JOURNAL OF VIROLOGY, Sept. 2005, p. 11952–11961 Vol. 79, No. 18
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.18.11952–11961.2005

Virion Instability of Human Immunodeficiency Virus Type 1 Reverse Transcriptase (RT) Mutated in the Protease Cleavage Site between RT p51 and the RT RNase H Domain

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Received 11 February 2005/Accepted 18 June 2005

Each of the human immunodeficiency virus type 1 (HIV-1) *pol*-encoded enzymes, protease (PR), reverse transcriptase (RT), and integrase (IN), is active only as a dimer (or higher-order oligomer in the case of IN), but only RT comprises subunits of different masses. RT is a heterodimer of 66-kDa and 51-kDa subunits. The latter is formed by HIV PR-catalyzed cleavage of p66 during virion maturation, resulting in the removal of the RNase H (RNH) domain of a p66 subunit. In order to study the apparent need for RT heterodimers in the context of the virion, we introduced a variety of mutations in the RT p51-RNH protease cleavage site of an infectious HIV-1 molecular clone. Surprisingly, rather than leading to virions with increased RT p66 content, most of the mutations resulted in significantly attenuated virus that contained greatly decreased levels of RT that in many cases was primarily p51 RT. IN levels were also reduced in several mutants. However, most mutants showed normal levels of the $Pr160^{gag\text{-}pol}$ precursor polyprotein, suggesting that reduced virion RT arose from proteolytic instability rather than decreased incorporation. Mutant virion p24 Gag levels were equivalent to wild type, indicating that Gag incorporation and processing were not affected. Repeated passage of MT-2 cells exposed to mutant viruses led to the appearance of virus with improved replication capacity; these virions contained normally processed RT at near-wild-type levels. These results imply that additional proteolytic processing of RT to the p66/p51 heterodimer is essential to provide proteolytic stability of RT during HIV-1 maturation.

The human immunodeficiency virus type 1 (HIV-1) genome encodes a variety of different proteins, including the essential viral enzymes protease (PR), reverse transcriptase (RT), and integrase (IN). These viral enzymes are translated not as discrete units but rather as segments of a much larger polyprotein termed $Pr160^{gag\text{-}pol}$, and nascent virions assemble using these polyprotein precursors. The individual active enzymes are subsequently formed by proteolytic cleavage at specific sites on $Pr160^{gag\text{-}pol}$ during virion assembly and budding, a "maturation" process catalyzed by PR (25, 34).

The monomeric subunits of the HIV-1 enzymes are inactive; each enzyme must oligomerize to at least a dimer for enzymatic activity (3, 16, 64). PR and IN are homodimers (or perhaps higher-order homo-oligomers in the case of IN) with subunits of the size predicted from their genes. The mature active form of HIV-1 PR is a symmetrical homodimer released from $Pr160^{gag\text{-}pol}$ upon autoprocessing carried out by the polyprotein form of the enzyme (45). RT and IN are formed after activation of PR, but it is still unclear whether the PR-mediated proteolytic processing of these Pol proteins occurs in *cis*, in *trans*, or in some combination of these (56, 57). Like HIV-1 PR, active HIV-1 IN is at least a homodimer, although higher-order homo-oligomers may play a role in the multiple activities of this enzyme (30, 31). In contrast, RT in mature infectious virions is a heterodimer with subunits of 66 kDa (p66) and 51 kDa (p51) (70), even though the gene for HIV-1 RT encodes only a protein of 66 kDa. The smaller p51 subunit is derived from the larger p66 subunit by proteolytic cleavage between RT amino acid residues F440/Y441 during virion maturation (9, 18, 27, 71). Although both p66 and p51 subunits have identical amino acid sequences, their folding in the context of the active RT heterodimer differs, resulting in an asymmetric dimer structure (37, 76). The catalytic activities of HIV-1 RT, namely, DNA polymerase and RNase H (RNH), are carried out solely by the p66 subunit of the RT heterodimer (40). The function of the p51 subunit is not entirely clear, but it may play a primarily structural role (1).

Thus, formation of mature active RT from the $Pr160^{gag\text{-}pol}$ polyprotein precursor in HIV-1 virions requires an additional internal PR-catalyzed cleavage event compared to the formation of active IN or PR. This suggests that the heterodimeric form of HIV-1 RT is essential for virus replication. However, recombinant RT p66/p66 homodimers have enzymatic activities (DNA polymerase and RNH) comparable to RT p66/p51 heterodimers (5, 20). While functional PR and IN are released as separate entities in all retroviruses, the proteolytic events that generate RT appear to differ. Moloney murine leukemia virus consists of a single subunit (29), suggesting that the catalytically active enzyme is a monomer or perhaps homodimer. In addition, chimeras constructed from the first 425 amino acid residues of HIV-1 RT and the last 200 amino acid residues of Moloney murine leukemia virus RT have a single subunit composition and possess substantial enzymatic activity (48). Further, RT isolated from avian sarcoma leucosis virus (ASLV) virions includes both heterodimers and homodimers (21, 26), neither of which are processed internally between the polymerase and RNH domain.

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So why does maturation of HIV-1 RT require an additional internal proteolytic processing step to convert p66 to p51 to form an active heterodimeric enzyme? To address this question, we introduced into molecular clones of HIV-1 a variety of mutations in the proteolytic cleavage site for the conversion of RT p66 to p51 (p51-RNH) in an attempt to block this PR-dependent processing step. Unexpectedly, we found that all amino acid substitutions tested resulted in much-reduced levels of virion RT. As well, when present, the major antibody-reactive RT in the mutant virions was almost exclusively the p51 form. Our data suggest that the additional proteolytic processing step for RT during virion maturation may be needed to stabilize the conformation of this viral enzyme to optimize its essential function in viral replication.

MATERIALS AND METHODS

Reagents and plasmid mutagenesis. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health: HeLa-CD4-LTR/β-galactosidase cells (HCB) obtained from Michael Emerman (35); $CD4^+$ MT-2 lymphocytoid cells from Douglas Richman; HIV-1_{SF2} p24/25 Gag and anti-HIV-1_{SF2} p24/25 immunoglobulin G monoclonal antibody (MAb) (76C) from Kathelyn Steimer, Chiron Corporation; and HIV-1 PR antiserum from Division of AIDS, National Institute of Allergy and Infectious Diseases, by Biomolecular Technologies. Anti-HIV-$1_{HTLV\text{-}IIIB}$ RT and anti-HIV-$1_{HTLV\text{-}IIIB}$ RNH (2F2) immunoglobulin G MAb were previously generated in our laboratory against recombinant p66/51 RT (42), as was anti-IN polyclonal mouse Ab against recombinant IN. Homopolymeric template-primers, poly(rA)-oligo$(dT)_{12\text{-}18}$, poly(dC)-oligo$(dG)_{12\text{-}18}$, [^{3}H]TTP, [^{3}H]dGTP, and goat anti-mouse-horseradish peroxidase secondary MAb were all purchased from Amersham Pharmacia Biotech (Piscataway, NJ). The SuperPico ECL substrate system for detection of peroxidase-labeled antibody was obtained from PIERCE (Rockford, IL). Sequencing primers and mutation-containing oligonucleotides were purchased from Invitrogen (Carlsbad, CA). COS-7 cells were obtained from the American Type Culture Collection (Rockville, MD). HIV-1 p24 antigen enzyme-linked immunosorbent assay kits were obtained from SAIC-Frederick (Frederick, MD).

