Endoproteolytic processing of integrin pro-α subunits involves the redundant function of furin and proprotein convertase (PC) 5A, but not paired basic amino acid converting enzyme (PACE) 4, PC5B or PC7

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Several integrin α subunits undergo post-translational endoproteolytic processing at pairs of basic amino acids that is mediated by the proprotein convertase furin. Here we ask whether other convertase family members can participate in these processing events. We therefore examined the endoproteolysis rate of the integrin subunits pro-α5, α6 and αv by recombinant furin, proprotein convertase (PC)5A, paired basic amino acid converting enzyme (PACE)4, PC1, PC2 and PC7 *in vitro* and/or *ex vivo* after overexpression in LoVo cells that were deficient in furin activity. We found that 60-fold more PC1 than furin was needed to produce 50 % cleavage of pro-α subunit substrates *in vitro*; the defective pro-α chain endoproteolysis in LoVo cells was not rescued by overexpression of PC1 or PC2. No endoproteolysis occurred with PC7 either *in vitro* or *ex vivo*, although similar primary sequences of the cleavage site are found in integrins and in proteins efficiently processed by PC7, which suggests that a particular conformation of the cleavage site is required for optimal convertase–substrate interactions. *In vitro*, 50 % cleavage of pro-α subunits was obtained with one-third of the amount of PC5A and PACE4 than of furin. In LoVo cells, PC5A remained more active than furin, PACE4 activity was quite low, and PC5B, which differs from PC5A by a C-terminal extension containing a transmembrane domain, was very inefficient in processing integrin α-subunit precursors. In conclusion, these results indicate that integrin α-subunit endoproteolytic processing involves the redundant function of furin and PC5A and to a smaller extent PACE4, but not of PC1, PC2, PC5B or PC7.

Key words: cellular expression, integrin processing, proteolytic cleavage, vaccinia virus.

INTRODUCTION

Endoproteolytic cleavages at sites comprising basic amino acids contribute to the post-translational processing of many proteins, polypeptide hormones, growth factors and neuropeptides. They are also critical for the functional activation of several cell-surface receptors, adhesion molecules and extracellular-matrix proteins [1,2]. The search for mammalian homologues of kexin, a yeast endoprotease that cleaves at dibasic residues, has resulted in the discovery of a family of mammalian Ca^{2+}-dependent serine endoproteases related to bacterial subtilases known as the proprotein convertases (PCs) [1,2]. These enzymes mediate peptide-bond hydrolysis C-terminal to arginine in a consensus site requiring additional basic amino acids in position P2, P4, P6 or P8. These enzymes (reviewed in [1,2]) exhibit the same structural organization consisting of: (1) an N-terminal pro-domain that undergoes autocatalytic cleavage and has to dissociate from the remainder of the molecule for the enzyme to be active; (2) a subtilisin-like catalytic domain sharing between 51 % and 75 % amino acid sequence identity within the family; and (3) a P-domain needed for the proper folding and function of the molecule. Two main subsets of these enzymes can be distinguished. The first includes convertases such as PC2 and PC1/PC3, expressed mainly in endocrine and neural tissues including brain, which are sorted into secretory granules of the regulated exocytic pathway [1,2]. These enzymes have the key role of releasing hormones and neuropeptides from biologically inactive precursors [1–3]. The second subset of convertases, which includes furin, paired basic amino acid converting enzyme (PACE)4, PC5/PC6 and PC7/LPC/PC8 [1,2], shows a wider tissue distribution in the body. Except for PC1, PC2 and PC7, these enzymes contain a C-terminal cysteine-rich region. In addition, furin, PC7 and PC5B (a splice variant of PC5A [1,2]) are type I membrane-bound convertases with a C-terminal transmembrane domain and a cytosolic tail that contains motifs involved in the regulation of trafficking within the *trans*-Golgi (TGN) per cell surface/endosomal network [4–7]. Generally, this subset is believed to be involved in the processing of proteins exported along the constitutive exocytic pathway such as growth factors and their receptors [7–11], extracellular matrix proteins [12,13], proteinases [14,15] and viral coat proteins [16,17].

