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Processing of prodynorphin by the prohormone convertase PC1 results in high molecular weight intermediate forms

Cleavage at a single arginine residue

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Abstract

Processing of rat prodynorphin (proDyn) by the mouse prohormone convertase PC1 was investigated. Recombinant vaccinia virus vectors were used to coexpress proDyn and PC1 in rat PC12 pheochromocytoma and mouse AtT-20 corticotroph cells. In vitro experiments were also conducted by co-incubating purified proDyn and PC1. The results demonstrate that PC1 cleaves proDyn at pairs of basic residues to yield 10 and 16 kDa high molecular weight (HMW) intermediates. Additionally, PC1 cleaves proDyn at a single arginine residue to yield an 8 kDa product and the C-peptide. This demonstrates that PC1 cleaves proDyn at single and pairs of basic residues.

Key words: Vaccinia virus; Dynorphin; Processing; Overexpression; PC12 cell

1. Introduction

ProDyn is the precursor molecule of three leucine-enkephalin (Leu-Enk) extended opioid peptides known as dynorphin A_{1-17} (Dyn A_{1-17}), dynorphin B_{1-13} (Dyn B_{1-13}) and α-neo-endorphin (α-NE) [1]. In the rat, proDyn is expressed in the central nervous system (CNS) and periphery (i.e. striatum, hypothalamus, hippocampus, cerebral cortex, pituiatry, adrenal, gut and testis) [2] and is processed in a tissue specific manner to yield different combinations of end products [3]. In the pituitary, proDyn derived products are localized in anterior lobe gonadotrophs [4], intermediate lobe melanotrophs [5] and neural lobe neurosecretory terminals [6]. Interestingly, gonadotrophs store high levels of partially processed high molecular weight (HMW) intermediates and only low levels of final products such as Dyn A_{1-17} or Dyn B_{1-13} [7].

All neuropeptides are derived through enzymatic processing from biologically inactive precursor proteins. Processing of the pro-neuropeptides occurs at the carboxy- (C-) terminal of basic residues by the action of an endopeptidase, followed by the trimming action of carboxypeptidase E (CPE) and sometimes by C-terminal amidation [8]. The most common processing site is the pair of basic amino acids 'lysine-arginine' (KR) [9]. Other paired basic cleavage sites are processed, such as 'KK', 'RR' or 'RK', as well as single basic residues such as 'R'. Six processing enzymes, generally referred to as the prohormone convertases (PCs) have been discovered in mammalian species. They are PC1 [10] (also named PC3 [11]), PC2 [10,12], furin [13] (also named PACE for Paired dibasic Amino acid-Cleaving Enzyme [14]), PC4 [15,16], PACE4 [17] and PC5 [18] (also named PC6 [19]).

ProDyn has 4 KR and 2 single R sites important for the formation of Dyn peptides (Fig. 1). Cleavage at a single R site between Dyn B_{1-13} and the C-peptide is necessary for the production of Dyn B_{1-13}, while cleavage at the single R site within the Dyn A_{1-17} peptide results in the formation of Dyn A_{1-8}. Furthermore, three potential cleavage sites (RR or RK) are found following the Leu-Enk sequences in the proDyn precursor. Cleavage at these sites could result in Leu-Enk being formed. Our previous studies looking at the processing of proDyn in the anterior pituitary had characterized HMW intermediates in this tissue [7]. In the present study we examined the direct actions of PC1 on proDyn. Our data demon-

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strates that PC1 cleaves proDyn at both single and pairs of basic residues to produce HMW intermediates.

2. Materials and methods

2.1. Vaccinia virus (VV)

The full-length proDyn cDNA clone, pFLRD3 [20], was the generous gift of Dr. J. Douglass (Portland, Oregon). The *Eco*RI fragment containing the entire coding region of proDyn was blunt-ended and subcloned into the VV vector pMJ601 into the *Sma*I site. Full-length PC1 was subcloned into the VV vector pVV3 as previously described [21]. PC12 and AtT-20 cells were infected with a mixture of VV:proDyn and either VV:WT (wild-type control) or VV:PC1 at various multiplicities of infection for 2 h. The medium (DMEM) was then replaced and the cells were harvested following a post-infection period of 16 h.

