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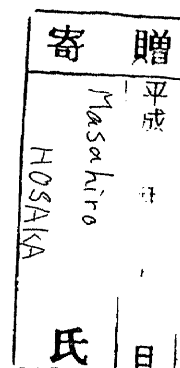

STUDIES ON STRUCTURE AND FUNCTION OF THE PRO-PROTEIN PROCESSING ENDOPROTEASES, FURIN, PC3, AND PACE4

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ABBREVIATIONS

POMC	:	proopiomelanocortin
SSC	:	0.15M sodium chloride - 0.015M sodium citrate
SDS	:	sodium dodecyl sulfate
RSV	:	Rous sarcoma virus
SV40	:	simian virus 40
DMEM	:	Dulbecco's modified Eagle's medium
FCS	:	fetal calf serum
RER	:	rough endoplasmic reticulum
PCR	:	polymerase chain reaction
CHO	:	Chinese hamster ovary
NDV	:	Newcastle disease virus
HIV:	:	Human immunodeficiency virus
RT-PCR	:	reverse transcriptase-polymerase chain reaction
vWF	:	von Willebrand factor

SUMMARY

I describe here the isolation and characterization of mouse and human cDNAs for furin, PC3, and PACE4, which are member of the Kex2 family of mammalian pro-protein processing endoproteases. Among them, furin and PACE4 are expressed in a variety of tissues and cell lines and proposed to be responsible for processing of precursors for various proteins within the constitutive secretory pathway. While neuroendocrine-specific endoprotease, PC3, cleaves precursors of biologically active peptides at paired basic residues, Lys-Arg and Arg-Arg, extensive studies have revealed that furin cleaves pro-proteins at sites marked by the consensus motif Arg-X-Lys/Arg-Arg (RXK/RR). Moreover, co-expression experiments in non-endocrine cells of PACE4 with pro-vWF, complement pro-C3, or a prorenin mutant ($M2R^{-4}$) indicate that although PACE4 can cleave precursors at sites marked by the Arg-X-Lys/Arg-Arg consensus motif within constitutive secretory pathway, its specificity is somewhat different from that of furin.

GENERAL INTRODUCTION

Many peptide hormones and proteins are synthesized as high molecular weight precursors which undergo a series of post-translational modifications to yield biologically active materials (12,40,43). The processes involved in the maturation of polypeptide precursors are now beginning to be understood at the molecular level. The knowledge obtained from signal sequence, the signal recognition particle and its receptor (12,35,39,40,43), and their possible function serve to focus attention on the coupled nature of protein biosynthesis and the intracellular routing processes (39,65). Once the newly formed secretory proteins are translocated into the cisternae of rough endoplasmic reticulum (RER), a poorly defined migration begins from the RER to the lamellar Golgi complex via transfer vesicles issued from transition elements of the RER and then to the secretory granules possibly via receptor-mediated segregation into clathrin-coated vesicles (65,74). During this transit, many proteins are modified by processes that include glycosylation, proteolytic conversion to bioactive peptides via selective cleavage at pairs of basic amino acid residues, NH_2-terminal acetylation, COOH-terminal amidation, and NH_2- and COOH-terminal trimming by amino- and carboxy-peptidase (12,40,43). Only a few of the enzymes responsible for prohormone and pro-protein maturation have been characterized: carboxypeptidase H capable of removing Lys and Arg residues from the COOH-terminus of peptides (42) has been cloned, the enzyme responsible for catalyzing NH_2-acetylation has been characterized (26), and the enzyme that carries out the oxidative amidation of the COOH-terminal Gly residue has been cloned (15).

However, little is known about the endoproteases responsible for the initial cleavage at the dibasic recognition sites. Although a number of processing endoproteases have been described with different properties, including pH optima and substrate specificities (7,16,17,41,42,44,50,51,52,61,63,90), most of them have not yet been authenticated. The only exception well characterized at the molecular and biochemical levels is the Kex2 protease of the yeast *Saccharomyces cerevisiae*. It is a

Ca^{2+}-dependent serine protease with a bacterial subutilisin-like catalytic domain and is involved in processing of pro-α-mating factor and pro-killer toxin at dibasic sites (24,25,34,53,54,59,60,72,73,76,77,81,87,93). Thomas *et al.* (87) have recently demonstrated that the Kex2 protease can function in mammalian cells; when the protease and proopiomelanocortin (POMC), a precursor of adrenocorticotropin, β-endorphin, α-MSH, etc. (59), are co-expressed by DNA transfection in mammalian cells, in which the precursor is not processed, mature hormones are produced through cleavage at the paired basic sites. This evidence leads to the speculation that mammalian Kex2-like proteins, if present, could function as prohormone processing endoproteases.

Recently, a human cDNA (25,93) encoding a protein, furin, highly homologous to the Kex2 protease in catalytic domain sequence has been identified. The protein is a product of the *fur* gene located in the upstream region of the c-*fes*/*fps* proto-oncogene (72). Based on the reason mentioned above, it has been proposed that furin is a mammalian prohormone processing endoprotease (25,93). However, no direct evidence is currently available about the function of furin.

In CHAPTER 1, to address this possibility, I have cloned a mouse furin cDNA, examined expression of its mRNA in various mouse tissues and cell culture cell lines, and co-expressed furin and a prohormone in a cell line by DNA transfection.

Northern blot analysis in CHAPTER 1 have revealed the presence of not only a 4.0-kb furin mRNA but also a 3.0-kb furin-like mRNA in mouse pituitary AtT-20 cells. It was possible that the product of the latter mRNA could be a prohormone processing endoprotease, since it is understood that various prohormones are processed at paired basic sites in these cells. In CHAPTER 2, to address this possibility, I have cloned a cDNA corresponding to the 3.0-kb mRNA, and examined the endoprotease activity of the product of the cDNA.

In CHAPTER 1, I have shown that furin cannot cleave a precursor, prorenin, at dibasic site in mammalian cells. However, I have reported in CHAPTER 3 that the Arg-

Lys/Arg-Arg (RXK/RR) sequence at the cleavage site is conserved in various constitutively processed precursors and that furin and PC3 cleave precursors at the RXK/RR and the dibasic sites, respectively.

In CHAPTER 4, I have used the polymerase chain reaction to identify a mouse cDNA, designated PACE4, which represented a new member of a growing class of mammalian endoproteases homologous to the yeast Kex2 protease involved in the processing of precursor proteins. And I have examined its substrate specificity by co-transfection of the PACE4 expression vector with those of precursor proteins into cultured cells.

CHAPTER 1

Furin, a Mammalian Protein Homologous to the Yeast Kex2 Protease, is Not Involved in Prohormone Processing

ABSTRACT

I have cloned and sequenced a mouse cDNA encoding the 793-residue sequence of furin, which is homologous to the yeast Kex2 protease. Its entire amino acid sequence is 94% identical to that of human furin. It contains a 289-residue sequence of a subutilisin-like catalytic domain. Within this region, 99, 64, and 53% of the amino acids are identical to those of human furin, human PC2 (the other Kex2-like protein), and the yeast Kex2, respectively. It has been proposed that furin is a mammalian prohormone processing endoprotease which cleaves precursors at paired basic amino acids, based on the fact that the Kex2 protease is responsible for processing of pro-α-mating factor and pro-killer toxin at basic sites. However, Northern blot analysis has revealed that a furin mRNA transcript is present in all tested mouse tissues and culture cell lines, including those known not to process prohormones. Moreover, when furin and a prohormone, prorenin, have been co-expressed in mammalian cells by DNA transfection, no processing has been observed. These observations suggest that furin is not involved in prohormone processing at paired basic amino acids, Lys-Arg.

EXPERIMENTAL PROCEDURES

Isolation of a Human fur Gene Fragment

To amplify genomic DNA by polymerase chain reaction (PCR), primers corresponding to the nucleotide sequences flanking a codon for the active site Ser residue of human furin were designed. The primers used were as follows: 5'-GGCAACCAGAATGAGAGCA-3' corresponding to nucleotide residues 317-336 and 5'-TCCAGGGTGAGAGAGCAATGAT-3' complementary to residues 883-902 of the human *fur* gene (73). DNA amplification by PCR using a GeneAmp Amplification Kit (Perkin-Elmer Cetus) was performed in a Thermal Cycler (Perkin-Elmer Cetus) for 30 cycles of denaturation (94℃, 1.5min.), annealing (50℃, 2.5min.), and extension (72 ℃, 3.5min.). After agarose gel electrophoresis, the corresponding DNA fragment was extracted from the gel, blunt-ended with T4 DNA polymerase, and ligated into the *Sma*I site of the pUC119 vector. The nucleotide sequence of the cloned DNA fragment was confirmed by the chain termination method (77).

cDNA Cloning of Mouse Furin

Total RNA isolated from male ICR mouse kidney by the guanidium/CsCl method (76) was subjected to oligo(dT)-cellulose chromatography (76) to select poly(A)$^+$ RNA. Double-stranded cDNA was synthesized from the poly(A)$^+$ RNA using the cDNA Synthesis System (Amersham), and inserted into the *Eco*RI site of the λgt10 vector. Four X10^5 clones derived from the cDNA library were screened by hybridization with the ^{32}P-labeled human *fur* gene fragment. Eleven positive clones were plaque-purified, then the inserts were separately subcloned into the pBluescript-II vector (Stratagene). The sequence of both strands of a clone, K-1, with the longest insert were determined.

Northern Blotting

Total RNAs (20μg) isolated from various mouse tissues and culture cell lines were denatured with glyoxal (76), fractionated on a 1.2% agarose gel, and blotted onto a GeneScreen Plus membrane (Du Pont-New England Nuclear). The blot was hybridized in 1M NaCl, 10% dextran sulfate, 1% SDS and 0.1 mg/ml salmon sperm DNA with the following ^{32}P labeled mouse furin cDNA fragments: a mixture of 639-bp *Bgl*II-*Stu*I and 177-bp *Stu*I-*Stu*I fragments covering the sequence highly conserved between furin and PC2 (81), encoding the polypeptide region containing the active site Asp and His residues (probe A), and a 924-bp *Bam*HI-*Bam*HI fragment covering the unconserved sequence encoding the COOH-terminal region and a portion of 3'-untranslated sequence (probe B). The blot was then washed sequentially in 2 X SSC (76) (room temperature, 10min), 2 X SSC/1% SDS (60℃, 1h), and 0.1 X SSC (room temperature, 1h).

Plasmid Construction

The furin cDNA insert was subcloned behind the Rous sarcoma virus (RSV) promoter of the pAGE123 vector (60), which has a selection marker, the neomycin phosphotransferase gene (see Fig.4B). A cDNA fragment covering the entire coding sequence of human preprorenin (32) was subcloned behind the RSV promoter of the pSGA1 vector; the vector was constructed by ligation of *Xho*I-*Cla*I fragment derived from the pAGE123 including the RSV promoter, the multiple cloning site and SV40-derived splicing and polyadenylation signals, the *Bam*HI-*Bam*HI fragment from the pMSG (Pharmacia-LKB Biotechnology) including the xanthine-guanine phosphoribosyltransferase gene, and the *Nae*I-*Pvu*II fragment from the pGEM-1 (Promega) including the replication origin and the β-lactamase gene (see Fig.4A). The resulting furin and prorenin expression plasmids were designated pAGEFur and pSGAHRn, respectively.

Cell Culture and DNA Transfection

Rat pituitary GH4C1 cells and AtT-20/D16v cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% dialyzed fetal calf serum (FCS) at 37℃ in 5 and 15% CO_2, respectively. The cells were transfected with pSGAHRn alone or in combination with pAGEFur using a CellPhect Transfection Kit (Pharmacia-LKB Biotechnology), and stable transfectants were then selected by the method of Wiren *et al.* (97) in the presence (for the transfectant containing both pSGAHRn and pAGEFur) or absence (for the transfectant containing pSGAHRn alone) of 0.4mg/ml G418 (Gibco/BRL), a neomycin analog.

Renin Assay

Culture media were assayed for the active renin and total renin (active renin, and prorenin after activation with 0.1 mg/ml trypsin at 24℃ for 1 h) activities by the angiotensin I (AngI) generating method using hog angiotensinogen as a substrate as described previously (27). Prorenin levels were deduced from the difference between these two measurements.

Radiolabeling and Immunoprecipitation

Radiolabeling and immunological identification of renin molecules were carried out as described previously (60). Briefly cells at about 70% confluence in a 35-mm dish were incubated in 0.5 ml of methionine-free DMEM with 0.2 mCi [^{35}S] methionine (> 6600 Ci/mmol, Du Pont-New England Nuclear) and 10% dialyzed FCS for 10 h. The culture medium was then collected, and immunoprecipitated with anti-human renin antiserum. The precipitates were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography.

