JOURNAL OF VIROLOGY, June 1996, p. 3763–3769 Vol. 70, No. 6
0022-538X/96/$04.00+0

Second Locus Involved in Human Immunodeficiency Virus Type 1 Resistance to Protease Inhibitors

LOUISE DOYON, GILBERT CROTEAU, DIANE THIBEAULT, FRANCIS POULIN, LOUISE PILOTE, AND DANIEL LAMARRE*

Department of Biochemistry, Bio-Méga/Boehringer Ingelheim Research Inc., Laval, Québec H7S 2G5, Canada

Received 27 November 1995/Accepted 23 February 1996

Protease inhibitors are potent antiviral agents against human immunodeficiency virus type 1. As with reverse transcriptase inhibitors, however, resistance to protease inhibitors can develop and is attributed to the appearance of mutations in the protease gene. With the substrate analog protease inhibitors BILA 1906 BS and BILA 2185 BS, 350- to 1,500-fold-resistant variants have been selected in vitro and were found not only to contain mutations in the protease gene but also to contain mutations in Gag precursor p1/p6 and/or NC (p7)/p1 cleavage sites. Mutations in cleavage sites give rise to better peptide substrates for the protease in vitro and to improved processing of p15 precursors in drug-resistant clones. Importantly, removal of cleavage site mutations in resistant clones leads to a decrease or even an absence of viral growth, confirming their role in viral fitness. Therefore, these second-locus mutations indicate that cleavage of p15 is a rate-limiting step in polyprotein processing in highly resistant viruses. The functional constraint of p15 processing also suggests that additional selective pressure could further compromise viral fitness and maintain the benefits of antiviral therapies.

Human immunodeficiency virus type 1 (HIV-1) encodes a small homodimeric aspartic protease required for the maturation of the structural proteins p17, p24, p2, p7, p1, and p6 and the viral enzymes protease, reverse transcriptase (RT), RNase, and integrase (reviewed in reference 26). To exert its function, the protease must first recognize and then cleave nine different cleavage sites in Gag and Gag-Pol precursor polyproteins (26). In HIV-1 polyproteins, only limited sequence similarity between the nine cleavage sites, giving rise to qualitatively different substrates for the protease, is observed. By using peptides corresponding to natural cleavage sites, it was determined that p2/p7 and TF/PR are the most rapidly processed cleavage sites, while p7/p1 and p1/p6 are the most slowly cleaved (6, 34, 38).

The protease plays an essential role in HIV-1 replication, since point mutations or deletions that abolish its activity do not give rise to infectious particles (15, 25). Inhibitors of this enzyme have therefore been developed and have proven to be potent antiviral agents against HIV-1 (5, 37). As observed with RT inhibitors, however, inhibitors of the protease have been met with the emergence of resistant variants due to the high replication rate of the virus and the low fidelity of the RT enzyme (10, 30, 36). Moderate resistance (2- to 100-fold) to protease inhibitors has indeed been obtained both in vivo (4, 18) and in vitro (18) and has been attributed to the appearance of mutations in the protease gene. Not surprisingly, since many of these mutations are located in the active site of the enzyme, drug resistance mutations also have considerable impact on protease activity. This is reflected by impaired processing of Gag precursors in protease-mutated virions and by decreased in vitro catalytic efficiency of the protease towards peptides representing natural cleavage sites (16, 17, 20, 24, 32). The development of high levels of resistance to protease inhibitors, possibly requiring multiple mutations in the active site of the protease, was therefore expected to be limited by the functional constraints of the enzyme, which must cleave all precursor cleavage sites.

BILA 1906 BS and BILA 2185 BS are substrate analog protease inhibitors that have potent antiviral activity with limited cytotoxicity (7). With these inhibitors, highly resistant HIV-1 variants were obtained in vitro. These variants contain multiple mutations in the protease gene and also contain mutations in one or two cleavage sites. These results not only involve a second locus in the development of high-level resistance to protease inhibitors but suggest a limit to the mutability of the protease.

MATERIALS AND METHODS

Cells and viruses. Cell lines C8166 (obtained from J. Sullivan), 293 (American Type Culture Collection), and H-9 (chronically infected with HIV-1 strain IIIB) (28, 29, 31) (AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases; obtained from Robert Gallo) were used. All cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 10^{-5} M β-mercaptoethanol, 2 mM L-glutamine, and 10 μg of gentamicin per ml. Viral stocks were prepared from chronically infected H-9 cells. Proviral DNA pNL4.3 (1) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, from Malcolm Martin.

Protease inhibitors. BILA 1906 BS and BILA 2185 BS are pipecolinic acid derivatives whose chemical structures and HIV-2 protease-bound conformations have been described previously (33). BILA 1906 BS and BILA 2185 BS correspond to inhibitors 1 and 4, respectively, described in reference 33.

Selection of HIV-1 protease inhibitor-resistant variants. Variants were obtained by infecting 10^6 C8166 T cells with HIV-1 strain IIIB (multiplicity of infection of 1) in the presence of challenging concentrations of BILA 1906 BS or BILA 2185 BS. At each passage (3 to 4 days), the cell-free culture supernatant was used to infect fresh cells with a greater concentration of the drug if a cytopathic effect persisted. The final drug concentrations were 2 μM for BILA 1906 BS passage 33 and 1.6 and 7 μM for BILA 2185 BS passages 37 and 58, respectively. The drug concentration giving 50% inhibition of viral replication (50% effective concentration [EC_{50}]) was determined by extracellular p24 antigen (Ag) production (Coulter HIV-1 p24 Antigen Assay) in acutely infected C8166 cells.

Protease and cleavage site sequence determination. For sequence determination, genomic DNA of HIV-1 variant-infected C8166 cells was amplified by PCR with one of five sets of primers. Primer set 1 (5′-CCCCATATGCCTCAGGTC ACTCTTTGG-3′ and 5′-CCCGGATCCTCAAAAATTTAAAGTGCAACC-3′

* Corresponding author. Mailing address: Department of Biochemistry, Bio-Méga/Boehringer Ingelheim Research Inc., 2100 Cunard, Laval, Québec H7S 2G5, Canada. Phone: (514) 682-4640. Fax: (514) 682-8434.

