In Vitro Processing of HIV-1 Nucleocapsid Protein by the Viral Proteinase: Effects of Amino Acid Substitutions at the Scissile Bond in the Proximal Zinc Finger Sequence†

József Tözsér,‡,§,|| Sergey Shulenin,‡,§ John M. Louis,⊥ Terry D. Copeland,‡ and Stephen Oroszlan*,‡

National Cancer Institute, Frederick, Maryland 21701, and Laboratory of Chemical Physics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

Received September 15, 2003; Revised Manuscript Received January 23, 2004

ABSTRACT: The human immunodeficiency virus type 1 (HIV-1) nucleocapsid protein flanked by Gag sequences (r-preNC) was expressed in *Escherichia coli* and purified. HIV-1 proteinase cleaved r-preNC to the "mature" NCp7 form, which is comprised of 55 residues. Further incubation resulted in cleavages of NCp7 itself between Phe16 and Asn17 of the proximal zinc finger domain and between Cys49 and Thr50 in the C-terminal part. Kinetic parameters determined for the cleavage of oligopeptides corresponding to the cleavage sites in r-preNC correlated well with the sequential processing of r-preNC. Mutations of Asn17 were introduced to alter the susceptibility of NC protein to HIV-1 proteinase. While mutating Asn17 to Ala resulted in a protein which was processed in a manner similar to that of the wild type, mutating it to Phe or Leu resulted in proteins which were processed at a substantially higher rate at this site than the wild type. Mutation of Asn17 to Lys or Gly resulted in proteins which were very poorly cleaved at this site. Oligopeptides containing the same amino acid substitutions at the cleavage site of the proximal zinc finger domain were also tested as substrates of the proteinase, and the kinetic parameters agreed well with the semiquantitative results obtained with the protein substrates.

HIV-1[1] is the causative agent of acquired immunodeficiency syndrome (AIDS; *1*, *2*). As for all replication competent retroviruses, the essential role of the proteinase (PR) of HIV-1 in the late phase of the virus life cycle is well-established (*3*−*5*). The highly specific processing of Gag, and Gag-Pro-Pol polyproteins mediated by the PR, is critical for the conversion of noninfectious immature virus particles into mature infectious virions. Therefore, the PR of HIV has been recognized as a primary target for antiviral therapy (*6*). Structural and substrate specificity studies of the protease have provided the rationale for the design of potent inhibitors (*7*). Currently, there are several transition state analogue PR inhibitors in clinical use for treating HIV-infected patients with or without full-blown AIDS. As reported, the clinical benefits are better than the initial expectations despite the emergence of drug resistant variants during treatment (*8*−*11*).

Retroviral NC proteins are small basic proteins that contain one or two zinc fingers as in mammalian type C viruses or lentiviruses, respectively. They can bind both ssRNA and ss/dsDNA and can be involved in both specific (requiring secondary structure) and nonspecific (primarily electrostatic) interactions with nucleic acids and proteins. An expanded repertoire of possible functions of NC includes its effect on cDNA synthesis, dimerization, maturation and packaging of genomic RNA, virus assembly, and nucleic acid chaperone activity (*12*). The role of NC protein in virus replication has been extensively studied by site-directed mutagenesis. It has been shown that certain mutations within the highly conserved zinc finger motifs or substitution of basic amino acid residues outside these domains renders the virus deficient in RNA packaging, resulting in noninfectious particles (*13*−*16*). Other mutations may cause partial or complete loss of infectivity without overtly affecting packaging (*17*).

In earlier studies, we purified structurally intact core particles of equine infectious anemia virus (EIAV), a lentivirus related to HIV. We found that the viral PR is located inside the core together with reverse transcriptase (RT), integrase (IN), NC protein, and RNA encapsulated by

† Research sponsored in part by the National Cancer Institute, Department of Health and Human Services under contract with ABL, by the Hungarian Science and Research Fund (OTKA T 43482), U.S.-Hungarian Science and Technology Fund Grant 320, and by a grant from the Intramural AIDS Targeted Program of the Office of the Director of the National Institutes of Health. The contents of this publication do not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

* To whom correspondence should be addressed: HIV Drug Resistance Program, NCI-Frederick, P.O. Box B, Bldg. 536, Room 6, Frederick, MD 21702-1201. Telephone: (301) 846-1355. Fax: (301) 846-1354. E-mail: oroszlans@ncifcrf.gov.

‡ National Cancer Institute.

§ These authors contributed equally to this paper.

|| Present address: Department of Biochemistry and Molecular Biology, Research Center for Molecular Medicine, Medical and Health Science Center, University of Debrecen, H-4012 Debrecen, Hungary.

⊥ National Institutes of Health.

[1] Abbreviations: AIDS, acquired immunodeficiency syndrome; EIAV, equine infectious anemia virus; HIV-1, human immunodeficiency virus type 1; AMV, avian myeloblastosis virus; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid.

10.1021/bi035625z
Published on Web 03/16/2004

the capsid (CA) protein that forms the core shell (*18*, *19*). In addition, we identified two cleavage sites and the complete biochemical pathway for a controlled *in situ* fragmentation of the NC protein resulting in three well-defined polypeptides. The cleavage required the presence of a chelating agent, EDTA, for removal of the bound zinc, but it also occurred during reverse transcription, at the same time as progression of cDNA synthesis (*19*). On the basis of these results, we have proposed a role for the retroviral protease in the early phase of virus replication.

As an extension of our initial studies of EIAV NC protein−protease interaction, we first used short synthetic peptides derived from the highly homologous HIV-1 NC as substrates for PR. On the basis of these studies, we predicted that the cleavage site located in the proximal portion (N-terminal zinc finger) of HIV-1 NC should be the Phe16−Asn17 peptide bond (*20*). This has been confirmed using a synthetic NCp7 as a substrate (*21*). To understand further HIV-1 NCp7 fragmentation and its possible role in the early phase of virus replication, we used a recombinant protein (r-preNC) consisting of all 55 amino acids of NCp7 and flanking sequences of the Gag to encompass the cleavage sites at the p2−NC, NC−p1, and p1−p6 junctions. In this paper, we show that NCp7 has two PR cleavage sites, one located in each zinc finger. We also report the time course of the processing of the wild-type and mutant NC proteins. Point mutations introduced at the P1′ position (notation according to ref *22*) of the cleavage site (Asn17) with certain amino acids resulted in proteins that were equal or better substrates than the wild-type NC protein, while other mutations showed resistance to cleavage.

MATERIALS AND METHODS

Plasmid Construction and Site Specific Mutagenesis. The pMM4XB2 plasmid containing an infectious proviral clone of HIV-1_{HXB2} was a kind gift of R. Swanstrom (Department of Biochemistry and Biophysics, Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC). A *Hin*dIII−*Eco*RV fragment (nucleotides 1712−2979 in GenBank entry HIVHXB2CG) from pMM4HXB2 was cloned into a *Hin*dIII- and *Sma*I-digested pTZ19U vector (USB, Cleveland, OH). The resulting vector, pNCH1, was mutated by site-directed mutagenesis using the Muta-Gene Phagemid In Vitro Mutagenesis Kit, version 2 (Bio-Rad, Hercules, CA). Oligonucleotides used to introduce mutation in the NC coding region were as follows: 5′-CCACATC-CGAAACACTTAACAA-3′ for Asn17 → Gly, 5′-CCA-CATTTAAAACACTTAACAA-3′ for Asn17 → Lys, 5′-GCCACAAAAAAAACACTTAAC-3′ for Asn17 → Phe, 5′-GCCACATAAAAAACACTTAAC-3′ for Asn17 → Leu, and 5′-CCACATGCGAAACACTTAACAA-3′ for Asn17 → Ala. PCR amplification of mutant and wild-type DNA utilizing the primer set with 5′-CTGAAGCAATGAGCT-CAGTAACAAATTC-3′ and 5′-TCTCTACCCCAGACTC-GAGGCTCTCTTCTGGTG-3′ was used to generate the NC coding region for cloning into the pET23b bacterial expression vector (Novagen Inc., Madison, WI). These primers were designed for amplification of the HIV Gag sequence (nucleotides 1896−2174) with the appropriate restriction sites (*Sac*I and *Xho*I) for cloning the gene in frame with the initiator sequence and the six-His coding region of pET23b (see Figure 1 for the encoded sequence).

