Replication of HIV-1 viruses in the presence of the Portland α 1-antitrypsin variant (α 1-PDX) inhibitor

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The Portland α 1-antitrypsin variant (α 1-PDX) inhibits gp160 cleavage into gp120 and gp41 by different prohormone convertases (PCs) including furin, PC5 and PC7. Jurkat cells stably transfected with this inhibitor (J-PDX cells) and, as controls, Jurkat cells transfected with the empty vector (J-pcDNA3) were tested for their susceptibility to HIV-1 infection. We found that HIV-1 replication was significantly impaired in J-PDX cells. However, the analysis of the infectivity of HIV-1 viruses produced in J-PDX cells on different days during the infection indicated that they recovered infectivity starting from 13 days post-infection. The sequencing of viruses collected earlier and later from J-PDX cells revealed no mutations in envelope-glycoprotein precursor (Env) maturation sites or in the N-terminal sequence of gp41 fusion peptide, which plays a key role in membrane fusion. Although conserved mutations were detected at the

C-terminus of the gp41 fusion peptide and ectodomain, the replication of mutant HIV-1 viruses produced on day 20 in J-PDX cells was inhibited at a similar level to wild-type viruses after a second passage in J-PDX cells. We then investigated the expression of the α 1-PDX protein, and found that HIV-1 replication activated its proteolysis since the 54 kDa cleaved form became predominant later on in the infection. In contrast, the expression of PC7, a protein that is transported through the secretory pathway, was unaltered in HIV-1 infected cells. We conclude that recovered HIV-1 infectivity in J-PDX cells was due to a loss of α 1-PDX activity via its extensive processing by intracellular proteases that cleave it through the substrate pathway.

Key words: gp160 mutation, gp160 processing, prohormone convertase, serine endoprotease inhibitor.

INTRODUCTION

The infection process of enveloped viruses requires genome entry into target cells. Viral surface glycoproteins are involved in this key step and control both viral binding and penetration of the virus into the target cell (reviewed in [1]). These glycoproteins are synthesized as inactive precursors and sorted through the constitutive secretory pathway of the infected cells where they are cleaved at the C-terminus of a highly conserved basic consensus sequence (Arg-Xaa-Lys/Arg-Arg; reviewed in [2]). This cleavage leads to the formation of two subunits, surface (SU) and transmembrane (TM) glycoproteins, that remain associated by non-covalent interactions, as in the case of the gp120–gp41 trimers of HIV-1, or through disulphide-bridge formation, e.g. the haemagglutinin 1–haemagglutinin 2 complex of the influenza virus. The infection process requires first that the SU glycoprotein recognizes the host-cell receptors, and then the envelope complex undergoes a conformational change that leads to exposure of the N-terminal fusion peptide of the TM glycoprotein (gp41 for HIV-1), which promotes virus-cell fusion (reviewed in [1]). Therefore the cleavage or maturation of the envelope glycoproteins is a crucial step for the propagation of viral infection [3,4]. Several studies have suggested the participation of several enzymes belonging to the prohormone convertase (PC) family in the maturation process of HIV-1 gp160. These include furin, PC7, PACE4 (paired amino acids-cleaving enzyme 4) and PC5 (reviewed in [2]). In fact, these enzymes recognize the cleavage consensus sequence and cleave it specifically both *in vitro* and upon their concomitant overexpression with gp160. On the other

hand, Ca^{2+} -independent enzymes that have diverged from the PC family have also been reported to specifically cleave gp160 into gp120 and gp41. They include at least two enzymes, virus envelope maturase (VEM), purified from MOLT4 cells [5], and VEM-like protease (VLP), which we purified recently from peripheral blood lymphocytes [6]. It is thus thought that at least two divergent protease families are implicated in the HIV-1 gp160 maturation process.

