Identification of the Paired Basic Convertases Implicated in HIV gp160 Processing Based on in Vitro Assays and Expression in CD4⁺ Cell Lines*

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The human immunodeficiency virus HIV envelope glycoprotein gp160 is synthesized as an inactive precursor, which is processed into its fusogenic form gp120/gp41 by host cell proteinases during its intracellular trafficking. Kexin/subtilisin-related endoproteinases have been proposed to be enzyme candidates for this maturation process. In the present study, 1) we examined the ability of partially purified precursor convertases and their isoforms to cleave gp160 in vitro. The data demonstrate that all the convertases tested specifically cleave the HIV envelope glycoprotein into gp120 and gp41. 2) We demonstrated that a 19-amino acid model peptide spanning the gp120/gp41 junction is cleaved by all convertases at the same gp160 site as that recognized in HIV-infected cells. 3) In an effort to evaluate specific convertase inhibitors, we showed that the α1-antitrypsin variant, α1-PDX, inhibits equally well the ability of the tested convertases to cleave gp160 in vitro. 4) Three lymphocyte cell lines were screened by reverse transcription polymerase chain reaction in an effort to identify which are the convertases expressed in the most common HIV target, the CD4⁺ lymphocytes. The data demonstrate that furin, PC5/6, and the newly cloned PC7 are the main transcribed convertases, suggesting that these proteinases are the major gp160-converting enzymes in T4 lymphocytes.

The lymphotropic human immunodeficiency virus (HIV)† has been etiologically linked with the expanding epidemic of AIDS (1). CD4⁺ target cells infection requires the fusion of the viral envelope lipid bilayer and the host cell membrane, allowing the introduction of the viral capsid into the cell cytoplasm. Fusion is mediated by the viral surface envelope glycoprotein encoded by the HIV-1 Env gene. The envelope glycoprotein is synthesized as an inactive precursor (gp160) and sorted to the host cell constitutive secretory pathway (2, 3). During its cellular transit, gp160 is proteolytically processed into the non-covalently linked gp120/gp41 (3, 4). While gp120 initiates the interactions with the cellular CD4 receptor, the non-covalently associated membrane-bound gp41 is responsible for the fusion process (3, 5). The cell surface glycoprotein is either incorporated into a new budding virion or promotes syncytia formation with neighboring CD4⁺ cells, thereby contributing to the depletion of the T cell repertoire (for review, see Ref. 6).

In spite of the variable primary sequence of the envelope glycoproteins, gp160 cleavage occurs in a highly conserved structure, RFEKR | AV (7). Thus far, the host cellular proteinase(s) responsible for the HIV-1 gp160 precursor processing have not been unequivocally identified. The HIV-1 gp160 cleavage recognition sequence, containing basic residues at positions P1, P2, and P4, is shared with HIV-2 and a number of other viral glycoproteins (8–10). Site-directed mutagenesis has suggested that such basic amino acids play a crucial role in determining the rate of the cleavage reaction (11–13). Because processing of gp160 occurs in the Golgi apparatus, likely in the trans-Golgi network, and is a calcium-dependent process (2, 14, 15), the recently discovered precursor convertases (PCs) of the subtilisin/kexin type are considered likely candidates for the intracellular processing of the HIV envelope glycoprotein (16–19). Thus far, seven mammalian PCs are known: furin (also called PACE), PC1 (also called PC3), PC2, PACE4, PC4, PC5/6-A and its isoform PC5/6-B, and the newly discovered PC7 (also called LPC and PC8) (20–30). These serine proteinases cleave at the C terminus to either single basic residues or pairs of basic residues in a wide variety of membrane-bound and secreted proteins (31).

Furin was first shown to cleave gp160 intracellularly into gp120/gp41 by Hallenberger et al. (16). However, recent studies reveal the involvement of other mammalian PCs in this process. This was based on the ability of PC1 (17) and PACE4 (18) to process gp160 in vitro and the ability of furin, PACE4, and PC5/6-B to cleave this glycoprotein ex vivo in cell lines coinfected with VV recombinants (18). Furthermore, since processing of gp160 also occurs in cells devoid of furin activity (18, 32, 33), the existence of other gp160 convertases was inferred. In view of the reported abrogation of infectivity of HIV-1 upon mutation of the gp160 processing site (4), a number of laboratories began to design inhibitors of convertases as a novel
antiviral strategy (16, 17, 34). Accordingly, the variant serpin α1-antitrypsin Portland (α1-PDX) (34) was shown to inhibit gp160 processing by all PCs within constitutively secreting cells (18).

