Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 6612–6616, September 1988
Biochemistry

Human immunodeficiency virus has an aspartic-type protease that can be inhibited by pepstatin A

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Communicated by Maurice R. Hilleman, May 19, 1988

ABSTRACT **The protease encoded by the human immunodeficiency virus (HIV) processes the viral gag and gag–pol protein precursor by posttranslational cleavage. In this study we have demonstrated by site-specific mutagenesis (Asp → Thr) and by pepstatin A inhibition that the recombinant HIV protease is an aspartic-type protease. Furthermore, incubation of HIV-infected H9 cells with pepstatin A inhibited part of the intracellular processing of the HIV gag protein yet had no apparent toxicity on HIV-infected cells during 48 hr of incubation.**

Inhibition of retrovirus-encoded enzymes offers a way to interfere with the replication and propagation of these viruses. Most studies have focused on inhibiting the reverse transcriptase. This led to the application of 3-azido-3-deoxythymidine (AZT), a reverse transcriptase (RT) inhibitor, as a chemotherapeutic agent for patients infected with the human immunodeficiency virus (HIV) (1, 2).

There is, however, growing interest in inhibiting the second HIV-encoded enzyme, a protease. A virus-encoded protease has been detected in avian and murine retroviruses (3, 4) and has been shown to process the gag protein precursor to yield mature gag proteins that are required for assembly of infectious virus particles (refs. 5 and 6; for review, see ref. 7). The retroviral proteases appear to be highly substrate specific; they process their own gag protein precursor, they process the gag precursor of only some closely related retroviruses, but they do not process gag precursors of distantly related retroviruses (8–10). Other proteins are not recognized as a substrate unless they are denatured (11, 12).

Perhaps an inhibitor specifically directed against the retrovirus protease could block the processing of the viral gag proteins and thereby prevent the production of infectious virus without interfering with host-cell proteolytic activities. A murine retrovirus with a defective protease has been shown to produce immature virus particles lacking infectivity (5, 6).

To obtain suitable inhibitors of the virally coded proteases, the functional groups in the active site of the enzyme must be identified. Examination of the amino acid sequences of retroviral proteases has revealed a conserved Asp-Thr-Gly (Asp-Ser-Gly) sequence, similar to that of aspartic proteases (7, 13, 14), and a structural model for a viral aspartic protease has been proposed (14). Further, the proteolytic activities of bovine leukemia virus, Moloney murine leukemia virus, and human T-cell leukemia virus type I can be inhibited *in vitro* by pepstatin A, an aspartic protease inhibitor, although at a relatively high drug concentration (15). These data suggest that these retroviral proteases are closely related to the class of aspartic proteases, and we wanted to determine whether the HIV protease also belonged to this class of proteases.

Here we describe studies on the inhibition *in vitro* and in part *in vivo* of the HIV protease, confirming the suggestion (7, 13, 14) that the HIV-encoded protease is an aspartic-type protease and demonstrating that it can be inhibited by pepstatin A.

MATERIALS AND METHODS

Cells and Virus. HIV isolates BRU (16) and BH10 (17) were grown in H9 cells (18). Virus was obtained from the culture supernatant of HIV-producing H9 cells and purified by pelleting and sucrose-density-gradient centrifugation.

Bacteria, Plasmids, and Recombinant Clones. The following constructs of recombinant HIV protease plasmid DNA were made: (*i*) a *Bgl* II–*Sca* I fragment (see Fig. 1) from the HIV (BRU) DNA clone [subclone of λJ19 (16)] containing the beginning (19) of the *pol–orf* sequence, the viral protease (*prt*), and the N-terminal part of the RT; or (*ii*) a longer *Bgl* II–*Eco*RI fragment, containing the sequence of the protease, RT, and part of the endonuclease. These constructs were inserted into the polylinker of the plasmid pTZ19R (Pharmacia). The plasmid was digested by *Bam*HI and *Hin*dII or by *Bam*HI and *Eco*RI, respectively, prior to the insertion of the HIV DNA. The resulting recombinant proteins have a N-terminal "fusion" end of 32 plasmid-derived amino acids, mainly from the polylinker (not from the β-galactosidase sequence), upstream of the indicated *Bgl* II site of HIV (Fig. 1). The recombinant plasmids were transfected into JM 105 cells (20) and selected in ampicillin according to standard procedures (21). The short recombinant plasmid was named pTZprts and the long one was pTZprtl.

