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cDNA structure of the mouse and rat subtilisin/kexin-like PC5: A candidate proprotein convertase expressed in endocrine and nonendocrine cells

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ABSTRACT **By using reverse transcriptase/PCR and oligonucleotide sequences derived from conserved segments (including the conserved RRGDL sequence) of the known proprotein convertases (PCs) PC1, PC2, furin, and PC4, we identified a subtilisin/kexin-like PC called PC5 in both mouse and rat tissues. The composite structure (2.85 kb) was deduced from the analysis of the reverse transcription/PCR products combined with the sequence from a clone isolated from a cDNA library made from corticotropin-activated mouse adrenocortical Y1 cells. The deduced cDNA structures of mouse PC5 and rat PC5 showed that the closest homologue is PACE4. Furthermore, like furin, *Drosophila melanogaster* (d) dfurin2, and PACE4, PC5 shows the presence of a C-terminal Cys-rich domain containing either 5 (PC5 and PACE4) or 10 (dfurin2) repeats of the consensus motif Cys-Xaa$_2$-Cys-Xaa$_3$-Cys-Xaa$_{5-7}$-Cys-Xaa$_2$-Cys-Xaa$_{8-15}$-Cys-Xaa$_3$-Cys-Xaa$_{9-16}$. The richest sources of rat PC5 mRNA (3.8 kb) are the adrenal and gut, but it can also be detected in many endocrine and nonendocrine tissues. Corticotropin-stimulated adrenocortical Y1 cells showed an increased expression of PC5 mRNA, suggesting an upregulation by cAMP. *In situ* hybridization of rat brain sections demonstrated a unique distribution of PC5 compared to PC1, PC2, and furin.**

Recently, five mammalian subtilisin/kexin-like proteinases have been implicated in prohormone and proprotein activation (1, 2). These include PC1 (3–5), PC2 (3, 6), furin (7), PACE4 (8), and PC4 (9, 10). Based on their tissue distribution, we can subdivide these proprotein convertases (PCs) into three groups: furin (7) and PACE4 (8), which exhibit a ubiquitous tissue distribution; PC1 and PC2 (3–6, 11), which are mostly found in neural and endocrine cells; and PC4, which is primarily expressed within testicular germ cells (9, 10) and ovaries (10). Cellular coexpression of furin, PC1, and PC2 with polypeptide precursors revealed that these enzymes exhibit exquisite selectivity of cleavage after pairs of basic residues (1, 2, 12–15).

We have identified (2) a 450-bp cDNA fragment of a rat PC that we called PC5, and here we present the complete cDNA sequences from mouse (m) and rat (r).§ PC5 exhibits a C-terminal Cys-rich domain and is most similar to PACE4. In contrast, it is not as homogeneously expressed as PACE4 or furin and its mRNA is upregulated by cAMP.

MATERIALS AND METHODS

Reverse Transcription (RT)/PCR and Isolation of PC5 Clones. The PCR protocol and oligonucleotides used in the RT/PCR for the isolation of the 450-bp rat testis PC5 were the same as those used in ref. 10. This cDNA sequence allowed the synthesis of a 62-mer sense primer, 5′-TCCCCCTCACCCGGCAAGCCTTTGAGAATGGTGTGAGAATGGGTCGGAGAGGCCTTGGTTCT-3′. The degenerate consensus reverse primer 5′-GTGAATTCGAT(C/A/G)(T/G)(T/G/C)(A/C/G)AGGTC(C/T/A)CC(A/T/G)CGGCG-3′ was based on the conserved RRGDL sequence of the PCs (1, 2). These primers were used in RT/PCR on total RNA isolated from mouse adrenocortical Y1 cells stimulated for 24 h by 0.1 μM corticotropin [(ACTH)-(1–24)] and from rat adrenal tissue. This allowed the identification of a 728-bp segment of PC5. Using an active site Asp sense primer based on the structure of mPC1 (4) and an antisense primer, 5′-AGGTC(T/C)CC(G/T)CGGCGTGGGTGTGTGATGGTAAT-3′, containing the RGD motif, we isolated a 1080-bp cDNA. By using an internal primer, 5′-TGCGCTCCATCTACAAAGCCTCAGGCTGCTCGGATAATC-3′, and an antisense oligo(dT) adaptor, 5′-GACTCGAGTCGACATCGATCGT$_{17}$-3′, we amplified a 1.4-kb segment encompassing the 3′ end of the PC5 cDNA (nt 1489–2847). We also obtained an oligo(dT)-primed custom-made library in λ Zap II vector (Stratagene) using 10 μg of poly(A)$^+$ RNA isolated from Y1 cells stimulated with 0.1 μM ACTH-(1–24). The screening of this library allowed the characterization of the mPC5 cDNA sequence from nt 373 up to the poly(A) tail (Fig. 1). The 5′ ends of PC5 were finally obtained using RT/PCR on total RNA isolated from mouse and rat duodenum, using an antisense oligonucleotide, 5′-TCGCAACTTGCCAGAGCATCGTAG-3′, and a sense oligonucleotide, 5′-GGCGGGCGAAGGCGGCGAAGCG-3′, based on the sequence of the 5′ end of a cDNA coding for mPC6 obtained from a very recent inclusion in GenBank deposited by Nakagawa *et al.* (accession no. D12619). All PCR fragments were subcloned in PCRII vector (Stratagene) and at least three clones were completely sequenced, using T7, SP6, and specific 5′-end-labeled fluorescent primers and a Pharmacia automatic ALF DNA sequencer.