RESULTS AND DISCUSSION

cDNA clones specific for mouse furin were isolated from a mouse kidney cDNA library by hybridization with a human *fur* gene fragment covering the sequence encoding the polypeptide region around the active site Ser residue of human furin; the fragment was isolated by PCR amplification of human genomic DNA (see EXPERIMENTAL PROCEDURES). Eleven clones thus isolated carried cDNA inserts whose restriction patterns were the same, although the insert lengths of different from one another. In addition, inserts of cDNA clones isolated from a mouse liver cDNA library in a similar manner also had the same patterns (data not shown). Therefore, the nucleotide sequence of a clone, K-1, which carried the longest kidney cDNA insert, was determined.

Fig.1 shows the 3,978-nucleotide sequence of the cloned cDNA and the amino acid sequence deduced for furin. The mouse furin polypeptide consists of 793-amino acid residues. Mouse and human (93) furin share 94% amino acid identity in the entire amino acid sequence, and especially 99% identity in the putative catalytic domain sequence homologous to the subutilisin serine protease family (Fig.2A). Moreover, its catalytic domain sequence is 53% identical to that of the yeast Kex2 protease, which cleaves the pro-α-factor and pro-killer toxin at dibasic sites, and 64% identical to that of the other mammalian Kex2-like protease, human PC2 (81), whose cDNA has been recently isolated from an insulinoma cDNA library. The amino acid sequences around the proposed active site Asp, His, and Ser residues, and around the Asn residue probably involved in binding to substrates, are also highly conserved between furin and other subutilisin family members (Fig.2B). Based on the similarity in the catalytic domain sequence between furin and Kex2, as well as on the fact the Kex2 protease can correctly process a prohormone, proopiomelanocortin (POMC), in a mammalian cells (87), it has been proposed that furin is a prime candidate for mammalian prohormone processing endoprotease (25,93).

To examine this possibility, I initially performed Northern blot analysis of total RNAs from various mouse tissues and culture cell lines. When hybridized with probe A, which covers the sequence conserved furin and PC2, RNAs from all tested tissues and culture cell lines, with two exceptions of mouse pituitary AtT-20 cells and testis, give rise to a single band with an estimated mRNA sized of ~4.0-kb. (Fig.3A). The size is similar to those of the K-1 cDNA insert and of human and rat furin mRNAs reported by Roebroek *et al.* (72). Among these cell lines, some, such as rat pituitary GH4C1 (8,45,62,78,80,85,87), rat pheochromocytoma PC12 (8,62,78,80), African green monkey kidney BSC-40 (45,85,87) and Chinese hamster ovary cells (68,69), cannot process foreign prohormones expressed by DNA transfection. On the other hand, in the case of RNAs from mouse pituitary AtT-20 cells are known to process endogeneous POMC and various foreign prohormones (8,45,60,69,78,80,85,87,89) at paired basic sites. There is a possibility that the 3.0-kb band may be derived from PC2-like mRNA based on the following reasons: a) Only the 4.0-kb band was detected (Fig.3B) when hybridized with probe B, which covers the region unconserved between furin and PC2 cDNA. b) Southern blot analysis of mouse genomic DNA digested with various restriction enzymes revealed that two bands and a single band were detected with probe A and B, respectively, indicating the presence of at least two furin-like genes (data not shown). c) It has been reported that human insulinoma RNA gives rise to two bands of 5.0 and 2.8-kb when hybridized with PC2 cDNA (81).

Data demonstrating the presence of the 4.0-kb furin mRNA in all tested tissues and culture cell lines, including those unable to cleave prohormones, lead to the speculation that furin may be not involved in prohormone processing. To support this speculation, GH4C1 cells, in which various foreign prohormones are not cleaved (16,34,42,44,65) and the Kex2 protease can function (32), were transfected with the human prorenin expression plasmid (pAGEHRn, Fig.4A) alone or in combination with that of mouse furin (pAGEFur, Fig4B). Prorenin was chosen as the substrate since enzymatically active renin, an aspartyl protease, is produced from it through cleavage at a Lys-Arg pair mainly in the kidney, from which we isolated the furin cDNA, and

when expressed by DNA transfection in some cell lines including AtT-20, it is cleaved at the pair to yield renin (60,69,89). As shown in Fig.4C, GH_4C_1 cells transfected not only with pSGAHRn alone but also with both pSGAHRn and pAGEFur secreted mostly enzymatically inactive prorenin, although Northern blotting revealed that the mRNA transcript from the introduced pAGEFur was indeed present in the co-transfectant (data not shown). Radiolabeling experiments also revealed that co-transfection with these two plasmids did not give rise to the conversion of prorenin to renin (Fig.4D). On the other hand, AtT-20 cells transfected with pSGAHRn secreted a significant amount of active renin (Fig.4C and D) as described previously (60). These results indicate that furin cannot process prorenin at the Lys-Arg pair in GH_4C_1 cells.

