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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

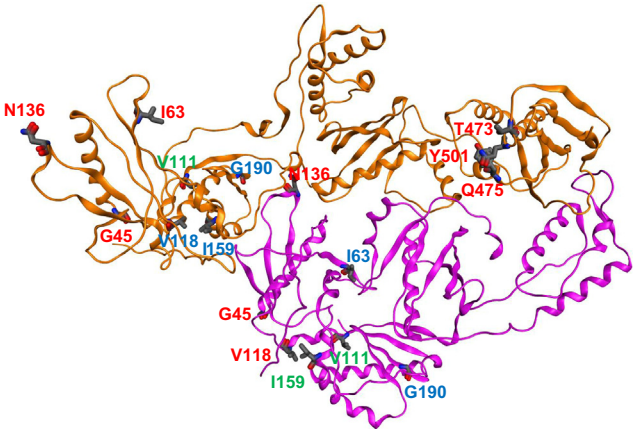


Fig. 1. The positions, in HIV-1 RT, of the amino acids where mutations caused RT to become sensitive to PR. The figure shows a ribbon diagram of the mature HIV-1 RT heterodimer. The larger subunit, p66, is shown in orange, the smaller subunit, p51, is shown in purple. The side chains of the amino acids of interest are shown. The labels of the amino acids are color coded: Red is surface exposed, blue is buried, and green is semi-buried. With the exception of the three amino acids in the RNase H domain (T473, Q475, and Y501), the amino acids are present in both subunits.

was also some variation in the amount of p55, and the processing intermediate, in the various WT controls used in the blots (Fig. 2).

We also analyzed the effects of two mutations in the fingers, G45L and I63S, both of which appeared to have, in virions, lower amounts of at least one of the RT subunits than WT (Fig. 2). In contrast, the drug resistance mutation L74V, which is also in the fingers, did not appear to cause any increased degradation of RT (data not shown). Of the mutations we analyzed in the palm subdomain, three, V111S, N136Q, and I159S, appeared to lead to a partial degradation of RT in virions (Fig. 2); however, virions containing RTs carrying the palm subdomain mutants Q91A, L92A, G93A, and P157A, appeared to have normal, or nearly normal amounts of undegraded RT (data not shown). We also analyzed one additional mutation in the palm (G190E) that is known to affect the susceptibility of the virus to nonnucleoside RT inhibitors (NNRTIs); RTs carrying this mutation were previously shown to be degraded in virions (Huang et al., 2003).

Based on the results of the analysis of the mutations in the thumb of HIV-1 RT, we expected that many of the protease-sensitive RT mutants would be TS. All of the mutants that showed signs of RT degradation were tested for their ability to infect cells with viruses grown at either 37 °C or at 32 °C (Table 1). With the possible exception of the G45L mutant, for which the difference in the infectivity of the viruses at the two temperatures is relatively small, all of the viral mutants which showed signs of RT degradation were TS for replication. Because the Q475A mutant was TS, we did not test the N474A/Q475A double mutant. The effects of reducing the temperature on the ability of the virus to replicate were, in some cases, quite dramatic, particularly for the mutants that showed the greatest amounts of RT degradation when the

