Differences in the autocatalytic cleavage of pro-PC2 and pro-PC3 can be attributed to sequences within the propeptide and Asp³¹⁰ of pro-PC2

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PC2 and PC3 are subtilisin-like proteases involved in the maturation of prohormones and proneuropeptides within neuroendocrine cells. They are synthesized as zymogens that undergo autocatalytic maturation within the secretory pathway. Maturation of pro-PC2 is slow ($t_{1/2} > 8$ h), exhibits a pH optimum of 5.5 and is dependent on calcium ($K_{0.5}$ 2 mM), while pro-PC3 maturation is relatively rapid ($t_{1/2}$ 15 min), exhibits a neutral pH optimum and is not calcium dependent. These differences in the rates and optimal conditions for activation of the proteases may contribute to the diversity of products generated by these proteases in different cell types. Although highly similar, there are two major differences between pro-PC2 and pro-PC3: the presence of an aspartate at position 310 in pro-PC2 compared with asparagine at the equivalent position in pro-PC3 (and all other members of the subtilisin family), and the N-terminal propeptides, which exhibit low sequence identity (30 %). With a view to establishing the structural features that might be responsible for these differences in the maturation of pro-PC2 and pro-PC3, Asp³¹⁰ in pro-PC2 was mutated to Asn, and Asn³⁰⁹ in pro-PC3 was mutated to Asp. Chimaeric proteins were also

made consisting of the pro-region of PC2 fused to the mature portion of PC3 and the pro-region of PC3 fused to the mature region of PC2. The wild-type and mutant DNA constructs were then transcribed and translated in an *in vitro* system capable of supporting maturation of pro-PC2 and pro-PC3. The results demonstrated that Asp³¹⁰ of pro-PC2 is responsible for the acidic pH optimum for maturation. Thus changing Asp³¹⁰ to Asn shifted the pH optimum for maturation to pH 7.0. However, changing Asn³⁰⁹ of pro-PC3 to Asp had no effect on the optimum pH for maturation of pro-PC3. A chimaeric construct containing the propeptide of pro-PC2 attached to PC3 shifted the pH optimum for maturation from pH 7.0 to 6.0 and slowed down the rate of maturation ($t_{1/2} > 8$ h). When attached to PC2, the pro-region of pro-PC3 had no effect on the optimum pH for maturation (pH 5.5–6.0), but it did accelerate the rate of maturation ($t_{1/2}$ 2 h). These results demonstrate that Asp³¹⁰ and the pro-region of pro-PC2 contribute to the acidic pH optimum and low rate of maturation of this zymogen relative to its closely related homologue PC3.

INTRODUCTION

PC2 and PC3 are serine proteases that belong to a large family of enzymes related to subtilisin, an alkaline serine protease produced from the bacteria *Bacillus subtilis* [1]. The first member of the eukaryotic family to be characterized was Kex2, isolated from the yeast *Saccharomyces cerevisiae* [2]. Kex2 is responsible for the processing of pro- α -factor and pro-(killer toxin) at pairs of basic amino acid residues, resulting in the maturation of these proteins [3]. Since the isolation of Kex2, a number of members of the mammalian subfamily have been isolated, including furin [4–6], PC2 [7,8], PC3/PC1 [9,10], PC4 [11], PACE4 [12], PC5A/6A [13], PC5B/6B [14] and PC7 [15–17].

These members of the subtilisin family all possess a signal peptide for translocation into the endoplasmic reticulum, a propeptide (pro-region), a catalytic domain containing the Asp/His/Ser triad typical of serine proteases, and a middle or P-domain. They are responsible for the cleavage of many precursor proteins, including prohormones and other polypeptides [18]. Endoproteolytic cleavage of these precursor forms occurs at pairs of basic, or in some cases at single basic [19], amino acids.

Expression of PC2 and PC3 is restricted to neuroendocrine cells, where they are thought to be involved in the maturation of prohormones and proneuropeptides within the regulated secretory pathway. Both enzymes are synthesized as inactive zymogens that undergo autocatalytic processing to generate the

mature forms [20,21]. Pro-PC2, a 75 kDa glycosylated protein, is cleaved at the sequence RKRR¹⁰⁹ to generate the mature 68 kDa enzyme [21,22]. Pro-PC3, a 88 kDa glycosylated protein, is cleaved at the amino acid sequence RSKR¹¹⁰ to generate the mature 80 kDa enzyme [23,24]. Both polypeptides can undergo cleavage at a secondary site within the propeptides KRRR⁸¹ in pro-PC2 and RSRR⁸¹ in pro-PC3 [25]. Further processing of PC3 can occur at the C-terminus to generate a 66 kDa form [26–28].

