Nature of the N-terminal Amino Acid Residue of HIV-1 RNase H is Critical for the Stability of Reverse Transcriptase in Viral Particles

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Abstract

Reverse transcriptase (RT) of Human Immunodeficiency Virus type 1 (HIV-1) is synthesized and packaged into the virion as a part of the GagPol polyprotein. Mature RT is released by the action of viral protease. However unlike other viral proteins RT is subject to an internal cleavage event leading to the formation of two subunits in the virion – a p66 subunit and a p51 subunit that lacks the RNase H domain. We have previously identified RNase H as an HIV-1 protein that has the potential to be a substrate for the N-end rule pathway that is an ubiquitin dependent proteolytic system in which the identity of the N-terminal amino acid determines the half-life of a protein. Here we examined the importance of the N-terminal amino acid residue of RNase H in the early life cycle of HIV-1. We show that changing this residue to a structurally different amino acid than the conserved residue leads to degradation of RT and in some cases integrase in the virus particle and this abolishes infectivity. Using intravirion complementation and in vitro protease cleavage assays we show that degradation of RNase H N-terminal mutant RT occurs in the absence of active viral protease in the virion. Our results also indicate the importance of the RNase H N-terminal residue in the dimerization of RT subunits.
Importance

HIV-1 proteins are initially made as part of a polyprotein that is cleaved by the viral protease into the proteins that form the virus particle. We were interested in one particular protein RNase H that is cleaved from reverse transcriptase. In particular we found that the first amino acid of RNase H never varies in over 1850 isolates of HIV-1 that we compared. When we changed the first amino acid we found that the reverse transcriptase in the virus was degraded. While other studies have implied that the viral protease can degrade mutant RT proteins we show here that this is may not be the case for our mutants. Our results suggest that presence of active viral protease is not required for the degradation of RT in RNase H N-terminal mutants suggesting a role for a cellular protease in this process.
Introduction

Like all retroviruses Human Immunodeficiency Virus Type 1 (HIV-1), the causative agent of AIDS, synthesizes and packages its main structural and enzymatic proteins as precursor polyproteins. For HIV-1 these polyproteins are p55 (Gag) and p160 (GagPol). Gag is the most abundant polyprotein and is translated from a genomic length mRNA that contains the Gag and GagPol open reading frames. The synthesis of GagPol requires a ribosomal frameshift leading to a Gag:GagPol ratio of about 20:1 in the virus particle (1). Individual mature viral proteins are generated following viral assembly as a result of a series of proteolytic cleavage events at specific positions catalyzed by the viral protease which is synthesized as a part of GagPol (2).

One protein that is released as a result of proteolytic processing of GagPol is reverse transcriptase (RT). RT catalyzes the reaction for the conversion of viral RNA to double stranded DNA (3). In contrast to the other viral enzymes encoded by the pol gene, RT functions as a heterodimer of two subunits, p66 and p51 (4-6). Formation of this heterodimer requires the proteolytic cleavage of the RNase H domain from one of the p66 subunits resulting in p51 that is associated with the p66 to form the heterodimer (4). RNA dependent DNA polymerase, and RNase H activities of HIV-1 RT are mainly carried out by the p66 subunit, while p51 was thought to be enzymatically inactive and only serving a structural role (5, 7-10). However, recent structural and biochemical evidence suggests that C-terminal end of the p51 subunit is involved in hydrolysis and
positioning of the RNA/DNA hybrid formed during the reverse transcription process (11-13).

Retroviral RNase H is a member of a family of enzymes that are found in all domains of life (14). It functions as an endonuclease that degrades RNA from the RNA/DNA hybrid formed during the first phase of reverse transcription. This function is crucial for the processing and completion of reverse transcription, as it creates an RNA primer for plus strand DNA synthesis and as it facilitates the first and second jump by removing the 5' end of viral RNA and tRNA respectively (8, 15, 16). In the virus particle RNase H is found both as a part of p66 and as a free protein (4). However it is not definitively established whether the RNase H species that is generated by the viral protease has any specific function.

The N-end rule pathway is an ubiquitin dependent proteolytic system in which the identity of the N-terminal amino acid determines the half-life of a protein. Since proteolytic cleavage of viral polyproteins can result in N-terminal residues that dictate a short half-life for the cleaved protein we have recently examined the involvement of the N-end rule pathway in the retroviral life cycle. Using N-end rule mutant cells and N-terminal amino acid substitution mutants we studied the effects on the mature integrase protein that bears a highly conserved destabilizing residue (17). Our results showed an impact of the N-end rule pathway on HIV-1 but not on the life cycle of MLV. However the interaction of the N-end rule machinery on the HIV-1 pathway was not at the level of
the integrase N-terminal residue (17). One of the differences in the protein composition of HIV-1 and MLV is that unlike the heterodimeric RT of HIV-1, MLV RT functions as a monomer of about 75kDa protein, hence doesn't require a proteolytic cleavage to remove the RNase H domain (18, 19). Due to this difference between HIV-1 and MLV and the fact that HIV-1 RNase H also has the potential to be a substrate for the N-end rule pathway, we turned our attention to this protein.

In this study we investigated the role of the N-terminal amino acid residue of HIV-1 RNase H. Here we show that the N-terminal residue of RNase H is highly conserved and changing this residue to an amino acid that is structurally different to the WT residue leads to degradation of RT and in some cases integrase in the virus particle. Notably this degradation in the virus particle is independent of the N-end rule that would be manifest in the target cell. We demonstrate that this degradation is processive and does not extend to WT RT species that are added in trans into the viral particle. We observed that liberation of RT from GagPol or a Vpr fusion poly-protein is required for the degradation of RNase H N-terminal mutants. Moreover we show that an N-terminal RNase H mutant RT molecule that is degraded in the viral particle is not degraded \textit{in vitro} in the presence of excess viral protease. We also present evidence that some RNase H N-terminal mutants have a defect in forming a RT heterodimer. Finally we show that RT in the RNase H N-terminal mutant viruses are degraded even if the viral protease is inactivated. Taken together these results suggest a possible role for one or more cellular proteases in the degradation of mutant RT in the virus.
**Materials and Methods**

**Reagents and Cell Culture**

293T and Jurkat cells were obtained from the American Type Culture Collection (ATCC). 293T cells were maintained in Dulbecco’s Modified Eagle Medium (Cellgro) supplemented with 10% Fetal Bovine Serum, FBS (Gemini Bioproducts). Jurkat cells were maintained in Iscove's Modified Dulbecco's Medium (ATCC) supplemented with 20% FBS.