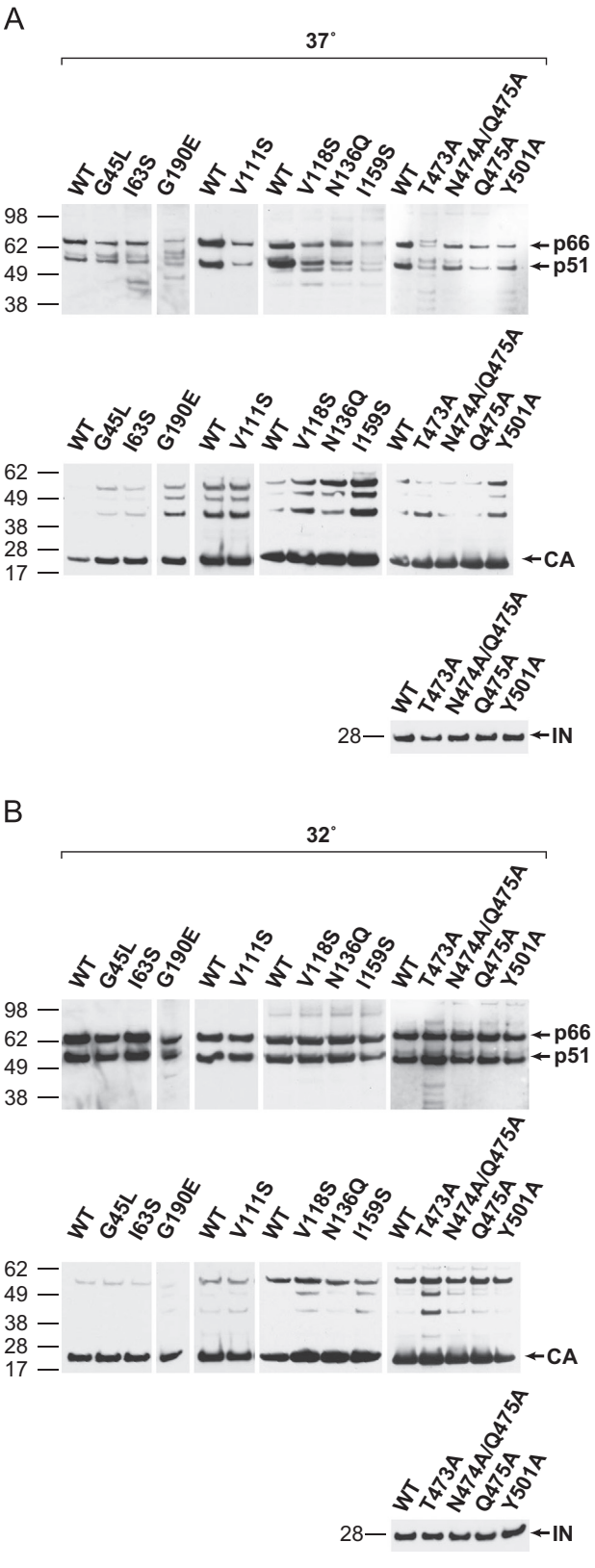


Fig. 2. The HIV-1 RT mutants are more extensively degraded in virions at 37 °C than at 32 °C. Virions were harvested from cells grown at either 32 °C or 37 °C (see Methods). The virions were collected by sedimentation, lysed, and the proteins fractioned using SDS-PAGE. The proteins were transferred to nitrocellulose, and the blots were probed with antibodies that react with HIV-1 proteins. Panel A. Western blots from virions grown at 37 °C. In the portion of the experiment shown at the top, the blots were probed with a mixture of monoclonal antibodies that react with HIV-1 RT. The positions of migration of molecular weight markers are indicated on the left; the positions where the p66 and p51 subunits migrate are indicated on the right. The panel shows data from four different blots; there is a separate WT control for the data from each of the four blots. In the center of the panel is a western blot done with anti-CA antisera; this serves as a loading control and shows that PR was able to properly process Gag. At the bottom of the panel is a blot done with anti-IN antisera. To do the analysis of the processing of IN and CA, the RT blots were stripped and reprobed with the appropriate antibodies. Panel B. The western blots correspond to the western blots shown in Panel A, except that the virions were grown at 32 °C. As in Panel A, there were four separate blots done. For all of the mutants, and all of the blots, the 37 °C and 32 °C samples were processed together. The western blots were repeated three times, and representative data are shown.

Table 1
Mutant RTs lead to a temperature sensitive phenotype for viral replication. The table lists the mutants, their location within RT, and the relative titer at the permissive and non-permissive temperatures. The relative titers of the mutants are expressed as the percentage of the relative titer of the WT.

Mutant	Location (p66/p51)	Relative Titer 37° (SD)	Relative titer 32°(SD)
G45L	Fingers (surface exposed; surface exposed)	61.6 (23.5)	80.0 (14.7)
I63S	Fingers (surface exposed; buried)	1.5 (0.8)	88.0 (11.8)
V111S	Palm (semi-buried; semi-buried)	30.6 (10.6)	65.0 (24.6)
V118S	Palm (buried; surface exposed)	1.3 (0.5)	55.3 (4.5)
N136Q	Fingers (surface exposed;buried)	1.5 (1.1)	89.0 (15.6)
I159S	Palm (buried;semi-buried)	2.0 (1.6)	70.6 (21.1)
G190E	Palm (buried;buried)	2.7 (1.4)	84.0 (16.1)
T473A	RNase H (surface exposed)	2.5 (1.5)	80.0 (2.4)
Q475A	RNase H (surface exposed)	9.6 (2.6)	71.3 (19.5)
Y501A	RNase H (surface exposed)	12.2 (4.5)	98.0 (2.8)

