Volume 317, number 1,2, 167–172 FEBS 12095 February 1993

Interaction between a membrane-associated serine proteinase of U-937 monocytes and peptides from the V3 loop of the human immunodeficiency virus type 1 (HIV-1) gp120 envelope glycoprotein

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Received 8 December 1992; revised version received 22 December 1992

A trypsin-like proteinase which is inhibited by recombinant gp120 and by synthetic peptides of various lengths spanning the conserved sequence of the V3 loop has been purified and partially characterized from a U-937 cell membrane extract. V3 loop peptides behave as competitive inhibitors of the enzyme, while gp120 exerts a tight-binding inhibition, reacting in stoichiometric amounts with the proteinase to provide significant inhibition. Though the properties of the U-937 membrane proteinase towards gp120 and synthetic peptides of the V3 loop resemble those of the Molt-4 T-cell tryptase TL2, these two proteinases differ by their physicochemical properties and their susceptibility to other inhibitors of serine proteinases. These results give support to the concept of a membrane-associated proteinase as a complementary or alternative receptor to the CD4, for allowing virus to enter host cells and thus spreading HIV infection.

HIV1; gp120 V3 loop; Proteinase; Proteinase inhibitor

1. INTRODUCTION

HIV infection is mediated by an initial interaction between the gp120 envelope glycoprotein of the virus and the membrane CD4 receptor of target cells [1]. However, other cell surface components may play a role in the interaction and membrane fusion, as several types of cells lacking the CD4 receptor can be infected by HIV, while several cell lines expressing the CD4 receptor are not susceptible to infection [2–6]. Accordingly, major neutralizing antibodies are not directed against the binding region to CD4 [7–9] and do not inhibit binding to CD4. [7–9] and do not inhibit binding to CD4. These antibodies recognize a region in the V3 loop of gp120 that appears to play a fundamental role in infection [7,8,10–15]. This loop has a conserved fragment, GPGRAF, resembling the structure of the reactive site of Kunitz-type inhibitors of serine proteinases [12]. Kido et al. [12,16] first suggested that a protease receptor might interact with this region of the V3 loop. They demonstrated the presence of a membrane-associated serine proteinase at the surface of Molt-4 T-cells, that was inhibited by gp120 through interaction with its V3 loop domain [16–18]. However, several lines of evidence suggest that HIV interacts with monocytes and T-cells differently [19], addressing the question of the occurrence of this interaction in monocytes, and as a general mechanism in HIV infection.

Mononuclear phagocytes are important vectors for the spread of HIV infection, since they are target cells for the virus and regulatory cells carying the virus and controlling its dissemination [19–21]. This study shows that a membrane-associated proteinase is present in the U-937 promonocytic cell lines [22]. This enzyme could interact with viral glycoproteins through the V3 loop of gp120, thus explaining the importance of this region in monocyte infection.

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Abbreviations: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); Z-Lys-SBzl, *N*-α-Cbz-L-Lysine-thiobenzylester·HCl; STI, Soybean trypsin inhibitor; pNPGB, *p*-nitrophenyl-*p*′-guanidinobenzoate; PMSF, phenylmethylsulfonyl fluoride; E64, L-3-carboxy-*trans*-2,3-epoxypropyl-leucylamido-(4-guanidino)butane; TLCK, *N*-α-*p*-tosyl-L-lysine-chloromethylketone·HCl; MCA, 7-amino-4-methylcoumarin; Boc, *t*-butyloxycarbonyl; TFA, trifluoroacetic acid.

2. MATERIAL AND METHODS

2.1. *Purification of membrane-associated proteinase from U-937 monocyte*

A human promonocytic line (U-937) [22] was cultured in RPMI-1640 medium (Gibco) supplemented with 10% foetal calf serum, penicillin G (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml) (Gibco) at 37°C with 5% CO_2. About 10^{10} cells were washed twice with PBS (0.01 M sodium phosphate buffer pH 7.4 containing 0.15 M NaCl) resuspended in relax buffer (10 mM piperazine-*N*-*N*′-bis (2-ethane sulfonic acid)·HCl pH 6.8, 3.5 mM $MgCl_2$, 0.1 M KCl, 1.25 mM ethylenediaminetetraacetic acid) and then compressed under N_2 to 1064 psi for 15 min at 4°C and suddenly decompressed. The resulting crude N_2-cavitation lysate (about 70 ml)

