Comparative cellular processing of the human immunodeficiency virus (HIV-1) envelope glycoprotein gp160 by the mammalian subtilisin/kexin-like convertases

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We present here the pulse and pulse-chase analysis of the biosynthesis of the envelope glycoprotein gp160 and its intracellular processing by the subtilisin/kexin-like convertases furin, PACE4, PC1, PC2, PC5 and its isoform PC5/6-B. We demonstrate that furin and to a much lesser extent PACE4, PC5/6-B and PC1 are candidate enzymes capable of processing gp160 intracellularly. Furthermore we show that furin can also process gp160/gp120 into gp77/gp53 products by cleavage at the sequence RIQR↓GPGR just preceding the conserved GPGR structure found at the tip of the hypervariable V3 loop. The results show that processing into gp120 could occur at or before

the *trans*-Golgi network (TGN) where sulphation of the oligosaccharide moieties of gp160 was detected. In contrast, the formation of gp77/gp53 by furin is a late event occurring after exit from the TGN. Our data also revealed that the α -glucosidase I inhibitor *N*-butyldeoxynojirimycin, although affecting the oligosaccharide composition of gp160, does not impair the processing of either gp160 or gp120 by either furin or PACE4. Finally, the co-expression of the [Arg³⁵⁵, Arg³⁵⁸]- α -1-antitrypsin Portland variant was shown to potently inhibit the processing of both gp160 and gp120 by these convertases.

INTRODUCTION

Viral envelope glycoproteins mediate the entry of the viral capsid into the cytoplasm of cells by a process implying both the fixation of the virion to a cellular receptor and the fusion of the viral membrane with that of the host cell. Viral surface glycoproteins are usually synthesized as inactive precursors that are cleaved and activated by one or more host-cell proteinases. In the HIV virus, the endoproteolytic cleavage of gp160 occurs within the Golgi apparatus at the conserved REKR↓AVGI sequence, thereby generating the two fragments gp120 and gp41. The N-terminal fragment (gp120) can interact with a specific CD4⁺ cellular receptor mediating infection of lymphocytes, and might allow entry of HIV into macrophages and monocytes, whereas the membrane-anchored C-terminal segment (gp41) contains an N-terminal hydrophobic sequence capable of promoting the fusion of the virion with cellular membranes. Both fragments are highly *N*-glycosylated with the gp120 and gp41 containing 24 and 4 *N*-glycosylation sites respectively [1]. The gp120 molecule is composed of about 512 amino acids and contains five conserved segments (C1 to C5) separated by five hypervariable loops (V1 to V5) [2]. In spite of the primary-sequence variability of gp120, its structural and functional domains are highly conserved. It is now accepted that gp120 contains at least four functional domains: (i) one recognizing the CD4⁺ receptor [3]; (ii) N- and C-terminal segments responsible for the association of gp120 with gp41 [4,5]; (iii) the junction of gp120 and gp41, which represents the preferred intracellular cleavage site of gp160 at the sequence REKR↓AV; and (iv) the hypervariable V3 loop (amino acids

296–331) constituting the principal immunoneutralization domain of HIV-1. This domain, which was shown to be important in the HIV-1 infection process [6], syncytia formation and the control of the viral replication rhythm, contains a very conserved GPGR sequence [6,7]. It has been shown that the V3 loop contains proteinase-sensitive sites close to the conserved GPGR sequence, which when cleaved generates a disulphide-linked gp70/gp50-like molecule. However, little is known about the human proteinase(s) responsible *in vivo* for the production of gp70/gp50. *In vitro*, the V3 loop is cleavable by tryptase, dipeptidyl peptidase IV and other serine proteinases [8–10], and such secondary processing seems to occur at the surface of CD4⁺ cells. It was suggested that the processing of the V3 loop may cause conformational changes that enhance the interaction of the fusion domain and the cellular membrane [11,12]. Co-expression of gp160 and furin in insect cells results in the cleavage of gp160 into gp120/gp41 and further processing of gp120 within the V3 loop into a gp60/45 product [11]. However, in the latter study neither the exact site of cleavage nor the importance of the carbohydrate moieties of gp160 (which are different in insect cells) in the processing of this precursor were defined.

The fact that processing of gp160 at the REKR↓ site into gp120 and gp41 was reported to occur within the Golgi apparatus, and also that the enzyme(s) involved seem to be calcium-dependent [13], suggested that the recently discovered prohormone and proprotein convertases of the subtilisin/kexin family (for reviews see [14–18]) may be good candidates as intracellular processing enzymes of gp160. The convertases proposed to be implicated in the processing of gp160 into

Abbreviations used: BFA, brefeldin A; Endo, endoglycosidase; ER, endoplasmic reticulum; h, human; m, mouse; NB-DNJ, *N*-butyldeoxynojirimycin; α 1-PDX, α 1-antitrypsin Portland; p.f.u., plaque-forming units; TGN, *trans*-Golgi network.

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gp120/gp41 include furin [19,20], PC1 [21] and possibly other enzymes found in cells devoid of furin activity such as LoVo cells [16,21,22] and the chinese hamster ovary (CHO)-derived cell line FD11 [23].

The aim of the present study is to define which of the following six possible subtilisin/kexin-like convertases could be involved in the intracellular processing of gp160 into gp120/gp41: furin (also known as PACE), PC1 (also known as PC3), PC2, PACE4 and PC5 (also known as PC6) and its isoform PC5/6-B. Because some of these convertases are constitutively secreted or could transit to the cell surface, we investigated their possible participation in the formation of gp70/gp50. The present results obtained from the co-expression of gp160 and each convertase in constitutively secreting or in regulated cells suggest that more than one enzyme can process gp160 into gp120 and gp41. In addition, the data revealed that furin is the most efficient convertase capable of producing gp70/gp50-like proteins. This study also identified the cleavage site in the V3 loop and investigated the role of carbohydrates in the processing of gp160 and gp120. Finally, α 1-antitrypsin Portland was shown to inhibit the convertase-catalysed formation of either gp120/gp41 or gp70/50.

