Retroviral proteases (PRs) cleave the viral polyprotein precursors into functional mature proteins late during particle release and are essential for viral replication. Unlike most retroviruses, β-retroviruses, including Mason-Pfizer monkey virus (M-PMV), assemble immature capsids within the cytoplasm of the cell. The activation of β-retroviral proteases must be highly regulated, because processing of the Gag-related polyprotein precursors occurs only after transport of immature capsids to the plasma membrane and budding. Several β-retroviral proteases have unique C-terminal extension sequences, containing a glycine-rich motif (G-patch), which specifically binds in vitro to single-stranded nucleic acids. In M-PMV PR, the G-patch is removed in vitro as well as in vivo by autoproteolytic processing to yield truncated active forms of PR. To investigate the role of the G-patch domain on the virus life cycle, we introduced mutations within the C-terminal domain of protease. We found that the G-patch domain of M-PMV PR is not required for the processing of viral polyproteins, but it significantly influences the infectivity of M-PMV, the activity of reverse transcriptase, and assembly of immature capsid within the cells. These results demonstrate for the first time that the G-patch domain of M-PMV PR is critical for the life cycle of β-retroviruses, and its evolutionary conservation within members of this genus suggests its importance for retroviruses that display D-type morphology.

The maturation process of retroviruses is mediated by a virus-encoded protease (PR) that functions to cleave the viral polyproteins into the mature structural proteins and enzymes found in infectious particles. The retroviral protease is translated as a domain of the Gag-related polyproteins. For most retroviruses, Gag and Gag-Pol polyproteins assemble into an immature particle at the plasma membrane during viral budding, in a process referred to as “C-type” viral assembly. Gag polyproteins preassembly in the cytoplasm of the host cell, away from the plasma membrane, is termed “B/D-type” assembly and is unique to the β-retroviruses (B- and D-type retroviruses), and the spumaviruses.

The RNA Binding G-patch Domain in Retroviral Protease Is Important for Infectivity and D-type Morphogenesis of Mason-Pfizer Monkey Virus*

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A bioinformatic analysis of proteins containing RNA binding sequences revealed a conserved glycine-rich module, termed the G-patch, in M-PMV gag-polypeptides within the C-terminal sequence of protease (11). Recently, the C-terminal domains in M-PMV protease and the protease of mouse intracisternal A-type particles endogenous retrovirus have been demonstrated to specifically bind in vitro to single-stranded DNA and RNA oligonucleotides (9). A similar domain was identified in over 100 eukaryotic proteins many of which are involved in RNA processing (11).

Here, we report an analysis of the influence of the C-terminal domain of the M-PMV protease on the life cycle of the virus. We show that although this C-terminal G-patch-containing sequence is not necessary for virus maturation, it is important for its infectivity and an activity of the reverse transcriptase. Surprisingly, this domain affects the morphogenesis of viral particles.

**MATERIALS AND METHODS**

**Plasmid Construction and Mutagenesis**—Mutations of single amino acids were introduced using the QuickChange site-directed mutagenesis kit (Stratagene) into the previously described bacterial phagemid pBP (12) harboring DNA sequences encoding a 28-kDa N-terminally extended precursor of protease from M-PMV. The primer sequences were as follows: A1114R, 5′-GCCCCAAATGACATAATCTGTCATATTGTTAGCCCC-3′ and A1114R-rev, 5′-GGGCTAACAATTGACAGTAGTTATGCAATTTGAGGCCC-3′ to generate vector pBP-P1114R; Q115I-rev, 5′-CATAGATCTGCTATTATGTTAGCCCC-3′ and Q115I-for, 5′-TGGGCTAACAATATGACAGTATATGACG-3′ to generate vector pBP-Q115I; N109I-for, 5′-GGATGTGTAGCCCCATTGACATGACAGTATATGACG-3′ and N109I-rev, 5′-GAATGTGTAGCCCCATTGACATGACAGTATATGACG-3′ to generate vector pBP-N109I; A114R-for, 5′-GATGTGTAGCCCCATTGACATGACAGTATATGACG-3′ and A114R-rev, 5′-GAATGTGTAGCCCCATTGACATGACAGTATATGACG-3′ to generate vector pBP-A114R; and SarmXba-rev, 5′-GTAATCCTGAGGGCTGGAGCCCTGGGCGT-3′ to generate vector pBP-SarmXba. All new plasmids were identified by restriction analysis, sequencing confirmation, and checking colony hybridization.

