Inhibition of HIV-2_{ROD} replication in a lymphoblastoid cell line by the α1-antitrypsin Portland variant (α1-PDX) and the decRVKRcmk peptide: comparison with HIV-1_{LAI}

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ABSTRACT – We investigated the effects of α1-antitrypsine Portland variant (α1-PDX) and decanoylRVKRchloromethylketone (decRVKRcmk) on HIV-2_{ROD} replication in the Jurkat lymphoblastoid cell line. To this end, cells were stably transfected with the α1-PDX (J-PDX) and used as targets for HIV-2_{ROD} infection. Controls were prepared with the empty vector (J-pcDNA3). HIV-2_{ROD} and HIV-1_{LAI} replications were significantly inhibited and delayed in the presence of the α1-PDX protein. When decRVKRcmk was used at 35 μM, inhibition rates were 70–80% for HIV-2_{ROD} and HIV-1_{LAI}, while total inhibition occurred at 70 μM. Control peptides consisting of decanoylRVKR and acetylYVADcmk had no effect. In the presence of the α1-PDX or the decRVKRcmk at 35 μM, the infectivity of HIV-2_{ROD} and HIV-1_{LAI} produced was 3–4-fold lower. Both molecules inhibited syncytium formation by HIV-2_{ROD} and HIV-1_{LAI} to a considerable extent. Finally, the inhibition of viral replication was correlated with the ability of the decRVKRcmk at 35 and 70 μM and of the α1-PDX, to inhibit the processing of envelope glycoprotein precursors. The α1-PDX protein and the decRVKRcmk peptide at 35 μM inhibited HIV-2 and HIV-1 to a similar level suggesting that identical or closely related endoproteases are involved in the maturation of their envelope glycoprotein precursors into surface and transmembrane glycoproteins. The significant inhibition observed with α1-PDX indicates that furin or furin-like endoproteases appear to play a major role in the maturation process.

α1-PDX / decRVKRcmk / HIV / env processing / lymphocyte

1. Introduction

Envelope glycoproteins of the human immunodeficiency viruses, HIV-1 and HIV-2, are essential for the adsorption and penetration of the virus into host cells. They are encoded by the *env* gene as non-functional precursors (gp160 for HIV-1 and gp140 for HIV-2) which are then cleaved along the secretory pathway by cellular endoprotease(s) to produce the functional subunits of the retroviral envelope [1]. This cleavage occurs C-terminally to the RXKR sequence to produce surface gp120 (SU) and transmembrane gp41 (TM) for HIV-1 [2, 3] and gp125 (SU) and gp36 (TM) for HIV-2 [4, 5]. They then combine into oligomers that are stabilized by non-covalent bonds on the viral envelope and on the surface of infected cells. SU specifically binds to the CD4 receptor and to the recently characterized chemokine receptors on target cells includ

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ing CCR5 and CXCR4 [6–9]. This interaction is followed by a conformational change of SU and TM that exposes the hydrophobic N-terminal extremity of TM involved in the mechanism of fusion between the virus and host cell membranes [10–12].

A number of reports have shown that processing of HIV envelope glycoprotein precursors occurs at the trans-Golgi level. Furin-like endoproteases belonging to the family of serine prohormone convertases (SPCs) have been advanced as candidates for gp160 processing [13–17]. This family currently contains seven members including furin (or PACE), PC1 (or PC3), PC2, PC4, PACE4, PC5 isoforms (or PC6) and PC7 (also known as PC8 or LPC). The activity of these endoproteases is Ca^{2+}-dependent and results in the cleavage of proteins at the C-terminus of the consensus sequence [K/R]X-[K/R]R↓ [18–22]. Furin and PC7 have attracted particular attention, since they were shown to be expressed in T4 lymphocytes, the major target for HIV infections [17, 23]. The possibility of SPCs being the unique candidates for gp160 processing in vivo, however, has been questioned by the fact that Ca^{2+}-independent enzymes divergent from SPC can also cleave gp160 into SU and TM in Molt-4 [24], and in CHO-RPE40 cells [25]. Finally, a more recent study has demonstrated the occurrence of gp160 processing activity after emergence from the trans-Golgi network (TGN) [26]. Taken together, these data indicate that the identity of the enzyme(s) involved in gp160 processing in vivo remains controversial.

Envelope glycoprotein precursor processing is a required event for HIV to be infectious [27]. The identification and characterization of processing enzymes and characterization of the cleavage site have led to the development of inhibitors of this reaction. Two types that have been tested are peptides and proteins. The former are decanoyl-RXKR-chloromethylketone (decRVKRcmk) derivatives reported to inhibit over-expressed gp160 processing and HIV-1 replication ex vivo [16]. The decanoyl facilitates the peptide entry into cells, whereas the cmk group mediates an irreversible inhibition of the target enzyme by covalent linkage. The latter is a serpin (serine proteinase inhibitor) variant known as α1-PDX derived from alpha-1 antitrypsin (α1-AT). Two mutations have been reported in the α1-AT reactive site loop. The first is natural and converts $A_{355}XXM_{358}$ to $A_{355}XXR_{358}$ yielding the protein known as alpha-1 antitrypsin Pittsburg variant (α1-PIT) that was found to be a potent inhibitor of thrombin [28, 29]. The second was engineered by site-directed mutagenesis and converts α1-PIT $A_{355}XXR_{358}$ to $R_{355}XXR_{383}$ which is the minimal sequence required for furin activity. This mutant is known as alpha-1 antitrypsin Portland variant or α1-PDX [30]. The α1-PDX protein has been shown to inhibit gp160 processing into SU and TM and to alter the gp160 syncytium-inducing capacity in non-lymphoid cell lines [13, 29]. Another serpin variant, α2-rM, derived from α2-macroglobulin by specific bait region $GFYE_{686}$ SD to $RSKR_{686}$ SL mutations, was also shown to inhibit furin-mediated gp160 cleavage in vitro [31]. Recently, the proteinase inhibitor 8 (PI-8) characterized as a member of the ovalbumin family [32], was found to be an irreversible inhibitor of furin activity in vitro [33].