Activation of the proprotein convertases involves two distinct stages. First, cleavage occurs at the primary site, involving an autocatalytic reaction which may take place in the endoplasmic reticulum, as in the case of furin [29,30] and PC3 [31], or later in the secretory pathway, as in the case of PC2 [25,32,33]. This is followed by an activation step, which for furin has been shown to involve dissociation of the inhibitory pro-region from the mature protease following its cleavage at the secondary cleavage site [34]. We have previously shown, using a *Xenopus* egg extract translation system, that cleavage of pro-PC2 at the primary cleavage site occurs slowly ($t_{1/2} = 8$ h), at an optimal pH of 5.5, and is dependent on calcium ($K_{0.5}$ 2–4 mM) [25]. Although there may be subtle differences between the maturation of pro-PC2 in the *Xenopus* egg extract system and in neuroendocrine cells, these conditions are consistent with maturation occurring in a late secretory compartment, such as immature granules, that would have a low pH [35] and a high calcium concentration. Maturation

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of pro-PC3, on the other hand, occurs rapidly ($t_{1/2} = 15$ min), at an optimal pH of 7.0–8.0, and is not dependent on added calcium [25]. These conditions are compatible with the maturation of pro-PC3 in an early secretory pathway compartment, such as the endoplasmic reticulum, where the pH would be close to neutral and the calcium concentration is lower than that found in later secretory compartments [36].

The differences in the rates of activation of pro-PC2 and pro-PC3 may contribute to the diversity of peptide products generated through limited proteolysis of a single precursor protein [23]. Moreover, changes in the milieu within the secretory pathway may affect the extent of pro-PC2 or pro-PC3 maturation and lead in turn to the secretion of inappropriately, or partially, processed products, as seen for example for proinsulin in non-insulin-dependent diabetes mellitus [37]. We were therefore interested in elucidating the structural basis for the differences in the maturation of the zymogen forms of PC2 and PC3. Although the two propolypeptides exhibit a high degree of sequence identity (approx. 57 % in the catalytic region and 55 % in the C-terminal region), there are two major differences between them: (i) within the pro-region, where the identity drops to 30 %; and (ii) the presence of an Asp residue at position 310 within pro-PC2; in pro-PC3 (and all other members of the subtilisin family) the equivalent amino acid is Asn. This amino acid residue is thought to be important in stabilizing the enzyme–substrate transition complex. The approach used in the present study was to swap pro-regions between pro-PC2 and pro-PC3 and to change Asp³¹⁰ of pro-PC2 to Asn, and the equivalent residue Asn³⁰⁹ of pro-PC3 to Asp. The effects of these changes on the pH optima and rates of maturation of the various constructs were then studied in an *in vitro* translation system comprising *Xenopus* egg extracts [38], which has been shown previously to efficiently process pro-PC2 and pro-PC3 to the mature enzymes [21,25].

MATERIALS AND METHODS

Animals

Xenopus laevis were purchased from Blades Biologicals, Edenbridge, Kent, U.K.

Chemicals and reagents

Radiochemicals were purchased from Amersham International, Little Chalfont, Bucks., U.K. Full-length cDNAs encoding human PC2 and mouse PC3 were provided by Dr. D. F. Steiner, Howard Hughes Medical Institute, University of Chicago, Chicago, IL, U.S.A. Rabbit reticulocyte lysates were purchased from Promega UK Ltd., Southampton, Hamps., U.K. Oligonucleotides were purchased from Alta Bioscience, University of Birmingham, Birmingham, U.K. SP6 RNA polymerase, BSA (DNase- and RNase-free), nucleotide triphosphates (ATP, CTP, UTP and GTP) and RNase Guard were purchased from Pharmacia Biotech, St. Albans, Herts., U.K. m⁷G(5')ppp(5')G cap structure was purchased from New England Biolabs Ltd., Hitchin, Herts., U.K. All other chemicals were purchased from Sigma–Aldrich Co. Ltd., Poole, Dorset, U.K. Elongase DNA polymerase and amplification system were purchased from Life Technologies Ltd., Paisley, Scotland, U.K.

DNA constructs

PC2M5, in which the oxyanion hole Asp³¹⁰ has been altered to an Asn, has been described previously [25].