**Bacterial Expression and Purification of M-PMV Protease**—Expression of PR and its mutants in Escherichia coli BL21 (DE3) from pBP phagemids were carried out in LB (Luria-Bertani) medium containing ampicillin (at a final concentration of 100 μg/ml). The cells were harvested 2 h postinduction by centrifugation. The PR and all of its mutants were isolated from the inclusion bodies by solubilization in 50 mM Tris-HCl buffer, pH 7.5, with 8 M urea, followed by dialysis against 50 mM Tris-HCl buffer, pH 7.5, with 0.1 M mercaptoethanol and by batch chromatography on QAE-Sephadex A25 under the same conditions (6).

**Autocatalytic Cleavage of Protease in Vitro**—The protease at a concentration of 0.2 mg/ml was dialyzed into 50 mM acetate buffer, pH 5.3, although containing 0.3 M NaCl, 0.1% mercaptoethanol and then incubated at 37 °C for different time (0–10 days). The aliquots were then analyzed by SDS electrophoresis in 15% polyacrylamide gel.

**Cell Cultures and Transformation**—COS-1 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Transformation of the DNA constructs into COS-1 cells was performed by using the FuGENE 6 reagent according to the instruction manual.

**Radiolabeling and Immunoprecipitation**—At 20 h posttransfection, cells were starved for 30 min in Cys/Met-free Dulbecco’s modified Eagle’s medium and then labeled for 30 min in Cys/Met-free Dulbecco’s modified Eagle’s medium containing 35S-labeled methionine-cysteine (100 μCi/ml) (ICN). Pulse-labeled cells were lysed in lysis buffer A (0.025 M Tris-HCl, 1% Triton X-100, 1% sodium deoxycholate, 0.05 M NaCl, pH 8). For the chase, the cells were incubated in complete Dulbecco’s modified Eagle’s medium for an additional 4 h and then lysed in lysis buffer A. Nuclei were then pelleted from both the pulse and chase cell lysates at 13,000 rpm in a microcentrifuge. After addition of SDS to the final concentration of 0.1% the viral proteins were immunoprecipitated with goat anti-M-PMV antiserum or with rabbit anti-CA antiserum. The chase-plate culture medium containing radiolabeled viral particles was filtered through a 0.45-μm pore size filter. It was then
G-patch Domain of M-PMV Protease

FIGURE 2. Sequence alignment of proteases of M-PMV, human immunodeficiency virus, type-1 (HIV-1), and mouse mammary tumor virus (M-MTV). Arrows indicate the sites of autocatalytic cleavage in M-PMV protease. Amino acids influencing the autocatalytic processing of M-PMV PR are shown on a black background. Conservative Gly residues of the G-patch domain of M-PMV protease are shown on a light gray background, aromatic residue essential for binding nucleic acid on a dark gray background; conservative hydrophobic residues are marked by * and conservative small residues by °.

RESULTS

Mutations within the C-terminal Domain of M-PMV Protease Do Not Affect Specificity of Protease and Rate of Virion-associated Proteolysis—Previous studies showed that proteolytic processing of the 17-kDa form of M-PMV PR into the truncated form both in vitro and in vivo is a time-dependent process. The freshly released mature particles contain only 17PR and the truncated 13PR appears in virions within 2 h after their release (6). In vitro experiments confirmed that the first autocatalytic processing of 17PR occurs at the C terminus in position Ala114, Gln115, and it is followed by cleavage in position Ser107-Pro108 yielding the 13 and 12 kDa forms of M-PMV PR, respectively (see Fig. 2). To determine an influence of the G-patch and the rate of its processing on the virus life cycle, we introduced several mutations into the C-terminal domain of PR (see Fig. 2). Asn109, which forms the P2’ position of the 12PR/13PR processing site, was mutated into Ile, Asp, Lys, Ser, Glu, Thr, Met, and Val. Furthermore, Ala114 in the P1 position of the 13PR/17PR processing site was replaced by Arg. Gln115 and it is followed by cleavage in position Ser107-Pro108 yielding the 13 and 12 kDa forms of M-PMV PR, respectively (see Fig. 2). To determine an influence of the G-patch and the rate of its processing on the virus life cycle, we introduced several mutations into the C-terminal domain of PR (see Fig. 2). Asn109, which forms the P2’ position of the 12PR/13PR processing site, was mutated into Ile, Asp, Lys, Ser, Glu, Thr, Met, and Val. Furthermore, Ala114 in the P1 position of the 13PR/17PR processing site was replaced by Arg. Gln115 and it is followed by cleavage in position Ser107-Pro108 yielding the 13 and 12 kDa forms of M-PMV PR, respectively (see Fig. 2).
contained only the shortest form of PR. In contrast, Ile introduced into the P2' position of the 12/13 cleavage site decreased the autoprocessing of 13PR into 12PR (data not shown). To obtain a mutant of PR that yielded a more stable 13PR, the double mutations (N109I and Q115I) were cloned into the protease processing sites. Mutations A114R, Q115I, and N109I/Q115I influencing the C-terminal autoprocessing of M-PMV PR were then cloned into the infectious proviral clone pSARM4. In addition, proviral clones containing truncated PR mutants (PR/H11032118-145) and (PR/H11032111-145) were constructed. Finally, a mutation Y121S in the PR, which was shown to significantly decrease binding of nucleic acids to the G-patch in vitro (9), was introduced in pSARM4.

