Role of β-Cell Prohormone Convertase (PC)1/3 in Processing of Pro-Islet Amyloid Polypeptide

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Islet amyloid polypeptide (IAPP) (amylin), the major component of islet amyloid, is produced by cleavage at the COOH- and NH_2-termini of its precursor, proIAPP, likely by the β-cell prohormone convertases (PC) 1/3 and PC2. Mice lacking PC2 can process proIAPP at its COOH- but not its NH_2-terminal cleavage site, suggesting that PC1/3 is capable of initiating proIAPP cleavage at its COOH-terminus. To determine the precise role of PC1/3 in proIAPP processing, Western blot analysis was performed on islets isolated from mice lacking PC1/3 ($PC1/3^{-/-}$). These islets contained not only fully processed IAPP as in $PC1/3^{+/+}$ islets, but also elevated levels of a COOH-terminally unprocessed intermediate form, suggesting impaired processing at the COOH-terminus. Next, GH3 cells that do not normally express proIAPP or detectable levels of PC1/3 or PC2 were cotransduced with adenoviruses expressing rat proIAPP and either PC2 or PC1/3. As expected, in GH3 cells transduced to express only proIAPP, no processing was observed. Coexpression of proIAPP and PC2 resulted in production of mature IAPP, whereas in cells that coexpressed proIAPP and PC1/3 only a 6-kDa intermediate was produced. We conclude that PC1/3 is important for processing of proIAPP at the COOH-terminus, but in its absence, PC2 can initiate complete processing of proIAPP to IAPP by cleaving the precursor at either its NH_2- or COOH-terminal cleavage sites.
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Type 2 diabetes is characterized by peripheral insulin resistance and progressive loss of β-cell mass accompanied by the deposition of amyloid in the pancreatic islets (1–3). Islet amyloid polypeptide (IAPP) (amylin) (4,5), the major component of islet amyloid deposits, is a peptide hormone that is colocalized with insulin in the secretory granules of β-cells and cosecreted in a molar ratio (IAPP to insulin) of ~1:100 (1,6,7). Islet amyloid, caused by aggregation of IAPP, is thought to be toxic to β-cells (8) and may therefore contribute to the progressive loss of insulin secretion in type 2 diabetes (1,7). Factors responsible for aggregation of IAPP and formation of islet amyloid in type 2 diabetes are still not well known. Both hypersecretion of IAPP associated with increased demand for insulin in type 2 diabetes and the presence of an amyloidogenic amino acid sequence in the human IAPP molecule have been implicated as important factors, but they are likely not sufficient for islet amyloid formation (1,9,10). Because proinsulin processing is impaired in type 2 diabetes (11,12) and proIAPP is processed in parallel with proinsulin, we (1,13) and others (14,15) have proposed that impaired processing of the IAPP precursor molecule proIAPP by islet β-cells may lead to hypersecretion of unprocessed or partially processed forms of proIAPP that may have a higher tendency for aggregation than mature IAPP (16,17). Determination of the enzymes responsible for processing of proIAPP and their cleavage products may therefore lead to the identification of potential amyloidogenic intermediates and a better understanding of the mechanism of islet amyloid formation.

Normal processing of proinsulin, the insulin precursor molecule, is initiated in β-cells by cleavage at the B-chain/C-peptide junction ($Arg^{32} \downarrow Glu^{33}$), preferentially by the prohormone convertase enzyme PC3 (also known as PC1 or PC1/3), resulting in the formation of des 31,32 proinsulin after removal of the two COOH-terminal arginine residues (Arg^{31}-Arg^{32}) from the B-chain by carboxypeptidase E (CPE). Cleavage of des 31,32 proinsulin at the C-peptide/A-chain junction ($Arg^{65} \downarrow Gly^{66}$) by PC2 followed by removal of the COOH-terminal basic residues (Lys^{64}-Arg^{65}) of C-peptide by CPE leads to production of mature insulin and C-peptide (18–21). Both subtilisin-like proprotein convertases PC2 and PC1/3 are expressed along with proinsulin and proIAPP in β-cell secretory granules (20,22). As with proinsulin, normal processing of human proIAPP (a 67–amino acid peptide) depends on the cleavage at two well-conserved dibasic sites: Lys^{10}-Arg^{11} at the NH_2-terminus and Lys^{50}-Arg^{51} at the COOH-terminus (Lys^{13}-Arg^{14} and Lys^{53}-Arg^{54} in mice) (23). In a previous study, we demonstrated that proIAPP processing is blocked at the NH_2-terminal cleavage site in mice lacking active PC2, leading to elevated levels of an NH_2-terminally extended, partially processed, proIAPP intermediate form (24). This study clearly showed that PC2 is essential for

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Ad-LacZ, adenovirus expressing β-galactosidase; Ad-rIAPP, adenovirus expressing rat pro–islet amyloid polypeptide; CPE, carboxypeptidase E; DMEM, Dulbecco's modified Eagle's medium; IAPP, islet amyloid polypeptide; KRB, Krebs-Ringer bicarbonate; PC, prohormone convertase; PMSF, phenylmethylsulphonyl fluoride.

processing of proIAPP at its NH_2-terminus in vivo. Moreover, our finding that cleavage at the COOH-terminus of proIAPP was not markedly impaired in PC2 null mouse islets suggested that another convertase enzyme(s) in β-cell secretory granules, most likely PC1/3, must be responsible for cleavage at the COOH-terminal processing site of proIAPP. Mice lacking active PC1/3 have recently been generated (21,25) and have been shown to have a severe impairment in the processing of proinsulin to insulin, manifested as elevated levels of intact proinsulin and des 64,65 proinsulin, an intermediate form produced by the action of PC2. Unlike PC2 null mice, plasma glucose levels and processing of other islet hormones including proglucagon and prosomatostatin appear normal in PC1/3 null mice (21,25). In the present study, we used this model to investigate the precise role of PC1/3 in normal processing of proIAPP in pancreatic β-cells in vivo.