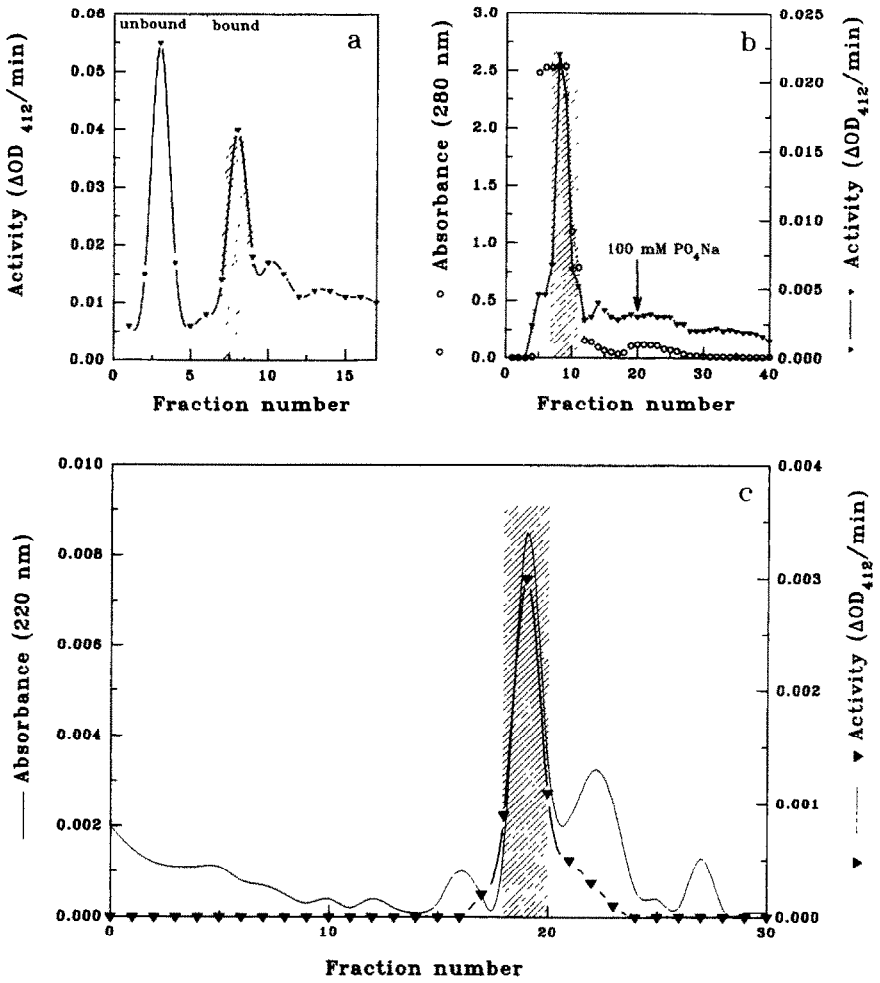

Fig. 1. Purification of membrane-associated proteinase from monocyte U-937. Panel a: Elution from the *p*-aminobenzamidine-agarose column. The bound material corresponding to the hatched area was fractionated on hydroxyapatite (Panel b). The fractions with an activity higher than 0.005 ΔOD_{412}/min (hatched area) were pooled and passed twice through a Superose 12 column. Panel c shows the elution profile after the second chromatography. Active fractions (hatched area) were pooled, concentrated and kept frozen at −70°C.

was centrifuged at 5,000 × *g* (Beckman J2-21, JA-20 rotor) for 20 min at 4°C and the supernatant was used for membrane isolation. The supernatant was centrifuged at 100,000 × *g* for 60 min at 4°C. The membrane pellet was suspended in buffer A (0.01 M Bis-Tris-HCl pH 6.1, 2 M NaCl, 0.05 g/l Thesit), sonicated for 20 s and centrifuged at 25,000 × *g* for 20 min at 4°C. The precipitate was re-extracted once by sonication in the same buffer and the combined supernatants (about 10 ml) were used for enzyme isolation. Total serine proteinase activity was separated from the membrane extract by affinity chromatography on *p*-aminobenzamidine-agarose (Pierce). The affinity column (2 ml) was washed with 10 volumes of buffer A at 4°C and loaded with about 4 ml of supernatant. The unbound material was washed out with buffer A and the column was eluted with 10 ml of buffer B (0.01 M Bis-Tris-HCl pH 6.1, 2 M NaCl, 0.15 M benzamidine). All eluate fractions were assayed for esterolytic activity using Z-Lys-SBzl (Bachem) as a substrate; those with activity were assayed for their susceptibility to inhibition by the R13K peptide. The inhibited fractions were pooled and dialysed against buffer C (5 mM sodium phosphate pH 6.2, 2 M NaCl) for hydroxyapatite chromatography.