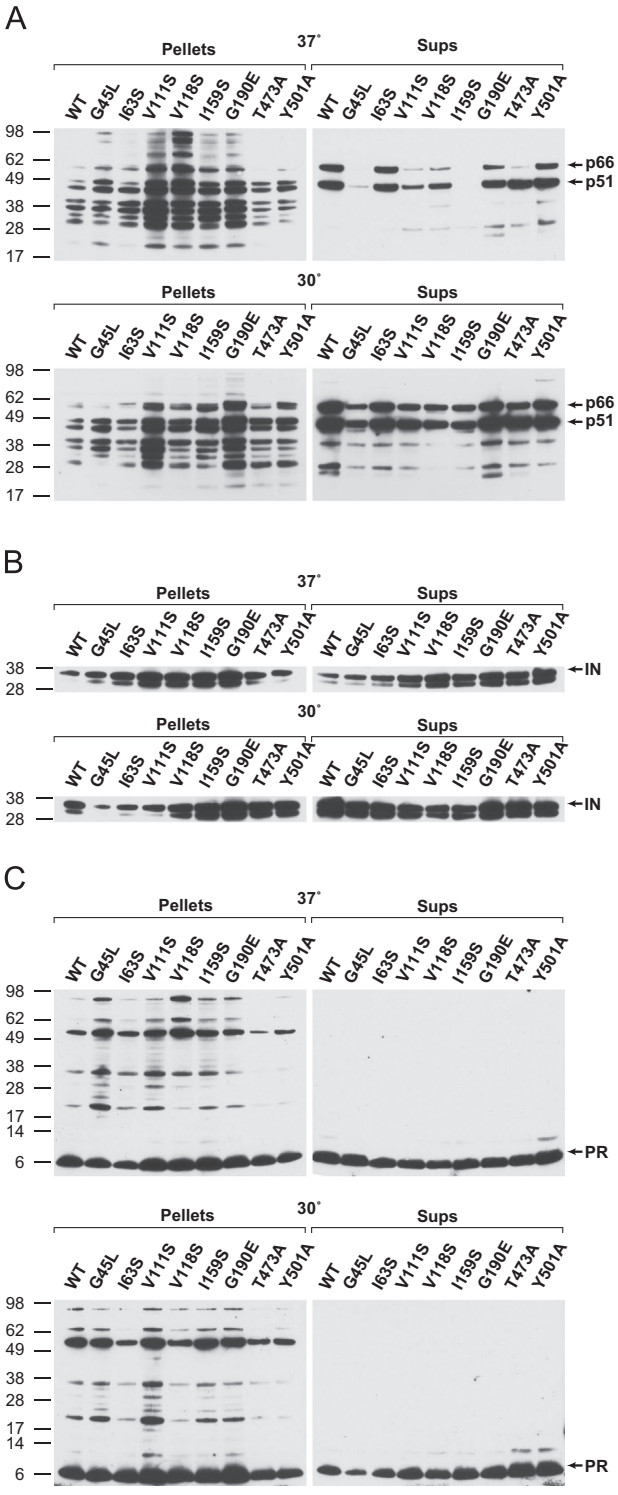
virions were grown at 37 °C (for example I63S, G190E, and T473A). As would be expected, based on the increase in viral titer at the permissive temperature, there was also an increase in the amount of undegraded RT in the virions when the mutant virions were grown at 32 °C. Conversely, G45L and V111S had much more modest effects on both stability and titer. There were two mutations (V118S and N136Q), both of which had a strong effect on the titer at the non-permissive temperature, that appeared to have a more modest effect on the amount of intact p66 and p51 in virions. However, it should be remembered that multiple RTs must cooperate to complete viral DNA synthesis and that reducing the amount of polymerase activity in virions by more than about half had a profound impact on viral titer (Julias et al., 2001). When the mutant viruses were grown at 32 °C, only the RT that carried the T473A mutation still showed clear signs of proteolytic degradation, but there was, for this mutant RT, a substantial increase in the amount of both intact p66 and intact p51 that was present in virions (Fig. 2).

We previously showed, for a number of other RT mutants that had a TS phenotype, that the proteolytic processing of Gag-Pol was reasonably normal, in terms of the maturation of both PR and IN (Dunn et al., 2009). Because the RNase H mutations were much nearer IN than any of the mutations we previously analyzed, we checked the effects of the three single RNase H mutations (T473A, Q475A, and Y501A) and the double mutation (N474A/Q475A) to see if the virions containing these RT mutations also contained the normal amount of processed IN. As judged by western blot, when virions containing each of these mutations in RT were grown at either 37 °C or at 32 °C, the virions contained normal, or nearly normal, amounts of processed IN (Fig. 2).

Fig. 3. The mutant RTs are degraded when Pol is expressed in *E. coli* at 37 °C; IN and PR are appropriately cleaved form Pol. Pol polyproteins that contained either WT or mutant RTs were expressed in *E. coli*. The bacteria were grown at either 37 °C (top), or 30 °C (bottom) and collected by centrifugation. The bacteria were lysed and the lysates separated into insoluble and soluble fractions. Aliquots were denatured in SDS, fractionated on SDS-PAGE, transferred to nitrocellulose, and Western blotted with antibodies that could detect RT (Panel A), IN (Panel B) and PR (Panel C). In the IN blot, the upper band corresponds to full length IN and the lower band corresponds to an *E. coli* protein detected by the rabbit polyclonal IN antiserum. The blots for RT (Panel A) and IN (Panel B) gave dramatically stronger signals so the exposure shown in the figure for panel A was made using an additional film to reduce the signal. The exposure was 20 s. One additional film was used to reduce the signal for Panel B and the exposure was for 2 min. The exposure for panel C had no additional blocking film and was for 66 min. The blots were initially reacted with the anti-PR antibody then were stripped and re-probed with the anti-IN antibody. The blots were stripped a second time and re-probed with the anti-RT antibodies. The Western blot data shown in Fig. 2 for the three antibodies (to IN, PR, and RT) were done with a single batch of *E. coli* extracts. The experiments were repeated four times, with different extracts, and representative data are shown in the figure.

The HIV-1 RT mutants that are PR-sensitive in virions are also PR-sensitive when expressed in E. coli.

