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Proinsulin processing by the subtilisin-related proprotein convertases furin, PC2, and PC3

(prohormone/Kex2 protease/vaccinia vectors/islets of Langerhans)

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ABSTRACT **Experiments using recombinant vaccinia viruses expressing rat proinsulin I coinfected into COS-7 cells with recombinant vaccinia virus expressing human furin, human PC2, mouse PC3 (subtilisin-related proprotein convertases 1–3, respectively), or yeast Kex2 indicate that in this system both Kex2 and furin produce mature insulin, whereas PC2 selectively cleaves proinsulin at the C-peptide–A-chain junction. This is a property consistent with its probable identity with the rat insulinoma granule type II proinsulin processing activity as described by Davidson *et al.* [Davidson, H. W., Rhodes, C. J. & Hutton, J. C. (1988) *Nature (London)* 333, 93–96]. PC3 generates mature insulin but cleaves preferentially at the proinsulin B-chain–C-peptide junction. This pattern of cleavage by PC3 is similar, but not identical, to that of the highly B-chain–C-peptide junction-selective type I activity as described by Davidson *et al.*, perhaps due to the presence of a P4 arginine residue near the C-peptide–A-chain junction unique to the rat proinsulins. These results along with data presented on the expression of both PC2 and PC3 in islet β cells strongly support the conclusion that these proteases are involved in the conversion of proinsulin to insulin *in vivo*.**

Highly specific proteolysis at sites marked by basic amino acids is an important requirement for the maturation of a large number of protein precursors (1, 2). Proinsulin was the first of these precursors to be identified and characterized (3). Since that time, >150 proteins and peptides have been found to require such processing in eukaryotic organisms as diverse as yeast and humans (2). These proproteins include virtually all neuropeptides and peptide hormones, as well as a large and diverse family of membrane and viral glycoproteins, growth factors, and plasma proteins (4–6).

Despite intensive efforts, it is only recently that several endoproteases that carry out some of these cleavages have been identified and characterized. Progress in this field was stimulated by the identification in yeast of the Kex2 protease, a membrane-bound dibasic amino acid-specific endoprotease required for maturation of the pro-α-factor and killer toxin peptides (7, 8). More recently, a superfamily of mammalian serine proteases has been identified in animal cells, all members of which have structural similarity to the Kex2 endoprotease (9). Interestingly, both the yeast and mammalian proteases contain catalytic domains that are organized similarly to those of the bacterial subtilisins, i.e., having the characteristic order and spacing of the catalytically important aspartate, histidine, and serine residues of this special class of serine proteases (10, 11). Two of these mammalian enzymes, PC2** and PC3** (also called PC1), are expressed specifically in pancreatic islets, pituitary, and other neuroendocrine cells (12–16) and have been shown by heterologous gene transfer experiments to be capable of cleaving selectively at the Lys-Arg and Arg-Arg processing sites within precursors such as proopiomelanocortin and prorenin (17–20).

A third member of this superfamily, furin** (also called PACE), has also been shown to correctly process a variety of precursors, including the β-nerve growth factor and von Willebrand factor precursors, both of which have processing recognition sequences consisting minimally of Arg-Xaa-Xaa-Arg (4, 5, 21–23). However, in contrast to PC2 and PC3, furin/PACE is expressed in many tissues of the body and appears to be predominantly localized to the Golgi region in cells (4, 23). Like Kex2, furin has a membrane-spanning sequence near the C terminus that is necessary for its Golgi retention (6, 24, 25). Since many of its substrates are moved to the cell surface or secreted by small constitutive (or unregulated) vesicles, furin—or enzymes like it, such as the recently identified PACE4 (26)—appears to account for the widespread ability of cells to process growth factor and receptor precursor molecules while lacking the ability to efficiently process most prohormones.