Until now, two types of molecule that inhibit HIV-1 gp160 processing have been described. The first are chloroalkylketone-derivatized peptides consisting of an Arg-Glu-Lys-Arg core, linked to chloroalkylketone function at the C-terminus, and a lipophilic group, such as decanoyl, at the N-terminus [7]. These molecules have been reported to efficiently inhibit envelope-glycoprotein precursor (Env) maturation and HIV-1 replication [7]. The second type of inhibitor consists of engineered protein inhibitors derived from the serine proteinase inhibitors (serpins), which inhibit their cognate targets by forming a highly stable complex resistant to strong denaturing factors, thus acting as suicide substrates [8,9]. This serpin feature makes them good tools for the design of new protease inhibitors. Anderson et al. [10] exchanged the catalytic site of the α 1-antitrypsin inhibitor for the consensus sequence recognized by convertases, generating the (Arg-355–Arg-358) α 1-antitrypsin Portland variant (α 1-PDX). This serpin variant has been shown to have the capacity to block the syncytium-inducing properties of gp160 and processing into gp120 and gp41 in different cell lines [10–14]. Moreover, α 1-PDX inhibits gp160-mediated cleavage by several members of the PC family, including furin, PC7, PACE4 and

Abbreviations used: D, day; Env, envelope-glycoprotein precursor; PC, prohormone convertase; RT, reverse transcriptase; α 1-PDX, α 1-antitrypsin Portland variant; SU, surface glycoprotein; TM, transmembrane glycoprotein; TCID₅₀, 50% tissue-culture infectious dose.

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PC5/6. In addition, Jean et al. [15] and Dufour et al. [16] have shown that $\alpha 1$ -PDX inhibits furin by forming a highly stable complex that can be detected under reducing conditions.

Although several sets of data are available about the capacity of $\alpha 1$ -PDX to block cleavage of overexpressed gp160 into gp120 and gp41 in different cell lines, little is known about its efficiency in the model of HIV-1 infection. In fact, the HIV-1 genome contains six regulatory genes that can interfere with the expression of different cellular genes (reviewed in [17]). We have reported recently [14] that HIV-1 replication is significantly blocked in Jurkat cells stably transfected with, and stably expressing, $\alpha 1$ -PDX cDNA (J-PDX cells), as compared with Jurkat cells transfected with the empty vehicle vector (J-pcDNA3). The results indicated that the $\alpha 1$ -PDX protein did not interfere with HIV-1 gene expression, but had the capacity to inhibit syncytium formation and HIV-1 gp160 processing into gp120 and gp41 [14].

In the present study, the analysis of HIV-1 viruses collected on different days post-infection in J-PDX cells revealed that they were not very infectious 7 days post-infection, that they recovered infectivity on day (D) 13, and that they had nearly the same infectious capacity on D20 post-infection as viruses produced in J-pcDNA3 cells. We thus characterized the origin of this escape by analysing the *env* sequence around the cleavage sites, and the rate of $\alpha 1$ -PDX expression on different days of the infection. The data show that HIV-1 replication in J-PDX cells is not affected by mutations within the HIV-1 *env* gene, but by specific inactivation of the $\alpha 1$ -PDX inhibitor.

MATERIALS AND METHODS

Cells

Jurkat cells stably transfected with the $\alpha 1$ -PDX cDNA cloned into the pcDNA3 vector (J-PDX cells; Invitrogen) or with empty vector (J-pcDNA3) have been used previously to demonstrate the inhibitory effect of $\alpha 1$ -PDX on Notch I receptor activation [18] and on HIV-1 replication [14]. These cells were cultured at 37 °C in 5% CO₂ in RPMI 1640 medium (Eurobio, Les Ulis, France) supplemented with 10% heat-inactivated foetal calf serum (ATGC, Orleans, France), 2 mM glutamine, antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) and 0.3 mg/ml neomycin as the selective marker.

Viruses and infection

The wild-type viruses described in our first study [14] were used here. They were obtained from Diagnostic Pasteur (Marnes La-coquette, France) as a supernatant from the CEM cell line containing 10⁵ TCID₅₀ (50% tissue-culture infectious dose)/ml. The infectivity titre of the virus was calculated according to the procedure of Reed and Muench (reviewed in [19]).

