Incomplete Processing of Proinsulin to Insulin Accompanied by Elevation of Des-31,32 Proinsulin Intermediates in Islets of Mice Lacking Active PC2

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The prohormone convertases PC2 (SPC2) and PC3/PC1 (SPC3) are the major precursor processing endopeptidases in a wide variety of neural and endocrine tissues. Both enzymes are normally expressed in the islet beta cells and participate in proinsulin processing. Recently we generated mice lacking active PC2 due to a disruption of the PC2 gene (Furuta, M., Yano, H., Zhou, A., Rouillé, Y., Holst, J. J., Carroll, R. J., Ravazzola, M., Orci, L., Furuta, H., and Steiner, D. F. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6646–6651). Here we report that these PC2 mutant mice have elevated circulating proinsulin, comprising 60% of immunoreactive insulin-like components. Acid ethanol extractable proinsulin from pancreas is also significantly elevated, representing about 35% of total immunoreactive insulin-like components. These increased amounts of proinsulin are mainly stored in secretory granules, giving rise to an altered appearance on electron microscopy. In pulse-chase experiments, the mutant islets incorporate lesser amounts of isotopic amino acids into insulin-related components than normal islets. In both wild-type and mutant islets, proinsulin I was processed more rapidly to insulin, reflecting the preference of both PC2 and PC3 for substrates having a basic amino acid positioned four residues upstream of the cleavage site. The overall halftime for the conversion of proinsulin to insulin is increased approximately 3-fold in the mutant islets and is associated with a 4–5-fold greater elevation of des-31,32 proinsulin, an intermediate that is formed by the preferential cleavage of proinsulin at the B chain-C-peptide junction by PC3 and is C-terminally processed to remove Arg31 and Arg32 by carboxypeptidase E. The constitutive release of newly synthesized proinsulin from both mutant and wild-type islets during the first 1–2 h of chase was normal (<2% of total). These results demonstrate that PC2 plays an essential role in proinsulin processing in vivo, but is quantitatively less important in this regard than PC3, and that its absence does not influence the efficient sorting of proinsulin into the regulated secretory pathway.

The efficient production of insulin in the pancreatic beta cell requires that it be processed as completely as possible from its precursor form, proinsulin, into fully active hormone before it is stored and secreted. Conversion of proinsulin to insulin involves cleavages at both junctions of the connecting segment that links the B and A chains in the prohormone to liberate insulin and C-peptide (1). The recognition sites for cleavage by the converting endopeptidases PC3 and PC2 include minimally six residues (2) at the B chain-C-peptide junction (residues 29 through 35) and at the C-peptide-A chain junction (residues 62 through 68) of proinsulin. Initial cleavage occurs within these sites between residues 32 and 33 and residues 65 and 66, respectively, and the resulting C-terminal basic residue pairs Arg31-Arg32 and Lys64-Arg65 are then removed by the action of carboxypeptidase E to complete the formation of the native beta cell products. In the islet beta cells, both PC2 and PC3 are present in the secretory granules, and these two enzymes are believed to cooperate in processing proinsulin (3–5). Earlier studies from this laboratory showed that PC3 cleaves preferentially at the B-C junction, while PC2 prefers the C-A junction (4). This was in keeping with the assignment of these two convertases as the Type 1 and Type 2 calcium-dependent prohormone processing activities, respectively, as described by Davidson, et al. (6). Subsequent studies have shown that either enzyme is capable of cleaving proinsulin at both junctions to bring about its complete conversion to insulin (7). In addition, Rhodes, et al. (8) suggested that PC3 might first act at the B-C junction to produce the intermediate des-31,32 proinsulin, which they showed was a preferential substrate for PC2 (having an approximate 5-fold faster cleavage rate than intact proinsulin). According to their scheme, lack of PC2 should lead to the generation of very large amounts of the partially cleaved intermediate, des-31,32 proinsulin, which could accumulate from the action of PC3. Since des-31,32 proinsulin is the major intermediate form found along with proinsulin in the circulation normally and is increased in the serum of many patients with type II diabetes (non insulin-dependent diabetes mellitus), it has been suggested that this indicates a relative lack of PC2 action (9). We recently generated a PC2 null strain of mice that lack active PC2 altogether (10). In the present study, we have examined the effect of this mutation on the biosynthesis and processing of proinsulin in islets from the mutant mice.

