The Prohormone Convertase Enzyme 2 (PC2) Is Essential for Processing Pro-Islet Amyloid Polypeptide at the NH_2-Terminal Cleavage Site

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Impaired processing of pro-islet amyloid polypeptide (proIAPP), the precursor of the β-cell peptide islet amyloid polypeptide (IAPP) (amylin), has been implicated in islet amyloid formation in type 2 diabetes. The prohormone convertase enzymes PC3 (also known as PC1) and PC2 are localized to β-cell secretory granules with proIAPP and proinsulin and are responsible for proinsulin processing. To determine whether PC2 might be essential for proIAPP processing, we performed Western blot analysis of freshly isolated islets from normal mice and mice lacking active PC2. As expected, the primary species of IAPP immunoreactivity in islets from wild-type mice was fully processed (4-kDa) IAPP, with only small amounts of the 8-kDa precursor (unprocessed proIAPP) present. Islets from heterozygous PC2 null mice were identical to wild-type animals, suggesting that half the normal complement of PC2 is sufficient for normal proIAPP processing. By contrast, in islets from homozygous PC2 null mice, the predominant IAPP-immunoreactive form was of intermediate size (~6 kDa), with no detectable mature IAPP and slightly elevated amounts of the 8-kDa precursor form present. Thus, in the absence of PC2, proIAPP processing appears to be blocked at the level of a proIAPP conversion intermediate. Immunofluorescence of pancreas sections and immunoblotting using antisera raised to the NH_2- and COOH-terminal flanking regions of mouse proIAPP demonstrated that the 6-kDa intermediate form was an NH_2-terminally extended proIAPP conversion intermediate (processed only at the COOH-terminus). These data indicate that PC2 is essential for processing of proIAPP at the NH_2-terminal cleavage site in vivo and that PC3 is likely only capable of processing proIAPP at the COOH-terminal cleavage site. *Diabetes* 50:534–539, 2001

Along with insulin, the β-cells of the pancreatic islet cosecrete a 37–amino acid peptide named islet amyloid polypeptide (IAPP, or amylin) (1,2). The physiological role of IAPP is still unknown (3), but in type 2 diabetes, this peptide aggregates to form islet amyloid deposits that may destroy β-cells (4), thus playing a major role in the progressive loss of insulin secretion that occurs in this disease (5,6). Like insulin, IAPP is first synthesized as a larger precursor molecule, pro-islet amyloid polypeptide (proIAPP), which is processed within β-cell secretory granules to produce the major 37–amino acid form IAPP (7). Processing of proinsulin in β-cells is known to be mediated by the action of two subtilisin-like proprotein convertase enzymes, PC3 (also known as PC1) and PC2 (8–11). These enzymes, part of a family of convertases involved in proprotein processing (12,13), are widely expressed in endocrine cells and neurons, including β-cells and other endocrine cells in the pancreatic islet (13,14). During normal proinsulin processing, PC3 is thought to cleave proinsulin preferentially at the insulin B-chain/C-peptide (B-C) junction on the COOH-terminal side of a pair of basic residues (Arg^{31}-Arg^{32}). After removal of these basic residues by carboxypeptidase E (CPE), PC2 cleaves the resulting proinsulin conversion intermediate (des 31,32 proinsulin) at the C-peptide/A-chain (C-A) junction, again on the COOH-terminal side of dibasic residues (Lys^{64}-Arg^{65}), resulting in equimolar production of insulin and C-peptide after removal of the basic residues by CPE (9–11). As with insulin, normal production of IAPP depends on the processing of proIAPP at two well-conserved dibasic cleavage sites: Lys^{10}-Arg^{11} in humans (Lys^{13}-Arg^{14} in mice) at the NH_2-terminal cleavage site and Lys^{50}-Arg^{51} (Lys^{53}-Arg^{54} in mice) at the COOH-terminus. Because both proIAPP and proinsulin are processed in β-cell granules in which PC2 and PC3 reside, it seems likely that either or both of these enzymes will similarly be responsible for processing proIAPP in β-cells. Indeed, recent in vitro data obtained by incubating synthetic human proIAPP with recombinant convertase enzymes suggest that both PC2 and PC3 are capable of cleaving proIAPP at either site (15).

