Effect of alpha-1 antitrypsin Portland variant (α 1-PDX) on HIV-1 replication

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The present work investigated the potential role of alpha-1 antitrypsin Portland variant (α 1-PDX), a bioengineered serine proteinase inhibitor (serpin), in the interference with the viral replication of HIV-1, induction of syncytia and maturation of envelope glycoprotein gp160 to gp120 and gp41. A Jurkat lymphoid cell line transfected with a plasmid containing the α 1-PDX cDNA (J-PDX) and expressing the protein in a stable manner was infected with HIV-1_{Lai}. Controls were Jurkat cells transfected with the same vector pcDNA3 without the cDNA insert (J-pcDNA3). The results showed that viral replication of HIV-1 was significantly inhibited with a delay in replication kinetics in J-PDX cells as compared with J-pcDNA3 cells. In addition, a comparison of the infectious capacity of viruses produced in the presence and absence of α 1-PDX revealed that

this capacity differed. It was found that α 1-PDX exerts its effect by interfering with the formation of syncytia between J-PDX cells infected with gp160 recombinant vaccinia virus, or after infection by HIV-1 and co-culture with uninfected Molt-4 cells. In contrast, when the same experiments were performed with J-pcDNA3 cells, a large number of syncytia was obtained. Analysis of viral proteins by Western blotting and densitometry showed that the inhibition of the cytopathic effect of HIV-1 and viral replication was correlated with the capacity of α 1-PDX to interfere with the maturation of gp160 to gp120 and gp41.

Key words: endoprotease, gp160 processing, serine endoprotease inhibitor.

INTRODUCTION

The envelope glycoproteins of HIV-1, HIV-2 and simian immunodeficiency virus (SIV) are synthesized in the form of precursors gp160 for HIV-1 and gp140 for HIV-2 and SIV. The precursors are then cleaved, primarily at the trans-Golgi level, to external glycoproteins and transmembrane glycoproteins [1]. One or several cellular endoproteases are responsible for this cleavage, yielding mature viral-envelope glycoproteins: gp120 and gp41 in the case of HIV-1. Glycoproteins gp120 and gp41 are present on the surface of viral particles and infected cells as oligomers linked by non-covalent bonds [2]. As a result of its high affinity to CD4 receptors ($K_d = 10^{-9}$ M) [3], gp120 is responsible for the tropism of the virus for CD4 [4] present on the surface of T4 lymphocytes, monocytes and macrophages and for co-receptors CXCR4 and CCR5 [5–7]. The gp120–CD4 interaction is followed by a conformational change of gp120 and gp41 that results in exposure of the hydrophobic N-terminal extremity of gp41 [8]. This mediates fusion between the virus and the host cell membranes [9].

Proteolytic activation of viral-envelope glycoprotein precursors by cellular endoproteases is a required step for the activation of viruses. This has been shown for Sendai virus protein F [10] and avian influenza virus haemagglutinin glycoproteins [11]. Results of work on Newcastle virus [12], Rous Sarcoma virus [13] and other viruses have shown that the endoproteolytic cleavage of envelope glycoprotein precursors is also a required step for the acquisition of fusogenic potential and thus of infectious capacity of viral particles [14]. As in the case of viral glycoprotein precursors, the expression of activity of

some bacterial toxins requires maturation by an endoprotease belonging to the family of subtilisin-like protoxin prohormone convertases (SPCs) [15]. This family of SPCs, which shares some structural and functional properties with the yeast endoprotease Kex2p has seven members known at the present time: furin (also known as PACE), PC1 (also known as PC3), PC2, PC4, PACE4, PC5A and B (also known as PC6) and PC7 (also named PC8 or LPC) [16]. The activity of these SPCs is calcium-dependent and results in the cleavage of precursor proteins at the C-terminus of the consensus sequence Lys/Arg-Xaa-Lys/Arg-Arg [17]. For HIV-envelope glycoprotein precursors, this maturation occurs within the constitutive secretory pathway where SPCs such as furin, PC5-B and PC7 are localized [18–20]. On the other hand, PC1, PC2 and in some cases PC5A exert their maximal activity in acidic compartments such as in immature secretory granules within the regulated pathway [18,21]. Among the proprotein convertases, furin has been characterized extensively. Localization studies using Northern-blot, immunocytochemical and *in situ* hybridization analyses in a large variety of tissues and cell lines revealed that furin is expressed ubiquitously, albeit at different levels, depending on the cell type [22]. Thus it has been found primarily in the trans-Golgi membrane [23], but has also been detected on the surface of cells [19] and in the extracellular compartment [24]. Previous studies have shown the capacity of furin to correctly cleave HIV-1 gp160 [25,26] and other proproteins [15,27]. However, the fact that gp160 maturation can be obtained in LoVo and CHO-RPE40 cells which are deficient in furin activity [28,29] supports the hypothesis that other cellular endoprotease(s) could participate in the maturation of HIV-1 viral glycoproteins [25,30–33]. In addition, our group has shown

Abbreviations used: SPC, subtilisin-like protoxin prohormone convertase; VEM, viral glycoprotein maturase; α 1-AP, alpha-1 antitrypsin Pittsburgh variant; α 1-PDX, alpha-1 antitrypsin Portland variant; serpin, serine proteinase inhibitor; FCS, fetal calf serum; TCID, tissue-culture-infective dose; RT, reverse transcriptase; X-Gal, 5-bromo-4-chloroindol-3-yl β -D-galactopyranoside; m.o.i., multiplicity of infection; LTR, long terminal repeat sequence.

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previously that the HIV-1 gp160 precursor is processed at the same Arg-Glu-Lys-Arg site by Kex2p [34], a yeast serine protease belonging to the family of the subtilisin-like endoproteases differing from furin by the fact that a basic amino acid in position P4 is not required [17]. We have also shown that the lymphocyte endoprotease responsible for gp160 maturation seems to be calcium-dependent [35]. However, Kido et al. [36] purified an endoprotease from Molt-4 cells, named viral glycoprotein maturase (VEM), that does not belong to the family of subtilisin-like endoproteases, is calcium-independent and correctly matures HIV-1 gp160 *in vitro*. It is notable that no direct evidence is yet available for the ability of VEM to process this precursor *ex vivo*.