The experiments presented here suggest that furin is not involved in prohormone processing in mammalian cells. Furin may be involved in constitutive processing of other protein precursors, such as those of albumin (55), some growth factors (5), and some viral envelope glycoproteins (10), since they are known to cleaved at dibasic sites also in non-endocrine cells. At present, I cannot examine this possibility, since all tested cell lines express furin. However, the present data do not completely exclude the possibility that furin can function as a prohormone processing enzyme from the following reasons: a) Intracellular localization of furin may differ from one cell to another, that is, in some cells in which prohormones are not processed, it may not locate in the compartment(s) where the processing take place (28,86); b) Furin could be involved in processing of some prohormones but not of others including prorenin, although studies concerning expression of foreign prohormones in some endocrine cells, such as AtT-20, have suggested that processing enzyme(s) have a broad substrate specificity for prohormones.

```
                                                                                         CGGG    4
ACCCTGGCACTGAGCAGGCACGTGGCAGCCAAGACCCTGTGACCAGGCCAGGGAGACAGGCGCTCTGGGGTTCCAGTCACCTGCCCCCCC   94

ATGGAGCTGAGATCCTGGTTGCTATGGGTGGTCGCAGCAGCAGGAGCCGTGGTCCTGCTGGCAGCTGATGCTCAAGGCCAGAAGATCTTC  184
 M  E  L  R  S  W  L  L  W  V  V  A  A  A  G  A  V  V  L  L  A  A  D  A  Q  G  Q  K  I  F   30
                                                                       ↑

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CCTCAAGTAAAGTGGCTGGAGCAGCAGGTAGCCAAGCGAAGAGCCAAGAGGGACGTGTATCAGGAGCCCACGGACCCCAAGTTCCCCCAG  454
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CAGTGGTACCTGTCTGGTGTCACTCAGCGAGACCTGAATGTGAAGGAGGCCTGGGCCCAGGGCTTCACAGGCCATGGCATTGTGGTCTCC  544
 Q  W  Y  L  S  G  V  T  Q  R  D  L  N  V  K  E  A  W  A  Q  G  F  T  G  H  G  I  V  V  S  150

ATCCTGGATGACGGCATTGAGAAGAATCATCCCGACCTAGCAGGCAATTATGACCCTGGAGCCAGTTTTGACGTGAATGACCAGGACCCC  634
 I  L  D  D  G  I  E  K  N  H  P  D  L  A  G  N  Y  D  P  G  A  S  F  D  V  N  D  Q  D  P  180
       ▲

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                                          ▲

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GGCCTGAATCCCAACCACATCCACATCTACAGCGCCAGCTGGGGCCCTGAGGACGACGGCAAGACCGTGGATGGACCAGCCCGGCTCGCT  904
 G  L  N  P  N  H  I  H  I  Y  S  A  S  W  G  P  E  D  D  G  K  T  V  D  G  P  A  R  L  A  270

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 E  E  A  F  F  R  G  V  S  Q  G  R  G  G  L  G  S  I  F  V  W  A  S  G  N  G  G  R  E  H  300
                                                                          *

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                      ▲                                                          ---------

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 E  G  Y  S  L  H  Q  K  S  C  V  Q  H  C  P  P  G  F  I  P  Q  V  L  D  T  H  Y  S  T  E   630
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 G  E  R  T  A  F  I  K  D  Q  S  A  L                                                        793
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CCGGGGCTGCCTGGGCTGTTGGCAGTTACAGTACAGGAGCCCCTGGGTGGGTGGTGGGGAGGCACGGTGGCCCAGCCGGCTCCTCCCGCC 3244
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TTTTTTTTTCCTTGTAATTTAAACAGGCCTGGCATTGTTGGTTCTATTTAATGGACACGAGATAATGTTAGAGGTTTTAAAGTGATTAAA 3964
CGTGCAGACTATGC                                                                             3978
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Fig. 1. The cDNA sequence for mouse furin and its deduced amino acid sequence. The amino acid sequence deduced for furin is shown under the nucleotide sequence. The putative cleavage site of signal peptide is indicated by an arrow. The proposed active site Asp, His, and Ser residues, and the Asn residue probably involved in binding to substrates are indicated by arrowheads and an asterisk, respectively. The putative transmembrane segment sequence is underlined. Consensus sequences for N-glycosylation are doubly underlined.

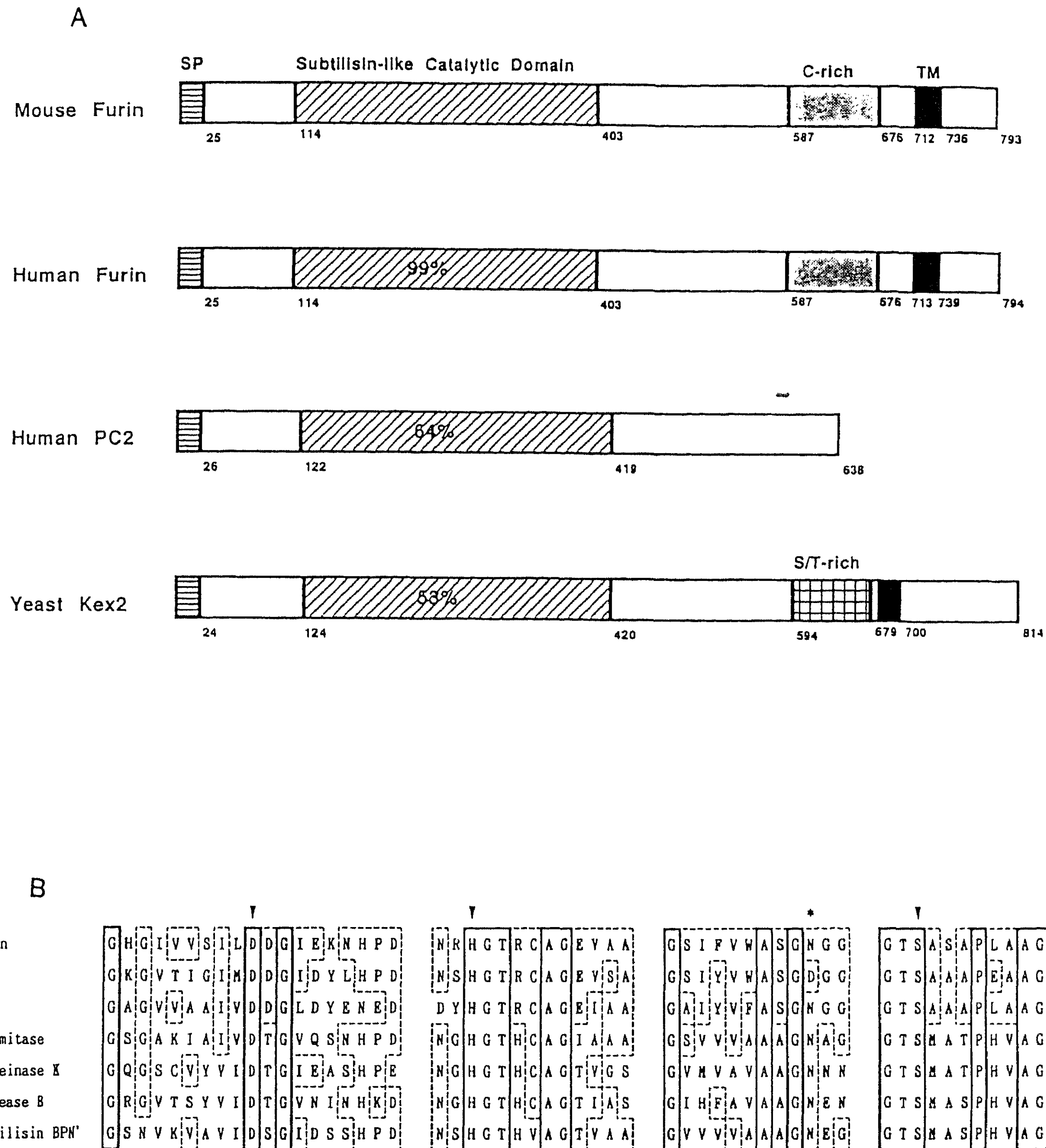

Fig.2. Comparison of the structure of mouse furin and other subutilisin family members. A, schematic representation of the structure of mouse and human furin, human PC2, and yeast Kex2. SP, Signal peptide; C-rich, Cys-rich region; TM, transmembrane segment; S/T-rich, Ser/Thr-rich region. B, conservation of active site Asp, His, and Ser residues, and Asn residue probably involved in binding to substrates are indicated by arrowheads and an asterisk, respectively. Residue conserved in all sequences are boxed with bold lines, and those conserved between furin and at least one other sequence are boxed with broken lines. Thermitase, *Termoactinomyces vurlgaris* thermitase; Proteinase K, *Tritirachium album Limber* proteinase K, Protease B, *S. cerevisiae* protease B; Subutilisin BPN', *Bacillus amyloliquefaciens* subutilisin BPN'.

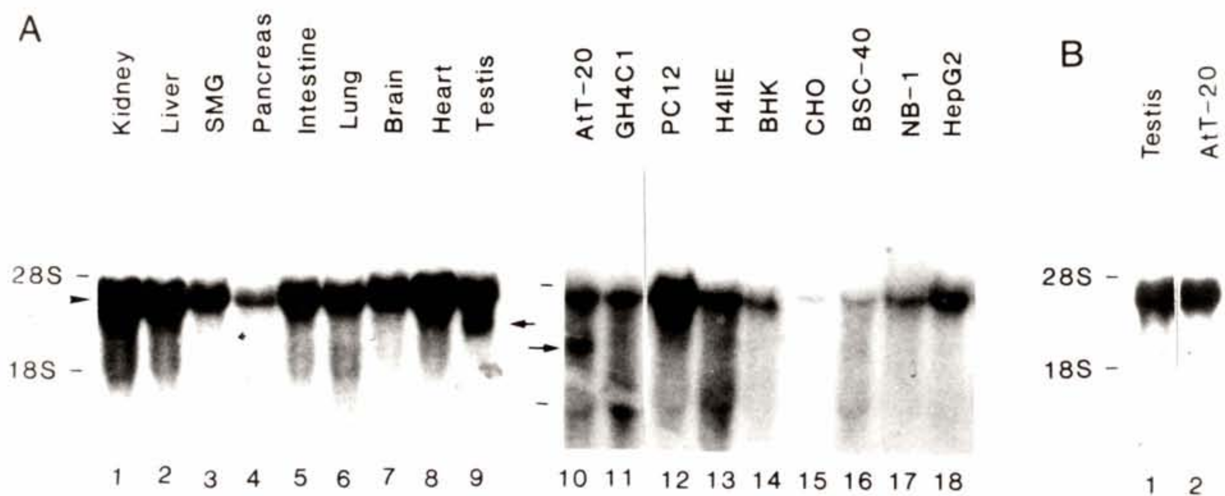

Fig.3. Northern blot analysis of total RNAs from mouse tissues and culture cell lines using probe A (A) and probe B(B). Experimental details are described under "EXPERIMENTAL PROCEDURES". Arrows and arrowheads indicate the position of 4.0- and 3.0-kb mRNAs, respectively. SMG, submandibular gland; AtT-20, a mouse pituitary cell line; GH4C1, a rat pituitary cell line; PC12, a rat pheochromocytoma cell line; H4IIE, a rat hepatoma cell line; BHK, baby hamster kidney cells; CHO, Chinese hamster ovary cells; BSC-40, an African green monkey kidney cell line; NB-1, a human neuroblastoma cell line; HepG2, a human hepatoma cell line.

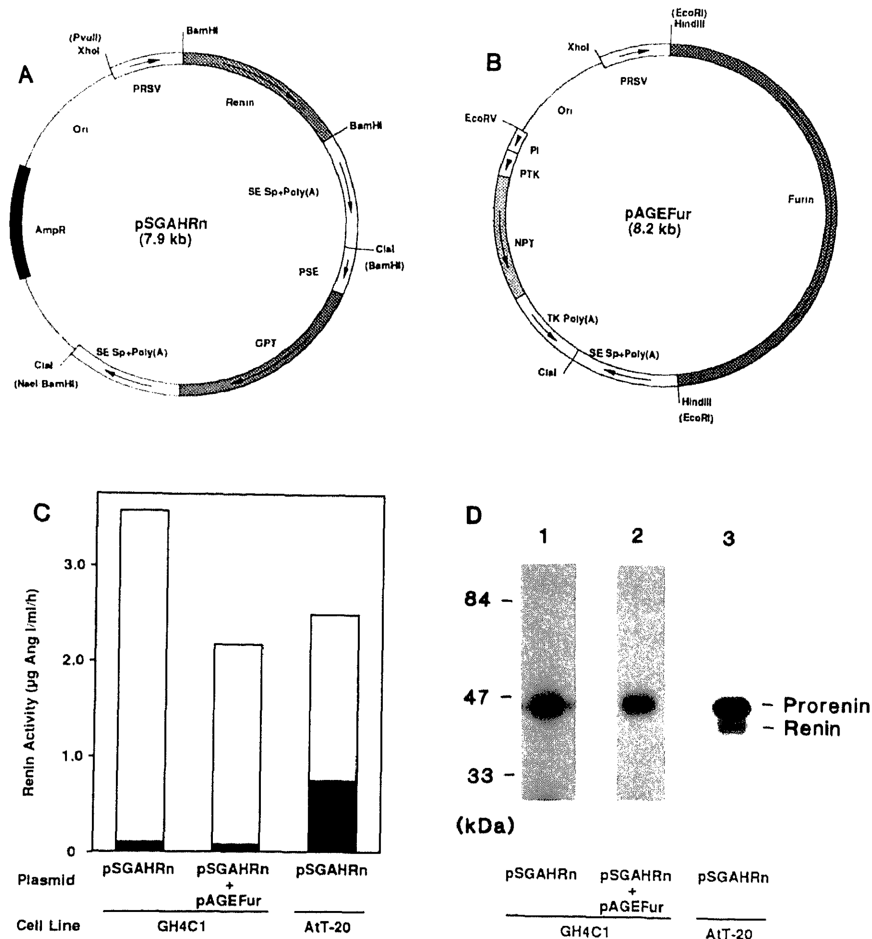

Fig.4. Co-expression of furin and prorenin in GH4C1 cells. A, structure of the expression plasmid of human prorenin. PRSV, RSV promoter; Renin, human preprorenin cDNA; SE, Sp+Poly(A), the splicing and polyadenylation signals from SV40 early gene; PSE; SV40 early promoter; GPT, *Escherichia coli* xanthine-guanine phosphoribosyltransferase gene; AmpR, pBR322 β-lactamase gene; Ori, the replication origin from pBR322. B, structure of the expression plasmid of mouse furin. Furin, mouse furin cDNA; TK Poly(A), the polyadenylation signal from herpes simplex virus thimidine kinase gene; NPT, Tn5 derived neomycin phosphotransferase gene; PTK, thymidine kinase promoter; P1, pBR322 P1 promoter. C, Renin activities in the culture media of the transfected cell lines. Cells transfected with the expression plasmid(s) as indicated were incubated at about 70% confluence in 60-mm dishes in 3 ml of DMEM with 10% dialyzed FCS for 24 h, then the culture media were assayed for active renin (filled column) and prorenin (open column) as described under "EXPERIMENTAL PROCEDURES". D, radiolabeling and immunological identification of renin molecules secreted from the transfected cell lines. Experimental details are described under "EXPERIMENTAL PROCEDURES".

CHAPTER 2

Cloning and Functional Expression of a Novel Endoprotease, PC3, Involved in Prohormone Processing at Dibasic Sites

ABSTRACT

I cloned and sequenced a cDNA from a library of mouse pituitary AtT-20 cells which are known to cleave an endogeneous and various foreign prohormones at dibasic sites. This cDNA encodes a novel 753-residue protein, named PC3, which is structurally related to the yeast Kex2 protease involved in precursor cleavage at dibasic sites and to recently identified mammalian Kex2-like proteins, furin and PC2. Among examined cell lines and tissues, PC3 mRNA was only detected in AtT-20 cells. The substrate specificity of PC3 expressed in mammalian cells was similar to that observed in AtT-20 cells. I conclude that PC3 is a resident prohormone processing endoprotease in AtT-20 cells.

EXPERIMENTAL PROCEDURES

Cloning and Sequencing of PC3

Double-stranded cDNA was synthesized from AtT-20 cell poly(A)$^+$RNA and inserted into the λgt10 vector as described in CHAPTER 1 vector. Two X 10^5 phages were screened by hybridization with the ^{32}P-labeled mouse furin probe A (see CHAPTER 1). Twelve out of 91-positive clones were plaque-purified and the inserts were subcloned into the pBluescript-II vector. The sequence of both strands of the clone with the longest insert, whose restriction patterns differed from those of the furin cDNA, were determined by the chain termination method (77).

Northern Blotting

Total RNAs (10μg) isolated from various culture cell lines and mouse tissues were electrophored in agarose gel and blotted onto a membrane as described in CHAPTER 1. The blot was hybridized with a ^{32}P-labeled 231-bp *Kpn*I-*Hind*III PC3 cDNA fragment covering the unconserved sequence encoding the COOH-terminal region and a portion of the 3'-untranslated sequence, and washed under the conditions described in CHAPTER 1.

Site-directed Mutagenesis and Plasmid Construction

A cDNA fragment covering the entire coding sequence of PC3 were subcloned behind the Rous sarcoma virus (RSV) promoter of the pAGE123 vector. cDNA fragments covering entire coding sequence of *Ren-2* prorenin and its mutants (Fig.5, 72) were separately subcloned behind the RSV promoter of the pSGA1 vector. Site-directed mutagenesis of *Ren-2* prorenin was performed using a oligonucleotide-directed *in vitro* mutagenesis system (Amersham). The oligodeoxyribonucleotide primers used for mutagenesis are shown in Fig.5.

DNA Transfection, Radiolabeling and Immunoprecipitation

Rat pituitary GH4C1 cells at ~70% confluence in a 35-mm dish were transfected with the expression plasmid of *Ren-2* prorenin alone or in combination with that of PC3 using the CellPhect transfection kit. After 48h of incubation, the cells were labeled with [^{35}S]methionine for 10 h as described in CHAPTER 1. The culture medium was then collected, and immunoprecipitated with anti-*Ren-2* renin antiserum. The precipitates were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography.

RESULTS AND DISCUSSION

In CHAPTER 1, I have failed to show a role for furin in prohormone processing at dibasic sites (28). However, I have shown that a furin-like mRNA distinct from that of furin is found in mouse pituitary AtT-20 cells which are known to cleave endogeneous POMC and various foreign prohormones at dibasic sites (5,10,55,86). Hence, it is possible that the protein encoded by this furin-like mRNA could be a processing endoprotease of AtT-20 cells.

To address this possibility, I cloned a cDNA for the furin homologue. Fig.6 shows the 2,446-nucleotide sequence of the cloned cDNA and the 753-amino acid sequence deduced for the encoded protein, designated PC3. A part (residue 214-478) of deduced sequence is identical to that from a partial cDNA (mPC1) recently isolated from mouse pituitary library (79). The hydropathy profile of the deduced sequence revealed only one hydrophobic segment of the putative signal peptide, indicating that PC3 enters the secretory pathway and is not membrane-bound. PC3 contains a putative catalytic domain (residue 122-455) homologue to the subutilisin serine protease family. Within this region, 57, 64, and 48% of amino acids are identical to those of PC2, furin, and Kex2, respectively (Fig.7A). The sequence around the proposed active site Asp, His, and Ser residues are closely related to those of other subutilisin family members (Fig.7B). Moreover, the NH_2- and COOH-terminal regions flanking the catalytic domain are moderately conserved between PC3 and other Kex2 family members (Fig.7A), suggesting that these regions may have important function(s) in these process. The NH_2-terminus of mature PC3 is assumed to begin at the residue 111 behind the Arg-Ser-Lys-Arg sequence, with fits the Arg-X-Lys/Arg-Arg (RXK/RR) consensus motif which I have proposed as a precursor cleavage signal within the constitutive secretory pathway as will be described in CHAPTER 3. These facts make it likely that these processing protease themselves may be initially synthesized as a pro-forms, and then conserved to mature forms through cleavage at the RXKR site. This speculation is supported by the fact that carboxypeptidase H, which moves COOH-

terminal basic residues from various peptide processing intermediates, is also produced from its precursor through cleavage at a site marked by this motif (19,71). There is another structual feature that the Arg-Gly-Asp sequence, which is involved in the receptor recognition of extracellular matrix proteins (75), is conserved in all mammalian members (Fig.7C), although it is unknown if this sequence is functional.

I next examined the expression of PC3 mRNA by Northern blotting of RNAs from various culture cell lines and mouse tissues (Fig.8). PC3 mRNA was detected only in AtT-20 cells and not in other endocrine cells such as rat insulinoma Rinm5F which can also cleave prohormones at dibasic sites (80,86,88), nor in endocrine tissues such as brain and pancreas. However, Seidah *et al.* have shown that PC3 mRNA is also present in hypothalamus, pituitary, brain, and adrenal.

To examine whether PC3 has endoproteolytic activity toward dibasic sites, I performed co-expression experiments in mammalian cells. I used rat pituitary GH4C1 cells as the host, since they are endocrine cells with no processing activity toward dibasic sites, and the Kex2 protease expressed in these cells has been shown to cleave POMC at dibasic sites (24,28,86). As shown in Fig.9B, when PC3 was co-expressed with prorenin, which is cleaved by a Lys-Arg site to yield mature renin in AtT-20 cells (22,38,56), a significant amount of renin was produced. I next examined the sequence requirements for processing by PC3 using various prorenin mutants at the dibasic cleavage site as the substrates (Fig.9C). PC3 cleaved native prorenin and its mutant with Arg-Arg (M2RR) instead of the native Lys-Arg pair. However, it did not cleave mutant prorenins with other combinations of pairs of basic residues (M2KK and M2RK), those with a single basic residue (M2QK and M2KQ), nor that with a Pro residue next to Lys-Arg pair in place of the native Ser (M2P). This sequence preference for prorenin processing by PC3 is consistent with that observed in AtT-20 cells (56).

Data presented here indicate that PC3 functions as a dibasic amino acid-specific endoproteases for at least one and perhaps other prohormones, and is a resident processing endoprotease in AtT-20 cells. In other endocrine cells, PC2 or other unknown endoproteases may be involved, since PC2 mRNA is present in insulinoma

cells, and other endocrine cells and tissues (81,79). These imply that processing endoproteases with different substrate specificities could be expressed in a cell type-specific manner, supported by the following data: Tissue-specific use of certain basic pairs is often observed (14); The sequence preference, among all four combinations of basic residues, for precursor cleavage observed in one cell line is different from that observed in another (11); The cleavages of proinsulin at two distinct dibasic sites are catalyzed by two distinct processing endoproteases (9).

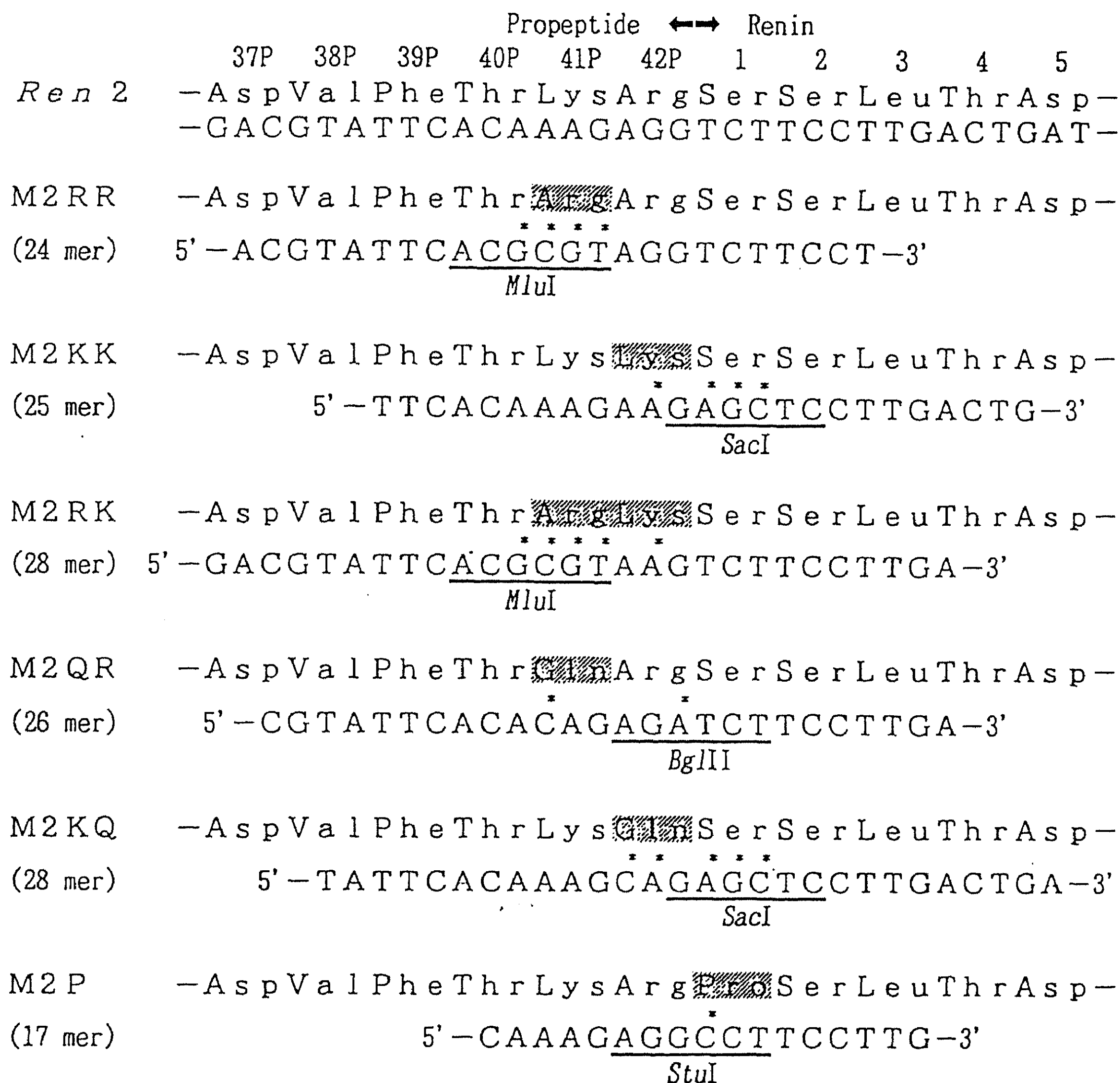

Fig.5. Nucleotide sequences of the pertinent region of mouse *Ren-2* preprorenin cDNA and the mutagenic primers. The deduced amino acid sequences are shown over the nucleotide sequences. The replaced nucleotide are indicated by asterisks, and the substituted amino acids are shadowed. The nucleotide sequences of restriction sites induced to facilitate identification of mutated cDNAs are underlined.

