Current Biology *16*, 1565–1570, August 8, 2006 ©2006 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2006.06.055

Report

Murine Retrovirus Escapes from Murine APOBEC3 via Two Distinct Novel Mechanisms

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Summary

APOBEC3G (A3G) is an antiretroviral host factor that functions by deaminating dC to dU in retroviral cDNA [1–5]. HIV-1 Vif protein counteracts A3G via a ubiquitin-proteasome pathway [6–12]. In the case of a simple retrovirus such as the murine leukemia virus (MLV), it remains unclear why it can replicate in cells expressing APOBEC3 (A3) even though it doesn't possess any accessory proteins such as Vif [2, 13]. In this study, we demonstrate that MLV escapes from murine A3 (mA3) via two distinct novel mechanisms. First, viral RNA (vRNA) blocks the binding of mA3 to Gag, resulting in the exclusion of mA3 from MLV virions. Second, viral protease (vPR) cleaves mA3 after maturation of virions. Here, we suggest that each virus has its own strategy to escape from A3 proteins and that these mechanisms might be used by other viruses that do not possess Vif-like protein. On the other hand, mice possess another form of mA3, Δexon5, that escapes from the cleavage by vPR to show more antiviral activity than the wild type mA3. This also suggests that battles between host intrinsic immunity and viruses have led to the evolution of proteins on both sides.

Results and Discussion

vRNA Specifically Blocked the mA3 Incorporation into MLV Virions by Inhibiting Its Binding to Gag Nucleocapsid Protein

We previously reported that mA3 could not inhibit MLV because it was specifically excluded from MLV virions [13]. To elucidate this mechanism, we first tested the interaction of mA3 with MLV Gag because human A3G (hA3G) had been reported to bind HIV-1 Gag Nucleocapsid (NC) for its incorporation into HIV-1 virions [14–18]. Immunoprecipitation assays clearly demonstrated that mA3 as well as hA3G could bind to the p65 Gag precursor protein ($p65^{Gag}$) (Figure 1A). GST pull-down assays also showed that GST-MLV Gag NC efficiently precipitated mA3, as well as hA3G, to similar extents (Figure S1A). These results were apparently different from those reported by the Cullen laboratory, which demonstrated that this specific exclusion is attributed to the inability of mA3 to bind MLV Gag [19]. We assume that this discrepancy might originate from the difference in GST-fusion proteins that each study used. Furthermore, when we prepared virus-like particles (VLPs) without transfer vectors, we clearly detected the mA3 incorporation into MLV VLPs (Figure 1B, lane 8). These data indicate that mA3 can be incorporated into MLV VLPs by binding to MLV Gag NC as seen with HIV-1.

Because MLV VLPs could efficiently package mA3 whereas MLV virion excluded it, we suspected that vRNA might block the mA3 incorporation. Expression of vRNA efficiently reduced the mA3 incorporation into MLV virions while barely affecting that into HIV-1 virions (Figure 1B). In contrast, expression of vRNA slightly reduced the hA3G incorporation into MLV virions but not into HIV-1 virions. Dose-escalation studies revealed that expression of MLV vRNA inhibited the incorporation of hA3G and mA3 into MLV virions in a dose-dependent manner, and the exclusion of mA3 from virions was much stronger than that of hA3G from virions (Figure 1C). In contrast, expression of HIV-1 vRNA facilitated the hA3G incorporation into HIV-1 virions as previously reported [16–18], whereas it did not affect the mA3 incorporation into HIV-1 virions (Figure 1D). These findings suggest that the exclusion of APOBEC3 by vRNA is specifically detected in MLV virions. To further investigate the mechanism, we tested several transfer vectors with different RNA-packaging capacities (Figure 1E). Comparable levels of luciferase expression in producer cells suggested that comparable levels of vRNA and mRNA were transcribed (data not shown). Luciferase activities in target cells demonstrated that only vRNA produced with pDON/Luc was efficiently packaged into MLV virions. Reduced luciferase activities with more transfer vectors from a certain point was seen even without mA3, suggesting that too many transfer vectors affect the retroviral titer itself (data not shown). Only cotransfection of pDON/Luc efficiently excluded mA3 from MLV virions in a dose-dependent manner, indicating that only packagable RNA could exclude mA3 from MLV virions. This suggests that the ability of vRNA to bind to MLV Gag clearly correlates with the capacity of MLV virions to exclude mA3.