2.2. Peptide analysis

Cells were washed 2 times in fresh medium and extracted in acidified methanol (methanol:0.1 N HCl; 1:1 v/v) [7]. After centrifugation at $15,000 \times g$ for 30 min at 4°C, the supernatants were collected and lyophilized. The samples were resuspended and submitted to reverse phase-high pressure liquid chromatography (RP-HPLC) or size exclusion chromatography. For RP-HPLC, the samples were resuspended in 0.1% trifluoroacetic acid (TFA) and separated using a 5 μm C_{18} column (300A), 25 × 0.46 cm (Chromatographic Sciences Co.) using a 0–60% acetonitrile gradient increasing at a rate of 0.5% /min. Gel chromatography is performed using Sephadex G-50 swollen in 0.1% formic acid, 0.1% BSA [7]. Fractions were collected, dried, resuspended and submitted to RIA. Protein standards run under the same conditions were used to determine the elution times of the peptide of interest.

2.3. In vitro studies

Rat proDyn was overexpressed in dhfr$^-$ Chinese hamster ovary (CHO) cells as previously described [22]. The protein was purified from 200 ml of filtered 24 h conditioned Opti-MEM (Gibco) containing 100 μg/ml aprotinin using RP-HPLC, first with a semi-preparative column eluted with TFA/acetonitrile, (Vydac C-4, 250 × 10 mm), during which proDyn eluted at 52% acetonitrile, followed by an analytical column (Vydac C-4, 250 × 4.6 mm) eluted in 0.2% heptafluorobutyric acid/acetonitrile during which proDyn eluted at 56% acetonitrile. Elution of proDyn was monitored by Western blotting of fractions using a mixture of antisera directed against the N- and C-termini of proDyn. Purity of the final product was confirmed by the presence of a symmetrical peak by absorbance at 215 nm; a single 30 kDa band upon Coomassie staining of a 15% polyacrylamide gel; and microsequencing, which revealed a single amino-terminus derived sequence of 20 amino acids corresponding exactly to those of rat proDyn (data not shown). The presence of an intact C-terminus was verified by Western blotting using C-terminally directed antiserum. The yield was about 500 μg. 87 kDa mPC1 was purified using fast protein liquid chromatography from conditioned medium obtained from overexpressing CHO cells as previously described [23]. Various amounts (1–25 μg) of purified proDyn were incubated with 50 ng of purified PC1. The incubations were carried out in 100 mM sodium acetate, pH 5.5, containing 5 mM $CaCl_2$ and 0.1% Brij for 16 h at 37°C. The samples were then submitted to G-50 chromatography and the fractions analyzed by RIA. In the case of Dyn B_{1-13} and α-NE RIA analysis, an aliquot of each fraction was pretreated with carboxypeptidase B before RIA in order to remove the C-terminal basic amino acid residues and thus permit RIA detection. The C-peptide-ir fractions were collected and microsequenced on an Applied Biosystems sequenator.

2.4. RIAs

The RIAs used in the present study for Dyn B_{1-13}, C-peptide and α-NE have been extensively characterized [7]. The Dyn B_{1-13} and α-NE antibodies are carboxy-terminus directed and under RIA conditions, neither antibody can recognize HMW intermediates containing a hindered antigenic site at the carboxy-terminus. The C-peptide antibody recognizes any pro-Dyn derived HMW intermediate with a C-peptide in its carboxy-terminus.

3. Results

We examined the processing capability of PC1 on the proDyn precursor by coinfecting PC12 and AtT-20 cells with VV:proDyn and VV:PC1. The cells were extracted and submitted to RP-HPLC (Fig. 2) or size exclusion chromatography (Fig. 3). The fractions obtained were analyzed for proDyn C-terminal peptide and Dyn B_{1-13} immunoreactivities (ir). In Fig. 2A, extracts of PC12 cells infected with VV:proDyn demonstrated high expression levels of proDyn precursor and very little processed products, as detected with the C-peptide RIA. A purified proDyn precursor standard (see section 2) was injected in a separate chromatogram under the same conditions. ProDyn eluted at 50% acetonitrile at the identical position to the C-peptide-ir detected in VV:proDyn PC12 cells. In Fig. 2B, processed products were observed in PC12 cells co-infected with VV:proDyn and VV:PC1. C-peptide-ir eluted at 22% acetonitrile, corresponding to the elution of a synthetic C-peptide standard. C-peptide-ir was also observed eluting at 32% acetonitrile. This C-peptide-ir most likely corresponds to a HMW intermediate described in Fig. 3.

