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Partial Inhibition of the Human Immunodeficiency Virus Type 1 Protease Results in Aberrant Virus Assembly and the Formation of Noninfectious Particles

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The production of infectious particles by human immunodeficiency virus type 1 is dependent on the accurate cleavage of its Gag and Gag/Pol precursors by a virally encoded protease. In the absence of protease activity, morphologically abnormal particles which are noninfectious are formed. Recently, inhibitors of the protease of human immunodeficiency virus type 1 have been developed as potential therapeutic agents. We have examined the basis for the loss of infectivity at the limiting inhibitor concentrations that are likely to be achieved in clinical settings. We found that subtle defects in processing are correlated with profound deficits in infectivity. Further, we correlated this partially disrupted processing with an altered virion morphology. These data suggest that accurate and complete processing is essential to the formation of infectious, morphologically normal virions and that the pathway by which these precursors are processed and assembled is sensitive to partial inhibition of the protease by an inhibitor disproportionate to the effect of the inhibitor on the viral protease itself.

The production of infectious retrovirus particles is dependent on the proper assembly of the core particle. The initial steps in assembly for most retroviruses, including human immunodeficiency virus type 1 (HIV-1), involve the association of the Gag and Gag/Pol precursors with the inner face of the membrane of the infected cell, followed by interaction of the precursors with each other (reviewed in reference 6). The viral protease is part of the larger Gag/Pol precursor and is only functional as a dimer. This arrangement both allows the mature proteins which compose the capsid to arrive at the membrane in a coordinated manner and is largely successful in preventing premature activation of the protease. The membrane-based association of the precursors precedes cleavage of the precursors by the viral protease and budding of the mature virus particle which ultimately contains a collapsed, centrally placed, electron-dense core structure.

Accurate cleavage of the Gag and Gag/Pol precursors by the viral protease is required for infectivity. In the absence of protease activity, as in the case of a virus containing a mutation of the protease active site, morphologically abnormal virus particles which have a distended capsid structure and are noninfectious are formed (5, 9, 14, 17, 26, 32). Recently, inhibitors of the protease of HIV-1 have been developed as potential therapeutic agents. Virus particles produced by infected cells treated with these inhibitors contain unprocessed precursors and are noninfectious (1, 7, 12, 15, 16, 21, 22, 29, 30).

Although it has been well established that protease-deficient retroviruses fail to adopt a wild-type morphology and are noninfectious, the role played by precursor processing in virus assembly and the mechanism of the loss of infectivity remain obscure. We have examined the basis for the loss of infectivity at the limiting protease inhibitor concentrations which only partially disrupt processing, a situation that is likely to be achieved when these inhibitors are used in clinical settings. We found that subtle defects in processing are correlated with profound deficits in infectivity. Further, we correlated this partially disrupted processing with altered virion morphology and with a reduced capacity to carry out DNA synthesis. These data suggest that the viral protease plays an important role in virus assembly and that accurate and complete processing is essential to the formation of fully infectious, morphologically normal virions.

MATERIALS AND METHODS

Cells and virus. CEM cells, a human T-cell lymphoma cell line (a gift of M. Cloyd, University of Texas, Galveston), were maintained in RPMI 1640 containing 10% (vol/vol) fetal calf serum. HIV-1_G which was isolated from a patient infected with HIV-1 was the virus isolate used in these experiments (a gift of M. Cloyd and R. Buckheit). CEM cells chronically infected with HIV-1 were isolated from acutely infected cells as described previously (13). Briefly, CEM cells acutely infected with HIV-1_G were pelleted daily by low-speed centrifugation; the supernatant medium was removed, and the cells were resuspended daily in fresh RPMI 1640 medium with 10% fetal calf serum. A chronically infected cell line grew out after 2 to 3 weeks. For drug treatment, cells were placed in a T25 flask at a concentration of 5×10^4 cells per ml of medium. They were grown for 4

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days in either 0.1% dimethyl sulfoxide (DMSO) or 0.1% DMSO with various concentrations of the protease inhibitor A-77003 (12). The cells were then placed in fresh medium and grown for another 24 h at the same inhibitor concentration. The supernatant from each culture was used as a source of virus. Virion proteins were recovered from aliquots of the supernatant by centrifugation at 100,000 × *g* for 1.5 h.