The column of hydroxyapatite (1 × 10 cm; Bio-Rad) was equilibrated in buffer C. Unbound material, which had most of the esterolytic activity, was collected, pooled and concentrated on a microconcentrator (Centricon 10, Amicon).

This material was then fractionated twice by high pressure liquid chromatography (HPLC) on Superose 12. A 1 ml sample was applied to the column and eluted with buffer C at 30 ml/h. The fractions with serine-esterase activity were pooled, concentrated on Centricon10 (Amicon) then stored at −70°C until use. The apparent M_r of the proteinase was determined by SDS-PAGE (12,5%) according to Laemmli [23], using the Phastsystem (Pharmacia). Proteins were stained with silver staining [24].

2.2. *Synthetic peptides*

Peptides SP88226 (TRKSIRIQRGPGRAFVK) and SP89487 (TRKSIRIQRGP), corresponding to fragments (308–323 and 308–318, respectively) of the V3 loop sequence of HIV-1 *Lai*, were obtained from the Agence Nationale de Recherches sur le SIDA (ANRS). Peptides R13K (RKSIRIQRGPGRK), L10K (LVGGQVVAGK) and R30C (NNTRKSIHIGPGRAFYTTGEIIGDIRQAHC), the latter corresponding to the consensus sequence reported by LaRosa et al. [25], were synthesized by the solid phase method of Merrifield [26] using an automated Applied Biosystems 431A peptide synthesizer. After cleavage and deprotection, peptides were purified on an Aquapore RP300 CB column by high pressure liquid chromatography using a linear (0–60%) acetonitrile gradient in 0.07% TFA. Their purity was

checked by N-amino terminal sequencing on an Applied Biosystems 477A gas-phase protein apparatus.

All peptides were assayed kinetically for their capacity to interact with the purified membrane proteinase.

2.3. *Kinetic assays*

Serine-protease activity was measured throughout purification using the chromogenic thiobenzylester substrate Z-Lys-SBzl [27]. A 15 mM stock solution was prepared in dimethylformamide. Enzyme activity was assayed in 0.10 M Tris-HCl buffer pH 8.2, 0.35 mM DTNB. Proteinase (0.1 to 10 μl) was mixed with 80 μl activator buffer (final volume) at 25°C, and the reaction started by adding 2 μl or 5 μl substrate (respectively 370 μM or 940 μM final). The rate of hydrolysis was calculated by substracting the non-enzymatic rate of absorbance increase at 412 nm for the enzyme-catalyzed rate; an extinction coefficient change of 13,600 $M^{-1} \cdot cm^{-1}$ was used for the hydrolysis reaction [15].

The kinetic parameters K_m, V_m and k_{cat}/K_m were determined by varying the amount of substrate (18 to 940 μM final) and using the Hanes linear plot (S/v vs. S) [28].

The *N*-methyl coumarylamide fluorogenic substrates, Pro-Phe-Arg-MCA, and Boc-Gln-Gly-Arg-MCA (Bachem), were assayed under standardized conditions as previously described [29].

Commercial inhibitors, of serine proteinases (STI, Boehringer Mannheim; pNPGB, Merck; Aprotinin, Boehringer Mannheim; PMSF, Sigma; TLCK, Bachem; Benzamidine, Sigma; Leupeptin, Bachem), cysteine proteinases (E64, Bachem) and aspartyl proteinases (Pepstatin, Bachem) were assayed by incubating a fixed amount of proteinase (2.3 nM final) with a molar excess of inhibitor for 15 min at 25°C. Residual activity against Z-Lys-SBzl was determined as before.

The purified membrane proteinase was titrated using STI, the concentration of which was first determined with pNPGB-titrated trypsin [30]. STI was prepared as a $3.5 \cdot 10^{-8}$ M solution in 0.1 M Tris-HCl buffer pH 8.2. Purified enzyme (5 μl) was incubated for 15 min at 25°C with increasing amounts of STI before starting the reaction with Z-Lys-SBzl.