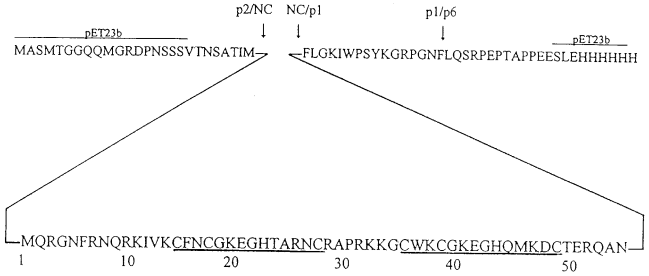

FIGURE 1: Primary structure of the precursor form of the wild-type NC fusion protein (r-preNC) expressed in *E. coli*. The pET23b coding regions are marked above the sequence. The maturation cleavage sites are denoted with arrows. The mature 55-residue NCp7 protein is numbered starting at the N-terminal Met. The sequences of the proximal and distal zinc fingers are underlined.

Expression, Purification, and Refolding of r-preNC Proteins. Expression of the recombinant fusion proteins in BL21-(DE3) *E. coli* cells (Novagen) was induced by the addition of 1 mM isopropyl thio-β-galactoside to the cultures and incubation at 37 °C for 4 h. Purification of the fusion proteins under denaturing conditions was performed according to the manufacturer's specification using the Xpress Purification System, version 2 (Invitrogen, San Diego, CA), with some modifications. Briefly, Triton X-100 (final concentration of 0.1%) was added to bacterial cells previously lysed with lysozyme, and the suspensions were treated with 100 μg/mL DNAse I (Sigma, St. Louis, MO) for 15−20 min at 4 °C in the presence of 2 mM $MgCl_2$. The insoluble fraction was collected by centrifugation and resuspended in 20 mM sodium phosphate buffer (pH 7.15) containing 500 mM NaCl, 5 mM β-mercaptoethanol, 0.1% Triton X-100, and 8 M urea. Samples were clarified by centrifugation and then applied to the nickel-charged ProBond resin. After extensive consecutive washing with two buffers having the same composition as the loading buffer, but at pH 6.15 and 5.95, respectively, the recombinant proteins bound to the resin through six-His tags were eluted with 20 mM sodium phosphate buffer (pH 4.2) containing 500 mM NaCl, 5 mM β-mercaptoethanol, 8 M urea, and 10 μM zinc chloride. Fractions containing the recombinant protein (as determined by SDS−PAGE) were combined and diluted to a final concentration of 1 mg/mL with the elution buffer. The purified proteins were refolded by overnight dialysis at 4 °C, against 20 mM sodium phosphate buffer (pH 7.0) containing 150 mM sodium chloride, 5 mM β-mercaptoethanol, and 10 μM zinc chloride. Refolded proteins were concentrated to 5−10 mg/mL by ultrafiltration on a Centricon-10 instrument (Amicon, Beverly, MA). Glycerol was added to a final concentration of 10%, and the proteins were stored at −70 °C in small aliquots. Protein concentrations were determined by the Bradford spectrophotometric method (Bio-Rad).

Cleavage of r-preNC Proteins by HIV-1 Proteinase. Wild-type or mutant r-preNC (1.5 μL, final concentration of 47 μM) in phosphate buffer [20 mM sodium phosphate (pH 7.0) containing 150 mM sodium chloride, 5 mM β-mercaptoethanol, and 10 μM zinc chloride] was mixed with 3 μL of 2× incubation buffer [500 mM potassium phosphate (pH 5.85) containing 2 M ammonium sulfate, 10 mM β-mercaptoethanol, 20% glycerol, and 2 mM EDTA], 1.3 μL of recombinant HIV-1 PR [final concentration of 2.0 μM (*23*)],

and 0.2 μL of water or 0.2 μL of 3 mM protease inhibitor UK 88.947. After the mixtures had been incubated for 16 h, reactions were stopped by the addition of an equal volume of 2× Tricine−sodium dodecyl sulfate (SDS) sample buffer (Novex, San Diego, CA). To follow the time course of the reaction, 13−14 μL (final concentration of 48−55 μM) of wild-type or mutant r-preNC was mixed with 24 μL of 2× incubation buffer and 10−11 μL (final concentration of 1.9−2.0 μM) of recombinant HIV-1 PR to give a final volume of 48 μL. Aliquots (6 μL) of the reaction mixture were withdrawn after various periods of incubation and reactions stopped by the addition of equal volume of 2× Tricine−SDS sample buffer. The samples were analyzed using a 16% or 10 to 20% gradient SDS−polyacrylamide Tricine-buffered gel (Novex). Molecular masses of the fragments were estimated using Rainbow molecular mass markers (Amersham, Arlington Heights, IL). For HPLC separation of fragments, 40 μL of wild-type r-preNC was mixed with 80 μL of 2× incubation buffer and 40 μL of recombinant HIV-1 PR. Aliquots (40 μL) were withdrawn, mixed with 100 μL of 8 M guanidine-HCl and 60 μL of 2.5% trifluoroacetic acid (TFA), and subjected to reversed-phase HPLC (Nova-Pak C_{18} column, 3.9 mm × 150 mm). The substrate and its cleavage products were separated using an increasing water−acetonitrile gradient (from 0 to 100%) in the presence of 0.05% TFA.

Protein Sequencing. Fragments of the NC protein cleaved by PR were separated by SDS−polyacrylamide gel electrophoresis (PAGE) and transferred to a PVDF (ProBlott, Applied Biosystems, Inc., Foster City, CA) membrane according to the method from ref *24*. N-Terminal amino acid sequence analysis was carried out by stepwise Edman degradation in a model 470A gas-phase sequencer (Applied Biosystems). Cleavage products from lyophilized HPLC fractions were identified by N-terminal sequencing as well as by FAB mass spectrometry.

Oligopeptide Synthesis and Characterization. Oligopeptides were synthesized by solid-phase peptide synthesis on a model 430A automated peptide synthesizer (Applied Biosystems, Inc.) using 9-fluorenylmethyloxycarbonyl chemistry and were purified by RP-HPLC. The amino acid composition of the peptides was determined with a Beckman 6300 amino acid analyzer. Stock solutions and dilutions were made in 5 mM dithiothreitol (DTT), and the peptide concentrations were determined by amino acid analysis.