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH; p24 Monoclonal Antibody (183-H12-5C) from Dr. Bruce Chesebro and Kathy Wehrly, HIV-1 HXB2 Integrase Antiserum (aa 23-34) from Dr. Duane P. Grandgenett, HIV-1 RT polyclonal antibody, pEGFP-Vpr (cat# 11386) from Dr. Warner C. Greene and Ritonavir. Monoclonal antibody for RT (1.149 B6) was previously isolated and characterized (20). Mouse monoclonal (IgG2a) antibody against HIV-1 vpr was obtained Cosmo Bio USA (Carlsbad, CA). Secondary p24 antibody for ELISA was collected from a hybridoma cell line obtained from the ATCC (HB-9725). Antibody isolation from the hybridoma cell line was performed using standard protocols as described previously (17). Secondary antibody for ELISA; goat-anti-mouse-HRP IgG2A was obtained from Southern Biotech (Birmingham, AL). Goat-anti-mouse-horseradish peroxidase and goat-anti-rabbit-horseradish peroxidase (HRP) secondary antibodies and West Femto enhanced chemiluminescent (ECL) HRP substrate were obtained from Thermo Scientific (Rockford, IL).
Plasmid Constructs and Mutagenesis

Plasmids used for VSVg pseudotyped HIV-1 production were: CSII-EGFP; an HIV-1 based vector encoding for GFP driven by EF-1a promoter. ΔNRF; encodes for gag, pol, rev, tat and vpu of HIV-1. pMDg; encodes for vesicular stomatitis virus glycoprotein. RNase H N-terminal mutations were introduced into ΔNRF by PCR mutagenesis (PCR Primers available upon request). PCR products, which contain the specific mutations, were cut with KpnI and ligated back into KpnI digested ΔNRF plasmid. Viral protease inactivating D25A mutation was introduced into ΔNRF by overlap PCR (Primers available upon request). Final PCR product was cut with SacII and SbfI, and cloned into ΔNRF. For the vpr complementation assays, a mammalian expression plasmid, pRK5, was used to clone the vpr fusion proteins. Vpr was amplified from pEGFP-Vpr using following primers; forward; 5' CTCGGATTCCACCGCCATGGAACAAGCCCCAGAAGAC 3', reverse; 5' CGC GAA GCT TCA GTT CCA GAT CTG AGT AGG ATC TAC TGG CTC CAT TTC TT 3'. PCR products were digested with BamHI and HindIII and cloned into pRK5. This construct, pRK5-vpr, was used for cloning WT or RNase H mutant RT or RT-Integrase. pRK5-vpr-RT constructs were generated by amplifying RT sequence including the protease cleavage site between Protease and RT from WT or RNase H mutant ΔNRF constructs using following primers; Forward 5' GCT CAA GCT TAC TTT AAA TTT TCC CAT TAG TCC 3', Reverse 5' GCT CAA GCT TTT ATA GTA CTT TCC TGA TTC 3'. PCR products were then digested with HindIII and cloned into pRK5-vpr. Same strategy was used for generating pRK5-vpr-RT-IN constructs, the following reverse primer together with forward primer above was used for generating the PCR product including RT and Integrase; 5' GCT CAA GCT TTT AAT CCT CAT CCT GTC...
TAC 3’. pLR2P-vpr-p51-IRES-p66 was previously described and was a kind gift from Dr John Kappes (21). RNase H mutants were cloned into this plasmid by first amplifying the ΔNRF construct with the corresponding mutation with the following primers; 5’ CAG TAA ATT TAA AGC CCG GGA TGG ATG G 3’ and 5’ GGA TCT CGA GTT ATA GTA CTT TCC TGA T 3’. PCR products were digested with XmaI and XhoI and cloned into pLR2P-vpr-p51-IRES-p66. pLR2P-vpr-pro50-p51-IRES-p66 was generated by first amplifying a region of ΔNRF containing 150 nucleotides of viral protease and p51 subunit of RT using the following primers; 5’ TAG ATC AGA TCT AAT TGG AGG TTT TAT CAA AGT AG 3’ and 5’ ATC TAC ACG CGT TTA GAA AGT TTC TGC TCC TAT 3’. PCR products were digested with BglII and MluI and ligated into pLR2P-vpr-p51-IRES-p66 cut with the same enzymes. pLR2P-vpr-p51-IRES-p66 with XmaI and XhoI and then re-ligating the plasmid after generating blunt ends of the 5’ overhangs with T4 DNA polymerase.

pRK5-GagPol was generated by first PCR amplifying GagPol from ΔNRF using the following primers; 5’ TCG ATT GAA TTC GCC ATG GGT GCG AGA GCG TCG G 3’ and 5’ GCT CCT GTC GAC TTA ATC CTC ATC CTG TCT 3’. PCR products were digested with EcoRI and SalI and cloned into pRK5. pRK5-GagPol-FS was generated by overlap PCR using following primers: Left pair 5’ GGC AAA GAA GGG CAC ACA GCC 3’ and 5’ CCC TGA GGA AGT TAG CCT GTC TCT CAG TAC 3’, right pair 5’ GGC TAA CTT CCT CAG GGA AGA TCT GGC CTT CC 3’ and 5’ GTT GAC AGG TGT AGG TCC TAC 3’. Final product was digested with Apal and BclI and cloned into pRK5-GagPol. RNase H mutants were cloned into pRK5-GagPol-FS from the ΔNRF containing the corresponding mutation using BsrGI digestion.
**Virus production and infectivity assays**

