

Effects of L- and D-REKR amino acid-containing peptides on HIV and SIV envelope glycoprotein precursor maturation and HIV and SIV replication

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The aim of the present study was to evaluate the capacity of synthetic L- and D-peptides encompassing the HIV-1_{BRU} gp160 REKR cleavage site to interfere with HIV and simian immunodeficiency virus (SIV) replication and maturation of the envelope glycoprotein (Env) precursors. To facilitate their penetration into cells, a decanoyl (dec) group was added at the N-terminus. The sequences synthesized included dec5D or dec5L (decREKRV), dec9D or dec9L (decRVVQREKRV) and dec14D or dec14L (TKAKRRVVQREKRV). The peptide dec14D was also prepared with a chloromethane (cmk) group as C-terminus. Because L-peptides exhibit significant cytotoxicity starting at 35 μ M, further characterization was conducted mostly with D-peptides, which exhibited no cytotoxicity at concentrations higher than 70 μ M. The data show that only dec14D and dec14Dcmk could inhibit HIV-1_{BRU}, HIV-2_{ROD} and SIV_{mac251} replication

and their syncytium-inducing capacities. Whereas peptides dec5D and dec9D were inactive, dec14Dcmk was at least twice as active as peptide dec14D. At the molecular level, our data show a direct correlation between anti-viral activity and the ability of the peptides to interfere with maturation of the Env precursors. Furthermore, we show that when tested *in vitro* the dec14D peptide inhibited PC7 with an inhibition constant $K_i = 4.6 \mu$ M, whereas the peptide dec14L preferentially inhibited furin with a $K_i = 28 \mu$ M. The fact that PC7 and furin are the major prohormone convertases reported to be expressed in T4 lymphocytes, the principal cell targets of HIV, suggests that they are involved in the maturation of HIV and SIV Env precursors.

Key words: envelope processing, prohormone convertase, serine endoprotease inhibitor, synthetic D-peptide.

INTRODUCTION

The envelope glycoprotein (Env) of HIV is synthesized as a fusion-inactive precursor (gp160) that is cleaved in the biosynthetic pathway to generate the mature, non-covalently associated surface glycoprotein (SU) [gp120 for HIV-1, gp125 for HIV-2 and gp105 for simian immunodeficiency virus (SIV)] and transmembrane (TM) subunits (gp41 for HIV-1, gp36 for HIV-2 and gp32 for SIV) [1]. The SU binds with a high affinity ($K_d = 10^{-9}$ M) to the CD4 receptor on host cells, primarily T4 lymphocytes, monocytes and macrophages. In order to mediate fusion, which leads to viral penetration, an additional interaction with co-receptor molecules such as CXCR4 and CCR5 is required [2]. The interaction of the co-receptors gp120 and CD4 causes conformational changes, resulting in the exposure of the hydrophobic N-terminal sequence of gp41, which is believed to initiate fusion by direct insertion into the target cell membrane. The endoproteolytic maturation of the Env precursor is a crucial step for the production of infectious viral particles. At present, this key step in the viral cycle is an increasingly attractive target for inhibitor design [3]. To date, two types of molecules, including native or modified peptides or proteins, have been developed as inhibitors: inhibitors of virus penetration and inhibitors of the maturation of the Env precursor. The former interferes with the formation of the gp41 trimer-of-hairpins structure and so prevents fusion [4]. Peptides derived from the C-terminal region of the gp41 ectodomain, referred to as C-

peptides, are in fact effective inhibitors of HIV-1 infection. The binding of these peptides to the N-terminal region of the pre-hairpin structure during the fusion process prevents the formation of the gp41 trimer-of-hairpins, ultimately leading to the irreversible loss of the capacity for membrane fusion. These peptides are active by blocking virus penetration. One of them, T20, which is now in clinical trials, has exhibited anti-viral activity in human beings [4]. In addition, small cyclic D-peptides, which specifically target a prominent pocket on the surface of the N-terminal coil of the pre-hairpin intermediate of gp41, have been reported to strongly inhibit HIV-1 entry [5]. More recently [6], Root et al. have reported the design and activity of the protein 5-helix, which binds the C-peptide region of gp41 with high affinity. On the basis of this mechanism, they showed that peptides modelling the C-terminal region of gp41 are able to block membrane fusion, by interacting with the N-terminal 3-helix structure and thus blocking the interaction of the N- and C-terminal regions. In fact, this interaction is necessary to bring the two regions of gp41 into close proximity and thus allow the contact of the viral and cell target membranes. On the other hand, Callebaut et al. [7] have shown that peptides that contain the RP motif, which is highly conserved in the third hyper-variable loop (V3), are able to inhibit HIV-1 entry by interacting with a 95 kDa cell surface protein.

The inhibitors of the maturation of the Env precursor interfere with Env precursor processing in SU and TM. This maturation

Abbreviations used: Boc, *t*-butyloxycarboxyl; cmk, chloromethane; dec, decanoyl; DIPCdi, *N,N*-di-isopropylcarbodiimide; Env, envelope glycoprotein; ESI-MS, electrospray ionization MS; Fmoc, fluorenyl-methoxycarbonyl; FCS, foetal calf serum; Pmc, pentamethylchromane-6-sulphonyl; RT, reverse transcriptase; SIV, simian immunodeficiency virus; SU, surface glycoprotein; SPC, subtilisin-like protoxin prohormone convertase; TFA, trifluoroacetic acid; TM, transmembrane; VEM, virus envelope maturase; VLP, VEM-like protease; VV, vaccinia virus.

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occurs at the N-terminus of the consensus sequence R-X-K/R-R. This consensus sequence is also found in a variety of cellular proteins (proteins, proreceptors, proneuropeptides and pro-hormones), bacterial proteins and viral glycoproteins [8,9]. Several endoproteases have been implicated in the maturation of HIV-1 gp160 precursor, including furin, PC1/3, PACE4, PC5/6 isoforms, PC7 and members of the family of subtilisin-like protoxin prohormone convertases (SPCs) [10]. The activity of the SPCs is Ca²⁺-dependent, but primarily furin and PC7 have been reported to fulfil the requirements of the endoproteases responsible for *in vivo* activation of HIV Env precursor. These two SPCs are in fact the major endoproteases expressed in HIV host cells [11,12]. A second family of Ca²⁺-independent endoproteases has been reported, which are different from SPCs and correctly cleave HIV-1 gp160. It includes virus envelope maturase (VEM), purified by Kido et al. [13], and the VEM-like protease (VLP) serine endoprotease that we have recently purified [14], which cleaves HIV-1 gp160 to gp120 and gp41. The identification and characterization of the processing enzymes and their substrates have led to the development of specific inhibitors. Two types that have been tested include peptides and proteins. The former are decanoyl REKR chloromethane (decRXXRcmk) derivatives, reported to inhibit overexpressed gp160 processing and HIV-1 replication [15]. Using a similar sequence, decRVKRcmk, we recently showed its anti-viral activity on HIV-2_{ROD} infection [16]. The latter molecules that inhibit Env processing also involve proteins derived from natural serpins. Anderson et al. [17] engineered an antitrypsin variant by introducing the RXXR sequence required for furin activity in its active site. This mutant is called α 1-anti-trypsin Portland variant protein, and it shows a remarkable potency to inhibit overexpressed gp160 processing and its fusogenic properties [1,17]. We have recently demonstrated [16,18] the ability of the α 1-antitrypsin Portland variant protein to block the replication, syncytium induction and maturation of Env precursors of both HIV-1_{BRU} and HIV-2_{ROD}.