In PC2M9, the pro-region of PC2 is replaced with the pro-region of pro-PC3. The mature region of PC2 was amplified by

PCR such that a *Bgl*II site was introduced at the sequence corresponding to amino acids Arg¹⁰⁶–Lys¹⁰⁷, using the primer 5' AGATCTAAGCGAGGTTACCGAGACATCAATGAG 3' and a primer in the vector sequence downstream of the cDNA. This mutation changes Lys¹⁰⁷ to Ser¹⁰⁷. The amplified fragment was subcloned into pCR3 (Invitrogen, Leek, The Netherlands) and sequenced using the T7 Sequenase version 2.0 DNA sequencing kit (Amersham). The PCR product was cut from pCR3 using the newly created *Bgl*II site and a unique 3' *Bam*HI site, and was used to replace an equivalent fragment from pPC3-B64T, in which a *Bgl*II site was engineered at the sequence corresponding to amino acids Lys¹⁰⁷–Ser¹⁰⁸. The resulting chimaeric protein contained the sequence Lys–Glu–Arg–Ser–Lys–Arg¹⁰⁹ at the primary cleavage site.

PC2M9/M5 contains the Asp → Asn mutation at position 310 and the pro-region swap. This was achieved by replacing a *Stu*I/*Bam*HI fragment of PC2M9.64T with an equivalent *Stu*I/*Bam*HI fragment of PC2M5.64T. *Stu*I cuts at a unique site corresponding to Ala²⁵².

PC3M5 was constructed by site-directed mutagenesis using the primer 5' GCTTCAGGGGATGGGGGTGGTGAG 3'. This primer changes Asn³⁰⁹ to an Asp.

In PC3M9 the propeptide of PC3 is replaced with that of PC2. Wild-type PC3.SP64T had silent mutations introduced at Arg²⁹–Gln³⁰ and Arg¹⁰⁷–Ser¹⁰⁸ to encode an *Eag*I and a *Bgl*II site respectively. The pro-region of PC2 was then amplified by introducing an *Eag*I site at amino acids 27–28 and a *Bgl*II site at amino acids 106–107 using the primers 5' CTGAGCGCCG-GTCTTCACGAA 3' and 5' GCTTAGATCTGTCAAATCC-TTCCTG 3'. An *Eag*I/*Bgl*II fragment containing the pro-region of pro-PC2 was used to replace an equivalent *Eag*I/*Bgl*II fragment from the mutated PC3-64T plasmid. This resulted in a chimaeric protein with the sequence Phe–Asp–Arg–Ser–Lys–Arg¹¹⁰ at the primary cleavage site.

PC3M9/M5 was constructed by ligating an *Apa*I/*Stu*I (Gly²⁶⁹–Gly³³⁸) fragment of PC3M5.64T into *Apa*I/*Stu*I-cut PC3M9.64T. The construct resulted in a double mutant containing both the M5 and M9 mutations.

In vitro transcription/translation

All cDNAs were subcloned into the plasmid SP64T for transcription and mRNA was synthesized from *Bam*HI-linearized vector as described previously [25]. *Xenopus laevis* egg extract was prepared using the method of Matthews and Colman [38]. *In vitro* translations were performed by the addition of mRNA (~50–100 ng) to a 50 μ l aliquot of egg extract containing 10 % (v/v) rabbit reticulocyte lysate, 10 μ M phosphocreatine, 0.2 μ M spermidine and 1 mCi/ml [³⁵S]methionine. Samples were left at room temperature for 30–60 min (termed the pulse period), to allow translation products to accumulate, after which RNase A was added to a final concentration of 100 μ g/ml to prevent further translation. In pH profile experiments, the pH of the samples was adjusted by the addition of 1 M Mes (pH 5.0–6.5) or 1 M Tes (pH 7.0–8.0) to a final concentration of 0.1 M. CaCl₂ was added to some of the samples after the initial pulse (final concentration 10 mM). To determine the optimal calcium concentration for maturation of each of the constructs, CaCl₂ was added to give the indicated concentrations.

The samples were chased for a given time (termed the chase period). After the chase period, samples were resuspended in a 10 % (w/v) sucrose/PBS solution and centrifuged at 12000 *g* for 15 min at 4 °C. The supernatant was removed and the pellet washed and centrifuged again. The pellet was finally resuspended in a 10 % (w/v) sucrose/1 % (v/v) Triton X-100/PBS solution.

Samples were analysed by SDS/PAGE and fluorography as described previously [25]. To allow quantification of the extent of maturation, autoradiographs were scanned on to disc using a Deskan II imager and the extent of maturation was analysed using Phoretix. Autoradiographs shown are representative of at least two experiments.

RESULTS

In order to determine the structural basis for the different maturation profiles of PC2 and PC3, various domain-swap mutants were constructed. These included an exchange of the oxyanion-hole residues and an exchange of the pro-regions, either individually or as a double mutation. The wild-type and mutant cDNAs were cloned into the plasmid pSP64T for transcription [39] and mRNA was translated in a *Xenopus laevis* egg extract translation/translocation system in order to study the maturation profiles of wild-type and mutant PC2 and PC3 proteins.