Finally, we examined the effect of vRNA on the binding of mA3 to MLV Gag. Immunoprecipitation and GST pull-down assays in the presence of vRNA revealed that expression of vRNA clearly reduced the binding of mA3 to Gag more efficiently than it reduced hA3G binding to Gag (Figure 1A, lanes 5 and 6 and Figure S1A, lane 4), which corresponded to the extent of these proteins' exclusion from MLV virions (Figure 1C). These findings indicate that vRNA specifically blocks the incorporation of mA3 into MLV virions by inhibiting its binding to Gag NC. In addition, we performed GST pull-down assays with RNase treatment, but RNase treatment eliminated all the binding of mA3 to Gag; this result suggested that the binding of mA3 to Gag was RNA dependent (Figure S1B).

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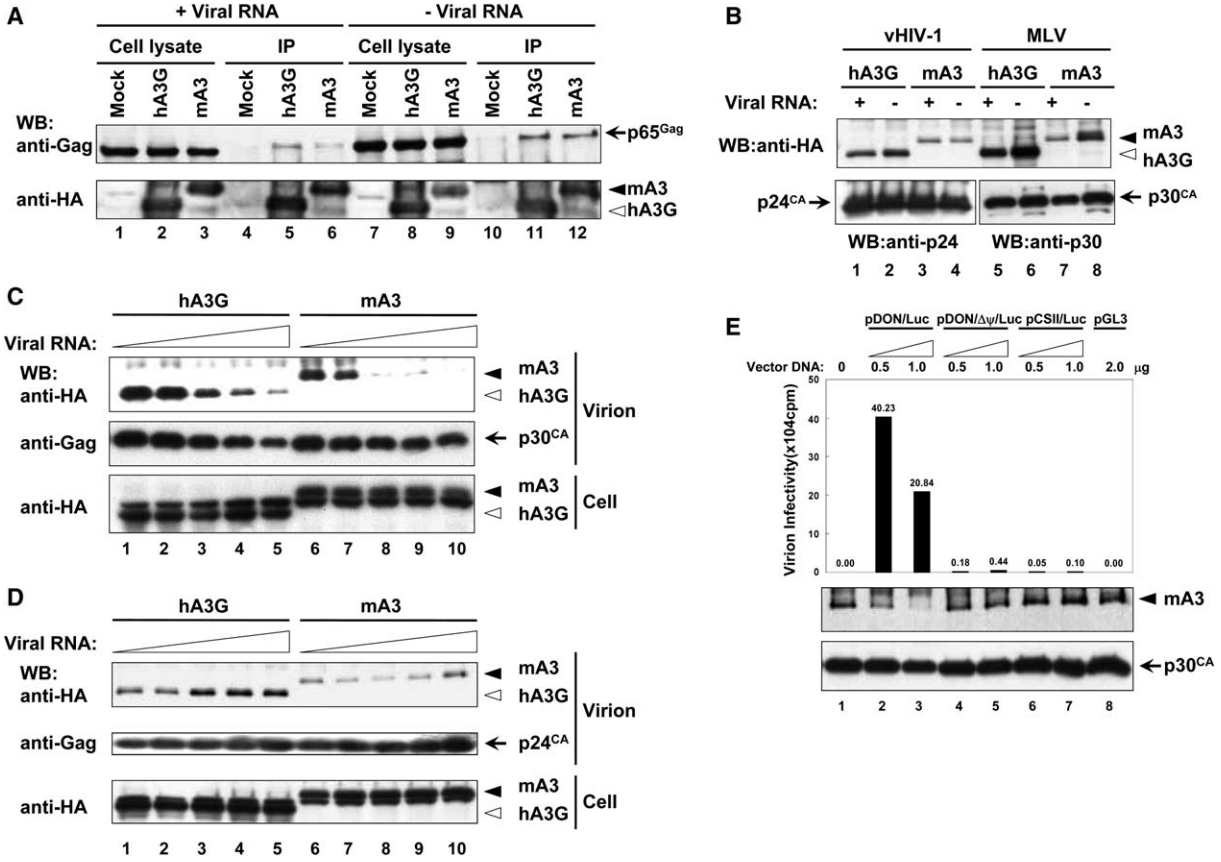