We previously described an *E. coli* system, based on the expressing of the Pol portion of the HIV-1 Gag-Pol polyprotein (Dunn et al., 2013) that can be used to monitor the expression and proteolytic processing of the RT, PR, and IN proteins; the system we use is similar to one developed earlier by Wrobel et al. (1998). The synthesis of the Pol polyprotein can be induced in bacteria that are being grown at either 30 °C or at 37 °C. This makes it



possible to compare the processing of Pol and the stability of RT in the presence of PR at these two temperatures. We used 32 °C and not 30 °C for the experiments in which we measured the amount of intact RT in virions only because we were not able to grow the human cells at 30 °C. After the Pol proteins have been expressed for two hours, the bacteria are collected, washed, and lysed as described previously (Dunn et al., 2013) with the exception that 50 mM DTT was added to all lysis solutions. The lysate was separated into a soluble and an insoluble fraction by sedimentation. Both the soluble and the insoluble fractions were treated with SDS and the proteins separated by SDS-PAGE; the Pol proteins (PR, IN, and RT) were detected by western blot. Using this system, we previously showed that the WT Pol polyprotein is appropriately expressed and processed by PR at both temperatures (Dunn et al., 2013); that result was reproduced in the experiments we report here. We also showed, in this E. coli expression system, that PR is responsible for degrading the protease-sensitive RT at the non-permissive temperature. The mature forms of WT RT, PR, and IN can be seen in the soluble fraction of the *E. coli* lysates at both 30° and 37° (Fig. 3). As is the case in virions, PR carries out the initial processing of the Pol proteins that contain the protease-sensitive mutations in RT at both the permissive and nonpermissive temperatures, judged by the presence, in the soluble portion of the bacterial extracts, of the mature forms of IN and PR. About half of the mature IN is insoluble when Pol is expressed and processed in *E. coli*; this matches what we reported previously. A portion of the processed PR is insoluble, and in the bacteria, some of the insoluble PR became crosslinked-giving rise to the dimers, trimers, tetramers, and some larger forms that were seen in the gels; this was also seen previously in the experiments done with WT RT and other RT mutants.

With some exceptions, which are discussed below, there is good agreement between the impact of the mutations on the stability of RT in virions, and in the E. coli expression system. With the possible exception of I63S, all of the RTs that were protease sensitive in virions also showed clear signs of instability at 37 °C in the *E. coli* expression system. For example, one of the mutations that had the greatest impact on RT stability in virions (I159S) also had the greatest impact on RT stability in the *E. coli* expression system. Several of the other mutations that had a profound effect on the titer at 37 °C also had an obvious effect on the stability of RT in the *E. coli* system (V111S, V118S, and T473A) When we looked at the impact of the mutations in the RNase H domain, which are present only in the p66 subunit, we found that the Y473A mutation primarily destabilized the p66 subunit in the *E. coli* Pol expression system. However, the Y473A mutant did not have an obvious selective effect on the stability of the p66 subunit in virions. Moreover, there were other mutations in RT (V111S and V118S) that also seemed to have greater impact on the stability of p66, relative to p51, in the *E. coli* expression system; these same mutations did not appear to selectively sensitize the p66 subunit to degradation by PR in virions. The most obvious disconnect between the *E. coli* stability data and the virion data involved G45L and I63S. Looking at the residual titer at 37 °C (Table 1), it would appear that the I63S mutation had the greater impact on RT, although a western blot of lysates of virions carrying either of these mutants grown at 37 °C showed evidence of intact p66 and p51. However, in the *E. coli* system, the G45L mutant was much less stable at 37 °C than was I63S. Because these samples were run side by side in the virion experiments and the *E. coli* expression experiments, we checked the sequences of all of the underlying plasmids, and repeated all the experiments. The results matched the data shown in Figs. 2 and 3 and in Table 1. There are several possible explanations for these discrepancies. First, we are comparing the processing of Gag-Pol and Pol, and the interior of the virion and *E. coli* are quite different. In addition, the experiments were done with two slightly different versions of RT. In the virions, the RT was derived from NL4–3; the *E. coli* Pol was derived from BH10. However, the only difference in these RTs is that, in BH10, position 529 is a V, and in NL4–3 it is an I. Thus, with some specific exceptions, there is reasonably good agreement between the impact of the mutations on the stability of RT in virions, and in an *E. coli* expression system that produces PR and RT as part of a Pol polyprotein. This strengthens and confirms our previous conclusion that the primary factor that determines how sensitive the various mutant RTs are to degradation by PR is the degree to which the individual RTs are unfolded at 37 °C.

PR-sensitive mutations are found both on the surface of RT, and buried in the protein.

In our previous analysis of PR-sensitive mutations in the thumb subdomain, most of the mutations that caused RT to become PR (and temperature) sensitive were in residues that were buried in the thumb (Dunn et al., 2009). In many cases, the PR-sensitive mutations in the thumb involved the substitution of a hydrophilic side chain for a hydrophobic side chain. The PR-sensitive phenotype is a consequence of the mutant RT unfolding at a lower temperature than WT RT, which makes it susceptible to degradation by PR. It is not surprising, therefore, that introducing a hydrophilic residue into a buried hydrophobic region of both of the subunits of RT tended to make it less stable, and more readily degraded by PR (for example I257T, L264S, I274T, L279S, L310S). There were mutations (L289A, A299L) in which substituting one hydrophobic residue for another gave a similar phenotype. We also showed that there were hydrophilic residues that could be mutated to cause protease sensitivity (R78K, N265D, D256N, E302Q). With the exception of R78K, these changes involved the substitution of an amino acid with an acidic side chain with one that has a basic side chain, or vice-versa. Some of the mutations that led to protease sensitivity also either increased the size of the side chain (G152A, G285A), or could have affected the preferred orientation of the amino acid backbone (P150G), all of which could interfere with the proper folding of RT.

