Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 340–344, January 1991
Biochemistry

Identification of a cDNA encoding a second putative prohormone convertase related to PC2 in AtT20 cells and islets of Langerhans

(precursor processing/proinsulin/kex2 protein/polymerase chain reaction)

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ABSTRACT **PC2 and furin are two recently identified members of a class of mammalian proteins homologous to the yeast precursor processing protease kex2 and the bacterial subtilisins. We have used the polymerase chain reaction to identify and clone a cDNA (PC3) from the mouse AtT20 anterior pituitary cell line that represents an additional member of this growing family of mammalian proteases. PC3 encodes a 753-residue protein that begins with a signal peptide and contains a 292-residue domain closely related to the catalytic modules of PC2, furin, and kex2. Within this region 58%, 65%, and 50% of the amino acids of PC3 are identical to those of the aligned PC2, furin, and kex2 sequences, respectively, and the catalytically important Asp, His, and Ser residues are all conserved. On Northern blots, PC3 hybridizes to two transcripts of 3 and 5 kilobases. Tissue distribution studies indicate that both PC2 and PC3 are expressed in a variety of neuroendocrine tissues, including pancreatic islets and brain, but are not expressed in liver, kidney, skeletal muscle, and spleen. The high degree of similarity of PC3, PC2, and furin suggests that they are all members of a superfamily of mammalian proteases that are involved in the processing of prohormones and/or other protein precursors. In contrast to furin, PC3, like PC2, lacks a hydrophobic transmembrane anchor, but it has a potential C-terminal amphipathic helical segment similar to the putative membrane anchor of carboxypeptidase H. These and other differences suggest that these proteins carry out compartmentalized proteolysis within cells, such as processing within regulated versus constitutive secretory pathways.**

A large number of peptide hormones, neuropeptides, and other biologically important peptides and proteins are synthesized as larger precursors which require limited proteolysis to liberate their active forms (1–6). In many cases this activation is initiated by endoproteolytic cleavage at paired basic residues within the precursor. The conversion of proinsulin to insulin, for example, begins in the early secretory granules, where cleavage at Lys-Arg and Arg-Arg residues releases the connecting peptide after it has functioned to promote the correct folding of the hormone during the initial phases of its synthesis (7). Similarly, precursors such as proopiomelanocortin (POMC) that contain several distinct biologically active peptides may undergo tissue-specific cleavage at selected dibasic and/or monobasic processing sites, giving rise to mixtures of different peptides from the same precursor.

Although widespread in mammals, this mechanism of proteolytic processing is not limited to higher eukaryotes. In the yeast *Saccharomyces cerevisiae* the α mating factor is translated in tandem copies which must first be cleaved at Lys-Arg residues to be released (8, 9), while maturation of pro-killer factor requires cleavage at both Lys-Arg and Arg-Arg residues (10, 11). The endoprotease involved in this processing has been mapped to the *KEX2* locus (12) and subsequent characterization has identified the encoded protein to be a Ca^{2+}-dependent serine protease related to the bacterial subtilisins (13–15). kex2 has also been found to be capable of processing proinsulin expressed in yeast (16) as well as processing POMC when transfected into POMC-secreting cells (17).

We have recently identified a cDNA that encodes a protein structurally related to the yeast processing protease kex2 from a human insulinoma and have called it PC2 (18). In addition, the human *FUR* gene, which was identified by its proximity to the *fes/fps* protooncogene (19), has also been shown to encode a protein (furin) which is also related to PC2 and the kex2/subtilisin family of proteases (18, 20, 21). We have now used the polymerase chain reaction (PCR) to identify and clone a third member of this growing family, which we have designated PC3, and we present here its nucleotide sequence† and data on its tissue distribution.

MATERIALS AND METHODS

Materials. Oligonucleotides were synthesized on an Applied Biosystems model 380B DNA synthesizer. RNA was isolated from AtT20 mouse anterior pituitary cells (a generous gift from R. Mains and B. Eipper, Johns Hopkins University), human insulinoma (18), βTC3 mouse cells (kindly provided by D. Hanahan, University of California, San Francisco), and rat pancreatic islets (Sprague–Dawley) by the guanidinium isothiocyanate/CsCl method (22). Rat tissue RNA samples were a generous gift of M. Gupta (Univ. of Chicago).