with *Nde*I and *Bam*HI sites) generated a 300-bp fragment encoding the protease flanked by initiation and termination codons. Primer set 2 (5′-CCCCCCGAATTCCAGGGCCCCTAGGAAA-3′ and 5′-CCCCCCTCTAGAAGTATACTTCCTCCTGAAGTC-3′ with *Apa*I and *Bst*1107I sites) generated a 930-bp fragment containing the protease gene and flanking regions in p7, p1, p6, and RT. Primer set 3 (5′-GGGCAAGCAGGGAGCTAG-3′ and 5′-GGGTGGCTCCTTCTG-3′) generated a fragment containing the p17/p24 cleavage site, primer set 4 (5′-ATCCAGTGCATGCAG-3′ and 5′-GCCTGTCTCTCAGTA-3′) generated a fragment containing the p24/p2/p7 cleavage sites, and primer set 5 (5′-CCACCTGGATTCCTGAGT-3′ and 5′-CACTAGCCATTGCTCTCC-3′) generated a fragment containing the RT/RNase/integrase cleavage sites. PCR was carried out by using the Perkin-Elmer GeneAmp PCR kit (catalog no. N801-0055) with 25 cycles of 1 min at 94°C, 30 s at 45°C, and 2 min 10 s at 72°C followed by one cycle of 3 min at 72°C. PCR fragments were subcloned in the pCRII vector (TA cloning kit; InVitrogen) and sequenced with the T7 sequencing kit (Pharmacia Biotech Inc.). Protease sequences were obtained from 10 independent clones derived from each viral population by using either primer set 1 or 2, while cleavage site sequences were determined from at least 3 independent DNA clones generated by using primer sets 2 to 5.

Molecular clone constructs. Plasmid NL4.3, containing the full-length HIV-1 proviral DNA, was modified to delete the *Bst*1107I restriction site located in the 5′ cellular flanking region. Briefly, the 6,027-bp *Eco*RI-*Stu*I and 8,195-bp *Eco*RI-*Bst*1107I fragments of NL4.3 were ligated to generate a 14,222-bp modified plasmid (called 2.12) containing a unique *Bst*1107I site at position 2927 of the HIV-1 sequence. To construct molecular clones, the *Apa*I (A)-*Bst*1107I (B) DNA fragment of plasmid 2.12 was replaced with A-B fragments derived by PCR from variant DNA. Chimeric clones were constructed by the PCR overlap method (9) with primer sets 2 and 6 (5′-AGAAGCAGGAGCCGA-3′ and 5′-TCGGCTCCTGCTTCT-3′) and cloned in the 2.12 proviral vector. Molecular clones (2 μg of DNA) were transfected by the calcium phosphate method (8) in the human kidney 293 cell line. Virus-containing culture supernatants were harvested 3 days after transfection and filtered on 0.22-μm-pore-size Millex-GV Millipore membranes. Viral titers were determined by cytopathic effect on C8166 cells and by p24 Ag quantification of transfection supernatants. Following these quantifications, it was determined that all clones produced similar amounts of p24 Ag upon transfection and that all infectious clones had similar p24 Ag/viral titer ratios (7).

Growth kinetics studies. For growth studies, 10^6 C8166 cells were infected with viral supernatant containing 10 ng of p24 Ag, washed, and maintained in 10 ml of supplemented RPMI 1640 medium at 37°C for 7 days. Aliquots of the culture supernatant were harvested every day and assayed for p24 Ag content as described above.

Western blot (immunoblot) analysis of viral proteins. Culture supernatants containing virions were harvested 3 days after transfection of molecular clones, centrifuged at low speed, and filtered on 0.22 μm-pore-size Millex-GV Millipore membranes. The p24 Ag levels in the supernatants were then determined as described above, and the supernatants were centrifuged at 20,000 × *g* for 2 h to pellet virus particles. The pellets were resuspended in 1× Laemmli sample buffer (0.06 M Tris, 2% sodium dodecyl sulfate [SDS], 10% sucrose, and 5% β-mercaptoethanol) and heated at 100°C for 5 min. Equivalent amounts of samples were run on discontinuous (10 to 16% polyacrylamide) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) or on SDS–10% PAGE, transferred onto nitrocellulose membranes, and hybridized with monoclonal antibodies to p6 (M35/278 [35]) or to p24 (Cellular Products Inc., no. 0801080) followed by a ^{125}I-labeled sheep anti-rat antibody (Amersham; no. IM132) or a ^{125}I-labeled sheep anti-mouse antibody (Amersham; no. IM131), respectively. The blots were exposed on a PhosphorImager screen (Molecular Dynamics Inc.). The M35/278 antibody does not recognize the p6 protein from NL4.3 (or clone 2.12). Therefore, an HIV-1 IIIB/2.12 clone containing the A-B fragment of HIV-1 IIIB subcloned in the 2.12 proviral vector was constructed and used for anti-p6 Western blot analysis.

Enzymatic studies. The 300-bp DNA fragments generated with PCR primer set 1 and coding for HIV proteases were subcloned in the pET-11a expression vector (Novagen) and expressed in *Escherichia coli* BL21(DE3)pLysS. Protease expression was induced by incubation with 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 2 h at 37°C. Enzyme preparations consisted of partially purified HIV-1 (22) or refolded mutant enzymes isolated from inclusion bodies. Inclusion bodies were prepared by lysing cells in buffer A (10 mM Tris [pH 8.0], 5 mM EDTA, 5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) containing 0.1% Nonidet P-40, 10 mM $MgCl_2$, and 4 U of DNase I per ml. The lysates were sonicated and centrifuged (10,000 × *g* for 20 min) to pellet the insoluble fraction containing inclusion bodies. The inclusion bodies were then washed three times in buffer A containing 2 M urea and 1% Triton X-100, solubilized in buffer A containing 8 M urea, diluted to 200 μg/ml, and dialyzed at 4°C against three changes of refolding buffer (25 mM sodium acetate [pH 4.5], 2 mM dithiothreitol, 1 mM EDTA, and 10% glycerol). Enzyme concentrations were determined by active-site titration with reversible, tightly binding inhibitors. The decapeptide substrates RPGNFLQSRP, RPGNFFQSRP, ERQANFLGKI, and ERRVNFLGKI were synthesized by using a standard solid-phase methodology (2). Peptidic substrates (200 μM) were incubated at 37°C in the presence of 100 nM HIV proteases in 100 mM morpholinoethanesulfonic acid (MES)–300 mM KCl, 5 mM EDTA–1 mg of bovine serum albumin per ml (pH 5.5) for periods varying from 5 to 360 min. The reaction was terminated by addition of 100 or 200 μl of 1% trifluoroacetic acid–H_2O. The cleavage products and substrate were then separated by reverse-phase high-pressure liquid chromatography (2) on a Perkin-Elmer 3×3CR C8 column. For all decapeptide substrates, the specificity of cleavage was established by using synthetic product peptides that comigrated with the products observed (not shown). Catalytic efficiency was determined from measurements of initial velocity.