Infectivity plaque assay. The focal immunoassay as described by Chesebro and Wehrly (4) was used to determine the titer of virus recovered from chronically infected cells treated with different concentrations of A-77003. The amount of pelletable viral protein in each sample was determined by comparing dilutions of each sample by Western blot (immunoblot) analysis. An aliquot of 200 μl of medium containing equivalent amounts of viral protein was added to a well of a 24-well plate containing the $CD4^+$ HeLa cell clone 1022. After 3 days, infected cells were identified by the presence of syncytia and the coincident immunostaining of cells with serum from an HIV-infected person and a peroxidase-conjugated goat anti-human antibody.

Detection of viral DNA. Aliquots of the virus stocks grown in various concentrations of A-77003 were pelleted by ultracentrifugation (100,000 × *g* for 90 min through a 15% sucrose cushion). The pellets were resuspended in an amount of fresh medium such that the concentration of viral protein was normalized in each specimen, and these were used to infect cultures of CEM cells (2×10^6) in duplicate. The cells and virus were placed at 37°C for either 6 or 10 h, and the cells were then removed by centrifugation and resuspended in RIPA buffer (0.15 M NaCl, 20 mM Tris-HCl [pH 7.4], 2 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 2 μg of aprotinin per ml, 2 μg of leupeptin per ml, 1 μg of pepstatin per ml, 100 μg of phenylmethylsulfonyl fluoride per ml). These samples were subjected to low-speed centrifugation to remove the nuclei. The polymerase chain reaction was then performed on these supernatants, which represent combined cytoplasmic and membrane fractions.

To detect viral DNA in infected cells, 25 cycles of polymerase chain reaction amplification using a primer pair specific for the R/U5 region of the viral long terminal repeat (M667-AA55) was performed as previously described (34, 35). This primer pair can be used to amplify the first 140 bp of viral DNA that is synthesized following the initiation of reverse transcription. Briefly, DNA isolated by extraction with phenol-chloroform was amplified with a single radioend-labelled primer (M667) along with the unlabelled antisense primer AA55. Denaturation was accomplished by heating to 94°C for 1 min; annealing and extension were accomplished with 65°C for 2 min. Radiolabelled amplified products were resolved on a 6% polyacrylamide gel, and the dried gels were subjected to autoradiography. HIV-1 DNA standards were amplified in parallel to quantitate viral DNA. The standards consisted of cloned HIV-1 JR-CSF DNA (18) digested with *Eco*RI. This DNA was diluted into uninfected peripheral blood lymphocyte DNA (10 μg/ml) as a carrier.

RESULTS

Moderate defects in processing result in profound deficits in infectivity. The Gag precursor of the HIV-1 structural proteins is processed in an ordered manner (10, 13, 23), and although infectious HIV-1 contains completely processed precursors, the degree of processing required for infectivity is unknown. We were interested in characterizing the effects of low concentrations of a protease inhibitor on processing and virion assembly. To approach this problem, we treated CEM cells chronically infected with HIV-1 with several concentrations of the protease inhibitor A-77003 for 4 days. At the end of this time, the cells were placed in fresh medium containing the inhibitor at the same concentration. These cells were grown for 16 h, at which time they were removed from the medium by centrifugation and prepared for analysis by electron microscopy. Aliquots of the supernatant were assayed for virus titer by the HeLa-CD4 syncytium assay (4). Virus was recovered from the rest of the supernatant by centrifugation, and the degree of processing of virion proteins was evaluated by Western analysis.