The expression of M-PMV-specific proteins was analyzed 20 h after transfection of the respective plasmids into COS-1 cells. Both cell-associated and released virion-associated proteins were analyzed by immunoprecipitation with rabbit anti-M-PMV antiserum (Fig. 4). Similar levels of Gag, Gag-Pro, and Gag-Pro-Pol precursor proteins were synthesized in cells transfected with truncated mutants as well as with wild-type genomes (Fig. 4a), confirming that deletion of sequences encoding a substantial part of the C-terminal domain of protease did not disturb the ribosomal frameshifts. Following virus release, Gag precursors were processed by wt PR as well as by the protease mutants (Fig. 4b).

The truncation of the most C-terminal domain (mutants PRΔ118-145 and PRΔ111-145) did not affect the proteolytic processing of protein precursors in released viral particles. Similar processing of Gag polyproteins and a comparable amount of the capsid protein in the released virions was also detected for the G-patch mutant, Y121S. Viral particles of constructs Q115I and N109I/Q115I contained, besides the mature proteins, a low level of unprocessed Gag polyprotein even after a 24 h incubation of virions at 37 °C (Fig. 4b). In some cases we also observed the other viral precursors, Gag-Pro (Pr95) and Gag-Pro-Pol (Pr180) in these particles (data not shown). The presence of unprocessed polyproteins suggests that the cleavage of Gag-polyproteins by these PR mutants was impaired compared with the wild type. In contrast, the in vitro autoproteolytic cleavage of the isolated 17-kDa protease containing Q115I and N109I/Q115I mutations was significantly faster than the wild type, confirming that these mutations affect in vivo substrate specificity of PR and not the activity per se.

Mutations within C Terminus of Protease Decrease Virus Infectivity—To analyze the effect of the mutations introduced into the C terminus of the protease region on the virus infectivity, virus particles released from the COS-1 cells transiently transfected by mutant and wild-type viral constructs were normalized to the content of the capsid protein, and equivalent amounts of viral particles were used for infection of the fresh COS-1 cells. The level of CA protein released into the medium was determined by Western blot analysis using polyclonal rabbit anti-CA antiserum (Fig. 5). Mutant A114R retained ~70% of the infectivity of wt virus (Fig. 5), but a significant decrease in infectivity was observed for mutants displaying accelerated C-terminal autoprocessing (Q115I, N109I/Q115I) as well as for the C-terminally truncated protease mutants (PRΔ111-145, PRΔ118-145). Importantly, the G-patch mutant, Y121S, with defective nucleic acid binding retained only ~20% infectivity of the wt virus, confirming that this region plays an important role in virus infectivity.
G-patch Domain of M-PMV Protease

The C-terminal Domain of Protease Is Important for the Activity of Reverse Transcriptase—As the decrease of virus infectivity caused by deletion or substitution mutations within the C-terminal protease domain was not because of impaired protease activity or virion maturation, we analyzed the virion-associated RT activity of all protease mutants described here (Fig. 6). Mutant and wild-type viral particles were isolated from media of transiently transfected COS-1 cells, and the rate of incorporation of $[^{35}]$S)dATP in an RT reaction was measured. As expected, replacement A114R had no significant influence on the RT activity, which is in agreement with the results obtained from the infectivity assay. However, for constructs Q115I, N109I/Q115I, and Y121S, RT activity was reduced by 50% and deletion the G-patch region (PRΔ111-145, PRΔ118-145) decreased RT activity by 70% in comparison with wt. These results demonstrate that the G-patch domain of PR is necessary for the activity of M-PMV reverse transcriptase. Both the virus infectivity and the activity of reverse transcriptase of all the constructs presented in Figs. 5 and 6 are average values of three independent experiments.

