Differences between the catalytic properties of recombinant human PC2 and endogenous rat PC2

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Human prohormone convertase PC2 was expressed in *Xenopus* oocytes and its properties were compared with those of the Type-2 endopeptidase of rat insulin secretory granules, previously identified as PC2 [Bennett, Bailyes, Nielson, Guest, Rutherford, Arden and Hutton (1992) J. Biol. Chem. 267, 15229–15236]. Recombinant PC2 had the same substrate specificity as the Type-2 endopeptidase, cleaving at the CA-junction (Lys⁶⁴, Arg⁶⁵) of human des-31,32-proinsulin to generate insulin; little activity was found toward human des-64,65-proinsulin or proinsulin itself. Recombinant PC2 was maximally active in 5–7 mM Ca²⁺ ($K_{0.5}$ = 1.6 mM) whereas the Type-2 endopeptidase was maximally active in 0.5–1 mM Ca²⁺ ($K_{0.5}$ = 40 μ M). Both enzymes had a pH optimum of 5.0–5.5 but the Type-2 endopeptidase was

active over a wider pH range. Two molecular forms of recombinant PC2 (71 kDa and 68 kDa) were found, both had an intact C-terminus but differed by the presence of the propeptide. The endogenous PC2 comprised several overlapping forms (size range 64–68 kDa), approximately two-thirds of which lacked C-terminal immunoreactivity. Part of the size difference between recombinant and endogenous PC2 was attributable to differences in N-glycosylation. The different post-translational proteolytic modifications of recombinant and endogenous PC2 did not account for the different pH and Ca²⁺ sensitivities shown by the enzymes. A modulating effect of carbohydrate on enzyme activity could not be excluded.

INTRODUCTION

The maturation of many proteins that enter the secretory pathway involves specific endoproteolytic cleavage(s), often at sites containing two or more basic amino acids [1]. Significant progress in identification of the converting enzymes has come from the discovery that Kex-2, a yeast proprotein-processing endopeptidase [2,3], has homologues in higher eukaryotes (see [4,5]). This subtilisin-related family was identified by molecular cloning and contains furin [6], PC2 [7,8], PC1/PC3 [9,10], PACE4 [11], PC4 [12] and PC5/PC6 [13,14]. The ubiquitous distribution of furin suggests that it is involved in the maturation of proproteins common to many cell types, whereas the localization of PC2 and PC1/PC3 to neuroendocrine cells suggests that these proteases are involved in the processing of prohormones and proneuropeptides.

Transfection of PC2 into mammalian cells has shown that it encodes an active endopeptidase that can cleave *in vivo* at sites containing two or more basic amino acids in various proproteins including pro-opiomelanocortin (POMC) [15,16] and pro-enkephalin [17] but not prorenin expressed in rat pituitary somatomammotrophs [18] or prosomatostatin expressed in monkey kidney cells [19].

In the pancreatic β -cell, proinsulin is converted into insulin by excision of the C-peptide at Arg³¹, Arg³² and Lys⁶⁴, Arg⁶⁵. Morphological studies indicate that processing occurs after the immature granule has separated from the *trans* Golgi network and correlates with acidification and concentration of Ca²⁺ within the nascent granule [20–22]. Two endopeptidases with acidic pH optima have been identified in rat insulin granule

extracts *in vitro* [23]. Type-1 endopeptidase, which cleaves at the BC-junction (Arg³¹, Arg³²), is activated by millimolar Ca²⁺ concentrations, and Type-2 endopeptidase, which cleaves predominantly at the CA-junction (Lys⁶⁴, Arg⁶⁵), is activated by micromolar Ca²⁺ concentrations. The Ca²⁺ and acidic pH requirements of these proteases are thus consistent with their being responsible for proinsulin maturation *in vivo*.

The Type-1 endopeptidase and mouse recombinant PC1/PC3 (expressed in *Xenopus* oocytes) are indistinguishable with respect to substrate specificity, pH activity profile (optimum pH = 5.5) and Ca²⁺ sensitivity ($K_{0.5}$ = 2.5–3 mM) [24]. The Type-2 endopeptidase has been identified as PC2 by immunoprecipitation studies and by co-chromatography of Type-2 enzyme activity and PC2 immunoreactivity [25].

Our initial study of the kinetic properties of human PC2 expressed in *Xenopus* oocytes, using small fluorogenic peptide substrates [26], was consistent with PC2 corresponding to one of the insulin granule proinsulin-processing endopeptidases. The protease inhibitor profile matched that of the granule enzymes [23] and the expressed enzyme had a pH optimum of 5.5 and was activated by Ca²⁺ in the millimolar range ($K_{0.5}$ = 1 mM). The preference of the recombinant PC2 for the substrate containing a Lys, Arg pair was consistent with the proinsulin CA-junction specificity (Lys⁶⁴, Arg⁶⁵) and with the peptidyl protease inhibitor profile of the Type-2 endopeptidase [27]. However, the millimolar Ca²⁺ requirement of the recombinant PC2 resembled that of the Type-1 endopeptidase.

