Levels of the conversion endoproteases PC1 (PC3) and PC2 distinguish between insulin-producing pancreatic islet β cells and non-β cells

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PC1 (PC3) and PC2, members of the mammalian family of proprotein convertases homologous to the yeast Kex2 gene product, are both expressed in pancreatic islets of Langerhans. Recent studies have suggested that PC1 and PC2 are responsible for the conversion of proinsulin to insulin and connecting peptide (C-peptide) in the islet β cells. However, the insulin-secreting β cells are not the only cells present in these complex micro-organs, prompting us to evaluate the expression of PC1 and PC2 in islet β and non-β cells. Rat islet cells were sorted by autofluorescence-activated flow cytometry to separate β cells from non-β cells, and conversion endoprotease levels were analysed by Western blotting. The immunolabel ratio of PC1/PC2 in β cells was 2.6. Non-β cells displayed much lower levels of PC1 than β cells, but twice as much PC2 (PC1/PC2 = 0.05). Post-translational modification of the convertases themselves was found to differ between the cell types. In particular, a 75 kDa precursor form of PC2 (pro-PC2) was found to accumulate in β cells, whereas only the fully processed 67 kDa form was detected in the non-β cells. Finally, the quantification of PC1 and PC2 and their precursor forms in transformed cells (insulin-producing β-TC and glucagon-producing α-TC) showed that transformation appeared to be accompanied by unusually high levels of the precursors.

INTRODUCTION

Proprotein cleavage at dibasic amino acids is characteristic of a family of subtilisin-like proteases related to the yeast Kex2 gene product [1]. Several mammalian homologues to Kex2 have recently been identified. Amongst these, furin, which is ubiquitously expressed, is thought to be responsible for proprotein processing in the constitutive pathway [2,3], while PC1 (also known as PC3) and PC2, which are expressed only in neuroendocrine and endocrine cells, are thought to cleave precursors in the regulated pathway [4–10]. One such precursor, proinsulin, which is synthesized in pancreatic islet β cells, has been studied in some detail. This propeptide is cleaved at two distinct sites to release the mature insulin molecule from the connecting peptide (C-peptide) [11]: a Type 1 activity cleaves between the B-chain and C-peptide, and a Type II activity cleaves at the A-chain/C-peptide junction [12]. Recent studies have provided evidence to suggest that PC1 is responsible for Type I activity, cleaving after Arg^{31}-Arg^{32} [13,14], and that PC2 is equivalent to the Type II endoprotease, specifically cleaving after Lys^{64}-Arg^{65} [15].

Although both PC1 and PC2 have been shown to be expressed in pancreatic islets [14], insulin-secreting β cells are not the only cells present in these complex micro-organs. Non-β cells, including principally α cells producing glucagon, δ cells producing somatostatin and PP cells synthesizing pancreatic polypeptide, are found at the periphery of the islet surrounding a core of β cells [16]. The peptides produced by non-β cells are also initially synthesized as larger precursors which require endoproteolytic cleavages (typically, but not exclusively, after pairs of basic residues) to produce the active hormone. We have separated β and non-β cells by autofluorescence-activated cell sorting (FACS) and then measured the relative activities of PC1 and PC2 in the two cell populations by quantitative Western blot analysis. The data show differential levels of expression of the two enzymes. Native (primary) rat β cells express much higher levels of PC1 than non-β cells, but only half as much PC2. Two mouse cell lines, α- and β-TC cells, secreting glucagon [17] and insulin [18] respectively, were found to faithfully reflect their primary rat cell counterparts in terms of their relative levels of PC1 and PC2.

MATERIALS AND METHODS

Materials

Standard chemicals were from Fluka (Buchs, Switzerland) or Sigma (St. Louis, MO, U.S.A.).

