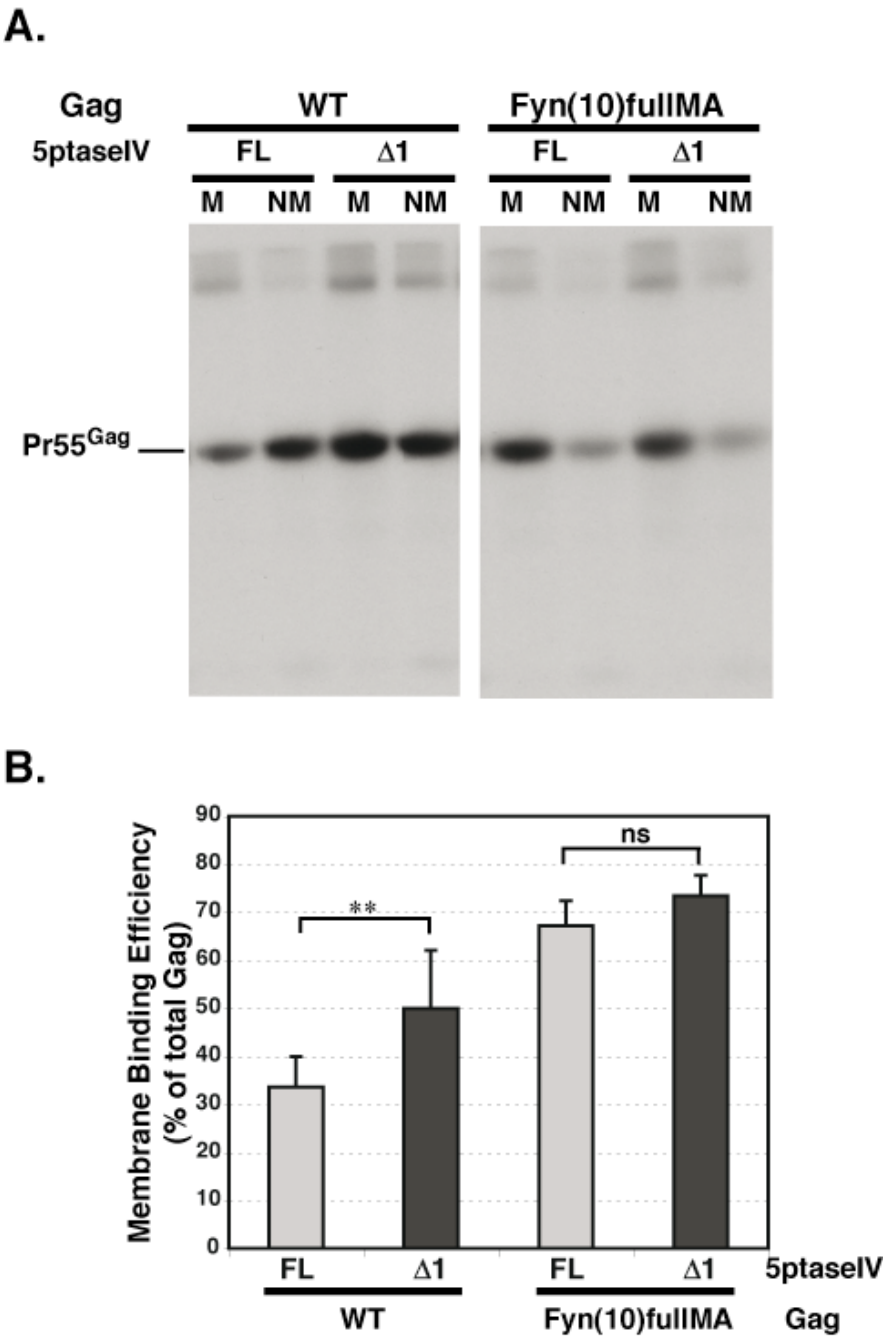

Fig. 2.2. Membrane binding of WT Gag is reduced in 5ptaseIV-overexpressing cells
HeLa cells expressing WT or Fyn(10)fullMA Gag along with full-length 5ptaseIV (FL) or the Δ1 mutant were pulse-labeled for 5 min and chased for 20min. Post-nuclear supernatants of cell homogenates were subjected to membrane flotation centrifugation. A) Membrane (M)- and non-membrane (NM)-bound Gag was recovered by immunoprecipitation and subjected to SDS-PAGE. B) The labeled Gag protein was quantified by phosphorimager analysis and the membrane binding efficiency was calculated. Data from six (WT) or four [Fyn(10)fullMA] different experiments are shown as means ± standard deviation. *p* values were determined using Student's *t*-test. (ns=not significant, ** $p<0.005$)

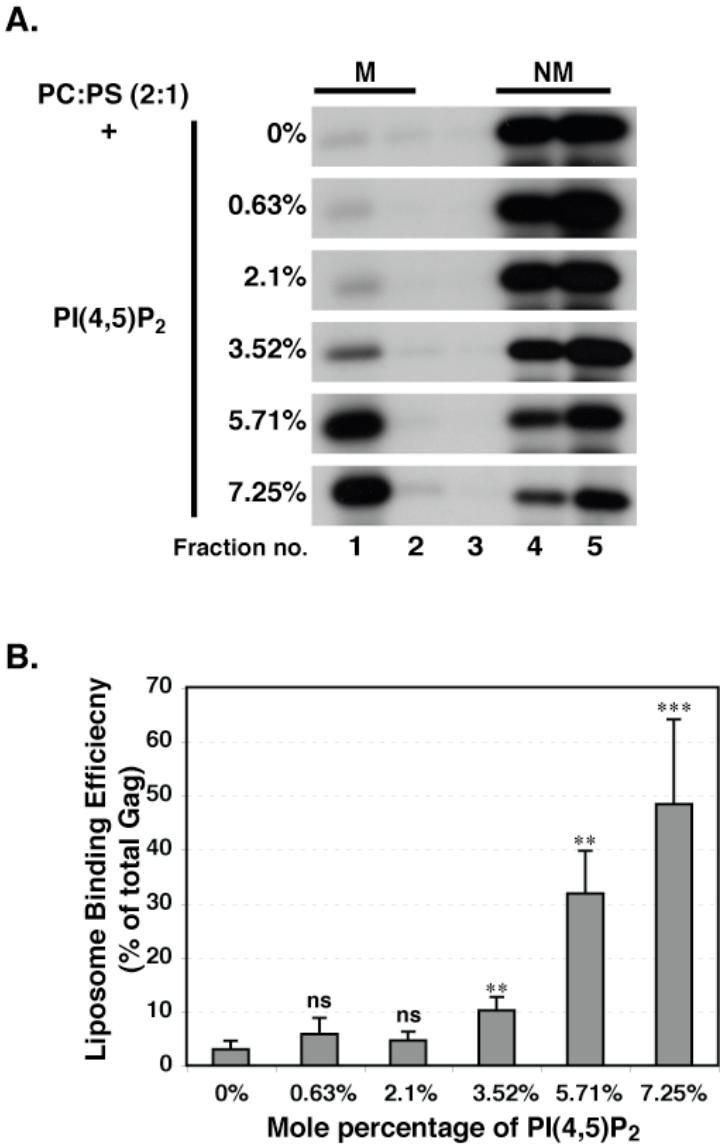

Fig. 2.3. Gag binds PI(4,5)P_2 in a concentration-dependent manner
[^{35}S]-labeled WT Gag was synthesized in the *in vitro* transcription and translation system by using reticulocyte lysates and was incubated with PC:PS (2:1) liposomes containing varying amounts of PI(4,5)P_2. The reactions were subjected to membrane flotation centrifugation, and five 1-ml fractions were collected from each tube. M, liposome-bound; NM, non-liposome-bound. Fractions were subjected to SDS-PAGE followed by autoradiography (A). The amount of labeled Gag in each fraction was quantified using a phosphorimager and the percentage of labeled Gag in the membrane-bound fraction versus total amount of labeled Gag was calculated (B). For B, data from seven (0 and 7.25 mol%), five (0.63 mol%) and four (2.1, 3.52, and 5.71 mol%) different experiments are shown as means ± standard deviation. *p* values were determined between 0% PI(4,5)P_2 and various percentages of PI(4,5)P_2 using Student's *t*-test (ns=not significant, ** $p<0.005$, *** $p<0.001$).

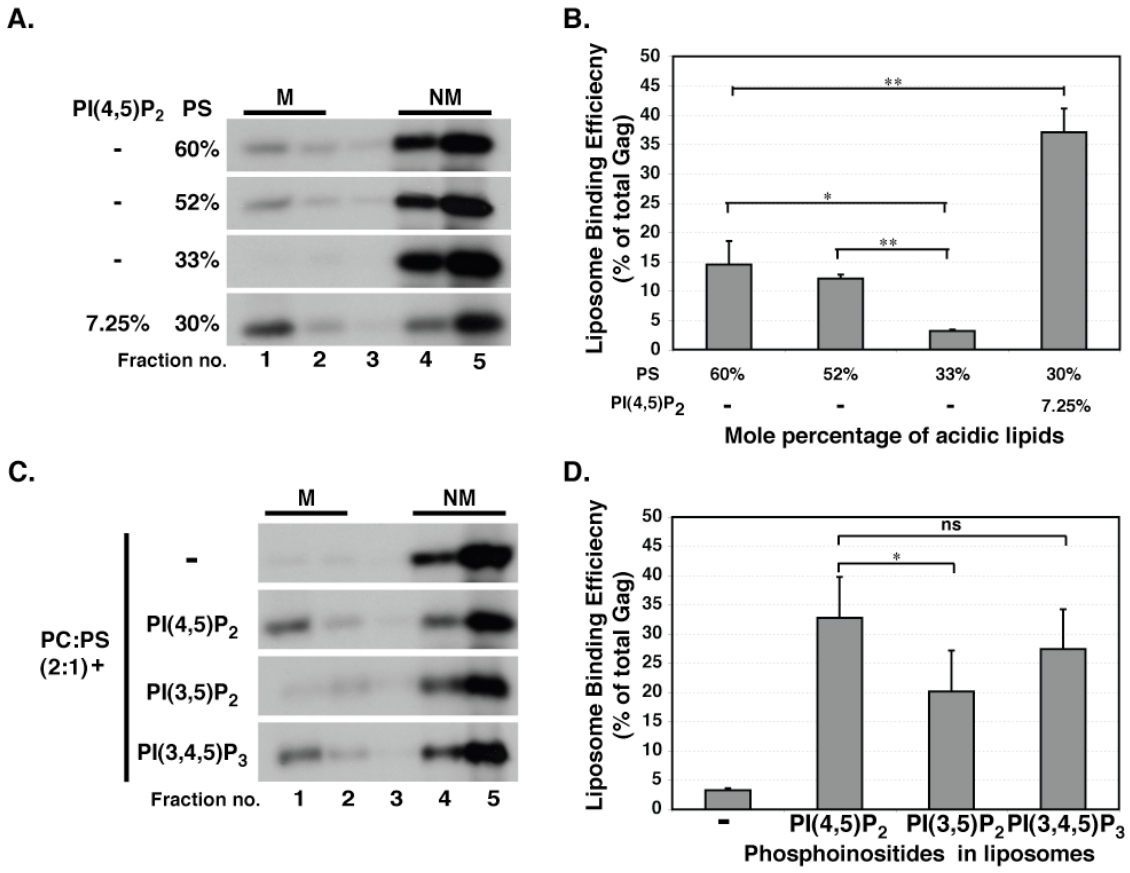

Fig. 2.4. Gag-PI(4,5)P_2 interaction is head-group specific and not merely electrostatic

[^{35}S]-labeled WT Gag was synthesized in the *in vitro* transcription and translation system by using reticulocyte lysates and was incubated with liposomes containing varying amounts of acidic phospholipids. In A and B, liposomes containing increased levels of PS were compared with liposomes containing 62.75 mol% PC, 30 mol% PS, and 7.25 mol% PI(4,5)P_2. In C and D, 7.25 mol% of PI(4,5)P_2, PI(3,5)P_2, and PI(3,4,5)P_3 were compared in the context of the PC:PS (2:1) liposomes. The fractions were collected and analyzed as in Fig. 2.4. M, liposome-bound; NM, non-liposome-bound. For B and D, data from three and four different experiments, respectively, are shown. (ns=not significant, *$p<0.05$, **$p<0.01$)

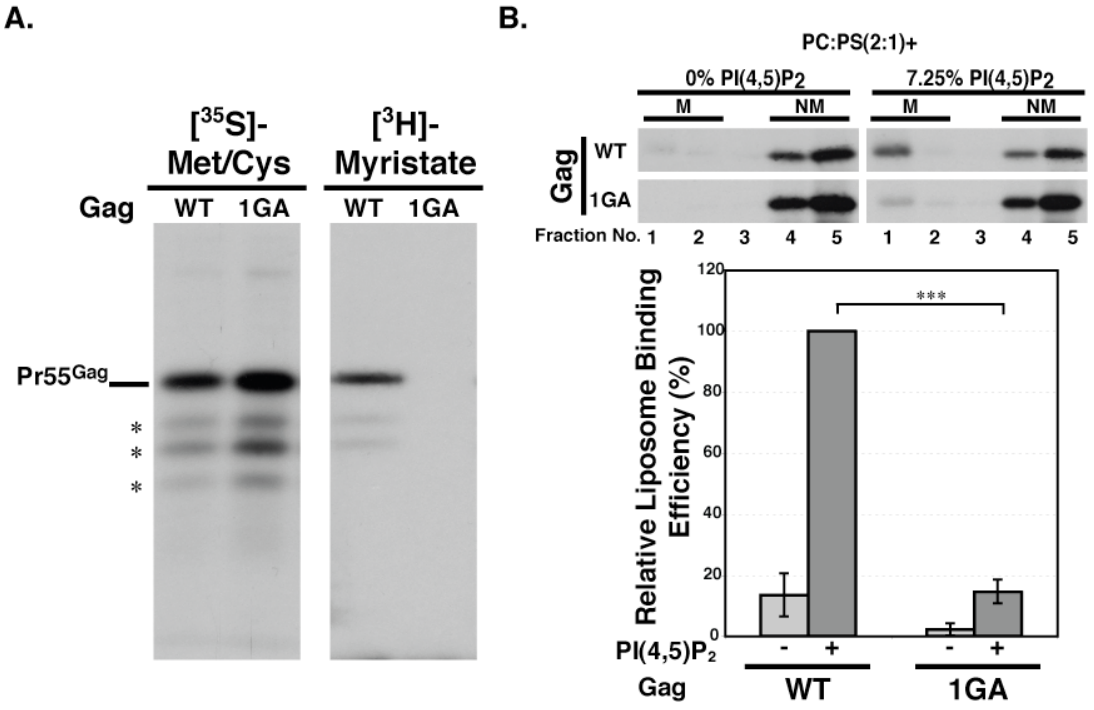

Fig. 2.5. Myristoylation-defective mutant Gag does not bind liposomes efficiently even in the presence of a high percentage of PI(4,5)P_2

A) WT or 1GA Gag were synthesized in the reticulocyte lysate system in the presence of [^{35}S]-methionine/cysteine or [^{3}H]-myristic acid. The major band (labeled Pr55^{Gag}) comigrated with Gag immunoprecipitated from lysates of HeLa cells transfected with pNL4-3/PR^{-} in a parallel experiment (data not shown). In addition to the Pr55^{Gag} band, three minor bands (asterisk) were detected in the *in vitro* translation reaction of WT Gag performed in the presence of the [^{35}S]-labeled amino acids. Two of the minor bands that are also labeled with ^{3}H likely represent Gag species arising from premature termination of translation, whereas the one band labeled only with ^{35}S may be formed by internal ribosomal entry. In vitro translation reactions for 1GA Gag as well as other Gag derivatives used in this study (data not shown) contained the same minor bands with similarly low abundance. B) [^{35}S]-labeled WT Gag or myristoylation mutant (1GA) Gag was synthesized as in Fig 2.3 and incubated with liposomes containing or not containing PI(4,5)P_2. The reactions were subjected to membrane flotation centrifugation and fractions were analyzed as in Fig. 2.3. The relative liposome binding efficiency for each condition was calculated after setting the binding efficiency of WT Gag with PI(4,5)P_2-containing liposomes to 100%. Data from three different experiments are shown as means ± standard deviation. *p* values were determined using Student's *t*-test (***p<0.001).