RESEARCH DESIGN AND METHODS

Avertin, collagenase (Type XI), DNase, BSA, phenylmethylsulphonyl fluoride (PMSF), dextran, dithizone, and aprotinin were obtained from Sigma-Aldrich (Oakville, ON, Canada); Hanks' balanced salt solution, Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, fetal bovine serum from Invitrogen (Burlington, ON, Canada), and protein G Sepharose beads from Amersham Biosciences (Baie d'Urfe, QC, Canada). Rodent IAPP 1–37 was obtained from Bachem (Torrance, CA) and [^{3}H]leucine from American Radiolabeled Chemicals (St. Louis, MO). All electrophoresis chemicals were from Bio-Rad Laboratories (Mississauga, ON, Canada).

Antisera and recombinant adenoviruses. Anti-rodent IAPP antibody (RGG-7323) was obtained from Peninsula Laboratories (Belmont, CA). Antisera specific for the NH_2-terminal (V3) and COOH-terminal (J2) regions of murine proIAPP were generated as previously described (24). These antisera were raised in rabbits against peptides corresponding to amino acids 1–14 (NH_2-terminal) and 52–65 (COOH-terminal) of mouse proIAPP. Rabbit antiserum against the last 15 amino acids of the COOH-terminal of the PC2 molecule and adenoviruses expressing PC2 (Ad-PC2) or PC1/3 (Ad-PC1/3) were generated as previously described (26,27). Rabbit antiserum against PC1/3, recognizing the NH_2-terminus of the mature enzyme, was kindly provided by Dr. Iris Lindberg (New Orleans, LA).

Animals. Mice lacking active PC1/3 were generated previously (25) by deleting a portion of the PC1/3 promoter and the first exon from the PC1/3 gene. Age-matched (4–5 months) male homozygous PC1/3 null mice and their wild-type littermates were used in all experiments. The animals were cared for in accordance with the National Institutes of Health and University of Chicago institutional guidelines. Mice lacking active PC2 were generated previously (20) and were bred in the animal facility unit of the BC Research Institute for Children's and Women's Health (24). The PC2 knockout mice were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care.

Islet isolation. Islets of Langerhans were isolated as previously described (21). For immunoblot experiments ~300 freshly isolated islets pooled from 2–3 homozygous PC1/3 null mice or their wild-type littermates were lysed in 40 μl lysis buffer containing 50 mmol/l Tris-HCl (pH 8.0), 150 mmol/l NaCl, 0.02% sodium azide, 0.1% SDS, 1 mmol/l PMSF, 10 μg/ml aprotinin, 1% Nonidet P-40, and 0.5% sodium deoxycholate for 25 min on ice and vortexed every 5 min. Samples were centrifuged (15,000*g*, 10 min, 4°C) and the supernatants frozen at −80°C until assayed. Protein concentration in the lysates was measured using the BCA assay (Pierce, Rockford, IL).

Transduction of GH3 cells with recombinant adenovirus. GH3 (rat anterior pituitary) cells were grown in DMEM containing 25 mmol/l glucose and supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. Cells at ~70% confluency in 25-cm^2 flasks were transduced with adenovirus expressing β-galactosidase (Ad-LacZ) as control at multiplicity of infection (moi) 10 or adenovirus expressing rat proIAPP (Ad-rIAPP) (moi 15.8) or cotransduced with Ad-rIAPP (15.8) and either Ad-PC2 (2.7) or Ad-PC1/3 (23) in 1 ml DMEM for 2 h at 37°C. For cotransduction, cells were incubated with fresh medium for 4 h to allow recovery before the second transduction (18). The level of PC2 protein expression was determined by Western blot of cell extracts. To assess transduction efficiency, cells transduced with Ad-LacZ were washed and fixed in 0.5% glutaraldehyde 24 h following adenovirus infection and the proportion of cells expressing β-galactosidase determined following incubation with the substrate X-gal (5-bromo-4-chloro-3-indole-β-D-galactoside). The transduction efficiency with Ad-LacZ was ~70%.

Metabolic labeling. Twenty-four hours post-transduction, cells were washed and preincubated with Krebs-Ringer bicarbonate (KRB) buffer containing 10 mmol/l HEPES (pH: 7.4), 16.7 mmol/l glucose, and 0.25% BSA (KRB-G16.7) for 15 min at 37°C. Cells were then labeled in 1 ml KRB-G16.7 buffer containing 200 μCi/ml [^{3}H]leucine (specific activity 110 Ci/mmol/l; American Radiolabeled Chemicals) for 2 h, washed with PBS, harvested with trypsin-EDTA and lysed in 200 μl lysis buffer as described above.

Immunoprecipitation. Cell extracts (780–800 μg) were precleared by incubation with 50 μl protein G-Sepharose beads (Amersham) for 45 min at 4°C. The supernatants were incubated with 4 μg anti-rodent IAPP IgG purified antibody (RGG-7323, Peninsula) for 2 h followed by 1.5 h incubation with 50 μl protein-G Sepharose beads at 4°C. The protein G-Sepharose immunocomplex was washed three times with lysis buffer and used for SDS-PAGE.