```
                                                                                AGCTTTAGTGAGCGCTCGCTCTCGCCGCCCAGCCTCTCCCAGTGAGCCTCTA   42
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 M  E  Q  R  G  W  T  L  Q  C  T  A  F  A  F  F  C  V  W  C  A  L  N  S▲ V  K  A  K  R  Q  F  V  N  E  W  A  A  E  I  P  G  G  Q  E  A    45
GCCTCTGCCATCGCCGAAGAACTGGGGTATGACCTTTTGGGTCAGATTGGATCACTTGAAAATCACTATTTATTCAAACACAAAAGCCATCCTCGGAGGTCCCGAAGAAGCGCTCTTCATATCACTAAGAGGTTA  457
 A  S  A  I  A  E  E  L  G  Y  D  L  L  G  Q  I  G  S  L  E  N  H  Y  L  F  K  H  K  S  H  P  R  R  S  R  R  S  A  L  H  I  T  K  R  L    90
TCTGATGATGATCGTGTGACGTGGGCTGAACAACAGTATGAAAAAGAGAGAAGTAAACGTTCAGTTCAAAAAGACTCAGCATTGGATCTCTTCAATGATCCAATGTGGAATCAGCAGTGGTACTTGCAAGATACC  592
 S  D  D  D  R  V  T  W  A  E  Q  Q  Y  E  K  E  R  S  K  R↑ S  V  Q  K  D  S  A  L  D  L  F  N  D  P  M  W  N  Q  Q  W  Y  L  Q  D  T   135
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 R  M  T  A  A  L  P  K  L  D  L  H  V  I  P  V  W  E  K  G  I  T  G  K  G  V  V  I  T  V  L  D  D  G  L  E  W  N  H  T  D  I  Y  A  N   180
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 Y  D  P  E  A  S  Y  D  F  N  D  N  D  H  D  P  F  P  R  Y  D  L  T  N  E  N  K  H  G  T  R  C  A  G  E  I  A  M  Q  A  N  N  H  K  C   225
GGGGTTGGAGTTGCATATAATTCCAAAGTTGGAGGCATAAGAATGCTGGATGGCATTGTAACTGATGCCATTGAGGCTAGTTCAATTGGATTCAACCCTGGCCATGTGGATATTTACAGTGCAAGCTGGGGCCCT  997
 G  V  G  V  A  Y  N  S  K  V  G  G  I  R  M  L  D  G  I  V  T  D  A  I  E  A  S  S  I  G  F  N  P  G  H  V  D  I  Y  S  A  S  W  G  P   270
AATGATGATGGAAAAACTGTGGAGGGGCCTGGCAGACTAGCCCAGAAGGCATTTGAATATGGTGTCAAACAGGGGAGACAAGGGAAAGGCTCCATCTTTGTCTGGGCTTCAGGGAATGGGGGTCGTCAGGGAGAT 1132
 N  D  D  G  K  T  V  E  G  P  G  R  L  A  Q  K  A  F  E  Y  G  V  K  Q  G  R  Q  G  K  G  S  I  F  V  W  A  S  G  N  G  G  R  Q  G  D   315
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 N  C  D  C  D  G  Y  T  D  S  I  Y  T  I  S  I  S  S  A  S  Q  Q  G  L  S  P  W  Y  A  E  K  C  S  S  T  L  A  T  S  Y  S  S  G  D  Y   360
ACAGACCAGCGAATAACAAGCGCTGACCTGCACAATGACTGCACAGAGACCCACACAGGCACCTCGGCTTCAGCACCCCTGGCTGCTGGTATCTTTGCTCTGGCCTTGGAGGCAAACCCAAATCTTACCTGGAGA 1402
 T  D  Q  R  I  T  S  A  D  L  H  N  D  C  T  E  T  H  T  G  T  S  A  S  A  P  L  A  A  G  I  F  A  L  A  L  E  A  N  P  N  L  T  W  R   405
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 D  M  Q  H  L  V  V  W  T  S  E  Y  D  P  L  A  S  N  P  G  W  K  K  N  G  A  G  L  M  V  N  S  R  F  G  F  G  L  L  N  A  K  A  L  V   450
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 D  L  A  D  P  R  T  W  R  N  V  P  E  K  K  E  C  V  V  K  D  N  N  F  E  P  R  A  L  K  A  N  G  E  V  I  V  E  I  P  T  R  A  C  E   495
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 S  K  N  A  L  S  K  Q  S  P  K  K  S  P  S  A  K  L  S  I  P  Y  E  S  F  Y  E  A  L  E  K  L  N  K  P  S  K  L  E  G  S  E  D  S  L   720
TACAGTGACTATGTTGATGTATTCTATAACACAAAACCTTATAAGCATAGAGATGACAGGCTGCTGCAAGCTCTCATGGACATCCTAAATGAGGAGAATTAAAATAAGTGTGTTGCCTG                 2466
 Y  S  D  Y  V  D  V  F  Y  N  T  K  P  Y  K  H  R  D  D  R  L  L  Q  A  L  M  D  I  L  N  E  E  N                                        753
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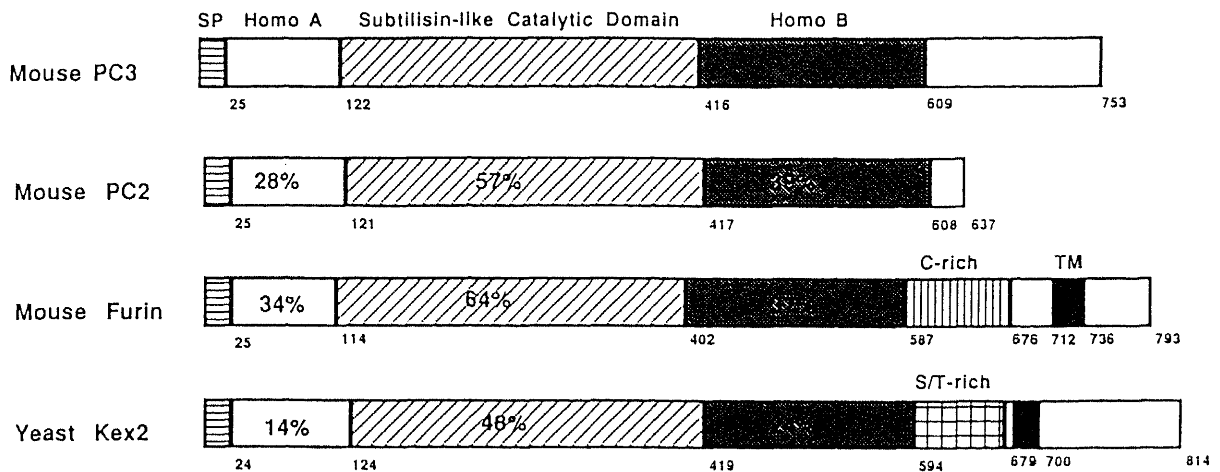
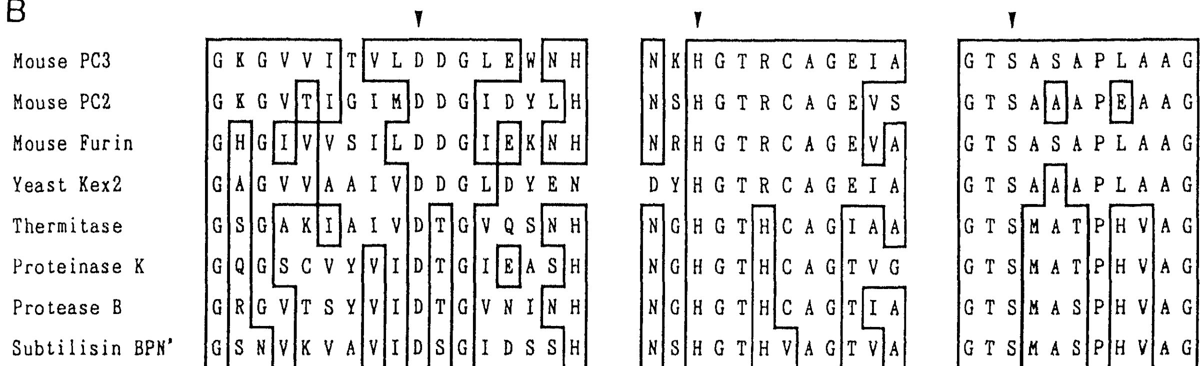
Fig.6. Nucleotide and deduced amino acid sequences of PC3. The amino acid sequence deduced for PC3 is shown under the nucleotide sequence. The putative cleavage site of signal peptide is indicated by an arrow. The proposed active site Asp, His, and Ser residues, and the Asn residue probably involved in binding to substrates are indicated by arrowheads and an asterisk, respectively. Consensus sequences for N-glycosylation are doubly underlined. The Arg-Gly-Asp sequence is underlined with a wavy line.