Maturation of PC2 from the 75 kDa precursor form to the 68 kDa mature form was optimal at acidic pH, i.e. pH 5.5–6.0 (Figure 1A). This is in marked contrast with the maturation of pro-PC3, which occurred optimally at pH 7.0–8.0 (Figure 1B).

Pro-PC2 is the only member of the subtilisin family of

Figure 1 pH profiles of pro-PC2 and pro-PC3 processing

PC2 (A) and PC3 (B) mRNAs were translated in the egg extract for 2 h (A) or 30 min (B), after which translation was inhibited by the addition of RNase (100 μ g/ml, final concentration). Aliquots were then adjusted to give the pH values shown by the addition of Mes or Tes buffer (final concentration 100 mM) at the appropriate pH. CaCl_2 (final concentration 10 mM) was also added in (A) to enhance maturation. The samples were then incubated at 21 $^{\circ}\text{C}$ for a further 6 h (A) or 3 h (B) before being prepared as described in the Materials and methods section and analysed by SDS/PAGE and fluorography. Track M denotes ^{14}C -labelled protein markers; molecular masses (kDa) are indicated by arrowheads. Abbreviation: a.a., amino acids.

Figure 2 pH profiles of pro-PC2M5 and pro-PC3M5 processing

PC2M5 (A) and PC3M5 (B) mRNAs were translated in the egg extract for 1 h (A) or 30 min (B), after which further translation was prevented by the addition of RNase. Aliquots were prepared by the addition of either 1 M Mes or 1 M Tes to give a final concentration of 0.1 M and the pH values shown. Samples were incubated for a further 8 h (A) or 3 h (B) at 21 $^{\circ}\text{C}$, and then prepared as described in the Materials and methods section and analysed by SDS/PAGE and fluorography. Track M denotes ^{14}C -labelled protein markers; molecular masses (kDa) are indicated by arrowheads. Abbreviation: a.a., amino acids.

proprotein convertases to undergo maturation at acidic pH, and it is notable that it is also the only member of the family to have an Asp residue in the oxyanion hole. Maturation of PC2M5, in which the oxyanion residue Asp³¹⁰ was mutated to an Asn, was therefore studied. The results for PC2M5 showed that processing of this mutant was optimal over a broad pH range of 6.5–7.5, contrasting with the acidic pH required for the maturation of PC2 (Figure 2A). Thus the oxyanion Asp of pro-PC2 may be important for efficient maturation under acidic conditions. However, when the oxyanion-hole Asn of pro-PC3 was altered to an Asp, there was no observed effect on the optimal pH of maturation (Figure 2B), with PC3M5 maturing optimally at pH 7.5, similar to wild-type PC3. This result suggests that, although the PC2 oxyanion-hole aspartic acid is at least partially necessary for the maturation of PC2 in acidic environments, it is not sufficient to confer an acidic maturation profile on to PC3.

Next we examined the maturation profiles of the pro-region swap mutants PC2M9 and PC3M9. PC2M9 contains the PC3 pro-region attached to the mature region of PC2, with the sequence around the primary cleavage site of this chimaeric protein being KERSKR¹⁰⁹. PC3M9 contains the pro-region of PC2 attached to the mature form of PC3, with the chimaeric protein having the sequence FDRSKR¹¹⁰ at the primary cleavage site. The pH profile of the maturation of PC2M9 resembled that for wild-type PC2, with optimal maturation occurring at pH 5.5–

Figure 3 pH profiles of pro-PC2M9 and pro-PC3M9 processing

PC2M9 (**A**) and PC3M9 (**B**) mRNAs were translated in the egg extract for 1 h, after which translation was inhibited by the addition of RNase. Aliquots were then adjusted to give pH values between 5.0 and 8.0 by the addition of Mes or Tes buffer, and CaCl_2 (final concentration 10 mM) was also added to enhance maturation. The samples were then incubated at 21 °C for a further 6 h, and prepared for analysis by SDS/PAGE and fluorography as described in the Materials and methods section. Track M denotes ^{14}C -labelled protein markers; molecular masses (kDa) are indicated by arrowheads.

6.0 (Figure 3A). However, maturation appeared to be partial, involving cleavage only at the secondary cleavage site, resulting in formation of the 71 kDa form of the chimaeric protein rather than the fully processed 68 kDa form. This was confirmed by radiomicrosequencing (results not shown). This suggests that the catalytic site of PC2 is unable to recognize the PC3 primary cleavage site KERSKR¹⁰⁹ which this mutant contains, preferring instead the secondary cleavage site PRRSRR⁸¹.