In this study, we have characterized human recombinant PC2 using proinsulin, its conversion intermediates (des-31,32-proinsulin and des-64,65-proinsulin) and the neuroendocrine pro-

Abbreviations used: ECL, enhanced chemiluminescence; TPCK, tosylphenylalanylchloromethane; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; CGA, chromogranin A; POMC, pro-opiomelanocortin; BC-junction, B-chain/C-peptide junction in proinsulin or des-64,65-proinsulin; CA-junction, C-peptide/A-chain junction in proinsulin or des-31,32-proinsulin.

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protein chromogranin A (CGA) as substrates and compared its kinetic and molecular properties with those of the rat insulin granule Type-2 endopeptidase. The relationship between the various post-translational modifications and the kinetic properties of PC2 is addressed.

EXPERIMENTAL

^{125}I -labelled human proinsulin, ^{125}I -des-31,32-proinsulin (proinsulin lacking residues Arg³¹ and Arg³²) and ^{125}I -des-64,65-proinsulin (proinsulin lacking residues Lys⁶⁴ and Arg⁶⁵) (monoiodinated at Tyr¹⁴ of the A-chain) were gifts from Lilly Research Laboratories, Indianapolis, U.S.A. Their specific activities were 1 mol of ^{125}I /mol of polypeptide.

Preparation of recombinant human PC2

Human PC2 was expressed in *Xenopus* oocytes as described in [28]. In brief, mRNA was synthesized from human PC2 cDNA and *Xenopus* oocytes were isolated and injected with 50 nl of PC2 mRNA (25 ng) or 50 nl of water. Oocytes were incubated at 20 °C in Barth's modified saline containing 5% (v/v) dialysed fetal-calf serum. After 48 h, the media were collected and stored at -70 °C. The media were used in the enzyme assays without further purification. In the text, the medium collected from oocytes injected with PC2 is referred to as PC2-injected oocyte medium and medium from the corresponding batch of oocytes injected with water at the same time is referred to as control medium.

Preparation of insulin granules and solubilized extracts

Insulin secretory granule fractions were prepared from a transplantable rat insulinoma and stored in liquid nitrogen [29]. The endopeptidase activities from these fractions were solubilized with Triton X-100 as described in [30]. This extract was used without further purification as a source of Type-2 endopeptidase activity in the pH and Ca^{2+} experiments.

Enzyme assays

The assays with proinsulin or the proinsulin conversion intermediates were conducted essentially as described in the previous study on recombinant mouse PC1/PC3 [24]. Samples (5–20 μl of oocyte medium or 5–15 μl of solubilized granules) were incubated at 30 °C for 3–23 h with ^{125}I -labelled substrate (human proinsulin, des-64,65-proinsulin or des-31,32-proinsulin). Unless stated otherwise, the assay was performed at pH 5.5, in 5 mM Ca^{2+} in the presence of protease inhibitors [10 μM E64, 10 μM pepstatin A, 100 μM tosylphenylalanylchloromethane (TPCK) and 1 mM PMSF]. The reaction products were analysed either by alkaline urea/PAGE, followed by autoradiography and densitometric scanning using a Joyce Loebl Chromoscan 3 densitometer [23] or by the A6 [30] or ANT-1 monoclonal antibody immunoabsorbent separation method [31]. The A6 assay format measures proteolysis at the BC-junction and the ANT-1 assay format measures proteolysis at the CA-junction. Monoclonal antibody A6 binds proinsulin and des-64,65-proinsulin in the region of the BC-junction and does not bind des-31,32-proinsulin or insulin. Monoclonal antibody ANT-1 binds des-31,32-proinsulin in the region of the CA-junction and does not bind insulin [32]. The extent of processing of ^{125}I -labelled des-31,32-proinsulin was determined by adding ANT-1 immunoabsorbent to the assay cocktails at the end of the enzyme incubation to precipitate unreacted substrate. The radioactivity remaining in solution (insulin) was measured and expressed as a percentage of the total