For the construction of gag recombinant protein a *Sac* I–*Hin*dII fragment from the HIV (BRU) DNA clone (16) containing the sequence of the entire gag open reading frame was inserted into the polylinker of plasmid pUC18. The recombinant plasmid referred to as pUCgag was transfected into JM 105 cells and selected in ampicillin. The resulting recombinant gag protein contains no β-galactosidase fusion sequences because two stop codons of the HIV sequence precede the *gag–orf* initiation codon.

Site-Specific Mutagenesis. The site-specific mutation was made on the pTZprtl recombinant plasmid as described (22). The 19-mer oligonucleotide used was centered around the indicated conserved (Asp-Thr-Gly) codons containing an ACU codon (threonine) instead of a GAU codon (aspartic acid) (Fig. 1). The recombinant mutant protease is referred to as pTZprtl$^-$ (in contrast to the wild-type pTZprtl$^+$).

Preparation of Recombinant Protein. The *prt*- and the *gag*-containing plasmid bacterial clones were grown to a midlogarithmic phase density at OD_{550} of 0.4. The *lac* promoter was induced by 2 mM isopropyl β-D-thiogalactoside and cells were incubated for 60 min at 37°C. The cells were

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Abbreviations: HIV, human immunodeficiency virus; Me_2SO, dimethyl sulfoxide; PCMB, 4-chloromercuribenzoic acid; RT, reverse transcriptase.

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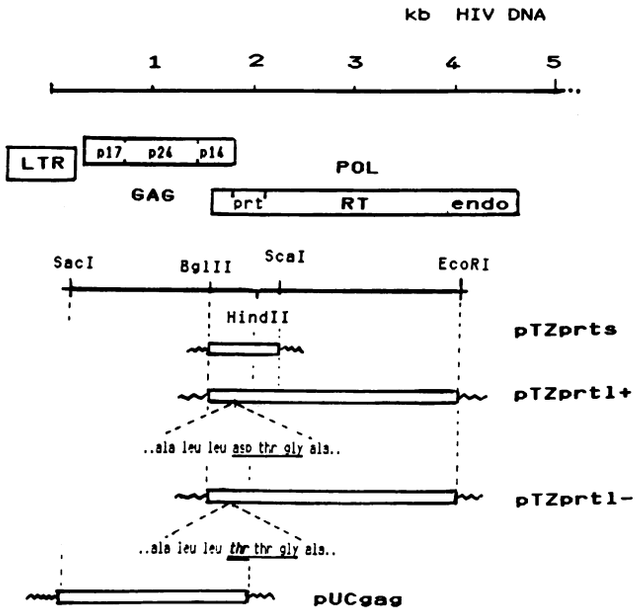

FIG. 1. Schematic representation of the *gag* and *pol* region in the HIV map and of the recombinant HIV constructs. For the recombinant HIV prt plasmid, the indicated *Bgl* II–*Sca* I or *Bgl* II–*Eco*RI fragments of the HIV DNA (BRU) (16) were excised and inserted into a polylinker of plasmid pTZ19R (Pharmacia) digested with *Bam*HI/*Hin*dII or *Bam*HI/*Eco*RI. The recombinant plasmids were referred to as pTZprts and pTZprtl$^+$. The Asp → Thr mutant was derived by site-specific mutagenesis from the pTZprtl clone and referred to as pTZprtl$^-$. For the recombinant HIV gag plasmid, the indicated *Sac* I–*Hin*dII fragment in the HIV DNA (BRU) was excised and inserted into a polylinker of plasmid pUC18 and referred to as pUCgag. kb, Kilobase(s).

collected by centrifugation and lysed by sonification in PNTE buffer (50 mM Pipes, pH 6.5/150 mM NaCl/0.2% Triton X-100/1 mM EDTA). The lysates were centrifuged and the supernatants (referred to as *Escherichia coli* lysates in this paper) were used as source of proteolytic activity or of gag substrate. Lysates had an average protein concentration of ≈10 mg/ml.