RESULTS

HIV-1 variants highly resistant to BILA 1906 BS and BILA 2185 BS contain multiple mutations in the protease gene and mutations in one or two cleavage sites. To determine if and how a high level of resistance to protease inhibitors could be obtained in vitro, HIV-1 strain IIIB was serially passaged in the presence of increasing concentrations of BILA 1906 BS or BILA 2185 BS. BILA 1906 BS and BILA 2185 BS are pipecolinic acid derivatives that exert potent antiviral activity against HIV-1 strain IIIB, with mean EC_{50}s of 1 and 2 nM, respectively (7). Variants showing 500-fold-reduced susceptibility to BILA 1906 BS were selected after 33 passages in the presence of this drug, while variants 350- and 1,500-fold resistant to BILA 2185 BS were selected after 37 and 58 passages, respectively (Table 1). PCR amplification followed by DNA sequencing identified mutations V-32→I (V32I), M46I/L, and A71V and the double nucleotide mutation I84A in the protease of BILA 1906 BS-resistant variants and up to eight mutations (L10F, L23I, V32I, M46I, I47V, I54M, A71V, and I84V) in the protease of BILA 2185 BS-resistant variants (Table 1). An examination of the crystal structure of the protease highlights the locations of most of these mutations in or around the active site, except for A71V, which is located on the outer side of the structure (Fig. 1). BILA 1906 BS and BILA 2185 BS have been shown to have protease-bound conformations that differ predominantly in the S2 and S3 subsites, which could explain the somewhat different mutational patterns selected by these inhibitors. Regardless, it is clear that the protease can tolerate considerable amino acid modifications despite its small size and the functional constraint of recognizing multiple cleavage sites in the Gag and Gag-Pol precursor polyproteins.

Interestingly, sequencing of the cleavage sites of the variants highly resistant to BILA 1906 BS and BILA 2185 BS also revealed mutations. Indeed, an L→F mutation in the P1′ position (as described in footnote *c* of Table 1) of the p1/p6 cleavage site was observed in 17 of 19 DNA clones (Table 1). This mutation remained even upon removal of the drug for 20 passages (BILA 1906 BS R10 and R20 in Table 1). This mutation was not observed, however, in variants from passages showing only low-level resistance to protease inhibitors or in wild-type viruses cultured without drugs for several passages (Table 1 and data not shown). A second cleavage site modification involving mutations in the P3 and P2 residues of the p7/p1 junction (QA→RV) was also observed but only in BILA 2185 BS-resistant variants containing seven or eight mutations in the protease gene and showing 1,500-fold resistance to the drug. Both of these cleavage site mutations are located within the p15 precursor. All other Gag and Pol cleavage sites in highly resistant variants were sequenced, and no other mutations (residues P5 to P5′) in these sites were observed (data not shown).

Cleavage site mutations improve growth of molecular clones containing multiple mutations in the protease gene. HIV-1 molecular clones representing either a variant obtained after 33 passages in the presence of BILA 1906 BS (clone 1906^{r33}, containing mutations V32I, M46I, A71V, and I84A in the protease and L→F in the p1/p6 cleavage site) or a variant

TABLE 1. Characterization of BILA 1906 BS- and BILA 2185 BS-resistant HIV-1 variants containing mutations in the protease and in cleavage sites

Drug	Passage	Fold EC_{50}[a]	Cleavage site sequence[b]	Protease sequence
			p7/p1 ↓ p1/p6 ↓	
None	0		EROAN FLGKIWPSYKGRPGNF LQSRP	Wild type
	12		- - - - - - - - - - - - - -	Wild type
BILA 1906 BS	33	520	- - - - - - - - - - - - F - -	M46L-A71V-I84A
			- - - - - - - - - - - - F - -	V32I-M46I-A71V-I84A
	R10[c]	200	- - - - - - - - - - - - F - -	M46I,L-A71V-I84A
	R20[c]	7	- - - - - - - - - - - - F - -	M46I-A71V-I84V
BILA 2185 BS	37[d]	350	- - - - - - - - - - - - F - -	L23I-M46I-I84A
			- - - - - - - - - - - - F - -	L10I-L23I-M46I-I84V
	58	1,500	- RV - - - - - - - - - - F - -	L23I-V32I-M46I-I47V-I54M-A71V-I84V
			- RV - - - - - - - - - - F - -	L10F-L23I-V32I-M46I-I47V-I54M-A71V-I84V

[a] Fold EC_{50} towards the drug against which the variant was selected. BILA 1906 BS and BILA 2185 BS have mean EC_{50}s of 1 and 2 nM, respectively, against HIV-1 strain IIIB.

[b] Cleavage site sequences are underlined, and scissile bonds are indicated by arrows. The identification of amino acids involved in cleavage sites is according to the convention of P1 to P5 going from the scissile bond towards the amino terminus and P1′ to P5′ going towards the carboxy terminus (5, 26, 36). A hyphen indicates no change from the original sequence.

[c] R10 and R20 are viral populations derived from passage 33 and maintained in culture for 10 (R10) or 20 (R20) passages without drug.

[d] Three clones from this passage had a 12-amino-acid duplication involving residues P1 to P11′ of the p1/p6 cleavage site (21). Only one of these clones had the L→F mutation.

obtained after 58 passages in the presence of BILA 2185 BS (clone 2185^{r58}, containing mutations L23I, V32I, M46I, I47V, I54M, A71V, and I84V in the protease, QA→RV in p7/p1, and L→F in p1/p6) were constructed for further studies (Fig. 2, parental clones). Growth kinetics studies with these clones showed that both 1906^{r33} and 2185^{r58} grew significantly less than the wild-type virus (clone 2.12) on C8166 T cells (Fig. 3). Indeed, upon infection of the very permissive cell line C8166 by wild-type virus 2.12, viral growth could be monitored for only 3 to 4 days, after which p24 Ag levels in the supernatants had peaked. In contrast, in cultures infected with mutant viruses, p24 levels were consistently lower than those in wild-type cultures and growth could easily be monitored until day 7. Sequencing of emerging viruses in 1906^{r33} and 2185^{r58} cultures showed that the highly mutated genotypes were stable, since no reversion of protease or cleavage site mutations could be detected in these experiments. Moreover, mutant viruses harvested from cultures at day 7 and used in a second round of replication on C8166 cells showed the same slow growth phenotype as in the first experiment, confirming the absence of

FIG. 1. Stereo diagram of the crystal structure at 1.7 Å (0.17 nm) of HIV protease in complex with BILA 1906 BS. Dimer subunits are in green and blue. Highlighted areas are side chains of mutated residues either present in both BILA 1906 BS- and BILA 2185 BS-resistant variants (white) or present only in BILA 2185 BS-resistant variants (red). Details of the crystal structure have been described elsewhere (33).