To our knowledge, this study is the first to investigate and to report the effects of α1-PDX and decRVKRcmk on HIV-2_{ROD} replication in a CD4-positive derived lymphoblastoid cell line. The aim of this work was to compare the extent of inhibition of viral replication and envelope glycoprotein precursor processing by α1-PDX and decRVKRcmk. The data show that both α1-PDX serpin and decRVKRcmk peptide can inhibit HIV-2_{ROD} replication by blocking envelope glycoprotein precursor processing in the Jurkat lymphocyte cell. They thus suggest that lymphocyte gp160 processing endoprotease(s) sensitive to α1-PDX inhibition is involved in HIV-2_{ROD} env processing to a significant extent. In addition, they do not exclude that other minor protease(s) may also be active in this process. We therefore suggest that envelope glycoprotein precursors of HIV-2_{ROD} and HIV-1_{LAI} are processed by identical or closely related endoproteases.

2. Materials and methods

2.1. Cells

The Jurkat lymphoid cell line stably transfected with full-length α1-PDX cDNA cloned into a pcDNA3 vector (J-PDX) or with the empty vector (J-pcDNA3), previously described, was used to show that α1-PDX inhibits notch I receptor maturation [34]. FACS and immunoblotting analysis showed that: i) the J-pcDNA3 and J-PDX clones expressed similar levels of CD4 and CXCR4 receptors; ii) the α1-PDX protein is stably expressed intracellulary in J-PDX cells, and is secreted into the medium (data not shown). The introduced α1-PDX gene has no effect on cell viability as found by growth curves performed for the parental cells, the J-pcDNA3 and the J-PDX cells (data not shown).

Lymphoid cells including J-PDX, J-pcDNA3 and Molt-4, were grown at 37 °C in 5% CO_2 in RPMI-1640 medium (Eurobio, Les Ulis, France) supplemented with 10% heat-inactivated FCS (ATGC, Orleans, France), 2 mM L-glutamine, 50 µg/mL streptomycin and 50 IU/mL penicillin (Eurobio, Les Ulis, France). For transfected Jurkat cells, geneticin G418 (France Biochem, Meudon, France) was added at 0.26 mg/mL. HeLa CD4-LTR/β-gal cells, a gift from Dr P. Charneau (Institut Pasteur, Paris, France), are HeLa cells stably transfected with human CD4 cDNA and the bacterial *lacZ* gene under the control of HIV-1 LTR promoter [35]. They were grown in complete Dulbecco's modified Eagle medium (DMEM) (Eurobio, Les Ulis, France) in the presence of 1 mg/mL of geneticin.

2.2. Viruses

All recombinant vaccinia viruses used in the present study were gifts from Transgène (Strasbourg, France). They included VV-TG-9.1-1139 encoding HIV-1_{LAI} gp160 (VV-gp160) and VV-TG-9.1-2180 encoding HIV-2_{ROD} gp140 (VV-gp140). Wild-type HIV-1_{LAI} and HIV-2_{ROD} were obtained from Diagnostics Pasteur (Marnes La-Coquette, France) as supernatants from infected CEM cells containing 10^5 50% tissue culture infective doses ($TCID_{50}$) per mL.

2.3. HIV-2 infection of J-PDX and J-pcDNA3 cells

J-PDX and J-pcDNA3 cells (10^6) were infected with 100 μL of the HIV-2 viral stock diluted to 1:100 (10^2 $TCID_{50}$) and 1:1 000 (10 $TCID_{50}$). After 1 h at 37 °C, unbound viral particles were removed by washing twice with FCS-free medium. Infected cells were cultured at 37 °C in 10% FCS-RPMI medium for the indicated time, with sampling every 3 days.

2.4. Peptide experiments

The peptides used were decRVKRcmk, acYVADcmk (Bachem Biochimie SARL, Voisins-le-Bretonneux, France), and decRVKR. They were dissolved at 5 mM in FCS-free culture medium, and aliquots were frozen until use. Peptide effects on HIV-1_{LAI} and HIV-2_{ROD} replications were determined by infecting 3×10^6 J-pcDNA3 cells as described above with undiluted viral stock such that an increase in reverse transcriptase (RT) activity could be observed during one week. DecRVKRcmk peptide (35 and/or 70 μM) was then added once per HIV replication cycle (20 h). To determine whether the effect of decRVKRcmk is related to its peptide sequence and/or cmk group, the peptides decRVKR and AcYVADcmk were tested under the same conditions at 70 μM.

In over-expression experiments using vaccinia viruses (VV), the peptides were added once per 12 h during infections. At the time of each peptide replenishment, an equal volume of culture medium without peptides was added to infected and uninfected cells. At the end of incubation, cells were collected by low-speed centrifugation, and the protein content was analysed immunologically for gp160- and gp140-related products as described below.

2.5. RT assay

RT activity microassay involves the direct measurement of RT activity in 50 μL of culture supernatant. The procedure and the buffers are the same as those previously described [36]. Assays were carried out in triplicate in two independent experiments.

2.6. HIV-infectivity assay

Free-cell supernatants of equal RT activity collected from infected J-pcDNA3 and J-PDX cells on the 20th day post-infection (20 days p.i.) were incubated with 2×10^5 HeLaCD4-LTR/β-gal cells for 1 h at 37 °C. After washing with FCS-free medium, cells were incubated at 37 °C for 20 h. By monitoring over a single viral replication cycle (20 h), this assay enabled us to determine the ability of the Tat produced to activate expression of the *lacZ* gene as previously described by Charneau et al. [35]. This assay was used to evaluate viral infectivity of HIV-2 and HIV-1 produced in the presence or absence of α1-PDX and/or 35 μM decRVKRcmk. Supernatants collected from cells infected in the presence of 70 μM decRVKR and AcYVADcmk were used for comparison with those incubated with decRVKRcmk. All the tested peptides had been found to have no effect on virus infection of HeLaCD4-LTR/β-gal cells during one cycle (20 h), when they were added at the same time as wild-type viruses (data not shown).

2.7. Syncytium formation assay

2.7.1. Effect of decRVKRcmk

HeLaCD4 cells were infected with recombinant vaccinia viruses at a multiplicity of infection (MOI) of 5 PFU/mL. After 1 h of incubation at 37 °C, unbound viruses were eliminated by washing and cells were cultured in 2% FCS medium for 24 h at 37 °C in the presence or absence of 35 and 70 μM decRVKRcmk. Peptide controls involved testing decRVKR and AcYVADcmk at 70 μM.

2.7.2. Effect of α1-PDX protein

J-pcDNA3 and J-PDX cells (10^6) were infected with 50 μL of HIV-1_{LAI} or HIV-2_{ROD} viral stock, and decRVKRcmk was added to J-pcDNA3 cells as described above. After 3 days of incubation, 2×10^4 J-pcDNA3 or J-PDX cells were co-cultured with 8×10^4 uninfected CD4-positive Molt-4 cells at 37 °C. Syncytia were assessed microscopically after 12 h. In other experiments, peptides were added for 3 days and incubations were continued for another 3 days. Co-cultures with uninfected Molt-4 cells were then carried out.