Assays of Proteolytic Activity, Inhibitors, and *in Vitro* Inhibition. Lysates (3–10 μl, as indicated in Figs. 2 and 3) of recombinant protease were incubated in PNTE lysate buffer with ≈5 μg of purified Triton X-100 (1%)-disrupted virus or with 10 μl of lysates (pUCgag) containing the gag recombinant protein for 20 min at 37°C. Protease inhibitors were added as indicated. Pepstatin A (23), phenylmethylsulfonyl fluoride, and E-64 were from Boehringer Mannheim, 4-chloromercuribenzoic acid (PCMB) was from Sigma; the pepsin inhibitor Val-D-Leu-Pro-Phe-Phe-Val-D-Leu (24) and renin inhibitor Pro-His-Pro-Phe-His-Leu-Val-Ile-His-Lys (25) were from Protogen, Laeufelfingen, Switzerland; and cystatin was prepared as described (26).

Analysis of Proteolytic Reaction Products. The reaction mixtures were analyzed immediately after the indicated incubation period by immunoblotting. The reaction products were separated on a $NaDodSO_4$/PAGE (12.5% or 15%), electroblotted to nitrocellulose filters (27), and incubated with HIV-positive human sera recognizing the HIV gag proteins (p55, p40, p24, and p17) or RT.

Reaction of Pepstatin A with HIV-Infected Cells. Pepstatin A was freshly dissolved in dimethyl sulfoxide (Me_2SO) at 7 mM. It was very slowly diluted (1:100) into the medium of HIV-infected H9 suspension cultures so that no pepstatin A precipitated (final concentration, 70 μM pepstatin A and 1% Me_2SO), and the cultures were incubated without change of culture medium for 48 hr. As control, uninfected H9 cells were also incubated with pepstatin and in addition HIV-infected and uninfected cells were incubated with 1% Me_2SO but without pepstatin; no alteration of viability was observed in any of the cells evaluated by trypan blue staining.

After the incubation period cells were disrupted in hypotonic buffer plus 1% Triton X-100 and the cell lysate was analyzed by immunoblotting, as described above. Virus from the culture medium was pelleted and the pellet was subjected to immunoblotting for preliminary virus analysis.

RESULTS

To determine if the HIV-encoded protease has an aspartic acid residue as part of the active site, we created a site-specific mutation *in vitro* in this protease by replacing the aspartic acid residue in the conserved Asp-Thr-Gly sequence with a threonine residue. The GAU codon (aspartic acid) was changed to an ACU codon (threonine) (Fig. 1) in the pTZprtl clone that contained a *Bgl* II–*Eco*RI fragment of an HIV DNA clone (LAV) (16). The wild-type recombinant plasmid was