Figure 1. vRNA Blocked mA3 Incorporation into MLV virions by Inhibiting its Binding to MLV Gag

(A) MLV Gag protein bound to mA3 as well as hA3G and vRNA inhibited this binding. Expression vectors for HA-hA3G or HA-mA3 were cotransfected with pMLVg/p in the presence (+) or absence (−) of transfer vector pDON/Luc. Complexes were immunoprecipitated with HA polyclonal antibody and subjected to immunoblot with MLV Gag (top panel) or HA (bottom panel) mAbs. In the absence of transfer vectors, both hA3G and mA3 coprecipitated $p65^{Gag}$ (lanes 11 and 12, respectively), whereas cotransfection of transfer vectors reduced the binding of A3 to $p65^{Gag}$ (lanes 5 and 6). An arrow indicates $p65^{Gag}$, a filled arrowhead indicates mA3, and an open arrowhead indicates hA3G.

(B) MLV VLPs efficiently packaged mA3, but MLV virions did not. HIV-1 vector (vHIV-1) virus and its VLPs were prepared by cotransfection of pMDLg/p plus pVSV-G and pRSV-Rev with pcDNA3/HA-hA3G or pcDNA3/HA-mA3 in the presence (+) or absence (−) of transfer vector pCSII/Luc, respectively. MLV and its VLPs were also prepared by cotransfection of pMLVg/p plus pVSV-G with pcDNA3/HA-hA3G or pcDNA3/HA-mA3 in the presence (+) or absence (−) of transfer vector pDON/Luc, respectively. Precipitated virions and VLPs were subjected to immunoblotting with HA mAb (top panel) and with p24 mAb for HIV-1 and MLV Gag mAb for MLV (bottom panel). An arrow indicates $p30^{CA}$.

(C) Cotransfection of MLV transfer vectors reduced the incorporation of hA3G and mA3 into MLV virions in a dose-dependent manner and inhibited the incorporation of mA3 into MLV virion more efficiently than it inhibited incorporation of hA3G. MLV virions were prepared by cotransfection of pMLVg/p plus pVSV-G with 30 ng of pcDNA3/HA-hA3G or pcDNA3/HA-mA3 in the presence of increasing amounts of MLV transfer vector pDON/Luc (with control vectors making up the balance). Precipitated virions were subjected to immunoblotting with HA (top panel) and MLV Gag mAbs (middle panel). Cell lysates were also subjected to immunoblotting with HA mAb (bottom panel).

(D) Cotransfection of HIV-1 transfer vectors facilitated the incorporation of hA3G into HIV-1 virion, but it did not affect incorporation of mA3 into HIV-1 virion. vHIV-1 virions were prepared by cotransfection of pMDLg/p plus pVSV-G with 30 ng of pcDNA3/HA-hA3G or pcDNA3/HA-mA3 in the presence of increasing amounts of HIV-1 transfer vector pCSII/Luc (with control vectors making up the balance). Precipitated virions were subjected to immunoblotting with HA (top panel) and p24 mAbs (middle panel). Cell lysates were also subjected to immunoblotting with HA mAb (bottom panel). An arrow indicates $p24^{CA}$.

(E) Only packagable RNA blocked the mA3 incorporation into virions. MLV virions were prepared by cotransfection of pMLVg/p and pVSV-G plus pcDNA3/HA-mA3 with different transfer vectors (pDON/Luc, pDON/Δψ/Luc, pCSII/Luc, and pGL3-control). Luciferase activities of target cell lysates challenged with the produced virions (top panel) were measured as indicated. Precipitated virions were subjected to immunoblotting with HA (middle panel) and MLV Gag mAbs (bottom panel). An arrow indicates $p30^{CA}$.

vPR Cleaves mA3

When we prepared VLPs, we detected, in addition to the 52 kDa full-length form, a protein band with an approximate molecular weight of 32 kDa (Figure S2A, lane 6). Similar experiments with mA3 containing different tags also showed similar results (Figure S2B), suggesting that mA3 might be cleaved into approximately 30 kDa N-terminal and 25 kDa C-terminal fragments in MLV VLPs. HIV-1 also contained this fragment but HTLV-1 did not, suggesting the specificity of this cleavage (Figure 2A).

