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Mutations in human immunodeficiency virus type 1 reverse transcriptase that make it sensitive to degradation by the viral protease in virions are selected against in patients

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ABSTRACT

Mutations in the thumb subdomain of reverse transcriptase (RT) of HIV-1 can cause this enzyme to be degraded in virions by the viral protease (PR). Many of these mutations confer a temperature-sensitive phenotype on RT and viral replication. The degradation of RT by PR appears to take place after Gag-Pol has been processed. We show here that mutations in other parts of RT, including the RNase H domain, can make RT PR-sensitive and temperature-sensitive. These data explain why some mutations in the RNase H domain, which had little or no effect on the polymerase activity of purified recombinant RT, had a profound effect on viral titer. Because the PR-sensitive phenotype significantly reduced viral titer, we previously suggested that these mutations would be selected against in patients. We also show that RT mutations that are known to confer a temperature sensitive phenotype are rarely found in the Stanford database.

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Introduction

The reverse transcriptase (RT), protease (PR), and integrase (IN) of HIV-1 are the three essential virally encoded enzymes that are the primary targets for anti-HIV drug therapy. RT carries out the conversion of the single-stranded RNA genome found in virions into a double-stranded DNA form that is inserted into the host genome by IN. This conversion requires both of the enzymatic activities of RT; a DNA polymerase that can copy either an RNA or a DNA template, and an RNase H that cleaves RNA if (and only if) it is part of an RNA:DNA duplex. Although there are two broad classes of drugs that block the reverse transcription of the viral RNA genome, both block the polymerase step. One class includes the nucleoside analogs, or NRTIs, all of which lack a 3' OH and, when incorporated by RT, block the synthesis of viral DNA. The second class, nonnucleoside RT inhibitors (NNRTIs), bind in a hydrophobic pocket of RT, about 10 Å from the polymerase active site. This binding distorts RT in a way that pushes the end of the viral DNA away from the polymerase active site, preventing polymerization (Das et al., 2012).

Unfortunately, HIV-1 has been able to develop resistance to all of the available drugs, including all of the NRTIs and NNRTIs. Thus it is important to understand the limitations of the variability of HIV-1 RT, because it is the variability of the viral components that are drug targets that underlies the ability of the virus to develop drug resistance. In this sense, it is important to understand not only the mutations that arise in RT in response to drug selection, but also to try to better understand why other changes in RT are not commonly seen.

As part of an analysis of the RNase H domain of HIV-1 reverse transcriptase (RT), we made alanine substitution mutations to some of the residues in RNase H that helps to properly position the nucleic acid heteroduplex for cleavage: the RNase H primer grip (Julias et al., 2002). Three single mutations, T473A, Q475A, Y501A, and a double mutation, N474A/Q475A, all had a substantial impact on the ability of a viral vector to replicate in a one round assay. The T473A mutation blocked viral replication and the Q475A, Y501A, and N474A/Q475A mutations reduced the titer 5–10 fold (Julias et al., 2002). The ability of viral vectors carrying either the Y501A mutation, or the N474A/Q475A double mutation, to synthesize viral DNA was measured in infected cells; both of these mutant vectors made much less viral DNA than the corresponding WT vector. Moreover, for both of these mutants, the magnitude of the defects seen in the synthesis of RU5, U3, *gag*, and plus-strand transfer DNA (late stage viral DNA) were similar, suggesting that a problem occurred early in viral DNA synthesis, perhaps during the initiation of minus-strand DNA. However, analysis done in parallel with purified recombinant RTs showed that all four of these

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mutant RTs were able to synthesize DNA at levels, comparable to WT RT, using a DNA primer and a DNA template, and that they were also approximately equivalent to WT in a polymerization assay using an RNA template and a tRNA lys3 primer that was designed to mimic the initiation of minus strand DNA synthesis (Rausch et al., 2002). Although it was possible, as we suggested, that the conditions in the virion core made it particularly difficult for these RT mutants to initiate viral DNA synthesis in an infected cell, the behavior of the purified mutant RTs did not match, or explain, the defect in the ability of viruses carrying these same mutations to replicate. Why did these mutant RTs appear to behave differently in vitro and in infected cells?

During the maturation of the virus, the viral protease (PR) cleaves a variety of sequences within the Gag and Gag-Pol polyproteins. However, not all of the sequences in these polyproteins are treated equally by PR. To cite an obvious example, in the maturation of HIV-1 RT, there is only limited processing of a site in HIV-1 RT near the boundary between the polymerase and RNase H domains. HIV-1 RT is a heterodimeric enzyme. The two subunits, p66 and p51, share a common N-terminus; however, the larger subunit is 560 amino acids long, and the smaller subunit is 440 amino acids long. Thus, only half of the RT precursors are cleaved by PR to produce p51. The cleavage site used to produce the p51 subunit, which is present in the p66 subunit, is not cleaved in p66, suggesting that the structure (folding) of the RT subunits can affect whether a site is cleaved or not. This idea is supported by a scan of the sequences in the p66 subunit, which suggests that there are additional sites in RT that could be cleaved if PR had access to them, and by experiments which showed that RT could be degraded by PR at low pH (Chou et al., 1996; Tomasselli et al., 1993). These data support the idea that PR might degrade an unfolded, or partially unfolded, RT much more extensively than the properly folded heterodimeric enzyme.