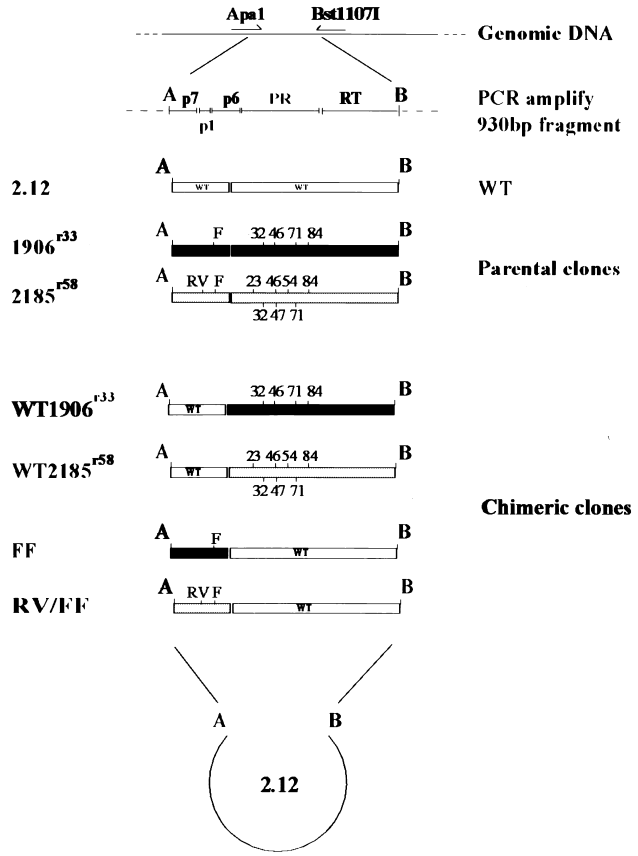


FIG. 2. Construction of HIV-1 molecular clones. To construct molecular clones, the *Apa*I (A)-*Bst*1107I (B) DNA fragments of variants were cloned into the unique A-B restriction sites of a modified NL4.3 proviral vector called 2.12. Molecular clones representing a variant obtained from passage 33 in the presence of BILA 1906 BS (1906^{r33}) or a variant from passage 58 in the presence of BILA 2185 BS (2185^{r58}) are referred to as parental clones in the text. Chimeric clones ($WT1906^{r33}$, $WT2185^{r58}$, FF, and RV/FF) were constructed by replacing the 200 most 5′ nucleotides of the A-B fragments of parental clones with corresponding sequences from clone 2.12, 1906^{r33}, or 2185^{r58} encoding wild-type or mutated cleavage sites. The numbers represent positions of amino acid mutations in the protease sequence, while F is the p1/p6 cleavage site mutation and RV is the p7/p1 cleavage site mutations.

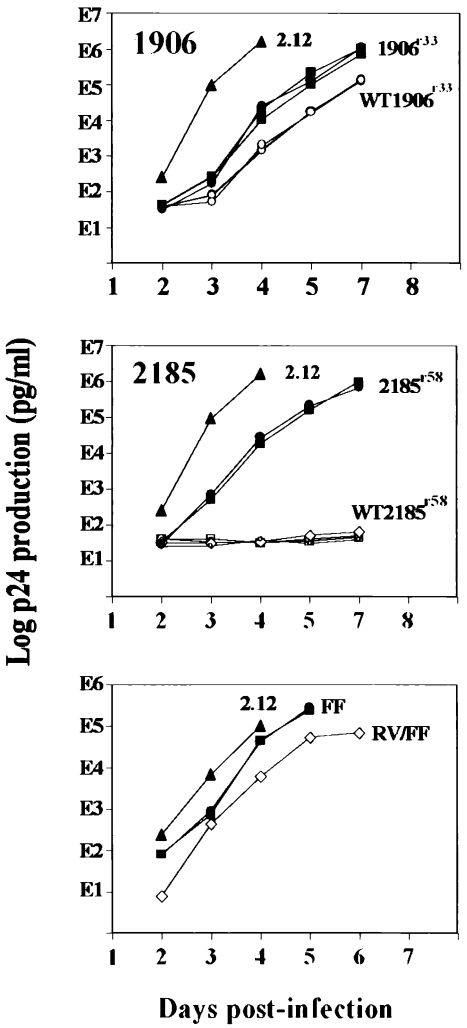


FIG. 3. Growth kinetics of viral molecular clones. C8166 cells were infected at day 0 with the viral molecular clones described in Fig. 2. Viral growth was then assessed daily by quantification of p24 Ag in the culture supernatants. (Top panel) Three independent 1906^{r33} clones and three independent $WT1906^{r33}$ clones; (middle panel) two independent 2185^{r58} clones and five independent $WT2185^{r58}$ clones; (bottom panel) wild-type clone 2.12 and clones FF and RV/FF containing wild-type proteases and mutated cleavage sites. Experiments have determined that for all infectious clones, p24 Ag levels correlated with levels of viral progeny (see Materials and Methods).

revertants (data not shown). Therefore, viruses containing multiple mutations in the protease and cleavage sites have slower growth kinetics than wild-type virus.

The contribution of the cleavage site mutations in this phenotype was studied by constructing molecular chimeras in which the p7/p1 and p1/p6 cleavage sites were replaced with corresponding sequences from wild-type virus (Fig. 2, chimeric clones). Removal of the p1/p6 cleavage site mutation in parental clone 1906^{r33} (to give $WT1906^{r33}$) further reduces growth compared with that of the wild-type virus, since p24 Ag levels in $WT1906^{r33}$ cultures did not increase until day 4 and still had not reached wild-type levels at day 7 (Fig. 3). Removal of both the p7/p1 and p1/p6 cleavage site mutations in clones 2185^{r58} has an even more dramatic effect, since growth was reduced to undetectable levels even 7 days postinfection of T cells with $WT2185^{r58}$, which contains wild-type cleavage sites (Fig. 3). Mutations in cleavage sites therefore compensate for the impaired ability of mutant viruses to replicate. The effect of cleavage site mutations in the absence of protease mutations was also studied by constructing chimeras containing wild-type proteases and mutated p1/p6 (FF) or p1/p6 and p7/p1 (RV/FF) cleavage sites (Fig. 2). Although both of these chimeras were shown to be infectious (Fig. 3, bottom panel), the presence of two cleavage site modifications in the RV/FF virus somewhat reduced its growth kinetics compared with that of wild-type virus.

TABLE 2. Characterization of viral molecular clones

Viral clone	EC_{50} (nM)	
	BILA 1906 BS	BILA 2185 BS
2.12	3.4	8.7
FF	1.1	6.3
RV/FF	2	4
1906^{r33}	428	ND[a]
$WT1906^{r33}$	326	ND
2185^{r58}	ND	>1,251

[a] ND, not determined.