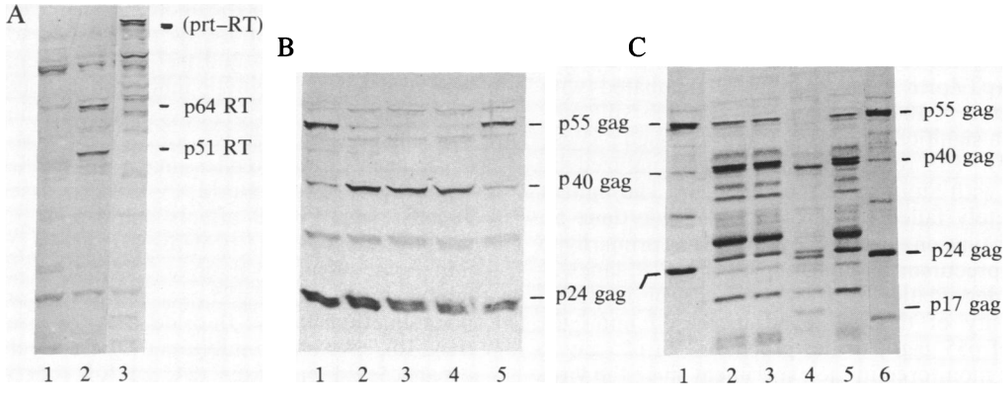

FIG. 2. Function of the recombinant HIV protease. (*A*) Intramolecular cleavage of prt–pol recombinant protein. Lanes: 1, 10 μl of pTZ19R lysate (i.e., plasmid without HIV DNA insertion); 2, 10 μl of pTZprtl$^+$ lysate; and 3, 10 μl of pTZprtl$^-$ lysate. Lysates were incubated for 20 min at 37°C. The incubation mixtures were analyzed by immunoblotting with human HIV antiserum reacting with RT. (*B*) Processing of viral gag precursor protein by recombinant protease. Purified, Triton X-100-disrupted HIV (5 μg) was incubated for 20 min at 37°C (except for lane 4 for 40 min at 37°C) as follows: Lanes: 1, 10 μl of pTZ19R lysate (i.e., vector alone); 2, 5 μl of pTZprtl$^+$ lysate; 3 and 4, 10 μl of pTZprtl$^+$ lysate; 5, 10 μl of pTZprtl$^-$ lysate. Reaction products were analyzed by immunoblotting with human HIV antiserum against p24 gag. (*C*) Processing of recombinant gag protein by recombinant protease. Lanes: 1 and 6, HIV as protein marker; 2–5, 10 μl of pUCgag lysate. Lanes: 2, no incubation; 3, incubation with 3 μl of pTZ19R lysate (i.e., vector alone); 4, incubation with 3 μl of pTZprtl$^+$ lysate; 5, incubation with 3 μl of pTZprtl$^-$ lysate. Incubations were at 37°C for 20 min. Reaction products were analyzed by immunoblotting as in *B* with antiserum against gag.

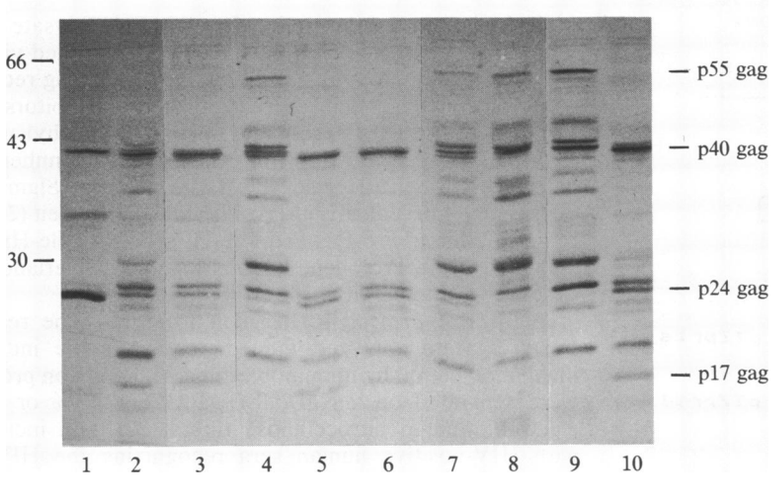

FIG. 3. Inhibition of recombinant protease by inhibitors of aspartic, cysteine, and serine proteases. pTZprts lysate (3 μl) was added to 5 μg of purified Triton X-100-lysed HIV as protein markers (lane 1) or to 10 μl of pUCgag lysate (lanes 2–10) and incubated for 20 min at 37°C. Addition of inhibitors was as follows. Lanes: 2, no inhibitor; 3, 100 μM PCMB; 4, 1 mM PCMB; 5, 1 mM cystatin; 6, 1 mM E-64; 7, 50 μM pepstatin A; 8, 250 μM pepstatin A; 9, 1 mM pepstatin A; 10, 5 mM phenylmethylsulfonyl fluoride. All reactions were analyzed by immunoblotting as in Fig. 2.

