Severe Defect in Proglucagon Processing in Islet A-cells of Prohormone Convertase 2 Null Mice*

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Mice homozygous for a deletion in the gene encoding prohormone convertase 2 (PC2) are generally healthy but have mild hypoglycemia and flat glucose-tolerance curves. Their islets show marked alpha (A)-cell hyperplasia, suggesting a possible defect in glucagon processing (Furuta, M., Yano, H., Zhou, A., Rouille, Y., Holst, J., Carroll, R., Ravazzola, M., Orci, L., Furuta, H., and Steiner, D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6646–6651). In this report we have examined the biosynthesis and processing of proglucagon in isolated islets from these mice via pulse-chase labeling and find that proglucagon undergoes essentially no processing in chase periods up to 8 h in duration. Only a small percent of cleavage at the sensitive interdomain site (residues 71 and 72) appears to occur. These observations thus conclusively demonstrate the essentiality of PC2 for the production of glucagon in the islet A-cells. Ultrastructural and immunocytochemical studies indicate the presence of large amounts of proglucagon in atypical appearing secretory granules in the hyperplastic and hypertrophic A-cells, along with morphological evidence of high rates of proglucagon secretion in PC2 null islets. These findings provide strong evidence that active glucagon is required to maintain normal blood glucose levels, counterbalancing the action of insulin at all times.

The subtilisin-like proprotein convertases PC2 and PC3 (a.k.a. PC1) are expressed extensively in mammalian neural and endocrine cells and play a major role in the proteolytic processing of both neuropeptide and peptide hormone precursors (1–5). They are members of a larger family of converting proteases of the secretory pathway and have specialized features directing their sorting into the acidic dense core vesicles of the regulated secretory pathway. PC2 and PC3 act either together or independently in selected cell populations throughout the brain and neuroendocrine system (2, 6). Thus, in the islets of Langerhans both enzymes are expressed in the beta (B)-cell and participate together in the processing of proinsulin, whereas only PC2 is expressed in the islet A-, D-, and pancreatic polypeptide-cells (7–9).

We recently generated a strain of mice lacking active PC2 (10). Mice homozygous for this defect grow normally and are generally healthy but have altered carbohydrate metabolism characterized by mild hypoglycemia and flattened glucose-tolerance curves. They have elevated intact proinsulin levels and increased levels of des 31,32 proinsulin, an intermediate cleavage form generated by the action of PC3 (11). The production of mature glucagon and somatostatin is also impaired as might be anticipated if PC2 plays a role in proglucagon processing to selectively release glucagon, as postulated by Rouillé et al. (12, 13).

The mammalian proglucagon protein contains three homologous hormonal sequences, glucagon, glucagon-like peptide 1 (GLP-1), and glucagon-like peptide 2 (GLP-2) (14, 15). These are arranged in this order in proglucagon but are separated by two intervening peptides, IP-1 and IP-2, and preceded by an N-terminal extension called glicentin-related polypeptide (GRPP) (see Fig. 1). At either end of each peptide, pairs of basic amino acids (Lys-Arg or Arg-Arg) are present representing sites of cleavage by the prohormone convertases PC2 or PC3/PC1 in the proglucagon-expressing islet A-cells or intestinal L-cells, respectively. In the islets of Langerhans, proglucagon is synthesized in the A-cells and processed to glucagon, GRPP, IP-1, and major proglucagon fragment (MPGF), which contains the unprocessed GLP-1, IP-2, and GLP-2 sequences (12, 13, 16). Only very low levels of GLP-1 are generated in the A-cells, whereas GLP-1 and -2 are major products of proglucagon processing in L-cells, along with glicentin, the intact N-terminal domain that contains unprocessed glucagon (17–19).

