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Defining the Level of Human Immunodeficiency Virus Type 1 (HIV-1) Protease Activity Required for HIV-1 Particle Maturation and Infectivity

JASON R. ROSÉ,[1] LILIA M. BABÉ,[2]† AND CHARLES S. CRAIK[2,3]*

Departments of Pharmacology,[1] Pharmaceutical Chemistry,[2] and Biochemistry and Biophysics,[3] University of California, San Francisco, California 94143-0446

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The human immunodeficiency virus type 1 (HIV-1) protease is the enzyme required for processing of the Gag and Gag-Pol polyproteins to yield mature, infectious virions. Although the complete absence of proteolytic activity prevents maturation, the level of activity sufficient for maturation and subsequent infectivity has not been determined. Amino acid substitutions that reduce catalytic activity without affecting substrate recognition have been engineered into the active site of the HIV-1 protease. The catalytic efficiency (k_{cat}) of the HIV-1 protease is decreased 4-fold when threonine 26 is replaced by serine (T26S) and approximately 50-fold when alanine 28 is replaced by serine (A28S). Genes containing these mutations were cloned into a proviral vector for analysis of their effects on virion maturation and infectivity. The results show that virions containing the T26S protease variant, in which only 25% of the protease is active, are very similar to wild-type virions, although slight reductions in infectivity are observed. Virions containing the A28S protease variant are not infectious, even though a limited amount of polyprotein processing does occur. There appears to be a linear correlation between the level of protease activity and particle infectivity. Our observations suggest that a threshold of protease activity exists between a 4-fold and 50-fold reduction, below which processing is insufficient to yield infectious particles. Our data also suggest that a reduction of protease activity by 50-fold or greater is sufficient to prevent the formation of infectious particles.

The protease encoded in the human immunodeficiency virus type 1 (HIV-1) genome plays a crucial role in the life cycle of the virus, cleaving the $p55^{gag}$ and $p160^{gag\text{-}pol}$ precursors into their mature and functional forms (16). Viruses in which the protease has been mutated to remove the active-site aspartic acid residues will assemble in the absence of proteolytic activity, but the resultant virions are noninfectious and morphologically aberrant (8, 16, 29). The development of inhibitors of the HIV-1 protease has been pursued as a means of preventing viral replication, and many inhibitors which can block viral maturation in tissue culture (24, 43, 44) and slow viral replication in vivo (21) now exist.

The exact requirements of the maturation step for protease activity are not clear. Although it is known that the elimination of activity prevents maturation, detailed information regarding the minimal proteolytic activity required for formation of an infectious virion is not available. This is an important issue in determining how potent an inhibitor of the protease must be to prevent viral replication. It has been reported that in the presence of limiting concentrations of protease inhibitors it is possible to partially inhibit the protease, resulting in virions with small defects in processing and large decreases in infectivity (15). We wished to analyze the effect of specific decreases in protease activity on viral maturation and infectivity and to determine if a correlation between protease activity and infectivity exists.

Site-directed mutagenesis has been carried out on the HIV-1 protease to determine the effects of mutation on substrate specificity and activity. A general rule derived from saturation mutagenesis of the protease is that the residues within the active site are highly sensitive to mutation and generally cannot be changed without severely compromising protease activity (19, 20). More-directed mutations within the active site have generally been able to affect the protease's ability to recognize substrates (36). Only one mutant in which the primary effect has been to alter the rate of proteolytic cleavage without significant effects on substrate binding has been reported (13).

We proposed to examine the requirement for protease activity in the maturation process by studying protease mutants with specifically diminished catalytic activities. Three mutations that resulted in reduced proteolytic activity, Thr-26→Ser (T26S), Ala-28→Ser (A28S), and Asp-25→Asn (D25N), were engineered in the active-site region of the protease. These variant enzymes were expressed in *Escherichia coli* by using a bicistronic expression system and analyzed kinetically for effects on substrate binding and catalytic efficiency.

For analysis of the effect of the protease mutants on viral function, genes containing the single protease mutations were cloned into an HIV proviral vector, HIV-gpt, which produced all of the viral gene products except envelope (27). Transfection of this vector into mammalian cells permitted the formation of viral capsids, which were analyzed with regard to RNA content, polypeptide composition, and degree of maturation. By cotransfection of envelope-encoding plasmids, it was possible to generate infectious particles to analyze the effects of altered polyprotein processing on viral infectivity. Our results indicate that although lowering the level of protease activity does not appear to affect the assembly process, reduction of activity by 50-fold or greater is sufficient to prevent the formation of infectious virions.

* Corresponding author. Phone: (415) 476-8146. Fax: (415) 476-0688.

† Present address: Khepri Pharmaceutical Inc., South San Francisco, CA 94080.