The existence of a truncated form of mA3 that is only detected in VLPs prompted us to test the possible involvement of vPR. We examined the presence of this truncated form in VLPs that was prepared with several mutants of packaging plasmids (Figure 2B). The presence of a truncated mA3 was supported by the existence of the p30 product of Gag ($p30^{CA}$) (Figure 2C).

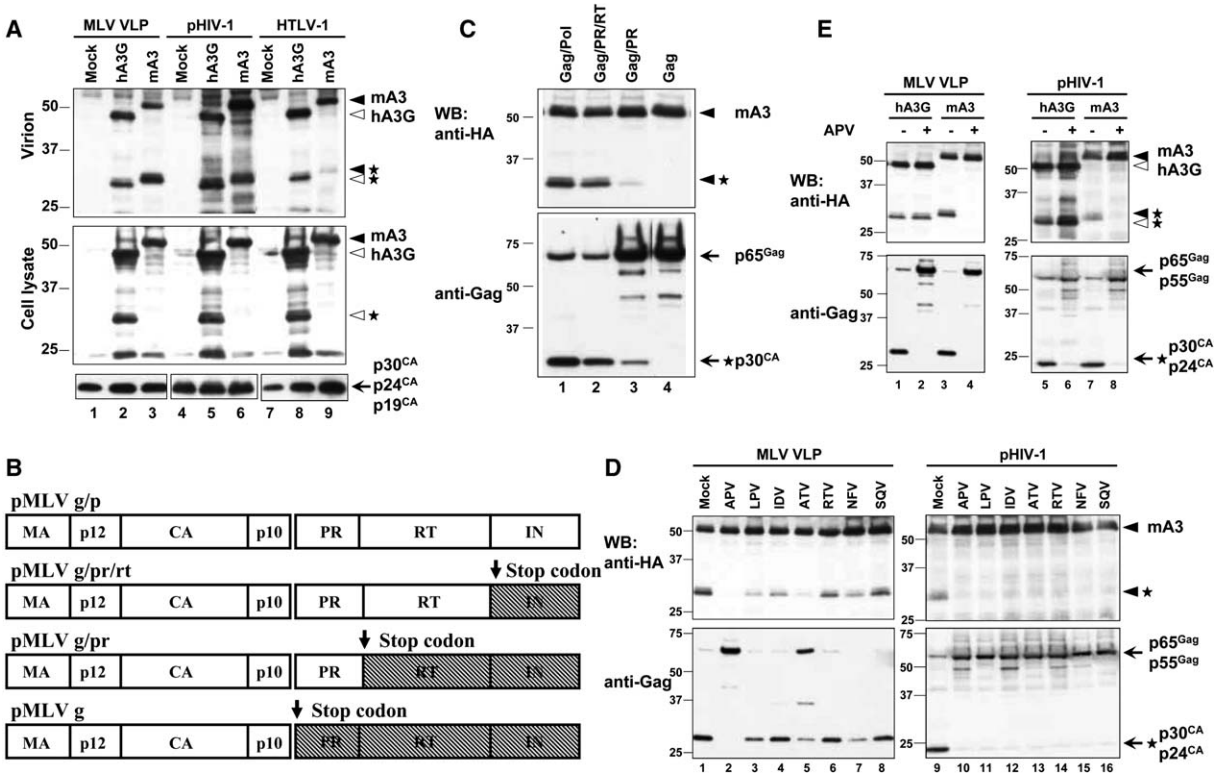

Figure 2. mA3 Was Cleaved in MLV and HIV-1 Virions, and vPR Was Involved in the Cleavage of mA3

(A) Small products of mA3 were specifically detected in MLV and HIV-1 but not in HTLV-1. Viruses were prepared by transfection with pMLVg/p plus pVSV-G for MLV, pNL43/ΔEnvΔvif-Luc plus pVSV-G for pHIV-1, or K30 for HTLV-1 with pcDNA3/HA-hA3G or pcDNA3/HA-mA3. Precipitated virions (top panel) and cell lysates (middle panel) were subjected to immunoblotting with HA mAb.