MATERIALS AND METHODS

Vaccinia virus recombinants

The recombinant vaccinia virus of VV:gp160 was a gift from Dr. B. Moss (NIH). The purified recombinant vaccinia viruses expressing mouse (m) mPC1, mPC2 and human (h) hfurin were previously described [24,25]. The recombinants VV:hPACE4 and VV:mPC5 have also been recently described [26]. The VV:mPC5/6-B was obtained from the cDNA of mPC5/6-B kindly provided by Dr. K. Nakayama [27]. The VV: α 1-antitrypsin Portland (α 1-PDX) recombinant vaccinia virus was recently reported [28].

Cellular infection

In this work, we used four cell types: the regulated mouse pituitary corticotroph AtT20 cells and mouse pancreatic β -cell line β -TC3, which contain secretory granules, and two constitutively secreting cell lines: BSC40 cells, an African green monkey kidney epithelial cell line, and LoVo-C5 cells, a human colon carcinoma cell line that is defective in proprotein processing caused by a deletion of one nucleotide within the P-domain of hfurin [22].

AtT20, β -TC3, BSC40 and LoVo cells were grown in 25 cm² Petri dishes in media (Gibco) consisting of Dulbecco's modified Eagle's medium for the AtT20 and β -TC3, minimum essential medium for BSC40 cells, or F10 for the LoVo-C5 cells (a gift from Dr. Jean-Claude Lissitzky, Marseilles, France). Each medium was supplemented with 10% (v/v) fetal calf serum and 28 μ g/ml gentamycin at 37 °C in 10% CO₂ and grown to 70% confluency. The vaccinia infections were then performed at a multiplicity of 1–4 plaque-forming units (p.f.u.) per cell for each recombinant virus.

Metabolic labelling of cells

After vaccinia virus infection and 17 h of incubation, AtT20, β -TC3, BSC40 or LoVo cells were washed and preincubated for 1 h in media depleted of one of methionine/cysteine, sulphate, valine or isoleucine (RPMI 1640, Gibco). The cells in each 25 cm² dish were then washed and pulse-labelled with about 150 μ Ci of L-[³⁵S]methionine or 300 μ Ci of Na₂³⁵SO₄, [³H]valine or [³H]isoleucine in RPMI media depleted of the corresponding

unlabelled amino acid. The radiolabelled products were then analysed directly or the labelling was followed by chase periods in the presence of 10 mM unlabelled methionine. At the end of the pulse or pulse-chase periods, the media were collected and centrifuged for 10 min at 300 g to remove any cells and debris. To the supernatant was then added a cocktail of protease inhibitors consisting of 100 g/ml PMSF, 2 μ g/ml aprotinin and 1 mM EDTA. The cells were washed once in PBS-D (2.7 mM KCl, 1.5 mM KH₂PO₄, 137 mM NaCl and 4.2 mM Na₂HPO₄), and then extracted for 30 min at 4 °C with 350 μ l of RIPA lysis buffer containing 50 mM Tris buffer, pH 7.5, 150 mM NaCl, 1% (w/v) NP40, 0.5% sodium deoxycholate and 1% (w/v) SDS. The cells were centrifuged and the supernatant was collected for immunoprecipitation. In experiments performed with *N*-butyl-deoxynojirimycin (NB-DNJ), a concentration of 0.2 mg/ml (2 mM) of this α 1-glucosidase inhibitor was added during both the preincubation and labelling/chase periods. In experiments involving brefeldin A (BFA) (2.5 μ g/ml) and tunicamycin (5 μ g/ml) the drugs were present during the preincubation and labelling periods. In the experiments with NaF/AlCl₃ (30 mM/0.05 mM) and A23187 (5 μ M, in Ca²⁺-free medium) these agents were only present during the radiolabelling period.

Analysis of radiolabelled gp160 and related processed products

To study the secreted and cellular form of gp160 and its processed forms radiolabelled with L-[³⁵S]methionine or Na₂³⁵SO₄, media and cell extracts were immunoprecipitated as described [26] with a pool of inactivated (0.5% Triton X-100 and heating at 56 °C for 30 min) sera obtained from four Belgian seropositive individuals who were selected for their high reactivity on Western blots with HIV-1 viral glycoproteins (Institut Pasteur du Brabant, Dr. J. Cogniaux). Immunoprecipitated radiolabelled proteins were then resolved by SDS/PAGE on an 8% T, 2.7% C polyacrylamide gel. Prestained SDS/PAGE standards were obtained from Bio-Rad. For analytical purposes, the gels were treated with Entensify as specified by the manufacturer (Du Pont), dried and autoradiographed on Reflection NEF films (Du Pont). For preparative purposes, identical gels were sliced (1 mm), and the radiolabelled proteins were passively eluted (in 7.5 mM ammonium carbonate, pH 7.4, containing 0.05% SDS) and subjected to microsequence analysis on an Applied Biosystems model 470A sequenator, as previously described [20,26].

Digestion of gp160 *in vitro* by furin and PACE4

[³⁵S]Methionine-labelled gp160 was partially purified from VV:gp160 infected CV-1 cells, as previously described [20], except that gp160 was further purified by SDS/PAGE, and the electroeluted gp160 was equilibrated with the incubation buffer by passage on a 1 ml Sephadex G-25 column. The soluble form of human furin that lacks the Cys-rich domain [29] called BCRD-furin (before Cys-rich domain), as well as PACE4, were obtained from media of vaccinia-virus-infected GH4C1 cells and their purification was performed essentially as described [30]. Incubations *in vitro* were performed in 100 μ l of 50 mM Tris/acetate buffer, 1% (w/v) Triton X-100, 2 mM CaCl₂, pH 7.5. The reaction buffer contained 10 μ l of ³⁵S-labelled gp160 (5000 c.p.m.) and an appropriate amount of convertase activity measured by the release of the fluorogenic 7-amino-3-methylcoumarin group from pGluArgThrLysArg-MCA, as described [31]. After overnight digestion at 25 °C, SDS/PAGE was performed on an 8% T, 2.7% C polyacrylamide gel, followed by autoradiography.