2.8. Over-expression experiments

HIV-1_{LAI} gp160 and HIV-2_{ROD} gp140 processing was analysed in the cells infected with VV-gp160_{LAI} or VV-gp140_{ROD}. Lymphoid cells (10^7) were infected at an MOI of 20 PFU/cell. After 1 h, to allow viral adsorption, cells were harvested by low-speed centrifugation. The inoculum was removed, and cells were resuspended in 2% FCS medium. Infected cells were then incubated at 37 °C in a humidified CO_2 atmosphere for 24 h. DecRVKRcmk previously dissolved at 5 mM in FCS-free RPMI medium was added to 70 μM at 12-h intervals. Supernatants were collected and tested for secreted gp120 and gp125 as described below. Cell pellets were lysed in lysis buffer (50 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 1% Triton X-100, 3 mM EDTA, and 2 mM phenylmethanesulfonyl fluoride (Sigma-ALDRICH, Saint-Quentin Fallavier, France)) for 15 min at 4 °C and centrifuged at 15 000 rpm. Solubilized proteins were then separated on 8% acrylamide by SDS-PAGE under reducing conditions and transblotted electrophoretically to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany).

Equivalent amounts of total cellular proteins were analysed using Coomassie blue and Red Ponceau staining. The membranes were then saturated with PBS containing 5% non-fat milk for 1 h at room temperature. After washing, they were incubated for 2 h with a 1:500 dilution of a monoclonal anti-HIV-1 gp120 antibody (A105.34) (ANRS, Paris, France), human anti-HIV-1 and HIV-2 polyclonal sera collected from seropositive patients (Hôpital Pitié Salpitière, Paris, France). After washing, membranes were incubated for 1 h with peroxidase-coupled rabbit anti-human antibodies (Dakko S.A, Trappes, France). After further washing, the peroxidase substrate diaminobenzidine (Sigma ALDRICH, Saint Quentin Fallavier, France) was added. To analyse SU (gp120 or gp125) secreted into the medium, 500 μL of cell-free supernatants from infected and uninfected cells were mixed with anti-HIV polyclonal sera or monoclonal anti-gp120 diluted to 1:500, and with protein A-sepharose. Then they were incubated overnight

at 4 °C with shaking. Unbound proteins were eliminated by washing three times, then the protein A-anti-SU-SU complexes were analysed by SDS-PAGE and Western blotting as described above.

The epitope reactivity of gp160, gp140, gp120, gp41 and gp36 was scanned with a densitometer, and the percent cleavage was calculated by using the formula 100 × (intensity of cell-associated SU or TM)/[(intensity of uncleaved precursor) + (intensity of cell-associated SU or TM)].

3. Results

In order to test the inhibitory effect of decRVKRcmk peptide during multiple HIV-1_{LAI} and HIV-2_{ROD} replication cycles, we first assessed the cytotoxic effects of decRVKRcmk on the cultured cells. The cytotoxic effect of peptides was determined on uninfected cells by incubating them with 35, 70 and 100 μM for 10 days. The peptides were added at these concentrations once every 24 h. Cell mortality was determined daily by a trypan blue dye exclusion assay and staining total cell proteins with Coomassie blue after SDS-PAGE on 10% acrylamide gels. No cytotoxicity was observed during cell incubations with peptide concentrations ≤ 70 μM. Cells were then infected with a high MOI of HIV-2_{ROD} or HIV-1_{LAI} (10^4 $TCID_{50}$) enabling the maximum RT activity after 7 days of infection to be observed. The data (*figure 1A,B*) show an efficient inhibition of HIV-2_{ROD} and HIV-1_{LAI} replications at 70 μM decRVKRcmk. At 35 μM decRVKRcmk, HIV-1_{LAI} and HIV-2_{ROD} were inhibited by 70–80%, indicating a dose-dependent effect. Of note, the cell count by trypan blue staining during HIV infection experiments (*figure 1*) in the presence of decRVKRcmk and in J-PDX cells showed no significant difference relative to controls (data not shown), thus reflecting that reductions in HIV replication were not due to alterations in cell machinery.

In order to test the specificity of this inhibition, equal doses of decRVKR, acYVADcmk were tested under the same conditions. These latter peptides were found incapable of altering HIV-1_{LAI} and HIV-2_{ROD} replication, thus reflecting the importance of irreversible inhibition of serine proteases mediated by cmk. We therefore conclude that the decRVKRcmk effect on HIV-2 and HIV-1 replication was specifically associated with the combination of the peptide sequence and the cmk group (see *table I*).

In parallel, J-PDX (Jurkat cells stably expressing α1-PDX) and J-pcDNA3 (Jurkat cells stably transfected with the empty vector) were infected with the same doses of HIV-2_{ROD} and HIV-1_{LAI} to determine whether they replicate these viruses at a similar level. The results (*figure 1A,B*) showed that α1-PDX inhibited the viral replication of HIV-1_{LAI} by 65% (*figure 1A*) and of HIV-2_{ROD} by 70% (*figure 1B*). Since α1-PDX serpin was not cytotoxic to the stably transfected cells, we then infected J-pcDNA3 and J-PDX cells with HIV-2_{ROD} at a lower MOI (10^2 and 10 $TCID_{50}$). The kinetics of infection were monitored for 20 days by sampling cell supernatants every 3 days and determining viral production by assaying RT activity. The results show that the peak of RT activity in J-PDX cells was at least 4-fold lower (*figure 1C,D*). The second visible effect of α1-PDX was the difference in replication kinetics as shown by a delay in the appearance of RT activity in J-PDX cells (*figure 1C,D*). The α1-PDX protein could thus clearly inhibit and delay HIV-2_{ROD} replication. Similar results have been obtained with HIV-1_{LAI} [37].

