Enzymic characterization of murine and human prohormone convertase-1 (mPC1 and hPC1) expressed in mammalian GH_4C_1 cells

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Prohormone convertase-1 (PC1), an endopeptidase that is structurally related to the yeast subtilisin-like *Kex2* gene product, has been proposed to be involved in mammalian tissue-specific prohormone processing at pairs of basic residues. To better study this enzyme, a rat somatomammotroph cell line, GH_4C_1, was infected with vaccinia virus recombinants of murine PC1 (mPC1) and human PC1 (hPC1). An enzymically active form of each protein was secreted into the cell medium and partially purified by anion-exchange chromatography. The 80–85 kDa enzyme was shown to be Ca^{2+}-dependent and exhibited a pH optimum of 6.0 when assayed against a synthetic fluorogenic substrate, acetyl-Arg-Ser-Lys-Arg-4-methylcoumaryl-1-amide. mPC1 and hPC1 displayed identical cleavage selectivity towards a number of fluorogenic substrates, and those incorporating an Arg at the P_4 site were most favoured. Synthetic peptides, encompassing the junction between the putative pro-region and the active enzyme, and between the pro-region and the biologically active parathyroid hormone, were shown to be recognized and cleaved specifically at the pair of basic residues by both enzymes. Group-specific proteinase inhibitors such as metal ion chelators and *p*-hydroxymercuribenzoate, but not phenylmethanesulphonyl fluoride and pepstatin, strongly inhibit the PC1-associated activity. In addition, it is shown that an enzyme activity displaying identical properties is present in the cell medium of uninfected corticotroph AtT-20 cells and that its level is increased following stimulation of secretion by the secretagogue 8-bromo cyclic AMP.

INTRODUCTION

Application of modern molecular biology techniques in conjunction with advances in yeast genetics have led to the recent discovery of candidate enzymes for prohormone processing at pairs of basic amino acids. Consistent with this substrate specificity, all of these enzymes, also known as convertases, share a common role in the cleavage of prohormone and/or proprotein precursors into a myriad of biologically active peptides and proteins. Whereas their involvement in prohormone processing has long been suspected [1,2], little is known concerning their physiological functions, except for the *Kex2*-encoded enzyme (kexin), which has been demonstrated by genetic means to be responsible for the processing of pro-α-factor and pro-killer toxin at paired basic residues. Kexin has been shown to be a Ca^{2+}-dependent serine proteinase with a subtilisin-like catalytic domain, and has a strong preference for substrates containing pairs of basic amino acids [3,4]. A growing number of serine proteinases structurally related to yeast kexin have now been identified by cDNA cloning. These include furin [5], prohormone convertase-1 (PC1; also known as PC3) [6–8], PC2 [7,9], PC4 [10,11], PACE-4 [12] and PC5 [13]; J. Lusson, D. Vieau, J. Hemelin, R. Day, M. Chrétien and N. G. Seidah, unpublished work), all of which are expressed to various degrees in mammalian tissues. Their important role has been further indicated by the observed conservation of amino acid sequences in related enzymes present in *Drosophila melanogaster* [14,15], *Caenorhabditis elegans* [16] and *Hydra vulgaris* [17].

Apart from their structural relatedness to kexin, further evidence in favour of a role for these enzymes in precursor processing in either the constitutive or the regulated pathway of secretion comes from co-expression and localization studies. Thus expression of PC1 or PC2 together with pro-opiomelanocortin in mammalian cells led to specific processing of this precursor at the C-terminal side of pairs of basic residues [18–21]. In the same vein, furin when expressed in both endocrine and non-endocrine cell types can also process various precursors at sites characterized by the presence of an Arg-Xaa-Lys/Arg-Arg sequence [5,22–25]. Furthermore, PC1 and PC2 transcripts were localized in neuronal and endocrine tissues [6,7,26] and PC4 transcripts were found in germ cells [10,11], whereas furin, PACE-4 and PC5 transcripts were found to be more widely distributed [12,13,27,28].

At the protein level, the presence of these enzymes in endocrine tissues has been ascertained by partial amino acid sequencing and/or by immunological techniques. Thus the presence of PC2 was demonstrated in insulinoma tissues [29] and in anglerfish pancreatic tissues [30], whereas both PC1 and PC2 were localized in adrenal chromaffin granules [31,32], in the pituitary gland [32] and in insulinoma tissues [29]. In addition, immunoreactive PC1 and PC2 were also shown to be secreted from adrenal chromaffin secretory granules into the cell medium after stimulation by carbamoylcholine chloride [33].

In order to better understand the physiological role as well as the molecular characteristics of these enzymes, it is mandatory to analyse them at the protein level, a task which has proven to be arduous. Therefore the use of expression systems offers a reasonable alternative in order to circumvent the problems of low expression levels and the presence of contaminating enzymes. This paper describes the use of a recombinant vaccinia virus

Abbreviations used: PC1, prohormone convertase 1 (the prefixes m and h indicate murine and human PC1 respectively); MCA, 4-methylcoumaryl-1-amide; Z, benzyloxycarbonyl; Boc, t-butoxycarbonyl; PTH, parathyroid hormone; AMC, 7-amino-4-methylcoumarin; r.i.a., radioimmunoassay; TFA, trifluoroacetic acid; SC, semicarbazone.

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containing a full-length PC1 insert to express either the human or the murine convertase in mammalian GH_4C_1 cells. We also present the partial purification of the secreted convertases in the cell medium and their enzymic characterization.

EXPERIMENTAL

Materials

For fluorometric assays, commercial peptidyl substrates were used, with the exception of the tetrapeptide acetyl-Arg-Ser-Lys-Arg-MCA, which was synthesized in our laboratory using a novel approach (F. Jean, A. Basak and C. Lazure, unpublished work; details available on application to the authors). The peptides used included the following: Arg-MCA, benzyloxycarbonyl(Z)-Arg-Arg-MCA, Z-Val-Leu-Arg-MCA, t-butoxycarbonyl(Boc)-Gly-Lys-Arg-MCA, Z-Lys-Lys-Arg-MCA, Z-Ala-Arg-Arg-MCA, Boc-Leu-Arg-Arg-MCA, Z-Ala-Lys-Lys-MCA, Z-Ala-Lys-Arg-MCA, Z-Val-Lys-Lys-Arg-MCA, Boc-Arg-Val-Arg-Arg-MCA and pGlu-Arg-Thr-Lys-Arg-MCA. These were purchased either from the Peptide Institute Inc., Tokyo, Japan, or from Enzyme System Products, Livermore, CA, U.S.A.

