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An Active-Site Mutation in the Human Immunodeficiency Virus Type 1 Proteinase (PR) Causes Reduced PR Activity and Loss of PR-Mediated Cytotoxicity without Apparent Effect on Virus Maturation and Infectivity

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Infectious retrovirus particles are derived from structural polyproteins which are cleaved by the viral proteinase (PR) during virion morphogenesis. Besides cleaving viral polyproteins, which is essential for infectivity, PR of human immunodeficiency virus (HIV) also cleaves cellular proteins and PR expression causes a pronounced cytotoxic effect. Retroviral PRs are aspartic proteases and contain two copies of the triplet Asp-Thr-Gly in the active center with the threonine adjacent to the catalytic aspartic acid presumed to have an important structural role. We have changed this threonine in HIV type 1 PR to a serine. The purified mutant enzyme had an approximately 5- to 10-fold lower activity against HIV type 1 polyprotein and peptide substrates compared with the wild-type enzyme. It did not induce toxicity on bacterial expression and yielded significantly reduced cleavage of cytoskeletal proteins in vitro. Cleavage of vimentin in mutant-infected T-cell lines was also markedly reduced. Mutant virus did, however, elicit productive infection of several T-cell lines and of primary human lymphocytes with no significant difference in polyprotein cleavage and with similar infection kinetics and titer compared with wild-type virus. The discrepancy between reduced processing in vitro and normal virion maturation can be explained by the observation that reduced activity was due to an increase in K_m which may not be relevant at the high substrate concentration in the virus particle. This mutation enables us therefore to dissociate the essential function of PR in viral maturation from its cytotoxic effect.

The catalysis of cleavage of retrovirus structural polyproteins within the nascent virus particle is performed by the viral proteinase (PR; for nomenclature of retroviral proteins, see reference 26). Viral polyprotein processing has been shown to be essential for productive virus infection (16, 32; for review, see references 23, 25, and 44). The main structural components of the retroviral core (Gag proteins) as well as the replication enzymes (Pol proteins, including PR itself) are synthesized and assembled into immature particles as polyprotein precursors. Proteolytic processing by the virion-associated PR takes place during and after budding of these particles from the plasma membrane and precedes morphological conversion (maturation) into the infectious mature virion (23). Tight regulation of PR activity appears essential for virus replication since premature or too rapid polyprotein processing prevents particle release (11, 20, 21, 28) or leads to aberrant particle morphology (36) while too low activity of PR abolishes viral infectivity (14, 16, 21). However, few comparative analyses of mutant PR kinetic properties and corresponding phenotypes of mutant viruses regarding polyprotein processing and specific infectivity have been reported (33), and the upper and lower boundaries of PR activity which are compatible with productive virus infection have not been defined in quantitative terms. In addition to its essential role in viral replication, PR of human immunodeficiency virus (HIV) also causes pronounced cytotoxic effects in prokaryotic and eukaryotic cells, presumably by cleaving cellular proteins. Cellular substrates of HIV type 1 (HIV-1) PR include cytoskeletal proteins (vimentin, actin, troponin, tropomyosin [10, 37, 42] and microtubule-associated proteins [2, 43]) as well as calmodulin and the precursor of the transcription factor NF-κB (31). On the basis of its toxicity in a variety of cells, it has been suggested that HIV PR may also play a role in the HIV-induced cytopathic effect both in tissue culture and in vivo (13, 37, 41, 42).

Retroviral PRs belong to the family of aspartic proteases which contain two copies of the amino acid triplet Asp-Thr-Gly (DTG; amino acids 25 to 27 in the case of HIV PR) in their active site. The viral enzymes are synthesized as monomeric subunits on the Gag-Pol polyprotein and require dimerization to achieve catalytic competence. Each of the monomers contributes one DTG triplet to the symmetric active site. A particular feature of the active site of aspartic proteases, revealed by analysis of their three-dimensional structures (4, 29, 45), is the interaction between the side chain of the active-site Thr (Thr-26 in the case of HIV) and the main chain amide of the active-site Thr (Thr-26′) from the other subunit. A similar interaction takes place between the side chain of this Thr (Thr-26) and the main chain carbonyl of the preceding Leu (Leu-24′) from the other subunit. The resulting symmetrical pairs of hydrogen bonds are presumed to stabilize the active-site geometry and are dependent on the side chain hydroxyl of the Thr residues. This elaborate hydrogen bond network has been given the descriptive name "fireman's grip" (references 4,

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6, and 12 and references therein). The only sequence divergence in the catalytic triplet of active aspartic proteases is a Thr→Ser substitution, leading to a DSG triplet in the PRs of avian retroviruses, foamy viruses, and some *Saccharomyces cerevisiae* retrotransposons (40). Serine with a side chain hydroxyl similar to threonine is the only other small amino acid capable of forming the fireman's grip. It is conceivable that the Thr→Ser substitution, while maintaining the essential hydrogen bond network, may play an important role in regulating PR activity. Interestingly, avian retroviruses encode their PR as part of the Gag polyprotein, which leads to higher levels of PR synthesis compared with other retroviruses. Since avian retroviral PRs are considerably less active than those of mammalian retroviruses (7, 17–19), it is tempting to speculate that down-regulation of PR activity may serve to counteract upregulation of PR expression and may in part be due to the unusual DSG triplet.