The identity of the prohormone convertases responsible for the A-cell phenotype has been investigated in several laboratories (6, 8, 9). Proglucagon processing has been examined in a number of cell lines expressing PC2, PC3, or both convertases with proglucagon by coexpression techniques, and these studies have suggested that PC2 generates the A-cell-processing phenotype (12, 13). However, other investigators have reported that PC2 acting alone is not able to generate mature glucagon (20, 21). Some of these discrepancies may have arisen because of low levels of expression of PC2 obtained in transfection experiments or low levels of activity in partially purified preparations of the recombinant enzyme used for in vitro studies.

To circumvent some of these problems we report here studies on the biosynthesis of glucagon in the islets of PC2 null mice. Our earlier studies of these mice indicated the presence of high levels of circulating glucagon-related peptides but little or no...
mature glucagon (10). We also noted an increased number (hyperplasia) of the A-cells consistent with a block in the production of glucagon. The present studies provide a more detailed analysis of the structural changes and the nature of the proglucagon processing defects in the A-cells in the absence of active PC2.

**MATERIALS AND METHODS**

**Animals**—The PC2 null mutant mouse line was generated as described previously (10). For all experiments, 8–12-week-old mutant mice (PC2−/−) and control littermates (PC2+/+) of the same age were used. The care of animals used in these studies was in accordance with the National Institutes of Health and Institutional guidelines.

**Antisera**—Antisera prepared in rabbits against glucagon (P7) was kindly provided by Dr. K. Polonsky (University of Chicago) and against GLP-1 (K2135) by Dr. J. J. Holst (Panum Institute, University of Copenhagen). Both antisera recognize extended forms of their respective targets. They were used for immunoprecipitation. Antisera used for immunocytochemistry were rabbit anti-porcine C-terminal glucagon (code 15k from R. H. Unger, Dallas, Texas) and rabbit antiporcine N-terminal glucagon (code K6248 from L. Heding, Novo Nordisk, Copenhagen, Denmark). Antibodies were diluted 1:100 and 1:200, respectively.

**RNA Analysis**—Islet total RNA was prepared using the acid-guanidinium thiocyanate-phenol-chloroform procedure. Because PC2−/− islets are larger than PC2+/+ islets due to the thick mantle of A-cells the amounts of total RNA extracted from the same number of islets from each genotype were different. Total RNA from PC2−/− islets was about 10-fold more abundant than from PC2+/+ islets. 15 μg of total RNA was analyzed by gel electrophoresis through a 1.4% agarose, 0.66 M formaldehyde gel, blotted onto nitrocellulose filters, hybridized with random-primed mouse glucagon cDNA, amplified, and labeled with [32P]dCTP by polymerase chain reaction using specific primers for mouse glucagon. After overnight hybridization at 42 °C, the filter was washed with washing buffer (0.1× SSC, 0.1% SDS) at 55 °C and autoradiographed. Band intensity was measured in the PhosphorImager (Molecular Dynamices, Inc.).

**Metabolic Labeling**—Islets of Langerhans were isolated as described (22). Isolated islets were cultured overnight in RPMI medium containing 5.5 mM glucose and 10% fetal calf serum. After overnight culture, control and mutant islets were labeled in groups of 300–400 in 100 μl of pulse medium (11) containing 2.8 mM glucose, 500 units/ml Trasylol, and 1 mM EDTA. The sonicate was analyzed by gel electrophoresis through a 1.4% agarose, 0.66 M formaldehyde gel, blotted onto nitrocellulose filters, hybridized with random-primed mouse glucagon cDNA, amplified, and labeled with [32P]dCTP by polymerase chain reaction using specific primers for mouse glucagon. After overnight hybridization at 42 °C, the filter was washed with washing buffer (0.1× SSC, 0.1% SDS) at 55 °C and autoradiographed. Band intensity was measured in the PhosphorImager (Molecular Dynamices, Inc.).

**Immunoprecipitation**—Immunoprecipitations were performed on isolated islets, some processing of proglucagon into glucagon and GLP-1 antibodies. After the antibody was added, samples were incubated overnight at 4 °C. After incubation, 30 μl of sample buffer (50 mM Tris-HCl, 4% SDS, 12% glycerol, 5% 2-mercaptoethanol, pH 6.8) and analyzed by SDS-polyacrylamide gel electrophoresis (12) followed by fluorography.