Peptide synthesis was carried out on a semi-automated peptide synthesizer (LKB-Biolynx model 4175; Pharmacia, Baie d'Urfée, Canada) using procedures already described [34]. The pro-parathyroid hormone (pro-PTH) tridecapeptide, representing positions -6 to $+7$ of the human prohormone, was kindly synthesized and purified by Dr. S. Sakakibara (Peptide Institute, Osaka, Japan).

DEAE-BioGel-A (100–200 mesh) and reagents used to perform the Bradford protein assay were obtained from Bio-Rad, Richmond, CA, U.S.A. The reverse-phase h.p.l.c. columns used, Vydac 218TP54 and Vydac 218TP510, were purchased from The Separation Group, Hesperia, CA, U.S.A.

The murine PC1 (mPC1) N-terminus-recognizing antibody was developed against peptides representing amino acids 84–100 of mPC1 [6] and was kindly provided by Dr. Iris Lindberg, (Louisana State University, New Orleans, LA, U.S.A.). The antibody recognizing the C-terminal region was obtained by immunization with an mPC1-derived peptide encompassing residues 629–726 produced from a fusion protein (A. Boudreault, unpublished work).

Vaccinia virus recombinants of mPC1 and hPC1

The purified recombinant vaccinia virus expressing mPC1 using the full-length cDNA insert [6] (VV:mPC1) and under the control of the vaccinia promoter has been previously described [18]. The recombinant vaccinia virus expressing hPC1 was prepared using the full-length cDNA insert [35] (VVtm1:hPC1) and the pTM-1 transfer vector [36]. In the latter case, expression of the recombinant protein is under the control of the T7 promoter and thus requires co-infection with a vaccinia virus (VTF7-3) expressing the bacteriophage T7 RNA polymerase as described [37].

Cell culture and viral infection

The murine corticotroph cell line AtT-20 and the rat somatomammotroph cell line GH_4C_1 were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum and 28 μg/ml gentamycin at 37 °C in 10% CO_2. Cell stimulation in the case of the AtT-20 cells was carried out by incubation in the presence of 5 mM 8-bromo cyclic AMP and the medium was collected following a 3 h stimulation period. Infection of 70% confluent GH_4C_1 cells in 10 or 15 cm dishes was performed using either the wild-type virus (VV:wt) or the recombinant virus (VV:mPC1) as described [38], with a multiplicity of infection of 1 plaque-forming unit/cell. For VVtm1:hPC1, cells were co-infected with 1 plaque-forming unit/cell of both VVtm1:hPC1 and VTF7-3 viruses.

Following the appropriate infection period, the inoculum was replaced with the cell culture medium and the cells were incubated for 17 h at 37 °C. After removal of the medium, the cells were then washed twice with unsupplemented Dulbecco's modified Eagle's medium or minimum essential medium. Following the wash, the cells were further incubated for the time indicated in each experiment in unsupplemented cell culture medium with or without 0.01% (w/v) BSA.

Isolation, purification and characterization of the recombinant mPC1 and human PC1 (hPC1)

The medium from vaccinia virus-infected GH_4C_1 cells as obtained above following two 3 h incubation periods (typically 100 ml) was combined with 5 ml of a 0.1 M disodium EDTA in 0.1 M $KHCO_3$, concentrated and dialysed using a Centriprep cartridge equipped with a membrane having a 30 kDa cut-off down to a volume of approx. 8.0 ml by repeated centrifugation at 1109 *g* for 30 min at 4 °C.

The concentrate was immediately loaded on a DEAE-BioGel-A column (13 cm × 1.5 cm) previously equilibrated with 50 mM sodium acetate buffer, pH 5.5, using a flow rate of 0.3 ml/min. Upon completion of the sample application, the column was first washed with the equilibrating buffer (3–4 column volumes) and then the retained material was eluted with a step gradient using the same buffer containing 0.6 M NaCl. Fractions of 3.0 ml were automatically collected. All operations were conducted at 4 °C.

Each fraction from the ion-exchange column was assayed for protein content using the Bradford protein assay, for immunoreactive PC1 by radioimmunoassay (r.i.a.), and for proteolytic activities by fluorometric assays as described in the following sections.

Chemical synthesis and characterization of the pro-mPC1 junction peptide

The sequence D-Tyr-Arg-Ser-Lys-Arg-Ser-Val-Gln-Lys-Asp of the synthetic peptide substrate represents the putative pro-junction [19] of mPC1, except for the inclusion of a D-Tyr residue at the N-terminus. The sequence Arg-Ser-Lys-Arg contained within this peptide was also used as a model for preparation of the fluorogenic substrate and of the inhibitors described in this study.

At the end of the synthesis, the pro-mPC1 derivative was cleaved from the resin and fully deprotected by treating the resin for 2 h with trifluoroacetic acid (TFA) in the presence of 8% (v/v) thioanisole. Since this deprotection procedure did not prove satisfactory, the peptide was finally obtained by treating a second aliquot of the partially deprotected mixture with reagent K [39] for 5 h.

The crude deprotected synthetic peptide was purified by reverse-phase h.p.l.c. using a semi-preparative Vydac 218TP510 C_{18} column (25 cm × 1.0 cm). The buffer system consisted of an aqueous 0.1% (v/v) TFA solution and an organic phase containing 0.1% (v/v) TFA in acetonitrile. Elution was carried out by using a linear gradient from 5 to 60% organic phase in 60 min

following a 5 min isocratic step at 5% organic phase; the flow rate was 2.0 ml/min. The purification of the peptide was followed by monitoring its u.v. absorbance at 225 nm.

The purified peptide was characterized by amino acid analysis as described [34], yielding the following amino acid composition: $Tyr_{0.9}$, $Arg_{2.2}$, $Ser_{1.5}$, $Lys_{2.4}$, $Val_{0.9}$, $Gln_{1.1}$ and $Asp_{0.9}$. It was also characterized with a mass spectrometer (MS-50 HMTCTA) operating in a positive ion fast-atom bombardment mode. The mass spectrum yielded a stable and sharp molecular ion peak at m/z 1267 $(M+H)^+$, together with numerous identifiable fragmentation peaks (results not shown).

Digestion of pro-PTH and pro-mPC1 synthetic peptides by PC1

Incubations with synthetic peptide substrates were conducted for various time intervals with 20 μl of purified hPC1 fraction, representing on average 2.41 nmol of 7-amino-4-methylcoumarin (AMC) released/h, at 37 °C in a total volume of 350 μl; the final incubation mixtures contained 50 mM sodium acetate and 5.0 mM $CaCl_2$, and the pH was adjusted to 5.5.