HIV-1 molecular clone preparation, transfection, and virus culture. Plasmid pSVC21-BH10 encodes an infectious molecular clone of the HTLV-IIIB (HxB2) strain of HIV-1 and carries a simian virus 40 origin of replication for expression in COS-7 cells (19). Mutations in the region corresponding to the RT p51-RNH cleavage site (residues 437 to 443) were introduced using the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). For the purpose of studying $Pr160^{gag\text{-}pol}$ incorporation, a catalytic inactivating mutation (D25A) was similarly introduced into the PR coding region of each of these clones. The presence of the expected mutations was verified by sequencing.

All cell lines were maintained in either Dulbecco's modified Eagle medium (COS-7 and HCB) or RPMI 1640 medium (MT-2) containing 10% (vol/vol) heat-inactivated fetal bovine serum, 2 mM L-glutamine, penicillin G (100 U/ml), and streptomycin (100 U/ml, Gibco-BRL/Life Technologies, Gaithersburg, MD). Culture medium for HCB cells was additionally supplemented with gentamicin (G418; 200 μg/ml) and hygromycin B (100 μg/ml). Virus was prepared by transfection of COS-7 cells using Lipofectamine Plus (Invitrogen, Carlsbad, CA). Virus-containing culture supernatants were harvested 60 h posttransfection, clarified by centrifugation (1,500 × *g*, 1 h at 4°C), and quantified by analyzing the levels of HIV-1 p24 antigen. Aliquots of virus preparations were stored at −80°C until use.

Infectivity of virions produced by transfection of COS-7 cells was determined by using HCB or MT-2 cells exposed to inocula of the various virus mutants normalized for HIV-1 p24 antigen content. Single-cycle viral infectivity was assessed using a multinuclear activation of galactosidase indicator (MAGI) assay (35). Briefly, HCB cells were plated into 24-well culture plates at 4×10^4 cells/well. The cells were infected the following day by replacing the culture medium from each well with virus (1 μg or 100 ng HIV-1 p24) in the presence of DEAE-dextran (20 μg/ml). After 48 h, the culture medium was removed and the cell monolayer was fixed with 1% formaldehyde–0.2% glutaraldehyde in phosphate-buffered saline (PBS) for 5 min, washed twice with PBS, and incubated for 50 min at 37°C in 250 μl of staining solution (4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM $MgCl_2$, and 0.4 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-Gal]). The staining solution was then removed, cells were washed with PBS, and the number of blue cells was assessed microscopically.

The median tissue culture infective dose ($TCID_{50}$/ml) of the various mutant virus stocks was determined as previously described (63). Briefly, clarified supernatants from transfected COS-7 cells were diluted in replicates of six in 96-well microtiter culture plates followed by the addition of MT-2 cells (10^5 cells/well). Culture medium was diluted twofold after 3 days of virus exposure to prevent overgrowth, and infections were scored by microscopic examination for the presence of syncytia 7 days postinfection.

Multiple-round viral replication was assessed by inoculation of MT-2 lymphoblastoid cells (1 μg or 50 ng of HIV-1 p24 per 10^5 cells) followed by daily microscopic observation of HIV-1-induced syncytium formation, as previously described (7, 51). After appearance of significant levels of HIV-induced cytopathic effect (CPE) (up to 30 days for certain mutants), the virus was expanded for 5 days by infection of fresh MT-2 cells. Virus-containing culture supernatants and cells were separated by centrifugation. Chromosomal DNA was extracted from the infected MT-2 cells using a QIAamp DNA Mini kit (QIAGEN Inc., Valencia, CA). The HIV-1 Pol-encoding region was then amplified by PCR and cloned into pCR-T7/CT TOPO (Invitrogen, Carlsbad, CA), and selected clones were analyzed by sequencing.

Immunoblot analysis of viral proteins. Clarified virus-containing supernatants (1 to 5 μg viral p24) were purified and concentrated by ultracentrifugation through 20% (wt/vol) sucrose cushions (275,000 × *g*, 1 h at 4°C). Virus pellets were lysed in 35 μl radioimmunoprecipitation assay lysis buffer (20 mM Tris-Cl, pH 8.0, containing 120 mM NaCl, 2 mM EDTA, 0.5% deoxycholate, and 0.5% NP-40 as well as 2 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml apoprotein, and 10 μg/ml pepstatin A). Virion particle protein composition was assessed by subjecting the lysate proteins to sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis resolution and Western blotting. Western blots were incubated with either anti-HIV-1 RT (5 μg/ml) or anti-HIV-1 p24 (5 μg/ml) monoclonal antibodies or mouse anti-IN polyclonal serum (1:50) followed by incubation with secondary horseradish peroxidase-conjugated goat anti-mouse antibody (1:3,000). Immunoreactive protein bands were visualized by enhanced chemiluminescence on a VersaDoc Imaging System and quantitated by densitometry under subsaturating exposure conditions using Quantity One v4.3.0 software (Bio-Rad, Hercules, CA). To assess the level of $Pr160^{gag\text{-}pol}$ incorporation, RT p51-RNH mutant viruses containing inactivated PR (D25A) were prenormalized by $Pr55^{gag}$ content prior to ultracentrifugation and Western blot analysis.