Northern Blot and *in Situ* Hybridization Analyses. Northern blot analysis (11) was carried out using an antisense 837-base (residues 1089–1925) ^{32}P-labeled rPC5 RNA probe and 5 μg of total RNA obtained from various tissues. *In situ* hybridization was carried out as described (11) using the same RNA probe labeled with ^{35}S-labeled UTP.

RESULTS

cDNA Sequence Analysis of rPC5 and mPC5. The composite cDNA sequence of mPC5 and rPC5 is shown in Fig. 1. Calculations reveal that within the deduced sequences, mPC5 and rPC5 exhibit an overall 98% protein identity and 95% nucleotide sequence identity. According to the von Heijne

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Abbreviations: PC, proprotein convertase; ACTH, corticotropin; RT, reverse transcriptase; m, mouse; r, rat; d, *Drosophila*.

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§The sequences reported in this paper have been deposited in the GenBank data base (accession nos. for mPC5 and rPC5, L14932 and L14933, respectively).

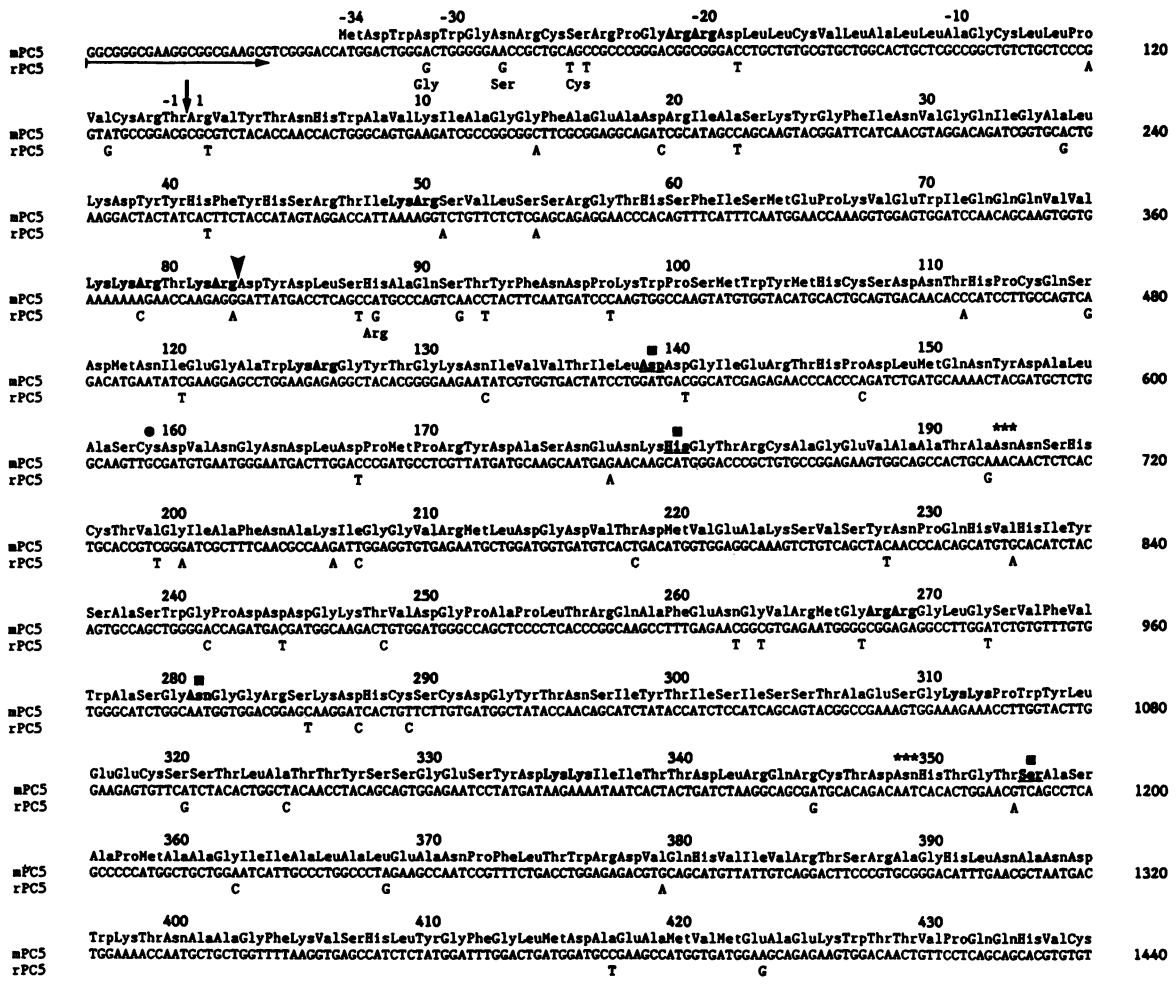

FIG. 1. (*Figure continues on the opposite page.*)

criteria (16), a 34-aa signal peptide is predicted (Fig. 1), resulting in an 881-aa enzyme. Alignment of PC5 and the other PCs revealed that, as in PC1, PC2 (13), furin, PACE4, and PC4, the PC5 sequence contains a Lys-Arg^{82} pair of basic residues at the presumed N-terminal zymogen activation site (Fig. 1). The mature enzyme, obtained after the excision of the 82-aa prosegment at Arg-Thr-Lys-Arg^{82}, therefore, would contain 799 aa and end either with Gly or a C-terminally amidated Gln. The three active-site residues Asp^{139}, His^{180}, and Ser^{354} and the catalytically important Asn^{281} (17) are