An examination of the virus titer compared with the extent of protein processing of the viral proteins at each of the inhibitor concentrations tested is shown in Fig. 1. Virus produced from untreated cells had a titer of approximately 7.4×10^3 syncytium-forming units/ml (Fig. 1A), and the viral proteins recovered from this sample were essentially completely processed when evaluated with monoclonal antibodies directed against either of the processed Gag products p24 (the viral capsid protein) (Fig. 1B, lane 2) and p17 (the matrix protein) (Fig. 1B, lane 1). A small amount of the unprocessed p55 Gag precursor was identified by a monoclonal antibody to the Gag carboxy-terminal p6 protein (Fig. 1B, lane 3). When chronically infected cells were grown in the presence of 0.1 μM inhibitor, the supernatant virus titer was reduced by 80%. At this concentration, Western blot analysis revealed that between one-half and two-thirds of the Gag-derived capsid protein was present as the completely processed p24 form (Fig. 1B, lane 5). Most of the remaining capsid protein was present as part of a processing intermediate of approximately 39 kDa. Staining with a monoclonal antibody directed against p6 (33) demonstrated that, at this low concentration of inhibitor, there was a small amount of the p15 processing intermediate composed of the Gag nucleocapsid protein and p6; however, most of the p6 was still completely processed (Fig. 1B, lane 6). When the cells were grown in the presence of 0.33 μM inhibitor, approximately 50% of the virion-associated capsid protein was present as the 39-kDa species and between 25 and 50% was present as the completely processed p24 (Fig. 1B, lane 8). In addition, we estimate that 50% of the matrix protein was present as completely processed p17 (Fig. 1B, lane 7). At this concentration of inhibitor, however, the virus titer was decreased between 80- and 100-fold. Finally, at drug concentrations higher than 0.33 μM, there were substantial inhibition of processing and no measurable virus titer. The relative amounts of the various processing intermediates present in each sample were evaluated by Western blot analysis of serial dilutions of each virus-containing supernatant. The amount of virus used in the infectivity assays was normalized to the amount of viral protein by comparing dilutions of the different samples by Western blot analysis (data not shown). In order to assess whether the differences in the amount of precursor observed could be due to a differential affinity of the monoclonal antibodies for the precursor, we repeated the Western analysis with antiserum from an HIV-1-infected patient and obtained equivalent results (data not shown). These results demonstrate that under conditions in which infectivity is reduced nearly 100-fold, there is only modest inhibition of Gag precursor processing.

A Western blot stained with a monoclonal antibody to the *pol* gene product reverse transcriptase (RT) is shown in Fig. 1C. The results are comparable to those seen with the Gag precursor; at low concentrations of the inhibitor, the predominant species were the completely processed p66 and p51 forms of the mature RT heterodimer. However, at higher

