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Conditional Human Immunodeficiency Virus Type 1 Protease Mutants Show No Role for the Viral Protease Early in Virus Replication

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The human immunodeficiency virus type 1 protease plays a critical role in the proteolytic processing of precursor polyproteins during virion maturation. Contradictory evidence has been obtained for a possible role for the protease early after infection, i.e., during DNA synthesis and/or integration. We have reexamined this question by using conditional mutants of the protease. In one set of experiments, protease mutants that confer a temperature-sensitive phenotype for processing were used to assess the need for protease activity early after infection. No significant difference from results with wild-type virus was seen when infections were carried out at either 35 or 40°C. In a separate set of experiments, infections were carried out in the presence of a protease inhibitor. In this case, both wild-type virus and a drug-resistant variant were used, the latter as a control to ensure a specific effect of the inhibitor. Infection with either virus was not inhibited at drug concentrations that were up to 10-fold higher than those needed to inhibit intracellular processing by the viral protease. The results obtained by both of these experimental protocols provide evidence that the human immunodeficiency virus type 1 protease does not play a role early after infection.

The retroviral protease (PR) plays a central role late in the replication cycle of the virus. Virus particles are assembled largely from the Gag and Gag-Pro-Pol polyprotein precursors, and the PR is responsible for cleaving these precursor proteins into their mature forms (reviewed in reference 23). If PR activity is blocked, either by mutation or with a specific inhibitor, virions are produced with unprocessed proteins and the virus particles are noninfectious (2, 4, 5, 7, 8, 13, 16, 20, 25, 31, 33, 34).

Mature nucleocapsid cores of equine infectious anemia virus have been purified (30). Incubation of this protein complex in vitro results in the cleavage of the mature viral nucleocapsid protein (NC) to smaller products (29). This observation raised the possibility that such a cleavage mediated by the viral PR occurs during the early phase of the replication cycle. Evidence indicating a role for proteolytic cleavage has been obtained by using PR-specific inhibitors to block the early steps of human immunodeficiency virus type 1 (HIV-1) replication (3, 36). However, similar experiments with the PR-specific inhibitor Ro 31-8959 (saquinavir) failed to show an effect on the early stages of infection (10), although higher concentrations of this inhibitor were found to block infection (22).

We have reexamined the role of the HIV-1 PR early in infection by using conditional mutants of the PR. In one set of experiments, we used viruses containing mutations in the PR that were shown previously to confer temperature sensitivity to PR-mediated protein processing (19). In another set of experiments, we used a virus containing two mutations that confer reduced sensitivity to the PR inhibitor A-77003 (12). We compared the infectivity of the mutant and wild-type viruses under permissive and nonpermissive conditions, measuring the early steps of replication by the MAGI cell assay (15). We found no significant difference in infectivity under conditions of reduced PR activity. Thus, our experiments do not indicate a role for the viral PR during the early steps of replication.

MATERIALS AND METHODS

Cells and viruses. MAGI cells (15) were obtained from Michael Emerman. Virus was generated from cloned viral DNA after transfection of purified DNA into HeLa cells (32). In the experiments designed to test the effect of temperature on infectivity, virus was generated after transfection of HeLa cells at 35°C. The virus was grown in either CEM cells or CEMX174 cells, using RPMI 1640 containing 2 mM glutamine and 10% (vol/vol) fetal calf serum as the growth medium. Virus infectivity in the culture supernatant was measured by the MAGI cell assay as described previously (15). For this assay, the cells were infected at approximately 40% confluence.

Mutant viruses were generated after the introduction of mutations into cloned viral DNA. Mutations within the PR coding domain to change the valine codon at position 56 to a glycine codon (V56G) or the proline codon at position 79 to a threonine codon (P79T) were introduced separately into the HXB2 clone of HIV-1 (27). The HXB2 clone was modified to remove the cellular sequences flanking the 3′ long terminal repeat. Mutations to alter the valine codon at position 32 to an isoleucine codon (V32I) and the valine codon at position 82 to an isoleucine codon (V82I) were introduced together into the NL4-3 clone of HIV-1 (1) to create the V32I/V82I mutant. In each case, the presence of the mutation in the full-length clone was confirmed by DNA sequence analysis. Details of the cloning procedures are available on request.

Infections under nonpermissive conditions. In the experiments designed to assess the effect of temperature on infectivity, cells were maintained at either 35 or 40°C. These temperatures were chosen on the basis of the ability of CEM cells to support wild-type virus replication at each of these temperatures (18a). In the MAGI cell assay, cells were transferred from a 37°C incubator to the indicated temperature 3 h prior to infection and the infection was done with virus-containing culture supernatants that had been prewarmed to the same temperature as the cells.