Assay of virion-associated RT activity. Virion-associated RT RNA-dependent DNA polymerase (RDDP) activity was measured using [^{3}H]TTP and poly(rA)-oligo$(dT)_{12\text{-}18}$ as template-primer, and RT DNA-dependent DNA polymerase (DDDP) activity was measured using [^{3}H]dGTP and poly(dC)-oligo$(dG)_{12\text{-}18}$ as template-primer. Reaction mixtures (total volume, 50 μl) contained 50 mM Tris-Cl (pH 7.9), 5 mM $MgCl_2$, 150 mM KCl, 0.5 mM EGTA, 0.05% (vol/vol) Triton X-100, 2% (vol/vol) ethylene glycol, 5 mM dithiothreitol, 0.5 mM glutathione, 50 μg/ml poly(rA)-oligo$(dT)_{12\text{-}18}$ or poly(dC)-oligo$(dG)_{12\text{-}18}$, and 20 μCi [^{3}H]TTP or 10 μCi [^{3}H]dGTP. Reactions were initiated by the addition of virus-containing culture supernatant (8 ng HIV-1 p24), incubated at 37°C for 5 h, and then quenched with 250 μl of ice-cold 10% trichloroacetic acid containing 20 mM $NaPP_i$. Quenched samples were left on ice for 30 min, filtered through glass fiber Type C filter multiwell plates (Millipore Corporation, Bedford, MA), and washed sequentially with 10% trichloroacetic acid containing 20 mM $NaPP_i$ followed by ethanol. The extent of radionucleotide incorporation was determined by liquid scintillation spectrometry.

RESULTS

Selection of mutations in the p51-RNH cleavage site in HIV-1 RT. Previous work has shown that while HIV-1 PR does not recognize a consensus sequence, the specificity of HIV-1 PR-catalyzed cleavages depends strongly on sequence context (6, 54, 55, 58, 60, 61, 72). The scissile peptide bonds recognized by HIV-1 PR lie within a 7-amino-acid segment, and the specificity of cleavage depends on the structural and conformational context of this segment rather than the primary sequence itself (75). The sequence of the RT p51-RNH cleavage site is $AETF^{440} \downarrow Y^{441}VD$. Our choice of mutations to introduce into this sequence (Table 1) was based on three factors: the PR context-dependent specificity, the stringency around

TABLE 1. Mutations introduced into the RT p51-RNH cleavage site

Abbreviation[b]	Sequence[a] (P4-P3′)	Substrate position[b]	Classification[c]	Comments[d]
WT	AETF ↓ YVD	N/A	N/A	Wild-type sequence
A437I	***I***ETF ↓ YVD	P4	Radical	Longer P4 to disrupt interactions with PR flap regions
V442S	AETF ↓ Y***S***D	P2′	Radical	Polar residue substitution
F440W	AET***W*** ↓ YVD	P1	Conservative	Maintain hydropathicity and scissile bond aromatic symmetry
F440V	AET***V*** ↓ YVD	P1	Radical	Nonpreferred β-branched residue
T439S/V442G	AE***S***F ↓ Y***G***D	P2/P2′	Conservative	Diminish side chain interactions of these corroborative positions
Y441I/V442K	AETF ↓ ***IK***D	P1′/P2′	Radical/Conservative	Disrupt hydropathicity/longer positive residue
F440A	AET***A*** ↓ YVD	P1	Radical	Nonpreferred small side chain
F440A/Y441A	AET***A*** ↓ ***A***VD	P1/P1′	Radical	Nonpreferred small side chain
F440W/Y441W	AET***W*** ↓ ***W***VD	P1/P1′	Conservative	Maintain hydropathicity and scissile bond aromatic symmetry
E438N	A***N***TF ↓ YVD	P3	Conservative	Removal of γ-carbonyl to diminish H-bond interactions with PR

[a] Amino acid substitutions introduced at various positions flanking the RT p51-RNH scissile bond are in bold italics and are underlined.
[b] Abbreviation and substrate position(s) of the mutation(s) are given. N/A, not applicable.
[c] Conservative or radical mutation based Dayhoff's ranking of functionally conserved amino acid groups, whereby chemical similarity is not necessarily as valuable as size (15).
[d] Basis for the mutation with respect to known commonalities and determinants of HIV-1 PR context-dependent specificity (23, 54, 58, 72).

PR-recognized scissile bonds ranging from low (P1′) to restricted (P4, P1, P2′) to high (P2), and the type of amino acid substitution (conservative or nonconservative).

RT content of mutant virions. We expected that the mutations chosen would provide HIV-1 virions containing various ratios of RT p66 and p51, depending on the contribution of the specific mutated residue to PR recognition of the cleavage sequence (Table 1). Surprisingly, most of the RT p51-RNH cleavage site mutations significantly diminished virion RT levels (Fig. 1A). Different mutations provided different perturbations of virion RT p66/p51 heterodimer content (Fig. 1A), ranging from an increased p51-to-p66 ratio relative to wild type (A437I and V442S) to only p51 (F440A/Y441A and E438N) to virtually complete loss of all immunoreactive RT protein (F440V, T439S/V442G, and Y441I/V442K). All mutant virions with detectable immunoreactive RT possessed dramatically increased levels of low-molecular-weight RT fragments (Fig. 1A2).

Effect of RT p51-RNH cleavage site mutations on HIV virion content of other Pol proteins. The appearance of low-molecular-weight RT fragments in many of the mutant virions suggested that aberrant PR activity might be a factor in the observed phenotype. However, it was also possible that the mutations affected virion incorporation of $Pr160^{gag\text{-}pol}$. To test this, we probed mutant virions for IN p32. Mutant virions with the most pronounced defects in RT p66/p51 content also showed reduced IN p32 levels (Fig. 1B), although IN levels were not reduced to nearly the same extent as that of RT. We were unable to probe for levels of virion PR, as the anti-PR polyclonal antibody on hand had insufficient specificity. However, the mutants showed wild-type levels of Gag Pr55 processing (Fig. 1C), suggesting nearly normal levels of PR activity in the virions. To better evaluate whether RT p51-RNH cleavage site mutations affected virion incorporation of $Pr160^{gag\text{-}pol}$ during virus assembly, we inactivated HIV-1 PR by introducing the D25A mutation into the protease gene (39, 44) in our RT p51-RNH cleavage site mutants. As shown in Fig. 2, levels of $Pr160^{gag\text{-}pol}$:$Pr55^{gag}$ in most mutants (with the exception of E438N and T439/V442G) were similar to those in wild-type HIV-1 particles. The lack of degradation products in the PR-inactivated RT p51-RNH cleavage site mutants suggests that the degradation of RT noted in the analogous PR-active mutants was due to HIV-1 PR activity and not to any cellular proteases that may have been carried into the nascent virion particles. Analysis of $Pr160^{gag\text{-}pol}$ processing intermediates in virions produced in the presence of various concentrations of ritonavir suggested that the RT p66 species rather than higher-mass intermediates was most susceptible to degradation (data not shown).