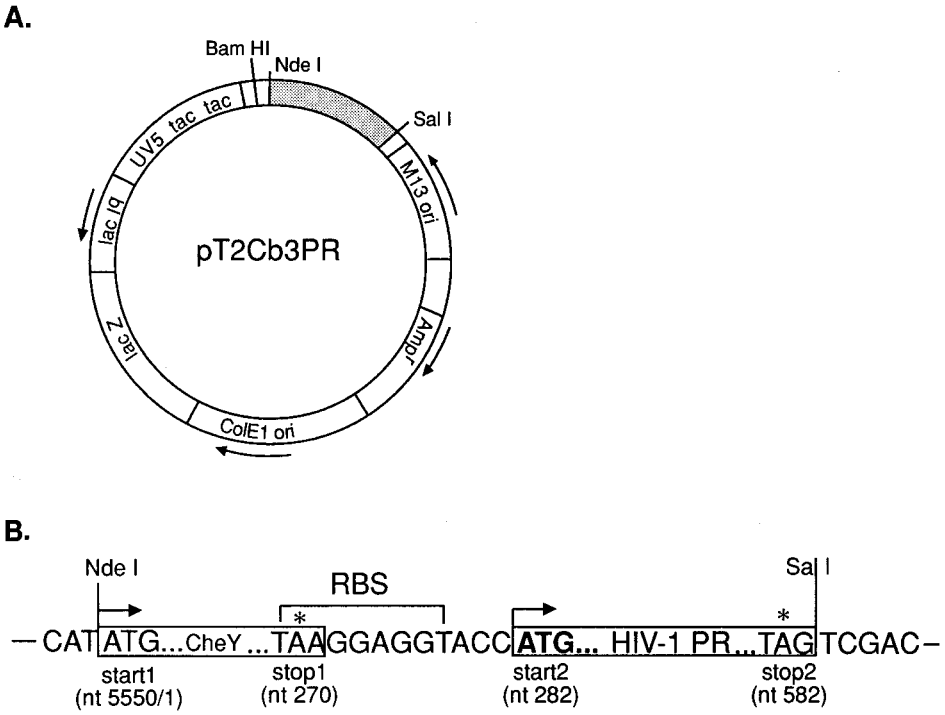

FIG. 1. Bacterial expression system for HIV-1 protease and salient features of the bicistronic expression vector. (A) Features of the plasmid backbone derived from pTacTac include an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible dual *tac* promoter, *lacI*^{q} for promoter repression, and an M13 origin (ori) for single-stranded DNA rescue. (B) Bicistronic gene arrangement used to express mutant proteases. Arrows and asterisks indicate translation start and stop sites, respectively. Numbers in parentheses indicate nucleotide positions of the *cheY* and HIV-1 protease (PR) genes. The start and stop codons of the HIV-1 protease gene are shown in boldface. RBS, ribosome binding site.

MATERIALS AND METHODS

Bacterial strains, vectors, and constructions. The *E. coli* strains used for cloning and expression were JM-101 [*supE thi-1* Δ(*lac-proAB*) F′(*traD36 proAB lacI*^{q}ZΔM15)] and X90 [F′ *lacI*^{q} *lacZY proAB*/Δ(*lac-pro*) *ara nalA argE*(Am) *thi* Rif^r]. The original expression plasmid, which encodes a gene fusion between human superoxide dismutase and a 179-amino-acid precursor of the wild-type HIV-1 protease, is designated SOD/PR179 (30). The expression vector pTacTac has been used previously (34). The HIV-1 protease gene used for bacterial expression was a synthetic gene which had been optimized for yeast codon usage and which coded for the 99-amino-acid protease (strain HXB2). A fragment of the *cheY* gene comprising the first 270 nucleotides (nt) was obtained from the vector pCheY15LOX (41). PCR was used to introduce an *Nde*I site into the 5′ end and a synthetic ribosome binding site at the 3′ end of the *cheY* gene. This gene fragment was ligated to the protease gene to generate the bicistronic construction shown in Fig. 1B. The expression plasmid containing this construction was designated pT2Cb3 PR, for dual tac vector (T2), *cheY* gene, bicistronic, 3-nt spacing after the ribosome binding site, and PR (for the HIV-1 protease gene) (Fig. 1A).

Site-directed mutagenesis. Mutagenesis was performed by standard methods (17). The T26S mutation was introduced into the bacterial expression system by using the oligonucleotide 5′-CTTTGTTGGAC**T**C**A**G**GTGCTG**ACGA-3′, which introduced two mismatches (underlined) and an *Alw*NI site for restriction endonuclease screening (boldface). The A28S mutation was introduced by using the oligonucleotide 5′-GGACACC**GGATCT**GACGACACC-3′, and mutants were identified by screening with *Bst*YI. The D25N mutation was introduced by using the oligonucleotide 5′-GAAGCTTTGTTG**A**ACAC**AGGTGCTG**ACG-3′, and screening was with *Alw*NI. All mutants identified by restriction endonuclease screening were sequenced to ensure no other mutations had occurred in the protease gene. Active-site mutants to be analyzed in vitro were made in the context of the Q7K mutation, which stabilized the enzymes to autoproteolytic digestion without affecting their kinetic parameters (34).