Figure 1 SDS/PAGE analysis of gp160 processing in regulated and constitutively secreting cells in the absence or presence of furin

Each cell line (AtT20, β -TC3, BSC40 and LoVoC5) was infected with either (A) VV:gp160 (4 p.f.u.) or (B) VV:gp160 (2 p.f.u.) plus VV:hfurin (4 p.f.u.) and then pulse-labelled for 30 min (P30) with [³⁵S]methionine followed by a chase of 90 (P30C90) and 180 min (P30C180). The media and cell extracts were immunoprecipitated with a gp160 antiserum, analysed by SDS/PAGE (8% T, 2.7% C) and fluorographed as described in the Materials and methods section. On the basis of the migration of the molecular standards obtained from Bio-Rad, the migration positions of gp160, gp150 (*), and gp120, gp41, as well as gp77 and gp53, are shown. In AtT20 cells gp41 is less visible because it co-migrates with a non-specific protein (see Figure 3).

Endoglycosidase H and F treatments

After 2 h of metabolic labelling of AtT20 cells with [35 S]methionine and immunoprecipitation procedures, the antigen-antibody complexes were subjected to SDS/PAGE analyses. The total immunoprecipitated proteins from the media or the [35 S]methionine-labelled 160, 150, 120, 110, 77 and 53 kDa proteins eluted from the gel were divided into three equal fractions. Each reaction was performed in 100 μ l at 37 °C for 17 h. To one fraction endoglycosidase H (Endo H) incubation buffer (100 mM sodium citrate, pH 5.5) and Endo H (10 m-units) were added. The second fraction was digested with 1.5 units of peptide-N-glycosidase F (N-glycanase; Endo F; Oxford Glyco-Systems) in a buffer containing 20 mM sodium phosphate, pH 7.5, and 50 mM EDTA. The third sample served as untreated control and did not contain enzyme. All reaction products were then analysed by SDS/PAGE as described above.

RESULTS

Comparative processing of gp160 in regulated and constitutive cells

It has been reported that vaccinia virus expression of gp160 in the constitutive CV-1 [19], BSC40 [28] or LoVo [21] cells resulted in the processing of this precursor into gp120 and gp41 by endogenous cellular convertases. However, because HIV is reported to possibly infect neuronal cells (for review see [31]) that contain secretory granules, it was of interest to compare the processing of gp160 in both constitutively secreting and regulated cells. Accordingly, we co-infected the two constitutive LoVo and BSC40 cells and the regulated AtT20 and β -TC3 cells with either VV:gp160 or VV:gp160/VV:hfurin. The infected cells were then subjected to a 30 min pulse-labelling period with [35 S]methionine followed by a chase of either 90 or 180 min, and the cell extracts and media were analysed for gp160 processing. As shown in Figure 1(A), all four cell lines gave similar processing patterns, albeit to a different extent, in the absence of exogenous furin, suggesting that each cell type contains one or more gp160 convertases. In the four cell extracts and media, immunoprecipitated gp120 was detected starting from the 90 min chase period with an increasing level observed at 180 min. We note that the [35 S]methionine-labelled gp41 that is also presumably produced (see Figure 5) migrates at the same position as that of a non-specific intracellular protein in AtT20 cells (compare Figure 1A and Figure 3), but is more visible in β -TC3, BSC40 and LoVo cells. In a similar experiment using a 15 min pulse followed by chase periods of 30 and 60 min we could detect gp120 both intracellularly and in the medium at 60 min of chase (results not shown). We also note that in AtT20 cells the first detectable form of the glycoprotein migrates with an apparent molecular mass of 150 kDa (gp150*), which is gradually transformed into a 160 kDa form (gp160) during chasing (Figure 1A). As is especially evident in AtT20 cells, at the 30 min pulse period (P30) we note the initial intracellular formation of a gp110 protein the level of which diminished during chasing, whereas the amount of secretable gp120 increased with chasing. In the presence of exogenously expressed furin, the initial formation of gp110 at P30 was quite visible (Figure 1B). Furthermore, in AtT20 cells, although the upper gp160 form disappeared completely in the presence of exogenous furin, the level of the lower gp150 form diminished relatively much less with chase times. This result was the first indication that the 150 kDa form may be localized in a cellular compartment that is not favourable for processing. In contrast, the data of Figure 1 suggest that the 160 kDa form, which can be processed intracellularly by furin,

Figure 2 Microsequence analysis of gp77 and gp53

Radiolabelled gp77 (A) and gp53 (B,C) were obtained from the media of AtT20 cells co-infected with VV:gp160 and VV:hfurin and pulsed for 2 h with either (A,B) [3 H]valine or (C) [3 H]isoleucine. The labelled proteins were separated by SDS/PAGE (8% T, 2.7% C), and the gel was then cut into 1 mm slices and the eluted proteins migrating at the position of gp77 and gp53 were sequenced for 20 cycles. Cycle 0 corresponds to a full sequencing cycle performed in the absence of the coupling reagent phenylisothiocyanate. The numbers above the peaks represent the deduced sequence positions of (A) valine in gp77, (B) valine in gp53 and (C) isoleucine in gp53.

represents the convertase-competent form that may have reached the *trans*-Golgi network (TGN) where processing normally occurs [32] and where furin has been localized [33].