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The abbreviations used are: PC, prohormone convertase; SPC, subtilisin-like proprotein convertase (29); BSA, bovine serum albumin; mP I and mP II, mouse proinsulins I and II, respectively; CPB, carboxypeptidase B; HPLC, high performance liquid chromatography.

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2 Q.-M. Gong, Y. Rouillé, and D.F. Steiner, unpublished results.
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 litter mates (PC2+/+) of the same age were used. The care of all animals used in these studies was in accordance with the National Institutes of Health and University of Chicago institutional guidelines.

Insulin Biosynthesis—Islets of Langerhans were isolated as described (11). Isolated islets were cultured overnight in RPMI medium containing 11 mM glucose and 10% fetal calf serum. After overnight culture, islets were labeled in groups of 300–400 in 100 μl of pulse medium (12) containing 27.7 mM glucose, 3 µg/ml glucagon and 500 µCi (1 µCi = 37 GBq) each of [35S]methionine (1000 Ci/mmol), [3H]leucine (300 Ci/mmol) (Amersham Corp.). After 30 min of preincubation at 10 °C, islet incubations were continued at 37 °C in a water bath for 1 h (pulse). Islets were then washed, divided into four batches and incubated for 1, 2, and 3 h in medium containing 5 mM glucose and 20 µg/ml of unlabeled methionine and leucine. After the pulse and chase incubations, the islets were washed, resuspended in immunoprecipitation buffer (0.05 M Tris-HCl, 0.1 M NaCl, 2.5 mg/ml BSA, 1% Triton X-100, pH 7.6) containing a mixture of protease inhibitors, and sonicated. The supernatants, after centrifugation for 2 min at 12,000 × g, were then treated with an immunospecific absorbent consisting of guinea pig anti-insulin IgG fraction coupled to Bio-Rad Affi-Gel 10 agarose beads (Bio-Rad) (13). Insulin and proinsulin-immunoreactive proteins were eluted from the beads with 30% acetonitrile, 1 M acetic acid.

HPLC Procedure—HPLC was carried out by a modification of the method of Davidson, et al. (6, 14) on a Waters System using a Lichrosphere 100 RP18 column, 4.6 × 250 mm, 5 μm particle size with prefilter (Altech). Buffers were 0.05 M phosphoric acid, 0.10 M sodium perchlorate, 0.01 M heptanesulfonic acid in water (A) and 90% acetonitrile in water (B). Elution was begun with 42.2% buffer B isocratic for 25 min, followed by a linear gradient increasing buffer B from 42.2 to 48.6% over 112.5 min (total time = 137.5 min). (In some experiments the gradient was ended after 75 min; total time = 100 min) Samples were dissolved in 100 μl of 50% acetic acid and filtered through a 0.45 μm Ultrafree-MC filter units (Millipore Corp.).

Proinsulin and Insulin-like Immunoreactive Products in Serum and Pancreas—Pancreatic extracts were prepared as described previously (15). Blood was taken from the retro-orbital sinus. One ml of serum was pooled and stored at −80 °C until analyzed. Serum was applied to Sep-pak® C18 cartridge (Millipore Corp.), and extracted protein containing proinsulin and insulin-like products (16) were lyophilized, dissolved in 3 μl acetic acid and applied to a 1 × 50 cm Bio-Gel P-30 column eluted with 3 μl acetic acid containing 50 μg/ml of BSA. Fractions containing proinsulin or insulin were combined, dried, and resolved with immunoprecipitate buffer following radioimmunoassay.