radioactivity. Since the immunoabsorbent separation method measures epitope destruction, it is important to ensure that the unbound radioactivity has not been generated by non-specific proteolysis of the substrate. Accordingly, the ANT-1 immunoabsorbent assay was validated for the measurement of recombinant PC2 activity by comparing the values obtained by this assay with those obtained by using alkaline urea/PAGE to separate the reaction products. Eight samples, in which total conversion of des-31,32-proinsulin ranged in value from 27.2% to 77.0% in the immunoabsorbent assay, were also analysed by PAGE. The immunoabsorbent values averaged $100.5 \pm 3.9\%$ of the values determined by PAGE. In all the enzyme assays, the matched water-injected oocyte medium was used as control. The control medium contained little or no activity towards des-31,32-proinsulin (< 2% substrate conversion in 21 h) and the activity in the PC2-injected oocyte medium was typically 30-fold above that in the control medium. However, using the A6 assay format, three different batches of control medium had measurable BC-junction-directed activity (4–11% substrate conversion in 21 h) but the activity in the corresponding PC2-injected oocyte medium was only 0.9–1.8-fold of the control values. Our previous study on the expression of mouse PC1/3 in the oocyte system also found that some control medium had significant proinsulin BC-junction-directed activity [24]. The CGA processing assay was performed as described in [33]. Samples (10 μl of medium from micro-injected oocytes or solubilized insulin granule proteins) were incubated with 4 μg of bovine CGA at 30 °C overnight in 50 μl (final volume) of 100 mM sodium acetate, pH 4.8, containing 5 mM CaCl_2 , 0.1% Triton X-100, 10 μM E64, 10 μM pepstatin A, 0.1 mM TPCK and 1 mM PMSF. The samples were separated by SDS/PAGE and stained for protein with Coomassie Brilliant Blue R-250.

Determination of Ca^{2+} and pH sensitivity

Des-31,32-proinsulin conversion assays were analysed by the ANT-1 immunoabsorbent separation method. In the pH studies (see Figure 4a), a buffer system composed of 30 mM sodium acetate, 30 mM Mes and 30 mM *N*-ethylmorpholine, adjusted with HCl or NaOH, was used. In the Ca^{2+} studies (see Figure 4b), 2 mM EDTA with variable concentrations of CaCl_2 was used to generate the ionized Ca^{2+} concentrations [23]. The solubilized granule extract used as a source of endogenous endopeptidase activity was diluted into medium to ensure comparable results.

Immunoblot analysis

SDS/PAGE and the Western blotting of PC2 shown in Figures 5(a), 6 and 8 were conducted as described in [25]. The Western blotting of Figures 5(b) and 7 used the enhanced chemiluminescence (ECL) detection system (Amersham Life Science). Quantification of the Western blotting was done by densitometric scanning (see Enzyme assays, above). The catalytic domain antiserum was raised to a bacterial fusion protein containing rat PC2 amino acids 168–380 [25]. The proPC2 domain antiserum was raised to a bacterial fusion protein containing rat PC2 amino acids 87–104. These antisera were incubated with Sepharose coupled to bacterial proteins (soluble plus the glutathione S-transferase fusion partner) to remove antibodies to the fusion partner. The antiserum to the C-terminal 19 amino acids (616–634) of mouse PC2 [34] was a kind gift from Dr. Ruth Hogue-Angeletti (Albert Einstein College of Medicine, New York, U.S.A.). All antisera were raised in rabbits.

N-glycanase treatment

Recombinant PC2 (20 μ l of medium) and granule extract (5 μ l) were precipitated by trichloroacetic acid and re-dissolved in 10 μ l of 20 mM sodium phosphate, pH 7.5, 50 mM EDTA, 0.02 % sodium azide, 0.5 % SDS, 50 mM 2-mercaptoethanol, plus protease inhibitors (10 μ M E64 and pepstatin A, 0.1 mM TPCK, 1 mM PMSF). Samples were heated at 100 °C for 4 min and cooled on ice. Aliquots (1.5 μ l) of 20 % (w/v) Triton X-100 and 0.5 unit of recombinant N-glycanase (Genzyme Corp, Cambridge MA, U.S.A.) were added. Samples were incubated at 30 °C for 17 h and analysed by Western blotting using the anti-C-terminal serum. Control incubations omitted N-glycanase.

Immunoprecipitation of granule PC2 with anti-C-terminal serum

Insulin granules (125 μ l, 0.75 mg of protein) were solubilized with 250 μ l of buffer A [50 mM Tris/HCl, pH 7.6, 1 % (w/v) Triton X-100, 0.5 % sodium deoxycholate, 5 mM EDTA] containing protease inhibitors (20 μ M E64 and pepstatin A, 0.2 mM TPCK, 2 mM PMSF). The lysate was centrifuged at 48000 g (max.) for 30 min and the supernatant was retained. Protein A-Sepharose (Sigma) was equilibrated with buffer A and 50 μ l samples of packed resin were incubated overnight at 4 °C with 30 μ l of a 1:3 dilution of the anti-C-terminal or pre-immune serum. Unbound protein was washed away with buffer A and the immunoadsorbents were incubated with 50 μ l of the granule extract for 24 h at 4 °C. The immunoadsorbents were centrifuged and the supernatants (50 μ l) were removed. The immunoadsorbents were washed (4 $\times$ 50 μ l) and the washes and supernatants were combined. Samples of the pooled material were Western blotted with C-terminal and catalytic domain antisera.