In the absence of exogenous furin, the media of the four cell lines reveal the predominant presence of gp120, further confirming the ability of their endogenous enzymic machinery to process gp160 into gp120. In contrast, when furin was over-expressed together with gp160, two proteins migrating with apparent molecular masses of 77 (gp77) and 53 (gp53) kDa were observed in cell extracts and media, the levels of which were

Figure 3 The cellular processing of gp160 by subtilisin/kexin-like convertases

AtT20 cells were either infected with wild-type vaccinia virus (Wt) or co-infected with VV:gp160 and one of VV:Wt, VV:mPC1, VV:mPC2, VV:hFurin, VV:hPACE4, VV:mPC5 or VV:mPC5/6-B. The cells were then pulse-labelled for 2 h with [³⁵S]methionine. The SDS/PAGE analysis of the gp160-immunoprecipitated cell extracts and media and the autoradiography conditions were identical with those in Figure 1. It should be noted that the exposure time of the cell extract X-ray film is 1 d, whereas for the media it represents a 5 d exposure.

highest in the media of AtT20 cells. This suggests that aside from the reported role of furin in the processing of gp160 into gp120/gp41, we now show that it can also produce gp77/gp53. Such processing could be achieved either directly from gp160 to form gp77, gp53 and gp41 or through the intermediate formation of gp120, which is then further processed into gp77/gp53. Confirmation of the nature of gp77 was provided by the microsequence of the [³H]valine-labelled protein (Figure 2A). It is clear that the Val^{6,8,12} sequence corresponds to that expected from the N-terminal sequence of either gp160 or gp120, confirming that gp77 is the N-terminal segment of gp120, starting just after the predicted signal peptidase cleavage site [34]. Because processing of the hypervariable V3 loop of gp120 has been reported to produce similar products *in vivo* [8,10,34], it was of interest to define the exact site of cleavage by microsequencing the radiolabelled gp53 form after a 2 h pulse with either [³H]valine (Figure 2B) or [³H]isoleucine (Figure 2C). The data demonstrated that gp53 contains a Val⁷ and Ile^{9,12} N-terminal sequence. On the basis of the primary structure of gp160 [33], this suggests that the major site of gp120 processing occurs in the sequence **ArgIleGlnArg**GlyProGlyArgAlaPheVal⁷ThrIle⁹GlyLysIle¹²Gly. Interestingly, the deduced cleavage site is just before the conserved, β -turn-containing, GPGR sequence found within the V3 loop [8,10,35]. We note that the gp77 form migrates with an estimated molecular mass about 7 kDa greater than that reported for gp70 [8]. This discrepancy is either due to a difference in glycosylation between the recombinant gp160 and that reported with the native virus [8] or may be dependent on the electrophoresis conditions used.

Which subtilisin/kexin-like convertase processes gp160?

Recently we have shown that *in vitro* both furin and PC1 can process gp160 into gp120 and gp41 [20]. Furthermore, Ohnishi et al. [21] also reported that LoVo cells, which are devoid of furin activity [22], can process gp160 into gp120 and gp41. Because LoVo cells express PACE4 [16] but not PC1, PC2 or PC5, it was

plausible that PACE4 and/or another as yet unidentified enzyme could also process gp160. Accordingly, in Figure 3 we compare the processing of gp160 in AtT20 cells that have been co-infected with both gp160 and one of the six subtilisin/kexin convertases: furin, PC1, PC2, PACE4, PC5 or its isoform PC5/6-B. Clearly, in the presence of exogenous furin the intracellular gp160 is almost completely processed. However, in the presence of furin we also detected the formation of gp77/gp53, especially in the medium. We note that intracellularly PACE4, PC5/6-B and PC1 are also capable of processing gp160 into gp120 but that these enzymes are less efficient than furin, as evidenced by the level of unprocessed cellular gp160. Furthermore, as shown in the media, these enzymes form the gp77/gp53 products to a much smaller extent than furin. Quantification of the data by scanning the autoradiograms of both cells and media demonstrated that overall, under the conditions used, furin, PACE4, PC5/6-B and PC1 are able to process gp160 into gp120 + gp77 + gp53 above its background processing by the endogenous AtT20 convertases by 39%, 26%, 14% and 10% respectively. These results are in agreement with the suggestion that more than one enzyme can process gp160 [20–22], and point out that PC2 and PC5 do not process gp160. The absence of processing by PC2 or PC5 was not due to their lack of activity because similar experiments performed with other precursor substrates such as pro-opiomelanocortin for PC2 [25] and pro7B2 for PC5 [26] revealed that these are potent convertases.

As a further test for the observed gp160 cleavage by more than one intracellular convertase, we digested overnight, *in vitro*, purified gp160 with human furin [20] and PACE4 obtained from vaccinia virus expression systems. As shown in Figure 4A, both furin and PACE4 can process gp160 *in vitro* to generate gp120/gp41. Interestingly, at higher enzyme concentrations and for equivalent pGluArgThrLysArg-MCA fluorogenic substrate cleavage activity (see the Materials and methods section) we note that the soluble furin lacking the Cys-rich domain [29] called BCRD-furin can further generate gp77/gp53, whereas PACE4 cannot (Figure 4B).

Figure 4 Processing of gp160 *in vitro* by human furin and PACE4

Purified [^{35}S]methionine-labelled gp160 was digested for 18 h with the specified activities [either at or below 31 units as in (A) or at 60–62 units as in (B)] of BCRD-furin and PACE4. One enzymic unit represents the number of nmol of 7-amino-3-methylcoumarin released from pGluArgThrLysArg-MCA in 18 h at 37 °C [29]. The reaction products were then analysed by SDS/PAGE (8% T, 2.7% C) followed by fluorography [20]. The control reaction (Ctl) performed in the absence of enzyme is also shown.

Is the 160 kDa form of gp160 the precursor of gp120?

The results in Figures 1 and 3 demonstrate the cellular presence of a major [^{35}S]methionine-labelled gp150 protein whose intensity did not seem to change appreciably with chase periods. This suggested that this isoform represents the gp160 protein still remaining within the endoplasmic reticulum (ER). This is in agreement with reports showing that only 5–40% of gp160 is processed, the remaining gp160 being possibly retained in the ER or eventually degraded [19,32]. To define further the processing pathway of the proportion of gp160 that reaches the TGN, we exploited the fact that recent data showed that some of the complex type N-linked oligosaccharide chains of gp160, gp120 and gp41 are sulphated within the Golgi apparatus [36].

