A Single Amino Acid Substitution in HIV-1 Reverse Transcriptase Significantly Reduces Virion Release

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HIV-1 protease (PR) mediates the proteolytic processing of virus particles during or after virus budding. PR activation is thought to be triggered by appropriate Gag-Pol/Gag-Pol interaction; factors affecting this interaction either enhance or reduce PR-mediated cleavage efficiency, resulting in markedly reduced virion production or the release of inadequately processed virions. We previously showed that a Gag-Pol deletion mutation involving the reverse transcriptase tryptophan (Trp) repeat motif markedly impairs PR-mediated virus maturation and that an alanine substitution at W401 (W401A) or at both W401 and W402 (W401A/W402A) partially or almost completely negates the enhancement effect of enfuvirenz (a nonnucleoside reverse transcriptase inhibitor) on PR-mediated virus processing efficiency. These data suggest that the Trp repeat motif may contribute to the PR activation process. Here we demonstrate that due to enhanced Gag cleavage efficiency, W402 alanine or leucine substitution significantly reduces virus production. However, W402 replacement with phenylalanine does not significantly affect virus particle assembly or processing, but it does markedly impair viral infectivity in a single-cycle infection assay. Our results demonstrate that a single amino acid substitution at HIV-1 RT can radically affect virus assembly by enhancing Gag cleavage efficiency, suggesting that in addition to contributing to RT biological function during the early stages of virus replication, the HIV-1 RT tryptophan repeat motif in a Gag-Pol context may play an important role in suppressing the premature activation of PR during late-stage virus replication.

In the late stage of human immunodeficiency virus type 1 (HIV-1) replication, thousands of viral capsid precursor (Pr55(090)) molecules assemble into virus particles and bud out from the plasma membrane (42). During or soon after virus budding, Pr55(090) is cleaved by viral protease (PR) into four major products: matrix (MA) (p17), capsid (CA) (p24), nucleocapsid (NC) (p7), and the C-terminal p6 domain (42). PR is encoded by pol, which is initially translated as the polypeptide precursor Pr160(090-pol). It is generally believed that Pr160(090-pol) is incorporated into assembling virions via interaction with Pr55(090) through its N-terminal Gag determinants (10, 11, 19, 22, 40, 41). However, some researchers have demonstrated that HIV-1 and murine leukemia virus (MLV) Pol can be packaged into virions at a reasonable efficiency despite an absence of Gag-Pol formation (3, 7). The proteolytic processing of Pr160(090-pol) gives rise to PR, reverse transcriptase (RT), and integrase (IN) in addition to Gag cleavage products. Blocking PR activity does not significantly affect virus particle assembly and release, but it does eliminate viral infectivity (17, 27, 36).

The reading frame of HIV-1 pol partially overlaps that of gag. During Gag translation, a −1 ribosomal frameshift event occurs at a frequency of 5%, resulting in an approximately 1:20 expression level of Pr160(090-pol) to Pr55(090) (24). An artificial overexpression of Pr160(090-pol) or PR-containing chimeric proteins results in a significant reduction in virion release, presumably due to the premature cleavage of Pr55(090) as mediated by PR (1, 20, 28, 35, 38, 48). Accordingly, both the PR expression level and PR activation timing with respect to the proteolytic processing of Gag and Gag-Pol are critical to virus assembly. The molecular mechanism behind PR activation is not entirely clear. It is generally accepted that Pr160(090-pol) dimerization or multimerization triggers PR activation, and therefore, sequences upstream or downstream of PR may affect PR-mediated virus maturation by interfering with Gag-Pol multimerization. Consistent with this suggestion, deletions of sequences upstream of PR (11, 52) or mutations in downstream pol sequences can significantly affect PR-directed virus particle maturation (4, 30, 37).