The Abbott PR inhibitor A-77003 (14) was also used to assess the effect of an inhibitor of the PR early after infection. In these experiments, cells were prein-

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cubated with various concentrations of inhibitor for the indicated time prior to infection. The inhibitor was present during the 2-h exposure of cells to virus and throughout the entire period after infection. The inhibitor was prepared at stock concentrations up to 3 mM in dimethyl sulfoxide. The concentration of dimethyl sulfoxide in the culture medium never exceeded 1%.

Analysis of viral proteins. For comparison of viral Gag polyprotein processing at different temperatures, HeLa cells were transfected with wild-type or mutant DNA clones of HIV-1 in duplicate 100-mm-diameter plates (11) and then incubated at 37°C for 18 h. At this time, the plates were placed at either 35 or 40°C and starved for 4 h in prewarmed Dulbecco's modified Eagle's medium with a high concentration of glucose (4,500 mg/liter) without methionine. The cells were then labeled with 100 μCi of [^{35}S]methionine per ml in methionine-free medium for 18 h at either 35 or 40°C. At the end of the labeling period, the cells were washed twice in ice-cold phosphate-buffered saline and scraped into 400 μl of RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 50 mM Tris [pH 7.5], 2 μg of leupeptin per ml, 1 μg of pepstatin A per ml, 100 μg of phenylmethylsulfonyl fluoride per ml, 2 μg of aprotinin per ml). Nuclei and cellular debris were pelleted by centrifugation of the extract at 3,000 × *g* for 5 min. The supernatant fraction was incubated with human serum from an HIV-1-infected individual. The serum had first been preabsorbed with an unlabeled HeLa cell extract and then absorbed to protein A-Sepharose to collect the antibodies from the unlabeled extract. The Sepharose-antibody complex was incubated with the labeled extract from infected cells for 4 h, and then the Sepharose-antibody-protein complexes were washed five times in washing buffer (250 mM NaCl, 1% Nonidet P-40, 0.5% sodium dodecyl sulfate [SDS], 50 mM Tris [pH 7.5], 2 μg of leupeptin per ml, 1 μg of pepstatin A per ml, 100 μg of phenylmethylsulfonyl fluoride per ml, 2 μg of aprotinin per ml), resuspended in 50 μl of SDS sample buffer, heated to 100°C for 5 min, and loaded onto an SDS–12% polyacrylamide gel. Following electrophoresis, the gel was fixed and prepared for fluorography (En^{3}Hance; DuPont). Radioactive analytic imaging (AMBIS, San Diego, Calif.) was used to quantitate the relative amounts of processed p24 capsid protein (CA) generated at 35 and 40°C by the wild-type and mutant viruses.

For Western blot (immunoblot) analysis, nearly confluent 100-mm plates of MAGI cells were preincubated with A-77003 at various concentrations for 24 h. The cells were then transfected with a molecular clone of either wild-type or mutant HIV-1 DNA. The inhibitor was present during the transfection and until the cells were harvested for Western blot analysis. At 48 h after the initial transfection, the cells were washed four times with PBS; 100 μl of RIPA buffer was then added to the plate, and the cells were scraped. Western blot analysis with serum from an HIV-1-positive patient as the primary antibody was carried out as described by Pettit et al. (26).

RESULTS

***ts* phenotype of PR mutants for processing.** We have previously described a series of HIV-1 PR mutants that have a temperature-sensitive (*ts*) phenotype for PR-mediated processing of a linked Pol polyprotein domain when expressed in *Escherichia coli* (19). The mutants showed a range of activity at the permissive temperature and in each case showed a moderate decrease in activity at the nonpermissive temperature relative to the permissive temperature. We chose two of these PR mutants, V56G and P79T, to characterize in the context of HIV-1 replication. In the bacterial processing assay, the V56G mutant showed very low processing at 37°C and intermediate processing at 32°C; the P79T mutant showed near-wild-type activity at 32°C and intermediate activity at 37°C (19). The mutated PR coding domains were introduced into a full-length infectious DNA clone of HIV-1, and a virus stock was generated for each mutant after transfection of the DNA into HeLa cells. Both mutants produced virus particles after infection.

Infectious DNA clones were used in a transfection procedure to express transiently the wild-type and mutant genomes at either 35 or 40°C in an effort to determine the extent of processing in the mutant viruses at the different temperatures. Viral proteins were labeled over an 18-h period, and then the cell-associated viral proteins were isolated by immunoprecipitation with a patient antiserum. The labeled proteins in the immunoprecipitate were separated by polyacrylamide gel electrophoresis and detected by autoradiography and radioactive analytic imaging. Cells transfected with the wild-type virus showed extensive processing of the Pr55^{Gag} precursor and the presence of the processing product p24^{CA} at both temperatures (Fig. 1, lanes c and d). A PR active-site mutant (D25A) contained only unprocessed Pr55^{Gag} precursor (lanes e and f). The V56G mutant contained Pr55^{Gag} at both temperatures, but p24^{CA} was clearly evident only at 35°C (lanes g and h). The P79T mutant contained Pr55^{Gag} and p24^{CA} at both temperatures, but more p24 was evident at 35°C (lanes i and j).