Enzyme Assay with Synthetic Peptide Substrates. The assay was performed in 0.25 M potassium phosphate buffer (pH 5.6) containing 7.5% glycerol, 1 mM EDTA, and 5 mM DTT, in the presence of 2 M NaCl. The reaction mixture was incubated at 37 °C for 1 h, and the reaction was stopped by the addition of guanidine-HCl (final concentration of 6 M). The solution was acidified by the addition of TFA, and an aliquot was injected onto a Nova-Pak C_{18} RP-HPLC column (3.9 mm × 150 mm). Substrates and the cleavage products were separated using an increasing water−acetonitrile gradient (from 0 to 100%) in the presence of 0.05% TFA. The composition of the cleavage products was determined by amino acid analysis after perchloric acid oxidation. Kinetic parameters were determined at less than 20% substrate turnover by fitting the data to the Michaelis−Menten equation using the Fig. P program (Fig. P Software Corp., Durham, NC). The range of substrate concentrations was 0.02−2.0 mM, depending on the approximate K_m values. Active site titration was performed for the HIV-1 PR with compound 3 (*25*). The standard errors were less than 20%.

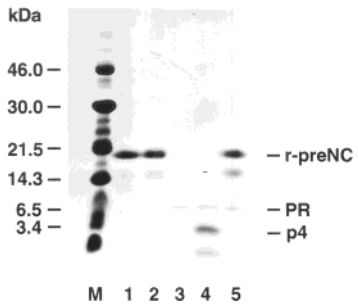

FIGURE 2: Proteolytic processing of the wild-type NC fusion protein (r-preNC) by the HIV-1 proteinase. Purified r-preNC protein and its cleavage products were analyzed by SDS−polyacrylamide gel electrophoresis: lane 1, r-preNC without incubation; lane 2, r-preNC after incubation for 16 h; lane 3, HIV-1 PR after incubation for 16 h; lane 4, r-preNC and HIV-1 PR after incubation for 16 h; and lane 5, r-preNC and HIV-1 PR after incubation for 16 h in the presence of 100 μM PR inhibitor UK 88.947.

RESULTS

Expression, Purification, and Proteolytic Processing of the Wild-Type r-preNC Protein. The NC protein (p7) of HIV-1_{HXB2} was cloned and expressed as a precursor encompassing the p2−NC, NC−p1, and p1−p6 Gag maturation cleavage sites (*26*, *27*) as shown in Figure 1. The yield of r-preNC was ∼7% of the total protein content (not shown). The affinity-purified recombinant precursor exhibited an apparent molecular mass of 19 kDa (Figure 2, lane 1), migrating much slower in the gel than the calculated molecular mass of 13.5 kDa. The anomalous migration could be due to the six-His tag.[2] N-Terminal sequence analysis of the protein indicated its N-terminus was Ala as expected upon removal of the initiator Met. Purified r-preNC preparations also contained a variable amount of a 15 kDa protein (Figure 2, lanes 1, 2, and 5). N-Terminal sequence analysis of this protein (RNQRKIVKXF) indicated that it is a truncated form of the precursor (r-preNC) cleaved between residues Phe6 and Arg7 of the mature NC protein (numbering from the N-terminal Met of the mature NCp7; see Figure 1) during expression and purification, most likely by a bacterial proteinase. Overnight incubation with HIV-1 PR resulted in complete processing of the 19 and 15 kDa proteins, to a major 4 kDa protein as detected by SDS−PAGE (Figure 2, lane 4). The incubation mixture contained EDTA, at a level at least 1 mM in excess over the zinc content, which has been shown to be sufficient for removal of bound zinc from the NC protein (*19*, *21*). Reducing agents prevented oxidation of free SH groups. A specific HIV-1 PR inhibitor (UK 88.947) completely prevented processing of the r-preNC by HIV-1 PR (Figure 2, lane 5). N-Terminal analysis of the 4 kDa cleavage product revealed that a cleavage occurred between Phe16 and Asn17 of the mature NCp7 protein (see the numbering in Figure 1), within the proximal zinc finger, in accordance with previous observations (*20*, *21*).

[2] *The QIA expressionist*, summer 1992 edition, p 29, QIAGEN Inc., Chatsworth, CA. The similarly tagged 19 kDa dihydrofolate reductase also migrates in SDS−PAGE with an apparent molecular mass approximately 7 kDa higher than the calculated value.

Design, Expression, and Processing of Cleavage Site Mutant r-preNC Proteins. To test the effects of substitutions in the proximal zinc finger of NCp7 on the susceptibility to cleavage by the HIV-1 PR, mutations were introduced into the P1′ position (Asn17) of the cleavage site. These mutations only at the P1′ position were selected for the following reasons. (i) Substitutions in the P2−P2′ positions could prevent substrate hydrolysis (*28*). (ii) The two cysteines in the P2−P2′ region are required for proper structure and function of the zinc fingers (*14*, *29*, *30*). (iii) Phe16 at the P1 position is essential for the packaging of viral RNA (*16*).

The rationale for the substitutions with different amino acids (Phe, Leu, Ala, Lys, and Gly) was based on extensive specificity studies performed by us and others, as well as the interactions of the HIV PR with the substrate that are mainly hydrophobic (*4*, *31*, *32*). The S1 and S1′ subsites of HIV-1 PR are large, and are able to accommodate large side chains. Phe at P1 satisfies this requirement; indeed, P1 Phe substitution in a MA/CA cleavage site peptide provided the highest specificity constant (*28*). However, Asn at P1′ is far from being optimal, since it could not provide sufficient hydrophobic interactions with residues forming the S1′ subsite. Its substitution with amino acids having bulky side chains was expected to yield better substrates, as found with substitution of the P1′ residue of a particular substrate (*33*). Substitution of Leu at P1′ also could provide a large but aliphatic side chain and therefore was expected to improve processing. Substitution with Ala was expected to provide a substrate similar to the wild type. This Ala substitution in the virus was previously reported to result in infectious virion; however, the proteolytic processing of the NC was not studied (*16*). Substitution of Asn at P1′ to Lys was expected to decrease the rate of substrate hydrolysis. Lys substitution in the P2−P2′ region of a peptide substrate prevented hydrolysis (*28*) and has not been found in the P1′ position of known substrates (*34*). Elimination of the P1′ side chain by Gly substitution also was expected to prevent or at least substantially diminish the level of substrate hydrolysis, as found with another substrate for HIV-1 and AMV PR (*33*).

Mutant r-preNC proteins were purified in a manner similar to that of the wild-type protein and analyzed by SDS−PAGE (Figure 3, left panel). Cleavage patterns of these proteins are also presented in Figure 3 (right panel). Uncleaved purified NCp7 was used as a marker. As expected, the major part of the Asn17Gly and Asn17Lys mutant proteins was not cleaved in the proximal zinc finger cleavage site. A smaller amount of the 4 kDa fragment was produced from the Asn17Gly mutant protein than from the Asn17Lys mutant protein. Replacement of Asn17 with Phe and Leu increased cleavage efficiency relative to that of the wild type. Incubation of the Asn17Ala mutant with the HIV-1 PR provided processing somewhat better than that of the wild-type protein.