For infection, 10⁶ J-PDX or J-pcDNA3 cells were incubated with 100 µl of the undiluted viral stock for 1 h at 37 °C. The input viruses were then washed off, and the cells cultured for the indicated times in RPMI medium containing 10% (v/v) foetal calf serum. Every 3 days, supernatants were collected for reverse transcriptase (RT) activity measurements according to the method described by Schwartz et al. [20].

To determine virus infectivity, equal amounts of RT activity from infected J-PDX and J-pcDNA3 cells were used to infect non-transfected Jurkat cells as mentioned above. In order to determine that HIV-1 replication in J-PDX cells was not due to resistant new variants, we used the viruses collected 20 days

post-infection from J-PDX cells to re-infect J-pcDNA3 and J-PDX cells. The inhibition rate was then compared with that obtained with the wild type (the initial stock) [14].

RT-PCR and sequencing

Primers used for the isolation of HIV-1 *env* sequences included: sense 1, 5'-GCA CCC ACC AAG GCA AAG AGA-3'; sense 2, 5'-GTG GAG GGG AAT TTT TCT ACT GT-3'; and anti-sense, 5'-GGT GAA TAT CCC TGC CTA ACT C-3'. The combination of sense 1 and antisense leads to amplification of the HIV-1_{Lai} *env* sequence from nt 7271 to 7902, while sense 2 and antisense leads to that from nt 6797 to 7902. RNA virus was isolated from equal volumes of infected cell supernatants using the Qiaamp viral RNA mini kit (Qiagen). The synthesis of cDNA was primed by random hexamers, and performed using the first-strand cDNA synthesis kit (Amersham Pharmacia Biotech). Then the cDNA was tested by PCR in the presence of 100 ng of the sense and antisense primers for 35 cycles at 90, 60 and 72 °C for 1 min each. We used total RNA of non-infected cells as a negative control and HIV-1 of the initial stock as a positive control. The products were resolved on a 1% (w/v) agarose gel, ligated into PCR 2.1 (Invitrogen) or pGEMT Easy (Promega) vectors, and then sequences were determined by automated sequencing. At least six clones were tested for each sample by using a combination of the two primer pairs.

Detection of HIV-1 virions

Virions were precipitated from infected cell supernatants of equal RT activity that were collected during the initial experiment of HIV-1 infection of J-PDX and J-pcDNA3 cells, according to the protocol described in [21]. The supernatants were first precleared by centrifuging at 4000 g for 15 min to suppress cell debris. Then the collected supernatants were centrifuged at 40000 g for 5 h at 4 °C to pellet viruses. Virion pellets were lysed directly in 1 × Laemmli buffer (see below). All of the lysates were separated by SDS/PAGE [8% (w/v) acrylamide] after boiling for 5 min, and then immunoblotted with anti-(HIV-1) human serum (Institut Pasteur, Paris, France) as described below.

Western blotting

Cells were lysed in lysis buffer [2% (v/v) Tween in PBS], and centrifuged at 16000 g for 30 min at 4 °C. Three parts of the resulting supernatants were mixed with one part of 4 × Laemmli buffer (0.5 M Tris/HCl, pH 6.8, 10% glycerol, 2% SDS, 5% mercaptoethanol and 0.05% Bromophenol Blue), and then heated for 5 min. Proteins were separated by SDS/PAGE [8% (w/v) acrylamide] and the fractionated proteins were then transferred on to a nitrocellulose membrane. For detection of stably expressed $\alpha 1$ -PDX protein, we used polyclonal anti-($\alpha 1$ -antitrypsin) antibodies (ICN Pharmaceuticals, OH, U.S.A.). To analyse secretion of the $\alpha 1$ -PDX protein and HIV-1 gp120, media from J-PDX cells were concentrated on a Centripeg-10 concentrator (Amicon), mixed with Laemmli buffer (3 vol.: 1 vol., respectively), boiled for 5 min and separated by SDS/PAGE (8% gels). The separated proteins were then analysed by anti-($\alpha 1$ -antitrypsin) antibodies and anti-(HIV-1) human serum, respectively. PC7 was detected by using specific antibodies described previously [22]. As an internal control, aliquots of the cell lysates were analysed simultaneously by anti-actin antibodies (Santa Cruz Biotechnology). The detection of specific antigens

was carried out by secondary antibodies coupled to horseradish peroxidase (Dako, Trappes, France).