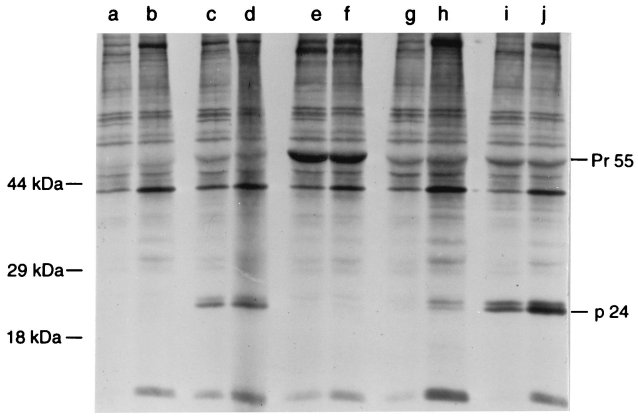

FIG. 1. *ts* proteolytic processing by two HIV-1 PR mutants. HeLa cells transfected with wild-type or mutant HIV-1 DNA clones were labeled for 18 h at either 35 or 40°C. At the end of the labeling period, viral proteins were isolated from cell lysates by immunoprecipitation followed by polyacrylamide gel electrophoresis and fluorography. Lanes a, c, e, g, and i, labeling at 40°C; lanes b, d, f, h, and j, labeling at 35°C; lanes a and b, mock transfection. Transfection was carried out with the following HIV-1 DNA clones: wild type (lanes c and d), PR active-site mutant (D25A) (lanes e and f), PR V56G mutant (lanes g and h), and PR P79T mutant (lanes i and j). The positions of molecular mass markers are shown on the left, and the positions of the Pr55^{Gag} precursor and the p24^{CA} processing product are shown on the right.

The amount of p24 in each lysate was compared at each temperature. A correction was made based on the wild type, which produced approximately twice as much labeled p24 at 35°C as at 40°C (Fig. 1, lanes c and d). After subtracting the twofold difference seen with the wild type, we found that both mutants produced more p24 at the lower temperature than at the higher temperature. The V56G mutant produced 11 times more p24 at 35°C than at 40°C, whereas the P79T mutant produced twice as much. These results confirm the *ts* phenotype of these mutants.

We also documented the *ts* phenotype of these mutants by producing virus particles at different temperatures and then quantitating the amount of virus produced at each temperature. Quantitation of the infectious virus particles produced from cells transfected at either temperature was performed by the MAGI cell assay at 37°C (15). In this assay, the early steps of virus replication up through the expression of *tat* must occur to score as an infectious event. For the wild-type virus, more infectious virus was present in the culture kept at the higher temperature than at the lower temperature. After correcting for the increase seen with the wild type, we found a reduction in titer to one-fifth for the V56G mutant and a reduction to one-half for the P79T mutant when particles were produced at the higher temperature compared with those produced at the lower temperature (data not shown). These results again confirm the *ts* phenotype of these mutants. However, the V56G mutant virus failed to grow when the virus stock was passaged in CEM cells. The P79T mutant virus grew poorly in CEM cells at 37°C, and we were unable to demonstrate a *ts* phenotype for virus spread.

Effect of temperature on the ability of viruses encoding mutant PRs to infect MAGI cells. Mutant and wild-type viruses

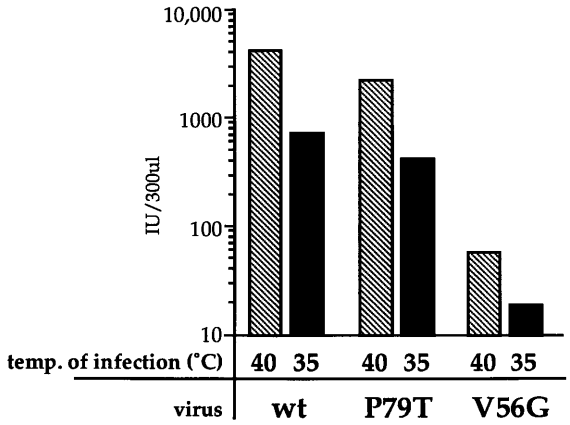

FIG. 2. Effect of temperature on the infectivity of HIV-1 PR mutants. Virus produced after transfection at 35°C was used to infect MAGI cells at either 35 or 40°C. At 2 days after infection, the cells were stained for the presence of β-galactosidase activity (15) and the number of staining cells was quantitated. IU, infectious units. Hatched bars, titer of virus after infection at 40°C; solid bars, titer of virus after infection at 35°C; wt, wild type.