Electrophoresis and immunoblotting. Immunoprecipitated samples (GH3 cells) or aliquots of protein (10 or 15 μg) from islet lysates were heated (100°C) in Laemmli's sample buffer for 5 min. Islet extracts were electrophoresed on a polyacrylamide gel using Tris-tricine buffer for separation of small proteins (28) and transferred to 0.45-μm PVDF membranes (15 V, 20 min) using a Bio-Rad semidry electrophoretic transfer cell (Trans-Blot SD). The membranes were blocked with 5% skim milk for 1 h at room temperature and washed and incubated for 1 h with appropriate antisera (or IgG purified antiserum) at the following dilutions: anti-rodent IAPP at 1:1,000 (2 μg/ml), V3 (NH_2-terminal proIAPP antiserum) and J2 (COOH-terminal proIAPP antiserum) at 1:100 at room temperature, followed by 1 h incubation with horseradish peroxidase–conjugated anti-rabbit IgG (Amersham) diluted 1:5,000 at room temperature. Immunodetection was performed using an enhanced chemiluminescence detection kit (Amersham). Protein bands on the films (Kodak X-OMAT) were analyzed by densitometry using "Quantity One" quantitation analysis software program. Immunoprecipitated proteins from GH3 cell extracts were electrophoresed on a polyacrylamide gel in Tris-tricine buffer as described and the separated proteins detected by fluorography using EN3HANCE (Perkin Elmer Life Sciences, MA) followed by exposure to Kodak X-OMAT film at −70°C for 5 days.

Statistical analysis. Values are expressed as the means ± SE. Statistical analysis was performed using ANOVA followed by a Newman-Keuls test. $P < 0.05$ was taken as level of significance.

RESULTS

Processing of proIAPP is impaired but not blocked in islets from PC1/3 null mice. Western blot analysis performed on islet extracts from wild-type mice (PC1/3$^{+/+}$) using an antiserum that was raised against mature IAPP and detects both unprocessed and mature forms of (pro)IAPP showed that the major species of IAPP immunoreactivity in islets from wild-type mice was fully processed IAPP (~4 kDa) (Fig. 1*A*). Small amounts of partially processed proIAPP (~6 kDa) and very low levels of unprocessed proIAPP (~8 kDa) were also detectable in normal islets. Densitometric analysis of blots from three independent experiments demonstrated that ~66% of total IAPP immunoreactivity was composed of mature IAPP, whereas ~25 and 9% were composed of partially processed and unprocessed forms of proIAPP, respectively (Fig. 1*B*). Interestingly, islets from homozygous PC1/3 null mice also contained predominantly fully processed IAPP but a partially processed (~6 kDa) form(s) of proIAPP was found to be markedly elevated (Fig. 1*A*). In addition, islets from PC1/3$^{-/-}$ mice had a slight but significant increase in unprocessed proIAPP as compared with wild-type islets (Fig. 1). Densitometric analysis revealed that ~40% of total IAPP immunoreactivity in the islets of PC1/3$^{-/-}$ mice was comprised of a partially processed proIAPP intermediate of ~6 kDa (Fig. 1*B*). Therefore, in the absence of PC1/3, proIAPP processing is impaired but not completely blocked at either one or both of its two cleavage sites, resulting in the accumulation of a partially processed intermediate form(s).

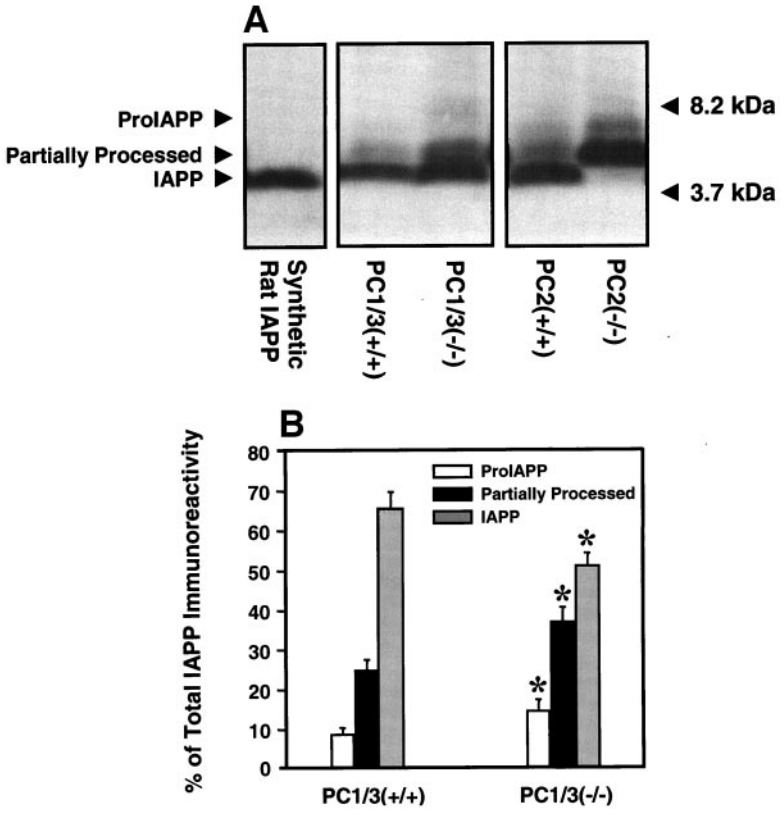

FIG. 1. Impaired processing of proIAPP in islets from mice lacking active PC1/3. *A*: Western blot analysis was performed on the islet extracts (10 μg) from homozygous PC1/3 null mice (PC1/3$^{-/-}$) and wild-type mice (PC1/3$^{+/+}$) followed by immunoblot using IgG-purified anti-rodent IAPP antibody (RGG-7323). A partially processed form(s) of (pro)IAPP with a molecular weight of ~6 kDa was markedly increased in islets from PC1/3$^{-/-}$ mice. Immunoblot of islets from age- and sex-matched PC2$^{-/-}$ mice with the same antisera is shown on the right side for comparison. Note that unlike PC3$^{-/-}$ mice, islets from PC2$^{-/-}$ mice are unable to process proIAPP to mature ~4-kDa IAPP. *B*: Densitometric analysis of immunoblots from three independent experiments. Results are presented as the percentage of each IAPP-immunoreactive molecular form, with total IAPP immunoreactivity taken as 100%. Data are expressed as means ± SE. *Significantly different from corresponding molecular form in PC1/3$^{+/+}$ islets ($P < 0.05$, ANOVA).