Virion-associated RT activity, infectivity, and viral replication kinetics of RT p51-RNH cleavage site mutants. Equivalent amounts (8 ng of HIV-1 p24) of virion-containing cell-free supernatants of transfected COS-7 cells were assessed for RT RDDP and DDDP activities. All mutants showed significantly decreased RT activity (Fig. 3) relative to wild-type virus. The diminution of RT activity approximately correlated with the severity of the loss of p66/p51 heterodimeric RT in the various mutant virions (Fig. 1A). Similarly, mutant virus infectivity in single-cycle MAGI cell infectivity assays was attenuated to different extents (Fig. 4A) and correlated well with virion RT content, composition, and activity.

We also assessed virus replication kinetics during long-term propagation in MT-2 cells. MT-2 cells were inoculated with high input (1 μg HIV-1 p24 per 10^5 cells) of COS-7-derived virions, and HIV-1-induced cytopathic effect was monitored over time (Fig. 4B). Mutants with relatively high RT p66/p51 heterodimer content (A437I and V442S) showed replication kinetics similar to wild type when cells were exposed to a large inoculum of virus. However, the same mutants showed delayed replication kinetics compared to the wild type when smaller viral inocula were used (data not shown), consistent with the lower $TCID_{50}$/p24 values of these mutants relative to the wild type (Table 2). Interestingly, the F440W and F440V mutants showed only slight delays in replication with large inocula of virus, despite significantly decreased virion RT content (Fig. 1A2). Both of the mutants retained some level of RT p66, suggesting the presence of some heterodimeric enzyme. This may account for the observed replication capacity of the mutant virions. All other mutants showed significantly delayed or no detectable replication even after 35 days of passage.

With 6 of the 10 mutants, repeated passage of infected MT-2 cells resulted in the appearance of mutant virus with improved replication capacity. Four mutants (T439S/V442G, Y441I/V442K, F440A, and F440W/Y441W) did not recover replica-

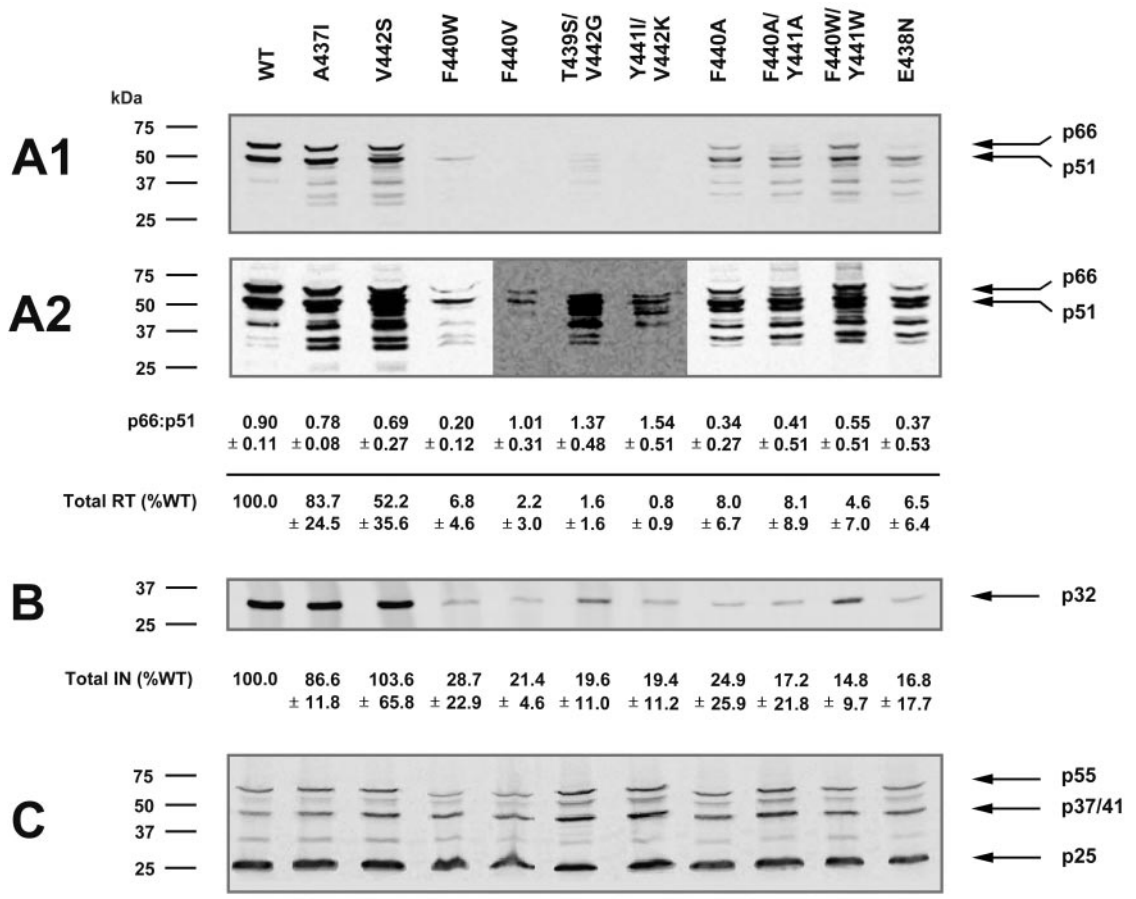

FIG. 1. Western blot analysis of viral protein expression of p51-RNH cleavage site mutant viruses. Approximately 5 μg of standardized viral p24 lysate was loaded into each lane. Viral RT subunits (A1 and A2), p32 IN (B), and Gag proteins Pr55, p48, p37/41, and p24/25 (C) were detected by probing with appropriate primary and secondary antibodies followed by enhanced chemiluminescence (ECL) as indicated in Materials and Methods. A1 and A2 represent under- and overexposures of viral RT content, respectively. Contrast was enhanced in A2 to enable visualization of protein bands in certain mutants. The relative proportion of p66 RT to p51 RT (p66:p51) and the total viral content of RT and IN were determined from multiple experiments ($n = 3$) by densitometric scanning analysis of ECL-exposed blots under subsaturating conditions.

tion competence despite passaging for extended periods of time. Viruses inducing discernible CPE 30 days postinfection (12 cell passages) were harvested, amplified for 5 days, and then assessed for infectivity. All of the "recovered" viruses showed near-wild-type replication kinetics both in single-cycle infectivity assays (Fig. 4C) and in virus spread assays in MT-2 cells (Fig. 4D). Analysis of the protein composition of the "recovered" mutant viruses showed nearly normal RT content and RT p66/p51 ratio (Fig. 5A) as well as wild-type levels of IN (Fig. 5B). Sequencing analysis of proviral DNA produced by these "recovered" mutants indicated that in one group of mutants (E438N and F440A/Y441A) the mutated RT p51-RNH cleavage site had reverted to the wild-type sequence (Table 3). Another group of mutants (A437I, V442S, and F440W) retained the original cleavage site mutations but also contained a number of additional mutations for which there was no consensus. It is of interest to note that these cleavage site mutants were initially only marginally attenuated in infectivity. One recovered mutant (F440V) retained the original cleave site mutation and also showed a single additional mutation, T477A, in all clones sequenced. We are currently characterizing the effect of T477A in restoring normal RT processing and viral infectivity in the presence of RT p51-RNH cleavage site mutations.