at similar relative positions to those found in PC1 (3–5), PC2 (3, 6), furin (7, 15), PACE4 (8), PC4 (9, 10), kexin (18), and subtilisins (19). We also note the presence of 56 Cys; 2 Lys-Arg, 2 Arg-Arg, 2 Arg-Lys, and 4 Lys-Lys pairs of basic residues; and 1 Lys-Lys-Arg tribasic sequence. It is interesting that PC5 (Fig. 1) and kexin (18) contain a Cys residue (indicated by a solid circle in Fig. 1) between the Asp^{139} and His^{180} of the active site. Furthermore, there are six Asn glycosylation sites, one of which is unique to PC5 and PACE4 (8) and is present between the active site His^{180} and the catalytically important Asn^{281}, in a predicted β-bend secondary structure (19). A similar site is also found in *Drosophila melanogaster* (d) dfurin1 (20). Within a predicted extended β-sheet (19), a second N-glycosylation site is found 5 residues before the active site Ser^{354}, 4 aa away from a similar site found in PC2 (3, 6). Assuming ≈1.5 kDa for each of the six N-glycosylation chains, the predicted molecular mass of mature PC5 is ≈97 kDa.

Alignment of the catalytic domain (segment 83–571) of mPC5 with the other PCs revealed that PACE4, furin, PC4, PC1, PC2, kexin, and subtilisin BPN[1] exhibit 69.5%, 60.2%, 53.1%, 52.8%, 48.9%, 40.5%, and 27.4% amino acid sequence identity, respectively. The presence of conserved amino acids surrounding these four active site residues (19) strongly argues that PC5 is a member of the subtilisin/kexin family of PCs. As in all mammalian paired basic amino acid PCs reported to date (1–10), PC5 also exhibits a conserved RRGDL structure, which contains the RGD sequence necessary for binding of certain proteins to cell surface integrins (21).