Integrins are transmembrane, cell-surface α/β heterodimeric adhesion receptors [18]. The ligand-binding function of integrins, which seems to be constitutive in most cell types, is regulated in leucocytes by intracellular signals triggered by growth factors or chemokines (inside-out signalling) [19]. On interaction with ligands in the extracellular matrix or on the cell surface of neighbouring cells, integrins form internal connections with the

Abbreviations used: AMC, 7-amino-4-methylcoumarin; BTMD, before transmembrane domain; HRP, horseradish peroxidase; mAb, monoclonal antibody; Mca, 4-methylcoumarin-7-amide; PACE, paired basic amino acid converting enzyme; PC, proprotein convertase; pfu, plaque-forming unit; TGN, *trans*-Golgi network; VV, vaccinia virus.

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cytoskeleton and stimulate various signalling pathways that participate in the control of cell movement, gene expression and cell survival (outside-in signalling) [20]. Of the 13 α subunits known so far, 10 (α3, α4, α5, α6, α7, α8, α9, αv, αE and αIIb) undergo post-translational endoproteolytic cleavage at a site comprising pairs of basic amino acids. Recent results with leucocytic cells suggest that endoproteolytic cleavages are required for $\alpha6\beta1$-integrin activation but not for the outside-in signalling events leading to adhesion [21]. However, the possibility has not been excluded that they are required for more sophisticated aspects of integrin function such as the regulation of cell migration and/or signalling.

The α-subunit cleavage site consists of a dibasic (Lys/Arg-Arg) sequence with an additional basic residue at P4 (Arg, in α3, α6, α7, αIIb and αE) or P6 (His, in α4, α5, α8 and αv). Except for α4, where it is located in the middle of the molecule, the cleavage site is found extracellularly near the transmembrane domain such that the mature molecule comprises a membrane-bound 25–35 kDa C-terminal light chain disulphide-bonded to the N-terminal heavy chain. It is most likely that furin participates in integrin endoproteolysis, because pro-α-subunit cleavage, which does not take place in LoVo cells that are deficient in furin activity [22], can be achieved by the recombinant enzyme [23]. Furin becomes fully activated on reaching the TGN, after which it is delivered to the plasma membrane and recycled back to the TGN [24]. Thus proprotein cleavage by furin can take place along the constitutive exocytic and endocytic pathways. Pulse–chase experiments have shown that integrin α-subunit cleavage takes place rapidly after translocation to cell compartments lying downstream of the endoplasmic reticulum [23]. Because integrins are endocytosed from the surface at a slow pace, these kinetics suggest that cleavage takes place either at the level of the TGN or during exocytosis.

Owing to the wide-ranging cleavage specificity of convertases and their overlapping expression in different tissues [1,2], it is possible that more than one convertase is involved in the endoproteolysis of a particular substrate. Answering this question might lay the foundation for selective therapeutic intervention with specific convertase inhibitors such as those developed by combinatorial peptide chemistry [25] or the engineering of naturally occurring inhibitors [26–28] or convertase prodomains [29,30]. Here we analyse this issue for integrins by examining the endoproteolysis rate of pro-α5, α6 and αv integrin subunits by recombinant PC1, PC2, PACE4, PC5A, PC7 and furin both *in vitro* and/or by overexpression in LoVo cells infected with vaccinia virus (VV) recombinants containing the coding sequences of the corresponding convertases. The results demonstrate that PC5A and to a much smaller extent PACE4, but not PC7, PC5B, PC1 or PC2, can function similarly to furin in integrin α-chain endoproteolysis.

MATERIALS AND METHODS

Recombinant vaccinia viruses and recombinant enzyme production

The VV recombinants of α5, αv and β1 [31] as well as those of the PCs [16,17,27] have already been described. Secreted mouse PC1, human PACE4, mouse PC5A, mouse PC5B, before transmembrane domain (BTMD) human furin (BTMD-furin) and rat PC7 (BTMD-PC7) were purified from the media of VV-infected GH_4C_1 cells, as described [16,32]. The specific activities of the enzyme preparations obtained were measured with the fluorogenic peptide pERTKR-4-methylcoumarin-7-amide (pERTKR-Mca) at 100 mM (Peptides International, Louisville, KY, U.S.A.) as described [16], on the basis of the increase of fluorescence generated by the cleavage of this substrate by the enzymes in 0.06 M Tris/HCl (pH 6.8)/1 % (w/v) n-octyl glucoside. After incubation (1 h, 37 °C) in the presence of 2 mM Ca^{2+} or 5 mM EDTA, the fluorescence of released 7-amino-4-methylcoumarin (AMC) was measured at 460 nm (excitation at 370 nm) and evaluated by plotting on a standard calibration curve with known amounts of free AMC. The Ca^{2+}-dependent release of AMC was used to calculate the activity of the enzymes as nmol of fluorogenic peptide hydrolysed/h per μl of enzyme.