In Fig. 3, AtT-20 cells were infected with VV:proDyn or coinfected with VV:proDyn and VV:PC1. The fractions obtained were analyzed with the C-peptide (Fig. 3 A,B) and Dyn B_{1-13} (Fig. 3C,D) RIAs. Large amounts of proDyn precursor were observed eluting from the column at the void volume (V_o) as detected with the C-peptide RIA (Fig. 3A). Very small amounts of processed products were observed at an M_r of 10 kDa and at the position of the C-peptide. This may be due to the endogenous processing enzymes found in AtT-20 cells, which express PC1 and lower levels of PC2 and furin [24]. Co-infection of AtT-20 cells with VV:proDyn and VV:PC1 resulted in large increases in the processed products at an M_r of 10 kDa and at the position of the C-peptide (Fig. 3B). The same two sets of fractions were analyzed with the Dyn B_{1-13} RIA (Fig. 3C,D). Very little ir-Dyn B_{1-13} was observed when AtT-20 cells were infected with VV:proDyn only (Fig. 3C). The proDyn precursor eluting at the void volume was poorly detected because of the strong requirement of the Dyn B_{1-13} antibody for a free C-terminus. Therefore, most of the ir-Dyn B_{1-13} detected represents peptide products in which

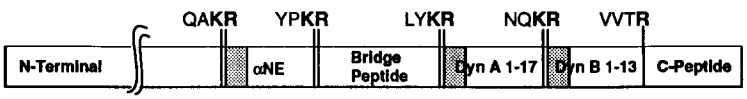

Fig. 1. Schematic representation of the proDyn precursor.

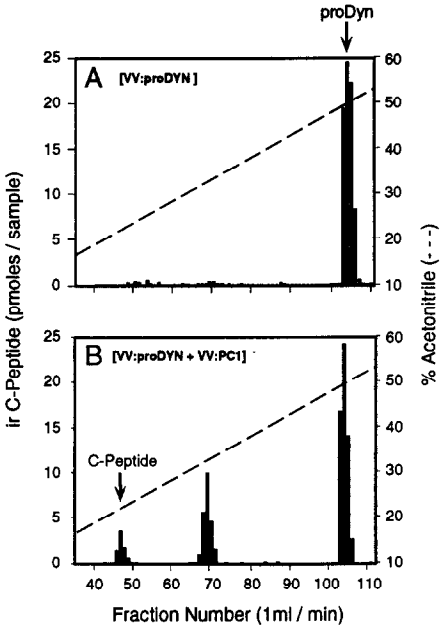

Fig. 2. RP-HPLC analysis of PC12 cells infected with (A) VV:proDyn and (B) VV:proDyn and VV:PC1. The fractions were collected, lyophilized, resusupended and analyzed with the C-peptide RIA. The arrows indicate the elution positions of the standard peptides. The experiment was repeated 2 times.

the C-peptide has been cleaved. Dyn B_{1-13}-ir was detected in AtT-20 cells that were coinfected with VV:proDyn and VV:PC1 (Fig. 3D). The Dyn B_{1-13}-ir eluted at an M_r of approximately 8 kDa. The sum of the data presented in Fig. 3 indicates that PC1 cleaves proDyn into HMW intermediates of 8 and 10 kDa. The difference between these two peptide products resides in the absence or presence of the C-peptide, as analyzed with the C-peptide RIA. We did not observe Dyn B_{1-13}-ir corresponding to the elution postion of Dyn AB or Dyn B_{1-13}, suggesting that PC1 does not further cleave the observed HMW intermediates.

We also examined the processing of proDyn by PC1 under in vitro conditions. Recombinant proDyn precursor was purified from the medium of overexpressing Chinese hamster ovary cells [22]. Recombinant PC1 was purified from a similar system [23]. Between 15 and 30 μg of proDyn were incubated with PC1. The samples were submitted to size exclusion chromatography and the fractions obtained were analyzed using the C-peptide (Fig. 4A), Dyn B_{1-13} (Fig. 4B) and α-NE (Fig. 4C) RIAs. The C-peptide RIA analysis (Fig. 4A) demonstrated that nearly 80% of the ir eluted at positions other than that of the intact proDyn precursor, indicating an almost complete cleavage by PC1 in vitro. The majority of the C-peptide-ir eluted at an M_r of 10 kDa, similar to that observed in the VV infection experiments. Furthermore, the synthetic C-peptide-ir was also detected at the position where the C-peptide standard elutes. Analysis of these fractions with the Dyn B_{1-13} RIA (Fig. 4B) demonstrated the formation of the 8 kDa intermediate, which was not detectable with the C-peptide RIA (Fig. 4A). Further analysis of these fractions with the α-NE RIA demonstrated the presence of a HMW intermediate at