In the experiments reported here, we demonstrate that PC2 and PC3 are localized in the pancreas to the islets of Langerhans and that both proteases selectively process specific cleavage sites in proinsulin when coexpressed with rat proinsulin I by means of vaccinia virus vectors. These results support the conclusion that these proteases are involved in the conversion of proinsulin to insulin in the maturing secretory granules and lend further support to our claim that PC2 and PC3 represent a core of endoproteases responsible for prohormone maturation at paired basic residue sites in a number of neuroendocrine cells (17).

MATERIALS AND METHODS

Antisera. Antisera were raised to peptides corresponding to amino acids 65–77 and 108–120 of preproPC2 and amino acids 95–108 and 110–122 of preproPC3. The two PC2 or the two PC3 peptides were both conjugated as a mixture to keyhole limpet hemocyanin via added C- or N-terminal cysteine residues and were then injected with adjuvants for immunization into rabbits, essentially as described (27). Sera collected 10 days after each booster injection were assessed

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**To avoid confusion due to wide usage of the designation "PC," we have recently proposed that these proteinases be designated substilisin-related proprotein convertases (SPCs): i.e., SPC1, furin; SPC2, PC2; SPC3, PC3; SPC4, PACE 4 (51).

for binding activity either to the original peptides immobilized in 96-well titer plates by ELISA (28) or to PC2 or PC3 preparations derived from a human insulinoma or cultured AtT20 cells, respectively, by Western blot analysis (29). Immunoreactivity was detected using the ECL chemiluminescence reaction (Amersham).

Vaccinia Viruses. The Rat I preproinsulin cDNA (30) was cloned into the vector pVZneo (31) and the resulting pVZneo:rInsI construct was used to introduce the Rat I preproinsulin sequence into the vaccinia virus genome (strain WR) by homologous recombination as described (32). Recombinant vaccinia viruses encoding human PC2, mouse PC3, human furin, and Kex2 have been described (17).

Cell Culture and Expression. COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal calf serum and incubated in a humidified incubator at 37°C and 5% CO_2/95% air. For expression studies, COS-7 cells were plated at 1×10^6 cells per 100-mm plate and infected with recombinant vaccinia virus at a multiplicity of infection of 5 as described (17). After incubation for 6 hr, medium was removed and replaced with DMEM minus cysteine but supplemented with 100 μCi of [^{35}S]cysteine (1 Ci = 37 GBq), and the cells were incubated further for 18 hr. The medium was then collected, any remaining cells and debris were removed by centrifugation, and the insulin-related material was immunopurified using guinea pig anti-insulin antiserum coupled to Affi-Gel 10 agarose beads (Bio-Rad). Briefly, 10 ml of labeled medium from each 100-mm plate was mixed with 0.2 ml of a 50% slurry of antiserum-coupled beads in phosphate-buffered saline (PBS) in a sealed Econo column (Bio-Rad) and gently rocked overnight at 4°C. Medium was then drained from the columns and the resin was washed sequentially with one column volume of PBS/0.1% Triton X-100, PBS, and water. Immunoreactive material was eluted by two 0.4-ml washes of 30% (vol/vol) acetonitrile/1 M acetic acid. Samples were then dried, resuspended in 50% (vol/vol) acetic acid, and analyzed.

Peptide Analysis. Peptides were resolved by HPLC on a C_4 reverse-phase column (Vydac) using a linear 27–36% acetonitrile gradient containing 0.1% trifluoroacetic acid developed over 75 min at a flow rate of 1.0 ml/min; 1.0-ml fractions were collected. Low pressure chromatography was used to verify the composition of peptides resolved by HPLC, as follows. Mature insulin was separated from proinsulin and the des-(31-32)- and des-(64-65)-proinsulin processing intermediates by P-30 gel chromatography in 3 M acetic acid/bovine serum albumin (50 μg/ml) as described (33). Cleavage at one or both processing sites within proinsulin was assessed by the presence of the normal or modified B- or A-chains of insulin determined after oxidative sulfitolysis of the samples and chromatography on Sephadex G-75 in 50% acetic acid as described (34). Human insulin, proinsulin, and the des-(31-32)- and des-(64–65)-proinsulin intermediates were used as standards (kindly provided by Bruce Frank and Ronald Chance, Eli Lilly).