Immunocytochemistry—Mice were fixed by vascular perfusion with 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Samples of pancreatic tissue were processed for the conventional electron microscopy by postfixation with OsO4, dehydration with alcohol, and embedding in Epon. For light microscopic immunofluorescence, fragments of tissue were prepared as above but by omitting the postfixation step. Semi-thin (1 μm thick) sections were treated for Epon removal (17), and incubated for 2 h at room temperature with mouse monoclonal anti-proinsulin antibodies (18, 19) diluted at 0.1 μg/ml and then for 1 h with goat antiserum conjugated with 10-nm gold particles. Sections were stained with 2% uranyl acetate followed by 30 min of exposure to goat antimouse IgG conjugated with 2% methylcellulose containing 10% gold particles. Sections were checked for Epon removal (17) and incubated for 10 min before being washed and desiccated in 0.1 M NaCl. In vitro conversion of aliquots of this labeled mouse proinsulin was carried out using purified recombinant PC2 and PC3 (kindly supplied by Dr. Iris Lindberg, Louisiana State University Medical Center, New Orleans, LA). Reactions were carried out in 0.01 M sodium acetate, pH 5.5, containing 0.1% Triton X-100, 50 μM diithiothreitol, 5.0 mM CaCl2 (reaction buffer). Five μl of recombinant PC2 or PC3 (0.25 μg) was incubated in 80 μl of reaction buffer at 37 °C for 1 h. An aliquot of labeled mouse proinsulin (~80,000 dpm) was then added and incubation continued for 3 h. The reaction mixtures were neutralized by addition of 10 μl of 1 M Tris buffer, pH 8.4, an aliquot (1.4 μg) of carboxypeptidase B (CPB) (Boehringer Mannheim) was then added, and the tubes were incubated for an additional 5 min at 37 °C. The reactions were then stopped by acidification with glacial acetic acid to pH ~2.0, and the samples were analyzed by HPLC as described above. The results are shown in Fig. 1. In this system, mouse proinsulin II (mP II), which contains both 35S and 3H, elutes earlier than mouse proinsulin I (m P I) (Fig. 1A). Fig. 1, B and C, show the effects of recombinant PC2/CPB. A PC2 prep having very low activity (panel B) produced only a small amount of des-64,65 mP II (migrating between mP I and mP II), but not of mP II, indicating the more rapid attack of PC2 at the A-C junction in mP I, very likely due to the presence of the arginine at the P4 position (fourth residue upstream from cleavage site). Panel C shows the results using a more active preparation of PC2; des-64,65 intermediates of both mP I and II are now evident, but no significant amounts of any other intermediates are detectable. However, significant amounts of both insulins I and II have been produced, but relatively more of insulin I, indicating again that mP I is more rapidly processed by PC2 at both junctions (both junctions have P4 basic residues in mP I). Panel D shows the effect of PC3/CPB. No significant production of des-64,65 intermediates or of insulin is evident, but the expected des-31,32 mP intermediates can be readily identified, and here again, there is more rapid processing of mP I at the B-C junction by PC3. These
results thus establish the relative elution positions of all the important mouse proinsulin I and II conversion intermediates and insulins in this HPLC system.

RESULTS

Initial light microscopic studies of the islets of the PC2 null mice revealed marked hyperplasia of the alpha and delta cells and some diminution in beta cell mass, as was also indicated by the reduced pancreatic content of insulin to approximately 40% of normal values (10). The results of measurements of plasma proinsulin-related and insulin components in extracts of pancreas or in serum are summarized in Table I. The proportion of proinsulin was increased to 35% in pancreas, indicating a significant block in proinsulin maturation in the storage granule compartment. In serum, proinsulin values were elevated to

![Graph showing conversion of proinsulin to insulin via intermediates in a pulse-chase format.](http://www.jbc.org/)

**Table I**

Proinsulin/insulin ratio in pancreata and serum from PC2<sup>+/+</sup> and PC2<sup>−/−</sup> mice

Whole pancreata and serum pooled from animals of each genotype (age 3 months) were extracted and fractionated by gel filtration followed by radioimmunoassay of fractions. See "Materials and Methods" for details.

| Genotype | Pancreas | Serum |
|----------|----------|-------|
| PC2<sup>+/+</sup> | 0.06 | 0.15 |
| PC2<sup>−/−</sup> | 0.35 | 0.60 |

60% of total immunoreactivity, but this greater increase merely reflects the prolonged plasma half-life of proinsulin, as compared with insulin, in the circulation.

We then carried out a series of biosynthetic studies to characterize proinsulin and insulin-related proteins in the islets and to examine the kinetics of conversion of the two mouse proinsulins into their respective insulins. The two mouse proinsulins differ at five positions, three in the C-peptide and two in the insulin (21). Mouse proinsulin II contains a single methionine residue substituting for the more typical lysine at position 29 in the B chain domain, which allows it to be readily distinguished from mouse proinsulin I in dual labeling experiments using [35S]methionine and 3H-leucine (see Fig. 3). Our initial studies confirmed the existence of a partial block in insulin maturation but also revealed a dramatic difference in the incorporation into proinsulin/insulin-related components in the islets of the mutant mice. The overall incorporation of both amino acid precursors on a per islet basis was reduced to 10% or less of the normal control islet values (data not shown), a much greater reduction than indicated by the pancreatic morphometry or the assessments of insulin content, as mentioned above. This very large decrease in incorporation persisted after overnight culture of islets in 11.1 mM glucose. One possible source for this discrepancy may be the very thick mantle of
alpha and delta cells that surrounds the more centrally located beta cells (10) and that may prevent substrate access. We could partially circumvent this problem by preincubating islets in the labeling buffer at low temperature (5–10 °C) for 30 min prior to beginning the 37 °C pulse to allow diffusion of labeled amino acids into the islet core.