To analyze the role of the Thr or Ser residue in the catalytic triplet of retroviral PRs in regulating enzyme activity, we generated a point mutation changing Thr-26 of HIV-1 PR into Ser-26 and analyzed its phenotype regarding viral infectivity, polyprotein and peptide processing, and PR-mediated toxicity. Interestingly, this mutation caused a 5- to 10-fold reduction in PR activity against viral and cellular protein and peptide substrates with an apparent loss of PR-mediated cytotoxic effects while no significant effect on particle maturation or virus infectivity was observed.

MATERIALS AND METHODS

Cells, transfections, and infections. COS-7 cells were maintained in Dulbecco modified minimal Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM glutamine. Transfections were performed as described previously (27). Approximately 5×10^6 cells suspended in 0.1 ml of phosphate-buffered saline (PBS) were electrotransfected with 20 μg of DNA with a Bio-Rad gene pulser set at 150 V, 960 μF, and 100-Ω resistance. Cells were diluted into fresh medium, plated, and harvested 48 h after transfection. To normalize for transfection efficiency, 2.5 μg of an expression vector containing the *lacZ* gene under control of the cytomegalovirus immediate early promoter/enhancer was cotransfected and cell extracts were normalized for β-galactosidase activity (35).

HIV-1 permissive MT-4 (8), C8166 (34), and AA-2 cells (3) were maintained in RPMI 1640 with the supplements described above. Cells were adjusted to 2×10^5 cells per ml 24 h before infection. For analysis of infection kinetics, culture media from transfected COS cells were filtered through a 0.45-μm-pore-size filter and cells were infected with equal amounts of wild-type (wt) and mutant viruses (normalized for CA antigen). The inoculum was removed 2 h postinfection (p.i.), and cells were washed and resuspended in fresh supplemented medium. Aliquots were removed at the times indicated; cells and media were analyzed by indirect immunofluorescence and enzyme-linked immunosorbent assay (ELISA), respectively. When indicated, the HIV-1 PR inhibitor Ro 31-8959 (32) was added to a final concentration of 5 μM at 6 or 30 h p.i. For titration of 50% tissue culture infectious dose, 100 μl of MT-4 or AA-2 cells was seeded in microtiter plates at a concentration of 2.5×10^5 cells per ml 24 h before infection and quadruplicate cultures were infected with 100 μl of filtered virus suspensions, serially diluted in complete medium. Infectivity was scored by indirect immunofluorescence at 10 days p.i. The infectious titer (50% tissue culture infectious dose) is defined as the dilution of virus yielding productive infection in 50% of the culture wells. For radioactive labeling of virus particles, fresh MT-4 cells were cocultivated with infected MT-4 cells for 24 h, resuspended in RPMI 1640 lacking cysteine, and labeled with 50 μCi of [^{35}S]Cys per ml for 5 and 24 h.

Human peripheral blood lymphocytes (PBLs) were purified on Ficoll-Hypaque gradients (Pharmacia) and resuspended at a concentration of 2×10^6 cells per ml in supplemented RPMI 1640. One aliquot of PBLs was directly mixed (without any further treatment) with an equal volume of filtered culture medium from transiently transfected COS cells (normalized for CA antigen). Input virus was removed after 3 days, and cells were washed and cultivated in the presence of 30 U of interleukin 2 (IL-2) per ml and 0.25 μg of phytohemagglutinin (PHA) per ml. A second aliquot of PBLs was PHA–IL-2 treated for 3 days prior to infection and subsequently infected by addition of transfection-derived virus and treated as described above. Aliquots were removed every 2 to 3 days and analyzed by indirect immunofluorescence and ELISA.

Expression plasmids. The mutations were made in a subclone of the infectious provirus pNL4-3 (1), containing the *Apa*I-to-*Pst*I fragment (nucleotides 2006 to 2839 of pNL4-3) in a pBluescript vector (Stratagene). Site-directed mutagenesis was performed on a single-stranded DNA template by the method of Kunkel (24). Mutation D25A changed the GAT triplet of the PR active-site Asp-25 (nucleotides 2325 to 2327) to a GCT (Ala) codon whereas mutation T26S altered the ACA codon (nucleotides 2328 to 2330) to a TCA (Ser) codon. The entire HIV-specific region was sequenced, and the respective *Apa*I-to-*Pst*I fragments were cloned into pNL4-3 to give plasmids pNL43-D25A and pNL43-T26S.

To construct a vector for large-scale preparation of HIV-1 PR (T26S), the segment of pNL4-3 (T26S) encoding PR and the preceding 21 codons of the p6* sequence was amplified by PCR, adding a 5′-flanking Met codon and *Nde*I restriction site and two 3′-flanking stop codons and an *Eco*RI site. The resulting *Nde*I-*Eco*RI fragment was completely sequenced and cloned into the bacterial expression vector pET11 (Novagen) to give pET11-PR(T26S).