Inhibition by peptides of the V3 loop and by gp120 *Lai* (Transgene) was studied by incubating the proteinase (5.7 nM final) for 15 min at 25°C in the same buffer as before with increasing amounts of either peptide or, with gp120.

The inhibition constant (K_i) for V3 loop peptide R13K, but not for gp120 which bound too tightly to the proteinase to obey Michaelian kinetics, was calculated by the method of Dixon [31], plotting $1/v$ versus I, at two substrate concentrations (0.31 and 0.93 mM).

3. RESULTS

3.1. *Extraction of serine protease activity from cell membranes*

The soluble material extracted by sonication of the membrane pellet from U-937 cells was first fractionated by affinity chromatography on immobilized benzamidine (Fig. 1a). Only a minor part of the serine proteinase activity initially present in the membrane supernatant after the centrifugation at 100,000 × *g* was bound to the column under the conditions used. Most of this activity, but not that in the unbound material, was inhibited by the peptide R13K, which is a conserved sequence of the HIV-1 V3 loop. Once eluted from the benzamidine column, the material with esterolytic activity was fractionated on hydroxyapatite (Fig. 1b). The fractions containing an esterolytic activity that was inhibited by R13K, were further fractionated twice on a Superose 12 column (Fig. 1c). A single peak that hydrolyzed Z-Lys-SBzl was obtained. The fractions were pooled, aliquoted and kept frozen at −70°C. All these steps were carried out in 2 M sodium chloride to preserve the serine protease activity extracted from the U-937 cell membranes. Under these conditions, full enzymatic activity was recovered after several weeks of storage. After concentration, electrophoresis on SDS gels and silver staining, the protease eluted from the Superose 12 column appeared as a major band of M_r (app) 49,000 (Fig. 2).

The esterase activity of the fractionated material represented approximately 8% of the total esterase activity initially present in the crude membrane extract (Table I) and the purification yield was about 25%. The total amount of purified material was determined by active site titration with soybean trypsin inhibitor, the most potent inhibitor of the protease. A linear titration curve was obtained at inhibitor and enzyme concentrations in the 10^{-9} molar range, indicating a K_i value below 10^{-10} M [32]. The protease is also inhibited by aprotinin, PMSF and chloro-methyl ketone, but not by pepstatin or E64 (Table II). Hence, it is undoubtly a serine protease. Unlike the tryptase TL2 from T-cell membranes [16] however, the proteinase was not inhibited by leupeptin (Table II). Kinetic measurements were made using the thiobenzylester substrate with trypsin-like specificity, Z-Lys-SBzl, which is the most sensitive we found for this protease. The kinetic parameters for the hydrolysis of Z-Lys-SBzl by the monocyte membrane protease are shown in Table III. The protease showed a maximal activity at pH 8.2 and was inactive at pH 7 and below. Calcium chloride (10 mM) did not influence the activity. Fluorogenic amide substrates such as Pro-Phe-Arg-MCA and Boc-Gln-Gly-Arg-MCA, the latter

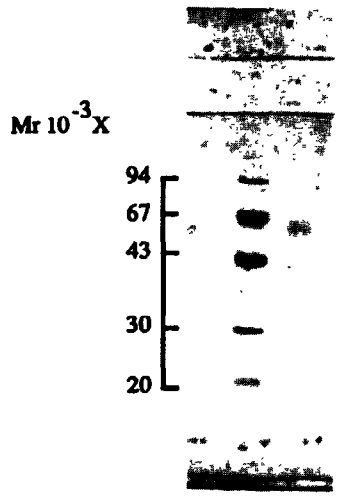

Fig. 2. Acrylamide (12.5%) gel electrophoresis in the presence of SDS of membrane-associated proteinase from monocyte U-937 after the last step of purification. Conditions were as described in section 2. Serine-proteinase (about 1 ng) was incubated for 3 min with sample buffer containing 2-mercaptoethanol prior to electrophoresis. The gel was stained with silver staining. Molecular weight markers are indicated on the left.

Table I

Purification of U-937 promonocytic serine proteinase

Material	Volume (ml)	Activity* (Δ OD_{412}/min)
Crude homogenate	6	0.065
Benzamidine-agarose fractions	unbound: 5	0.054
	bound: 3	0.040
Hydroxyapatite fractions 8,9,10	4.5	0.023
Superose 12 fractions 18,19,20	1	0.030

*Activity in Z-Lys-SBzl esterase assay for whole sample. The rate of hydrolysis was calculated after subtracting the non-enzymatic increase in absorbance at 412 nm from the enzyme catalyzed rate.

being the preferred substrate of tryptase TL2 [16], were poorly hydrolysed by the protease as compared to the thiobenzylester substrate.