RESULTS

The original identification and isolation of PC2 from a human insulinoma (12) and the finding by Northern blot analysis that PC2 and PC3 are expressed in both insulinoma tissue and isolated islets of Langerhans (13) led us to investigate the role of these enzymes in the proteolytic maturation of proinsulin. Immunohistochemical analysis of rat pancreas sections showed that both the PC2 and PC3 antisera react specifically with the majority of cells within islets of Langerhans, the site of synthesis and proteolytic processing of a number of peptide hormones including proinsulin, proglucagon, and prosomatostatin (Fig. 1 *A* and *B*). No immunostaining was observed within the exocrine pancreas. The diffuse islet staining pattern observed is consistent with the expression of both PC2 and PC3 in the β cells, which are the most abundant cell type within islets. Electron microscopic immunolocalization revealed PC2 staining within β-cell insulin-containing secretory granules (Fig. 1*C*). Western blots indicated the presence of processed forms (see refs. 36 and 37) of both PC2 (69 and 71 kDa) and PC3 (68 kDa) in mouse islets of Langerhans (Fig. 2, lanes 2 and 5), whereas PC3 was much less abundant in a human insulinoma (see refs. 12 and 13) (Fig. 2, lane 4) and PC2 was not detectable in AtT20 cells,

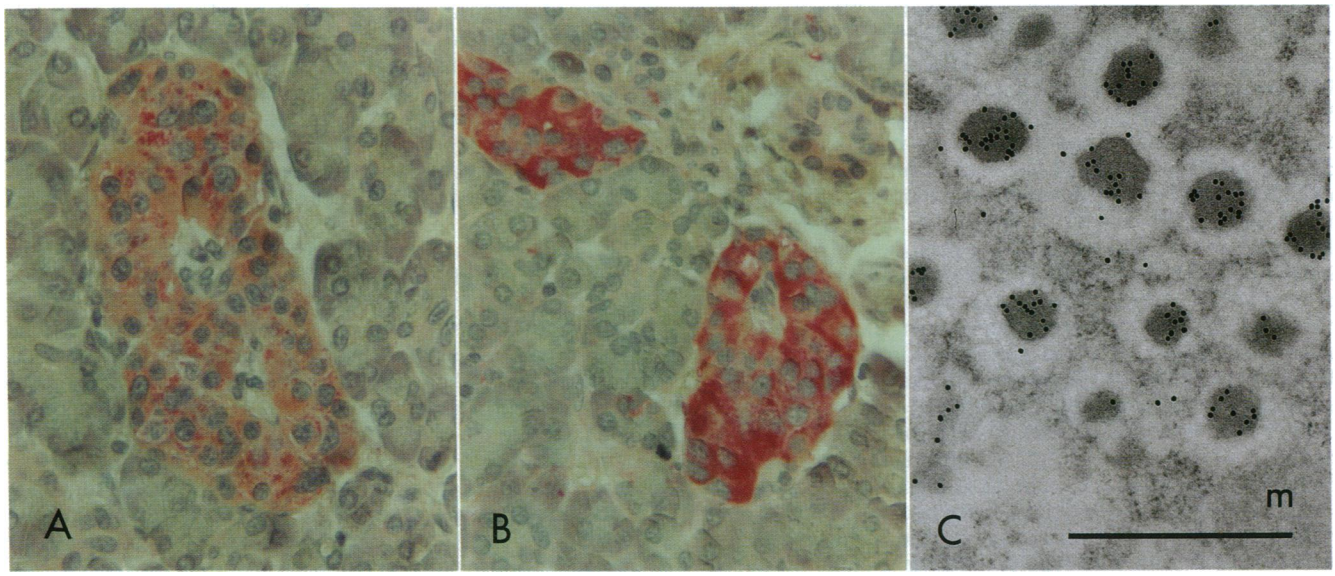