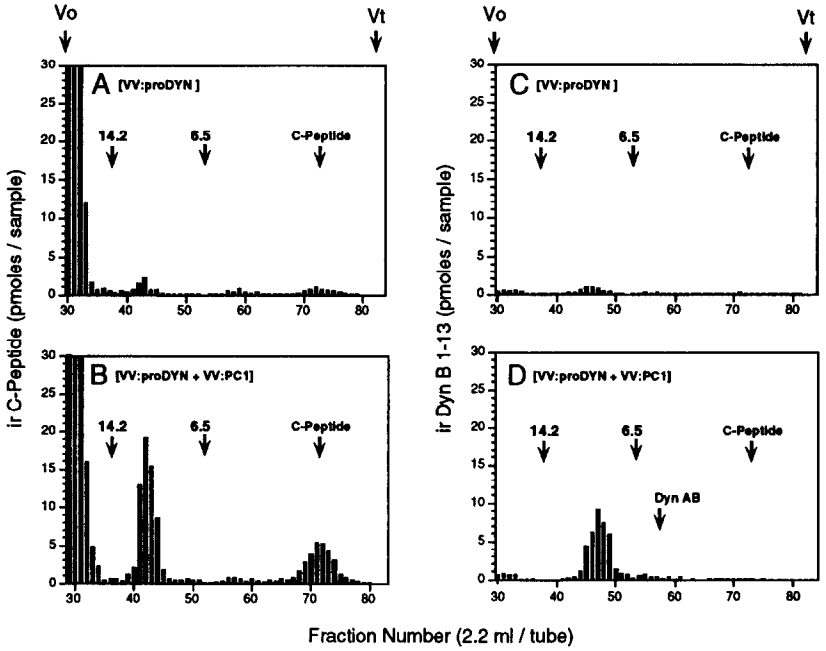

Fig. 3. Size exclusion chromatography of extracts from AtT-20 cells infected with (A,C) VV:proDyn and (B,D) VV:proDyn and VV:PC1. The fractions were analyzed with the (A,B) C-peptide and (C,D) Dyn B_{1-13} RIAs. The molecular weight markers are α-lactalbumin (14.2 kDa) and aprotinin (6.5 kDa). The void volume (V_o) is determined with Dextran blue and the total volume (V_t) is determined with cobalt chloride. Other standards are indicated by arrows include the C-peptide and Dyn AB. The experiment was repeated 2 times.