Following incubation, aliquots were acidified using 50 μl of acetic acid and analysed on a Vydac 218TP54 C_{18} column (25 cm × 0.46 cm). Elution was carried out using the buffer system and the linear gradient described above, except for the flow rate, which was set at 1.0 ml/min. The elution pattern was monitored by measuring the u.v. absorbance at 225 nm. The peptides detected were automatically collected and subsequently analysed by amino acid analysis.

Fluorometric assays and determination of kinetic constants K_m and $V_{max.}$

Each fluorogenic substrate was kept as a 2 mM stock solution in dimethyl sulphoxide at −20 °C. For each assay, the substrate (at a final concentration of 170 μM unless otherwise stated) was incubated with proteinase samples (20 μl) in a total volume of 350 μl containing 50 mM sodium acetate (pH 5.5) and 5.0 mM $CaCl_2$. Incubations for determination of kinetic constants were typically carried out in the presence of various concentrations (corrected for peptide content) of fluorogenic peptide substrates for 2.5 h at 37 °C before stopping the reaction by addition of 50 μl acetic acid. The values of K_m and $V_{max.}$ were determined using a computer-assisted algorithm (Enzfitter; Elsevier Science Publishers, Amsterdam, Holland). All fluorescence measurements were made with a Perkin–Elmer MPF-3L spectrofluorometer using an excitation wavelength set at 370 nm and an emission wavelength set at 460 nm in order to measure the AMC released, as described previously [40].

Inhibitor profile and K_i determination

The various inhibitors were preincubated with 20 μl of hPC1 or mPC1 for 30 min at 25 °C in 50 mM sodium acetate, pH 5.5, containing 5.0 mM $CaCl_2$ prior to the addition of acetyl-Arg-Ser-Lys-Arg-MCA substrate. The amount of AMC released relative to the control (done in parallel but without added inhibitor) was measured after a 3 h incubation period at 37 °C. Stock solutions of inhibitors were prepared according to the manufacturer's instructions.

The inhibitors Arg-Ser-Lys-Arg-SC and Arg-Lys-Lys-Arg-SC (SC is semicarbazone) were synthesized in our laboratory essentially according to procedures described previously [41]. The preincubation was done using various concentrations of inhibitors (determined by amino acid analysis) and the K_i values were determined graphically as described [42].

Determination of calcium- and pH-dependence of hPC1

Stop-time assays for the determination of Ca^{2+}-dependence were done after overnight incubations of 20 μl of hPC1 with concentrations of $CaCl_2$ varying from 0.01 mM to 100 mM in a total reaction volume of 350 μl of 50 mM sodium acetate, pH 5.5. The Ca^{2+} concentration for half-maximal activation was determined using a computer-assisted algorithm (Enzfitter). For determination of the pH-dependence of hPC1, a buffer system composed of 30 mM sodium acetate, 30 mM Mes and 30 mM *N*-ethylmorpholine, adjusted to the desired pH with HCl or NaOH, was used.

RESULTS

Detection of endogenous mPC1 in AtT-20 cell culture medium

As PC1 sequestered within secretory granules might be released upon stimulation of secretion, we investigated whether the medium of uninfected and stimulated corticotroph AtT-20 cells might represent a useful source of PC1. It was found that an enzymic activity recognizing the acetyl-Arg-Ser-Lys-Arg-MCA substrate was secreted into the medium following stimulation with the secretagogue 8-bromo cyclic AMP. Indeed, measurement of the enzymic activity released into the medium revealed a 2.2-fold increase following stimulation when compared with the activity released by unstimulated cells. This increase was only seen when the activity was measured at pH 5.5; no increase was noted at pH 7.5. An identical ratio (2.19), of values measured before and after stimulation, in PC1 content was measured by r.i.a. prior to and after stimulation, confirming that the increase in activity is mostly due to the release of PC1 into the medium. Furthermore, an identical ratio (2.15) was obtained when assaying by r.i.a. the adrenocorticotrophic hormone-(1–39) content in the cell culture medium before and after stimulation. This correlation appears to indicate that PC1 can be secreted from secretory granules and is enzymically active. Furthermore, the behaviour of the secreted enzyme upon ion-exchange chromatography as revealed by r.i.a., its pH- and Ca^{2+}-dependence as well as its sensitivity towards enzyme inhibitors were identical to those of the enzyme obtained from vaccinia virus recombinant expression (results not shown).

However, in view of the complexity of the medium contents, and especially of the very low amount of activity detected, it was decided to use the vaccinia virus expression system in conjunction with mammalian cell lines. Somatomammotroph GH_4C_1 cells were chosen because, unlike AtT-20 cells, they do not contain endogenous PC1 transcripts, as determined by Northern blot analysis [7]. Thus GH_4C_1 cells were subsequently infected with VV:wt, VV:mPC1 and VVtm1:hPC1 in combination with VTF7-3.

Expression of mPC1 and hPC1 in recombinant vaccinia virus-infected GH_4C_1 cells

The secretion of mPC1 and hPC1 in the cell medium was monitored both by measuring the increases in enzyme activities in the presence of added Ca^{2+} at pH 5.5 using fluorogenic substrates, and by r.i.a. Thus monitoring the enzymic activity using tripeptide fluorogenic substrates revealed the presence of a measurable enzyme activity in the media from control and infected cells; however, this activity did not increase significantly