Production and purification of recombinant proteins. Wild-type HIV-1 protease was expressed from the plasmid SOD/PR179 in *E. coli* X90 and purified as described previously (34). Protease mutants containing the Q7K mutation were purified by a modified two-column protocol. Clarified lysates of *E. coli* were adsorbed onto phosphocellulose (Whatman Paper Ltd., Maidstone, England) equilibrated in 100 mM Tris (pH 8.0)–1 mM EDTA–1 mM dithiothreitol. After extensive washing, the protease was eluted with a gradient of 0 to 750 mM NaCl. Active fractions were pooled and chromatographed over Pepstatin-A agarose (Sigma, St. Louis, Mo.) as described previously (34). The yield from this procedure was approximately 3 mg of HIV-1 protease from 3.5 g of *E. coli*. Protease activity was monitored by using a peptide cleavage assay which has been described previously (31).

Mammalian cells and viral constructs. COS-7 and HeLaT4 cells were maintained in Dulbecco's modified Eagle's medium H21 supplemented with 10% fetal calf serum (GIBCO, Grand Island, N.Y.), 100 U of penicillin per ml, and 100 μg of streptomycin per ml. COS-7 cells were obtained through the American Type Culture Collection. HeLaT4 cells were obtained from R. Axel through the AIDS Research and Reference Program, Division of AIDS, National Institute of Allergy and Infectious Diseases. HeLaT4 cells were grown in the presence of G-418 (300 μg/ml) to maintain CD4 expression.

The HIV-gpt proviral vector consists of the HIV-1 HXB2 sequences (the *env* gene, with nt 6402 to 7620 replaced with the drug-selectable *E. coli gpt* gene) cloned into the pBS plasmid (pBluescript; Stratagene, San Diego, Calif.) (27). The HXB2-env vector encodes the gp160 envelope sequences from the HXB2 strain (nt 5999 to 8896) cloned into a simian virus 40 expression vector (27). These plasmids were obtained from D. Littman.

For introduction of mutations into the provirus, site-directed mutagenesis was carried out with the Bluescript-based phagemid pSPR, which contains a 918-bp *Bgl*II-*Eco*RV fragment (nt 1639 to 2557) (nucleotide designations are based on the numbering scheme of the HIV-1 HXB2 genomic sequence [33]) spanning the protease gene. The T26S mutation was introduced by using the oligonucleotide 5′-GCTCTATTA**GATTCT**GGAGCAGATGAT-3′ and identified by restriction endonuclease screening with *Hin*fI. The A28S mutation was introduced by using the oligonucleotide 5′-TTAGATACA**GGATCCG**ATGATACAG-3′ and screened with *Bam*HI. The D25N mutation was introduced by using the oligonucleotide 5′-GAAGCTCTATTA**A**A**C**AC**C**G**G**AGCAGATG-3′ and screened with *Hpa*II. All mutations were sequenced through the protease gene prior to cloning from pSPR. Mutants were recovered from pSPR by a two-step process. The *Bgl*II-*Eco*RV fragment from pSPR was cloned into a second shuttle vector, pSpol, which contains the 4,315-bp *Spe*I-*Sal*I (nt 1051 to 5366) fragment of the HIV-1 HXB2 clone. The *Spe*I-*Sal*I fragment was then cloned into the HIV-gpt vector to generate the complete viral vector.

Transfection. Viral capsids were produced by transfection of COS-7 cells by the calcium phosphate procedure (10) with the following modifications. Thirty micrograms of HIV-gpt DNA was used per 10-cm-diameter dish of approximately 30% confluent cells, and 60 μg of HXB2-env was included with the HIV-gpt DNA to generate infectious particles.

Infection. Viral titers were determined by drug selection on monolayers of HeLaT4 cells as previously described (27).

Isolation of viral particles. Viral particles were purified from transfected-cell supernatants over 20% sucrose cushions as previously described (27). The bottom 500 μl of the cushion was collected and stored at −20°C.

Quantitation of p24. To determine levels of particle-associated p24, pelleted virions were diluted in phosphate-buffered saline (PBS) and assayed by using an enzyme-linked immunosorbent assay (ELISA) kit (DuPont NEN, Wilmington, Del.) which is sensitive to processed forms of the major capsid antigen (p24 and p25) but not to the $p55^{gag}$ or $p160^{gag\text{-}pol}$ precursor. For determination of the total amount of p24 derived from all precursors, purified capsids were diluted fourfold in HIV-1 protease activity buffer (50 mM Na acetate [pH 5.5], 1 M NaCl, 1 mM dithiothreitol, 1 mM EDTA) containing 0.1% Triton X-100 and were then vortexed to disrupt capsids. The samples were processed in vitro by incubation at 37°C following the addition of 100 ng of recombinant HIV-1 protease.

Virion stability studies. Viruses were harvested at 50 h posttransfection and purified by sucrose cushion sedimentation. Samples were adjusted to 50 ng of total Gag protein by ELISA and incubated at 37°C for 1 h in PBS with or without 0.5% Triton X-100 and 2.5 mM EDTA. Following treatment, the samples were layered over 3.5-ml cushions of 20% sucrose containing the same detergent conditions as for the experimental treatment and were centrifuged for 1 h at 170,000 × *g*. Fractions of 500 μl from the top and the bottom of the gradient were collected, precipitated with trichloroacetic acid, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% acrylamide gels. Proteins were transferred to 0.2-μm-pore-size nitrocellulose (Schleicher & Schuell, Keene, N.H.) and visualized by immunoblotting with a primary rabbit polyclonal anti-p24 antibody (Intracel, Cambridge, Mass.) followed by a secondary horseradish peroxidase-conjugated goat antibody to rabbit immunoglobulin G (Pierce, Rockford, Ill.).