Since partial viral replication persisted in J-PDX cells on the 20th day (*figure 1C,D*) and in cells treated with 35 μM decRVKRcmk on day 7 postinfection (p.i.) (*figure 1A,B*), it was of interest to determine whether these viruses had the same infectious capacity as viruses produced in J-pcDNA3 or in the presence of the peptide controls including decRVKR and acYVADcmk. To this end, equal amounts of RT activity from supernatants collected on 20 days p.i. (*figure 1C*) were used to infect HeLa CD4-LTR/β-gal cells for one replication cycle (20 h). The data indicate that both HIV-2_{ROD} and HIV-1_{LAI} produced in the presence of α1-PDX protein were 3–4-fold less infectious than viruses produced by J-pcDNA3 cells (*figure 2A,B*). Similar results were obtained with HIV-1 and HIV-2 produced by Jurkat cells treated with decRVKRcmk at 35 μM. It was found that the infectivity of viruses produced in the presence of 70 μM decRVKR and acYVADcmk was similar to viruses released by control cells. Furthermore, the addition of decRVKRcmk peptide to the wild-type virus inoculum used for HeLa indicator cell infection did not alter the extent of LTR transactivation during one viral replication cycle (20 h), suggesting that this inhibitor did not interfere with virus penetration or with viral Tat activity. Taken together, these results indicate that both decRVKRcmk and α1-PDX protein probably interfere with viral replication by the production of non or less infectious viral particles in comparison to those produced by controls. The effects of α1-PDX serpin and decRVKRcmk peptide were further analysed for the inhibition of membrane fusion. In the first experiment, HeLaCD4 cells were infected with recombinant vaccinia viruses expressing HIV-2_{ROD} gp140 (VV-gp140) and HIV-1 gp160 and examined for syncytium formation. The results presented clearly show that syncytium induction mediated by VV-$gp140_{ROD}$ was completely blocked by 70 μM decRVKRcmk (*figure 3B,3*) in comparison to controls (*figure 3B,1*), while smaller syncytia were observed less frequently at 35 μM (*figure 3B,2*), thus confirming the dose-dependent effect. Similar results were obtained with VV-gp160 (data not shown). In the second experiment, J-PDX and J-pcDNA3 cells with or without the indicated peptide doses were infected with HIV-2_{ROD} (*figure 3A*) for 3 days and then co-cultured with uninfected Molt-4 cells. HIV-2_{ROD}-induced syncytia were again completely inhibited by the peptide decRVKRcmk at 70 μM (*figure 3A,4*), while they persisted to a lower extent and with a smaller size in the presence of α1-PDX (*figure 3A,2*) or with 35 μM decRVKRcmk (*fig 3A,3*) compared to controls (*figure 3A,1*). Again, no apparent alteration in syncytium induction was observed with peptides decRVKR (*figure 3A,5* or *3B,5*), and acYVADcmk (*figure 3A,6* or *3B,6*), thus supporting the finding that the effect of decRVKRcmk was mediated both by its peptide sequence and the cmk group (*table I*). These results reflect a significant correlation between the effects of decRVKRcmk or

