Role of the prohormone convertase PC2 in the processing of proglucagon to glucagon

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Abstract Proglucagon is alternatively processed to glucagon in pancreatic α-cells, or to glucagon-like peptide-1 in intestinal L cells. Here, the specificity of PC2, the major prohormone convertase of α-cells, was examined both in vivo and in vitro. Adenovirus-mediated co-expression of proglucagon and PC2 in GH_4C_1 cells resulted in a pattern of processing products very similar to that observed in α-cells. Oxyntomodulin, an intermediate in the processing of proglucagon, was quantitatively converted to glucagon in vitro by purified recombinant PC2, in combination with carboxypeptidase E. It is concluded that PC2 is able to act alone in the pancreatic pathway of proglucagon processing.

Key words: Proglucagon; Prohormone processing; Islet of Langerhans; Adenovirus vector; α-Cell

1. Introduction

The recent discovery of a novel family of mammalian subtilisin-like intracellular endopeptidases, all related to the yeast kexin, has shed new light on the molecular mechanisms of limited proteolysis associated with the post-translational processing of secreted proteins and peptides [1–3]. Two members of this family, PC2 (SPC2) and PC3 (SPC3, also known as PC1), are expressed in neural and endocrine tissues, and are active in the regulated secretory pathway of these cells. PC2 and PC3 have been implicated in the processing of a growing number of pro-hormones and pro-neuropeptides [4–17]. Their specificities towards various cleavage sites appear to be distinct, although overlapping, and variations in their levels of expression may be responsible for the differential processing of a given precursor, as suggested for the processing of proopiomelanocortin [18–20].

Proglucagon (PG) presents another interesting example of such a differential processing, leading to the alternative secretion of two hormones having opposite biological activities (Fig. 1). In mammals, the same precursor is initially synthesized in both α-cells in the islets of Langerhans of the pancreas, and endocrine L cells of the intestinal mucosa [21,22]. However pancreatic α-cells secrete glucagon, which stimulates glycogenolysis and gluconeogenesis in the liver and counterbalances the action of insulin on the blood glucose level, whereas intestinal L cells release upon stimulation a very potent insulinotropic hormone [23,24], recently identified as a truncated form of GLP-1 [25].

In intestinal L cells, proglucagon processing mainly results in the formation of glicentin (PG 1–69), GLP-1, IP-2, GLP-2 [26,27]. Glicentin is partially cleaved into GRPP (PG 1–30) and oxyntomodulin (PG 33–69), but not to glucagon [28]. An additional monobasic cleavage at Arg^{77}, which only occurs in the intestine, yields a shortened form of GLP-1, known as truncated GLP-1, or tGLP-1 [25]. In contrast, in the pancreas, α-cells mainly process proglucagon to glucagon, GRPP, IP-1, and the major proglucagon fragment (MPGF) (PG 72–158), a 10-kDa peptide encompassing the GLP-1, IP-2, and GLP-2 sequences [29–31]. In addition a small fraction of the MPGF is further processed to inactive N-terminally extended GLP-1 [31]. This differential processing is thought to result from the expression of different convertases in α- and L cells. Pancreatic α-cells express high levels of PC2 but low levels, if any, of PC3 [10,32–34], while intestinal L cells have immunoreactive PC3 but not PC2 [35]. Accordingly, several recent reports have suggested that PC2 and PC3 are involved in the pancreatic and intestinal phenotypes of processing, respectively [10,36–41].

However, it has also been proposed that PC2 alone would be unable to perform all the cleavages required for the processing of proglucagon to glucagon [39–41]. According to these authors, the formation of glucagon in α-cells would require an additional as yet unidentified convertase. In particular, they reason that PC2 would be unable to perform the cleavage at the Lys^{62}-Arg^{63} site, that is located at the C-terminus of glucagon in the precursor and is essential for the production of mature active glucagon (see Fig. 1). Here we show that purified recombinant PC2 is indeed able to cleave this site in oxyntomodulin, and thus converts this peptide into glucagon in vitro. Furthermore, we found that the co-expression of proglucagon and PC2 in GH_4C_1 cells reconstitutes the processing of proglucagon observed in αTC1-6 and pancreatic α-cells. These results further support the conclusion that PC2 is the endoprotease responsible for the processing of proglucagon to glucagon in the α-cell, and suggest that this enzyme is able to act alone in this processing pathway.