Precipitated virions were also subjected to immunoblotting with MLV Gag mAb for MLV, p24 mAb for HIV-1, and HTLV-1 Gag mAb for HTLV-1 (bottom panel). A filled arrowhead indicates mA3; an open arrowhead indicates hA3G; a filled arrowhead with an asterisk indicates a small fragment of mA3; an open arrowhead with an asterisk indicates a small fragment of hA3G; and an arrow indicates $p30^{CA}$ (lanes 1–3), $p24^{CA}$ (lanes 4–6), and $p19^{CA}$ (lanes 7–9).

(B) A schematic figure of pMLVg/p and its mutants.

(C) Cleavage of mA3 corresponded to the cleavage of Gag. MLV VLPs were prepared by cotransfection of pMLVg/p or its mutants (pMLVg/pr/rt, pMLVg/pr, and pMLVg) plus pVSV-G with pcDNA3/HA-mA3. Precipitated VLPs were subjected to immunoblotting with HA (top panel) and MLV Gag (bottom panel) mAbs. A filled arrowhead indicates mA3; a filled arrowhead with an asterisk indicates a truncated form of mA3; an arrow indicates $p65^{Gag}$; and an arrow with an asterisk indicates $p30^{CA}$.

(D) Protease inhibitors specifically blocked the cleavage of mA3 as well as the cleavage of Gag. MLV VLPs and pHIV-1 were prepared as described above and treated with protease inhibitors as indicated. Precipitated VLPs and virions were subjected to immunoblotting with HA mAb (top panel) and MLV Gag mAb for MLV or p24 mAb for HIV-1 (bottom panel). A filled arrowhead indicates mA3; a filled arrowhead with an asterisk indicates a truncated form of mA3; an arrow indicates $p65^{Gag}$ for MLV and $p55^{Gag}$ for HIV-1, respectively; and an arrow with an asterisk indicates $p30^{CA}$ for MLV and $p24^{CA}$ for HIV-1, respectively. APV, amprenavir; LPV, lopinavir; IDV, indinavir sulfate; ATV, atanazavir sulfate; RTV, ritonavir; NFV, nelfinavir; SQV, saquinavir.

(E) APV blocked the cleavage of mA3 but not the cleavage of hA3G. MLV VLPs and pHIV-1 were prepared as described above and treated with APV as indicated. A filled arrowhead indicates mA3; a filled arrowhead with an asterisk indicates a truncated form of mA3; an open arrowhead indicates hA3G; an open arrowhead with an asterisk indicates a truncated form of hA3G; an arrow indicates $p65^{Gag}$ for MLV and $p55^{Gag}$ for HIV-1, respectively; and an arrow with an asterisk indicates $p30^{CA}$ for MLV and $p24^{CA}$ for HIV-1, respectively.

We suspect that the introduction of a stop codon at the initiation of the coding sequence of RT might affect the enzymatic activity of vPR and result in a small amount of a truncated form and $p30^{CA}$. We further examined the assay in the presence of several HIV-1 PR inhibitors (PIs). All the PIs showed anti-HIV-1 PR activity as expected, whereas only APV and ATV could block the activity of MLV PR (Figure 2D, bottom panel). In parallel, all the PIs inhibited the appearance of a truncated form of mA3 in HIV-1, whereas treatment with APV and ATV only showed the disappearance of a truncated form in MLV (Figure 2D, top panel). These data indicate that the truncated form in VLP is a cleaved product of mA3 and that vPR is involved in this cleavage. In contrast, a small product of hA3G was detected even in cell lysate (Figure 2A), and treatment with APV could not reduce this product of hA3G in MLV and HIV-1. These results suggest that this product was made in producer cells and incorporated into virions as an already-truncated form. This indicates that the cleavage by vPR is specific for mA3.