were produced after transfection of HeLa cells at 35°C and used immediately to infect MAGI cells at either 35 or 40°C. After a 3-h infection of the MAGI cells, the virus-containing medium was removed and replaced with prewarmed medium. The respective cultures were maintained for 2 days at the two temperatures. At that time, the cells were stained to assess the virus titer. In all cases, the virus titer was higher at 40°C than to 35°C (Fig. 2). For the wild-type virus and the P79T mutant, the increase in titer at the higher temperature compared with the lower temperature was comparable, approximately sixfold. The titer of the V56G mutant also increased at the higher temperature, although in this case the increase was only threefold. Taken together, these results suggest that the higher temperature had little to no effect on the ability of the mutant viruses encoding a *ts* PR to carry out the early steps of virus replication compared with the wild-type virus.

Protein processing of wild-type and mutant viruses in the presence of a PR inhibitor. Previous studies have used PR inhibitors to assess the role of the HIV-1 PR in the early phases of virus replication, with disparate results (3, 10, 22, 36). We sought to define an experimental protocol which would allow us to document the effectiveness of the inhibitor on PR activity and also modulate the sensitivity of the virus to the inhibitor. We chose to infect MAGI cells with virus in the presence of the PR inhibitor A-77003 (14) and to use either wild-type HIV-1 or a mutant of HIV-1 that is partially resistant to this inhibitor (12). If the viral PR is important during an early step in replication, the inhibitor should block infection of MAGI cells, and the two viruses should show different sensitivities to the inhibitor.

In an effort to establish the effectiveness of the inhibitor and the relative sensitivity of the two viruses to the inhibitor, we examined the extent of proteolytic processing of viral proteins expressed in MAGI cells. The cells were pretreated for 24 h either with no A-77003 or with 0.1, 0.3, or 1.0 μM inhibitor. The infectious viral DNA represented either the wild-type genome or the mutant genome encoding the PR mutations valine at position 32 to isoleucine and valine at position 82 to isoleucine (V32I/V82I). The V32I/V82I mutant virus encodes a PR that has a 20-fold increase in the K_i for A-77003 (12). Inhibitor was maintained in the medium after the transfection. Cells were harvested at 48 h after transfection, and cell-associated viral proteins were examined by Western blot analysis. The wild-type virus showed both $Pr55^{Gag}$ and $p24^{CA}$ in the absence of inhibitor, with an excess of p24 (Fig. 3, lane b). At 0.1 μM A-77003, there was an increase in the amount of the $p25^{CA}$ intermediate and of an approximately 40-kDa intermediate (lane c). At 0.3 μM inhibitor, very little $p24^{CA}$ was generated, and at 1.0 μM, only the $Pr55^{Gag}$ precursor was apparent and all processed products were essentially absent (lanes d and e). This experiment defined conditions in MAGI cells in which all activity of the wild-type PR was lost, as measured by processing of the Gag precursor.

The V32I/V82I mutant virus showed reduced sensitivity to the inhibitor compared with the wild type. In the absence of inhibitor, there was evidence of some incomplete processing with the presence of the $p25^{CA}$ intermediate (Fig. 3, lane f). However, this pattern was unchanged in the presence of either 0.1 or 0.3 μM inhibitor (lanes g and h). Only at the highest concentration of inhibitor (1.0 μM) was there significant inhibition of processing, and even here the inhibition was incomplete (lane i). Thus, as expected, the mutant was less sensitive to the effects of the inhibitor.

Effect of a PR inhibitor on the infectivity of wild-type and mutant viruses. We used the conditions defined in the transfection experiment to assess the ability of a PR inhibitor to block the initial steps of HIV-1 infection. Wild-type and V32I/V82I mutant viruses were used to infect MAGI cells that had been pretreated with inhibitor for 48 h. The inhibitor was maintained throughout the 48-h period after infection, at which time the number of successful infectious events was determined at each drug concentration.