**Immunocytochemistry and Immunocytochemistry**—Pancreatic tissue was obtained from wild type and PC2−/− mice fixed by vascular perfusion with 2% OsO4, dehydrated in ethanol, and embedded in Epon. Tissue sections containing islets of Langerhans were stained with uranyl acetate and lead citrate and examined with a Philips CM10 electron microscope. For immunofluorescence, the postfixation with OsO4 was omitted. Semithin sections, about 1 μm thick, were etched to remove the epoxy resin (23) and then incubated with antigliucagon antibodies followed by fluorescein isothiocyanate-conjugated anti-rabbit IgG. Cell size was estimated on immunofluorescent sections by measuring the diameter of A-cells sectioned across their nucleus. Values were expressed as mean ± S.E. of 50 cells in each wild type and mutant mouse.

For cryoimmunocytochemistry, islets of Langerhans were dissected from the exocrine pancreatic tissue, embedded in 12% gelatin, and cryoprotected with 2.3 M sucrose before freezing with liquid nitrogen and sectioning with a cryoultramicrotome. Thin sections were incubated with the primary antibodies for 1 h at room temperature, washed, and labeled with protein A-gold for 30 min.

**RESULTS**

**Proglucagon Processing in PC2 Null Mouse Islets**—Groups of isolated islets were metabolically labeled for 30 min followed by chase incubations for 2, 4, and 8 h. Glucagon-related peptides produced during these pulse-chase studies were immunoprecipitated and identified after electrophoresis and fluorography. Both antisera used for immunoprecipitation recognize N- and C-terminally extended forms of either glucagon or GLP-1. In the PC2−/− islets, some processing of proglucagon into glucagon-related components was already evident at the end of the 30-min pulse period (see Fig. 2, panel A). The number and size of the bands observed corresponded well to those identified previously in a TC1–6 cells (12), consisting of 18-kDa proglucagon, 9-kDa glicentin, 7.5-kDa GRPP-glucagon, 4.5-kDa oxyntomodulin, and 3.4-kDa glucagon (see Fig. 1). The amount of all the processed peptides reached a peak after 2 h of chase incubation and then gradually decreased.

In contrast, in PC2−/− islets a major proglucagon band appeared that persisted throughout the 8-h chase period (Fig. 2, panel B). In addition trace amounts of a 14.3-kDa peptide tentatively identified as a C-terminally extended form of glicentin (Gli-GLP-1) appeared at 2 h, followed by small amounts of glicentin, which reached a maximum at 4 h and then decreased. GRPP-glucagon, oxyntomodulin, or glucagon were not detected even after 8 h of chase incubation, in either islets (Fig. 2).
Proglucagon Processing in PC2 Null Mice

The components of islet immunoprecipitates using GLP-1 antisera are shown in Fig. 2, panel B. In the PC2+/+ islets, large amounts of 8-kDa MPGF were produced, reaching a maximum after 2 h of chase. In PC2−/− islets, the major band at all times was proglucagon (Fig. 2, panel B). The 14.3-kDa extended form and 8-kDa MPGF, which both peaked at 4 h of chase, were also evident but at much lower levels. No GLP-1 was seen. In the medium of the mutant islets large amounts of proglucagon and a small amount of MPGF were seen, peaking at 4 h. Because the conditions used were stimulatory to secretion from A-cells (2.8 mM glucose), it is very likely that this represents regulated release of proglucagon from secretory granules (see below). The lack of detectable amounts of glucagon in the PC2+/+ chase medium (Fig. 2, panel C), which contains trace amounts of proglucagon and glicentin, may be because of losses of mature glucagon during fixation of gels or to its greater susceptibility to nonspecific proteolytic degradation after secretion. The tendency of secreted proglucagon to peak at 4 h of chase in both PC2+/+ and PC2−/− islet media is most consistent with regulated secretion, although a small component of constitutive secretion of the prohormone cannot be ruled out.

