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Retroviral Gag protein - RNA interactions: Implications for specific genomic RNA packaging and virion assembly

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Abstract

Retroviral Gag proteins are responsible for coordinating many aspects of virion assembly. Gag possesses two distinct nucleic acid binding domains, matrix (MA) and nucleocapsid (NC). One of the critical functions of Gag is to specifically recognize, bind, and package the retroviral genomic RNA (gRNA) into assembling virions. Gag interactions with cellular RNAs have also been shown to regulate aspects of assembly. Recent results have shed light on the role of MA and NC domain interactions with nucleic acids, and how they jointly function to ensure packaging of the retroviral gRNA. Here, we will review the literature regarding RNA interactions with NC, MA, as well as overall mechanisms employed by Gag to interact with RNA. The discussion focuses on human immunodeficiency virus type-1, but other retroviruses will also be discussed. A model is presented combining all of the available data summarizing the various factors and layers of selection Gag employs to ensure specific gRNA packaging and correct virion assembly.

Keywords

Retroviruses; Gag; RNA; gRNA packaging; Assembly

1. Introduction

Retroviruses possess an RNA genome that is reverse transcribed into cDNA and integrated into the host genome of the infected cell. The integrated DNA (the provirus) is then transcribed by host cellular machinery. The spliced and unspliced transcripts encode the necessary suite of viral proteins, while full-length RNAs serve as the genetic material in the newly assembling virions. While different retroviruses encode distinct accessory proteins, the Gag polyprotein is present in all retroviral genera [1]. Gag serves as the main structural protein for retroviruses and is the primary driver of virus particle assembly [2]. Indeed, expression of Gag alone is sufficient to lead to the formation of virus-like particles (VLPs) in cell culture [1]. The role of Gag in assembly involves interactions with a variety of different factors including lipid membranes, other viral and cellular proteins, and RNAs.

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This review focuses on Gag-RNA interactions and their role in regulating specific genomic RNA (gRNA) packaging and virion assembly.

While each retroviral Gag protein has distinct features, there are three key domains found in all Gag proteins (listed N- to C-terminus): the matrix (MA) domain, which is primarily associated with membrane binding capability, although MA is also known to interact with nucleic acids (NAs); the capsid (CA) domain, which mediates numerous Gag-protein and Gag-Gag interactions; and the nucleocapsid (NC) domain, which is the primary NA binding motif and is involved in specifically packaging the gRNA (Fig. 1) [2]. In addition to these three domains, various spacer peptides (such as SP1 and SP2 in human immunodeficiency virus type-1 (HIV-1)), enzymes (such as protease (PR) in Rous sarcoma virus (RSV)), and protein recruitment domains (such as p6 in HIV-1), are also found in Gag proteins. In this review, we will examine the known Gag domain interactions with RNA and the ways these interactions regulate the correct assembly of viral proteins and RNAs into a fully infectious particle. Based on all available published data, we will summarize the mechanisms employed by Gag to specifically package its gRNA over the vast excess of cellular and non-genomic viral RNAs present in the cell.

2. NC-RNA interactions

The primary NA-binding domain on Gag proteins is NC. NC is a multi-functional protein involved in a number of specific and non-specific interactions with NAs, as well as chaperoning NA annealing and rearrangements throughout the reverse transcription process. Several comprehensive reviews of NC's chaperone activity have been written [3–6], and this aspect of its function will not be covered in detail here. Instead we will focus on its role in specific packaging of viral gRNA in the context of Gag.

All retroviral NC proteins contain one or two zinc finger motifs [1]. The 55-amino acid HIV-1 NC protein is highly basic, possessing 15 cationic amino acids and two Cys-Cys-His-Cys-type zinc finger motifs that are critical for many of its activities, including gRNA packaging [5]. Specific packaging of gRNA into retroviral particles is accomplished, at least in part, through NC interactions with a cis-acting RNA element, which has been termed “Psi”, within the viral genome [7]. Early NMR structures of HIV-1 NC confirmed the presence of the zinc binding domains and revealed that the protein is largely unstructured when not bound to NAs [8]. Subsequent studies examining the structures of other unbound retroviral NC proteins demonstrated a largely similar architecture of the zinc finger domains, although differences in the distal zinc finger structure were observed [9–12]. Several structures of HIV-1 NC bound to RNAs derived from Psi helped to define the changes that occur to the protein structure upon NA binding. Structures in complex with the stem-loop 3 (SL3) RNA of Psi (Fig. 2C) revealed how the initially disordered N-terminus adopts a 3_{10} helix conformation to pack against the RNA, with four of the conserved basic residues making non-specific contacts with the phosphate groups on the backbone of the RNA stem [13]. These structures also demonstrated the role of the zinc finger domain residues in ordering the aromatic Phe¹⁶ and Trp³⁷ residues to specifically interact via stacking interactions with the nucleobases G⁹ and G⁷, respectively, of the SL3 tetraloop [13]. Both the proximal and distal zinc fingers contain binding pockets apparently optimized to

accommodate G residues. Site-directed mutagenesis experiments confirmed many of these structural observations, and interestingly found that the Phe¹⁶ residue in the first zinc finger was the most important for binding SL3 RNA [14]. Additional studies found that mutation of the aromatic residues in the zinc fingers only modestly reduced RNA binding affinity [15], while mutation of the N-terminal basic residues resulted in a more pronounced defect [16]. An NMR structure of an NC-stem-loop 2 (SL2) RNA complex was also solved, showing many of the same features of NC bound to SL3 [17]. Interestingly, the amino acid residues used to contact the SL2 and SL3 RNAs were not identical, underscoring the ability of NC to adapt its structure to bind various RNA sequences. In addition to NAs, HIV-1 NC is also capable of binding negatively charged membranes [18]. The structures of the NC domains from murine leukemia virus (MLV) [19] and RSV [20] bound to their cognate Psi RNAs have also been solved by NMR, revealing a similar mechanism of stacking contacts made between aromatic amino acids in the protein and nucleobases in the RNA in optimized binding pockets. Thus, despite differences in their exact structures, retroviral NC proteins possess a highly conserved structural basis for binding RNAs.