Biological activation of many circulating plasma-protein precursors is physiologically regulated by specific protease inhibitors which account for up to 10% of plasma proteins [37]. They control a variety of critical events associated with connective-tissue turnover, coagulation, fibrinolysis and inflammatory reactions. In addition, at least one of the inhibitors, alpha-2 macroglobulin, plays an important secondary role in backing up the primary function of the other inhibitors [37]. Many plasma inhibitors are reported to be congenitally deficient in specific individuals. When this occurs, there is an associated development of specific disease, as in the case of alpha-1 antitrypsin. The function of alpha-1 antitrypsin is known because its genetic deficiency leads to the development of severe bleeding disorders and pulmonary emphysema [38]. In fact, alpha-1 antitrypsin, a 53-kDa glycoprotein, is the natural and physiological inhibitor of neutrophil elastase [37]. After a natural mutation of Met³⁵⁸ to Arg at the active site, the mutant produced, named alpha-1 antitrypsin Pittsburgh variant (α 1-AP), lost the ability to inhibit elastase and became a powerful inhibitor of thrombin [39]. This observation opened the way to engineering mutations of the active site. Considering the importance of arginine residues in positions P1 and P4 in substrates recognized by furin and other SPCs in general, Anderson et al. [40] introduced a second mutation at position 356 of α 1-AP by site-directed mutagenesis. The Ala³⁵⁶ → Arg α 1-AP mutant, known as alpha-1 antitrypsin Portland variant (α 1-PDX), creates an SPC-recognition site with high affinity for furin and PC5 [15,20,30,32,40] as well as significant affinity for PC7; in addition, it inhibits these activities *in vitro* and *ex vivo* [20,30,32]. Interest in controlling the activation by SPCs of several pathogenic agents has led to the development of peptides mimicking the consensus sequence recognized by SPCs [25–27] or proteins that can specifically block these endoproteases. Protein inhibitors include several natural and bioengineered serine proteinase inhibitors (serpins). A representative native serpin is PI8 [41], a protein whose reactive site contains two furin consensus sites, Arg-Asn-Ser-Arg³³⁹ and Arg³³⁹-Cys-Ser-Arg³⁴². Even though this cytosolic inhibitor is not expected to meet the furin inside the cell, it exhibits a potent inhibitory activity towards this convertase. In addition to this native serpin and α 1-PDX, using site-directed mutagenesis two other engineered inhibitors of furin-like enzymes have been reported: (i) α 2-macroglobulin mutant, Gly-Phe-Tyr-Glu³⁸⁶ to Arg-Ser-Lys-Arg³⁸⁶ [42], and (ii) the turkey ovomucoid third-domain mutant, Ala-Cys-Thr-Leu¹⁸ to Arg-Cys-Lys-Arg¹⁸ [43], the reactive-site loop of which contains the minimum furin-recognition sequence Arg-Xaa-Xaa-Arg [12].

We conducted the present study to evaluate the extent of the implication of the SPC-related endoproteases during HIV-1 infection using the α 1-PDX serpin variant. This was tested on a recently developed CD4-positive Jurkat cell line stably expressing α 1-PDX in which it was shown clearly that this inhibitor effectively blocks intracellular Notch I-receptor processing by furin-like enzymes [44]. We report here that the α 1-PDX serpin

variant significantly impaired HIV-1 infection, since the viral replication was reduced and delayed as a consequence of inhibition of gp160 precursor processing. We suggest that proteases sensitive to α 1-PDX, presumably furin and/or related proteases, are implicated in this process.

MATERIALS AND METHODS

Cells

Jurkat cells stably transfected with full-length α 1-PDX cDNA cloned into the pcDNA3 vector (Invitrogen; named J-PDX) or with the empty pcDNA3 vector (named J-pcDNA3) were established as described previously [44]. Lymphoid cell lines, including 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 fetal calf serum (FCS) (ATGC, Orleans, France), 2 mM glutamine and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin). For J-pcDNA3 and J-PDX cells, 0.26 mg/ml neomycin was added to the culture medium. HeLa cells, a gift from Dr P. Charneau (Pasteur Institute, Paris, France), are stably transfected with the bacterial *lacZ* gene under the control of HIV-1 LTR (long terminal repeat sequence), and with human CD4 (HeLaCD4-LTR/ β -gal). HeLaCD4-LTR/ β -gal cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 1 mg/ml neomycin.

Viruses

Wild type HIV-1_{Lai} was obtained from Diagnostic Pasteur (Marnes La-Coquette, France) as a supernatant from infected cells of the lymphocytic CEM cell line containing 10⁵ tissue-culture-infective doses (TCID₅₀)/ml. For titration of HIV-1, 10⁵ CEM cells were cultured in 200 µl of RPMI 1640 medium in wells of flat-bottomed 96-well tissue-culture plates. Serial 1/10 dilutions (10 µl) of HIV-1 initial stock were added to six replicates. After 6 days, the CEM culture supernatants were screened for the presence of reverse transcriptase (RT) activity as described below. The infectivity titre of the HIV-1 stock (TCID₅₀) was expressed as the viral dose required to infect 50% of the six replicate samples. TCID₅₀ was calculated according to the procedure of Reed and Muench (reviewed in [45]). The vaccinia virus expression vector recombinant for HIV-1_{Lai} gp160 (VV.TG.9-1, VV-gp160) was a gift from Transgene (Strasbourg, France).

HIV-1 infection

J-PDX and J-pcDNA3 cells (10⁶) were incubated with a titrated HIV-1 viral stock diluted to 1/100 and 1/1000 for 1 h at 37 °C. After washing and eliminating unbound viral particles, cells were cultured for 3 weeks in 24-well plates at 37 °C in 1 ml of RPMI medium supplemented with 10% FCS, glutamine and antibiotics. Every 3 days, half of the supernatant was changed and aliquots were tested for subsequent determination of viral production using RT assay.

RT assay

The RT-activity microassay involves the direct measurement of RT activity in 50 µl of HIV-1-infected cell-culture supernatants. The procedure and the buffers were the same as those described by Schwartz et al. [46]. Briefly, 10 µl of solution A (0.5 M KCl/50 mM dithiothreitol/0.5% Triton X-100), 40 µl of buffer

B (10 mM EGTA in 0.5 M Tris/HCl, pH 7.8), 1 μ l of 0.5 M $MgCl_2$, 3 μ l of [3H]dTTP (2'-deoxythymidine 5'-bisphosphate; Amersham), 10 μ l of poly(rA)-poly(dT) at 5 absorbance units/ml and 16 μ l of water were added to the culture supernatants in the wells. After 1 h at 37 °C, the reaction was stopped by adding 20 μ l of 12 mM $Na_4P_2O_7$ in 60% trichloroacetic acid. After 15 min at 4 °C, precipitates were filtered on skatron filters and radioactivity was determined by liquid scintillation spectrometry. Assays were carried out in triplicate in two independent experiments.