Thus, although several convertases have been proposed to be implicated in the proteolytic maturation of gp160 into gp120/gp41 and of furin in the processing of gp120 into gp70/gp53 (18), no systematic analysis of the kinetic parameters governing the relative ability of the PCs to cleave either gp160, gp120, or other cryptic sites within gp120 has been reported. Furthermore, kinetic data are lacking regarding the cleavability of the second cryptic site located 8 amino acids N-terminal to the major cleavage site PTKKRRVQRE at α2 VGGIG, which seems to be partially processed in gp120 in vivo (19, 19).

However, the above cited biochemical data by themselves are not a definitive proof of the direct role of the PCs in gp160 processing in vivo, and their corroboration with other analyses is needed to define the physiologically relevant convertases.

In this work, four (PC1, furin, PACE4, and PC5/6A as well as its isoform PC5/6B) of the seven known convertases were tested as gp160 convertases in vitro. This involved the comparison of their relative activities in the in vitro processing of gp160 and of a model 19-amino acid peptide spanning the junction between gp120 and gp41 (35). This synthetic peptide allowed us to measure the kinetics of the REKR_{α2} VGGIG cleavage. In addition, in vivo data are presented describing the direct inhibitory action of α1-PDX on each convertase. Finally, in an effort to identify the relevant gp160 convertases, semiquantitative reverse transcription polymerase chain reaction (RT-PCR) defined the mRNA expression levels of the PCs in three different CD4⁺ lymphocyte cell lines.

**EXPERIMENTAL PROCEDURES**

**Vaccinia Virus Recombinants**—The recombinant vaccinia virus gp160 (VV:gp160) was a gift from Dr. B. Moss (National Institutes of Health). The purified recombinant vaccinia virus, expressing mouse PC1, PC2, PC5/6-A, and PC5/6-B and human PACE4 and furin as well as its truncated form before the cysteine-rich domain (BCRD-furin), were gifts from G. Thomas (Vollum Institute, Portland, OR) (34, 40). Vaccinia virus cellular infections were performed on 70% BTMD-hfurin, BCRD-hfurin) were purified from the medium of VV: gp160 infected cells by centrifugation and were used at an appropriate dilution in inhibition experiments. Inhibitors were preincubated each time for 10 min at 20°C before addition of gp160.

**Peptide Synthesis**—The 1–19 peptide of sequence PTKKRRVQREKAVGGIG was synthesized by solid phase methods using an Applied Biosystems model 431-A synthesizer. The synthesis of 1–19 peptide was accomplished by Fmoc (N-(9-fluorenylmethoxycarbonyl) chemistry using 2-[(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and 1H-benzotriazole (1H-BTZ) as activating agent. Purification was performed by RP-HPLC on a Vydac C18 column. Elution was carried out with a linear gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid at 0.1 ml/min. Emerging peaks, monitored by absorbance measurement at 214 nm, were collected, and the identity of the peptide fragments was determined by amino acid composition.

**Determination of the Michaelis-Menten Constant (K_m) and the V_max**—Incubations with increasing concentrations of the 1–19 synthetic peptide (15–252 μM) were conducted for 1 h at 37°C with an appropriate convertase concentration (generating 0.1 to 2.5 nmol of 7-amino-4-methylcoumarin from the fluorogenic substrate pERTKR-MCA) were incubated with 8 pmol of the 1–19 substrate for 1 h at 37°C in 100 μl of buffers identical to those used for gp120 digestion. Following the incubation period, the digests were acidified using 5 μl of glacial acetic acid and analyzed by RP-HPLC on a Vydac C18 column. Elution was carried out with a linear gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid and 0.01% triethylamine over 70 min at a flow rate of 1 ml/min. Emerging peaks, monitored by absorbance measurement at 214 nm, were collected, and the identity of the peptide fragments was determined by amino acid composition.