HIV-1 vectors were generated by transiently transfecting three plasmids into 293T cells as described previously (22, 23). 15µg of CSII EGFP, 10µg of ΔNRF and 5µg of pMDG were transfected using the method of Chen and Okoyama (24). For vpr complementation assays 10 µg of the corresponding vpr fusion protein expressing plasmids were used for transfections. 72 hours after transfection virus was collected and filtered through a 0.45 µM membrane. Filtered virus was concentrated by ultracentrifugation (100,000 x g, 2 hours at 4°C). Viral pellet was resuspended in Phosphate buffer saline (PBS) and aliquots were stored at -80°C. Concentrated virions were normalized to WT virus using p24 ELISA as described previously (17). MOI for WT was determined by infecting 1 × 10^5 Jurkat cells with 10 fold dilutions of the viral preparation. Infections for mutant or vpr complemented viruses were done by using p24 equivalent levels of concentrated viral supernatants (based on p24 ELISA) that correspond to the MOI value for WT virus. 72 hours after the infections EGFP expression was quantified by flow cytometry on a Becton-Dickinson FACScalibur.

**In vitro transcription and translation assays**

Transcription and translation of WT and RNase H mutant pRK5-GagPol-FS was performed using the TNT coupled Reticulocyte Lysate System with SP6 polymerase (Promega) according to the manufacturer's instructions. 4 µL of the translation product was used for western blotting. For the external HIV-1 protease processing reactions 4 µL of translation products were incubated with 1 µg of HIV-1 protease (Abcam) in
phosphate buffer (25 mM NaCl, 25 mM Na₂HPO₄, 1 mM dithiothreitol and pH 7.0) in 20 µL reaction volumes at 30°C for 2 hours. The entire reaction volume was used for western blotting.

**Analysis of viral proteins by immunoblotting**

Viral pellets and *in vitro* transcription/translation products were dissolved in loading buffer (0.25M Tris-HCl, pH 6.8, 15% SDS, 50% glycerol, 25% β-mercaptoethanol, 0.01% bromophenol blue) and proteins were separated by SDS-PAGE on a 12% polyacrylamide gel and subjected to immunoblotting using the antibodies indicated.

For the analysis of protein expression from vpr-pro50-p51-IRES-p66, 5 x 10⁵ 293T cells which were cotransfected with this plasmid together with ΔNRF D25A were lysed in cell lysis buffer (20 mM Tris-HCl [pH 8.0], 1 mM CaCl₂, 150 mM NaCl, 1% Triton) and proteins were separated by SDS-PAGE on a 12% polyacrylamide gel and analyzed by immunoblotting as described above.

**Co-Immunoprecipitation (Co-IP) Assay**

293T cells were co-transfected with ΔNRF D25A and pLR2P-vpr-p51-IRES-p66wt, pLR2P-vpr-p51-IRES-p66pro, pLR2P-vpr-p51-IRES-p66leu, pLR2P-vpr-p51-IRES-p66met, pLR2P-vpr-p51-IRES-p66gly, pLR2P-vpr-p51-IRES-p66ala or pLR2P-vpr-p51ΔΔp66 as described above. 24 hours after transfection 5 x 10⁶ cells were lysed in immunoprecipitation buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton and 0.5% sodium deoxycholate). 5% of this lysate was used as an input control for the
assay. The remaining lysate was incubated with the Vpr antibody for 1 hour at 4°C. Protein G beads (Bio-world) were added and incubated for 2 hours at 4°C. Lysate-bead mixture were concentrated in a microfuge and washed with the immunoprecipitation buffer 3 times. Immunoprecipitated proteins were eluted from the beads with loading buffer (0.25M Tris-HCl, pH 6.8, 15% SDS, 50% glycerol, 25% β-mercaptoethanol, 0.01% bromophenol blue) separated by SDS-PAGE and immunoblotted as described above.

Results

N-terminal amino acid residue of HIV-1 RNase H is highly conserved

In our previous study we have reported on 7 HIV-1 mature proteins as having primary destabilizing residues as defined by the N-end rule (17). Further analysis indicated that only 4 of these proteins (p1, Trans frame octapeptide, RNase H and Integrase) have a conserved residue at the N-terminus. Here we investigate the role of the N-terminal residue of RNase H during HIV-1 infection. Comparison of the sequence of the cleavage site between the p51 subunit of RT and RNase H in HIV-1 isolates present in the Los Alamos HIV sequence database reveals that the sequence for the N-terminal amino acid residue of RNase H is absolutely conserved for all the isolates found in this database (Figure 1). This suggests a hypothesis that changing this residue would have deleterious effects on viral infectivity.