Transactivation assay

HeLaCD4-LTR/ β -gal cells were used to quantify the amount of infectious viruses produced by Jurkat cells in the presence of α 1-PDX (J-PDX) or in its absence (J-pcDNA3). By monitoring over a single viral replication cycle (20 h), this assay enabled us to determine the ability of *Tat* produced to activate the expression of the *lacZ* gene. In this assay, cell supernatants of equal RT activity (50 μ l) from J-PDX- and J-pcDNA3-infected cells were added to 2×10^4 HeLaCD4-LTR/ β -gal cells in 96-well plates, and incubated for 1 h at 37 °C. Unbound virus was removed by washing and the cells were cultivated in Dulbecco's modified Eagle's medium containing 10% FCS, glutamine and antibiotics. Cells were then washed twice with PBS (0.5 mM $MgCl_2$ /1 mM $CaCl_2$), fixed with 0.5% glutaraldehyde for 10 min, and washed twice with the same buffer. The cells were then incubated for 3 h in a reaction mixture containing 1 mg/ml β -galactosidase substrate (X-Gal, 5-bromo-4-chloroindol-3-yl β -D-galactopyranoside) in PBS containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM $MgCl_2$. X-Gal had been dissolved previously in DMSO at 40 mg/ml. The enzymic reaction was stopped by removing the X-Gal reaction mixture. Stained cells corresponding to infected cells were counted microscopically.

Syncytium formation

Syncytium formation was assayed according to the following protocols. (i) J-pcDNA3 or J-PDX cells (10^6) were infected with VV-gp160 (VV.TG.9-1) at a multiplicity of infection (m.o.i.) of 20 plaque-forming units/ml. After 1 h of incubation at 37 °C, unbound virus was eliminated by washing and the cells were cultured at 37 °C. After 24 h, multinucleated cells were observed. (ii) J-pcDNA3 or J-PDX cells (10^6) were infected with 50 μ l of HIV-1_{Lai} viral stock as described above. After 3 days, 2×10^4 J-pcDNA3 or J-PDX cells were co-cultured with 8×10^4 CD4-positive uninfected Molt-4 cells at 37 °C. After 18 h, syncytia were observed microscopically.

RT-PCR

RT-PCR was performed to amplify the HIV-1 gp160 sequence in the vicinity of the maturation site that contains nucleotides +7271 to +7902. Briefly, HIV-1 RNA was extracted from equal volumes of cell-free supernatants collected from infected J-pcDNA3 and J-PDX cells by using the Qiamp viral RNA Mini kit (Qiagen). The cDNA-synthesis reaction primed by random hexamers was performed using the First-Strand cDNA synthesis kit (Pharmacia Biotech). Then, cDNA was assayed by PCR with 100 ng of a sense primer (5'-GCA-CCC-ACC-AAG-GCA-AAG-AGA-3') and 100 ng of an antisense primer (5'-GGT-GAA-TAT-CCC-TGC-CTA-ACT-3'). PCR was performed for 35 cycles at 94, 60 and 72 °C for 1 min at each temperature. The products were resolved on a 1% agarose gel, stained with

ethidium bromide, and visualized on a UV transilluminator. The positive control was HIV-1_{Lai} gp160 cDNA ligated into the pcDNA3 vector and cDNA obtained from the HIV-1 stock used for the initial experiments. Total RNAs obtained after triZol extraction from uninfected J-PDX or J-pcDNA3 cells were used as negative control. In addition, water was used instead of RNA or cDNA to determine whether cross-contamination was likely to occur. The sequencing of the gp160 portion around the cleavage site of the viruses obtained from J-PDX and J-pcDNA3 cells was performed by ligating amplified cDNA into the PCR 2.1 vector according to the TA cloning method (Invitrogen). Nucleotide sequences were determined using an automated sequencer.

Analysis of gp160 maturation

Intracellular HIV-1 gp160 processing was analysed at various times in Jurkat cells infected with gp160 recombinant vaccinia virus or after infection with HIV-1_{Lai}.

Analysis of overexpressed gp160 maturation

For this purpose, 10^7 J-pcDNA3 and J-PDX cells were infected with recombinant VV-gp160 (VV.TG.9-1) at an m.o.i. of 20 pfu/cell. After 1 h, cells were harvested by low-speed centrifugation and resuspended in 1 ml of fresh culture medium. Infected cells were then incubated at 37 °C in a humidified CO₂ atmosphere. After centrifugation, supernatants were collected and stored at -20 °C until they were assayed. The cells in the pellet were lysed in 200 μ l of lysis buffer (50 mM Tris/HCl, pH 7.4/0.15 M NaCl/1% Triton X-100/10 mM EDTA/2 mM PMSF) for 15 min at 4 °C and centrifuged at 16000 g. Supernatants were heated for 5 min at 100 °C in 4 $\times$ Laemmli buffer (0.5 M Tris/HCl, pH 6.8/10% glycerol/2% SDS/5% mercaptoethanol/0.05% Bromophenol Blue). Proteins were then separated on 8% acrylamide by SDS/PAGE. The fractionated proteins were transblotted on to a nitrocellulose membrane. The membranes were then saturated with PBS containing 5% non-fat milk for 1 h at room temperature. After washing, the membranes were incubated for 3 h with a 1/500 dilution of human serum from a seropositive patient. After further washing, membranes were incubated for 1 h with peroxidase-coupled rabbit anti-human antibodies (Dako, Trappes, France). After washing, the peroxidase substrate diaminobenzidine was added. The analysis of gp120 secreted in cell supernatants was investigated by immunoprecipitation using Protein A-Sepharose. Briefly, 500 μ l of cell supernatant were incubated with the HIV-1-positive serum diluted to 1/500 and Protein A-Sepharose overnight at 4 °C with shaking. After washing to eliminate unbound proteins, Protein A-anti-gp120-gp120 complexes were analysed by SDS/PAGE and Western blotting as described above.

Analysis of the maturation of gp160 in HIV-1-infected J-PDX and J-pcDNA3 cells

Cells (10^7) were infected with 50 μ l of the HIV-1 stock diluted to 1/10. On the 13th day post-infection, cells were centrifuged and the pellet lysate and cell-free supernatants were analysed for gp160 processing by Western blotting as described above.