RESULTS

The J-pcDNA3 and J-PDX cells were found to express similar levels of the primary receptor CD4, and the transfected $\alpha 1$ -PDX protein had no effect on cell viability or on the kinetics of cell growth as observed by Trypan Blue dye-exclusion assays and growth curves for the parental cells for both clones (results not shown). J-PDX and J-pcDNA3 cells were infected with the undiluted viral stock (10^5 TCID₅₀), and viral replication was assessed by measuring RT activity on different days post-infection. Our findings revealed an inhibition of HIV-1 replication in J-PDX cells, as shown by a delay in viral replication and a 3-fold reduction of the RT peak (Figure 1). In order to assess viral infectivity, equal RT activities of viruses produced on different days in the presence or absence of the $\alpha 1$ -PDX in the previous experiment were used to re-infect naive Jurkat cells. We found that viruses produced on D7 in J-PDX cells were incapable of replicating, as shown by the absence of RT activity during 17 days of infection (Figure 2A). In contrast, the viruses produced on D13 (Figure 2B), D17 (Figure 2C) and D20 (Figure 2D) had increasing capacities for replication, reflecting the fact that HIV-1 viruses from J-PDX cells were recovering their infectivity. Indeed, the data in Figure 3(A) show that gp120 was associated with HIV-1 viruses precipitated from J-PDX cells on D20 (Figure 3A, lane 3), indicating that gp160 maturation was occurring intracellularly. In contrast, no gp120 was detected with the non-

infectious viruses precipitated on D7 from these cells (Figure 3A, lane 1). The control corresponds to viruses produced in J-pcDNA3 cells (Figure 3A, lane 2). In addition, the results depicted in Figure 3(B) show the effect of $\alpha 1$ -PDX on the processing of gp160 to mature gp41 (TM glycoprotein) and secreted gp120 (SU glycoprotein). A significant amount of mature gp120 was found in the virus-free supernatant of cells infected in the absence of the $\alpha 1$ -PDX inhibitor (Figure 3B, lane 2) or with supernatants of HIV-1 viruses that were resistant to the $\alpha 1$ -PDX inhibitory effect on D20 (Figure 3B, lane 3). By contrast, gp120 secretion was reduced by a significant level in supernatants collected from HIV-1-infected J-PDX cells on D7 (Figure 3B, lanes 1 and 4). On the other hand, one can note that the amount of gp120 associated with the pelleted viruses remained much lower than that found in the infected cells (see Figure 4B). This apparent discrepancy is mainly due to the extent of gp120 shedding from the oligomeric non-covalently associated gp120–gp41 complexes [23]. The release of HIV-1 gp120 is a common phenomenon observed during preparation of retroviruses by ultracentrifugation. Furthermore, it has been shown previously that when the maturation of gp160 is inhibited (for instance, by mutations in the maturation site), the incorporation of the resultant uncleaved gp160 precursors into budding viruses is greatly reduced [24–26]. These data may explain the rarity and/or the absence of uncleaved gp160 in viral pellets isolated on D7 post-infection of J-PDX cells (Figure 3A, lanes 1 and 4).