The aim of the present study was to develop inhibitors that could interfere with the maturation of Env precursor to external-surface glycoprotein and transmembrane subunits. This maturation step occurs at an R-X-K/R-R consensus site and is in fact a key step in the viral cycle. When it is blocked, viral infectivity is either low or zero. To this end, we modelled synthetic consensus peptides encompassing one or two potential maturation sites of HIV-1_{BRU} gp160 (TKAKRRVVQREKRV). To increase the resistance of peptides to protease activities, the peptide sequence was assembled with non-natural D-amino acids. Transmembrane passage was facilitated by the N-terminal addition of a dec group. Finally, the inhibitory capacity of the peptide was improved by the replacement of the C-terminal group by a cmk group. Once the peptide is recognized by the active site of the target enzyme, this modification enables the peptide to establish an irreversible covalent bond with one of the nitrogen atoms of the histidine ring, which along with aspartate and serine residues constitutes the catalytic triad of the active site of serine proteases. Hence this type of inhibitor is regarded as a suicide substrate.

In the present study, we report the design and activity of D-peptides that can inhibit cell fusion mediated by HIV-1_{BRU}, HIV-2_{ROD} and SIV_{mac251}. Their anti-viral activities *in vivo* are strongly correlated with their ability to inhibit the activity of cellular enzymes *in vitro* (Furin and PC7), which can convert gp160 to gp120 and gp41 and have been previously described as the major SPCs expressed in CD4+ lymphocytes and HIV and SIV target cells [11,12]. Only D-peptides that encompass the first cleavage site (KAKRR₅₀₃) are active, indicating that the second matu-

ration site (REKR₅₁₂) may not be the sole structure required for Env cleavage into SU and TM.

MATERIALS AND METHODS

Cells

Lymphoid cells (Jurkat and Molt-4 cells) were cultured at 37 °C under 5% CO₂ in RPMI 1640 medium (Eurobio, Les Ulis, France), supplemented with 10% heat-inactivated foetal calf serum (FCS; ATGC, Orleans, France), 2 mM glutamine, antibiotics (50 µg/ml streptomycin and 50 units/ml penicillin). HeLa-CD4-LTR/ β -gal cells, a gift from Dr P. Charneau (Pasteur Institute, Paris, France), were HeLa cells that were stably transfected with human CD4 cDNA and the bacterial *LacZ* gene under the control of the HIV-1 LTR promoter. They were grown in complete Dulbecco's modified Eagle's medium in the presence of 1 mg/ml of geneticin.

Peptide synthesis and peptide cytotoxicity

The peptide sequences tested were derived from the HIV-1_{BRU} gp160 sequence downstream from the maturation site (Table 1). Peptide synthesis (dec5L, dec5D, dec9L, dec9D, dec14L and dec14D) was performed manually on a PAL (5-[4-(9-fluoromenyl-methyloxycarbonyl)aminoethyl-3,5-dimethoxyphenoxy]-valeric acid) handle-derivatized *p*-methylbenzhydrylamine resin, by using the standard fluoren-9-ylmethoxycarbonyl (Fmoc)/*t*Bu protocol [19,20]. The following side chain-protecting groups were used: *t*-butyloxycarboxyl (Boc) for Lys, *t*Bu for Thr and Glu residues, pentamethylchromane-6-sulphonyl (Pmc) for Arg residue. Decanoic acid was coupled using *N*,Ni-di-isopropylcarbodiimide (DIPCDI) as the coupling reagent in dimethylformamide. Peptides were cleaved from the resin and deprotected with trifluoroacetic acid (TFA)/Et₃SiH for 2 h followed by repeated washing with diethyl ether [21]. The pellets were dissolved in aq. 10% (v/v) acetic acid and freeze-dried. The peptides were characterized by HPLC, amino acid analysis and electrospray ionization MS (ESI-MS; results not shown). Synthesis was performed with either L- or D-amino acids, and the sequences obtained included: 14L or 14D (TKAKRRVVQREKRV); 9L or 9D (RVVQREKRV); 5L or 5D (REKRV) (Table 1). dec14Dcmk was prepared by DIPCDI/3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine-mediated coupling [22,23] of D-valine cmk and the *N*-dec derivative of the corresponding all-D 13 amino acid-protected peptide: dec-Thr(*t*Bu)-Lys(Boc)-Ala-Lys(Boc)-Arg(Pmc)-Arg(Pmc)-Val-Val-Gln(*Ot*Bu)-Arg(Pmc)-Glu(*Ot*Bu)-Lys(Boc)-Arg(Pmc)-OH, which was prepared using the solid-phase approach [24] on a high-acid-labile resin [20]. Peptide synthesis was performed manually on a 4-(4-hydroxy-methyl-3-methoxyphenoxy)-butyric acid handle-derivatized [24] poly(ethylene glycol) graft polystyrene resin, by using a standard Fmoc/*t*Bu procedure [20]. The following side chain-protecting groups were used: Boc for Lys, *t*Bu for Thr and Glu, Pmc for Arg. Decanoic acid was coupled using DIPCDI as the coupling reagent in dimethylformamide. Peptides were cleaved from the resin by alternating washes of 1% TFA in CH₂Cl₂ for 20 s (2 ml of solution each time; total volume, 16 ml). This operation was repeated five times. The washes were collected in 16 ml of water (each fraction). The organic solvent of the fractions was evaporated under vacuum, with concomitant formation of a solid, which was filtered. The solid was dissolved in dioxan and evaporated under vacuum to eliminate the remaining TFA (three times). The resulting solid was crystallized in diethyl ether. Synthesis of D-valine cmk was performed by the method of Angliker et al. [25].

Table 1 Sequences of the peptides synthesized with D-amino acids

Cell viability was assessed with the Trypan Blue-exclusion test by incubating uninfected cells with various amounts of peptides (1–100 μ M). Survival was calculated as the percentage of unstained cells. Percent cytotoxicity is the difference between controls (100%) and the percent survival.

Peptide sequence	Properties	Cytotoxic effect of peptides used at 100 μ M on cells in culture
Dec <u>REKR</u> \downarrow V	5d (maturation site of HIV-1 gp160)	–
Dec <u>R</u> VQ <u>REKR</u> V	9d (maturation site + P6* + P8*)	–
decT <u>KAKRR</u> VVQ <u>REKR</u> V	14d (two potential cleavage sites)	–
decT <u>KAKRR</u> VVQ <u>REKR</u> Vcmk	14dcmk (cmk at the C-terminus)	–

* P indicates the position of the amino acid in the gp160 sequence. The arrow indicates cleavage of gp160 into gp120 and gp41. The principal peptide properties are underlined.

