Inhibition of U-937 membrane-associated cathepsin G by GP120 (IIIB) and V3 loop-derived peptides from several strains of HIV-1

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Received 19 April 1995; revised version received 15 May 1995

Abstract A cell surface-associated cathepsin G has been reported to be a possible complementary factor for HIV-1 infection of U-937 cells. The effect of recombinant gp120 (IIIB) and a series of V3 loop peptides derived from the sequence of different strains of HIV on the activity of U-937 cathepsin G was assayed. The sequence on the N-terminal side of the highly conserved GPGRAF V3 loop segment was required for interaction with cathepsin G. The inhibition was stable for several hours and there was no cleavage of the peptides derived from the HIV-1(IIIB) strain. Recombinant gp120 (IIIB) also remained uncleaved after incubation with cathepsin G for 3 h, but some cleavage occurred, generating 2 fragments (50 kDa and 70 kDa), after 16 h. Linear peptides derived from HIV-1 Mal, ELI, MN, CDC4 and SF162 strains, and consensus V3 peptides all had inhibitory properties towards cathepsin G, although they were significantly cleaved after one hour. The cleavage site was at the carboxy-terminus of Tyr^{323} which is conserved in all these HIV-1 strains but not in HIV-1(IIIB). There was no cleavage at the Arg residue of the GPGRAF sequence, whatever the V3 peptide sequence, the amount of proteinase, or the incubation time. We conclude that the inhibition of membrane-associated cathepsin G of U-937 cells by the gp120 V3 loop of HIV-1 does not occur via a Kunitz-type mechanism, and that the proteinase-V3 loop interaction does not result in a significant cleavage of the V3 loop, though it has been suggested that this event is required for the entry phase of the virus.

Key words: Cathepsin G; HIV-1; gp120; V3 loop; U-937 cell

1. Introduction

It has been suggested that a variety of membrane components at the surface of HIV-1 permissive cells, including negatively charged components [1–7], proteinases [8–14], lectins [15] and integrins [16,17], can act as alternative or complementary factors to CD4, to allow the binding and entry of HIV-1.

The main epitope involved in neutralizing HIV-1 is located within the V3 loop of the gp 120 molecule and discrete mutations at the crown of this loop may dramatically alter virus tropism and syncytium formation [18–22]. Hence, this fragment probably takes part in the process of virus–cell interaction at a later stage than CD4 binding [23].

A characteristic feature of the V3 loop, which could explain its importance in virus infection, is the presence at its tip of a conserved GPGRAF sequence which has a structure similar to the proteinase inhibitors of the Kunitz-2 family [24]. This peculiar feature explains why a membrane associated proteinase was suggested to participate in HIV-1 binding at the surface of sensitive cells [9]. Since then, several proteinases that may interact with peptides mimicking V3 loop sequences, have been identified, but there is as yet no proof that the interaction with a proteinase is a prerequisite for virus binding and fusion [8–14]. The proteolytic cleavage of the V3 loop by a membrane or endosomal proteinase also remains to be demonstrated. The identification of a preferential cleavage site at the arginyl residue of the GPGRAF sequence supports the proposal that V3 loop may behave as a Kunitz-type inhibitor. This cleavage has, however, been observed only using recombinant gp120 expressed in CHO cells, or in vitro upon incubation with large amounts of thrombin or lung tryptase [25,26]. No cleavage products have been identified during virus-cell fusion or during syncytium formation [8,23]. The involvement of a proteinase to cleave the V3 loop and allow virus entry and infection, would indicate that the enzyme(s) responsible has a broad specificity and can act upon all HIV-1, and possibly HIV-2, strains. Tryptase TL2 from Molt4 lymphocytes, and membrane cathepsin G from U-937 cells both have a combined trypsin-like and chymotrypsin-like specificity, and both are inhibited by recombinant gp120 via a V3 loop-dependent mechanism [9,14]. Supporting these observations, peptides reproducing the sequence at the tip of the V3 loop and including the conserved GPGRAF sequence inhibited tryptase TL2 and cathepsin G [9,11]. There was no indication however, that the GPGRAF sequence is involved in the interaction, or that a proteolytic cleavage within the V3 loop sequence occurs as a result of the interaction. We have therefore investigated this interaction using U-937 membrane cathepsin G and V3 loop-derived peptides of various lengths and sequences, to evaluate the capacity of membrane cathepsin G to interact with, and cleave the V3 loop of several strains of HIV-1.

