Involvement of the Matrix and Nucleocapsid Domains of the Bovine Leukemia Virus Gag Polypeptide Precursor in Viral RNA Packaging

Huating Wang, Kendra M. Norris, and Louis M. Mansky

Molecular, Cellular and Developmental Biology Graduate Program, Ohio State University, and Department of Molecular Virology, Immunology and Medical Genetics, Center for Retrovirus Research, Comprehensive Cancer Center, Ohio State University Medical Center, Columbus, Ohio 43210

Received 18 March 2003/Accepted 14 June 2003

The RNA packaging process for retroviruses involves a recognition event of the genome-length viral RNA by the viral Gag polypeptide precursor (PrGag), which acts to initiate the morphogenesis of virus particles (see reference 17 for review). The mechanism underlying this genome recognition event is poorly understood, but many biochemical and genetic analyses have revealed that this event involves the interaction between stable RNA secondary structures at the 5′ end of the viral genome and, in many cases, amino acids in the nucleocapsid (NC) domain of PrGag (1, 2, 4, 6, 7, 9, 10, 13, 15, 29, 30, 32, 36, 37).

The genome recognition event is an important step in the morphogenesis of infectious retrovirus particles. This recognition event leads to the predominant packaging (encapsidation) of the genome-length viral RNA into assembling particles (38). This discrimination process, which is primarily a viral RNA-protein interaction, is known to strongly favor the full-length viral RNA over spliced viral RNAs and cellular mRNAs (38). In general, the RNA sequences necessary and sufficient for the viral RNA over spliced viral RNAs and cellular mRNAs (38). Presently, there is limited information regarding PrGag-RNA interactions involved in RNA packaging for the deltaretroviruses, which include bovine leukemia virus (BLV) and human T-cell leukemia virus types 1 and 2 (HTLV-1 and -2, respectively). To address this, alanine-scanning mutagenesis of BLV PrGag was done with a virus-like particle (VLP) system. As predicted, mutagenesis of conserved basic residues as well as residues of the zinc finger domains in the BLV NC domain of PrGag revealed residues that led to a reduction in viral RNA packaging. Interestingly, when conserved basic residues in the BLV MA domain of PrGag were mutated to alanine or glycine, but not when mutated to another basic residue, reductions in viral RNA packaging were also observed. The ability of PrGag to be targeted to the cell membrane was not affected by these mutations in MA, indicating that PrGag membrane targeting was not associated with the reduction in RNA packaging. These observations indicate that these basic residues in the MA domain of PrGag influence RNA packaging, without influencing Gag membrane localization. It was further observed that (i) a MA/NC double mutant had a more severe RNA packaging defect than either mutant alone, and (ii) RNA packaging was not found to be associated with transient localization of Gag in the nucleus. In summary, this report provides the first direct evidence for the involvement of both the BLV MA and NC domains of PrGag in viral RNA packaging.

The NC proteins of all retroviruses share the characteristics of a high percentage of basic residues and zinc binding domains involved in RNA packaging and in many instances alter the specificity of RNA binding (8, 12, 14, 17, 21, 23, 33, 36, 37). It is not clear whether NC actually confers all of the selective packaging properties of viral RNA into virus particles. The NC proteins of all retroviruses share the characteristics of a high percentage of basic residues and zinc binding domains involved in RNA packaging and in many instances alter the specificity of RNA binding (8, 12, 14, 17, 21, 23, 33, 36, 37). It is not clear whether NC actually confers all of the selective recognition of the viral genomic RNA (4, 32). The p2 spacer peptide between CA and NC of human immunodeficiency virus type 1 (HIV-1) PrGag has been suggested to play a role in selective RNA packaging (20). However, previous studies...
with HIV-1 have indicated that MA does not play a role in RNA packaging (32).

Bovine leukemia virus (BLV), as well as other deltaretroviruses, replicates to low titers in animals and is poorly infectious in cell culture. Cocultivation is typically used to infect permissive host cells. Because of these difficulties, information regarding the molecular details of the virus life cycles, including virus assembly, is limited. To more easily study the RNA packaging step in the virus assembly pathway of BLV, we have used a virus-like particle (VLP) model system to identify the protein determinants of BLV RNA packaging (39). As expected, many of the basic amino acid residues and all zinc finger residues mutated in the NC domain of PrGag led to significant reductions in RNA packaging. Interestingly, many of the basic residues in MA were also found to significantly influence RNA packaging. Together, these observations provide the first evidence that both the MA and NC domains of BLV PrGag are involved in viral RNA packaging.