Biologically active RT is assumed to be present in the form of a p66/p51 heterodimer (12, 31). A hydrophobic cluster consisting of six tryptophan (Trp) residues has been identified in the connection subdomain of the HIV-1 RT subunit (codons 398 to 414). This Trp repeat motif is highly conserved among primate lentiviral reverse transcriptases (2). It has been shown that substitution mutations of HIV-1 RT Trp repeat motif residues can markedly impair RT dimerization in vitro and RT-RT interactions. Although the extent to which the RT domain contributes to Gag-Pol multimerization is unknown, some data support the idea that the RT sequence (the Trp repeat motif in particular) may affect PR activation by favoring Gag-Pol multimerization. First, RT truncation mutations involving the Trp repeat motif significantly impair PR-mediated Gag processing (30). Second, enfuvirenz (EFV)—a nonnucleoside reverse transcriptase inhibitor (NNRTI) that greatly enhances HIV-1 RT dimerization in vitro (46, 47)—is capable of suppressing virion production by enhancing the efficiency of PR-mediated Gag and Gag-Pol cleavage (14, 45). However, a W401A alanine substitution mu-
tation that abrogates RT dimerization in vitro (49) almost completely negates the EFV inhibitory effect on virion production (9). The lack of W401A susceptibility to this inhibitory effect is most likely due to a defect in Gag-Pol/Gag-Pol interaction that impedes the EFV enhancement of Gag-Pol multifunctionalization and consequently nullifies the EFV enhancement of Gag processing.

Based on our previous finding that a W401A-imposed lack of susceptibility to the EFV enhancement effect on Gag processing is partially mitigated by an additional W402 alanine substitution, the W402 mutation may affect the Gag-Pol multifunctionalization required for effective PR activation (9). Here we demonstrate that an alanine or leucine substitution for W402 results in enhanced Gag processing efficiency, which is associated with a dramatic reduction in the released-virion level.

MATERIALS AND METHODS

Plasmid construction. The parental HIV-1 proviral plasmid used in this study was derived from HXB2. RT mutations were generated by PCR-based overlap extension mutagenesis using HIVgpt as a template. RT W401A and W401A/ W402A mutants were as described previously (9). Primers (forward) for engineering the W402A, W402L, and W402F mutations were 5′-GAAAATGGGG AAACCTGGCCAGAGGTAT-3′, 5′-GAAACATGGGAAACATGGTACAGACAGATGAT-3′, and 5′-GAAAATGGGG AAACCTGGCCAGAGGTAT-3′, respectively. The reverse primer was 5′-GAAATGGGATCCAGGCACTGCGTACCTG-3′.

Amplified DNA fragments were digested with a combination of EcoRV and BsrGI and ligated into HIVgpt. The following constructs have all been described in detail in previous reports: HIV-1 RT mutants (W401A and W401A/W402A) (9); Pol-truncated mutants containing inserted stop codons at the designated IN or RT residue positions (RN198, R560, R515, and R425); plasmids expressing the RT subunits p66 and p51, coexpressed with the W402A mutant (Fig. 1C, lane 7 versus lane 8). These constructs were prepared as described previously (8). Numbers of drug-resistant colonies were determined as previously described (9).

Cell culture, transfection, and infection. 293T cells and HeLa cells were maintained in Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum. Confluent 293T cells were trypsinized, split 1:10, and seeded onto 10-cm dishes 24 h before transfection. For each construct, 293T cells were transfected with 20 μg of plasmid DNA by the calcium phosphate precipitation method; 50 μM chloroquine was added to enhance transfection efficiency. For infection, 10 μg of wild-type (wt) or mutant HIVgpt was cotransfected with 5 μg of the vesicular stomatitis virus G (VSV-G) protein expression vector (pCMV5G-Flag), coexpressing IN and/or Myc-tagged p66RT expression vector were collected and subjected to immunoprecipitation analysis as previously described (9). Immunoprecipitate-associated agarose beads were pelleted, washed three times with RIPA buffer, washed two times with phosphate-buffered saline (PBS), eluted with 1X sample buffer with 5% β-mercaptoethanol, boiled for 5 min, and subjected to SDS-10% PAGE as described above.