RNase H N-terminal mutations impact intravirion protein levels and viral infectivity
To test the role of the N-terminal residue of RNase H in the viral life cycle of HIV-1 we changed this residue to several different amino acids. Selection of mutants was based on N-end rule designation or structure of the specific amino acid as compared to the wild type (WT) residue of Tyrosine (Table 1). We first analyzed the effect of these N-terminal RNase H mutations on viral protein packaging and maturation by immunobloting. We observed that viral pellets of most of the RNase H mutants contained drastically reduced levels of RT when compared to WT viral pellets (Figure 2A). Extended exposure of the immunoblot (using enhanced chemiluminescence for detection) revealed that some mutant (i.e. Proline (Pro) and Lysine (Lys)) virions contained barely detectable levels of RT, while others contained diminished amounts of p51 (Figure 2B). In contrast, two of the mutants (Tryptophan (Trp) and Phenylalanine (Phe) containing structurally similar amino acids to the WT tyrosine residue had slightly reduced levels to WT in terms of their RT content (Figure 2A, top blot). We further probed our RNase H mutants for the presence of other HIV-1 proteins, and this analysis revealed that some of the mutants also had diminished levels of integrase (Figure 2A, middle panel). The mutants that had the most effect on the RT levels (i.e. Pro and Lys) showed the most pronounced decrease in virion integrase levels as well. We further probed the RNase H mutant virions for viral capsid protein (p24) to determine whether the mutations we introduced caused any gross aberrations in assembly or processing. As shown in the bottom panel of Figure 2A, a product of the Gag processing, p24, is found in all mutants suggesting WT levels of protease activity on the Gag polyprotein in virions. This is reflected in the analysis of p24 expression in producer cells where stable
and unstable mutants show similar levels of expression (Figure 2C). Gag-Pol incorporation is also detected at similar levels in virions (Figure 4).

Next, we tested the infectivity of the N-terminal RNase H mutants of HIV-1. As shown in Figure 3, infectivity was slightly decreased (by a factor of 0.75) for Trp and Phe mutants that were not compromised for RT levels in the virions. Infectivity was undetectable in all the other mutants tested except for the Met mutant that showed a 100 fold decrease in infectivity compared to WT.

Packaging and processing of viral polyproteins with RNase H N-terminal mutations

Despite the correct processing of Gag to p24 in all of the N-terminal RNase H mutants tested (Figure 2 bottom blot), diminished levels of RT and integrase in some mutants indicate the possibility of aberrant packaging or aberrant processing of GagPol. To better assess this, we analyzed the presence of GagPol, Gag and other proteolytic processing products in the mutant virions. We produced WT and RNase H mutant virions (Met and Leu which are typical for residual RT of other mutants) in the presence of varying amounts of ritonavir, an HIV-1 protease inhibitor. Following concentration, virions were analyzed by immunoblotting. As shown in Figure 4 (bottom panel), WT and mutant virions showed similar patterns of cleavage intermediates when probed for p24. As the concentration of ritonavir increased the levels of processed p24 decreased with a corresponding increase in the amounts of higher molecular weight products such as Gag and GagPol (Figure 4, bottom panel). Notably at the highest concentration of
ritonavir the Gag and GagPol levels were similar in all virions suggesting that the mutants are not compromised in the assembly of Gag and GagPol into virion particles. When probed with RT antibody, cleavage patterns of WT and mutant virions are only comparable at the higher ritonavir concentrations (Figure 4, top blots). For virions produced in the presence of 1 or 0.5 µM of ritonavir, WT virus shows the predicted processing intermediates consistent with previous reports (25, 26). There is a loss of immunoreactive epitopes in the two mutants examined (Figure 4 top panel). From this analysis we cannot conclude whether a higher molecular weight intermediate or the RT p66 species is mainly susceptible to the degradation observed in the mutants. However the appearance of similar levels of GagPol and Gag in both mutants and WT at the highest ritonavir level indicates correct packaging of these polyproteins in RNase H N-terminal mutants.

**Delivery of RT independent of GagPol into HIV-1 virions**

Our analysis of proteolytic processing in RNase H mutants did not allow us to conclude whether this degradation requires only the RT p66 species or a higher molecular processing intermediate. In addition we wondered if this degradation could affect WT RT p66 species that may dimerize with a mutant RT if both were present in the virion. To examine these questions we delivered p66 synthesized independent of GagPol into virions containing WT or RNase H mutant GagPol. This was accomplished by using fusions to the HIV-1 accessory protein vpr that is packaged into the virion by binding to the p6 domain of Gag (27). We generated constructs by fusing vpr to WT or RNase H
mutant RT-Integrase (Figure 5A and B). The protease cleavage site (PC) between protease and RT was retained in the vpr fusion to enable cleavage and release of RT-Integrase in the virus particle. We generated WT or N-terminal RNase H mutant viral particles in the presence or absence of vpr fusion constructs. Concentrated virions were analyzed by immunoblotting following p24 ELISA to enable equivalent loading based on viral capsid concentration. As shown in Figure 6 top panel, when probed with a vpr antibody, WT or mutant viruses complemented with vpr-RT-IN wt fusion constructs contain the predicted 4 bands as a result of protease cleavage at 3 different sites; between vpr and RT, p51 and RNase H and RNase H and integrase (Figure 6 top panel: lanes marked +vpr-RT-IN wt). When we probed vpr-RT-IN complemented mutant virions with an RT antibody we observed the almost complete rescue of both RT subunits only when the vpr constructs contained WT or Trp mutant RNase H fusions (Figure 6A and C middle panel). RNase H N-terminal mutants that are structurally different than the WT showed either diminished levels of RT subunits (Figure 6C) or the appearance of only low levels of p51 (Figure 6A). Notably the vpr fusion polyproteins appear to be at similar levels in virion particles (Figure 6 top panel and middle panel) and only the RT subunits are subject to degradation suggesting that these are the substrates for proteolysis. Similar to the results obtained with the vpr-RT-Integrase fusions, we observed the appearance of both RT subunits only in the virions complemented with vpr-RTwt or vpr-RTtrp when we delivered vpr-RT fused RNase H mutants (data not shown). These results support the idea that the mutant RT p66 is the substrate for proteolysis once it is liberated from the polyprotein (GagPol or from the vpr fusion).
Infectivity of Vpr-RT-Integrase complemented viruses

Since the vpr-RT-IN complemented RNase H N-terminal mutants showed rescue of intravirion RT when complemented with WT or Trp mutants, we tested if this molecular rescue also extends to infectivity of the RNase H mutants. We infected Jurkat cells with WT or mutant vpr-RT-IN complemented viruses (Figure 7). As predicted by the immunoblotting data, infectivity of Leu, Met and Pro mutants were rescued to similar levels by complementation with vpr-RT-INwt (Figure 7B, C and D). We also observed rescue of infectivity by vpr-RT-INtrp, albeit at different levels for each mutant (Figure 7B, C and D). In contrast, complementation with vpr-RT-IN constructs containing RNase H mutants that had diminished levels of RT in the virion did not rescue infectivity in any of our mutants (Figure 7B, C and D). Of note there is a general slight decrease in infectivity in WT virions that are complemented with different vpr constructs.