P₁₄ P₂ P₁ P₁
TKAKRRVVQREKR \downarrow V
 First Second
 cleavage cleavage
 site (maturation site)

For experiments, the corresponding peptides were dissolved in 2% FCS/RPMI 1640 medium or Dulbecco's modified Eagle's medium at 5 mM and aliquots were frozen until use. Cytotoxicity was determined on uninfected cells, including HeLa, CEM_{x174}, Jurkat and Molt-4 cells by incubating with each peptide for 7 days at 10–100 μ M. Each day, cell mortality was determined with the Trypan Blue dye-exclusion assay and/or the determination of growth curves for cells cultured in the presence or absence of the peptides. To determine the ability of peptides to enter cells, peptide molecules (1 mg) were coupled with FITC as described previously [26]. The FITC-coupled peptides were purified by gel-exclusion chromatography and incubated with 10⁵ CEM_{x174} and/or Jurkat cells overnight at doses of 1–35 μ M. At the end of incubation, cells were pelleted and washed three times with PBS 1 \times (0.5 mM MgCl₂/1 mM CaCl₂) by 10 min centrifugations at 1000 g, then they were fixed with 1% formaldehyde in PBS and revealed under a confocal microscope (Zeiss LSM 410) equipped with an argon laser having a line at 488 nm. The excitation wavelength was 488 nm. Images were captured every 5 s.

Viruses

Wild-type HIV-1_{BRU}, HIV-2_{ROD} and SIV_{mac251} were produced, titrated and stocked as supernatants containing 10⁵ tissue-culture infective doses (TCID₅₀)/ml. HIV and SIV were titrated by the method of Muenich and Reed in [27]. Vaccinia viruses (VV) encoding Env precursors of HIV-1_{BRU} (VV-gp160) were a gift from Transgène (Strasbourg, France), whereas the VV encoding the prohormone convertases, including furin (VV-FUR), PC7 (VV-PC7), VV-PC5 and VV-PC1 were described previously [28–30].

Replication assay

To test the inhibitory effect of peptides, 3 \times 10⁶ CEM_{x174} or Jurkat cells were incubated in 24-well culture plates, with 5 \times 10³ TCID₅₀ of SIV_{mac251}, HIV-1_{BRU} or HIV-2_{ROD} in 500 μ l of RPMI 1640 medium for 2 h at 37 °C. Cells were collected by low-speed centrifugation (1000 g for 10 min), washed twice with FCS-free medium, resuspended in 500 μ l of 2% FCS/RPMI 1640 medium with or without different doses of the peptides and cultured in duplicate, for 3 days. At the end of incubation, viral replication was assessed by determining the reverse transcriptase (RT) activity in 50 μ l of cell-free supernatants collected by the method of Benjouad et al. [31].

Syncytium formation

CEM_{x174} and Jurkat cells were infected with SIV_{mac251} and HIV-1_{BRU} respectively, as described in the Replication assay section. At 3 days post-infection, cells were pelleted and washed twice with FCS-free medium at 1000 g for 10 min, and 1 part was co-cultured with 4 parts of uninfected CD4+ Molt-4 cells. The co-cultures were incubated for 20 h and then examined for syncytium formation. On the other hand, syncytium induction in CEM_{x174} cells infected with SIV_{mac251} in the presence or absence of the peptides was directly recorded, since syncytia are readily visible.

Infectivity assay

HeLa-CD4-LTR/ β -gal cells (6 \times 10⁴) were infected for 20 h (1 viral replication cycle) with supernatants of equivalent RT activities that were collected from cells infected with HIV-1_{BRU} in the presence or absence of different doses of the peptides (5–35 μ M). HIV-1 Tat-mediated activation of β -galactosidase activity in the infected cells was detected as described previously [18,32]. In this assay, infected cells are coloured blue after addition of the enzyme substrate.

Production of Env

The statement rate of Env products was determined in cells infected with SIV_{mac251} or HIV-1_{BRU} in the presence or absence of active peptides. In this assay, cells were first infected for 2 days in 2% FCS/RPMI 1640 medium in the presence of 70 μ M dec14d, which was added once per replication cycle (20 h). Infected cells were then collected by low-speed centrifugation at 1000 g for 10 min and washed twice with FCS-free medium. Incubations were continued for an additional 6 days, without the peptides, in 2% FCS/RPMI 1640 medium. On D2, D4 and D6 post-infection after peptide arrest, the infected cells were pelleted at 1000 g for 10 min, washed with PBS 1 \times and lysed in an equal volume of 2% (v/v) Tween 20 in PBS for 30 min at 4 °C. The lysates were then centrifuged at 16000 g for 20 min at 4 °C to remove cell debris and membranes. Equal volumes of the collected supernatants were mixed in the ratio of 3 : 1 (v/v) with 4 \times Laemmli buffer [0.5 M Tris/HCl (pH 6.8)/10% glycerol/2% SDS/5% 2-mercaptoethanol/0.05% Bromophenol Blue], boiled for 5 min and separated by SDS/PAGE (8% gel). Proteins in the gel were electroblotted on to the nitrocellulose membranes at 60 mA overnight at 4 °C. For HIV-1_{BRU}, Western-blot assays

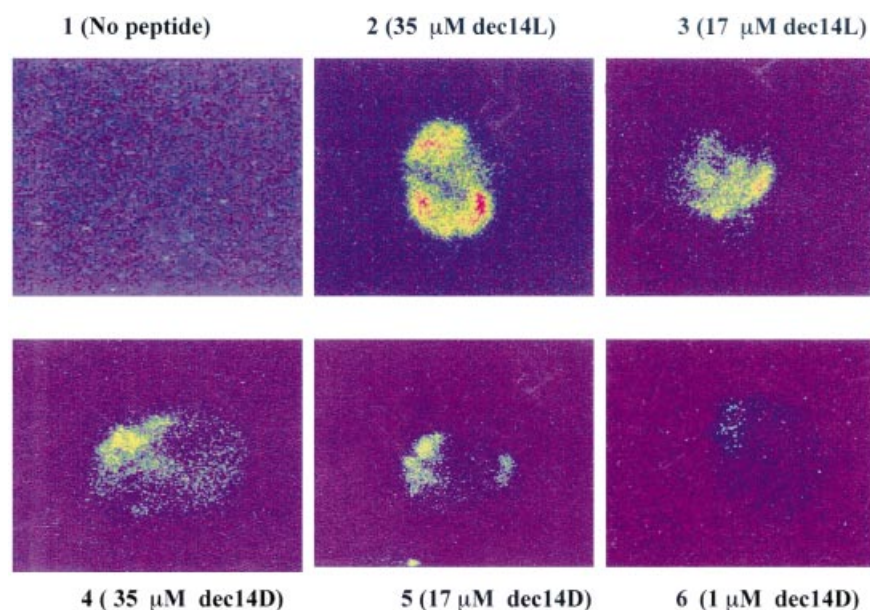


Figure 1 Penetration of dec14L and dec14D into cells

CEM_{x174} (10^6) and/or Jurkat cells were incubated overnight with dec14L-FITC and dec14D-FITC at doses ranging from 1 to 35 μ M. Cells were pelleted and washed three times with PBS by centrifugation at 1000 *g* every 10 min. The cells were fixed with 1% formaldehyde, and aliquots were analysed by confocal microscopy. Legends indicated at the top of each image show the peptide dose tested.

were performed with anti-gp160 polyclonal antibodies, by using both HIV-1-positive human sera and anti-gp160 polyclonal antibodies produced in rabbits immunized with soluble recombinant gp160 emulsified in Freund's adjuvant, as described previously [31]. For SIV_{mac251} and HIV-2_{ROD}, Env products were revealed by a pool of monoclonal antibodies directed against epitopes within SIVmac gp105 and gp32, as described previously [33]. To ensure that equal amounts of cellular proteins were loaded on to the gel, actin was used as an internal standard by testing aliquots of the cell lysates with anti-actin polyclonal antibodies diluted to the ratio of 1:100 (Santa Cruz Biotechnology, Heidelberg, Germany).