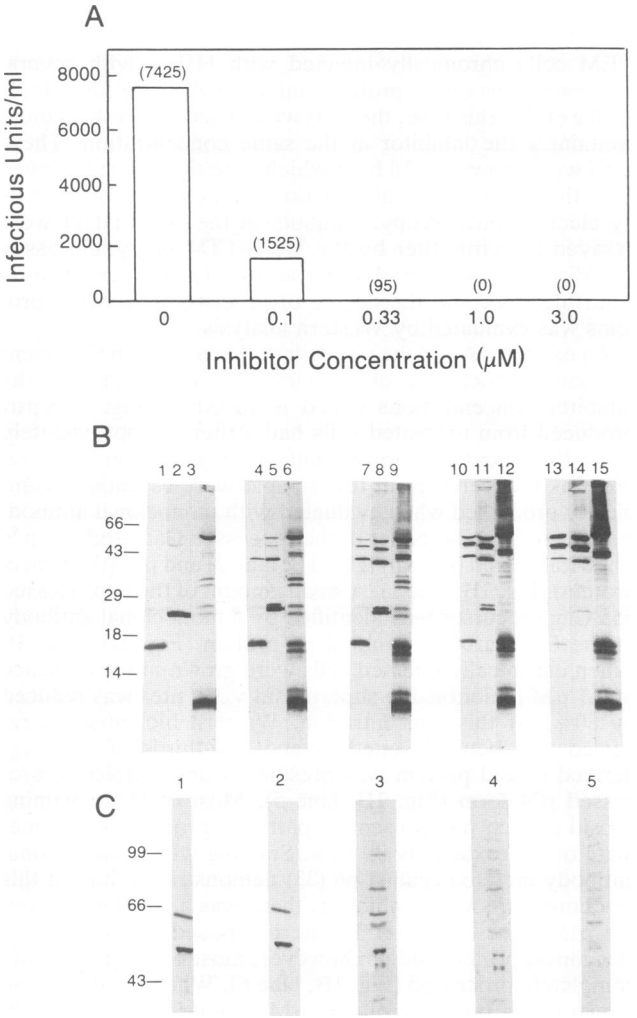

FIG. 1. Moderate defects in processing result in profound deficits in infectivity. CEM cells chronically infected with HIV-1 were isolated from acutely infected cells as described in Materials and Methods and were used for these experiments. The chronically infected cells were grown for 4 days in either 0.1% DMSO or 0.1% DMSO with various concentrations of the protease inhibitor A-77003. The supernatant from each culture was harvested and used as a source of virus. Pelletable viral protein was recovered from aliquots of the supernatant by ultracentrifugation at 100,000 × *g* for 1.5 h. (A) Infectivity. The focal immunoassay as described by Chesebro and Wehrly (4) was used to determine the titer of virus recovered from chronically infected cells treated with different concentrations of A-77003. The number of infectious units per milliliter was calculated and is shown graphically. The absolute number of infectious units per milliliter is shown in parentheses. (B) Extent of Gag processing. Cell-free supernatant was recovered from infected cells as described above, and equivalent amounts of pelleted virion-associated protein were subjected to Western analysis. The samples were either from untreated cells (lanes 1 to 3) or from cells treated with different concentrations of A-77003: 0.1 (lanes 4 to 6), 0.33 (lanes 7 to 9), 1.0 (lanes 10 to 12), or 3 (lanes 13 to 15) μM. The samples were then stained with monoclonal antibodies to either the N-terminal p17 matrix protein (lanes 1, 4, 7, 10, and 13), the p24 capsid protein (lanes 2, 5, 8, 11, and 14), or the C-terminal p6 Gag protein (lanes 3, 6, 9, 12, and 15). Monoclonal antibodies to p17 and p24 were purchased from DuPont (Wilmington, Del.). Monoclonal antibody to p6 was graciously supplied by F. Veronese (33). (C) Extent of processing of RT. Equivalent amounts of pelleted virion-associated protein from each sample were analyzed by Western blot and stained with monoclonal antibody directed against the RT of HIV-1 (DuPont). Pelleted virion-associated protein is from untreated cells (lane 1) or cells treated with 0.1 (lane 2), 0.33 (lane 3), 1.0 (lane 4), or 3 (lane 5) μM A-77003.

concentrations of the inhibitor processing intermediates began to appear. At least 50% of RT was present in its completely processed forms under conditions in which infectivity was reduced nearly 100-fold. Therefore, the processing events required to generate the mature RT do not appear to be more sensitive to the inhibition than the processing of Gag, and the modest reduction in processing seen is inconsistent with the more dramatic drop in infectivity.

Partial inhibition of processing results in aberrant virion morphology. If precursor processing is completely blocked, for example, with a high concentration of inhibitor (30) or with a mutation in the protease domain (9, 17, 26), budded virions assume a well-described distended or open-circle shape when thin sections of virus particles are analyzed by electron microscopy. This is in contrast to the electron-dense, concentrically placed core seen in mature virions. We were interested in determining the morphology of virions with subtle defects in processing but profound deficits in infectivity. The infected cells which were used in the experiments described above were fixed and examined by electron microscopy for the presence of extracellular virus (Fig. 2). A variety of virion morphologies exists in both the treated and the untreated samples, and different forms predominate at different inhibitor concentrations (Fig. 2A to H). The virus produced from untreated cells contains the characteristic centrally placed electron-dense core structure (Fig. 2A and E). At an inhibitor concentration of 0.1 μM, there is a mix of normal-appearing virions and virus particles containing eccentrically located cores which appear condensed but remain attached to the inner face of the viral envelope. At 0.33 μM, virus particles with these eccentrically placed cores predominate (Fig. 2B and F), and at the higher concentrations, C shapes and the open forms are most common (Fig. 2C, D, G, and H). In addition to the open forms, there are also some partially open rings as well as structures that seem to contain double open rings. These structures have been observed previously at high concentrations of protease inhibitors (30). The frequency with which each of these distinct virion morphologies appears at each inhibitor concentration is tabulated in Table 1. In this analysis, we see a series of morphologies which correspond to the variable extent of processing. The virtual absence of infectivity in those samples in which the aberrant forms predominate suggests that none of the virus particles with altered morphology is infectious.