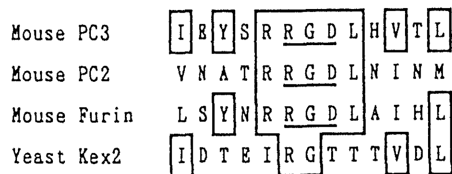


Fig.7. Structure comparison of PC3 with other subutilisin family members. A, schematic representation of the structure of PC3 and other Kex2 family members. The percentage of amino acid identity in each region of each protein with PC3 is shown. SP, Signal peptide; Homo A and Homo B, NH_2- and COOH-terminal homologous regions, respectively; C-rich, Cys-rich region; TM, transmembrane segment; S/T-rich, Ser/Thr-rich region. B, conservation of active site residues between PC3 and other subutilisin family members. The active site Asp, His, and Ser residues are indicated by arrowheads. Thermitase, *Termoactinomyces vurlgaris* thermitase; Proteinase K, *Tritirachium album Limber* proteinase K, Protease B, *S. cerevisiae* protease B; Subutilisin BPN', *Bacillus amyloliquefaciens* subutilisin BPN'. C, conservation of the Arg-Gly-Asp motif (underlined) in mammalian Kex2 family members. In B and C, residues conserved between PC3 and at least one of other members are boxed.

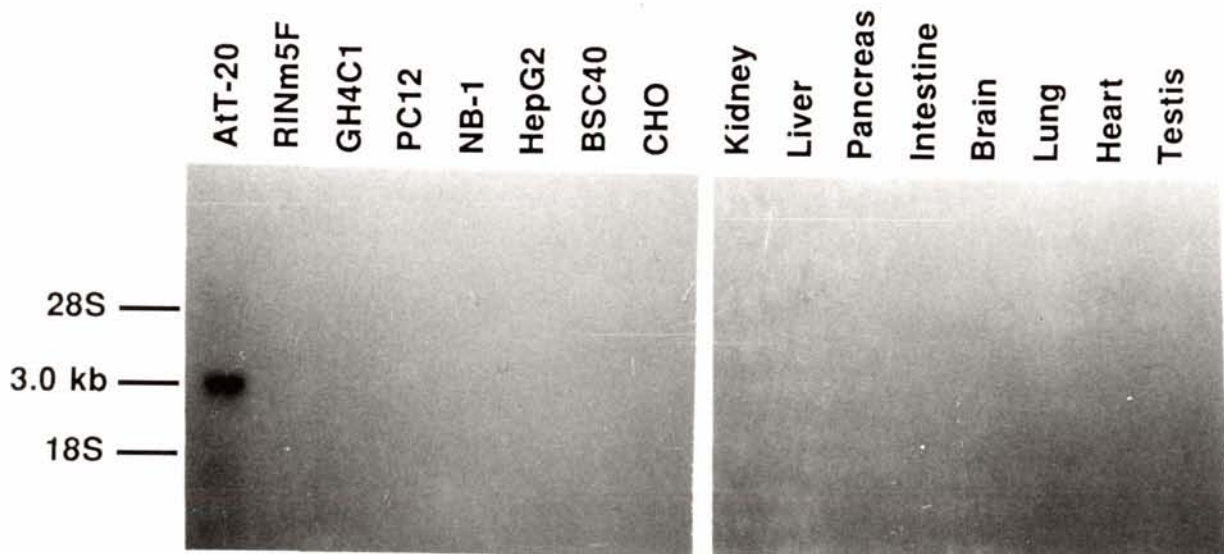


Fig.8. Northern blot analysis of total RNAs from mouse tissues and culture cell lines using the 231-bp *Kpn*I-*Hind*III PC3 fragment. Experimental details are described under "EXPERIMENTAL PROCEDURES".Cell lines are derived from: AtT-20, a mouse pituitary cell line; GH4C1, a rat pituitary cell line; PC12, a rat pheochromocytoma cell line; NB-1, a human neuroblastoma cell line; HepG2, a human hepatoma cell line; BSC-40, an African green monkey kidney cell line; CHO, Chinese hamster ovary cells.

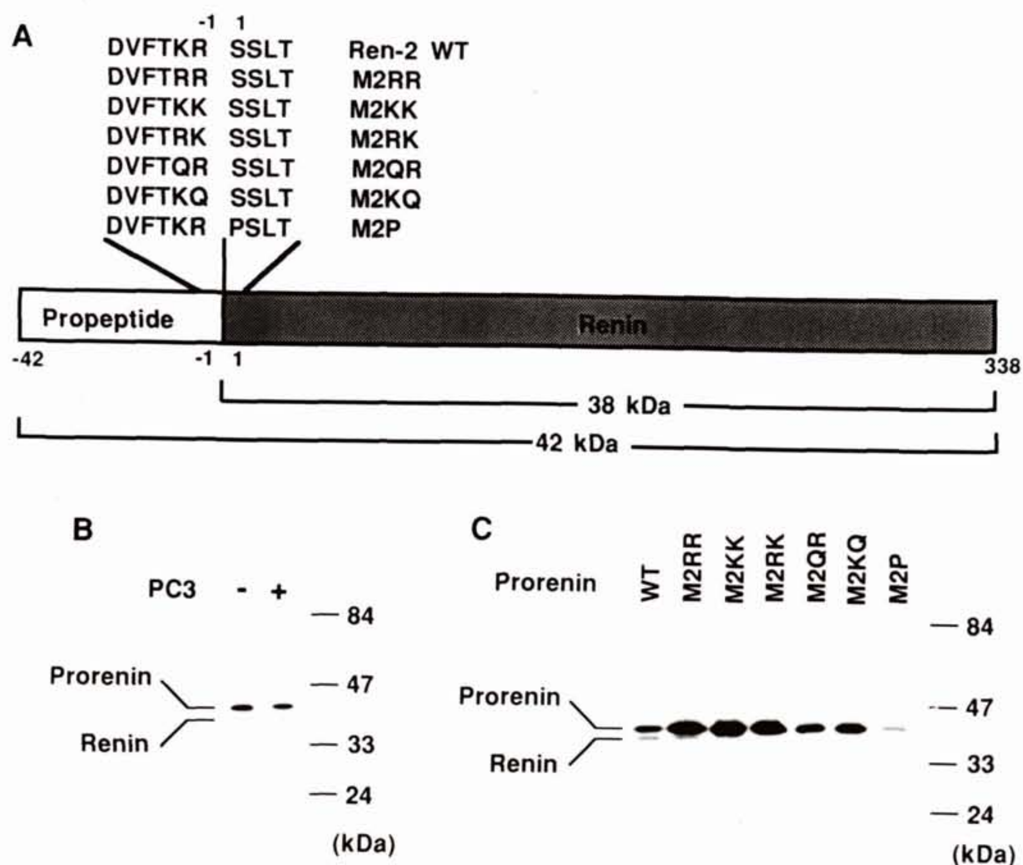


Fig.9. Co-expression of PC3 and prorenin in GH4C1 cells. A, schematic representation of the structures of wild type (WT) mouse *Ren-2* prorenin and its mutant at the Lys-Arg cleavage site. Site-directed mutagenesis to generate these mutants has been described under "EXPERIMENTAL PROCEDURES" and Fig.5. B, processing activity of PC3. GH4C1 cells at 70% confluence in 35-mm dish were transfected with the expression plasmid of Ren-2 prorenin in combination with that of PC3 (+) or the control plasmid lacking the cDNA insert (-), and cultured for 2 days. The cells were then labeled with [^{35}S]methionine as described under "EXPERIMENTAL PROCEDURES". The culture medium was subjected to immunoprecipitation with anti-*Ren-2* renin antiserum and SDS-PAGE analysis. The expression plasmids of PC3 and prorenin were constructed by subcloning of their cDNAs behind the Rous sarcoma virus promoter of pAGE123 vector. C, sequence requirements for prorenin processing by PC3. GH4C1 cells were transfected with the expression plasmid of PC3 in combination with that of wild type *Ren-2* prorenin or its mutant shown in A. Renin molecules secreted into the culture medium were analyzed as described in CHAPTER 1 and "EXPERIMENTAL PROCEDURES"

CHAPTER 3

Arg-X-Lys/Arg-Arg Motif as a Signal for Precursor Cleavage Catalyzed by Furin within the Constitutive Secretory Pathway

ABSTRACT

Many peptide hormones are produced from larger precursors by endoproteolysis at pairs of basic amino acids (*e.g.* Lys-Arg and Arg-Arg) within the regulated secretory pathway in endocrine cells. However, many other secretory and membrane proteins appear to be produced from precursors through cleavage at multiple, rather than paired, basic residues within the constitutive secretory pathway in non-endocrine cells. By surveying various precursors processed constitutively, we noticed that most of them have the consensus sequence, Arg-Lys/Arg-Arg (RXK/RR), at the cleavage site. When expressed in endocrine and non-endocrine cells, a precursor with the RXKR sequence was cleaved in both types of cells, whereas that with the Lys-Arg pair was cleaved only in the endocrine cells. When the RXKR precursor was co-expressed with furin and PC3, both of which are mammalian homologues of the yeast precursor-processing endoprotease Kex2, in non-endocrine cells, enhancement of the precursor cleavage by furin but not by PC3 was observed. By contrast, when the Lys-Arg precursor was co-expressed with the two mammalian proteases in endocrine cells with no endogeneous processing activity at dibasic sites, it was cleaved only by PC3. These results indicate that the basic pair and the RXK/RR sequence are the signals for precursor cleavages catalyzed by PC3 within the regulated secretory pathway and by furin within the constitutive path way, respectively.

EXPERIMENTAL PROCEDURES

Plasmid Constructions

Site-directed mutagenesis to replace the Phe residue at the fourth residue upstream of the cleavage site (position -4) of mouse *Ren-2* prorenin by Arg was performed using the primer in shown Fig.11A in a manner described in CHAPTER 2. Mutagenesis to replace the Lys residue at position -2 by Gln was described in CHAPTER 2. cDNA fragments covering the entire coding sequence of native and mutant prorenins, furin (28), and PC3 were subcloned behind the SV40 promoter of the pSVD vector (29) for expression in Chinese hamster ovary (CHO) cells or behind the RSV promoter of the pAGE123 vector (60) for expression in AtT-20 and GH_4C_1 cells.