Cleavage site mutations do not contribute to viral resistance to protease inhibitors. Since cleavage site mutations were present in viruses selected in the presence of protease inhibitors (Table 1), the contribution of these mutations to the resistance phenotype was also determined. Molecular clones and chimeras were tested for their in vitro susceptibilities to protease inhibitors BILA 1906 BS and BILA 2185 BS. Table 2 shows that in viruses containing wild-type proteases, the presence of mutations in the p1/p6 or p7/p1 cleavage sites did not affect susceptibility to protease inhibitors (viruses 2.12, FF, and RV/FF). Likewise, in the presence of four mutations in the protease (viruses 1906^{r33} and $WT1906^{r33}$), a mutation in the p1/p6 cleavage site did not significantly increase the EC_{50} of BILA 1906 BS (428 versus 326 nM, respectively). However, all molecular clones containing mutations in the protease gene (1906^{r33}, $WT1906^{r33}$, and 2185^{r58}) showed decreased susceptibility to protease inhibitors compared with the wild-type virus. These results suggest that mutations in the protease rather than in cleavage sites confer resistance to protease inhibitors.

Cleavage site mutations improve Gag p15 processing in protease-mutated virions. To determine if altered cleavage sites were increasing growth by improving Gag processing, proteins from parental and chimeric viral clones were analyzed by Western blotting with an anti-p6 antibody (Fig. 4A). Wild-type and parental clones 1906^{r33} and 2185^{r58} express immunoreactive 55- and 6-kDa proteins as well as intermediate precursor proteins of approximately 24 and 39 kDa (Fig. 4A). These represent the full-length $Pr55^{Gag}$ precursor, the mature p6 protein, a p24-p2-p7-p1-p6 polyprotein precursor, and a 24-kDa protein of unknown origin (19). Removal of the cleavage site mutations (clones $WT1906^{r33}$ and $WT2185^{r58}$) gives rise to an additional but prominent 15-kDa band, representing the p7-p1-p6 precursor (19). The accumulation of this precursor only in chimeric viruses indicates that efficient processing of p15 requires mutations of cleavage sites in highly resistant viruses. Interestingly, in $WT1906^{r33}$ but not $WT2185^{r58}$ viruses, an increased immunoreactivity of a 7-kDa protein, which could result from cleavage of the p15 precursor at the wild-type p7/p1 junction, is observed. Cleavage site mutations specifically affect p15 processing, since maturation of the p24 capsid protein is equally productive in parental and chimeric clones (Fig. 4B). However, an anti-p24 Western blot shows that in clones con-

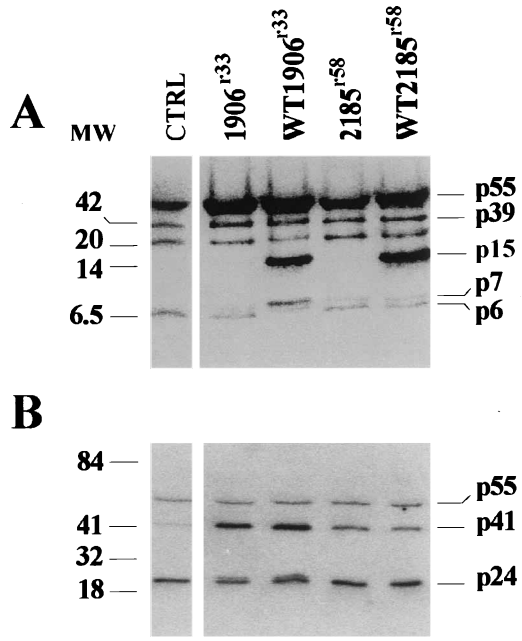


FIG. 4. $Pr55^{Gag}$ processing in HIV-1 molecular clones. MW, molecular weights in thousands. (A) Virus produced upon transfection of 293 cells was pelleted by high-speed centrifugation, lysed in 2% SDS buffer, run on discontinuous SDS–10 to 16% PAGE, and analyzed by Western blotting with an anti-p6 antibody. One representative clone of each construct is shown. The control (CTRL) is viral protein from a virus consisting of the A-B fragment of an HIV-1 IIIB wild-type virus inserted in the 2.12 proviral vector. The positions of the p55, p39, and p15 precursors (19) and the p6 protein are indicated on the right. The band of approximately 24,000 molecular weight, although already reported (13, 35), does not correspond to any known precursor. (B) The same samples were run on SDS–10% PAGE and analyzed by Western blotting with an anti-p24 antibody. The positions of the precursors p55 and p41 and the p24 capsid protein are indicated on the right.

taining mutated proteases there is accumulation of a p41 protein and that an additional p25 protein is detected in 1906^{r33} clones. These bands probably reflect the presence of p17-p24-p2 and p24-p2 uncleaved precursors, respectively. These results indicate that although it is productive, p24 processing is inefficient in clones containing mutated proteases. Of interest is that comparable levels of RT activity and immunoreactive p17, gp160, and gp41 were also detected in all parental and chimeric viruses (7).

Cleavage site mutations improve enzymatic activity towards peptide substrates. The mutations observed in the p7/p1 and p1/p6 regions of highly resistant viruses seem to give rise to better cleavage sites for the proteases. To address this, decapeptides representing either wild-type or mutated cleavage sites were assayed in vitro as substrates for HIV proteases (Table 3). Cleavage of peptides representing wild-type p1/p6 (RPGNF-LQSRP) or p7/p1 (ERQAN-FLGKI) cleavage sites was very low when the highly mutated protease 1906^{r33} or 2185^{r58} was used ($k_{cat}/K_m < 0.01\ \mu M^{-1}\ h^{-1}$). These peptides were well cleaved, however, by the wild-type HIV-1 enzyme ($k_{cat}/K_m = 0.60$ and $0.30\ \mu M^{-1}\ h^{-1}$, respectively). In contrast, peptides representing mutated cleavage sites were more efficiently cleaved by all proteases tested. Indeed, cleavage of the mutated p1/p6 peptide (RPGNF-FQSRP) increased to 0.17 $\mu M^{-1}\ h^{-1}$ with the 1906^{r33} protease and to 0.02 $\mu M^{-1}\ h^{-1}$ with the 2185^{r58} protease. Cleavage of the mutated p7/p1 peptide (ERRVN-FLGKI), on the other hand, was increased to 0.15 and 0.01 $\mu M^{-1}\ h^{-1}$, respectively, when these same enzymes were used. Altogether, mutations in cleavage site peptides gave rise to 2- to 10-fold increases in the catalytic efficiency values compared with cleavage of wild-type peptides. Despite these increases, however, cleavage of the mutated peptides by mutant proteases was not as efficient as cleavage of wild-type peptides by the wild-type HIV-1 enzyme.