FIG. 1. Immunochemical localization of PC2 and PC3 in human islets of Langerhans with antisera against PC2 (*A*) and PC3 (*B*). Briefly, 5-μm sections of paraffin-embedded human pancreas fixed in Bouin's solution were incubated (25°C, 14 h) with a 1:200 dilution of antibody RS8 or RS20, which recognizes the N termini, respectively, of mature PC2 or PC3. Immunostaining was detected by the avidin–biotin alkaline phosphatase method using indole-fast red as substrate (35). (×200.) (*C*) Electron micrograph of a portion of the cytoplasm in a mouse β-cell. Tissue was fixed in glutaraldehyde/paraformaldehyde/cacodylate and embedded in Lowicryl K4M. Sections were treated with PC2 antibody (RS8) diluted 1:100 with 0.5% bovine serum albumin; secondary antibody was goat anti-rabbit IgG labeled with 15-nm colloidal gold and then lightly counterstained with uranyl acetate. (Bar = 1 μm.)

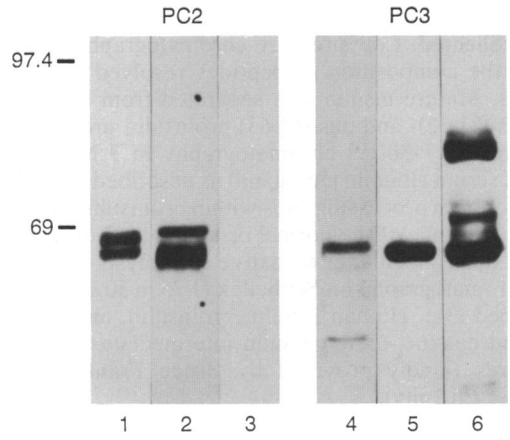

FIG. 2. Expression of PC2 or PC3 in human insulinoma (lanes 1 and 4), normal mouse islets (lanes 2 and 5), and AtT20 cells (lanes 3 and 6). Western blot analysis was carried out (29) using RS-22 (anti-PC2 raised to baculovirus-expressed holoprotein; diluted 1:1000) or an antiserum (MPC1 2B7) against the N terminus of mature PC3 (see ref. 37; diluted 1:1000; kindly provided by Iris Lindberg, New Orleans, LA). Samples analyzed were as follows: Lanes: 1 and 4, 100 μg of protein of a human insulinoma particulate fraction; 2 and 5, 100 mouse islets; 3 and 6, ≈100 μg of an AtT20-cell particulate fraction. Note that the radioautograph in lane 4 was exposed four to five times longer to intensify the band.

which express mainly 87- and 68-kDa forms of PC3 (see ref. 37) (Fig. 2, lanes 3 and 6).