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Abbreviations: GLP, glucagon-like peptide; IP, intervening peptide; GRPP, glicentin-related polypeptide; MPGF, major proglucagon fragment; tGLP-1, truncated GLP-1 (GLP-1-(7-37) and/or GLP-1-(7-36amide)); DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; CPE, carboxypeptidase E; TPCK, *N*-Tosyl-L-phenylalanine chloromethyl ketone; TLCK, *Nα-p*-Tosyl-L-lysine chloromethyl ketone; SPC, subtilisine-like proprotein convertase; PG, proglucagon

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2. Materials and methods

2.1. Cell culture

GH_4C_1 cells were obtained from Dr. D.F. Steiner (University of Chicago), and were cultured at 37°C in 5% CO_2-95% air with constant humidity, in DMEM complemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.2. Adenovirus constructions and infections

The preparation of the purified recombinant adenovirus expressing PC2 (Ad:PC2) was previously reported [42]. In order to construct a recombinant adenovirus expressing proglucagon (Ad:Glu), a 1-kbp *Dde*I fragment of syrian hamster preproglucagon cDNA [43] was subcloned in the plasmid pACCMV.pLpA [44]. Recombinant adenovirus expressing proglucagon was obtained by homologous recombination in 293 cells, and purified as previously described [42,44]. Val^{87} and Leu^{91} of proglucagon were replaced with Met residues by site directed mutagenesis [45], in order to improve the sensitivity of the fluorographic detection of the GLP-1-containing peptides. The mutated and wild type proglucagons were expressed in COS 7 cells and in AtT-20 cells, and their processing was assessed by continuous labeling and pulse-chase, respectively. No difference was observed regarding the levels of expression, the processing patterns, and the efficiency of the immunoprecipitations (data not shown).

GH_4C_1 cells (approximately 5×10^5 cells) were washed twice with prewarmed DMEM and infected with a minimal volume of 90% DMEM-10% FCS containing the virus, for 1 h at 37°C. Co-infections were carried out by sequential infections with Ad:PC2 at a multiplicity of infection of 5, and then with Ad:Glu at a multiplicity of infection of 100, separated by a recovery period of 4 h in normal growth medium. Following the contact with the virus, the inoculum was removed, the cells were washed twice with DMEM, and returned to normal growth medium.

2.3. Immunoblot analysis

Infected and control cells were extracted 24 h after infection. The proteins in the extracts were separated by SDS-PAGE and transferred to nitrocellulose membrane as described [32]. Immunodetection was performed with an enhanced chemiluminescence detection kit from Amersham (Amersham, Bucks., U.K.). Antibody dilutions and incubations were performed as previously described [32].

2.4. Metabolic labeling and immunoprecipitations

Infected and control cells were labeled with 0.1 mCi of [^{35}S]Met in 0.25 ml of Met-deficient medium for 30 min, and chased for 2 h in 0.5 ml of medium containing 1 mM Met. Pulse-chase experiments and immunoprecipitations were as described previously [10]. The monoclonal antibody Glu001 (from Novo-Nordisk) and the GLP-1 antiserum 2135 (generously provided by Dr. J.J. Holst, Panum Institute, Copenhagen), that were used for the immunoprecipitations, recognize N- and/or C-terminally extended forms of these peptides. Immunoprecipitates were analyzed by SDS-PAGE [46] and fluorography. Molecular weights were determined by comparison with the co-migration of prestained markers (Biorad), complemented with ^{125}I-labeled GLP-1-(1–37), and ^{125}I-labeled glucagon, as 4-kDa, 3.4-kDa markers, respectively.

2.5. In vitro conversion of oxyntomodulin to glucagon

Five micrograms of synthetic human oxyntomodulin (from Peninsula Laboratories) were incubated with 1 μg of purified recombinant PC2 (kind gift from Dr. I. Lindberg, Louisiana State University, New Orleans) and 0.25 μg of purified recombinant CPE (generously provided by Dr. L.D. Fricker, Albert Einstein College of Medicine, New York), or with CPE alone, in 0.1 M sodium acetate buffer, pH 5.0 containing 10 mM $CaCl_2$, 10 μM dithiothreitol, 0.05% bovine serum albumin, and 20 μM each of TPCK and TLCK. After a 4-h incubation period at 37°C, samples were frozen until HPLC analysis. HPLC was performed in a Vydac C_{18} column with a linear gradient of acetonitrile (0–60% over 60 min) in trifluoroacetic acid (0.05%). Synthetic glucagon and oxyntomodulin standards were chromatographed separately to determine their elution time.