TABLE 3. Catalytic efficiencies of wild-type and mutant proteases towards cleavage site peptides

Protease	k_{cat}/K_m ($\mu M^{-1}\ h^{-1}$)			
	p1/p6		p7/p1	
	RPGNF-LQSRP	RPGNF-FQSRP	ERQAN-FLGKI	ERRVN-FLGKI
Wild type	0.60	7.15	0.30	2.93
1906^{r33}	<0.01	0.17	<0.01	0.15
2185^{r58}	<0.01	0.02	<0.01	0.01

DISCUSSION

The data presented in this paper provide the first evidence of second-locus cleavage site mutations in HIV-1 protease inhibitor-resistant viruses. These mutations give rise to improved polyprotein processing in protease-mutated viruses and provide a mechanism by which HIV-1 could compensate for impaired protease activity. The first modification of the p1/p6 cleavage site in both BILA 1906 BS- and BILA 2185 BS-resistant variants identifies this cleavage as a probable rate-limiting step in polyprotein processing in virions containing mutated proteases. The presence in some variants of additional mutations at the nearby p7/p1 junction is further evidence that cleavage of p15 is compromised in resistant viruses. No mutations were observed in other cleavage sites, although there is evidence of inefficient processing at the p17/p24 and p24/p2 junctions (Fig. 4B). The latter results suggest that the elevated concentrations of substrates in immature virions could somewhat compensate for the loss of protease activity towards p17/p24 and p24/p2 cleavage sites or that protease activity is still above a theoretical threshold giving productive cleavage of the p17, p24, and p2 proteins. Inversely, the requirement for mutations in the p7/p1 and p1/p6 cleavage sites suggests that despite high polyprotein concentrations in immature virions, processing of p15 is severely limiting when the protease is mutated. In fact, cleavage site modifications correlate with the relative inefficiencies of p7/p1 and p1/p6 as Gag processing sites and peptide substrates (6, 34, 38). Therefore, it is not surprising that mutations in cleavage sites give rise to better peptide substrates for wild-type as well as mutant proteases (Table 3). Cleavage site mutations are expected to be fairly limited, however, since not much flexibility is allowed near the scissile bond (3, 27) and since sequences must remain generally hydrophobic (26). The p7/p1 cleavage site has an additional constraint in that its P1 residue and all of its P′ residues are encoded by nucleotide sequences involved either in the frameshift site or in the stem-loop structure needed for ribosomal frameshifting (12) in Gag-Pol expression.

The emergence of HIV-1 variants resistant to protease inhibitors in vitro depends on the capacities of viruses both to resist drugs and to grow efficiently. Enzymatic studies have shown that mutations in the active site of the protease confer drug resistance by increasing the inhibitor's K_i (11, 23, 24). However, the results in this study show that as a consequence, protease mutations also lead to significant growth reduction due to impaired polyprotein processing. Cleavage site muta-

tions, on the other hand, seem to be uniquely related to growth properties. Indeed, the results in Fig. 3 show that protease-mutated viral clones which contain cleavage site mutations grow much better than clones in which cleavage site mutations have been removed. Susceptibility to protease inhibitors, however, is not affected by cleavage site sequences (Table 2). In addition to reflecting impaired growth kinetics, the presence of cleavage site mutations in highly resistant HIV-1 variants also highlights a potential limit to the mutability of the protease. Indeed, the protease can probably tolerate only a certain number of active-site mutations before the limiting step in polyprotein processing is seriously compromised. When the enzyme can no longer sustain loss of activity ("dead-end protease"), concomitant modifications of both the protease and cleavage sites may be the only way for survival and outgrowth of viruses with higher levels of resistance to occur. Optimal growth of HIV-1 variants could be important during in vivo infection, according to recent reports on HIV-1 viral dynamics (10, 36). Indeed, any significant reduction in growth properties in vivo could lead to viral clearance by a boosted immune system.

Although variants containing cleavage site mutations have been selected in vitro by using two structurally related protease inhibitors, it is impossible to predict whether such mutations will appear following in vivo treatment of HIV-infected patients. While many factors may contribute to the emergence of variants with cleavage site mutations in vivo, concentrations of peptidomimetic inhibitors in plasma in human patients (14) do compare with the concentrations used in the present study. Therefore, the in vivo appearance of second-locus mutations could be expected to be associated with the emergence of resistant but growth-impaired variants. In this view, convergent therapies with potent first-generation protease inhibitors and mimics of mutated cleavage sites should confer a therapeutic advantage, targeting not only the wild-type protease but also dead-end proteases. Surviving viruses, if any, would be expected to have their rate-limiting p15 processing and growth kinetics further compromised, an outcome with sustained clinical benefits.