ProIAPP processing is impaired at the COOH-terminal cleavage site in PC1/3 null mice. To determine the site(s) at which proIAPP processing is impaired in the absence of PC1/3, we used antisera specific for the NH_2- and COOH-terminal flanking regions of proIAPP (24) in order to allow identification of the ~6-kDa intermediate form(s) of proIAPP that is increased in the islets of PC1/3 null mice. Western blot analysis using either of these antisera detected small amounts of a proIAPP-immunoreactive form of ~8 kDa in islets from both PC1/3$^{+/+}$ and PC1/3$^{-/-}$ mice (Fig. 2*A* and *C*). The COOH-terminal proIAPP antiserum also detected a partially processed form (~6 kDa), which was present in the PC1/3$^{-/-}$ islets but not detectable in PC1/3$^{+/+}$ islets (Fig. 2*A*). Moreover, a partially processed form was detected by the NH_2-terminal proIAPP antiserum in both PC1/3$^{-/-}$ and PC1/3$^{+/+}$ islets and appeared to be slightly higher in PC1/3$^{-/-}$ islets (Fig. 2*C*). Thus, using these antisera we observed elevated levels of a partially processed intermediate form in PC1/3$^{-/-}$ islets that contained immunoreactivity for the COOH-terminal flanking region of proIAPP, suggesting that cleavage at the COOH- but not the NH_2-terminus is impaired in the absence of PC1/3 in vivo. Unlike PC2$^{-/-}$ islets that contain elevated levels of NH_2-terminally unprocessed proIAPP (Fig. 2*D*), cleavage at the NH_2-terminus of proIAPP does not appear to be significantly impaired in PC1/3$^{-/-}$ islets (Fig. 2*C*).

PC2 can cleave proIAPP at both its NH_2- and COOH-terminal cleavage sites to form IAPP. The finding that mature IAPP is generated in islets of PC1/3 null mice suggested that another enzyme(s) in β-cell secretory granules can cleave proIAPP at its COOH-terminus in the absence of PC1/3. We therefore investigated whether the β-cell convertase enzyme PC2 is capable of cleavage at the COOH-terminus of proIAPP. GH3 (rat pituitary) cells have secretory granules but are normally unable to convert proinsulin to insulin because they express very low (or undetectable) levels of PC2 and PC1/3 (18). We transduced GH3 cells with a recombinant Ad-rIAPP alone or with adenoviruses expressing either PC2 (Ad-PC2) or PC1/3 (Ad-PC1/3). Consistent with previous reports, very low levels of PC2 and no PC1/3 were detected by Western blot in the GH3 cells used in this study (Fig. 3*A* and *B*). The adenovirus titers used in this study were determined empirically to maximize proIAPP expression in the absence of cell toxicity and to obtain levels of PC2 and PC1/3 expression comparable with INS-1 β-cells, which normally express both PC2 (Fig. 3*A*) and PC1/3 (Fig. 3*B*) and are able to completely process proIAPP to IAPP (Fig. 3*C*). Twenty-four hours after infection, cells were radiolabeled with [^{3}H]leucine for 2 h followed by immunoprecipitation and SDS-PAGE analysis of IAPP-related molecules in the cell lysates and fluorography. GH3 cells transduced with a recombinant Ad-LacZ were used to examine transduction efficiency and the possibility of expression of any undesired proteins by adenovirus infection. As expected, proIAPP (~8 kDa) was not processed to mature IAPP (~4 kDa) in GH3 cells that were transduced with Ad-rIAPP alone and lacked PC2 and PC1/3 expression and was only partially processed in GH3 cells cotransduced with Ad-rIAPP and Ad-PC1/3 (Fig. 4*A* and *B*). By contrast, cotransduction of GH3 cells with Ad-rIAPP and Ad-PC2 resulted in the formation of mature IAPP (~4 kDa) (Fig. 4*C* and *D*). The faint ~6-kDa band observed in GH3 cells transduced with Ad-rIAPP alone is likely a small amount of partially processed proIAPP intermediate due to the presence of very low levels of endogenous PC2 in these cells. These data indicate that PC2, when expressed at levels compa-