Reverse transcriptase assays. Reverse transcriptase activity was determined by using the RT-Detect kit from DuPont NEN. Briefly, virions were purified directly from the medium by centrifugation at 100,000 × *g* for 2 h. Virions were lysed with 0.3% Triton X-100, and the endogenous reverse transcriptase was used to extend a synthetic RNA template. Following alkaline hydrolysis of the RNA template, the cDNA was detected by sandwich hybridization (32). A biotinylated capture probe complementary to one region of the cDNA was used to immobilize synthesized strands, which were detected by a second probe, complementary to a different region of the cDNA, and conjugated to horseradish peroxidase. Detection was performed with 3,3′,5,5′-tetramethylbenzidine. For comparison of results, the lysed virions were digested with HIV-1 protease, and p24 was quantitated by ELISA as described above. The amount of cDNA synthesized was normalized to the total amount of Gag protein present in the viral samples.

RESULTS

Expression and characterization of mutant proteases. Recombinant HIV-1 protease has been expressed previously in our laboratory as a fusion of the human superoxide dismutase gene and a virally derived gene fragment encoding 179 amino acids encoded by the *pol* gene. The protease is efficiently released from the fusion protein by autoproteolytic processing (2, 30). When the active-site mutation T26S was introduced into the protease in this system, the fusion protein was not processed efficiently and was present in cells as insoluble aggregates (data not shown).

To overcome the requirement for autoprocessing, an attempt was made to express a synthetic gene which coded for the 99-amino-acid protease. No protease expression was observed when this gene was cloned into the pTacTac vector (data not shown). A bicistronic system was engineered on the basis of the model of Schoner et al. (38, 39) to create a transcriptional but not a translational fusion between the protease and a highly expressed gene. This construction is shown in Fig. 1B and was designed to couple translation of the protease gene to that of a highly expressed upstream gene (primary cistron) without requiring fusion of the polypeptides. The primary cistron chosen coded for an N-terminal fragment of the bacterial protein CheY (22). The stop codon for CheY and 6 nucleotides downstream (TAAGGAGGT) form a sequence which is complementary to the 3′ end of the 16S rRNA (40) and serves as a ribosome binding site for the downstream HIV-1 protease gene. This construction, pT2Cb3 PR, was highly effective at producing soluble T26S protease (T26S PR) with a molecular weight corresponding to that of the mature 99-amino-acid protease. The purified enzymes were subjected to amino-terminal sequence analysis and shown to be lacking the initiator methionine, which was presumably removed by endogenous methionine aminopeptidase activity (3).

TABLE 1. Kinetics of wild-type and mutant proteases[a]

Protease	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} μM^{-1})
WT	820 ± 20	990 ± 50	0.82 ± 0.04
Q7K	1,030 ± 30	1,300 ± 60	0.81 ± 0.03
Q7K/T26S	240 ± 10	900 ± 60	0.26 ± 0.02
Q7K/A28S	17 ± 2	1,100 ± 140	0.015 ± 0.003
Q7K/D25N	0	0	0

[a] k_{cat} and K_m were determined by using the substrate NH_2-ATLNFPISPW-COOH in standard assay buffer (50 mM Na acetate [pH 5.5], 1 M NaCl, 1 mM EDTA, 1 mM dithiothreitol). Results are reported as means ± standard deviations for three determinations. WT, wild type.

A kinetic analysis of the mutant proteases was carried out by using a decapeptide substrate whose sequence is based on the protease-reverse transcriptase cleavage site. The mutation Q7K was included in all of our studies as a means of minimizing autoproteolytic degradation of the protease (34). The effects of the T26S and A28S mutations were primarily to decrease the catalytic rates (k_{cat}) of the enzymes without any significant effect on substrate binding (K_m) (Table 1). Q7K/T26S PR showed a 4-fold decrease in k_{cat}, whereas in Q7K/A28S PR, catalysis decreased nearly 50-fold. Q7K/D25N PR was expressed but showed no detectable activity when purified, confirming that this mutant was totally inactive.

The structures of Q7K/T26S PR and Q7K/A28S PR were determined and compared with the structure of the Q7K variant. No significant changes in the residues composing the substrate binding pocket were observed, further suggesting that only catalytic activity had been affected by these mutations (34a).

Effect of mutants on polyprotein processing. To assess the effect of reduced proteolytic activity on viral polyprotein processing, the mutants were introduced into a tissue culture system for analyzing HIV virion assembly. Protease genes containing the three single mutations T26S, A28S, and D25N were cloned into the HIV-gpt vector. Following transfection in COS-7 cells, capsids were labeled with [^{35}S]methionine and collected by sucrose cushion sedimentation. The presence of T26S PR, A28S PR, and D25N PR had a dramatic effect on the degree to which viral polyproteins were processed (Fig. 2). After a 12-h pulse-chase, wild-type virions contain predominantly mature capsid protein (p24), with a small amount of a less mature form (p25). In T26S PR capsids, the capsid protein exists largely in the p25 form, and additional precursors are visible at 39 and 55 kDa. The A28S PR and D25N PR capsids do not show any significant processing and contain the immature $p55^{gag}$ precursor almost exclusively. Small amounts of processing intermediates could be observed in A28S PR samples only after extended periods of time (data not shown).