called pTZprtl$^+$ and the mutant was pTZprtl$^-$. When the wild-type construct (pTZprtl$^+$) was expressed in *E. coli* and the lysates were incubated *in vitro*, immunoblot analysis (Fig. 2*A*, lane 2) showed the formation of processed polypeptides of 64 and 51 kDa [similar to those reported for the viral RT (28)] and no polypeptide of the predicted length of 98 kDa encoded by the HIV DNA insert was detected.

With the mutationally altered construct (pTZprtl$^-$), however, the immunoblot showed a large 98-kDa protein (Fig. 2*A*, lane 3). This protein had the predicted size of the unprocessed, primary translation product of the mutant recombinant construct, indicating that the Asp → Thr mutation blocked the virus-specific proteolytic activity that normally processes the prt–pol recombinant protein. Without *in vitro* incubation of the lysates (i.e., lysates were analyzed immediately after the bacteria were lysed) results identical to those in Fig. 2*A* were obtained (data not shown), but the experiment (Fig. 2*A*) was done as described to ensure that even with the additional *in vitro* incubation the mutant protease did not function. The 98-kDa recombinant protein of the mutant protease lysate (pTZprtl$^-$) could, however, be processed to the 64- and 51-kDa RT proteins (p51 and p64) when lysate with the wild-type protease (pTZprts) was incubated with the mutant enzyme (data not shown), indicating that the Asp → Thr mutation did not render the protein uncleavable but blocked the active enzymatic site.

Next, the proteolytic activity of the mutant toward its specific substrate, the 55-kDa gag precursor (p55 gag), was examined. *E. coli* lysates containing the wild-type (pTZprtl$^+$) or the mutant (pTZprtl$^-$) protease were incubated with purified and Triton X-100-disrupted HIV, which still normally contained a significant amount of unprocessed p55 gag (Fig. 2*B*, lane 1). The reaction product was analyzed by immunoblotting with anti-gag serum (Fig. 2*B*). The mutant protease completely failed to cleave the p55 gag (lane 5), whereas the wild-type enzyme processed p55 gag primarily to an intermediate precursor of 40 kDa (p40) (Fig. 2*B*, lanes 2–4); nonspecific degradation of p55 gag could be excluded since the intensity of the p40 band increased upon the disappearance of p55 gag. Further processing of p55 gag to p24 could not be monitored in the experiment since the virus particles, used as the source of the p55 gag substrate, contained an abundance of p24. Thus to analyze the entire recombinant protease processing of the gag protein *in vitro*, a recombinant p55 gag protein was used as substrate. For this purpose a recombinant plasmid was made with a *SacI–HindII* fragment of the HIV DNA (19) spanning the entire gag protein sequence (see Fig. 1*B*) referred to as pUCgag.

The expression of this construct in *E. coli* yielded a protein of 55 kDa comigrating with the HIV gag precursor protein and reacting with anti-gag sera in an immunoblot (Fig. 2*C*, lane 2). Some smaller polypeptides reacting in the immunoblot with anti-gag serum were also expressed in the *E. coli* lysate; they are probably due to incorrect start of translation in *E. coli* at internal gag AUG codons (29). When the recombinant gag precursor was incubated with recombinant protease (pTZprtl$^+$) (Fig. 2*C*, lane 4), p55 gag was processed to proteins reacting with anti-gag serum and comigrating with viral p40 and p24 and to a protein migrating slightly slower than authentic viral p17. This processed recombinant protein probably migrated more slowly because, in contrast to the viral p17 (30), it lacks myristoylation; it appears only in lane 4 (in which processing occurred) and is different from the unprocessed double band in lanes 2, 4, and 5, which is smaller than viral p17. The lysate containing the mutant protease did not cleave the recombinant gag precursor (Fig. 2*C*, lane 5).