The examples cited above reflect specific NC-RNA interactions, but also support the structural plasticity of NC to adapt to different NA sequences. Early in vitro studies showed HIV-1 NC had the strongest affinity and specificity for polyG sequences and bound with especially high affinity to poly(T/U)G sequences, while binding much more weakly to polyA sequences [21–23]. Simian immunodeficiency virus (SIV) and bovine leukemia virus (BLV) NC were found to possess the same nucleotide (nt) binding preference as HIV-1, although there was less variability in BLV NC specificity [24, 25]. Interestingly, human T-cell leukemia virus type-1 (HTLV-1) NC displays little nt preference, only modestly preferring T/U-rich sequences, and binds NAs more weakly than other retroviral NC proteins [26, 27]. The lack of high-affinity HTLV-1 NC binding appears to be compensated for by the expanded role of HTLV MA domains in NA binding and chaperone activities [28, 29].

Selective 2' hydroxyl acylation analyzed by primer extension (SHAPE) experiments were used to investigate gRNA binding sites of HIV-1 NC in mature virions, revealing that NC bound numerous single-stranded G-rich bulges, primarily in the Psi domain of the gRNA 5' untranslated region (5'UTR) [30]. Thus, specific NC binding to Psi appears to reflect its preference for binding to G-rich single-stranded RNA sequences and helps to explain why this RNA element is so crucial for specific gRNA packaging. A recent NMR structure of HIV-1 Psi revealed that many of the G-rich bulges were exposed and thus, accessible for NC interaction [31]. A cross-linking immunoprecipitation-sequencing (CLIP-Seq) study confirmed that Gag in the cytoplasm of infected cells and NC in mature virions interacted with G/U-rich sequence motifs [32]. However, unexpectedly, membrane-associated Gag and Gag in immature virions was found to bind primarily to G/A-rich RNA sequences (of both viral and cellular origin), indicating that a switch in the nt binding specificity of Gag occurs during virion genesis. Interestingly, the altered binding preference of Gag for A-rich sequences mimics the A-rich composition of the HIV-1 gRNA [33], suggesting the possibility that the genome evolved to facilitate selective packaging. It remains to be determined what causes this change in binding preference, which could be related to changes in the ability of NC to interact with RNAs in the context of a multimeric Gag lattice

vs. lower order Gag monomers or dimers. A recent study reported that Gag bound more tightly to G/A-rich sequences than to G/U-rich sequences; this effect depended on the presence of the p6 domain of Gag and the ability of Gag to multimerize [34]. Additional studies are needed to elucidate the mechanistic basis for this shift in HIV-1 Gag specificity, and to investigate if such changes also occur in other retroviral Gag proteins.

In vitro, NC binds many NAs with similar apparent affinity (low nM); at physiological salt concentrations, the strength of Gag binding to Psi RNA is not significantly different from binding to non-Psi sequences [35]. Early studies found that when HIV-1 NC bound its preferred oligonucleotide sequences, such as polyTG, these complexes were more resistant to salt dissociation than binding to other NAs [22]. This is consistent with certain NC-RNA binding interactions involving more specific, non-electrostatic stacking interactions, whereas others use non-specific charge-charge contacts, which are more susceptible to disruption by salt. A fluorescence anisotropy salt titration binding assay examined HIV-1 NC and Gag p6 (Gag lacking the p6 domain) binding to Psi vs. non-Psi RNAs. In this assay, protein-RNA interactions are assessed at increasing salt concentrations, and the parameters $K_{d(1M)}$ and Z_{eff} can be determined. $K_{d(1M)}$ corresponds to the strength of the non-electrostatic component of binding, while Z_{eff} reports on the number of charges mediating the protein-RNA interaction [36]. Thus, this analysis can reveal if a protein is employing more of the specific interactions (e.g., base stacking) vs. non-specific electrostatic contacts to bind an RNA. Similar to the earlier study using short oligonucleotides, this study revealed that Gag p6 bound Psi RNAs with much higher salt resistance than non-Psi RNAs [35]. Moreover, the salt-resistant, specific binding to Psi RNA depended critically on intact NC zinc finger structures, confirming the necessity of the ordered aromatic residues for specific RNA interactions. A more recent study confirmed that the binding of Gag p6 to non-Psi RNA was more easily reduced by salt competition compared to binding to Psi RNA, and also found that the presence of competitor tRNA had a greater ability to disrupt the Gag:non-Psi complex than the Gag:Psi complex [37]. Interestingly, all of these studies found that in the absence of high salt or competitor RNA, Gag p6 binding to Psi vs. non-Psi RNAs was not significantly different, certainly not enough to explain the observed selectivity of gRNA packaging.