There were two considerations in choosing the inhibitor concentrations. First, on the basis of the transfection experiment, we expected that 0.3 μM inhibitor would block wild-type PR activity but not mutant PR activity, and that 1.0 μM inhib-

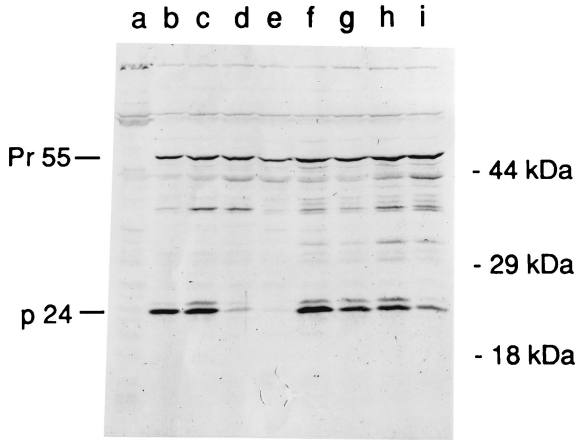

FIG. 3. Proteolytic processing by wild-type and mutant PR in the presence of a PR inhibitor. MAGI cells were pretreated with various concentrations of the HIV-1 PR inhibitor A-77003 and then transfected with a full-length HIV-1 infectious DNA representing either the wild-type genome (WT, NL4-3) or a mutant with codons 32 and 82 of the PR coding domain encoding isoleucine instead of valine (V32I/V82I). At 24 h after transfection, the cells were lysed and the presence of viral proteins was assessed by Western blot analysis with serum from an HIV-1-positive patient as the primary antibody. Lanes: a, cell lysate from cells that were neither transfected nor treated with inhibitor; b to e, cells transfected with the wild-type HIV-1 DNA; f to i, cells transfected with the V32I/V82I mutant HIV-1 DNA. The cells were treated with the PR inhibitor as follows: no inhibitor (lanes b and f), 0.1 μM inhibitor (lanes c and g), 0.3 μM inhibitor (lanes d and h), and 1.0 μM inhibitor (lanes e and i). On the right are the positions of the molecular mass markers, and on the left are the positions of the $Pr55^{Gag}$ precursor and the $p24^{CA}$ processed product.

TABLE 1. Effect of a PR inhibitor on the early steps of HIV-1 replication

Virus[a]	% Infectivity at A-77003 concn (μM) of[b]:						% Infectivity with AZT (1.0 μM)
	0 (control)	0.1	0.3	1.0	3.0	10.0	
Wild type							
Expt 1	100 (120)	71	68	93	—	—	—
Expt 2	100 (1,680)	—[c]	85	98	84	—	6
Expt 3	100 (1,353)	—	—	—	—	106	—
V32I/V82I							
Expt 1	100 (169)	83	142	154	—	—	—
Expt 2	100 (924)	131	106	92	—	—	14
Expt 3	100 (1,671)	—	108	114	122	116	8

[a] Infections of MAGI cells were carried out with the indicated virus: wild type, NL4-3; V32I/V82I, NL4-3 with two mutations in the PR coding domain.

[b] Cells were pretreated with the indicated concentration of A-77003 for 48 h prior to infection, and the inhibitor concentration was maintained throughout infection. The control infection was carried out in the absence of any inhibitor treatment. The numbers in parentheses are the total numbers of blue cells counted in the control well. The number of blue cells (infectious events) was set at 100% for the infection in the absence of inhibitor. The number of infectious events in the presence of inhibitor divided by the number in the control infection in the absence of inhibitor times 100 is recorded as the percent value for the rest of the infections.

[c] —, that this concentration of inhibitor was not included in the experiment.

itor would block both the wild-type and mutant PRs. Therefore, infections were done in the presence of these concentrations of inhibitor. We also considered the possibility that the inhibitor would enter infected cells to a higher level than it would enter uninfected cells, causing us to underestimate the amount of inhibitor needed to effect PR inhibition. Thus, we also tested inhibitor concentrations that were 10-fold higher (3.0 and 10 μM); we chose not to use higher concentrations because of concerns of low level toxicity. As seen in Table 1, neither the wild-type virus nor the V32I/V82I mutant showed any decrease in infectivity when the infection was done in the presence of the PR inhibitor at concentrations between 0.1 and 10.0 μM. By contrast, zidovudine, which inhibits viral DNA synthesis (21), significantly reduced infectivity in this assay. These results show that in the presence of concentrations of inhibitor at which PR activity is inhibited as measured in the context of virus assembly and maturation late in the virus life cycle, there is no inhibition of the early steps of virus replication. This was true for both the wild-type and mutant viruses. As with the *ts* viruses, these results indicate no role for the PR early after infection.

DISCUSSION

The retroviral PR plays a central role in the virus life cycle. Viruses with mutations in the PR assemble noninfectious particles composed of unprocessed Gag and Gag-Pro-Pol precursors (5, 8, 13, 16, 25, 33, 34). Thus, PR activity has a major impact at a late step in the virus life cycle, during the assembly and maturation of virus particles. The particle that is released must carry out the early steps of virus replication (penetration, DNA synthesis, and integration) in the absence of any additional viral proteins. The particle that is produced late in infection must be competent to carry out all of the necessary steps early after infection.