Mice lacking active PC2 have recently been generated and used to investigate the role of PC2 in proprotein processing in different endocrine tissues, including pancreatic islets (16,17). These mice have been shown to have severe impairments in processing of islet hormones, in-

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B-C, B-chain/C-peptide; BSA, bovine serum albumin; C-A, C-peptide/A-chain; CPE, carboxypeptidase E; HBSS, Hanks' balanced salt solution; IAPP, islet amyloid polypeptide; PBS, phosphate-buffered saline.

cluding an absolute loss of proglucagon and prosomatostatin processing (16). Although proinsulin is still converted to insulin and C-peptide in PC2 null mice, proinsulin processing is considerably retarded compared with wild-type mice and is characterized by elevated levels of the des 31,32 proinsulin conversion intermediate (17). These findings underscore the importance of PC2 in normal prohormone processing in islets, particularly in non–β-cells, and confirm that PC3 is capable of fully processing proinsulin on its own (11) and is likely more important than PC2 in proinsulin processing (17,18). In the present study, to help elucidate the role of PC2 and PC3 in normal IAPP production, we assessed proIAPP processing in islets of mice lacking active PC2.

RESEARCH DESIGN AND METHODS

Materials. Collagenase (Type XI), DNase, bovine serum albumin (BSA), Ficoll, and dithizone were obtained from Sigma (St. Louis, MO); Hanks' balanced salt solution (HBSS) from Canadian Life Technologies (Burlington, ON, Canada), and rodent IAPP 1-37 from Bachem (Torrance, CA).

Animals. Mice lacking active PC2 because of deletion of exon 3 in the pro-PC2 gene were generated previously (16) and maintained by breeding heterozygous PC2 null pairs. Offspring were genotyped by polymerase chain analysis of genomic tail DNA using primers described previously (16). Age- (3–4 months) and sex-matched animals were used in all experiments. All animal studies were approved by the University of British Columbia Committee on Animal Care in accordance with guidelines of the Canadian Council on Animal Care.

Islet isolation. Islets were isolated from mice by collagenase digestion. In brief, after the mice were euthanatized by cervical dislocation, the pancreas was harvested and minced (2 min) in ice-cold HBSS and incubated in 2 mg/ml collagenase (Type XI) for 15 min at 37°C, followed by a second incubation of 10–15 min in collagenase plus 1.3 mg/ml DNase. The digestion was stopped by dilution in ice-cold HBSS plus 0.1% BSA. After partial purification on a Ficoll gradient, the islets were stained with dithizone and handpicked twice under a dissecting microscope. Islets from two to four mice of the same genotype were pooled and washed twice by centrifugation in HBSS, and the pellet was frozen until Western blot analysis.

Antisera. Antiserum number 8342 (provided by Dr. S.E. Kahn, University of Washington) was raised in a rabbit against rodent IAPP (19). The IgG fraction of this serum was purified before use by protein A affinity chromatography. Glucagon antiserum (raised in guinea pig) was purchased from Linco Research (St. Charles, MO). Antisera specific for the NH_2- and COOH-terminal regions of murine proIAPP were generated in rabbits using peptides corresponding to amino acids 1–14 (NH_2-terminal) and 52–65 (COOH-terminal) of mouse proIAPP conjugated to keyhole limpet hemocyanin. Antibody titers were assessed by enzyme-linked immunosorbent assay using the synthetic peptides as standard. Two antisera, V3 (to the NH_2-terminal region of proIAPP) and J2 (to the COOH-terminal region), were used in all experiments.