3. Results

3.1. Processing of proglucagon in GH_4C_1 cells co-expressing PC2

In order to study PC2-mediated processing of proglucagon in an appropriate cellular environment, proglucagon and PC2 were co-expressed with recombinant replication-defective adenovirus vectors in GH_4C_1 cells. This rat pituitary derived cell line, was chosen, because it had previously been reported that they possess a regulated secretory pathway formed by classical dense core granules [47], without expressing detectable levels of the convertases PC2 or PC3, usually associated with the regulated secretory pathway of endocrine cells [3]. Accordingly, they do not efficiently process transfected proglucagon [36,48]. Using an E1-deleted adenovirus as a vector allowed the overexpression of the transgene, with reduced expression of the viral proteins, thus preventing the cell toxicity associated with the viral replication [42,44]. Following infection with Ad:PC2, the expression of PC2 was assessed by immunoblot analysis (Fig. 2). Uninfected cells and cells infected with Ad:Glu alone were devoid of detectable PC2-immunoreactive material, whereas cells co-infected with Ad:Glu and Ad:PC2 displayed a strong signal for the 75-kDa pro-PC2 and a weaker signal for the 68-kDa mature form of PC2. This pattern has regularly been observed when PC2 is expressed without 7B2, its specific chaperone [49–51]. Using lower m.o.i. did not significatively change the relative amounts of pro and mature forms [42].

The processing of proglucagon was studied by pulse-chase experiments followed by immunoprecipitation of glucagon- and GLP-1-containing peptides with specific antibodies. After SDS-PAGE, and fluorography Fig. 3 shows that, in the ab-

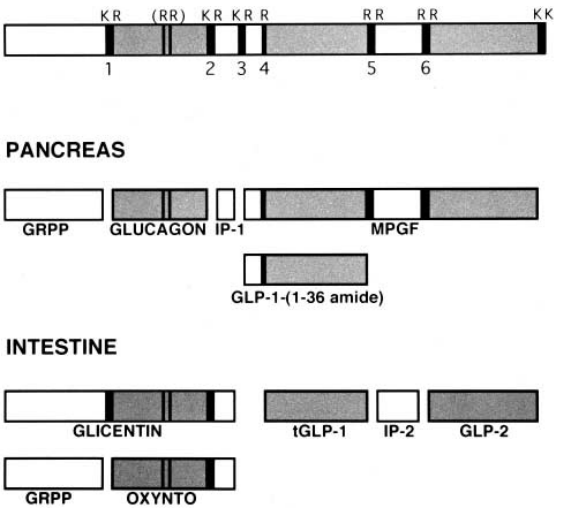

Fig. 1. Schematic representation of proglucagon and its processing products in the pancreas and the intestine. Homologous sequences are shown as shaded areas. Monobasic and dibasic sites that are cleaved by the convertases are shown as black bars and numbered from 1 to 6. A pair of arginines, that is present in the glucagon moiety and is not cleaved in the pancreas nor in the intestine, is also represented. Note that about 10 to 20% of the MPGF is converted in the pancreas to a 4-kDa N-terminally extended form of GLP-1, which is not found in the intestine. GRPP, glicentin-related pancreatic polypeptide; MPGF, major proglucagon fragment; GLP, glucagon-like peptide; tGLP-1, truncated GLP-1; IP, intervening peptide; OXYNTO, oxyntomodulin. Sites: 1, Lys^{31}-Arg^{32}; 2, Lys^{62}-Arg^{63}; 3, Lys^{70}-Arg^{71}; 4, Arg^{77}; 5, Arg^{109}-Arg^{110}; 6, Arg^{124}-Arg^{125}.