Ion-exchange chromatography

Insulin granules were solubilized and fractionated on a DEAE-cellulose column at pH 5.5 as described in [25], except that the volume of the 0–0.6 M NaCl gradient used to elute PC2 was doubled to increase resolution.

RESULTS

Characterization of recombinant PC2 enzymic activity

Substrate specificity

The proinsulin conversion intermediate des-31,32-proinsulin was hydrolysed by recombinant PC2 at a rate which approximated linearity, provided that the total substrate conversion did not exceed 25 % (Figure 1). Beyond this, the reaction rate declined due to depletion of substrate (data not shown). The activity of PC2 towards the BC-junction site in proinsulin (Arg³¹, Arg³²) was examined using the A6 immunoadsorbent assay. Three separate batches of PC2, incubated to give 23–29 % conversion of des-31,32-proinsulin, generated only 0–2 % conversion of the BC-junction in proinsulin under the same conditions, indicating that PC2 had little or no activity towards the BC-junction. The formation of insulin from des-31,32-proinsulin was confirmed by alkaline urea/PAGE (Figure 2). This analysis also showed that proinsulin itself was not a good substrate for PC2. The activity of PC2 towards proinsulin and des-64,65-proinsulin was only 0.46 % and 0.4 % substrate conversion per hour respectively, whereas the corresponding rate towards des-31,32-proinsulin was 6.5 % per hour [determined from an earlier time point (4 h) not shown on this gel]. Proinsulin conversion by PC2 (whether at the BC-junction or the CA-junction) thus amounted to less than 7 % of its des-31,32-proinsulin conversion activity.

Figure 1 Time course of conversion of des-31,32-proinsulin into insulin by PC2

Recombinant PC2 (20 μ l of medium) was incubated with ¹²⁵I-des-31,32-proinsulin for the times indicated. Unreacted substrate and product were separated and quantified using the ANT-1 immunoadsorbent. The results shown are the mean and range of duplicates.

Rat insulin granule PC2 catalyses the processing of CGA, an acidic glycoprotein of molecular mass 48 kDa (70–100 kDa by SDS/PAGE), widely distributed in neuroendocrine cells [33]. Incubation of recombinant PC2 with CGA converted the 80 kDa bovine precursor into peptides of 55 and 18 kDa, plus a small amount of a 16 kDa peptide (Figure 3a). Similar peptides were produced when CGA was processed by endogenous PC2 of rat insulin granules (Figure 3b) (production of the 65 kDa CGA peptide by the recombinant PC2 is masked by the serum albumin of the oocyte medium) (Figure 3a). The 65 and the 55 kDa peptides are overlapping intermediates in the conversion pathway of CGA, the 18 and 16 kDa peptides are final products [33].

pH and Ca²⁺ activity profiles

Recombinant PC2 was optimally active at pH 5.0–5.5, with little or no activity at pH 4.5 and below, or at pH 6.5 and above. The insulin granule Type-2 endopeptidase was also maximally active at pH 5.0–5.5 but gave a broader profile, with 30 % of the activity remaining at pH 4, and 40 % at pH 6.5 (Figure 4a).

The Ca²⁺ profiles of the two enzyme activities were markedly different (Figure 4b). Recombinant PC2 was essentially inactive at Ca²⁺ concentrations below 0.5 mM, had a *K*_{0.5} of 1.6 mM and did not reach maximum until 5–7 mM Ca²⁺. The insulin granule extract, in contrast, was active at micromolar levels of Ca²⁺, with a *K*_{0.5} for Ca²⁺ of 40 μ M and maximal activity at 0.5 to 1 mM. These differences could not be accounted for by the presence of endogenous Ca²⁺ in either the oocyte medium or the granule extract as these were compensated for by the Ca²⁺/EDTA buffer used in the assay cocktail.