We have shown previously that the maturation of pro-PC2 is dependent on millimolar concentrations of calcium, whereas pro-PC3 maturation is calcium independent [25]. Preliminary experiments using the mutants PC3M9 and PC3M9/5 showed that these chimaeric proteins required 10 mM calcium for efficient maturation (results not shown). The pH profiles of maturation of these proteins were therefore assessed in the presence of calcium. The maturation of PC3M9 differed from that of wild-type PC3, with optimal maturation occurring at pH 6.0 instead of pH 7.0 (Figure 3B). This result suggests that the pro-region of PC2 provides a dominant and transferable signal that confines the maturation of the precursor to acidic pH. Replacement of this signal with an alternative pro-region from a related proprotein convertase was not sufficient to prevent maturation at acidic pH, implying that there are, in addition, other factors within the mature PC2 molecule that determine its acidic pH of maturation. The time courses for maturation of the pro-region swap mutants

Figure 4 Time courses of maturation of pro-PC2 and pro-PC2M9

PC2 (**A**) and PC2M9 (**B**) mRNAs were translated in the egg extract for 1 h, and then translation was terminated by the addition of RNase. The samples were adjusted to pH 5.5 and 10 mM calcium and incubated at 21 °C, and aliquots were removed at 0, 1, 2, 4, 8 and 20 h. Samples were prepared as described in the Materials and methods section and analysed by SDS/PAGE. Track M denotes ^{14}C -labelled protein markers; molecular masses (kDa) are indicated by arrowheads.

were also different from those of the parent proteins. PC2M9 underwent processing to its mature form more rapidly than wild-type PC2 ($t_{1/2}$ of 2 h, compared with in excess of 8 h for wild-type PC2; Figure 4), whereas the maturation of PC3M9 was delayed markedly in comparison with wild-type PC3 ($t_{1/2}$ of in excess of 8 h, compared with 15 min for wild-type PC3; Figure 5).

The PC2M9/5 mutant contains both the PC3 propeptide and the oxyanion-hole asparagine; conversely, PC3M9/5 contains both the PC2 propeptide and the oxyanion-hole aspartic acid mutation. The maturation profile of PC2M9/5 (Figure 6A) was similar to those of PC2 and PC2M9, with optimal maturation occurring in acidic conditions (pH 5.5). The double mutant PC3M9/5, like PC3M9, had a pH profile of maturation similar to that of PC2, maturing at acidic pH (4.5–5.0) (Figure 6B).

DISCUSSION

The prohormone convertases PC2 and PC3 have very similar amino acid sequences, they perform the same catalytic reactions, cleaving at pairs of basic amino acids, and they are both optimally active at acidic pH, requiring the presence of calcium. The conditions required for their conversion from the precursor

Figure 5 Time courses of maturation of pro-PC3 and pro-PC3M9

PC3 (**A**) and PC3M9 (**B**) mRNAs were translated in the egg extract for 1 h, and translation was terminated by the addition of RNase. In (**A**) the samples were unaltered, but in (**B**) the samples were adjusted to pH 5.5 and 10 mM calcium. The reactions were incubated at 21 °C and aliquots were removed at the times indicated. Samples were prepared as described in the Materials and methods section and analysed by SDS/PAGE. Track M denotes ¹⁴C-labelled protein markers; molecular masses (kDa) are indicated by arrowheads.

forms into the mature forms of enzyme, however, differ substantially. The present study identifies particular key features of these two enzymes that may be responsible for their different maturation profiles. Thus it was found that the oxyanion-hole residue Asp is an important determinant of the acidic maturation profile of pro-PC2; in addition, the propeptide contains a transferable signal conferring acidic maturation properties. The propeptide also appears to contribute towards the delayed maturation observed for pro-PC2.

PC2 is the only member of the subtilisin-like family of serine proteases to possess an aspartic acid residue in the oxyanion-hole position in place of the asparagine found in PC3 and the other members of the family [1]. When Asp³¹⁰ of PC2 was mutated to an Asn, maturation of the PC2 mutant was optimal in neutral conditions, suggesting that the Asp is important for the acidic maturation profile shown by PC2. A PC3 mutant containing an Asp in the oxyanion hole did not, however, acquire the ability to mature at acidic pH, suggesting that the oxyanion-hole Asp of PC2 does not play a dominant role in confining maturation to acidic environments. In the present study we have not assessed the activity of our mutant proteins *in trans*; however, similar mutations of PC2 by other workers have produced conflicting results. Benjannet et al. [40] indicated that D309N (mouse) PC2 did not undergo as efficient maturation or secretion from vaccinia-virus-infected AtT20 cells as did wild-type PC2, and