Antibodies

The following antibodies were used for the immunoprecipitation of integrins: monoclonal antibody (mAb) 69-6-5 against the αv integrin subunit [33] (purchased from Immunotech, Marseille, France), anti-α3 (clone C3VLA3), anti-α5 (clone SAM1) and anti-α6 (clone GoH3). Antibodies raised against the C-terminal part of the α subunit were used for integrin α-chain detection after immunoblotting: anti-(C-terminal α6A) mAb (clone 4E9G8; Immunotech) and anti-α5 polyclonal Ab produced by us in rabbits by serial injections of a 21-residue cysteinyl-C-terminal α5 peptide coupled to BSA.

Cellular infection

LoVo cells were grown to subconfluence in Dulbecco's modified Eagle's medium containing 10 % (v/v) fetal calf serum on 75 cm^2 culture dishes, rinsed in PBS, and infected as indicated with recombinant viruses at 1 plaque-forming unit (pfu) per cell in 5 ml of PBS/0.01 % BSA for 30 min at room temperature. After being washed in culture medium, cells were left overnight in the cell culture incubator.

Pro-α-integrin hydrolysis *in vitro* by recombinant convertases

$\alpha5\beta1$ and $\alpha v\beta1$ integrins were obtained from LoVo cells co-infected with the VV recombinants VV:β1 and VV:α5 or VV:αv [31] each at 1 pfu per cell. Endogenous $\alpha6\beta4$ integrins were obtained from non-infected cells. Cells infected with αv and β1 viruses were labelled metabolically for 18 h at 37 °C with 150 μCi of Tran^{35}S-label™/ml in Dulbecco's modified Eagle's medium containing 1/10 the usual concentrations of methionine and cysteine and 10 % (v/v) dialysed fetal calf serum. After being washed with PBS, cells were lysed in 50 mM Tris/HCl (pH 8)/150 mM NaCl/0.5 % BSA/1 mM EDTA/5 mM iodoacetamide/1 % (v/v) Triton X-100 containing the protease inhibitors aprotinin, leupeptin, pepstatin and α_2-microglobulin (each at 1 μg/ml) and 1 mM PMSF. After centrifugation at 15000 ***g*** for 10 min, extracted proteins were incubated with anti-integrin mAbs (2 μg) for 18 h at 4 °C and with the Protein G–agarose or magnetic beads coated with anti-rat or anti-mouse immunoglobulins (Dynal, Compiègne, France) for a further 2 h. After being washed, the beads were resuspended in 0.06 M Tris/HCl (pH 6.8)/1 % (w/v) *n*-octyl glucoside. Equal fractions of the immunoprecipitates were digested for 1 h at 37 °C with serial dilutions of the convertase in the presence of 5 mM Ca^{2+} or EDTA, as indicated. Digestions were stopped with a 5-fold concentrate of reducing SDS/PAGE sample buffer and analysed by electrophoresis on two-layered SDS/polyacrylamide gels; the polyacrylamide concentration was 6 % (w/v) in the upper layer of resolving gels and 12 % (w/v) in the lower layer. After electrophoresis, the gels loaded with $\alpha v\beta1$ integrin material were impregnated with Amplify (Amersham, Les Ulis, France), dried and autoradiographed on KodaK X-O-Mat AR film at −80 °C. The gels loaded with $\alpha5\beta1$ or $\alpha6\beta4$ integrins were electrotransferred to nitrocellulose membranes (Hybond C+; Amersham) and blocked with PBS/5 % (w/v) non-fat dried

milk. α-subunit C-terminal immunoreactive material was detected by incubation with mAb 4E9G8 (2 μg/ml) or purified anti-α5 polyclonal immunoglobulins (5 μg/ml). After being washed, blots were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit immunoglobulins (Immunotech, Marseille, France), as required. Bound HRP–antibodies were revealed with an HRP chemiluminescent substrate (Pierce, Interchim, Montluçon, France), in accordance with the supplier's instructions.