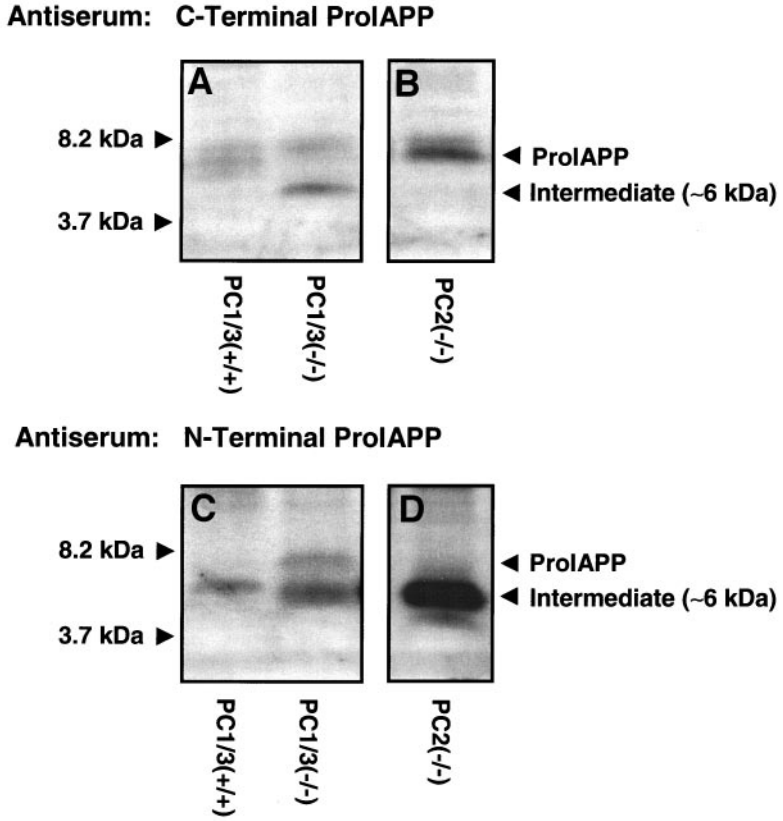

FIG. 2. Processing of proIAPP at NH_2- and COOH-terminal cleavage sites detected by immunoblot in islets from PC1/3 null mice. Western blot analysis of islet extracts (15 μg) from homozygous PC1/3 null mice and their wild-type littermates followed by immunoblot using antisera raised against the NH_2- (V3) or COOH-terminal (J2) flanking regions of mouse proIAPP (*A* and *C*). A representative blot from three independent experiments is shown. Immunoblot with J2 antiserum detected a marked increase in the COOH-terminally unprocessed intermediate form of (pro)IAPP in PC1/3$^{-/-}$ islets (*A*), whereas the level of the NH_2-terminally unprocessed form detected by V3 antiserum was only slightly higher in PC1/3$^{-/-}$ than PC1/3$^{+/+}$ islets (*C*). Note that the blot in *C* has been exposed longer to allow the detection of the low levels of NH_2-terminally unprocessed form present in PC1/3$^{+/+}$ and PC1/3$^{-/-}$ islets. Immunoblots of islets from age- and sex-matched PC2$^{-/-}$ mice with the same antisera are shown for comparison (*B* and *D*). As previously reported (24), the NH_2-terminally unprocessed intermediate form of proIAPP is markedly elevated in PC2$^{-/-}$ mouse islets (*D*).

rable with those observed in INS-1 β-cells (Fig. 4*C*), is able to process proIAPP at both its NH_2- and COOH-terminal cleavage sites to produce IAPP.

We then examined whether islets of mice lacking PC1/3 express normal levels of mature PC2, by Western blot analysis. Immunoreactivity for the mature (67 kDa) form of PC2 was observed at comparable levels in both PC1/3$^{+/+}$ and PC1/3$^{-/-}$ mouse islets (Fig. 5*A*), although it should be noted that since PC2 is also expressed in α-cells, our immunoblot analysis of islet extracts may not directly reflect PC2 protein levels in islet β-cells. By contrast, PC1/3 protein expression was somewhat decreased in PC2$^{-/-}$ islets (Fig. 5*B*), likely because of the low proportion of β-cells relative to α-cells in those islets (20,24).

DISCUSSION

The mechanism(s) by which proIAPP, the IAPP precursor molecule, is processed in vivo is still not completely understood, but it is likely that the prohormone convertases PC1/3 and PC2, which are responsible for processing proinsulin to insulin and C-peptide (18,21,27,29), also mediate proIAPP processing (24,30,31). We have previously shown that mice lacking PC2 are unable to process proIAPP at its NH_2-terminal cleavage site, indicating that PC2 is essential for proIAPP processing at the NH_2-terminus in vivo (24). The finding that PC2 null mice were capable of processing proIAPP at its COOH-terminus suggested that another enzyme(s) in β-cell secretory granules must mediate proIAPP processing at this site. In the present study, we demonstrate that PC1/3 is indeed important for processing proIAPP at its COOH-terminal cleavage site in vivo. Using islets from PC1/3 null mice, we found that the processing of proIAPP was impaired in the absence of PC1/3, manifested as elevated levels of a partially processed (~6 kDa) form. We further showed that this intermediate form was COOH-terminally extended, suggesting that cleavage at the COOH- but not the NH_2-terminus is impaired in the absence of PC1/3 in vivo.

One of the important findings of this study is that unlike islets from PC2 null mice, in which proIAPP processing is

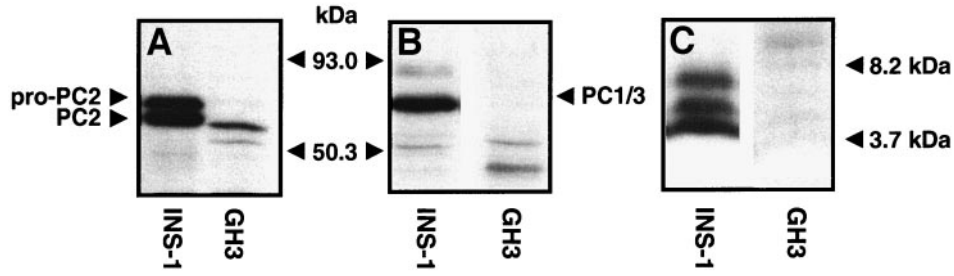

FIG. 3. Comparison of PC2, PC1/3, and (pro)IAPP protein levels in GH3 and INS-1 cells. Western blot analysis of cell lysates was performed on a 10% polyacrylamide gel for PC2 and PC1/3 (10 μg) or a Tris-tricine gel for (pro)IAPP (30 μg) followed by immunoblot using appropriate antisera as described in RESEARCH DESIGN AND METHODS. Very low levels of PC2 (*A*) and no PC1/3 (*B*) or (pro)IAPP (*C*) were detected in GH3 cells. Note that films of GH3 cell immunoblots were exposed longer than those for INS-1 cells to maximize likelihood of detection.