Time Course of the Processing of r-preNC. To study the kinetics of r-preNC processing and identify intermediate and final products, samples of the wild-type r-preNC protein were analyzed by SDS−PAGE after various times of incubation with PR (Figure 4A). A short period (1−8 min) of incubation resulted in the ordered appearance within minutes of 15.3, 8.5, and 7 kDa proteins, comprised of 93, 71, and 55 amino acids, respectively (Figure 4A). N-Terminal analysis (Table 1 and Figure 5) showed that these proteins were derived by

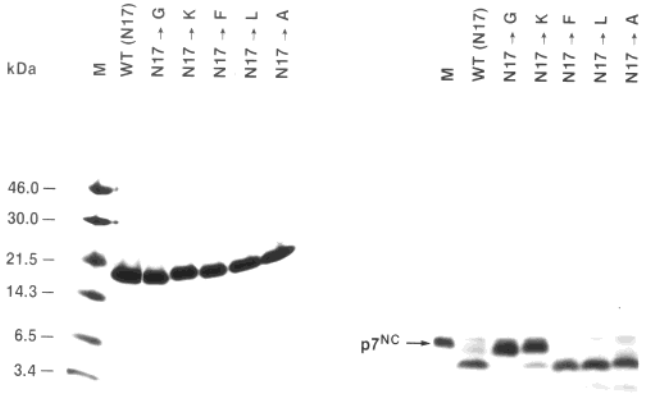

FIGURE 3: Comparison of the degree of processing of wild-type and mutant recombinant preNC proteins by HIV-1 proteinase. Wild-type r-preNC, labeled WT(N17), and its mutants were analyzed on SDS−PAGE before (left panel) and after incubation for 16 h (right panel) with HIV-1 PR: M, molecular mass markers; $p7^{NC}$, 55-residue p7 nucleocapsid protein.

Table 1: Analysis of the Full-Length r-preNC Protein and Its Proteolytic Fragments by SDS−Polyacrylamide Gel Electrophoresis and N-Terminal Sequencing

incubation time	molecular mass (kDa) obsd	calcd	length[a]	N-terminal sequence	source of the protein
L[b]	18	13.370	118	A-S-M-T-G-G-Q-Q-M-G	wild type
S	15.3	10.859	93	M-Q-R-G-N-F-R-N-Q-R	wild type
S	8.5	8.274	71	M-Q-R-G-N-F-R-N-Q-R	wild type
S	7	6.425	55	M-Q-R-G-N-F-R-N-Q-R	wild type
L	4.9	4.418	39	N-X-G-K-E-G-H-T-A-R-N-R	wild type
L	4	3.718	33	N-X-G-K-E-G-H-T-A-R-N-R	wild type
S	18	8.795	77	F-X-G-K-E-G-H-T-A-R	N17F
S	12	6.210	55	F-X-G-K-E-G-H-T-A-R	N17F
L	4	3.661	33	F-X-G-K-E-G-H-T-A-R	N17F
L	6.5	5.669	49	M-Q-R-G-N-F-R-N-Q	N17G

[a] Number of residues. [b] L, long incubation time (1−16 h); S, short incubation time (2−60 min). The N-terminus of the full-length protein was determined from a sample incubated for 16 h in the absence of proteinase, and it lost the N-terminal Met.

the cleavage at the p2−NC site, followed by subsequent cleavages at the p1−p6 and NC−p1 sites (Figure 5). The 7 kDa (55-residue) protein exhibited a migration pattern identical to that of purified viral NCp7 (Figure 4A, lane 10). The apparent molecular sizes of the two smaller proteins comprised of 71 and 55 amino acids are similar to their expected values (8.3 and 6.4 kDa, respectively), in good agreement with the loss of the six-His tag, due to the C-terminal processing at the p1−p6 site (Table 1 and Figure 5). On the basis of the protein profile, the preference of cleavages at the maturation sites is as follows: p2−NC > p1−p6 > NC−p1. Although our experiments were carried out at a high ionic strength, the order of maturation cleavages we observed is in good agreement with that previously found at low ionic strengths (*35*).

A longer (several hours) incubation with PR yielded further processing (Figure 4A). The N-terminal sequence of the doublet bands at 4 kDa showed that the cleavage of the NC protein occurred in the proximal zinc finger between Phe16 and Asn17 (Figure 5), producing a 39-residue fragment with a molecular mass of 4.9 kDa (see Table 1 and Figure 5). To complement the results obtained with the SDS−PAGE analysis of the samples, RP-HPLC was used to identify the fragments which were not recovered from the gel. Peaks from

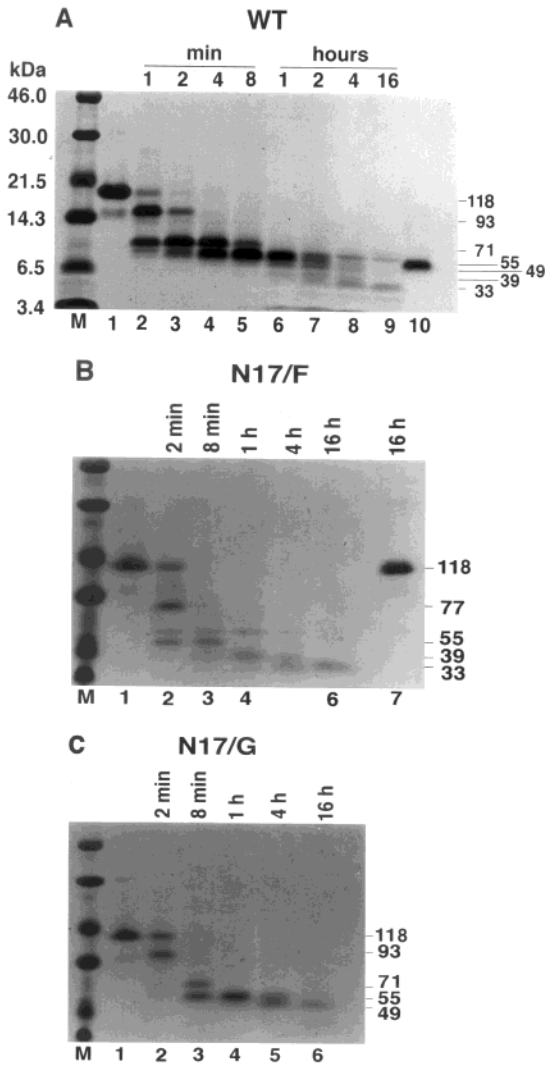

FIGURE 4: Time-dependent processing of preNC by HIV-1 proteinase. The purified precursor form of wild-type r-preNC protein (A) was incubated in the absence of HIV-1 PR for 16 h (lane 1) or with HIV-1 PR (lanes 2−9) for the time indicated above the lanes and analyzed by SDS−polyacrylamide gel electrophoresis followed by Coomassie staining. Rainbow molecular mass markers (M) were used to determine the size of the fragments. The numbers of amino acid residues in fragments deduced for the size of the bands and from protein sequencing are given at the right. The 55-residue NCp7 purified from virus was used as a size marker (lane 10) for the mature NC protein. Mutant r-preNC proteins containing Phe (B) and Gly (C) in place of Asn17 were incubated in the absence of HIV-1 PR for 16 h (lane 1) or with HIV-1 PR (lanes 2−6) for the time indicated above the lanes. The N17F mutant was also incubated in the presence of the proteinase inhibitor (lane 7).