As shown in Figure 5, analysis of the immunoprecipitated proteins of AtT20 (or BSC40; results not shown) cells pulse-labelled for 30, 90 or 180 min with $^{35}\text{SO}_4$ and expressing gp160 either alone (Figure 5, upper panel) or in combination with furin (Figure 5, lower panel) revealed that gp160, gp120, gp77 and gp41 are sulphated, whereas no $^{35}\text{SO}_4$ -labelled protein was observed migrating at either 53 kDa (as is especially apparent in the media) or 150 kDa. As controls, on expression of either VV:gp160 alone or VV:hfurin alone no protein was immunoprecipitated with a normal human serum or the immune human serum respectively (results not shown). Note that gp41 is more extensively labelled with $^{35}\text{SO}_4$ than are gp120 or gp77. That furin is also able to process gp160 in AtT20 cells is evident from the quantitative scanning of the autoradiograms (Figure 5). For example, at the 3 h pulse we estimate that furin causes a 16-fold increase in the intracellular ratio of sulphated (gp41+gp77+gp120)/gp160 varying from 2.4 in the absence of furin (Figure 5, upper panel) to 38 in its presence (Figure 5, lower panel). Furthermore, at the 30, 90 and 180 min pulse periods we respectively calculate 3-, 10- and 15-fold increases in the intracellular ratio of (gp120+gp41)/gp160 in the presence as against the absence of exogenous furin. This result provided the needed evidence that furin can process the 160 kDa form of gp160 into gp120/gp41 intracellularly. Furthermore whereas endo-F treatment released the sulphated moieties, arylsulphatase

Figure 5 Sulphation of gp160

AtT20 cells were co-infected either with VV:gp160 and VV:Wt or with VV:gp160 and VV:hfurin (gp160/Fur). The cells were then labelled with $\text{Na}_2^{35}\text{SO}_4$ for 30, 90 and 180 min. The SDS/PAGE analysis of the gp160-immunoprecipitated media and cell extracts and the autoradiography conditions were identical with those in Figure 1.

did not (results not shown). This suggests that gp160 and its derived products gp120, gp77 and gp41 are sulphated on one or more carbohydrate chains but not on a Tyr residue [37].

Ca^{2+} dependence and subcellular site(s) of gp160 processing

To define the subcellular site where processing of gp160 cleavage starts, AtT20 cells infected with either VV:gp160/VV:Wt (Figure 6A) or VV:gp160/VV:hfurin (Figure 6B) were pulse-labelled for 2 h with [^{35}S]methionine in the absence (control) or presence of a number of drugs. These included BFA, which causes the redistribution of the *cis* and medial Golgi stacks to the ER [38], a mixture of NaF/AlCl_3 , which inhibits the formation of secretory vesicles from the *trans*-Golgi [39], the calcium ionophore A23187 [40], or the N-glycosylation inhibitor tunicamycin [41]. Furthermore we performed the same experiment at 20 °C, a treatment that prevents the budding of immature secretory granules from the TGN [42]. The results of Figure 6 demonstrate that in the absence or presence of overexpressed furin the effects of these various treatments are very similar.

Treatment with BFA led to the formation of a product migrating with an apparent molecular mass of 110 kDa (gp110)

Figure 6 Definition of the subcellular localization and Ca^{2+} dependence of gp160 processing

AtT20 cells were either **(A)** infected with VV:gp160 (4 p.f.u.) alone or **(B)** co-infected with VV:gp160 (2 p.f.u.) and VV:hfurin (4 p.f.u.). The cells were then pulse-labelled for 2 h with [³⁵S]methionine in the presence of BFA, NaF/AICl₃, the Ca^{2+} ionophore A23187, or tunicamycin. The SDS/PAGE analysis of the gp160-immunoprecipitated cell extracts and media and the autoradiography conditions were identical with those in Figure 1.

that is more apparent in the presence of excess furin (Figure 6B). The formation of this immature form of gp120 in the presence of BFA suggests that cleavage of gp160 at the REKR↓AV sequence can start before the gp160 reaches the TGN. However, the formation of gp77/gp53 was virtually abolished by this treatment (Figure 6B), suggesting that the RIQR↓GPGR cleavage in the V3-loop occurs later than the cleavage needed for the formation of gp120/gp41. In agreement with these data, analysis of the incubations at 20 °C revealed that at this temperature processing of gp160 to gp120 does occur at a lower rate, but the gp77/gp53 forms are no longer detected. The conclusion reached from these data is that processing of gp160 into gp120/gp41 occurs at or

before the TGN, whereas the further processing of gp120 is favoured in a post-TGN organelle, possibly in the more acidic immature secretory granules.

The results of the A23187 and NaF/AICl₃ experiments show that these drugs led to an accumulation of gp150 with no further processing into gp160, gp120/gp41 or gp77/gp53. Interestingly, this treatment also prevented secretion of any gp160-derived proteins, as uncleaved gp150 is anchored to cellular membranes. Finally, tunicamycin treatment reduced the molecular mass of gp160 to about 90 kDa and prevented processing of this unglycosylated precursor. Because no secretion is observed, the absence of processing of this 90 kDa protein may

Figure 7 Endo H and Endo F sensitivity of gp160 and its products

AtT20 cells were either infected with VV:gp160 (4 p.f.u.) alone or co-infected with VV:gp160 (2 p.f.u.) and VV:hfurin (4 p.f.u.). The cells were then pulse-labelled for 2 h with [35 S]methionine. The immunoprecipitated cellular gp160, gp150, gp120, gp110, gp77 and gp53 were purified by SDS/PAGE and the individual forms were then divided into three equal fractions: (i) a control sample, (ii) a sample digested overnight with Endo H and (iii) another treated with Endo F. The total immunoprecipitated media were also divided into three equal fractions and treated in the same way as the cellular extracted forms. The digestion products of each fraction were then analysed by SDS/PAGE as in Figure 1.

be due to its inability to leave the ER and be processed by either the cellular enzymes (Figure 6A) or furin (Figure 6B). In addition, tunicamycin treatment led to the formation of lower-molecular-mass proteins, e.g. a doublet at the 35 kDa position with one of the bands also detected with BFA treatment. These proteins probably represent degradation products of gp160, as over-expression of furin did not significantly increase their level.