The results of a typical pulse-chase study are shown in Fig. 2, which shows HPLC profiles for immunopurified proinsulin/insulin components after a 1-h pulse (used to enhance incorporation) followed by a 3-h chase. Notably in the case of the wild-type islets, both proinsulins (components g and h) rapidly disappear and are replaced by the two insulins (components a and b). In contrast, the proinsulin peaks decrease more slowly in the case of the mutant islets, and the two insulins appear more slowly. These results, summarized in Figs. 3 and 4, indicate that mouse proinsulin I is more rapidly processed to insulin than mouse proinsulin II in both the wild-type and mutant islets. In the wild-type islets, low levels of des-31,32 proinsulin intermediates are detectable, but no significant levels of des-64,65 intermediates. In mutant islets this pattern is similar, but the levels of both des-31,32 intermediates I and II are increased about 5-fold to levels comprising up to 25% of the total insulin-related proteins (Fig. 4). Interestingly, while both proinsulins are essentially completely processed to insulin in wild-type islets, conversion slows during the 3rd h of chase so that about 80% of mP I is converted while only slightly more than half of the mP II is converted to insulin. These values are in good agreement with the immunoassay data, which indicated about 35% stored proinsulin in mutant pancreas (Table I). Since most of this material is in secretory granules, it is evident that conversion of proinsulin essentially stops after 4–5 h of granule maturation. Secreted levels of labeled proinsulin/insulin after 1 h of chase incubation amounted to less than 2% of total islet proinsulin/insulin radioactivity in these experiments, indicating that there was no significant elevation of constitutive secretion from either wild-type or mutant islets.

The data from the foregoing experiments are plotted in Fig. 5. Assuming pseudo-first-order initial conversion rates (1), half-lives of 0.36, 0.7, 0.8, and 2.5 h can be assigned, respectively, to PC2+/− mP I, PC2+/− mP II, PC2−/− mP I, and PC2−/− mP II. These data show that the processing of mP I is approximately 1.9-fold faster than mP II in the PC2+/− islets, while in the PC2−/− islets, it is about 3.1-fold faster. However, the rate of proinsulin processing between wild-type and mutant islets is 2.2-fold slower for mP I and 3.5-fold slower for mP II. Since mP II makes up 70% of the total proinsulin in mice, the overall retardation attributable to the lack of PC2 is approximately 3-fold.

The subcellular localization of these increased amounts of proinsulin was examined by light and electron microscopic immunocytochemistry. In contrast to normal beta cells where proinsulin is localized to the Golgi apparatus and maturing secretory granules, proinsulin immunostaining in the beta cells of the mutant mice extends to the majority of the secretory granules (Fig. 6 and 7). The secretory granules are abundant in these cells, but most of them have a pale, homogeneous appearance and lack the typical well defined central, dense crystalline core (Fig. 8). Elevated proinsulin/insulin ratios have been shown to inhibit the crystallization of insulin in vitro (22). The formation of the typical dense crystalline beta granule cores appears to be similarly inhibited by elevated granule proinsulin in vivo (for another example, see Ref. 23). All these findings are thus consistent with the incomplete state of processing of

\[ \text{FIG. 3. Kinetics of proinsulin processing in wild-type and mutant islets. Panel } A \text{ represents the results for mP I, and panel } B \text{ represents the results for mP II from the experiment shown in Fig. 2. Symbols are as follows: solid squares = intact proinsulin; closed triangles = des-31,32 intermediate; and open squares = mature insulin. See "Materials and Methods" for experimental details.} \]

\[ \text{FIG. 4. Time course of accumulation of des-31,32 proinsulin intermediates in wild-type and mutant islets during a 3-h chase following a 1-h pulse. Note the much greater accumulation of this intermediate in the mutant (closed symbols) versus wild-type (open symbols) islets. Also note that the level of des-31,32 intermediate of proinsulin I (diamonds) rises and falls faster than that of proinsulin II. Data are compiled from Fig. 3.} \]
the secretory granule contents (19), but do not indicate any alteration in the sorting of proinsulin into secretory granules in the beta cells of the mutant mice.

**FIG. 5.** Semilog plot of the disappearance rates of proinsulin-like components during a 3-h chase in islets of PC2<sup>+/+</sup> (open symbols) or PC2<sup>−/−</sup> (closed symbols) mice.