In a typical experiment, about 10 μg purified active protease were obtained from a starting homogenate containing 10^{10} cells.

3.2. *Inhibition by peptides from the V3 loop*

A synthetic peptide reproducing part of the V3 loop sequence of the gp120 of HIV-1 was used to identify the protease at each stage of the purification procedure among proteolytic activities present in U-937 cell membranes. Only a minor part of the total proteolytic activity in cell membranes was inhibited by the so-called R13K peptide, showing the relative enrichment in the protease of interest during the course of the purification (Table I). Whereas the R13K peptide inhibited about 80% of the esterolytic activity of the purified protease, only 10% of the total activity was inhibited in the initial extract under the same experimental conditions.

The inhibition activity of several peptides reproducing fragments of the conserved sequence of the V3 loop of HIV1 *Lai*, and a peptide corresponding to the V3 consensus sequence [25] were tested with a constant amount (5.7 nM final) of purified proteinase. All the peptides, including the GPGRAF conserved fragment, inhibited the U-937 proteinase, though with different efficiencies. Peptide SP 89487, which includes only the first two residues of the sequence GPGRAF, still inhibited the protease, demonstrating that all this conserved segment is not required for interaction. The consensus sequence, which includes the GPGRAF sequence but differs by adjacent residues, also inhibited the membrane proteinase. Variations in the primary structure of the V3 loop of gp120 in the vicinity of its GPGRAF fragment, would not therefore prevent the viral protein from interacting with this proteinase. A K_i value of 42 μM for the interaction of the membrane-associated proteinase with R13K was calculated from Dixon plots, which also indicated that inhibition was competitive (not shown). Incubation with an unrelated peptide that inhibits cysteine proteinases L10K [33] had no effect on the U-937 proteinase activity. No peptide cleavage was observed during the time of the experiment. However R13K and SP 88226 peptides were cleaved after several hours of incubation with proteinase.

A far more potent inhibition of the U-937 proteinase was obtained using recombinant gp120 (Table IV). There was significant inhibition at a 1:1 molar ratio in the nanomolar range, making gp120 a tight binding inhibitor of the proteinase. This also means that the secondary and/or the tertiary structure of gp120 could be a major factor in its interaction with a putative enzymic receptor. As reported above for the V3 peptides, no proteolytic cleavage of gp120 was observed after 15 min of incubation with the proteinase, but fragmentation of the molecule occurred after overnight incubation.

4. DISCUSSION

Several lines of evidence suggest that receptors other than the CD4 are involved in the interaction between the gp120 of HIV-1 and host cells. Neural cells lack this receptor, but may still be infected [5] while other brain-derived cells which express the receptor remain susceptible to infection in the presence of anti-CD4 antibodies [2,6,34]. HIV binding to mononuclear phagocytes is poorly inhibited by anti-CD4 antibodies, suggesting that another receptor is present at the surface of these cells [2].

It has recently been shown that the infectivity of HIV for mononuclear phagocytes may be determined by a region of gp120 encompassing the V3 loop, and therefore distinct from the CD4 binding domain [35]. Though the nature of the receptor and the mechanism of interaction remain unknown, a trypsin-like proteinase associated with the membrane of Molt-4 T-cells has been shown to interact with the V3 loop of gp120 [16,18]. This proteinase is therefore a possible addi-

Table II

Inhibition spectrum of U-937 promonocytic serine proteinase

Inhibitor	Concentration	Inhibition (%)
Soybean trypsin inhibitor	1 μM	100
pNPGB	1 μM	36
Aprotinin	1 μM	40
PMSF	2 mM	89
TLCK	2 mM	31
Benzamidine	2 mM	31
Leupeptin	10 mM	0
Pepstatin	10 mM	0
E64	1 mM	0

The enzyme (2.3 nM final) was incubated with inhibitors for 15 min at 25°C in 80 μl of 0.10 M Tris-HCl, pH 8.2 containing 0.35 mM 5,5'-dithiobis(nitrobenzoic acid) before starting the reaction with 5 μl of Z-Lys-SBzl (0.37 mM final): ΔOD_{412} was recorded for 5 min.