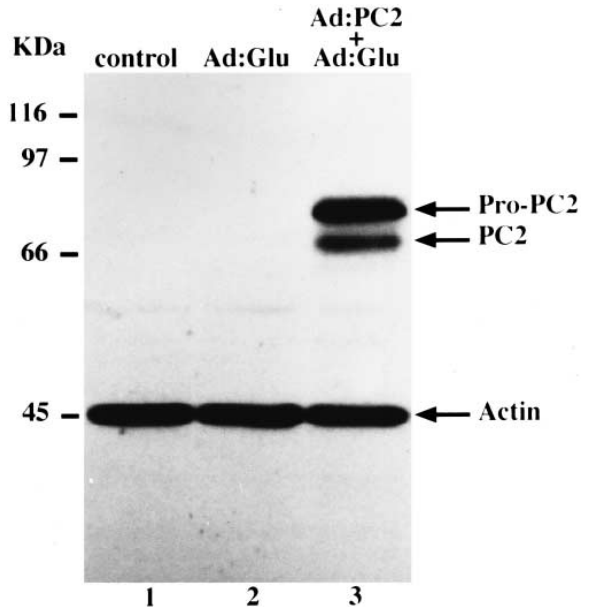

Fig. 2. Immunoblot analysis of PC2 expression in GH_4C_1 cells following infection with adenovirus vectors. GH_4C_1 cells were mock infected (lane 1), infected with Ad:Glu at m.o.i. = 100 (lane 2), or co-infected with Ad:Glu at m.o.i. = 100 and Ad:PC2 at m.o.i. = 5 (lane 3). Cells were extracted 24 h later, and the extracts submitted to SDS-PAGE and transferred to a nitrocellulose membrane. The blot was developed with a mixture of anti-actin and anti-PC2 antisera. The migration of molecular weight markers is indicated.

sence of co-expressed PC2, the 19-kDa proglucagon was the major glucagon- and/or GLP-1-containing peptide that had accumulated in the cells after 2 h of chase. Lower amounts of a 9-kDa glucagon-containing peptide and of a 8-kDa GLP-1-containing peptide were also detected. These two peptides were previously identified as glicentin and MPGF, respectively [10]. These results indicated that proglucagon was partly cleaved at the interdomain site Lys^{70}-Arg^{71} by endogenous convertases of GH_4C_1 cells, as previously observed with a vaccinia virus vector [36]. Very low amounts of a truncated form of MPGF with an apparent molecular mass of about 7 kDa, and of 3.4-kDa GLP-1 were also detected. In contrast, when PC2 was co-expressed, levels of proglucagon and glicentin were markedly lowered, and amounts of MPGF were increased. Two other peptides were immunoprecipitated, that were not detected when PC2 was omitted: a 3.4-kDa glucagon-containing peptide and a 4-kDa peptide precipitated by the GLP-1 antiserum, that were previously identified as being mature glucagon and GLP-1-(1–36 amide), respectively [10]. It should be noted that glucagon contains only one methionine residue, as opposed to the four present in the mutated proglucagon used during this study; the molar ratio of glucagon relative to proglucagon is thus underestimated by a factor of four. After overexposure, lower levels of a 4.5-kDa glucagon-containing peptide were also detected. This compound was previously identified as oxyntomodulin and was shown to be an intermediate in the processing of proglucagon to glucagon in αTC1-6 cells [10]. These results indicated that, when co-expressed in GH_4C_1 cells, PC2 processed proglucagon mainly to glucagon and MPGF, with partial conversion of MPGF to N-terminally extended GLP-1, a set of peptides seen in αTC1-6 cells and in pancreatic α-cells in very similar proportions [10,31]. Interestingly, using a higher multiplicity of infection for Ad:PC2 resulted in higher levels of PC2, and consequently in a higher enzyme to substrate ratio, but did not significantly change the relative proportions of glucagon, MPGF, and GLP-1 (data not shown).