RESULTS

The maturation of envelope glycoprotein gp160 to gp120 and gp41 of HIV-1 virus by cellular endoprotease(s) is a required step in the viral cycle; if this step is blocked, the resulting virus is no

Figure 1 Effect of $\alpha 1$ -PDX on HIV-1 replication

J-PDX (○) and J-pcDNA3 (●) cells (10^6) were infected in 24-well plates with 50 μ l of HIV-1 stock (10^5 TCID₅₀/ml) diluted to 1/100 (A) and 1/1000 (B). Virus and cells were incubated for 1 h at 37 °C. After washing and centrifugation to eliminate unbound virus, the cells were resuspended in 1 ml of RPMI medium containing 10% FCS and cultured for 20 days. Every 3 days, half the cell supernatant was collected and assayed for RT activity. As controls, non-infected cells treated in the same conditions gave an RT activity value of 450 ± 50 c.p.m. The results are the means $\pm$ S.D. of three assays in two independent experiments.

longer infectious [47]. Hence the importance of the development of specific inhibitors of the endoprotease(s) implicated in this maturation. The cell model used to study this step was the CD4-positive Jurkat cell line stably transfected with J-PDX. Controls involved the same Jurkat cell line transfected in the same conditions but with J-pcDNA3. In the initial characterization of J-PDX and J-pcDNA3 cell lines, flow cytometry was used to show that they expressed the same levels of membrane CD4, and the Western-blot assays showed that $\alpha 1$ -PDX was expressed by J-PDX cells (results not shown).

We then analysed viral replication in J-pcDNA3 and J-PDX cells. For this purpose, the cells were infected in the same conditions by HIV-1_{Lai} viral stock at dilutions of 1/100 and 1/1000. Infection was monitored for 3 weeks by sampling cell supernatants at 3-day intervals and determining viral production by assaying RT activity and testing infectious power directly. The results showed that there was a clear-cut difference in viral replication between J-PDX and J-pcDNA3 cells, shown by a peak of RT activity that was 5–6-fold lower in the presence of the $\alpha 1$ -PDX protein (Figure 1). The second visible effect of $\alpha 1$ -PDX was the difference in replication kinetics, as shown by a delay in

Figure 2 Comparison of HIV-1 infectivity produced in the presence (J-PDX) or absence (J-pcDNA3) of $\alpha 1$ -PDX

(A) HeLaCD4-LTR/ β -gal cells were infected for one viral cycle (20 h) with equal RT activity in infected cell supernatants collected at the peak of RT activity as described in the Materials and methods section. Cell infection and viral replication were correlated directly with the transactivation of the *lacZ* gene by the early translated HIV *Tat* gene. β -Galactosidase activity was visualized by incubating cells with the substrate X-Gal which stained cells blue after being degraded. (B) The same amount of HIV-1 virus (equal RT) produced at the peak of RT activity was used to infect naive Jurkat cells for 3 weeks under the same conditions as described in Figure 1. Viral replication was assessed by monitoring RT activity in cell supernatants collected at 3-day intervals. The results are the means $\pm$ S.D. of three assays in two independent experiments. Black bar and ●, J-pcDNA3; white bar and ○, J-PDX.

the appearance of the peak of RT activity. Of note is that the peak of RT activity with the virus diluted to 1/1000 (130000 c.p.m.) was greater than that obtained with a 1/100 dilution (100000 c.p.m.; Figure 1). This apparent discrepancy could be due to the greater cytotoxic effects of the virus at the higher m.o.i., which could lead to rapid killing of HIV-1-infected replicating cells.

It was then asked if the virus produced in J-PDX cells had the same infectious power as that produced in J-pcDNA3 cells. Equivalent virus doses (equal RT activity) of HIV-1 produced in the presence of $\alpha 1$ -PDX (J-PDX) or in its absence (J-pcDNA3) were used to infect HeLaCD4-LTR/ β -gal cells expressing human CD4 and stably transfected with a plasmid containing the *lacZ* gene under the control of the LTR promoter of HIV-1. Viral replication was analysed over a single viral cycle, i.e. 20 h after infection. The results (Figure 2A) showed that the HIV-1 produced in the presence of $\alpha 1$ -PDX was nearly 5-fold less infectious. This decreased infectious power of HIV-1 produced in

Figure 3 Effect of α 1-PDX on the induction of syncytia after cell infection with either gp160-recombinant vaccinia virus or after HIV-1 infection

J-pcDNA3 and J-PDX cells (10^6) were infected with VV-gp160 at an m.o.i. of 20 pfu/cell. After adsorption and elimination of unbound virus, multinucleated cells were investigated microscopically after 24 h. (A) and (B) correspond to syncytia in J-pcDNA3 and J-PDX infected with VV-gp160, respectively. (C) and (E) correspond respectively to J-pcDNA3 and J-PDX cells infected with 50 μ l of HIV-1 stock diluted to 1/10, and co-cultured with Molt-4 cells for 18 h at 37 °C. (D) and (F) correspond to uninfected Molt-4 and J-pcDNA3 cells, respectively.

J-PDX cells were characterized further during multiple viral cycles by infecting naive Jurkat cells for 3 weeks with equal viral doses collected in the presence or absence of α 1-PDX by using the same protocol as in Figure 1. Again, the infection with the HIV-1 collected from J-PDX cells led to 4-fold lower RT activity (Figure 2B). The data in both assays are strongly consistent and reflect a clear defect in HIV-1 infectivity in the presence of α 1-PDX serpin.

The α 1-PDX protein may interfere with viral replication by blocking the maturation of HIV-1 gp160 by furin-like endoproteases. If this is the case, the virions produced should be incapable of penetrating the host cells since fusion between the viral envelope and the cell membrane is blocked. We assessed this possible mechanism by two experiments. In the first, J-PDX and J-pcDNA3 cells were infected with the same dose of envelope glycoprotein precursor (gp160)-recombinant vaccinia virus. After 24 h of infection, cells were observed microscopically. A large number of syncytia was observed in J-pcDNA3 cells (Figure 3A) as compared with J-PDX cells, which aggregated without leading to syncytia formation (Figure 3B). In the second experiment, J-PDX and J-pcDNA3 cells were initially infected with HIV-1_{Lai} viral stock for 3 days and then co-cultured with 4-fold of Molt-4 cells. After 20 h of co-culture, large syncytia were observed with J-pcDNA3 (Figure 3C). In contrast, this phenomenon was attenuated in J-PDX cells, although several smaller and less numerous syncytia were observed (Figure 3E). No multinucleated