Amplification of DNA Fragments Encoding PC3. cDNA templates for PCR were synthesized with 5 μg of rat pancreatic islet total RNA by using Moloney murine leukemia virus reverse transcriptase as described by the supplier (Bethesda Research Laboratories), and 1/10th of the cDNA reaction mixture was used for each PCR. Reaction mixtures for PCR (0.1 ml) contained cDNA template, 100 pmol each of the degenerate primers SQ-2 and SQ-4 (Table 1), and 2.5 units of *Taq* DNA polymerase (Perkin–Elmer/Cetus) in 10 mM Tris·HCl, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$ containing dNTPs at 0.2 mM each and 0.01% gelatin. Reactions were carried out in a Perkin–Elmer/Cetus thermal cycler for 30 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min), and extension (72°C, 2 min). After fractionation on 1% agarose gels, the PCR products of interest were electroe-

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Abbreviations: POMC, proopiomelanocortin; PCR, polymerase chain reaction.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M58507).

Table 1. Oligonucleotide primers used for PCR

Primer	Sequence*	Positions†
Degenerate		
	Thr Trp Arg Asp Met Gln	405–410
SQ-2	3′-TGN ACC GCN CTP TAC GTC	1300–1317
	His Gly Thr Arg Cys Ala	208–213
SQ-4	5′-CAQ GGN ACN AGP TGQ GC	709–725
PC3		
DR	3′-GA CAT GAC CTA CTA CCG AAC C	677–697
R-14	3′-CTA ATG TGT CTG GTC GCT TA	1261–1280
Rhoda	5′-GAC ATT TAT GCC ATT TAT GA	712–731
PC2		
360	5′-CC AAC AAC AAT ATC TGT	746–762
150	3′-C ATG CCG TTG ACG TGA	1203–1218
Vector		
T3	5′-AAT AAC CCT CAC TAA AG	‡

*P = G or A; Q = T or C; N = A, C, G, or T.
†For degenerate primers, amino acid and nucleotide sequence positions correspond to PC2. Other positions are from PC3 (Fig. 1) or PC2 (18) as indicated.
‡Corresponds to the bacteriophage T3 RNA polymerase promoter sequence in the Lambda ZAP vector.

luted, and the ends were made blunt with T4 DNA polymerase and ligated into the *Eco*RV site of pBluescript (Stratagene).

Anchored PCR was carried out as described above except using oligonucleotides T3 and DR as primers (Table 1) and 10^7 phage from the amplified cDNA library as the template. To determine the size of the amplified DNA, 20 μl of this reaction mixture was fractionated on a 1% agarose gel, blotted to nitrocellulose, and probed. The products of interest were isolated from the remaining PCR reaction mixture and subcloned as described above.

Construction and Screening of the AtT20 cDNA Library. Poly(A)$^+$ RNA was selected from total RNA by using a commercial kit (5 Prime → 3 Prime, Inc.). Double-stranded cDNA was prepared with a cDNA synthesis kit (Pharmacia), followed by size fractionation with Sepharose 4B and cloning in the *Eco*RI site of Lambda ZAP (Stratagene). One-half of the library (complexity 2×10^6) was plated directly and the remaining half was amplified and stored. For screening, duplicate filters were prepared, hybridized [2× SSC (1× SSC is 150 mM NaCl/15 mM sodium citrate, pH 7.0)/50% (vol/vol) formamide, 37°C for 18 hr] with pPCR3 labeled by PCR, and washed (2× SSC/0.1% sodium dodecyl sulfate, 65°C for 1 hr) as described (23, 24). After plaque purification, the phage inserts were subcloned in the *Eco*RI site of pBluescript.

Northern Blotting. RNA was fractionated on 1% agarose/formaldehyde gels, transferred to nitrocellulose, hybridized [2× SSPE (1× SSPE is 180 mM NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA)/50% formamide, 37°C, 18 hr] with PCR labeled probes specific for PC2 or PC3 synthesized using primers 360/150, or Rhoda/R-14, respectively (Table 1), washed (0.5× SSPE/0.1% sodium dodecyl sulfate, 60°C, 1 hr), and autoradiographed with intensifying screens as described (23, 24).