The salt-titration binding assay has also been used to investigate RSV Gag PR (Gag lacking the protease domain) binding to its Psi RNA. RSV Gag PR, like HIV-1 Gag p6, bound its Psi RNA in a much more salt-resistant manner relative to a non-Psi RNA construct [38]. A similar study of the SIV NC protein found that it too displayed salt-resistant behavior when binding HIV-1 Psi but not a non-specific RNA [39]. Taken together, the specificity of NC-Psi interactions is readily observed in competition studies in vitro [35, 37–39]. Moreover, even though Psi and non-Psi RNA could equally associate with Gag assemblies on liposomes in the absence of excess competitor RNA, only Psi RNA persisted in its ability to associate with Gag assemblies in the presence of the competitor [40]. Importantly, it has recently been shown in cell-based assays that when Gag is expressed at levels that mimic those in a genuine infection, the presence of gRNA actually enhances virion production relative to virions produced in the absence of gRNA [41]. However, when Gag is expressed at higher levels, the presence of gRNA has a less pronounced effect on virion production efficiency. Together, these studies suggest that the capability of specific Gag/NC-RNA

interactions at the membrane to resist competition from other RNAs is an important feature of selective gRNA incorporation.

In addition to specific Gag/NC-RNA interactions with Psi RNA discussed so far, nonspecific interactions also play a role in the viral life cycle [42]. The generally accepted model is that RNAs can serve as a scaffold for Gag proteins to assemble on during the process of virion genesis. In support of this, deletion of the NC domain of MLV Gag completely abolishes virus production [43]. In contrast, the HIV-1 NC domain can be deleted, and virions are still produced as long as Gag-Gag and Gag-membrane interactions are unperturbed, although these variants package substantially less RNA overall [44, 45]. This suggests that, at least in the case of HIV-1, Gag-Gag and Gag-membrane interactions can compensate for the loss of the scaffold and Gag can still multimerize, while MLV Gag is more reliant on an RNA scaffold to facilitate this process. If the NC domain of HIV-1 Gag is replaced by a protein multimerization motif, such as a leucine zipper, then virions are produced that appear morphologically normal but do not contain RNA [46, 47]. In vitro, any NA is capable of initiating Gag VLP formation in the case of HIV-1 [48, 49] and RSV [50, 51], as long as the constructs are at least 20–40 nt in length. Furthermore, in the absence of gRNA, morphologically normal virions are still produced that contain levels of RNA roughly consistent with their cytoplasmic prevalence [52, 53], although a more recent analysis revealed that there is a slight preference for mRNAs with longer 3'UTRs [54]. In cells, overexpression of microRNAs 18–22 nt in length inhibited Gag assembly and viral production, suggesting that excess RNA of insufficient length to support assembly can inhibit the process [55]. Taken together, these data support the model that generic NC-RNA interactions may play an important role in facilitating Gag assembly, although there are retroviral-specific differences. Although many RNAs can be passively incorporated into virions and support assembly (albeit not as efficiently as gRNA [41]), the fact that RNAs substantially shorter than authentic gRNA can nonetheless support assembly suggests that the need for “scaffolding” in this process may not extend beyond the initial oligomerization of a few Gags that serve to “nucleate” virus assembly at the plasma membrane (PM).

3. MA-RNA interactions

The MA domain is associated with the membrane binding capability of most retroviral Gag proteins. A discussion of Gag/MA interactions with membranes, the molecular determinants that govern the interactions in different retroviruses, and mechanistic/structural details of these interactions will not be covered in detail in this article, but the reader is referred to recent reviews on this topic [56, 57]. Here, we will focus on the RNA binding capabilities of MA.

It has long been appreciated that retroviral MA domains possess the capability to bind NAs [58, 59], and more recently the functional implications of these interactions are becoming clear. The three-dimensional structures of MA domains from HIV-1 [60–62], HTLV-2 [63], SIV [64], BLV [65], and Mason-Pfizer monkey virus (MPMV) [66] have all been determined, revealing a remarkable similarity in the overall protein architecture despite highly divergent primary sequences. All of these MA domains possess a patch of surface-exposed basic residues and have also been found to trimerize. The basic patch of trimeric

MA generates a large cationic electrostatic surface involved in binding acidic lipids in the inner leaflet of the PM. The MA domain of some retroviruses is also post-translationally myristoylated, and this modification is typically involved in enhancing MA-membrane interactions. The MA highly basic region (HBR) has been primarily implicated in the protein's interaction with NAs [67–69]. Mutagenesis experiments further confirmed that removal of the N-terminus of MA, where the HBR is located, or mutation of critical lysine residues in the HBR abolished HIV-1 MA-NA interactions [70, 71].

The physiological role for MA-NA interactions in the context of Gag is still an area of active investigation and appears to vary between retroviruses. Efforts exploring the functional redundancy of the HIV-1 MA and NC domains found that substituting MA for NC led to defects in both virion assembly and RNA incorporation [72]. Consistent with the idea that the primary role of HIV-1 MA was to mediate membrane interactions, it was demonstrated that specific phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂)-containing liposomes could strongly outcompete even a tightly bound RNA for MA binding [73]. Subsequent studies showed that RNA was able to efficiently block HIV-1 Gag binding to membranes lacking PI(4,5)P₂ by preferentially interacting with MA, while PI(4,5)P₂-containing membranes could still interact with MA pre-bound to RNA [74, 75]. RNA could also block RSV MA binding to membranes, but less effectively compared to HIV-1 [76]. The prevalence of PI(4,5)P₂ in the inner leaflet of the PM [56] together with the requirement for PI(4,5)P₂ to initiate and maintain the process of HIV-1 virion assembly [77], collectively suggest a model in which RNA serves to prevent Gag from interaction with intracellular membranes, which would lead to non-productive assembly, and to re-direct Gag to assemble preferentially on the PM.