DISCUSSION

Each of the three HIV-1 $Pr160^{gag\text{-}pol}$-derived enzymes PR, RT, and IN is active only as a dimer (or possibly a higher-order oligomer in the case of IN). However, of the three, only RT is a heterodimer. The formation of the RT p66/p51 heterodimer thus requires an additional proteolytic cleavage event during virion maturation. We have previously demonstrated that the kinetics of RT processing follow an ordered sequential pathway from a truncated Pol polyprotein expressed in bacteria (67). In this system, following synthesis of the polyprotein precursor, RT p66 is excised first. After a buildup of p66, p51 begins to appear. The levels of p51 increase over time concomitant with a decrease in p66, until the levels of the two RT subunits are approximately equal. We also noted a similar pattern of RT processing intermediates that arise from full-length $Pr160^{gag\text{-}pol}$ in virions produced by transfected COS-7

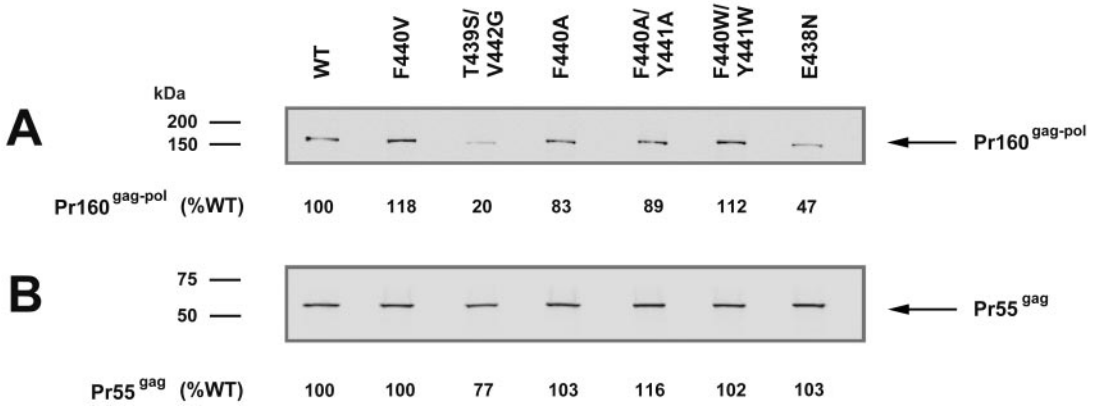

FIG. 2. Western blot analysis of $Pr160^{gag\text{-}pol}$ incorporation in immature p51-RNH cleavage site mutant viruses that contain an inactivated HIV-1 PR (D25A). Immature viruses, previously standardized for $p55^{gag}$ content by densitometry, were ultracentrifuged, lysed, and loaded into each lane. Relative viral content of (A) $Pr160^{gag\text{-}pol}$ and (B) $Pr55^{gag}$ were determined by probing separate blots with anti-RT and anti-p24 monoclonal antibodies, respectively, followed by densitometry scanning analysis of ECL-exposed blots under subsaturating conditions.

cells cultured in the presence of various concentrations of the HIV-1 PR inhibitor ritonavir (67). However, because isolated recombinant HIV-1 RT p66/66 homodimers possess enzymatic activity in vitro similar to that of recombinant RT p66/51 heterodimers (5, 20, 24, 28), the need for the RT heterodimer structure in HIV-1 virions is unclear. One possibility is that the initially processed RT homodimer is in a quasistable conformation, unlike that of purified recombinant RT homodimers, and that formation of p66/p51 RT heterodimers proceeds through this quasistable p66/66 RT homodimer intermediate (66, 67).

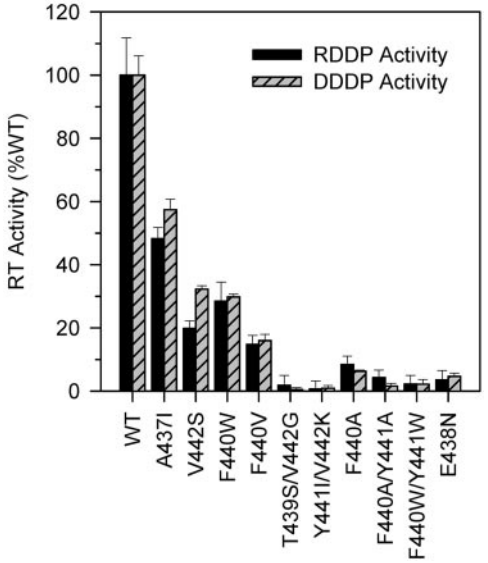

FIG. 3. Virus particle-associated RT activities. Clarified virus-containing culture supernatants (8 ng viral p24) were assayed separately for RDDP activity and DDDP activity by the incorporation of [^{3}H]dTTP into poly(rA)-oligo(dT)$_{12\text{-}18}$ and [^{3}H]dGTP into poly(dC)-oligo(dG)$_{12\text{-}18}$, respectively. Reaction mixtures were prepared as described in Materials and Methods, incubated at 37°C for 5 h, and quenched with 10% trichloroacetic acid–$NaPP_i$. Values are expressed as a percentage relative to wild-type virus. Each bar represents the average of three separate measurements.

Analysis of RT p51-RNH cleavage site mutations in synthetic peptides (72) or in the context of RT polyproteins expressed in bacteria (27, 33, 49) has generally shown that these mutations reduce or eliminate HIV-1 PR cleavage at this site. In the present study, we analyzed the effect of these mutations on intravirion processing, a more biologically relevant approach. Our results show that mutations within the 7-amino-acid protease recognition sequence that defines the p51-RNH cleavage site in the RT p66 subunit do not lead to an accumulation of p66 RT, as would be predicted from previous studies, but rather lead to significant defects in virion RT processing such that the mutant virions contain much-reduced levels of RT that is primarily p51 and smaller-mass products (Fig. 1).