DNA Transfection

Transfections of the pSVD-derived prorenin expression plasmids into CHO/DXB-11 cells and of the pAGE-derived ones into AtT-20/D16v cells were performed using a CellPhect Transfection Kit. Stable transfectants of CHO (29) and AtT-20 (60) cells were then selected under previously described conditions. In the co-expression experiments, CHO or GH_4C_1 cells at ~70% confluence in a 35-mm dish were transfected with the expression plasmid of either native or mutant prorenin alone or in a combination with the that of either furin or PC3, incubated for 48h, and used for following experiments.

Renin Assay

Active renin and latent prorenin activities in the culture medium were determined by the angiotensin I-generating method, as described in CHAPTER 2.

Radiolabeling and Immunoprecipitation

Radiolabeling and immunological identification of renin molecules were carried out as described in CHAPTERs 1 and 2.

RESULTS AND DISCUSSION

In endocrine cells, many peptide hormones are produced from larger precursors through cleavage at paired basic amino acids, primarily Lys-Arg and Arg-Arg, within the regulated secretory pathway (4,46,86). However, many other secretory and membrane proteins appear to be produced from precursors through cleavage at multiple, rather than paired, basic residues within the constitutive secretory pathway in non-endocrine cells.

By surveying the sequences around the cleavage site of various precursors that known or thought to be processed constitutively, we noticed that most of them have an Arg residue at position -4 besides basic residues at position -1 and -2 (Fig.10). There are precursors with various functions, such as those of hormones, growth factors, plasma proteases, receptors, and viral glycoproteins. Genetic evidence and studies using site-directed mutagenesis have also suggested the importance of the Arg^{-4} residue, as well as the basic pair in the proteolytic processing, although nobody has pointed out the conservation of the RXK/RR motif at the cleavage site of various constitutively processed precursors. The facts are as follows. A point mutation at the Arg^{-4} resulting in expression of an unprocessed factor IX precursor causes hemophilia B (1). Envelope glycoprotein precursors with the RXK/RR sequence of virulent stains of influenza virus and Newcastle disease virus (NDV) are processed in cells throughout the organisms, whereas those without the sequence of avirulent stains are processed only in limited types of cells (37). Human immunodeficiency virus (HIV) gp160 (2) and human cytomegalovirus glycoprotein B (83) mutated at either basic residue in the RXK/RR sequence are not cleaved. For processing of the Fo protein simian virus 5, which has 5 successive Arg residues at the cleavage site, at least 4 successive residues are essential (67). The protein C precursor with a Lys-Arg cleavage site is processed more efficiently if an Arg residue is present at position -4 (18). Prosomatostatin mutant with an Arg-Glu-Lys-Arg or Arg-Glu-Arg-Arg sequence but not the native precursor

with Arg-Glu-Arg-Lys is processed in non-endocrine cells (84). Taken together , it is possible that precursors with the RXK/RR motif are cleaved constitutively by common or limited types of endoprotease, which differ from those involved in prohormone processing at dibasic sites within the regulated pathway.

To address this possibility, I expressed mouse *Ren-2* prorenin, which is cleaved at a Lys-Arg site to yield enzymatically active renin, and its mutants with an Arg residue at position -4 ($M2R^{-4}$; Fig.11A) in non-endocrine and endocrine cells; CHO cells, which have only the constitutive pathway and secrete the renin molecule as prorenin (21,29); and mouse pituitary AtT-20 cells, which have both secretory pathways and cleave endogeneous POMC and various foreign prohormones, including prorenin, at dibasic sites within the regulated pathway (4,22,55,56,86). As shown in Fig.11B, CHO cells expressing native *Ren-2* prorenin secreted mostly inactive prorenin, whereas those expressing the $M2R^{-4}$ mutant secreted a significant amount of active renin. Moreover, the ratio of active renin to inactive prorenin secreted from AtT-20 cells expressing $M2R^{-4}$ was much higher than that from cells expressing native prorenin. The renin activity data were confirmed by immunological identification of renin molecules (Fig.11C).

To examine whether the processing of $M2R^{-4}$ in AtT-20 cells occurs within the regulated or constitutive pathway, I performed pulse-chase experiments. As shown in Fig.12, cells expressing native prorenin secreted mainly prorenin for the labeling period, and for the chase period, they secreted renin in response to 8-Br-cAMP, which stimulates release via the regulated pathway (55,56). By contrast, cells expressing $M2R^{-4}$ secreted mostly renin throughout the labeling and chase periods, although the renin release was also stimulated by secretagogue. These results indicate that processing of $M2R^{-4}$ prorenin in AtT-20 cells occurs within both of the constitutive and regulated pathways.

In CHAPTER 1, I have shown that furin cannot cleave prorenin at the dibasic site (28). Moreover, I have shown that furin mRNA is expressed in all examined tissues

and cell lines, including those unable to cleave precursors at dibasic sites. Since some precursors with the RXK/RR, such as pro-nerve growth factor (13), proactivin A (31), and the F_0 protein of NDV virulent strains (57), are cleaved in all examined cells whether they are derived from endocrine or non-endocrine cells, I speculated that furin may be involved in precursor cleavage at RXK/RR sites within the constitutive pathway. To confirm this speculation, I examined the effect of furin expression on the cleavage of native and $M2R^{-4}$ prorenins by co-expression in CHO cells. As shown in Fig.13A, co-expression of furin and $M2R^{-4}$ prorenin resulted in complete conversion of the precursor to the mature form. By contrast, processing of native prorenin by furin was barely detectable.

As shown in CHAPTER 2, I identified a novel Kex2-like protein, designated PC3 in AtT-20 cells and proposed that it is a resident processing protease of these cells. To examine this possibility, I performed co-expression experiments. Co-expression of PC3 with native or $M2R^{-4}$ prorenin in CHO cells did not result in conversion of the precursor (Fig.13A). I then used rat pituitary GH_4C_1 cells as the host, since they are endocrine cells with no endogeneous processing activity at dibasic sites (28,86,87), the Kex2 protease expressed in these cells has been shown to cleave co-expressed POMC at dibasic sites (87), and I have shown that furin expressed in these cells cannot cleave co-expressed prorenin (28). As shown in Fig.13B, PC3 cleaved native prorenin in these cells. By contrast, it did not cleave a mutant prorenin with Gln-Arg ($M2Q^{-2}$) instead of the native Lys-Arg pair. Similar results were obtained when another mutant with Lys-Gln was used as a substrate (data not shown).

Most recently, furin has been shown to cleave the von Willebrand factor precursor (94,98) and pro-nerve growth factor (3) and has been proposed to be involved in precursor cleavage at dibasic sites. However, the cleavage site sequence (Arg-Ser-Lys/Arg-Arg) of these precursors fits the RXK/RR motif. Taken together with the data shown in CHAPTERs 1 and 2, I conclude that furin and PC3 are involved in precursor cleavage at RXK/RR sites within the constitutive pathway and at dibasic sites within

the regulated pathway, respectively. This conclusion is supported by the data concerning processing of *Aplysia* pro-egg laying hormone (82), which has a tetrabasic Arg-Arg-Lys-Arg sequence and several basic pairs at the cleavage sites; an initial cleavage occurs at the tetrabasic site, probably in the *trans*-Golgi network, and later cleavages occur at the dibasic sites after the processing inter-mediates have left the compartment.

It is of note that all mammalian Kex2 homologues, furin (25,28,93), PC2 (79,81), and PC3 (CHAPTER 2), have a RXKR sequence in the NH_2-terminal region of their polypeptide sequences. These facts make it likely that these processing proteases themselves may be initially synthesized as pro-forms and then converted to mature forms through cleavage at the RXKR site. This speculation is supported by the fact that carboxypeptidase H , which removes COOH-terminal basic residues from various peptide-processing intermediates, is produced from its precursor through cleavage at a site marked by this motif (19,71).

The fact that furin cleaves precursors with the RXK/RR sequence is of great interest from the viewpoint of antiviral therapy. Envelope glycoproteins of many viruses, including HIV, which causes acquired immunodeficiency syndrome, are matured through cleavage at the RXK/RR site (see Fig.10). Since the mature, but not immature, glycoproteins can induce fusion between the viral envelope and host cell membrane, thereby enabling entry of the viral genome into host cells (86), it is possible that specific inhibitors for this protease, if discovered, could function as antivirals, although there are some problems to be settled such as those concerning side effects and drug delivery.

Precursor	Sequence around cleavage site	
	−4 −1 ⬇	1
Hormones and growth factors		
Human pro-PTHrP	G S L R R L K R	A V
Aplysia pro-egg laying hormone	E S H S R R K R	S V
Porcine proendothelin-1	W R P R R S K R	C S
Human pro-transforming growth factor β1	L Q S S R H R R	A L
Human proactivin A	H P H R R R R R	G L
Mouse pro-nerve growth factor	N R T H R S K R	S S
Porcine pro-BDNF	N M S M R V R R	H S
Human pro-platelet-derived growth factor	E S L A R G R R	S L
Plasma proteins		
Human proalbumin	R G V F R R	D A
Human factor VII precursor	G V L H R R R R	A N
Human factor IX precursor	K I L N R P K R	Y N
Human factor X precursor	N I L A R V T R	A N
Bovine factor X precursor	R V L Q R A R R	A N
Human prothrombin precursor	S L L Q R V R R	A N
Human protein C precursor	Q V L R I R K R	A N
Bovine protein S precursor	Q V L I R R R R	A N
Human von Willebrand factor precursor	P L S H R S K R	S L
Human complement C3 precursor	Q P A A R R R R	S V
Human complement C4 precursor	E K T T R K K R	N V
Human complement C5 precursor	K E I L R P R R	T L
Receptors		
Human insulin proreceptor	P R P S R K R R	S L
Human IGF-I proreceptor	P R P E R K R R	D V
Human LRP precursor	T T S N R H R R	Q I
Human platelet glycoprotein IIb precursor	A H H K R D R R	Q I
Mouse E-cadherin precursor	P G L R R Q K R	D W
Chicken N-cadherin precursor	S H L K R Q K R	D W
Viral envelope glycoproteins		
HIV-1 gp160	R V V Q R E K R	A V
HTLV-1 gp46/p20	T L G S R S R R	A V
Human cytomegalovirus glycoprotein B	L T H N R T K R	S T
Simian virus 5 F_0	I P T R R R R R	F A
Mumps virus F_0	P S G R R H K R	F A
Measles virus F_0	A S S R R H K R	F A
NDV F_0 (a virulent strain)	S G G R R Q R R	F I
Avian influenza A virus HA (a virulent strain)	P S K K R E K R	G L

Fig. 10. Alignment of the amino acid sequences around the cleavage site of precursors processed constitutively. Basic residues are underlined. PTHrP, parathyroid hormone-related protein; BDNF, brain-derived neurotrophic factor; IGF-I, insulin-like growth factor-I; LRP, low density lipoprotein receptor-related protein; HTLV-1, human T-cell leukemia virus type 1.