Pro-α-integrin hydrolysis *ex vivo* by recombinant convertases

LoVo cells were infected at 1 pfu per cell with VV recombinants of α5, β1 and one of each convertase: PC1, PC2, PACE4, furin, PC5A, PC5B, PC7 or the wild-type VV control. After 18 h, cells were washed with PBS containing 1 mM Mg^{2+} and labelled with sulphosuccinimidobiotin-EZ-link (Pierce) at 0.5 mg/ml in ice-cold PBS/1 mM Mg^{2+} for 30 min at 4 °C. After the cells had been washed, cell lysates were prepared and divided into equal fractions for immunoprecipitation of integrin with anti-α5, αv and α6 antibodies, as described above. Immunoprecipitates were analysed by SDS/PAGE [6 % (w/v) gel] under non-reducing or reducing conditions. After electrotransfer of the gel contents to nitrocellulose, the blots were blocked with 50 mM Tris/HCl buffer (pH 8)/10 % (w/v) glycerol/1 M glucose/0.5 % BSA/0.1 % (v/v) Tween 20. Biotinylated proteins were detected with HRP–streptavidin and chemiluminescence. Band intensities on chemiluminograms or autoradiograms were quantified by image analysis (Herold Lab, Osis, France).

RESULTS

Analysis *in vitro* of pro-α-integrin cleavage by recombinant convertases

It has previously been shown that endoproteolytic processing of integrin α subunits is defective in LoVo cells [23]. Therefore integrin pro-α-chains immunoprecipitated from these cells were used as substrates for hydrolysis by a panel of convertases *in vitro*. Sufficient amounts of pro-α6 subunit, which contains a dibasic consensus cleavage site with an Arg residue at P4, are constitutively produced by the cells to permit an analysis of the endoproteolysis of this precursor [23]. In contrast, because of the low expression by LoVo cells of pro-αv or pro-α5 subunits, which exhibit an **H**XXX**KR** ↓ cleavage motif, we co-expressed in these cells their VV recombinants together with VV:β1. Cleavage of immunoprecipitated pro-α subunits was evaluated by measuring the production of the 35 kDa light chain after reducing SDS/PAGE and immunoblotting with antibodies directed against the C-terminus of the α6 or α5 subunits.

Figure 1 shows that a Ca^{2+}-dependent cleavage of the 140 kDa pro-α6 subunit with the corresponding production of the C-terminal light chain was obtained with PC5A, furin and PACE4 but not with PC7. Experiments done with 2-fold serial dilutions of the enzymes showed a dose-dependent cleavage of the α6 subunit precursor by furin, PACE4, PC5A and PC1 (Figure 2A), whereas no cleavage occurred with PC7 (results not shown). A similar pattern was found with the α5 subunit precursor, which was hydrolysed by furin, PACE4 and PC1 (Figure 2B) but not by PC7 (results not shown). Interestingly, both pro-α6 and pro-α5 subunits were least efficiently cleaved by PC1 (Figure 2). This was ascertained more precisely by considering pro-α-subunit cleavage as a function of the ability of the enzyme preparations to hydrolyse the fluorogenic peptide substrate pERTKR-Mca. Thus 50 % cleavage of the pro-α6 subunit required relative amounts of pERTKR-Mca-cleavage activities of 1, 0.3, 0.3 and 60 for furin, PC5A, PACE4 and PC1 respectively (Figure 3A). Endoproteolytic sensitivity of the α5 and α6 subunit precursors to furin was not significantly different and cleavage selectivities were similar because, in comparison with furin, the cleavage of pro-α5 was also obtained with approx. one-third the amount of PACE4 and 100-fold more PC1 (Figure 3B). No cleavage was observed with PC7, although it displayed the highest hydrolysis rates of pERTKR-Mca of all enzyme preparations (0.7 nmol/h per μl). This convertase was therefore at most 1/200 as potent as furin for endoproteolysis of the α6 or α5 subunit precursors. Digestion by convertases of immunoprecipitated, overexpressed ^{35}S-labelled αvβ1, α5β1 or α4β1 integrins yielded results quite similar to those obtained for pro-α5 and pro-α6 subunits (results not shown). No digestions were performed with PC2, because this enzyme is only active in secretory-granule-containing and 7B2-containing cells [1,2], which is not true of constitutively secreting cells expressing integrins.