Inhibition of Env precursor cleavage

Env cleavage into SU and TM was examined by the Western-blot assay as described above. Cells were infected with the undiluted SIV_{mac251} or HIV-1_{BRU} stock as described above, and the peptides were added once per 24 h until D2. At D4 post-infection, total viral proteins with similar p25 (SIV_{mac}) antigen titres were analysed for Env statement as described above. The extent of Env cleavage was evaluated qualitatively by comparing the intensities of the Western-blot bands.

Stability of the effects of D-peptides

The stability of D-peptides in infected cell cultures was investigated by monitoring syncytium induction of infected cells previously treated with D-peptides. Cells were infected with the viruses in the presence or absence of peptides added once every 24 h until D2 post-infection, and incubations were continued for an additional 6 days without peptides. Syncytium induction was recorded on D3 and D6 post-infection.

In vitro inhibition of prohormone convertases

The purification of active PC5, PC1, furin and PC7 from the concentrated medium of cells infected with VV-PC5, VV-PC1, VV-FUR and VV-PC7 respectively has been described previously [28–30,34]. The inhibition constants K_i and IC_{50} of the D-peptides used in the present study were determined in a fluorometric assay using the synthetic peptide PyroGlu-RTKR-7-amino-4-methylcoumarin as substrate, as described previously [28–30].

RESULTS

Characterization of peptides: synthesis, purification, modification and cytotoxicity

Peptides dec5L/D, dec9L/D and dec14L/D were synthesized with a solid-phase method using Fmoc amino acids. Peptides were purified by HPLC and homogeneity varied from 80 to 95%. The correct composition of the peptides was determined by analysing the amino acid composition after hydrolysis using 6 M HCl followed by MS. The results of these analyses showed that the composition of the peptides synthesized was correct in terms of amino acids and their molecular masses, and they agreed with expected theoretical values.

The synthesis of dec14Dcmk was performed in three steps. The first step corresponds to the synthesis of the 13 amino acid-protected peptides (compound 1): dec-Thr(*t*Bu)-Lys(Boc)-Ala-Lys(Boc)-Arg(Pmc)-Arg(Pmc)-Val-Val-Gln(*O**t*Bu)-Arg(Pmc)-Glu(*O**t*Bu)-Lys(Boc)-Arg(Pmc)-OH, which was prepared using the solid-phase approach on a high-acid-labile resin as described in the Materials and methods section. The protected peptide was characterized by HPLC (> 95% purity in a reverse-phase C-18 column) amino acid analysis (Thr_{0.8}Glu_{2.0}Ala_{0.9}Val_{1.9}Lys_{3.1}Arg_{3.9}) and ESI-MS (calculated M_{avg} for C₁₆₂H₂₆₉N₂₈O₃₈S₄ was

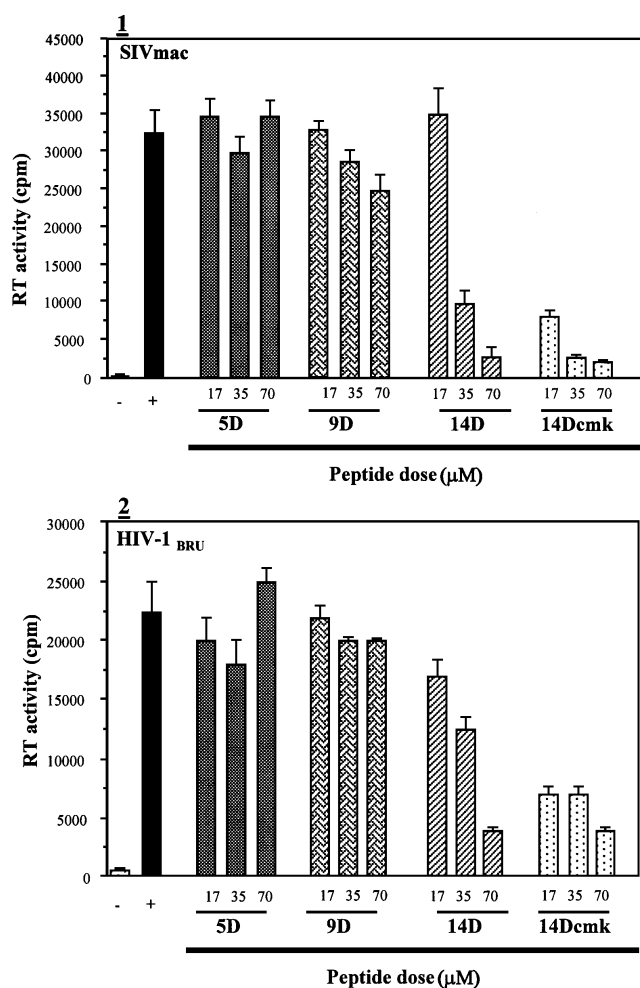


Figure 2 Replication of SIV_{mac251} and HIV-1_{BRU} viruses in the presence of peptides with D-amino acid sequences derived from HIV-1_{BRU} gp160 around the cleavage sites

CEM_{x174} and Jurkat cells were infected with SIV_{mac251} (1) and HIV-1_{BRU} (2) respectively, and cultured for 3 days in the presence or absence of different amounts of peptides added once per day. At the end of incubation, RT activity was determined in 50 μl of cell-free supernatants. The corresponding L-peptides are cytotoxic to cells in culture, and so the RT-activity assay was not performed. Values are the means ± S.D. for three experiments.

3345.34; M was determined using the estimated value of $m/z = 3345.2 \pm 0.5$). In the second step, we synthesized D-valine cmk (compound 2). The cmk-modified valine was characterized by HPLC (90% pure and 95% yield in a reverse-phase C-18 column) and by ESI-MS (calculated M_{avg} for C₆H₁₂NOCl was 149.06; $M+H$ was determined using the estimated value of $m/z = 150.1 \pm 0.5$). In the last step, synthesis of dec14D peptide cmk (compound 3) was prepared by DIPCDI/3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine-mediated coupling of the *N*-dec derivative of the corresponding all-D 13 amino acid-protected peptide (compound 1) and D-valine cmk (compound 2). The unprotected peptide was purified by medium-performance liquid chromatography (0.94 mg; 16% yield) and characterized by HPLC (> 95% purity in a reverse-phase C-18 column), amino acid analysis (Thr_{0,7}Glu_{2,1}Ala_{0,9}Val_{1,9}Lys_{3,2}Arg_{3,8}) and ESI-MS (M_{avg} calculated for C₈₅H₁₅₉N₂₉O₂₀Cl₁ was 1941.20; M found using $m/z = 1941.3 \pm 0.5$). The peptide sequences are listed in Table 1. Finally, to test the capacity of dec14L/D peptides for

transmembrane passage, these peptides were labelled by FITC (peptide dec14D-FITC or 14L-FITC) and then incubated with Jurkat or CEM_{x174} cells at doses between 1 and 35 μM. Peptide penetration was determined by following intracellular fluorescence with confocal microscopy. The results show that the peptide dec14D-FITC traversed the cell membrane. In addition, fluorescence seemed to be localized specifically to one cell pole. In cells incubated with peptide dec14L-FITC, on the other hand, fluorescence was more intense, suggesting its rapid degradation in the cell culture (Figure 1). The cytotoxic effect of peptides was determined on uninfected cells by incubating them with 1, 35, 70 and 100 μM for 7 days. The peptides were added once every 24 h. Cell mortality was determined daily by Trypan Blue dye-exclusion assay. No cytotoxicity was observed during cell incubation with D-peptide concentrations ≤ 100 μM (Table 1).