The question of whether specific cellular RNAs were responsible for HIV-1 MA interactions was addressed by several recent studies. CLIP-Seq was used to identify RNAs that interact with the different domains of Gag throughout the viral life cycle. Surprisingly, this study revealed that the majority of RNAs bound to Gag in cells were tRNAs, and that the MA domain of Gag bound almost exclusively to tRNAs [32]. The study further confirmed that RNase treatment of cells led to an increase in membrane-associated Gag, consistent with the model that RNAs act to downregulate Gag-membrane binding. It was later reported that certain tRNAs were capable of inhibiting Gag binding to liposomes lacking PI(4,5)P₂, and that this RNA-specific inhibition required the NC domain of Gag but not the specific sequence of the MA HBR [78]. It had been demonstrated earlier that the specific sequence of the HBR was important for PI(4,5)P₂ recognition, whereas only the total overall charge was required for RNA interactions [69], implying that MA HBR-RNA interactions are largely non-specific. Salt-titration binding assay results supported this idea, showing that Gag, but not MA, displayed specificity for tRNA^{Pro} over tRNA^{Lys3} [78]. It is possible that the NC domain facilitates loading of specific tRNAs onto MA. Based on the available data, it is not clear whether aminoacyl-tRNA synthetases are also part of the Gag-tRNA complex in cells. Consistent with this possibility is the observation that the site of MA cross-linking to the tRNA occurred in the D loop [32], which is solvent exposed when tRNAs are bound to tRNA synthetases [79].

It is well established that the NC domain of Gag plays a dominate role in determining the specificity of gRNA packaging in most retroviral systems (reviewed in [7]), but more recently it has begun to emerge that MA also contributes to this process. Salt-titration binding assays first revealed that the HIV-1 MA domain interacts with non-Psi RNAs in the context of Gag, but does not appear to interact with Psi-containing RNAs [35]. In addition, Gag lacking the MA domain was less able to distinguish Psi from non-Psi RNA. These findings are consistent with the earlier observation that, in the absence of membrane mimetics, HIV-1 Gag was capable of binding poly(TG) DNAs simultaneously with the MA and NC domains [80]. A recent study also found that neutralizing the basic residues of the MA HBR resulted in a greater loss of affinity to non-Psi RNA than to Psi RNA [37]. This study also reported that tRNA more easily outcompeted non-Psi RNA binding to Gag relative to Psi RNA binding in a MA HBR-dependent manner, suggesting that MA-tRNA interactions are likely critical to the role of MA in upregulating Gag specificity for gRNA.

MA-RNA interactions can also modulate association of Gag-gRNA assemblies on membranes. For example, tRNA had a more pronounced effect on blocking Gag-non-Psi RNA assembly on PI(4,5)P₂-containing liposomes than Gag-Psi RNA assemblies [40]. Additionally, it was reported that RNAs containing SL1 of the Psi domain (Fig. 2C), but not viral-derived RNAs lacking this motif, were capable of blocking Gag binding to liposomes lacking PI(4,5)P₂ [78]. Thus, even in the absence of tRNA, binding to Psi-derived RNAs prevents Gag binding to non-PI(4,5)P₂ membranes (i.e., non-productive assembly). Taken together, these data suggest a model whereby MA-RNA interactions enhance the specificity of Gag-gRNA interactions in the cytoplasm, and ensure assembly on PI(4,5)P₂-containing membranes.

The role of MA and the nature of its interactions with RNAs varies between different retroviruses. First, the relative affinity of retroviral MA domains for NAs varies quite widely. The HIV-1 MA domain displays higher NA binding affinity (~400 nM) than HTLV-1 MA (~1–3 μM) and the much more weakly binding RSV MA (~17 μM), but lower affinity than the BLV and HTLV-2 MA domains (~20–100 nM) [29, 81, 82]. In addition, whereas in most retroviral genera it has been demonstrated that NC plays the dominate role in specifically interacting with and packaging the gRNA, deltaretroviruses (e.g., BLV and HTLVs) also appear to use the MA domain in this capacity. For example, mutations to BLV MA that did not perturb BLV Gag membrane binding were nonetheless found to impair gRNA encapsidation [28]. In addition, BLV MA bound very tightly to RNA sequences derived from its packaging signal, suggesting a direct role for MA in gRNA recognition and packaging [82]. Subsequent studies of another deltaretrovirus, HTLV-2, revealed that its MA domain also specifically interacted with RNA stem loops from the putative HTLV-2 packaging signal. Moreover, when HIV-1 MA was mutated to more closely resemble HTLV-2 MA, it could partially restore gRNA packaging of a NC HIV-1 virus in cell-based assays [29]. Therefore, in contrast to HIV-1, it is possible that deltaretroviruses have evolved a general mechanism to employ both the NC and MA domains of Gag to package gRNA.