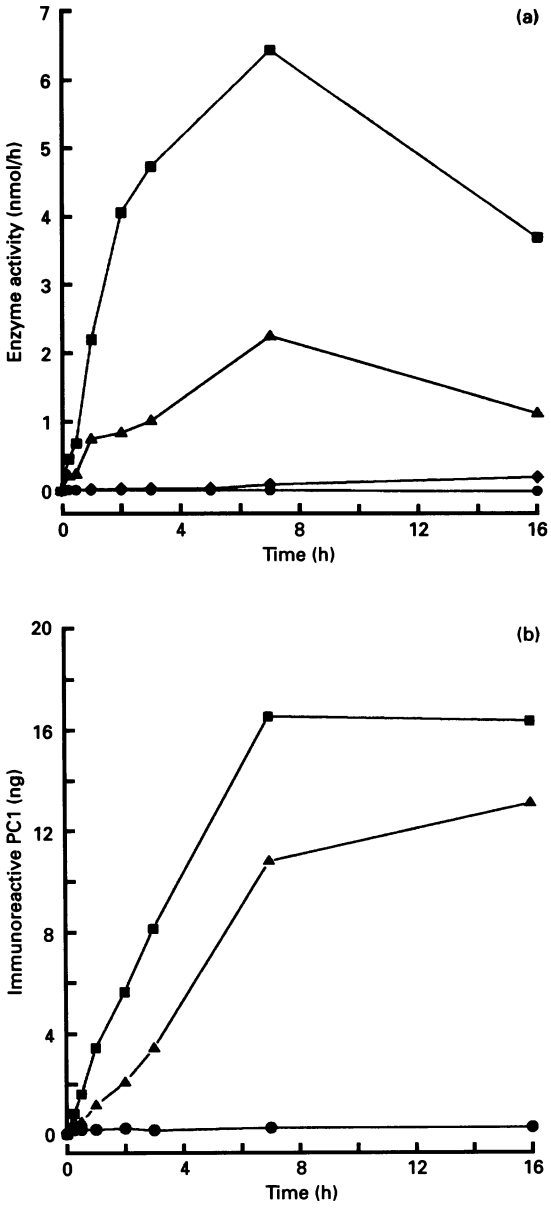

Figure 1 Release of mPC1 and hPC1 into the cell culture medium from AtT-20 and GH_4C_1 cells

(**a**) The enzyme activity released, measured using acetyl-Arg-Ser-Lys-Arg-MCA as substrate, in the unfractionated cell culture medium from unstimulated and uninfected AtT-20 cells (◆) and from GH_4C_1 cells infected with VV:wt (●), VV:mPC1 (▲) or VVtm1:hPC1 (■) is shown as a function of medium retrieval time. (**b**) Determination of immunoreactive PC1 enzymes using the C-terminus-recognizing antibody in the unfractionated cell culture medium as a function of medium retrieval time. Symbols are identical to those described in (**a**).

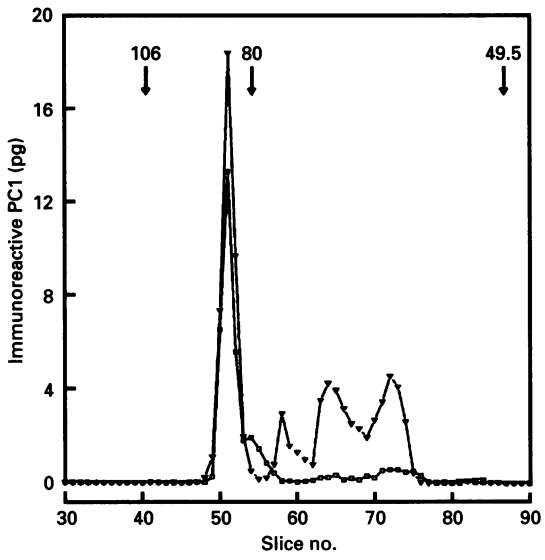

Figure 2 SDS/PAGE analysis of the mPC1 molecular forms released into the cell medium of VV:mPC1-infected GH_4C_1 cells after an incubation time of 3 h (□) or 6 h (▼)

Detection of the immunoreactive forms was accomplished by manually cutting the gel into slices and detecting the enzyme content by r.i.a. using the N-terminus-recognizing antibody. Molecular masses (kDa) of protein standards are indicated by arrows.

with time following infection. Using the tetrapeptide acetyl-Arg-Ser-Lys-Arg-MCA, it was possible to detect a released enzymic activity which increased over time, especially when compared with the amount released by uninfected control GH_4C_1 cells or by uninfected and unstimulated AtT-20 cells (Figure 1a). In parallel with the production of this activity, an increase in immunoreactive PC1 was also seen over time (Figure 1b). The differences in the amount of enzyme activity released and the immunoreactivity of hPC1 compared with mPC1 are correlated with the promoter used in the construction of the recombinants VVtm1:hPC1 and VV:mPC1 used. However, due to amino acid substitutions in the sequence of hPC1 within the region recognized by both anti-mPC1 antibodies used, it is not possible to accurately determine the yield of secreted hPC1. Nevertheless, mPC1 expression under the control of the 7.5 kDa vaccinia promoter appears to be lower than hPC1 expression, which is under the control of the T7 promoter. These graphs also indicate that the amount of activity present in the medium does not parallel the increase in immunoreactive material. Based on the ratio of enzyme activity to amount of immunoreactive material, it is evident that the maximal value is obtained at short secretion times. Thus for hPC1 the highest ratio was obtained after 2 h of incubation, and for mPC1 this maximum was obtained after 1 h. Nevertheless, the secretion of enzymically active and immunoreactive hPC1 and mPC1 continued to increase up to 7 h (24 h post-infection), after which time there was a net decrease in enzyme activity and a plateau in production.

The observed loss of enzyme activity could be due to either intrinsic instability of the enzyme or transformation of the active enzyme into an inactive form following protein degradation. The molecular forms of the secreted mPC1 enzyme were examined by SDS/PAGE and immunodetection using the N-terminus-directed antibody. Thus a highly active fraction (based on the ratio of enzyme activity to enzyme content) was obtained following a 3 h secretion period and contained, as shown in Figure 2, mainly an immunoreactive form of mPC1 with an apparent molecular mass of 80–85 kDa. Based on the same criterion, a less active fraction, obtained after a 6 h period, contained this form together with other lower-molecular-mass forms migrating as proteins of 75, 69 and 60 kDa (Figure 2). This result, and the observed decrease in the ratio of enzyme activity to immunoreactivity during this period, suggest that further processing and/or degradation into enzymically inactive (at least against the substrate used) smaller forms occurs following a longer period of time prior to harvesting the medium. These

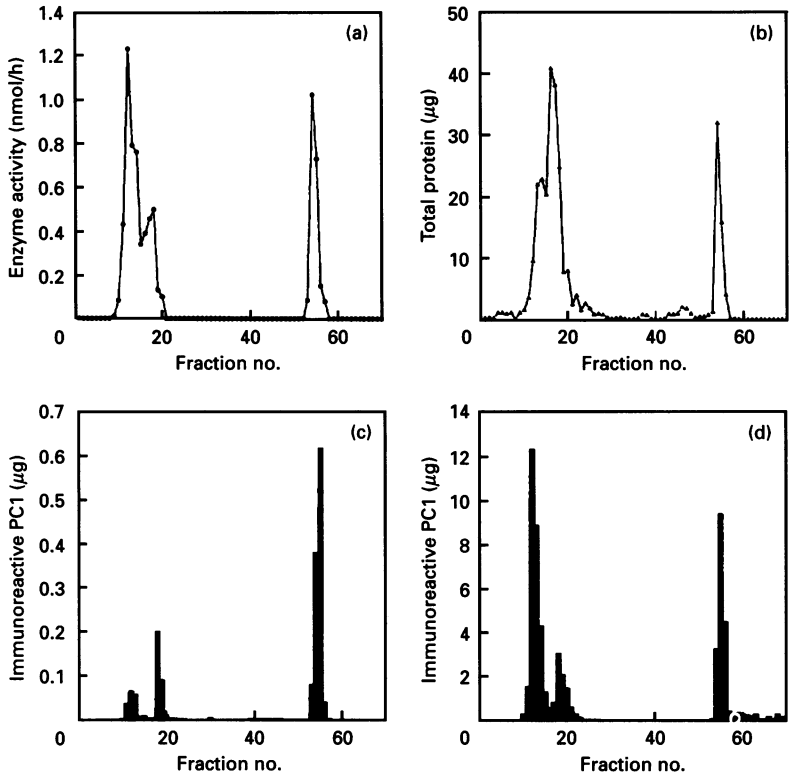

Figure 3 Analysis of the mPC1 content in the cell culture medium of VV:mPC1-infected cells after two combined 3 h secretion periods