By using the recombinant protease pTZprts (see Fig. 1) instead of pTZprtl$^+$, identical processing of gag (as in Fig. 2 *B* and *C*) was obtained (data not shown).

Next, the *in vitro* system of recombinant viral protease and gag precursor was used to test the effect of various protease inhibitors (Fig. 3). When such inhibitors were added to an incubation mixture of *E. coli* lysates containing prt (pTZprts) and gag (pUCgag) recombinant proteins (as in Fig. 2), the proteolytic activity was significantly inhibited (Fig. 3, lanes 7–9) by pepstatin, an aspartic protease inhibitor. At a concentration of 5×10^{-5} M, partial inhibition of protease activity is indicated as the uncleaved p55 gag band remained; at 2.5×10^{-4} M more of p55 gag band remained and significantly less p24 and p17 were present; and at 1 mM pepstatin A the proteolytic inhibition seemed to be complete since neither p24 nor p17 was detected. These data indicate that pepstatin A has an IC_{50} of 25×10^{-5} M. The processing of p55 gag was inhibited less markedly by PCMB (lanes 3 and 4), because, even at 1 mM, PCMB-processed p24 could still be observed. PCMB affects sulfhydryl groups but is not selective for cysteine proteases; specific cysteine protease inhibitors, such as cystatin and E-64 (lanes 5 and 6), did not inhibit processing of p55 gag. The serine protease inhibitor phenylmethylsulfonyl fluoride did not affect the protease (lane 10). Two other inhibitors that effectively inhibited other aspartic acid proteases (24, 25) did not inhibit the HIV protease at a concentration of 10^{-3} M (data not shown).

Finally, in a preliminary experiment, the effect of pepstatin A on the processing of the gag precursor was tested in infected cells and its possible toxicity to uninfected or HIV-infected cells was also examined. Pepstatin, which is poorly water soluble, was dissolved in Me_2SO and diluted into the culture medium of noninfected or HIV-infected H9 cells (a final concentration of 1% Me_2SO in the medium is tolerated by the cells). After incubation for 48 hr, cell viability, determined by trypan blue staining (data not shown), of

uninfected and infected cells was not significantly altered by pepstatin A treatment as compared to the untreated control cells. The intracellular processing of HIV-specific gag protein was, however, affected by pepstatin A, as demonstrated in Fig. 4, lanes 1 and 2, and the densitometer scan. The processing of the gag precursor, p55 gag, to the intermediate precursor p40 was markedly decreased. A significant decrease of p24 or p17 was not anticipated in this experiment because of the steady-state pool of intracellular mature viral gag protein.

When lysates of uninfected H9 cells treated and not treated with pepstatin A were analyzed by immunoblot analysis with anti-gag serum, evidently no specific bands were visible; but, upon overexposure of the immunoblot, nonspecific cell-derived protein bands appeared (Fig. 4, lanes 3 and 4). No difference between pepstatin A-treated and untreated cells was detected, suggesting that pepstatin A did not principally affect cellular protein synthesis and probably did not cause severe cell toxicity.

There was no significant reduction in the number of virus particles released from culture cells after pepstatin A treatment compared to untreated cells, as judged by the amount of HIV antigen pelleted after aliquots of culture medium were centrifuged and immunoblotted (data not shown). RT activity was not quantified at this time because it would not necessarily reflect a lack of infectivity of released virus (see refs. 5 and 6).

DISCUSSION

The hypothesis that the HIV-encoded (gag processing) protease is an aspartic or aspartic-like protease is confirmed and extended by the following results: (*i*) Site-directed mutagenesis of the aspartic acid residue in the presumed active site (conserved Asp-Thr-Gly sequence) to a threonine residue abolished the proteolytic activity of the recombinant HIV protease. (*ii*) An inhibitor of aspartic proteases clearly affected the *in vitro* activity of the recombinant protease whereas a typical serine protease inhibitor (phenylmethylsulfonyl fluoride) did not; the inhibition by PCMB reflects an unspecific interaction with a sulfhydryl residue of the protease protein but is not likely to be characteristic for cysteine proteases, because cystatin and E-64, both very specific thiol protease inhibitors, did not affect the protease activity.