PR purification and determination of enzyme kinetics. For large-scale bacterial expression, freshly transformed *Escherichia coli* BL21(DE3) cultures (39) were grown to an A_{600} of 0.8 and induced by addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Upon induction, precursor proteins accumulated in the cytoplasm as inclusion bodies and mature PR was autocatalytically released after refolding. Cells were harvested 4 h after induction and disrupted, and inclusion bodies were isolated, washed, and dissolved in 10 M urea as described previously (17). After dialysis against 0.01 M Tris (pH 7.5), insoluble material was removed and the supernatant was incubated with quaternary aminoethyl-Sephadex A25 (Pharmacia) equilibrated at pH 8.6. Unbound material was adjusted to pH 7.5 and applied to a Sephadex C50 CM column (Pharmacia) equilibrated with 0.05 M Tris (pH 7.5) containing 4 mM EDTA, 0.1% mercaptoethanol, and 20% glycerol. PR was eluted by an NaCl gradient (0 to 200 mM) and concentrated with Centricon concentrators (Amicon).

Concentrations of wt HIV-1 PR and T26S PR were determined by active-site titration with the tight binding inhibitor BocPhe[CH(OH)CH_2]PheGlnPhe-NH_2 (27). The purity of the products was determined to be >90% by polyacrylamide gel electrophoresis (PAGE) and amino acid analysis. The yield of active enzyme was approximately 60 to 80% of total protein in the pure preparation and was similar for wt and T26S PR.

The chromophoric peptide substrates shown in Table 1 were synthesized by the solid-phase method and purified by high-pressure liquid chromatography (30). Hydrolysis of peptide substrates was monitored with an Aminco DW 2000 spectrophotometer at 305 nm and ambient temperature with a reaction time of 20 min in most instances. The buffer was 0.1 M MES (morpholineethanesulfonic acid) (pH 6.5) with 4 mM EDTA and 0.3 M NaCl in a final volume of 500 μl with the final enzyme concentration between 2 and 10 nM. Initial rates of hydrolysis were measured for at least eight substrate concentrations, and kinetic constants were derived from the computer fit of the data with the EnzFitter program. All K_m and k_{cat} values are the means of at least two separate determinations with separately purified batches of enzyme.

In vitro cleavage of protein substrates. Cleavage of particle-derived pr55^{gag} was performed as described previously (17). Extracellular particles were collected from cleared media of 33/4 cells (22) by centrifugation through a cushion of 20% (wt/wt) sucrose and resuspended in 1 mM Tris (pH 7.5)–100 mM NaCl–5 mM dithiothreitol–0.4% Triton X-100. Incubations were performed at a final concentration of 50 mM MES (pH 6.5) with 0.3 or 1 M NaCl as indicated in a total volume of 20 μl for 60 min at 37°C. Reactions were terminated by adjusting samples to 1% sodium dodecyl sulfate (SDS) followed by immunoblot analysis.

Cleavage of cytoskeletal proteins was performed in a total volume of 100 μl in 10 mM Tris (pH 7.0)–1 mM phenylmethylsulfonyl fluoride–5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] containing 25 μg of mouse vimentin, porcine glial fibrillary acidic protein, and porcine desmin and the indicated amounts of active-site titrated PR or T26S PR. Reaction mixtures were incubated at 37°C for 60 min and were terminated by addition of 5× protein loading buffer and analyzed on SDS gels.

Analysis of expression products. HIV antigens were detected with a CA-specific capture ELISA based on monoclonal antibody from hybridoma cell line 183 (clone H12-5C). The antibody was purified from ascites fluid by successive precipitation with caprylic acid and ammonium sulfate (9) and used for coating

TABLE 1. Kinetic constants for proteolytic cleavage of peptide substrates by wt and T26S PR[a]

Peptide sequence	wt			T26S		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}/\mu M$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}/\mu M$)
KARVLNphEAL	60	10	$16.7 \cdot 10^4$	405	10	$2.5 \cdot 10^4$
KARVNleNphEANle*	215	30	$14.0 \cdot 10^4$	619	17	$2.7 \cdot 10^4$
ATHQVYNphVRKA*	11	17	$154.6 \cdot 10^4$	139	8	$5.7 \cdot 10^4$

[a] Peptides were incubated with purified HIV-1 wt and T26S PR in 0.1 M MES buffer (pH 6.5) containing 4 mM EDTA and 0.3 M NaCl. Peptide hydrolysis was monitored spectrophotometrically as described in Materials and Methods. The identity of cleaved products was confirmed by amino acid analysis and N-terminal sequencing. The sequence specificity was identical for wt and T26S PR.

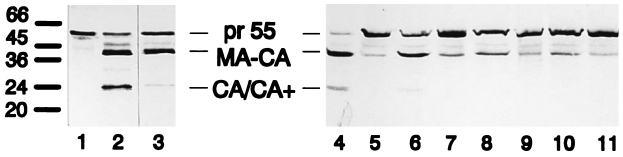

FIG. 1. Western blot (immunoblot) analysis of products after in vitro cleavage of pr55^{gag} by wt and T26S PR. Viruslike particles containing uncleaved pr55^{gag} were purified from a constitutive expression line, stripped of their lipid envelope, and incubated with wt and T26S PR. Lane 1 corresponds to a control incubation without PR. Concentration of wt HIV-1 PR was 45 nM (lanes 2, 4, and 6) or 9 nM (lanes 5 and 7); concentration of T26S PR was 36 nM (lane 3), 45 nM (lanes 8 and 10), or 9 nM (lanes 9 and 11). Incubations were performed in the presence of 0.3 M salt (lanes 1 to 5, 8, and 9) or 1 M salt (lanes 6, 7, 10, and 11). Cleavage reaction mixtures were analyzed by immunoblotting with antiserum against HIV-1 CA. Molecular mass standards (in kilodaltons) are indicated on the left; HIV-specific precursor proteins and cleavage products are identified between the two panels. CA/CA+ indicates heterogeneity in C-terminal processing, giving rise to several species of CA with slightly different electrophoretic mobilities.