We next examined the cleavage of human-murine chimeras as shown in Figure 3A to determine the exact cleavage site in mA3. Because chimeras containing a human linker region flanked by the two catalytic sites (chimeras 1, 2, 5, 8, and 9) was cleaved even in producer cells, as seen with hA3G, and incorporated into virions, we could not make any assessment about these

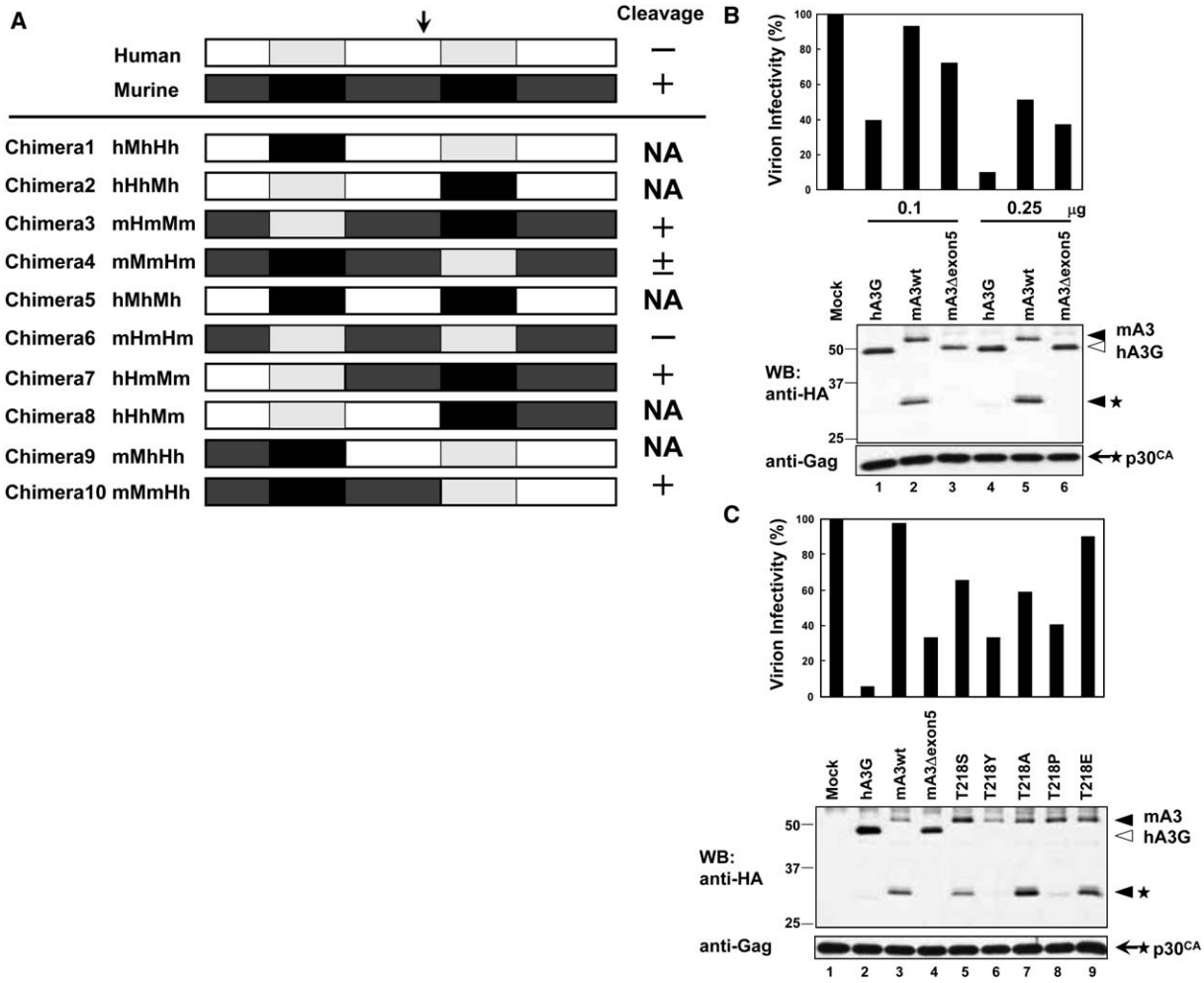

Figure 3. A Cleavage Site in mA3

(A) mA3G was cleaved at the site in the linker region flanked by the two catalytic sites. A schematic figure of human-murine chimera mutants is shown. A plus sign indicates the presence of the cleaved product only in VLPs, whereas a minus sign indicates the absence of the cleaved product. NA indicates "not assessed" because of the presence of a nonspecific cleaved product even in producer cell lysates. Capital letters in chimera names indicate two catalytic sites. h(H) is for human, and m(M) is for murine. An arrow indicates a predicted cleavage site.