Islet isolation and FACS of islet cells

Islets were obtained from the pancreas of male Sprague–Dawley rats weighing 200–250 g by collagenase digestion and then gently digested with trypsin in order to obtain individual cells as described previously [19]. These cells were sorted according to their FAD autofluorescence plotted against their forward light scatter using a FACStar Plus from Becton–Dickinson (Erembodegem, Belgium). The sorting procedure has been described in detail previously [19]. Analysis of insulin and glucagon immunoreactivity by double antibody cytochemistry revealed that one population contained more than 93% non-β cells (of which approx. 80% were α cells) and the other more than 95% β cells [19].

Insulin- and glucagon-secreting cell lines

β-TC cells, secreting insulin (from Dr. David Gross, Jerusalem, Israel), and α-TC-6 cells producing glucagon (from Dr. Edward Leiter, Bar Harbor, MN, U.S.A.) were grown in Dulbecco's modified Eagle's medium, 10% foetal calf serum, 15 mM Hepes and 16.7 mM glucose.

Western blot analysis of PC1/3 and PC2

Rabbit antiserum 2B7 against PC1, recognizing the N-terminus of the mature enzyme [20], was kindly provided by Dr. Iris Lindberg, New Orleans, LA, U.S.A. We are grateful to Dr. Chris

Abbreviation used: FACS, autofluorescence-activated cell sorting.

Rhodes, Boston, MA, U.S.A. for the gift of rabbit antiserum 'Thumpa', directed against the last 15 residues of the C-terminal tail of PC2 [21], and to Dr. G. Gabbiani, Geneva, Switzerland, for the anti-actin antibody. SDS/PAGE was performed according to Laemmli [22]. All cell types were extracted in sample buffer (62.5 mM Tris/HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% Bromophenol Blue), boiled for 5 min and loaded on a 4% stacking gel/7.5% resolving gel. Approx. 0.2×10^6 cells were loaded per lane. After having been run overnight at 7 mA, the gels were electrotransferred for 5 h at 30–50 V on to nitrocellulose (Schleicher and Schuell). Immunodetection was performed using the ECL detection procedure from Amersham International (Amersham, Bucks., U.K.). Antibody dilutions and incubations were as previously described [14]. Densitometry measurements were performed by scanning the films using a flat-bed scanner (Macintosh) and quantifying the bands of interest using the Image 1.33g program (Macintosh). Band density was shown to be linearly related to antigen quantity in a control experiment in which increasing amounts of cell extracts (50000 to 250000 cells) were immunodetected with the three specific antisera: sorted β-cells were used for detection of PC1 and actin, whereas α-TC cells were used for PC2 blotting.

RESULTS

PC1 and PC2 levels in islet cell types

Rat islet cells were sorted by FACS as described in the Materials and methods section. One population ('β cells') contained more than 95% β cells, whereas the other ('non-β cells') contained more than 93% non-β cells, of which some 80% were glucagon-producing α cells [19]. The levels of the conversion endoproteases PC1(3) and PC2 were determined by Western blotting. Figure 1 is a representative blot of three independent experiments showing the expression of PC1 and PC2 in transformed α (α-TC) and β (β-TC) cells from the mouse, sorted non-β cells, sorted β cells and whole rat islets. High levels of expression of the mature forms of both PC2 (67 kDa) and PC1 (66 kDa) were found in whole rat islets (lane 5) in addition to significant levels of higher-molecular-mass forms (approx. 75 kDa for PC2 and 87 kDa for PC1). The distribution of these two convertases between non-β cells (lane 3) and β cells (lane 4) was quite striking. The β cells expressed much higher levels of PC1 than non-β cells, in the face of lower levels of PC2. This differential expression was also seen in mouse β-TC and α-TC cells (lanes 1 and 2). To ensure that band density was proportional to the amount of antigen in the experimental conditions used for this study, increasing amounts of cell extracts (50000 to 250000 cells) were blotted with the specific antisera against PC1, PC2 and actin. Figure 2 shows band density as measured using the Macintosh Image 1.33g program, expressed as a function of cell number for all three antisera. Similar graphs were obtained for four different exposures of the same blot. The values used to calculate PC1/PC2 ratios were in linear parts of