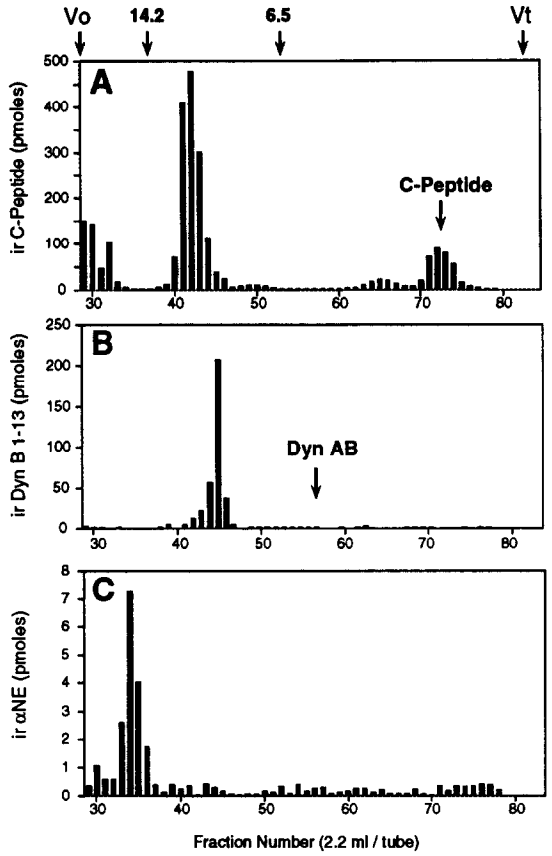

Fig. 4. Size exclusion chromatography of proDyn precusor incubated with PC1 under in vitro conditions. The fractions obtained were analyzed with (A) C-peptide (B) Dyn B_{1-13} and (C) α-NE RIAs. Prior to RIAs with Dyn B_{1-13} and α-NE, aliquots of each fraction were treated with CPB to remove C-terminal basic amino acids, and thus permit access to antigenic determinants. No ir-Dyn B_{1-13} or ir-α-NE is observed prior to CPB treatment. The markers are identical to those described in Fig. 3.

approximately 16 kDa (Fig. 4C). This intermediate was present at much lower levels in comparison to the intermediates observed in Fig. 4A and B. A summary of the data in Fig. 4 is presented in Table 1.

In a separate experiment, we incubated 5 μg of proDyn precursor and PC1. The sample was submitted to RP-HPLC under the same conditions shown in Fig. 2. The fractions were analyzed with the C-peptide RIA. The fractions containing C-peptide-ir eluting at 22% acetonitrile (same as C-peptide standard) were collected and submitted to sequence analysis. Nine cycles were carried out to obtain the N-terminal sequence of this C-peptide-ir. The following sequence was obtained: X-X-E-N-P-N-T-Y-S. The first two residues (X), given the low signal and the initial high background could not be identified unequivocally. The subsequent 7 cycles were clearly identified. The full rat C-peptide sequence is: S-Q-E-N-P-N-T-Y-S-E-D-L-D-V. This data demonstrates cleavage by PC1 at the post-single **R** site as follows: V-V-T-**R**- ↓ S-Q-E-N-P-N-T-Y-S-E-D-L-D-V.

4. Discussion

The present study demonstrates the processing of proDyn by PC1 in a cellular environment (PC12 and AtT-20 cells) and under in vitro conditions. The two studies yielded similar results, namely that PC1 cleaves proDyn both at pairs of basic residues and at a single R cleavage site to yield HMW intermediates.

We propose the following model for the cleavage of 26 kDa proDyn by PC1 (Fig. 5). The major site of cleavage by PC1 is the KR site at the N-terminal of α-NE (site 1). PC1 efficiently cuts this site since 61% of all processed products obtained are found at the elution position of the 10 kDa HMW intermediate (Table 1). The 2nd most important site of cleavage for PC1 is the single R residue (site 2). This cleavage results in the formation of the 8 kDa HMW intermediate and the C-peptide. Under in vitro conditions, we observe almost equal quantities of the 8 kDa HMW intermediate and of the C-peptide (350 and 400 pmol, respectively), which together represent 33% of processed proDyn molecules. Finally, a minor cleavage C-terminal to the KR pairs of α-NE (site 3), results in the formation of the 16 kDa HMW intermediate (Fig. 4C), and represents only 1% of the total processed products. The ratios of processed HMW intermediates obtained in these in vitro experiments are consistent with those observed in the cellular studies and to those obtained by examining the steady state levels of proDyn products in the anterior pituitary [7].

The proDyn HMW intermediates (8, 10 and 16 kDa) produced by PC1 are similar to the proDyn forms we previously described in the anterior pituitary [7]. This correlation is interesting for the following reasons. The anterior lobe expresses high levels of PC1 but much lower levels of PC2 or furin [24]. Our recent specific analysis of anterior lobe gonadotrophs, the cells which express proDyn, demonstrated high levels of PC1 and much lower levels of PC2 ([25]; and unpublished observation). This suggests that in the anterior lobe gona-

Table 1

Ir levels of proDyn peptide products from in vitro experiments

Product	ir C-peptide (pmol)	ir Dyn B 1-13 (pmol)	ir αNE (pmol)
PrDyn (unprocessed)	450	–	–
16 kDA	–	–	16 (1%)
10 kDA	1400 (61%)	–	–
8 kDA	–	350 (15%)	–
C-Peptide	400 (18%)	–	–
Other	100 (4%)	20 (1%)	2 (0.1%)

In parentheses are shown the amounts of processed products as a percentage of total processed products. Total processed products are equal to the sum of all the values shown, except that of unprocessed proDyn (450 pmol).