As originally observed in the structure of furin (7), *D. melanogaster* dfurin2 (22), and PACE4 (8), PC5 exhibits a Cys-rich region at its C terminus, beginning at residue 604 (Fig. 1). This large domain, which contains 44 Cys residues, appears to be composed of five repeats of the consensus motif: Cys-Xaa$_2$-Cys-Xaa$_3$-Cys-Xaa$_{5-7}$-Cys-Xaa$_2$-Cys-Xaa$_{8-15}$-Cys-Xaa$_3$-Cys (Fig. 2), with 9–16 aa separating each repeat. PACE4 and dfurin2 also exhibit 5 and 10 equivalent motives, respectively (Fig. 2). Notice that the repeats 6–10 of dfurin2 are unique to this *D. melanogaster* PC and that repeat 1 in all enzymes lacks the third Cys residue. In mammalian furin, only the second of the two Cys repeats (7) exhibits a match of 7 out of the 8 Cys residues (22). Four N-glycosylation sites are found in motifs 1, 3, 4, and 5 of PC5 and only the last one is conserved in PACE4.

As originally proposed by Roebroek *et al.* (22) for dfurin2, the C-terminal domain of PC5 shows some homology to Cys-rich domains of other proteins (Fig. 2). These included the *Giardia lamblia* surface antigen CRP170 (23), the let-23 surface-expressed tyrosine kinase from *Caenorhabditis ele-*