**Enzymatic Assays**—The analysis of the cleavage of the 1–19 peptide by purified convertases was performed as follows: increasing convertase concentrations (generating 0.1 to 2.5 nmol of 7-amino-4-methylcoumarin from the fluorogenic substrate pERTKR-MCA) were incubated with 8 pmol of the 1–19 substrate for 1 h at 37°C in 100 μl of buffers identical to those used for gp120 digestion. Following the incubation period, the digests were acidified using 5 μl of glacial acetic acid and analyzed by RP-HPLC on a Vydac C18 column. Elution was carried out with a linear gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid and 0.01% triethylamine over 70 min at a flow rate of 1 ml/min. Emerging peaks, monitored by absorbance measurement at 214 nm, were collected, and the identity of the peptide fragments was determined by amino acid composition.

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Convertisces were produced using a VV expression system and purified on a DEAE column. The enzyme activity was measured on the pERTKR-MCA fluorogenic substrate in an overnight stop-time assay.

| Convertase       | Optimal pH (activity >80%) | Optimal calcium concentration (activity >80%) | 2 mm EDTA inhibition (activity <10%) | Molecular mass by Western blot |
|------------------|----------------------------|-----------------------------------------------|--------------------------------------|--------------------------------|
| Shed-furin       | 6.5–8.0                    | 0.4–4                                         | Yes                                  | ~80                            |
| BTMD-furin       | 6.5–8.0                    | 0.5–10                                        | Yes                                  | ~82                            |
| BCRD-furin       | 6.5–8.5                    | 2.0–10                                        | Yes                                  | ~60                            |
| PACE4            | 7.0–8.5                    | 0.5–10                                        | Yes                                  | ND                             |
| PC1              | 5.5–6.0                    | 4.0–10                                        | Yes                                  | ~84                             |
| PC5/6-A          | 7.0–8.0                    | 0.2–2.5                                       | Yes                                  | ~120<sup>b</sup>               |
| Shed-PC5/6-B     | 7.5–8.5                    | 0.2–1                                         | Yes                                  | ~170<sup>b</sup>               |

<sup>a</sup> The major band was identified using a C-terminal antibody. A minor band at 66 kDa could also be detected using PC1 N-terminal antibody.

<sup>b</sup> The major band was identified. We also detected minor bands at 85 and 80/35 kDa for PC5/6-A and shed-PC5/6-B, respectively.

Table I

Precursor convertase characterization

RESULTS

**Determination of Calcium and pH Dependence of Recombinant Convertases**—It was previously reported (17, 42) that GH<sub>H</sub>C<sub>1</sub> cells infected with VV:PC1 and VV:furin secreted active enzymes in the cell media. Accordingly, we infected GH<sub>H</sub>C<sub>1</sub> cells with recombinant VV expressing each of the convertases: PC1, PACE4, PC5/6-A, PC5/6-B, and furin (hfurin, BTMD-hfurin, and BCRD-hfurin). Enzymatic activity was detected in each case in the 50-fold-concentrated media using the fluorogenic substrate pERTKR-MCA. In contrast, this approach did not reveal significant enzymatic activity in the media of GH<sub>H</sub>C<sub>1</sub> cells infected with either VV:WT, VV:PC2 or VV:PC7 (data not shown). In the case of PC7, the absence of a secreted form is in contrast with furin and suggests that the protein is firmly anchored to the trans-Golgi network membrane, as previously proposed (22), and that PC7 does not undergo internal processing and hence cannot release a shed form. In the case of PC2, the absence of activity may be related to the lack of coexpression of B2B, which is a specific PC2-binding protein (38). Following partial purification on a DEAE column, only the media obtained from recombinant viruses expressing the convertases showed activity, whereas the medium resulting from VV:WT infection did not. Table I summarizes the characterization of each PC using the fluorogenic substrate and Western blots with their relevant antibodies. In contrast to all other enzymes, PC1 is characterized by its narrow acidic pH optimum (pH 5.5–6) and a greatly reduced activity at neutral pH (42). The convertases PC5/6-A, soluble PC5/6-B, PACE4, and the three soluble forms of furin show a broad pH dependence curve with optimal activity at neutral to weakly basic pHs. These enzymes still exhibit at least 40% of their activity at pH 6.5. Another marked difference between PC1 and the other convertases is their Ca<sup>2+</sup> requirement for optimal activity. Thus, whereas PC1 requires at least 4 mM Ca<sup>2+</sup> to reach 80% of its maximal activity, only 0.2–2 mM concentrations are needed for the other convertases. Furthermore, PC5/6-A, PC5/6-B, and shed-furin were partially inhibited at calcium concentrations above 4 mM. As expected, calcium chelating agents, such as EDTA and EGTA, inhibit the activity of all subtilisin-like enzymes. Finally, we confirmed the presence of these convertases in the isolated fractions by Western blots using specific antibodies, which revealed the molecular masses of each enzyme (Table I).