Longer time periods were necessary to examine the abilities of the less active proteases to process viral capsids. The accumulation of mature capsid protein was monitored over 130 h by p24 ELISA of particle-associated material (Fig. 3A). Although the T26S PR particles display a short, 24-h lag relative to wild-type HIV-gpt, significant levels of p24 and p25 are present in particles at later time points. Very low levels of p24 and p25 are present in cells transfected with the A28S PR clone, indicating that the processing of polyprotein precursors is dramatically diminished even after 130 h. The order of appearance of Gag processing intermediates in the T26S PR and A28S PR capsids over longer time periods was similar to the

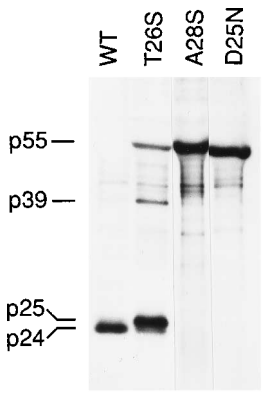

FIG. 2. Contents of viral capsids. Cells were transfected with 30 μg of the various HIV-gpt plasmid DNAs and grown for approximately 48 h after transfection. Cells were labeled for 6 h and then subjected to a 6-h cold chase. Capsids were isolated by sucrose cushion sedimentation as described in Materials and Methods and precipitated by treatment with trichloroacetic acid. Proteins were analyzed by SDS–12% PAGE and visualized by autoradiography. WT, wild type.

order in which they have been observed in other systems (6, 9, 14), suggesting that the selection of cleavage sites had not been altered in the mutant capsids.

Effect of mutants on viral infectivity. The effect of altered kinetics of polyprotein processing on viral infectivity was analyzed. Infectious particles were generated by cotransfecting the wild-type and mutant-bearing HIV-gpt vectors with an HIV-1 gp160-expressing plasmid, HXB2-env. Samples from four independent transfections were analyzed at 48 h and gave the results shown in Table 2. At a point at which p24 levels are approximately equal for wild-type and T26S PR virions, the T26S PR clones are consistently three- to fourfold less infectious than the wild type. The A28S PR and D25N PR clones produced no infectious particles at this time point or later time points up to 130 h (Fig. 3B). It was observed that infectious titers of wild-type and T26S PR virions declined after 72 h despite a continuous increase in p24 levels, thus limiting the use of the system to time points at 72 h or earlier.

Effect of mutants on viral assembly. To determine if the protease mutations affected other aspects of the assembly process, RNA encapsidation and envelope association were analyzed. To measure RNA levels, purified particles were normalized for the amount of total Gag protein and dot blots were performed with a 900-nt HIV-1 HXB2-specific probe encompassing a region of the *gag* and *pol* genes. Association of the envelope glycoprotein was assessed by separating purified virions on 4 to 12% gradient SDS-polyacrylamide gels and immunoblotting with anti-gp160 antibodies. The results indicated that levels of gp120/gp160 and viral RNA were comparable for the three virion samples (data not shown).

Effect of mutants on virion stability. Since the previous data suggested that the viruses contained all of the components necessary for replication, we surmised that inefficient uncoating of the viral nucleoprotein complex may be occurring in unprocessed particles. To examine relative virion stabilities, wild-type and mutant virions which had been purified over sucrose cushions were treated with Triton X-100 and 2.5 mM EDTA to cause dissociation. Treated and untreated samples were purified a second time over sucrose cushions (Fig. 4). As has been observed for HIV and avian leukosis virus capsids, wild-type virions were totally disrupted by detergent treatment, whereas immature virions were unaffected (28, 42). T26S PR capsids, which appeared to be 85 to 90% processed, were