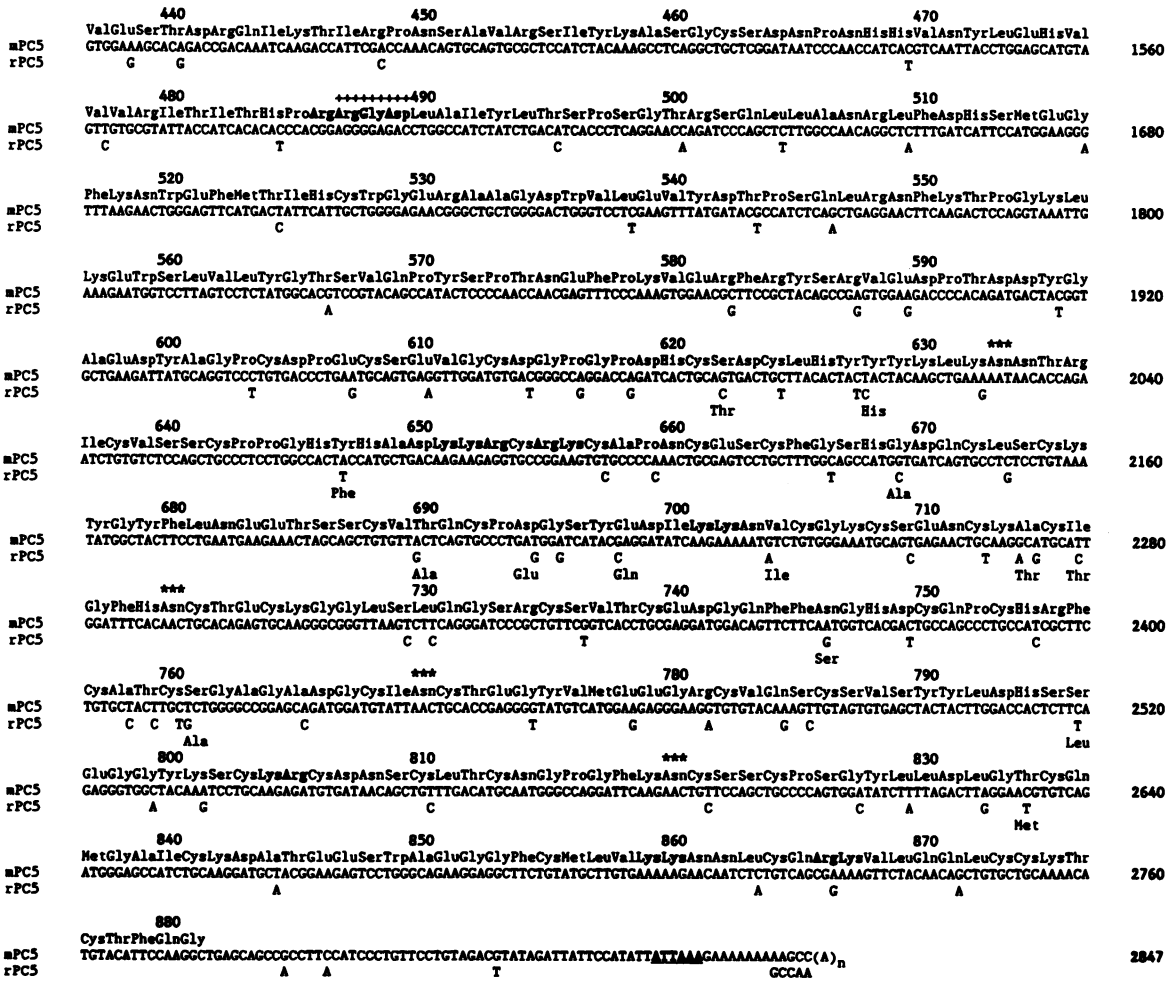

FIG. 1. cDNA and deduced amino acid sequences of mPC5 and rPC5. The active site Asp (■), His (■), and Ser (■), the important Asn (■) residue, the six potential N-glycosylation sites (***), the RGD (Arg-Gly-Asp) sequence, and a specific Cys (●) are emphasized. Only the differences between the rat and mouse sequences are shown. The variant polyadenylylation signal ATTAAA is underlined. The predicted sites of the signal peptidase cleavage (16) and zymogen activation (1, 2, 13) are depicted by an arrow and an inverted triangle, respectively. Pairs of basic and tribasic residues are shown in boldface type. The horizontal arrow at the top emphasizes the mPC5 oligonucleotide used to obtain the rat 5′-end sequence.

gans (24), and the epidermal growth factor receptor homologue (DER) from *D. melanogaster* (25). No conservation of amino acid sequence is apparent between the Cys residues.

Northern Blot Analysis of the Tissue Distribution of rPC5 and *in Situ* Hybridization in the Central Nervous System. Essentially the same results were obtained when a 390-bp (residues 805–1195), an 837-bp (residues 1089–1925), or a 1355-bp (residues 1090–2445) complementary RNA probe was used. As shown in Fig. 3, PC5 is widely distributed over many rat tissues, with a high abundance in the gut, the adrenal glands, ovaries, and lungs. The richest sources are the gut, the duodenum, jejunum, and ileum. Although not shown, PC5 transcripts were localized mainly within the epithelial cells of the small intestine (M.K.-H. Schäfer, personal communication). We also note the presence of rPC5 mostly in the anterior lobe of the pituitary and in the thyroid. In general, within the central nervous system, the levels of PC5 are lower than those observed for PC1 and PC2 (3, 4, 11). Notice the very low levels of PC5 in liver. The major form of rPC5 observed migrated with an apparent molecular size of 3.8 kb. Minor forms of ≈6.5 and ≈7.5 kb were also observed, the ratio of which varies tissue-specifically. For example, in the gut we mainly observed the 7.5-kb form and in the lung we mainly observed the 6.5-kb form, whereas both forms are equally expressed in the adrenal gland. We also note that within the reproductive organs the levels of PC5 are more elevated in the female (ovaries) than in the male (testis). From the other tissues examined (some of which are not shown in Fig. 3), we deduced that PC5 is also expressed mainly in the adrenal cortex, oviduct, heart atria and ventricles, and esophagus, with very low levels in seminal vesicles, kidney, thymus, spleen, muscle, submaxillary gland, and pancreas.

We have also examined the distribution of PC5 within a number of cell lines including AtT-20, GH3, βTC3, BSC40, and Y1 cells. In general we find low levels of PC5 in most of these cells, except for the Y1 cells, where significant amounts of PC5 mRNA are detected, especially after stimulation with ACTH (Fig. 3*F*), suggesting a regulation by cAMP (26).

Fig. 4 depicts a comparative autoradiogram of coronal sections of rat brain hybridized with complementary RNAs encoding PC1, PC2 (11, 27), furin (27), or PC5. The *in situ* hybridization of rat brain tissues demonstrated a unique distribution of PC5 compared to PC1, PC2, and furin. This is especially evident from its abundance within the CA3 region of the hippocampus, with lower levels in the dentate gyrus. Notice the high levels of PC5 within the amygdaloid nucleus (Fig. 4*D*).