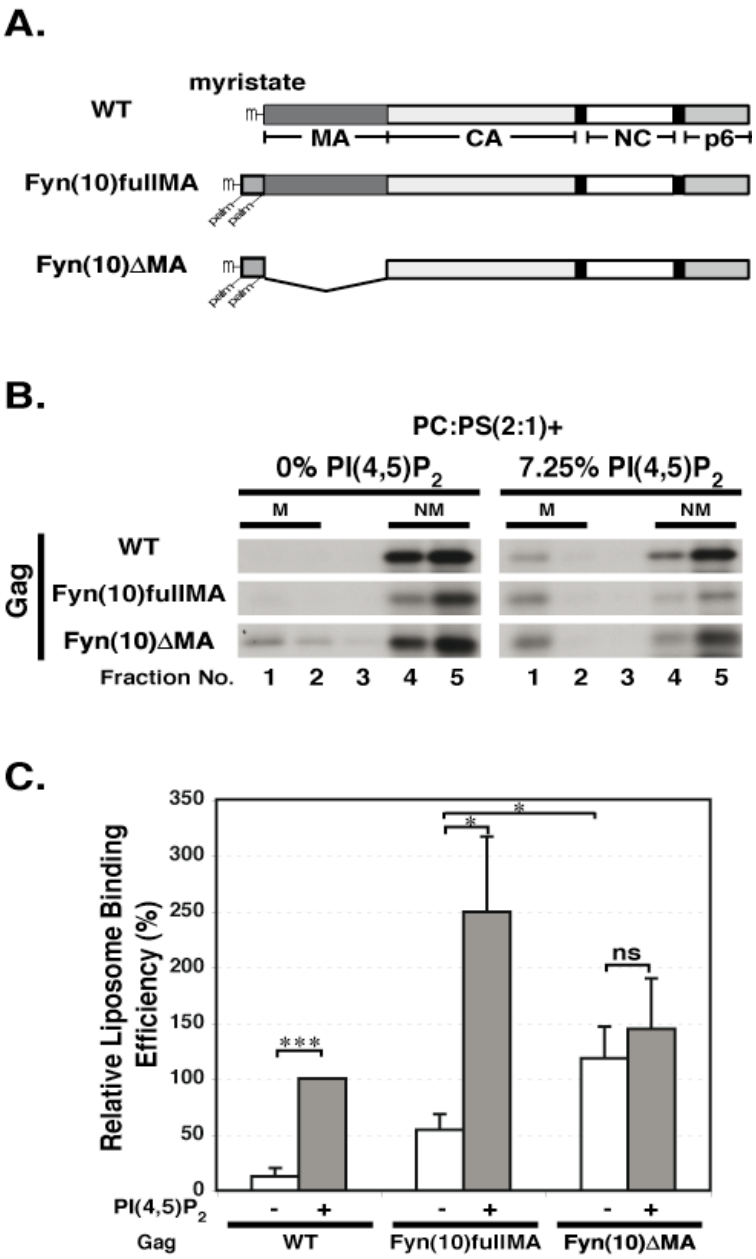

Fig. 2.6. Addition of an efficient membrane binding sequence does not eliminate the requirement for PI(4,5)P_2 in Gag-liposome binding.

A) Schematic representation of Gag derivatives with the Fyn N-terminal sequence. Myristoylation (m) and palmitoylation (palm) sites are shown. B) [^{35}S]-labeled WT Gag, Fyn(10)fullMA Gag, and Fyn(10)ΔMA Gag, were synthesized and incubated with liposomes as in Fig. 2.4. The reactions were subjected to membrane flotation centrifugation and the fractions were analyzed as in Fig. 2.3. C) The amount of labeled Gag in each fraction was quantified using a phosphorimager, and statistical analysis was performed as in Fig. 2.4. Data from four different experiments are shown as means ± standard deviation. *p* values were determined using Student's *t*-test (***p<0.001, * p<0.05, ns= not significant).

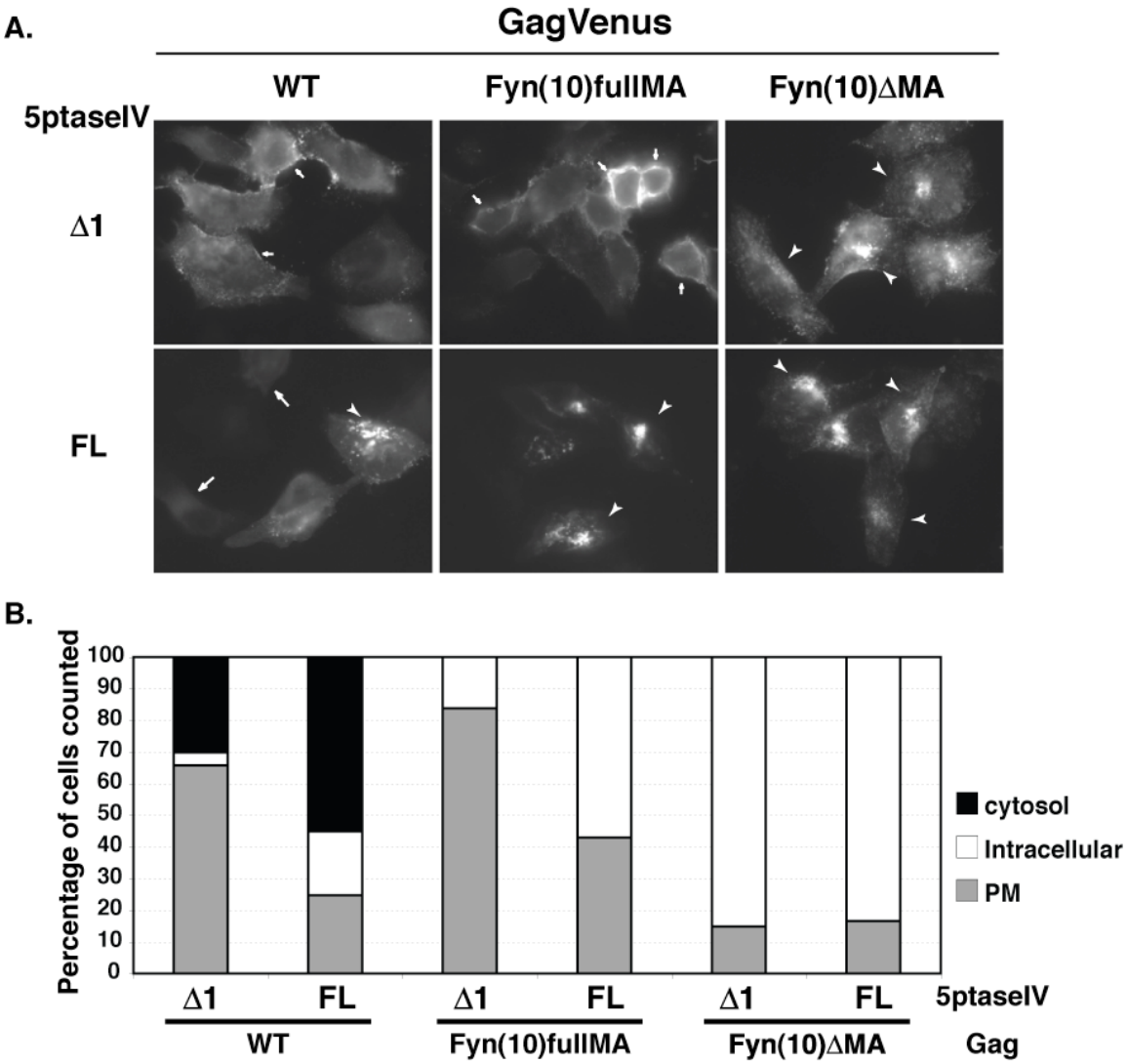

Fig. 2.7. PM localization of Fyn(10)fullMA Gag is still sensitive to PI(4,5)P_2 depletion in cells

A) HeLa cells expressing WT, Fyn(10)fullMA, or Fyn(10)ΔMA GagVenus along with full-length 5ptaseIV (FL) or the Δ1 mutant (Δ1) were immunostained with anti-Myc antibody and analyzed with a Nikon TE2000 microscope. B) The number of cells with 1) Gag localized at the PM (small arrows/grey); 2) Gag localized predominantly at intracellular compartments (arrow heads/white); and 3) Gag localized only in the cytosol (large arrows/black), were counted as in Fig. 2.1. Note that, for cells expressing Fyn(10)ΔMA Gag, a majority of cells showing Gag signals at the intracellular vesicles (category 2) also displayed Gag signals at the plasma membrane.

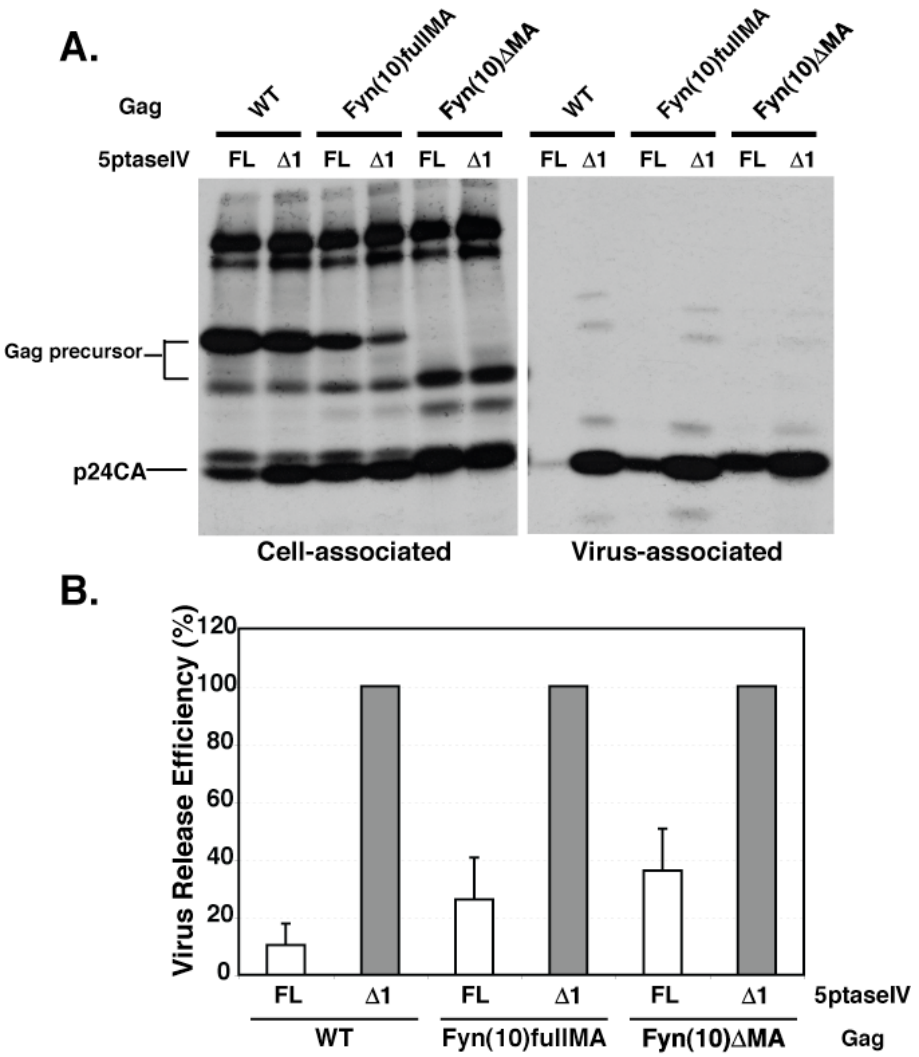

Fig. 2.8. Virus release of Fyn(10)fullMA Gag is still sensitive to PI(4,5)P_2 depletion in cells

A) HeLa cells expressing WT, Fyn(10)fullMA, or Fyn(10)ΔMA Gag, along with full-length 5ptaseIV (FL) or Δ1 mutant (Δ1) were metabolically labeled for 120 min. Cell- and virus-associated Gag were recovered by immunoprecipitation and analyzed by SDS-PAGE. B) Signal intensity of labeled Gag was quantified by phosphorimager analysis. Virus release efficiency was calculated as the amount of virus-associated Gag as a fraction of total Gag synthesized during the labeling period and normalized to the virus release efficiency in 5ptaseIVΔ1–expressing cultures. The average virus release efficiencies by cells expressing WT, Fyn(10)fullMA, and Fyn(10)ΔMA Gag along with 5ptaseIVΔ1 are 15.6%, 27%, and 21.3%, respectively. Data from four different experiments are shown as means ± standard deviation. *p* values were determined using Student's *t*-test (***p<0.001, **p<0.005, * p<0.05).

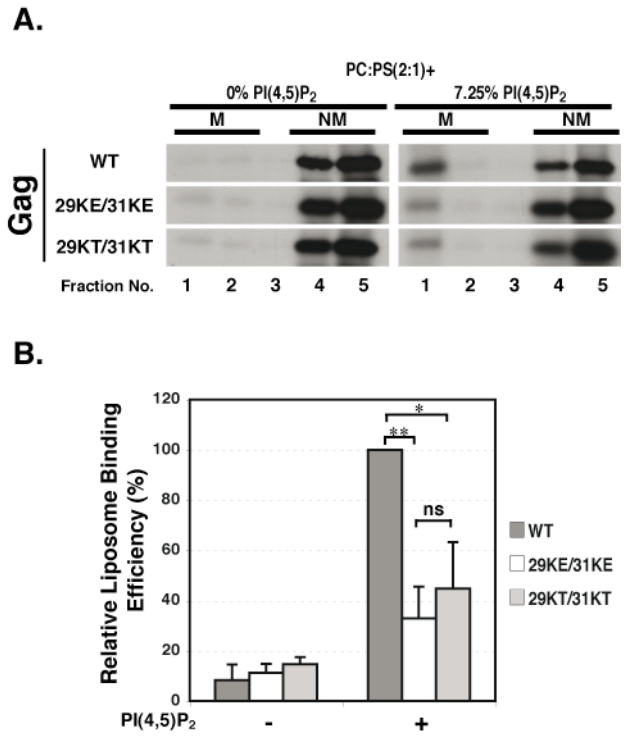

Fig. 2.9. The basic domain of MA is important for efficient Gag-PI(4,5)P_2 interaction.

[^{35}S]-labeled WT Gag and basic domain mutants (29KE/31KE or 29KT/31KT) were synthesized and incubated with liposomes as in Fig. 2.5. A) The reactions were subjected to membrane flotation centrifugation and the fractions were analyzed as in Fig. 2.3. B) The amount of labeled Gag in each fraction was quantified using a phosphorimager and the statistical analysis was performed as in Fig. 2.5. Data from three different experiments are shown as means ± standard deviation. *p* values were determined using Student's *t*-test (**p<0.005, * p<0.05, ns=not significant).

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CHAPTER III

Opposing mechanisms involving RNA and lipids regulate HIV-1 Gag membrane binding through the highly basic region of the matrix domain

ABSTRACT

Membrane binding of Gag, a crucial step in HIV-1 assembly, is facilitated by bipartite signals within the matrix (MA) domain: N-terminal myristoyl moiety and the highly basic region (HBR). The data from chapter II has shown that Gag interacts with a plasma-membrane-specific acidic phospholipid, phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P_2], via the HBR, and that this interaction is important for efficient membrane binding and plasma membrane targeting of Gag. Generally, in protein-PI(4,5)P_2 interactions, basic residues promote the interaction as docking sites for the acidic headgroup of the lipid. In this study, toward better understanding of the Gag-PI(4,5)P_2 interaction, we sought to determine the roles played by all the basic residues in the HBR. We identified three basic residues promoting PI(4,5)P_2-dependent Gag-membrane binding. Unexpectedly, two other HBR residues, Lys25 and Lys26, suppress membrane binding in the absence of PI(4,5)P_2 and prevent promiscuous intracellular localization of Gag. This inhibition of non-specific membrane binding is likely through suppression of myristate-dependent hydrophobic interaction since mutating Lys25 and Lys26 enhances

binding of Gag with neutral-charged liposomes. These residues were previously reported to bind RNA. Importantly, we found that RNA also negatively regulates Gag membrane binding. In the absence but not presence of $PI(4,5)P_2$, RNA bound to MA HBR abolishes Gag-liposome binding. Altogether, these data indicate that the HBR is unique among basic phosphoinositide-binding domains, as it integrates three regulatory components, $PI(4,5)P_2$, myristate, and RNA, to ensure plasma membrane specificity for particle assembly.

INTRODUCTION

HIV-1 assembly is a multi-step process mediated primarily by the viral structural protein, Gag. This protein, synthesized as a polyprotein precursor, $Pr55^{Gag}$, contains four major domains: matrix (MA), capsid (CA), nucleocapsid (NC) and p6. CA and NC contain determinants for Gag multimerization, whereas p6 facilitates release of virus particles [1, 2]. In most cell types, Gag assembles on the cytoplasmic leaflet of the plasma membrane (PM). MA is required for this PM-specific targeting as well as the lipid bilayer binding of Gag.