Mutation of P4 (A437I) and P2′ (V443S) had only a minor effect on the formation of the RT p66/p51 heterodimer. Conservative mutations at P3 (E438N), P1 (F440W), or P1/P1′ (F440W/Y441W) resulted in virions containing primarily RT p51, as did radical changes at P1 (F440A and F440V) and P1/P1′ (F440A/Y441A). Mutations at P2/P2′ (T439S/V422G) and P1′/P2′ changes (Y441I/V442K) appeared to lead to a virtually complete loss of virion RT. Our choice of the type and position for the mutations was based on available data for HIV-1 PR context-dependent cleavage specificity (23, 54, 58, 72). However, our findings appear to indicate that there is no clear correlation between the type of mutation, its position, and the degree of loss of RT in the virion. The unexpected phenotype of RT overprocessing suggests that in addition to the primary sequence determinants for HIV-1 PR recognition of the cleavage site (58, 65, 72), other structural factors, such as conformational attributes, may be important in the regulation of the p51-RNH cleavage site in RT.

Since $Pr160^{gag\text{-}pol}$ incorporation in the mutant virions was in most cases similar to that of wild-type virions, it seems that the p66/66 RT homodimer (or perhaps the RT p66 monomer) may not be proteolytically stable in the virion. Proteolytic removal of one of the RNH domains may thus be essential for providing a conformation of RT that is refractory to further proteolytic events within the virion, thereby stabilizing the protein. Our data also clearly show that the diminution of mutant virion RT content is due to overprocessing by HIV-1 PR, since no aber-

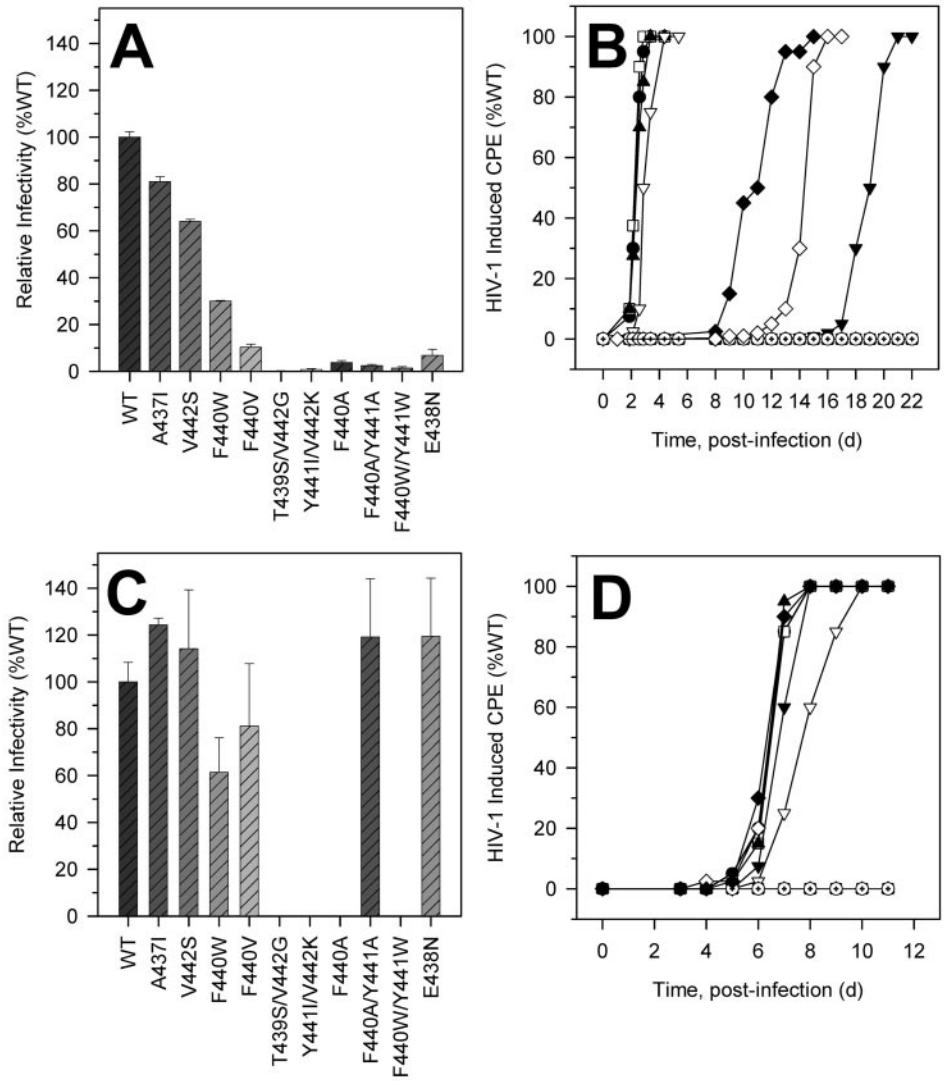

FIG. 4. Replication analysis of p51-RNH cleavage site mutant viruses. (A and C) Single-cycle (MAGI) viral infectivity. HeLa-CD4-LTR/β-galactosidase (MAGI) cells (4×10^4) were infected separately with (A) COS-7-generated mutant virions (1 μg viral p24) or (C) MT-2 cell-generated mutant viruses derived after 35 days of culture (100 ng viral p24). MAGI cells were stained and quantitated for β-galactosidase gene expression at 48 h postinfection. Percent infectivity is expressed relative to wild type (WT), with each bar representing the average of two separate measurements. (B and D) Viral replication kinetics analysis. MT-2 lymphocytoid cells (10^5) were (B) infected with COS-7-generated mutant virions (1 μg viral p24) or (D) reinfected with MT-2 cell-generated mutant viruses derived after 35 days of culture (50 ng viral p24). Cultures were split every 3 days to prevent overgrowth, and HIV-1-induced CPE was scored daily as percent syncytium formation. WT (●), A437I (□), V442S (▲), F440W (▽), F440V (◆), T439S/V442G (○), Y441I/V442K (■), F440A (△), F440A/Y441A (▼), F440W/Y441W (⊙), E438N (◇).

rant processing of RT was noted when HIV-1 PR was inactivated (Fig. 2) in mutant constructs that showed severe processing defects with active PR. It is also possible that the mutations may induce instability in processing intermediates between the full-length $Pr160^{gag\text{-}pol}$ polyprotein and the RT p66 form, especially in intermediates comprising RT-IN fusions. This possibility could account for the decreased levels of IN in some of the mutant virions.