**MATERIALS AND METHODS**

**VLP vector and mutagenesis.** The vector used to produce VLPs, pGag-HA, has a hemagglutinin (HA) epitope tag fused to the C terminus of the BLV PrGag. The construction of this vector and the ability of the vector to produce VLPs have been described previously (39). This vector transcribes a single RNA transcript and no spliced RNA. The RNA is translated to express PrGag in the absence of the viral protease. Therefore, the VLPs do not have mature cores. VLPs have been described previously (39). This vector transcribes a single RNA transcript from the gag gene. All in vitro transcripts were DNase I treated and tested for DNA contamination before RNA packaging. A series of NC mutants were made in pGag-HA and Transfected cells were grown on coverslips, fixed with 4% paraformaldehyde, and permeabilized with Triton X-100, both diluted in phosphate-buffered saline. The cells were then incubated with anti-HA antibody followed by incubation with Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes). Images were collected with a Bio-Rad MRC 600 confocal microscope.

**RESULTS**

**Effects of mutations in the NC domain of PrGag on RNA packaging.** In order to determine the influence of NC on RNA packaging, a series of NC mutants were made in pGag-HA and tested for their ability to influence RNA packaging. Alanine residues were introduced in place of basic amino acid residues and in place of the conserved residues in each of the two zinc finger binding domains (Fig. 1A). The panel of NC mutants was analyzed in parallel with the parental vector. Figure IB shows the RNA packaging efficiencies of the mutants. There were three groups of mutant phenotypes. The first group of mutants had no RNA packaging defects compared to the parental vector. These mutants included K17A and R18A. A second group of mutants had mild RNA packaging defects (40 to 70% reduction in VLP production) (i.e., R12A, K17A/ R18A, K59A, K66A, and K68A). The third group of mutants had severe RNA packaging defects compared to the parental vector (85 to 95% reduction) (i.e., C24A, C27A, R35A, C49A, H57A, and R60A). For many of the mutants, VLP production was reduced two- to threefold that of the parental vector (i.e., R12A, K17A, R18A, K17A/R18A, R35A, and C49A). This reduction is within the linear detection range for the immuno blot assay used (described below). As predicted, many of the basic amino acid residues that were mutated led to significant reductions in RNA packaging efficiencies. The mutated basic residues that resulted in the greatest reductions were R35A and R60A. Interestingly, each of these residues lies between

Detection of the RNA transcript that expresses PrGag in cells and its incorporation into VLPs due to the RNA packaging signal was done by quantitative real-time RT-PCR analysis. Quantitative real-time RT-PCR was carried out with the Qiagen RT-PCR Master SYBR Green kit in a 20-μl reaction volume. RNA templates were reverse transcribed at 50°C for 25 min followed by a denaturing step at 95°C for 15 min. PCR was then performed with the primers +BLV979 (5′-AACGGCATGTTGCTTGGCCA-3′) and –BLV1183 (5′-CGTTCAGCGGGCGGATTGTC-3′). These primers amplify an approximately 200-bp-long fragment within the gag gene.

The PCR protocol consisted of 50 cycles of denaturation (95°C for 1 s), annealing (60°C for 20 s), and extension (72°C for 10 s). For each cycle, the temperature transition rate was 20°C/ s. The PCR was monitored with a Light Cycler (Roche Diagnostics). Standard RNA was synthesized by in vitro transcription of a linearized plasmid containing a BLV proviral DNA representing the 5′ end of the gag gene. All in vitro transcripts were DNase I treated and tested for DNA contamination.

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the conserved histidine and cysteine residues in the first (i.e., R35A) and second (i.e., R60A) zinc finger domains.