The viral proteins reverse transcriptase and integrase are incorporated into the virion to be available for DNA synthesis and integration, respectively, early after infection. The structural proteins encoded within Gag function both late (in assembly) and early in the replication cycle, as shown by the existence of Gag mutants that form virions but fail to proceed through the early stages of replication (9, 17, 28, 35, 38, 40). Like all of the proteins in the Gag and Gag-Pro-Pol precursors, the PR is included in the budded virus particle (18, 37, 39) and thus is presumably present, like reverse transcriptase and integrase, during the early stages of infection after carrying out proteolytic processing during the preceding late stage of virion assembly. The observation that the equine infectious anemia virus PR cleaves its NC protein during incubation of virus cores in vitro led to the suggestion that such a cleavage might occur during the early stages of virus replication as a required part of successful DNA synthesis and/or integration (29).

Evidence for a role for the HIV-1 PR early after infection was obtained by using PR inhibitors to block the early steps (3, 36). However, contradictory results were obtained by other workers (10), although these subsequent negative results were attributed to the use of insufficient inhibitor (22). One concern in interpreting results with inhibitors is the potential for toxicity that would reduce the ability of the cell to support virus replication. Toxicity that would affect virus replication is likely to be apparent at concentrations of inhibitor that are below the levels that result in cell killing, making the choice of a cytotoxicity assay problematic.

On the basis of these considerations, we chose several approaches to address the question of the role of PR early after infection. We used two viruses with mutations in the PR that confer *ts* processing (Fig. 1) and found no significant effect of carrying out the early stages of infection at an elevated temperature (Fig. 2), a result that does not support a role for PR early after infection. A limitation in this experiment is the possibility that the *ts* phenotype is expressed during folding of the PR and not on the folded enzyme. In this case, the production of wild-type and mutant viruses at a permissive temperature should result in equally infectious particles when tested subsequently at either a permissive or nonpermissive temperature. At this time, we do not know the basis for the *ts* phenotype of these mutants and therefore cannot distinguish between these two possibilities. Another limitation with this genetic approach is that less enzyme activity may be needed early after infection than that required during the late steps of infection, when the effects of these mutations were assessed.

The alternative approach was to use a PR inhibitor during the early steps of infection. We chose inhibitor concentrations that blocked the intracellular processing of the Gag precursor by PR and also used concentrations 10-fold higher to ensure that we had established conditions under which the PR would be inhibited. k_{on} rates for protease inhibitors have been measured in the range of 10^7 s^{-1} M^{-1} (6, 24), so that we would expect PR to bind inhibitor rapidly after entering the cells which had been pretreated with inhibitor at micromolar levels. As a control against potential toxic effects, we compared the infectivity of a wild-type virus with that of a virus that has a modest level of resistance to the PR inhibitor used (Fig. 3). Our expectation was that if a decrease in infectivity were due to toxicity, it would affect both viruses equally, but that if the effect were due to inhibition of PR, the wild-type virus would be more sensitive than the mutant. Over the range of inhibitor concentrations used, we saw no decrease in infectivity with either the wild-type or mutant virus (Table 1). This result suggests that in these experiments, the PR inhibitor was not toxic at the concentrations used and that it did not inhibit the early steps of virus replication. It is difficult to reconcile these negative results with the positive results previously published (3, 22, 36) other that to raise concern over possible low levels of cell toxicity at the concentrations of inhibitor that were used previously. Our results, designed to validate the inhibitor con-

centration needed to inhibit PR intracellularly, showed no role for PR activity early after infection.