The mPC1-containing fraction was chromatographed on a DEAE-BioGel-A column, and the eluate was monitored for (**a**) enzyme activity determined with acetyl-Arg-Ser-Lys-Arg-MCA at pH 5.5 in the presence of 5.0 mM Ca^{2+}, (**b**) total protein content as determined using the Bradford assay, and immunoreactivity content as determined using (**c**) the N-terminus-specific antibody and (**d**) using the C-terminus-specific antibody.

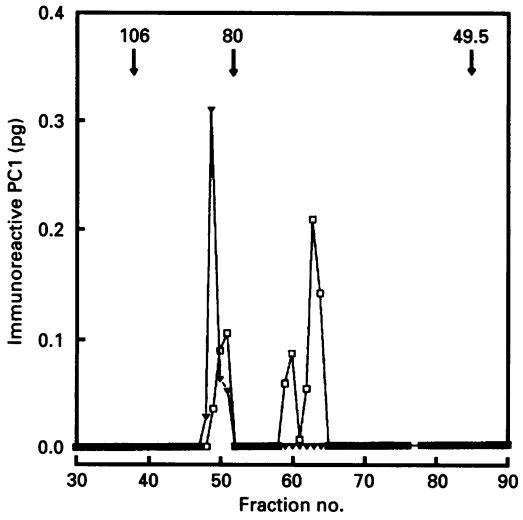

Figure 4 SDS/PAGE analysis of the molecular forms of mPC1 in the retained fraction (fraction 54, Figure 3) containing the highest activity on the DEAE-Biogel-A column (▼) and in the slightly retained fraction (fraction 8, Figure 3, □)

The mPC1 content was determined after cutting the gel in slices by r.i.a. using the N-terminus-recognizing antibody. Molecular masses (kDa) of protein standards are indicated by arrows.

lower-molecular-mass forms represent C-terminally truncated forms, since they were detected solely with the N-terminus-directed antibody. However, we cannot rule out the possibility that the shorter forms could also be active, albeit to a lesser extent.

Partial purification of recombinant mPC1

Partial enzymic purification was needed in order to better characterize the secreted protein and to remove the contaminant enzymic activity detected in the media of control and infected cells. The medium from two combined 3 h secretion periods contained 2.15 mg of protein and approx. 10 μg of mPC1/100 ml (0.12 amol/cell), as estimated by r.i.a. with the N-terminus-directed antibody, thus yielding a specific activity of 4.28 pmol/h per μg of protein. Concentration and dialysis led to a fraction containing 1.35 mg of protein with a specific activity of 9.2 pmol/h per μg of protein, which was fractionated on a DEAD-Biogel-A column. A representative summary of the results is illustrated in Figure 3; as observed in more than 20 runs, the separation always yielded a slightly retained fraction eluting with the equilibration buffer and a well retained fraction that was eluted with 0.6 M NaCl in the appropriate buffer. The former fraction (770 μg of protein; activity 8.0 pmol/h per μg), containing much of the albumin still present in the medium, contained an enzymic activity detected with the control cells that was predominant around neutral pH values. In addition, this fraction contained immunoreactive mPC1 when assayed with the C-terminus-directed antibody (Figure 3d), but there was less detection with the N-terminus-directed antibody (Figure 3c). The fraction eluted by 0.6 M NaCl was well recognized by both antibodies (Figures 3c and 3d), and displayed high enzyme activity towards Lys-Arg-containing substrates. This fraction

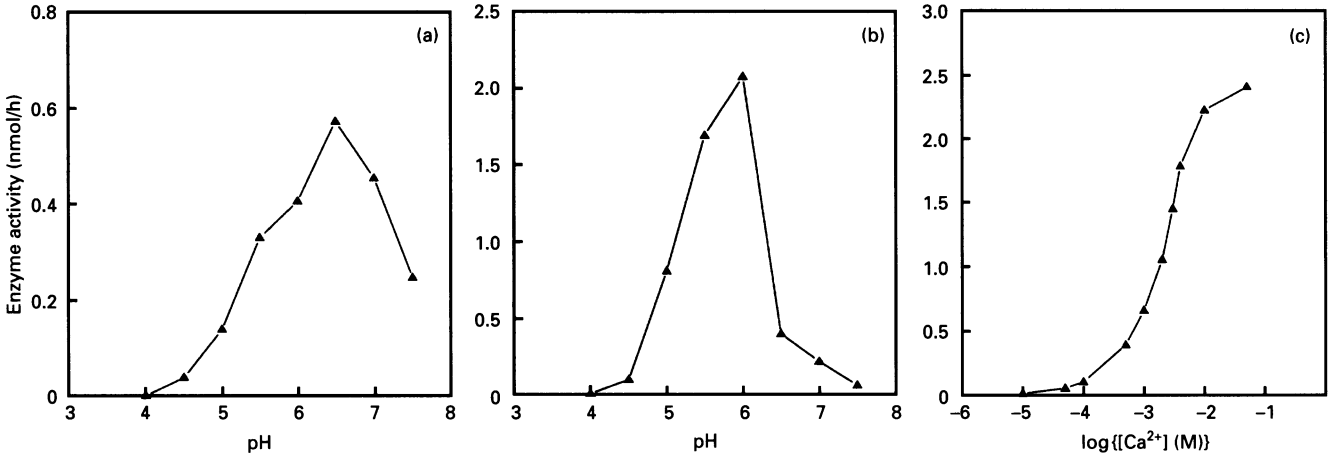

Figure 5 pH- and Ca^{2+}-dependence of secreted mPC1

(**a**) pH profile of the total enzyme activity against acetyl-Arg-Ser-Lys-Arg-MCA present in the DEAE-BioGel-A unretained fraction (fraction 8, Figure 3) arising from the chromatography of the cell culture medium of VV:mPC1-infected GH_4C_1 cells. (**b**) pH profile of the mPC1 enzyme activity against acetyl-Arg-Ser-Lys-Arg-MCA in the DEAE-BioGel-A retained fraction (fraction 54, Figure 3). (**c**) Ca^{2+}-dependence of the DEAE-BioGel-A retained fraction (fraction 54, Figure 3) arising from the cell culture medium of VV:mPC1-infected GH_4C_1 cells.