(B) mA3Δexon5 was not cleaved in virions and showed more antiviral activity on MLV. MLV reporter viruses were prepared by cotransfection of pDON/Luc plus pVSV-G and pMLVg/p with the indicated amounts of expression vectors for hA3G, mA3wt, and mA3Δexon5. The infectivity of these viruses was presented as the percentage infectivity relative to that of MLV without expression of A3 proteins (top panel). Precipitated virions were subjected to immunoblotting with HA (middle panel) and MLV Gag (bottom panel) mAbs. A filled arrowhead indicates mA3; a filled arrowhead with an asterisk indicates a truncated form of mA3; an open arrowhead indicates hA3G; and an arrow with an asterisk indicates $p30^{CA}$.

(C) The mutants that were not cleaved in virions showed more antiviral activity on MLV. MLV reporter viruses were prepared by cotransfection of pDON/Luc plus pVSV-G and pMLVg/p with expression vectors for hA3G, mA3wt, mA3Δexon5, and the indicated mutants. The infectivity of these viruses was presented as described above (top panel). Precipitated virions were subjected to immunoblotting with HA (middle panel) and MLV Gag (bottom panel) mAbs. A filled arrowhead indicates mA3; a filled arrowhead with an asterisk indicates a truncated form of mA3; an open arrowhead indicates hA3G; and an arrow with an asterisk indicates $p30^{CA}$.

chimeras (data not shown). In contrast, chimeras 3, 4, 7, and 10 had a cleaved product only in VLPs. Taking this information together with the size of a cleaved product, we predicted that the cleavage site was located in the linker region of mA3 (Figure 3A, arrow). Mice possess two types of A3: a full-length form (mA3wt) and an alternative splicing form (mA3Δexon5). We and others reported that mA3Δexon5 shows more antiviral activity (10%–20%) on MLV as well as HIV-1 than mA3wt (Figure 3B; data not shown). Because the region encoded by *exon5* contained the predicted cleavage site of mA3, we tested the cleavage of mA3Δexon5 in virions. The truncated form was not detected in virion preparation with mA3Δexon5 (middle panel, lanes 3 and 6), and its absence resulted in more incorporation of mA3Δexon5 into MLV virions. These findings suggest that mA3Δexon5 possesses more antiviral activity on MLV and HIV-1 than mA3wt because it is not cleaved by vPR and that the amount of incorporated mA3 corresponds to its antiviral activity. Because *exon5* encoded amino acids from 199 to 231, we predicted a MLV and HIV-1 vPR cleavage site between Leu-217 and Thr-218. We prepared several mutants with a mutation on Thr-218 and tested their cleavage in virions and their antiviral activity on MLV. A truncated form was detected in virion preparation with T218S, T218A, and T218E but not

in that with T218Y and T218P (Figure 3C). Antiviral activity of these mutants clearly correlated with their cleavage; T218Y and T218P showed more activity than other mutants with a cleaved form. This suggests that vPR cleaves mA3 directly between Leu-217 and Thr-218.

In this study, we demonstrate that MLV escapes from an antiretroviral host factor A3 by two distinct novel mechanisms: exclusion by vRNA and cleavage by vPR. This is quite intriguing because a simple retrovirus is resistant to its cognate A3 by using not an accessory protein like HIV-1 Vif but by using its basic structural elements such as vRNA and vPR. We suggest that each virus has its own strategy to protect itself from A3 proteins and that these novel mechanisms might be used by other viruses that do not possess Vif-like proteins.