Effects of mutations in the MA domain of PrGag on RNA packaging. Previous studies with other retroviruses have provided little support for a role of the MA domain in retroviral RNA packaging (32). To test whether basic residues in the BLV MA domain influence RNA packaging, a panel of mutants that changed basic residues in MA to alanine was created (Fig. 2A). The RNA packaging efficiencies of the mutants were then analyzed in parallel with the parental vector. Figure 2B shows a representative protein analysis of Gag from cells and VLPs. The K52A and K53A MA mutants led to the lowest levels of VLPs (8 and 17%, respectively). These reductions are within 13-fold of the level of the parental vector and are within the linear range of detection for the assay, which was at least 16-fold (Fig. 2C). Figure 2D shows the resulting RNA packaging efficiencies for the panel of MA mutants tested. As with the NC mutants, there were three general groups of mutant phenotypes. Several basic residues that are not conserved among BLV, human T-cell leukemia virus type 1 (HTLV-1), and HTLV-2 led to no or small changes in RNA packaging efficiencies (i.e., R31A, H48A, K53A, R69A, and R72A). A second group led to modest reductions in RNA packaging efficiencies (i.e., R27A and K52A). Finally, a third group had significant reductions in RNA packaging (66 to 92%) compared to that of the parental vector (K41A and H45A). The H45A MA mutant had a reduction in RNA packaging efficiency (92%) that was as great as any of those in the individual NC mutants, indicating that BLV MA plays an important role in RNA packaging. The K49 residue was not analyzed in this study.

In other retroviruses, such as HIV-1, basic amino acid residues in MA can influence PrGag membrane targeting and are proposed to form a positively charged patch that promotes membrane binding (16, 27, 40, 41). To determine whether MA mutants that significantly reduced RNA packaging also influenced PrGag membrane localization, the cellular distribution of Gag was analyzed by confocal microscopy. Figure 2E shows the effects of several basic amino acid mutants on Gag distribution in cells. These mutants (i.e., R27A, K41A, H45A, K52A, and K53A) had a cellular distribution of Gag that was comparable to that observed with the wild type. Specifically, Gag expression yielded a stippled appearance along the plasma membrane.
FIG. 2. Mutagenesis of basic residues in the BLV MA domain of PrGag results in RNA packaging defects. (A) The BLV MA domain of PrGag. Amino acid residues in boldface indicate basic residues, and boldface residues that are underlined indicate basic and/or charged residues conserved among BLV, HTLV-1, and HTLV-2. The α-helices in MA are indicated, based upon nuclear magnetic resonance solution structure analysis of BLV MA (28). (B) Quantitation of VLP production. Preparation of lysates from VLP-producing cells and VLPs and immunoblot analysis are described in Materials and Methods. Representative results from the immunoblot analysis are shown. WT, wild type. (C) Linear range of detection for immunoblot analysis. Protein was diluted (1:1, 1:2, 1:4, 1:8, 1:5, 1:10, and 1:16) and was subjected to immunoblot analysis. The band intensity (arbitrary units) for each dilution was determined with the Quantity One software package of the Chemi Doc 2000 Documentation System (Bio-Rad) and plotted against the amount of protein used for immunoblot analysis (16, 8, 4, 3.2, 2, 1.6, or 1) to determine if protein detection within the dilution range was linear. (D) Relative RNA packaging efficiencies of BLV MA mutants. RNA packaging efficiencies were determined by the ratio of viral RNA to the amount of Gag from VLPs (see Materials and Methods for details). The RNA packaging efficiency with wild-type BLV MA was set at 100, and the values for the MA mutants (± standard deviation) are relative to that for the wild type. Mutants were tested in parallel with the parental construct, and each experiment was done in triplicate. (E) Cellular distribution of PrGag for selected MA mutants. Cells stably transfected with VLP constructs containing mutations in MA were grown on coverslips, fixed, and incubated with an anti-HA Ig followed by incubation with Alexa Fluor 488-conjugated antimouse Ig. Images were obtained with a confocal microscope.
membrane for the parental Gag and for these mutants (Fig. 2E).