Figure 4 Effect of α 1-PDX on gp160 processing after VV-gp160 infection

J-pcDNA3 and J-PDX cells (10^7) were infected with VV-gp160 at an m.o.i. of 20 pfu/cell. The process was stopped at the indicated times post-infection and equal amounts of proteins from cell lysates (A) or supernatants (B) from infected J-pcDNA3 and J-PDX cells were analysed by SDS/PAGE and Western blotting. gp160-related proteins were immunodetected with a human serum containing anti-HIV-1 antibodies. Lanes 1, HIV-1-infected HeLa CD4-positive cells (positive control). Lanes 2 and 5, uninfected J-pcDNA3 and J-PDX cells, respectively. Lanes 3 and 4, VV-gp160-infected J-PDX and J-pcDNA3 cells at 24 h post-infection, respectively. Lanes 6 and 7, VV-gp160-infected J-PDX and J-pcDNA3 cells at 48 h post-infection, respectively. Secreted gp120 was analysed after immunoprecipitation using anti-gp120 antibodies and Protein A-Sepharose.

cells were observed in the uninfected Molt-4 (Figure 3D) and J-pcDNA3 cells (Figure 3F).

To exclude the possibility that the inhibition of HIV-1 replication and syncytium formation in J-PDX cells were due to genetic alterations in the HIV-1 cleavage site (KAKRRVVQREKR; where underlining indicates potential cleavage sites) in the presence of α 1-PDX serpin, we performed its sequencing from both infected J-PDX and J-pcDNA3 cells. No mutations were detected in the cleavage site, which lead us to suggest that α 1-PDX interferes with gp120/gp41-dependent membrane fusion through the inhibition of gp160 maturation and allowed us to analyse the rate of gp160 processing in the presence and absence of α 1-PDX.

This was addressed by infecting J-PDX and J-pcDNA3 cells with gp160 recombinant vaccinia virus (VV-gp160, VV.TG.9-1) for 24 and 48 h. Processing of gp160 was analysed in cell lysates and in supernatants of infected cells. Following SDS/PAGE and electrotransferral, gp160-related proteins were detected with human anti-HIV-1 antibodies from a seropositive patient. The overexpression experiments showed no difference in the rate of gp160 production between J-pcDNA3 and J-PDX cells, indicating that the α 1-PDX protein did not impair VV-gp160 expression, whereas the densitometric measurements showed that the uncleaved gp160/secreted gp120 ratio was 3-fold higher at 24 h (Figures 4A and 4B; lanes 3 versus 4) and 2.5-fold higher at 48 h (Figures 4A and 4B; lanes 6 versus 7) in J-PDX cells than in J-pcDNA3 cells (Table 1). These data indicate that protease(s) sensitive to the α 1-PDX inhibition play the major role in the

Table 1 The cleavage ratio (uncleaved gp160/secreted gp120) in VV-gp160-infected J-PDX and J-pcDNA3 cells in Figure 4Means $\pm$ S.D. are shown.

Ratio	Time post-infection	
	24 h	48 h
gp160 _{J-PDX} /gp120 _{J-PDX}	6 $\pm$ 0.5	2.5 $\pm$ 0.2
gp160 _{J-pcDNA3} /gp120 _{J-pcDNA3}	2.1 $\pm$ 0.4	1.2 $\pm$ 0.1

Figure 5 Effect of α 1-PDX on gp160 processing after HIV-1 infection

J-pcDNA3 and J-PDX cells (10^7) were infected with 50 μ l of HIV-1 viral stock. After the 13th day post-infection (peak of RT activity), cells and supernatants were analysed by Western blotting using human HIV-1 seropositive serum. Lanes 1 and 4, immunoprecipitated gp120 from HIV-1-infected J-pcDNA3 and J-PDX cell supernatants, respectively. Lanes 2 and 3, equal amounts of viral proteins, as assessed by p24 measurement, in cell lysates from infected J-pcDNA3 and J-PDX cells, respectively.

gp160 maturation process. Therefore, our results showed a direct correlation between gp160 processing inhibition and the absence of syncytium induction in VV-gp160-infected J-PDX cells.

To test that the inhibition of HIV-1 replication was correlated specifically with the inhibition of gp160 processing as suggested by the overexpression experiments, we analysed the extent of gp160 processing in HIV-1-infected cells. For this purpose, HIV-1-infected J-PDX and J-pcDNA3 cell lysates with equal amounts of viral protein, obtained with equal amounts of p24 antigen, were analysed for gp160-related products. Only the extent of gp160 processing was altered in HIV-1-infected J-PDX cells. The uncleaved gp160/secreted gp120 ratio was 5-fold higher in J-PDX cells than in J-pcDNA3 cells (Figure 5, lanes 3 and 4 versus 1 and 2). Indeed, gp120 secretion was 4-fold lower in

J-PDX cells (Figure 5, lane 4) compared with J-pcDNA3 cells (Figure 5, lane 1). Also, cellular membrane-bound gp120 was reduced in J-PDX cells and coincided with a greater accumulation of uncleaved gp160 (Figure 5, lane 3). These data indicated again that the effect of α 1-PDX protein was significantly correlated with a significant defect in gp160 processing but not to inhibition of HIV-1 protein expression. Such a defect can explain further the lower ability of the virus to replicate in J-PDX cells. In these experiments, we were unable to quantify cellular membrane-bound gp41 envelope-cleavage product since it has been thought to co-migrate with HIV-1 41-kDa gag-encoded protein intermediate [47]. This could provide an explanation for our observation that the reduction in gp41 expression was much less than that of gp120 in HIV-1-infected J-PDX cells. Conversely, when gp160 was expressed alone in the overexpression experiments, such an observation was not made, thus supporting our explanation (Figure 4). Our findings therefore demonstrate a functional relationship between α 1-PDX inhibition of gp160 processing and the reduction of both viral replication and cytopathogenicity.