Immunostaining. Pancreas samples from killed mice were fixed in 4% paraformaldehyde in 0.1 mol/l phosphate buffer (pH 7.5) for ~1 h, washed in 70% ethanol, dehydrated, and embedded in paraffin. Sections (5 μm) were washed in 0.05 mol/l phosphate-buffered saline (PBS) plus 0.25% Triton X-100, blocked in the same buffer containing 2% normal goat serum (Vector Laboratories, Burlingame, CA), and incubated overnight (4°C) with the NH_2- or COOH-terminal proIAPP antisera at a dilution of 1:100 in 0.05 mol/l PBS containing 0.25% Triton X-100 and 1% BSA. The sections were then washed and incubated for 1 h with anti-rabbit secondary antibody conjugated to Texas Red (Jackson Immunoresearch Laboratories, West Grove, PA) at a dilution of 1:200. After washing, the sections were incubated overnight with the glucagon antibody at a dilution of 1:100, followed by 1 h in the presence of anti–guinea pig antibody conjugated to the green fluorophore, Alexa Fluor 488 (Molecular Probes, Eugene, OR). The slides were mounted in Cytoseal 60 Mounting Medium (Stephens Scientific, Kalamazoo, MI) and viewed using a Zeiss Axioplan 2 microscope equipped for epifluorescence with a Sensys high-performance charge-coupled device (HCCD) camera (Photometrics, Tucson, AZ) and Quips Pathvysion imaging software (Applied Imaging, Santa Clara, CA). Images were captured using appropriate filters, pseudo-colored, and merged to give the final image.

Western blot analysis. Approximately 200 islets from mice of each genotype were lysed in 0.06 mol/l Tris (pH 6.8) containing 2% SDS, 10% glycerol, and 0.025% bromophenol blue; sonicated; and electrophoresed on 17.5% polyacrylamide gels using Tris-tricine buffer for separation of small proteins (20). After transfer to nitrocellulose membranes (overnight at 30 mA), the blots were blocked with 5% skim milk, washed, and incubated for 1 h with appropriate antisera at the following dilutions: antisera number 8342 (rodent IAPP 1-37) at 1:1,000, V3 (NH_2-terminal proIAPP) at 1:100, and J2 (COOH-terminal proIAPP) at 1:100. The blot was then incubated with anti–rabbit IgG linked to horseradish peroxidase and immunoreactivity visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). The relative amount of each molecular form of IAPP (as a percentage of total IAPP immunoreactivity) was determined from films using the public domain NIH Image program (version 1.59) developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image. Data obtained from three separate experiments are expressed as means ± SE. Statistical comparison of the effect of PC2 genotype on proIAPP processing was made by one-way analysis of variance.