Anti-viral activity of synthetic D-amino acid peptides

The anti-viral activity of peptides was determined using two complementary tests. In the first, we tested the capacity of peptides dec5D, dec9D, dec14D and dec14Dcmk to block the viral replication of HIV-1_{BRU} and SIV_{mac251} by determining RT activity in the supernatants of infected cells. In the second test, peptides were screened for their capacity to block the formation of syncytia between cells expressing Env and non-infected cells expressing CD4 and the co-receptor CXCR4. The results show that the peptide dec14Dcmk had a high anti-viral activity against the replication of HIV-1_{BRU} and SIV_{mac251}. At 17 μM, peptide dec14Dcmk inhibited the replication of SIV_{mac251} by 75% (Figure 2-1) and that of HIV-1_{BRU} by 65% (Figure 2-2). At this concentration, the peptide dec14D, which was not modified by cmk had very low anti-viral activity, although it became effective at 70 μM and inhibited the replication of SIV_{mac} by 92% (Figure 2-1) and that of HIV-1_{BRU} by 85% (Figure 2-2). However, analogues dec5D and dec9D, which contain only the second REKR cleavage site, had no anti-viral activity even at the highest concentration of 70 μM (Table 2). Similar results were obtained with HIV-2_{ROD} (results not shown). In parallel, peptides dec5L, dec9L and dec14L assembled from natural L-amino acids were tested under the same conditions, but their considerable cytotoxicity, starting at 35 μM, precluded further examination.

Inhibition of syncytia formation by the peptides

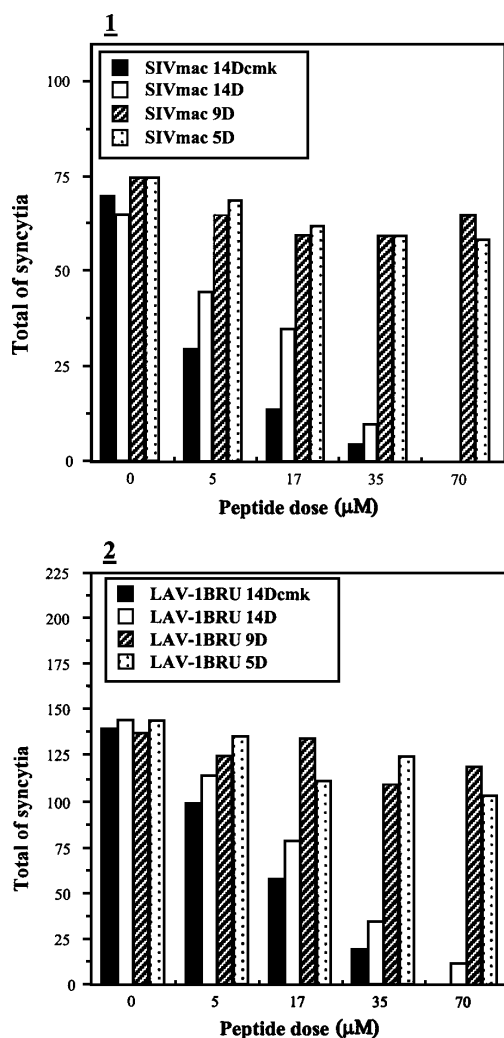
In agreement with the results of inhibition of viral replication, the incubation of peptides dec14D and dec14Dcmk with infected cells led to a significant and dose-dependent block of syncytia formation, although it did not completely prevent the synthesis of viral proteins (Figure 3). Total inhibition was obtained at the dose of 70 μM of dec14D and dec14Dcmk. At the concentration of 35 μM, the peptides dec14Dcmk and dec14D inhibited the formation of syncytia, induced by SIV_{mac251}, by 95 and 85% respectively (Figure 3-1), and these peptides inhibited the formation of HIV-1 by 90 and 80% respectively (Figure 3-2). Similar results were obtained with HIV-2_{ROD} (results not shown). Here again, the peptides dec5D and dec9D had no significant anti-viral activity (Table 2). It is also worth mentioning that the infectivity of HIV-1_{BRU} viral particles, produced in the presence of 35 μM of peptide dec14D or dec14Dcmk, was at least four times lower than that of wild-type viruses when equivalent doses of virus, corresponding to equal amounts of RT activity from virus supernatants produced in the presence or absence of peptides, were tested over one viral cycle (20 h) in the HeLa-CD4-LTR-LacZ cell system (Figure 4).

Table 2 Similarities between the effects of peptides on HIV-1_{BRU}, HIV-2_{Rod} and SIV_{mac251} viruses

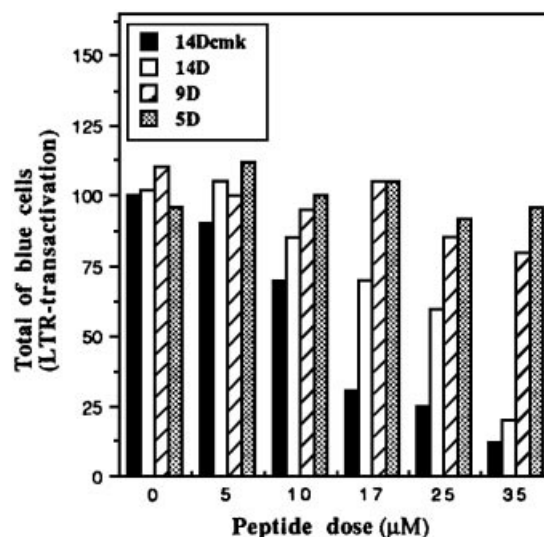
+, indicates an inhibitory effect; —, indicates no inhibitory effect. Note that the peptides are decanoyled at the N-terminus.

Viral properties	SIV _{mac}				HIV-2 _{Rod}				LAV-1 _{BRU}			
	5d	9d	14d	14dcmk	5d	9d	14d	14dcmk	5d	9d	14d	14dcmk
Viral replication (RT activity rise)	—	—	+	+	—	—	+	+	—	—	+	+
Syncytium induction	—	—	+	+	—	—	+	+	—	—	+	+
Viral infectivity*	—	—	+	+	—	—	+	+	—	—	+	+
Env processing into SU and TM	—	—	+	+	—	—	+	+	—	—	+	+

* Viral infectivity was determined by infecting HeLaCD4-LTR/ β -gal cells with equal RT activities of HIV-1_{lai} (or HIV-2_{Rod}) viruses collected in the presence or absence of 35 μ M peptides. Since SIV_{mac} does not infect HeLaCD4-LTR/ β -gal cells, viral infectivity was determined by infecting CEM_{x174} cells for 3 days with equal RT activities of SIV_{mac251} viruses collected in the presence or absence of peptides, and syncytium sizes and frequencies were then recorded.