Figure 6 pH profiles of pro-PC2M9/5 and pro-PC3M9/5 processing

PC2M9/5 (**A**) and PC3M9/5 (**B**) mRNAs were translated in the egg extract for 1 h, after which translation was inhibited by the addition of RNase. The pH was then adjusted to give a range of pH values between 5.0 and 8.0 by the addition of Mes or Tes buffer. CaCl₂ was added to give a final concentration of 10 mM, and samples were incubated for a further 8 h at 21 °C before analysis by SDS/PAGE and fluorography. Track M denotes ¹⁴C-labelled protein markers; molecular masses (kDa) are indicated by arrowheads.

showed a significantly decreased ability to cleave prepro-opiomelanocortin to β -endorphin. Zhou et al. [41], however, stably expressed a similar mutant (rat) PC2 in AtT20 cells and showed that it underwent maturation and catalysed the subsequent processing of prepro-opiomelanocortin in a manner similar to wild-type PC2. These differences may be due either to species differences or to differences in the level of expression acquired. While the role of the oxyanion residue for activity *in trans* is still a matter for debate, our results would suggest that it is important for the production of maturation-competent PC2 at acidic pH, and hence may help to confine maturation of this protein to immature granules.

The rate-limiting step in the maturation of any of the subtilisin-like endoproteases is likely to be the correct folding of the protease into a maturation-competent state. The propeptides of a number of bacterial members of the subtilisin family have been shown to have a role in the folding and transport of the protein, as well as acting as endogenous inhibitors of enzyme activity [42–44]. The pro-regions of PC2 and PC3 are of similar lengths, but there are notable differences in their primary amino acid sequences. One such difference is the higher number of histidine residues, including pairs and triplets, in the propeptide of PC2. In acidic conditions these histidines will have a greater positive charge and could lead to a stronger interaction between the propeptide and the negatively charged amino acids of the catalytic region. It has been proposed that the charged propeptide could

act as a potential sorting signal, as pro-PC2, but not mature PC2, has been shown to associate with membranes [45]. However, other studies suggest that this function may be carried out by sequences situated towards the C-terminus of the polypeptide [46].

Studying the role of the propeptide in maturation and secretion has proved difficult due to its importance in protein folding. Mutations in the propeptide of subtilisin effectively destroyed subtilisin folding and activity [47], and when regions of the propeptide of PC2 were deleted and the constructs expressed in Cos7 cells, the mutant PC2 proteins were invariably retained either in the endoplasmic reticulum or in an early Golgi compartment [48]. In the present study the role of the propeptide in the maturation of PC2 and PC3 was investigated by creating domain-swap mutants. The maturation of chimaeric proteins, similar to the ones used in this study, has been studied previously in transfected AtT20 cells [41]. There it was found that the pro-regions of these two proteases were not interchangeable and that the chimaeric proteins did not undergo maturation, being retained in the endoplasmic reticulum. The *in vitro* system used in the present study offers the advantage that the environment can be manipulated more easily than in cell transfection studies, allowing enforcement of a *trans*-Golgi network- or granule-type environment on the proteins in order to study their maturation without having to escape the quality control system of the endoplasmic reticulum. In this way it has been shown that the pro-region of PC2 provides a dominant, transferable signal that allows maturation at acidic pH. It may be that the higher incidence of histidine residues within the PC2 propeptide requires an acidic environment to obtain sufficient charge to provide favourable interactions with the catalytic domain, leading to cleavage of the propeptide and maturation. Replacing the PC2 pro-region with that of PC3 did not, however, lead to more efficient maturation at neutral pH. Instead maturation occurred at acidic pH, similar to wild-type PC2, but at a more rapid rate. Thus, although the PC3 pro-region is able to direct the folding of PC2, folding into a maturation-competent state only occurred at acid pH. Our results also suggest that the slow rate of maturation of PC2 is a property of the pro-region, since exchanging PC2's propeptide with that of PC3 led to a more rapid maturation of the PC2 chimaeric protein. This mutant protein, however, also contained the sequence KDRSKR at the primary cleavage site, as opposed to the sequence FDRKKR found in wild-type PC2. Thus slow maturation of wild-type PC2 may indicate an inefficiently recognized primary cleavage site. However, other factors must also be involved, since the chimaeric protein consisting of the mature portion of PC3 and the pro-region of PC2 had a slower rate of maturation compared with wild-type pro-PC3, despite containing the normal PC3 primary cleavage site.