Several of the additional mutants we describe here, which are in the RNase H domain or subdomains of the polymerase domain of RT other than the thumb, fall into these same groups as were seen with the thumb mutants: for example, hydrophobic to hydrophilic (I63S, V111S, I159S). Although the G190E mutation causes a substantial increase in the size of the side chain, it is likely, based on the properties of other G190 mutants (for example, G190A and G190S) (Huang et al., 2003) that it is the introduction of a charged residue into an interior portion of RT that is largely hydrophobic, not the size of the side chain of the new amino acid, that causes the G190E mutant to be PR sensitive. However, the T473A, T475A and Y501A mutations are surface residues, which are present only in the p66 subunit. In the WT enzyme, these residues interact with the primer strand, and help to position an RNA/DNA duplex for proper processing of the RNA strand by RNase H. The fact that substitutions of these residues can make RT PR-sensitive broadens the types of mutations that can confer this phenotype. Not only do mutations located at positions either buried in one or both of the subunits of the protein, mutations in residues that reside on the surface can also confer PR-sensitivity. Moreover, a variety of different types of amino acid substitutions can confer this phenotype.

There are extensive opportunities for mutations to arise in RT in patients

Errors are made during the replication of HIV-1 in cultured cells at a frequency of approximately 10^{-5}; this number is based on experiments that were done in cells that do not appear to express significant amounts of the APOBEC proteins that are

known to cause additional mutations in the HIV genome (Abram et al., 2010; Mansky and Temin 1995; Mansky et al., 2003). Setting aside the possibility that the host APOBEC proteins could increase the overall error rate, this means, when the virus replicates, that there is approximately one mutation in every 10 newly synthesized viral genomes. Based on an analysis of mutations that arise in one round of replication in a reporter gene carried by an HIV vector, all mutations are not equally likely to occur. For example, the majority of the mutations are base substitutions, and transitions occur more frequently than transversions. Errors occur much more frequently at some positions than at others. However, it has been estimated that, in untreated patients, approximately 10^9 new cells are infected every day (Ho et al., 1995; Wei et al., 1995). Assuming that the overall mutation rate for HIV is similar in patients and in cells in culture, this suggests that, in patients, approximately 10^{10} new viral mutants are generated every 3 months. Thus the virus has, in patients, the opportunity to broadly sample the genetic space that is available to it in a relatively short time. This accounts for the fact that, even though the majority of people are infected by a single virus, within a few years, the single virus that initiates the infection evolves into a swarm, and when the RNA genomes in the blood of patients in whom the virus has been allowed to replicate for several years are sequenced, the sequences are all different.

However, despite the fact that the sequences of these viral genomes are all different, only a small subset of the changes that could have occurred (and probably did occur) in the viral genome are frequently seen in patients. The changes that are seen in patients are, in general, those that have a relatively modest impact on the ability of the virus to replicate. In some cases, specific sequence changes are selected for, either because there is selective pressure on the sequence of one (or more) of the viral proteins from the host's immune system and/or from drug therapy. Generally speaking, mutations that are not seen frequently have a significant deleterious effect on viral replication. For example, deletions and insertions in regions encoding the viral proteins, particularly deletions and insertions that cause frameshifts, are relatively rare. In thinking about the viral sequences that are well-conserved, there are portions of the viral proteins that are essential for their proper function; to cite a trivial example, the catalytically critical residues of the active sites of the three viral enzymes, RT, IN, and PR are all quite well conserved. In addition, there are conserved regions of the viral genome that do not encode proteins but that have functions that are essential for viral replication.

The protease-sensitive mutations we identified in RT are rarely seen in patient samples

Although a substantial fraction of the mutations in RT that are rarely seen in patients have a strong negative impact on one or both of the enzymatic activities of RT, it now appears that mutations in RT that make it protease-sensitive in virions can also limit the mutational changes that are available to the virus. We looked at the protease-sensitive mutations in RT we report here, and determined both the fraction of the sequences in the Stanford database that have any amino acid change at the position of interest, and also the fraction of the mutations at each of the positions of interest in which the mutation matched the specific protease-sensitive substitutions we studied. It is of course possible that some of the other amino acid substitutions that could have arisen at the position of interest could also be protease-sensitive. The fraction of the total sequences that are mutant is low for most of the positions (45, 63, 111, 118, 136, 159, 473, 475 and 501). Only at positon 190, a position where NNRTI therapy selects for mutations, is the ratio of sequences that are mutant to those that are WT above 1–100 (0.067). Moreover, in most cases, not only is the overall fraction that is mutant low, but, in addition, the percentage of the sequences that carry any mutation at the position of interest that is the identified protease sensitive mutation is also quite low. This percentage can be zero (these mutations that we identified as protease-sensitive were never seen in patients: V111S, V118S, I159S, Q475A, and Y501A); it can be less than 1% of the mutations at the position of interest: G45L, I63S, N136Q, and T473A and it is 2.4% for G190E. There is only one case (T473A) in which a large fraction of the substitutions at the designated position are the identified protease-sensitive mutation (40%). As has already been noted, having any amino acid substitution at position 473 is quite rare; overall the percentage of viruses in the Stanford database that carry this specific protease-sensitive mutation is approximately 0.1%.