Other Methods. The entire DNA was sequenced in both directions by the dideoxy chain termination method, using the Sequenase kit (United States Biochemical). Protein structural predictions were done by using the MacVector software (International Biotechnologies). PCR labeled probes were prepared as described previously (18). All other methods were as described (23, 24).

RESULTS

Identification and Cloning of PC3. To identify new mammalian members of the kex2/subtilisin-like family of proteases we designed degenerate PCR primers that were complementary to stretches of amino acid sequences highly conserved among PC2, furin, and kex2. Alignment of these proteins indicated that the closely related catalytic domains contained the most useful sequences for this purpose. These included one region adjacent to the active site histidine and a second C-terminal to the catalytically important serine (see *Materials and Methods*). After PCR amplification of cDNA prepared from rat pancreatic islet total RNA, the expected 600-base-pair (bp) products were subcloned in pBluescript and individual plasmid DNAs were probed on Southern blots with PC2. Of 12 DNAs analyzed only 1 did not hybridize with the PC2 probe. Analysis of the sequence of this clone (pPCR3) revealed that while the predicted amino acid sequence was highly similar to the corresponding regions of PC2, kex2, and furin—including the presence of a putative serine active site region—it was nevertheless distinct from these previously characterized proteases. This newly identified clone was designated PC3.

Since preliminary Northern blot analysis indicated that PC3 was present in relatively high levels in the mouse AtT20 pituitary cell line (see Fig. 3), a cDNA library was prepared from these cells to isolate a full-length cDNA clone. Three clones were identified upon screening 150,000 plaques with pPCR3, and one of these had an insert of 2.4 kilobase (kb) pairs). Comparison of the deduced amino acid sequence of this clone with the sequences of PC2, furin, and kex2 indicated that it was missing approximately 40–45 residues at its N terminus, including an initiator methionine (ATG). To isolate the remainder of this cDNA, anchored PCR was carried out with oligonucleotide primers complementary to PC3 (DR) and the bacteriophage T3 RNA polymerase promoter sequence within the Lambda ZAP vector (T3). This resulted in the isolation of a 700-bp DNA which contained the missing 126 bp of coding sequence as well as 186 bp of 5′ untranslated cDNA, bringing the total size of the cloned cDNA to 2615 bp.

The complete sequence of the PC3 cDNA is shown in Fig. 1. It contains an open reading frame of 2259 bp and is predicted to encode a protein of 753 amino acids which contains a signal peptide, a catalytic domain similar to the domains of PC2, furin, and kex2, and two potential sites for N-glycosylation, Asn-401 and Asn-645. *In vitro* translation of PC3 in the cell-free reticulocyte system gave a band of the predicted size of 84 kDa (not shown). While this manuscript was in preparation a cDNA partial sequence corresponding to nucleotides 826–1621 of PC3 was reported (25).

Comparison of PC3, PC2, Furin, and kex2. Alignment of the amino acid sequences of PC3, PC2, furin, and kex2 illustrates the similarity among these proteins (Fig. 2). Each begins with a signal peptide followed by a subtilisin-like catalytic domain. Within this 292-residue active site region PC3 shows 58%, 65%, and 50% amino acid sequence identity with PC2, furin, and kex2, respectively. While in all of these comparisons the catalytically important Asp, His, and Ser residues align exactly, it should be noted that PC3, like furin and kex2, has an Asn residue at a position (Asn-309) that has been found to be both highly conserved and catalytically important in the subtilisins (26, 27). In contrast, PC2 has an Asp residue at the analogous position (Asp-310). While such a substitution has been shown to reduce the catalytic efficiency in the subtilisins (26), the presence of this Asp in PC2 may serve to restrict its activity to acidic environments (see *Discussion*).