of ELISA plates at a concentration of 50 ng per well. Wells were blocked with gelatin in PBS, and appropriate dilutions of cleared medium from transfections or infections, chosen to be in the linear response range of the ELISA, were added. Antigen was detected by incubation with polyclonal rabbit antiserum against HIV-1 CA (27) and purified on DEAE Affi-Gel Blue (Bio-Rad), followed by horseradish peroxidase-conjugated goat antiserum against rabbit immunoglobulin G (Dianova). Enzyme activity was detected with tetramethylbenzidine as substrate, and quantitative analysis was derived from the absorbance readings with known amounts of purified HIV-1 CA protein (5) as standard.

For radioimmunoprecipitation, labeled cells or particles, sedimented through a cushion of 20% (wt/wt) sucrose, were adjusted to 2% SDS and heated for 10 min at 80°C. Samples were diluted 10-fold in 1% Triton X-100–0.5% deoxycholate in 10 mM Tris (pH 7.5)–150 mM NaCl and incubated with polyclonal antiserum against HIV-1 CA (27) and protein A-Sepharose (Pharmacia). For protein analysis, cell or particle extracts or immune complexes were separated on SDS-polyacrylamide gels containing 17.5% polyacrylamide (200:1 ratio of acrylamide:*N*,*N*-methylenebisacrylamide). Radioactive samples were quantitated by PhosphorImager (Molecular Dynamics) analysis. For immunoblot analysis, proteins were transferred to nitrocellulose (Schleicher & Schuell) membranes by electroblotting and reacted with polyclonal antisera against HIV-1 CA, reverse transcriptase (RT), or integrase (IN) followed by alkaline phosphatase-conjugated second antiserum (Jackson Immunochemicals Inc.). Immune complexes were visualized by color detection. Antisera against CA (dilution, 1:500) and RT (dilution, 1:250) have been described previously (27). A polyclonal rabbit antiserum against HIV-1 IN was generated by immunization with purified IN protein containing six His residues as N-terminal extension and was used at a dilution of 1:250.

RESULTS

Construction of PR mutants and analysis of proteolytic activity. To analyze a potential regulatory role of the Thr or Ser residue in the active site of retroviral PRs, we generated a point mutation changing Thr-26 of HIV-1 PR to Ser-26. As a control, an inactive mutant changing the active-site Asp-25 to Ala-25 was also made. For quantitative determination of proteolytic activity, the wt and mutated PR domains with a segment of the preceding p6* domain were cloned into a bacterial expression vector. Upon expression, the uncleaved products accumulated in inclusion bodies, and in the case of enzymatic activity, cleaved mature PR was purified following denaturation and refolding of these inclusions, yielding approximately 0.5 to 1 mg of >90% pure enzyme per liter of induced bacterial culture (data not shown).

For analysis of PR activity, wt and mutated enzymes were incubated with the HIV-1 precursor polyprotein pr55^{gag} obtained from viruslike particles. Detergent-disrupted particles were incubated in the absence (Fig. 1, lane 1) or presence (Fig. 1, lanes 2 and 3) of wt or T26S PR. Specific cleavage of pr55^{gag} to a MA-CA intermediate and the two species of CA (CA/CA^{+}) was observed in both cases, but the mutated PR exhibited lower activity (compare lanes 2 and 3). For a more detailed analysis of relative PR activities, particle-derived pr55^{gag} was incubated with two different concentrations of active-site titrated wt and T26S PR (45 and 9 nM) at low and high salt concentrations. At the lower salt concentration, wt PR was approximately fivefold more active than T26S PR (Fig. 1, compare lanes 5 and 8). In agreement with our previous observations (17), both wt and T26S PR are inhibited by high salt (1 M [Fig. 1, lanes 6, 7, 10, and 11]) compared with incubation at low salt (0.3 M [Fig. 1, lanes 4, 5, 8, and 9]). Similar to wt PR, T26S PR also showed lower activity at pH 7.2 (data not shown).

For kinetic characterization of the purified enzymes, we used two synthetic chromogenic peptides derived from a processing site on the HIV-1 polyprotein (30) and a chromogenic peptide derived from the consensus sequence of the PR processing sites on the Gag and Pol polyproteins of avian sarcoma and leukemia viruses (38). The kinetic data summarized in Table 1 indicate that the k_{cat} values for hydrolysis of these substrates by both enzymes are either identical (substrate KARVLNphEAL, Table 1, first row) or lower for the mutated enzyme by a factor of 2 (substrates KARVNleNphEANle and ATHQVYNphVRKA, second and third rows, respectively). The major difference between wt and mutated PR was found in the K_m values, which are 4 to 13 times higher for the T26S PR than for the wt enzyme (Table 1). The values given in the table were determined at pH 6.5, which is reasonably close to the physiological value and was also used for analysis of polyprotein cleavage (compare with Fig. 1). Interestingly, the mutated and wt PRs showed very different pH profiles with the difference in K_m increasing with increasing pH and a much smaller difference at more acidic pH (unpublished observations). In addition, T26S PR was significantly less stable than wt PR and lost activity much more rapidly (data not shown).