HPLC fractions of samples taken at various time intervals of incubation were identified by N-terminal analysis, as well as mass spectral analysis (Table 2). A six-residue fragment was recovered by RP-HPLC (Table 2), and its sequence suggested a cleavage between Cys49 and Thr50 (see Figure 5A). The EIAV NC protein was also found to be cleaved at both zinc fingers (*19*). Interestingly, as for EIAV, the HIV-1 NC processing occurs through two intermediate products, although the second cleavage occurs at the last Cys of the distal zinc finger, instead of the first one. The N-terminal cleavage in NCp7 occurs inside the proximal zinc finger, and this cleavage was shown to require the removal of zinc (*21*). The C-terminal site utilizes the last Cys residue of the

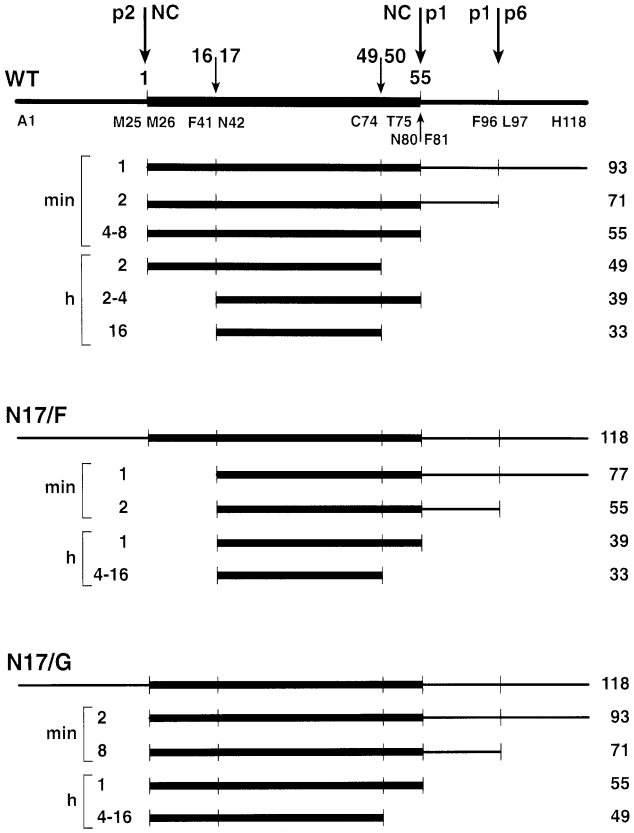

FIGURE 5: Schematic diagram of the processing of wild-type and mutant r-preNC proteins.

Table 2: Analysis of Proteolytic Fragments of the r-preNC Protein Separated by Reversed-Phase HPLC

molecular mass (kDa)			
obsd[a]	calcd	length[b]	N-terminal sequence of the protein
2.529	2.529	25	A-S-M-T-G-G-Q-Q-M-G-R-D-P-N-S
2.606	2.606	22	L-Q-X-X-P-E-P-T-A-P
1.867	1.867	16	F-L-G-K-I-X-P-S-Y-K
ND[c]	0.718	6	T-E-R-Q-A-N

[a] Sizes of these fragments (recovered by HPLC) were determined by FAB mass spectrometry. [b] Number of residues. [c] Not determined.

distal zinc finger as residue P1. Since the retroviral proteinases bind substrates in an extended conformation, a rigid conformation with the coordinated zinc is incompatible with the cleavage. In good agreement with this prediction, addition of 0.6 mM zinc chloride over the final EDTA concentration completely prevented the internal processing of NCp7 (not shown).

The patterns of cleavage of Asn17Phe and Asn17Gly mutant proteins were different from that of wild-type NC protein. Like the wild type, these mutant proteins were incubated with HIV-1 PR for various time intervals, and most of the products were analyzed by protein sequencing after SDS−PAGE. A short (2 min) incubation of the Asn17Phe mutant with the PR revealed an intermediate band comprising 77 amino acid residues but migrating as an 11.5 kDa polypeptide (Figure 4, lane 2) instead of the major 15.3 kDa cleavage product that was observed in the case of the wild type (Figure 4A, line 2). N-Terminal sequence analysis of this fragment corresponds to the cleavage product in the

Table 3: HIV-1 Protease-Catalyzed Hydrolysis of Oligopeptides Representing Cleavage Sites in the Recombinant NC Precursor Protein (r-preNC)

	site	peptide	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
1	p2−NC	TATIM*MQRGN	0.05	3.7	74.0[a]
2	NC−p1	ERQAN*FLGK	ND[b]	ND[b]	0.2[c]
3	p1−p6	RPGNF*LQSRP	1.20	0.98	0.80
4	NC-1	KIVKCF*NCGK	0.43	0.02	0.04
5	NC-2	HQMKDC*TERQA	2.01	0.08	0.04

[a] These values have been reported previously (28). [b] Increasing the substrate concentration above 0.2 mM caused a substantial decrease in activity. [c] Determined in the concentration range of 0.02−0.15 mM, in which the activity was a linear function of the substrate concentration.

proximal zinc finger site (Table 1). These data suggest that the rates of processing at maturation and mutated sites are comparable (see Figures 4B and 5 and Table 1). Further, C-terminal processing followed a similar pattern as found with the wild-type protein (Figures 4B and 5). The Asn17Leu mutant exhibited a processing pattern very similar to that of the Asn17Phe mutant; however, the cleavage at the mutated site was somewhat slower (not shown). The time-dependent processing of the Ala mutant was very similar to that of the wild type (not shown). While the processing pattern of the Asn17Gly mutant was similar to that of the wild type during a short (2−8 min) incubation period, during a longer incubation period only the C-terminal processing of the NCp7 occurred, and the cleavage was substantially impaired in the proximal zinc finger site (Figures 4C and 5 and Table 1).

Hydrolysis of Oligopeptides Having the Sequence of Cleavage Sites in Wild-Type and Mutant r-preNC. Oligopeptides representing the cleavage sites in wild-type r-preNC were analyzed as substrates for the HIV-1 PR (Table 3). The obtained kinetic parameters are in good agreement with the observed processing profile of r-preNC. The highest k_{cat}/K_m value was obtained for the p2−NC cleavage site peptide (the Met−Met bond), which previously was found to be one of the best substrates of HIV-1 PR among the HIV-1 Gag cleavage sites (*36*). The specificity constants (k_{cat}/K_m) for the peptides representing the p1−p6 and NC−p1 sites were substantially lower. The NC-1 peptide representing the cleavage site in the proximal zinc finger exhibited a 5-fold lower specificity constant than the peptide representing the least efficient NC−p1 "maturation site". Similarly, a 3-fold difference in the k_{cat}/K_m values was calculated for these cleavage sites by protein substrates at high ionic strengths (*21*). Although the catalytic constants for this peptide suggest that it is not an efficiently cleaved substrate of HIV-1 PR, it should be noted that similar or even lower catalytic constants were found previously for some peptides representing maturation cleavage sites of HIV-1 (*36*), EIAV (*20*), and AMV (*37*). The oligopeptide representing the cleavage site at the end of the distal zinc finger (NC-2) was also hydrolyzed, with a specificity constant similar to that of the other slow (NC-1) cleavage (Table 3).

Phe, Leu, Ala, Lys, and Gly substitutions were also introduced into the sequence of the NC-1 cleavage site peptide, and the peptides were assayed as substrates of HIV-1 proteinase. Kinetic parameters for these peptides are shown in Table 4. In good agreement with the findings with recombinant proteins, exchanging Asn at P1′ with Phe and Leu resulted in good substrates (Table 4). The specificity

Table 4: HIV-1 Protease-Catalyzed Hydrolysis of Substituted Oligopeptides Representing the Proximal Zinc Finger Site of the Nucleocapsid Protein

	site	peptide	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
6	P1′ N → F	KIVKCF*FCGK	0.02	0.9	59.6
7	P1′ N → L	KIVKCF*LCGK	0.17	0.2	1.2
8	P1′ N → A	KIVKCF*ACGK	0.32	0.06	0.2
9	P1′ N → K	KIVKCF*KCGK	0.86	0.005	0.006
10	P1′ N → G	KIVKCF*GCGK	1.34	0.002	0.001

constant for the Phe-containing peptide was comparable to those of the most easily hydrolyzed peptides representing naturally occurring maturation cleavage sites (*36*). Changing Asn at P1′ to Ala provided a substrate that was somewhat better than the unsubstituted one, in agreement with its also small but more hydrophobic side chain. Substitution with Lys and Gly reduced the k_{cat}/K_m values by 6- and 40-fold, respectively. It appears that the hydrophobic interactions provided by the flexible side chain of Lys could somewhat compensate the unfavorable positive charge at the S_1' subsite.

