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Immunological detection of prohormone convertases in two different proglucagon processing cell lines

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Abstract

The distribution of the prohormone convertases, PC1/3, PC2 and PC5/6, was determined by immunoblotting in two cell lines. In αTC1–6 cells, the proglucagon processing occured according to the pancreatic A-cell type. In STC-1 cells, proglucagon was processed in a manner reminiscent of the intestinal L-cell type. PC1/3 was undetectable in both proglucagon processing cell lines whereas PC2 displayed a strong immunostaining in the αTC1–6 cells and was barely detectable in the STC-1 cells. PC5/6 was detected as a 70 kDa protein in both cell lines. These results suggest a possible role of PC2 in the processing of proglucagon into glucagon in the A-cells, whereas in L-cells it would require still undetermined endoproteases.

Key words: Glucagon; Oxyntomodulin; αTC1–6 cell; STC-1 cell; Prohormone convertase; Immunoblot; Radioimmunoassay

1. Introduction

A single gene encoding proglucagon is expressed in A-cells of the pancreatic islets, in intestinal L-cells, in a few cells of the gastric mucosa and in neurons of the hypothalamus and brainstem [1–3]. Results of analysis of these tissues have provided evidence for a tissue-specific post-translational processing of proglucagon. In A-cells, proglucagon maturation produces essentially glucagon and the major proglucagon fragment (Fig. 1) [4,5] whereas in L-cells and in the brain, it is processed into the glucagon-containing peptides glicentin, oxyntomodulin [1,6] and in two glucagon-like peptides 1 and 2 (GLP-1 and GLP-2) [1,5,7]. GLP-1 is further processed by N-terminal deletion of six amino acids into the biologically active peptides (tGLP-1) [8,9]. This remarkable tissue-specific processing of proglucagon, which provides a mechanism to increase the diversity of bioactive molecules generated from a single precursor, might be determined by the restriction of different processing enzymes to defined cell types.

Several endopeptidases implicated in the processing of precursor proteins have been identified by cDNA cloning and belong to the subtilisin family of serine proteinases [10]. Two main endopeptidases, PC1/3 and PC2 have been described so far in endocrine and neuroendocrine cells [11–13] and were proposed to be implicated in the maturation of several prohormones [14–16]. Furthermore, it has been suggested that cell-specific prohormone processing is likely to be the result of differential expression of these two prohormone convertases [15]. A new member of this family, PC5/6, mainly localized in the epithelial cells of the small intestine and in the adrenal [17,18] might also be responsible for the maturation of gastrointestinal peptides instead of PC1/3 [18]. To address the enzymatic basis for the tissue specificity of proglucagon maturation, the distribution of the prohormone convertases, PC1/3, PC2 and PC5/6, were compared in two cell lines that process proglucagon differently. Both cell lines are derived from endocrine tumors arising in transgenic mice harboring a hybrid gene construct linking hormone promoter to a potent oncogene [19]. The αTC1–6 cells process proglucagon in a manner similar to that found in pancreatic A-cells [20,21], whereas the STC-1 cells were claimed to express a set of peptides reminiscent of the intestinal L-cells [22,23].

2. Materials and methods

2.1. Peptides

Synthetic GLP-1(7–36)amide (tGLP-1) was purchased from Peninsula Laboratories (San Carlos, CA). Partially purified glicentin and synthetic oxyntomodulin were prepared in our laboratory as previously described [24]. Glucagon was obtained from Novo Nordisk A/S (Bagsvaerd, Denmark). Mono[^{125}I]peptides (tGLP-1 and glucagon) were prepared as described [25].

2.2. Cell culture

αTC1–6 cells [26] and STC-1 cells were kindly provided by S. Efrat (Albert Einstein College of Medicine, Bronx, NY) and D. Hanahan (University of California, San Francisco, CA). For reference purposes,

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the hyperplastic β-cell line, βHC9 [27] was kindly donated by F. Radvanyi (Ecole Normale Supérieure, Paris, France) and the mouse pituitary AtT20 cells were obtained from X. Bertagna (CHU, Cochin-Port-Royal, Paris, France). The cell lines were cultured in DMEM (αTC1–6, βHC9), DMEM/Ham's F12 (AtT20) or RPMI-1640 (STC-1) media supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin in an atmosphere of 5% CO_2 (7% CO_2 for AtT20) in air, at 37°C.

2.3. Cell extraction and HPLC

αTC1–6 and STC-1 cells were harvested from 10-cm Petri dishes in phosphate buffered saline, 0.7 mM EDTA, boiled for 3 min and extracted in 0.5 M acetic acid. Cell extracts were subjected to high performance liquid chromatography (HPLC) as previously described [27]. Fractions were dried using a Speed-Vac concentrator and reconstituted in the radioimmunoassay buffer.