It is possible that the fact that some of the protease-sensitive mutations would take two nucleotide changes could have some effect on the frequency at which they arise. However, as has already been discussed, when HIV replicates in patients, it has an opportunity to sample the available mutational space in a relatively short time. This idea is supported by the fact that there are well-known drug resistance mutations that require two nucleotide changes. For example T215Y, which is a primary mutation for AZT resistance, arises frequently when patients were given AZT monotherapy. In considering that result, it is important to remember that, even if the drug is given alone, it still reduces the overall replication of the virus, which will reduce the viral load, and the virus's ability to sample amino acid substitutions in RT. Thus, it is likely that the virus has a better opportunity to test the amino acid substitutions in RT that require two nucleotide changes in the absence of drug therapy than in the presence of drug therapy. Because the T215Y mutation is readily selected by AZT monotherapy, it is likely that other amino acid changes that require two nucleotide changes arise in the absence of drug therapy.

The results obtained with the G190E mutation are instructive for two reasons. First, because it is a mutation that can confer resistance to NNRTIs, it is a mutation that would be selected during treatment with this class of drugs. However, the impact of this mutation on the stability of HIV-1 RT appears to limit the frequency with which it is selected in patients. There are other amino acid substitutions at position 190 that are known to confer NNRTI resistance (for example, G190A and G190S) that are seen much more frequently. Because the selection pressure for NNRTI resistance is strong, the limitation on the selection of G190E is not absolute. However, the RTs that carry the G190E mutation have been reported to carry a number of additional changes that appear, collectively, to be able to compensate for the presence of the G190E mutation (Huang et al., 2003). We looked at the Stanford database to see if we could find mutations that were present much more frequently in the presence of the G190E mutation than in its absence. Although there were a number of mutations that appeared frequently (mutations that were present in $>33\%$ of the sequences in the database that carried the G190E mutation), all of these substitutions were also seen quite frequently in the absence of the G190E mutation; thus all could be classified as polymorphisms, not mutations (Table 2). However, of the 11 polymorphisms that were on the on the list we derived from the Stanford database, 6 (K122E, T200A, R211K, A272P, T286A, and E297K) were listed as compensatory mutations in the RT of a patient derived virus that carried the G190E mutation. This virus had, relative to WT, a reduced, but significant, residual replication capacity that was much greater than an RT that carried only G190E (Huang et al., 2003). The data reported by Huang et al, and to a lesser extent, the data in the Stanford database, are heavily biased towards the amino terminal two-thirds of the RT coding region, and there might be additional polymorphisms/mutations that

Table 2
Prevalence, in the Stanford database, of the mutations that make RT sensitive to PR. The table lists the number of WT and mutant sequences for the positions of interest in the Stanford database, and also lists any mutations that are frequently seen in conjunction with the mutation of interest. SSS means small sample size.

Mutation	Location of the mutation	Number of WT sequences at that position in the Stanford database	Total number of mutant sequences at the position in the Stanford database	Ratio of mutant to WT sequences at the position	Percent of mutations at the position that are the specified change	Other mutations associated with the specified change (> 33.3%)
G45L	Fingers	100,856	292	0.0029	0.3%	SSS
I63S	Fingers	101,165	143	0.0014	0.7%	SSS
R78K	Fingers	101,137	222	0.0022	79.3%	V35T (50.6%) K122E(51.7%) Q174K (34.7%) D177E (47.2%) T200A (43.2%) R211K (48.3%) V245Q (36.6%) A272P (41.8%) T286A (68.5%) P294T (33.7%) G335D (63.5%) R356K (54.0%) M357K (40.0%) G359S (62.0%) I375V (35.6%) A400T (44.7%) T403M (45.7%)
		101,137	222	0.0022	79.3%	SSS
V111S	Palm	100,456	910	0.0091	0.0%	NA
V118S	Palm/ Fingers	92,569	8796	0.0095	0.0%	NA
N136Q	Fingers	101,226	163	0.0016	0.6%	SSS
V148S	Fingers/ Palm	101,193	190	0.0019	0.0%	NA
I159S	Palm	100,764	605	0.0060	0.0%	NA V35T (39.3%) K122E (64.4%) D177E (36.2%) T200A (40.8%) R211K (45.4%) A272P (60.8%) T286A (40.4%) E297K (39.4%) M357T (32.0%) T386I (37.5%) A400T (50.0%)
G190E	Palm	94,931	6383	0.067	2.4%	SSS > 400
D256N	Thumb	61,831	83	0.0013	4.8%	SSS
L264S	Thumb	57,263	47	0.0008	4.3%	SSS
I274T	Thumb	55,320	563	0.010	1.4%	SSS
L279S	Thumb	55,047	280	0.005	0.4%	SSS
L310S	Thumb	34,666	402	0.012	1.2%	SSS
T473A	RNase H	5679	15	0.0027	40%	SSS
Q475A	RNase H	5687	2	0.0004	0.0%	NA
Y501A	RNase H	5333	5	0.0094	0.0%	NA

would appear on both lists if we had more complete information. One simple explanation for the prevalence of these mutations/polymorphisms in the Stanford database is that some or all of these mutations, and perhaps others that are normally thought of as polymorphisms, increase the stability of RT, allowing it to carry mutations that would otherwise make it PR-sensitive. However, if this is the correct explanation, it would appear that no single polymorphism is sufficient, and that several changes must collaborate to compensate for the effects of a PR-sensitive mutation like G190E.