DISCUSSION

The viral-envelope glycoprotein of several viruses is synthesized in the form of a precursor that requires maturation by a cellular endoprotease(s) in order to yield infectious viruses. In the case of HIV-1, any block in this maturation step yields viruses that are not infectious [47]. This explains the importance of developing specific inhibitors that can control endoprotease activities in cases of a genetic deficit or following infection by pathogenic agents (bacteria or viruses). In this context, Hallenberger et al. [26] tested peptide inhibitors mimicking the cleavage site recognized by SPC to block the maturation of HIV-1 gp160 by overexpressed furin and endogenous cellular endoprotease(s). These experiments were carried out with peptides in a cell system of co-infection with gp160-recombinant (VV-gp160) and furin-recombinant (VV-furin) vaccinia virus and upon HIV-1 infection of a CD4-positive cell line. Penetration of peptides into cells is facilitated by the presence of a decanoyl (dec) group at the N-terminal extremity, and 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. The use of dec-Arg-Glu-Lys-Arg-cmk and dec-Arg-Ala-Lys-Arg-cmk peptides in these experiments showed that the inhibition of intracellular gp160 maturation and of viral replication were of the order of 60–70% [26]. Similar results have been reported by others using analogues of these peptides [25]. However, the major limitation of chloromethylketone peptides as SPC inhibitors is related to their cytotoxic effects by interference with the biosynthesis of many important cellular proteins when used at active concentrations [40–43]. In this framework, we decided to assess the capacity of α 1-PDX, a bioengineered variant, to interfere with HIV-1 replication. We determined the capacity of α 1-PDX to inhibit directly the viral replication of HIV-1, maturation of the envelope glycoprotein precursor and induction of the cytopathic effects. The experimental procedure involved the use of CD4-positive Jurkat cells stably transfected with a plasmid containing the α 1-PDX cDNA. The same cells transfected with the same plasmid but without the gene of interest were used as controls. The results describe clearly the inhibitory effect of α 1-PDX on viral replication in terms of both kinetics and viral load. Indeed, α 1-PDX was able to inhibit HIV-1 replication and to delay it. It was expected that some viral production persists in J-PDX cells, since the assembly and budding of viral particles can occur with immature precursors, as

reported by McCune et al. [48]. These authors showed that when the gp160 cleavage site was mutated to a site specific for chymotrypsin, non-infectious viral particles were produced whose electron-microscopic morphology was similar to and indistinguishable from that of wild-type particles. However, the produced viruses became infectious only after chymotrypsin was added to the medium [48]. We then asked whether HIV-1 produced in J-PDX cells had the same infectious capacity as the virus from J-pcDNA3 cells, and found that the viral infectivity was impaired in the presence of α 1-PDX protein expression.

To determine the level at which α 1-PDX acted on viral replication, Jurkat cells that did or did not express α 1-PDX and were infected previously with gp160-recombinant vaccinia virus or with HIV-1 and co-cultured with uninfected Molt-4 lymphocytes, were tested for their capacity to form syncytia. In VV-gp160 infection, only cells infected in the absence of α 1-PDX formed syncytia. In contrast, when J-pcDNA3 or J-PDX cells were infected with HIV-1 and co-cultured with Molt-4, large syncytia were observed in J-pcDNA3 cells compared with J-PDX cells, which exhibited a much weaker capacity for syncytium induction. These structures result from membrane fusion, the mechanism by which HIV penetrates the cytoplasm of host cells and which occurs only if there is maturation of gp160. In fact, comparison of the gp160/gp120 ratio in VV-gp160- and HIV-1-infected J-pcDNA3 and J-PDX cells showed clearly a significant defect in gp160 processing while no alteration was observed in the expression level of the total viral proteins.

It is evident from the gp160-expression experiments that processing enzymes are not completely inhibited in the presence of α 1-PDX serpin. The α 1-PDX protein has been reported to exert its inhibitory activity in the trans-Golgi network, hence raising the possibility that gp160 maturation might have occurred after exit from the trans-Golgi network in J-PDX cells. Recent studies support this hypothesis: (i) gp160-processing activity was detected in trans-Golgi-network-derived vesicles [49] and (ii) Kido and co-workers reported that gp160 processing could occur by a Ca^{2+} -independent enzyme VEM [36] and/or extracellularly by plasmin [50]. On the other hand, the lack of syncytium induction in J-PDX cells could be due to the much lower rate of gp120 production and/or to the formation of hetero-oligomers between uncleaved gp160 and gp120 on the cell surface and on the HIV-1 envelope. As a consequence, these hetero-oligomers may be less functional in inducing fusion and virus penetration in target cells.

It has been shown recently that α 1-PDX inhibits the maturation of the precursors pro7B2 and POMC (prepro-opiomelanocortin) by different convertases in the constitutive secretory route [30]. The enzymic mechanism of this inhibition was investigated recently. Jean et al. [15] demonstrated the selectivity of α 1-PDX to furin and PC5A. The *in vitro* interaction between bacterially produced α 1-PDX and furin resulted in a stable complex as judged by its resistance to temperature and SDS denaturation. Under these conditions *in vitro* α 1-PDX seems to act as a suicide substrate. Both *in vitro* and *ex vivo* experiments demonstrated that upon overexpression of α 1-PDX in cell lines this cellularly produced serpin can inhibit the processing activity of furin, PC5, PACE4 and PC7 [30,32]. Indeed, in our hands the *in vitro* inhibitory IC_{50} value of α 1-PDX overproduced in mammalian cells is only 3-fold for PC7 versus furin (N. G. Seidah and J. S. Munzer, unpublished work). Therefore, overexpression of this serpin in Jurkat cells, although more specific for furin and PC5A, can still inhibit considerably the activity of endogenous PC7. Since both furin and PC7 could play a role in the processing of gp160 [31,33], the overproduced serpin would have the combined effect of interfering with the membrane-bound SPCs that are

found within the constitutive secretory pathway [16]. Therefore, the data of the present work suggest that furin-like protease(s) are implicated predominantly in the HIV-1 gp160-processing mechanism, and are in accordance with the previous reports that have localized furin and PC7 in the CD4+ lymphocyte.

As a result of the specificity of α 1-PDX as a suicide substrate for prohormone convertases and its inhibitory effect on HIV-1 replication by interfering with envelope precursor processing and thus with membrane fusion and viral infectivity, α 1-PDX could have potential therapeutic applications, principally in the control of SPC-dependent viral and bacterial infections.