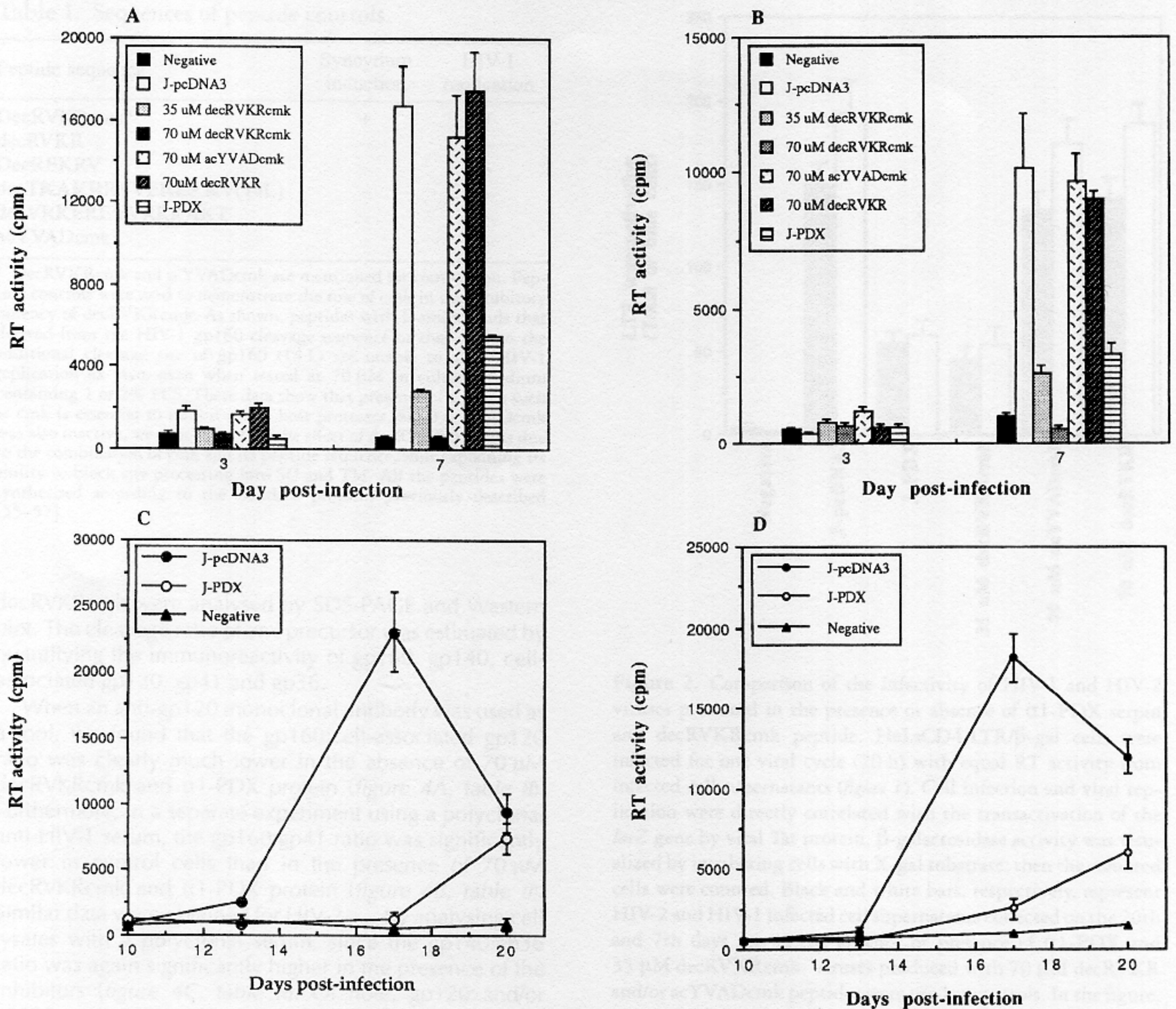


Figure 1. Effect of α1-PDX serpin and decRVKRcmk peptide on HIV-2_{ROD} and HIV-1_{LAI} replications. To test the decRVKRcmk effect, 3×10^6 J-pcDNA3 cells were infected with 50 μL of undiluted viral stock of HIV-1_{LAI} (5×10^3 $TCID_{50}$) (A) and HIV-2_{ROD} (5×10^3 $TCID_{50}$) (B) for 7 days in the presence or absence of 35 or 70 μM decRVKRcmk. At the same time, cells were also infected in the presence of peptide controls including 70 μM decRVKR and acYVADcmk in order to determine whether the effect of decRVKRcmk at the indicated doses was specific. J-pcDNA3 and J-PDX cells (10^6) were infected with 100 μL of HIV-2 viral stock diluted to 1:100 (10^2 $TCID_{50}$) (C) and 1:1000 (10^3 $TCID_{50}$) (D) for 20 days. In the figure, 'negative' represents the uninfected cells. Every 3 days, half of the cell supernatants were collected and assayed for RT activity. The results are the mean of three assays in two independent experiments ± standard deviation.

α1-PDX protein on syncytium induction and inhibition of viral replication.

One notable finding was the importance of the continuous presence of peptide decRVKRcmk at 70 μM for the inhibition of syncytia. When Jurkat cells were infected with HIV-1_{LAI} or HIV-2_{ROD} in the presence of 70 μM decRVKRcmk, syncytium induction was in fact completely blocked, as mentioned above. In contrast, when the peptide was omitted on day 3 p.i. and the syncytia assay was performed after an additional 3 days of incubation, cells recovered their capacities to induce syncytia (*figure 3C*). This experiment is an additional argument that peptide decRVKRcmk was not cytotoxic at 70 μM. On the other hand, the frequency of syncytium formation increased in controls or in cells infected in the presence of decRVKR and acYVADcmk at 70 μM (*figure 3C*).

We further investigated the existence of a direct correlation between the inhibition of viral replication and syncytium formation with processing of env precursors into SU and TM. To this end, lysates and supernatants of J-PDX and J-pcDNA3 cells infected by VV-$gp160_{LAI}$ or VV-$gp140_{ROD}$ in the presence or absence of 70 μM

Table I. Sequences of peptide controls.

Peptide sequence	Syncytium induction	HIV-1 replication
DecRVKRcmk[a]	+	+
decRVKR	–	–
DecREKRV	–	–
decT**KAKR**RVVE**REKR**V(14L)	–	–
decVRKEREVVRRKAKT	–	–
acYVADcmk[a]	–	–

[a] DecRVKRcmk and acYVADcmk are mentioned for comparison. Peptide controls were used to demonstrate the role of cmk in the inhibitory potency of decRVKRcmk. As shown, peptides with L-amino acids that derived from the HIV-1 gp160 cleavage sequence or that contain the additional cleavage site of gp160 (14 L) are unable to alter HIV-1 replication ex vivo, even when tested at 70 μM in culture medium containing 1 or 2% FCS. These data show that presence of a group such as cmk is essential to inhibit active host proteases. Since acYVADcmk was also inactive, we concluded that the effect of decRVKRcmk was due to the combination of cmk and its peptide sequence, thus explaining its ability to block env processing into SU and TM. All the peptides were synthesised according to the standard protocol previously described [55–57].

decRVKRcmk were analysed by SDS-PAGE and Western blot. The cleavage ratio of env precursor was estimated by quantifying the immunoreactivity of gp160, gp140, cell-associated gp120, gp41 and gp36.

When an anti-gp120 monoclonal antibody was used as a tool, we found that the gp160:cell-associated gp120 ratio was clearly much lower in the absence of 70 μM decRVKRcmk and α1-PDX protein (*figure 4A, table II*). Furthermore, in a separate experiment using a polyclonal anti-HIV-1 serum, the gp160:gp41 ratio was significantly lower in control cells than in the presence of 70 μM decRVKRcmk and α1-PDX protein (*figure 4B, table II*). Similar data were obtained for HIV-2_{ROD} by analysing cell lysates with a polyclonal serum, since the gp140:gp36 ratio was again significantly higher in the presence of the inhibitors (*figure 4C, table II*). Of note, gp120 and/or gp125 secretion occurred at a considerable extent in the absence of decRVKRcmk and α1-PDX protein (bottom panels). Both anti-HIV-1_{LAI} and anti-HIV-2_{ROD} polyclonal sera detect SU only in supernatants, which could reflect the rapid shedding of the SU into medium. These findings, by using both polyclonal antibodies and a monoclonal anti-env antibody in independent experiments, lead us to conclude that both decRVKRcmk at 70 μM and α1-PDX protein inhibited env processing into SU and TM. We noted that inhibition of env cleavage was higher at 70 μM decRVKRcmk compared to that in J-PDX cells. As expected, lysates of cells incubated with the control peptides including decRVKR and acYVADcmk had gp160:gp41 or gp140:gp36 ratios that were similar to those of J-pcDNA3 (*table II*). These data strongly suggest that both α1-PDX and decRVKRcmk compete with gp160 for furin-like serine endoproteases, and significantly reduce its extent of maturation at the multibasic cleavage site.

Peptide decRVKRcmk at 35 μM and the α1-PDX protein significantly inhibit ex vivo HIV-1 and HIV-2 replications (70–80% inhibition, see *figure 1*), in addition, this inhibitory potency remained correlated with the inhibition