**Figure 3** Induction of syncytium formation in the presence or absence of the peptides tested

CEM_{x174} or Jurkat cells (3×10^6) were infected with SIV_{mac251} (1) and HIV-1_{BRU} (LAV-1BRU) (2) respectively for 3 days in the presence or absence of different amounts of peptides added once per day. Syncytium formation was then directly recorded on CEM_{x174} cells, whereas 1 part of HIV-1 infected Jurkat cells was co-cultivated with 4 parts of uninfected Molt-4 cells before assessing syncytium formation. These data were reproduced in at least three independent experiments. Data are presented for one representative experiment.

**Figure 4** Infectivity of HIV-1_{BRU} viruses produced in the presence or absence of the peptides tested

HeLaCD4-LTR/ β -gal cells (6×10^4) were infected for 20 h (one viral replication cycle) with equal amounts of HIV-1 viruses, corresponding to equal amount of RT activity (10^4 c.p.m.), collected in the presence or absence of 5–35 μ M of the α -peptides. The supernatants were discarded and Tat-mediated activation of β -galactosidase activity was revealed by the addition of the enzyme substrate as described previously [18]. Infected cells that were coloured blue were then counted. These data were reproduced in at least three independent experiments. Data are presented for one representative experiment.

Effects of peptides on the production and maturation of Env

To elucidate the mechanism of inhibition by peptide dec14D, we determined its effect on the level of Env maturation in CEM_{x174} and Jurkat cells infected by SIV_{mac251} and HIV-1_{BRU} respectively. This evaluation was interpreted qualitatively by comparing the intensities of the Western-blot bands. Peptide dec14D was maintained in the culture for 2 days, and infection was continued in the absence of the peptide for 6 days. Infected cells were removed on D2, D4 and D6 post-infection after peptide arrest, and the quantity of Env products was evaluated by Western-blot analysis. The results of this study clearly show that in the presence of peptide dec14D, the quantity of Env products of SIV_{mac251} (Figures 5-1 and 6-1) and HIV-1_{BRU} (Figures 5-2 and 6-2) was

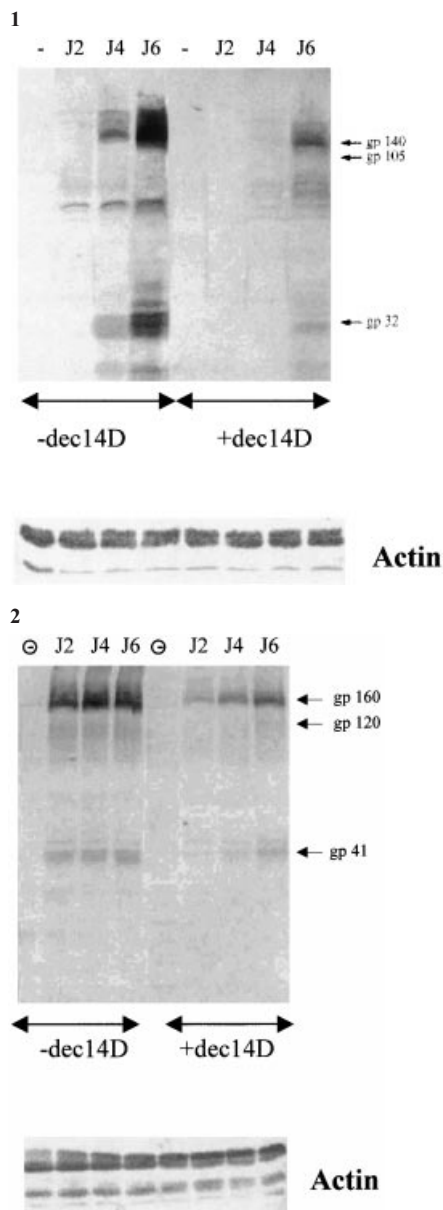


Figure 5 Effects of the peptides on the rate of Env precursor production

CEM_{x174} and Jurkat cells were infected with SIV_{mac} and HIV-1_{BRU} viruses respectively for 2 h at 37 °C, and then cultured for 2 days in the presence or absence of 70 μ M dec14D peptide added once per day. The addition of the peptide was stopped and the cells were incubated for an additional 6 days. To analyse the rate of production of Env precursor, post-incubation cells were collected on different days after peptide arrest, and were lysed, and tested by Western-blot analysis using antibodies specific for SIV_{mac} and HIV-1_{BRU} Env precursor-related products. Upper panels, (5-1) represents SIV_{mac} and (5-2) represents HIV-1_{BRU}. Lane -: uninfected cells; lane J2: 2 days after peptide arrest; lane J4: 4 days after peptide arrest; lane J6: 6 days after peptide arrest. The absence (-) or presence (+) of dec14D during 48 h of infection before its removal from cell cultures are indicated below the arrows in the Figure. Lower panels, equal volumes of cell lysates, previously quantified by Bradford assay, were analysed for the rate of actin production by Western-blot analysis using anti-actin polyclonal antibodies. The legends are the same as in the upper panels.

significantly inhibited in infected cells treated with the peptide dec14D at 70 μ M. It is also noteworthy that elimination of the peptide from the culture medium was accompanied by an increase in the quantity of SIV_{mac251} (Figure 5-1, lane J6) and HIV-1_{BRU}

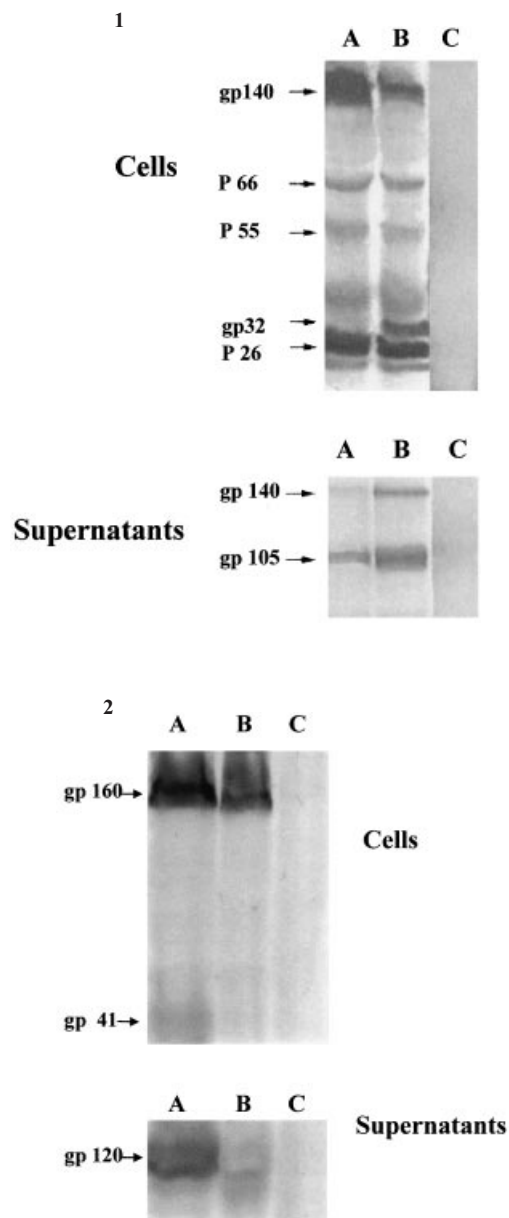


Figure 6 Env precursor cleavage into SU and TM in the presence or absence of 70 μ M peptide 14D