While the greatest similarity among all these proteins occurs within the catalytic module, they are also related throughout most of their amino acid sequences. Of the first 598 amino acids of PC3, 82%, 81%, and 74% are either identical or similar to PC2, furin, or kex2, respectively. Beyond this point, which marks the end of PC2, the sequences of these proteins diverge; furin contains a Cys-rich

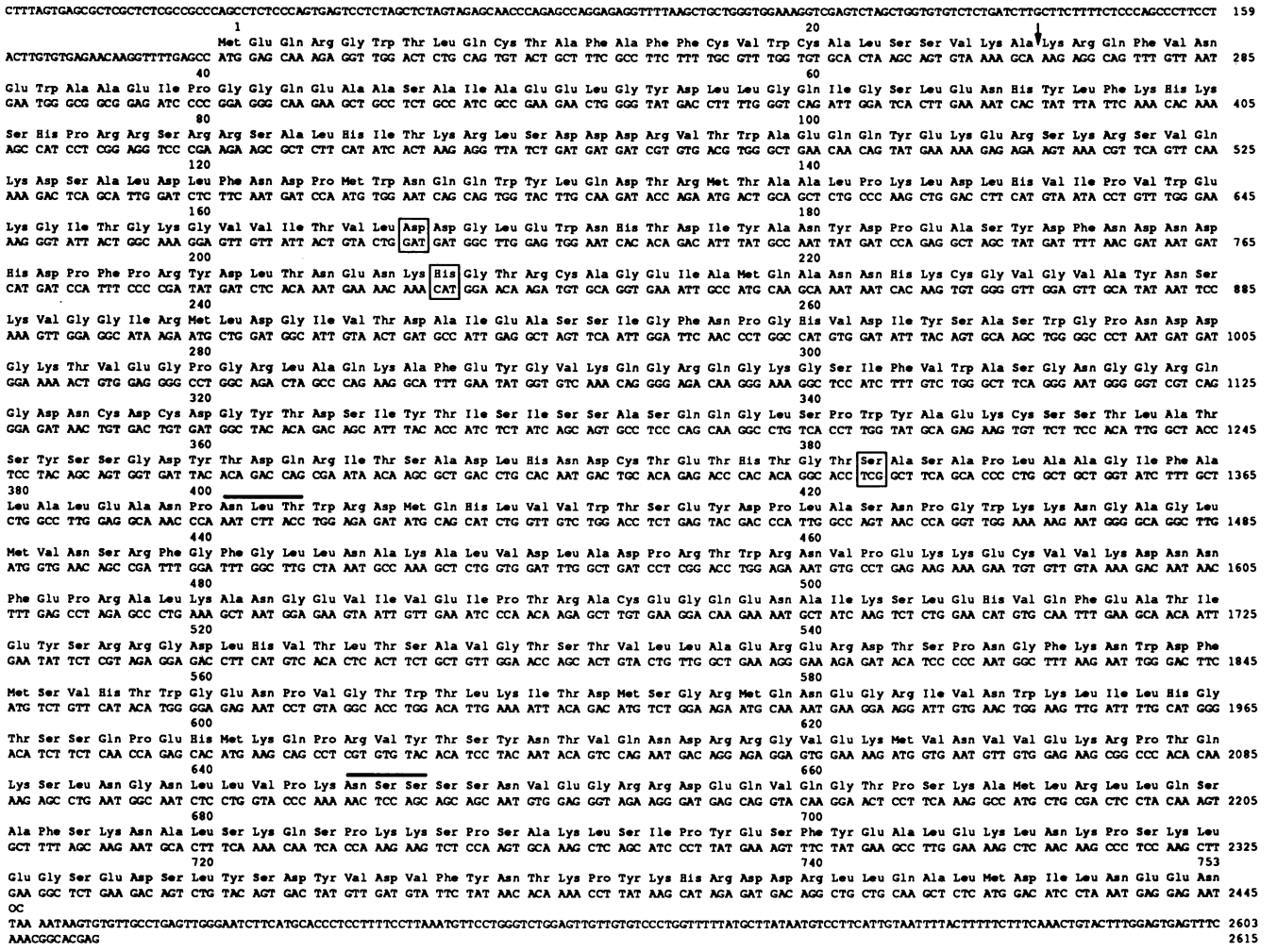

FIG. 1. Nucleotide and predicted amino acid sequence of PC3. The number of the nucleotides is indicated at the end of each line. The arrow indicates the putative cleavage site of the signal peptide. The proposed active site Asp, His, and Ser residues are boxed. Solid bars indicate consensus sequences for N-glycosylation.

region similar to those found in the receptors for insulin, insulin-like growth factor, and epidermal growth factor, while kex2 contains a Ser/Thr-rich domain believed to be the site of O-glycosylation. Although both furin and kex2 contain membrane-spanning domains, neither PC3 nor PC2 possesses such regions. However, both of these proteins contain potential C-terminal amphipathic α-helical segments extending from Glu-621 to Ile-634 in PC2, and Asp-739 to Glu-751 in PC3 (Fig. 2), which may function as membrane anchors as recently proposed for carboxypeptidase H (28).