**In Vitro Digestion of gp160 by the Convertases**—The widespread tropism of HIV suggests that its gp160 could be processed by more than one convertase, which could vary with cell type. Previous reports suggested that furin is important but not essential for the proteolytic maturation of the HIV envelope glycoprotein gp160 (32, 33). To determine which are the convertases potentially involved in the processing of gp160, we first compared, in vitro, the ability of each convertase to cleave gp160 into gp120 and gp41. <sup>35</sup>S-Radiolabeled gp160 was expressed in the constitutively secreting CV-1 cells using a recombinant vaccinia virus. The protein gp160 was partially purified by an affinity column, taking advantage of the strong binding of its carbohydrate chains to lentil-lectin (17). This method allowed us to obtain a fraction enriched with the full-length gp160 (>90%) with low amounts of processed gp120/ gp41 (Fig. 1, CTL). In vitro digestions, using increasing enzyme concentrations, demonstrated that each convertase tested can process gp160 into gp120 and gp41 (Fig. 1). The absence of contaminating bands provided evidence for the specificity of gp160 cleavage by all convertases tested. Control experiments using concentrated supernatant media of VV:WT-infected cells indicate that the proteolytic activity observed with the recombinant viruses does not result from a contaminating proteinase secreted by the VV-infected GH<sub>H</sub>C<sub>1</sub> cells. Furthermore, we confirmed that all gp160-cleaving activities were completely inhibited by a Ca<sup>2+</sup>-chelating agent such as EDTA (data not shown). Finally, we have microsequenced the <sup>3</sup>H-leucine-labeled gp41 fragment produced by each convertase and confirmed that the deduced <sup>3</sup>H-leucine sequence corresponds to the expected gp41 N-terminal sequence (data not shown). In addition to the major production of gp120/gp41, we also observed that high concentrations of BCRD-furin, and to a lesser extent, shed-furin and PC5/6-A, generate secondary digestion products that upon SDS-polyacrylamide gel electrophoresis migrate with apparent molecular masses of 77 and 53 kDa. The increase in the level of the gp77 and gp53 products coincided with the decrease in the level of gp41, suggesting that they are derived from the latter. The molecular masses of the generated products correspond to those previously described following the intracellular furin-mediated cleavage of the V3 loop sequence RIQR↓GP within gp120 (18).
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In Vitro Digestion of a Model Synthetic Peptide (1–19) Spanning the gp120/gp41 Cleavage Site—To study in more detail the specificity and kinetics of cleavage of the favored gp120/gp41 site, we synthesized a model 19-amino acid peptide (PTKAKRRVVQREKR) encompassing the sequence at the junction of gp120 and gp41. In addition, this model peptide contains 14 amino acids N-terminal to position 512 because it was reported that up to 20% of gp120 is further cleaved in vivo at position 504 (10, 19). Fig. 2A shows a typical HPLC separation of the original peptide and its cleavage products at various BCRD-furin activities. At low concentrations, the cleavage of the model 1–19 peptide (retention time, 38 min) by each convertase generated two major products characterized by retention times of 26 and 29 min. Amino acid analysis of the products demonstrated that they correspond to AVGIG and PTKAKRRVVQREKR, respectively. Thus, the preferred cleavage site in this model peptide corresponds to the physiological cleavage site of gp160. At higher convertase/peptide ratios, further proteolytic cleavage occurs, as revealed by the appearance of products eluting at 25 min (RVVQREKR) and an uncharacterized product at 28 min at the expense of the peptide eluting at 29 min. Our data suggest that the second, less favored gp160 cleavage site (KAKR) can be recognized at high furin, PACE4, and PC5/6 enzyme concentrations. In contrast, the 1–19 peptide digestion by PC1, which also generates both the AVGIG and PTKAKRRVVQREKR peptides, revealed a similar further processing at a much lower relative enzyme activities (data not shown), suggesting that PC1 discriminates to a lesser extent between the two processing sites as compared to the other convertases.