2.4. Radioimmunoassays

Glucagon-containing peptides were determined using antisera raised in rabbits. GAN antiserum and GOL antiserum are directed against the C-terminal and the central part of the glucagon molecule, respectively [24]. FAN antiserum recognizes the C-terminal region of oxyntomodulin/glicentin molecules [28]. tGLP-1 was measured using an antiserum (HER) produced by immunization with the synthetic N-terminal heptapeptide of tGLP-1, coupled to keyhole limpet hemocyanin (KLH) according to [28]. HER antiserum displayed 2% cross-reactivity with secretin and 5% with glucagon but no cross-reactivity with the full length GLP-1 nor with pituitary adenylate cyclase activating polypeptide and vasoactive intestinal peptide. In all 4 radioimmunoassays, bound and free ligand were separated with dextran-coated charcoal and the intra- and inter-assay variance were within 15% for all assay systems. Quantitative evaluations of the final processed peptides were performed using GAN antiserum for glucagon, FAN antiserum for glicentin and oxyntomodulin and HER antiserum for tGLP-1.

2.5. Antisera to PCs and immunoblotting

Rabbits were immunized against synthetic peptides coupled to KLH via a cysteine residue. For PC1/3, the N-terminal region corresponding to amino acids 84–93 [13], for PC2 the C-terminal amino acid sequence 592–608 [12] and for PC5/6 the putative N-terminal region corresponding to amino acids 117–126 [17] were used. Cell and mouse intestinal mucosa samples were electrophoresed on 10% SDS-polyacrylamide gels and then electroblotted onto nitrocellulose membranes. PC products were detected by incubation with the antisera and revealed, using [^{125}I]protein A, by autoradiography.

3. Results

The HPLC profiles of αTC1–6 and STC-1 cell extracts (Fig. 2) analyzed by the different sequence-specific antisera to glucagon-related peptides clearly demonstrated the differential processing of proglucagon in these cell lines. In the αTC1–6 cells (Fig. 2A), glucagon represented about 80% of the final processed peptides from the N-terminal moiety of the proglucagon precursor. Oxyntomodulin (recognized by FAN and GOL antisera) accounted for 10% of the glucagon-containing peptides. Two additional peaks, with retention times of 29.5 min and 33 min were also detected. The processing of the C-terminal part of the precursor, as assessed by tGLP-1 measurement, was weak and represented only 1% of the glucagon-containing peptides. In the STC-1 cells (Fig. 2B), the N-terminal moiety of the proglucagon precursor was partially processed into glicentin (32%), oxyntomodulin (37%), both recognized by GOL and FAN antisera, and glucagon (25%). In these cells, tGLP-1 represented

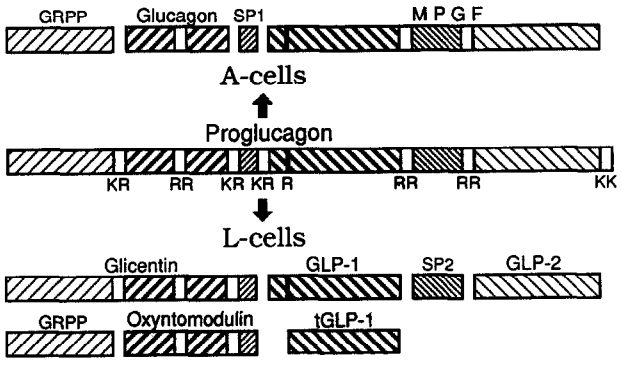


Fig. 1. Schematic representation of the cell-specific processing of proglucagon in pancreatic A-cells and in intestinal L-cells. End products in A-cells are glucagon and major proglucagon fragment (MPGF) and in L-cells, glicentin, oxyntomodulin, glucagon-like peptides 1 and 2 (GLP-1 and GLP-2) and truncated GLP-1 (GLP-1(7–37) and GLP-1(7–36)amide). GRPP, glicentin-related pancreatic peptide; SP1, SP2, spacer peptides 1 and 2. The basic amino acids at the potential cleavage sites are also shown.

46% of the glucagon-containing peptides. The overall amount of proglucagon-derived peptides in STC-1 cells was about 30-fold lower than those found in αTC1–6 cells.

Immunoblotting with an antiserum against the PC1/3 enzyme (Fig. 3, top panel) identified a major band at 66 kDa and a slower moving band (88 kDa) in both reference cell lines, βHC9 cells and AtT20 cells, but neither in the two proglucagon processing cell lines, αTC1–6 and STC-1 nor, in the ileal mucosa preparation.