Aberrantly assembled particles produce reduced amounts of viral DNA upon infection of susceptible cells. Although it has long been appreciated that retroviruses which lack functioning proteases are noninfectious, the exact block to infection has not been fully characterized. We examined newly infected cells in order to determine whether reverse transcription of the viral genome occurs. We began by measuring the activity of RT in the virus preparations by an exogenous RT assay (Table 2). RT activity was roughly correlated with the degree of processing; however, as in the case with the Gag precursor processing, infectivity decreased out of proportion to the modest decline in RT activity.

An analysis of the viral DNA synthesized after infection with virus particles produced at two concentrations of A-77003 was performed (Fig. 3). Our initial experiments used polymerase chain reaction primers designed to detect the initial product of reverse transcription. It has been suggested that the viral protease acts early in infection, just after the virus particle enters the target cell (28). To avoid a carryover effect from the inhibitor present in the superna-

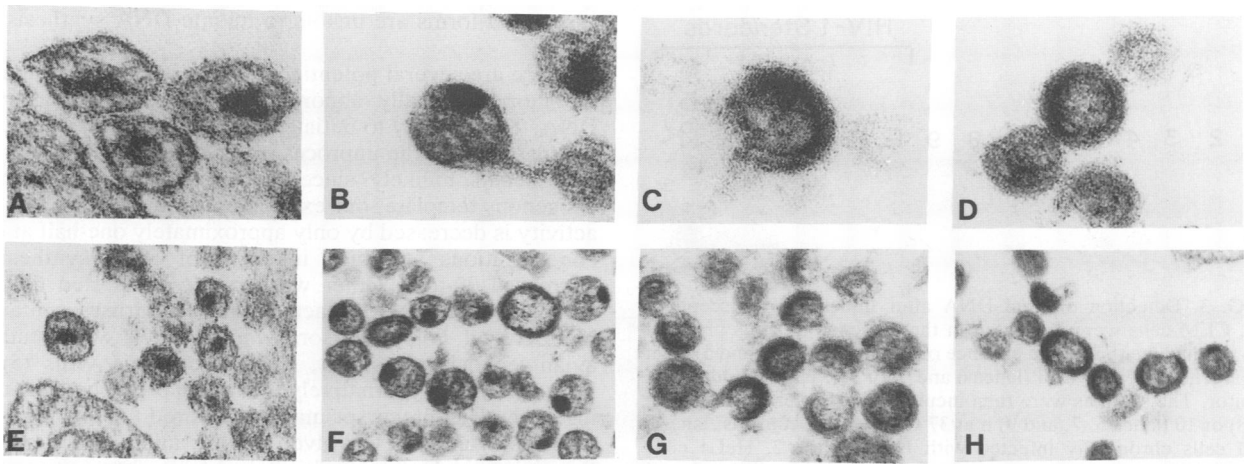

FIG. 2. Virion morphology is altered by incomplete processing of the Gag precursor. The chronically infected CEM cells used to produce supernatant for the experiment described in the legend to Fig. 1 were fixed, embedded, and sectioned for electron microscopy. Cells were grown for 5 days either in 0.1% DMSO (A and E) or in 0.1% DMSO and various concentrations of the inhibitor A-77003: 0.33 (B and F), 1.0 (C and G), and 3.0 (D and H) μM.