Although the RSV NC domain appears to be the dominant determinant of specific gRNA recognition and packaging [83], the RSV MA domain has been found to contribute in a variety of ways. Salt-titration binding studies revealed that, just as with HIV-1, the RSV MA

domain upregulated the ability of RSV Gag to distinguish Psi RNA from non-Psi RNA [38]. Interestingly, RSV and HIV-1 Gag chimeras with swapped MA domains possessed the same ability to recognize their cognate Psi RNAs, suggesting a functional equivalency of MA domains, at least between these two retroviral species. Additional studies are required to ascertain if other retroviral MA domains or a non-specific RNA binding domain could also mediate specific Psi recognition. RSV Gag has been shown to traffic back into the nucleus after translation, where it is proposed to bind gRNA for packaging [84]. RSV MA was found to bind importin-11 to facilitate the nuclear import of Gag, and interestingly, RSV Psi RNA was able to compete for importin-11-MA binding [85]. This suggested that a negative feedback mechanism could be at play, whereby gRNA, once successfully exported from the nucleus in complex with Gag, maintains interactions with MA to prevent further Gag nuclear import.

The capability of RNA to modulate MA-membrane interactions has also been investigated in different retroviruses. One study reported that RSV MA was less susceptible than HIV-1 MA to RNA-mediated membrane binding inhibition, but that this depended to a large degree on liposome composition [76]. A subsequent report investigating a larger panel of retroviral MA proteins concluded that HIV-1 and RSV MA were susceptible to RNA-mediated binding inhibition to liposomes lacking PI(4,5)P₂, while the HTLV-1, MLV, and human endogenous retrovirus K (HERV-K) MA domains were not [69]. These differences were largely attributable to differences in the HBR regions on the different retroviral MA proteins.

In summary, a significant degree of variability exists in the functions of retroviral MA-RNA interactions. While some general trends have begun to emerge, a number of questions still remain. Of particular interest would be a high-resolution structure of MA bound to RNA. This could potentially reveal how MA may (or may not) be capable of achieving sequence specificity for particular tRNA species. The precise mechanism by which MA modulates specific Psi RNA recognition in HIV-1 and RSV is also unclear. In addition, it remains to be determined whether other cellular factors, such as aminoacyl-tRNA synthetases, play a role in mediating MA-RNA interactions in different retroviruses.

4. Factors contributing to specific gRNA packaging by Gag

Despite decades of study, a mechanistic explanation for how retroviral Gag proteins selectively encapsidate their gRNAs remains elusive. A variety of possibilities have been explored, and it is likely that multiple mechanisms collectively contribute to the observed specificity.

Even though all retroviruses are ultimately capable of specifically packaging a single, dimeric copy of their gRNA, there is a considerable degree of variability in how well defined the Psi packaging element is in different retroviruses. For example, in the case of RSV and MLV, the RNA element necessary and sufficient to direct gRNA packaging appears to be well established [86–89] (Fig. 2A & 2B). Mutation of these RNA elements leads to dramatic reductions in gRNA packaging and these elements are capable of directing packaging of non-genomic RNAs into virions [86, 89]. However, in the case of lentiviruses such as HIV-1 and SIV, the picture is less clear. Relative to the previous examples, mutation of lentiviral Psi

RNA sequences has a much less dramatic effect on gRNA packaging, and a minimal element that can direct heterologous RNA packaging has not yet been defined [7, 90]. A comprehensive discussion of what constitutes the minimal RNA packaging element in HIV-1 is outside of the scope of this review; however, the reader is referred to several recent reviews that focus on this topic [54, 91–93]. Here, we will focus on Gag interactions with the HIV-1 5'UTR. Despite some disagreement as to what constitutes the minimal packaging signal in HIV-1, there is general agreement that this region contains important RNA elements required for gRNA packaging.

What structural features of Psi RNAs are responsible for Gag selectivity? In the case of simple retroviruses such as MLV and RSV, significant insights into this question have been obtained in recent years. The structure of the 132-nt MLV Psi element has been solved by NMR in monomer, dimer, and NC-bound states, revealing that four conserved UCUG motifs, which bind tightly to MLV NC were exposed only in the RNA dimer [19, 94, 95]. SHAPE probing of this region of RNA in immature virions showed exposure of these UCUG motifs and confirmed the NMR results (Fig. 2A) [96]. Mutation of the G residue in this motif to an A was sufficient to significantly disrupt MLV NC and Gag binding, and to reduce MLV gRNA packaging by ~100-fold, similar to passive levels of RNA incorporation [97]. The overall results from these studies suggest that at the level of initial Gag-RNA recognition, at least in some simple retroviruses such as MLV, the exposure of several high-affinity NC binding sites upon RNA dimerization explains selective Gag recognition of gRNA.

The structure of the 82-nt minimal RSV Psi RNA (μ Psi) bound to a single RSV NC has also been solved by NMR. Interactions were observed between the first NC zinc finger and the UGCG loop of SL-C and the second zinc finger and the linker between SL-A and SL-B (Fig. 2B, boxed) [20]. Mutation of the UGCG loop of SL-C or the A197 nt in the linker region resulted in a loss of RSV NC binding affinity and defects in viral replication [20, 98]. Earlier studies had implicated the linker between SL-B and SL-C as important for RSV gRNA packaging but did not observe any effect upon UGCG loop mutation [99, 100]. A recent salt titration binding study found that mutation of the linker nt had the greatest impact on RSV Gag binding specificity, while no defect was associated with mutation of the SL-C loop [38]. Clearly, more work is required to precisely define the residues in RSV Psi RNA that are critical for specific Gag binding and gRNA packaging.