CEM_{x174} (6-1) or Jurkat cells (6-2) were infected with SIV_{mac251} and HIV-1_{BRU} respectively, in the presence or absence of dec14D peptide at 70 μ M added once per day for 2 days. The cells were incubated for an additional 2 days in the absence of the peptide and lysed. Equal amounts of total viral proteins in the presence or absence of the peptide (equivalent intensities of p25 antigen) were analysed by an anti-HIV-2_{ROD} polyclonal serum collected from infected humans or anti gp160 polyclonal antibodies produced in rabbits. Upper panel, cell lysates; lower panel, immunoprecipitated soluble gp105 or gp120 SU proteins present in the supernatant. Lane A, cells infected in the absence of peptide 14D; lane B, cells infected in the presence of peptide 14D; lane C, uninfected cells.

Env products (Figure 5-2, lanes J2–J6). The weak intensities of the bands corresponding to gp105 (Figure 5-1) and gp120 (Figure 5-2) are expected because they are related to soluble secreted proteins, whereas in this assay we analyse mainly cell-associated proteins. In addition, the fact that viral replication resumed after the peptide was removed on D2 post-infection indicates that the peptide dec14D at this concentration exerts a specific and selective

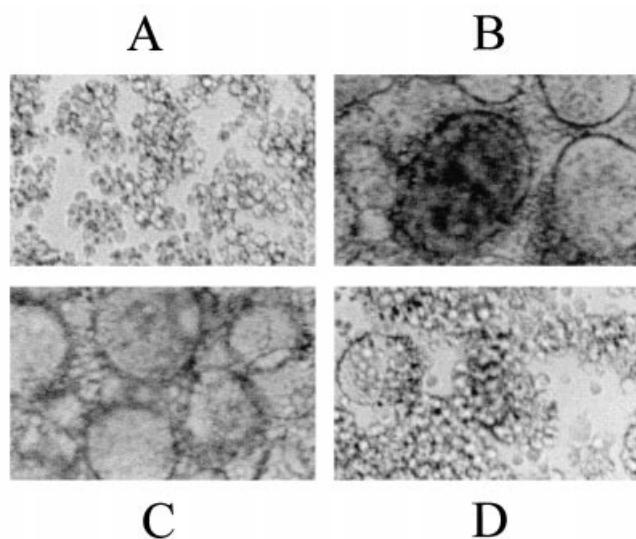


Figure 7 Stability of the inhibitory effect of peptide dec14D

CEM_{x174} cells were infected with SIV_{mac251} for 2 days in the presence of dec14D, dec9D and dec5D peptides added once per day. Peptide replenishment was arrested and cells were cultured for an additional 6 days post-infection (D6 p.i.). (A) 3 days after dec14D removal; (B) 3 days after dec9D removal; (C) 3 days after dec5D removal; (D) 6 days after dec14D removal from cell cultures.

effect on viral replication, but had no cytotoxic effect on CEM_{x174} and Jurkat target cells. Then we analysed the effect of peptide dec14D on the maturation of Env precursors to SU and TM. This analysis was conducted on the same quantity of core viral proteins (p25) associated with cells infected in the presence or absence of 70 μ M peptide dec14D. The results show that in the presence of the peptide dec14D, there is a substantial accumulation of gp140, precursor of SIV_{mac} or HIV-1 Env (Figures 6-1 and 6-2, lane A). This inhibition of precursor gp140 maturation is also correlated with the low levels of gp105 (SU) found in supernatants of infected cells. In the absence of peptide dec14D, on the other hand, maturation of the SIV and HIV glycoprotein precursors occurred normally as shown by the relatively weak intensity of the gp140 and gp160 bands (Figures 6-1 and 6-2, lane B). This result is consistent with the detection of higher amounts of both cell-associated gp32 (TM) and secreted gp105 (SU) of SIV in the absence of dec14D peptides.

The specificity of action of peptide dec14D on blocking syncytia formation was characterized further in the following experiment in which cells infected by SIV_{mac} (or HIV-1_{BRU}) were incubated

with peptide dec14D for 2 days and grown for an additional 6 days in the absence of the peptide. Syncytia were analysed on D3 and D6 post-infection after peptide arrest. The results (Figure 7A) show that on D3, even in the absence of peptide dec14D, cells could not form syncytia, whereas on D6 they had recovered a partial capacity for syncytia formation (Figure 7D). Similar results were obtained with HIV-1 (results not shown).

Among the PC members, including furin (or PACE), PC1/PC3, PC2, PACE4, PC5/PC6, PC7 (or LPC), primarily furin and PC7 are expressed in CD4⁺ lymphocytes, the principal targets of HIV and SIV. Hence, we investigated the capacity of peptide dec14D and its analogues to inhibit the enzymic activity of furin and PC7 *in vitro*. As a control, we also tested the inhibitory activity of these peptides on PC5 and PC1. Briefly, the substrate used was a fluorogenic peptide PyGlu-RTKR-MCA and the K_i of the peptides (dec14L/D, dec9L/D and dec5L/D) was determined in the presence of PC7, furin, PC5 and PC1. The results (Table 3) show a selective inhibition by peptides dec14L and dec14D. The peptide dec14L selectively inhibited furin with a $K_i = 28 \pm 3.2 \mu$ M and peptide dec14D selectively inhibited PC7 with a $K_i = 4.6 \pm 0.6 \mu$ M. The incapability of peptides dec5D and dec9D to inhibit furin and PC7 suggests the importance of the presence of two potential cleavage sites for the production of inhibitory activity. It should also be noted that no inhibition of PC1 and PC5 was obtained with any of the peptides, including dec14L and dec14D. This underlines the heterogeneity of specificity in the family of prohormone convertases, although they cleave after a basic amino acid, their recognition and interaction with the substrate is apparently dependent on other structural parameters.

DISCUSSION

HIV Env maturation to SU and TM subunits by endoproteolytic cleavage is a key step in the viral cycle of HIV. Blocking this maturation leads to the production of viral particles that cannot reinitiate a new replication cycle [8,9]. This property is also shared by several enveloped viruses, such as Influenza [35], Measles [36], Ebola [37], bovine leukaemia virus [38] and Borna disease virus [39]. It has been reported that the total block of Env precursor cleavage yields a non-functional protein that cannot induce membrane fusion [40], the mechanism by which HIV penetrates target cells.