MA has bipartite entities that facilitate membrane binding of Gag: (i) the N-terminal myristoyl moiety; and, (ii) a highly basic region (HBR) comprising of residues 17 to 31[3-5]. The N-terminal myristate is normally sequestered within the globular head domain of MA[6], which also includes the HBR. A structural change, either through Gag multimerization or phosphatidylinositol-(4,5)-bisphosphate [$PI(4,5)P_2$] binding to MA, is required to expose the myristate and enhance membrane binding[6, 7]. This mechanism is known as the myristoyl switch[8, 9]. Structural and biochemical studies suggest that

the HBR of HIV-1 MA and analogous regions of other retroviral MA are apposed to face the cytoplasmic leaflet of membranes and facilitate membrane binding by interacting with acidic lipids [4, 10-14]. In addition, MA, especially the HBR, has been shown to interact with RNA [15-19] although the role of this interaction in virus assembly is less well understood. The HBR is also implicated in other steps of virus replication such as the postentry process (for review, see [20]).

Many cellular proteins that interact peripherally with the cytoplasmic leaflet of membranes have basic amino acid-rich domains [21]. The subcellular localization of these proteins generally depends on the specific binding of their basic domains with acidic lipids of target membranes. For example, the pleckstrin homology domain of phospholipase C δ1($PH_{PLC\delta1}$) has basic residues located on the surface of a binding pocket for $PI(4,5)P_2$, a PM-specific acidic lipid. These basic residues specifically interact with the phosphates on the inositol headgroup of $PI(4,5)P_2$[22]. This interaction allows $PH_{PLC\delta1}$ to localize to the PM. Similarly, basic residues of other phosphoinositide-binding domains play crucial roles in specific interactions with target membranes.

Previously, we showed that depleting cellular $PI(4,5)P_2$ reduces virus release of HIV-1 significantly and relocalizes Gag from the PM to cytosol or intracellular compartments [23, 24]. Not only HIV-1, but other retroviruses such as HIV-2, murine leukemia virus, and Mason-Pfizer monkey virus also require $PI(4,5)P_2$ or other phosphoinositides for efficient virus release [25-28]. Several studies using various *in vitro* techniques, including NMR, protein foot printing, liposome binding, and monolayer membrane binding suggest that MA interacts specifically with $PI(4,5)P_2$[7, 24, 29, 30]. A recent lipid analysis study demonstrated that $PI(4,5)P_2$ is enriched in the HIV envelope in

an MA-dependent manner, confirming the MA-PI(4,5)P_2 interaction *in vivo* [26]. These studies suggest that MA basic residues, in particular those in the HBR, promote PI(4,5)P_2 interaction and membrane binding, as observed for basic residues in other phosphoinositide-binding domains. However, the contribution of each basic residue in the HBR to the interaction between full-length Gag and PI(4,5)P_2-containing membrane remains to be examined.

Previous reports showed that mutating lysines 29 and 31 to threonines (29KT/31KT) within the HBR relocalizes Gag from the PM to intracellular compartments, as observed for wild-type (WT) Gag in PI(4,5)P_2-depleted cells[31]. Consistent with the altered localization, the *in vitro* liposome binding assay showed that 29KT/31KT Gag binds PI(4,5)P_2 less efficiently compared to WT Gag[24]. However, the Gag-PI(4,5)P_2 interaction was not completely abolished, suggesting a role for other basic amino acids in this interaction.

In this study, towards better understanding of the Gag-PI(4,5)P_2 interaction, we sought to identify all the basic residues in the HBR important for this interaction. We confirmed that the HBR as a whole is indeed essential for PI(4,5)P_2 interaction and efficient membrane binding of Gag. Surprisingly, analysis of a panel of Gag mutants revealed that a part of the HBR actually suppresses membrane binding in the absence of PI(4,5)P_2. This suppression is likely due to inhibition of the myristate-dependent hydrophobic interaction. Furthermore, RNA bound to the HBR also inhibits Gag-membrane binding through inhibition of acidic lipid binding and to a lesser extent via suppression of myristate exposure. Altogether, these results showed that the HBR regulates membrane binding in both positive and negative manners. Data presented in

this chapter suggest a novel, RNA-dependent mechanism by which HIV-1 Gag ensures specific binding to the PI(4,5)P_2-containing membrane, i.e., the PM. To our knowledge, this is the first example of a protein-phospholipid interaction directly suppressed by RNA.

MATERIALS AND METHODS

Cells and Plasmids

HeLa cells were cultured and maintained in DMEM supplemented with 5% fetal bovine serum. HIV-1 molecular clone pNL4-3[32], its derivative encoding YFP-tagged Gag (pNL4-3/GagVenus) and a Gag expression vector for *in vitro* translation reaction, pGEMNLNR, were previously described[24]. pNL4-3/1GA, pNL4-3/25KT/26KT, pNL4-3/17KT/19RL, pNL4-3/29KT/31KT, pNL4-3/19RL, pNL4-3/21RL were kind gifts from E. Freed [33, 34]. Other MA changes were introduced into pNL4-3 by PCR mutagenesis. MA changes in pNL4-3 derivatives were also introduced into pGEMNLNR and pNL4-3/GagVenus using standard molecular biology techniques. pGEMNLNR/delNC was constructed by replacing BssHII to PflMI fragment (nt 711-5297) with that of pNL4-3/delNC (a kind gift from D. Ott)[35].

Liposome binding assay

Liposome binding assay was performed as previously described [24]. Briefly, radiolabeled Gag is synthesized using in vitro transcription and translation coupled reaction mix containing rabbit reticulocyte lysates (Promega). Liposomes containing corresponding lipids were added after 30 minutes at 30°C and incubated further for 90

minutes. Then, the liposome-bound and non-liposome-bound Gag were separated by equilibrium flotation centrifugation.

For RNase treatment experiments, the above protocol was modified as following. Gag synthesis was performed at 30°C for 90 minutes. Subsequently, 1 μl of RNase, which is a mixture of pyrimidine-specific endoribonucleases from bovine pancreas (Cat. No: 11119915001, Roche Applied Science), was added to 25 μl of the reaction mix and incubated for 20 minutes at 37ºC. Liposomes were then added and incubated for 15 minutes at 30ºC before equilibrium flotation centrifugation was performed.

The top two fractions were considered liposome-bound and the bottom three as non-liposome-bound Gag. The liposome binding efficiency was calculated as the amount of liposome-bound Gag as a fraction of total Gag.

Virus release assay and statistical analysis

Virus release assay was performed as previously described[23]. Statistical analysis was performed as previously described[23].

Quantification of RNA bound to Gag in rabbit reticulocyte lysate

The protocol described by Peritz et al was adapted for purifying RNA bound to Gag[36]. Briefly, rabbit reticulocyte lysate containing S-35 labeled Gag was diluted with lysis buffer [100mM KCl, 5mM $MgCl_2$, 10mM HEPES, 0.5% NP-40, 1mM DTT, 100U/ml rRNasin (Promega) and 1 tablet/50ml Complete protease inhibitor cocktail (Roche)] and precleared at 4 degrees for 1 hour with protein A sepharose beads. The precleared lysate was incubated with HIV-Ig - protein A complex at 4 degrees overnight. The immunoprecipitated complex was washed four times with lysis buffer, resuspended in lysis buffer containing 0.1% SDS and proteinase K (0.3mg/ml) and incubated at 50

degrees for 30 minutes. The supernatant was mixed with ethanol and added to Qiagen RNasy column, washed and eluted as described by the manufacturer. The RNA was then quantified using Invitrogen Quant-iT Ribogreen RNA reagent as per the manufacturer's protocol. The amount of RNA was normalized to the levels of radioactive Gag immunoprecipitated and the RNA bound to WT Gag was set to 100 percent.

RESULTS

The HBR of the MA domain is essential for PI(4,5)P_2-dependent membrane binding of Gag

To examine whether the HBR of MA is indeed important for Gag-PI(4,5)P_2 interaction, we mutated all the positively charged residues (6A2T) within the HBR(Fig. 3.1). This mutant was then analyzed for PI(4,5)P_2-dependent membrane binding by previously described *in vitro* liposome binding assay[24]. In this assay, myristoylated full-length Gag is synthesized using rabbit reticulocyte lysates and incubated with liposomes. Liposome-bound and non-liposome-bound Gag proteins are separated by sucrose gradient membrane flotation centrifugation. As shown in Fig. 3.2, compared to WT Gag, binding of 6A2T Gag to PI(4,5)P_2-containing liposomes was significantly reduced. To examine the effect of the 6A2T change on the localization of Gag in cells, we introduced the same mutations in a Gag derivative tagged with Venus, a variant of yellow fluorescent protein (GagVenus) [24, 37]. Consistent with the weak liposome binding, 6A2T GagVenus showed a hazy, cytosolic signal in most cells. Additionally, perinuclear localization of GagVenus was observed in some cells (Fig. 3.3). This is in contrast to WT GagVenus that localizes predominantly on the PM. The virus release was

undetectable for the 6A2T mutant. Altogether, these results suggest that the HBR likely forms a binding site for PI(4,5)P_2 and that this interaction is important for efficient Gag membrane binding and virus release.

MA basic residues Lys25 and Lys26 in the HBR suppress PI(4,5)P_2-independent membrane binding and inhibit promiscuous localization of Gag

To pinpoint the residues essential for Gag-PI(4,5)P_2 interaction, we analyzed liposome binding of Gag mutants with single or double amino acid substitutions of the basic residues in the MA HBR by using *in vitro* liposome binding assay (Fig. 3.1 and Fig.3.2). Several of these mutants have been previously studied with respect to other proposed MA functions [19, 20, 33, 34]. Overall, the mutants could be categorized into three different phenotypes: (i) reduced PI(4,5)P_2 binding [17KT, 17KT/29KT, and 29KT/31KT], (ii) increased PI(4,5)P_2-independent binding [25KT/26KT], and (iii) no change compared to WT [all of the other mutants]. Altogether, these experiments suggest that MA basic residues 17, 29, and 31 promote MA-PI(4,5)P_2 interaction.

Among the Gag mutants examined, the 25KT/26KT mutant, where lysines 25 and 26 were changed to threonines, showed no significant change in binding to PI(4,5)P_2-containing liposomes. Unexpectedly, however, this mutant had significantly elevated binding to control liposomes that lack PI(4,5)P_2(Fig. 3.2). These results suggest that, in addition to facilitating membrane binding through PI(4,5)P_2 interaction, some basic residues in the HBR inhibit PI(4,5)P_2-independent membrane binding.

To characterize the 25KT/26KT mutant Gag further, we analyzed both virus release and localization using cell-based assays as described previously[24]. Consistent with the lack of requirement for PI(4,5)P_2 observed in the in vitro liposome binding assay

(Fig. 3.2), the virus release efficiency of 25KT/26KT was substantially augmented compared to WT (Fig. 3.3B). Notably, 25KT/26KT GagVenus displayed promiscuous membrane binding in cells, localizing both at the PM and intracellular compartments (Fig. 3.3A). This promiscuous localization of Gag is not due to protein aggregation as intact virus particles bud from both PM and intracellular compartments for this 25KT/26KT mutant (Fig. 3.4). Altogether, these results indicate that MA basic residues 25 and 26 inhibit PI(4,5)P_2-independent membrane binding of Gag.

RNA inhibits liposome binding of Gag

Previous studies showed that the residues Lys25 and Lys26 can interact with RNA[17-19]. Therefore, it is conceivable that the 25KT/26KT mutations abolish RNA binding and allow other MA basic residues to freely interact with acidic lipids thereby enhancing promiscuous membrane binding. To examine whether RNA does interfere with Gag membrane binding, we first tested the effect of RNase treatment on the liposome binding of WT Gag. Remarkably, treating WT Gag with RNase before incubation with liposomes greatly enhanced interaction of Gag with control liposomes containing phosphatidylcholine (PC) and phosphatidylserine (PS) [PC+PS] (Fig. 3.5). A similar increase was observed with PI(4,5)P_2-containing liposomes [PC+PS+PI(4,5)P_2]. Notably, however, as we reported previously[24], a substantial amount of Gag was bound to PI(4,5)P_2-containing liposomes even without RNase treatment (Fig.3.5). These results indicate that Gag-membrane binding is suppressed almost completely by RNA unless PI(4,5)P_2 is present.

To determine if the phenotype of 25KT/26KT Gag can be explained by the loss of RNA binding, we examined the effect of RNase treatment on liposome binding of this

mutant Gag. The enhancement of membrane binding by RNase treatment was smaller than that observed for WT, especially in the absence of $PI(4,5)P_2$ (Fig. 3.5). These data suggest that the 25KT/26KT mutation and RNase treatment enhance membrane binding by overlapping mechanisms.

The 25KT/26KT changes increase the hydrophobic interaction with membranes

Efficient membrane binding of Gag requires both the myristoyl moiety and the HBR. It is still possible that the 25KT/26KT mutant enhances membrane binding by constitutively exposing the myristoyl moiety. To test this hypothesis, we analyzed the binding of 25KT/26KT Gag to liposomes containing PC alone. Because PC is a neutral lipid, binding of Gag to these liposomes would likely be mediated by hydrophobic interaction through N-terminal myristate moiety. Consistent with the previous findings that the myristate moiety is sequestered inside the globular head of MA, and that $PI(4,5)P_2$ binding is required for facilitating myristate exposure [2, 6, 7], WT Gag was unable to bind well to PC liposomes (Fig.3.6). However, the double mutant 25KT/26KT was able to bind efficiently to liposomes containing only PC (Fig. 3.6). This increased membrane binding of 25KT/26KT was myristate dependent as it was completely abolished when myristoylation signal was mutated on Gag (data not shown). These results suggest that the 25KT/26KT mutant readily exposes the myristate. Therefore, it is likely that lysines 25 and 26 suppress non-specific membrane binding by regulating hydrophobic interactions dependent on the myristate.

RNA bound to MA but not NC inhibits liposome binding of Gag

As described above, RNase treatment enhanced membrane binding of both WT and to a lesser extent 25KT/26KT Gag (Fig. 3.5). As RNA-mediated inhibition of

membrane binding may represent a unique mode of regulation in virus assembly, we sought to understand the mechanism of this inhibition. Coimmunoprecipitation experiments showed that both MA HBR and NC facilitate RNA binding to Gag in reticulocyte lysates (Fig. 3.7). To test if RNA bound to NC is involved in the inhibition of membrane binding, we analyzed NC-deleted Gag (delNC) for RNase sensitivity. Liposome binding of delNC Gag was increased upon RNase treatment to a similar extent as WT Gag (Fig 3.8). These results suggest that at least in the liposome binding assay, NC is not required for RNA-mediated inhibition of membrane binding of Gag.

RNase treatment increases both the interaction of MA basic residues with acidic lipids and the myristate-dependent hydrophobic interaction

The results presented above are consistent with the possibility that RNA bound to MA HBR blocks the interaction of the HBR basic residues with acidic lipids and interferes with Gag membrane binding. However, it is also conceivable that RNA binding to the HBR might reduce myristate exposure thereby suppressing membrane binding of Gag. Both of these possibilities are consistent with the observation that membrane binding of 25KT/26KT Gag that has readily exposed myristate (Fig. 3.6) is enhanced upon RNase treatment markedly but not as much as WT Gag (Fig. 3. 5). Hence, to further understand the mechanism by which RNA inhibits membrane binding, we analyzed the myristoylation-defective Gag mutant (1GA). Interestingly, although RNase treatment did increase membrane binding of 1GA Gag, the extent of increase depended on the composition of liposomes used in the assay. 1GA Gag bound PI(4,5)P_2-containing liposomes as efficiently as WT Gag upon RNase treatment (Fig. 3.9A). This strongly

supports the model that interaction of basic residues in the HBR with acidic lipids is increased upon RNase treatment.

In contrast, RNase treatment increased binding of 1GA Gag to PC+PS liposomes only modestly compared to that of WT Gag. These results are consistent with the possibility that RNA also inhibits membrane binding partially by sequestration of myristate. Indeed, WT Gag did show a modest increase in PC-liposome binding upon RNase treatment, whereas 1GA Gag showed no increase (Fig. 3.9B). These data suggest that removal of RNA has an impact on the myristoyl switch as well. Altogether, these results indicate that RNA inhibits membrane binding both by preventing the electrostatic interaction between basic residues and acidic lipids and by suppressing the myristate-dependent hydrophobic interaction.