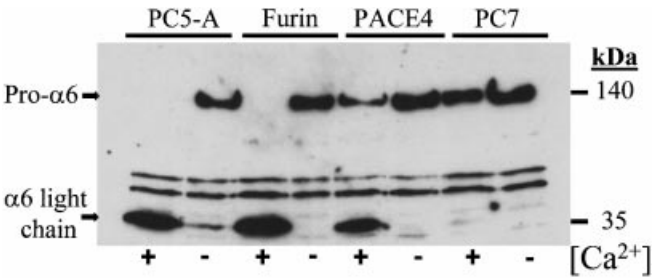

Figure 1 Ca^{2+}-dependent cleavage of the integrin pro-α6 *in vitro* by selected proprotein convertases

α6β4 integrins immunoprecipitated from LoVo cells were incubated for 1 h at 37 °C with 20 μl of recombinant PC5A, BTMD-furin (Furin), PACE4 or BTMD-PC7 (PC7) in the presence of 5 mM Ca^{2+} (+) or EDTA (−). The specific activities of the enzyme preparations evaluated by cleavage of the fluorogenic peptide pERTKR-Mca were 0.15, 0.17, 0.025 and 0.7 nmol/h per μl respectively. After digestion, reducing SDS/PAGE [6–12 % (w/v) gradient polyacrylamide gel] and electrotransfer to nitrocellulose, the presence of a C-terminal immunoreactivity of the pro-α6 subunit (140 kDa) or the α6 light chain (30 kDa) was evaluated with a mAb directed against a 23-residue peptide corresponding to the C-terminal sequence of α6. HRP-conjugated anti-mouse immunoglobulins served as the secondary antibodies, after which the blots were revealed by chemiluminescence. The unlabelled double bands correspond to the heavy and light chains of the immunoglobulins used for immunoprecipitation.

Pro-α-integrin cleavage *ex vivo* by overexpressed convertases in LoVo cells

Besides expression levels and hydrolysis efficiencies, the functional specificity of convertases involves the regulation of their zymogen activation, along with the targeting of enzymes and substrates to the same cellular microdomain [34]. Therefore the overexpression of integrin α5β1 in LoVo cells served as a model for further cellular analysis of the selectivity of pro-α-integrin subunit cleavage by convertases. At 18 h after infection with VV recombinants, the cell surface was biotinylated and the cleavage of integrin α subunits was assessed after immunoprecipitation, reducing SDS/PAGE and immunoblotting with HRP-conjugated streptavidin (Figure 4). No processing of pro-α subunits was observed in control cells infected with the wild-type virus. In these cells we observed the association of overexpressed pro-α5 and β1 chains (Figure 4A), endogenously expressed pro-α6 with the endogenous β4 or overexpressed β1 (Figure 4B), and the endogenous pro-αv with β5 or β6 subunits (Figure 4C). α-subunit precursors were all cleaved completely by overexpressed PC5A, significantly by furin and not at all by PC1, PC2 or PC7

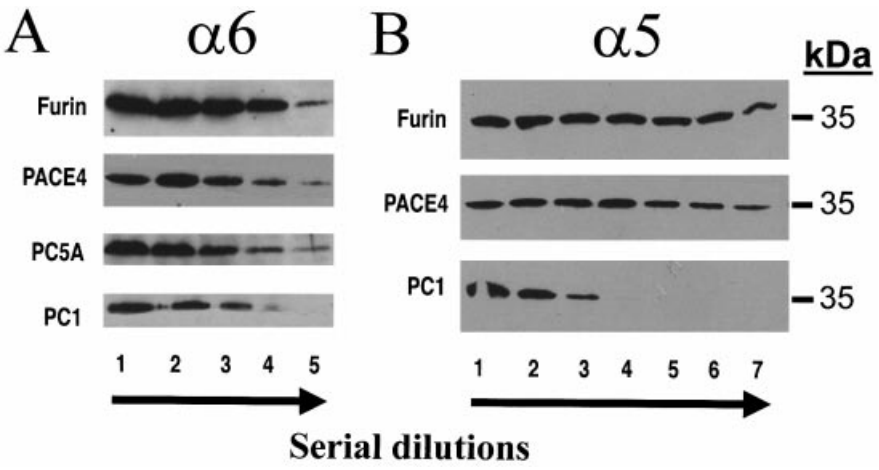

Figure 2 Dose–response profile of pro-α6 and pro-α5 integrin subunit cleavage by recombinant convertases *in vitro*