Table II summarizes the kinetic parameters deduced from the quantitation of the processing products at low enzyme concentrations. We have excluded PC1, since we could not detect the primary processing products independently from the secondary ones, as stated above. In the absence of a specific active site titrant for the PCs, we performed $K_m$ and $V_{max}$ measurements. The relative $V_{max}$ values were normalized with respect to convertase activity on the representative pERTKR-MCA peptide. The data show that the $K_m$ of PACE4, soluble PC5/6-B, and the three soluble forms of furin are very similar, suggesting that the catalytic sites of these enzymes have similar binding affinity for the gp160 sequence present within the 1–19 peptide.

$\alpha_1$-Antitrypsin PDX Inhibits All Convertases—Cellular expression studies have previously shown that $\alpha_1$-PDX but not $\alpha_1$-PIT can inhibit the proteolytic processing of gp160 by endogenous cellular enzymes and consequently prevents the formation of syncytia (34). This result suggested that, in vivo, the convertases responsible for the gp160 processing are inhibitable by $\alpha_1$-PDx. In agreement with this result, recent coexpression studies demonstrated that this inhibitor blocks the furin-, PACE4-, and PC5/6-B-mediated processing of gp160 (18). However, because these cellular experiments did not prove the direct interaction of the inhibitor and the cognate convertase(s), we compared in vitro the inhibitor potency of $\alpha_1$-PDX on furin-, PACE4-, PC5/6-, and PC1-mediated gp160 cleavage. $\alpha_1$-PDx and $\alpha_1$-PIT were obtained from a 200-fold-concentrated supernatant of VV:$\alpha_1$-PDx- and VV:$\alpha_1$-PIT-infected GH4C1 cells. Fig. 3 shows that increasing $\alpha_1$-PDx concentrations progressively inhibited the in vitro ability of all convertases to process gp160 into gp120/gp41. In contrast, $\alpha_1$-PIT used as control does not show such inhibition pattern (data shown only for BTMD-furin). In Fig. 3B, we further show that $\alpha_1$-PDx inhibits the formation of gp77/gp53 by BCRD-furin at lower concentrations than those needed to block the formation of gp120/gp41. The latter in vitro result is in agreement with the ex vivo data reported for the effect of $\alpha_1$-PDx in the furin-mediated processing of gp160 in AT20 cells (18).
with the gp160 substrate at some point during their intracellular trafficking. Because the above and previous results (18) demonstrated that more than one enzyme can process gp160, it is necessary to define the candidate convertases expressed in CD4⁺ lymphocytes, which are the major targets of HIV infection. We exploited the technique of semiquantitative RT-PCR to define the mRNA levels of PCs in three human cell lines as model CD4⁺ cells.

Accordingly, PCR sense primers were designed around the conserved exon/intron boundaries, located about 40 amino acids before the RGD sequence, and antisense primers within the divergent C-terminal part of the convertases (Table III). RT-PCR conditions were optimized using as positive control an RNA pool from human pituitary and HT29 cells (control). Fig. 4 shows that in this control RT-PCR, products are obtained at the expected molecular mass for all convertases and for the housekeeping ribosomal protein L27. Each control PCR product was further authenticated by digestion with restriction endonucleases cutting at a unique position within the PCR products (Table III), resulting in each case in restriction fragments migrating at the predicted size (data not shown). The sensitivity limits of the PCR method were also tested, using serial dilution of the control RNA template. RT-PCR amplification products of the positive control were detected in all cases in at least 64-fold dilutions of the control template.