It would appear, therefore, that PC2 has evolved with an inherent difficulty in folding, such that folding to a maturation-competent conformation occurs only in acidic conditions, and possesses an intramolecular chaperone that ensures that folding, adequate for maturation and subsequent activation of protease activity, is delayed until the protein reaches the *trans*-Golgi network or a later compartment in the secretory pathway. These may represent protective mechanisms that the cell has devised to prevent premature activation of this protease. Maturation and activity of PC2 is often considered to be linked to the proposed chaperone 7B2 [49–52]. Recent evidence, however, suggests that 7B2 associates with pro-PC2 only after the latter has folded [53], implying that 7B2 does not function as a traditional chaperone to aid folding, but may function later in the maturation/activation process. The results of the present study suggest that,

in the *Xenopus* egg extract system, efficient maturation and activation (results not shown) of pro-PC2 can occur in the absence of 7B2.

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REFERENCES

- 1 Siezen, R. J., de Vos, W. M., Leunissen, J. A. M. and Dijkstra, B. W. (1991) *Protein Eng.* **4**, 719–737
- 2 Mizuno, K., Nakamura, T., Ohshima, T., Tanaka, S. and Matsuo, H. (1988) *Biochem. Biophys. Res. Commun.* **156**, 246–254
- 3 Fuller, R. S., Brake, A. and Thorne, J. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1434–1438
- 4 Roebroek, A. J. M., Schalken, J. A., Leunissen, J. A. M., Onnekink, C., Bloemers, H. P. J. and Van de Ven, W. J. M. (1986) *EMBO J.* **5**, 2197–2202
- 5 Van den Ouweland, A. M. W., Van Duijnhoven, H. L. P., Keizer, G. D., Dorssers, C. J. and Van de Ven, W. J. M. (1990) *Nucleic Acids Res.* **18**, 664
- 6 Barr, P. J., Mason, O. B., Landsberg, K. E., Wong, P. A., Kiefer, M. C. and Brake, A. J. (1991) *DNA Cell Biol.* **10**, 319–328
- 7 Smekens, S. P. and Steiner, D. F. (1990) *J. Biol. Chem.* **265**, 2997–3000
- 8 Seidah, N. G., Gaspar, L., Mion, P., Marcinkiewicz, M., Mbikay, M. and Chretien, M. (1990) *DNA Cell Biol.* **9**, 415–424
- 9 Smekens, S. P., Avruch, A. S., LaMendola, J., Chan, S. J. and Steiner, D. F. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 340–344
- 10 Seidah, N. G., Marcinkiewicz, M., Benjannet, S., Gaspar, L., Beaubien, G., Mattei, M. G., Lazure, C., Mbikay, M. and Chretien, M. (1991) *Mol. Endocrinol.* **5**, 111–122
- 11 Nakayama, K., Kim, W. S., Torii, S., Hosaka, M., Nakagawa, T., Ikemizu, J., Baba, T. and Murakami, K. (1992) *J. Biol. Chem.* **267**, 5897–5900
- 12 Keifer, M. C., Tucker, J. E., Joh, R., Landsberg, K. E., Saltman, D. and Barr, P. J. (1991) *DNA Cell Biol.* **10**, 757–769
- 13 Lussan, J., Vieau, D., Hamelin, J., Day, R., Chretien, M. and Seidah, N. G. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 6691–6695
- 14 Nakagawa, T., Hosaka, M., Torii, S., Watanabe, T., Murakami, K. and Nakayama, K. (1993) *J. Biochem. (Tokyo)* **113**, 132–135
- 15 Bruzzaniti, A., Goodge, K., Jay, P., Taviaux, S. A., Lam, M. H., Berta, P., Martin, T. J., Moseley, J. M. and Gillespie, M. T. (1996) *Biochem. J.* **314**, 727–731
- 16 Seidah, N. G., Hamelin, J., Mamabachi, M., Dong, W., Tardos, H., Mbikay, M., Chretien, M. and Day, R. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 3388–3393
- 17 Meerabux, J., Yaspo, M. L., Roebroek, A. J., Van de Ven, W. J. M., Lister, T. A. and Young, B. D. (1996) *Cancer Res.* **56**, 448–451
- 18 Halban, P. A. and Irminger, J. C. (1994) *Biochem. J.* **299**, 1–18
- 19 Dupuy, A., Lindberg, I., Zhou, Y., Akil, H., Lazure, C., Chretien, M., Seidah, N. G. and Day, R. (1994) *FEBS Lett.* **337**, 60–65
- 20 Goodman, J. and Gorman, C. M. (1994) *Biochem. Biophys. Res. Commun.* **201**, 795–804
- 21 Matthews, G., Shennan, K. I., Seal, A. J., Taylor, N. A., Colman, A. and Docherty, K. (1994) *J. Biol. Chem.* **269**, 588–592
- 22 Shennan, K. I., Seal, A. J., Smekens, S. P., Steiner, D. F. and Docherty, K. (1991) *J. Biol. Chem.* **266**, 24011–24017
- 23 Benjannet, S., Reudelhuber, T., Mercure, C., Rondeau, N., Chretien, M. and Seidah, N. G. (1992) *J. Biol. Chem.* **267**, 11417–11423
- 24 Zhou, Y. and Lindberg, I. (1993) *J. Biol. Chem.* **268**, 5615–5623
- 25 Shennan, K. I., Taylor, N. A., Jermany, J. L., Matthews, G. and Docherty, K. (1995) *J. Biol. Chem.* **270**, 1402–1407
- 26 Vindrola, O. and Lindberg, I. (1992) *Mol. Endocrinol.* **6**, 1088–1094
- 27 Rufaut, N. W., Brennan, S. O., Hakes, D. J., Dixon, J. E. and Birch, N. P. (1993) *J. Biol. Chem.* **268**, 20291–20298
- 28 Coates, L. C. and Birch, N. P. (1997) *J. Neurochem.* **68**, 828–836
- 29 Leduc, R., Molloy, S. S., Thorne, B. A. and Thomas, G. (1992) *J. Biol. Chem.* **267**, 14304–14308
- 30 Molloy, S. S., Thomas, L., Vanslyke, J. K., Stenberg, P. E. and Thomas, G. (1994) *EMBO J.* **13**, 18–33
- 31 Lindberg, I. (1994) *Mol. Cell. Neurosci.* **5**, 263–268
- 32 Benjannet, S., Rondeau, N., Paquet, L., Boudreau, A., Lazure, C., Chretien, M. and Seidah, N. G. (1993) *Biochem. J.* **294**, 735–743
- 33 Shen, F. S., Seidah, N. G. and Lindberg, I. (1993) *J. Biol. Chem.* **268**, 24910–24915
- 34 Anderson, E. D., Vanslyke, J. K., Thulin, C. D., Jean, F. and Thomas, G. (1997) *EMBO J.* **16**, 1508–1518
- 35 Urbé, S., Ditté, A. S. and Tooze, S. A. (1997) *Biochem. J.* **321**, 65–74
- 36 Anderson, R. G. W. and Orci, L. (1988) *J. Cell Biol.* **106**, 539–543
- 37 Rhodes, C. J. and Alarcon, C. (1994) *Diabetes* **43**, 511–517
- 38 Matthews, G. and Colman, A. (1991) *Nucleic Acids Res.* **19**, 6405–6412