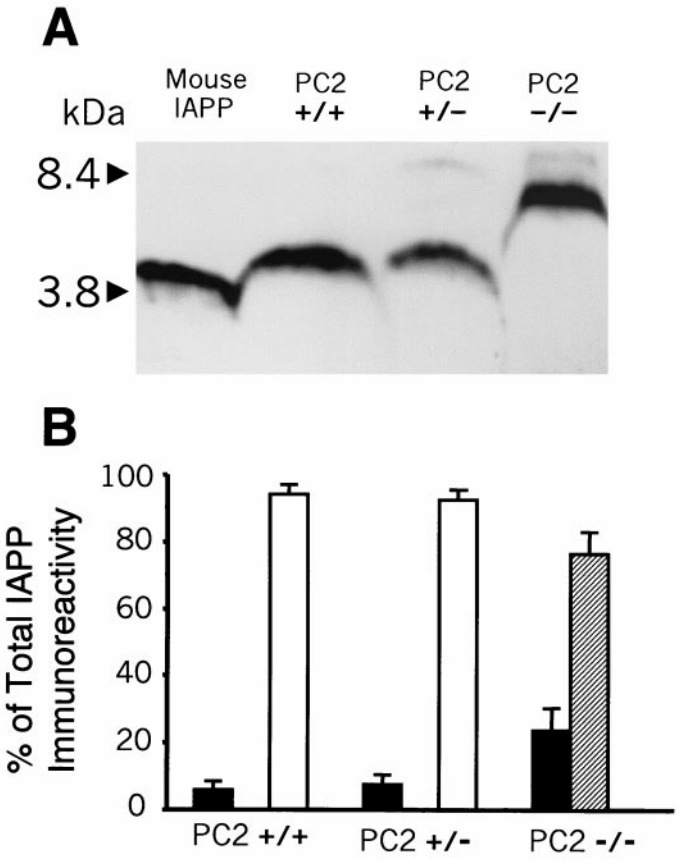

FIG. 1. Accumulation of a partially processed form of proIAPP in islets of mice lacking active PC2. *A*: Western blot analysis of lysates of islets isolated from homozygous PC2 null mice ($PC2^{-/-}$), heterozygous PC2 null mice ($PC2^{+/-}$), and wild-type mice ($PC2^{+/+}$). Immunoblotting was performed using an antiserum (number 8342) raised to rodent IAPP 1-37. A representative blot (repeated on two occasions) is shown. *B*: Densitometric analysis of three independent immunoblots. Data are expressed as the proportion of total IAPP immunoreactivity comprised of each molecular form (means ± SE). □, 8-kDa proIAPP; ▨, 6-kDa intermediate; ■, 4-kDa IAPP. Note the markedly increased levels of the ~6-kDa form of IAPP immunoreactivity in $PC2^{-/-}$ mouse islets.

RESULTS

Processing of proIAPP is impaired in mice lacking active PC2. Western blot analysis revealed that the major IAPP-immunoreactive form in islets isolated from either wild-type or heterozygous PC2 null mutant mice was a 4-kDa form (Fig. 1*A*)—likely mature IAPP 1-37. Small amounts of the 8-kDa precursor were usually present, but no intermediate forms were ever detectable (Fig. 1*A*). Strikingly, in islets from homozygous PC2 null mice, no 4-kDa IAPP immunoreactivity was detectable. The primary species of IAPP immunoreactivity in these mice was of