Both we and others have shown that there are mutations in HIV-1 RT that allow it to be extensively degraded in virions by the viral PR (Dunn et al., 2013, 2009; Huang et al., 2003, 1998; Takehisa et al., 2007; Wapling et al., 2005; Wang et al., 2010). Although the degree to which RT is degraded can vary, depending on which mutation is present, the mutations that have a profound effect on the amount of intact RT in virions also have a profound negative impact on the ability of the virus to replicate. Most of the protease-sensitive mutations that we previously described are in the thumb subdomain (Dunn et al., 2013, 2009). Many of these protease-sensitive mutations also caused viral replication (and the underlying degradation of RT) to be temperature sensitive (TS). In a one-round assay, the mutant viruses replicated much better at 32 °C than at 37 °C. In virions carrying the TS RT mutants that were grown at 37 °C, there was, judged by a western blot, less intact RT than in WT virions, suggesting that the higher temperature allowed RT to partially unfold, exposing sites that could be cleaved by PR. In some cases, when the virions that carried the mutant RTs were grown at 37 °C, they contained proteolytic fragments of RT. Although RT was degraded (or partially degraded) in the mutant virions, the amounts of full-length PR and IN were normal, or nearly normal. Thus, the degradation of RT by PR appeared to take place after the initial processing of Gag-Pol.

We purified recombinant forms of some of the mutant RTs and showed that the mutant RTs were partially unfolded at the non-permissive temperature (Dunn et al., 2013). Parallel analysis of the mutant RTs in virions showed that the mutant RTs were degraded by the viral PR. The polymerase domains of both the p51 and p66 subunits are encoded by the same segment of the viral genome. Thus, any mutation that arises in the polymerase domain will be present twice in the mature heterodimeric enzyme, once in the large subunit (p66) and in once the small subunit (p51). We prepared recombinant forms of RT that had TS mutations present in only one of the two subunits, and showed that having the amino acid change in either p66 or p51 contributed to the propensity of RT to partially unfold at a the higher (non-permissive) temperature (Dunn et al., 2013).

In the experiments described here, we were particularly interested in the three RNase H primer grip mutants, not only because these mutants had a change in only one of two subunits of RT (p66), but also because these mutants represented particularly well-characterized examples in which data obtained with purified recombinant RTs and viral vectors carrying the same mutations did not appear to agree (Julias et al., 2002; Rausch et al., 2002). We show here that the RNase H mutants have a TS phenotype and that these mutant RTs are degraded in virions at 37 °C. That is similar to the PR-sensitive thumb mutants we previously described. Because most of the mutants we previously analyzed were in the thumb subdomain, we also analyzed some additional mutants that have changes in the fingers and palm subdomains, and showed that they also have a TS phenotype, and that this phenotype is linked to the degradation of RT in virions. We tested the effects of these additional RT mutations, both in the RNase H domain and in the polymerase domain, on the processing of a Pol polyprotein expressed in *E. coli*, and used this system to show that these mutations in RT directly affect its susceptibility to the viral PR, outside of the context of the virion. This shows that mutations that cause HIV-1 RT to become PR-sensitive can occur in the RNase H domain, and in several of the subdomains of the polymerase domain. These data confirm and extend our previous observations, showing that mutations in any part of HIV-1 RT can affect the susceptibility of RT to PR.

Because mutations that make RT PR-sensitive can have a profound effect on viral replication, we previously proposed that these mutations can constrain the spectrum of amino acid changes that HIV-1 can tolerate in RT (Dunn et al., 2009). In support of this idea, we analyzed the frequencies at which the mutations that make RT susceptible to PR appear in viruses in patients, using the Stanford database. As we predicted, the PR-sensitive mutations in RT make up only a small fraction of the amino acids found at their respective positions in RT.

Results and discussion

Mutations in the fingers and palm subdomains and in the RNase H domain of HIV-1 RT can lead to the proteolytic degradation of RT in virions

We began by investigating the effects of three mutations in a portion of RNase H called the primer grip: T473A, Q475A, and Y501A, on the stability of RT in virions. The positions, in HIV-1 RT, of the amino acids where mutations caused RT to become sensitive to PR are shown in Fig. 1. Virions were prepared from cells grown at 37 °C; the amount of intact RT was monitored by doing western blots. We also tested one double mutant in the primer grip, N474A/Q475A. As described in the Introduction, all of these mutations had been shown to interfere with the ability of the virus to initiate viral DNA synthesis, but none of the mutations had much effect on the polymerase activity of the corresponding purified recombinant RTs (Julias et al., 2002; Rausch et al., 2002). All four of these RNase H mutants showed signs, in a western blot, of RT degradation in virions (diminished levels of RT, and/or the presence of proteolytic fragments of RT, see Fig. 2). A western blot, performed for the virion protein capsid (CA), showed that the approximately same amount of virions were present in the lysates that were used in the western blots of RT, and that Gag was appropriately processed by PR. There was some variation in the amount of p55 Gag, and a Gag processing intermediate, in some of the samples. However, there