Our experiments were done with recombinant protease protein similar to that reported by others (28, 29). We found it extremely difficult to prepare native HIV protease, because of the minute amounts present in virus particles and probably because of its potential hydrophobic character. The fidelity and specificity of the recombinant protease in processing the gag precursor was high; the native viral p55 gag precursor, in addition to the recombinant gag protein (29, 31, 32), was cleaved by the recombinant enzyme. The intermediate processed product p40, which reacted with anti-gag serum (Fig. 2*B*), appears to be relatively stable because it is still present in virus particles (Fig. 2*B*, lane 1); it is probably the uncleaved form of p24 plus p17 (see ref. 30) since it reacts with monospecific anti-p17 (data not shown). Analogous intermediate precursors have also been found in other retroviruses (3, 4, 6, 12) and in HIV (30).

Further processing to proteins comparable to the mature virion proteins p24 and p17 was shown by using recombinant gag precursor protein; we suggest that the *in vitro*-processed recombinant gag protein of molecular weight slightly larger than viral p17 is p17 but migrated more slowly because of its lack of myristoylation. Processed p14 protein could not be detected because the antiserum used in the immunoblot analysis does not recognize p14.

The results with the site-specific mutagenesis directly confirmed earlier suggestions (28) that the recombinant HIV-encoded protease is responsible for cleaving the RT (p64 and p51) out of a recombinant protein containing the prt and RT sequences of the HIV *pol* gene (Fig. 2*A*); they corroborate indirectly the autocatalytic processing of the protease (29). This has interesting implications for future drug design, because blocking the protease activity at the level of the gag–pol precursor stage might abolish both protease and RT activity.

It was demonstrated that the initial cleavage step of p55 gag → p40 is truly catalyzed by the viral protease (Fig. 2*B*, lanes 2–4, and *C*, lane 4) as compared to the control (Fig. 2*B*, lane