Expression of PC2 and PC3 mRNA Is Restricted to Neural and Endocrine Cells. To determine the tissue distribution of PC2 and PC3, we carried out Northern blot analysis of total RNA isolated from a variety of tissues and cultured cell lines. As shown in Fig. 3, both PC2 and PC3 mRNAs are readily detectable in rat pancreatic islets and brain, mouse (βTC3) and human insulinomas, and the mouse AtT20 pituitary cell line. However, PC2 and PC3 transcripts were not detectable in liver, kidney, heart, intestine, skeletal muscle, or spleen. Interestingly, in tissues where they are expressed, the levels of PC2 and PC3 transcripts appear inversely related; where PC2 mRNA levels are high—such as in brain, islets, and the insulinomas—PC3 levels are low. Conversely, in the AtT20 cell line, PC3 mRNA levels are high and PC2 levels are low. The possible significance of this relationship is discussed below.

The Northern blots indicate that PC2 and PC3 hybridize to two mRNAs in virtually all tissues tested; PC3 hybridizes to 3- and 5-kb transcripts (Fig. 3*B*), whereas PC2 hybridizes to 2.8- and 5-kb transcripts (Fig. 3*A*) as reported previously (18). These larger transcripts are also apparent when AtT20 or human insulinoma poly(A)$^+$ RNA is probed with PC3 (not shown) or PC2 (18), respectively, and thus cannot be due to nonspecific hybridization of the probes to 28S ribosomal RNA (Fig. 3). Moreover, when Northern blots of AtT20 RNA were hybridized with probes specific for the N-terminal, C-terminal, or catalytic domains of PC3, both the 3- and 5-kb transcripts hybridized with each probe (not shown), suggesting that both are derived from the same gene. The molecular basis for this size difference is unknown but may be due to differences in the length of the 3′ untranslated region of the transcripts. Divergence in the 3′ untranslated region has been observed for a number of mRNAs in both normal (29) and tumor (30) cells and has been shown to be capable of influencing both the efficiency of translation (31) and the stability of the mRNA (32). On the other hand, the larger transcripts might represent alternatively spliced mRNA, which may include forms containing a transmembrane domain.

DISCUSSION

We have used PCR to identify a cDNA (PC3) from mouse AtT20 anterior pituitary cells that represents another member of a growing class of mammalian subtilisin-like proteases that appear to represent cellular precursor processing enzymes.