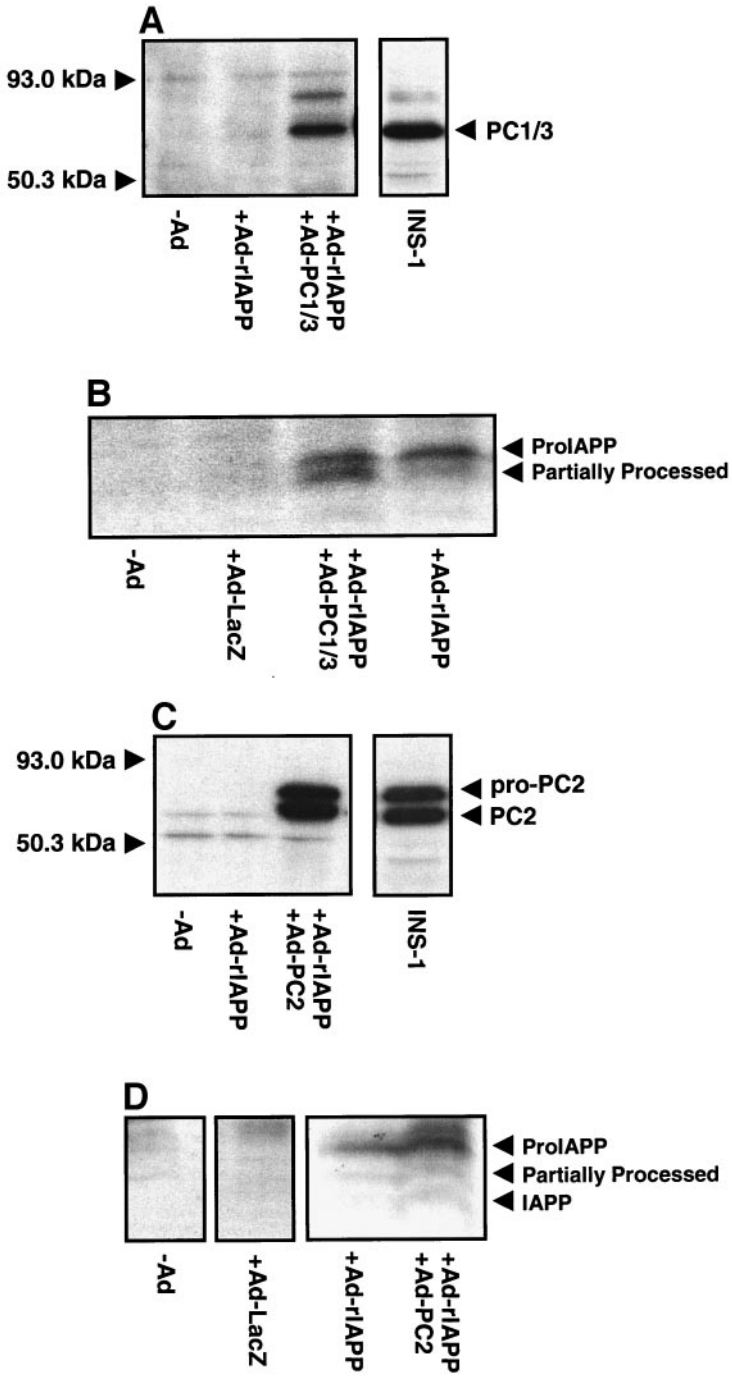

FIG. 4. Processing of proIAPP in GH3 cells transduced with Ad-rIAPP and Ad-PC1/3 or Ad-PC2. PC1/3 and PC2 protein levels in GH3 cells transduced with Ad-PC1/3 (moi 23) or Ad-PC2 (2.7) were comparable with those seen in INS-1 β-cells (*A* and *C*). GH3 cells transduced with Ad-rIAPP (15.8) and Ad-PC1/3 (*B*) or Ad-PC2 (*D*) were radiolabeled with [^{3}H]leucine (2 h) 24 h after infection, followed by immunoprecipitation and SDS-PAGE analysis. GH3 cells transduced with Ad-LacZ were used as a control to examine transduction efficiency and nontransduced labeled GH3 cells as a control to detect nonspecific labeling. Coexpression of proIAPP and PC2 (but not PC1/3) in GH3 cells resulted in the complete processing of proIAPP (~8 kDa) to mature IAPP (~4 kDa).

completely blocked at the NH_2-terminus and no mature IAPP (~4 kDa) is detectable, PC1/3 null mice islets are able to process proIAPP to mature IAPP in the absence of PC1/3, albeit not as efficiently as normal mouse islets. This finding suggests that PC2 alone must be capable of processing proIAPP at both its NH_2- and COOH-terminal cleavage sites to form mature IAPP in β-cells in vivo.