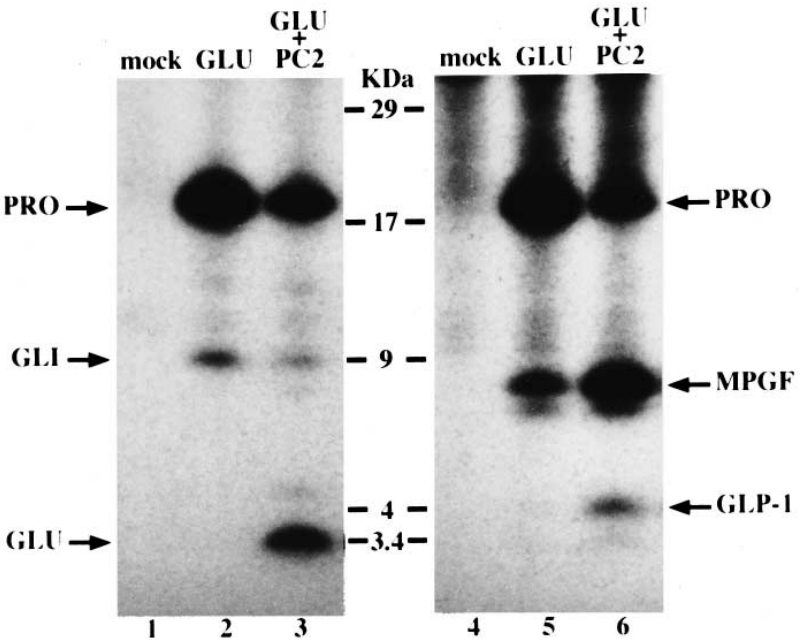

Fig. 3. PC2-mediated processing of proglucagon in GH_4C_1 cells co-infected with adenovirus vectors. GH_4C_1 cells were mock infected (lanes 1 and 4), infected with Ad:Glu at m.o.i. = 100 (lanes 2 and 5), or co-infected with Ad:Glu at m.o.i. = 100 and Ad:PC2 at m.o.i. = 5 (lanes 3 and 6). The day after, cells were labeled for 30 min with [^{35}S]Met and chased for 2 h. Cells were lysed and lysates were immunoprecipitated with an anti-glucagon antibody (lanes 1 to 3), or an anti-GLP-1 antiserum (lanes 4 to 6). Immunoprecipitated material was analyzed by SDS-PAGE and fluorography. The migration of the molecular weight markers is indicated. PRO, proglucagon; GLI, glicentin; GLU, glucagon; MPGF, major proglucagon fragment; GLP-1, glucagon-like peptide-1.

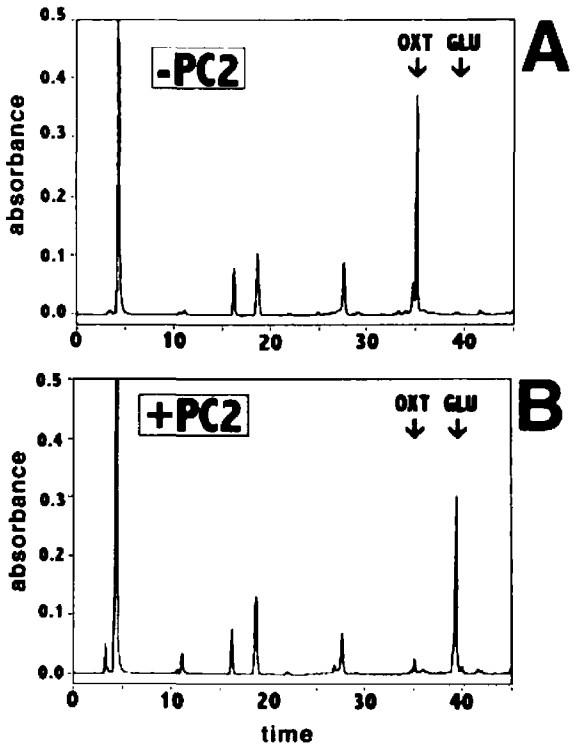

Fig. 4. In vitro conversion of oxyntomodulin to glucagon by recombinant purified PC2 and CPE. Oxyntomodulin (5 μg) was incubated with CPE (0.25 μg) (A), or with both PC2 (1 μg) and CPE (0.25 μg) (B) in sodium acetate buffer 0.1 M, pH 5.0 at 37°C for 4 h. Peptides present at the end of the incubation were identified by their elution time in HPLC. Elution times of synthetic oxyntomodulin (OXT), and glucagon (GLU) are indicated by arrows.

3.2. *In vitro conversion of oxyntomodulin to glucagon by recombinant PC2*

The data above show that PC2 is important for the processing of proglucagon to glucagon in vivo. This finding is consistent with a previous report indicating that immunoprecipitated PC2 is indeed able to cleave proglucagon at both Lys^{31}-Arg^{32} and Lys^{70}-Arg^{71} sites in vitro [39], two cleavages involved in the processing of proglucagon to glucagon. To directly determine whether PC2 could cleave at Lys^{62}-Arg^{63}, which is the third cleavage essential for the formation of glucagon (see Fig. 1), synthetic oxyntomodulin was incubated with purified recombinant PC2 and CPE, followed by separation of the products by HPLC (Fig. 4). A single peak, exhibiting the same retention time as synthetic glucagon, was observed, indicating the cleavage of oxyntomodulin by PC2 at the Lys-Arg site C-terminal to glucagon. Despite a near complete conversion to glucagon, no cleavage occurred at an internal Arg-Arg pair present in both oxyntomodulin and glucagon, demonstrating the specificity of cleavage.