The mechanism by which HIV-1 PR cleaves only one of the RT p66 subunits to form the RT p66/51 heterodimer has not been unequivocally established. Numerous crystal structures of the RT p66/p51 heterodimer show that the 7-amino-acid p51-RNH cleavage sequence in the p66 subunit is buried (37, 46, 76) and therefore presumably inaccessible to PR. Some investigators propose that the p66/66 RT homodimer is an asymmetric dimer similar to the RT p66/51 heterodimer but with the RNH domain of the soon-to-be p51 subunit unfolded to an extent that allows PR-mediated cleavage at the p51-RNH junction (14, 27, 46, 71, 76). This may be the result of energy derived from RT subunit dimerization that induces strain in one of the RNH domains that is relieved by unfolding along the tether region towards the p51-RNH cleavage site (14, 22, 27). Other studies, supported by circular dichroism data, suggest that RT p66/p66 may be a symmetrical homodimer but that removal of the RNH domain from one of the p66 subunits induces conformational changes in the other p66 subunit that

TABLE 2. Infectivity of recombinant WT and mutant HIV-1[a]

Strain or mutation	p24 (ng/ml)		$TCID_{50}$/ml		$TCID_{50}$/p24		
	Mean	SD	Mean	SD	Mean	SD	% WT
WT	299.6	57.8	21,728.0	3,979.2	79.8	11.3	100.0
A437I	175.9	89.7	4,728.2	1,700.0	24.3	8.4	30.4
V442S	145.6	83.1	3,281.2	459.8	19.9	2.2	24.9
F440W	203.7	69.2	504.3	52.7	2.4	0.2	3.0
F440V	241.1	33.7	ND	ND	ND	ND	ND
T439S/V442G	177.7	0.9	ND	ND	ND	ND	ND
Y441I/V442K	208.0	7.0	ND	ND	ND	ND	ND
F440A	181.6	18.5	ND	ND	ND	ND	ND
F440A/Y441A	182.5	60.0	ND	ND	ND	ND	ND
F440W/Y441W	219.0	36.2	ND	ND	ND	ND	ND
E438N	182.6	69.0	ND	ND	ND	ND	ND

[a] $TCID_{50}$/ml of COS-7 cell-generated virions assayed as described in Materials and Methods. Data represent the means ± standard deviations (SD) from four separate experiments. The ratio $TCID_{50}$/p24 represents the relative infectivity per virion, calculated upon dividing the mean $TCID_{50}$/ml by the amount of produced viral p24 in units of ng/ml. Virus-containing supernatants whose titers were not able to be determined due to low infectivity are indicated by ND (not determined).

protect this subunit from similar cleavage at its p51-RNH junction (2, 50). Unfortunately, no structure for the RT p66/p66 homodimer is yet available, so it is unclear whether the p51-RNH cleavage sequence is accessible in only one or in both subunits of the homodimer or whether the homodimer possesses different inter- or intrasubunit contacts with this region. It is possible that the p51-RNH cleavage site mutations may have altered surface accessibility of this region to allow processing of alternative nearby cleavage site(s) in both RT p66 subunits, leading to extensive destabilization and subsequent degradation of the protein. Even though HIV-1 PR is a rather adaptable enzyme, able to tolerate a variety of substrate specificities in extended substrate conformations (23), alternative or cryptic cleavage sites may be recognized in cases of partial substrate folding or unfavorable intramolecular interactions (71, 75).

While our present studies do not allow us to unequivocally differentiate between these models, we favor a model in which the virion RT p66/p66 homodimer initially exists in a "quasi-stable" symmetrical conformation and that it is this form of the RT homodimer that can undergo proteolytic processing to the p66/p51 heterodimer. We have been unable to generate significant amounts of RT p66/p51 heterodimer by in vitro treatment of purified recombinant p66/p66 RT with HIV-1 PR in *trans* under physiological pH and enzyme-substrate concentrations (data not shown). This suggests that the conformation of the purified RT p66/p66 homodimer may be very different from that formed during HIV-1 PR-mediated processing of the $Pr160^{gag\text{-}pol}$ polyprotein in the virion. Interestingly, introduction of certain RT p51-RNH cleavage site mutations into a truncated Pol polyprotein followed by expression in bacteria does not lead to aberrant proteolytic cleavage of RT but rather results in the appearance of the expected p66 RT only (data not shown). Instability of RT mutated in the p51-RNH cleavage sequence thus seems to be a function of the intravirion milieu. Many in vitro expression systems are unable to provide significant late-stage processing of full-length Gag-Pol due to limitations in substrate concentration, conformation, and dimerization efficiency (45, 56, 57). In addition, the absence of extra-PR domains (57, 62, 78), IN (8), and ancillary factors (4, 47) in the truncated models, as well as the need for progressive,

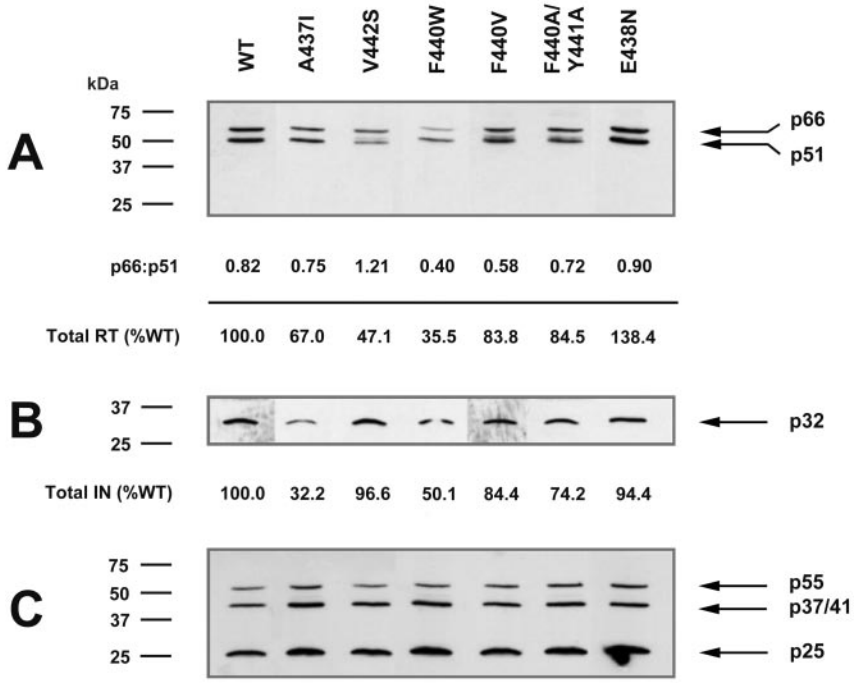

FIG. 5. Western blot analysis of viral protein expression of p51-RNH cleavage site mutant viruses after multiple rounds of MT-2 cell infection. Approximately 100 ng of standardized viral p24 lysate was loaded into each lane. (A) RT; (B) p32 IN; (C) Gag p24-reactive protein. The relative proportion of p66 RT to p51 RT (p66:p51) and the total viral content of RT and IN were determined by imaging analysis.