RESULTS AND DISCUSSION

W402A mutant is severely assembly defective and can transdominantly inhibit virion production. To determine whether an alanine substitution of RT codon W402 results in a phenotype that differs from those resulting from the alanine substitution mutations W401A and W401A/W402A, a constructed W402A mutant and the two other alanine substitution mutants were transiently expressed in 293T cells and analyzed by Western immunoblotting. To our surprise, the W402A transfectants expressed readily detectable Gag but produced barely detectable virus-associate d Gag, suggesting a severe defect in virus assembly or release (Fig. 1A, lane 4, and 1B). Similar results were observed across several repeated, independent experiments. In contrast, both W401A and W401A/W402A transfectants produced virus particles at approximately the wt level and displayed partially degraded or barely detectable virus-associated RT (Fig. 1A, lanes 3 and 5), which is consistent with previously reported results (9, 49). Intracellular Gag and RT were readily detected for the W402A mutant, indicating that it had no major impact on Gag and Gag-Pol expression. Notably, the W402A mutant showed a relatively low cellular Pr55Gag/p24Gag ratio compared to the wt and other mutants (Fig. 1A, upper panel, lanes 7 to 10), implying that the W402A-associated virion production defect may be due to enhanced Pr55Gag cleavage efficiency.

To test whether the W402A mutant can affect virion production by wt or assembly-competent mutants in trans, we coexpressed it with the wt, the W401A/W402A mutant, or an HIV-1 protease-defective (PR−) mutant and observed that virus-associated p24Gag was readily detected when PR− was coexpressed with the W402A mutant (Fig. 1C, lane 7 versus lanes 8 to 9). Virus yields from the wt or mutants were significantly reduced by the W402A mutant in a dose-dependent manner (Fig. 1C, lower panel), with a noticeable decrease in the cellular Pr55Gag/p24Gag ratio (Fig. 1C, lower panel). These data suggest that the W402A mutant can provide functional PR to mediate Gag cleavage in trans and provide support for the assumption that the W402A-incurred virion assembly defect is primarily due to enhanced Gag cleavage efficiency.

W402A virus assembly defect is PR activity dependent. To determine if the W402A virus assembly defect is directly associated with viral protease activity, we treated W402A transfectants with an HIV-1 PR inhibitor (PI). As expected, virus-associated W402A Gag (Pr55Gag, p41Gag, and mature p24Gag) and pol-derived products (IN, RT subunits p66/p51, and RT-associated precursors, such as Pr160gag-pol) that were previously undetectable or barely detectable became readily detectable when PI concentrations were gradually increased (Fig. 2, lanes 5 to 8). Similar results were observed in repeat independent experiments. These data support the assumption that the

papers. After washing and drying, RT activity was determined using a Beckman scintillation counter.

Coimmunoprecipitation assay. 293T cells transfected with the HA-tagged and/or Myc-tagged p66RT expression vector were collected and subjected to immunoprecipitation analysis as previously described (9). Immunoprecipitate-associated agarose beads were pelleted, washed three times with RIPA buffer, washed two times with phosphate-buffered saline (PBS), eluted with 1X sample buffer with 5% β-mercaptoethanol, boiled for 5 min, and subjected to SDS-10% PAGE as described above.
W402A virus assembly defect is dependent on PR activity. Additional verification is the observation that the introduction of a PR-defective mutation (PR−) into the W402A mutant restored virus production to a level comparable to that of the PR− mutant, with expressed Pr55gag and Pr160gag-pol levels comparable to those of the PR− mutant (data not shown).

We then tested the idea that replacement of W402 with another amino acid residue (in this case Leu or Phe) can produce a phenotype similar to that of the W402A mutant. The resulting mutants (designated W402L and W402F) were transiently expressed in 293T cells. We also determined that the W402L mutant phenotype was similar to that of W402A, with virus-associated Gag and RT barely detectable in transfectant supernatant samples (Fig. 3A and B, lanes 3 and 4). Similar to the case with the W402A mutant, the effect of an HIV-1 PR inhibitor on PR activity resulted in substantial amounts of virus-associated Gag and Gag-Pol being released from W402L transfectants (data not shown).

In contrast, the W402F mutant exhibited a Gag processing profile similar to that of the wt and a virus-associated Gag level comparable to that of the wt (Fig. 3B, lane 5). This suggests that an aromatic residue at RT codon 402 plays an important role in preventing premature Gag cleavage. We found that both W402A and W402L, mutants (hereafter referred to as W402A/L) had relatively low levels of cellular Gag, presumably due (at least in part) to enhanced proteolysis mediated by PR. In addition, we consistently noted that the p24gag/Pr55gag ratios of W402A/L mutants were significantly higher than those displayed by the wt and the W402F mutant (Fig. 3D), thus supporting our proposal that the W402A/L mutations triggered enhanced PR-mediated Gag cleavage. Reduced virion release as a result of PR-mediated Gag cleavage has also been ob-
Past studies have confirmed that most amino acid substitution mutations at W398, Y405, or W410 result in a phenotype not shown). Further work is required to determine if substitution mutations at W398, Y405, and W410 have no significant effect on RT dimerization (yeast two-hybrid system and in vitro binding assays) (44). The results of our coimmunoprecipitation experiments suggest that the W402A mutation does not significantly affect p66RT self-interaction (data not shown). Further work is required to determine if substitution mutations at W398, Y405, and W410 result in a phenotype similar to that of the W402A/L mutation.