-
- 39 Krieg, P. A. and Melton, D. A. (1984) *Nucleic Acids Res.* **12**, 7057–7070
- 40 Benjannet, S., Lusson, J., Hamelin, J., Savaria, D., Chretien, M. and Seidah, N. G. (1995) *FEBS Lett.* **362**, 151–155
- 41 Zhou, A., Paquet, L. and Mains, R. E. (1995) *J. Biol. Chem.* **270**, 21509–21516
- 42 Silen, J. L. and Agard, D. A. (1989) *Nature (London)* **341**, 462–464
- 43 Shinde, U. and Inouye, M. (1994) *J. Biochem. (Tokyo)* **115**, 629–636
- 44 Eder, J. and Fersht, A. R. (1995) *Mol. Microbiol.* **16**, 609–614
- 45 Shennan, K. I., Taylor, N. A. and Docherty, K. (1994) *J. Biol. Chem.* **269**, 18646–18650
- 46 Creemers, J. W., Usac, E. F., Bright, N. A., Van de Loo, J. W., Jansen, E., Van de Ven, W. J. M. and Hutton, J. C. (1996) *J. Biol. Chem.* **271**, 25284–25291
- 47 Shinde, U., Li, Y., Chatterjee, S. and Inouye, M. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 6924–6928
- 48 Taylor, N. A., Shennan, K. I. J., Cutler, D. F. and Docherty, K. (1997) *Biochem. J.* **321**, 367–373
- 49 Braks, J. A. and Martens, G. J. (1994) *Cell* **78**, 263–273
- 50 Chaudhuri, B., Stephan, C., Huijbregts, R. P. and Martens, G. J. (1995) *Biochem. Biophys. Res. Commun.* **211**, 417–425
- 51 Zhu, X. and Lindberg, I. (1995) *J. Cell Biol.* **129**, 1641–1650
- 52 Benjannet, S., Savaria, D., Chretien, M. and Seidah, N. G. (1995) *J. Neurochem.* **64**, 2303–2311
- 53 Muller, L., Zhu, X. and Lindberg, I. (1997) *J. Cell Biol.* **139**, 625–638
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