DISCUSSION

The major precursor form of HIV-1 NC protein(s) is the Gag polyprotein Pr55, present in the immature virion. Proteolytic processing first yields an intermediary smaller precursor p15 from which the final products, NCp9 and NCp7, are derived (see Figure 6). While both are found in the mature virus particles, p9 is present at ∼10% of the level of p7. Both p9 and p7 have been studied structurally and biochemically, and it has been suggested that NCp9 comprised of 71 amino acids (p7 + p1) may have all the properties of NCp7 and an additional function (*38*).

In this study, we identified two cleavage sites in the HIV-1 NCp7, one located between Phe16 and Asn17 of the proximal zinc finger and the other between Cys49 and Thr50 of the distal zinc finger. These cleavages occurred after removal of the tetrahedrally coordinated zinc with a chelating agent, EDTA, and the complete processing of NCp7 yielded three fragments (Figure 6): (a) N-terminal 16-residue fragment (p1.8) containing the first Cys of the proximal zinc finger, (b) a 33-amino acid residue fragment p4 (from residue Asn17 to Cys49), and (c) p0.7, comprised of the six C-terminal amino acids. Polypeptides p4 and p0.7 were derived through an intermediary product p5 (see Figure 6).

We have used a mutational analysis to assess the effects of amino acid substitutions on PR processing of NC protein of HIV-1. The mutations in the NC coding region were designed so that the role of the NC protein in the late phase (i.e., RNA packaging) would not be abolished, yet its processing by PR would be altered. Asn17 was chosen because it occupies the P1′ position of the PR cleavage site, and mutations of this residue could alter the ability of PR to cleave this substrate. PR processing of Asn17Ala was similar to that of the wild type, while the cleavage of Asn17Leu and Asn17Phe appeared to be more efficient than that of the wild type. On the other hand, as expected from substrate specificity analysis, cleavage of Asn17Gly and Ans17Lys was impaired. Only a small amount of cleaved product appeared after 16 h.

In vitro studies using synthetic peptides or the protein suggested that NC protein may function during reverse

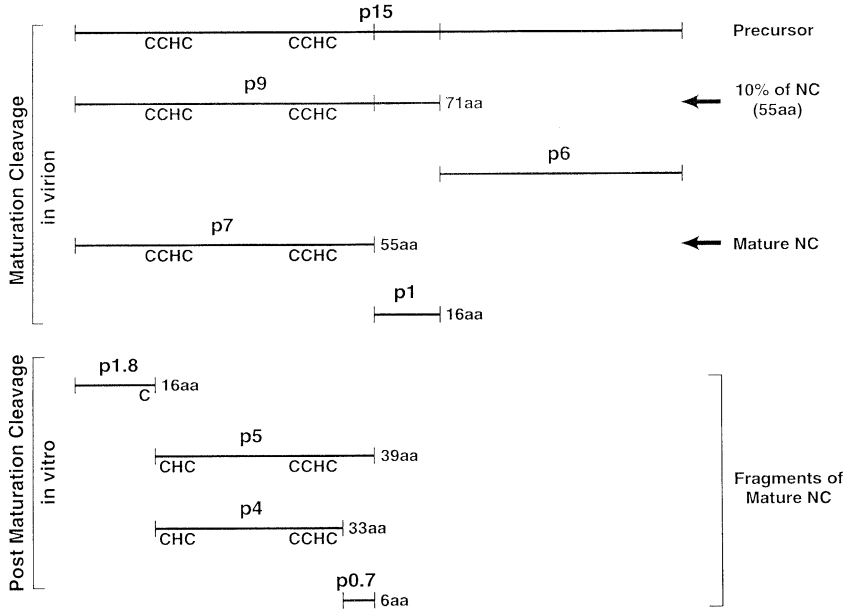

FIGURE 6: Various forms of HIV-1 NC generated by proteolysis.

transcription by enhancing the strand transfer reactions (*39*, *40*). Mutants of NC protein that produce virions that are defective in the early phase of replication suggest that NC is functional during that phase (*30*). Recent studies also indicated that the wild-type NC is also required for protection of newly synthesized viral DNA and initial integration processes (*41*).

On the basis of the NC cleavage observed in the EIAV capsid, we hypothesized that the PR is active in the early phase of the virus life cycle and that the NC protein is one of the targets of PR processing (*18*, *42*, *43*). Several research groups, including ours, found an inhibitory effect of PR inhibitors in the early phase of viral replication (*44*−*47*), supporting this assumption, while others did not observe such an effect (*48*−*50*). When the required zinc removal and the acidic pH optimum of the PR are taken into account, the likely place of the early-phase NC processing is the endosome for viral particles entering the cells through receptor-mediated endocytosis. Therefore, one possible explanation of the apparent discrepancy in results obtained with PR inhibitors is the different entry pathways of the tested isolates in the various assay systems.

It has been commonly accepted that unlike most enveloped viruses, including some retroviruses using the receptor-mediated endocytotic route, HIV-1 enters the cells only through a pH-independent manner, by fusion of the viral lipid with that of the cells (*51*). However, it has been recently demonstrated that endocytotic entry of HIV-1 into cells can lead to viral integration and gene expression (*52*). The use of direct fusion and the endocytotic route depends on the viral isolates, the type of virus-producing cells, and the target cells (*52*). Primary macrophages mostly take up HIV-1 by macropinocytosis, a receptor-independent process (*53*). Following uptake, a large part of the macropinocytosed virions were degraded, likely because of the endosomal/lysosomal fusions, but productive infection also occurred (*53*). The infection route used by the virion may have a profound effect on the proteolytic events involved in the early phase of viral replication (reviewed in ref *11*). Since the viral core of HIV-1, like the core of EIAV (*18*), entering the cells contains the active protease (*54*), its activity could be boosted by the acidic pH of the endosomes, and this could lead to protease-mediated cleavage of viral proteins. Recent *in vitro* and *in vivo* studies of Mason-Pfizer monkey virus (MPMV) as well as HIV indicated that the viral capsid protein is a substrate of the respective PR in the early phase of viral replication, and this cleavage requires acidic pH (*55*, *56*). In the case of NC, the acidic environment of the endosome also could lead to the loss of the coordinated zinc, which is required for the complete processing, since the affinity of zinc for the NC zinc fingers is substantially reduced below pH 6 (*57*). Our hypothesis about the early-phase effect of PR also implied that the NC protein of lentiviruses and/or its proteolysis fragments are active in the early phase (*42*, *43*, *45*). In addition to a possible role in reverse transcription (*12*, *58*), the NC protein or the cleaved fragments may be active after reverse transcription, either to protect the cDNA from host nucleases in the transport of the infectious entity to the nucleus or in integration events. The proteolytic fragments retained nucleic acid binding and chaperone activity (*12*; A. Rein and J. Levin, personal communications). Characterization of mutant viruses harboring the mutations described in this paper is in progress and supports the important biological role of the P1′ residues of the NC cleavage site in the proximal zinc finger. On the basis of the virological studies, only viruses having an "optimal" (wild-type) level of NC cleavage susceptibility (wild type and Asn17Ala) produced highly infections virions; those having noncleavable (Ans17Gly) or "supercleavable" (Asn17Phe) NC mutations exhibited severely impaired infectivity (R. J. Gorelick et al., manuscript in preparation).