Mutations within the C-terminal Domain of PR Change the Type of Assembly—To analyze the effect of mutations within the C terminus of protease on the morphogenesis and morphology of the viral particles, ultrathin sections were prepared and analyzed by transmission electron microscopy (Fig. 7). The wt M-PMV particles assembled within the cytoplasm and were subsequently transported to the plasma membrane, representative of D-type morphogenesis as described by Chopra and Mason (15). Unexpectedly, all constructs with a mutated C-terminal domain were not because of impaired protease activity or virion maturation, we analyzed the virion-associated RT activity of all protease mutants described here (Fig. 6). Mutant and wild-type viral particles were isolated from media of transiently transfected COS-1 cells, and the rate of incorporation of $[^{35}]$S)dATP in an RT reaction was measured. As expected, replacement A114R had no significant influence on the RT activity, which is in agreement with the results obtained from the infectivity assay. However, for constructs Q115I, N109I/Q115I, and Y121S, RT activity was reduced by 50% and deletion the G-patch region (PRΔ111-145, PRΔ118-145) decreased RT activity by 70% in comparison with wt. These results demonstrate that the G-patch domain of PR is necessary for the activity of M-PMV reverse transcriptase. Both the virus infectivity and the activity of reverse transcriptase of all the constructs presented in Figs. 5 and 6 are average values of three independent experiments.

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**DISCUSSION**

Proteases from several endogenous retroviruses and β-retroviruses (MIA-14, HERV K10, rabbit endogenous retrovirus H, M-PMV) undergo autoprocessing from the C terminus both in vitro (16–19) and in vivo (6) yielding proteolytically active proteases shorter by ~4 kDa. However, the significance of the C-terminally extended domain of PR and its processing for their life cycle has not been elucidated. The first active form of the M-PMV protease is released as a dimer of two 152 amino acid subunits (17PR), which undergoes further autoprocessing from its C terminus yielding proteolytically active homodimers that contain 114 (13PR) or 107 (12PR) amino acid monomers (6). Importantly, the RNA-binding module, whose function is in most eukaryotic proteins associated with RNA processing and also with specific protein-protein interactions (11, 20), is present at the C terminus (amino acid residues 108–152) of M-PMV 17PR as well as in closely related simian retroviruses I and II (SRV) and in squirrel monkey retrovirus (SMRV) PRs. The G-patch is a region of ~45 amino acids, which contains six highly conservative glycines and a number of hydrophobic and basic residues (see Fig. 2). The position following the first Gly (Gly120 in M-PMV PR) is occupied almost invariably by an aromatic residue (Tyr121 in M-PMV PR) that has been shown to be critical for interaction with nucleic acid bases (21). Recently, we demonstrated that mutation Y121S in M-PMV PR prevented binding of single-stranded DNA and RNA oligonucleotides to the protease (9). Interestingly, a phylogenetic analysis showed that the G-patch is present in most endogenous retroviruses of class II that are clustered with M-PMV PR and/or its time-dependent
release might regulate the proteolytic activity of PR and might prevent the premature processing of the Gag-polyproteins within the immature particle. However, the experiments with mutated M-PMV proviral genomes presented here demonstrate that truncation of M-PMV PR does not affect the maturation of viral particles and that the C-terminal domain of PR is not necessary for proteolysis. Even the activity of the PR mutant Δ111-145, corresponding to the shortest variant, 12 PR, which has been never detected in released particles (6), was sufficient for maturation of M-PMV when subcloned into the proviral genome. Unexpectedly, virion-associated proteins of Q115I and N109I/Q115I mutants contained a low level of unprocessed Gag-polyproteins (Fig. 4b), and a fraction of the released particles was immature as documented by electron micrographs (Fig. 7), even though the in vitro autoprocessing of proteases containing these mutations was accelerated. We can hypothesize that the mutated 13/17PR processing site in PR is preferentially cleaved within the embedded polyprotein and yields a conformationally altered Gag-product with a sterically less accessible N-terminal cleavage site of the protease domain. Because the initial proteolytic cleavage within the Gag-Pro precursor is primarily directed at the N terminus of the protease, release of the protease could be altered in the Q115I and N109I/Q115I mutants and processing of the Gag-polyproteins might be decreased. The results presented here clearly confirm that the G-patch domain does not affect maturation of released viral particles.

Importantly, we demonstrate that deletion of the C-terminal domain in PRΔ118-145 and PRΔ111-145 mutants, acceleration of the C-terminal processing of PR in Q115I, N109I, and Q115I mutants, and/or mutation of the RNA binding sequence (Y121S mutant) decreased the activity of RT in released virions by 50–70% and the infectivity of the virus by 80–95%. The M-PMV reverse transcriptase domain is expressed by the −1 ribosomal frameshift at the 3′-end of the protease reading frame.