TABLE 3. Sequencing analysis of recovered RT p51-RNH cleavage site mutant virions

Abbreviation[a]	Sequence[a] (P4-P3′)	Recovery[b]	Reversion[c]	Second site mutations[c]
WT	AETF ↓ YVD	Yes	N/A	N/A
A437I	***I***ETF ↓ YVD	Yes	No	R4631 (1/3), D498G (1/3), E516V (1/3), E523G (1/3)
V442S	AETF ↓ Y***S***D	Yes	No	Y427N (1/3), R463G (1/3), 1542L (2/3), V548L (3/3)
F440W	AET***W*** ↓ YVD	Yes	No	E404Q (1/3), N418D (1/3), Q487L (1/3), Q500H (1/3)
F440V	AET***V*** ↓ YVD	Yes	No	T477A (3/3)
T439S/V442G	AE***S***F ↓ Y***G***D	No	N/A	N/A
Y441I/V442K	AETF ↓ ***IK***D	No	N/A	N/A
F440A	AET***A*** ↓ YVD	No	N/A	N/A
F440A/Y441A	AET***A*** ↓ ***A***VD	Yes	Yes	N/A
F440W/Y441W	AET***W*** ↓ ***W***VD	No	N/A	N/A
E438N	A***N***TF ↓ YVD	Yes	Yes	N/A

[a] Abbreviation and amino acid substitution(s) originally introduced at various positions flanking the RT p51-RNH scissile bond are in bold italics and are underlined.
[b] Recovery of viral replication and/or phenotype of RT.
[c] Reversion of the RT p51-RNH cleavage site and the frequency of second site mutations observed in sequenced clones. N/A, not applicable.

regulated processing from the full-length Gag-Pol polyprotein, may limit efficient processing in these in vitro models.

It is interesting that many of the p51-RNH site mutations resulted in virions containing primarily p51 RT. This may be due to cleavage at proposed alternative sites (5, 9, 71) near the p51-RNH junction that do not induce the necessary conformational changes in the other p66 subunit of the putative homodimer intermediate that protect this subunit from similar cleavage at its p51-RNH junction. Alternatively, the mutations may not in themselves prevent cleavage at the p51-RNH junction but instead prevent the attainment of a stable form of the RT p66/p51 heterodimer that is refractory to additional PR cleavage. Our attempts to identify the C terminus of the p51 RT in mutant virions have been unsuccessful due to the limited amounts of virion protein attainable.

The reduced levels of IN in some of the mutant virions are also intriguing. It is possible that the p51-RNH cleavage site mutations resulted in proteolytic instability of certain intermediates in the path between the full-length $Pr160^{gag\text{-}pol}$ polyprotein and the RT p66 subunit. Intermediates comprising PR-RT and/or RT-IN fusion polyproteins could lead to the mature p66/p51 RT in vivo, and this is supported by studies using heterologous expression systems (33, 41) and protease inhibitor-treated virions (43, 68). Although recombinant IN (73) and its core domain (17) are resistant to proteolysis, p51-RNH cleavage site mutations may expose predicted (13) internal IN cleavage sites, leading to its degradation. Our inability to detect any IN fragments may have been due to the loss of antibody-specific epitopes in the degraded protein.

Virion RT content has been estimated at 20 to 100 molecules of RT heterodimer (52, 53, 69), but in theory a single molecule of RT p66/p51 could complete reverse transcription. Certain p51-RNH cleavage site mutants (e.g., A437I and V442S) showed relatively equivalent levels of virion RT p66 and p51 subunits, although the level of RT was reduced relative to wild-type virus (Fig. 2A). The infectivity of these mutants was significantly attenuated (Table 2). This implies that the virion needs a substantial quantity of the RT heterodimer for efficient viral replication. Our data are consistent with previous studies using phenotypically mixed virions (32) that showed virus infectivity correlated with the amount of active RT, especially RT DNA polymerase activity, in the virus inoculum. The apparent "excess" of RT in the virion may be important to compensate for the relatively low processivity of HIV-1 RT, thereby facilitating completion of viral DNA synthesis.

The complete proteolytic release of *pol*-encoded enzymes is believed to be a late event in the ordered pathway of $Pr160^{gag\text{-}pol}$ polyprotein processing (38, 56, 57, 68, 79). Formation of the RT p66/p51 heterodimer requires proteolytic cleavage at three different sites, the N-terminal PR-RT junction, the C-terminal RT-IN junction, and the internal p51-RNH junction. However, few studies have attempted to define the functional consequences at the virus level of mutating these PR-recognized cleavage sites in the Pol polyprotein. Mutation of the PR-RT junction prevented cleavage at this site and resulted in virions containing RT subunits of 77 kDa (corresponding to the PR-RT p66 fusion) and 62 kDa (corresponding to the PR-RT p51 fusion) (11, 12). The mutant virions showed wild-type levels of RT activity and were only slightly attenuated in infectivity. Other retroviruses, such as prototype foamy virus, a human spumaretrovirus, contain PR-RT fusions. RT in prototype foamy virus virions consists of a completely processed 80-kDa PR-RT subunit and an unprocessed 127-kDa PR-RT-IN (Pro-Pol) subunit (59). RT activity appears to be associated with both homodimer forms (p80/p80 and p127/p127) as well as the p127/p80 heterodimer (36).

We have recently mutated the RT-IN junction and found that the ensuing virions contain RT subunits of 98 kDa (corresponding to the expected RT p66-IN fusion) and 51 kDa (RT p51), as well as IN subunits of 98 kDa (RT p66-IN) and approximately 40 kDa (corresponding to an RNH-IN fusion) (M. E. Abram and M. A. Parniak, unpublished data). Mutant virions containing the RT-IN fusions retain wild-type levels of RT activity. RT-IN fusions are found in virions of ASLV (74). ASLV RT exists as subunits of 63 kDa (α subunit, corresponding to the mass defined by the RT gene) and 95 kDa (β subunit, corresponding to an RT-IN fusion). RT activity is present in both αα and ββ homodimers as well as the αβ heterodimer form of ASLV RT (10, 77).

Thus, mutations in the cleavage sites defining the N and C termini of HIV-1 RT p66 result in stable fusion proteins of the expected mass, with relatively normal RT DNA polymerase activity. However, cleavages within the RT subunit seem to be

unique to lentiviruses such as HIV-1. We examined the effect of a large number of conservative and nonconservative mutations throughout the 7-amino-acid p51-RNH cleavage site in HIV-1 RT. None of these resulted in the expected virion RT p66/p66 homodimer phenotype but instead resulted in dramatic alterations in PR processing of the RT protein, suggesting that the phenotype arising from this internal cleavage is essential for ensuring adequate virion levels of active RT.

ACKNOWLEDGMENTS

This work was supported in part by NIH GM066671.

We thank N. Sluis-Cremer and D. Arion for useful discussions in the early stages of this work.

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