ACKNOWLEDGMENT

We thank Louis Henderson for the NCp7 protein, Cathy Hixson and Suzanne Specht for their help with the amino acid analysis, Patrick Wesdock and Eva Majerova for the peptide synthesis, Young Kim for protein sequencing, Terry Sumpter for the FAB mass spectral analysis, and Carol Shawver for preparing the manuscript.

REFERENCES

1. Barré-Sinoussi, F., Chermann, J. C., Rey, F., Nugeyre, M. T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vezinet-Brun, F., Rouzioux, C., Rozenbaum, W., and Montagnier, L. (1983) Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS), *Science 220*, 868−871.
2. Popovic, M., Sarngadharan, M. G., Read, E., and Gallo, R. C. (1984) Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS, *Science 224*, 497−500.
3. Coffin, J. M., Hughes, S. H., and Varmus, H. E. (1997) *Retroviruses*, Cold Spring Harbor Laboratory Press, Plainview, NY.
4. Oroszlan, S., and Luftig, R. B. (1990) Retroviral proteinases, *Curr. Top. Microbiol. Immunol. 157*, 153−185.
5. Katz, R. A., and Skalka, A. M. (1994) The retroviral enzymes, *Annu. Rev. Biochem. 63*, 133−173.
6. Oroszlan, S. (1990) Biosynthesis and proteolytic processing of retroviral proteins: an overview, in *Current communications in molecular biology: viral proteinases as targets for chemotherapy* (Krausslich, H. G., Oroszlan, S., and Wimmer, E., Eds.) pp 87−100, Cold Spring Harbor Laboratory Press, Plainview, NY.
7. Wlodawer, A., and Erickson, J. W. (1993) Structure-based inhibitors of HIV-1 protease, *Annu. Rev. Biochem. 62*, 543−585.
8. Merigan, T. C. (1995) A quarter-century of antiviral therapy, *N. Engl. J. Med. 333*, 1704−1705.
9. Coffin, J. M. (1995) HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy, *Science 267*, 483−489.
10. Swanstrom, R., and Eron, J. (2000) Human immunodeficiency virus type-1 protease inhibitors: therapeutic successes and failures, suppression and resistance, *Pharmacol. Ther. 86*, 145−170.
11. Tözsér, J., and Oroszlan, S. (2003) Proteolytic events of HIV-1 replication as targets for therapeutic intervention, *Curr. Pharm. Des. 9*, 1803−1815.
12. Rein, A., Henderson, L. E., and Levin, J. G. (1998) Nucleic-acid-chaperone activity of retroviral nucleocapsid proteins: significance for viral replication, *Trends Biochem. Sci. 23*, 297−301.
13. Méric, C., and Goff, S. P. (1989) Characterization of Moloney murine leukemia virus mutants with single-amino-acid substitutions in the Cys-His box of the nucleocapsid protein, *J. Virol. 63*, 1558−1568.
14. Aldovini, A., and Young, R. A. (1990) Mutations of RNA and protein sequences involved in human immunodeficiency virus type 1 packaging result in production of noninfectious virus, *J. Virol. 64*, 1920−1926.
15. Dupraz, P., Oertle, S., Méric, C., Damay, P., and Spahr, P. F. (1990) Point mutations in the proximal Cys-His box of *Rous sarcoma* virus nucleocapsid protein, *J. Virol. 64*, 4978−4987.
16. Dorfman, T., Luban, J., Goff, S. P., Haseltine, W. A., and Göttlinger, H. G. (1993) Mapping of functionally important residues of a cysteine-histidine box in the human immunodeficiency virus type 1 nucleocapsid protein, *J. Virol. 67*, 6159−6169.
17. Gorelick, R. J., Gagliardi, T. D., Bosche, W. J., Wiltrout, T. A., Coren, L. V., Chabot, D. J., Lifson, J. D., Henderson, L. E., and Arthur, L. O. (1999) Strict conservation of the retroviral nucleocapsid protein zinc finger is strongly influenced by its role in viral infection processes: characterization of HIV-1 particles containing mutant nucleocapsid zinc-coordinating sequences, *Virology 256*, 92−104.
18. Roberts, M. M., and Oroszlan, S. (1989) The preparation and biochemical characterization of intact capsids of equine infectious anemia virus, *Biochem. Biophys. Res. Commun. 160*, 486−494.
19. Roberts, M. M., Copeland, T. D., and Oroszlan, S. (1991) *In situ* processing of a retroviral nucleocapsid protein by the viral proteinase, *Protein Eng. 4*, 695−700.
20. Tözsér, J., Friedman, D., Weber, I. T., Bláha, I., and Oroszlan, S. (1993) Studies on the substrate specificity of the proteinase of equine infectious anemia virus using oligopeptide substrates, *Biochemistry 32*, 3347−3353.
21. Wondrak, E. M., Sakaguchi, K., Rice, W. G., Kun, E., Kimmel, A. R., and Louis, J. M. (1994) Removal of zinc is required for processing of the mature nucleocapsid protein of human immunodeficiency virus, type 1, by the viral protease, *J. Biol. Chem. 269*, 21948−21950.
22. Schechter, I., and Berger, A. (1967) On the size of the active site in proteases. I. Papain, *Biochem. Biophys. Res. Commun. 27*, 157−162.
23. Louis, J. M., McDonald, R. A., Nashed, N. T., Wondrak, E. M., Jerina, D. M., Oroszlan, S., and Mora, P. T. (1991) Autoprocessing of the HIV-1 protease using purified wild-type and mutated fusion proteins expressed at high levels in *Escherichia coli*, *Eur. J. Biochem. 199*, 361−369.
24. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350−4354.
25. Grobelny, D., Wondrak, E. M., Galardy, R. E., and Oroszlan, S. (1990) Selective phosphinate transition-state analogue inhibitors of the protease of human immunodeficiency virus, *Biochem. Biophys. Res. Commun. 169*, 1111−1116.
26. Henderson, L. E., Sowder, R. C., Copeland, T. D., Oroszlan, S., and Benveniste, R. E. (1990) Gag precursors of HIV and SIV are cleaved into six proteins found in the mature virions, *J. Med. Primatol. 19*, 411−419.
27. Henderson, L. E., Bowers, M. A., Sowder, R. C., II, Serabyn, S. A., Johnson, D. G., Bess, J. W., Jr., Arthur, L. O., Bryant, D. K., and Fenselau, C. (1992) Gag proteins of the highly replicative MN strain of human immunodeficiency virus type 1: posttranslational modifications, proteolytic processings, and complete amino acid sequences, *J. Virol. 66*, 1856−1865.
28. Tözsér, J., Weber, I. T., Gustchina, A., Bláha, I., Copeland, T. D., Louis, J. M., and Oroszlan, S. (1992) Kinetic and modeling studies of S3−S3′ subsites of HIV proteinases, *Biochemistry 31*, 4793−4800.
29. South, T. L., Blake, P. R., Sowder, R. C., III, Arthur, L. O., Henderson, L. E., and Summers, M. F. (1990) The nucleocapsid protein isolated from HIV-1 particles binds zinc and forms retroviral-type zinc fingers, *Biochemistry 29*, 7786−7789.
30. Gorelick, R. J., Nigida, S. M., Jr., Bess, J. W., Jr., Arthur, L. O., Henderson, L. E., and Rein, A. (1990) Noninfectious human immunodeficiency virus type 1 mutants deficient in genomic RNA, *J. Virol. 64*, 3207−3211.
31. Dunn, B. M., Gustchina, A., Wlodawer, A., and Kay, J. (1994) Subsite preferences of retroviral proteinases, *Methods Enzymol. 241*, 254−278.
32. Tomasselli, A. G., and Heinrikson, R. L. (1994) Specificity of retroviral proteases: an analysis of viral and nonviral protein substrates, *Methods Enzymol. 241*, 279−301.
33. Cameron, C. E., Grinde, B., Jacques, P., Jenoft, J., Leis, J., Wlodawer, A., and Weber, I. T. (1993) Comparison of the substrate-binding pockets of the *Rous sarcoma* virus and human immunodeficiency virus type 1 proteases, *J. Biol. Chem. 268*, 1171−1720.
34. Chou, K. C., Tomasselli, A. G., Reardon, I. M., and Heinrickson, R. L. (1996) Predicting human immunodeficiency virus protease cleavage sites in proteins by a discriminant function method, *Proteins 24*, 51−72.
35. Tritch, R. J., Cheng, Y. S. E., Yin, F. H., and Erickson-Viitanen, S. (1991) Mutagenesis of protease cleavage sites in the human immunodeficiency virus type 1 *gag* polyprotein, *J. Virol. 65*, 922−930.
36. Tözsér, J., Bláha, I., Copeland, T. D., Wondrak, E. M., and Oroszlan, S. (1991) Comparison of the HIV-1 and HIV-2 proteinases using oligopeptide substrates representing cleavage sites in Gag and Gag-Pol polyproteins, *FEBS Lett. 281*, 77−80.
37. Tözsér, J., Bagossi, P., Weber, I. T., Copeland, T. D., and Oroszlan, S (1996) Comparative studies on the substrate specificity of avian myeloblastosis virus proteinase and lentiviral proteinases, *J. Biol. Chem. 271*, 6781−6788.
38. Cameron, C. E., Ghosh, M., Le Grice, S. F., and Benkovic, S. J. (1997) Mutations in HIV reverse transcriptase which alter RNase H activity and decrease strand transfer efficiency are suppressed by HIV nucleocapsid protein, *Proc. Natl. Acad. Sci. U.S.A. 94*, 6700−6705.
39. Lapadat-Tapolsky, M., De Rocquigny, H., Van Gent, D., Roques, B., Plasterk, R., and Darlix, J. L. (1993) Interactions between HIV-1 nucleocapsid protein and viral DNA may have important functions in the viral life cycle, *Nucleic Acids Res. 21*, 831−839.
40. You, J. C., and McHenry, C. S. (1994) Human immunodeficiency virus nucleocapsid protein accelerates strand transfer of the terminally redundant sequences involved in reverse transcription, *J. Biol. Chem. 269*, 31491−31495.
41. Buckman, J. S., Bosche, W. J., and Gorelick, R. J. (2003) Human immunodeficiency virus type 1 nucleocapsid Zn^{2+} fingers are required for efficient reverse transcription, initial integration