DISCUSSION

PC5 represents a member of the family of kexin/subtilisin-like PCs, with PACE4 being the closest homologue. Both

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                *  *  *  *     *  *              *     *
mPC5     CDPECSEV--GCDGPGPDHCSDCLHYYYKLKNNTRI--CVSS----C
hPACE4   CHPECGDK--GCDGPNADQCLNCVHFSLGSVKTSRK--CVSV----C
dfur2    CDAECDSS--GCYGRGPTQCVACSHYRLD-----NT--CVSR----C
mPC5     C-RKCAPNCESCPGSHGDQCLSCKYGYFLNEETSS---CVTQ----C
hPACE4   C-RRCHKGCETCSSRAATQCLSCRRGFYHHQEMNT---CVTL----C
dfur2    C-WPCHDTCETCAGAGPDSCLTCAPAHLHVIDLAV---CLQF----C
mPC5     C-GKCSENCKACIG--FHNCTECKGGLSLQGSR-----CSVT----C
hPACE4   C-LKCHPSCKKCVDEP-EKCTVCKEGFSLARGS-----CIPD----C
dfur2    C-VPCEPNCASCQDHP-EYCTSCDHHLVMHEHK-----CYSA----C
mPC5     C-QPCHRFCATCSGAGADGCINCTEGYVMEEGR-----CVQS----C
hPACE4   C-GECHHTCGTCVGPGREECIHCAKNFHFHDWK-----CVPA----C
dfur2    C-AFCHSTCATCNGPTDQDCITCRSSRYAWQNK-----CLIS----C
mPC5     C-KRCDNSCLTCNGPGFKNCSSCPSGYLLDLGM-----CQMGAI--C
hPACE4   C-RRCDENCLSCAGSS-RNCSRCKTGFTQLGTS-----CITNHT--C
dfur2    C-MPCQEGCKTCTSN--GVCSECLQNWTLNKRDK----CIVSGSEGC
dfur2    C-RPCHASCGSCNGPADTSCTSCPPNRLLEQSR-----CVSG----C
         C-SPCLHTCSQCVS--RTNCSNCSKGLELQNGE-----CRTT----C
         C-AKCYLSCHTCSGPRRNQCVQCPAGWQLAAGE-----CHPE----C
         C-QKCHHYCKTCNDAGPLACTSCPPHSMLDGGL-----CME-----C
         C-KTCHDSCRSCFGPGQFSCKGCVPP-LHLDQLNSQ--CVS-----C
hFurin   C-APCHASCATCQGPAPTDCLSCPSHASNDPVEQT---CSRQSQSSR
CRP170   C-SKCDGTCLTCETSAA-QCTSCPEGKYLKGDKS----CVNNNG---
         C-EACGANCATCTQAGNDKCTKCKPGFFMKGNGPTGE-CVA-----C
let-23   C-ERCSPECETCNGLGELDCLTCRHKTLYNSDFGNRMECVHD-----
DER      C-KICHPECRTCNGAGADHCQECVHVRDGQH-------CVSE----C
Consensus
  Motif  Cx xCxxxCxxC(x)5-7 CxxC    (x)8-15    C x x x C
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FIG. 2. Amino acid alignment of the repeats found in the Cys-rich region of PC5 with those in other proteins. The Cys-rich sequences of human PACE4 (hPACE4) (8), dfurin2 (dfur2) (22), human furin (hFurin) (7), CRP170 (23), let-23 (24), and DER (25) are compared to those found in mPC5. In the consensus motif, Cys (boldface type) and the number of in-between residues are marked with an asterisk and x, respectively. The repeats are separated by 9–16 aa.

enzymes have five repeats within their Cys-rich domain (Fig. 2). Furthermore, the C-terminal amino acid of both enzymes is Gly, suggesting the possibility that within cells that synthesize the amidation enzyme (27, 28), these two putative PCs could be amidated at the C terminus. So far, the cleavage specificity of PC1, PC2, and furin (1, 2) demonstrated that these PCs are capable of cleaving precursors in a distinct fashion at specific pairs of basic residues (12–15) and sometimes at monobasic residues (29, 30). The large degree of similarity in the catalytic regions predicts that PC5, PACE4, and PC4 should exhibit cleavage specificities similar to PC1, PC2, and furin.