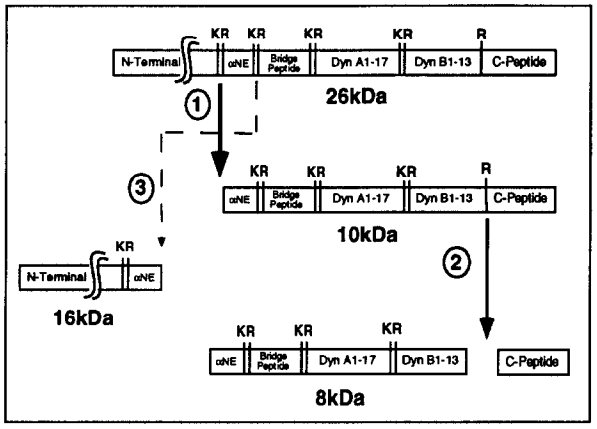

Fig. 5. Proposed processing of proDyn by PC1. Preference of cleavage is in the following order: Site 1 > Site 2 > Site 3.

dotrophs, the observed HMW proDyn intermediates are primarily derived from cleavage by PC1. The results of the present study support this notion since the proDyn precursor was processed to similar HMW intermediates by PC1. It should be noted that further processing to Dyn AB or Dyn B_{1-13} was not observed in the present study either in a cellular or in vitro environment. However, the analysis of anterior lobe gonadotrophs demonstrated low levels of Dyn AB as well as Dyn B_{1-13} [7]. This suggests that further processing in anterior lobe gondotrophs is occurring; this may be due to the presence of other enzymes, such as the low levels of PC2 or furin. As an additional correlation, it is interesting to note that other proDyn expressing tissues such as the striatum [26], hypothalamus [7] or spinal cord [7], process the proDyn precursor much more completely than the anterior lobe. These tissues express higher levels of PC2 [27] than the anterior lobe gonadotrophs [24]. In preliminary experiments, we tested proDyn processing by PC2 using the VV overexpression system and observed the formation of Dyn B_{1-13} (data not shown). Taken together, our data indicate that PC1 cleaves proDyn to HMW intermediates and that further processing to final opioid peptide end products might require the actions of PC2 and/or other enzymes.

The processing of proDyn to HMW intermediates by PC1 is reminiscent of the actions of the PCs on another opioid peptide precursor, pro-opiomelanocortin (POMC) [21]. PC1 was shown to cleave POMC to β-LPH and ACTH, but PC2 is required to obtain α-MSH and β-endorphin. Once again, this suggests that PC1 is responsible for the formation of larger intermediate molecules, while PC2 cleaves POMC to smaller peptide products. Furthermore, our recent studies [28] examining the processing of proenkephalin indicate that PC1 is responsible for the formation of larger 'enkephalin' containing molecules, including Peptide B, but PC2 enhances the production of free enkephalins. Thus, an interesting association can be made in cells which express the same precursor but differential levels of PC1 and PC2. Tissue-specific processing would therefore be highly dependent on the expression levels of each enzyme, and the resulting biological activity of the processed precursor could be modified.

One of the unique features of the present study is the processing of proDyn by PC1 at a single R cleavage site. This is the first such demonstration with an endogenous substrate. Our data supporting cleavage at the single R residue, include the observation of the formation of the C-peptide in cellular experiments (Figs. 2 and 3) and under in vitro conditions (Fig. 4). Furthermore the N-terminal sequence of the observed C-peptide-ir unequivocally proves cleavage at this site. At the present time, no recognition motif has been described for PC1 (or PC2), as has been demonstrated for furin [29]. However, a recent study [30] has proposed rules and tendencies for the cleavage at single basic residues. These criteria are met for the single R cleavage site in proDyn. The fact that PC1 cleaves this single R according to these rules and tendencies, suggests that the family of kexin/subtilisin-like proteases may be responsible or partly responsible for cleavage at single basic residues. Indeed, we have observed that PC2 and furin can produce, although less efficiently than PC1, the C-peptide product, indicating their capacity to cleave at the same single R residue. The actions of two other members, PC5 and PACE4, are untested. We cannot exclude that other processing enzymes, not part of the kexin/subtilisin-like proteases, are also responsible for cleavage at single basic residues, as has been suggested previously [31].

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