Effect of the T26S mutation on polyprotein processing in virus replication and on virus infectivity. Since T26S PR showed a 5- to 10-fold lower activity against polyprotein and peptide substrates in vitro, it was of interest to analyze its phenotype regarding polyprotein processing in virus replication and regarding viral infectivity. We therefore constructed complete proviral clones containing either the D25A or the T26S mutation (pNL43-D25A and pNL43-T26S, respectively). Following transient transfection of COS-7 cells with the respective proviral clones, HIV-permissive MT-4 cells were infected with the respective viruses and cell and particle extracts were analyzed by radioimmunoprecipitation with antiserum against HIV-1 CA (Fig. 2A). Particle extracts from media of transiently transfected COS cells were also analyzed by immunoblot with antisera against HIV-1 CA, RT, and IN proteins (Fig. 2B). The wt provirus yielded mostly processed Gag- and Pol-derived proteins, both in the infected cells (Fig. 2A, lane 1) and in extracellular particles (Fig. 2A, lanes 3 and 5; Fig. 2B, lanes 1, 4, and 7). Less than 1% of CA-reactive proteins in the particle preparation remained as uncleaved polyprotein after a 24-h labeling period (Fig. 2A, lane 3) or when particles were collected following pulse-chase labeling (5-h labeling, 5-h chase [Fig. 2A, lane 5]). In contrast, the PR-defective provirus (pNL43-D25A) yielded uncleaved pr55^{gag} and pr160$^{gag\text{-}pol}$ polyproteins (Fig. 2B, lanes 2, 5, and 8). Particles derived from pNL43-T26S yielded a similar pattern as observed for wt provirus (Fig. 2A, compare lanes 3 and 4 and lanes 5 and 6), again showing almost exclusively processed Gag (Fig. 2A, lanes 5 and 6, and Fig. 2B, lane 3) and Pol (Fig. 2B, lanes 6 and 9) proteins with small amounts of remaining polyproteins. Quantitative determination of CA-reactive proteins in the particle preparation indicated that approximately 2% of immunoreactive protein remained as uncleaved polyprotein after a 24-h labeling

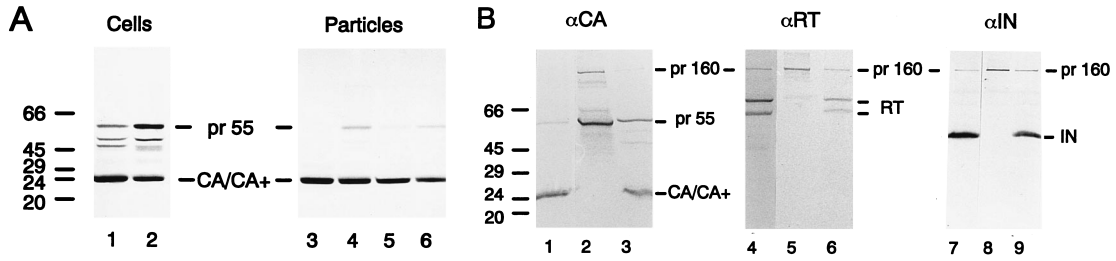

FIG. 2. Analysis of *gag* and *pol* gene products from proviral plasmids. (A) MT-4 cells were infected with filtered medium from COS-7 cells transfected with pNL4-3 (lanes 1, 3, and 5) or pNL43-T26S (lanes 2, 4, and 6). Immunoprecipitation of infected cells (lanes 1 and 2) was performed after a 24-h labeling period; isolated particles were immunoprecipitated following 24 h of labeling (lanes 3 and 4) or following pulse-labeling for 5 h and a chase period of 5 h (lanes 5 and 6). (B) Extracellular particles from COS-7 cells transfected with pNL4-3 (lanes 1, 4, and 7), pNL43-D25A (lanes 2, 5, and 8), or pNL43-T26S (lanes 3, 6, and 9) were analyzed by immunoblot with antiserum against CA (lanes 1 to 3), RT (lanes 4 to 6), or IN (lanes 7 to 9). Molecular mass standards (in kilodaltons) are indicated on the left; HIV-specific precursor proteins and cleaved products are identified on the right. CA/CA+ indicates heterogeneity in C-terminal processing. RT corresponds to the heterodimer of HIV-1 RT; pr160 denotes the complete Gag-Pol polyprotein of HIV-1.

period (Fig. 2A, lane 4) or following pulse-chase labeling (Fig. 2A, lane 6). In contrast to the particle preparation, a larger difference between wt- and mutant-infected samples was observed in labeled cell extracts: almost 90% of CA-reactive proteins in wt-infected cells corresponded to mature CA protein with the remaining 10% as precursor and intermediate proteins (Fig. 2A, lane 1). Considerably less intracellular processing was observed in pNL43-T26S-infected cells, with approximately 70% of CA-reactive protein corresponding to mature CA (Fig. 2A, lane 2).