2. Material and methods

2.1. Materials

Enzymes. Membrane-associated cathepsin G was purified from U-937 monocyte-like cells as previously described [11,14]. Commercial neutrophil cathepsin G (E.C. 3.4.21.20) was obtained from ICN Biochemicals.

Recombinant glycoproteins. Human immunodeficiency virus type 1 gp120 (IIIB isolate) expressed in the baculovirus system, was obtained from American Biotechnologies Inc. (Cambridge, MA) via the U.K. Medical Research Council (MRC) AIDS Directed Program-Reagent

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Abbreviations: CHO, Chinese Hamster Ovary; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, Dithiothreitol; h.p.l.c., high pressure liquid chromatography; NaCl/Pi, Phosphate buffered saline; Z-Lys-SBzl, *N*-α-Cbz-L-lysine-thiobenzylester, HCl.

SSDI 0014-5793(95)00571-4

Project. Recombinant HIV-1 gp 120 (SF2 isolate) was expressed in Chinese hamster ovary (CHO) cells and obtained from the MRC.

Synthetic peptides. Peptides R13K, V3 ELI, V3 MAL, V3 MN, SF162, V3 Cons and V3 Af. Cons were synthesized by the solid phase method of Atherton and Sheppard [27] using an automated Applied Biosystems 431 A peptide synthesizer. The cleaved and deprotected peptides were purified on an Aquapore RP300 CB column by high pressure liquid chromatography using a linear (0–60%) gradient of acetonitrile in 0.07% trifluoroacetic acid. Their purity was checked by amino-terminal sequencing on an Applied Biosystems 477 A peptide sequencer.

Peptides SP88226, SP89046, SP89047, SP89364, SP89487, SP89488 and V3 CDC4 were obtained from the Agence Nationale de Recherches sur le SIDA (ANRS), and V3b peptide was from Neosystem France (Strasbourg). V3 Cons and V3b peptides correspond to the consensus sequence reported by LaRosa et al. [28] and V3 Af. Cons peptide corresponds to the african consensus sequence deduced from the analysis of 108 isolates [29].

Synthetic peptides containing carboxy-terminal cysteine were treated with 1 mM DTT and 2 mM iodoacetamide before incubation with U-937-proteinase.

Monoclonal antibodies (MAb) (MN-A, 110-D, 110-H). These were obtained from F. Traincard (Pasteur Institute) and from the ANRS. They were raised against gp120 (IIIB) and recognized epitopes located in the sequences 315-322 (MN-A), 317-325 (110-H) and 381-394 (110-D).

2.2. Interaction between U-937 cathepsin G and HIV-1 gp120, or synthetic peptides spanning the V3 loop

Inhibition by HIV-1 gp120 (IIIB) was studied by incubating the U-937 or neutrophil cathepsin G (22.7 nM final concentration) for from one minute to three hours at 25°C in 0.10 M Tris-HCl buffer pH 8.2, 0.35 mM DTNB with increasing amounts of glycoprotein. The reaction was started by adding 20 μl Z-Lys-SBzl (0.38 mM final concentration) and the rate of hydrolysis was calculated by subtracting the background rate of absorbance increased at 412 nm from the enzyme-catalyzed rate; an extinction coefficient of 13,600 $M^{-1} \cdot cm^{-1}$ was used for the calculation of the hydrolysis rate [30].

The gp 120 cleavage products were analysed by immunoblotting using monoclonal antibodies raised against two epitopes of the glycoprotein spanning the tip of the V3 loop and one directed against an epitope (381–394) in the 50 kDa C-terminal fragment. Recombinant glycoproteins (IIIB or SF2) (270 nM final concentration) were incubated without or with U-937 cathepsin G (300 nM final concentration) for from 15 min to 16 h. Electrophoresis sample buffer (0.25 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.1% Bromophenol blue) was then added to completely inhibit proteinase activity. The samples were boiled and separated by SDS-PAGE (7.5%) using the Phastsystem (Pharmacia). They were electroblotted onto nitrocellulose (0.45 μm, Sartorius) by semi-dry electrotransfer for 15 min at 25 mA. The nitrocellulose sheet was incubated with NaCl/P_i (10 mM phosphate buffer, pH 7.4, 150 mM NaCl) containing 5% dried milk (W/V) at 37°C for 120 min and treated as previously described [31]. Monoclonal antibodies (10 μg/ml in NaCl/P_i containing 0.1% Tween-20 and 5% dried milk) were added and incubated overnight at 4°C. The membrane was then washed three times with NaCl/P_i containing 0.05% Tween-20 and incubated with anti-mouse IgG-peroxydase conjugate (Sigma) (1/500 in NaCl/P_i, 0.05% Tween-20, 1% dried milk) for 2 h at room temperature and washed as before. Antigens were detected by chemiluminescence (ECL, Amersham) according to the manufacturer's instructions.