The HIV-1 5'UTR has been shown to adopt at least two conformations [101–103]. In one conformation (dimerization competent), the palindromic dimerization initiation site (DIS) loop is exposed, whereas in the other, the DIS loop is sequestered via a pseudoknot interaction. A structure of the dimer competent conformation of the HIV-1 “core encapsidation signal” has been solved by NMR, revealing 17 unpaired or weakly paired G residues that could serve as NC binding sites [31]. The structure is consistent with specific Gag recognition and packaging of dimeric gRNA [104]. However, a structure of the DIS-sequestered conformation will be needed to understand how that conformation is discriminated against by Gag. While it may be that all of these G-rich sites are required to achieve specific gRNA packaging, recent analysis has suggested that some sites may be more important than others. Several binding studies have identified the G-rich elements of

SL1 and SL3 as being most critical [38, 105, 106], and CLIP-Seq data are also consistent with this observation [32].

In addition to the presence of exposed NC binding sites, differential Gag binding stoichiometry to different RNAs may also contribute to packaging. Isothermal titration calorimetry analysis revealed that when the HIV-1 5'UTR was in the DIS-exposed conformation, a greater number of NC proteins bound the RNA [101]. A similar analysis of Gag binding to short RNA oligonucleotides also showed that Gag bound with higher stoichiometry to G/A-rich RNA than G/U-rich RNA [34]. Another study examining Gag binding to longer RNAs corresponding to the first 600 nt of gRNA and spliced viral mRNAs found that only the gRNA construct supported higher (6 to 7) Gag binding stoichiometry [107]. Interestingly, the Gag-gRNA binding stoichiometry observed in this study is consistent with a Gag hexameric unit proposed to nucleate virion assembly [54]. Also consistent with the nucleation hypothesis, a salt titration binding study found that there were three critical binding sites on an RNA monomer (i.e., six on a dimer) derived from the SL1-SL3 region of the 5'UTR that led to the greatest Gag specificity defects [38] (Fig. 2C). Interestingly, the degree of specificity lost upon mutation of a single site was about the same as if all sites were simultaneously mutated [35]. Thus, it is plausible that gRNA is recognized and specifically packaged in large part because Psi serves as an optimal platform to assemble a Gag hexamer, poised to trigger further Gag oligomerization and virion formation at the PM. Additional experiments will be required to more directly probe the degree of Gag oligomerization on different RNAs, and to link this to the ability to initiate virion assembly.

In addition to conformational heterogeneity in the 5'UTR, Gag is also capable of adopting different conformational states. The individual domains of retroviral Gag proteins (Fig. 1) are connected by flexible linkers, and small angle neutron scattering data suggested that HIV-1 Gag existed in a predominantly bent conformation in its apo state [108]. The addition of both NA and a PI(4,5)P₂ mimetic (such as inositol hexaphosphate) was required to shift Gag to an extended conformation [80]. The extended conformation of Gag is found in immature viral particles with the correct morphology, while the bent conformation of Gag is associated with smaller, morphologically-aberrant VLPs, such as those that form around NAs alone [80, 108]. A single molecule Förster resonance energy transfer (FRET) study showed that Gag alone or in the presence of DNA existed in roughly equal proportions of bent and extended conformation [109]. However, simultaneous addition of both DNA and inositol hexaphosphate (a PI(4,5)P₂ mimetic) led to Gag exclusively adopting the extended conformation, a transition that occurs on the timescale of minutes [109]. In contrast, small-angle X-ray scattering analysis of MLV and RSV Gag proteins showed that they predominantly adopted an extended conformation in their apo states, although RSV Gag was still found to adopt a bent conformation when bound to membranes [110, 111]. Thus, in contrast to HIV-1 Gag, RSV and MLV Gag proteins may not require a major structural rearrangement to achieve the correct conformation to assemble into virions. The multiple high-affinity Gag binding sites found in HIV-1 Psi may be required to allow Gag to overcome the energetic barrier to adopting the extended conformation and thus proceed to assembly. Indirect evidence from salt titration studies suggests that both HIV-1 and RSV Gag contact non-Psi RNAs primarily with their MA and NC domains (presumably in a bent

conformation), while they interact with Psi RNAs with their NC domain (presumably in a more extended conformation) [35, 38]. Thus, the identity of the RNA may facilitate the switch of Gag to a conformation primed for virion assembly.

Early studies identified the SP1 linker peptide located between the CA and NC domain of HIV-1 Gag as being critical for proper assembly of the immature virion both in cells and in vitro, as its removal or mutation of certain residues led to defects specifically in formation of the immature Gag lattice [112–115]. SP1 mutations that negatively impacted Gag multimerization were also found to impair the ability of Gag to bind membranes [116]. These studies predicted that SP1 could adopt an α -helical structure, and that this motif may be involved in important contacts in the context of the immature Gag lattice [117, 118]. However, NMR studies of the SP1 region showed that it is predominantly unstructured in solution with only a weak propensity towards α -helical structure [119]. Circular dichroism analysis revealed that the SP1 domain is able to undergo a random coil to α -helix transition in a concentration-dependent manner, and molecular dynamics (MD) simulations showed that residues in the SP1 α -helix were involved in making inter-protein contacts [120, 121]. These studies suggested that SP1 could act as a molecular switch to trigger Gag assembly and potentially propagate information about the multimeric state of Gag bound to RNA to the CA domain, leading to further multimerization through CA-CA interactions. It is also possible that SP1 plays a role in regulating the bent/extended Gag conformation, as a recent MD study found interactions between MA and the CA-CTD domain (near SP1) in the bent conformation of Gag [122]. Thus, while more work will be required to determine the effect of specific RNA binding on the SP1 conformation, this peptide appears to play an important role in modulating Gag conformation and may be a key link between specific gRNA binding/packaging and virion assembly.