To compare the infectivity of wt and mutated viruses, we inoculated several HIV-permissive cell lines as well as human PBLs with culture media from transfected COS cells. Equal amounts of CA antigen were used to normalize for input virus. Productive infection was scored by indirect immunofluorescence of cells and ELISA determination of CA antigen in the medium. All cell lines studied were equally infected by wt and T26S virus while D25A virus was not infectious. The kinetics of virus production were identical or very similar for wt and T26S viruses in several established cell lines: infection of the rapid producer T-cell lines MT-4 (Fig. 3A) and C8166 (data not shown) yielded identical growth kinetics for both viruses. Infection of the CD4-positive human B-cell line AA-2 (Fig. 3B) and the T-cell line CEM-T4 (data not shown) also yielded very similar growth kinetics possibly showing a minor delay for the T26S virus. Immunofluorescence analysis also indicated similar relative numbers of MT-4 and AA-2 cells infected by wt and T26S virus at all time points analyzed (data not shown). Furthermore, end point titration of wt and T26S virus, derived from transfected COS cells and normalized for CA antigen, on MT-4 and AA-2 cells yielded very similar titers of 2.5×10^4 to 5×10^4 50% tissue culture infectious doses per ml. Analysis of infection in PBLs yielded virtually identical growth curves for the two viruses by using PBLs either first stimulated in vitro and subsequently infected (Fig. 3D) or first incubated with virus and subsequently stimulated after virus removal (Fig. 3C). Identical results were obtained for PBLs from two different donors (data not shown). Equal amounts of antigen-positive cells were observed for wt and T26S virus in PBLs at all time points analyzed (data not shown). To analyze for the stability of the T26S mutation and its potential for reversion, cell-free T26S virus was consecutively passaged three times at low multiplicity of infection on MT-4 and C8166 cells, and the presence of the mutation was confirmed in all cases by sequence analysis following PCR-mediated amplification of the PR region.

Analysis of PR-mediated cleavage of cytoskeletal proteins and cytotoxicity. Besides viral polyproteins, HIV-1 PR also cleaves cellular proteins including the cytoskeleton proteins vimentin, desmin, and glial fibrillary acidic protein (37, 43). Since PR-mediated cytotoxicity has been attributed to cleavage of these and other proteins (37) and T26S PR showed considerably reduced if any toxic effects when expressed in *E. coli* (data not shown), we hypothesized that T26S PR may be less effective in cleaving cellular proteins and may thus show reduced cytotoxicity. Equal amounts of active-site titrated wt and T26S PR were incubated with vimentin, desmin, and glial fibrillary acidic protein, and the resulting products were analyzed on SDS gels. Both enzymes induced cleavage of all three proteins, yielding the same products in each case, but T26S PR was considerably less active (Fig. 4A). Incubation with serially diluted PRs showed that T26S PR cleaved these substrates approximately 5- to 10-fold less effectively than wt PR (data not shown), similar to the observed relative activities on the viral polyprotein.

Since wt PR showed a higher activity against cytoskeleton

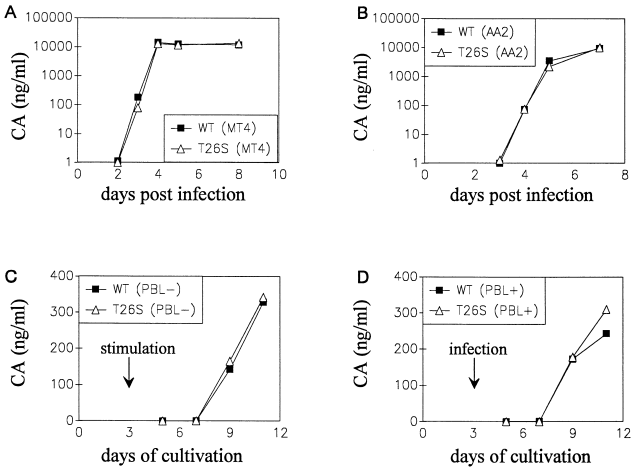

FIG. 3. Infectivity of wt and mutant virus particles. The CD4-positive cell lines MT-4 (A) and AA-2 (B) and human PBLs (C and D) were inoculated with equal amounts (normalized for CA antigen) of filtered culture medium from COS cells transfected with pNL4-3 (filled squares) or pNL43-T26S (open triangles). For panel C, PBLs were infected for 3 days and subsequently stimulated with PHA–IL-2, while for panel D, PBLs were PHA–IL-2 treated for 3 days and subsequently infected (on day 3 of cultivation). At the times indicated, release of progeny virus was determined by HIV-1 CA-specific ELISA of cleared medium from infected cells. In parallel, cells were analyzed by indirect immunofluorescence (not shown). Antigen concentrations are given as nanograms of CA protein per milliliter of culture medium. Note that in panels A and B a semilogarithmic scale is applied.