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REFERENCES

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59:**284–291.
2. **Ashorn, P., T. J. McQuade, S. Thaisrivongs, A. G. Tomasselli, W. G. Tarpley, and B. Moss.** 1990. An inhibitor of the protease blocks maturation of human and simian immunodeficiency viruses and spread of infection. Proc. Natl. Acad. Sci. USA **87:**7472–7476.
3. **Baboonian, C., A. Dalgleish, L. Bountiff, J. Gross, S. Oroszlan, G. Rickett, B. C. Smith, P. Troke, and J. Merson.** 1991. HIV-1 proteinase is required for synthesis of proviral DNA. Biochem. Biophys. Res. Commun. **179:**17–24.
4. **Bennett, R. P., S. Rhee, R. C. Craven, E. Hunter, and J. W. Wills.** 1991. Amino acids encoded downstream of *gag* are not required by Rous sarcoma virus protease during *gag*-mediated assembly. J. Virol. **65:**272–280.
5. **Crawford, S., and S. P. Goff.** 1985. A deletion mutation in the 5′ part of the *pol* gene of Moloney murine leukemia virus blocks proteolytic processing of the *gag* and *pol* polyproteins. J. Virol. **53:**899–907.
6. **Darke, P. L., S. P. Jordan, D. L. Hall, J. A. Zugay, J. A. Shafer, and L. C. Kuo.** 1994. Dissociation and association of the HIV-1 protease dimer subunits: equilibria and rates. Biochemistry **33:**98–105.
7. **Erickson, J., D. J. Neidhart, J. VanDrie, D. J. Kempf, X. C. Wang, D. W. Norbeck, J. J. Plattner, J. W. Rittenhouse, M. Turon, N. Wideburg, W. E. Kohlbrenner, R. Simmer, R. Helfrich, D. A. Paul, and M. Knigge.** 1990. Design, activity, and 2.8 A crystal structure of a C2 symmetric inhibitor complexed to HIV-1 protease. Science **249:**527–533.
8. **Gottlinger, H. G., J. G. Sodroski, and W. A. Haseltine.** 1989. Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **86:**5781–5785.
9. **Hsu, H. W., P. Schwartzberg, and S. P. Goff.** 1985. Point mutations in the P30 domain of the *gag* gene of Moloney murine leukemia virus. Virology **142:** 211–214.
10. **Jacobsen, H., L. L. Ahlborn, R. Gugel, and J. Mous.** 1992. Progression of early steps of human immunodeficiency virus type 1 replication in the presence of an inhibitor of viral protease. J. Virol. **66:**5087–5091.
11. **Kaplan, A., M. Manchester, and R. Swanstrom.** 1994. Analysis of the role of the HIV-1 protease in virion assembly. Methods Enzymol. **241:**58–69.
12. **Kaplan, A. H., S. F. Michael, R. S. Wehbie, M. F. Knigge, D. A. Paul, L. Everitt, D. J. Kempf, D. W. Norbeck, J. W. Erickson, and R. Swanstrom.** 1994. Selection of multiple human immunodeficiency virus type 1 variants that encode viral proteases with decreased sensitivity to an inhibitor of the viral protease. Proc. Natl. Acad. Sci. USA **91:**5597–5601.
13. **Katoh, I., Y. Yoshinaka, A. Rein, M. Shibuya, T. Odaka, and S. Oroszlan.** 1985. Murine leukemia virus maturation: protease region required for conversion from "immature" to "mature" core form and for virus infectivity. Virology **145:**280–292.
14. **Kempf, D. J., K. C. Marsh, D. A. Paul, M. F. Knigge, D. W. Norbeck, W. E. Kohlbrenner, L. Codacovi, S. Vasavanonda, P. Bryant, X. C. Wang, N. E. Wideburg, J. J. Clement, J. J. Plattner, and J. Erickson.** 1991. Antiviral and pharmacokinetic properties of C2 symmetric inhibitors of the human immunodeficiency virus type 1 protease. Antimicrob. Agents Chemother. **35:**2209–2214.
15. **Kimpton, J., and M. Emerman.** 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated β-galactosidase gene. J. Virol. **66:** 2232–2239.
16. **Kohl, N. E., E. A. Emini, W. A. Schleif, L. J. Davis, J. C. Heimbach, R. A. Dixon, E. M. Scolnick, and I. S. Sigal.** 1988. Active human immunodeficiency virus protease is required for viral infectivity. Proc. Natl. Acad. Sci. USA **85:**4686–4690.
17. **Lee, P. P., and M. L. Linial.** 1994. Efficient particle formation can occur if the matrix domain of human immunodeficiency virus type 1 Gag is substituted by a myristylation signal. J. Virol. **68:**6644–6654.
18. **Lillehoj, E. P., F. H. Salazar, R. J. Mervis, M. G. Raum, H. W. Chan, N. Ahmad, and S. Venkatesan.** 1988. Purification and structural characterization of the putative *gag-pol* protease of human immunodeficiency virus. J. Virol. **62:**3053–3058.

18a. **Manchester, M.** Unpublished observation.