ACKNOWLEDGMENTS

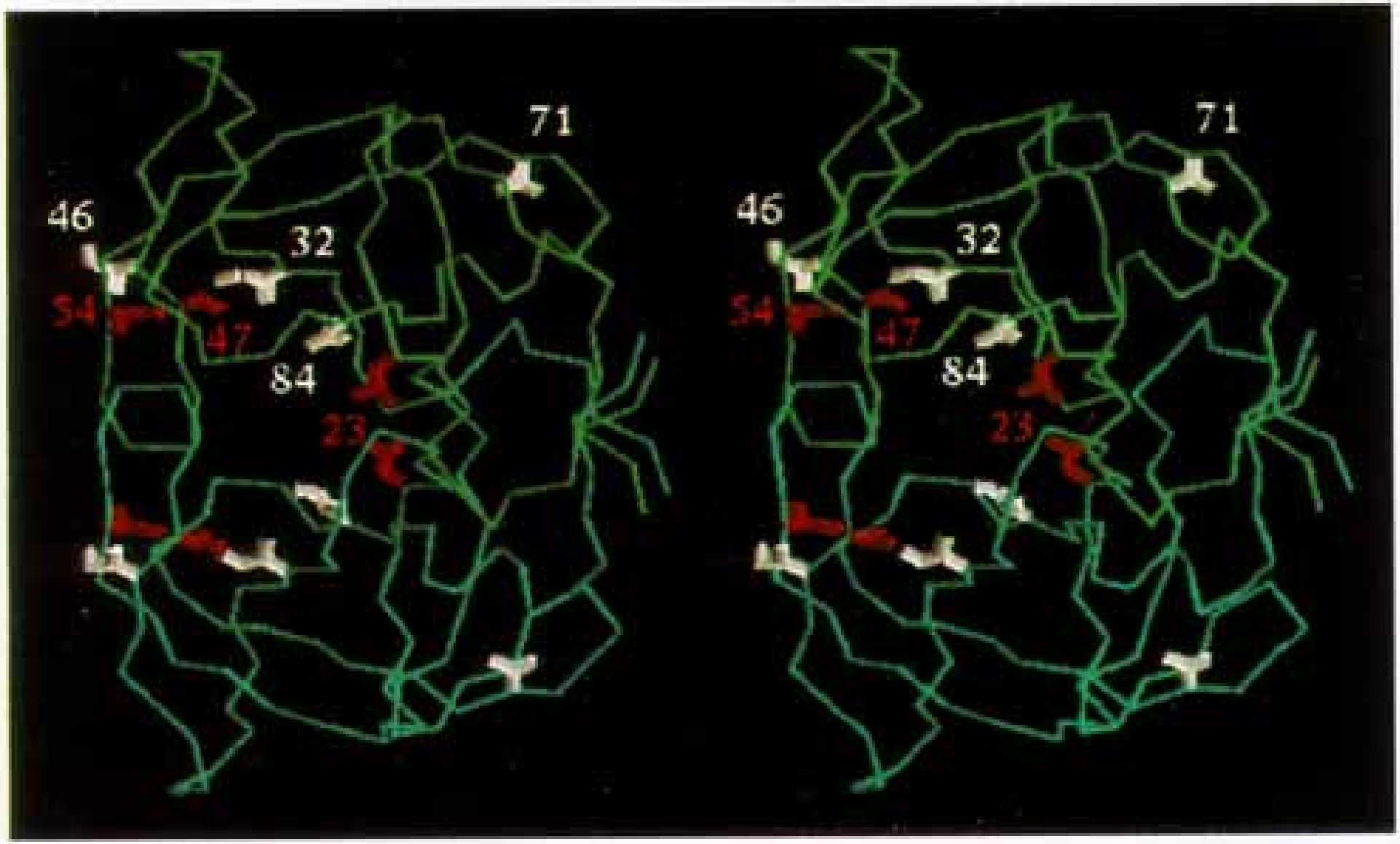
We thank C. Clouette for technical assistance; C. Yoakim, F. Soucy, P. Beaulieu, and C. Grand-Maître for BILA 1906 BS and BILA 2185 BS synthesis; and M.-A. Poupart and A. Abraham for decapeptide synthesis. We also thank M. G. Cordingley and P. C. Anderson for critical review of the manuscript. The crystal structure of HIV-2 protease was kindly provided by Liang Tong (Boehringer Ingelheim Pharmaceuticals Inc., Ridgefield, Conn.). The M35/2F8 anti-p6 monoclonal antibody was obtained from M. G. Sarngadharan (Advanced BioScience Laboratories Inc., Kensington, Md.).

Louise Doyon is the recipient of a University-Industry fellowship award from the Medical Research Council of Canada.

REFERENCES

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59:**284–291.
2. **Betageri, R., J. L. Hopkins, D. Thibeault, M. J. Emmanuel, G. C. Chow, M. T. Skoog, P. DeDreu, and K. A. Cohen.** 1993. Rapid, sensitive and efficient HPLC assays for HIV-1 proteinase. J. Biomed. Biophys. Methods **27:**191–197.
3. **Candotti, D., C. Chappey, M. Rosenheim, P. M'Pele, J. M. Huraux, and H. Agut.** 1994. High variability of the Gag/pol transframe region among HIV-1 isolates. C.R. Acad. Sci. **317:**183–189.
4. **Condra, J. H., W. A. Schleif, O. M. Blahy, L. J. Gabryelski, D. J. Graham, J. C. Quintero, A. Rhodes, H. L. Robbins, A. Roth, M. Shivaprakash, D. Titus, T. Yang, H. Teppler, K. E. Squires, P. J. Deutsch, and E. A. Emini.** 1995. In vivo emergence of HIV-1 variants resistant to multiple protease inhibitors. Nature (London) **374:**569–571.
5. **Darke, P. L., and J. R. Huff.** 1994. HIV protease as an inhibitor target for the treatment of AIDS. Adv. Pharmacol. **25:**399–454.
6. **Darke, P. L., R. F. Nutt, S. F. Brady, V. M. Garsky, T. M. Ciccarone, C. H. Leu, P. K. Lumma, R. M. Freidinger, D. F. Veber, and I. S. Sigal.** 1988. HIV-1 protease specificity of peptide cleavage is sufficient for processing of Gag and pol polyproteins. Biochem. Biophys. Res. Commun. **156:**297–303.
7. **Doyon, L., D. Thibeault, and D. Lamarre.** Unpublished data.
8. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52:**456.
9. **Higuchi, R., B. Krummel, and R. K. Saiki.** 1988. A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res. **16:**7351–7367.
10. **Ho, D. D., A. U. Neumann, A. S. Perelson, W. Chen, J. M. Leonard, and M. Markowitz.** 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature (London) **373:**123–126.
11. **Ho, D. D., T. Toyoshima, H. Mo, D. J. Kempf, D. Norbeck, C. M. Chen, N. E. Wideburg, S. K. Burt, J. W. Erickson, and M. K. Singh.** 1994. Characterization of human immunodeficiency virus type 1 variants with increased resistance to a C2-symmetric protease inhibitor. J. Virol. **68:**2016–2020.
12. **Jacks, T., M. D. Power, F. R. Masiarz, P. A. Luciw, P. J. Barr, and H. E. Varmus.** 1988. Characterization of ribosomal frameshifting in HIV-1 Gag-pol expression. Nature (London) **331:**280–283.
13. **Kaplan, A. H., J. A. Zack, M. Knigge, D. A. Paul, D. J. Kempf, D. W. Norbeck, and R. Swanstrom.** 1993. Partial inhibition of the human immunodeficiency virus type 1 protease results in aberrant virus assembly and the formation of noninfectious particles. J. Virol. **67:**4050–4055.
14. **Kempf, D. J., K. C. Marsh, J. F. Denissen, E. McDonald, S. Vasavanonda, C. A. Flentge, B. E. Green, L. Fino, C. H. Park, X. P. Kong, N. E. Wideburg, A. Saldivar, L. Ruiz, W. M. Kati, H. L. Sham, T. Robins, K. D. Stewart, A. Hsu, J. J. Plattner, J. M. Leonard, and D. W. Norbeck.** 1995. ABT-538 is a potent inhibitor of human immunodeficiency virus protease and has high oral bioavailability in humans. Proc. Natl. Acad. Sci. USA **92:**2484–2488.
15. **Kohl, N. E., E. A. Emini, W. A. Schleif, L. J. Davis, J. C. Heimbach, R. A. F. Dixon, E. M. Scolnick, and I. S. Sigal.** 1988. Active human immunodeficiency virus protease is required for viral infectivity. Proc. Natl. Acad. Sci. USA **85:**4686–4690.
16. **Kuroda, M. J., M. El-Farrash, S. Choudhury, and S. Harada.** 1995. Impaired infectivity of HIV-1 after a single point mutation in the POL gene to escape the effect of protease inhibitors in vitro. Virology **210:**212–216.
17. **Lin, Y., X. Lin, L. Hong, S. Foundling, R. L. Heinrikson, S. Thaisrivongs, W. Leelamanit, D. Raterman, M. Shah, B. M. Dunn, and J. Tang.** 1995. Effect of point mutations on the kinetics and the inhibition of human immunodeficiency virus type I protease: relationship to drug resistance. Biochemistry **34:**1143–1152.
18. **Mellors, J. W., B. A. Larder, and R. F. Schinazi.** 1995. Mutations in HIV-1 reverse transcriptase and protease associated with drug resistance. Int. Antiviral News **3:**8–13.
19. **Mervis, R. J., N. Ahmad, E. P. Lillehoj, M. G. Raum, F. H. R. Salazar, H. W. Chan, and S. Venkatesan.** 1988. The Gag products of human immunodeficiency virus type 1: alignment within the Gag open reading frame, identification of posttranslational modifications, and evidence for alternative Gag precursors. J. Virol. **62:**3993–4002.
20. **Moody, M. D., S. C. Pettit, W. Shao, L. Everitt, D. D. Loeb, C. A. Hutchison, and R. Swanstrom.** 1995. A side chain at position 48 of the human immunodeficiency virus type-1 flap provides an additional specificity determinant. Virology **207:**475–485.
21. **Myers, G., B. Korber, J. A. Berzofsky, R. F. Smith, and G. N. Pavlakis.** 1994. Human retroviruses and AIDS 1994. Los Alamos National Laboratory, Los Alamos, N.Mex.
22. **Pargellis, C. A., M. M. Morelock, E. T. Graham, P. Kinkade, S. Pav, K. Lubbe, D. Lamarre, and P. Anderson.** 1994. Determination of kinetic rate constants for the binding of inhibitors to HIV-1 protease and for the association and dissociation of active homodimer. Biochemistry **33:**12527–12534.
23. **Partaledis, J. A., K. Yamaguchi, M. Tisdale, E. E. Blair, C. Falcione, B. Maschera, R. E. Myers, S. Pazhanisamy, O. Futer, A. B. Cullinan, C. M. Stuver, R. A. Byrn, and D. J. Livingston.** 1995. In vitro selection and characterization of human immunodeficiency virus type 1 (HIV-1) isolates with reduced sensitivity to hydroxyethylamino sulfonamide inhibitors of HIV-1 aspartyl protease. J. Virol. **69:**5228–5235.
24. **Patick, A. K., R. Rose, J. Greytok, C. M. Bechtold, M. A. Hermsmeier, P. T. Chen, J. C. Barrish, R. Zahler, R. J. Colonno, and P. F. Lin.** 1995. Characterization of a human immunodeficiency virus type 1 variant with reduced sensitivity to an aminodiol protease inhibitor. J. Virol. **69:**2148–2152.
25. **Peng, C., B. K. Ho, T. W. Chang, and N. T. Chang.** 1989. Role of human immunodeficiency virus type 1-specific protease in core maturation and viral infectivity. J. Virol. **63:**2550–2556.
26. **Pettit, S. C., S. F. Michael, and R. Swanstrom.** 1993. The specificity of the HIV-1 protease. Persp. Drug Discov. Design **1:**69–83.
27. **Pettit, S. C., J. Simsic, D. D. Loeb, L. Everitt, C. A. Hutchison III, and R. Swanstrom.** 1991. Analysis of retroviral protease cleavage sites reveals two types of cleavage sites and the structural requirements of the p1 amino acid. J. Biol. Chem. **266:**14539–14547.