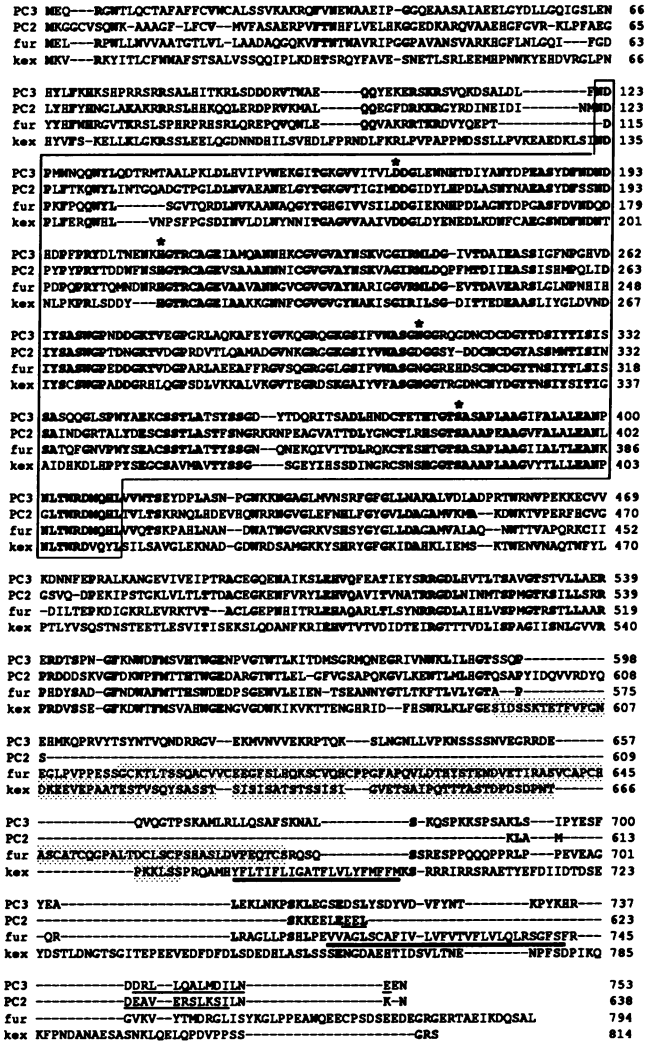

FIG. 2. Amino acid sequence alignments of PC3, PC2, furin, and kex2. The subtilisin-like catalytic domains are boxed and the catalytically important Asp, His, Asn(Asp), and Ser residues are indicated with asterisks. Shaded residues correspond to the Cys-rich and Ser/Thr-rich regions of furin and kex2, respectively. Transmembrane segments in furin and kex2 are indicated by heavy underline, whereas the potential α-helical amphipathic segments in PC3 and PC2 are lightly underlined. Amino acids are indicated by their single-letter symbols and are numbered for each sequence at the end of each line. Residues at each position that are identical between at least three of the proteins are indicated in boldface. Gaps introduced into the alignment are indicated by hyphens.

PC3 is a 753-residue protein that begins with a signal peptide and contains a 292-residue domain homologous to the catalytic modules of both kex2 and the bacterial subtilisins, as well as to the recently identified human gene products PC2 (18) and furin (20, 21). Within this region 84–88% of the amino acids of PC3 are either identical or similar to those of the aligned PC2, furin, and kex2 sequences. In addition, the similarities among these four proteins extend over the majority of their sequences where, within the first 598 amino acids, the percent of identical and conserved residues ranges from 74% to 82% for any pair of these proteins.

Tissue distribution studies show that expression of PC2 and PC3 is apparently restricted to neural and endocrine cell types, including pancreatic islets, brain, insulinoma, and AtT20 cells. This limited pattern of expression, as well as the structural similarity of PC2 and PC3 to the yeast precursor processing protease kex2, supports the notion that these proteases are involved in the maturation of prohormone

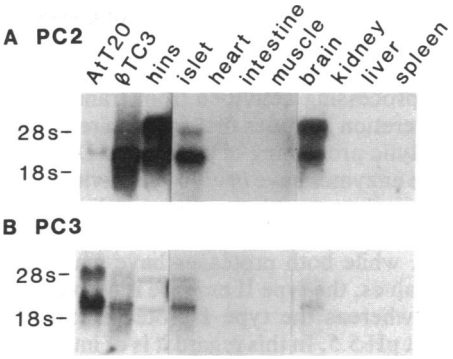

FIG. 3. Expression of PC2 and PC3 in various adult tissues and cell lines. Ten micrograms of rat pancreatic islet total RNA and 25 μg of total RNAs from each of the indicated tissues and cell lines were electrophoresed in duplicate on 1% agarose/formaldehyde gels and probed for PC2 (*A*) or PC3 (*B*). The mouse AtT20 and βTC3 cell lines and the human insulinoma (hins) are described in *Materials and Methods*. All other tissues are from rat. The positions of the 28S and 18S rRNAs are indicated.