Table 1
Inhibition of U-937 cathepsin G by gp120 IIIB

gp 120 IIIB (nM)	Enzyme (nM)	I/E	Inhibition (%)
22.7	22.7	1	27 ± 2
227.0	22.7	10	35 ± 2
380.0	22.7	16	50 ± 4

U-937 cathepsin G (22.7 nM) was incubated with gp120 IIIB for from one minute to three hours at 25°C in 0.1 M Tris-HCl, pH 8.2, containing 0.35 mM DTNB, before starting the reaction with 20 μl of thiobenzylester substrate (0.38 mM final concentration). Absorbance was recorded for 2 min at 412 nm.

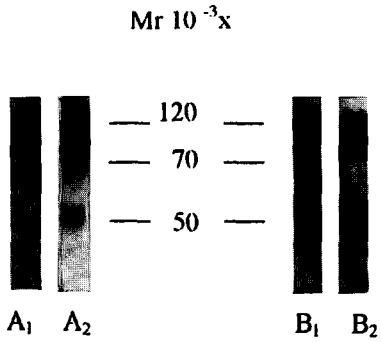

Fig. 1. Immunoblotting of gp120 (IIIB or SF2) incubated with U-937 cathepsin G. GP 120 IIIB (A_1, A_2) and SF2 (B_1, B_2) were incubated 16 h with (A_2, B_2) or without (A_1, B_1) U-937 cathepsin G. Mixtures were analyzed by SDS-PAGE and immunoblotting using monoclonal antibodies. Immunoreactive fragments were detected by chemiluminescence.

The K_i value for the interaction between the R13K inhibitory peptide and cathepsin G was determined using the Dixon plot [32] and inhibitory properties of other peptides spanning the V3 loop of gp120 of different strains of HIV-1, were compared to those of R13K using these peptides at a single concentration. For this purpose, U-937 cathepsin G (6.2 nM final concentration) was incubated with increasing amounts of R13K (0–40 μM) in 0.1 M Tris-HCl buffer, pH 8.2, 0.35 mM DTNB, for 15 min at 25°C before starting the reaction with Z-Lys-SBzl used at two different concentrations (125 and 380 μM). V3 loop-derived peptides were used at a single concentration (32 μM final concentration) and incubated with the same amount of cathepsin G (6.2 nM final) for 15 min, before adding the substrate (0.38 mM final concentration) and reading the absorbance at 410 nm.

All peptides were also incubated with cathepsin G for longer times (up to 3 h) to check their stability. Mixtures were analysed by HPLC on an Aquapore ODS 032 column using a linear (0–80%) gradient of acetonitrile in 0.07% trifluoroacetic acid. When partial hydrolysis occurred, cleavage sites were determined by amino-terminal sequencing. Fragments issuing from the 3-h incubation of the V3 cons. peptide with cathepsin G, were reequilibrated in the activation buffer and assayed for their inhibitory properties, and the results compared to those obtained with the full-length peptide.

3. Results

3.1. Interaction between U-937 cathepsin G and gp120 (IIIB)

The time-course of the interaction between purified cathepsin G and recombinant gp120 (IIIB) and any proteolytic cleavage of the molecule were studied using proteinase at 22.7 nM and varying the gp120 (IIIB)/cathepsin G molar ratio from 1 to 16 (Table 1). Maximal inhibition was obtained within one minute after mixing and it remained stable for at least 3 h at 25°C. An identical result was obtained using commercial cathepsin G purified from human neutrophils. Western blotting revealed that there was no significant cleavage of the recombinant gp120 (IIIB) within 3 h but cleavage into two fragments (70 kDa and 50 kDa) occurred after a 16 h incubation (Fig. 1). The $M_{r(app)}$ of these fragments was consistent with cleavage within the V3 loop of the protein, as previously shown using thrombin as a proteolytic agent [25] or spontaneously cleaved gp120 (SF2) produced in CHO cells [26]. However, the site of cleavage by cathepsin G was different from that of thrombin as shown using a monoclonal antibody directed against an