The conservation of the Cys motif found in PC5, PACE4, dfurin2, human furin, *G. lamblia* CRP170, *C. elegans* let-23, and *D. melanogaster* DER (Fig. 2) suggests that this motif could have a common functional role in these various proteins. It is worth noting that CRP170 (23), let-23 (24), and *D. melanogaster* DER (25) represent surface-exposed molecules (either a surface antigen or a receptor) in which the Cys-rich domains are in contact with the extracellular space. It has been suggested that this may protect the surface antigen CRP170 from oxidation and, hence, favor the survival of the trophozoite within the small intestine (23). The presence of a free Cys residue close to the active site His^{180} (19) should render the PCs susceptible to oxidation. However, this may be alleviated by the presence of a Cys-rich domain. Since the removal of the Cys-rich domain of furin did not affect its ability to intracellularly cleave proproteins (29, 31), this may mean that this domain in furin is not functionally important inside the cell. The absence of a transmembrane domain in PC5 and PACE4 suggests that these enzymes are secreted from the cell and, hence, may also have an extracellular enzymatic function. The Cys-rich domains may either provide additional protection and/or permit binding to certain proteins, allowing efficient extracellular action. This may involve the cleavage of certain proproteins known to be processed within the extracellular space. For example, it has been reported that the monobasic processing of proatrial natriuretic factor is achieved after its secretion from cardiac

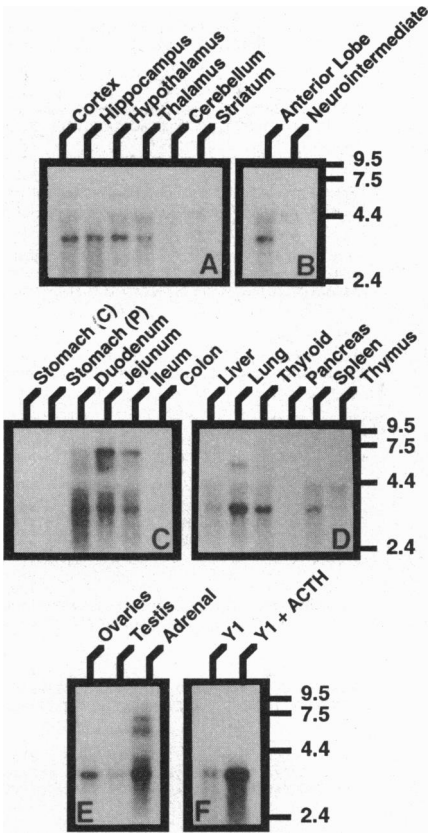

FIG. 3. Distribution of PC5 mRNA by Northern blot analysis. Autoradiograms show the tissue distribution of PC5 in brain (*A*), pituitary (*B*), digestive system (*C*), selected peripheral tissues (*D*), reproductive tissues (*E*), and adrenal (*F*) and PC5 from mouse adrenocortical cell line Y1 and Y1 cells treated for 24 h with 0.1 μM ACTH-(1–24). Total RNA (5 μg) was loaded into each lane. The major PC5 form observed is 3.8 kb. We also observed two other bands of ≈6 and ≈7.5 kb, the ratio of which varies across tissues. X-ray film exposure was 16 h. Indicated markers are in kilobases. Stomach (C) is cardiac; stomach (P) is pyloric.

myocytes, by a nonmyocytic atrial cell enzyme (32). This example shows that prohormone processing can also involve mechanisms in which the PC and the substrate originate from different cells.

Interestingly, a data bank search (GenBank, September 1992) revealed that mPC5 exhibits a 90.5% and 87.1% sequence identity at the protein and nucleotide levels, respectively, with a human-expressed sequence tag (EST02038) containing 319 nt and coding for an unknown human brain protein (accession no. M85522) (33). This observation suggests that this sequence represents the C terminus of human PC5.

Since PC5 exhibits a widespread tissue distribution, its physiological substrates are expected to be numerous. However, the abundance of PC5 within the epithelial cells of the small intestine speaks for a possible function in the cleavage of proproteins synthesized in the duodenum, ileum, and jejunum. These could include precursors of growth factors synthesized in epithelial cells and/or of the secretin/glucagon/vasoactive intestinal peptide family. In contrast to PC4 (9, 10), the expression of PC5 is at least 2- to 3-fold higher in female rather than in male reproductive tissues (Fig. 3*E*). In a preliminary study, testicular PC5 expression was primarily observed in Sertoli cells.