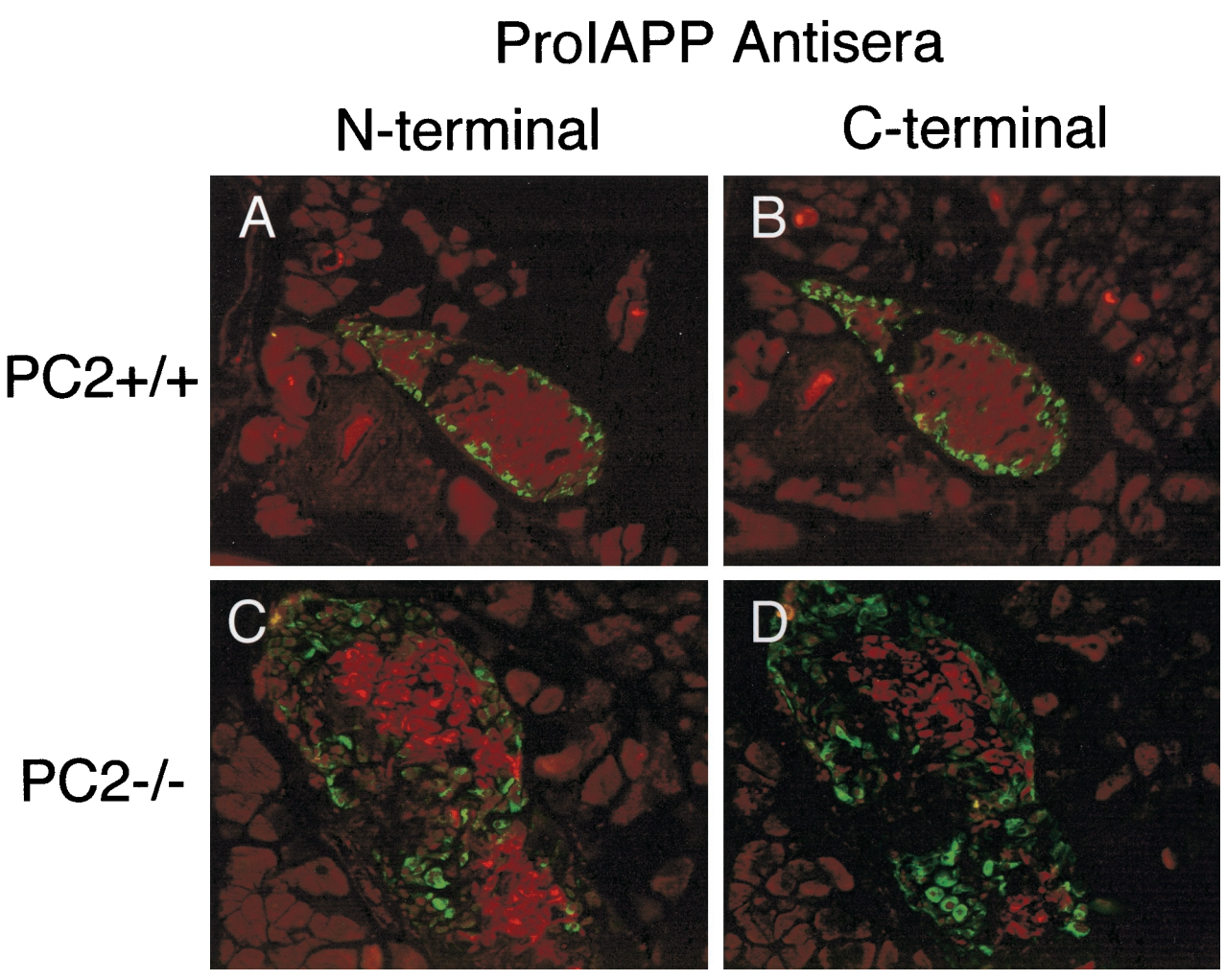

FIG. 2. Immunofluorescent staining of NH_2-terminal (N-terminal) and COOH-terminal (C-terminal) proIAPP immunoreactivity in pancreas of mice lacking active PC2. Serial sections of pancreas from wild-type ($PC2^{+/+}$) mice (*A* and *B*) and homozygous PC2 null ($PC2^{-/-}$) mice (*C* and *D*) were double-immunostained for the presence of (pro)glucagon (green) and either the NH_2-terminal (*A* and *C*) or the COOH-terminal (*B* and *D*) flanking region of proIAPP (red). Images were captured using green and red filters and merged to give the final image. Note the more intense immunostaining of $PC2^{-/-}$ relative to $PC2^{+/+}$ mice for proIAPP, particularly when immunostained with the NH_2-terminally directed antiserum. Islets of $PC2^{-/-}$ mice also have a marked increase in the number of glucagon-positive α-cells.

intermediate size (~6 kDa), with small amounts of the 8-kDa precursor present (Fig. 1*A*). Data from three separate experiments were quantified and are summarized in Fig. 1*B*. The distribution of IAPP immunoreactivity in wild-type and heterozygous PC2 null mice was almost identical. More than 90% of IAPP was fully processed in both wild-type and heterozygous PC2 null mice, in contrast to the homozygous PC2 null mutant mice, in which no 4-kDa IAPP could be detected. In the homozygous PC2 null mice, ~80% of IAPP immunoreactivity was comprised of the 6-kDa intermediate form. Full-length proIAPP comprised the remaining 20% of IAPP immunoreactivity, with levels about twofold higher in these animals relative to heterozygous and wild-type mice ($P < 0.05$). Thus, in the absence of PC2, proIAPP appears to be only partially processed to an intermediate form.

Identification of the proIAPP conversion intermediate in mice lacking active PC2. To identify the 6-kDa form of IAPP immunoreactivity accumulating in the islets of PC2 null mice, we generated antisera specific for the NH_2- and COOH-terminal flanking regions of mouse proIAPP. We first immunostained pancreas sections of homozygous PC2 null and wild-type mice with these antisera to assess their ability to stain β-cells and to determine whether the intensity of NH_2- or COOH-terminal proIAPP immunostaining would be increased in the PC2 null mice. Sections were co-immunostained using antiserum to (pro)glucagon. As previously reported, a marked α-cell hyperplasia was observed in the PC2 null islets (16), as demonstrated by the large increase in the number of glucagon-positive cells in the periphery of PC2 null mouse islets (Fig. 2*C* and *D*). Immunoreactivity for both the NH_2- and COOH-terminal regions of proIAPP was observed in all mice, localized to the central regions of the islet in which β-cells reside (Fig. 2). When compared with wild-type mice immunostained with the same antiserum at the same dilution, the intensity of immunofluorescence for the NH_2-terminal region of proIAPP consistently appeared greater in the β-cells of PC2 null mice (compare Fig. 2*C* with Fig. 2*A*). When immunostained with the COOH-terminal antiserum, the β-cells of PC2 null mice tended to have more intense immunofluorescence relative to wild-type animals, although this difference was less marked (compare Fig. 2*D* with Fig. 2*B*). Immunostains of heterozygous PC2 null mice pancreas