Three human CD4⁺ lymphocyte cell lines (SupT1, CEM-T4, and Jurkat) were then screened for the presence of mRNA coding for the presently known convertases. Fig. 4 demonstrates that whereas RT-PCR cDNA products (all of which were sequenced) corresponding to PC7 and furin were detected in the three cell lines tested, PC5/6 was only significantly detected in SupT1 and Jurkat cells. We did not detect PC5/6 in CEM-T4 cells, a subclone of CEM cells, whereas we detected PC5/6 in CEM cells (data not shown). In contrast, no PACE4, PC1, or PC2 mRNA transcripts were found in these cell lines. The expression of furin, PC7 and PC5/6 in SupT1 cells was con-

| Enzyme             | Km (mM) | Vmax (AUa) |
|--------------------|---------|------------|
| Shed-furin         | 30 ± 6  | 500 ± 40   |
| BTMD-furin         | 16 ± 7  | 430 ± 43   |
| BCRD-furin         | 9 ± 3   | 450 ± 30   |
| PACE4              | 13 ± 3  | 380 ± 26   |
| PC5/6-B            | 18 ± 5  | 330 ± 17   |

a AU, arbitrary unit.
confirmed by Northern blot analyses, which also failed to detect any PACE4 or PC2 mRNA in such cell line. We note that the RT-PCR reactions using the PC2-specific primers amplified two smaller cDNAs (340 bp in control and 300 bp in lymphocyte cell lines), which, when sequenced, turned out to be unrelated to PC2 (data not shown). Fig. 4*B shows the relative mRNA levels (normalized to those of the L27 control) after densitometric quantitation of the RT-PCR amplification products.

DISCUSSION

HIV envelope glycoprotein gp160 requires cellular proteolytic maturation into the functionally active form gp120/gp41 for infectivity (4). In this report, we have shown that enzymes belonging to the convertase family can directly cleave gp160 in vitro, yielding the expected products observed in vivo. The in vitro assay developed did not reveal significant differences between the abilities of the tested convertases to perform the gp120/gp41 cleavage. These data were further extended and confirmed using a 19-amino acid synthetic peptide encompassing the cleavage site (Table II). Only furin was able to effectively further process gp120 into gp77/gp53 (Fig. 1B). This result suggests a redundancy in the ability of the convertases to process gp160 into gp120/gp41. What, then, are the factors determining which are the physiological gp160 convertases? Some of these parameters include the tissue-specific expression of the convertases (22, 46) and their distinct intracellular localization (47, 48). Thus, it became important to define which are the convertases expressed in CD4+ cells, the major target of HIV infection. In this report, semiquantitative RT-PCR performed on RNA isolated from three lymphocytic CD4+ cell lines demonstrated that the newly discovered PC7 (22, 24, 28) and furin are the most abundant convertases in these cells, whereas PC5/6 expression is limited to two of the three cell lines (Fig. 4). In contrast, we could not detect transcripts for either PACE4, PC1, or PC2 in these cells. The data suggest

**FIG. 3.** Inhibition of the gp160 cleavage mediated by the convertases using α1-PDX. Fluorography of an SDS-polyacrylamide gel (8% under reducing conditions). 35S-labeled gp160 was digested in vitro by VV-produced convertases in the presence of increasing concentrations of the convertase inhibitor α1-PDX and of α1-PIT, used as a control. CTL, control.

**TABLE III**

| Enzyme                  | Sense | Oligo 5'–sequence–3' | Oligo length | PCR fragment length | Nucleotide number | Restriction enzyme | Generated fragment size |
|-------------------------|-------|----------------------|--------------|---------------------|------------------|-------------------|------------------------|
| PC1                     | S     | TGGCTTGCTAAATGCCAAAGCTC | 23           | 553                 | 1512             | EcoRI            | 137                    |
|                         | AS    | ATCCACCATCTTCTCCACCC | 21           | 2065                | 203              |                  | 416                    |
| PC2                     | S     | GTCCCTGTGAGGGTGCCAAC | 21           | 422                 | 1422             | HincII           | 219                    |
|                         | AS    | ACTCCTTCAGCAACCCCTCTC | 20           | 1844                | 203              |                  | 219                    |
| PC5/6-A or PC5/6-B      | S     | CTGCCGTTTAAAGGTGACCA | 22           | 403                 | 1304             | HindIII          | 175                    |
|                         | AS    | TCACAGCGACTTCTCTCTCT | 20           | 1707                | 228              |                  |                        |
| PC7                     | S     | CATCAATGGCTCCACACGCC | 22           | 500                 | 1449             | BglII            | 130                    |
|                         | AS    | ATGACTCATTCCCCGACATCC | 20           | 1949                | 370              |                  |                        |
| PACE4                   | S     | GGGTGAGGCAAGGGGTGCGTG | 23           | 440                 | 1501             | AvaI             | 186                    |
|                         | AS    | AGGCTCCATTCCTCTCTCT | 22           | 1941                | 254              |                  |                        |
| Furin                   | S     | TGGGGCTACGGGCTTTTGG | 19           | 399                 | 1486             | AvaI             | 124                    |
|                         | AS    | TCGGCTGGATTTTTTCAAATC | 22           | 1885                | 275              |                  |                        |
| Ribosome L27            | S     | ACAATCACTACACAGCCACAG | 23           | 186                 | 246              | EcoRV            | 152                    |
|                         | AS    | GCATCTAAAACCCGCACTT | 23           | 432                 | 34               |                  |                        |