Despite the possibility that there are compensatory mutations that can help to stabilize RT, all of the protease sensitive mutations that we describe here comprise only a small fraction of the RT in the Stanford database. More importantly, in almost every case, the mutations that we showed confer PR-sensitivity also comprise only a small fraction of the amino acid substitutions at the position (s) in question, which suggests that these protease sensitive mutations are selected against in patients. To confirm this idea, we also looked at the prevalence, in patients, of mutations at sites where we previously reported amino acid substitutions that can make RT PR-sensitive. The ratio WT to mutant amino acids is low at a number of these positions. The ratio is 1 in 100 or less at positions 78, 256, 264, 274, 279 and just over 1 in 100 at position 310. Moreover, the fraction of the mutations at most of these positions that are known to make RT PR-sensitive is also small. For example there are several PR-sensitive mutations that are present in less than 2% of the viral isolates that have any amino acid change at the position of interest (V148S, L274T, L279S, and L310S), and in two more (D256N, L264S) for which the known protease sensitive mutations comprise between 4% and 5% of the amino acid changes seen at these positions. These results support the idea that the protease-sensitive mutations in the RT coding region are rare in patient samples. There is only one additional case (R78K), in which a mutation that confers protease-sensitivity in RT

makes up a large fraction of the mutations seen at that position in patients. As was the case with T473A, although the R78K mutation represents the majority of the mutations that arise at this position, RTs carrying the R78K mutation make up a relatively small percentage (less than 1%) of the total RT sequences in the Stanford database. As might have been expected, R78K does not have the strongest protease-sensitive phenotype of the mutants we have tested. A vector that replicates using BH10 RT and carries the R78K mutation still has residual titer, and the virions have, based on a western blot, a substantial amount of intact p66 and P51. In addition, when the sequences of the RTs that were reported to carry the R78K mutation were analyzed, there were a number of positions at which there were secondary mutations; however, as was the case for the G190E mutation, all of these were polymorphisms that are present in the Stanford database at a high frequency in the absence of the R78K mutation. We considered the possibility that R78K could be selected for, at least under certain circumstances; however, this mutation has not been reported to be associated with resistance to any of the standard RT inhibitors, nor has it been reported to be in a CTL epitope (Yusim et al., 2002).

In considering the results obtained both in the analysis of the mutant RTs done in vitro, and in cell culture, it would appear that there are a large number of amino acid changes that make RT PR (and temperature) sensitive. Many of these mutations have a profound negative effect on the titer of the virus at the temperature at which the virus replicates in patients. Although there appear to be compensatory mutations (some of which have previously identified as polymorphisms) that allow the virus to carry a mutation in RT (for example, G190E) that would otherwise confer protease sensitivity in WT RT, it appears that the effects of a mutation on the stability of RT in virions is an important factor that helps to determine whether or not a mutation is likely to be present in RT. A search of the Stanford database supports the conclusion that this applies to viruses that are replicating in patients, based on the observation that not only do viral genomes that carry the identified PR-sensitive mutations make up only a small fraction of the total, but, in addition, with a small number of exceptions, the identified PR sensitive mutations make up only a very small portion of the mutations at their respective positions.

Materials and methods

Preparation of the expression plasmids and growth of the E. coli strains

The E. coli expression systems used to generate the various forms of the HIV-1 RT have been described (Boyer et al., 1994, 2001). The various mutations were introduced in the RT sequence in the various constructs using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies: La Jolla, CA). The vector that encodes and expresses the p66 subunit of RT and separately encodes and expresses PR was used to generate p66/p51 heterodimers. For the *E. coli* expression experiments, we used a plasmid that expresses the entire Pol coding region in *E. coli* (Dunn et al., 2013). The Pol region was derived from the HIV-1 clone BH10 (Genebank #HIVBH102). The plasmids that encode both p66 and PR, and the Pol expression plasmids express PR, which is toxic to the bacteria. These proteins are therefore expressed in the IPTG-inducible vector pT5m. To express Pol, a glycine codon located in p6* (nucleotide #1545) 22 codons from the beginning of the protease coding region was selected as the start position. PCR amplification was used to add a methionine initiation codon, and the sequence around the ATG was modified to create an NcoI site (CC ATG GGT AGA GAC…..). The same PCR reaction was used to add a HindIII site 3' of the termination codon at the end of the IN coding region (nucleotide #4454). The PCR product was digested with NcoI/HindIII and cloned into the expression vector pT5m. The vectors were transformed into Rosetta 2(DE3) Competent Cells (EMD Chemicals). *E. coli* from an overnight culture were inoculated 1:80 into pre-warmed NZY/amp (100 μg/ml) media for two hours at 30 °C or 37 °C. The bacteria that expressed p66 plus PR or Pol were induced with IPTG (Isopropyl-beta-D-thiogalactopyranoside at 2.5 mM final) for two hours at either 30 °C or 37 °C. Bacteria were collected by centrifugation for 5 min at 325*g*. Media was removed and the pellets were resuspended in PBS. For Western blot analysis, 4 ml cultures were used; the OD600 was measured and equal amounts of *E. coli* were collected by sedimentation for 5 min at 325*g*. The supernatant was removed and the pellet was resuspended by vortexing and stored at −70 °C.