DISCUSSION

Phosphoinositide-binding domains found in cellular proteins contain basic residues that are essential for interaction with acidic headgroups of phosphoinositides [21]. Similarly, the current study showed that some basic residues within the HBR (Lys17, Lys29, and Lys31) are important for efficient Gag membrane binding mediated by $PI(4,5)P_2$ (Fig. 3.2). However, our study also revealed that the HBR contains basic residues (Lys25 and Lys26) that suppress membrane binding of Gag (Fig 3.2). This negative regulation of membrane binding by Lys25 and Lys26 likely occurs via myristate sequestration (Fig. 3.6). Consistent with the increased $PI(4,5)P_2$-independent liposome binding, 25KT/26KT Gag localizes promiscuously in cells and releases virus particles

more efficiently than WT Gag (Fig. 3.3,3.4), a phenotype reminiscent of Gag derivatives with MA globular head deletions[38].

This work also identified RNA as a negative regulator of Gag membrane binding. Several *in vitro* studies showed that HIV-1 MA interacts with RNA via its HBR[17-19]. Therefore, competition between RNA and acidic lipids for binding to the HBR at least partly accounts for the RNA-mediated suppression of Gag membrane binding. Consistent with this hypothesis, in other in vitro experiments performed in the absence of RNA, binding of MA to PS has been readily observed [39-41], whereas in our system, Gag binding to the PC+PS liposomes was negligible without RNase treatment. Importantly, in contrast to PC+PS liposomes, substantial amount of Gag binds to PC+PS+PI(4,5)P_2 liposomes even without RNase treatment (Figs 3.5 and 3.9). Consistent with these data, Eric Barklis' group reported that PI(4,5)P_2-containing liposomes out-compete oligonucleotides for binding to beads coated with purified myristylated MA domain [42]. Thus, it is likely that PI(4,5)P_2, but not other acidic lipids such as PS, can bind the HBR in the presence of RNA. In addition, exposure of myristate may also be more dependent on PI(4,5)P_2 binding in the presence of RNA, because RNA binding to MA HBR also reduces myristate-dependent hydrophobic interaction (Fig. 3.9). In any case, our data suggest that RNA binding to MA represents a novel regulatory mechanism in Gag membrane binding.

It remains to be determined whether RNA bound to the HBR inhibits unregulated membrane binding in cells. In our *in vitro* system, the RNA concentration is estimated to be lower than that in the cytoplasm of HeLa cells. Nonetheless, the inhibition of membrane binding by RNA does not require NC, the major RNA binding domain (Fig.

3.8). However, in cells, it is likely that the viral genomic RNA bound to NC also binds MA because of their close proximity. This model is also consistent with a recently proposed folded-over conformation of Gag [43, 44] (not depicted in Fig. 3.10). Notably, two recent reports suggest that RNA export from the nucleus plays a key role in MA-mediated membrane binding of Gag [45, 46]. These authors observed that HIV Gag assembly defects linked to specific Gag mRNA export pathways could be rescued by increasing membrane binding ability of Gag, either by MA mutations or replacing MA with heterologous membrane binding domains [45, 46]. In light of the results presented here, it is possible that nuclear export pathways used by viral RNAs affect MA-RNA interactions, which in turn, influence Gag membrane binding. This hypothesis is also in agreement with the observed autoinhibition of Gag membrane binding by HIV-1 MA in murine cells [47, 48].

It has been long known that retroviral MA interacts with RNA[15-19, 49-53]. Although genomic RNA encapsidation does not require MA in the case of HIV-1 [54], a variety of roles have been suggested for the retroviral MA-RNA interaction in the later half of the virus life cycle. These include (i) regulation of viral mRNA translation [18, 55], (ii) encapsidation of genomic viral RNA [56], (iii) dimerization of encapsidated RNA[57, 58], and (iv) enhancement of Gag assembly[59, 60]. Thus, it is tempting to speculate that binding of the HBR to both PI(4,5)P_2 and RNA may play a key role in coordinating the four-way interactions between the PM, viral RNA, MA, and NC during the late phase of the retrovirus life cycle. For example, once Gag reaches the PM, the viral RNA bound to MA might get displaced by the HBR-PI(4,5)P_2 interaction. This displaced RNA may then act as a scaffold for NC-dependent Gag multimerization and

facilitate efficient particle assembly at the PM. Such a process might also require cellular proteins reported to play a role in Gag multimerization or RNA packaging, including ATP-binding cassette protein in the E subfamily (ABCE1)[61, 62] and Staufen-1[63]. Clearly, further studies are necessary for addressing interrelationships between events during the late phase.

In summary, our data indicate that the HBR within MA modulates membrane binding in three different ways: positively by interaction with $PI(4,5)P_2$, and negatively by suppression of myristate-dependent hydrophobic interaction and by interaction with RNA. RNA binding to MA HBR not only interferes with interactions between MA basic amino acids and acidic lipids, but also appears to reduce myristate exposure. Based on this and previous studies, we put forth the following model (Fig. 3.10). Both RNA binding and suppression of myristate exposure mediated by the HBR prevent Gag from premature or non-specific membrane binding. Once Gag reaches $PI(4,5)P_2$-containing membranes such as the PM, $PI(4,5)P_2$ binds the HBR, which then triggers myristate exposure and stabilizes binding to the lipid bilayer. However, if myristate sequestration is blocked (e.g., by the 25KT/26KT mutations) or if RNA detaches from the HBR (e.g., by RNase treatment), Gag binds membrane in a non-specific, $PI(4,5)P_2$-independent manner (Fig. 3.10). Co-operation of interactions with multiple membrane factors, which enhances the robustness and specificity of membrane binding, is prevalent in phosphoinositide-binding domains [21]. However, MA HBR is unique among these domains for integrating opposing regulatory mechanisms to ensure specificity for the target membrane that contains $PI(4,5)P_2$.

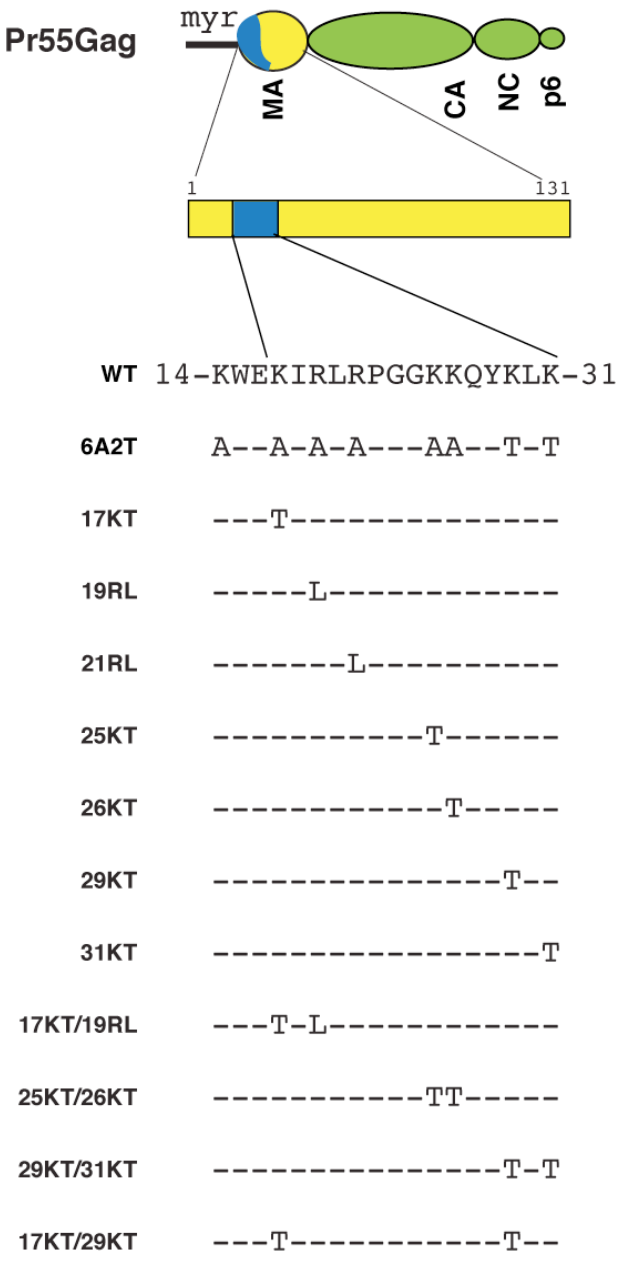

Fig. 3.1. Amino acid mutations in the MA HBR analyzed in this study
The wild type NL4-3 sequence is shown using single-letter amino acid code. The name of the mutant and the changes in the amino acid sequence are shown. Dashes represent unchanged sequence. myr- myristoyl moiety, Blue represents HBR

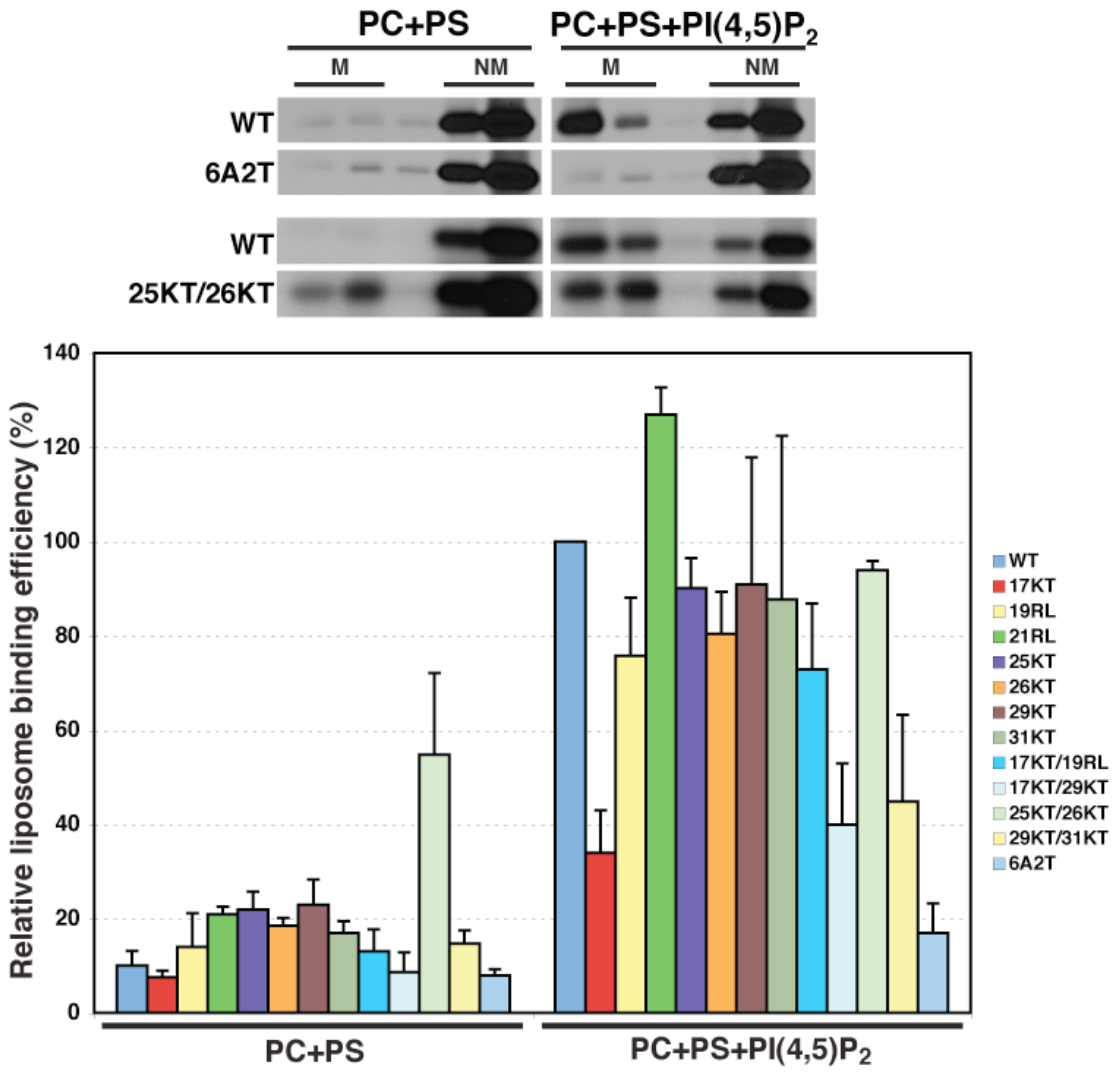

Fig. 3.2. Lys25 and Lys26 in the HBR inhibit membrane binding of Gag
[^{35}S]-labeled WT Gag and Gag mutants with indicated amino acid substitutions were synthesized by the *in vitro* transcription and translation system using reticulocyte lysates and incubated with liposomes containing or not containing PI(4,5)P_2. The reactions were subjected to membrane flotation centrifugation and five 1-ml fractions were collected from each tube. Representative gels are shown in the top panel. M, liposome-bound; NM, non-liposome-bound. The relative liposome binding efficiency was calculated in comparison to the binding efficiency of WT Gag with PI(4,5)P_2-containing liposomes for each experiment. Data from three independent experiments are shown as means ± standard deviation. *p* values were determined using Student's *t*-test as a comparison to WT (ns=not significant, * $p< 0.05$, ** $p<0.01$, *** $p<0.001$).

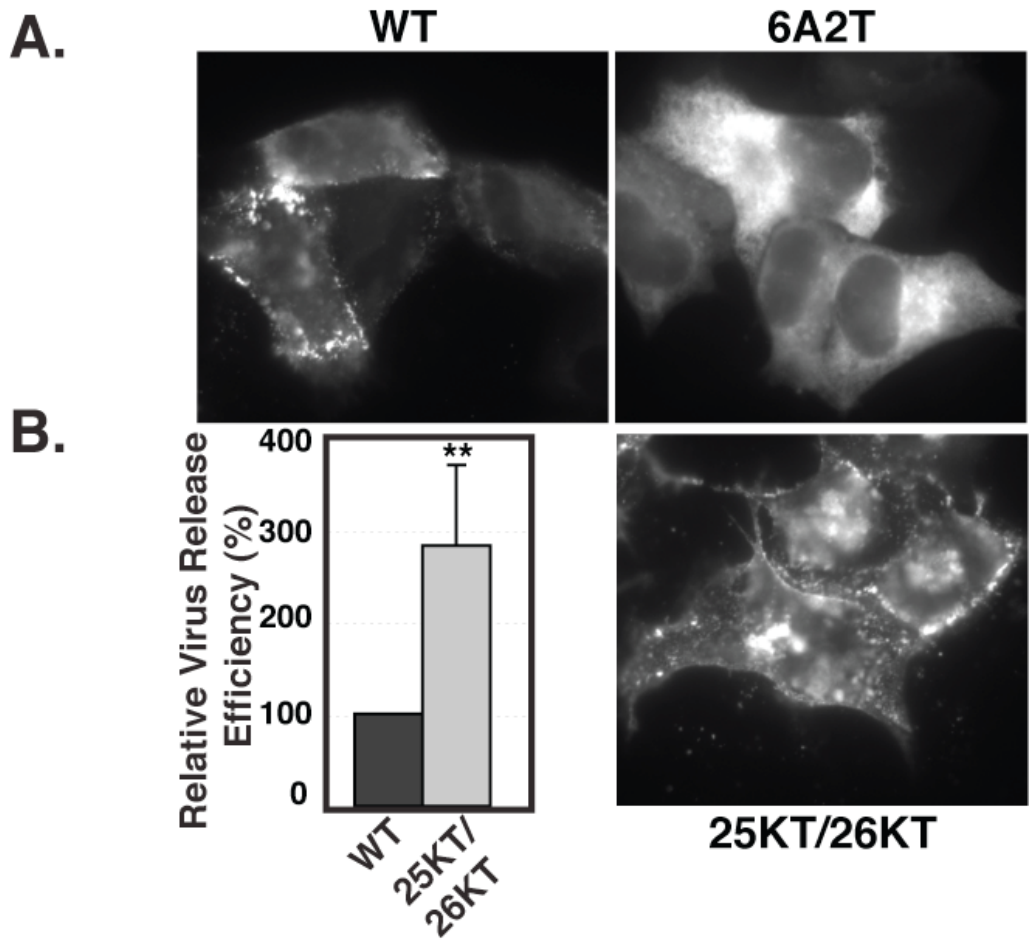

Fig.3.3 Mutations in Lys25 and Lys26 show promiscuous localization and enhanced virus release

A) HeLa cells expressing WT, 6A2T and 25KT/26KT GagVenus were analyzed using Nikon TE2000 microscope. B) HeLa cells expressing HIV molecular clones encoding WT or 25KT/26KT Gag were metabolically labeled for 2 h. Cell- and virus-associated Gag were recovered by immunoprecipitation. Virus release efficiency was calculated as the amount of virus-associated Gag as a fraction of total Gag synthesized during the labeling period and normalized to the virus release efficiency of WT. The average virus release efficiencies for WT and 25KT/26KT are 7.22% and 17.85%, respectively. Data from five different experiments are shown as means ± standard deviation. ** $p<0.01$.