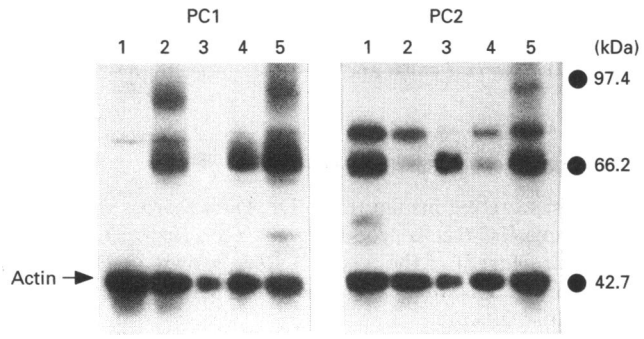

Figure 1 Expression of endoproteases PC1 and PC2 in primary (rat) and transformed (mouse) islet cell types

Western blot analysis was performed as described in the Materials and methods section. Approx. 0.2×10^6 cells were loaded per lane. The positions of the molecular size markers are shown on the right and that of the 43 kDa band detected using the anti-actin antiserum is shown on the left. Lane 1, α-TC; lane 2, β-TC; lane 3, sorted non-β cells; lane 4, sorted β cells; lane 5, whole rat islets.

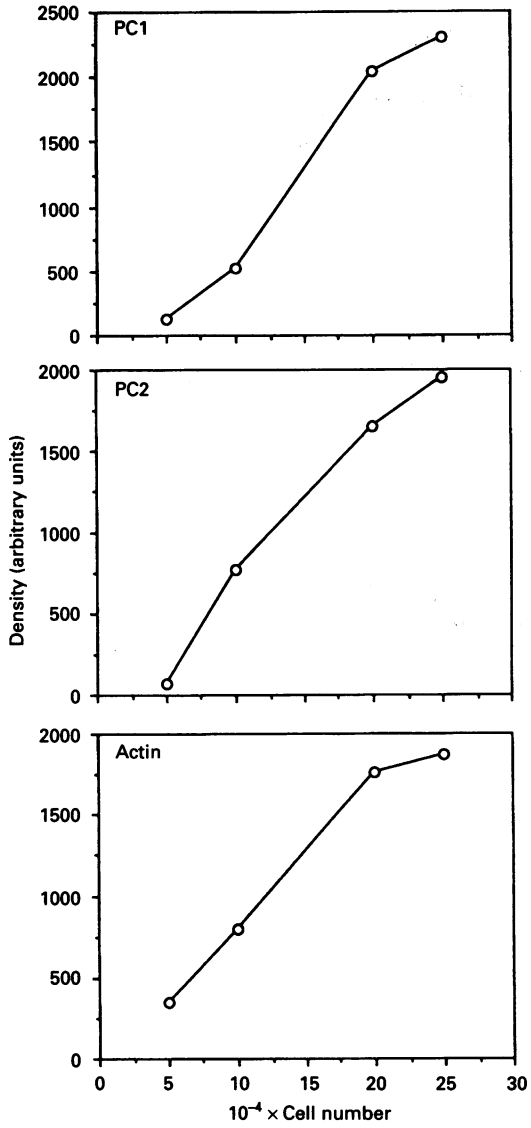

Figure 2 Linear relationship between band density and quantity of antigen (cell number) for PC1, PC2 and actin antisera

Western blot analysis was performed as described in the Materials and methods section with increasing amounts of cell extracts. Sorted β cells were used for PC1 and actin detection; α-TC cells were used for PC2 immunoblotting. The band density was measured (in arbitrary units) after scanning the films with a Macintosh flat-bed scanner using the Macintosh Image 1.33g program. Four different exposures of the same blot yielded similar results.

Table 1 PC1 and PC2 expression in whole islets, in primary β and non-β cells, and in β-TC and α-TC cells

Densitometric analysis of Western blots was performed as described in the Materials and methods section. The data are represented as means ± S.E.M. for three independent experiments and are expressed in arbitrary units normalized for actin content.