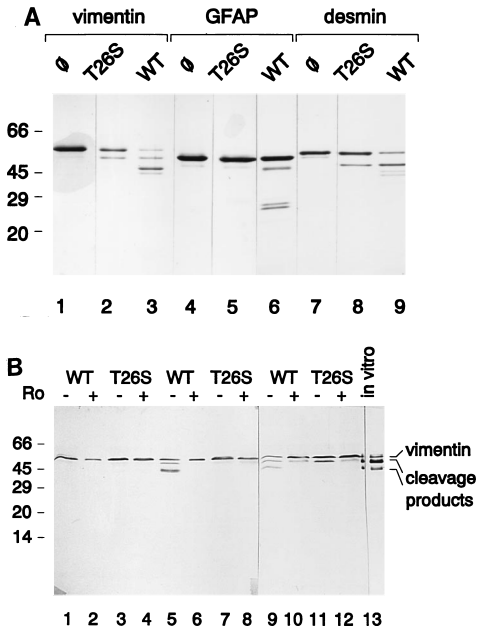

FIG. 4. (A) In vitro cleavage of the cytoskeletal proteins vimentin, desmin, and glial fibrillary acidic protein (GFAP) by T26S and wt PR. Purified proteins were incubated without addition of PR (lanes 1, 4, and 7), with 100 nM T26S PR (lanes 2, 5, and 8), or with 100 nM wt PR (lanes 3, 6, and 9). Aliquots of the reaction mixtures were analyzed by SDS-PAGE and stained with Coomassie blue. Molecular mass standards (in kilodaltons) are shown on the left. (B) Immunoblot analysis of vimentin cleavage in infected cells. C8166 cells were infected with filtered culture medium from COS cells transfected with pNL4-3 (WT; lanes 1, 2, 5, 6, 9, and 10) or pNL43-T26S (T26S; lanes 3, 4, 7, 8, 11, and 12). In the samples run in lanes 2, 4, 6, 8, 10, and 12 (marked +), the specific PR inhibitor Ro 31-8959 was added at 6 h p.i. at a concentration of 5 μM. Cells were harvested at 2 days (lanes 1 to 4), 3 days (lanes 5 to 8), or 4 days (lanes 9 to 12) after infection and analyzed by immunoblotting with goat antiserum against vimentin. For lane 13, the products of in vitro cleavage of purified vimentin by wt HIV-1 PR are run in parallel. Molecular mass standards (in kilodaltons) are indicated on the left; vimentin and its specific cleavage products are identified on the right.

proteins in vitro, it was of interest to analyze wt- and mutant-infected cells for PR-mediated cleavage of vimentin. Extracts from wt- and T26S mutant-infected C8166 cells were analyzed by immunoblotting with a polyclonal antiserum against vimentin. To rule out nonspecific degradation, the specific HIV PR inhibitor Ro 31-8959 (32) was added to one aliquot of infected cells 6 h after infection. Productive infection was scored by indirect immunofluorescence and indicated similar numbers of infected cells for wt and T26S virus. Since no infectious progeny virus was produced in inhibitor-treated cells, these samples showed approximately 2- to 10-fold fewer infected cells depending on the day of harvest. Specific degradation of vimentin was observed in wt HIV-infected cells 3 and 4 days p.i. (Fig. 4B, lanes 5 and 9). No vimentin cleavage was observed in T26S-infected cells on day 3 p.i. (Fig. 4B, lane 7), and very limited cleavage was observed on day 4 p.i. (Fig. 4B, lane 11). Intracellular cleavage products comigrated with those observed after in vitro cleavage of vimentin with HIV-1 PR (Fig. 4B, lane 13) and were never found in inhibitor-treated samples. Independent of the amount of vimentin cleavage, wt- and mutant HIV-infected C8166 cells were killed on day 5 to day 6 p.i. HIV-induced cell killing was also observed for the other CD4-positive cell lines described above (MT-4, AA-2, and CEM-T4), and no significant difference in cell killing was seen for wt- and mutant-infected cells. To analyze the relative contribution of PR-mediated cytotoxicity towards HIV-induced cell killing, MT-4 cells were infected with a high-titered stock of either wt or T26S virus and were left untreated or were treated with the inhibitor Ro 31-8959 at a concentration of 5 μM at 6 or 30 h after infection. Indirect immunofluorescence indicated that all cells were infected 2 days p.i. More than 80% of the cells had been killed by day 4 p.i. in all samples, and very little difference was observed between inhibitor-treated and untreated cells.

DISCUSSION

This study was performed to analyze the role and possible significance of having either a threonine or a serine in the active site of retroviral aspartic proteinases. Structural studies had indicated that this residue, and in particular its side chain hydroxyl, is necessary for formation of the fireman's grip, which is presumed to stabilize the geometry of the catalytic cleft (4, 6, 12). Since a Ser residue is observed, e.g., in the PR of avian retroviruses (40), and these enzymes are considerably less active than their mammalian counterparts with a threonine in this position, it was hypothesized that a Thr→Ser substitution in HIV-1 PR should maintain activity, albeit at a reduced level. In agreement with this hypothesis, serine can indeed functionally substitute for threonine in the active site of HIV-1 PR but causes a 5- to 10-fold reduction in activity. Additional evidence for the fireman's grip concept comes from recent experiments showing that substitution of Thr-26 by the structurally similar small amino acid alanine, which lacks a side chain hydroxyl group and is therefore unable to accept or donate a side chain hydrogen bond, results in inactivation of the enzyme (25a). While the observed results are consistent with a functional contribution of Thr-26 towards regulating PR activity, it should be noted that reverse mutations in the PRs of the *S. cerevisiae* transposon Ty3 and a foamy virus changing their active sites from DSG to the commonly found DTG triplet had little if any effect on viral polyprotein processing and replication (15, 16a). In these reports, however, only the processing of particle-associated polyproteins was analyzed and a change only in K_m as observed in the present study may not have been detected under these conditions.