Gel analysis of the recombinant RTs

Proteins were extracted from *E. coli* pellets that had been stored at −70 °C. The pellets were resuspended in 60 μl of 10 mM Tris-Cl, 1 mM EDTA, 25% sucrose, 5 mg/ml lysozyme, pH 8.0 and placed on ice 15 min. 82 μl of 50 mM Tris-Cl, 6.25 mM EDTA, 0.1% Triton X-100, 50 mM NaCl, 0.2 mM PMSF, 8U Benzonase (Novagen), 22 mM $MgCl_2$, pH 8.0 were added to the lysed bacterial pellets, which were placed on ice 15 min. Polymethyl sulfoxide (PMSF) was dissolved in ethanol at a concentration of 17.4 mg/ml (100 mM) and added to the buffer 15 min before the buffer was used. 180 μl of 50 mM Tris-Cl, 6.25 mM EDTA, 0.1% Triton X-100, 950 mM NaCl, pH 8.0, 0.2 mM PMSF were added and the lysates were sedimented at 16,000*g* for 1 h at 4 °C. The supernatants were removed from the pellet and the OD280 of the supernatants were measured. Equal amounts of supernatants and pellets (based on the OD of the supernatants) of the bacteria that expressed WT and the RT mutant proteins were mixed with loading buffer (final concentration of the loading buffer: 1× NuPage LDS sample buffer, with 50 mM dithiothreitol), aliquoted, and stored at −70 °C. The samples were heated to 95 °C for 5 min. Protein samples were fractionated on NuPage 4–12% Bis-Tris gels.

Western Blots

Following fractionation on a NuPage 4–12% Bis-Tris gel (Invitrogen) the proteins were wet blot transferred onto Hybond-ECL nitrocellulose (Amersham). Blots were incubated in block solution (5% dry milk in Tris-buffered saline-Tween) for more than 1 h. Primary antibody was diluted in 0.5% dry milk in Tris-buffered saline-Tween, and blots were incubated for 4–24 h. Blots were washed 10 times for 6 min (1 h total). Secondary antibody was diluted in block solution, and the blots were incubated for 2–4 h. The blots were washed 5 times for 6 min (30 min total), and the antibody was detected with either Super-Signal West Pico or Supersignal West Femto chemiluminescent substrates (Pierce). The individual mouse anti-RT antibodies have been described; the anti-RT MAb mix was prepared using equal amounts of MAbs 19, 21, 42, 48, and 50 (Ferris et al., 1990). The mouse anti-RT MAbs were diluted 1:500; mouse anti-PR antibody (Exbio #10-302-C100) was diluted 1:2000. The rabbit anti-IN antibody (kindly provided by Duane Grandgenett) was used at a dilution of 1:10,000. The experiments were repeated several times, with different extracts, and the data shown in the figures are representative.

Virus production and infection

293T cells were seeded in 100 mm plates at 1.2×10^6 cells per plate the day prior to transfection. Cell numbers were determined by counting in a hemocytometer. Cells were co-transfected with 12 μg of the plasmid encoding the one-round vector pNLNgo-MIVR-E-HSA, or 12 μg of plasmid encoding the RT mutants, and 3 μg of the plasmid pHCMV-g, which expresses VSV-G (obtained from Jane Burns, University of California at San Diego) using the calcium phosphate method. The encoded RTs are derived from NL4–3 which differs by one amino acid from the group M subtype B-strain BH10 (see text). At 7 h after the calcium phosphate precipitate was added, the cells were washed 3 times with phosphate-buffered saline (PBS), and fresh medium was added. At this point, one set of plates was incubated at 37 °C and the other at 32 °C for 40 h. The day prior to infection, 60 mm plates were seeded with 2×10^5 HOS cells, 4 plates were prepared for each of the mutants. The morning of infection, the viral supernatants were collected, clarified by sedimentation for 5 min at 1700*g*, and 4 mls of the clarified supernatant was added to one of the plates, and 4 mls of 1:5, 1:25, and 1:125 dilutions was added to three other plates. Samples were set aside for measuring the amount of each of the viruses by p24 ELISA. After 4 h, the virus was removed and fresh media added. At 24 h, plates were rinsed with 1 ml of Versene (Gibco). One ml Versene was added and incubated for 5 min. Cells were harvested with 3 ml of PBS and collected by sedimentation. Cells were resuspended in 100 μl PBS and incubated on ice for 30 min after the addition of 2 μl PE Rat Anti-Mouse CD24 Heat Stable Antigen (BD Pharmingen, BD Biosciences). Cells were washed twice with PBS, resuspended in 150 μl PBS, and added to 150 μl 4% paraformaldehyde. The relative virus titer was determined by counting the number of cells that express the mouse CD24 Heat Stable Antigen marker (HSA) and expressing the data relative to p24 ELISA data.

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