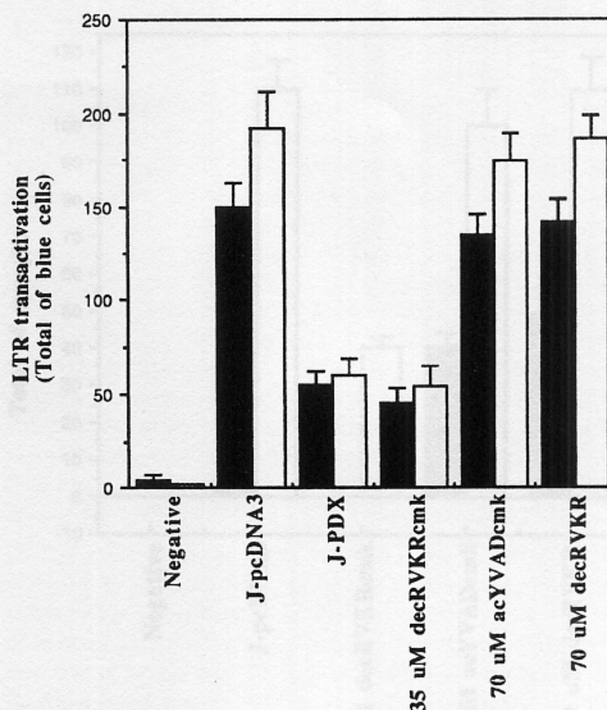
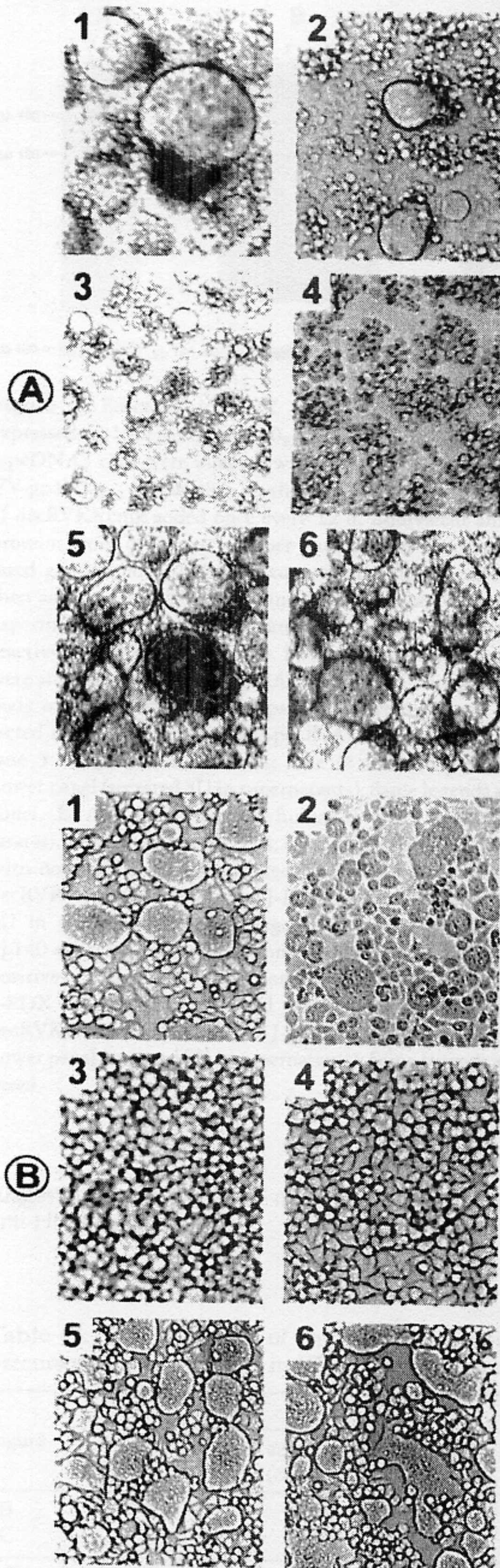


Figure 2. Comparison of the infectivity of HIV-1 and HIV-2 viruses produced in the presence or absence of α1-PDX serpin and decRVKRcmk peptide. HeLaCD4-LTR/β-gal cells were infected for one viral cycle (20 h) with equal RT activity from infected cell supernatants (*figure 1*). Cell infection and viral replication were directly correlated with the transactivation of the *lacZ* gene by viral Tat protein. β-galactosidase activity was visualized by incubating cells with X-gal substrate; then the coloured cells were counted. Black and white bars, respectively, represent HIV-2 and HIV-1 infected cell supernatants collected on the 20th and 7th days p.i. in the absence or presence of α1-PDX and 35 μM decRVKRcmk. Viruses produced with 70 μM decRVKR and/or acYVADcmk peptides were used as controls. In the figure, 'negative' represents supernatants collected from uninfected cells.

of env processing. In our experiment, the minimal dose of decRVKRcmk required to inhibit env processing is ≥ 35 μM (data not shown). These data directly indicate that the inhibition of viral replication observed is a consequence of the reported ability of the molecules tested to interfere with the maturation of env precursors [16, 30].

Examination of the amino acid sequences at the junction between SU and TM of the envelope glycoproteins in a number of HIV-1 and HIV-2 viruses directly isolated from infected patients reveals strong pressure to retain a cluster of dibasic sequences where cleavage of the envelope glycoprotein precursors will occur. It had been found previously that these cleavage sequences remain unchanged between primary and cell-line-adapted HIV-1 isolates [38]. In our hands, there is no difference in cleavage sites of HIV-1 viruses obtained early from culture supernatants and those multiply passaged in T-cell lines. Therefore, cleavage of env precursors by host proteases appears to play a major role in viral infectivity in vivo,