To assess the ability of PC2 and PC3 to cleave proinsulin, we utilized the vaccinia virus expression system (32, 38). Recombinant viruses were constructed containing the rat preproinsulin I (vv:rPI), human PC2 (vv:hPC2), or mouse PC3 (vv:mPC3) cDNA. Since earlier studies had shown that proinsulin expressed in yeast is partially processed to insulin by the endogenous Kex2 endoprotease (39), a recombinant virus containing this yeast protease (vv:kex2) was also utilized. In addition, a furin-containing virus (vv:hfur) (4) was used to assess the ability of this protease to cleave proinsulin. After infection of the COS-7 monkey kidney cell line by the purified viruses and metabolic labeling with [^{35}S]cysteine, the immunoreactive insulin-like material secreted into the media of these cultures was analyzed by HPLC. As shown in Fig. 3*A*, medium from cultures coinfected with vv:rPI and nonrecombinant wild-type virus (vv:wt) showed a major peak (P) eluting in fractions 40 and 41. Analysis of this material by Bio-Gel P-30 and Sephadex G-75 gel chromatography before and after sulfitolysis, respectively (data not shown), showed that it corresponded to intact proinsulin. Smaller secondary peaks (N1 and N2) were eluted after proinsulin in fractions 42 and 43 (N2) and fractions 48 and 49 (N1) (Fig. 3*A*). Analysis by Sephadex G-75 gel chromatography of sulfitolyzed material from peaks N1 and N2 in subsequent experiments (see below) indicated that these products corresponded to the des-(64-65)- and des-(31-32)-proinsulin intermediates, respectively. Thus, furin or a similar protease(s) associated with the COS-7 cells (see *Discussion*) is able to carry out limited cleavage of the virus-expressed proinsulin at both the B-chain–C-peptide and C-peptide–A-chain junctions. However, this cleavage is inefficient, since no discernible level of mature insulin was observed in these experiments. Infection of COS-7 cells with wild-type (vv:wt) virus alone produced no detectable insulin-related material (data not shown). As shown in Fig. 3*B*, COS cells coinfected with vv:rPI and vv:kex2 secrete predominately mature insulin (peak INS). This processing appears to be efficient as only low levels of proinsulin and the des-dipeptide intermediates remained unprocessed. The synthesis of mature insulin in these experiments indicates that carboxypeptidases associated with the COS-7 cells are able to efficiently remove C-terminal basic residues from the insulin B chain; incubation of products with carboxypeptidase B was without effects (data not shown).

The products secreted from cells coinfected with vv:PC3 included mature insulin and both des-(31-32)- and des-(64-65)-proinsulin intermediates (Fig. 3*D*, peaks INS, N1, and N2, respectively). The higher level of the des-(31-32)-proinsulin cleavage intermediate (N2) relative to the des-(64-65)-proinsulin intermediate suggests that PC3 is more selective for cleavage at the B-chain–C-peptide processing site in rat proinsulin I. Coinfection with vv:PC2, on the other hand, showed a distinctly different mode of cleavage of proinsulin; the major product observed was des-(64-65)-proinsulin intermediate (Fig. 3*C*, peak N1), indicating that cleavage by PC2 is highly selective for the C-peptide–A-chain junction. Only very low levels of mature insulin were produced by PC2, possibly via cleavage of N2 intermediates generated by the endogenous COS-7 protease(s) (Fig. 3*A*). Interestingly, when PC2 and PC3 were both expressed together with proinsulin (Fig. 3*E*), a significant decrease in intermediate N2 [des-(31-32)-proinsulin] was observed but not of N1 [des-(64-65)-proinsulin], suggesting that N2 is a good substrate for PC2 but that N1 may not be a particularly good substrate for PC3. This finding suggests that PC3 may act *in vivo* at a somewhat earlier stage in the maturation cycle than does PC2. Finally, coexpression of furin (vv:fur) with vv:rPI showed essentially complete processing of proinsulin to insulin (Fig. 3*F*), indicating that in this system furin, like Kex2, is able to cleave efficiently at both processing sites within the rat proinsulin I molecule.

DISCUSSION

The data presented here demonstrating the ability of PC2, PC3, and furin to cleave rat proinsulin suggest that one or more of these proteases may be involved in processing of proinsulin *in vivo*. However, furin is normally expressed mainly in nonendocrine cells, such as liver, and it efficiently processes a number of blood clotting factors, growth factors, and some receptor precursors at sites having the general sequence Arg-Xaa-Lys/Arg/Xaa-Arg (5, 40–43). We have observed that furin is expressed at very low levels in normal islets (unpublished data). Moreover, expression of proinsulin in a variety of nonendocrine cell lines, as well as *Xenopus* oocytes, has shown that in such constitutively secreting cells little, if any, processing of proinsulin and other prohormones normally occurs, as also was observed in these experiments with the COS-7 cells expressing proinsulin alone, despite the expression in most of these cells of furin or related enzymes (44).