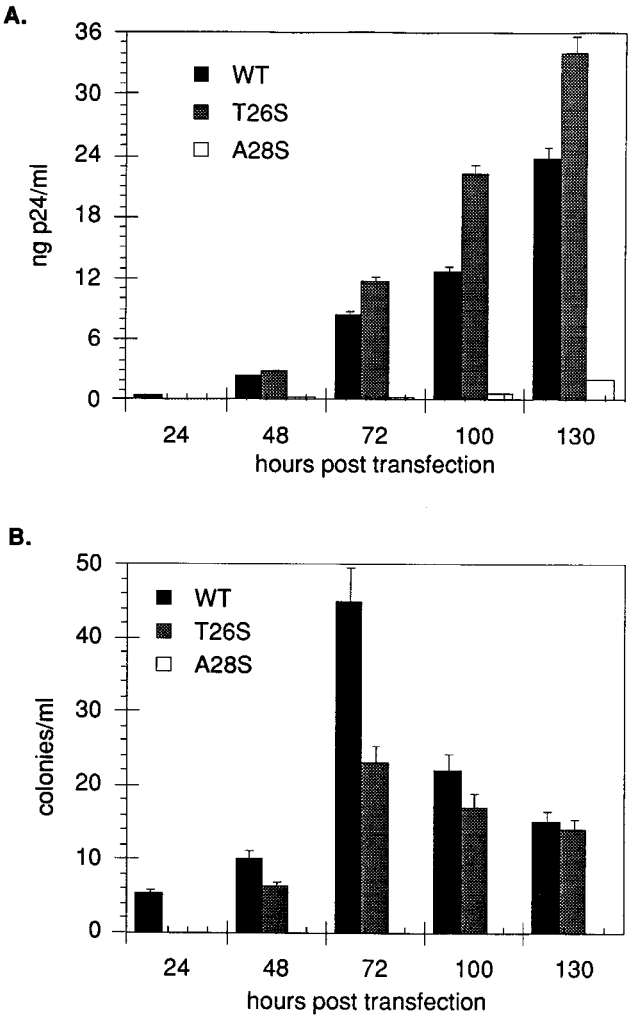

FIG. 3. Extended time course of mutant capsid processing. COS-7 cells were transfected with 30 μg of the various HIV-gpt plasmid DNAs and 60 μg of HXB2-env. Culture supernatants were harvested at 24, 48, 72, 100, and 130 h posttransfection. (A) Viral particles isolated over a sucrose cushion were disrupted by vortexing with 0.1% Triton X-100, and p24 levels from three serial dilutions were measured by ELISA (DuPont NEN). (B) Culture supernatants from the same experiment were filtered through 0.45-μm-pore-size filters and used to infect HeLaT4 cells for determination of viral titers. WT, wild type. Error bars indicate average errors for two determinations.

similar to wild-type virions in that they were completely disrupted by this treatment. Treated samples from A28S PR particles showed some dissociation, in that small amounts of p24/25 and p39/41 were found at the top of the gradient, while all of the protein precursors remained pelleted at the bottom of the cushion. This suggests that the A28S PR capsid samples contained a small number of particles which were sufficiently processed to allow particle dissociation, while the majority of the particles were immature.

Reverse transcriptase activity in mutant virions. To characterize the degree of activation of the reverse transcriptase enzyme, virions were harvested at 56 h posttransfection and purified directly from tissue culture supernatants by centrifugation at 100,000 × *g* for 2 h. Virion samples were assayed for total Gag protein content by ELISA, and reverse transcriptase activity was quantitated. As shown in Fig. 5, there was an approximately 10-fold difference in the levels of reverse tran-

TABLE 2. Infectivity of mutant viruses[a]

Expt	HeLaT4 colonies/ml/U of p24			
	WT	T26S	A28S	D25N
1	400 ± 15	131 ± 7	0	0
2	176 ± 8	64 ± 5	0	0
3	121 ± 11	31 ± 5	0	0
4	106 ± 5	40 ± 3	0	0

[a] For normalization of data for infectious particles to total particle levels, purified particles were disrupted with 0.1% Triton X-100–5 mM EDTA and digested with exogenous purified HIV-1 protease to convert all Gag proteins to their processed forms. The total amount of p24 in digested samples was determined by ELISA, and the colony numbers were divided by the ratio of mutant to wild-type p24 levels in order to generate the values shown. Results are expressed as means ± average errors for two determinations. WT, wild type.

scriptase activity observed in wild-type and D25N PR virions. T26S PR virions contained approximately the same amount of reverse transcriptase activity as wild-type virions, whereas levels in A28S PR virions were only 75 to 80% of those in the wild type.

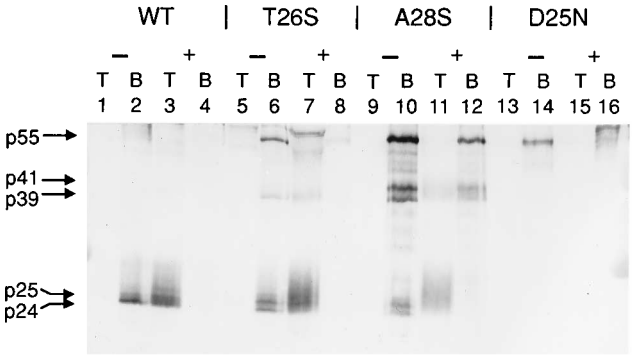

FIG. 4. Virion stability to detergent disruption. Viral particles were incubated at 37°C in the presence (lanes 3, 4, 7, 8, 11, 12, 15, and 16) or absence (lanes 1, 2, 5, 6, 9, 10, 13, and 14) of 0.5% Triton X-100 and 2.5 mM EDTA. Samples were loaded on 20% sucrose cushions, and particles were isolated again by centrifugation at 170,000 × *g* for 1.5 h. Samples from the top (T) and bottom (B) of the tubes were precipitated with trichloroacetic acid and separated by SDS–12% PAGE. Following transfer to 0.2-μm-pore-size nitrocellulose, proteins were visualized by p24 immunoblot. WT, wild type.

DISCUSSION

In vitro structure-function analysis. Although the crucial role of the HIV-1 protease in the viral life cycle was defined early through mutational studies (8, 16, 29) and later through protease inhibitor studies (1, 24), it is not clear how much of the protease activity present in a virion is necessary and sufficient for production of an infectious particle. We chose to adopt a mutagenic strategy to evaluate this question. This paper describes the development of systems for producing recombinant protease variants in bacteria for biochemical characterization and in tissue culture for analysis of viral assembly and infectivity.