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REFERENCES

- Wiley, R. L., Bonifacio, J. S., Potts, B. J., Martin, M. A. and Klausner, R. D. (1988) Biosynthesis, cleavage, and degradation of the human immunodeficiency virus 1 envelope glycoprotein gp160. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9580–9584
- Sattentau, Q. J. and Moore, J. P. (1991) Conformational changes induced in the human immunodeficiency virus envelope glycoprotein by soluble CD4 binding. *J. Exp. Med.* **174**, 407–415
- Fenouillet, E., Clerget-Raslain, B., Gluckman, J. C., Guétard, D., Montagnier, L. and Bahraoui, E. (1989) Role of N-linked glycans in the interaction between the envelope glycoprotein of human immunodeficiency virus and its CD4 cellular receptor. Structural enzymatic analysis. *J. Exp. Med.* **169**, 807–821
- Klatzmann, D., Champagne, E., Chamaret, S., Gruest, J., Guétard, D., Hercend, T., Gluckman, J. C. and Montagnier, L. (1984) T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. *Nature (London)* **312**, 767–768
- Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P. D., Wu, L., Mackay, C. R., Larosa, G. and Newman, W. et al. (1996) The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* **85**, 1135–1148
- Feng, F., Broder, C. C., Kennedy, P. E. and Berger, E. A. (1996) HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272**, 872–877
- Moore, J. P. (1997) Coreceptors: implications for HIV pathogenesis and therapy. *Science* **267**, 51–52
- Kowalski, M., Potz, J., Basiripour, L., Dorfman, T., Coh, W. C., Terwilliger, F., Dayton, A., Rosen, C., Haseltine, W. and Sodroski, J. (1987) Functional regions of the envelope glycoprotein of human immunodeficiency virus type 1. *Science* **237**, 1351–1355
- Freed, E. O., Myers, D. J. and Risser, R. (1989) Mutational analysis of the cleavage sequence of the human immunodeficiency virus type 1 envelope glycoprotein precursor gp160. *J. Virol.* **63**, 4670–4675
- Homma, M. and Ouchi, M. (1973) Trypsin action on the growth of Sendai virus in tissue culture cells. 3. Structural difference of Sendai viruses grown in eggs and tissue culture cells. *J. Virol.* **12**, 1457–1465
- Garten, W., Steineke, A., Shaw, E., Wikstrom, P. and Klenk, H.-D. (1989) Inhibition of proteolytic activation of influenza virus hemagglutinin by specific chloroalkyl ketones. *Virology* **172**, 25–31
- Sakaguchi, T., Matsuda, Y., Kiyokage, R., Kawahara, N., Kiyotani, K., Katunuma, N., Nagai, Y. and Yoshida, T. (1991) Identification of endoprotease activity in the trans Golgi membranes of rat liver cells that specifically processes *in vitro* the fusion glycoprotein precursor of virulent Newcastle disease virus. *Virology* **184**, 504–512
- Dong, J., Dubay, J. W., Perez, L. G. and Hunter, E. (1992) Mutations within the proteolytic cleavage site of the Rous sarcoma virus glycoprotein define a requirement for dibasic residues for intracellular cleavage. *J. Virol.* **66**, 865–874
- Watanabe, M., Hirano, A., Stenglein, S., Nelson, J., Thomas, G. and Wong, T. C. (1995) Engineered serine protease inhibitor prevents furin-catalyzed activation of the fusion glycoprotein and production of infectious measles virus. *J. Virol.* **69**, 3206–3210
- Jean, F., Stella, K., Thomas, L., Liu, G., Xiang, Y., Reason, A. J. and Thomas, G. (1998) α 1-antitrypsin Portland, a bioengineered serpin highly selective for furin: application as an antipathogenic agent. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 7293–7298