Proinsulins from most mammals have the sequence Lys-Xaa-Arg-Arg at the B-chain–C-peptide junction and Xaa-Xaa-Lys-Arg at the C-peptide–A-chain junction, whereas rat (or mouse) proinsulin I used in the experiments described here uniquely has Arg-Xaa-Lys-Arg at the C-peptide–A-chain processing site. The presence of a basic residue in the P4 position in both processing sites in rat proinsulin I may thus facilitate the processing of this proinsulin by furin (41). In this regard it is of interest that Sizonenko and Halban (45) have shown that rat proinsulin II, which has the sequence Met-Xaa-Arg-Arg, rather than Lys-Xaa-Arg-Arg, at the B-chain–C-peptide junction, is processed less efficiently at this site during insulin biosynthesis in normal rat islets. It will be of interest to examine the ability of furin, as well as PC2 and PC3, to cleave human proinsulin in comparison to the rat/mouse proinsulins I and II to resolve these issues arising from species differences in processing site sequences.

Davidson *et al.* (46) have described two proteolytic activities isolated from a secretory granule-rich fraction of a transplantable rat insulinoma that carry out the complete

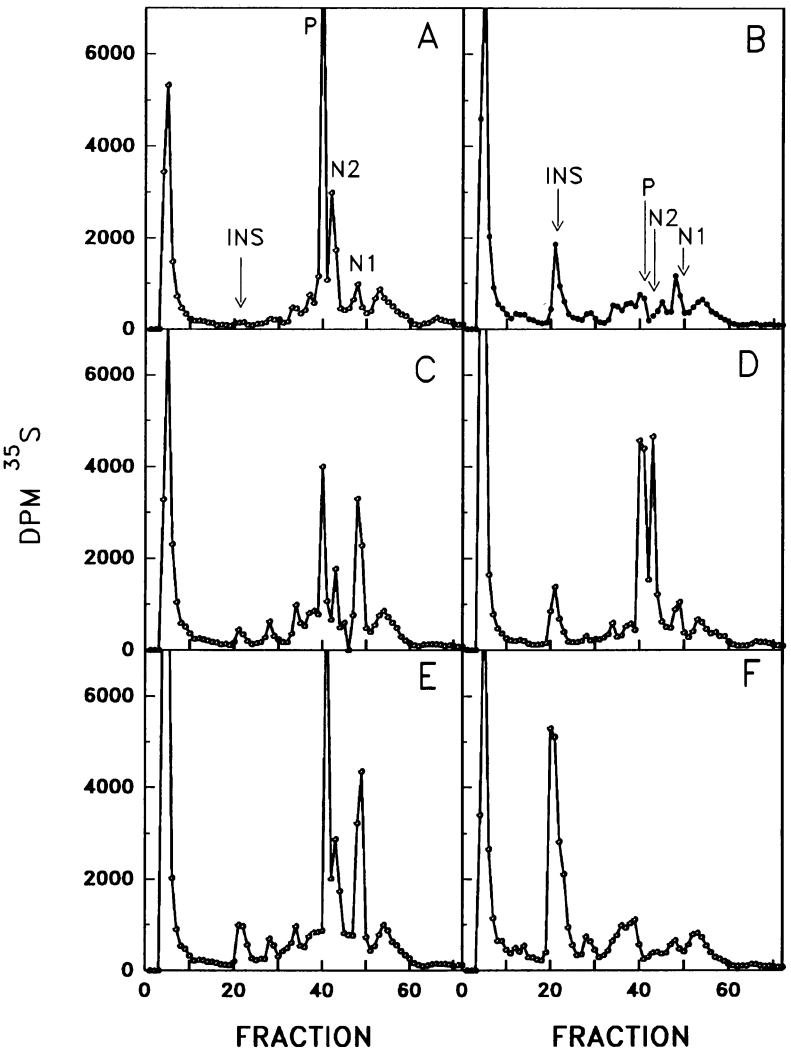

FIG. 3. Processing of rat proinsulin I in COS-7 cells by various subtilisin-related proprotein convertases. HPLC elution profiles of labeled proteins separated on Vydac C_4 columns are shown. (*A–F*) Rat proinsulin I virus-coinfected cultures are as follows. (*A*) Plus wild-type virus. (*B*) Plus *kex2* virus. (*C*) Plus PC2 virus. (*D*) Plus PC3 virus. (*E*) Plus PC2 and PC3 virus in equal amounts. (*F*) Plus furin virus. P, proinsulin; INS, insulin; N1, des-(64-65)-proinsulin intermediate; N2, des-(31-32)-proinsulin intermediate.

endoproteolytic processing of human proinsulin *in vitro*. Designated type I and II, these Ca-dependent proteases display a pH optimum of 5.5 and appear to be selective for cleavage at the B-chain–C-peptide and C-peptide–A-chain processing sites within proinsulin, respectively. The data presented here show that the cleavage selectivity of PC2 greatly favors the C-peptide–A-chain site of proinsulin; the same as that of the type II activity described by Davidson *et al.* (46). In addition, partial biochemical characterization of PC2 expressed in *Xenopus* oocytes after injection of *in vitro*-transcribed RNA has shown that PC2 is a Ca-dependent protease with a pH optimum of 5.5 and that it has a similar inhibitor profile to that of the type II activity (36, 47). In addition to the consistency of this biochemical data, Northern blot analysis has shown that the expression of PC2 is restricted to cell types known to be capable of processing prohormone precursors (13, 15). Thus with the immunohistochemical data presented here (Fig. 1) showing that the expression of PC2 in the pancreas is restricted to the endocrine islets of Langerhans, it seems likely that PC2 and the type II activity are either very closely related or identical proteases.