a S, sense; AS, antisense.

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that PC7, furin, and possibly PC5/6 may be the major processing enzymes of gp160 in CD4+ T lymphocytes.

In support of this conclusion, recent results in our laboratory demonstrated that this general conclusion is also valid for human CD4+ primary cells. Furthermore, we further demonstrated that in AT20 cell lines, rat PC7 can process gp160 to yield gp120/gp41 but not gp77/gp53. Thus, even though PACE4 is able to correctly process gp160 into gp120/gp41, both in vitro and ex vivo, in cell expression systems ("Results" and Ref. 18), the absence of its expression in CD4+ cells suggests that it may not play a significant role in infected T lymphocytes. However, the participation of PACE4 in the processing of gp160 in non-T cells cannot be excluded.

Further arguments favoring the above conclusions are based on the fact that furin (48, 49), PC5/6-B (50), and PC7 (42) are the major processing enzymes of the constitutive secretory pathway, which is the route of transit of gp160 and its products gp120 and gp41. Although PACE4 was reported to cleave precursors within the constitutive secretory pathway, including the von Willebrand factor (51), the neurotrophins (52, 53), and gp160 (18), its exact intracellular localization is not known. Furthermore, the neutral pH optima of all these enzymes suggest that unlike PC1 and PC2, which exhibit acidic pH optima (42, 54), they would exert their actions within weakly acidic to neutral subcellular locations, such as the Golgi apparatus or the cell surface. Finally, the localization of furin, PC7 (5), and PC5/6-B (50) in the trans-Golgi network further supports their role in the processing of constitutively secreted proteins, including gp160, which was shown to be processed by calcium-dependent enzymes in this compartment (2, 14, 15). In contrast, the granular localization of PC1, PC2 (47), and PC5/6-A (50) suggests that these enzymes exert their functions within acidic compartments and process mainly precursors within the regulated pathway.

Studies based on biochemical enrichment of a 26-kDa protein from subcellular Golgi fractions obtained from T lymphocytes implicated a protease unrelated to the calcium-dependent enzymes of the convertase family (55, 56). However, the low levels of calcium needed for maximal activity of furin, PACE4, PC5/6 (Table I), and PC7 (5) does not exclude their functions even in the presence of 10^{-7}–10^{-8} M A23187. Furthermore, at higher concentrations (10^{-7}–10^{-6} M) of A23187, two reports concluded that gp160 processing is completely inhibited (14, 18), clearly emphasizing the importance of calcium in gp160 processing. In addition, biochemical and genetic definition of the protease implied in gp160 processing in yeast suggest that it is an enzyme related to the kexin family (57).

The processing of gp120 at a second site, N-terminal to the main gp120/gp41 junction, has been reported to occur to an extent of about 10–20% (11, 19). Our in vitro studies, using a 19-amino acid model peptide, revealed that processing occurs preferentially at the physiological REKR2AV site, and only at high enzyme concentrations do we observe the second cleavage (Fig. 2). In agreement with these conclusions, the N-terminal sequence of the gp41 generated by all the PCs tested revealed a single N terminus corresponding to the physiological cleavage site (data not shown; Ref. 17).