The *N*-glycosylation state of gp160 and its products

To define in more detail the *N*-glycosylation state of the various forms of gp160 and its derived products we purified each cellular form by SDS/PAGE and used them as well as total media immunoprecipitates to probe the effects of the Endo F and Endo H. From the results presented in Figure 7 it is clear that the gp160, gp150, gp120, gp110, gp77 and gp53 cellular products are sensitive to both Endo H and Endo F, suggesting that each form probably contains both complex type oligosaccharides (Endo F-sensitive only) but also that some chains are of the high mannose type (Endo H- and Endo F-sensitive). Interestingly, whereas the

Figure 8 Effect of NB-DNJ on gp160 processing

AtT20 cells were co-infected with VV:gp160 and VV:Wt or VV:gp160 and VV:hfurin (Fur). The cells were then labelled for 120 min with [35 S]methionine in the absence or presence (+) of 0.2 mg/ml of NB-DNJ (DNJ). The SDS/PAGE analysis and autoradiography conditions were identical with those in Figure 1.

intracellular gp110 is processed by Endo H into a similar 60 kDa form to that obtained by treatment with Endo F, the intracellular gp120 is cleaved by Endo H to yield mostly a 75 kDa form compared with the 60 kDa form obtained by Endo F. This result agrees with the notion that gp120 is derived by carbohydrate trimming and remodelling of the gp110 form, because some of its carbohydrate chains are resistant to Endo H digestion. Only the gp120 is found in the media, and whereas its digestion with Endo H leads to the same 75 kDa form as the intracellular gp120, its Endo F digestion product migrates with an apparent molecular mass of about 95 kDa, which is much higher than the 60 kDa calculated for the equivalent product of the intracellular gp120. Clearly this suggests that the secreted gp120 is further processed to yield a more Endo F-resistant form than the intracellular gp120.

In the presence of overexpressed furin, the 77 and 53 kDa forms produced are also sensitive to both Endo H and Endo F, with the gp77 containing some Endo H-resistant chains, because its digestion product migrates at 55 kDa compared with the 36 kDa Endo F product. Again, Endo F treatment in the media gives rise to higher-molecular-mass products than in the cells. In conclusion, the multiple carbohydrate chains of gp120 are quite complex, exhibiting both Endo H-sensitive and Endo H-resistant characteristics.

Is the trimming of oligosaccharides needed for gp160 processing?

Over the past few years, a number of amino sugar derivatives capable of inhibiting glycoprotein processing have been reported to inhibit cellular cytopathicity *in vitro* and hence to attenuate

Figure 9 Effect of α 1-PDX on the processing of gp160 in the absence or presence of exogenous hfurin, hPACE4 or mPC5/6-B

VV:gp160 (1 p.f.u.)-infected AtT20 cells were co-infected with either VV:convertases (2 p.f.u.) and VV:Wt (5 p.f.u.) or with VV:convertases (2 p.f.u.) and VV: α 1-PDX (PDX) (5 p.f.u.). The cells were pulse-labelled for 30 min with [35 S]methionine and then chased for 2 h. The SDS/PAGE analysis of the immunoprecipitated cell extracts and media and autoradiography conditions were identical with those in Figure 1.

HIV infections [43–45]. These compounds include castanospermine and its derivatives 1-deoxynojirimycin (DNJ), *N*-alkylated derivatives of DNJ and other related amino sugars [43–46]. All these compounds are potent inhibitors of specific glucosidases involved in glycoprotein processing [47]. When a range of sugar analogues were screened for anti-HIV activity *in vitro*, the imino sugar NB-DNJ was found to be a potent inhibitor of infection and to exhibit minimal cytotoxicity. This compound was shown to inhibit *in vitro* the purified glycoprocessing enzyme α _{1,2}-glucosidase I with a *K*_i of 0.22 μ M and in intact cells its optimal antiviral concentration was found to be around 0.5 mM [45].

The envelope glycoprotein gp160 of the HIV virus is heavily *N*-glycosylated with the processed products gp120 and gp41 containing respectively, 20–25 and 4 potential N-linked glycosylation sites with the oligosaccharides contributing about 50 % of the protein mass [1]. The mechanism by which NB-DNJ and its congeners affect viral infectivity is not known. However, it has been demonstrated that treatment with NB-DNJ affects the surface charge of gp120 and hence decreases its ability to interact with other cellular and viral components and thereby to modulate infectivity. It is also possible that because glycosidase I inhibitors interfere with early processing of sugars, the change of conformation of gp160 could affect its processing by host-cell convertases. We therefore verified this hypothesis by evaluating the effect of NB-DNJ on the processing of gp160 in AtT20 cells by the convertases furin (Figure 8), PACE4, PC1 and PC5 (results not shown). As seen in Figure 8, the addition of NB-DNJ, as expected, elevated the molecular masses of gp160 and gp120 in both cellular extracts and media: they now migrated with apparent molecular masses of 165 and 130 kDa respectively. However, we note that the processing of gp160 by host-cell convertases or by excess furin was not significantly affected. Furthermore, as seen from the media results, the further cleavage of gp120 into gp77/gp53 by furin was also not affected by this

drug. Therefore the anti-HIV properties of NB-DNJ do not involve a difference in the processivity of gp160.