	PC1	PC2	PC1/PC2
Rat islets	1.3 ± 0.3	0.9 ± 0.3	1.4 ± 0.1
β cells	1.3 ± 0.1	0.5 ± 0.08	2.6 ± 0.2
Non-β cells	0.05 ± 0.05	1.1 ± 0.08	0.05 ± 0.04
β-TC	1.24 ± 0.4	0.4 ± 0.01	2.9 ± 0.7
α-TC	0.03 ± 0.01	1.36 ± 0.2	0.01 ± 0

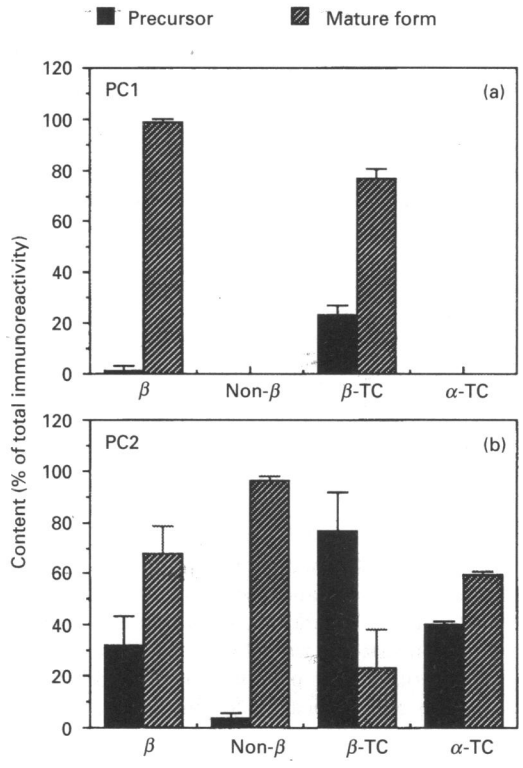

Figure 3 Processing of PC1 and PC2 precursors by islet cell types

The relative contributions of pro-PC1 (87 kDa) and mature PC1 (66 kDa) to total PC1 immunoreactivity (**a**), and of pro-PC2 (75 kDa) and PC2 (67 kDa) to total PC2 immunoreactivity (**b**), were calculated by densitometric analysis of Western blots as already described. The data are expressed as means ± S.E.M. for three independent experiments.

the curves, except for those cells (α-TC and sorted non-β cells) for which PC1 immunoreactivity was hardly detectable.

Table 1 shows the densitometric analyses performed on blots obtained from three independent experiments. All immunopositive bands for each enzyme were summed and normalized for actin content, ensuring that the amount of cells loaded for immunodetection by anti-PC1 and anti-PC2 was the same. In summary, PC1 levels were much higher in β than in non-β cells, whereas PC2 levels were lower. The transformed cells faithfully reflected their primary counterparts.

Post-translational processing of PC1 and PC2 in islet cells

In addition to their differential levels of expression of the two convertases, the two islet cell populations seemed to process the conversion enzymes themselves differently. Figure 3 represents data obtained from three independent observations in which the relative contributions of the precursor forms (87 kDa for PC1, 75 kDa for PC2) and the fully processed enzymes (66 kDa for PC1, 67 kDa for PC2) to the total immunoreactivity obtained for each endoprotease were determined. There was a striking accumulation of the PC2 75 kDa precursor in the native β cells (32.2 % ± 10.9 of total PC2 immunoreactivity), a form which is present at very low levels in sorted non-β cells (3.6 % ± 1.9) (Figure 3b). More generally, the transformed mouse cells accumulated larger amounts of the precursor molecules than of their native counterparts, and this was observed not only for PC2 but also for PC1 (Figure 3a).