The relationship of PC3, furin, and the type I activity described by Davidson *et al.* (46) remains less clear. Although the type I activity of rat insulinomas appears to be highly selective for cleavage at the B-chain–C-peptide junction of human proinsulin, our results indicate that both PC3 and furin are able to cleave rat proinsulin I at both processing sites. As discussed above however, the ability of furin to efficiently cleave at the C-peptide–A-chain junction of rat proinsulin I may be related to the P4 arginine at this cleavage site; i.e., the absence of this P4 arginine in human proinsulin may restrict processing to the B-chain–C-peptide junction. Similar considerations may apply to PC3 in that, while it is able to cleave at both sites in rat proinsulin I, it is more selective for the B-chain–C-peptide junction. Moreover, its clearcut localization to the islets of Langerhans supports a role for this protease in proinsulin processing. Recent studies of Bailyes *et al.* (48) lend additional support to the conclusion that the insulinoma type I processing activity is indeed identical to PC3.

The expression system used here does not fully reproduce the conditions within normal β cells in that COS-7 cells lack a regulated secretory pathway and processing must occur either in the *trans*-Golgi or in constitutive vesicles en route to the plasma membrane. This could favor the actions of furin and Kex2, which have more neutral pH optima (8, 49) and are retained within the cells, while being suboptimal for the dense core granule processing enzymes PC2 and PC3, which are secreted in substantial amounts as judged by Western blot

analysis (data not shown) and which require a more acidic environment for optimal activity (36, 48). The limited cleavage obtained with PC2 and PC3, however, more clearly defined their cleavage site preferences in rat proinsulin I. These results confirm the ability of these proteases to carry out proinsulin processing, but data from antisense, gene knockout, or selective mutation will be required for unambiguous proof of their involvement in particular processing events *in vivo* (1, 7). Experiments of this type indicate that PC3 is essential for proopiomelanocortin processing in AtT20 cells (50). In preliminary biosynthetic experiments using antisense oligonucleotides to reduce PC2 expression, we have been able to inhibit proinsulin processing by PC2 in normal islets, accompanied by significant reduction of des-(64-65)-proinsulin intermediates (data not shown). Similar results have not yet been achieved with antisense oligonucleotides against PC3 or furin.

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