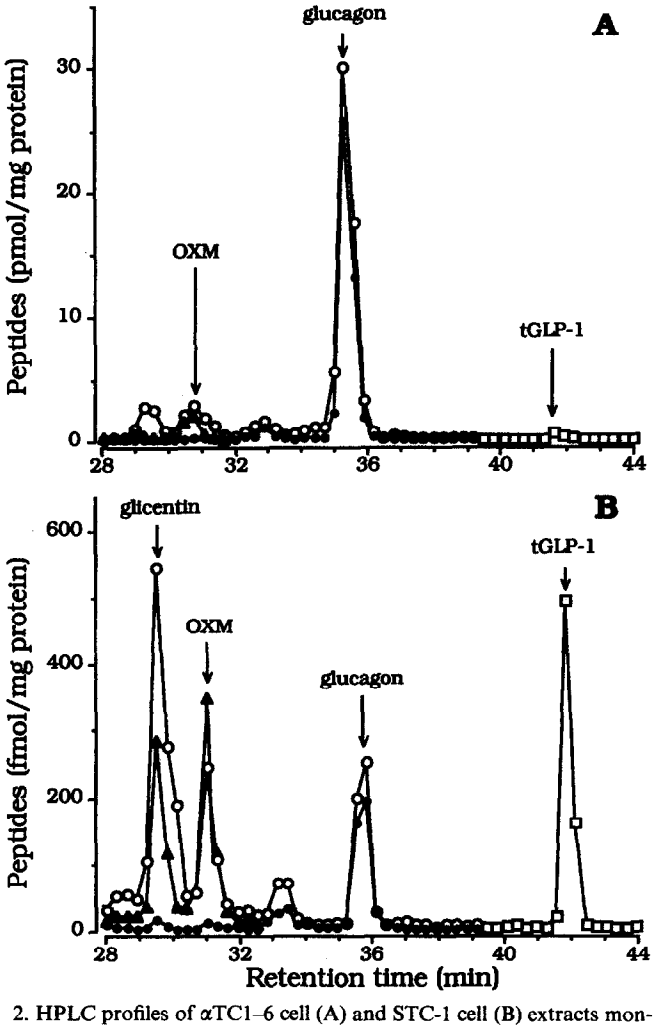
With the antiserum to PC2, a strongly labelled band (65 kDa) was observed in αTC1–6 cells and in βHC9 cells (Fig. 3, middle panel). In contrast, an analogous but faintly immunostained band was detected in STC-1 cells and AtT20 cells but not in ileal mucosa sample.

As shown in Fig. 3 (bottom panel), immunoblotting with PC5/6 antiserum revealed an immunoreactive band at 70 kDa in the different cell lines analyzed and in the ileal tissue sample. A 70 kDa protein was also detected with this antisera in adrenal and brain cortex extracts but not in the liver (data not shown). In the four cell lines, additional faster moving bands were apparent in the 30 kDa range that were not detected in the tissue extracts. The identity of these 30 kDa bands is not know; it may represent non-specific immunostaining artifacts or breakdown products of the enzyme.

4. Discussion

The conversion of proglucagon to glucagon in pancreatic A-cells and to glucagon-related peptides in intestinal L-cells is a multi-step mechanism involving endoproteolytic cleavages of the precursor molecule at several basic sites (Fig. 1). However, in both cell types, pro-

glucagon is cleaved at a common site between the glicentin and MPGF regions. Then, the extent to which subsequent cleavages occur differs between the cell types. In the αTC1–6 cell line, we show that glucagon is the major proglucagon-derived peptide produced, as previously reported with the uncloned αTC1 cells [21]. The HPLC profile of the glucagon-related peptides in αTC1–6 cells is consistent with the typical pattern described for pancreatic A-cells both in rat and human, including the presence of oxyntomodulin, the intermediary peptide between proglucagon and glucagon [1,6,28]. Thus, in αTC1–6 cells, there is little further processing of the C-terminal region of the precursor, as assessed in our study by tGLP-1 determination, when the N-terminal region was further cleaved at both ends of the glucagon molecule. In the other cell line, STC-1, the proglucagon molecule, is processed more extensively in a manner which appears intermediate between the intestinal L-cell type and the pancreatic A-cell type [1,6,28]. Indeed, the two glucagon-containing peptides, glicentin and oxyntomodulin, as well as tGLP-1, are predominant but, unlike the rodent L-cell type, glucagon is produced in similar amounts. In both cell lines, the differences in the respective amounts of the glucagon-containing peptides, as determined by the centrally directed antiserum (GOL) and by the C-terminal glucagon (GAN) or the oxyntomodulin/glicentin (FAN) antisera, suggest the presence of peptides incompletely processed at their C-terminal end by specific exopeptidases such as carboxypeptidase H that has been described in pancreatic A-cells [29].



2. HPLC profiles of αTC1–6 cell (A) and STC-1 cell (B) extracts monitored with RIA using a central glucagon antiserum (GOL; ○), a C-terminal glucagon antiserum (GAN; ●), a C-terminal oxyntomodulin /glicentin antiserum (FAN; ▲) and a N-terminal tGLP-1 antiserum (HER; □). The arrows indicate the elution times of the peptide markers. OXM, oxyntomodulin. Experimental details are described in section 2.