DISCUSSION

The recent discovery of a mammalian family of endoproteases homologous to the yeast Kex2 protease and responsible for the cleavage of proproteins and prohormones at dibasic sites [23–25] has led to the identification of the enzymes involved in the processing of a number of precursors including proinsulin [13,15]. While furin, a transmembrane endoprotease localized in the Golgi apparatus, is ubiquitously expressed, other members of this family, such as PC1 and PC2, have been found only in neuroendocrine and endocrine tissues equipped with the regulated secretory pathway. One example of such a tissue is the pancreatic islet of Langerhans, which expresses both proteases [14]. It has been thought up to now, based on analysis of transformed cells, that β cells show higher levels of expression of PC2 than PC1 [6,26]. Since on the one hand islets consist of several different endocrine cell types aside from β cells, and on the other transformed β cells may differ in their properties from primary cells, we wished to determine the levels of expression of these two endoproteases in primary islet β and non-β cells. To this end, rat islet cells were sorted into two populations ('β' and 'non-β'), and levels of PC1 and PC2 were monitored by quantitative Western blot analysis. For comparison with the primary cell populations, two well differentiated cell lines, β-TC and α-TC cells secreting insulin and glucagon respectively, were included in the study.

The measurement of PC1 and PC2 by Western blotting using two unrelated enzyme-specific antisera does not allow for comparison of the absolute amounts of each enzyme in a given cell type. It must, furthermore, be assumed that the level of PC1 or PC2 protein (as measured by Western blotting) faithfully reflects that of the corresponding enzymic activity. Despite these reservations, it is perfectly valid to compare the levels of PC1 or PC2 and their ratios in different cell types, and such a comparison reveals unexpected differences between β and non-β cells. The level of PC1 in β cells was > 20-fold higher than in non-β cells. By contrast, PC2 levels in non-β cells were approximately twice those found in β cells. This differential expression was found also in the transformed α-TC and β-TC cells from the mouse, indicating that, at least in terms of endoprotease expression (and assuming that the direct comparison of mouse and rat cells is valid in this context), the mouse β-TC cell line is more representative of the native β-cell than are INS [14] and RIN-m5F cells (M. Neerman-Arbez and P. A. Halban, unpublished work) derived from rat insulinomas.

The antisera used in this study are able to recognize both the precursor and mature forms of their cognate enzymes. Thus pro-PC1 is initially processed by removal of the first, N-terminal, 83

residues and thereafter by a C-terminal truncation [20,27]. The antiserum used in this study is directed towards the N-terminal region that is common to both processed molecules and present as an internal domain of the precursor. Pro-PC2 processing in other cell types has been suggested to involve only removal of the N-terminal pro-sequence, with no C-terminal truncation [27]. In keeping with this, in rat islets, pulse–chase experiments have shown processing to a mature 64–67 kDa form of PC2 [21,28], which appears to be recognized both by the antiserum used in this study (raised against the last 15 amino acids of the C-terminal tail) [21] and by an antiserum directed towards the catalytic domain (residues 158–391) [21,28]. Based upon these results, it is thus assumed that in the present study pro-PC2 and mature PC2 will be equally well recognized. It cannot, however, be totally excluded from the earlier studies that some limited truncation at the extreme C-terminus of PC2 may occur, and in this event such a processed form would not be detected by the antiserum used in this study. A more detailed analysis of the molecular forms detected by the anti-PC1 and -PC2 antisera in the various cell types revealed interesting differences in the post-translational modifications of the endoproteases themselves. As a general observation, the transformed cells accumulated larger amounts of the precursor forms of the conversion enzymes (87 kDa for pro-PC1 and 75 kDa for pro-PC2). In fact, for the β-TC cells, pro-PC2 represented more than 76% of the total immunoreactivity. Amongst the native sorted cells, β-cells accumulated larger amounts of pro-PC2 than did the non-β cells. It is not yet clear what these differences in convertase processing reflect. The biosynthesis and processing of PC2 has been studied in some detail in rat islets [28], and it appears that only the mature 66–67 kDa form is present in secretory granules and released after stimulation of regulated exocytosis by glucose, whereas both the 75 kDa and 67 kDa forms are found in endoplasmic reticulum- and Golgi-enriched fractions [28]. The post-translational processing of this precursor is relatively slow [27,28] and is believed to reflect an intrinsic property of pro-PC2 [27]. Non-β cells may thus provide a better environment and/or a more active enzyme (the enzyme responsible has not yet been identified, although furin seems to be excluded [27]) for pro-PC2 processing than β-cells. It is perhaps relevant to note in this context that β cells do seem able to process pro-PC1 quite efficiently, raising in turn the possibility that the processing machinery for the two endoproteases is not identical (as indeed suggested by the striking differences in processing kinetics [27]). It must be stressed, however, that measuring the steady-state levels of the various forms of PC1 and PC2 in cells does not provide any information on the precise kinetics of conversion of the endoproteases.