α 1-PDX inhibits the processing of gp160 by furin, PACE4 and PC5/6-B

Anderson et al. [28] demonstrated that the expression in BSC40 cells of an Ala/Arg³⁵⁵, Met/Arg³⁵⁸ variant of α 1-antitrypsin, known as α 1-PDX, inhibits the processing of both β -nerve growth factor and gp160 by the endogenous convertase(s). Because furin is the major convertase in BSC40 cells [16], and because α 1-PDX was shown to inhibit furin activity *in vitro* at a *K*_{0.5} of 0.03 μ g/ml [28], it was plausible that the observed processing inhibition in BSC40 cells was due to the blockage of the furin activity by α 1-PDX. We therefore sought to verify whether the gp160 cleavage by both furin and PACE4 was also inhibitable by the co-expression of α 1-PDX. Figure 9 shows the SDS/PAGE analysis of the co-expression in AtT20 cells of gp160 with either furin or furin/ α 1-PDX, and PACE4, PACE4/ α 1-PDX and PC5/6-B or PC5/6-B/ α 1-PDX after a 30 min pulse-labelling with [35 S]methionine and a 120 min chase. The data show that in both cells and media the co-expression of α 1-PDX does not completely inhibit the processing of the 160 kDa form of gp160 into gp120 by the endogenous AtT20 convertases and by overexpressed furin, PACE4 or PC5/6-B. However, the formation of gp77/gp53 seems to be more effectively, although not totally, abolished by this inhibitor. These data agree with the original report of Andersen et al. [28] and further show that PACE4 and PC5/6-B processing of gp160 as well as the formation of gp77/gp53 are also inhibitable by α 1-PDX. The 60 kDa band seen in the medium of the PACE4/PDX experiment is likely to be the secreted α 1-PDX itself, which sometimes co-immunoprecipitates with gp120, as it is also observed upon co-expression of gp160/ α 1-PDX but not gp160/Wt (results not shown). In addition, the co-expression of gp160 with either α 1-antitrypsin or

its Met/Arg³⁵⁸ variant known as α 1-antitrypsin Pittsburgh did not affect the proteolytic processing of gp160 (results not shown).

DISCUSSION

Whereas the polyprotein coded by the *gag/pol* gene of HIV is processed by the virally encoded HIV protease, the other polyprotein (gp160), encoded by *env*, is processed by host-cell proteinases. Mutational analysis of gp160 in the region of the cleavage site identified a crucial Arg⁵¹⁸ residue whose mutation to Thr blocks the gp160 cleavage, syncytium formation and viral infectivity [48–50]. Because envelope glycoproteins of many viruses, including HIV (cleaved sequence RVVQREKR↓AV), are produced from polyprotein precursors by cleavage at RX(K/R)R↓ sites, it was hypothesized that one or more members of the newly discovered serine proteinases belonging to the subtilisin/kexin family (for reviews see [14–18,51]) could be responsible for such a cleavage. Indeed, substrate mutational analysis demonstrated that one such member, furin, which is ubiquitously expressed in all tissues and cells [16], exhibits a preference for cleavage after RX(K/R)R↓ sequences [52]. The first evidence that furin was involved in the processing of gp160 was reported by Hallenberger et al. [19], who showed (using the vaccinia virus expression system) that in the constitutively secreting CV-1 cells, furin is capable of converting gp160 into gp120 and gp41. In view of the identification of six mammalian members of the subtilisin/kexin family (PC1, PC2, furin, PACE4, PC4 and PC5), five of which (excluding PC4) are potentially expressed in lymphocytes, it was of interest to compare the gp160 conversion efficiency of each convertase with that of furin. This was first done *in vitro*, where it was shown that PC1 is able to convert gp160 into gp120/gp41, albeit at a reduced efficiency when compared with furin [20]. Another clue as to the possible participation of other members in this reaction was provided from the ability of LoVo cells to process gp160 even though they are deficient in furin activity [22] and PC1 mRNA [16], but express PACE4 [17]. Therefore in this report we systematically examined the ability of each convertase furin, PACE4, PC1, PC2, PC5 and its isoform PC5/6-B to effect such intracellular processing in both constitutive and regulated cells.

The results presented in this work with the vaccinia virus expression system allowed us to conclude that furin and to a lesser extent PACE4, PC5/6-B and PC1 are the best candidate convertases for the processing of gp160, whereas PC2 and PC5 were clearly unable to process this precursor (Figure 3). Therefore, although PC5 and furin (but not PACE4) process haemagglutinin of virulent avian influenza virus 53, our data show that PC5 is not able to process gp160. The cellular expression results are in agreement with data from experiments *in vitro* that showed that furin and PC1 [20] as well as PACE4 (Figure 4) are all capable of processing gp160 into gp120/gp41. Interestingly, overexpression of furin in mammalian cells further led to the formation of gp77/gp53, an observation first reported in insect cells co-infected with recombinant baculoviruses expressing furin and gp160 [11]. However, we observed this second cleavage *in vitro* only at high levels of soluble furin (Figure 4). This suggests that the second cleavage is less favoured than that leading to gp120/gp41. Indeed, our data further demonstrate that whereas the gp120/gp41 can occur within the Golgi apparatus, the gp77/gp53 formation is a post-TGN event, as incubation at 20 °C blocked its occurrence (Figure 6). This second cleavage occurs at a site within the sequence RIQR↓GPGRF found in the V3 loop around the conserved GPGR structure. Interestingly, in contrast with the specificity of furin, PACE4 and PC5/6-B reported here, it was shown that, *in*