(**A**) Production of α6 light chain after incubation of α6/β4-β1 immunoprecipitates with 2-fold serial dilutions (lanes 1–5) of recombinant BTMD-furin (Furin), PACE4, PC5A and PC1. After digestion, the samples were analysed as described in the legend to Figure 1. The region of the chemiluminograms corresponding to the migration position of the α6 light chain is shown. (**B**) Production of α5 light chain after incubation of α5β1 immunoprecipitates with 2-fold serial dilutions (lanes 1–7) of recombinant furin, PACE4 and PC1. After digestion, samples were analysed, as described in the legend to Figure 1, with a polyclonal antiserum raised against a peptide corresponding to the C-terminal sequence of α5 integrin. The region of the chemiluminograms corresponding to the migration position of the 35 kDa α5 light chain is shown.

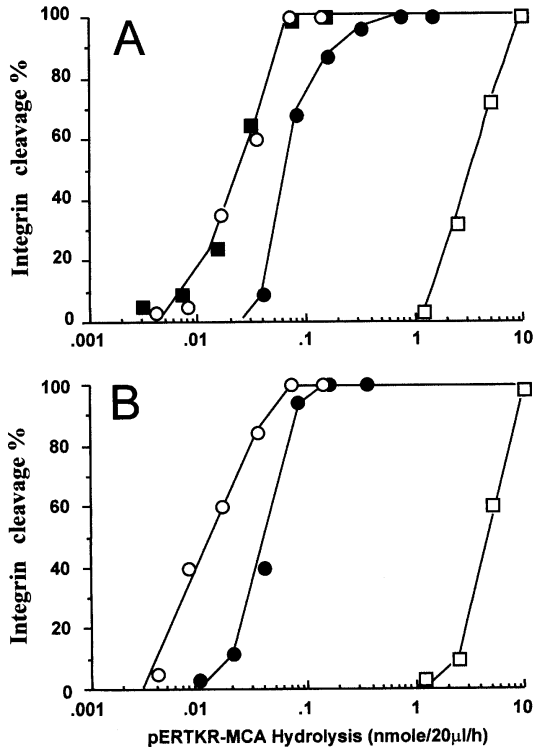

Figure 3 Dose–response profile of integrin of the hydrolysis *in vitro* of pro-α6 (A) and pro-α5 (B) by the recombinant convertases furin (○), PC5A (■), PACE4 (●) and PC1 (□)

Chemiluminograms such as those shown in Figure 2 were scanned and the percentage of pro-α-chain cleavage was plotted as a function of the amount of pERTKR-Mca cleavage activity (nmol/h per 20 μl) of each enzyme preparation. At the highest level of enzyme activity, the C-terminal immunoreactive labelling of pro-α subunits (140 kDa) had completely disappeared, indicating that the band intensity measured for the light chains of integrin corresponded effectively to 100% precursor hydrolysis (results not shown).

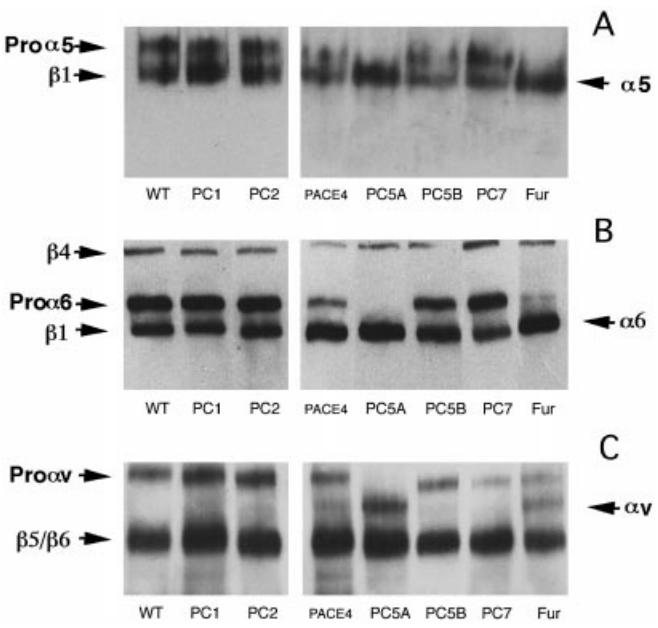

Figure 4 Endoproteolytic processing of integrin α-subunit in conjunction with convertase overexpression in LoVo cells