processes, and protection of newly synthesized viral DNA, *J. Virol. 77*, 1469−1480.
42. Roberts, M. M., Volker, E., Copeland, T. D., Nagashima, K., Cassell, M. B., Briggs, C. J., and Oroszlan, S. (1991) Regulated proteolytic processing within mature retroviral capsids, in *Advances in molecular biology and targeted treatment for AIDS* (Kumar, A., Ed.) pp 273−280. Plenum Press, New York.
43. Roberts, M. M., and Oroszlan, S. (1990) The action of retroviral protease in various phases of virus replication, in *Retroviral proteases, control of maturation and morphogenesis* (Pearl, L., Ed.) pp 131−139, Macmillan Press, London.
44. Baboonian, C., Dalgleish, A., Bountiff, L., Gross, J., Oroszlan, S., Rickett, G., Smith-Burchnell, C., Troke, P., and Merson, J. (1991) HIV-1 proteinase is required for synthesis of pro-viral DNA, *Biochem. Biophys. Res. Commun. 179*, 17−24.
45. Nagy, K., Young, M., Baboonian, C., Merson, J., Whittle, P., and Oroszlan, S. (1994) Antiviral activity of human immunodeficiency virus type 1 protease inhibitors in a single cycle of infection: evidence for a role of protease in the early phase, *J. Virol. 68*, 757−765.
46. Panther, L. A., Coombs, R. W., Aung, S. A., dela Rosa, C., Gretch, D., and Corey, L. (1999) Unintegrated HIV-1 circular 2-LTR proviral DNA as a marker of recently infected cells: relative effect of recombinant CD4, zidovudine, and saquinavir in vitro, *J. Med. Virol. 58*, 165−173.
47. Goto, T., Nakano, T., Kohno, T., Morimatsu, S., Morita, C., Hong, W., Kiso, Y., Nakai, M., and Sano, K. (2001) Targets of a protease inhibitor, KNI-272, in HIV-1-infected cells, *J. Med. Virol. 63*, 203−209.
48. Jacobsen, H., Ahlborn-Laake, L., Gugel, R., and Mous, J. (1992) Progression of early steps of human immunodeficiency virus type 1 replication in the presence of an inhibitor of viral protease, *J. Virol. 66*, 5087−5091.
49. Kaplan, A. H., Manchester, M., Smith, T., Yang, Y. L., and Swanstrom, R. (1996) Conditional human immunodeficiency virus type 1 protease mutants show no role for the viral protease early in virus replication, *J. Virol. 70*, 5840−5844.
50. Uchida, H., Maeda, Y., and Mitsuya, H. (1997) HIV-1 protease does not play a critical role in the early stages of HIV-1 infection, *Antiviral Res. 36*, 107−113.
51. Clapham, P. R., and McKnight, A. (2002) Cell surface receptors, virus entry and tropism of primate lentiviruses, *J. Gen. Virol. 83*, 1809−1829.
52. Fackler, O. T., and Peterlin, B. M. (2000) Endocytic entry of HIV-1, *Curr. Biol. 10*, 1005−1008.
53. Marechal, V., Prevost, M. C., Petit, C., Perret, E., Heard, J. M., and Schwartz, O. (2001) Human immunodeficiency virus type 1 entry into macrophages mediated by macropinocytosis, *J. Virol. 75*, 11166−11177.
54. Welker, R., Hohenberg, H., Tessmer, U., Huckhagel, C., and Krausslich, H. G. (2000) Biochemical and structural analysis of isolated mature cores of human immunodeficiency virus type 1, *J. Virol. 74*, 1168−1177.
55. Rumlova, M., Ruml, T., Pohl, J., and Pichova, I. (2003) Specific in vitro cleavage of Mason-Pfizer monkey virus capsid protein: evidence for a potential role of retroviral protease in early stages of infection, *Virology 310*, 310−318.
56. Tözsér, J., Shulenin, S., Kadas, J., Boross, P., Bagossi, P., Copeland, T. D., Nair, B. C., Sarngadharan, M. G., and Oroszlan, S. (2003) Human immunodeficiency virus type 1 capsid protein is a substrate of the retroviral proteinase while integrase is resistant toward proteolysis, *Virology 310*, 16−23.
57. Mely, Y., De Rocquigny, H., Morellet, N., Roques, B. P., and Gerad, D. (1996) Zinc binding to the HIV-1 nucleocapsid protein: a thermodynamic investigation by fluorescence spectroscopy, *Biochemistry 35*, 5175−5182.
58. DeStefano, J. J. (1996) Interaction of human immunodeficiency virus nucleocapsid protein with a structure mimicking a replication intermediate. Effects on stability, reverse transcriptase binding, and strand transfer, *J. Biol. Chem. 271*, 16350−16356.

BI035625Z