We demonstrate that mA3 can be incorporated into MLV VLPs by binding to MLV Gag and that MLV vRNA specifically excludes mA3 from MLV virions by inhibiting its binding to MLV Gag. This specific exclusion might be attributable to the sites responsible for binding of A3 and vRNA in MLV Gag NC. HIV-1 Gag NC has two Zinc domains responsible for vRNA binding, whereas the binding site of hA3G is determined around the N-terminal region of NC [14, 17]. In contrast, MLV Gag NC possesses only one Zinc finger domain for vRNA binding, and this domain might be overlaid by the binding site for A3 proteins. Further study on the A3 binding site in NC should be performed. We also demonstrate that not only mA3 but also hA3G is excluded from MLV virions by vRNA and that the exclusion of mA3 is more efficient than that of hA3G. There have been controversies about the incorporation of mA3 into MLV virions and the antiviral activity of hA3G on MLV. The report by the Landau laboratory first showed no anti-MLV activity of hA3G and mA3 despite their incorporation into MLV virions [6]. In contrast, we and others demonstrated that hA3G shows anti-MLV activity by being incorporated into MLV virions, whereas mA3 cannot inhibit MLV because it is not incorporated into virions [2, 3, 13]. We assume that these discrepancies might originate from differences in amounts of transfer vectors used in each study. The greater the amount of transfer vectors transfected, the more A3 proteins are excluded from MLV virions, resulting in less anti-MLV activity. Using a replication competent MLV system, the Cullen laboratory showed no incorporation into MLV virions and no anti-MLV activity of mA3 [19]; these results also support our data.

We also demonstrate that mA3 is specifically cleaved in MLV and HIV-1 but not in HTLV-1. We previously demonstrated that deletion mutants of hA3 easily lose their antiviral activity because of loss of dimerization activity [4]. We also confirmed this notion by conducting experiments that used mA3 deletion mutants (Figure S4). The experiments with packaging vector mutants and HIV-1 PIs show that vPR is involved in this cleavage. Although we cannot show the direct cleavage of purified mA3 by vPR because preparation of purified mA3 is quite difficult, the experiment with human-murine chimeras enables us to determine that the candidate cleavage site is located in the linker region flanked by the two active sites. Furthermore, the experiment with mA3 mutants, which have a mutation on the predicted cleavage site, clearly shows the direct cleavage by vPR. We and others

		MLV		HIV-1ΔVif	
mA3 wt	Exclusion by vRNA	+++	++++ Fully Resistant	-	+/- Slightly Resistant
	Cleavage by vPR	+		+/-	
mA3 Δexon5	Exclusion by vRNA	+++	+++ Moderately Resistant	-	- Fully Sensitive
	Cleavage by vPR	-		-	
hA3G	Exclusion by vRNA	+	+ Slightly Resistant	-	- Fully Sensitive
	Cleavage by vPR	-		-	

Figure 4. Summary of the Data

previously reported that mA3Δexon5 has more antiviral activity on MLV and HIV-1. However, the reason for this has been unclear. mA3Δexon5 is excluded from virions to similar extents as mA3wt (Figure S3) but cannot be cleaved by vPR, which results in more antiviral activity on both viruses. These findings suggest that the cleavage mechanism confers 10%–20% of the resistance of MLV to mA3 (Figure 3B) and that the remaining resistance is attributed to the exclusion mechanism. In light of these results, we conclude that exclusion of mA3 by vRNA is the main mechanism for the resistance of MLV to mA3 (Figure 4).

Simple retroviruses are becoming resistant to their cognate A3 by using vRNA and vPR. They have evolved to efficiently replicate in their host cells, perhaps through changes in the structure of Gag NC. Such changes may have resulted in the loss of the retroviruses' resistance to A3, they might have then had to obtain new genes such as *vif* and *bet* to antagonize A3. On the other hand, from the aspect of host cells, mA3Δexon5 can protect itself from vPR. Through the APOBEC family, these findings give us new insight into how battles between our intrinsic immunity and viruses have led to the evolution of our innate immune system and should help us to develop new strategies for controlling viral infection.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and four figures and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/15/1565/DC1/.

Acknowledgments

This study was partly supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. We are very grateful to Dr. S. Kawamata for MLV packaging vectors, to Cell Genesys and Dr. H. Miyoshi for the Lentivirus vector system, and to Dr. T. Kindt for HTLV-1 molecular clone K30, which was obtained through the AIDS Research and Reference Reagent Program.

Received: February 16, 2006
Revised: May 31, 2006
Accepted: June 13, 2006
Published: August 7, 2006

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