(**A**) Pro-α5 processing. LoVo cells were co-infected with 1 pfu per cell each of VV:α5, VV:β1 and VV recombinants of PC1, PC2, PACE4, PC5A, PC5B, PC7, full-length furin (Fur) or the wild-type virus (WT); 18 h after infection, cells were biotinylated and the molecular status of the α5β1 integrins was examined after immunoprecipitation with an anti-(α5-integrin) antiserum, followed by reducing SDS/PAGE and immunoblotting with HRP–streptavidin. The migration positions of the pro-α5, α5 and β1 subunits are indicated. (**B**) Pro-α6 processing. The experimental set-up was identical with that in (**A**) except that the molecular status of α6β1 and α6β4 integrin was examined after immunoprecipitation with anti-α6 integrin antibodies. The migration positions of the pro-α6, α6, β1 and β4 are indicated. (**C**) Pro-αv processing. LoVo cells were infected at 1 pfu per cell with VV recombinants of PC1, PC2, PACE4, PC5A, PC5B, PC7, furin (Fur) or the wild-type virus (WT); 18 h after infection, cells were biotinylated and the molecular status of αv/β5-β6 integrins was examined after their immunoprecipitation with anti-αv-integrin antibodies, reducing SDS/PAGE and immunoblotting with HRP–streptavidin. The migration positions of pro-αv, αv, β5 and β6 subunits are indicated.

(Figures 4A, 4B and 4C). The results are representative of the results obtained in multiple experiments ($n = 10$ for αv; $n = 6$ for α5 and α6). In summary, in all experiments pro-α subunits were completely cleaved by PC5A and never by PC1, PC2 or PC7. Cleavage by furin was more than 75% in two-thirds of the experiments and over 50% in the rest; this situation was observed for all integrin α subunits. Pro-α-chain hydrolysis by PACE4 was less than 50% in most experiments and that by PC5B was generally low, being no more than 25% in most experiments. These *ex vivo* results therefore corroborate the high efficiency of integrin α-subunit cleavage by PC5A and furin found *in vitro*, whereas the importance of the cellular role of PACE4 in integrin processing is attenuated and the low activity of PC1 *in vitro* is rendered negligible.

DISCUSSION

Some integrin α subunits undergo endoproteolytic processing at sequences comprising dibasic amino acids that are cleavage sites for serine proteinases belonging to the subtilisin/kexin-like convertase family. Accordingly, it has been shown that this processing might involve furin [23]. In view of the possibly redundant functions of some the PCs [2,35–37], we have asked here whether additional members of this enzyme family can also process integrin α chains. To answer this question, we studied the cleavage of pro-α subunits by recombinant convertases *in vitro* or *ex vivo* after overexpression in LoVo cells.

Digestions *in vitro* indicated that pro-α subunits were hydrolysed by PC5A and PACE4 with an efficiency even higher than that of furin, whereas PC1 was only slightly active and PC7 not at all. In addition, this profile seemed similar for all integrin α subunits, suggesting that the same convertases would be involved in the endoproteolytic processing of α subunits, such as pro-α6, that contain an R-X-(K/R)-R↓ motif at the cleavage site or those exhibiting a dibasic cleavage site with a P6 His residue, such as pro-α5 or pro-αv. It was unexpected to find that pro-α subunits were not cleaved by PC7 because it is thought that the cleavage site sequences required for optimal hydrolysis by PC7 are fairly similar to those best cleaved by furin [5,32] and to those that are actually found in pro-α subunits [33]. The discrepancy therefore suggests that some structural features other than the cleavage site sequence are critical for efficient substrate–convertase interactions and peptide bond hydrolysis. Those might include particular peptide sequences that surround the cleavage site or a precise positioning within the three-dimensional scaffold of the substrate. Accordingly, studies have shown that pro-α6 subunits are not cleaved when the position of the cleavage site in the molecule is artificially displaced a few amino acid residues towards the C-terminus [38].