Molecular forms of recombinant and granule PC2

The molecular forms of the two enzymes were examined for differences which might account for the different Ca²⁺ profiles. In the oocyte system, the 75 kDa precursor PC2 is converted intracellularly into a 71 kDa intermediate by proteolysis at the first tetrabasic site (amino acids 78–81) and proteolytic conversion to the final 68 kDa product occurs extracellularly [28]. In the batches of oocyte medium used in this study (Figure 5a), the 68 kDa form comprised 42 $\pm$ 2 % (S.E.M., *n* = 4) of the total PC2 immunoreactivity (Table 1). This indicated that the extent

Figure 2 Substrate specificity of PC2

Solubilized insulin granules (lanes 1, 4 and 7), control media (lanes 2, 5 and 8) and PC2-injected oocyte media (lanes 3, 6 and 9) were incubated with ^{125}I -proinsulin (lanes 1–3), ^{125}I -des-64,65-proinsulin (lanes 4–6) and ^{125}I -des-31,32-proinsulin (lanes 7–9) for 23 h and analysed by alkaline urea/PAGE.

Figure 3 Conversion of CGA by recombinant and endogenous PC2

(a) PC2-injected oocyte medium and control medium were incubated overnight with and without 4 μg of bovine CGA. (b) Bovine CGA (4 μg) was incubated with 2 μg of solubilized granule proteins for 0–18 h as indicated. The samples were separated by SDS/PAGE and stained for protein.

of conversion of the 71 kDa form into the 68 kDa product was constant despite great variation in the biosynthetic capacity of the different batches of oocytes. The granule PC2 consisted almost entirely of mature PC2 (64–66 kDa), with only trace amounts of the 75 kDa precursor PC2 present. An estimate of the specific activity of the recombinant PC2 relative to that of the endogenous PC2 was made by calculating the immunoreactivity and enzyme activity values relative to those of the endogenous PC2 in lane 9. Three batches of oocyte medium which had measurable PC2 immunoreactivity and PC2 enzyme activity were used for these calculations (Figure 5a, lanes 2, 6 and 7; Table 1). The enzyme activity of each batch of PC2 was determined in separate experiments and expressed relative to that of endogenous PC2 (Table 1). It was assumed for the purposes of these calculations that only the 68 kDa protein contributed enzyme activity since mutational studies indicated that the 71 kDa form is enzymically inactive [28]. Recombinant PC2 specific activities in the three batches where measurements were possible were 10–55 % of the rat granule enzyme specific activity,

Figure 4 pH-dependence and Ca^{2+} dependence of enzyme activity

The pH-dependence (a) and Ca^{2+} dependence (b) of PC2 (○) and the insulin granule Type-2 endopeptidase activity (●) were determined using des-31,32-proinsulin as substrate. The results show the mean $\pm$ S.E.M. ($n = 3$) for PC2 or mean $\pm$ range ($n = 2$) for the Type-2 endopeptidase activity and are expressed as percentages of the maximum substrate conversion attained by each enzyme. Similar results were obtained in two other separate experiments.

indicating that although the environment and route of biosynthesis in the oocyte differs greatly from that of the pathway of regulated biosynthesis in the β -cell, the 'specific' activity of the recombinant PC2 approaches that of the endogenous enzyme.

The size difference apparent between the recombinant PC2 and the endogenous PC2 (Figure 5a) was investigated further using antisera raised to different parts of PC2. The size of the recombinant PC2 forms did not vary with the antisera used. However, the C-terminal-reactive granule PC2 (65–68 kDa) was larger than the catalytic-domain-reactive PC2. As expected, only the 71 kDa form of recombinant PC2 had proPC2 immunoreactivity (Figure 5b, lane 4), consistent with the production of the 68 kDa form from the 71 kDa form by N-terminal processing.

N-glycanase treatment showed that the size difference between the C-terminal immunoreactive recombinant and endogenous PC2 appeared to be due to the more extensive N-glycosylation of recombinant PC2 (Figure 6). The size of both enzymes decreased to 58 kDa, consistent with our previous study on rat islet PC2 [35].

Molecular forms of PC2 in the insulin granule

The difference in mobility between the granule C-terminal immunoreactive PC2 (size range 65–68 kDa) and the catalytic domain immunoreactive PC2 (range 64–66 kDa) (Figure 5b) suggested that part of the granule PC2 was modified at the C-terminus. To test this possibility directly, solubilized granule

Figure 5 Comparison of the molecular forms of recombinant and granule PC2 by Western blotting

(a) PC2 catalytic domain antiserum. Six different batches of recombinant PC2 (lanes 2–7) and control (lane 1) medium (40 μ l) and detergent-solubilized insulin granule proteins (lanes 8 and 9) were separated by SDS/PAGE and Western-blotted with the PC2 catalytic domain antiserum. The solubilized insulin granule proteins in lanes 8 and 9 were extracted from 20 μ g and 40 μ g of total granule protein. (b) PC2 catalytic domain, C-terminal and pro-PC2 antisera. Recombinant PC2 (50 μ l of medium) and endogenous granule PC2 (detergent-extracted from 30 μ g of total granule protein) were subjected to SDS/PAGE and transferred to nitrocellulose. The lanes were cut down the middle to form the tracks shown and developed with different PC2 antisera. Recombinant PC2 is in tracks 3, 4, 5 and 6 and endogenous PC2 is in tracks 1, 2 and 7. The catalytic domain antiserum was used to develop tracks 2, 3 and 5. The C-terminal domain antiserum was used to develop tracks 1, 6 and 7. The pro-PC2 antiserum was used to develop track 4. The migration of the standard proteins is indicated in kDa. The ECL method of detection was used.