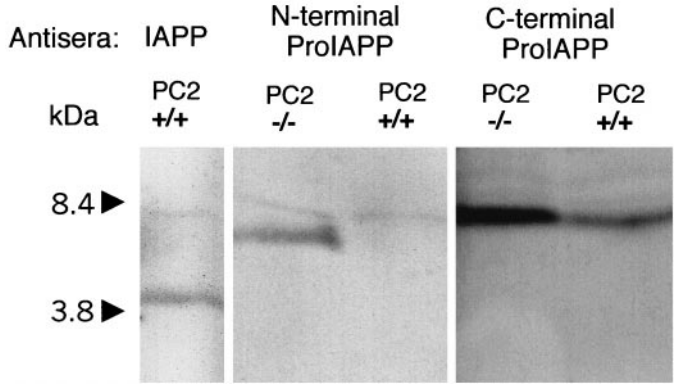

FIG. 3. Identification of the proIAPP conversion intermediate in islets of mice lacking active PC2 by immunoblot. Western blot analysis of lysates of islets isolated from homozygous PC2 null mice ($PC2^{-/-}$) and wild-type mice ($PC2^{+/+}$) is shown. Immunoblotting was performed using antisera raised to the NH_2-terminal (N-terminal) and COOH-terminal (C-terminal) flanking peptides of mouse proIAPP. For reference, lysate of wild-type mouse islets immunoblotted with antiserum number 8342 (to mature mouse IAPP) was also included (far left lane). A representative blot (repeated on two occasions) is shown. Both the NH_2- and COOH-terminal proIAPP antisera recognize unprocessed proIAPP (8 kDa) but not mature IAPP (4 kDa). Only the NH_2-terminal antiserum recognizes the 6-kDa proIAPP intermediate that is present in PC2 null mouse islets.

with the proIAPP and (pro) glucagon antisera appeared identical to those of wild-type animals (data not shown).

The intense immunofluorescence for NH_2-terminal proIAPP in the PC2 null mouse pancreas suggests that the identity of the 6-kDa proIAPP conversion intermediate is likely NH_2-terminally extended proIAPP (processed at the C-terminus) but is by no means conclusive. We therefore performed Western blot analysis of islets isolated from PC2 null and wild-type mice using the NH_2- and COOH-terminal proIAPP antisera. In lysates of wild-type mouse islets, both the NH_2-terminal and the COOH-terminal proIAPP antisera recognized unprocessed proIAPP (8 kDa) but not mature IAPP (4 kDa) (Fig. 3). This finding was expected because the NH_2- and COOH-terminal flanking regions that these antisera are raised to are present in unprocessed proIAPP but are cleaved out of mature IAPP. In lysates of PC2 null mouse islets, the NH_2-terminal proIAPP antiserum recognized not only the 8-kDa full-length form of proIAPP but also large amounts of a 6-kDa form. The COOH-terminal proIAPP antiserum, by contrast, detected only 8-kDa proIAPP in PC2 null mouse islets and not the 6-kDa form detected by the NH_2-terminal proIAPP antiserum. The 6-kDa IAPP-immunoreactive form that accumulates in PC2 null mouse islets therefore contains regions recognized by antisera raised to IAPP 1-37 (Fig. 1) and the NH_2-terminal region of proIAPP (Fig. 3) but not the COOH-terminal region of proIAPP (Fig. 3). These data strongly suggest that the 6-kDa IAPP-immunoreactive form is an NH_2-terminally extended proIAPP conversion intermediate (processed at the COOH-terminus only).