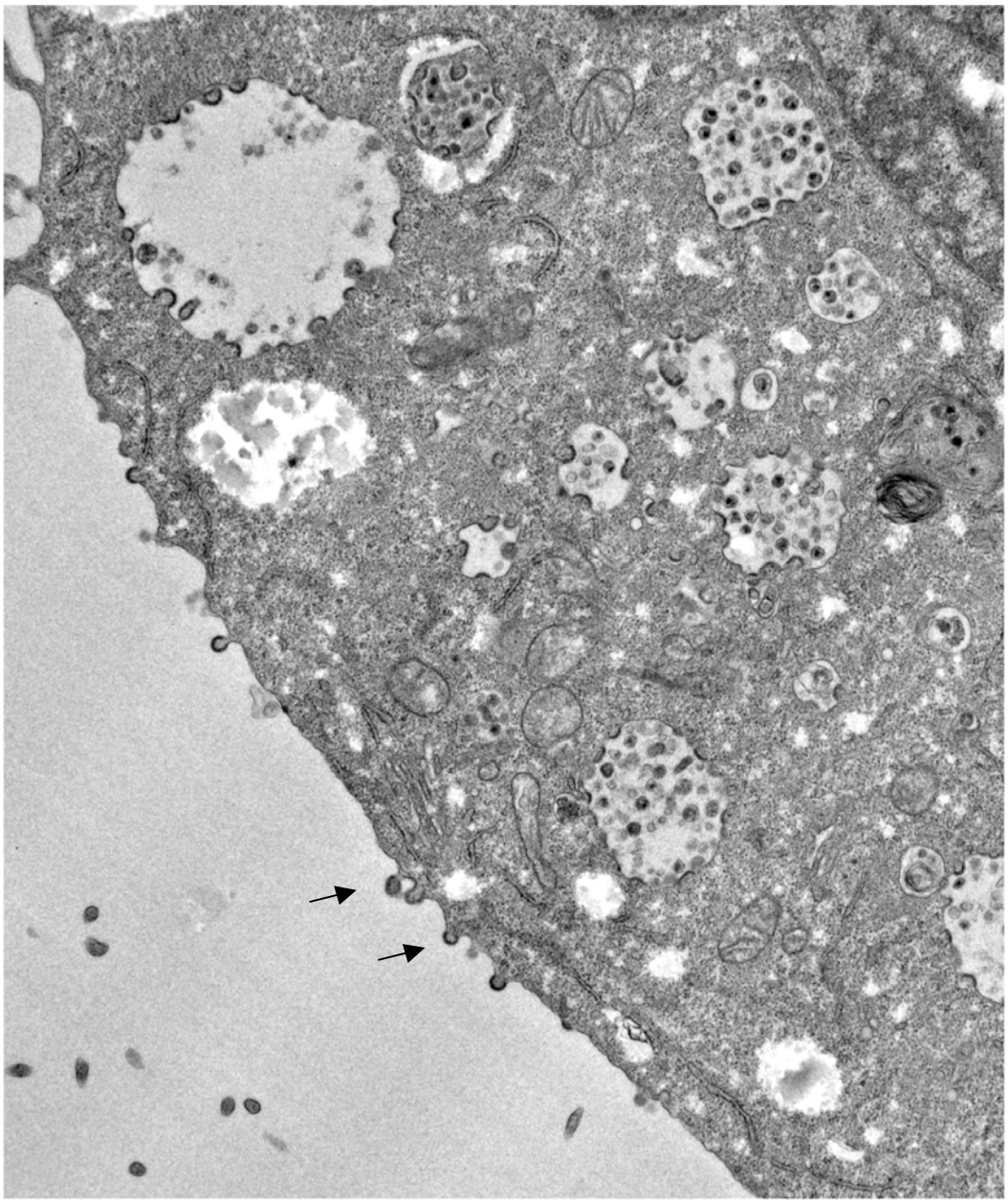

Fig.3.4 25KT/26KT mutant forms virus particles both at PM and intracellular compartments

HeLa cells were transfected with HIV molecular clone encoding 25KT/26KT Gag. 16 hours post-transfection, they were fixed and analyzed by transmission electron microscopy. Note that the virus particles form both at PM (black arrows) and intracellular compartments consistent with the localization of GagVenus in Fig. 3.3.

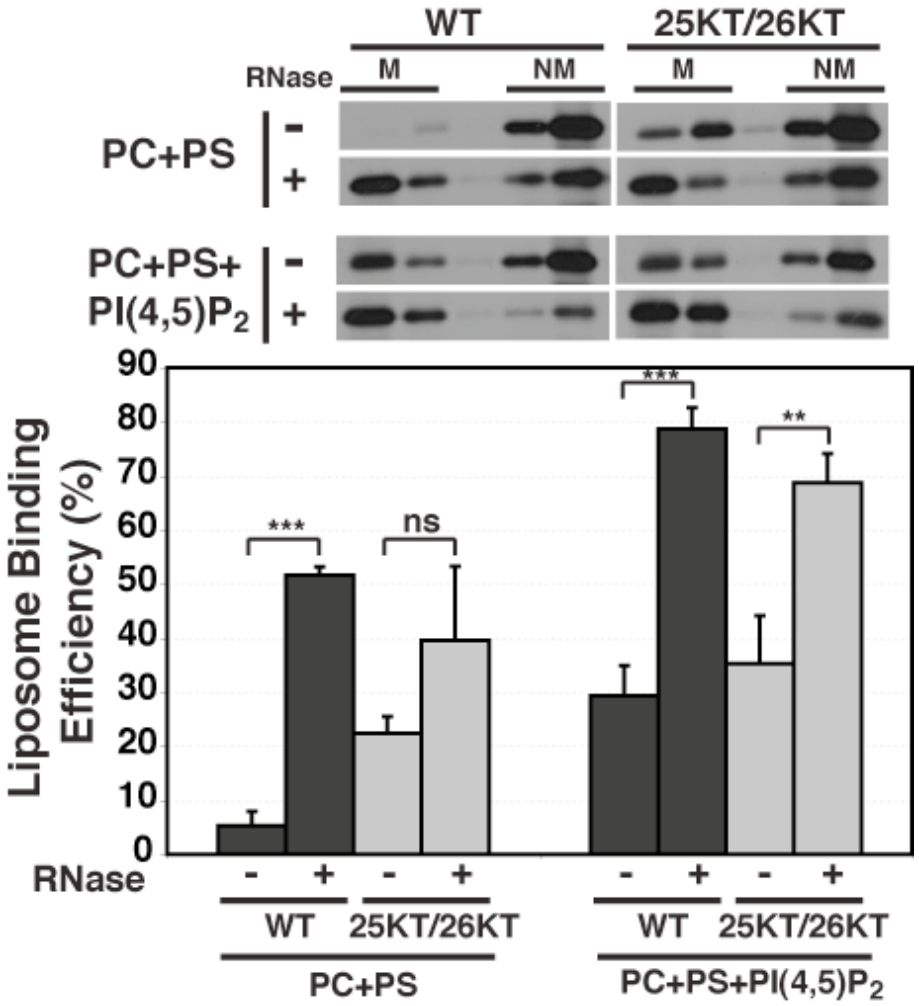

Fig.3.5. RNA inhibits membrane binding of Gag

WT and 25KT/26KT Gag were synthesized using rabbit reticulocyte lysates and either treated or not treated with RNase. Subsequently, liposomes containing indicated lipids were added, and the liposome binding efficiency was calculated as described in Materials and Methods. M, liposome-bound; NM, non-liposome-bound. Data from three (PC+PS) or four (PC+PS+PI(4,5)P_2) independent experiments are shown as means ± standard deviation. ns=not significant,** $p<0.01$, *** $p<0.001$. There is a statistically significant difference (*) in binding to PI(4,5)P_2-containing liposomes between RNase-treated WT and 25KT/26KT Gag.

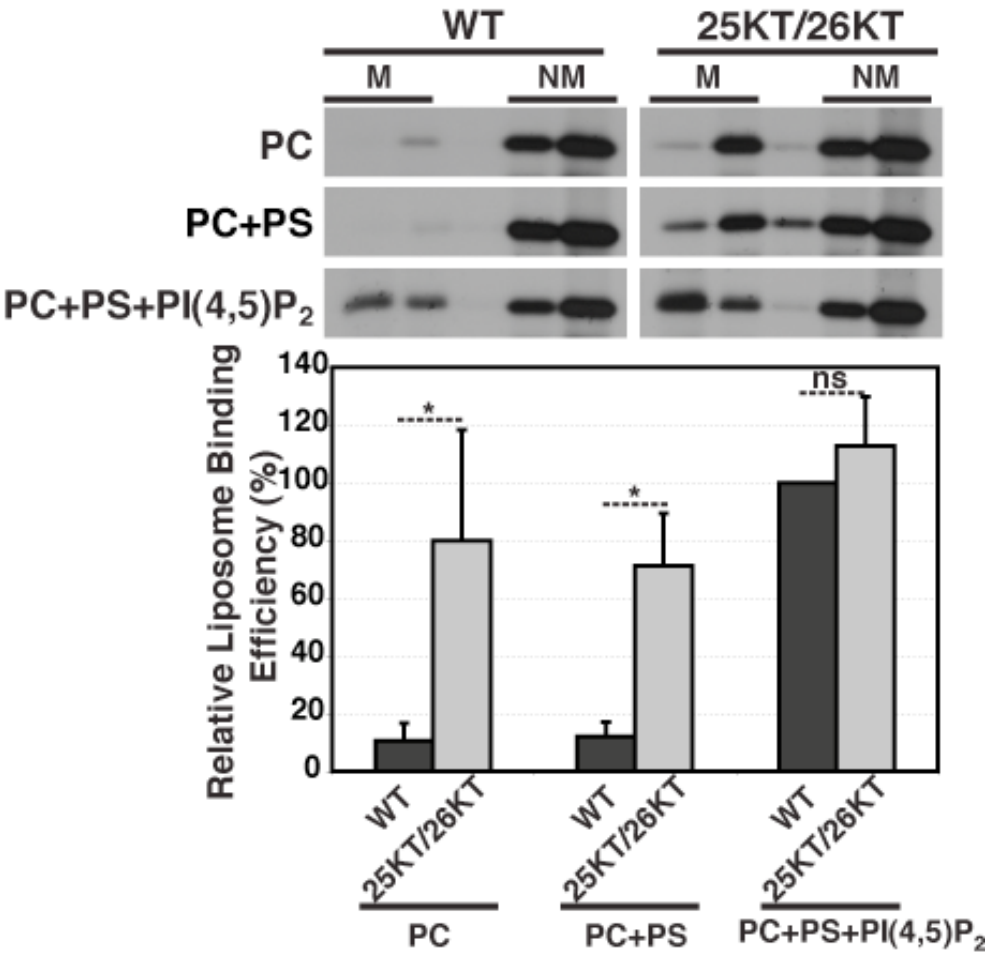

Fig.3.6. 25KT/26KT Gag binds efficiently to liposomes lacking acidic phospholipids
Binding of WT and 25KT/26KT Gag to liposomes containing indicated lipids were analyzed as in Fig. 3.2. Data from three independent experiments are shown as means ± standard deviation. ns=not significant,*$p<0.05$. There are no statistically significant differences between binding of 25KT/26KT to different liposomes.

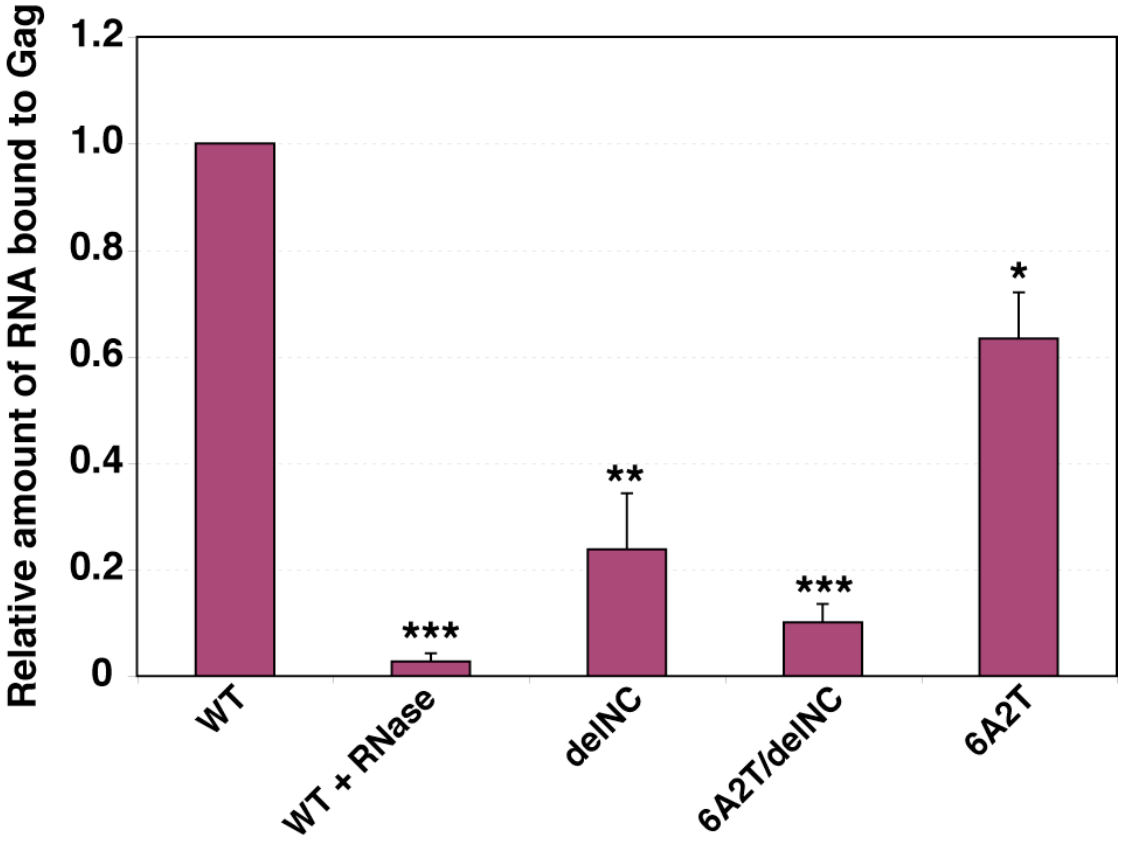

Fig. 3.7. Relative percentage of RNA bound to Gag in rabbit reticulocyte lysate
WT, delNC, 6A2T, 6A2TdelNC Gag were synthesized using rabbit reticulocyte lysate and either treated or not treated with RNase. Subsequently, Gag was immunoprecipitated and the amount of RNA coimmunoprecipitated was quantified as described in Materials and Methods. Relative percentage of RNA bound to Gag derivatives is shown after setting WT without RNase treatment as 100 percent. Data from three independent experiments are shown as means ± standard deviation. ***$p<0.001$, **$p<0.01$, *$p<0.05$.