RNase H N-terminal mutant RT is stable in vitro

Our observations revealed that the RT of the majority of N-terminal mutants of RNase H are degraded in virions and that this degradation is dependent on release of the mutant p66 from the polypeptide in which it is embedded (Figure 4 for GagPol, Figure 6 for vpr-RT-Integrase). However, it is not clear whether this degradation is caused by the viral protease (for example by the mutation uncovering cryptic target sites) or a cellular protease that is packaged into the virion. We first investigated this by using a rabbit reticulocyte lysate (RRL) in vitro transcription/translation system. Since Gag is the dominant protein species translated from full length viral mRNA this would interfere with
our ability to detect RT that is made from the frameshifted GagPol polyprotein (1).

Hence we used a GagPol transcript with an engineered frameshift that would produce only full length GagPol polyprotein (Figure 5C). We further introduced an N-terminal RNase H mutation (Pro) to this construct since it is the mutant with the most drastic effect on RT stability in the virion. Previous studies on in vitro translated GagPol polyprotein showed that the encoded protease cannot completely process the GagPol precursor to mature viral proteins (26, 28, 29). A strong initial cleavage event between p2 and nucleocapsid (NC) was observed followed by minor cleavages at other sites (26). However, addition of HIV-1 protease to the reaction has been shown to generate mature viral proteins (28). In our assay system we first translated the GagPol in vitro and then incubated the final product with exogenous HIV-1 protease and used immunoblot analysis to probe for RT release and stability. As shown in Figure 8, in the absence of exogenous protease we observe the predicted proteins that result from a single dominant cleavage of GagPol by the intrinsic protease (40 kDa, and an approximately 120 kDa). We also observed lower molecular weight peptides using this RT monoclonal antibody, consistent with several minor cleavage products observed previously (26, 29). Consistent with previous findings, addition of HIV-1 protease resulted in the processing of GagPol with the appearance of p24 for both WT and the RNase H proline mutant, when probed with a p24 antibody (Figure 8, top blot). We also observed the appearance of the p51 and p66 subunits of RT from WT GagPol following incubation with the viral protease (Figure 8, bottom blot). In contrast to the intravirion degradation of RT in the RNase H proline mutant, the in vitro maturation of GagPol containing the RNase H proline mutant resulted in stable RT subunits (p51 and p66) at
levels similar to WT (Figure 8, bottom blot). Addition of greater concentrations of viral protease or longer incubations times did not reveal any additional cleavages of the final products (data not shown). These results suggest that HIV-1 protease that is active and site specific for cleavage (i.e. competent to release p24 and the RT 51 and p66 subunits) does not degrade RNase H mutant RT.

Some RNase H N-terminal mutants lead to impairment of dimerization of RT subunits

While the above in vitro experiments suggest that viral protease may not be responsible for the degradation of intravirion RT of some RNase H N-terminal mutants, there remains the possibility that in the context of the viral milieu, concentrations of viral protease or specific interactions between the viral proteins inside the virion are required for the mutant RT to be degraded by the HIV-1 protease. To properly investigate these possibilities we packaged the p66 RT subunit into virions without the need to fuse it to any protein. This would enable a test of intravirion stability of RT in the absence of an active viral protease. To this end we utilized a previously described construct pLR2P-vpr-p51-IRE-p66 (vpr-p51/p66) (21). In this construct, vpr fused p51 subunit is packaged into the virion through the interaction of vpr with the p6 domain of Gag, while the p66 subunit is packaged via its interaction with the p51 subunit in the vpr-p51 fusion (21). Since the vpr-p51/p66 construct makes it difficult to distinguish between vpr-p51 fusion protein and p66 on immunoblots probed with an RT antibody (30), we generated a construct that contains 50 amino acids from the C terminus of HIV-1 protease
between the vpr and p51 (Figure 5D). This enables differentiation between vpr-pro50-p51 and p66 on immunoblots. To test for the involvement of viral protease in the degradation of RT in RNase H mutants, we inactivated the viral protease by introducing a D25A mutation into the protease gene in our viral production helper plasmid ΔNRF.

Since the packaging of p66 into the virus particle requires binding to the p51 subunit in the vpr-pro50-p51 fusion protein we tested the dimerization of RT subunits harboring the RNase H proline mutant using a co-IP assay. For this we incubated extracts of 293T cells co-transfected with ΔNRF D25A (protease inactive) and vpr-pro50-p51Δp66, vpr-pro50-p51/p66wt or vpr-p51/p66pro with a vpr antibody. Bound protein complexes were pulled down with Protein G conjugated beads and analyzed by immunoblotting. As shown in Figure 9A top panel, both vpr-pro50-p51 and p66 can be detected from the cells expressing WT p66 (lanes under pLR2P WT) in the input and IP extracts. In contrast, p66 is only present in the lysate and not in the immunoprecipitate of the cells expressing RNase H proline mutant p66 (Figure 9A top panel, lanes under pLR2P Pro).

These results indicate that dimerization of RT p66 with p51 is impaired when the N-terminal residue of RNase H is changed to Proline. To further examine the importance of RNase H N-terminal residue in the dimerization of RT subunits we tested the association of RT subunits in other RNase H mutants i.e. Glycine, Leucine, Alanine and Methionine substitutions. As shown in Figure 9B top panel, we detected both vpr-pro50-p51 and p66 in both lysate (Inp) and immunoprecipitate (IP) from the cells expressing RNase H glycine and leucine mutant p66 (lanes under pLR2P Gly and pLR2P Leu) in ratios similar to wild-type. In contrast p66 was only present in the lysate (Inp) of RNase H mutants alanine and methionine (lanes under pLR2P Ala and pLR2P Met). These
results highlight the importance of N-terminal RNase H residue in the dimerization of RT subunits.