proteins were incubated with an anti-C-terminal immunoadsorbent and the unbound PC2 immunoreactivity determined by Western blotting with the C-terminal and the catalytic domain

Figure 6 Deglycosylation of PC2

Recombinant PC2 (1, 4) and granule PC2 (2, 3) were incubated with (3, 4) or without (1, 2) N-glycanase and Western-blotted with the anti-C-terminal serum.

Figure 7 Immunoprecipitation of granule PC2 with C-terminal antiserum

Solubilized insulin granule proteins were incubated with an anti-PC2 C-terminal domain immunoadsorbent or a pre-immune immunoadsorbent and samples of the unbound material were analysed by Western blotting with PC2 catalytic domain antiserum (lanes 1 and 2, 40 μ l samples) and PC2 C-terminal antiserum (lanes 3 and 4, 30 μ l samples). Lanes 1 and 3 are the pre-immune immunoadsorbent supernatants and lanes 2 and 4 are the C-terminal domain immunoadsorbent supernatants. The ECL detection method was used and four different exposures of the blots were made and analysed by densitometry.

antisera. The immunoadsorbent removed all the C-terminal-reactive PC2 but 70% of the catalytic-domain-reactive PC2 remained in the supernatant (Figure 7). Similar results were obtained in two separate experiments, two different batches of insulin granules were used as the source of the endogenous PC2.

Table 1 Immunoreactivity and enzyme activity of recombinant and endogenous PC2

The tracks in Figure 5(a) were scanned densitometrically and the ratio of the 68 kDa form to the total PC2 immunoreactivity for each recombinant PC2 sample was determined. The immunoreactivity of the 68 kDa form of recombinant PC2 (lanes 2–7) is expressed relative to the granule PC2 immunoreactivity (lanes 8 and 9). The catalytic activities of the samples were determined in separate experiments and are expressed relative to the activity of the granule PC2. The 'relative specific activity' is relative catalytic activity divided by immunoreactivity. Abbreviations: N.D., not detected; N.A., not applicable.

PC2 sample	Mature:total immunoreactivity	Immunoreactivity (mature form)	Relative catalytic activity	Relative specific activity
Figure 5, lane 2	0.46	36.5	3.9	0.11
Figure 5, lane 3	N.D.	N.D.	0.18	—
Figure 5, lane 4	N.D.	N.D.	1.09	—
Figure 5, lane 5	0.43	26	N.D.	—
Figure 5, lane 6	0.39	37.5	7.85	0.21
Figure 5, lane 7	0.38	59	33.2	0.56
Figure 5, lane 8	N.A.	50	50	1
Figure 5, lane 9	N.A.	100	100	1

Figure 8 Fractionation of solubilized insulin granules by ion-exchange chromatography

Solubilized insulin granules were applied to an ion-exchange column and bound proteins were eluted with a NaCl gradient. (a) The Type-2 endopeptidase activity (■) and the PC2 immunoreactivity using the catalytic domain (□) and the C-terminal domain antisera (●) were determined (see the Materials and methods section). Neither were found in the void fractions and so only the gradient fractions containing activity are shown. The arbitrary densitometric units are plotted such that the C-terminal immunoreactivity comprises one-third of the catalytic domain immunoreactivity. The NaCl gradient is indicated by the straight line. (b) Selected fractions from (a) were assayed for Type-2 endopeptidase activity at the concentrations of Ca^{2+} shown. (c) Selected fractions from (a) were assayed in the presence of 5 mM Ca^{2+} , at pH 5.0 (●) or 0.2 mM Ca^{2+} at pH 4.0 (○). In (c) the results are given as a percentage of the maximum substrate conversion reached, in (a) and (b) the total substrate conversion is shown.

It therefore appeared that only one-third of the granule PC2 possessed an unmodified C-terminus.