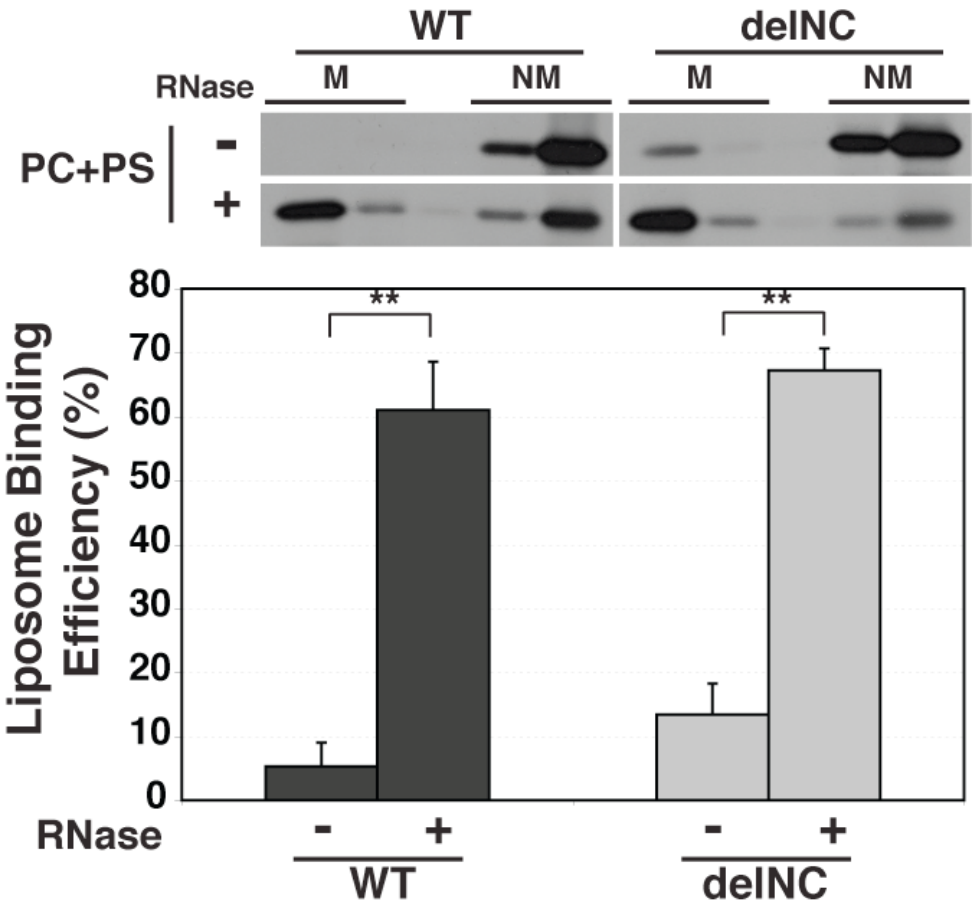

Fig.3.8. Nucleocapsid is not required for RNA-mediated inhibition of Gag-membrane binding

Liposome binding of WT and delNC Gag was analyzed as in Fig.3.5. using PC+PS liposomes. Data from three independent experiments are shown as means ± standard deviation. ** $p<0.01$. There is no statistically significant difference in membrane binding of RNase-treated Gag between WT and delNC

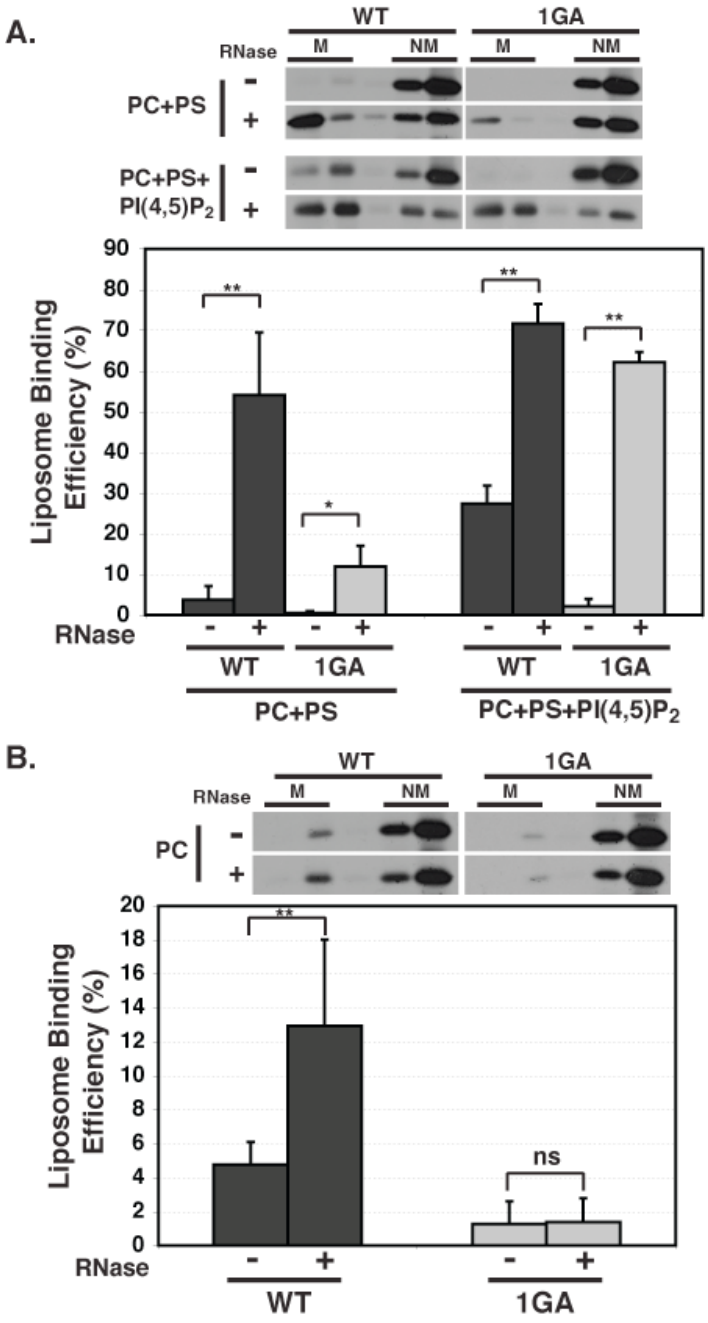

Fig.3.9. MA-bound RNA suppresses membrane binding of Gag by inhibiting interactions between basic residues and acidic lipids and by reducing the myristate-dependent interaction

A. and B. Liposome binding of WT and 1GA Gag was analyzed as in Fig. 3.5 using liposomes containing either PC+PS or PC+PS+PI(4,5)P_2 (A) or only PC (B). Data from three to five independent experiments are shown as means ± standard deviation. ns=not significant,* $p<0.05$,** $p<0.01$,*** $p<0.001$. There is a statistically significant difference (*) in binding to PC+PS liposomes but not PI(4,5)P_2-containing liposomes between WT and 1GA Gag treated with RNase.

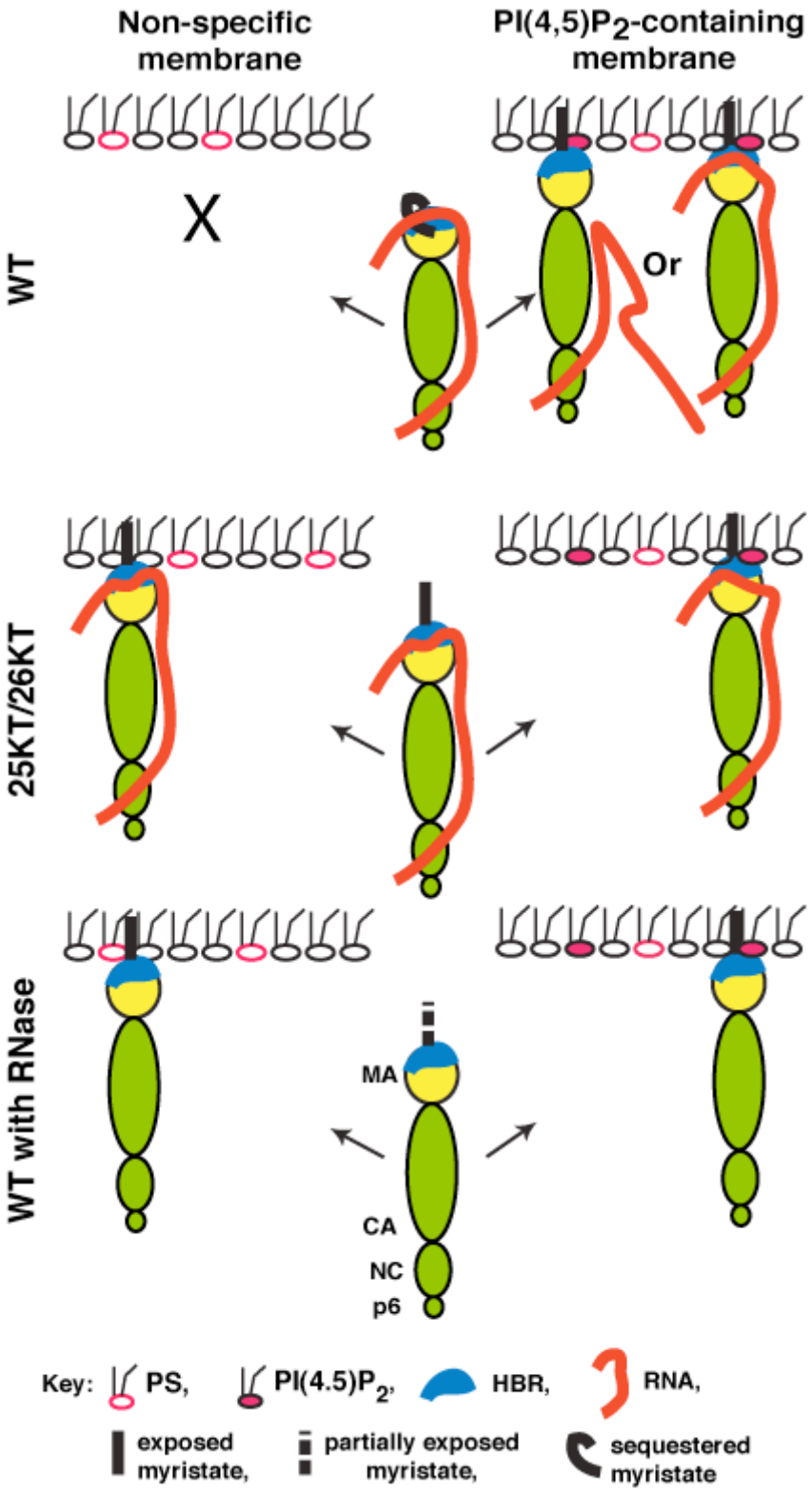

Fig.3.10. A model for the regulation of Gag-membrane binding specificity
PI(4,5)P_2-dependent and independent membrane binding of WT, 25KT/26KT and RNase-treated Gag are shown. See discussion for explanation.

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CHAPTER IV

Discussion

HIV particle production is a multi-step process that occurs specifically on the plasma membrane (PM) or in compartments that are continuous with the PM. How Gag directs this assembly process to the PM has been the focus of my thesis and this chapter will discuss the results from Chapter II and III and some of the future directions for the project. The first part of the discussion focuses on the data related to the role of $PI(4,5)P_2$ in HIV-1 assembly and how the data might be expanded to a broader range of physiologically relevant cell types. In addition, I will also discuss whether the use of phosphoinositides in virus assembly is a conserved process among other members of Retroviridae family. The second part of the discussion focuses on the role of RNA as a negative regulator of membrane binding of Gag. Being a new topic in the field of HIV assembly, there are a lot of unanswered questions. Does RNA-mediated inhibition of Gag membrane binding occur in cells? What is the identity of the RNA that mediates this regulation of membrane binding? Some of these questions will be discussed in light of recent preliminary data obtained. Additionally, I will speculate on how $PI(4,5)P_2$ and RNA together may help coordinate steps within the assembly process and the advantages associated with this regulation. I will conclude by highlighting how the data obtained in this thesis can aid in development of anti-retroviral drugs.

Interaction between MA highly basic region and PI(4,5)P_2 is important for PM targeting and efficient membrane binding of Gag

Summary of data

Previous work in the lab has shown that depletion of PI(4,5)P_2 in HeLa cells relocalizes Gag from the PM to intracellular compartments and significantly reduces virus release [1]. Thus, PI(4,5)P_2 is an important cellular factor that mediates PM association of Gag. The work in chapter II illustrates, that in addition to relocalizing Gag to intracellular compartments, PI(4,5)P_2 depletion also increases non-membrane bound Gag in HeLa cells. To better understand the role of PI(4,5)P_2 in membrane binding of Gag, we developed an *in vitro* liposome-binding assay. Previous studies analyzing Gag-PI(4,5)P_2 interaction used bacterially purified Gag constructs. However, only part of Gag can be synthesized in this system due to solubility issues. In contrast, the *in vitro* system used in this thesis utilizes rabbit reticulocyte lysates that are capable of synthesizing full-length myristoylated Gag. This multi-domain protein is what mediates assembly in cells. Thus, we are analyzing physiologically relevant Gag in our system compared to bacterial expression systems. In addition, liposomes are made with defined lipids allowing us to specifically analyze the interaction between Gag and lipids of interest. Using this assay, we were able to show that Gag interacts with PI(4,5)P_2 in a head-group specific manner and that this interaction is necessary for efficient liposome binding of Gag (Chapter II). Using mutagenesis, we were also able to show that the MA domain of Gag is important for this interaction. When the MA domain was removed and an exogenous membrane binding signal was added, Gag showed promiscuous localization in cells both in the presence or absence of PI(4,5)P_2. Interestingly, however, virus release was still reduced

when $PI(4,5)P_2$ is depleted, albeit to a lesser extent than WT. These data suggest that $PI(4,5)P_2$ may have an additional role in virus assembly and release independent of its function as a PM anchor for Gag. Consistent with this hypothesis, a recent study suggested that activation of inositol trisphosphate receptor by $PI(4,5)P_2$ hydrolysis and subsequent Ca^{2+} mobilization modulate endocytosis of Gag and thus is important for virus release [2]. Relationships between this indirect role of $PI(4,5)P_2$ and its role as a Gag binding partner remain to be elucidated.

To further understand the molecular details of Gag- $PI(4,5)P_2$ interaction, we tested single and double amino acid substitutions of basic residues within the MA highly basic region (HBR) for their ability to bind $PI(4,5)P_2$ using *in vitro* liposome binding assay (Chapter III). Using this assay, we were able to show that lysines at position 17, 29 and 31 within MA domain are important for efficient Gag-$PI(4,5)P_2$ interaction. Surprisingly, mutations of two other lysines at position 25 and 26 increased $PI(4,5)P_2$-independent membrane binding. Further analysis showed that this mutant is promiscuously localized in cells likely due to an exposed myristate. In summary, MA HBR has opposing dual role in the context of membrane binding. Some of the basic residues directly interact with the acidic lipid $PI(4,5)P_2$ and facilitate efficient membrane binding. In contrast, some other basic residues inhibit membrane binding, especially in the absence of $PI(4,5)P_2$, by sequestering myristate. Notably, NMR structural analysis suggests that when $PI(4,5)P_2$ binds to MA HBR it causes a structural change that induces myristate exposure [3]. Thus, $PI(4,5)P_2$ facilitates membrane binding of Gag not only by electrostatic interaction but also by the structural change that can increase the

hydrophobic interaction of Gag. Both of these interactions with membrane can synergistically enhance and stabilize binding of Gag to the plasma membrane.

What about the role of PI(4,5)P_2 in relevant cell types?

The work discussed above was mostly performed in HeLa cells. Similar results were also obtained using HEK293 and Cos-1 cells [4, 5]. Even though these cells are good model cells to study assembly due to the ease of transfection, HIV infects only human cells that have CD4 on the surface. Thus, it is important to confirm the role of PI(4,5)P_2 in these physiologically relevant cell types.

Recent work from our lab suggests that depletion of PI(4,5)P_2 in the natural host cells of HIV-1, CD4+ T cells, significantly reduces Gag-membrane association and virus release [6]. In addition to T cells, HIV also infects antigen-presenting cells like macrophages and dendritic cells. As mentioned in Chapter I, virus assembly occurs in sub-cellular compartments called virus-containing compartments (VCC) in these cell-types [7, 8]. Preliminary work on the role of PI(4,5)P_2 in primary macrophages suggests that PI(4,5)P_2 is necessary for efficient virus release and membrane binding of Gag as previously observed in HeLa cells (Fig. 4.1). Thus, PI(4,5)P_2 plays an important role in virus assembly not only in model cell lines but also in physiologically relevant cell types.

Does the requirement for PI(4,5)P_2 hold true for other types and sub-types of HIV?

As mentioned in Chapter I, subtype C is the most predominant subtype in the HIV pandemic accounting for 50% of the cases. Due to its efficient tissue culture adaptability, the subtype studied in the lab is subtype B that accounts for only 10% of the cases. Before developing therapeutics against the virus using the data obtained from the lab, it is

important to test that the data holds true for all types and strains of HIV. It is also of note that although most MA HBR residues are conserved, subtype C has methionine instead of lysine at position 29 within MA. This residue has been shown to be important for Gag-$PI(4,5)P_2$ interaction in HIV-1 subtype B. Lack of a basic residue at position 29 raises the possibility that either subtype C does not require $PI(4,5)P_2$ for assembly or that it has a different binding site for $PI(4,5)P_2$. Data from my work using a subtype C strain (MJ4) suggests that $PI(4,5)P_2$ depletion significantly reduces its virus release efficiency, although the defect is less severe (3 fold reduction) compared to the subtype B (7 fold reduction) (Fig. 4.2). Further studies are required to pinpoint the residues in subtype C that are involved in $PI(4,5)P_2$ interaction. Likewise, NMR study on HIV-2 suggests that even though $PI(4,5)P_2$ binds HIV-2 MA, it is unable to efficiently expose myristate as in HIV-1. Nevertheless, the virus release of HIV-2 is still significantly reduced upon $PI(4,5)P_2$ depletion [9]. Thus, even though there are some type-specific differences, overall, the requirement of $PI(4,5)P_2$ for efficient virus release holds true among different HIV types and subtypes tested.

What about other members of Retroviridae family?