**RNase H N-terminal mutant RT is still degraded in the absence of active viral protease**

We next examined the stability of RNase H N-terminal mutants in the presence and absence of viral protease. We used the glycine and leucine RNase H mutants since we have determined that association of the subunits is not impaired for these mutants. We produced virions with active or inactive viral protease complemented with WT or RNaseH mutant vpr-pro50-p51-IRES-p66, and analyzed them by immunoblotting. To avoid confusion of RT subunit bands contributed by GagPol and vpr-pro50-p51-IRES-p66 constructs in the immunoblot, we chose GagPol with RNase H proline mutant to produce viral protease active virions. This mutant completely lacks RT subunits inside the virion (Figure 2), so the only RT subunits observed are derived from the complementing vpr-pro50-p51-IRES-p66 construct. GagPol containing D25A mutation in the viral protease was used to produce protease inactive virions. We observed that both protease active and inactive virions with WT vpr-pro50-p51-IRES-p66 contain the expected peptides for vpr-pro50-p51 and p66 (Figure 10 top blot, pLR2P WT). In contrast, for both glycine and leucine mutants both vpr-pro50-p51 and p66 are almost completely absent with or without active HIV-1 protease (Figure 10 top blot, pLR2P Leu and pLR2P Gly). For these mutants the formation of the heterodimer independent of the HIV-1 protease (i.e. by expression of the subunits from the vpr-pro50-51 and IRES
expression of p66) leads to degradation of the heterodimer. These results suggest that presence of active viral protease may not be required for the intravirion degradation of RNase H N-terminal mutant RT.

Discussion

RNase H of HIV-1 is excised from half of the viral p66 RT species that are synthesized and this results in the association of heterodimeric subunits of RT, p51 and p66 (4). This proteolytic cleavage releases RNase H into the virion and this mature form of RNase H bears a conserved N-terminal amino acid residue that makes it a potential substrate for the cellular N-end rule pathway (Figure 1). In this study we set out to examine the role of the N-terminal residue of RNase H on the life cycle of HIV-1. To this end we generated virions containing mutations of the N-terminal RNase H tyrosine residue. We observed that most of the amino acid substitutions led to a complete or almost complete absence of RT in the virion (Figure 2). The nature of the amino acid substitution that leads to the absence of detectable RT in the virion was important since mutants that contained structurally similar amino acids to WT (i.e. Trp and Phe) at the N-terminus of RNase H had both subunits of RT at levels similar to WT. Similar observations were reported by another group following introduction of multiple mutations in the p51/RNase H cleavage site region (33). Since that study introduced multiple mutations that led to the degradation of RT in the virus particle, it was not possible to determine which mutation is responsible or what type of amino acids are tolerated at any specific residue. In this study we report on mutagenesis of only the N-terminal residue and our
results point to the importance of the nature of the amino acid at the N-terminal amino acid residue of the RNase H for the stability of RT in the virus particle. This is also corroborated by the high conservation of the Tyr residue across HIV-1 isolates (Figure 1).

In addition to the absence of RT, some RNase H mutants also contained diminished amounts of intravirion integrase (Figure 2A). There is also a concomitant appearance of a peptide around 25 kDa (Figure 2B). It is probable that a subset of RT mutations lead to a structural change in the RT-Integrase polyprotein that may expose an alternative protease cleavage site in integrase. It is important to note that some of the mutant virions did not contain significant amounts of this possible alternative cleavage product including the proline mutant that has the most drastic effect on RT stability. This suggests a different or additional mechanism for integrase degradation for these mutants. It is important to note that relative amounts of integrase correlated with the relative amounts of RT found in the RNase H mutants. This suggests a mechanism of degradation of higher molecular weight intermediate induced by presumably some structural change in the polyprotein.

The fact that we observed proteolytic processing of Gag to p24 by viral protease and the presence of at least some amounts of integrase in each mutant, combined with the observation that GagPol and Gag is expressed in the RNase H mutants leads us to conclude that deficiency in GagPol production and packaging cannot explain the drastically low levels of RT found in some of the RNase H N-terminal mutants.
Infectivity of the N-terminal RNase H mutants correlated with RT content in the virion. With viruses containing structurally similar amino acids to WT Tyr amino acid at the RNase H N-terminus (Trp and Phe) reporting only a slight decrease in infectivity while the other mutants resulted in complete or almost complete lack of infectivity (Figure 3). Even though some mutants retained a limited amount of p51 in the virus particle the presence of p66 is required for the reverse transcription process (8). One of our mutants (Met) showed limited levels of infectivity. Longer exposures of the immunoblots probed with monoclonal or polyclonal RT antibody reveals that the Met mutant contained limited amounts of p66 in the virion that correlates with the residual level of infectivity observed for this mutant (Figure 2B).

Despite the degradation of both RT and integrase, we do not observe a deficiency in intravirion incorporation of GagPol in our RNase H mutants. Moreover appearance of identical proteolytic cleavage patterns in Gag processing indicates that activity of viral protease is not impacted by mutations at the N-terminal amino acid residue of HIV-1 RNase H (Figure 4).