In contrast to the fivefold difference in in vitro processing of a variety of substrates, a comparable difference was not observed for polyprotein processing within virus particles. Cleavage of particle-associated polyproteins remained largely unaltered in T26S virus, and the kinetics of infection by wt and T26S virus were virtually identical or only marginally different in all target cells analyzed. Furthermore, no reversion was observed on multiple passages, indicating that there was no significant selective advantage for the wt sequence at least under rapid growth conditions in tissue culture. We suggest that the apparent paradox of reduced activity in vitro and virtually normal cleavage in the virion is resolved by the results of kinetic analysis. The major difference between wt and T26S PR at pH 6.5 was observed in the K_m value for all three substrates while the k_{cat} values were affected only slightly or not at all. These differences in K_m explain the reduced activity of T26S PR in in vitro assays, and the observed fivefold reduction in activity correlates well with the approximately 5- to 10-fold increase in K_m. In virus particles, on the other hand, the local concentrations of PR and its substrate are much higher and may reach saturating conditions. Assuming the diameter of a spherical retrovirus to be 120 nm and an average number of 2,000 Gag polyproteins and 50 PR dimer molecules per virion, their concentrations would be approximately 4 and 0.1 mM, respectively (compare reference 42). Thus, substrate concentrations would be saturating, and relative cleavage should be determined by the turnover number, which is similar for wt

and mutated PR. In contrast, substrate and enzyme concentrations in the cytoplasm of infected or transfected cells or in the in vitro assays are considerably lower and substrates are subsaturating. Under these conditions, the higher K_m value of T26S PR may be responsible for the less effective cleavage observed.

In a recent report, Rosé et al. (33) analyzed the phenotype of a similar mutant (Q7K; T26S) of HIV-1 PR, but these authors observed a difference mostly in the k_{cat} value of the mutated PR with no significant alteration in K_m. Moreover, virus particles carrying the mutation were slightly reduced in infectivity and appeared to exhibit slower kinetics of polyprotein processing (33). We believe that the apparent discrepancy in the results of kinetic analysis may be explained by a combination of several factors: as pointed out above, Rosé et al. (33) have introduced an additional mutation (Q7K) into PR, and the possibility that this mutation may also influence the kinetic properties cannot be ruled out. More importantly, however, these authors used a different peptide substrate and performed cleavage under very different conditions (pH 5.5, 1 M salt). At pH 5.5, the K_m value for our chromogenic substrate was only twofold higher compared with wt PR, and at even lower pH, there was virtually no difference. Considering the significant influence of pH on substrate binding and the fact that slight differences were also observed for the three substrates analyzed in our study, we believe that the results of Rosé et al. (33) can easily be reconciled with this study. It is likely, however, that our analysis of several substrates under conditions more closely related to those expected for virus replication may more appropriately reflect the activity of PR in vivo. Similarly, the minor differences in polyprotein processing and virus infectivity observed by Rosé et al. (33) may also be explained by the different experimental conditions used. However, even in their study the observed differences were very minor and may be summarized by saying that T26S PR has little if any influence on polyprotein processing in virions and on virus infectivity. This is in obvious contrast to the easily observed phenotype on polyprotein and peptide cleavage in vitro.

It is surprising that the T26S mutation, which leads to the loss of a single methyl group in a position that should not be in direct contact with the bound substrate, changed the binding constant in such a dramatic way. Analysis of the HIV-1 PR structure model suggests that the side chains of Thr-26 and Thr-26′ pack tightly to the side chains of Leu-97 and Leu-97′ in the C-terminal part of the molecule. In T26S PR, the missing methyl groups in the serine side chains putatively lead to an imperfect side chain packing, especially with the C-terminal Leu residues (Leu-97, Leu-97′), creating an apparent gap between these residues. Although the hydrogen bonding of the fireman's grip leads to a relatively rigid structure, the lack of support from the β-sheet formed by the amino and carboxy termini might explain the decreased activity and stability of the enzyme. Preliminary molecular dynamic analyses indicated that the removal of the side chain methyl groups may cause Ser-26 to fill the apparent gap and to pack tightly against the C-terminal Leu residues. This may cause an opening of the binding cleft and a distortion of the hydrogen bond network in the active site, which may also explain the different pH profiles of mutant and wt PRs and the lower stability of T26S PR.

HIV-1 PR causes a pronounced cytotoxic effect in eukaryotic and prokaryotic cells which is probably caused by PR-mediated cleavage of essential host cell proteins (37). In contrast, no cytotoxic effects were observed in bacteria harboring T26S PR and there was almost no cleavage of vimentin in T26S virus-infected T-cell lines. Moreover, T26S PR cleaved several cellular protein substrates much less effectively (if at all) than the wt enzyme on in vitro incubation. Although there is currently no evidence proving that vimentin cleavage is directly responsible for the cytotoxic effect of PR, it may be taken as a marker for PR-mediated cytotoxicity. Accordingly, the fivefold reduction in activity observed for T26S PR may be sufficient to abolish or significantly reduce the PR-mediated cytopathic effect. Importantly, however, T26S virus-infected cells are killed at similar rates and times as cells infected with wt HIV-1. Moreover, addition of a specific PR inhibitor to HIV-1-infected cultures did not significantly delay or reduce HIV-mediated cell killing. Several viral factors have been implicated in HIV-induced cytopathogenic effects including envelope glycoproteins, unintegrated viral DNA due to superinfection, and PR. Our results suggest that PR-mediated toxicity is not required for the cytopathic effect in tissue culture, although they do not rule out a possible contribution of PR to this effect.

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