FIGURE 7. Thin section electron microscopy of COS-1 cells transfected with individual proviral genomes. Cells producing wt or protease mutants 20 h were fixed in 2.5% glutaraldehyde and 2% osmium tetroxide. Scale bars, 200 nm. Immature particles assembled with D-type morphogenesis (within the cytoplasm of the host cells) are indicated by black arrows. White arrows indicate particles assembled with C-type morphogenesis (at the plasma membrane). Electron micrographs of released particles are shown in the last column.

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G-patch Domain of M-PMV Protease

The frequency of particles assembled with the C-type morphogenesis in COS-1 cells transfected with wt M-PMV and protease mutants

| Construct        | Frequency of C-type morphogenesis [%] |
|------------------|---------------------------------------|
| wt M-PMV         | <1                                    |
| A114R            | 20                                    |
| PRA111-145       | 35                                    |
| PRA118-145       | 46                                    |
| Q115I            | 44                                    |
| N109I/Q115I      | 43                                    |
| Y121S            | 60                                    |

A total of 300 – 400 viral capsid structures for each mutant were analyzed by transmission electron microscopy to yield the percentage for C- and D-type morphogenesis.

Insertion of M-PMV CTRS into the same region of murine leukemia virus (MoMuLV) is the only virus from the -retroviruses, allowing the polyproteins to be incorporated into stable, pelletable particles within the cytoplasm. The G-patch of the protease, which contains the G-patch Domain of M-PMV Protease

within the Gag-Pro-Pol polyprotein. In this long precursor the protease region is followed by the reverse transcriptase sequence. It is likely that the proteolytic digestion of this polyprotein yields the reverse transcriptase covalently linked at its N terminus with the G-patch sequence of PR, since Entin-Meir et al. (23) reported that mature mouse mammary tumor virus RT contained C-terminal flanking residues of the protease within virions. In vitro experiments with this extended RT confirmed that this transframe form of RT is active. However, mouse mammary tumor virus is the only virus from the -retroviruses that does not contain the C-terminal extension and the G-patch at the C terminus of the protease domain (see Fig. 2).

A surprising observation of this study was that the G-patch of the protease influences the type of virus assembly. Electron micrographs of all the mutants studied here show that morphogenesis is shifted, to different degrees, from the D-type to the C-type morphogenetic pathway. M-PMV as the prototypical representative of D-type retroviruses assembles very few particles (<1%) via the C-type pathway (24). In contrast, ~40% of PRA111-145, PRA118-145, Q115I, and N109I/Q115I mutant particles assembled at the plasma membrane and as many as 60% of Y121S particles displayed C-type morphology. M-PMV Gag precursors together with genomic RNA assemble into capsids at a pericentriolar region of the cytoplasm in a microtubule–dependent process through interactions between a short peptide signal, known as the cytoplasmic targeting-retention signal, and the dynein/dynactin motor complex (2, 25). The C- and D-type morphogenetic pathways appear distinct; however, a point mutation of Arg55 within the CTRS signal in the matrix domain, abrogates the pericentriolar targeting of Gag-polyproteins, and this mutant follows the C-type assembly pathway (3).

Insertion of M-PMV CTRS into the same region of murine leukemia virus (MoMuLV), which exhibits C-type morphogenesis, results in intracytoplasmic assembly of the Gag-polyproteins. However, only 20–30% of MoMuLV Gag-polyproteins containing M-PMV CTRS were incorporated into stable, pelletable particles within the cytoplasm (4), suggesting that efficient assembly of M-PMV immature capsids in the cytoplasm requires other domains that are present in the Gag-related polyproteins. The G-patch of the protease, which contains the basic as well as the hydrophobic amino acids might represent a signal that promotes the efficient intracytoplasmic assembly of immature capsids by both the binding of genomic RNA during the assembly process and/or by the protein-protein interaction with cellular proteins involved in intracellular trafficking. The influence of the G-patch on the ratio of incorporated Gag-Pro and Gag-Pro-Pol polyproteins into the immature particles might be also considered.

In summary, the observations presented here describe the so far uncharacterized function of the RNA binding domain in -retroviruses. We showed that this domain located within the C terminus of the protease does not affect the maturation; however, more surprisingly it influences the morphogenesis and the virus infectivity. The mutations that impair the function of the G-patch, which is evolutionarily conserved among endogenous retroviruses and -retroviruses, allow the polyprotein precursors to escape from the intracytoplasmic site of assembly and redirect them to the cytoplasmic membrane.

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J. Biol. Chem. 2005, 280:42106-42112.
doi: 10.1074/jbc.M508031200 originally published online October 27, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M508031200

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