The mechanism of the formation of the RT p51/p66 heterodimer is not clearly established and two working models have been proposed. In the concerted model, p66 and p51 subunits are separately cleaved out of different GagPol molecules and come together to form the heterodimer. In contrast, the sequential model posits that following the formation of an initial homodimer by two p66 subunits the RNase H domain of one of the subunits is cleaved to form the heterodimer of p66/p51. Even though most of the
crystallographic and functional evidence points to the sequential model, definitive
evidence is still missing. Since the RT of some our RNase H N-terminal mutants are
degraded in the virus particle, we explored the possibility of this degradation affecting a
non-mutant RT p66 species by delivering the RT as a GagPol independent vpr fusion
protein into the virus. While these results do not clearly distinguish between the two
models of RT heterodimer formation, they indicate that degradation of RT caused by the
RNase H N-terminal substitutions is confined to the specific p66 species that contains
the mutation. Hence we observed that trans-complementation with WT RT-Integrase vpr
fusion protein substantially rescued infectivity in our RNase H mutants.

Proteolytic processing of the precursor polyproteins of HIV-1 is an ordered series of
events carried out by the viral protease following the assembly of viral components. A
conformational change due to a mutation in the polyprotein may expose alternative
cleavage sites leading to additional cleavages of the viral polyprotein and result in loss
of infectivity. We tested the possibility that the RNase H N-terminal mutants were
degraded by the HIV-1 protease using both in an in vitro and an intravirion assay. In
vitro experiments relied on the ability of externally added viral protease to complete the
processing of in vitro translated GagPol precursor leading to the appearance of mature
viral proteins (28). With the caveat that in vitro conditions may not exactly represent the
conditions inside the virus particle we did not observe any degradation of RT for the
proline RNase H mutant (Figure 8). This led us to question the role of HIV-1 protease in
the degradation of RT in N-terminal RNase H mutants. An intravirion assay showed the
absence of RT p66 species bearing the proline RNase H mutant in the virions with
inactivated viral protease (data not shown) although its expression in the producer cell
was unaffected (Figure 9A). However further analysis using co-IP showed that the absence of RT p66 in these virions is caused by the impaired interaction between vpr-pro50-p51 and RNase H proline mutant p66 (Figure 9A). This indicates that dimerization of RT subunits is impaired in this RNase H mutant. To properly test the role of viral protease in the intravirion degradation of RNase H N-terminal mutants we sought to find an RNase H mutant that is not defective in association of RT subunits. This search led to identification of two RT mutants (glycine and leucine) that are not defective in RT subunit binding. Using these mutants we show that both p66 and vpr-pro50-p51 are degraded in the virus particle (Figure 10). While we didn't anticipate that degradation of RNase H mutant p66 would be accompanied by the degradation of vpr-pro50-p51, we hypothesize that delivery of these proteins to the virus particle as a heterodimerized complex leads to degradation of both subunits. Collectively these results indicate that viral protease may not be required for the degradation RT containing RNase H N-terminal mutants in the virus particle. Moreover our results suggest that one or more cellular proteases may be packaged into virus particles resulting in the degradation of RNase H N-terminal mutant RT.

Previous studies have shown that dimerization of RT subunits is a prerequisite for the activity of the enzyme (34-36). Moreover, mutations in the tryptophan repeat motif of RT has been shown to cause defects in dimerization (35, 36) and these results were later confirmed using the same pLR2p constructs utilized in this study (32). While some of these tryptophan motif mutants also showed decreased levels of RT in the virus particle the degradation was not as pronounced as the degradation we observed in RNase H N-terminal mutants.
terminal mutants. This suggests that the lack of dimerization per se may not be the initiating event for degradation by the extent of the structural change caused by the mutation. Moreover since some of our unstable RNase H mutants showed successful association of p66 and vpr-pro50-p51, degradation of RT in RNase H N-terminal mutants cannot be linked to the defect in RT subunit association. Others have speculated that the RT-RNase H cleavage site is not involved in the RT dimer formation (37). Here we provide evidence that at least one of the amino acids in this cleavage site can impact the association of RT subunits.

It is notable that while the results from the in vitro and intravirion assays indicate that viral protease may not be responsible for the degradation of RNase H proline mutant of RT p66, our vpr complementation and protease inhibitor experiments clearly indicate that release of the mutant RT p66 species from the polyproteins by viral protease cleavage is required, since GagPol and vpr fused polyproteins are stable even when harboring the unstable RNase H mutations. This observation suggests caution in ascribing the degradation of mutant RT to the action of HIV-1 protease based only on data from the inhibition if HIV-1 protease i.e. the action of HIV-1 protease is a prerequisite for degradation that may be mediated by another protease.

Recent advances in proteomic technology have enabled more detailed analysis of the protein content of HIV-1 virus particles. Indeed multiple studies to date have identified cellular proteins packaged into HIV-1 virions (38-41). Relevant to this study the presence of multiple cellular proteases have been reported. However there is no significant overlap in the identity of the proteases identified in these studies. Indeed the only protease that is common to two of these studies is carboxypeptidase D (38, 39). In
addition, Santos et al. found this protease only in viral particles generated from one of
the cell lines they used (39). While this indicates the importance of the specific cell line
and methodology used for these studies, we believe that RT degradation of mutants is
caused by a protease that is broadly expressed since the same phenomenon (of
degradation of RT mutants as result of mutations in this region) was observed when
monkey kidney cells (COS-7) and human T cells (MT-2) were used for virus production
(33).

Due to its importance as an anti-viral target reverse transcriptase of HIV-1 is one of the
most highly studied proteins. Several groups have introduced amino acid substitutions
at different subdomains of RT (11, 33, 35, 42-46). Many of these mutants led to
destabilization of the RT protein in the virus particle as well as loss of infectivity. Some
studies have concluded that the viral protease is responsible for the degradation of the
mutant RT. Our results indicate that liberation of the mutant subunit from the fusion
polypeptide is a prerequisite for degradation and previous results should be re-
evaluated in this light. This study could have implications for the development of small
molecules that distort the structure of the HIV-1 polyprotein in the Pol polypeptide that
signals destruction of essential viral enzymes.