To confirm that PC2 is indeed the enzyme that contributes to cleavage at the COOH-terminus of proIAPP in the absence of PC1/3, GH3 cells were cotransduced with recombinant adenoviruses expressing rat proIAPP and PC2 or PC1/3. GH3 cells do not normally express proIAPP or detectable levels of PC1/3 and PC2 but are equipped with the regulated secretory pathway (18). Since very high levels of PC1/3 or PC2 expression might induce cleavage at sites that would not normally be observed at physiological protein levels, we chose a virus titer that caused PC2 or PC1/3 to be expressed in GH3 cells at levels comparable with those observed in the insulin-secreting transformed β-cell line, INS-1. GH3 cells expressing rat proIAPP and PC2 were able to produce mature IAPP, thus confirming that PC2 alone can process proIAPP at both its NH_2- and COOH-terminal cleavage sites. Hence, PC2 is most likely the prohormone convertase that cleaves proIAPP at its COOH-terminus in the islets of PC1/3 null mice. By contrast, in GH3 cells expressing proIAPP and PC1/3, only a partially processed intermediate (~6 kDa) was produced, suggesting that PC1/3 can initiate but not complete

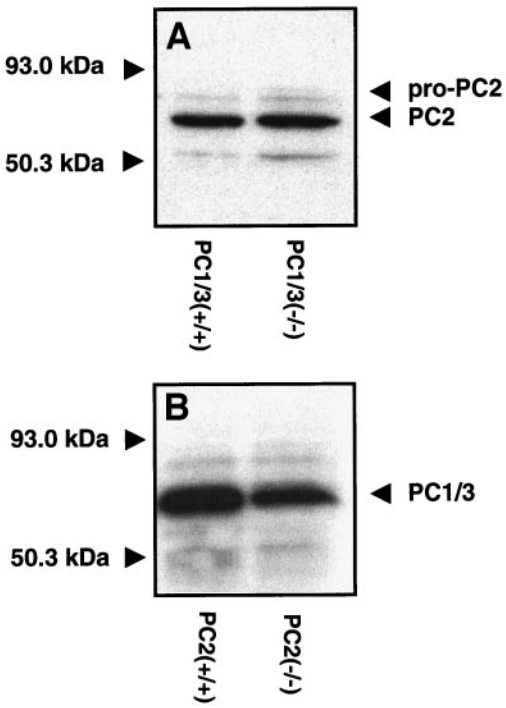

FIG. 5. Western blot analysis of the levels of PC1/3 or PC2 expression in mice lacking active PC1/3 or PC2. Aliquots of islet extracts (10 μg) were subjected to SDS-PAGE and immunoblot with appropriate antisera as described in RESEARCH DESIGN AND METHODS. Representative immunoblots of three independent experiments are shown. There was no detectable difference between PC2 protein levels in islets from PC1/3$^{+/+}$ and PC1/3$^{-/-}$ mice (*A*), whereas PC1/3 protein levels were lower in islets from PC2$^{-/-}$ mice compared with their wild-type littermates (*B*).

proIAPP processing. Note that the actual production of partially processed and mature IAPP by PC2 or PC1/3 is likely underestimated by the bands shown in Fig. 4 for two reasons. First, mature and NH_2-terminally unprocessed proIAPP have only four leucines compared with the eight in rat proIAPP, thus the radioactive signal for the mature and NH_2-terminally extended forms would only be one-half that of proIAPP, given the same amount of peptide. Second, because a 2-h radiolabeling period with no chase period was used to maximize signal, some newly synthesized proIAPP (in the last 30 min of labeling) would not have had sufficient time for processing by PC2 or PC1/3 to occur.

These data, taken together with our earlier findings in PC2 null mice (24) strongly suggest that PC1/3 cleaves proIAPP only at its COOH-terminus, whereas PC2 can cleave proIAPP at either site, although it preferentially cleaves at the NH_2-terminus in β-cells in vivo. Therefore, in PC2 null mice, cleavage at the NH_2-terminus is completely blocked (since only PC2 can cleave at this site) and cleavage at the COOH-terminus is mediated by PC1/3 and is normal, whereas in PC1/3 null mice, cleavage at the COOH-terminus is impaired (since PC1/3 preferentially cleaves at this site) but proIAPP is still processed to IAPP by the action of PC2 at both cleavage sites. These data are in agreement with earlier in vitro studies that suggested that PC2 can process proIAPP at its COOH-terminal cleavage site (30,31). Interestingly, the lack of active PC1/3 in PC1/3$^{-/-}$ islets was not accompanied by a compensatory increase in PC2 protein levels, implying that normal levels of PC2 are enough to process proIAPP at both sites. It should be noted, however, that the islet extracts used in these studies would contain both β- and non–β-cells, therefore our Western blot analysis of PC2 expression will reflect both β- and α-cell PC2 levels. Considering that both the proportion and morphology of α-cells as well as proglucagon processing are normal in PC1/3 null mice islets, with β-cells being the predominant cell type as in normal islets (21), it seems unlikely that changes in PC2 expression in α-cells would mask a major change in PC2 expression in β-cells. By contrast, PC1/3 protein expression was lower in PC2$^{-/-}$ mouse islets than in islets from their sex-matched wild-type littermates. Since PC1/3 is only expressed in β-cells and not in other islet endocrine cells, this finding likely reflects a true decrease in PC1/3 expression in the β-cells of PC2 null mice, possibly related to the apparent decrease in the proportion of β-cells observed in PC2$^{-/-}$ islets (20,24).