Importance of charged amino acid residues in MA on RNA packaging. The MA residues R27 and H45, when mutated, revealed significant reductions in RNA packaging efficiencies. To further analyze the importance of these positively charged amino acid residues on RNA packaging, mutations were made at either R27 or H45 to create either a conservative (i.e., R27H or H45R) or nonconservative (i.e., R27G or H45G) change at these residues. Figure 3A shows the effects of these amino acid substitutions on RNA packaging. R27G had a phenotype comparable to that of R27A, while R27H had a phenotype that was comparable to that of the parental vector. Furthermore, H45G had an RNA packaging defect similar to that of H45A, while H45R had a phenotype equivalent to that of the parental vector. These data indicate that there is a correlation between the absence of a positively charged amino acid at positions 27 or 45 and a defect in RNA packaging. The cellular distribution of Gag was not influenced by these mutations, indicating there was no correlation between the RNA packaging defects and Gag membrane localization (Fig. 3B).

Combined effect of MA and NC mutations on RNA packaging. To test for the potential interplay between MA and NC on RNA packaging, a mutant construct was created in which both MA and NC mutations were introduced into the same vector and then assayed for the ability to package RNA. In particular, the MA/NC double mutant MA H45A/NC C24A was tested. The combined mutant had a 10-fold reduction in VLP production and a severe defect in RNA packaging (98% reduction compared to that of the parental strain) that was significantly lower than the RNA packaging efficiency of either mutant alone (Fig. 4). This indicates that the combination of mutations together further reduced the efficiency of RNA packaging.

Lack of transient nuclear localization of Gag. A recent study with Rous sarcoma virus (RSV) revealed that RSV Gag protein enters the nucleus by a nuclear-targeting sequence in the MA domain and is subsequently transported to the cytoplasm by using a CRM1-mediated nuclear export pathway (35). Transient expression of a dominant-negative CRM1 or by treating cells with leptomycin B (LMB; a drug that attaches to the central domain of CRM1 to disrupt its interaction with nuclear export signals) resulted in the redistribution of Gag from the cytoplasm to the nucleus. The MA mutant, Myr1E, was insensitive to the effects of LMB treatment, apparently because it bypassed the nuclear compartment during virus assembly (35). Myr1E has a defect in RNA packaging, which implies that RSV nuclear localization of Gag might be involved in viral RNA-Gag interactions. Since we discovered BLV MA mutants with RNA packaging defects like Myr1E, we hypothesized that the viral RNA-Gag interactions involved in BLV RNA packaging might occur in the nucleus after transient nuclear localization of Gag. To test whether BLV Gag is localized in the nucleus, 293T cells transiently transfected with the BLV VLP construct PR+/H11001 (39) were treated with either LMB or transiently cotransfected with a dominant-negative CRM1 (ΔCAN). In addition, fetal lamb kidney cells chronically infected with BLV (FLK-BLV) were either treated with LMB or transiently transfected with ΔCAN.

FIG. 3. Requirement of charged amino acid residues in BLV MA for RNA packaging. RNA packaging efficiencies were determined by the ratio of viral RNA to the amount of Gag from VLPs (see Materials and Methods for details). The RNA packaging efficiency with wild-type (WT) BLV MA was set at 100, and the values for the MA mutants (± standard deviation) are relative to that for the wild type. (A) Mutants were tested in parallel with the parental construct, and each experiment was done in triplicate. (B) Cellular distribution of PrGag. Cells stably transfected with VLP constructs were grown on coverslips, fixed, and incubated with antibodies prior to confocal microscopy.

FIG. 4. Combined influence of NC and MA mutations on RNA packaging. RNA packaging efficiencies were determined by the ratio of viral RNA to the amount of Gag from VLPs (see Materials and Methods for details). The RNA packaging efficiency with wild-type BLV was set at 100, and the values for the mutants (± standard deviation) are relative to that for the wild type (WT). Mutants were tested in parallel with the parental construct, and these experiments were done in triplicate.

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When 293T cells were either cotransfected with an RSV Gag-green fluorescent protein (GFP) construct and ΔCAN or transfected with Gag-GFP and then treated with LMB, there was a distinct nuclear localization of Gag-GFP, comparable to that originally reported by Scheifele et al. (35). When 293T cells were either cotransfected with the BLV vector and ΔCAN or treated with LMB after transfection with the BLV vector, no BLV Gag was observed in the nucleus of cells (Fig. 5). Comparable results were obtained with LMB treatment or transfection of ΔCAN into FLK-BLV cells. One interpretation of these data is that BLV Gag does not enter the nucleus, implying that the genome recognition event occurs in the cytoplasm. However, it is possible that BLV Gag does enter the nucleus and is exported by a CRM1-independent pathway.