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References


Wapling J, Moore KL, Sonza S, Mak J, Tachedjian G. 2005. Mutations that abrogate human immunodeficiency virus type 1 reverse transcriptase
Figure 1. N terminal residue of HIV-1 RNase H is highly conserved. Sequence of the protease cleavage site between RT (p51) and RNase H was analyzed for 1850 isolates of HIV-1 and SIVcpz present in Los Alamos HIV Database (http://www.hiv.lanl.gov) using the web alignment tool. Amino acid that corresponds to the conserved sequence is shown at the bottom. The sequence logo at the top was generated using WebLogo (http://weblogo.berkeley.edu).

Figure 2. Changing the N terminal residue of HIV-1 RNase H leads to instability of RT and integrase in the virus particle. Equivalent p24 amounts of WT and RNase H N terminal mutant virions were analyzed by western blotting following production in 293T cells using helper plasmids and concentration by ultracentrifugation. Particles were probed with RT (top), integrase (middle) and p24 (bottom) antibodies. Extended exposure of the immunoblots from A. C. 293T cells transfected with helper plasmids for producing WT or RNase H mutant virions were lysed 48 hours after transfection and the protein content of equivalent amount of cells were analyzed by immunoblotting with the indicated antibodies.

Figure 3. Effect of N-terminal RNase H mutations on HIV-1 infectivity. Jurkat cells were infected with p24 equivalent amounts of VSVg pseudotyped WT or mutant RNase H mutant HIV-1 vectors. Infectivity was measured by flow cytometry 3 days post infection. MOI for WT was measured as indicated in the Methods section.
Figure 4. Intravirion processing of Gag and Gag-Pol polyproteins in HIV-1 RNase H N-terminal mutants. WT and two representatives of RNase H mutant HIV-1 virion particles were produced in the presence of varying concentrations of the HIV-1 protease inhibitor ritonavir and the cleavage pattern of the Gag and Gag Pol polypeptides were analyzed by immunoblot analysis. WT, as well as methionine (Met) and Leucine (Leu) RNase H mutants were probed with antibodies to RT (top) or p24 (bottom).

Figure 5. Constructs used for Vpr complementation and in-vitro studies. Position and identity of RNase H N-terminal substitutions are indicated. Frameshift causing mutation for pRK5-GagPol-FS is shown. PC: protease cleavage site between viral protease and RT.

Figure 6. Analysis of Vpr-RT-Integrase complemented HIV-1 virions. HIV-1 virions with GagPol containing WT (B) and RNase H N-terminal mutants Leu(A) or Pro (C) were produced in 293T cells with helper plasmids in the presence or absence of expression plasmids encoding for Vpr-RT-Integrase fusion proteins with WT or N-terminal RNase H mutant RT. Concentrated virions were normalized for p24 with ELISA and analyzed by western blotting using vpr (top), RT(middle), p24(bottom) antibodies.

Figure 7. Infectivity of Vpr-RT-Integrase complemented WT or RNase H N-terminal mutant viruses. Jurkat cells were infected with p24 equivalent WT (A) or N-terminal
RNase H Leu (B), Met (C), Pro (D), Trp (E) mutant VSVg pseudotyped HIV-EGFP containing WT or RNase H mutant vpr-RT-IN fusion proteins. Infectivity was determined by flow cytometry 3 days post infection. MOI for WT was measured as described in Methods.

Figure 8. In vitro analysis of the role of viral protease in the degradation of RNase H mutants. pRK5-GagPol-FS was used for in vitro transcription translation by RRL. Translation products were incubated at 30 °C for 2h with HIV-1 protease or phosphate buffer and analyzed via immunoblotting. Arrows indicate the identity of the RT, p24 and vpr antibody detected fragments. Antibodies used for each blot are indicated in bold with an α sign.

Figure 9. RNase H N-terminal residue is critical for RT subunit association A. 293T cells were co-transfected with vpr-pro-p51/p66 plasmids encoding WT or proline RNase H mutant p66 and ΔNRF D25A. Vprp51Δp66 was used as control. Co-IP was performed on cell extracts using vpr antibody and protein G beads. B. 293T cells were co-transfected with vpr-pro-p51/p66 plasmids encoding WT or different RNase H mutant p66 and ΔNRF D25A. Vprp51Δp66 was used as control. Co-IP was performed on cell extracts using vpr antibody and protein G beads. Antibodies used for each blot are indicated in bold with an α sign.

Figure 10. Intravirion analysis of the role of viral protease in the degradation RNase H mutants. HIV-1 virions with either Protease inactivating D25A mutant GagPol or RNase H N-terminal proline mutant GagPol (Labeled as GagPol Pro) were produced in 293T cells in the presence or absence of vpr-pro50-p51/p66 plasmids encoding WT
or RNase H N-terminal mutant p66. Concentrated virions were analyzed by western blotting using the indicated antibodies following normalization via p24 ELISA. Antibodies used for each blot are indicated in bold with an α sign.
### Table 1. HIV-1 RNase H N-terminal mutants

<table>
<thead>
<tr>
<th>Cleavage site (RT/RH)</th>
<th>N-end Rule Designation</th>
<th>Amino acid at P1'</th>
<th>Reason for selection</th>
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</thead>
<tbody>
<tr>
<td>AETF /YVD (WT)</td>
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<td>Tyrosine</td>
<td>WT</td>
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<tr>
<td>AETF /MVD</td>
<td>Stabilizing</td>
<td>Methionine</td>
<td>N-end rule</td>
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<td>Structurally conserved</td>
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<td>Threonine</td>
<td>N-end rule</td>
</tr>
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<td>Destabilizing</td>
<td>Leucine</td>
<td>N-end rule</td>
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<td>AETF /VVD</td>
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<td>Valine</td>
<td>Small hydrophobic side chain</td>
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</table>
A. pRK5-Vpr-RT

B. pRK5-Vpr-RT-IN

C. pRK5-GagPol-FS

D. pLR2P-vpr-pro50-p51-IRES-p66