We then put forward two hypotheses to explain this resistance: (i) mutations in the *env* gene or (ii) loss of $\alpha 1$ -PDX activity in J-PDX cells. The HIV-1 RNA was then isolated by RT-PCR on the different days indicated in Figure 4(A) from J-PDX and J-pcDNA3 cells. Env sequences of six clones collected on D7 (Figure 2A) were compared with six other clones obtained from viruses collected on D20 (Figure 2D). No mutations were detected in gp160 cleavage sites, including REKR⁵¹² and KAKRR⁵⁰⁴, nor the N-terminal sequence of the gp41 fusion peptide, which are required for HIV-1 infectivity. In addition, the data in Figure 4(B) show that HIV-1 gp160 had a similar processing rate in J-PDX and J-pcDNA3 cells on D20, and thus correlated well with the conservation of the cleavage sites that are the major elements required for gp160 processing. Nevertheless, two conserved mutations [FLG(A → T)⁵¹¹AGS ... TYSTLI(H → Y)⁶²⁹SLIEE] occurred distant from the maturation site in the gp41 sequence in all of the analysed clones on D20. Of note was the fact that these mutations occurred much less frequently on D7 since they were only found in one clone; hence they represent replication-competent genomes in J-PDX cells. While the mutated H⁶²⁹ in the gp41 ectodomain is variable among the known HIV-1 strains, the mutated A⁵¹¹ near the C-terminus of the gp41 fusion peptide is highly conserved. One could thus argue that at least the A → T mutation reflects HIV-1 adaptation in J-PDX cells.

The viruses isolated on D20 from J-PDX cells (Figure 3A, lane 3) were then used to re-infect J-PDX and J-pcDNA3 cells with the purpose of determining whether they would remain resistant to the $\alpha 1$ -PDX inhibition after a further passage in its presence. Interestingly, the data in Figure 5 revealed that replication was blocked, as these results matched those observed previously with wild-type viruses from the initial stock [14]. Furthermore, Figure 3(A) (lane 4) shows that gp120 was no longer associated with these viruses. In contrast, some uncleaved gp160 molecules were apparent, thus directly reflecting inhibition of gp160 maturation. Therefore, these mutations seemed not to be the major factors responsible for the restoration of gp160 processing in J-PDX cells on D20 post-infection. The other possibility was that HIV-1 replication might have interfered with $\alpha 1$ -PDX activity or

Figure 1 HIV-1 replication in the presence of stably transfected $\alpha 1$ -PDX in a CD4+ Jurkat lymphoblastoid cell line

Jurkat cells were stably transfected with $\alpha 1$ -PDX cDNA cloned into the pcDNA3 vector (J-PDX) or with the pcDNA3 vector alone (controls, J-pcDNA3). Then, 10^6 J-pcDNA3 and J-PDX cells were infected with $100 \mu\text{l}$ of the undiluted viral stock containing 10^5 TCID₅₀/ml. ●, J-pcDNA3 cells; ○, J-PDX cells. Results are the means $\pm$ S.D. from three assays in two independent experiments.

Figure 2 Infectivity assay of HIV-1 produced in the presence or absence of $\alpha 1$ -PDX

(A)–(D) show the results of infection of non-transfected Jurkat cells with equal RT activities of HIV-1 viruses collected from J-pcDNA3 cells (●) and J-PDX cells (○) on D7, D13, D17 and D20 during the experiment shown in Figure 1, respectively. Results are the means $\pm$ S.D. from three assays in two independent experiments.

synthesis. To this end we quantified $\alpha 1$ -PDX expression on different days in HIV-1-infected J-PDX cells.

It was reported previously [13] that the $\alpha 1$ -PDX protein is synthesized in constitutive cells as a precursor of 59 kDa, which is transformed rapidly into a glycosylated and secreted 62 kDa form. The 62 kDa form is then cleaved at the engineered RXXR site to a shorter form of 54 kDa [13]. Functional analysis showed that only the 62 kDa form exhibited anti-protease activities *in vitro* by forming stable complexes, resistant to strong denaturation, with furin [15,16] and PACE4 [27]. The 62 kDa active form

exhibits its activity in the *trans*-Golgi network [13]. The activity of $\alpha 1$ -PDX is described in the following scheme:

Where E is the enzyme, I is the inhibitor, I^* is its cleaved form, EI' is the enzyme–inhibitor intermediate and EI is the enzyme–inhibitor complex. Therefore the substrate and the inhibitor