Table 1 Cleavage of fluorogenic peptide substrates by mPC1 and hPC1

All substrates were used at a concentration of 170 μM based solely on a weight basis and were not corrected for the peptide content. All incubations were performed overnight at pH 5.5 as described in the Experimental section. The AMC released is expressed as a percentage of the amount of AMC released using either substrate XIII or substrate XI as indicated. All determinations represent the means of three separate experiments, each one done in triplicate. The 'medium' value was obtained using the crude medium prior to fractionation. The 'purified' value was obtained using the enzymatic activity present in the retained DEAE-BioGel-A fraction. ND, not determined.

	AMC released (%)			
	hPC1		mPC1	
Substrate used (170 μM)	Medium	Purified	Medium	Purified
Arg-MCA (I)	0	0	0	0
Z-Arg-Arg-MCA (II)	0.03	0	ND	0
Z-Val-Leu-Arg-MCA (III)	0	0	0	ND
Z-Lys-Lys-Arg-MCA (IV)	3.22	2.11	1.47	1.17
Z-Ala-Lys-Arg-MCA (V)	2.64	1.73	0.51	2.10
Z-Ala-Arg-Arg-MCA (VI)	0.07	0.07	ND	0
Z-Ala-Lys-Lys-MCA (VII)	0	0	ND	0
Boc-Gly-Lys-Arg-MCA (VIII)	0.69	0.99	0.42	0.91
Boc-Leu-Arg-Arg-MCA (IX)	1.41	2.31	ND	2.09
Z-Val-Lys-Lys-Arg-MCA (X)	ND	1.81	1.19	ND
Ac-Arg-Ser-Lys-Arg-MCA (XI)	95.24	77.40	100.00	79.41
Boc-Arg-Val-Arg-Arg-MCA (XII)	60.84	70.09	ND	56.83
pGlu-Arg-Thr-Lys-Arg-MCA (XIII)	100.00	100.00	ND	100.00

contained 68.1 μg of protein, yielding a specific activity of 48 pmol/h per μg of protein or 1.88 nmol/h per μg of immunoreactive-mPC1. Based upon enzyme activity, the recovery yield was 22.5%, and the enzyme was purified approx. 11.2-fold. However, it is worth noting that the presence of added BSA (0.01%, w/v) in the retrieval medium had no detrimental effect on enzyme activity, the immunoreactivity using either antibody or the purification yield (results not shown).

Analysis of both fractions on SDS/PAGE and using the N-terminus-directed antibody (Figure 4) revealed that the retained fraction contained solely the 80–85 kDa form, whereas the non-retained fraction contained, in addition to some 80–85 kDa form, significant amounts of lower-molecular-mass forms.

pH optimum and Ca^{2+}-dependence of enzyme activity

In order to obtain a representative pH-dependence curve for either mPC1 or hPC1, it was mandatory to purify either enzyme from the cell medium. Indeed, in the medium of control GH_4C_1 cells infected by VV:wt, one can detect an enzymic activity that hydrolyses the acetyl-Arg-Ser-Lys-Arg-MCA substrate as well as other fluorogenic substrates. The pH-dependence curve obtained with the unretained fraction of the ion-exchange chromatography is indicative of the presence of more than one enzyme (Figure 5a). On the other hand, both purified mPC1 and hPC1 exhibit identical pH-dependence curves, as shown in Figure 5(b); maximal activity was observed in the restricted range of pH 5.0–6.5,

with an optimum at pH 6.0. It is worth noting that the crude medium from uninfected and stimulated AtT-20 cells exhibited identical pH-dependence curves (results not shown).

Contrary to what was observed with their pH-dependence curves, mPC1 or hPC1 present in the crude cell medium or in purified fractions yielded identical Ca^{2+}-dependence curves. This suggests that, in the unfractionated cell medium, the contaminating proteinases are not Ca^{2+}-dependent enzymes. From the curve illustrated in Figure 5(c) it is possible to derive a concentration of Ca^{2+} causing half-maximal activation of 2.46 ± 0.05 mM.

Substrate specificity of hPC1 and mPC1 as defined using synthetic fluorogenic peptide substrates

As already alluded to, an enzymic activity able to cleave a hydrophobic-Arg sequence, as indicated in Table 1, was present in the less-retained fraction, in addition to the activity recognizing a pair of basic amino acids. However, this activity was well resolved from that mainly present in the retained fraction which exhibits a strong preference for the pentapeptide- and tetrapeptide-MCA substrates. Indeed, as indicated in Table 1, the amount of AMC released using the pentapeptide-MCA and/or tetrapeptide-MCA substrates was at least 25 times greater than that obtained using the best tripeptide-MCA substrate. For these substrates, labelled XI, XII and XIII, purified hPC1 exhibited apparent K_m values of 45 ± 4, 572 ± 56 and 20 ± 3 μM, and $V_{max.}$ values of 3.08 ± 0.08, 18.6 ± 0.8 and 2.2 ± 0.1 nmol of AMC released/h, yielding $V_{max.}/K_m$ ratios of 0.07, 0.03 and 0.11 nmol/h per μM respectively. It is worth noting that the presence of a bulky protecting group, the t-Boc moiety, in substrate XII appears to have a profound effect, since removal of this group by acid treatment led to an 8.4-fold reduction in catalytic efficiency ($V_{max.}/K_m$ of 0.004 nmol/h per μM).