- 16 Seidah, N. G., Mbikay, M., Marcinkiewicz, M. and Chrétien, M. (1998) The mammalian precursor convertases: paralogs of the subtilisin/kexin family of calcium-dependent serine proteinases, in *Proteolytic and Cellular Mechanisms in Prohormone Processing* (Hook, V., ed.), pp. 49–76. R. G. Landes Company, Georgetown
- 17 Barr, P. J. (1991) Mammalian subtilisins: the long sought dibasic processing endoproteases. *Cell* **66**, 1–3
- 18 De Bie, I., Marcinkiewicz, M., Malide, D., Lazure, C., Nakayama, K., Bendayan, M. and Seidah, N. G. (1996) The isoforms of protein convertase PC5 are sorted to different subcellular compartments. *J. Cell. Biol.* **135**, 1261–1275
- 19 Molloy, S. S., Thomas, L., VanSlyke, J. K., Stenberg, P. E. and Thomas, G. (1994) Intracellular trafficking and activation of the furin proprotein convertase: localization to the TGN and recycling from the cell surface. *EMBO J.* **13**, 18–33
- 20 Munzer, J. S., Basak, A., Zhong, M., Mamarbachi, M., Hamelin, J., Savaria, D., Lazure, C., Benjannet, S., Chrétien, M. and Seidah, N. G. (1997) *In vitro* characterization of the novel proprotein convertase PC7. *J. Biol. Chem.* **272**, 19672–19681
- 21 Malide, D., Seidah, N. G., Chretien, M. and Bendayan, M. (1995) Electron microscopic immunocytochemical evidence for the involvement of the convertases PC1 and PC2 in the processing of proinsulin in pancreatic beta-cells. *J. Histochem. Cytochem.* **43**, 11–19
- 22 Seidah, N. G., Chrétien, M. and Day, R. (1994) The family of Subtilisin/Kexin like pro-protein and pro-hormone convertases: divergent or shared functions. *Biochimie* **17**, 197–209
- 23 Bresnahan, P. A., Leduc, R., Thomas, L., Thorner, J., Gibson, H. L., Brake, A. J., Barr, P. J. and Thomas, G. (1990) Human fur gene encodes a yeast KEX2-like endoprotease that cleaves pro-beta-NGF *in vivo*. *J. Cell. Biol.* **111**, 2851–2859
- 24 Vidricaire, G., Denault, J.-B. and Leduc, R. (1993) Characterization of a secreted form of human furin endoprotease. *Biochem. Biophys. Res. Commun.* **195**, 1011–1018
- 25 Decroly, E., Vandenbranden, M., Ruyschaert, J.-M., Cogniaux, J., Jacob, G. S., Howard, S. C., Marshall, G., Kompelli, A., Basak, A. and Jean, F. et al. (1994) The convertases furin and PC1 can both cleave the human immunodeficiency virus HIV-1 envelope glycoprotein gp160 into gp120 (HIV-1 SU) and gp41 (HIV-1 TM). *J. Biol. Chem.* **269**, 12240–12247
- 26 Hallenberger, S., Bosh, V., Anglikar, H., Shaw, E., Klenk, H.-D. and Garten, W. (1992) Inhibition of furin-mediated cleavage activation of HIV-1 glycoprotein gp160. *Nature (London)* **360**, 358–361
- 27 Stieneke-Grober, A., Vey, M., Anglikar, H., Shaw, E., Thomas, G., Roberts, C., Klenk, H.-D. and Garten, W. (1992) Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. *EMBO J.* **11**, 2407–2414
- 28 Ohnishi, Y., Shioda, T., Nakayama, K., Iwata, S., Gotoh, B., Hamaguchi, M. and Nagai, Y. (1994) A furin-defective cell line is able to process correctly the gp160 of human immunodeficiency virus type 1. *J. Virol.* **68**, 4075–4079
- 29 Inocencio, N. A., Sucic, J. F., Moehring, J. M., Spence, M. J. and Moehring, T. J. (1997) Endoprotease activities other than furin and PACE4 with a role in processing of HIV-1 gp160 glycoproteins in CHO-K1 cells. *J. Biol. Chem.* **272**, 1344–1348
- 30 Benjannet, S., Savaria, D., Laslop, A., Munzer, J. S., Chrétien, M., Marcinkiewicz, M. and Seidah, N. G. (1997) α 1-Antitrypsin Portland inhibits processing of precursors mediated by proprotein convertases primarily within the constitutive secretory pathway. *J. Biol. Chem.* **272**, 26210–26215
- 31 Decroly, E., Benjannet, S., Savaria, D. and Seidah, N. G. (1997) Comparative functional role of PC7 and furin in the processing of the HIV envelope glycoprotein gp160. *FEBS Lett.* **405**, 68–72
- 32 Vollenweider, F., Benjannet, S., Decroly, E., Savaria, D., Lazure, C., Thomas, G., Chrétien, M. and Seidah, N. G. (1996) Comparative cellular processing of the human immunodeficiency virus (HIV-1) envelope glycoprotein gp160 by the mammalian subtilisin/kexin-like convertases. *Biochem. J.* **314**, 521–532
- 33 Hallenberger, S., Moulard, M., Sordel, M., Klenk, H.-D. and Garten, W. (1997) The role of eukaryotic subtilisin-like endoprotease for the activation of human immunodeficiency virus glycoproteins in natural host cells. *J. Virol.* **71**, 1036–1045
- 34 Moulard, M., Achstetter, T., Kiény, M.-P., Montagnier, L. and Bahraoui, E. (1994) Kex2p: a model for cellular endoprotease processing human immunodeficiency virus type 1 envelope glycoprotein precursor. *Eur. J. Biochem.* **225**, 565–572
- 35 Moulard, M., Montagnier, L. and Bahraoui, E. (1994) Effects of calcium ions on proteolytic processing of HIV-1 gp160 precursor and on cell fusion. *FEBS Lett.* **338**, 281–284
- 36 Kido, H., Kamoshita, K., Fukutomi, A. and Katunuma, N. (1993) Processing protease for gp160 human immunodeficiency virus type 1 envelope glycoprotein precursor in human T4+ lymphocytes. *J. Biol. Chem.* **268**, 13406–13413
- 37 Travis, J. and Salvesen, G. S. (1983) Human plasma proteinase inhibitors. *Annu. Rev. Biochem.* **52**, 655–709
- 38 Carell, R. and Travis, J. (1985) α 1-Antitrypsin and the serpins: variation and countervariation. *Trends Pharmacol. Sci.* **6**, 20–24
- 39 Lewis, J. H., Iammarino, R. M., Spero, J. A. and Hasiba, U. (1978) Antithrombin Pittsburgh: an alpha-1 antitrypsin variant causing hemorrhagic disease. *Blood* **51**, 129–137
- 40 Anderson, E. D., Thomas, L., Hayflick, J. S. and Thomas, G. (1993) Inhibition of HIV-1 gp160-dependent membrane fusion by a furin directed α 1-antitrypsin variant. *J. Biol. Chem.* **268**, 24887–24891
- 41 Dahlen, J. R., Jean, F., Thomas, G., Foster, D. C. and Kiesel, W. (1998) Inhibition of soluble recombinant furin by human proteinase inhibitor 8. *J. Biol. Chem.* **273**, 1851–1854
- 42 Van Rompey, L. V., Ayoubi, T., Van de Ven, W. and Marynen, P. (1997) Inhibition of intracellular proteolytic processing of soluble proproteins by an engineered α 2-macroglobulin containing a furin recognition sequence in the bait region. *Biochem. J.* **326**, 507–514
- 43 Lu, W., Zhang, W., Molloy, S. S., Thomas, G., Ryan, K., Chiang, Y., Anderson, S. and Laskowski, Jr., M. (1993) Arg15-Lys17-Arg18 turkey ovomucoid third domain inhibits human furin. *J. Biol. Chem.* **268**, 14583–14585
- 44 Logeat, F., Bessia, C., Brou, C., Leblat, O., Jarriault, S., Seidah, N. G. and Israël, A. (1998) The Notch1 receptor is cleaved constitutively by a furin-like convertase. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 8108–8112
- 45 Johnson, V. A. and Byington, R. E. (1990) Quantitative assays for virus infectivity, in *Techniques in HIV Research* (Aldovini, A. and Walker, B. D., eds.), pp. 71–76, Stockton Press, New York
- 46 Schwartz, O., Henin, Y., Marechal, V. and Montagnier, L. (1988) A rapid and simple colorimetric test for the study of anti-HIV agents. *AIDS Res. Hum. Retroviruses* **4**, 441–448
- 47 Mervis, R. J., Ahmad, N., Lillehu, E. P., Raum, M. G., Salazar, F. H., Chan, H. W. and Venkatesan, S. (1988) The gag gene products of human immunodeficiency virus type 1: alignment within the gag open reading frame, identification of posttranslational modifications, and evidence for alternative gag precursors. *J. Virol.* **62**, 3993–4002
- 48 McCune, J. M., Rabin, L. B., Feinberg, M. B., Lieberman, M., Mosek, J. C., Reyes, G. R. and Weissman, L. L. (1988) Endoproteolytic cleavage of gp160 is required for the activation of human immunodeficiency virus. *Cell* **53**, 55–67
- 49 Wouters, S., Decroly, E., Vandenbranden, M., Shober, D., Fuchs, R., Morel, V., Leruth, M., Seidah, N. G., Courtoy, P. J. and Ruyschaert, J. M. (1999) Occurrence of an HIV-1 gp160 endoproteolytic activity in low-density vesicles and evidence for a distinct density distribution from endogenously expressed furin and PC7/LPC convertases. *FEBS Lett.* **456**, 97–102
- 50 Okumura, Y., Yano, M., Murakami, M., Mori, S., Towatari, T. and Kido, H. (1999) The extracellular processing of HIV-1 envelope glycoprotein gp160 by human plasmin. *FEBS Lett.* **442**, 39–42

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