tants, virus was pelleted from the supernatant and resuspended in medium without inhibitor. However, we do not know whether the inhibitor remained tightly bound to some fraction of the protease molecules. In the absence of inhibitor, there were approximately 1,000 copies of viral DNA present in the target cells at 6 h after infection (Fig. 3, lane 4). At this same time, there were fewer than 50 copies of viral DNA in the cells infected with virus grown in the presence of 0.33 μM A-77003 and similar amounts of viral DNA in the cells grown at 3.0 μM (lanes 6 and 8). By 10 h after infection, the cells infected with the untreated virus contained approximately 5,000 copies of viral DNA (lane 5), whereas the cells newly infected with virus produced in the presence of 0.33 μM inhibitor contained 250 copies (lane 7). Target cells infected for 10 h with virus produced in the presence of 3.0 μM A-77003 contained only 50 copies of viral DNA (lane 9). Therefore, little viral DNA was detected following infection by samples grown in the presence of 3.0 μM A-77003, and there is an approximately 20-fold reduction in the initiation of viral DNA synthesis when susceptible cells are infected with virus grown in the presence of 0.33 μM inhibitor.

TABLE 1. Virus grown in the presence of different concentrations of A-77003 exhibits a range of morphologies

Concn (μM)[a]	Morphology[b,c]			
	Wild type	Eccentric	C-shaped	Open
0	123 (86)	14 (10)	4 (3)	2 (1)
0.1	49 (31)	84 (54)	17 (11)	6 (4)
0.33	5 (4)	78 (56)	52 (37)	4 (3)
1.0	0 (0)	9 (5)	56 (32)	110 (63)
3.0	0 (0)	2 (2)	9 (7)	109 (91)

[a] Cells chronically infected with HIV-1 were grown in the listed concentrations of A-77003 and fixed for electron microscopy.

[b] Four distinct morphologies were identified (Fig. 2) and quantitated. These forms are a condensed, centrally placed core (wild type [Fig. 2A]), a condensed core still associated with the viral membrane (eccentric [Fig. 2B]), C-shaped electron density associated with the viral membrane (C-shaped [Fig. 2C]), and a mix of open ring structures as well as structures containing double open rings (open [Fig. 2D]).

[c] The number of each form of virus seen in thin sections of cells grown in the indicated concentration of inhibitor is listed, and the percentage of the total is given in parentheses.

DISCUSSION

Thus far, the only clinically available treatments for HIV infection are the RT inhibitors 3′-azido-3′-deoxythymidine and 2′,3′-dideoxyinosine. Like RT, the viral protease is an attractive target for therapeutic intervention. Both are enzymes which play critical roles in a complex series of events in the virus life cycle: RT in the synthesis of double-stranded DNA from single-stranded RNA and protease in the processing of the Gag and Gag/Pol precursors during virus assembly. The multiple events involved in virus assembly are each potentially susceptible to inhibition, and to the extent that the individual events are interdependent, inhibition of one step may be amplified in its effect by disruption of required subsequent interactions with other components. Although the need for accurate processing in the production of properly assembled (i.e., morphologically normal) and infectious virus is well established, the impact of subtle defects in the processing pathway on virus assembly and infectivity has not been well studied.

TABLE 2. RT activity in the presence of various concentrations of A-77003

Concn (μM)[a]	Expt no.[b,c]	
	1	2
0	72,268	92,540
0.1	34,882	48,771
0.33	21,900	49,907
1.0	10,562	19,703
3.0	10,845	25,155

[a] Virus-containing supernatants were obtained from chronically infected CEM cells treated with the indicated concentrations of inhibitor and assayed for the presence of RT activity.

[b] Two representative experiments are shown.

[c] RT assays were performed as described previously and are reported as counts per minute of [^{3}H]TTP incorporated with poly(rA) · oligo(dT) as a template-primer (3).

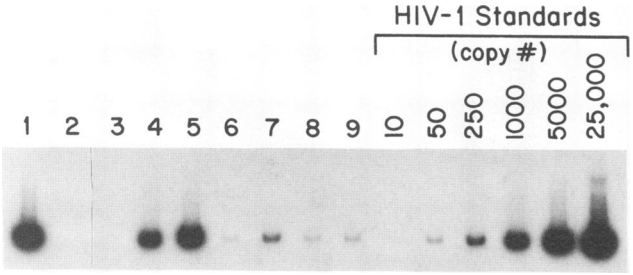

FIG. 3. Detection of viral DNA after infection of susceptible cells. CEM cells were incubated in the presence of virus preparations produced either in the absence of A-77003 (lanes 4 and 5) or in the presence of either 0.33 (lanes 6 and 7) or 3.0 (lanes 8 and 9) μM inhibitor. The samples were then incubated for either 6 (lanes 4, 6, and 8) or 10 (lanes 5, 7, and 9) h at 37°C. Negative controls: lane 1, CEM cells chronically infected with HIV-1; lane 2, HeLa cells incubated for 10 h with untreated virus; lane 3, CEM cells incubated in the presence of heat-killed HIV-1.