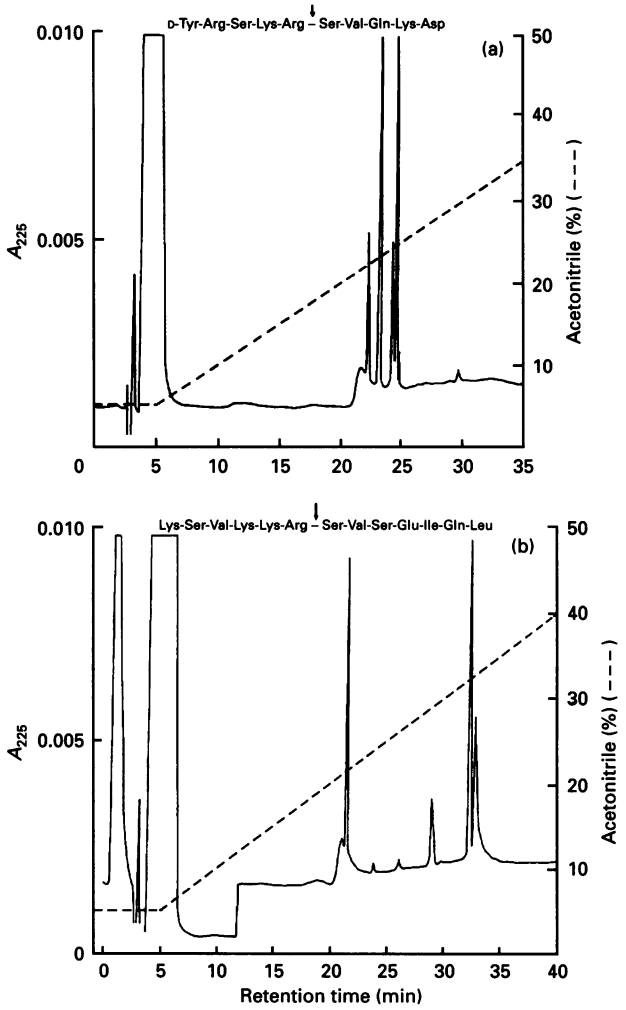

Figure 6 Proteolytic digestion of synthetic pro-mPC1 and pro-PTH by partially purified hPC1

(**a**) Reverse-phase h.p.l.c. of the digestion products arising from the incubation of the synthetic pro-mPC1 decapeptide (25 μg) with purified hPC1. Identification of the peaks was accomplished by amino acid analyis of each manually collected fraction; the first peak eluted (22 min) corresponds to Ser-Val-Gln-Lys-Asp, followed by D-Tyr-Arg-Ser-Lys-Arg, an undigested sulphonated derivative of the starting product and the remaining undigested starting product. The acetonitrile gradient used is indicated by the broken line. (**b**) Reverse-phase h.p.l.c. of the digestion products arising from incubation of the pro-PTH synthetic peptide (25 μg) with purified hPC1. Identification of the peaks was accomplished by amino acid analysis of the manually collected fractions; the first peak eluted (21 min) corresponds to Lys-Ser-Val-Lys-Lys-Arg, followed by an unidentified contaminant (present in the control incubation without enzyme), the Ser-Val-Ser-Glu-Ile-Gln-Leu fragment and the undigested remaining starting product. The acetonitrile gradient used is indicated by the broken line.

Table 2 Effect of proteinase inhibitors on the enzymic activities of hPC1 and mPC1

Inhibition of substrate XI (85.7 μM final concentration)-processing activity at pH 5.5 was assessed over a 3 h period at 37 °C. In control samples where no proteinase inhibitor was added, hPC1 and mPC1 were able to release 2.41 nmol of AMC/h and 1.19 nmol of AMC/h respectively under identical assay conditions. The effects of proteinase inhibitors are expressed as percentage inhibition compared with the control.

Inhibitor	Concentration (mM)	Inhibition (%) hPC1	mPC1
EDTA	10.0	99	98
CDTA	10.0	100	100
EGTA	10.0	86	86
1,10-Phenanthroline	1.0	0	0
Phenylmethanesulphonyl fluoride	1.0	0	0
Pepstatin A	0.1	0	0
Leupeptin	1.0	30	33
Antipain	1.0	67	68
p-Hydroxymercuribenzoate	1.0	98	92
Lys-Arg-chloromethyl ketone	0.1	96	92
Tos-Lys-chloromethyl ketone	0.1	13	12
Arg-oxime	1.0	56	45
$CaCl_2$	100	80	86
$CaCl_2$	200	100	98
NaCl	600	81	76

Effect of proteinase inhibitors on hPC1 and mPC1 activity

As shown in Table 2, both purified hPC1 and mPC1 exhibited identical inhibitor profiles. Both activities were completely inhibited by Ca^{2+}-complexing agents such as EDTA, CDTA or EGTA and were sensitive to thiol-reacting agents such as pHMB. Inhibition by the peptide-aldehyde inhibitors, leupeptin and antipain, is best explained by the presence of a C-terminal argininal moiety. This result was further confirmed by the inhibition with an Arg-oxime derivative, Arg-semicarbazone derivatives and Arg-chloromethyl ketone derivatives. In this last case it can be seen that the Lys-Arg-chloromethyl ketone derivative is 7-fold more potent than the tosyl(Tos)-Lys-

chloromethylketone. When assayed with hPC1, both of the peptidyl-Arg-semicarbazone derivatives synthesized (Arg-Ser-Lys-Arg-SC and Arg-Lys-Lys-Arg-SC) exhibited K_i values of 3.56 μM and 2.33 μM respectively. These K_i values are similar to those obtained with these inhibitors with another enzyme that preferentially cleaves at pairs of basic amino acids, namely plasma kallikrein [41]. Finally, a high concentration of Ca^{2+} or the presence of salt inactivates both enzymes, thus rendering mandatory their dilution and/or dialysis following ion-exchange chromatography prior to assay.

Synthetic pro-mPC1 and pro-PTH as potential substrates for mPC1 and hPC1

The region encompassing the putative cleavage site was synthesized in order to determine whether cleavage could be accomplished by PC1 itself. This was tested using a synthetic pro-mPC1 peptide and analysing the resulting cleavage products by reverse-phase h.p.l.c. It was possible to demonstrate cleavage of the synthetic peptide into two fragments identified by amino acid composition analysis as D-Tyr-Arg-Ser-Lys-Arg and Ser-Val-Gln-Lys-Asp. As shown in Figure 6(a), PC1 can efficiently accomplish this cleavage, whereas little cleavage was observed using the control cell medium. Indeed, similar to our observation that the use of a tetrapeptide-MCA substrate is much more discriminatory than is a tripeptide-MCA substrate towards PC1 activity, it seems that this synthetic peptide represents a suitable sequence for detecting PC1 activity in the cell medium. An identical result was obtained with this peptide following radiolabelling using either unfractionated cell medium or purified mPC1 or hPC1 enzymes and very short incubation periods (results not shown). Therefore this peptide sequence could be used in our recently proposed solid-phase assay [34], and its immobilization on an insoluble matrix would yield an efficient method for the screening of PC1 activity from various sources.