Endoproteolytic processing *in vivo* by convertases implies the targeting of the activated enzyme and the substrate proprotein to the same cellular compartment(s). Integrins are exported along the constitutive secretory pathway. It was therefore not surprising to find that the defective processing of α-subunit precursors in LoVo cells was not restored after the overexpression of PC1 or PC2 because full activation of these convertases is known to take place after their translocation into secretory vesicles [1,2,35]. Integrin α-subunit precursors were cleaved to some extent by all other overexpressed convertases except for PC7. However, the cleavage efficiency differed widely and the best results were obtained with PC5A, which seemed more active than furin. Thus the high cleavage capacity found for this convertase *in vitro* was maintained in the cellular environment. These results demonstrate that PC5A is probably involved in the processing of some proproteins exported via the constitutive exocytic pathway. In support of this, PC5A is reportedly involved in the endoproteolytic activation of the receptor tyrosine phosphatase μ [39], which is consistent with immunofluorescence studies showing that it is located in the TGN [40]. PC5A has also been found in secretory vesicles in some cell lines [40], participating in the endoproteolytic maturation of hormones such as neurotensin [41] and Müllerian inhibiting factor [42]. PC5A therefore seems unique among convertases in that it might function in both the constitutive and regulated exocytic pathways. Interestingly, PC5B, a splice variant isoform of PC5A that differs by a C-terminal extension containing a transmembrane domain and cytoplasmic tail that affect its trafficking [40], was very inefficient in processing integrin α-subunit precursors. This is to be contrasted with the similar cleavage efficacy of HIV gp160 by both isoforms [16,36]. Although the hydrolysis efficiency of integrins by PC5B *in vitro* was not studied, it is unlikely to differ much from PC5A [16]. In addition, experiments done with furin and PC7 have shown similar selectivities and cleavage efficiencies for full-length convertases and their respective soluble recombinant counterparts lacking either the transmembrane domain or both the cysteine-rich and transmembrane regions [33]. Although the low activity of PC5B might be a consequence of its insertion in the membrane, thus limiting diffusion of the enzyme and its encounter with integrins, this explanation seems insufficient if one considers that furin (also a membrane-bound convertase) is fairly efficient in processing pro-α-integrin subunits. It is therefore more realistic to assume that the low integrin-processing activity of PC5B results from the targeting of the two proteins to non-overlapping cellular compartments, as already observed in AtT20 cells [40]. The same reason can be advanced to explain why the functional activity of PACE4 was attenuated after cellular expression. Indeed, the integrin cleavage potency of PACE4 after overexpression in LoVo cells was generally lower than that of furin, whereas *in vitro* it was as high as that of PC5A and greater than that of furin. This result is consistent with the observation that integrin α-chain subunits are not processed in LoVo cells, even though PACE4 is expressed and presumably participates in the endoproteolysis of proteins such as E-cadherin, which remain unaffected by the furin deficiency [43]. It is therefore unlikely that PACE4 would participate in integrin processing under physiological conditions unless perhaps it were expressed at very high levels.

In summary, this study suggests that only two members of the convertase family, PC5A and furin, predominantly perform endoproteolytic processing of the integrin pro-α-chain. Although furin is expressed ubiquitously, albeit at different levels depending on the cell type [1,2,35], PC5A is widely expressed [35] but highly enriched in endothelial cells and in cardiac tissues [44]. The integrin vitronectin receptor αvβ3, which is prominently expressed in the endothelium and in human atherosclerotic plaques [45], has been implicated in a range of physiological processes such as cell migration, wound repair, angiogenesis, cancer invasion and metastasis (reviewed in [46]). Its expression increases on the acquisition of a migratory/invasive phenotype, as demonstrated in endothelial [47] and smooth-muscle [48] cells. Thus αvβ3 in these tissues might well be a physiological substrate for PC5. Interestingly, the serpin α_1-antitrypsin Portland inhibits furin and PC5 best [27,49], suggesting that these two enzymes have similar catalytic specificities. The integrin-cleaving ability of certain convertases (e.g. PACE4 and PC5B) is attenuated in the living cell, even though they traffic though the TGN in a similar fashion to that of furin and PC5A. This suggests that the TGN might be subdivided into different functional microcompartments or that processing occurs in post-Golgi vesicles. Accordingly, under certain conditions furin and PC7 have been shown to

localize to different post-Golgi vesicular compartments [50]. The particular features that favour the cellular interaction of integrins with PC5A and furin but not with PC5B, PACE4 and PC7 remain to be defined.

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