Studies have also investigated the location, timing, and dynamics of Gag-gRNA interactions in different retroviral systems. For example, as mentioned earlier, RSV Gag has been shown to traffic into the nucleus after it is synthesized. Nuclear trafficking is required for efficient gRNA incorporation, implying that this is the location of initial RSV Gag-gRNA interaction [123, 124]. In the case of HIV-1, the available evidence points towards the cytoplasm as the site of initial Gag-gRNA interaction, involving lower order Gag multimers [125]. Gag-gRNA recognition may occur soon after nuclear export in the vicinity of the nuclear envelope [126]. More recent live cell microscopy studies are consistent with Gag oligomerization in the cytoplasm occurring prior to PM association in an RNA-dependent manner [127, 128]. Interestingly, FRET measurements indicated that the spacing of the lower order Gag oligomers on the RNA were consistent with the distances found in the immature Gag lattice [128]. These observations are also consistent with the hypothesis that organization of Gag on specific gRNA nucleates virion genesis and ensures gRNA incorporation.

Once the Gag-gRNA complex is formed, there remains some debate as to the mechanism of its translocation to the PM. Gag-gRNA transport has been reported to be associated with dynein motor-directed transport [129], while another study reported that the complex moves by diffusion [130]. Different conclusions might be attributable to differences in the timing of the experiments or the pathway used by the complex, as it has been shown that Gag can

generate virions via an endosome-dependent route, as well as a cytoplasmic one [131], although the relevance of the former pathway is unclear. Interestingly, mis-localization of the gRNAs used to translate Gag leads to mis-localization of the sites of virion assembly in a Gag-Psi interaction-dependent manner, suggesting a link between the location of Gag synthesis and the pathway towards assembly on specific gRNA [132].

The dynamics of HIV-1 Gag association at the PM has also been investigated. Total internal reflection fluorescence (TIRF)-microscopy experiments revealed that virion assembly was nucleated by HIV-1 Gag molecules from the cytosolic pool or those that had recently attached to the membrane [133]. Microscopy studies comparing HIV-1 with HTLV-1 Gag also demonstrated strikingly different behavior with respect to membrane binding. HIV-1 Gag required concentrations of $\sim 0.5 \mu\text{M}$ to associate with the PM, while HTLV-1 Gag readily associated with the PM at all concentrations tested ($\sim 10\text{--}1000 \text{ nM}$) [134]. It appears that different retroviral Gag proteins have different propensities, and potentially different requirements, to stably associate with the PM to initiate assembly.

The location of gRNA dimerization has also been an area of active investigation. Early TIRF-microscopy studies of HIV-1 assembly observed that gRNA arrived at the PM to initiate assembly as a dimer associated with about 10 or fewer Gag proteins [135, 136]. Super-resolution microscopy experiments also confirmed the presence of dimeric gRNA in the cytosol [137]. A subsequent TIRF-microscopy study observed HIV-1 gRNA dimerization occurring on the PM in the presence of Gag protein [138], although it was unclear from this study whether dimerization also occurred in the cytosol. Clearly further work is required to precisely define the location of gRNA dimerization and to elucidate the site (or sites) of dimerization that are relevant to the initiation of virion assembly.

5. Proposed model for specific Gag-gRNA packaging

It appears unlikely that there is a single determinant that can explain the high degree of gRNA packaging specificity, but rather there appear to be multiple layers of selection involved in this process (Fig. 3). Based on the available data, we propose the following model for specific gRNA packaging leading to the nucleation of virion formation in HIV-1. Following Gag translation in the cytoplasm, Gag likely binds to one or more tRNAs, which are abundant and available around ribosomes. At the point of the initial interaction between HIV-1 Gag and gRNA, tRNA remains bound to the MA domain but NC interacts preferentially with Psi. A key feature of the specific NC domain-gRNA interaction is its ability to resist competition from salt, other RNAs, etc. Several factors could contribute to Gag-Psi resistance to competition, such as optimal Gag-gRNA binding stoichiometry due to multiple clusters of G-rich binding sites on Psi. In addition, specific RNA binding may modulate the bent/extended Gag conformational equilibrium, shifting it to the extended form, which facilitates Gag multimerization via CA-CA interactions (Fig. 3, left) [117, 118]. A coil to helix transition in the SP1 domain may also play a role in the Gag conformational switch and multimerization. This could lead to the formation of a sufficient cluster of Gag proteins on gRNA to allow for stable association only at PI(4,5)P₂-containing membranes. This association is directed by MA-tRNA binding, which prevents association with non-PI(4,5)P₂ membranes. Once at the membrane, Gag is specifically capable of retaining gRNA

while other RNAs can be lost. The shift in Gag binding preference at the membrane from G/U-rich to G/A-rich sequences may also play a role in specific retention of A-rich gRNA and allow assembly to proceed. In contrast to specific Gag-Psi binding, the lack of specific NC-RNA interactions on non-Psi RNA leads to suboptimal Gag binding and assembly (Fig. 3, right).