The amino acid substitutions were targeted to the vicinity of the protease active site, to produce as minimal a perturbation as possible in the substrate binding pocket. The development of the bicistronic expression system was crucial for the production of the mutant proteases. Previously the protease had been expressed as a fusion with human superoxide dismutase, from which it was freed by autoproteolytic cleavage. In the context of mutants which decreased or abolished all protease activity, no mature protease could be recovered. By using a bicistronic approach, the high translation initiation efficiency of the *cheY* gene could be exploited to direct HIV-1 protease expression without requiring posttranslational processing.

A limited number of mutations can be made which affect only the catalytic activity of the HIV-1 protease while leaving substrate recognition intact. Since we wished to isolate the activity of the protease (k_{cat}) from its substrate specificity (K_m), we were restricted to the two mutations described here: T26S and A28S. The T26S mutation was made to correspond to an amino acid substitution found in the Rous sarcoma virus protease, which has a specific activity lower than that of the HIV-1 protease (11). Relative to that of the wild-type protease, T26S PR had a specific activity which was reduced fourfold, within the range of that of the Rous sarcoma virus protease. This effect appeared to be due solely to a decrease in the catalytic efficiency of the enzyme and not to an altered affinity for the substrate. The second mutation, A28S, has been reported previously to diminish catalytic activity by approximately 50-fold, as well as shifting the pH optimum of the enzyme (13). Our results correspond well with this observation and again suggest that the catalytic rate of the enzyme, and not substrate binding, is affected. No activity was observed in D25N PR, which was used as a negative control for protease activity.

Virion maturation. The presence of the protease mutants had a dramatic effect on the rate at which viral proteins were processed following the assembly of virions. In pulse-chase studies of viral capsids, the wild-type protease was able to convert p55 to p24 in as little as 10 min, whereas even after 6 h capsids harboring T26S PR contained a mixture of Gag precursors. Although it was apparent that A28S PR retained some ability to produce processed Gag proteins, these appeared at a significantly decreased rate relative to those with both wild-type protease and T26S PR. The magnitude of the processing defect in virions was approximately correlated with the diminished k_{cat} values measured in vitro.

The infectivity of viral particles harboring the protease mutants was also measurably affected. No infectious T26S PR virions were detected until 48 h after transfection, presumably because of the increased time of processing that is required in the presence of the catalytically impaired protease. The infectivity of T26S PR virions remained lower than that of wild-type

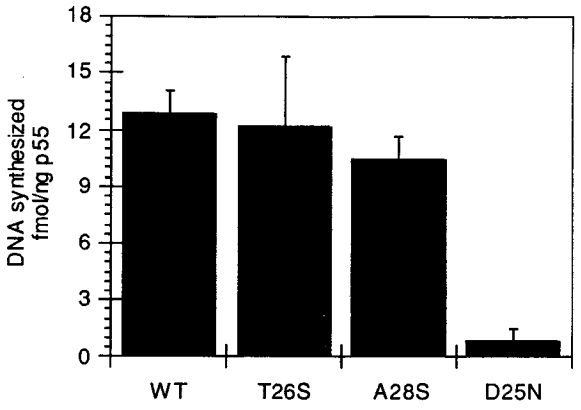

FIG. 5. Reverse transcriptase activity in mutant virions. Virions were purified directly from medium by centrifugation at 100,000 × *g* for 2 h, and cDNA was synthesized by using the endogenous reverse transcriptase. For comparison of results, the amount of cDNA synthesized was normalized to the amount of p55 present in the viral samples. WT, wild type. Error bars indicate standard deviations for three determinations.

virions at time points beyond 48 h, which was most likely due to the decreased infectivity of partially processed particles (15). In contrast, viruses containing A28S PR did not produce any infectious particles. This observation is in keeping with our data showing only minimal p24 production at 48 h. The general decrease in particle infectivity after 72 h might have contributed to the inability to detect infectious particles in A28S PR samples at later times.

Our data suggest that there is a direct correlation between the activity of the protease, as measured by the efficiency constant (k_{cat}/K_m), and the infectivity of the virus. There is a clear effect of the mutants on both the rate of polyprotein processing, as determined by quantitation of particle-associated p24 in transfected-cell supernatants, and the rate at which infectious particles are produced. The T26S PR virions, in which only 25% of the total wild-type protease activity is present, are still able to infect cells, despite a decreased rate of processing. Apparently this level of protease activity is sufficient to process the viral proteins within a time in which the virus is potentially viable. In the case of the A28S PR virions, processing proceeds at a 50-fold-slower rate, and no infectious particles are observed.

The data suggest that a fourfold reduction in proteolytic activity still permits polyprotein processing within a time in which the virus is potentially viable. However, when processing proceeds at a 50-fold-slower rate, the virus does not mature within this window of viability and infectious virions are not produced. This window may be defined by other aspects of the viral aging process, such as genome dimerization (4, 7) or loss of the envelope glycoprotein (23). Our data showing that the numbers of infectious particles drop markedly after 72 h, despite a continual rise in total particle production, suggest that instability of gp120 limits the lifetimes of these viruses in tissue culture. The instability of the gp160 envelope glycoprotein in the HXB2 laboratory strain may not be observed in naturally occurring isolates of HIV-1 from patients.