**Mutations at W402 significantly impair viral infectivity.** Although W402F had a virus-associated p66/p51RT level comparable to that of the wt and exhibited in vitro RT polymerase activity at a level near that of the wt (Fig. 4), according to results from a single-cycle infection assay, its infectivity was reduced 5-fold compared to that of the wt (Table 1). This suggests that the W402F mutation significantly impairs RT biological function during viral replication even though it does not exert any major effects on RT polymerase activity in vitro. In contrast, the very low levels of RT activity demonstrated by the W401A, W402A, and W402L mutants are compatible with their severely impaired infectivity. The relatively low level of virus-associated RT activity for W402A/L may be due in part to a Gag-Pol package defect as a result of premature Gag-Pol cleavage by PR. Consistent with this idea, relatively lower levels of p66/p51RT subunits were found in W402A virions than in wt virions when PR activity was partially inhibited (Fig. 2, lane 6 versus lane 2).

**W402A mutation significantly impairs Gag processing following a deletion in the RNase H subdomain.** Given that the W402A enhancement effect on Gag processing is mediated by influencing Gag-Pol/Gag-Pol interaction, additional C-terminal deletions in the pol sequence may incur a conformational Gag-Pol change and disrupt Gag-Pol/Gag-Pol interaction, thereby mitigating the W402A enhancement effect on Gag processing. We found that virion production for the assembly-competent Gag-Pol-truncated mutants RN198, R560, R515, and R425 (Fig. 5A and 5B, lanes 1 to 5) (30) was markedly reduced following the introduction of the W402A mutation (Fig. 5B, lanes 6 to 10). The one exception was R515/W402A (lane 9), which demonstrated readily detectable virus-associated Gag. Pr55<sup>goe</sup> and p41<sup>goe</sup> were identified as major species in both supernatant and cell samples (lane 4 versus lane 9). Our finding of impaired Gag cleavage accompanied by improved virus production is consistent with our hypothesis that the inhibitory effect of W402A on virion production is primarily due to enhanced Gag cleavage efficiency. The virus assembly defect characteristic of RN198/W402A, R560/W402A, and R425/W402A is also dependent on PR activity, since virus-associated Gag and Gag-Pol become readily detectable in the supernatant following treatment with a PR inhibitor (data not shown).

The W402A/L mutation may exert its virus assembly inhibitory effect in a fashion similar to that following EFV treatment (14)—that is, by triggering premature PR activity via Gag-Pol multimerization enhancement. However, at this point it is difficult to determine whether EFV or the W402A/L mutation enhances Gag-Pol multimerization efficiency, given that Gag-Pol multimerization largely depends on the N-terminal Gag domain and that most RT deletion mutations have no detectable effects on Gag-Pol multimerization (30). Together, the
observations that (i) EFV significantly reduced R515 virion production by enhancing Gag processing (data not shown) and (ii) the W402A mutation markedly impaired R515 Gag processing (Fig. 5) suggest that W402A triggers PR activation in a manner that is similar but not identical to that of EFV. It appears that the maintenance of an appropriate Gag-Pol con-

**FIG. 3.** Effects of W402 substitution mutations on virus assembly and processing. (A to C) 293T cells were transfected with wt or mutant HIVgpt plasmids carrying one of the following RT substitution mutations: W402A, W402L, or W402F. At 48 to 72 h posttransfection, cells and culture supernatant were collected and subjected to Western immunoblot analysis. HIV-1 Gag proteins were probed with an anti-p24CA monoclonal antibody. RT was detected with anti-RT serum. Indicated are positions of Pr160\textsuperscript{gag-pol}, RT p66 and p51 subunits, Pr55\textsuperscript{gag}, p41\textsuperscript{gag}, and p24\textsuperscript{gag}. The lower blot in panel B was derived from the panel A blot by extending the exposure. (D) Effects of the W402A and W402L mutations on Gag processing efficiency. Cellular Pr55\textsuperscript{gag} and p24\textsuperscript{gag} levels were quantified by scanning Pr55\textsuperscript{gag} and p24\textsuperscript{gag} band densities from immunoblots. Ratios of p24\textsuperscript{gag} to p55\textsuperscript{gag} were determined for the wt and each mutant. Bars indicate standard deviations. *P < 0.05.