Relative Abundance of Proglucagon mRNA—Our earlier findings indicated that the A-cell population in PC2−/− pancreatic islets is greatly expanded because of hyperplasia of these cells (10). Accordingly, it was of interest to determine the level of preproglucagon mRNA by Northern analysis. These results, shown in Fig. 3, indicate a marked increase in proglucagon mRNA abundance in mutant islets, being roughly 6- to 7-fold greater than in normal islets.

Western Blotting of (Pro)Glucagon and PC3 Levels—Islet lysates from wild type or PC3 null mice were electrophoresed on 12 or 7.5% polyacrylamide gels to examine proglucagon and its processed products, or PC3 levels. The results of immunoblotting with glucagon antiserum P7 are shown in Fig. 4A and confirm the results of the pulse-chase studies, indicating the presence in PC2 null islets only of large amounts of intact proglucagon and traces of the 14.3-kDa component described above. In contrast, in normal islet lysates, the proglucagon band is greatly reduced, and a faint mature glucagon band is also visible. This result was further confirmed by Coomassie Blue staining, which gave a visible proglucagon band only in PC2 null islet lysates (data not shown). Immunoblots for PC3 shown in Fig. 4B revealed significantly reduced levels of PC3 in

Fig. 3. Northern analysis of glucagon gene expression in PC2+/+ and PC2−/− islets. 15 μg of islet total RNA were applied to the gel. Hybridization was performed using mouse glucagon-derived [32P]dCTP-labeled probe. The blot was exposed for 1 day. See “Materials and Methods” for details. kb, kilobase.
PC2 null mice, indicating that a lack of active PC2 in the non-β-cell population of the islets does not result in induction of expression of PC3 in these cells. Indeed, the diminished levels seen in the null islets are consistent with the observed reduction in β-cell activity and mass in these chronically hypoglycemic animals (10, 11).

Morphologic and Immunocytochemical Findings—Studies by light and electron microscopy of A-cells in the mutant mice indicated hyperplasia of the A-cells (10). Here we found that the cells are also enlarged in size (hypertrophy). The diameter of mutant A-cells was 13.5 ± 0.3 μm versus 9.3 ± 0.2 μm in wild type. The increase of cell size in mutant mice was accompanied by the accumulation of large numbers of atypical secretory granules. These have a homogeneous content filling the granule space rather than the usual dense core surrounded by a clear peripheral halo (Fig. 5, A and B). Immunocytochemical analysis reveals that unlike mature granules of the wild type A-cells (Fig. 5C), the atypical granules react weakly to antisera specific for mature glucagon (Fig. 5D). However, these granules react strongly with antisera to glicentin/glucagon (Fig. 5F), indicating the presence of higher molecular weight forms. In addition, ultrastructural analysis provides evidence for intense rates of exocytosis of the proglucagon-rich granules from the PC2 null A-cells (Fig. 6).

DISCUSSION

The results presented here strongly support the conclusion that PC2 is the essential convertase in the processing of proglucagon to release glucagon and MPGF from the A-cells of the islets of Langerhans (12, 13). In the absence of active PC2 in the mutant mice, large amounts of glucagon-like immunoreactivity are present in the plasma, but fractionation of the plasma
reveals only larger precursor material and no detectable mature glucagon (data not shown). In keeping with the lack of circulating active glucagon, the animals are chronically hypoglycemic and have reduced and flattened glucose-tolerance curves (10). The hyperplasia and hypertrophy of the A-cells are interpreted as responses to feedback stimulation arising from the chronic hypoglycemia and/or hypoinsulinemia. The β-cell population is reduced, and the requirement for insulin is clearly significantly decreased in the absence of glucagon, indicating the existence of a normal tonic balance between the two hormones (24).