As shown in Figure 6(b), the peptide containing the pro-segment of pro-PTH is also efficiently cleaved into two components identified by amino acid composition as containing residues -6 to -1 and $+1$ to $+7$ respectively. This result, when compared with the low amount of cleavage observed (Table 1) with the tetrapeptide-MCA substrate Z-Val-Lys-Lys-Arg-MCA, suggests that the presence of a Lys residue at position -6 is beneficial in terms of cleavage efficiency.

DISCUSSION

The present study was aimed at describing the enzymic properties of one of the members of the newly discovered convertase family, namely PC1. We used the vaccinia virus expression system to infect somatomammotroph GH_4C_1 mammalian cells, which are devoid of endogenous PC1. We hereby demonstrate that the recombinant enzyme is produced and secreted in an 80–85 kDa active form into the GH_4C_1 cell culture medium. This result agrees with that obtained using uninfected AtT-20 cells, i.e. that these cells are able to secrete the enzyme in an active form and in a basal mode without any stimulation of secretion. Furthermore, we have shown that the amount of enzymic activity released is dependent upon type of promoter being used, as exemplified in the case of mPC1 which makes use of the usual vaccinia promoter as compared with hPC1 which utilizes the much more potent T7 promoter. The latter appears to be more advantageous, since greater amounts of activity and of immunoreactive protein are produced; it must be noted that the amount of hPC1 detected herein is probably an underestimate, as the extent of cross-reactivity between the human and murine forms is not known. For both mPC1 and hPC1, a significant decrease in the ratio of enzymic activity to enzyme content was observed at longer secretion times. In both instances this decrease coincided with the appearance in the medium of smaller forms whose enzyme activity, if any, remains to be demonstrated.

Recent studies on the expression of PC1 in microinjected *Xenopus* oocytes [43–46] identified PC1 as the type-1 proinsulin-converting enzyme originally described by Davidson et al. [47]. Based on the results described herein, it can be concluded that the activity of our vaccinia virus recombinant PC1 is indistinguishable from that activity and from the 87 kDa form described by Vindrola and Lindberg [48] (Y. Zhou and I. Lindberg, unpublished work), and also corresponds to the activity detected following secretion from chromaffin granules [33]. However, unlike in these previous studies, it was mandatory to purify the enzymic activity in order to resolve it from another activity capable of cleaving after a hydrophobic-Arg site. This activity appears not to be Ca^{2+}-dependent and to be considerably increased at neutral pH, in contrast to either mPC1 or hPC1 activity. The identity of this activity was not further documented, but its presence appears not to be related to the production of PC1, as it was found in the culture medium of cells infected with the wild-type virus. A contaminating enzyme arising from our use of Dulbecco's modified Eagle's medium supplemented with calf serum for cell cultures could be responsible for this activity. Another possibility is that GH_4C_1 cells are known to secrete an enzymic activity that is able to cleave prodynorphin at a single Arg-containing site in order to produce rimorphin (dynorphin B-13) [49]. The role of this enzymic activity, whatever its origin, in the transformation of the 80–85 kDa form of PC1 into smaller forms still remains to be assessed.

Another conclusion reached by this study concerns the characteristics of both the human and murine 80–85 kDa forms of PC1. Indeed, throughout this study these two enzymes have displayed virtually identical properties in terms of chromatographic behaviour, pH optimum, Ca^{2+}-dependency, inhibitor profiles, substrate specificities and molecular forms. This result is not unexpected considering the extremely high sequence conservation, especially within the catalytic region of both enzymes. Indeed, the entire mPC1 sequence (754 amino acids) exhibits a 92.6% identity to the hPC1 sequence, and both enzymes contain at least one Ca^{2+}-binding site, the Ca1 site present in all type-1 subtilases [50], and the lone half-cystine residue near the active-site histidine.

Concerning the peptide substrates used, we have shown that pGlu-Arg-Thr-Lys-Arg-MCA and acetyl-Arg-Ser-Lys-Arg-MCA are favoured substrates for the detection of PC1 activity. A similar conclusion was reached using Z-Arg-Ser-Lys-Arg-MCA as substrate while screening for the type-II proinsulin convertase [51]. Interestingly, these peptides also proved to be much better than another tetrapeptide substrate, Z-Val-Lys-Lys-Arg-MCA, to the point that the latter was no better than the tripeptide or dipeptide substrates used. However, and quite unexpectedly, this substrate when inserted into a longer peptide sequence, namely the synthetic pro-PTH peptide, proved to be as well cleaved as the Arg-Ser-Lys-Arg-containing pro-mPC1 peptide. This appears to indicate that the presence of an Arg residue at the P_4 position in the tetrapeptide substrates and a Lys at the P_6 position in the pro-PTH peptide might have a role in stabilizing the substrate in the active site, thus increasing the cleavage efficiency. Similar conclusions were reached in order to explain the cleavage of the BC-junction in proinsulin containing an Arg-Arg sequence by an enzyme which has been shown to cleave following a Lys-Arg pair [18,19,24,43]. Similar requirements

have also been proposed in the case of another enzyme belonging to the convertase family, namely furin [24,52], and also quite recently in the case of PC1 with respect to possible precursor cleavage at a single Arg residue [53]. Interestingly, the C-terminal region, which exhibits the greatest difference between the hPC1 and mPC1 sequences, does not seem to affect their properties towards small peptide-substrates. Clearly, further studies are necessary in order to delineate the requirements for substrate recognition and cleavage by PC1.

We took advantage of the k_m value of the enzyme for acetyl-Arg-Ser-Lys-Arg-MCA in designing the first synthetic inhibitors reported for hPC1 and mPC1. Thus semicarbazone derivatives incorporating either Arg-Ser-Lys-Arg or Arg-Lys-Lys-Arg sequences were prepared and shown to inhibit hPC1 activity towards the tetrapeptide-MCA substrate. Since these compounds can be used for affinity purification of serine proteinases [41], their applications in the context of PC1 purification are currently under investigation.

Finally, we also demonstrate that the synthetic peptide encompassing the junction between the pro form and the mature enzyme in the putative zymogen molecule is efficiently cleaved by the 80–85 kDa PC1 molecule. In addition to the partial sequence derived from PC1 extraction from chromaffin granules [31], and also from pulse–chase experiments [19], this result is further evidence (1) that PC1, in order to be active, must be cleaved at this junction, and (2) that this cleavage can be carried by PC1 itself in an autocatalytic manner. The cleavage of the synthetic pro-PTH peptide by PC1 is also of biological relevance, since PC1 and furin appear to share similar substrate requirements despite their different localization in the cell. Studies are currently in progress in our laboratories to identify by *in situ* hybridization and by Northern blot analysis the presence of PC1 and furin in the parathyroid gland.

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