Chromatography of the granule immunoreactive PC2 and Type-2 endopeptidase activity

To determine whether C-terminal processing affected enzyme activity, solubilized insulin granules were fractionated by DEAE cellulose chromatography and assayed for PC2 immunoreactivity and enzyme activity. All the PC2 immunoreactivity and Type-2 endopeptidase activity bound to the column and were eluted with a NaCl gradient (Figure 8a). There was a partial separation of the anti-C-terminal PC2 immunoreactivity (fractions 31–36) and

the anti-catalytic-domain PC2 immunoreactivity (fractions 32–38). The Type-2 activity eluted with the C-terminal-immunoreactive PC2 except that fractions 37–38 contained catalytic-domain-immunoreactive PC2 but little or no C-terminal-immunoreactive PC2. The column profile suggests that while both forms of the enzyme have enzyme activity, the specific activity of the C-terminal intact form is higher than that of the processed enzyme. The Ca^{2+} activity profile of column fractions containing various proportions of the two PC2 forms did not reveal any differences in sensitivity, which remained in the micromolar range (Figure 8b). Likewise, the activity measured at pH 4 and 0.2 mM Ca^{2+} co-eluted with that measured at pH 5.0 and 5 mM Ca^{2+} (Figure 8c). The difference in catalytic properties of the recombinant PC2 and the granule form could not therefore be due to the structural heterogeneity at the C-terminus. Moreover, there was no evidence for any other des-31,32-proinsulin conversion activity apart from that of PC2, nor were there any molecular forms of granule PC2 with the pH and Ca^{2+} sensitivity shown by the recombinant PC2.

DISCUSSION

There have been a number of studies on the expression of recombinant PC2 by transfection of cultured cells, but none on its kinetic properties *in vitro* with potential physiological substrates. Attempts to obtain enzymically active PC2 from the media of transfected cells have been unsuccessful [36], suggesting that the activation of PC2 requires specific post-translational modifications. In this study, the expression of human recombinant PC2 in *Xenopus* oocytes produced active enzyme which was characterized *in vitro* with human proinsulin and split proinsulin substrates. The substrate specificity of the recombinant PC2 was consistent with its identification as the Type-2 endopeptidase [25,33]. The preference for the CA-junction in des-31,32-proinsulin over the same site in proinsulin itself which was shown by recombinant and endogenous PC2 [31,37] implies that insulin production proceeds via des-31,32-proinsulin rather than des-64,65-proinsulin (see [38,39]) since the reaction rate of PC1/PC3 with the BC-junction is little affected by processing at the CA-junction [24]. This conversion route would explain why des-31,32-proinsulin is the predominant intermediate detected in biosynthetic studies of proinsulin conversion in human islets [40] and why des-31,32-proinsulin is the principal proinsulin conversion intermediate in the plasma of people with non-insulin-dependent diabetes mellitus (NIDDM) [41].

In the original characterization of the Type-2 endopeptidase activity, approximately 10% of the proinsulin conversion activity was found to be directed at the BC-junction [23]. In this study, a minor amount of BC-junction-directed activity on des-64,65-proinsulin was detected, both in the A6 immunoadsorbent assay (data not shown) and by urea/PAGE. However, the data must be viewed against the presence of a significant rate of BC-junction-directed processing activity in the control medium (see the Experimental section). The specificity of this *Xenopus*-derived activity (the CA-junction was unaffected) resembled the processing of human proinsulin at the BC-junction in the constitutive pathway of transfected cells, an activity which has been attributed to the preference of furin for substrates having a basic residue in the -4 position [42]. Although a *Xenopus* homologue of furin has not yet been cloned, the processing of foreign prohormones in *Xenopus* oocytes also requires the presence of the furin cleavage site consensus motif involving a basic residue at the -4 position [43]. It is therefore possible that the protease activity found in the control medium comes from furin-like enzyme(s) released from the oocyte and it is conceivable that more is produced when the

oocyte is engaged in the biosynthesis of foreign genes. Studies in which human PC2 and rat proinsulin I were transfected into COS-7 cells showed that proinsulin was converted mainly into des-64,65-proinsulin plus a small quantity of insulin [44]. Here again, this minor amount of BC-junction cleavage could have been brought about by the endogenous furin activity of the host cells. Clearly, further studies on the purified recombinant or endogenous enzyme are needed to resolve this issue.

The difference between the Ca^{2+} sensitivity of the recombinant PC2 and that of the Type-2 endopeptidase was striking. All the granule des-31,32-proinsulin-converting activity was active at micromolar Ca^{2+} ; there was no subfraction of the activity which corresponded to that of recombinant PC2 in terms of Ca^{2+} sensitivity or inactivity at low pH. This could reflect either an intrinsic difference in the properties of rat and human PC2s or a failure of the oocyte system to replicate the post-translation events that modify PC2 in the regulated pathway of secretion in the β -cell. Alternatively, it is possible that a granule component not present in the oocyte could modulate PC2 activity. One such protein is the precursor form of 7B2 which has been shown to inhibit PC2 but not PC1/PC3 activity [45]. However, it does not appear that the granule PC2 binds a protein that affects its Ca^{2+} sensitivity since its size on gel filtration corresponds to its size by SDS/PAGE [25].