The observation of much higher levels of PC1 in β than in non-β cells supports the hypothesis that this enzyme is important for proinsulin processing [14]. This hypothesis was based upon work by ourselves and others. The study of rat proinsulin I conversion in COS cells cotransfected with proinsulin and conversion endoproteases has shown that PC1 is able to convert proinsulin to fully processed insulin, whereas PC2 cleaves only at the C-peptide/A-chain junction [26]. Note, however, that COS cells release proteins only via the constitutive pathway and thus provide an unusual setting for these enzymes, which are normally restricted in their expression to cells with the regulated secretory pathway. In addition, we have demonstrated that in transformed β (INS) cells which show an abnormally low level of PC1, rat proinsulin conversion is significantly impaired [14], whereas it is rapid and efficient in AtT20 cells [29], which have very high levels of PC1 and vanishingly low quantities of PC2 [30]. The situation might be different, however, for human proinsulin, as studies *in vitro* have shown that PC1 cannot cleave the C-peptide/A-chain junction [13], whereas PC2 favours this site (albeit with a preference for des-31,32-split proinsulin rather than intact proinsulin as its substrate [31]). In human β cells, which remain to be characterized with regard to their endoprotease levels, PC2 might play a more essential role in the cleavage of this particular junction. One difference between human and rat proinsulins is the presence of a basic residue (Arg^{62}) in a -4 position preceding the C-peptide/A-chain junction of only the two rat proinsulins [32]. This basic residue may affect cleavage at this junction [32,33].

When compared with β cells, PC2 is the dominant regulated pathway conversion endoprotease expressed in non-β cells. Although the non-β population obtained by FACS is composed of a mixture of α, δ and PP cells, the majority (80%) are glucagon-producing α cells. It therefore remains possible that the low levels of PC1 detected in the non-β cell population may reflect expression limited to just one non-α/non-β cell subtype.

The high levels of expression of PC2 in glucagon-, somatostatin- and pancreatic polypeptide-producing cells are consistent with a possible role for this enzyme in the post-translational processing of the corresponding precursors. In most cases, the conversion sites consist of Lys-Arg sequences, which, at least in the proinsulin molecule, is the preferred dibasic cleavage site for PC2 [15,26]. It is interesting to note that, for proglucagon, only those sites which must be cleaved to release glucagon itself present the putative PC2 consensus substrate sequence, thereby possibly accounting for the production of this particular hormone in the α cells of pancreatic islets [34]. The generation of other active peptides from the proglucagon molecule, notably of glucagon-like peptide 1 from the major proglucagon fragment [34], involves cleavage at sites which are probably less suitable for PC2, i.e. Arg-Arg sites. It will be interesting to see whether PC1 or another related enzyme indeed dominates in the intestinal L-cells responsible for the secretion of this interesting peptide. If so, this would be a new example of different endoprotease levels being responsible for tissue-specific differential processing of prohormones.

In conclusion, rat islet β and non-β cells display differential levels of PC1 and PC2. The data confirm that PC1 is important for proinsulin conversion, with PC2 probably assuring the processing of the other islet prohormones, notably the tissue-specific conversion of proglucagon to glucagon.

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