Figure 3 Env expression in precipitated HIV-1 virions

Supernatants of equal RT activity were used to isolate HIV-1 viruses by ultracentrifugation as described in the Materials and methods section. The analysed supernatants were collected during the experiment described in Figure 1. **(A)** Detection of viral antigens was performed with anti-(HIV-1) human serum. Lane 1, HIV-1 in J-PDX on D7; lane 2, HIV-1 in J-pcDNA3 on D7 (control); lane 3, HIV-1 in J-PDX on D20; lane 4, HIV-1 on D7 from J-PDX cells infected with HIV-1 viruses shown in lane 3. **(B)** Detection of gp120 in virus-free supernatants. After centrifuging the supernatants to precipitate the viruses, the resultant supernatants were used directly for virus-free gp120 detection. Lanes are as for **(A)**.

pathways lead to the formation of E + I* and the stable complex EI*, respectively. The detection of stable EI* complexes in cells would indicate that the serpin variant interacts with the serine proteinase enzyme and acts as a suicide substrate.

In non-infected J-PDX cells cultured for 20 days or after multiple passages, we detected the expected products, including the 62 kDa form representing the full-length inhibitor (I), and the 54 kDa product, which corresponds to its cleaved form (I*; Figure 6A, lane 1); the 62 kDa/54 kDa intensity ratio was consistently > 1 in uninfected J-PDX cells. The detection of these products showed that α 1-PDX was cleaved specifically at the engineered RXXR site. Thus the fragments detected at the bottom of the gels may correspond to the missing 8 kDa C-terminal fragment of α 1-PDX. Interestingly and by contrast, the 62 kDa/54 kDa ratio significantly decreased with time in HIV-1-infected J-PDX cells since a clear depletion of the 62 kDa product and accumulation of the 54 kDa inactive cleaved product

Figure 4 Isolation of HIV-1 RNA and Env processing

(A) HIV-1 RNA was detected by RT-PCR with a combination of sense 1 and antisense primers (see the Materials and methods section). Infected J-PDX cells: lanes 1 (D7), 2 (D13), 3 (D17) and 4 (D20). Infected J-pcDNA3 cells: lanes 5 (D7), 6 (D13), 7 (D17) and 8 (D20). M, molecular size marker. Note the higher viral load in J-pcDNA3 supernatants, and increasing viral RNA amounts by infection time in J-PDX cells. **(B)** Western blotting of J-pcDNA3 and J-PDX cells infected with HIV-1 for 20 days shown in the experiment in Figure 1. Lane 1, gp120 in J-pcDNA3 cell medium; lane 2, J-pcDNA3 cells; lane 3, J-PDX cells; lane 4, gp120 in J-PDX cell medium.

were more apparent on D20 post-infection (Figure 6A, lane 3) as compared with D7 (Figure 6A, lane 2). These data cannot be due to variations in total proteins analysed as shown by the use of actin as an internal control (Figure 6A, actin panel), and suggest that HIV-1 replication enhanced proteolysis of the α 1-PDX 62 kDa product through the substrate pathway. In J-PDX cells analysed with the anti-(α 1-antitrypsin) antibodies, some bands of 130–140 kDa were apparent and may have indicated furin- α 1-PDX complexes.

We next analysed the secretion rate of α 1-PDX on different days post-infection. As the HIV-1 infection time was prolonged, the data revealed that the 54 kDa form became predominant in J-PDX cell medium on D20 (Figure 6B, lane 3). In contrast, the 62 kDa form was predominant in non-infected cells (Figure 6B, lane 1) and to a lesser extent on D7 (Figure 6B, lane 2). The data in Figure 6 thus reflect that degradation of the α 1-PDX is

Figure 5 Replication of mutant HIV-1 collected on D20 in J-PDX cells

The HIV-1 viruses (shown in Figure 3A, lane 3) produced in J-PDX cells after 20 days of incubation were used to re-infect J-pcDNA3 (●) and J-PDX (○) cells. RT activity was determined as described in the text. Results are the means $\pm$ S.D. from three assays in two independent experiments. On D7 post-infection, viruses from J-PDX cell supernatant were precipitated and analysed for Env glycoprotein expression (see Figure 3A, lane 4).

progressive and related directly to the course of HIV-1 replication.