Virion assembly and postentry events. The protease mutants studied here do not appear to affect virion assembly beyond altering the degree to which viral proteins are processed. By dot blot analysis, wild-type and mutant virions contained approximately the same amount of viral RNA when compared at the same time point. The lack of effect of protease mutations on RNA packaging has been observed with Rous sarcoma virus and murine leukemia virus (4, 42), in which virions containing inactive proteases package genomic RNA as efficiently as wild-type viruses. It does not, however, imply that the RNA has formed the appropriate dimeric structures. Stewart et al. reported that abnormal forms of the viral RNA were present in the mutant virions (42), and a similar defect in RNA dimerization, but not in total packaging, has recently been reported for HIV (7). The presence of the protease mutants reported here did not appear to cause a differential association of gp160 with the viral particle, as both A28S PR and T26S PR virions contained amounts of the envelope glycoprotein in the same proportion to total virion Gag protein as did the wild-type virions.

Since virions appear to assemble normally, it is possible that the loss of infectivity is due to perturbation of a postassembly event necessary for viral replication. We surmised that uncoating of viral RNA, a crucial step for freeing the nucleoprotein complex for reverse transcription, may be inefficient in the presence of unprocessed viral proteins. To address the issue of virion stability, virions containing each of the mutant proteases were examined for susceptibility to disruption by a nonionic detergent. The virions which were the most compromised in proteolytic processing activity, those containing D25N PR and A28S PR, were unaffected by this treatment, although a small amount of detergent-sensitive material was detected in A28S PR samples. The T26S PR and wild-type virions both displayed a high level of sensitivity to detergent disruption, despite the presence of several processing intermediate forms of p55 in the T26S PR virions. This suggests that the dissociation of viral cores requires a certain degree of polyprotein processing in order to take place.

Inefficient activation of reverse transcriptase by mutant proteases may also prevent the synthesis of viral DNA following uncoating of the viral core. Reverse transcriptase activity associated with unprocessed polyproteins has been reported at levels from 6- to 30-fold lower than that found in wild-type virions (8, 29, 37). Kaplan et al. demonstrated that protease inhibitor-treated viruses containing decreased amounts of processed polyproteins and reverse transcriptase activity are not able to direct efficient viral DNA synthesis after infection (15). In general, the amounts of RNA-directed DNA polymerase activity associated with the wild-type and T26S PR virions were equivalent when corrected for total amounts of Gag protein present, whereas this amount was slightly reduced (15 to 20%) in A28S PR virions and reduced nearly 90% in D25N virions. Although the reverse transcriptase present in mutant virions was functional in this simple polymerization assay, it may not efficiently carry out the multiple steps required to synthesize integration-competent viral DNA. Further studies on these processes in the cellular context of infection would help to elucidate the exact point at which infection is blocked when the proteolytic maturation process is incomplete.

Implications for antiprotease therapeutic intervention. It is clear that the rate of the maturation process can be diminished at least fourfold without seriously affecting the ability to form infectious particles. This suggests that a reservoir of protease activity exists in an immature virion and that this reservoir can absorb a decrease in protease activity induced by point mutations in the enzyme. Point mutations in the protease which affect the binding of peptidomimetic inhibitors by 5- to 10-fold have been obtained by in vitro selection of viruses (5, 12, 26). It has been noted in at least one case that a virus containing an escape mutation in the protease is considerably less infectious than the parent virus (5) and that protease function is impaired. Although the presence of these mutations would aid in slowing the rate of viral spread, it is also clear that second-site mutations can occur to restore catalytic competence while maintaining resistance to inhibitors (12). Clearly, the development of effective protease inhibitors must now take into account the ability of the enzyme to survive in the presence of point mutations that may disrupt the binding of small-molecule inhibitors. The effects of such mutations on the protease itself can be analyzed in systems similar to the one described here to provide a detailed understanding of the activity and specificity requirements of the viral maturation process.

The use of HIV-1 protease inhibitors to prevent the spread of viral infection depends upon the ability to stop the maturation process at a point at which the virus is noninfectious and to maintain the virus in this state until it is irreversibly inactivated. It has been observed that the HIV-1 protease can reactivate following diffusion of peptidomimetic inhibitors out of virions (24), although it is not entirely clear whether this event is sufficient to restore infectivity (18). Depending upon the pharmacodynamics of inhibitors used therapeutically, it is likely that a situation will exist in vivo in which some of the virion-associated proteases either will not be inhibited or may be reactivated. It therefore may be advantageous to develop inhibitors of the protease which would irreversibly inactivate the enzyme at essential active-site amino acid residues (25, 35).

This strategy may show promise in the development of effective antiproteolytic agents.

The observation that a 50-fold reduction in protease activity is sufficient to prevent the production of infectious virus is encouraging. It predicts that total inhibition of the protease will not be necessary to block the spread of the virus in a therapeutic situation. Consequently, methods that can reduce inhibitor sensitivity to point mutations in the protease and still inhibit the majority of protease molecules in a virion will yield effective inhibitors for the clinical therapy of HIV.

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