19. **Manchester, M., L. Everitt, D. D. Loeb, C. A. Hutchison III, and R. Swanstrom.** 1994. Identification of temperature-sensitive mutants of the human immunodeficiency virus type 1 protease through saturation mutagenesis. J. Biol. Chem. **269:**7689–7695.
20. **Meek, T. D., D. M. Lambert, G. B. Dreyer, T. J. Carr, T. J. Tomaszek, M. L. Moore, J. E. Strickler, C. Debouck, L. J. Hyland, T. J. Matthews, B. W. Metcalf, and S. R. Petteway.** 1990. Inhibition of HIV-1 protease in infected T-lymphocytes by synthetic peptide analogues. Nature (London) **343:**90–92.
21. **Mitsuya, H., K. J. Weinhold, P. A. Furman, C. M. St, S. N. Lehrman, R. C. Gallo, D. Bolognesi, D. W. Barry, and S. Broder.** 1985. 3′-Azido-3′-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus in vitro. Proc. Natl. Acad. Sci. USA **82:**7096–7100.
22. **Nagy, K., M. Young, C. Baboonian, J. Merson, P. Whittle, and S. Oroszlan.** 1994. Antiviral activity of human immunodeficiency virus type 1 protease inhibitors in a single cycle of infection: evidence for a role of protease in the early phase. J. Virol. **68:**757–765.
23. **Oroszlan, S., and R. B. Luftig.** 1990. Retroviral proteinases. Curr. Top. Microbiol. Immunol. **157:**153–185.
24. **Pargellis, C. A., M. M. Morelock, E. T. Graham, P. Kinkade, S. Pav, K. Lubbe, D. Lamarre, and P. C. Anderson.** 1994. Determination of kinetic rate constants for the binding of inhibitors to HIV-1 protease and for the association and dissociation of active homodimer. Biochemistry **33:**12527–12534.
25. **Peng, C., B. K. Ho, T. W. Chang, and N. T. Chang.** 1989. Role of human immunodeficiency virus type 1-specific protease in core protein maturation and viral infectivity. J. Virol. **63:**2550–2256.
26. **Pettit, S. C., M. D. Moody, R. S. Wehbie, A. H. Kaplan, P. V. Nantermet, C. A. Klein, and R. Swanstrom.** 1994. The p2 domain of human immunodeficiency virus type 1 Gag regulates sequential proteolytic processing and is required to produce fully infectious virions. J. Virol. **68:**8017–8027.
27. **Ratner, L., A. Fisher, L. L. Jagodzinski, H. Mitsuya, R. S. Liou, R. C. Gallo, and S. F. Wong.** 1987. Complete nucleotide sequences of functional clones of the AIDS virus. AIDS Res. Hum. Retroviruses **3:**57–69.
28. **Reicin, A. S., S. Paik, R. D. Berkowitz, J. Luban, I. Lowy, and S. P. Goff.** 1995. Linker insertion mutations in the human immunodeficiency virus type 1 *gag* gene: effects on virion particle assembly, release, and infectivity. J. Virol. **69:**642–650.
29. **Roberts, M. M., T. D. Copeland, and S. Oroszlan.** 1991. In situ processing of a retroviral nucleocapsid protein by the viral proteinase. Protein Eng. **4:**695–700.
30. **Roberts, M. M., and S. Oroszlan.** 1989. The preparation and biochemical characterization of intact capsids of equine infectious anemia virus. Biochem. Biophys. Res. Commun. **160:**486–494.
31. **Roberts, N. A., J. A. Martin, D. Kinchington, A. V. Broadhurst, J. C. Craig, I. B. Duncan, S. A. Galpin, B. K. Handa, J. Kay, A. Krohn, R. W. Lambert, J. H. Merrett, J. S. Mills, K. E. B. Parkes, S. Redshaw, A. J. Ritchie, D. L. Taylor, G. J. Thomas, and P. C. Machin.** 1990. Rational design of peptide-based HIV proteinase inhibitors. Science **248:**358–361.
32. **Sambrook, J., T. Maniatis, and E. F. Fritsch.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
33. **Sommerfelt, M. A., S. S. Rhee, and E. Hunter.** 1992. Importance of p12 protein in Mason-Pfizer monkey virus assembly and infectivity. J. Virol. **66:**7005–7011.
34. **Stewart, L., G. Schatz, and V. M. Vogt.** 1990. Properties of avian retrovirus particles defective in viral protease. J. Virol. **64:**5076–5092.
35. **Strambio de Castillia, C., and E. Hunter.** 1992. Mutational analysis of the major homology region of Mason-Pfizer monkey virus by use of saturation mutagenesis. J. Virol. **66:**7021–7032.
36. **Venaud, S., N. Yahi, J. L. Fehrentz, N. Guettari, D. Nisato, I. Hirsch, and J. C. Chermann.** 1992. Inhibition of HIV by an anti-HIV protease synthetic peptide blocks an early step of viral replication. Res. Virol. **143:**311–319.
37. **von der Helm, K.** 1977. Cleavage of Rous sarcoma viral polypeptide precursor into internal structural proteins in vitro involves viral protein p15. Proc. Natl. Acad. Sci. USA **74:**911–915.
38. **Wang, C. T., and E. Barklis.** 1993. Assembly, processing, and infectivity of human immunodeficiency virus type 1 *gag* mutants. J. Virol. **67:**4264–4273.
39. **Yoshinaka, Y., and R. B. Luftig.** 1977. Properties of a P70 proteolytic factor of murine leukemia viruses. Cell **12:**709–719.
40. **Yu, X., Q. C. Yu, T. H. Lee, and M. Essex.** 1992. The C terminus of human immunodeficiency virus type 1 matrix protein is involved in early steps of the virus life cycle. J. Virol. **66:**5667–5670.