It has been reported previously that α 1-PDX forms stable complexes with furin *in vitro* [15,16] and intracellularly upon their co-overexpression in COS-7 cells [16]. We extended these reported findings by analysing the expression pattern of PC7, an enzyme that is less sensitive to α 1-PDX inhibition *in vitro*. Like the α 1-PDX protein, PC7 transits through the secretory pathway where it is processed into the active form by auto-catalytic cleavage of its N-terminal prosegment [22]. In contrast to α 1-PDX, PC7 protein expression was unaltered by HIV-1 infection from D7 to D20 (Figure 7, lanes 2 and 3, respectively). Therefore, the loss of serpin variant activity did not reflect a global effect of the virus on the host-cell machinery as shown by the unmodified actin and PC7 patterns of regulation during cell infection (Figure 7, actin panel). Of note is that PC7 existed in J-PDX cells in a free form (E) since the bands detected with anti-PC7 antibodies did not correspond to PC7- α 1-PDX complexes. This finding strongly correlates with the ability of PC7 to cleave α 1-PDX through the substrate pathway. In conclusion, the increasing infectivity of HIV-1 viruses in J-PDX cells seemed to be associated directly with a loss of the inhibitor activity of α 1-PDX.

DISCUSSION

The processing of gp160 into gp120 and gp41 is a key step in the life cycle of HIV-1, and its blockage leads to the production of non-infectious particles [3,4]. Therefore the serpin-based strategy to inhibit cellular enzymes that catalyse this cleavage may be useful for blocking spread of the virus. The major finding of the

Figure 6 Analysis of α 1-PDX expression in cells and medium

(A) Detection of α 1-PDX in lysates of HIV-1-infected J-PDX cells. Lane 1, non-infected J-PDX on D20; lane 2, D7 post-infection; lane 3, D20 post-infection. Actin was used as an internal control, as shown in the separate panel. (B) Secretion of α 1-PDX during HIV-1 infection. Lane 1, non-infected J-PDX on D20; lane 2, D7 post-infection; lane 3, D20 post-infection.

present study was that α 1-PDX became negatively regulated at the protein level by HIV-1 infection, and thus could not be used stably to inhibit HIV-1 replication. This finding is supported by our observation that HIV-1 viruses recovered infectivity in the presence of the inhibitor starting at 13 days post-infection, and that the extent of gp160 processing into gp120 and gp41 became similar at D20 post-HIV-1 infection of J-pcDNA3 and J-PDX cells during the experiment shown in Figure 1.

In order to understand the mechanism by which the virus escapes the anti-viral effect of the α 1-PDX protein, two hypotheses were explored. In the first one, we investigated the possibility of the virus escaping by mutations within the potential cleavage sites. To this end, six clones from the viruses produced in J-PDX cells on D7 (less infectious viruses) and six other clones from viruses produced on D20 (more infectious viruses) were isolated by RT-PCR and sequenced. The sequence analysis revealed two conserved mutations distant from the maturation site [FLG(A $\rightarrow$ T)⁵¹¹AGST...YSTLI(H $\rightarrow$ Y)⁶²⁹SLIEE] in all six clones from D20. The two mutations were only found in a single clone on D7. Furthermore, they were not found in HIV-1 from J-pcDNA3 cells or from the initial stock. Thus they appear to be specific to HIV-1 replicating in J-PDX cells, and may reflect the existence of an alternative gp160-processing mechanism, probably by enzymes insensitive to the α 1-PDX protein's inhibitory effect that appears to play a minor role in the maturation process compared with furin. These mutations could not in fact explain the recovered infectivity of the HIV-1 viruses because a