**DISCUSSION**

This is the first report to provide direct genetic evidence that both the MA and NC domains of BLV PrGag are protein determinants of RNA packaging. The unique observation in this study is that the basic residues in MA that influenced RNA packaging had no effect on Gag membrane localization. This is in contrast to studies with HIV-1 that indicate a role for the basic amino residues of MA in membrane localization (by the formation of a charged patch that is thought to promote membrane binding) (11, 16, 27, 40, 41). However, our data support those from a previous study of the basic residues in the HTLV-1 MA, which found that HTLV-1 MA basic residue mutants led to normal transport of Gag to the membrane as well as cleavage of PrGag (22). Many of these HTLV-1 mutants had reduced infectivity. Our study found that several of the BLV MA basic residue mutants affected RNA packaging and did not influence Gag membrane targeting (although they did lead to reductions in virus particle production), suggesting that the reduced infectivity observed with the HTLV-1 mutants may be (in some cases) related to an RNA packaging defect.

The locations of the basic residues that led to defects in RNA packaging help to provide some mechanistic insights into potential viral RNA-protein interactions involved in genome recognition. Among the NC mutants, the residues that lie within the first and second zinc finger domains, including R35 and R60, had a significant impact on RNA packaging. Similar residues in the HIV-1 NC zinc finger have been implicated in interactions with SL3 RNA and have been shown to be important for RNA packaging by mutagenesis studies (9). The basic residues in the BLV MA that caused the greatest defects in
RNA packaging (i.e., K41 and H45) are located in α-helix B (28). Presently, no structural data are available regarding the potential viral RNA-MA protein interactions. However, it has been previously reported that BLV MA specifically interacts with the viral RNA in a region (18, 19) that spans the locations of SL1 and SL2 (26). This suggests that the contacts made by BLV MA with the viral RNA may also involve an interaction with SL1 and/or SL2. Interestingly, the HIV-1 MA protein has been found to bind to the viral RNA in the pol gene, via the basic amino acids in MA, and virus mutants that disrupted this RNA-protein interaction had delayed replication kinetics, although no role in RNA packaging was reported (34). The BLV MA mutants in this study that influenced RNA packaging do not overlap with the known RNA sequence of gag known to be involved in RNA packaging (25, 26). This indicates that the mutations introduced into the VLP vector influence RNA packaging by the amino acid change in MA and not the nucleotide changes in the RNA. This conclusion is supported by the data in Fig. 3, which show the importance of charged amino acid residues (and not just nucleotide changes) in RNA packaging.

The magnitude of the defects in RNA packaging described here in this study for the most extreme MA and NC mutants (i.e., a MA-NC double mutant) are in the range of those observed previously when SL1 or SL2 was mutated (24, 26). Taken together, these observations suggest that residues in both MA and NC make direct contacts with the packaging signal or perhaps adjacent sequences that would influence genome recognition. The interaction of NC with RNA sequences outside of those RNA secondary structures of the RNA packaging signal has been shown directly or implied with murine leukemia virus and spleen necrosis virus and is believed to play an important role in RNA packaging (3, 10).

The data in this report provide evidence for a role of both the MA and NC domains of Gag in BLV RNA packaging. This observation implies that distinct viral RNA-protein interactions occur in BLV genome recognition and RNA packaging to that of other retroviruses. Further studies will determine the precise RNA-protein interactions involved in BLV RNA packaging, the location in the cell at which genome recognition occurs, and whether both MA and NC play a role in RNA packaging of other deltaretroviruses.

ACKNOWLEDGMENTS
We thank M. Knowlton for technical assistance; K. Wolken for assistance with confocal microscopy; J. Wills, L. Parent, and T. Hope for sharing reagents; and M. Summers for helpful discussions.

This work was supported by the American Cancer Society (RPG 0027801).

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