DISCUSSION

This study demonstrates that the prohormone convertase enzyme PC2 is essential for normal proIAPP processing in islet β-cells. In the absence of PC2, proIAPP processing occurs only at the COOH-terminal cleavage site, resulting in production of a 6-kDa NH_2-terminally extended proIAPP intermediate form. The remaining processing step, cutting at the NH_2-terminal cleavage site, appears to be totally dependent on the presence of PC2. Thus, no mature IAPP is produced, and there is a marked accumulation of the NH_2-terminal proIAPP intermediate in the β-cells of mice lacking active PC2.

These findings highlight differences between the processing of proinsulin and proIAPP in β-cells. PC3 appears to be quantitatively more important in normal proinsulin processing because complete proinsulin processing still occurs (albeit more slowly) in islets of PC2 null mice (17). Thus, PC3 is fully capable of cleaving proinsulin at both the B-C and C-A junctions (11,17), although it has a preference for the B-C junction (9–11), and proinsulin processing is more efficient in the presence of PC2 (17). Assuming that PC3 mediates the partial cleavage of proIAPP that we observed in PC2 knockout mice, our data indicate that PC3 is capable only of cleaving proIAPP at the COOH-terminal processing site. This finding contrasts with recent data that showed that recombinant PC3 is capable of completely cleaving synthetic human proIAPP in vitro at either site (15). The reason for this discrepancy is unclear but could be related to differences between the milieu of the β-cell granule in vivo versus the conditions generated in vitro or possibly due to differences in the processing of human versus mouse proIAPP. In any case, unlike proinsulin, complete processing of proIAPP in vivo in mice is clearly dependent on the presence of PC2.

Our data further imply that normal proIAPP processing may be a two-step process, as is the case with proinsulin (18), in which one enzyme would preferentially cleave proIAPP at one site, followed by cleavage by the other convertase at the second site. The order of cleavage events in normal proIAPP processing cannot be ascertained from these experiments; however, we can speculate based on the present data and previous studies (see model in Fig. 4). We can likely rule out, for example, that PC2 cleavage is an important first step in proIAPP processing because if PC2 were essential for this step, the 8-kDa unprocessed precursor would have been the primary molecular form to accumulate in the PC2 knockout mice. Thus, PC3 cleavage of proIAPP at the COOH-terminal site may be the first step in normal proIAPP processing (as is the case for proinsulin at Arg^{31}-Arg^{32}), followed by PC2 acting at the NH_2-terminal cleavage site (Fig. 4). Indeed, recombinant PC3 appears to favor the COOH-terminal cleavage site of synthetic human proIAPP in vitro, whereas PC2 tends to favor the NH_2-terminal site (15). However, the lack of any fully processed IAPP in PC2 null mouse islets indicates that in vivo, only PC2 can cleave the NH_2-terminal site in proIAPP. Because no intermediate forms were detectable in wild-type mouse islets, the second step in normal proIAPP processing may be very rapid, implying that the first step (possibly cleavage by PC3 at the COOH-terminus) may be rate-limiting in proIAPP processing. It should be stressed, however, that these conclusions are based on Western blot analysis, which does not assess the kinetics of proprotein processing, and further, the detection of intermediate forms may have been below the sensitivity of our immunoblots.

The small but significant accumulation in full-length proIAPP in the islets of PC2 null mice (Fig. 1) suggests that the first step in proIAPP processing in these mice (cleavage at the COOH-terminus) is also somewhat impaired in