28. **Popovic, M., E. Read-Connole, and R. C. Gallo.** 1984. T4 positive human neoplastic cell lines susceptible to and permissive for HTLV-III. Lancet **ii:**1472–1473.
29. **Popovic, M., M. G. Sarngadharan, E. Read, and R. C. Gallo.** 1984. Detection, isolation and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science **224:**497–500.
30. **Preston, B. D., B. J. Poiesz, and L. A. Loeb.** 1988. Fidelity of HIV-1 reverse transcriptase. Science **242:**1168–1171.
31. **Ratner, L., W. Haseltine, R. Patarca, K. J. Livak, B. Starcich, S. F. Josephs, E. R. Doran, J. A. Rafalski, E. A. Whitehorn, E. Baumeister, L. Ivanoff, S. R. Petteway, Jr., M. L. Pearson, J. A. Lautenberger, T. S. Papas, J. Ghrayab, N. T. Chang, R. C. Gallo, and F. Wong-Staal.** 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature (London) **313:**277–283.
32. **Sardana, V. V., A. J. Schlabach, P. Graham, B. L. Bush, J. H. Condra, J. C. Culberson, L. Gotlib, D. J. Graham, N. E. Kohl, R. L. LaFemina, C. L. Schneider, B. S. Wolanski, J. A. Wolfgang, and E. A. Emini.** 1994. Human immunodeficiency virus type I protease inhibitors: evaluation of resistance engendered by amino acid substitutions in the enzyme's substrate binding site. Biochemistry **33:**2004–2010.
33. **Tong, L., S. Pav, S. Mui, D. Lamarre, C. Yoakim, P. Beaulieu, and P. C. Anderson.** 1995. Crystal structures of HIV-2 protease in complex with inhibitors containing the hydroxyethylamine dipeptidyl isostere. Structure **3:**33–40.
34. **Tozser, J., I. Blahah, T. D. Copeland, E. M. Wondrak, and S. Oroszlan.** 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.
35. **Veronese, F. D. M., R. Rahman, T. D. Copeland, S. Oroszlan, R. C. Gallo, and M. G. Sarngadharan.** 1987. Immunological and chemical analysis of p6, the carboxyl-terminal fragment of HIV p15. AIDS Res. Hum. Retroviruses **3:**253–264.
36. **Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, B. H. Hahn, M. S. Saag, and G. M. Shaw.** 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature (London) **373:**117–122.
37. **West, M. L., and D. P. Fairlie.** 1995. Targeting HIV-1 protease: a test of drug-design methodologies. Trends Pharm. Sci. **16:**67–75.
38. **Wondrak, E. M., J. M. Louis, H. de Rocquigny, J. C. Chermann, and B. P. Roques.** 1993. The Gag precursor contains a specific HIV-1 protease cleavage site between the NC (p7) and p1 proteins. FEBS Lett. **333:**21–24.


71
46
32
54
47
84
23
71
46
32
54
47
84
23