Fractionation of PC2 null pancreatic extracts by gel filtration reveals complex mixtures of high molecular weight products, some of which may have arisen through autolysis or through autophagy of proglucagon-containing α-granules (10). We have therefore examined the biosynthesis of glucagon in pulse-chase labeling experiments to ascertain the extent of the processing defect more accurately. The experiments presented here reveal that in chases up to 8 h, less than 5% of the proglucagon is cleaved at the interdomain site (between residues 71 and 72) to give rise to glicentin and MPGF. There is no detectable production of glucagon, oxyntomodulin, GLP-1, or other smaller processing products. Although other investigators have raised questions as to whether PC2 acting alone is capable of cleaving glucagon from proglucagon, especially cleavage at the N terminus of the glucagon sequence in the precursor (20, 21), these results indicate that the action of another convertase is not likely. If another convertase were required for cleavage after GRPP (see Fig. 1) relatively large quantities of glucagon extended at its C terminus would be expected. However, if prior cleavage at the interdomain site is necessary to produce fragments that are susceptible to this convertase, then one would expect that instead of glicentin, one would see oxyntomodulin and MPGF as the minor products, rather than glicentin and MPGF as we observe. Thus it seems highly unlikely that any other convertases will be found to be involved in the processing of proglucagon to glucagon in vivo.

Although it is conceivable that the small amounts of glicentin and MPGF seen in the pulse-chase experiments in mutant islets might be produced by very small amounts of active PC2 arising from the truncated pro-PC2 that is produced in the PC2−/− mice (10), it is more likely that low levels of other convertases such as PC3 or PC6A may be responsible (25). Proglucagon processing is similarly severely inhibited in mice lacking 7B2, a neuroendocrine secretory protein that is required for the production of active PC2 from pro-PC2 (26). Another important point emerging from the studies reported here is that proglucagon is secreted into the medium in large

**FIG. 6.** Thin sections of pancreatic A-cells from wild type (A) and PC2−/− (B–F) mice reveal morphologic differences of the secretory granules and exocytotic events. Secretory granules in wild type (A, arrowheads) have a distinct halo surrounding the dense core. The halo is absent in mutant mice (B, arrowheads), and the dense core extends to the secretory granules membrane. In the mutant mice, as compared with the wild type, the exocytotic figures are frequent (arrow). On electron micrographs, 39 exocytotic events were recorded in 117 A-cells of mutant mice, versus only two events in 109 A-cells of wild type mice. In addition to single granule exocytosis (B), images suggesting compound exocytosis involving several secretory granules are detectable (C, arrow). Consecutive serial sections (D–F) reveal the extent of the funnel-shaped plasma membrane deformation-delimiting compound exocytosis. NE, nerve ending; ES, extracellular space; BM, basement membrane. Bar, 0.5 μm.
amounts, and this seems to reflect the situation in vivo, where large amounts of proglucagon-related forms are present in the circulation in the null mice. This does not, however, mean that proglucagon is being secreted via constitutive pathways; rather the evidence from the morphological studies presented here indicate that the secretory granules contain very large amounts of proglucagon and other precursor-related products and that exocytotic events are markedly increased, indicating that this is the main source of the secreted precursor forms.

These findings indicate that the absence of active PC2 does not influence developmental processes in the neuroendocrine system but rather influences the postnatal production and maturation of multiple regulatory peptides, including the neuropeptides melanin-concentrating hormone (27), neuropeptide Y (28), the enkephalins (29), dynorphins (30, 31), and melanocortin (26). Processing of the precursors of all of the pancreatic islet hormones is affected, including insulin (11), islet amyloid polypeptide/amylin (32), glucagon (this report), somatostatin (26), and melanocortin (28). Further studies of other neuropeptides and/or hypothalamic-releasing factors in PC2 null mice should shed light on the extent of involvement of PC2 in other areas of normal central nervous system/endocrine function.

Acknowledgment—We thank Chunling Zhang for technical assistance and Rosie Ricks for expert secretarial assistance in the preparation of this paper.

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