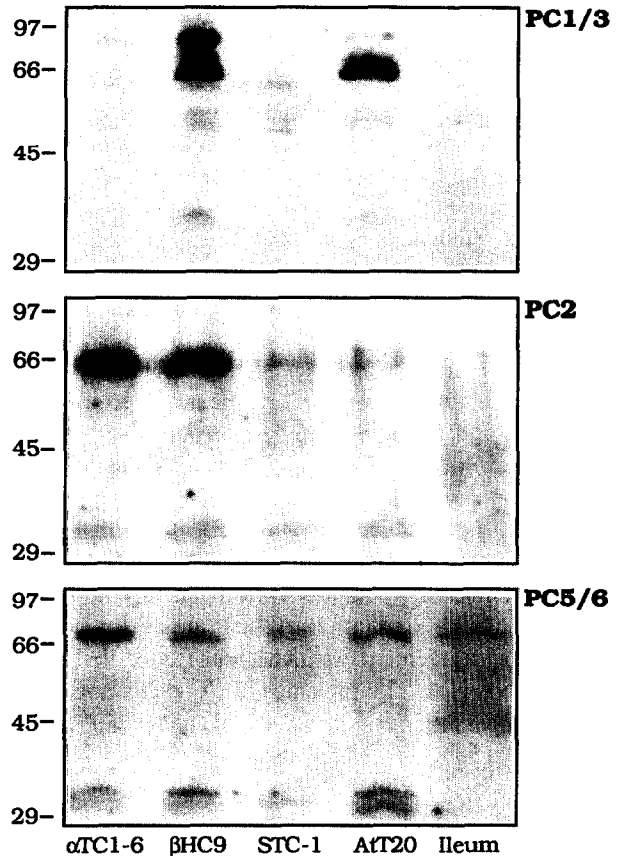


Fig. 3. Western blots with PC1/3 (top panel), PC2 (middle panel) and PC5/6 (bottom panel) antisera. Cells and tissue extracts were subjected to SDS-PAGE followed by immunoblotting. Amounts of proteins used were 100 μg each for the different cell lines and 800 μg for ileal mucosa. The migration of molecular size standards, expressed in kDa, are indicated.

Provided that the protein levels measured by immunoblot analysis reflect the activity of the respective enzymes, the present data clearly show that the distribution patterns of the three convertases are quite similar in the two cell lines except for PC2 which is much more abundant in the αTC1–6 cells than in the STC-1 cells. The high expression of PC2 along with the high glucagon content in the αTC1–6 cells suggests that this convertase might be implicated in the cleavage of proglucagon at both sides of the glucagon molecule.

PC1/3 is clearly revealed as two bands (88 and 66 kDa)

in both the insulin-secreting cells and the anterior pituitary cells, as previously reported [30,31]. This enzyme is not detectable in αTC1–6 and in STC-1 cells, suggesting that PC1/3 is not implicated in the maturation of proglucagon in the two proglucagon processing cell lines.

PC5/6, a new member of the subtilisin family identified by cDNA cloning [17,18], displays a widespread distribution, as assessed by Northern blot analysis, over many mammalian tissues, with a high abundance in the gut and the adrenal glands [17,18]. Low levels of PC5 mRNA have been reported in several cell lines, including AtT20 cells and the insulin-secreting βTC3 cells [18]. From the predicted amino acid sequence, we have raised an antiserum to the putative N-terminal sequence of the mature protein. By immunoblotting, this new antiserum reveals a protein band of 70 kDa which is present with similar intensities in the two proglucagon processing cell lines. The same protein band is also detected in AtT20 cells, in the ileal mucosa (Fig. 3, bottom panel) and (not shown) in the adrenal glands and the brain cortex but not in the liver, in agreement with the reported distribution of PC5 mRNA [18]. The presence of PC5/6 in both proglucagon processing cells suggests that the enzyme might be responsible for some of the observed cleavages at the level of dibasic sites which occur in both cell lines, such as the KR (Lys–Arg) between glicentin and MPGF regions in the precursor, and therefore it would not be implicated in the differential processing that takes place in these cells.

The present data suggest that PC2 might be responsible for the efficient processing of proglucagon into glucagon in the αTC1–6 cells, through the KR cleavages on both sides of the glucagon molecule, thus conferring to these cells their specificity as the A-cell type. The low level of PC2 in STC-1 cells would dramatically reduce the processing of the prohormone, revealing end products that were in negligible amounts, compared to glucagon, in the αTC1–6 cells. Alternatively, some as yet uncharacterized enzyme(s) might be involved in the further processing of the C-terminal moiety of proglucagon (MPGF) in STC-1 cells and in intestinal L-cells.

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