The slight but significant increase in the levels of proIAPP and its NH_2-terminally unprocessed intermediate in the islets of PC1/3$^{-/-}$ compared with PC1/3$^{+/+}$ mice indicates that the processing of proIAPP at its NH_2-terminus in PC1/3 null mice is also not as efficient as in wild-type animals. This finding suggests that processing of proIAPP might normally be initiated by PC1/3 at its COOH-terminus, and that the resulting COOH-terminally processed form might be a better substrate for PC2 than unprocessed proIAPP, as des 31,32 proinsulin has been shown to be a preferred substrate for PC2 compared with intact proinsulin (32). If true, these data would imply that like proinsulin processing (21,29,32,33), normal proIAPP processing in β-cells may be a two-step process in which IAPP production can be initiated by cleavage of proIAPP at its COOH-terminus by either PC1/3 or PC2, although initiation by PC1/3 is favored (Fig. 6). Cleavage of the NH_2-terminally unprocessed intermediate form of proIAPP by PC2 would then result in the formation of mature IAPP. Moreover, it appears that neither PC1/3 nor PC2 is essential for initiation of proIAPP processing, since the ~8-kDa unprocessed form is cleaved in both PC1/3 and PC2 (24) null mouse islets. It is important to emphasize, however, that these immunoblot data do not reveal the precise sequence of events in proIAPP processing and that further kinetic studies are required for this purpose.

These studies also point out two important differences between the processing of proIAPP and proinsulin in β-cells in vivo. The finding of elevated levels of des 64,65 proinsulin in mice lacking active PC1/3 (21) reflects the strong preference of PC1/3 for cleavage at the B-chain/C-peptide junction, whereas the accumulation of des 31,32 proinsulin in PC2 null mice indicates that PC2 preferentially cleaves at the C-peptide/A-chain junction (29). The presence of mature insulin and C-peptide in both PC1/3 and PC2 null mice suggests that either enzyme can process proinsulin completely in order to produce mature insulin. By contrast, only PC2 appears to be capable of complete processing of proIAPP at both its NH_2- and COOH-terminal cleavage sites in vivo. Moreover, although both PC1/3 and PC2 play an essential role in proinsulin processing in vivo, PC1/3 clearly is more important than PC2 for the processing of proinsulin in β-cells in both rodents (21) and humans (34,35). By contrast, PC2 (and not PC1/3) is the

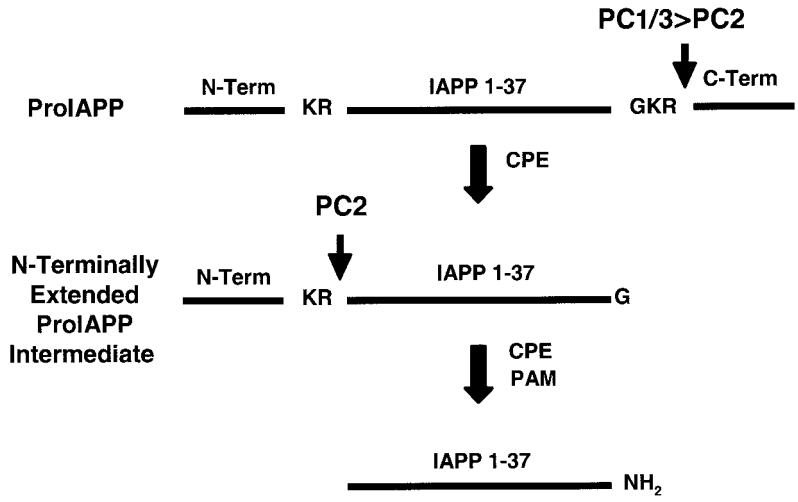

FIG. 6. Proposed model for normal processing of pro-IAPP in islet β-cells. ProIAPP processing is initiated by cleavage at its COOH-terminus by either PC1/3 or PC2, although cleavage by PC1/3 is favored at this site in β-cells. Cleavage of the NH_2-terminally unprocessed proIAPP intermediate by PC2 then results in the production of IAPP (~4 kDa). After cleavage by PC1/3 or PC2, the remaining dibasic residues are likely removed by CPE and mature IAPP is formed by removal of Gly at the COOH-terminus and amidation at this site by the peptidyl amidating mono-oxygenase complex (PAM) (37,38).

major enzyme responsible for proIAPP processing in vivo, although it appears that like proinsulin, PC1/3 and then PC2 work sequentially to process proIAPP to mature IAPP most efficiently. Finally, since the pathway for proIAPP processing closely resembles the pathway for normal processing of proinsulin in β-cells, it seems likely that as with proinsulin (12), processing of proIAPP will also be impaired in individuals with type 2 diabetes (7,13).

In type 2 diabetes, there is a disproportionate secretion of des 31,32 proinsulin from β-cells (12), likely due to either an intrinsic processing defect or decreased residence time in granules preventing adequate opportunity for PC2 cleavage of the des 31,32 proinsulin intermediate form (33,36). If the latter is true, we predict, based on our model for normal proIAPP processing, that the NH_2-terminally extended intermediate form will be disproportionately elevated in type 2 diabetes. While proIAPP has not yet been measured in human plasma, elevated levels of the NH_2-terminally extended intermediate form have been reported in human islets following prolonged culture in high glucose (15). Interestingly, immunoreactivity for the NH_2-terminally extended intermediate form of proIAPP has been reported in islet amyloid in pancreas of humans with type 2 diabetes (16), and we have previously reported that this NH_2-terminally extended form may have a high affinity for binding to heparan sulfate proteoglycans, a major component of islet amyloid (17). Identifying the mechanism by which proIAPP is processed and secreted from β-cells, in health and in type 2 diabetes, might therefore lead to a better understanding of islet amyloid formation and eventually to new approaches to prevent islet amyloid formation and preserve β-cell function in type 2 diabetes.

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