Not only HIV, but also other retroviruses including MLV and MPMV require $PI(4,5)P_2$ for efficient virus particle production [4, 10, 11]. However, not all retroviruses require $PI(4,5)P_2$. For example, although EIAV Gag that localizes both to the PM and intracellular compartments can bind $PI(4,5)P_2$ [12], this Gag seems to depend on $PI(3,5)P_2$ rather than $PI(4,5)P_2$ for efficient virus release and membrane binding [5]. Similarly, HTLV-1, which also localizes at the PM and intracellular compartments, is less sensitive to $PI(4,5)P_2$ depletion and is able to bind membranes efficiently even in the

absence of PI(4,5)P_2 [13]. Thus, different classes of retroviruses may have evolved with different requirements in terms of targeting to the site of assembly and virus particle production.

RNA bound to MA HBR inhibits PI(4,5)P_2-independent membrane binding of Gag

Summary of data

In addition to binding acidic lipids, several *in vitro* studies have shown that the MA HBR can bind RNA [14-20]. In Chapter III, we have shown that RNase treatment of *in vitro* synthesized Gag significantly increases its binding to control liposomes that lack PI(4,5)P_2 but still contain another acidic lipid, phosphatidylserine (PS). These data suggest that RNA inhibits membrane binding of Gag, especially in the absence of PI(4,5)P_2. In addition to increasing the binding of Gag to liposomes containing acidic lipids, RNase treatment of Gag also had a modest increase in its binding to liposomes containing only a neutral lipid, PC, in a myristate–dependent manner. Thus, RNA likely acts as a negative regulator of non-specific membrane binding by blocking both electrostatic interactions of Gag with acidic lipids and to a lesser extent hydrophobic interactions determined by myristate exposure. Based on these data, we hypothesize that RNA binding to MA necessitates the involvement of PI(4,5)P_2 for efficient membrane binding of Gag and is discussed later.

Does RNA-mediated inhibition of Gag membrane binding occur in cells?

To shift to a cell-based study on the role of MA-bound RNA in membrane binding, the question that needs to be addressed first is whether MA HBR interacts with RNA in cells. To examine this question, we quantified the amount of RNA bound to cell-

derived Gag mutants after immunoprecipitation. As expected, when NC, the major RNA-binding domain in Gag was removed, there was significantly less RNA co-immunoprecipitated with Gag compared to full-length Gag. The amount of RNA bound to Gag was also reduced when MA HBR was mutated, demonstrating that MA HBR binds RNA in cells (Fig. 4.3). To address whether the RNA bound to Gag in cells inhibits membrane binding, cytosolic Gag was isolated from digitonin-permeabilized HeLa cells transfected with HIV-1 molecular clones. The isolated Gag was then tested for its ability to bind liposomes with or without RNase treatment. As observed in chapter III using *in vitro* synthesized Gag, RNase treatment significantly enhanced binding of Gag to liposomes lacking PI(4,5)P_2 (Fig.4.4). This suggests that RNA bound to Gag in cells can block non-specific membrane binding of Gag. We are currently investigating whether this RNA-mediated inhibition occurs in intact cells using microinjection and protein transfection methods.

What is the identity of the RNA that inhibits membrane binding of Gag?

The RNA molecules that are present in the *in vitro* studies performed using rabbit reticulocyte lysates are Gag mRNA, tRNA and rRNA. Based on these data, we performed an *in vitro* functional-rescue experiment by adding back different RNA species to RNase-treated Gag. If there is any RNA specificity involved in the inhibition of membrane binding, specific RNA species are expected to inhibit membrane binding of Gag. However, preliminary studies suggest that any RNA, including Gag mRNA, tRNA or HeLa total RNA (that contains around 80% rRNA) is capable of inhibiting membrane binding of Gag (Fig.4.5). Assuming that a typical HeLa cell volume is $4x10^{-6}$ µl and that a cell has around 10-30 pg of RNA, there is about 2.5-7.5µg/µl of RNA in a HeLa cell.

Under normal physiological conditions, eighty percent of cellular RNA is rRNA, 15-20 % are tRNA or other small RNAs and the rest being mRNA. The concentration of RNA used in the functional-rescue assay is only around 0.11μg/μl, which is less than the physiological concentration. However, it is not known how much RNA is free (unbound RNA) in cells. It is conceivable that the RNA species bound to the MA HBR are restricted in cells, due to the limited amount of RNA that Gag encounters. In addition, it is possible that the same RNA that is bound to NC also binds MA. If this is the case, due to its inherent high affinity for RNA, NC may bring the RNA in close proximity to the MA HBR, which may then allow efficient MA-RNA interaction in cells. Further studies are required to address these possibilities. Overall, the results are consistent with the observation that several abundant RNA molecules interact non-specifically with Gag and are packaged into the virion [21].

What would be the implication of MA-RNA interaction in the viral life cycle?

The MA-viral RNA interaction has been shown to be important for genomic RNA dimerization, encapsidation, or nucleocytoplasmic transport of Gag in other retroviruses [22-25]. However, there are no reports indicating similar roles for MA-RNA interaction in the HIV-1 life cycle. Nevertheless, there are several recent studies functionally linking nuclear export of HIV-1 gRNA and MA-mediated membrane binding. It is known that HIV-1 assembly is blocked in murine cells [26-29]. However, altering the RNA export pathway, or introducing mutations in MA, which enhance Gag membrane binding [30-33], are sufficient to alleviate the block in HIV-1 assembly in murine cells [34]. Similar MA-dependent inhibition of assembly was also observed in human cells, when the native Rev response element (RRE) was replaced with Hepatitis B virus post-transcriptional

regulatory element (PRE) [35]. Thus, the membrane binding of Gag mediated by MA is functionally linked to the RNA export pathway used by HIV-1.

As mentioned in the introduction, Rev is important for nuclear export of unspliced RNA that is packaged into the virus as genomic RNA. Interestingly, a recent study has shown that excessive amount of spliced RNA in the cytosol reduces virus release and replication of HIV, likely by inhibiting membrane binding and assembly of Gag [36]. In addition, a viral RNA packaging signal (Ψ) mutant that packages more spliced RNA compared to viral genomic RNA had a second site mutation in MA [37]. This mutation, which is a change from an acidic residue to a basic residue, rescued the replication of Ψ mutant by reverting to normal levels of spliced to unspliced RNA ratio in the virion. Interestingly, the second site mutant specifically excluded spliced RNA incorporation into the virion but did not alter the amount of unspliced RNA. Based on these data, we can speculate that in normal circumstances unspliced viral RNA may interact with NC and due to close proximity may also bind to MA. However, in the absence of such interaction as observed in packaging signal mutant, MA may have higher affinity for spliced RNA. To avoid such excessive amount of spliced viral RNA incorporation, the second site mutation in MA that has an additional basic residue may alter the structure of MA such that MA has enhanced affinity for acidic lipids like $PI(4,5)P_2$. Higher affinity for $PI(4,5)P_2$ may then displace spliced RNA from MA, thus bringing back the spliced to unspliced RNA ratios in the virion to normal levels. On this note, it is interesting that another single amino acid mutation in MA that enhances $PI(4,5)P_2$ specificity can rescue the block in assembly seen in murine cells [34]. The nature of the link between RNA export, splicing and membrane binding needs to be elucidated.

MA-PI(4,5)P_2 binding may be important for coordinating Gag membrane binding with other assembly and post-assembly events

As mentioned earlier, both myristate and HBR are necessary for efficient membrane binding of Gag. Either one alone is not sufficient in the context of full-length Gag in cells, as supported by observations that both non-myristoylated Gag and Gag derivatives with substitutions of all basic residues in the HBR do not bind membranes efficiently [38-42]. However, in contrast to amino acid substitutions in the HBR, deletion of the entire MA globular head except for the myristoylation signal enhances Gag membrane binding [33, 43-46]. Treating non-myristoylated Gag with RNase also rescues binding of this otherwise defective Gag to membranes that contain PS, an abundant acidic phospholipid in the inner leaflet of cell membranes [42]. These observations suggest that membrane binding is inhibited by the globular-head-dependent sequestration of myristate and potentially by RNA bound to the HBR. In this context, it is intriguing that PI(4,5)P_2 appears to be capable of reversing both modes of inhibition. Unlike PS, PI(4,5)P_2 is able to bind MA HBR even in the presence of RNA [42, 47], perhaps because it binds to a site in MA not blocked by RNA or has a higher affinity for MA than the RNA. NMR studies on MA-PI(4,5)P_2 interaction suggest that binding of Gag to PI(4,5)P_2 causes a structural change that can expose myristate [3]. Thus, both targeting of Gag to PI(4,5)P_2-enriched membranes, i.e. PM, and efficient membrane binding are tightly linked.

NMR data further suggest that once the myristoyl moiety is exposed, the hydrophobic pocket of Gag is then occupied by the 2' acyl chain of PI(4,5)P_2 that is usually unsaturated [3]. This would leave two saturated acyl chains that mediate MA-membrane interaction; the remaining saturated 1' acyl chain of PI(4,5)P_2, and the N-

terminal myristoyl moiety of Gag (Fig. 4.6). The intriguing implication of this association is that it could enhance the affinity of Gag for PM microdomains known as lipid rafts, where saturated but not unsaturated acyl chains are preferred. Consistent with this model, replacing N-terminal myristate with an unsaturated analog significantly reduces Gag in detergent-resistant membrane fractions that are thought to originate from lipid rafts [48]. Lipid rafts have been previously shown to play a crucial role in virus assembly and infectivity of progeny virus particles [48-52] (for review see [53, 54]). Therefore, interaction with $PI(4,5)P_2$ not only promotes specific Gag localization to the PM and stable membrane binding, but may also confer an additional advantage to HIV-1 by associating assembly events to lipid rafts. The latter role for $PI(4,5)P_2$ has not been tested experimentally.

As mentioned earlier, in vitro studies indicate that RNA bound to MA HBR can block interactions with PS but not with $PI(4,5)P_2$ [42, 47]. Therefore, it is possible that the requirement for $PI(4,5)P_2$ could be a mere consequence of RNA-mediated inhibition of MA interaction with other acidic lipids. Conversely, RNA may be recruited to ensure specificity of the MA HBR for $PI(4,5)P_2$ and membranes containing this lipid (i.e., the PM). Indeed, HTLV-1 Gag membrane binding that is less dependent on $PI(4,5)P_2$ is also not inhibited by RNA, suggesting a correlation between RNA binding and $PI(4,5)P_2$-dependent membrane interaction[13]. Interestingly, a neutron scattering study showed that in solution, monomeric Gag adapts a conformation in which Gag is folded over, and MA and NC are in a close proximity [55]. When bound to either acidic membrane or oligonucleotides, Gag is still in a compact shape. However, when both acidic membrane and oligonucleotides are present, Gag forms the extended rod-like structure similar to the

structure observed in immature virion [56] (Fig. 4.6). An intriguing model proposed in these studies is that when bound to either the PM or RNA, Gag can do so via both basic regions in MA and NC simultaneously. When both the PM and RNA are present, however, MA-PM and NC-RNA interactions may occur in parallel. Thus, it is conceivable that once RNA-bound Gag reaches the PM, a high affinity of the MA HBR to $PI(4,5)P_2$ may help strip RNA from MA, but not NC (Fig. 4.6). Consistent with this possibility, *in vitro* RNA chaperone activity (annealing of tRNA to the primer-binding site of viral RNA) of NC in the context of Gag increased several fold when IP6 was added to Gag or when MA was deleted [57]. These data suggest that RNA binding of MA hinders NC activities in the absence of inositol phosphates. Based on the above data, we speculate that RNA-mediated regulation of membrane binding may be a way of coordinating several major assembly steps for HIV-1. Once the MA HBR is bound to $PI(4,5)P_2$ and releases RNA, NC may become capable of freely performing its functions, such as tRNA annealing and RNA-mediated Gag multimerization. However, it is important to note that this model assumes that the same RNA binds both MA and NC, an assumption that has to be tested experimentally.

Interestingly, NC-mediated multimerization has been shown to be important for localization of Gag to the rear-end protrusions known as uropods in polarized T cells [58]. Therefore, binding of $PI(4,5)P_2$ to MA might represent the transition from the MA-driven PM-targeting phase to the NC-dependent uropod localization phase. Notably, these Gag-containing uropods form frequent contacts with other T cells and participate in virological synapses [58, 59] at which cell-to-cell virus transmission is thought to take place efficiently[60-63](for review see [64-68]). Therefore, this polarized localization of

Gag on the PM likely facilitates efficient cell-to-cell transfer of the virus at the contact sites, which might represent another advantage associated with targeting of Gag to the PM. Further studies are required to better understand the connection between the MA-mediated regulation of targeting and membrane binding of Gag and the NC- mediated Gag multimerization step that drive Gag to specific sites within the plane of the PM.

Conclusions

Binding of Gag to the PM is the essential early step in HIV-1 assembly. These processes are likely modulated by MA in three different but coordinated ways: positively by interacting with $PI(4,5)P_2$, negatively by suppressing myristate-mediated hydrophobic interaction, and negatively by interacting with RNA. With the present knowledge on Gag-membrane binding, several types of drugs can be developed for inhibiting this process during HIV-1 assembly. Blocking N-terminal myristoylation of Gag or supplementing cells with unsaturated analogues of myristate have already been shown to significantly reduce infectious virus particle production [48, 69-72]. Similarly, as the Gag- $PI(4,5)P_2$ interaction plays an essential role in virus assembly, small molecule inhibitors that interfere with this interaction can be developed. Lately, RNA aptamers have been emerging as a new class of therapeutics for several different diseases [73]. With the recent evidence for the role of RNA in the inhibition of membrane binding of Gag, RNA aptamers that bind MA with higher affinity than $PI(4,5)P_2$ might prove useful as inhibitors of HIV assembly (for example, see reference [20]).

Mechanisms regulating both MA-$PI(4,5)P_2$ and MA-RNA interactions, as well as their potential roles in coordinating Gag membrane binding and other steps in assembly

and post-assembly processes remain to be elucidated. In addition, several events that precede Gag-membrane binding continue to pose outstanding questions: Where is Gag synthesized? How does Gag traffic to the PM at which $PI(4,5)P_2$ stabilizes its membrane binding? When and where does Gag encounter the RNA that inhibits MA-dependent membrane binding? How and why are RNA-export pathway and membrane binding linked? Addressing these questions will provide new strategies to inhibit HIV-1 replication at early stages of virus assembly.

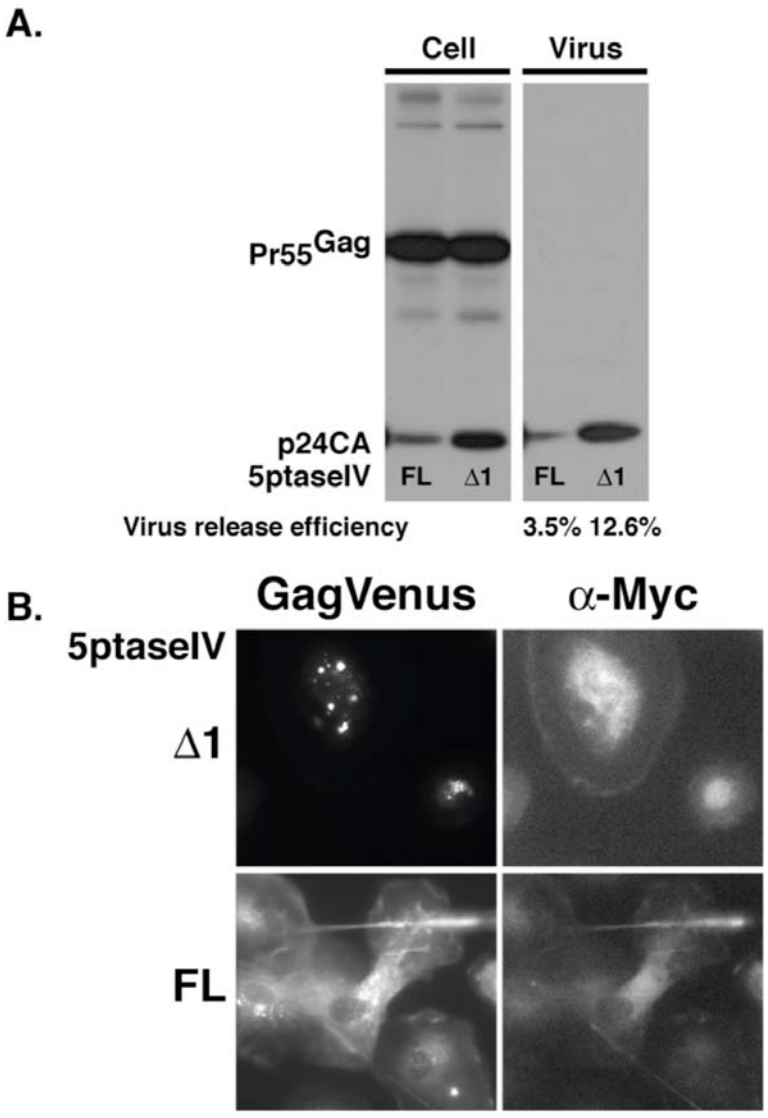

Fig.4.1 PI(4,5)P_2 is important for efficient virus release and membrane binding in macrophages

Primary monocyte-derived macrophages were infected with VSV-G pseudotyped HIV containing 5ptaseIV(FL) or 5ptaseIVΔ1(Δ1) in the place of Nef. A) 36 hours post-infection, virus-release assay was performed as in Fig. 2.8. Note that the virus release efficiency is reduced around 4 fold in 5ptaseIV expressing cells. B) Gag tagged with venus fluorescent protein was used for infection and 48 hours post-infection, the cells were fixed, permeabilized and stained with α-Myc antibody to visualize 5ptaseIV expression. Note that in 5ptaseIVΔ1-expressing cells, GagVenus shows strong VCC localization but in 5ptaseIVFL-expressing cells, Gag shows hazy cytosolic signal with very little VCC localization.