Figure 7 Detection of PC7

Lane 1, non-infected J-PDX cells; lane 2, J-PDX cells on D7 post-infection; lane 3, J-PDX cells on D20 post-infection; lane 4, non-infected J-pcDNA3 cells. Actin was used as an internal control, as shown in the separate panel.

similar pattern of inhibition was obtained when the mutant viruses isolated on D20 were used to re-infect J-PDX cells. The conservation of the cleavage sites indicated that either serine proteases that are related closely to furin or Ca^{2+} -independent proteases [5,6] are involved in HIV-1 gp160 maturation in J-PDX cells.

In the second hypothesis we investigated the modulation of α 1-PDX expression during HIV-1 infection of J-PDX cells. Interestingly, our findings showed a dramatic loss in α 1-PDX anti-viral activity at D20. This reduction in α 1-PDX activity was found to occur at the protein level since the inhibitor-cleaved form (I*) accumulated during the course of the infection both intracellularly and in supernatants. The 62 kDa/54 kDa ratio was similar to that found in non-infected cells on D7 and, by contrast, was much lower than that found on D20. These data lead us to suggest that HIV-1 enhanced the activity of endoproteases that cleave the α 1-PDX protein through the substrate pathway (see the scheme shown above). In fact, HIV-1-induced alterations of cellular gene expression have been reported previously [28]. For instance, HIV-1 Tat protein down-regulates Mn-superoxide dismutase [29] and interleukin-12 [30] expression, whereas Nef induces reduction in CD4 expression at the post-transcriptional level [31]. In addition, Tat has been found to stimulate the expression of other proteins, such as CXCR4 [32] and interleukin-10 [33].

The PC7 enzyme was principally immunodetected in a non-complexed form in J-PDX cells, and so may be one of the enzymes responsible for the rapid cleavage of the stably transfected inhibitor. In fact, anti-PC7 antibodies revealed no complexes, although serpins almost always form highly stable

complexes with their target proteases that are resistant to denaturation by heat and SDS. These complexes can be detected after SDS/PAGE and Western-blotting analysis, as shown in the present study and by others [8,15,16,27]. On the other hand, in addition to the 62 kDa (I) and 54 kDa (I*) proteins, the anti-(α 1-antitrypsin) antibodies revealed bands of 130–140 kDa in the lysates of J-PDX cells, which may correspond to furin- α 1-PDX complexes. The formation and stability of furin- α 1-PDX complexes have been shown in previous reports [15,16,27]. The rarity of such complexes in J-PDX cells may be due to proteolysis of furin in EI* complexes. In fact, Huttington et al. [9] have shown recently that binding of trypsin to the α 1-antitrypsin serpin leads to conformational changes that make it susceptible to proteolysis to a considerable extent. Secretion of the inhibitor into and detection of its cleavage products in the medium implied that it is able to inhibit furin at different levels, since this enzyme is known to cycle between the *trans*-Golgi network and the plasma membrane (reviewed in [34]). This correlated well with the strong inhibition observed during the first replication cycles of HIV-1.

Previous results indicate significant differences between furin and PC7: (i) stable EI* complexes that characterize serpin molecules have been reported for furin but not for PC7 [15,16]; (ii) PC7 is about 3-fold less sensitive to α 1-PDX than furin (N. G. Seidah and J. S. Munzer, unpublished work); (iii) PC7 cleaves protein substrates at the RXXR sites *in vitro* less efficiently than furin [11,35]; and (iv) co-expression of α 1-PDX and PC7 κ , which corresponds to native PC7 that was modified such that the DNA sequence surrounding its initiator methionine better fits Kozak's rule, results in complete destruction of the α 1-PDX protein [13]. This latter finding correlates directly with our observation that the activity of α 1-PDX was reduced by a considerable extent on D20.

Although furin appears to play the major role in HIV-1 gp160 processing, the present findings show that its selective α 1-PDX protein inhibitor cannot be used stably to inhibit its activity in HIV-1-infected cells.

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