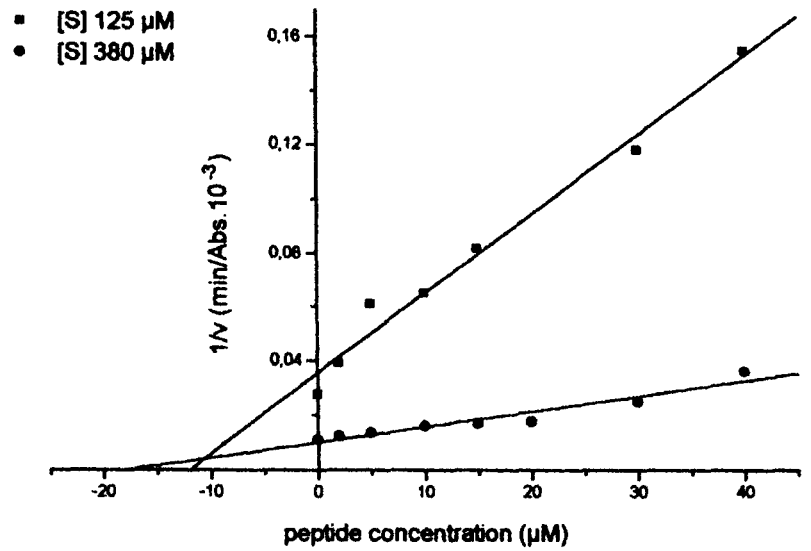

Fig. 2. Dixon plots for R13K peptide. U-937 cathepsin G (6.2 nM) was incubated for 15 min at 25°C with concentrations of R13K from 0–40 µM (final) before starting the reaction with Z-Lys-SBzl (125–380 µM) as described in Table 1. Kinetic constant (K_i) was determined according to Dixon ($1/V$ versus peptide concentration).

epitope encompassing the GPGRAF sequence that do not react anymore after cleavage of this sequence at its Arg site: Both the 50 kDa and the 70 kDa fragments were recognized in gp120 (IIIB) after cathepsin G cleavage and immunoprinting using a mixture of Mab-110-D (directed against the 50 kDa fragment) and Mab-110-H and Mab-MN-A (both directed against the GPGRAFVT epitope). Under the same conditions the 70 kDa fragment was not recognized using recombinant gp120 (SF2) spontaneously cleaved at the Arg site. Recognition of the 70 kDa fragment generated by cathepsin G also indicates that the cleavage is on the C-terminal side of the GPGRAF sequence.

3.2. *Interaction between cathepsin G from U-937 cells and V3 loop-derived peptides from gp120 (IIIB)*

The interaction of cathepsin G with the V3 loop of gp120 (IIIB) was examined using synthetic peptides that included all or part of the GPGRAF sequence. The mechanism of inhibition and the K_i were first determined using the peptide R13K. A competitive inhibition with a K_i value of 11 ± 1 µ was observed for the interaction between this peptide and U-937 cathepsin G, using the Dixon plot, and working at two different substrate concentrations (Fig. 2). Preliminary attempts using related peptides showed that most had inhibiting properties similar to those of R13K, so that experimental conditions were standardized to allow comparison between all peptides using a single inhibitor concentration (Table 2). Under these conditions, all peptides behaved similarly, except those lacking the N-terminal extension which includes a conserved dibasic doublet at position 309–310 (i.e. peptides SP89046 and SP89488). The results also show that the GPGRAF sequence was not required for inhibition, since peptide SP89487 which lacks the GRAF moiety, still inhibited cathepsin G, whereas peptide SP89046, which includes the whole sequence, did not (Table 2).

As for gp120 (IIIB), maximal inhibition by all inhibitory peptides occurred within one minute after mixing, and remained stable for at least 3 h. Reverse phase h.p.l.c. fractionation of the reaction products from incubations for 3 h and for 16 h, showed no significant cleavage of the peptides, confirming that the arginyl site of the GPGRAF sequence is resistant to cathepsin G hydrolysis (Fig. 3).