**FIG. 6.** Immunofluorescence localization of proinsulin in pancreatic islets from wild-type (A) and mutant (B) mice. Proinsulin labeling is represented by small bright spots located in the perinuclear region in normal mice (A) and in the mutant (B) by a granular immunofluorescence that extends throughout the cytoplasm. Bar = 20 μm.

**FIG. 7.** Immunocytochemical localization of proinsulin on frozen thin sections of pancreatic beta cells from normal (A) and mutant mice (B). In normal mice (A), proinsulin labeling shows the classic location over the Golgi complex and the maturing secretory granules (msg) associated with the Golgi apparatus. Mature granules (sg) appear virtually free of labeling. In mutant mice (B), proinsulin labeling is found over the Golgi complex and over the vast majority of secretory granules (sg). Bar = 0.5 μm.

**DISCUSSION**

The data presented here show that despite the lack of active PC2 about two-thirds of the proinsulin is processed to insulin in the maturing secretory granules of the islet beta cells in the PC2<sup>−/−</sup> mice. While earlier studies had indicated that PC3 cleaves preferentially at the B chain-C-peptide junction to generate des-31,32 proinsulin intermediates, these data confirm that PC3 acting alone is capable of converting proinsulin completely to insulin by processing at both junctions (7). However, these results also clearly demonstrate the preference of PC3 for the B chain-C-peptide junction in that the processing of both proinsulin I and II in the mutant islets is accompanied by significant elevations in des-31,32 proinsulin. In the absence of PC2, there is no detectable generation of des-64,65 intermediates, as would be anticipated (4, 7, 8). The level of PC3 in the mutant beta cells does not appear to be significantly altered (data not shown), and thus the results we have obtained reflect...
the normal roles of PC2 and PC3 in proinsulin processing in the beta cells.

It is of interest to compare these results on the generation of intermediate forms with those reported recently by Sizonenko and Halban (14) in studies on proinsulin processing in rat islets of Langerhans. While both rats and mice have two homologous proinsulin genes, there are differences in sequence surrounding the processing sites that affect their susceptibility to the convertases. As indicated in Fig. 9, rat proinsulin I has P4 basic residues at both positions while rat proinsulin II, like mouse proinsulin II, lacks the P4 lysine residue at the B-C junction, having methionine at this position instead. On the other hand, rat proinsulin II retains an arginine at the P4 position at the C-A junction, while mouse proinsulin II lacks basic P4 residues at both cleavage sites. Interestingly, in their study, Sizonenko and Halban (14) also found a slower rate of conversion of rat proinsulin I in the wild-type islets also indicates that PC3 prefers a P4 basic residue for cleavage at the B chain junction. The lack of any build up of mouse des-64,65 proinsulin II, but this was accompanied by a marked rise in the concentration of human des-64,65 proinsulin intermediate. A recent study on the processing of proinsulin in human islets supports these predictions (24). Moreover, the composition of human plasma proinsulin-related components, which consist predominantly of des-31,32 intermediate and intact proinsulin, but with little or none of the des-64,65 intermediate, are in accord with these considerations (25).

The extent to which prior cleavage at the B chain junction to generate intermediates that are preferentially cleaved by PC2 plays a role in the normal sequence of events in proinsulin processing will be best assessed in future studies using mice lacking PC3, when these become available. However, a recent report in the medical literature (26) of a patient who is a compound heterozygote for defects in the SPC3 gene (27) has indicated that insulin was not detectable among the circulating plasma immunoreactive insulin-like components, the more prominent being the des-64,65 proinsulin intermediate and large amounts of intact proinsulin. These findings taken altogether are consistent with the likelihood that, in both rodents and man, PC3 plays a quantitatively more important role in proinsulin processing than PC2. Even the most severe defect in PC2 production or action in the beta cell would be expected to produce only a partial block in the processing of proinsulin, but such a defect could be associated with diabetes. However, thus far no significant association of the PC2 gene locus with diabetes has been found although several polymorphisms have been noted (28). Mutations in the PC2 gene leading to lower PC2 expression or activity throughout the neuroendocrine system, on the other hand, would be less likely to be associated with diabetes in view of the much more drastic consequences of such mutations on the production of glucagon, a hormone which normally opposes the action of insulin. Thus in the PC2 null mice, blood sugar levels are low and there is no impairment of glucose tolerance despite the high proportion of circulating proinsulin-like materials (10).

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