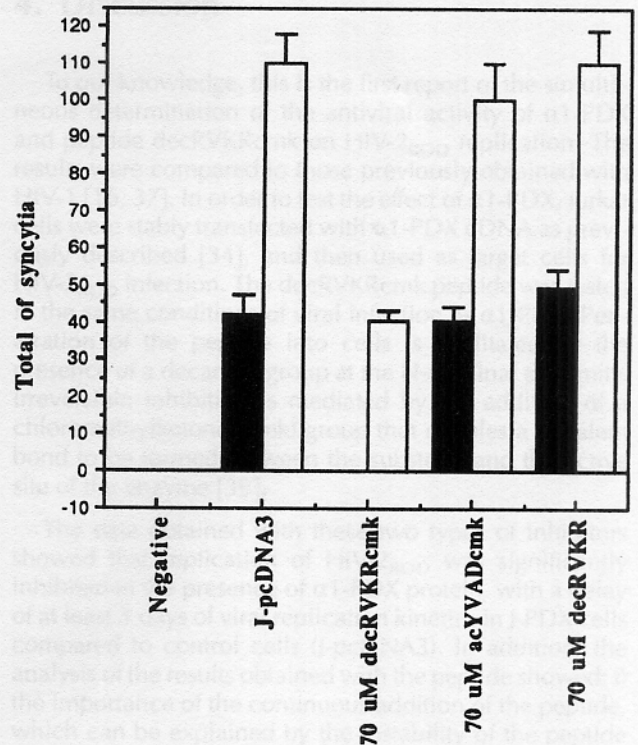


Figure 3. Effect of α1-PDX and decRVKRcmk on the induction of syncytia after HIV-2_{ROD} or VV-gp140_{ROD} infections. A, J-pcDNA3 and J-PDX cells were infected with 50 μL (5×10^3 $TCID_{50}$) of HIV-2 viral stock. Thirty-five micromolar (panel 3), 70 μM decRVKRcmk (panel 4), 70 μM decRVKR (panel 5), acYVADcmk (panel 6) were added to J-pcDNA3 cells once every 20 h. After 3 days, cells were co-cultivated with a 4-fold excess of uninfected Molt-4 cells at 37 °C for 12 h, and syncytia were counted microscopically. Panels 1 and 2, HIV-2-infected J-pcDNA3 and J-PDX cells, respectively. B, HeLaCD4 cells were infected with VV-gp140_{ROD} at 5 PFU/mL. After 1 h of incubation, unbound viruses were eliminated by washing, and cells were cultured in 2% FCS-DMEM medium for 24 h at 37 °C in the absence (panel 1) or presence of peptides added once every 12 h including 35 μM (panel 2), 70 μM decRVKRcmk (panel 3), 70 μM decRVKR (panel 5), 70 μM acYVADcmk (panel 6). The uninfected cells (panel 4). C, cells were infected as described above in the presence of peptides for 3 days (3 days p.i.) and incubations were continued for another 3 days in their absence (6 days p.i.). Co-cultures with uninfected Molt-4 cells were performed on 3 and 6 days p.i., and syncytia were counted microscopically. Black bars, 3 days p.i.; white bars, 6 days p.i.

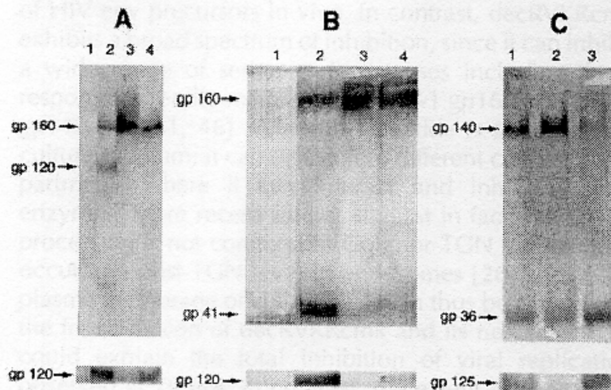


Figure 4. Effect of α1-PDX and decRVKRcmk on overexpressed $gp160_{LAI}$ and $gp140_{ROD}$ processing. J-PDX and/or J-pcDNA3 cells were infected with VV-$gp160_{LAI}$ (A and B) or VV-$gp140_{ROD}$ (C) for 48 h, in the absence or presence of 70 μM of decRVKRcmk added once every 12 h. Equivalent amount of proteins from cell lysates (upper panels), and immunoprecipitated gp120 and gp125 from supernatants (lower panels) were then analysed by SDS-PAGE and Western blot. Note that the experiments in *figures 4 A–C* are independent, and so epitope reactivities are not equivalent. Env precursor-related products were immunodetected using: (A) monoclonal anti-gp120 antibody named A105.34. Upper panel (cell lysates). Lane 1: uninfected cells; lane 2: infected J-pcDNA3 cells with no peptide; lane 3: 70 μM decRVKRcmk; lane 4: infected J-PDX cells. Lower panel (secreted SU in supernatants). Same legends as upper panel. (B) Anti-HIV-1 positive human serum. Upper panel (cell lysates). Lane 1: uninfected cells; lane 2: infected J-pcDNA3 cells with no peptide; lane 3: infected J-pcDNA3 cells with 70 μM decRVKRcmk; lane 4: infected J-PDX cells. Lower panel (secreted SU in supernatants). Same legends as upper panel. (C) The gp140-related proteins were immunodetected with anti-HIV-2-positive human serum. Upper panel (cell lysates). Lane 1: infected J-PDX cells, lane 2: infected J-pcDNA3 cells with 70 μM decRVKRcmk, lane 3: infected J-pcDNA3 cells with no peptide. Lower panel (secreted SU in supernatants): Same legends as upper panel.

suggesting these proteases might be a novel target for anti-HIV therapies.

4. Discussion

To our knowledge, this is the first report of the simultaneous determination of the antiviral activity of α1-PDX and peptide decRVKRcmk on HIV-2_{ROD} replication. The results were compared to those previously obtained with HIV-1 [16, 37]. In order to test the effect of α1-PDX, Jurkat cells were stably transfected with α1-PDX cDNA as previously described [34], and then used as target cells for HIV-2_{ROD} infection. The decRVKRcmk peptide was tested in the same conditions of viral infection as α1-PDX. Penetration of the peptide into cells is facilitated by the presence of a decanoyl group at the N-terminal extremity. Irreversible inhibition is mediated by the addition of a chloromethylketone (cmk) group that enables a covalent bond to be formed between the substrate and the active site of the enzyme [39].

The data obtained with these two types of inhibitors showed that replication of HIV-2_{ROD} was significantly inhibited in the presence of α1-PDX protein, with a delay of at least 3 days of viral replication kinetics in J-PDX cells compared to control cells (J-pcDNA3). In addition, the analysis of the results obtained with the peptide showed: i) the importance of the continuous addition of the peptide, which can be explained by the instability of the peptide and particularly the cmk group; ii) the inability of peptides decRVKR, and acYVADcmk being a cell-permeant peptide that has been reported to inhibit caspase and cathepsin B in cell cultures [40], to block viral replications underlines the importance of the simultaneous presence of the enzyme recognition sequence and the cmk group. The α1-PDX protein has been reported to inhibit furin [13, 41–43] and PACE4 [13, 40, 44], PC5 [13, 15] and PC7 [21] by acting as a suicide substrate. Experiments in vitro and ex vivo have shown that α1-PDX could inhibit gp160 processing [13, 23, 30] and that of the F0 precursor of measle virus, resulting in a blockade of its spread in adherent cell lines [45]. We have extended these findings to the natural HIV host, a derived CD4-positive lymphocyte cell line that is a better model for studying the role of endoproteases involved in HIV infection in vivo.

Given that α1-PDX protein was found to inhibit HIV-2_{ROD} gp140 processing, these data suggest furin and/or closely related proteases as candidates for the maturation

Table II. Determination of env processing ratio by densitometric measurements of the immunoreactivity of env precursor-related products in VV-$gp160_{HIV-1}$-and/or VV-$gp140_{HIV-2}$ -infected cells.