3.3. *Interaction between U-937 cathepsin G and V3 loop-derived peptides from HIV-1 CDC4, ELI, MAL, MN, SF162 strains and from consensus strains (V3b, V3 cons, V3 Af.Cons)*

We analysed the interaction between cathepsin G and V3 loop peptides from different strains of HIV to ensure that they all inhibited proteinase activity. All the peptides were long enough (at least 29 residues) to include both the GPGRAF sequence or its equivalent (since this sequence is not conserved in all HIV-1 strains) and a dibasic doublet lying N-terminally to this sequence. The experimental conditions were standardized as before.

As for gp120 (IIIB)-derived peptides, inhibition was stable and almost immediate whatever the peptide sequence, but the percentage of inhibition was slightly lower than with V3 (IIIB)-

Table 2
Inhibition of U-937 cathepsin G activity by peptides of gp120 IIIB spanning the crown of the V3 loop

Name	Sequence	Inhibition *(%)
	310.....320	
	| |	
R13K	RKSIRIQRGPGRK	56 ± 6
SP89047	TRPNNNTRKSIRIQRGPGRAFVT	58 ± 2
SP89364	CNTRKSIRIQRGPGRAFVTIGK	64 ± 6
SP88226	TRKSIRIQRGPGRAFV	58 ± 2
SP89487	TRKSIRIQRGP	40 ± 5
SP89046	IRIQRGPGRAFVTIGK	(n.s)
SP89488	GRAFVTIGK	(n.s)

U-937 cathepsin G (6.2 nM final) was incubated with peptides (32 µM final) as described in Table 1. n.s: non significant; *mean of at least three experiments.