As a result, the blocking of this first step of the viral cycle has considerable importance for therapeutic applications by developing molecules that can interfere with cleavage of the glycoprotein precursor. Even so, the development of these inhibitors requires the understanding of cell proteases involved in this maturation. Current knowledge would tend to show that the maturation of HIV Env occurring in the consensus sequences R-X-K/R-R

Table 3 Inhibition constants K_i (μ M) of prohormone convertases by the peptides

Inhibition constants were determined in a fluorometric assay by assessing cleavage of the PyE-RTKT-MCA peptide by the enzymes in the presence or absence of the peptides.

Enzyme	dec5L	dec5D	dec9L	dec9D	dec14L	dec14D*
Furin†	> 150	> 150	> 150	> 150	28 ± 3.2	> 150
PC7†	> 150	> 150	> 150	> 150	‡	4.6 ± 0.6
PC5	> 150	> 150	> 150	> 150	> 150	> 150
PC1	> 150	> 150	> 150	> 150	> 150	> 150

* The dec14D peptide inhibits PC7 at an IC_{50} of $22.4 \pm 1.2 \mu$ M and furin at $102 \pm 9 \mu$ M.

† These enzymes are expressed in the CD4⁺ lymphocytes, the major natural targets of HIV infection.

‡ A good substrate but not an inhibitor.

involves serine endoproteases belonging primarily to two sub-families: (i) furin and PC7, members of the subfamily of prohormone convertases (PCs) whose activity is Ca^{2+} -dependent. Among the members of this subfamily, furin and PC7 are the major ones expressed in CD4+ lymphocytes, monocytes and macrophages, the principal targets of HIV (these two PCs can cleave the precursor of the HIV envelope gp160 *in vitro* and *in vivo*); (ii) VEM [13] and VLP [14], lymphocyte endoproteases that can correctly mature HIV gp160 into gp120 and gp41 in the total absence of Ca^{2+} .

In the present study, we determined the inhibitory capacity of synthetic peptides assembled from L- or D-amino acids, containing only the second cleavage site (dec5D/L and dec9D/L) or both potential cleavage sites (14D/L). All peptides were synthesized with a hydrophobic N-terminal dec group to facilitate passage via the cytoplasmic membrane. The advantage of using peptides composed of D-amino acids is their capacity to resist degradation by cell proteases. Since peptide dec14D had the best anti-viral activity with no effect on cell viability, we decided to improve this activity by modifying it with a C-terminal cmk group. This modification transformed peptide dec14D into an irreversible inhibitor or suicide substrate.

Our results show that peptides dec14L-FITC or dec14D-FITC (fluorescein-labelled) can penetrate cells. Confocal microscopic analysis, however, revealed an apparent difference in fluorescence distribution inside the cell. Whereas peptide dec14D-FITC accumulated preferentially at one pole of the cell, the same analysis with dec14L-FITC showed more intense and more diffuse fluorescence throughout the cell. This could be explained by the greater susceptibility of peptide dec14L to degradation by cellular proteases. The polarized and stable fluorescence of dec14D peptide may indicate that it formed stable complexes with endogenous cellular proteases. In addition, cytotoxicity tests showed that peptide dec14L was highly cytotoxic at concentrations $\geq 35 \mu\text{M}$, whereas the D-peptide exhibited no cytotoxicity at the same doses. Therefore the entire study was conducted with D-peptides.

Our results on anti-viral activities against HIV-1_{BRU}, HIV-2_{ROD} and SIV_{mac251} showed anti-viral activity of peptide dec14D and dec14Dcmk, whereas no significant effect was obtained with peptides dec5D and dec9D. This suggests either the importance of the first cleavage site (KAKRR₅₀₃) or the importance of the entire region downstream from the REKR↓V cleavage site for endoproteases. In agreement with our results, the presence of the tetrabasic sequence R-X-K/R-R, although important, is apparently insufficient to provoke cleavage. Its recognition and cleavage probably require parameters of charge, accessibility and well-defined structures [40]. For example, the introduction of a negative charge at the second cleavage site Arg⁵¹⁰ → Glu or in the first Arg⁵⁰² → Glu has no effect on the maturation of HIV-1 gp160 into gp120 and gp41. The double mutation of Arg⁵¹⁰ and Arg⁵⁰² to Glu, on the other hand, results in the total block of gp160 maturation [40]. This suggests the importance of the charge of the first cleavage site in the maintenance of structural integrity by neutralizing the effect of the negative charge Arg⁵¹⁰ → Glu introduced in the main cleavage site. Bosch and Pawlita [8] reported that multiple non-conserved mutations in the first potential site (KAKRR₅₀₄) result in the inhibition of Env precursor maturation. Taken together, these results point out the importance of structure in the immediate vicinity of the principal cleavage site REKR. This suggestion is supported by the lack of consensus amino acid stretches flanking the basic residues. For example, it has been shown that some cleavage sites are associated with exposed structures in prohormones as β -turn [41–43] and Ω -loops [44].

When the active concentrations of peptides dec14D and dec14Dcmk are compared, the higher activity of peptide dec14Dcmk is seen: at $17 \mu\text{M}$, it leads to a considerable inhibition of HIV-1_{BRU}, HIV-2_{ROD} and SIV_{mac251} replication, whereas peptide 14D has no significant anti-viral activity at this concentration. This difference in activity thus seems to be related to the cmk group that alkylates histidine, one of the components of the catalytic triad of the active site of serine proteases, and that acts as an irreversible inhibitor or suicide substrate. It is also noteworthy that the infectivity of HIV-1 viral particles produced in the presence of $70 \mu\text{M}$ dec14D or $35 \mu\text{M}$ dec14Dcmk, in a single-round infectivity assay, is at least five times lower than that of wild-type particles. This anti-viral activity of the peptides is apparently related to their capacity to interfere with the maturation of Env precursors. In the presence of peptides dec14D or dec14Dcmk, there is a significant inhibition of the maturation of Env (HIV-1_{BRU} gp160 and SIV_{mac251} gp140), as shown by the increase in the gp160/gp120 and gp140/gp32 ratios in infected cells. These results agree with work showing that the inhibition of Env maturation produced non-functional glycoproteins in terms of their capacity to induce membrane fusions [15,18]. In addition, the maturation of the Env affects the efficacy of its incorporation into the envelope during the budding of viral particles on the membranes of infected cells [40].

Although furin was initially proposed as the cellular protease responsible for the maturation of gp160 into gp120 and gp41 [15,17], more recent work [11,14] has implicated other proteases, such as PC7, VEM and VLP, present in HIV target cells. In agreement with these potential candidate proteases, our results show that peptides dec14L and dec14D have different affinities for furin and PC7. The peptide dec14L preferentially inhibits furin with $K_i = 28 \pm 3.2 \mu\text{M}$, whereas peptide dec14D has nearly a 7-fold higher affinity for PC7 with a $K_i = 4.6 \pm 0.6 \mu\text{M}$. Peptide dec14L is cleaved at the principal cleavage site REKR↓ as expected, whereas peptide dec14D is recognized but not cleaved. This property was exploited by using peptide dec14D as an affinity ligand, enabling us to purify a new serine endoprotease from peripheral blood lymphocytes that correctly cleaves gp160 into gp120 and gp41, independent of Ca^{2+} [14]. All these results suggest that the maturation of HIV Env is probably ensured by an entire large family of endoproteases. This should be considered for the development of inhibitors of viral Env maturation for therapeutic applications.

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