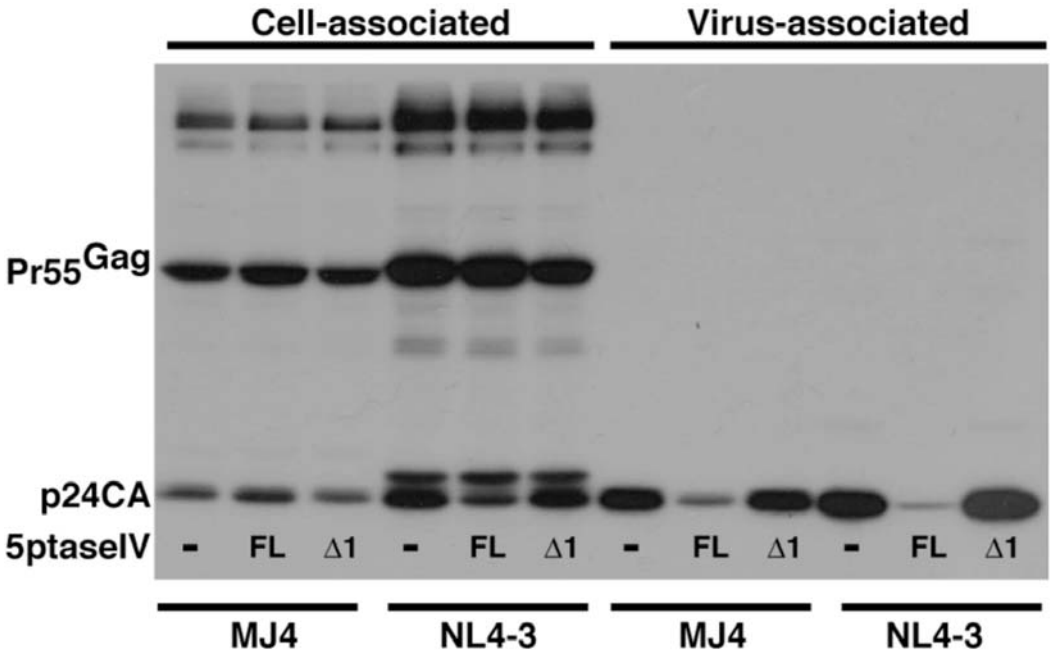

Fig.4.2. PI(4,5)P_2 is important for efficient virus release of HIV-1 subtype C.
HeLa cells were transfected with pMJ4 (HIV-1 subtype C) or pNL4-3 (HIV-1 subtype B) along with either pUC19(-) or 5ptaseIV(FL) or 5patseIVΔ1(Δ1). 16 hours post-transfection, virus release assay was performed as in Fig. 2.8. The virus release efficiency is reduced 3 fold for MJ4 and 7 fold for NL4-3 in 5ptaseIV expressing cells compared to 5ptaseIVΔ1 expressing cells.

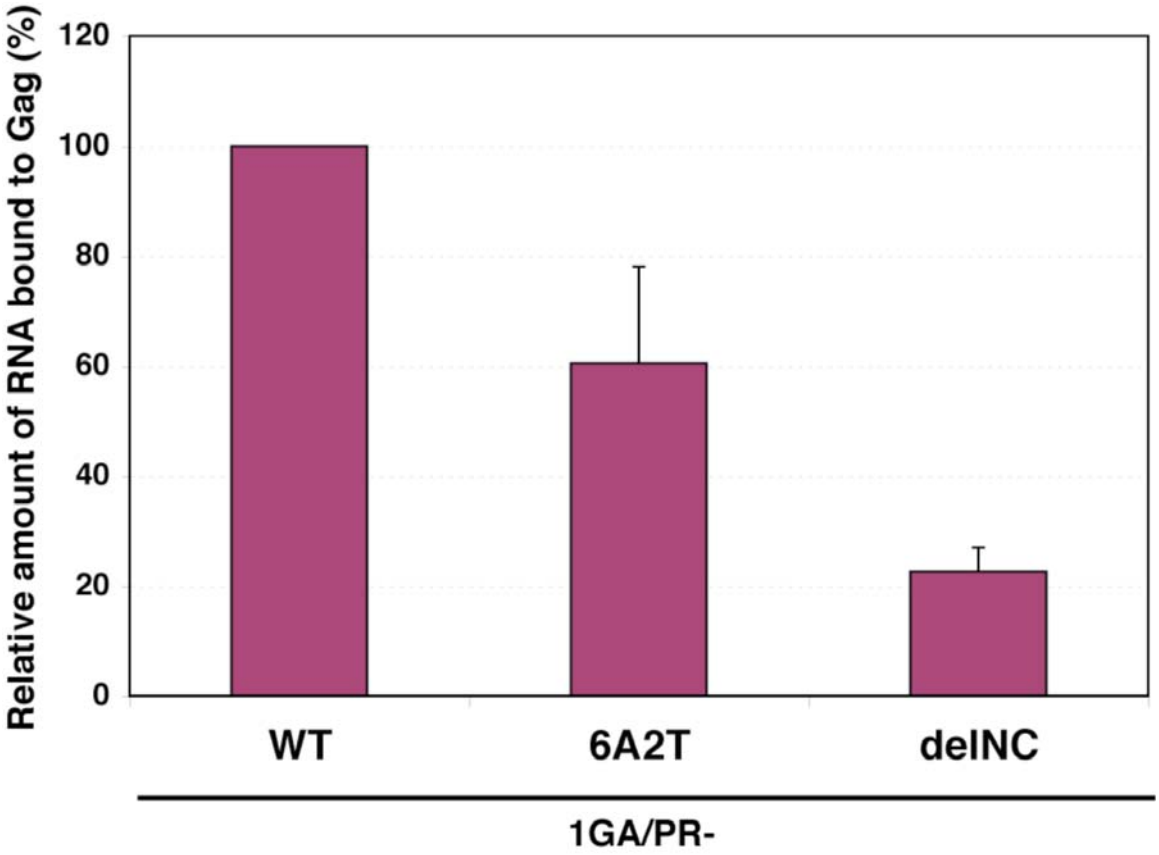

Fig.4.3. MA HBR interacts with RNA in cells
HeLa cells were transfected with pNL4-3/1GA/PR-, pNL4-3/1GA/6A2T/PR- or pNL4-3/1GA/delNC/PR- and radiolabeled overnight. Cells were lysed and immunoprecipitated with anti-HIV Ig antibody. The RNA bound to the immunoprecipitated samples was isolated using Qiagen miRNA isolation kit. The amount of RNA was quantified by fluorometry using ribogreen reagent. Simultaneously, the immunoprecipitated samples were analyzed on SDS-PAGE and the amount of Gag was quantified using phosphorscreen analysis. 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.

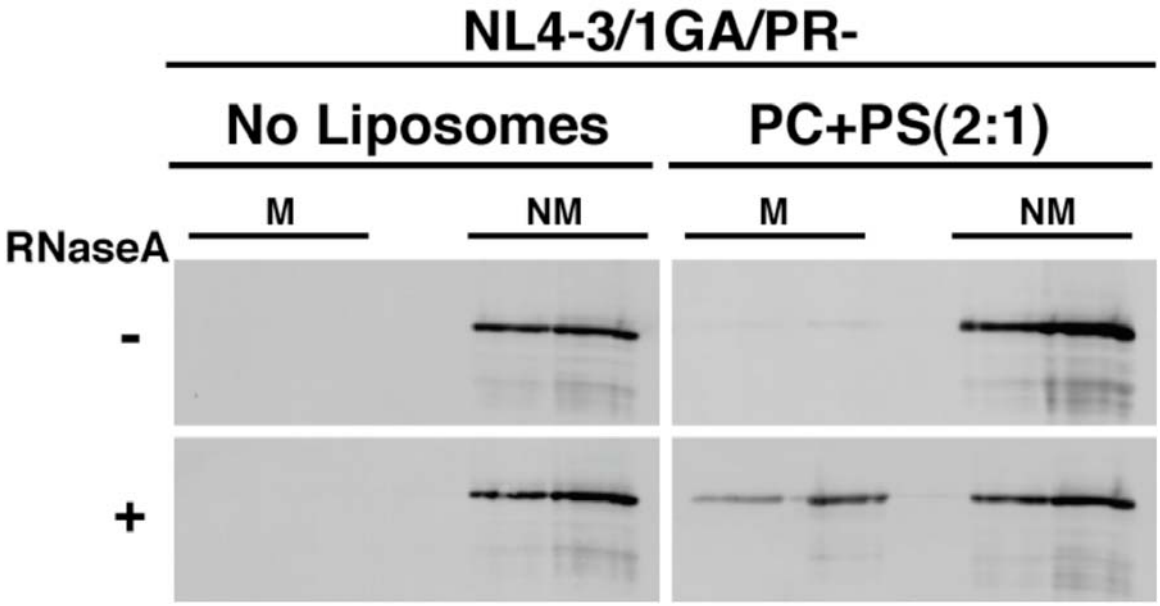

Fig.4.4. RNA inhibits membrane binding of cell-derived Gag
HeLa cells were transfected with pNL4-3/1GA/PR-.16 hours post-transfection, cells were treated with digitonin and centrifuged at high-speed. The supernatant containing cytoplasmic Gag was treated with 10 μg of RNase A or left untreated and incubated at 37° C for 20 minutes. Liposomes were then added and the reaction was further incubated for 15 minutes before performing sucrose gradient centrifugation. Five 0.8 ml fractions were collected and 50 μl of each fraction were loaded on gel and analyzed by western blotting using anti-HIV Ig primary antibody and anti-human Alexa Fluor488 secondary antibody. M: membrane bound, NM: non-membrane bound.

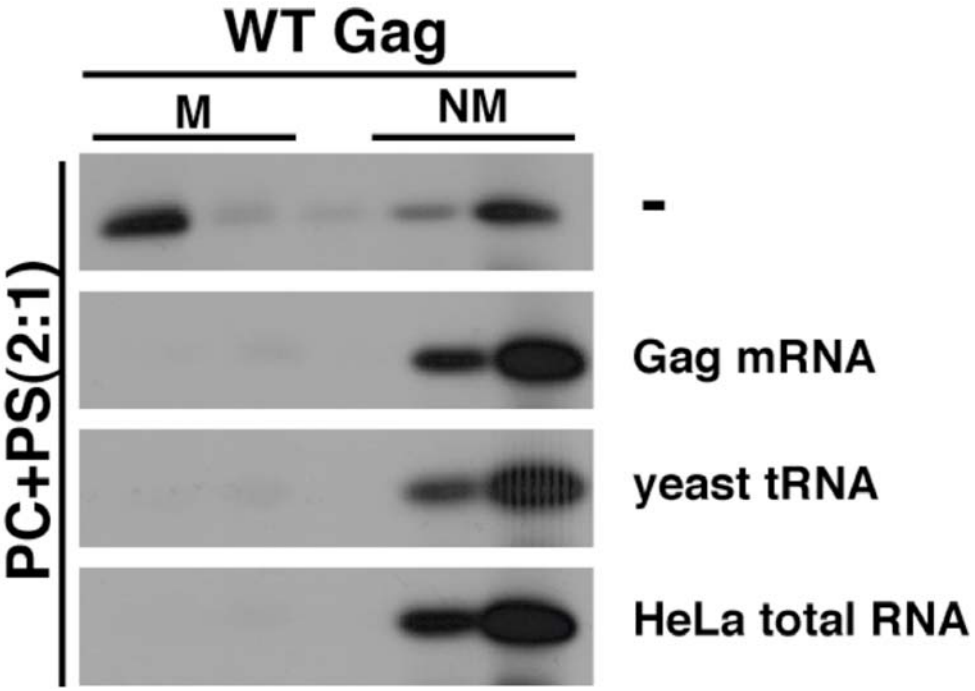

Fig. 4.5 Different RNA species can inhibit membrane binding of Gag
Radiolabeled Gag synthesized using rabbit reticulocyte lysates was treated with 400 ng of RNase A at 37°C for 20 minutes. RNase A was blocked using 10 μl of RNasin (40 U/μl) and 5 μg of different RNA or buffer (-) was added (final concentration of RNA is 0.11 μg/μl), and incubated at 30°C for 30 minutes. Liposomes were then added and incubated further for 15 minutes before performing sucrose gradient centrifugation. Five 1 ml fractions were collected and 25 μl of each fraction was loaded and analyzed on SDS-PAGE.

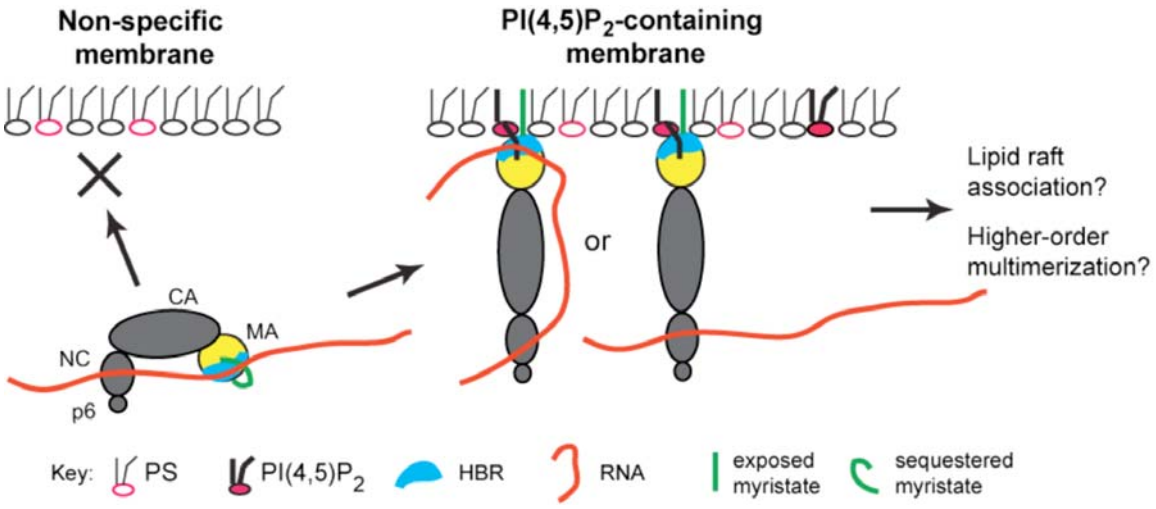

Figure 4.6 Regulation of Gag-PM binding via MA-PI(4,5)P_2 interaction and its possible impacts on subsequent assembly events

Both sequestration of the N-terminal myristoyl moiety and binding of RNA to the HBR inhibit non-specific membrane binding of matrix (MA) via hydrophobic and electrostatic interactions, respectively. Once Gag reaches the plasma membrane, PI(4,5)P_2 binds to the MA HBR thereby serving as a membrane anchor for Gag even when RNA is bound to the HBR. In addition, PI(4,5)P_2 binding to MA exposes myristate, further enhancing membrane binding. Altogether, these positive and negative regulation mechanisms ensure the plasma membrane specificity of Gag. Upon PI(4,5)P_2 binding, sequestration of 2' acyl chain of PI(4,5)P_2 into a hydrophobic pocket vacated by the myristoyl moiety may result in Gag association with membrane via two saturated acyl chains. This is likely to increase propensity of Gag to partition into lipid rafts. In addition, MA-PI(4,5)P_2 interaction might induce dissociation of RNA from MA, which in turn may facilitate NC-mediated functions, such as higher-order multimerization. Therefore, it is possible that MA-PI(4,5)P_2 interaction coordinates Gag membrane binding with other assembly processes.

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