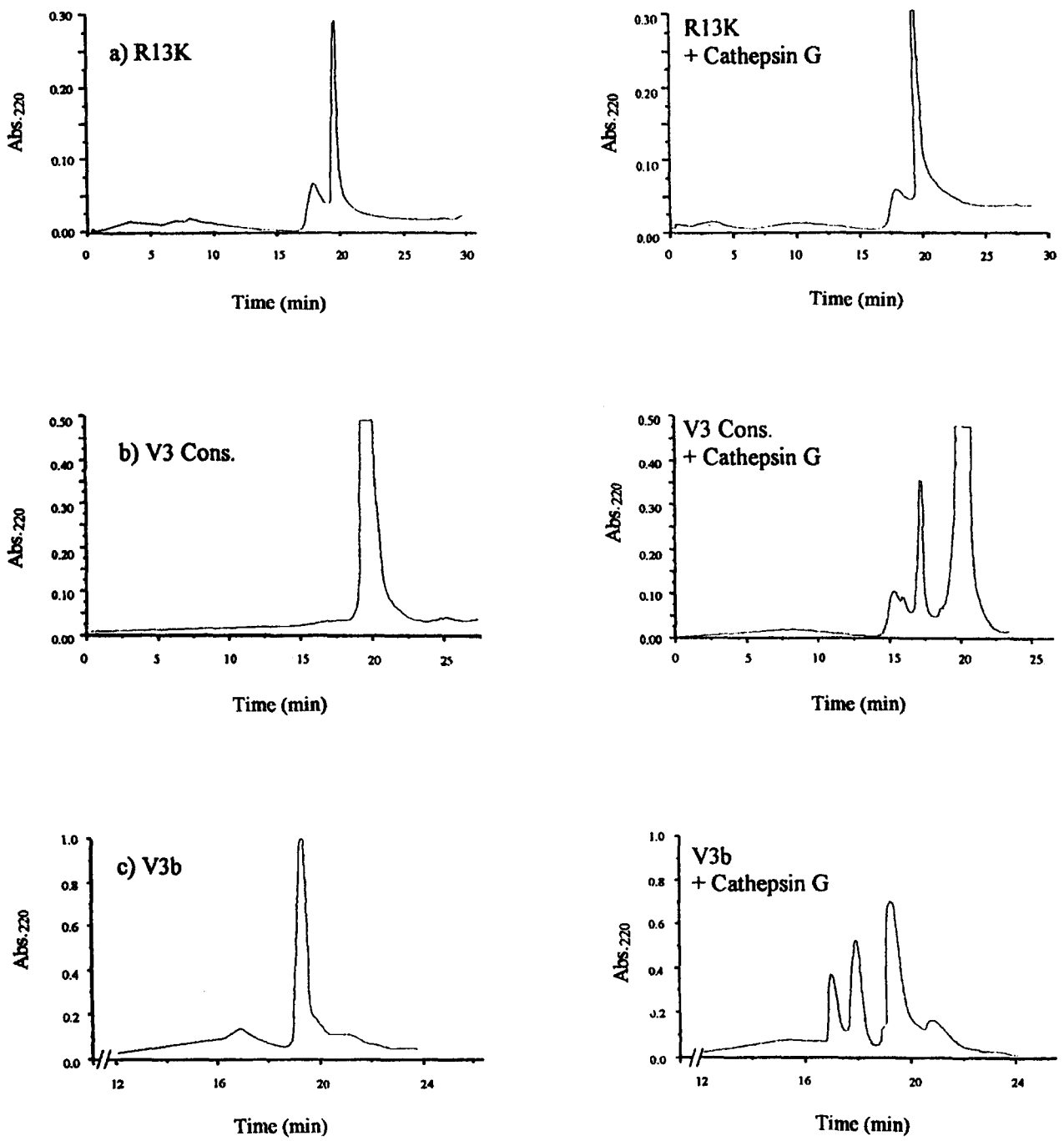

Fig. 3. HPLC analyses of the cleavage products of peptides, R 13K (a), V3 Cons (b) and V3b (c) deduced from the V3 loop sequence of gp120 IIIB (a) and from the consensus sequence of Larosa et al. (b, c) by U-937 cathepsin G. Each peptide (32 μM final) was incubated with U-937 cathepsin G (6.2 nM) for 60 min (a, b) or 16 h (c) at 25°C in 0.1 M Tris-HCl, pH 8.2. Mixtures were fractionated on a C18 HPLC cartridge.

derived peptides (Table 3). Unlike V3 (IIIB)-derived peptides, all peptides derived from other HIV-1 strains were significantly cleaved after one hour. These peptides differ from those derived from the HIV-1 (IIIB) strain by the presence of a Tyr residue at position 323, and the cleavage for all peptides was identified at this site by N-terminal sequencing. Prolonged incubation resulted in a minor cleavage on the C-terminal side end of Lys_{312}, when it was present in the conserved dibasic doublet. This agrees with the combined trypsin-like and chymotrypsin-like specificity of cathepsin G [14]. This cleavage induced no change in the percentage of inhibition, which remains stable for at least 3 h, indicating that cleavage is not essential for interaction. Accordingly, h.p.l.c. purified N-terminal fragment obtained from the hydrolysis of V3 Cons. peptide, still inhibited U-937 cathepsin G. The consensus disulfide-linked V3 peptide, which is more closely related structurally to the V3 loop within the gp120, was cleaved only after 16 h incubation with cathepsin G as was gp120 IIIB.

Table 3
Inhibition of U-937 cathepsin G by V3 loop peptides from different strains of HIV-1, or deduced from consensus sequences reported by Larosa et al. (V3 Cons.) and Myers et al. (V3 Af. Cons.)

Name	Sequence	Inhibition *(%)
	310.....320.....330	
V3 MN	NNTYNKRKRIHIGPGRAFYTTKNIIGTIRRQAHC	30 ± 2
V3 Mal	NNTRRGIHFGPGQALYTTGIVGDIRRAYC	31 ± 3
V3 ELI	YQNTRQRTPIGLGQSLYTTRSRSIIGQAHC	18 ± 2
V3 CDC4	CHTRKRVTL---GPGRVWYTTGE	15 ± 1
SF162	NTRKSITIGPGRAFYATGDI	15 ± 2
V3 Cons.	NNTRKSIHIGPGRAFYTTGEIIGDIRQAHC	24 ± 1
V3 Af. Cons	YNNTRQRTRIGPGQAFYTTGKIIGDIRQAHC	24 ± 2

U-937 cathepsin G (6.2 nM final) was incubated with peptides (32 μM final) as described in Table 1. *Mean of at least three experiments.