Figure	EPR[a]		
	J-pcDNA3 cells with no peptide (controls)	J-pcDNA3 cells with 70 mM decRVKRcmk	J-PDX cells (stably transfected with the α1-PDX protein)
4B	0.8	4	3
4C	0.5	1.7	1

[a] The env processing ratio (EPR) was calculated using the formula [100 × (intensity of cell-associated TM)]/[(intensity of uncleaved env precursor) + (intensity of cell-associated TM)] and/or by the ratio of (intensity of uncleaved env precursor)/(intensity of cell-associated TM) as shown in the table. When using a monoclonal anti-gp120 antibody (*figure 4A*), cell-associated gp120 was detected only in controls. The majority of SU was secreted into the medium, and this occurred to a much lesser extent in the presence of the inhibitors.

of HIV env precursors in vivo. In contrast, decRVKRcmk exhibits a broad spectrum of inhibition, since it can inhibit a wide range of serine endoproteases including those responsible for the maturation of HIV-1 gp160 and HIV-2 gp140 [30, 41, 46]. When this peptide is added in the culture medium, it can diffuse into different cellular compartments where it can interact and inhibit various enzymes. More recent studies suggest in fact that gp160 processing is not confined to Golgi or TGN but can also occur at a post-TGN level in endosomes [26] and at the plasma membrane of cells [47]. It can thus be argued that the free diffusion of decRVKRcmk and its non-selectivity could explain the total inhibition of viral replication observed at 70 μM. In contrast, the extent of processing inhibition obtained with α1-PDX (*table II*) would suggest that env processing occurs at a significant level in the TGN, the compartment where α1-PDX has been shown to exert its maximal activity [43]. These data underline the potential role of furin and furin-like proteases.

The characterization of HIV-1 and HIV-2 particles produced in the presence of the inhibitors, α1-PDX and 35 μM decRVKRcmk, showed that they are 3–4-fold less infectious. This impaired infectivity might be a result of the co-existence and association of hetero-oligomers of uncleaved env precursor and mature SU and TM on the viral envelope and on the infected cell surface. Such hetero-oligomerization might alter either the affinity to CD4 or chemokine receptors, or the flexibility of the N-terminal domain of transmembrane glycoprotein that is necessary for the induction of membrane fusion. The latter hypothesis is in agreement with the report of McCune et al. [27], who showed that replacement of the cleavage site of gp160 (REKR) with a chymotrypsin site results in the production of non-infectious viruses, even though their microscopic morphology is similar to and indistinguishable from that of wild-type viruses. These defective particles recover infectivity, however, after the addition of chymotrypsin to the culture medium. On the other hand, the partial inhibitory potency of α1-PDX protein and 35 μM decRVKRcmk on HIV-2 and HIV-1 replication may be due either to the existence of active processing enzymes that do not co-localize with α1-PDX (plasma membrane-associated or extracellular) or to enzymes present in intracellular compartments which are not accessible to decRVKRcmk peptide at doses lower than 70 μM.

Our data therefore show that both HIV-1 gp160 and HIV-2 gp140 maturation exhibited similar sensitivity to the endoprotease inhibitors, decRVKRcmk and α1-PDX. These data are supported by several findings: i) the higher conservation of amino acid sequence around the potential cleavage sites of HIV-1_{LAI} and HIV-2_{ROD} [48]; ii) correct maturation by furin of synthetic peptides representing the sequence around the cleavage sites of HIV-1_{LAI} and HIV-2_{ROD}; and iii) the conservation of secondary structure of the regions around the cleavage sites of HIV-1_{LAI} and HIV-2_{ROD} as revealed by ^{1}H-NMR (Bahroui E., unpublished results).

The biological activation of many circulating plasma protein precursors is physiologically regulated by specific proteinase inhibitors. They may account for as much as 10% of plasma proteins [49]. They control a variety of important events such as coagulation and inflammatory reactions. Specific diseases are associated with the congenital deficiency of some physiological inhibitors. One example of this is the severe bleeding disorders and pulmonary emphysema associated with the mutation of α1-AT (A_{356}) to α1-PIT (R_{356}) [28, 29]. These genetic or infectious diseases reinforce the importance of developing new protein inhibitors that may be used as therapeutic tools. Van Rompeay et al. [31] produced a new serpin variant, called α2-rM, by mutating α2-macroglobulin reactive site loop $GFTE_{386}$ to $RSKR_{386}$. In contrast to α1-PDX, α2-rM was unable to inhibit the processing of gp160 after its transfection in Cos-1 cells, although it inhibits furin in vitro as a suicide substrate. This inhibition defect may result from the fact that α2-rM transits in a pathway different from that of intracellular gp160 processing or that the required oligomerization of this inhibitor is rate limiting. These data thus directly indicate that in order to exhibit their inhibitory capacities as is the case of α1-PDX in the lymphoid cell line used here, serpins should co-localize with their cognate proteases.

The development of inhibitors directed against the host cell protease activity implicated in env precursor processing can lead to novel anti-HIV therapies. Elimination of host target will bypass the problems associated with HIV-1 and HIV-2 variations and with viral resistance to such inhibitors. These proteases are tangible targets for therapy based on the observations that: i) mammalian cells remain viable in their absence, such as in the cases of LoVo cells [50], CHO-K1 cells [25, 51, 52], yeast cells [53], and J-PDX cells used here; and ii) the env cleavage is a key step in viral replication in vivo as shown by the conservation of the dibasic cleavage site in primary HIV isolates [38]. The major limitations are specific-tissue expression of the inhibitors, and maintenance of its expression level near the efficient dose in infected cells. In this regard, serpin activity can be improved by controlling their cellular expression with specific promoters. For instance, the use of the HIV LTR promoter would allow serpin expression during viral replication, and so higher specific effects could be obtained. Expression of these inhibitors in vivo, even transiently, will lead to the blockade of multiple generational viral cycles. Furthermore, combination of these inhibitors with the currently used highly active antiretroviral therapy (HAART) may be useful, since protease inhibitors will block the resistant viruses' replication during and/or after HAART arrest [54]. In fact, resistance to protease or RT inhibitors should not affect compounds used against new targets. On the other hand, The specificity of peptides that inhibit the processing of env precursors can be improved by several modifications. An initial approach is to modify their structures by introducing additional amino acids downstream from the REKR cleavage site of gp160. Previous studies have in fact suggested that amino acids of the $KAKRR_{504}$ site positively alter gp160 processing into SU and TM [11]. However, peptides with L-amino acids, such as the 14L used in the present study (*table I*), are subject to rapid proteolysis, and thus the use of D-amino acids can be expected to raise their stability and specificity.

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