precursors and other proproteins secreted via the regulated secretory pathway. In support of this, preliminary results of light and electron microscopic immunocytochemical staining of pancreas sections, using antibodies developed against synthetic peptides derived from the PC2 sequence, indicates that PC2 is localized to the secretory granules of the β-cells within the islets of Langerhans but is not present in the exocrine pancreas (S.P.S., H. Swift, T. Montag, and D.F.S., unpublished results). In addition, while tissues expressing PC2 also express PC3 (and vice versa) their relative levels of expression vary in different neuroendocrine tissues (Fig. 3). This raises the interesting possibility that differences in the relative expression of PC2 and PC3, or of other enzymes related to them, may account for tissue-specific differences in the pattern of processing of the same precursor, such as POMC in the anterior versus the intermediate lobes of the pituitary (33). In contrast, while furin is also related to kex2, the finding that it is expressed in a wide range of tissues, including liver (34), suggests that it may act mainly in the trans Golgi on proteins secreted via unregulated or constitutive pathways. Such processing might include important early cleavages in precursors such as the egg-laying hormone of *Aplysia* (35), proalbumin, and related hepatic precursors (40, 41), as well as the processing of a diverse group of membrane glycoproteins such as the insulin proreceptor (36), human immunodeficiency virus gp160, and influenza hemagglutinin (37).

The hydrophobic transmembrane segments of furin and kex2 indicate that they are membrane-bound proteins. Indeed, recent studies have indicated that kex2 requires its transmembrane and cytosolic domains for optimal function (14, 38). Although the available data indicate that PC2 and PC3 lack a hydrophobic membrane-spanning segment, they may be associated with the membrane by other mechanisms. For example, Fricker *et al.* (28) have recently identified a membrane anchoring region within the highly charged C-terminal segment of the secretory granule processing enzyme carboxypeptidase H. This region is predicted to form an amphipathic helix which may interact in a pH-dependent manner with the secretory granule membrane (28). Structural predictions of PC2 and PC3 reveal that both proteins also contain potential C-terminal amphipathic α-helical regions (Fig. 2) which may function similarly as membrane anchors. However, it is not yet clear how such a mechanism of membrane association may enhance or modify the activity of these proteases. Whether PC2, PC3, and carboxypeptidase H, as well as other related posttranslational processing

enzymes, associate into "processing complexes" is also an interesting question.

Davidson *et al.* (39) have recently described two Ca^{2+}-dependent processing activities from transplantable rat insulinoma secretion granules that are both required for proper endoproteolytic processing of proinsulin. Designated types I and II, these enzymes have *in vitro* selectivity for the Arg-Arg and Lys-Arg cleavage sites of proinsulin, and they have maximal activities at 1.0 mM and 0.1 mM Ca^{2+}, respectively. In addition, while both proteases have maximal activity at acidic pH values, the type II enzyme is active over a broader pH range, whereas the type I protease exhibits a narrow maximum at pH 5.5. In this regard it is of interest to note that while PC3, furin, and kex2 contain conserved Asn residues at positions 309, 295, and 314, respectively (Fig. 2), PC2 contains an Asp at the analogous position (Asp-310). Studies with the subtilisins have shown that in addition to the Asp, His, and Ser residues of the catalytic triad, this Asn residue also plays an important role in the catalytic mechanism (26, 27). Following nucleophilic attack by the serine hydroxylate on the carbonyl of the peptide bond to be cleaved, the resulting transition-state complex is stabilized in part by hydrogen bonds formed between the enzyme and the oxyanion that develops from the carbonyl oxygen of the substrate. These hydrogen bonds are donated by the main-chain amide of the catalytic serine and δ amide of the conserved Asn residue. Substitution of Asp for this Asn has been shown to reduce catalytic efficiency in the subtilisins when they were assayed at their alkaline pH optimum (26). However, such a substitution in PC2 may represent a mechanism for restricting the activity of this enzyme to acidic pH by requiring that Asp-310 be protonated to donate the hydrogen bond necessary to stabilize the transition state intermediate. In agreement with this prediction our preliminary observations indicate that PC2 has a narrow acidic pH optimum similar to that observed for the type I insulinoma enzyme (K. Shennan, S.P.S., D.F.S. and K. Docherty, unpublished data). Similarly, if the Asn residue in PC3 allows this protease to function over a broader pH range, then it may well be that PC2 and PC3 represent the two endoproteolytic processing activities necessary for the conversion of proinsulin.

We are grateful to Dr. Graeme Bell for critical reading of this manuscript and helpful suggestions, Paul Gardner for oligonucleotide synthesis, Lisa Phillips and Raymond Carroll for expert technical assistance, and Florence Rozenfeld for help in preparing this manuscript. Work from this laboratory is supported by the Howard Hughes Medical Institute and by National Institutes of Health Grants DK 13914 and DK 20595.

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