4. Discussion

There is little doubt as to the importance of the V3 loop in HIV-1-cell fusion, but the way it participates in this process is still a matter of debate. One possibility is that the V3 loop interacts with cell surface components via its conserved sequences, but the precise role of putative candidates has not been elucidated. The V3 loop has an overall positive charge which may favour interaction with negatively charged compounds at the surface of host cells [5,33,34]. The V3 loop of gp120 could also interact with CD4 at a site different from the primary gp120 binding site [35], or with another cell surface protein able to accommodate a sequence as variable as that of the V3 loop [6,15–17]. It has also been recently suggested that the V3 domain could interact with other domains of gp120 (V1/V2) and thus modulate the conformation of the glycoprotein [36].

A cell surface proteinase has been proposed as the second receptor for HIV-1 because the crown of V3 loop has a conserved sequence that is similar to the inhibitory site of proteinase inhibitors of the Kunitz-2 family [24]. Consistent with this proposal, several inhibitors of serine proteinases and an antibody directed against the Molt4 surface proteinase tryptase TL-2, were shown to block syncytium formation [9]. Both Molt4 tryptase TL2 and U-937 cathepsin G are inhibited by gp120 and V3-loop derived peptides. Neither cathepsin G was found at the surface of Molt4 cells by flow cytometry, nor tryptase TL2 at the surface of U-937 cells (unpublished data). Whether the presence of different proteinases at the surface of cells of the lymphocyte or macrophage lineages is related to the different tropisms of HIV strains remains however to be demonstrated. The question of how a proteinase with a very restricted specificity can interact with the hypervariable sequence of the V3 loop of all HIV-1 strains has recently been addressed [37]. The presence of a proteinase with broad specificity or of several proteinases has been suggested [23]. Tryptase TL-2 and cathepsin G both partly satisfy these conditions as they have a combined trypsin-like and chymotrypsin-like specificity [8,14].

Another possibility would be that the proteinase does not recognize gp120 as a true substrate or inhibitor, but interacts with it through a less specific mechanism. Supporting this hypothesis, we found that the Kunitz-like sequence GPGRAF at the crown of V3 loop was not involved in the inhibition, since its C-terminal truncation does not alter the inhibitory properties of V3 loop peptides. In contrast, the sequence located N-terminally with respect to this fragment, which contains a conserved dibasic doublet, is involved in the interaction. This suggests that a charge effect could participates in the interaction, as it was previously deduced from the observation that syncytium-inducing isolates have a significantly higher positive V3 loop charge than those of non-cyncytium-inducing isolates [39]. In order to check this hypothesis, peptides are now being synthesized substituting R_{309} and K_{310} with structurally related, uncharged residues.

The reason why peptides reproducing the sequences of the different HIV-1 strains are less inhibitory than V3(IIIB)-derived peptides is not clear. Since these peptides, but not those derived from V3(IIIB), are also cleaved by cathepsin G, it could be that competition occurs between the C-terminal cleavage site and the N-terminal interaction site. Cleavage of Tyr-containing peptides caused no change in inhibitory properties, suggesting that inhibitory binding and hydrolysis are independent. Confirmation is provided by the finding that h.p.l.c.-purified N-terminal fragments of cleaved peptides remain inhibitory towards U-937 cathepsin G.

It is worth noticing that no cleavage at the R residue of the GPGR sequence was observed neither in recombinant gp120 (IIIB) nor in V3-derived peptides suggesting that this cleavage previously observed with heterologous proteinases, was a fortuitous event [9,25]. If the proteolytic function of cathepsin G is not directly involved in the interaction, the proteolytic cleavage of the V3 loop should not be essential for binding or the subsequent events leading to virus entry and infection. The results presented here agree with this proposal, since recombinant gp120 (IIIB) and peptides derived from its V3 loop sequence are resistant to proteolytic cleavage. The question is now addressed as to know whether inhibitors of HIV infection, such as polyanionic compounds [2–5,7], or betulinic acid derivatives [40], that are implicated at early, envelope-dependent stages, can inhibit cathepsin G at the surface of U-937 membranes.

Acknowledgements: We thank A. Baillou-Beaufils (CNRS URA 1334) for providing peptides derived from the CDC4, ELI, MAL, MN, SF162 strains and from consensus strains (V3b, V3 cons, V3 Af.Cons), N. Haigwood and the MRC AIDS Directed Programme Reagent Project for recombinant gp120, G. Lalmanach (CNRS URA 1334) for helpful discussions and F. Traincart (Pasteur Institute) for providing monoclonal antibodies. This work was supported by Grant 930815 from the Agence Nationale de Recherches sur le Sida.

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