vitro, thrombin and trypsin prefer the GPGR↓AF site [10]. We note that the site recognized by furin and PACE4 fits the minimal consensus sequence of furin processing, which is RXXR↓, emphasizing the importance of the P1 and P4 Arg residues for optimal furin activity [54,55]. Recently it was proposed that the P6 [56,57] and possibly P8 [58–61] positions could also improve the cleavage efficiency by furin or PC1, but to a smaller extent than the P4 Arg [54]. Cleavage of the gp120 within the V3 loop has been reported to be an essential step in the processes leading to virus–cell fusion [6,7,62]. It has also been reported that such cleavage could occur at the host cell surface [9]. The fact that the exogenous addition of furin leads to the cleavage of gp120 into gp70/50-like products may be due to the high levels of furin attained during such an experiment. Because intracellular furin levels are in general low [16,18], this may explain the observed reduced extent of cleavage of viral gp120. Interestingly, the virion protein Vpu was reported to stabilize gp160 against degradation during its transit through the infected cell [63]. It is therefore also possible that viral proteins affect the degree of processing of gp120 in HIV-infected cells and the access of furin to the V3 loop could thus be restricted during virion egress, as suggested by Morikawa et al. [11]. However, because it was reported that furin, via its transit from the TGN to the cell surface [28], is able to process extracellular precursors such as anthrax toxin [55] and diphtheria toxin [64], it is also likely that furin participates in the processing of gp120 at the cell surface. What would be the advantage gained by the HIV virus from such a cleavage? Is it possible that the exposure of the NH₂-GPGRF... sequence at the N-terminus of gp53 may enhance cellular infectivity? Very recent evidence was presented in favour of such a model, as it was reported by Yahi et al. [65] that a synthetic polymeric construction displaying eight GPGRF motifs radially branched to a polylysine core matrix interfered with the ability of HIV-1 entry into CD4⁺ and CD4⁺ cells, possibly by competing with the cellular binding of the V3 loop.

The data presented here imply that a certain redundancy between the functions of furin, PACE4, PC5/6-B and PC1 can be expected [16]. However, in view of the plasticity of the mRNA levels of furin [20] and possibly PACE4 [66], it is likely that the relative contribution *in vivo* of each enzyme to the gp160 processing is affected by factors regulating their cellular levels. So far, furin is the only known convertase that exhibits a ubiquitous distribution, but PACE4 and PC1 mRNAs were detected in both spleen and thymus [16], suggesting their possible expression within the haematopoietic system. However, it will be necessary in the future to verify the expression in CD4⁺ cells of PACE4 and PC5/6-B, or possibly of other as yet unidentified convertases, as was done for furin and PC1 [20].

The present results also suggest that the exit of the 150 kDa form of gp160 from the ER to the TGN is rather slow and that only a fraction reaches the TGN and becomes the mature sulphated gp160 which is a substrate for the convertases (Figure 5). The fraction that is not cleaved is either retained in the ER, as evidenced from its lack of sulphation (this work) and sialylation [67], or is misfolded, thereby preventing it from being processed [68].

It has been suggested that alteration of oligosaccharide structures could induce local changes in the gp120 protein conformation, resulting in the attenuation of viral infectivity [69]. Host-cell enzyme cleavage of gp160 into gp120/gp41 or HIV-1-induced syncytium formation were reported to be unaffected by treatment with 1-deoxymannojirimycin and swainsonine, which are inhibitors of the Golgi α -mannosidase I and II respectively [67,69]. Similarly, 1-deoxynojirimycin and castanospermine, which are inhibitors of endoglucosidase I, were

reported to markedly reduce syncytium formation and interfere with infectivity [43,70]. Based on these data, it was suggested that some of the derivatives of these glucosidase inhibitors may prove useful as potential therapeutic agents in the control of viral infections, including AIDS [45]. Ratner et al. [70] suggested that although treatment of CD4⁺ cells with NB-DNJ could decrease the rate of proteolytic processing of gp160 to gp120/gp41, neither the level of cell surface expression of gp120 nor the amount of gp120/gp41 in the progenitor virus particles seems to be affected by this drug. In the work presented here, we demonstrate that treatment of AtT20 cells with NB-DNJ does not affect the ability of furin or PACE4 to process either gp160 into gp120/gp41 or gp120 into gp77/gp53. Although the results are not shown, we also found that treatment of cells with NB-DNJ does not affect the ability of furin, PC1 or PC2 to process another precursor pro-opiomelanocortin (N. G. Seidah and S. Benjannet, unpublished work). Although NB-DNJ has proved to be a potential antiviral agent, on the basis the results presented here, we suggest that the impaired HIV-1 infectivity and cytopathogenicity caused by this drug is not related to the ability of the convertases to process gp160 or gp120.

In an effort to design inhibitors of the subtilisin/kexin-like convertases as potential novel antiviral agents, Anderson et al. [28] reported that α 1-PDX is a potent inhibitor of furin activity, because in cells expressing gp160 it can reduce their ability to exhibit syncytia formation. In this work we further demonstrate that α 1-PDX is an inhibitor of both furin- and PACE4-mediated processing of gp160 and gp120 (Figure 9), suggesting a wider spectrum of inhibitory activity for this serpin. In agreement with this hypothesis, we also found that α 1-PDX completely inhibits the furin- and PACE4-mediated processing of the PC2-specific binding protein pro7B2 [25] in LoVo cells (S. Benjannet and N. G. Seidah, unpublished work). To design inhibitors specific for each convertase, more mutagenesis is therefore needed and, as more information is gathered on the cleavage site specificity of each enzyme, more selective inhibitors can be made.

In conclusion, the data presented in this report demonstrate that furin and to a lesser extent PACE4, PC5/6-B and PC1 are the best candidates for subtilisin/kexin-like convertases capable of intracellular proteolytic cleavage of gp160. The intracellular processing of gp160 by other candidate proteinases not related to the subtilisin/kexin family [71] still awaits experimental confirmation.

We thank Dr. J. Cogniaux (Institut Pasteur du Brabant, Brussels, Belgium) for the gp160 sera, and Dr. Majambu Mbikay for critical reading of the manuscript; Dr. Jim Hicks and Dr. Kazuhisa Nakayama for generously providing us with the α 1-PDX vaccinia recombinant and the PC6-B cDNA respectively; Dr. Gary S. Jacob (Monsanto, St. Louis) for his support during this work and for providing the NB-DNJ; and Sylvie Emond for secretarial assistance. This work was supported by a research grant from MRC-Industry (UI-12378), by an MRC/PENCE network grant and by J. A. deSève Succession. F. V. is a recipient of a Fonds National Suisse (FNS) bursary. The Clinical Research Institute of Montréal is affiliated to the University of Montreal and Protein Engineering Network of Centres of Excellence.

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