Our data show that high concentrations of furin are needed to further process gp120 to generate pp77/pp53 in vitro. This observation was also previously reported in ex vivo cellular coexpression systems (18, 19), which revealed that the cleavage

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*E. Decroly, J. Cogniaux, J.-M. Ruysschaert, and N. G. Seidah, manuscript in preparation.*

*S. Benjannet, E. Decroly, and N. G. Seidah, manuscript in preparation.*

*S. Benjannet and N. G. Seidah, manuscript in preparation.*

*J. S. Munzer and N. G. Seidah, manuscript in preparation.*

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**Fig. 4. Identification of precursor convertase mRNA in CD4+ lymphocytes by RT-PCR.** Each RT-PCR amplification (28 cycles) was performed using convertase-specific primers together with two others specific for the control ribosomal L27 protein. A, agarose gel (1.8%) electrophoresis of PCR products; B, bar graph representing the quantitation of the relative amount of each convertase in the tested lymphocyte cells obtained by densitometric analysis and averaged over five independent RT-PCR experiments. CTL control.
site RIQR \rightarrow GPGR occurs within the V3 loop around the conserved GPGR structure. Processing of the gp120 within the V3 loop has been reported to be an essential postbinding step leading to virus cell fusion (58–60). The low degree of gp160 processing in HIV-1-infected T cells and macrophages suggests that the endogenous enzyme level is not sufficient to generate this V3 loop cleavage in vivo. Furthermore, gp120 reaching the cell surface and virion-bound gp120 are not cleaved within the V3 loop. The V3 loop proteolysis, proposed to be important for the fusion process, occurs at the cell surface following the binding of gp120 to the CD4 receptor (60). Because furin is known to cycle between the trans-Golgi network and the cell surface (48), it is likely to perform such cleavage during its transit to the plasma membrane. Since the gp120-CD4 interaction promotes gp120 conformational changes (61), possibly the cleavage site of gp160 affect the envelope glycoprotein processing of gp160, and more information is needed to identify selective convertase inhibitors, we tested the inhibition of the gp160 processing by peptidyl chloromethyl ketones containing sequences mimicking the cleavage site of gp160 affecting the envelope glycoprotein maturation process. However, the inhibitors tested thus far are not specific for one convertase to the exclusion of others (16, 17). In an effort to identify selective convertase inhibitors, we investigated whether gp120 bound to the CD4 receptor is more likely to be processed by furin into gp77/p53. If this is the case, such cleavage will be restricted to specific isolates, because the V3 loop is a hypervariable domain, and the furin consensus cleavage sequence seems to be only conserved in 20% of the known isolates.

Inhibition of gp160 processing was reported to block syncytia formation and results in noninfectious viral particles (4). Garten and co-workers (16) were the first to show that N-decanoyl-peptidyl chloromethyl ketones containing sequences mimicking the cleavage site of gp160 affect the envelope glycoprotein maturation process. However, the inhibitors tested thus far are not specific for one convertase to the exclusion of others (16, 17). In an effort to identify selective convertase inhibitors, we tested the inhibition of the gp160 processing by α1-antitrypsin-PDX, a newly designed convertase inhibitor reported to block cellular HIV envelope glycoprotein cleavage (34). Our ex vivo (18) and in vitro (this work) data demonstrate that this serpin inhibits the gp160 cleavage activity of PC1, PACE4, furin, and PC5/6. Therefore, the observed lack of specificity of α1-PDX toward the members of the convertase family makes it difficult to eliminate the candidacy of any one of these convertases in the in vivo processing of gp160, and more information is needed before such a choice is made.

Because neither the reported ex vivo coexpression studies nor our in vitro data allow us to unambiguously define the processing enzymes of gp160 in vivo, it is likely that a major selection will be made at the level of the expression of the convertases in the different cell types infected by HIV. It is clear from this work that PC7, furin, and PC5/6 are the best physiological candidates in CD4+ lymphocytes to date. This picture may be complicated by the plasticity of the cellular expression of the PCs, whereby viral infection and/or lymphocyte activation may influence the expression pattern of some of these convertases (17). In addition, the ability of HIV to infect cells devoid of CD4 receptors such as macrophages and glial cells (6) suggests that this virus is well adapted to variable cellular repertoires and could be activated by more than one enzyme.

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