tional or complementary receptor to the CD4. Whether this model applies to other cells susceptible to the virus is not known. We have demonstrated the presence of such a proteinase in membrane extracts of U-937 monocytes. This proteinase interacts with recombinant gp120, probably through its V3 loop as deduced from its reactivity with peptides spanning this sequence of the viral envelope glycoprotein. Hence the phenomenon first observed for T-cells could also apply to mononuclear phagocytes, in spite of their different mode of infection [19,21]. The U-937 monocyte serine proteinase activity responsible for this interaction represents about 10% of the total trypsin-like activity in membranes, as in Molt 4 T-cells [16]. As little as 10 μg of proteinase may be obtained from 10^{10} cells and the enzyme concentration in the stock solution is only about 10^{-8} M which, at the moment, prevents from further physicochemical or enzymic characterization. Nevertheless, M_r (app), esterolytic activity and susceptibility to inhibitors of the U-937 membrane proteinase suggest that it differs from tryptase TL2 isolated from Molt-4 T-cell membranes, in spite of their similar behaviour towards HIV-1 gp120 and peptides derived from the V3 loop.

Apart from the STI which was used for active site titration, recombinant gp120 appears to be the best inhibitor of the proteinase. Significant inhibition was obtained under stoichiometric conditions in the nanomolar range, ranging the viral envelope glycoprotein as a tight-binding inhibitor of the proteinase [32]. The initial concentration of enzyme was too low however, to allow accurate determination of the Ki value under conditions of tight binding [36]. The inhibition by peptides spanning the V3 loop of the gp120 shows that at least a part of the conserved sequence GPGRAF is involved in inhibition. Those peptides with inhibitory properties obeyed Michaelian kinetics, and a large excess of peptide over proteinase was required for significant inhibition. This differs from the tight-binding properties of gp120, and indicates that the conformation of the peptide in the V3 loop structure is essential for binding [37], or that other domains within the gp120 are involved in inhibition. We have observed that no significant cleavage of V3 peptides and gp120 occurred during the initial stage of the interaction, which leads to strong inhibition of the membrane proteinase. Proteolytic cleavage may appear however after a longer time of incubation. Such a cleavage has been reported as being an essential step for virus infection (reviewed in [38]). However, Kido et al [18] reported that gp120 was not cleaved upon interaction with tryptase TL2 from T4+ lymphocytes. Whether gp120 cleavage is necessary for virus infection remains therefore to be demonstrated. Our data support the hypothesis that a host cell membrane proteinase could interact with an inhibitory site of the V3 loop during the process of HIV infection. Such a conformational site would not depend on the sequence variability of HIV-1 strains and would therefore represent a consensus target to develop inhibitory site-directed antibodies of therapeutical and/or preventive use.

Table III

Kinetic parameters for U-937 promonocytic serine esterase hydrolysis of Z-Lys-SBzl

Substrate	K_m (μM)	V_m (μM·s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1}·s^{-1})
Z-Lys-SBzl	175	0.042	7	$40 \cdot 10^3$

Kinetic constants were calculated from Hanes linear plot [28] using at least seven substrate concentrations.

Table IV

Inhibition of U-937 promonocytic serine-proteinase activity by gp120 *Lai* and by synthetic peptides spanning the V3 loop structure

Inhibitor	Enzyme	Concentration	E/I	Inhibition (%)
gp120 (*Lai*)	5.7 nM	6.0 nM	0.95	20
"	"	45.8 nM	0.12	35
"	"	91.5 nM	0.06	48
R13K	"	58.2 μM	$9.8 \cdot 10^5$	80
SP88226	"	110.0 μM	$5.2 \cdot 10^5$	60
SP89487	"	62.5 μM	$9.1 \cdot 10^5$	25
Consensus sequence	"	41.7 μM	$1.3 \cdot 10^4$	30
L10K	"	40.0 μM	$1.4 \cdot 10^4$	0

Inhibition was measured with 5.7 nM purified enzyme, 0.94 mM Z-Lys-SBzl and 0.35 mM 5-5'-dithiobis(nitrobenzoic acid) in 0.10 M Tris-HCl, pH 8.2 as described in the legend of Table II.

Acknowledgements: We thank S. Brunet for technical assistance, A. Baillou-Beaufils for help in peptide synthesis and the Agence Nationale de Recherches sur le SIDA for providing V3 peptides. This work was supported by grant n° 92/DRS/V20 from the Agence Nationale de Recherches sur le SIDA. L.E.A. holds a fellowship from the Région Centre.

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