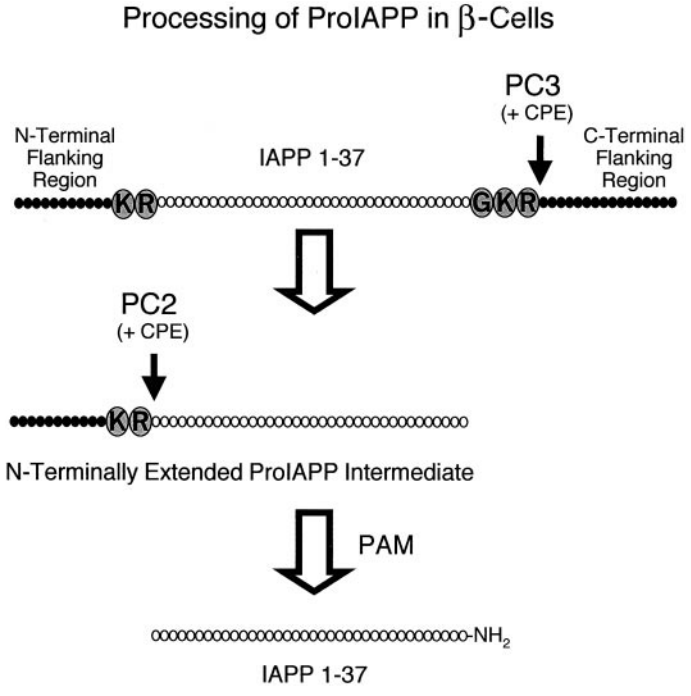

FIG. 4. Possible pathway for normal processing of proIAPP in β-cells. PC3 may cleave proIAPP first at the IAPP–COOH-terminal (C-terminal) cleavage site, followed by PC2 processing of the resulting intermediate at the NH_2-terminal (N-terminal)–IAPP cleavage site. In mice lacking PC2, the second step in this pathway is blocked, resulting in accumulation of the NH_2-terminally extended proIAPP intermediate. After cleavage by PC2 or PC3, the remaining basic residues are removed by CPE. Amidation of the COOH-terminus occurs via conversion of the COOH-terminal glycine residue by the peptidyl amidating mono-oxygenase complex (PAM) (26).

the absence of PC2. This possibility is further supported by our observation that immunoreactivity for the COOH-terminal region of proIAPP appears to be somewhat elevated in mice lacking PC2, whether determined by immunostaining (Fig. 2*D*) or Western blot analysis (8-kDa band in right panel of Fig. 3). The first step in proinsulin processing in PC2 null mice is similarly impaired (17) because not only des 31,32 proinsulin but also full-length proinsulin accumulates in the islets of PC2 null mice. This impairment in the initial step in proIAPP processing might be due to impaired action of PC3 in these mice, possibly because of substrate inhibition of the enzyme by the accumulation of the NH_2-terminal proIAPP conversion intermediate. Alternatively, it is possible that PC2 may also contribute to processing of proIAPP at the COOH-terminal cleavage site, as has been suggested by in vitro studies (15,21).

Based on our findings, we predict that if loss of PC2 activity is a manifestation of β-cell dysfunction in type 2 diabetes, secretion of NH_2-terminally extended proIAPP will be disproportionately elevated. Secretion of des 31,32 proinsulin (which is thought to be processed primarily by PC2) is known to be elevated in type 2 diabetes, although secretion of full-length proinsulin is similarly increased, suggesting that a generalized β-cell defect in prohormone processing exists in this disease rather than a specific defect in PC2 activity (22). Further, we found that heterozygous PC2 null mice, which presumably have half the normal complement of PC2, appear to be able to process proIAPP normally, suggesting that a profound defect in PC2 activity would be needed for increased secretion of NH_2-terminally extended proIAPP to occur. Nonetheless, the NH_2-terminally extended proIAPP intermediate has recently been shown to be markedly increased in cultured human islets after long-term exposure to high glucose, comprising more than half of total IAPP immunoreactivity (23). Such culture conditions are also associated with rapid islet amyloid formation in islets of transgenic mice expressing human proIAPP (24). Further, immunoreactivity for the NH_2-terminal (but not the COOH-terminal) flanking region of human proIAPP has been found in islet amyloid deposits in pancreas from individuals with type 2 diabetes (25). Partially processed NH_2-terminally extended proIAPP may therefore be an important molecule in islet amyloid formation, and we speculate that defective proIAPP processing by PC2 and increased production of NH_2-terminally extended proIAPP could play a role in amyloidogenesis. By expressing human proIAPP in the β-cells of mice lacking PC2, we can now test this hypothesis.

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