While much has been accomplished leading to an improved understanding of how HIV-1 Gag specifically packages a gRNA dimer, there are many questions that remain unanswered. For example, can specific RNAs trigger conformational changes in Gag? What is the mechanism of HIV-1 Gag binding specificity changes during virion genesis? Answers to these questions will improve our understanding of this dynamic and highly-regulated process. Moreover, the processes of virion assembly and Gag-RNA binding remain untargated by any FDA-approved therapeutics, motivating further work in this area. Understanding the molecular mechanisms of Gag-gRNA packaging and viral assembly also has implications beyond retroviral biology. For example, it has recently been shown that the neuronal *arc* gene is derived from a retrotransposon, encodes a Gag-like protein that forms viral-like capsid structures around arc mRNA, and is involved in neuronal signaling via transport across neurosynaptic junctions [139, 140]. Thus, enhancing our understanding of Gag-RNA interactions could potentially have a broad impact across multiple systems.

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Abbreviations

HIV-1	human immunodeficiency virus type-1
HTLV-1	human T-cell leukemia virus type-1
HTLV-2	human T-cell leukemia virus type-2
SIV	simian immunodeficiency virus
RSV	Rous sarcoma virus
MLV	murine leukemia virus
BLV	bovine leukemia virus
HERV-K	human endogenous retrovirus K
MPMV	Mason-Pfizer monkey virus
MA	matrix
CA	capsid

NC	nucleocapsid
PR	protease
SP	spacer peptide
HBR	highly basic region
SL	stem loop
DIS	dimerization initiation site
gRNA	genomic RNA
Psi	packaging signal
5'UTR	5' untranslated region
SL2	stem-loop 2
SL3	stem-loop 3
VLP	virus-like particle
NA	nucleic acid
nt	nucleotide(s)
PM	plasma membrane
PI(4,5)P₂	phosphatidylinositol 4,5-bisphosphate
CLIP-Seq	cross linking immunoprecipitation-sequencing
FRET	Förster resonance energy transfer
TIRF	total internal reflection fluorescence
SHAPE	selective 2' hydroxyl acylation analyzed by primer extension
MD	molecular dynamics

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Fig. 1.

Retroviral Gag domain organization. The domains present in all retroviruses, matrix (MA), capsid N-terminal domain (CA-NTD), capsid C-terminal domain (CA-CTD), and nucleocapsid (NC), as well as the various spacer peptides (SP) and other peptide sequences (p6 in HIV-1, p2 and p10 in RSV, p4 in BLV, and p12 in MLV) are illustrated. RSV Gag additionally contains the enzymatic protease (PR) domain located at its C-terminus. MA is shown in dark blue, CA-NTD in dark green, CA-CTD in light green, NC in maroon, SP1 and p2 in black, and p6 and PR in grey, while SP2, p10, SP, p4, and p12 are all shown in light blue.

Fig. 2. Proposed retroviral RNA Psi (Ψ) domains. Secondary structures of the known Psi domain of MLV (Shinnick strain) (A), RSV (Prague C strain) (B), and a portion of the HIV-1 Psi domain (NL4-3 strain) (C) are shown. The numbering corresponds to the nt number in the context of the genomic RNAs. The boxes indicate sites of NC interaction that are believed to be involved in recognition of the RNA element for specific packaging. In the case of RSV, the minimal 82-nt packaging element known as $\mu\Psi$ is boxed.

Fig. 3.

Working model for specific gRNA packaging in HIV-1. This model attempts to convey the many reported mechanisms that may function together to ensure that viral particle assembly preferentially occurs on Ψ^+ RNA, while proceeding less efficiently on Ψ^- RNA. HIV-1 Gag domains colored as in Fig. 1. Left: Interaction with Ψ^+ RNA shifts the Gag conformational equilibrium to an extended conformation wherein RNA interacts with the NC domain and the MA remains bound to cellular tRNA. The role of tRNA binding in facilitating the conformational switch of Gag is unclear. Multiple NC interaction sites in close proximity on the RNA (indicated by the asterisks) lead to optimal clustering of Gag and multimerization via CA-CA interactions. The SP1 domain of Gag undergoes a coil-to-helix transition possibly upon Gag-Psi binding and/or Gag multimerization. When Gag reaches the PI(4,5) P_2 -containing PM, it retains Ψ^+ RNA, releasing tRNA to allow MA-PM binding and further assembly. In the cytoplasm, tRNA-MA interactions prevent Gag-RNA complexes from binding non-specific (PI(4,5) P_2 -lacking) membranes. Right: In the case of Ψ^- RNA interactions, Gag interacts with RNAs (cellular or spliced viral RNAs) in either a bent or extended conformation via both the NC and MA domains. tRNA-MA interactions still prevent Gag assembly on PI(4,5) P_2 -lacking membranes. Ψ^- RNA lacks a sufficient cluster of NC interaction sites to facilitate Gag multimerization. Gag- Ψ^- RNA complexes can interact with PI(4,5) P_2 -containing membranes with either the MA domain alone or with both NA binding domains. In this model, higher Gag concentrations are required for the assembly on Ψ^- RNA to be as efficient as on Ψ^+ RNA.