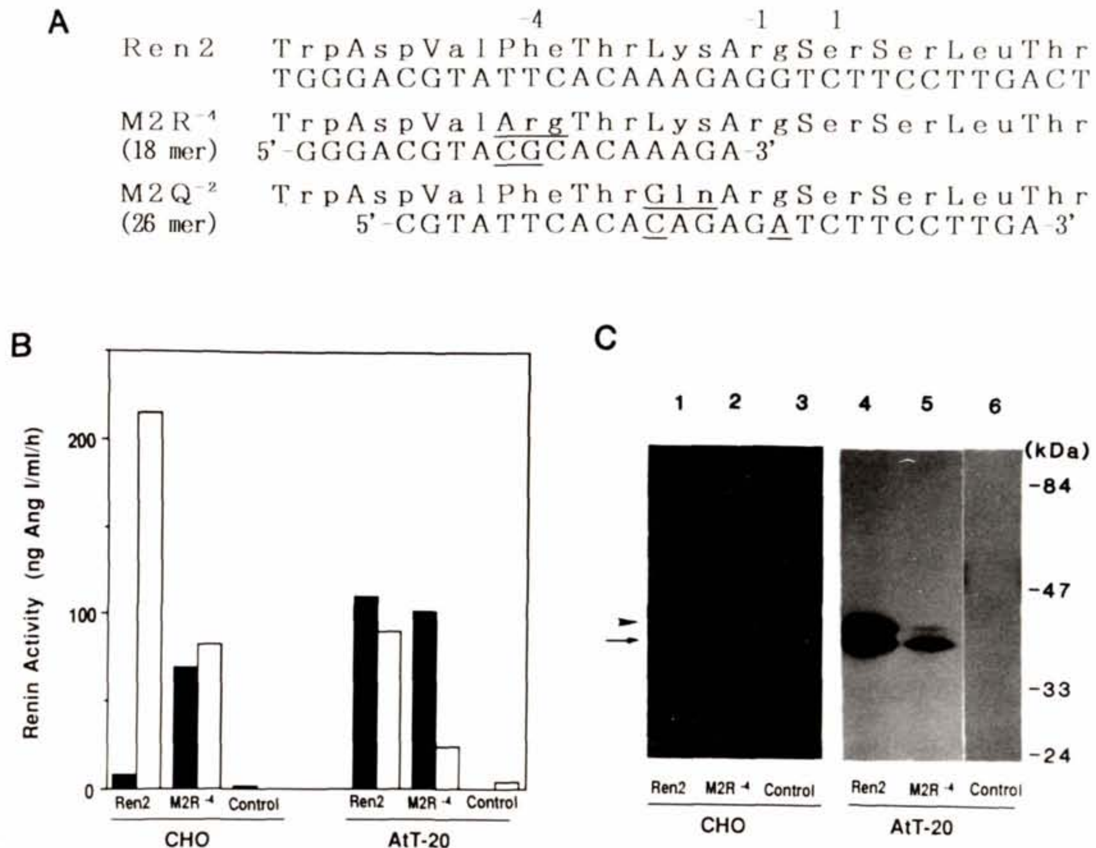


Fig.11. Expression of native *Ren-2* and M2R^{-4} mutant prorenins in CHO and AtT-20 cells. A, nucleotide sequences of the pertinent region of Ren-2 prorenin cDNA and mutagenic primers with their deduced amino acid sequences. The substituted nucleotides and amino acids are underlined. B, renin activities secreted from cells transfected with the native and M2R^{-4} prorenin plasmids and with the control plasmid lacking the cDNA insert. Closed and open columns represent active renin and latent prorenin activities, respectively. Ang I, angiotensin I. C, radiolabeling and immunological identification of renin molecules secreted from the transfected cells. The radiolabeled culture media from CHO (lanes 1-3) and AtT-20 (lanes 4-6) cells transfected with the native prorenin (lanes 1 and 4), M2R^{-4} (lanes 2 and 5), and control (lanes 3 and 6) plasmids were immunoprecipitated with anti-*Ren-2* renin antiserum and then SDS-PAGE analysis. The arrowhead and arrow indicate the positions of prorenin and renin, respectively.

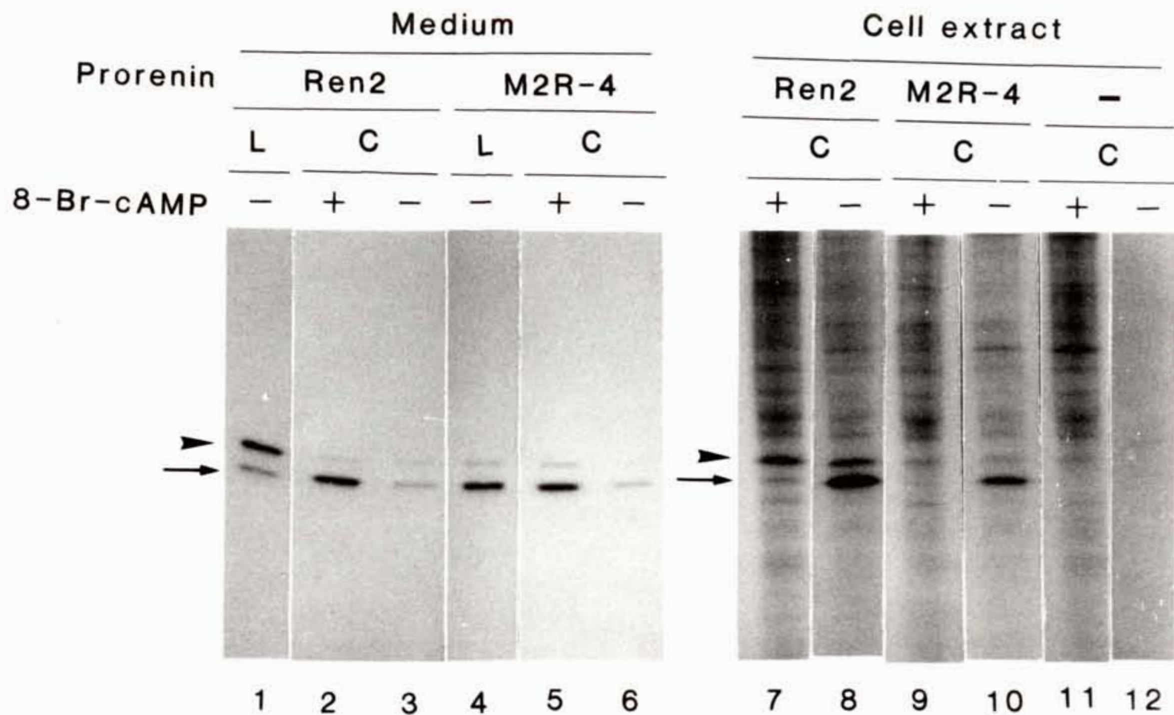


Fig.12. Studies on the secretory pathway of native *Ren-2* and M2R^{-4} prorenins in AtT-20 cells. The radiolabeled culture media (lanes 1-6) and cell extracts (lanes 7-12) of AtT-20 cells transfected with the native prorenin (lanes 1-3, 7, and 8), M2R^{-4} (lanes 4-6, 9, and 10), and control (lanes 11 and 12) plasmids were immunoprecipitated with anti-*Ren-2* renin antiserum and then SDS-PAGE analysis. Lanes 1 and 4, labeling period (L); lanes 2, 5, 7, 9, and 11, chase period (C) with 8-Br-cAMP; lanes 3, 6, 8, 10, and 12, chase period without 8-Br-cAMP. Arrowheads and arrows indicate the positions of prorenin and renin, respectively.

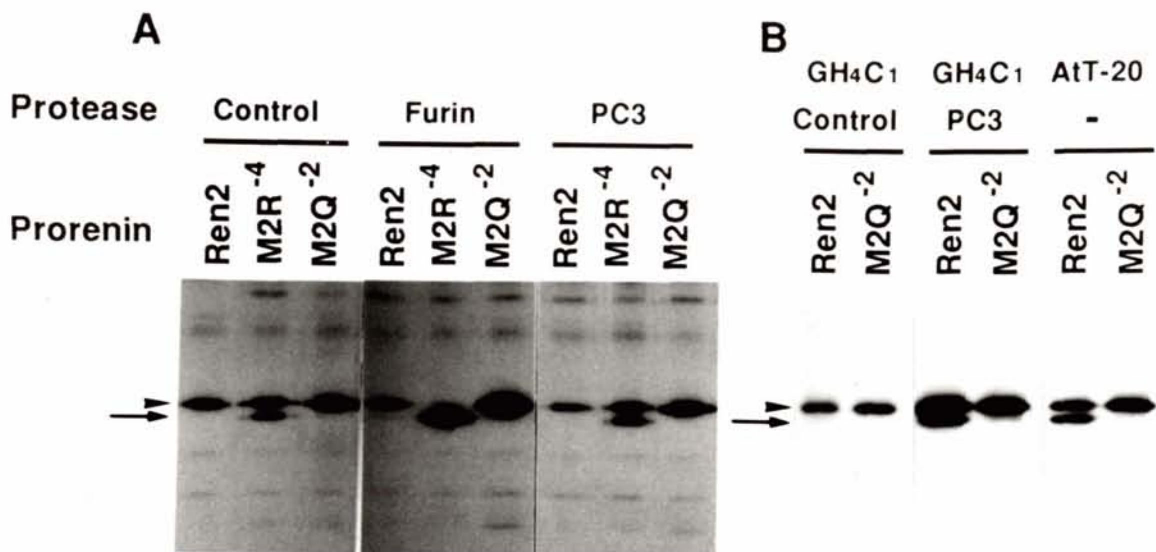


Fig.13. Co-expression of endoprotease and prorenin in CHO (A) and GH4C1(B) cells. The expression plasmids of endoprotease and prorenin as indicated were cotrnsfected into cells. Plasmids lacking the endoprotease cDNA insert were used as controls. Renin molecules secreted from the transfected cells were analyzed as described under "EXPERIMENTAL PROCEDURES". Arrowheads and arrows indicate the positions of prorenin and renin, respectively.

CHAPTER 4

Molecular Cloning and Substrate Specificity of a Kex2-like Processing Endoprotease, PACE4

ABSTRACT

I describe here the isolation and characterization of a mouse cDNA for an isoform of PACE4 (PACE4A), which is a member of the Kex2 family of mammalian pro-protein processing endoproteases. Mouse PACE4A is deduced to be synthesized as a 946-amino acid precursor with a 141-amino acid prepropeptide. It is highly homologous to rat and human PACE4A in the primary sequence, especially within the subtilisin-like catalytic domain (>97% identity). The PACE4A mRNA was detected in all examined tissues and cell lines like furin and PC6A mRNAs. Co-expression experiments in COS-7 cells of PACE4A with pro-von Willebrand factor, complement pro-C3, or a prorenin mutant indicate that although PACE4A can cleave precursors at sites marked by the Arg-X-Lys/Arg-Arg consensus motif, its specificity is somewhat different from those of furin and PC6A.

EXPERIMENTAL PROCEDURES

Cloning of Mouse and Human PACE4 cDNAs

A fragment of mouse PACE4 cDNA corresponding to nucleotides 829-1,268 shown in Fig.13 was obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) of mouse brain poly(A)$^+$ RNA with primers used for cloning of the mouse PC6 cDNA fragment in our previous study (58). cDNA libraries in the λgt10 vector of mouse brain, liver, and kidney, and of human hepatoma HepG2 cells were constructed as described in CHAPTER 1. At first, ~1.8 X 10^6 recombinant phages from the mouse brain library were screened using the ^{32}P-labeled PACE4 cDNA fragment cloned by RT-PCR. Since none of the 42 clones thus obtained covered the entire coding sequence, ~1.2 X 10^6, ~5.0 X 10^5, and ~1.8 X 10^6 phages from the mouse liver and kidney, and HepG2 cDNA libraries, respectively, were screened with a 158-bp cDNA fragment (nucleotides 146-303 in Fig.13) derived from the 5'-end of the longest clone from the brain library, and 20, 8, and 18 positive clones, respectively, were obtained. Although three HepG2 clones covered the entire coding sequence, no mouse liver or kidney clone included the full coding sequence; the longest insert of the clone isolated from liver (designated ML-1) started at nucleotide 1 in Fig.13.

Northern Blot Analysis

Ten μg of total RNAs from various mouse tissues and cell lines were separated on an agarose gel and blotted onto a GeneScreenPlus membrane (Du Pont-New England Nuclear) as described in CHAPTER 1. The blot was hybridized with a ^{32}P-labeled cDNA probe specific for PACE4A (a 794-bp *Sty*I fragment, nucleotides 2090-2887 in Fig.13), and washed as described in CHAPTER 1. The blot was analyzed using a BAS 2000 bioimaging analyzer (Fuji Photo Film Co.).

DNA Construction

Expression plasmids for $M2R^{-4}$ prorenin (30), rat complement pro-C3 (49), mouse furin (28) and PC6A (58), and human PACE4A (this study) were constructed by subcloning of cDNA fragments covering the overall coding sequence into the pRcCMV vector (Invitrogen). An expression plasmid (pSVLvWF) for human pro-von Willebrand factor (vWF) (94) was kindly provided by Dr. W.J.M. Van de Ven (University of Leuven, Belgium) and Dr. J.A. van Mourik (Central Laboratory of the Netherlands Red Cross).

DNA Transfection, Immunoprecipitation, and SDS-PAGE Analysis

To test the substrate specificity of processing endoproteases, monkey kidney COS-7 cells were transfected with the expression plasmid for the proprotein ($M2R^{-4}$ prorenin, pro-C3, or pro-vWF) alone, or in combination with that for the endoprotease (human PACE4A, mouse furin, or mouse PC6A) using a CellPhect transfection kit (Pharmacia-LKB Biotechnology). The processing products were analyzed as described in CHAPTER 1. Briefly, 48 h after transfection, cells at ~70% confluence in a 35-mm dish were labeled with 0.2 mCi/ml of $EXPRE^{35}S^{35}S$ (Du Pont-New England Nuclear) for 10 h. The culture medium was then collected and immunoprecipitated with an appropriate antiserum (anti-renin, anti-C3, or anti-vWF) and protein A-Sepharose (Sigma). The immune complex was denatured by boiling in the SDS-gel sample buffer, electrophoresed on an SDS-polyacrylamide gel (10% for renin or 5% for C3 and vWF) under reducing conditions, and analyzed using a BAS2000 bioimaging analyzer. The experiments were performed at least twice to confirm the reproducibility of the data. When indicated, the processing efficiency was estimated by determining the densities of the bands of the pro- and mature proteins using the BAS2000 analyzer.