**FIG. 4.** Relative reverse transcriptase activity levels of HIV-1 RT mutants. 293T cells were transfected with the indicated plasmid. The W401A/W402A double mutant, recognized as being severely defective in terms of RT activity (9), served as a control. At 48 to 72 h posttransfection, supernatant was collected, pelleted, and resuspended in PBS buffer. Equivalent aliquots were subjected to in vitro RT assays and Western immunoblot analyses. Ratios of RT activity to Gag protein levels obtained via immunoblot band density quantification were determined for each mutant and normalized to those of the wt. Values for each construct were derived from at least three independent experiments. Error bars indicate standard deviations.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Titer (CFU/ml)</th>
<th>Infectivity(^c) (%)</th>
<th>Mean infectivity(^c) ± SD</th>
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<tbody>
<tr>
<td></td>
<td>Mutant</td>
<td>Wild type(^a)</td>
<td></td>
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<tr>
<td>W401A</td>
<td>392</td>
<td>10,980</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>590</td>
<td>8,400</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>940</td>
<td>12,320</td>
<td>5.3 ± 2.0</td>
</tr>
<tr>
<td>W402A</td>
<td>48</td>
<td>10,980</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>58</td>
<td>8,400</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>280</td>
<td>12,320</td>
<td>6.0 ± 3.6</td>
</tr>
<tr>
<td>W402L</td>
<td>224</td>
<td>10,980</td>
<td>15</td>
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<tr>
<td></td>
<td>278</td>
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<td></td>
<td>652</td>
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<tr>
<td></td>
<td>272</td>
<td>12,320</td>
<td>11 ± 8.0</td>
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\(^a\) 293T cells were transfected with the indicated plasmid plus a VSV-G expression vector. At 48 to 72 h posttransfection, approximately 50% of the collected supernatant was subjected to Western immunoblot analysis. The remaining supernatants were aliquoted and used to infect HeLa cells. Supernatant amounts used for infections: wt and W402F mutant, 50 μl; W401A, W402A, and W402L mutants, 1 ml. Infection and selection of drug-resistant colonies were performed as described in Materials and Methods. Drug-resistant colonies were converted to titers (CFU/ml). No drug-resistant colonies were detected in the absence of VSV-G coexpression (data not shown).

\(^b\) Wild-type (HIVgpt) titers were determined in parallel experiments. Experiments were performed in triplicate.

\(^c\) Ratios of viral titers to Gag protein levels (obtained via immunoblot band density quantification) were determined for each mutant and normalized to those of the wt in parallel experiments. Mean and standard deviation values for viral infectivity are indicated.
formation is critical to the ability of W402A to trigger premature PR activation.

The roles of tryptophan and proline residues in protein-protein interactions have been well documented (26, 32). It is speculated that the large hydrophobic residue Trp (especially its aromatic side chain) may play a stabilizing role in Gag-Pol conformation and thus prevent accelerated Gag-Pol multimerization. Gag-Pol conformation instability as incurred by W402A virus may propel or accelerate Gag-Pol multimerization, resulting in premature PR activation. This may explain, at least in part, why a Phe substitution at W402 (which also contains an aromatic side chain) has no measurable effect on virus processing and assembly.

In conclusion, our results indicate that the HIV-1 RT W402 codon in the Gag-Pol context may play a crucial role in preventing PR from premature activation by stabilizing the Gag-Pol conformation during Gag-Pol/Gag-Pol interaction. A logical next step is to test whether the inhibition of virion production by a single amino acid substitution in the Trp repeat motif residue is also applicable to other primate lentiviruses. Our results point to a potential HIV-1 RT Trp repeat motif target in HIV/AIDS therapeutic interventions.

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