We have established that a relatively small amount of protease inhibition resulted in profound declines in virus infectivity. Krausslich recently noted similar results with another protease inhibitor (19). Furthermore, we have correlated this subtle inhibition of processing with aberrant and apparently noninfectious virion morphologies. The mechanism by which incomplete protease inhibition results in the observed morphological change is unclear. The Gag precursor is myristoylated at its amino terminus, and this myristoylation has been shown to be important for several different retroviruses in directing the precursor to the membrane (2, 9, 27, 31). The amino-terminal matrix protein remains associated with the viral membrane after processing (8). It may be that the eccentrically placed core structures are the result of incomplete processing at the cleavage site between the matrix protein and the adjacent capsid protein. This would be predicted to result in persistent membrane association of the otherwise condensed core. These forms predominate at an inhibitor concentration of 0.33 μM, and at this concentration, about 50% of the matrix protein was present as the mature p17 species with the rest of the matrix protein remaining linked to the adjacent capsid protein. Support for this explanation comes from studies in which the matrix-capsid cleavage site was blocked by mutation, leading to virus with eccentrically placed cores (9), although our results would suggest that only a fraction of the matrix protein needs to remain attached to the collapsed core to produce this phenotype. The persistent association of the core particle with the viral membrane is similar to that seen with the B- and D-type retroviruses. It is possible that incomplete cleavage of the C terminus of the matrix protein may also be responsible for the distinctive morphology of these viruses (6).

The exact nature of the block to infectivity is also obscure. The aberrantly assembled particles do not appear to be able to initiate viral DNA synthesis efficiently upon infection. Our data indicate that the particles produced from infected cells grown at a concentration of 0.33 μM A-77003 display an approximately 20-fold reduction in the initiation of viral DNA synthesis. This same virus stock has an infectivity which is decreased 80- to 100-fold compared with that of virus produced from untreated cells. Since 1 in 20 of the particles from the cells treated with this concentration is morphologically normal, it is possible that the viral DNA we identified is produced by these particles and that improperly assembled forms are unable to initiate DNA synthesis efficiently.

There are several potential explanations for the failure of the morphologically abnormal particles to produce viral DNA. The inability to initiate DNA synthesis may be due to the inactivity of the unprocessed or partially processed RT. This seems unlikely since, with an assay based on an exogenous template, our experiments indicate that total RT activity is decreased by only approximately one-half at drug concentrations at which initiation of DNA synthesis is decreased 20-fold. Other workers have reported differing amounts of residual RT activity in retroviruses containing unprocessed Pol precursors whether they were studying avian leukosis virus (32), murine leukemia virus (5), or HIV-1 (20, 25). Alternatively, complete proteolytic processing of the Gag precursor may be required for the release of proteins which are involved in the initiation of reverse transcription. For example, it has been demonstrated for Rous sarcoma virus that a fully processed nucleocapsid (NC) protein is important in maintaining the normal dimeric conformation of the viral genome (24, 32). It is unclear whether monomeric viral genomic RNA can undergo reverse transcription. Another possibility is that residual inhibitor carried in the treated virus stock inhibited the protease, which caused a failure to cleave the NC protein after infection. Such a cleavage shortly after infection has been suggested to enhance infectivity (28), although contradictory results have been obtained by others (11). Finally, persistent association of the viral core with the target cell membrane after fusion may prevent release of the core into the cytoplasm, where DNA synthesis normally occurs.

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ADDENDUM IN PROOF

Kageyama et al. have reported similar experiments (Kageyama et al., submitted for publication) and found that virions made in the presence of A-77003 contain, on average, less p24 antigen per virion particle when measured by three different commercially available antigen assay kits.

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