Human and rat preproPC2 are > 96% identical, differing in only 23 amino acids, 14 of which are found in the mature sequence. The three-dimensional structure of the mammalian subtilisins is not known, but comparison with the three-dimensional structure of the related bacterial subtilisin proteases has enabled structural predictions to be made for the catalytic domain. One of the three Ca^{2+} -binding sites found in the bacterial subtilisins occurs in PC2 [46] and one of the amino acid differences (Asn²²¹ in human, Ser in rat) is found in the region of the Ca^{2+} -binding domain. However, this residue is not predicted to be directly liganded by the Ca^{2+} ion and so should have little effect on the enzymes' Ca^{2+} -binding properties [47]. Furthermore, if this residue was involved in Ca^{2+} binding, the Asn substitution in the human enzyme should confer enhanced Ca^{2+} binding which would not be consistent with the higher Ca^{2+} requirement of human PC2 (R. Siezen, personal communication).

The oocyte expression system has been widely used to express biologically active foreign proteins. However, the correct post-translational modifications are not always achieved, not all proproteins are proteolytically processed correctly, and glycosylation has often been found to be aberrant (for review see [48]). Comparison of the molecular properties of the recombinant and human PC2 revealed some differences in post-translational proteolysis. Mature rat granule PC2 consists of enzyme cleaved after Arg¹⁰⁸ (after the second tetra-basic site, amino acids 105–108) and Arg¹¹¹ in approximately equal quantities [25]. Mutational analysis and N-terminal sequencing studies on recombinant PC2 showed that the 68 kDa final product is also produced by cleavage at the second tetrabasic site in the proprotein [28,49]. Differential N-terminal processing of PC2 cannot therefore explain the difference in Ca^{2+} sensitivity since approximately 50% of the granule enzyme has the same N-terminus as the oocyte-expressed enzyme.

The difference in C-terminal processing between the recombinant and endogenous PC2 did not provide an explanation of the difference in Ca^{2+} sensitivity. The C-terminal immunoreactive form of endogenous PC2 was most closely associated with enzyme activity, but was not the only source of enzyme activity in the fractions recovered after ion-exchange chromatography of insulin granule proteins. Consistent with this conclusion is the finding that a C-terminal truncated form of human

PC2 (23 amino acids deleted) expressed in oocytes retained enzyme activity (measured with a fluorogenic peptide substrate) [28]. The C-terminal processing may be a late event in PC2 maturation which occurs within the granule compartment since both C-terminal immunopositive and C-terminal immunonegative PC2 are secreted from rat islets in response to glucose (P. C. Guest, unpublished work). Although the structural basis for the loss of C-terminal immunoreactivity has not been determined, it was associated with only a small change in size. If processing occurred at the only paired basic amino acid site in the C-terminal region (Lys⁶¹⁴, Lys⁶¹⁵), it would remove 24 amino acids (2900 Da). There would also be a net removal of two acidic residues which might reduce the change in SDS/PAGE mobility.

The possibility that N-linked glycosylation affects pH and Ca^{2+} sensitivity requires consideration. The biological roles of N-glycosylation appear to range from the trivial to crucial (for review see [50]) but modulation of enzyme activity by varying degrees of N-linked glycosylation has been shown [51]. Unfortunately, the role of carbohydrate in the modulation of PC2 catalytic activity is not one that can easily be addressed. The incubation conditions required for the action of N-glycanase would irreversibly denature PC2 [25]. Prevention of N-glycosylation by tunicamycin treatment of cells expressing PC2 resulted in considerable intracellular degradation of the unglycosylated protein [52]. This suggests that carbohydrate addition might be required for successful transport of PC2 through the secretory pathway, but precludes the preparation of unglycosylated PC2 for enzymic analysis.

Of the other possible post-translational modifications, it has been shown that PC2 transfected into a rat somatomammotroph line undergoes tyrosine sulphation but the effect of this on enzyme activity is unknown [52]. The possibility that PC2 undergoes phosphorylation has not been addressed.

In conclusion, we have shown that the substrate specificity of human PC2 corresponds to that of the Type-2 endopeptidase, in agreement with its proposed role in human proinsulin maturation. The contrasting Ca^{2+} sensitivities of the human recombinant PC2 and the endogenous rat PC2 suggest that post-translational modifications may modulate the enzyme activity of PC2. However, a difference in properties related to an intrinsic species difference cannot be entirely ruled out. Further studies on the expression of recombinant PC2 in different cell lines possessing the regulated pathway of secretion followed by characterization of its post-translational processing and kinetic properties *in vitro* should enable these issues to be addressed.

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