4. Discussion

We have previously proposed that proglucagon is a two-domain precursor, and that its differential processing can be regarded as an alternative processing of its N- or C-terminal domains, following an initial cleavage at the interdomain site [1]. According to this model, the alternative processing of proglucagon would be the result of the actions of different convertases, with distinct specificities, in α-cells and in L cells. Evidence has been presented that PC2 is the convertase responsible for the pancreatic processing [10], and that PC3 is involved in the intestinal one [36,41]. This hypothesis is in agreement with the known expression of PC2, and lack of PC3, in pancreatic α-cells [10,32–34], and the immunological detection of PC3, but not PC2, in intestinal L cells [35].

However, the actual role of PC2 has been questioned [39–41]. It has been suggested that other convertases, such as PC3 or PC6A, may participate to this processing in addition to PC2. We now show that the co-expression of PC2 and proglucagon results in the formation of the processing products that are normally found in pancreatic α-cells and in αTC1-6 cells, including the partial cleavage of MPGF at the Arg^{109}-Arg^{110} site, that liberates limited amounts of 4-kDa GLP-1 [10,31]. Interestingly, the relative proportions between the processing products were remarkably similar to the ones naturally found in pancreatic α-cells and in αTC1-6 cells. This further supports the conclusion that PC2 alone is the endoprotease responsible for the α-cell processing pathway. These results corroborate our previous report showing that the expression of proglucagon in AtT-20 cells stably transfected with PC2 resulted in the formation of glucagon [36]. However, similar co-expression experiments with vaccinia virus vectors did not allow proglucagon to be processed to glucagon, but only to glicentin [41], suggesting that the use of a vaccinia vector for expressing PC2 did not allow the delivery of high amounts of active PC2 to the distal compartment of the regulated secretory pathway, where the conversion of glicentin to glucagon is thought to occur.

One of the major argument presented against the conclusion that PC2 alone is the endoprotease responsible for the formation of glucagon was its reported failure to cleave proglucagon at the Lys^{62}-Arg^{63} site in vitro [39]. However, in the present report, we show that purified recombinant PC2 is indeed able to cleave oxyntomodulin at this site. Oxyntomodulin is a natural intermediate in the conversion of proglucagon to glucagon. Pulse-chase experiments in αTC1-6 cells have demonstrated that proglucagon is initially processed to glicentin following cleavage at the Lys^{70}-Arg^{71} interdomain site. Glicentin is then cleaved either at the Lys^{62}-Arg^{63}, or at the Lys^{31}-Arg^{32} sites, to generate proglucagon-(1–61) and oxyntomodulin intermediates, respectively. Finally these secondary intermediates are cleaved at the Lys^{31}-Arg^{32} and, Lys^{62}-Arg^{63}, respectively, and this gives rise to mature glucagon [10]. Thus, the production of glucagon requires three successive cleavages, and perhaps the low activity of the PC2 preparation used by Rothenberg et al. [39], which only partially cleaved the Lys^{70}-Arg^{71} and Lys^{31}-Arg^{32} sites, accounts for its failure to cleave the third site in vitro.

In conclusion, the demonstration that PC2 is the endoprotease responsible for the formation of glucagon is now supported by several convergent lines of evidence: (1) Heterologous expression of proglucagon in various cell lines allowed correlations to be made between glucagon production and expression of PC2 [10,36,38]; (2) Expression of PC2 antisense RNA in αTC1-6 cells inhibited both PC2 production and proglucagon processing concomitantly [10]; (3) Co-expression of proglucagon and PC2 in AtT-20 cells [36], or in GH_4C_1 cells [this work] resulted in the efficient processing of proglucagon to glucagon, whereas parallel expression of proglucagon in control cells only produced very low levels of mature glucagon in AtT-20 cells, and no detectable glucagon in

GH_4C_1 cells; and (4) PC2 is able to cleave in vitro the Lys^{31}-Arg^{32}, Lys^{70}-Arg^{71} [39], and Lys^{62}-Arg^{63} [this work] sites of proglucagon, the cleavage of which leads to the production of glucagon in vivo.

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