PC5 expression is lower in brain than peripheral tissues. Within the various rat brain regions, PC5 is primarily expressed in the cortex, hippocampus, hypothalamus, and

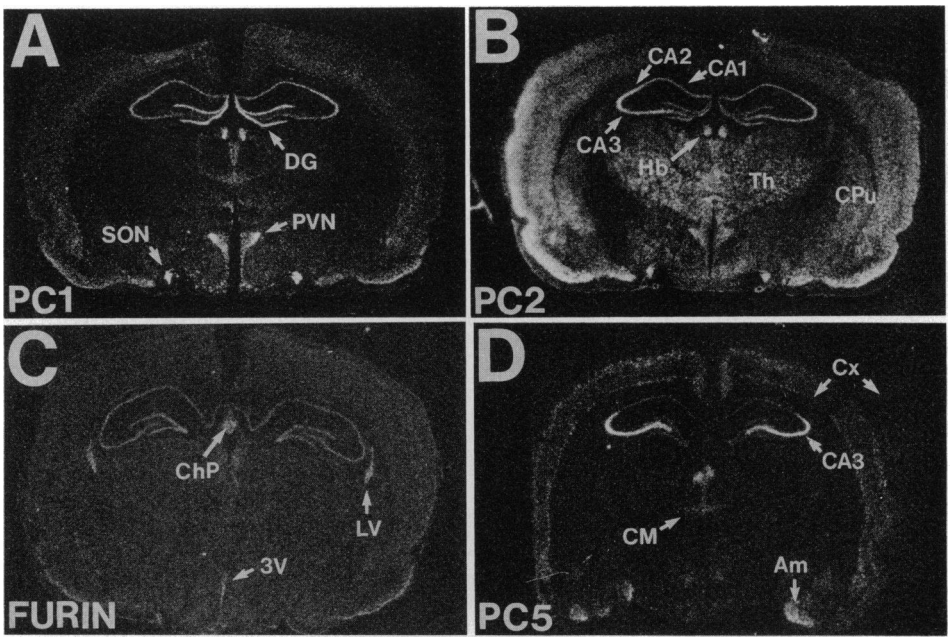

FIG. 4. *In situ* hybridization of PC1, PC2, furin, and PC5 in rat brain. Autoradiograms show the comparative distribution of mRNAs coding for the processing enzymes PC1 (*A*), PC2 (*B*), furin (*C*), and PC5 (*D*) in similar rat brain coronal sections. DG, dentate gyrus; SON, supraoptic nucleus; PVN, paraventricular nucleus; CA1, CA2, and CA3, regions of the pyramidal cells of the hippocampus; Th, thalamus; Hb, habenula; CPu, caudate putamen; ChP, choroid plexus; LV, lateral ventricle; 3V, third ventricle; Cx, cortex; CM, centromedial nucleus of the thalamus; Am, amygdaloid nucleus. X-ray film exposure was 96 h.

thalamus, with much lower levels in the cerebellum and the striatum (Fig. 3*A*). This distribution pattern is quite different from PC1, PC2, and furin (2, 11, 27), emphasizing the distinctiveness of PC5. In contrast to PC1, PC2, and furin, the *in situ* distribution of PC5 in the hippocampus is highest in the CA3 region.

ACTH stimulation of Y1 cells, which do not express PC1, leads to an upregulation of PC5 mRNA levels (Fig. 3*F*), implying mediation by cAMP (26), but no changes in the levels of the endogenous furin and PC2 were observed (data not shown). In the future, it will be important to identify the endogenous substrates of PC5 within Y1 cells and show whether these are coregulated with their PC, as is the case for PC1 and PC2 with proopiomelanocortin (11). Furthermore, we should also measure the level of PC5 in the adrenal cortex of humans suffering from ACTH-secreting tumors. One such example involves patients suffering from a corticotroph adenoma known as Cushing disease who exhibit hyperplasia of the adrenal cortex (34). It is tempting to speculate on the role of PC5 in the cellular proliferation of the adrenal cortex via the activation of growth factors by cleavage at either single or pairs of basic residues (31).

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