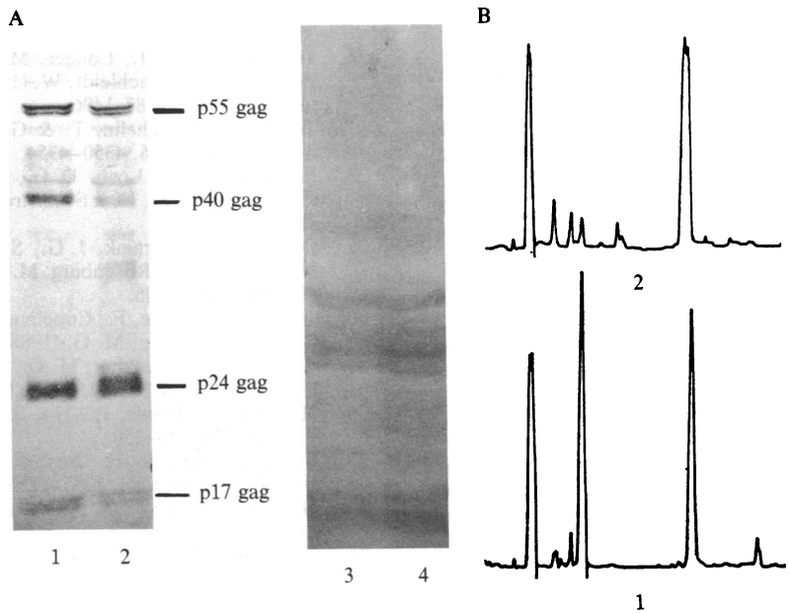

FIG. 4. Inhibition by pepstatin A *in vivo*. (*A*) Me_2SO was diluted 1:100 into cultures of HIV-infected H9 cells (lane 1). Pepstatin A, freshly dissolved at a final concentration of 7 mM in Me_2SO, was very slowly diluted 1:100 as it was added to cultures (lane 2). Me_2SO was diluted 1:100 into cultures of noninfected H9 cells (lane 3). Pepstatin A in Me_2SO was diluted 1:100 into cultures (lane 4). Cell cultures were incubated for 48 hr at 37°C, then lysed in 1% Triton X-100, and centrifuged, and the supernatant was analyzed by immunoblotting. (*B*) Densitomer scans of *A*, lanes 1 and 2. The left peaks represent p55 gag, the middle peak of lane 1 represents p40, and the right peaks represent p24.

1, and *C*, lane 3). This cleavage could be blocked by the Asp → Thr mutation of the recombinant protease or inhibited *in vitro* by pepstatin A (Fig. 3).

Some inhibitory effects of pepstatin A on the avian retroviral protease p15 *in vitro* (11) and on focus formation of murine sarcoma virus *in vivo* (33) have been reported.

In a preliminary study pepstatin A was added to cell cultures to determine whether pepstatin A had any effect on intracellular HIV gag processing and whether it would be toxic to the cells. The results show that pepstatin A is able to inhibit at least part of the intracellular HIV-specific gag processing (p55 gag → p40) and does not cause obvious toxic side effects for the cells (at least not during the 48-hr incubation).

Other data corroborating the low toxicity of pepstatin A have been reported: pepstatin A treatment at 2×10^{-4} M for 4 days showed no effect on the viability of Epstein–Barr virus-infected Raji cells (34); daily oral administration of 800 mg/kg to monkeys does not cause any sign of toxicity (23); the LC_{50} in mice is 1190 mg/kg, i.p. (35); oral medication of ulcer patients with pepstatin A at 700 mg/day for 6 weeks did not cause any side effects (36).

It was not the purpose of this preliminary study to determine accurately a decrease of released virus particles or the activity of the RT, because these parameters do not necessarily reflect viral infectivity. It has been reported that a protease mutant of Moloney murine leukemia virus that was deficient in processing of the gag and gag–pol precursor rendered the virus almost noninfectious, although viral particles containing unprocessed gag proteins were still produced, released, and had nearly normal levels of RT activity (5, 6).

The IC_{50} of pepstatin A for the HIV protease was found to be 7×10^{-5} M (*in vivo*) and 2.5×10^{-4} M (*in vitro*), comparable to concentrations described by Katoh *et al.* (15) for inhibiting other retroviral proteases. The IC_{50} values for the inhibition of other aspartic proteases by pepstatin A are 10^{-6}–10^{-8} M. Thus the inhibition of pepstatin A against the HIV protease is less efficient than against other aspartic proteases. However, pepstatin A is the only one of three aspartic acid inhibitors we tested that does inhibit the HIV protease; the other aspartic protease inhibitors do not inhibit the HIV protease.

This and the fact that the viral protease has less than half the molecular weight of other aspartic proteases (14) implies that the two types of enzymes must have a distinct molecular structure. The results of a second site-specific mutation of the aspartic acid residue close to the C-terminal end of the viral protease might reveal whether there is a likelihood for a dimer structure as proposed (14) for the active form of the viral enzyme.

The fact that the structure of the viral enzyme is distinct from other aspartic enzymes is, however, an intriguing motivation to search for more specific inhibitors.

Note Added in Proof. While this paper was in press, Mous *et al.* (37) published a comparable mutation also blocking the HIV protease activity. In the meantime, we have shown that a mutation of the C-terminal aspartic acid partly inhibits the protease activity.

We thank F. Deinhardt for his interest in and continuous support of this work; L. Gürtler and J. Eberle for kindly providing cultures of HIV-infected and noninfected H9 cells, part of the HIV used in this study, and HIV antiserum; S. Wain-Hobson for providing the HIV (BRU) DNA clone; H. Wolf and F. Schwarzmann for synthesis of oligonucleotides; and H. Fritz for helpful discussions and critical reading of the manuscript.

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