RESULTS AND DISCUSSION

I obtained 42, 20, and 8 cDNA clones of PACE4 from mouse brain, liver, and kidney libraries, respectively. However, none of these clones included the putative translation initiation codon. A further attempt to obtain the missing 5'-end of the mouse PACE4 cDNA by the 5'-RACE (rapid amplification of cDNA end) method (23) met with failure. I speculate that the reverse transcriptase and/or DNA polymerase reactions on the occasion of cDNA synthesis went wrong because of a very high GC-content in the missing region of the mouse PACE4 mRNA, in view of the fact that the corresponding region of human cDNA is extremely GC-rich (36). On the other hand, three full-length cDNAs for human PACE4 were obtained from the HepG2 library; the longest (Hep-1) covered the sequence from nucleotides -63 to 3,356 reported by Kiefer et al. (36).

Fig. 1 shows the nucleotide sequence of the longest mouse cDNA (ML-1) and the deduced amino amino acid sequence of mouse PACE4. Recently, it has been reported that there are at least four human PACE4 isoforms designated PACE4A, B, C, and D (36,91). The sequenced mouse cDNA encoded the PACE4A isoform. Determination of the 5'- and 3'-terminal sequences of all the isolated cDNAs revealed that at least 21 cDNAs (10, 9, and 3 cDNAs of brain, liver, and kidney, respectively), which covered the isoform-specific regions, encoded the PACE4A isoform; thus, I could not isolate cDNAs for other PACE4 isoforms (B, C, and D). However, the data do not exclude the possibility that other isoforms do not exist in mouse. Further analysis will be required to address this issue.

By the way, Johnson *et al.* (33) have reported the NH_2-terminal 50-amino acid sequence of mouse PACE4 along with the full-length sequence of rat PACE4A. In comparison with the rat and human full sequences and with the partial mouse sequence, it appeared that the region coding for the first 14-amino acids was missing in the ML-1 PACE4A cDNA (Fig.14). Based on the composite sequence, the overall PACE4A polypeptide was deduced to consist of 946-amino acids. The entire sequence was 98.3

and 92.6% identical with those of rat and human PACE4A, respectively. Especially within the subtilisin-like catalytic domain (amino acids 151-443), only 1 and 6 amino acids were different from those of rat and human PACE4A, respectively. The Cys topography in the Cys-rich domain at the COOH-terminus was completely conserved among the three species. Similar to other Kex2-like endoproteases as described in CHAPTER 3, I expect that mouse PACE4A would be first synthesized as a zymogen with a propeptide which is then cleaved after the Arg-Val-Lys-Arg sequence (amino acids 138-141); thus, mature PACE4A is deduced to consist of 805-amino acids.

I then examined the tissue distribution of mouse PACE4 by Northern blot analysis of RNAs from a variety of mouse tissues and cell lines, since there were conflicting reports on the PACE4 distribution; Kiefer *et al.* (36) and Tsuji *et al.* (92) reported that PACE4 is expressed ubiquitously, while Johnson *et al.* (33) reported the restricted distribution. As shown in Fig.15, a single ~4.0-kb transcript was detected in all examined tissues and cell lines, although the relative expression levels were different from one another. Thus, my data indicate that PACE4 is a ubiquitous protease like furin and PC6A (28,58). I speculate that Johnson *et al.* (33) could not detect the PACE4 mRNA in several tissues and cell lines because of the relatively low level of expression of the mRNA.

Recent data using pro-vWF and its mutants around the processing site as substrates (6,70) have suggested that PACE4A has overlapping but not identical sequence specificity to other ubiquitously expressed Kex2-like endoproteases, furin and PC6; while wild type pro-vWF with an RXKR cleavage site is processed efficiently by both furin and PACE4A, endoproteolytic processing of its mutant with an RXXR cleavage site by PACE4A is much less efficient than that by furin. Furthermore, pro-factor IX, which has an RXKR cleavage site and can be processed efficiently by furin, is not a good substrate of PACE4A (95). Therefore, I examined the substrate specificity of PACE4 by co-transfection experiments in COS-7 cells (Fig.16).

As shown in panel A, coexpressed PACE4A, like furin and PC6A, was able to cleave pro-vWF completely, while expression of pro-vWF alone resulted in ~35%

cleavage of the precursor, which is thought to be caused by endogenous furin (30); thus, I could reproduce the data of other researchers (6,70). I then examined the cleavage of complement pro-C3, which has an RXRR cleavage site and is known to be cleaved to yield α- and β-chains by furin (48). As shown in panel B, coexpression of PACE4A resulted in significant enhancement of pro-C3 cleavage. Unexpected results were obtained when M2R^{-4} prorenin, which is a mutant with an RXKR cleavage site of mouse Ren-2 prorenin and is known to be cleaved by furin and PC6A (30,58), was used as a substrate (panel C). The prorenin cleavage efficiency was ~25% when expression of prorenin alone, and was enhanced to ~50% by furin coexpression. In contrast, coexpression of PACE4A with the prorenin mutant did not give rise to increased processing of the precursor.

It is of interest that the expression of PACE4A did not affect processing of M2R^{-4} prorenin while were capable of cleaving pro-vWF and pro-C3. Taken together with the data of Wasley *et al.* (95) showing that PACE4A cannot cleave pro-factor IX at the RXKR site, my data suggest that PACE4A has similar but not identical substrate specificity to furin and PC6A. Residues outside the RXK/RR motif and/or higher orders of protein structure around the cleavage site may be responsible for discrimination of substrates for different processing endoproteases. In view of the fact that furin, PC6 and PACE4 are expressed ubiquitously, these processing endoproteases may play different physiological roles in the same tissues and cells.

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CCCCGGGCCGCCGATGTCGCGCGGGGCGCAGGGGCCGCCGGCCGGCACGGACTCCCGCCGCTCGCGCTGCGCCCC    75
P  R  A  A  D  V  A  R  G  A  G  A  A  G  R  H  G  L  P  P  L  A  L  R  P  (39)

TGGCGTTGGCTGCTTCTGCTCGCGCTGCCCGCCGCCTGCTCCGCGCTGCCGCCGCCGCGCCCCGTCTACACCAAC   150
W  R  W  L  L  L  L  A  L  P  A  A  C  S  A  L  P  P  P  R  P  V  Y  T  N  (64)

CACTGGGCAGTGCAAGTGCTGGGCGGCCCCGGCGCGGCGGACCGCGTGGCTGCGGCGCACGGCTACCTCAACTTG   225
H  W  A  V  Q  V  L  G  G  P  G  A  A  D  R  V  A  A  A  H  G  Y  L  N  L  (89)

GGCCAGATTGGAAACCTGGACGATTACTATCATTTTTACCACAGCAAGACCTTCAAGAGATCGACCTTGAGCAGC   300
G  Q  I  G  N  L  D  D  Y  Y  H  F  Y  H  S  K  T  F  K  R  S  T  L  S  S  (114)

AGGGGCCCCCACACCTTCCTCAGAATGGACCCACAGGTAAAATGGCTGCAACAACAGGAAGTGAAGCGCAGAGTC   375
R  G  P  H  T  F  L  R  M  D  P  Q  V  K  W  L  Q  Q  Q  E  V  K  R  R  V  (139)

AAGAGACAGGCGCGAAGTGACTCTCTTTATTTCAATGATCCCATTTGGTCTAACATGTGGTACATGCATTGTACT   450
K  R  Q  A  R  S  D  S  L  Y  F  N  D  P  I  W  S  N  M  W  Y  M  H  C  T  (164)

GATAAGAACAGTCGCTGTCGGTCAGAGATGAACGTCCAGGCGGCGTGGAAGCGTGGCTACACAGGAAAGAATGTG   525
D  K  N  S  R  C  R  S  E  M  N  V  Q  A  A  W  K  R  G  Y  T  G  K  N  V  (189)

GTCGTCACCATCCTCGATGACGGCATAGAAAGGAATCACCCAGACCTGGCCCCCAACTACGATTCCTATGCAAGC   600
V  V  T  I  L  D  D  G  I  E  R  N  H  P  D  L  A  P  N  Y  D  S  Y  A  S  (214)

TACGATGTCAATGGAAATGATTATGACCCATCCCCGAGATATGACGCCAGCAACGAGAACAAACATGGCACTCGC   675
Y  D  V  N  G  N  D  Y  D  P  S  P  R  Y  D  A  S  N  E  N  K  H  G  T  R  (239)

TGTGCGGGTGAAGTCGCTGCCTCGGCCAACAACTCCTACTGCATCGTGGGCATCGCATATAATGCAAAGATAGGA   750
C  A  G  E  V  A  A  S  A  N  N  S  Y  C  I  V  G  I  A  Y  N  A  K  I  G  (264)

GGCATCCGGATGCTGGACGGCGATGTGACCGATGTGGTGGAGGCCAAGTCTCTGGGCATCAGACCCAACTACATT   825
G  I  R  M  L  D  G  D  V  T  D  V  V  E  A  K  S  L  G  I  R  P  N  Y  I  (289)

GACATTTACAGCGCCAGTTGGGGACCCGATGATGACGGAAAGACAGTAGATGGACCAGGCCGGCTGGCTAAACAA   900
D  I  Y  S  A  S  W  G  P  D  D  D  G  K  T  V  D  G  P  G  R  L  A  K  Q  (314)

GCTTTCGAGTATGGCATTAAAAAGGGTCGCCAAGGTCTGGGCTCCATTTTTGTTTGGGCCTCTGGGAATGGTGGG   975
A  F  E  Y  G  I  K  K  G  R  Q  G  L  G  S  I  F  V  W  A  S  G  N  G  G  (339)

AGAGAAGGGGACCACTGCTCCTGTGATGGCTACACCAACAGCATCTACACCATCTCCGTAAGCAGCACCACTGAG  1050
R  E  G  D  H  C  S  C  D  G  Y  T  N  S  I  Y  T  I  S  V  S  S  T  T  E  (364)

AACGGCCACAAACCCTGGTACCTGGAAGAATGCGCTTCCACGTTGGCTACCACCTACAGCAGCGGGGCCTTCTAT  1125
N  G  H  K  P  W  Y  L  E  E  C  A  S  T  L  A  T  T  Y  S  S  G  A  F  Y  (389)

GAACGGAAGATCGTCACCACAGATCTGCGTCAGCGCTGCACCGACGGCCACACTGGGACATCAGTCTCAGCCCCC  1200
E  R  K  I  V  T  T  D  L  R  Q  R  C  T  D  G  H  T  G  T  S  V  S  A  P  (414)

ATGGTGGCTGGTATCATTGCCTTGGCTCTAGAAGCAAACAACCAGTTGACCTGGAGGGATGTGCAGCACCTGCTG  1275
M  V  A  G  I  I  A  L  A  L  E  A  N  N  Q  L  T  W  R  D  V  Q  H  L  L  (439)

GTGAAGACATCACGGCCAGCTCATCTGAAGGCGAGCGACTGGAAAGTCAACGGAGCTGGGCATAAAGTTAGCCAT  1350
V  K  T  S  R  P  A  H  L  K  A  S  D  W  K  V  N  G  A  G  H  K  V  S  H  (464)

CTCTATGGATTTGGCTTGGTGGATGCAGAAGCTCTGGTCCTGGAGGCGAGGAAGTGGACGGCAGTGCCCTCCCAG  1425
L  Y  G  F  G  L  V  D  A  E  A  L  V  L  E  A  R  K  W  T  A  V  P  S  Q  (489)

CACGTATGCGTGGCCACCGCAGACAAAAGGCCCAGGAGCATCCCCATAGTGCAGGTGCTACGGACCACAGCCCTG  1500
H  V  C  V  A  T  A  D  K  R  P  R  S  I  P  I  V  Q  V  L  R  T  T  A  L  (514)
```

```
ACCAACGCCTGTGCGGACCACTCAGACCAGCGTGTGGTGTACCTGGAGCATGTGGTTGTCCGAATCTCTATCTCA  1575
T  N  A  C  A  D  H  S  D  Q  R  V  V  Y  L  E  H  V  V  V  R  I  S  I  S   (539)

CACCCACGCCGGGGCGATCTCCAGATCCACCTGATTTCTCCCTCTGGAACCAAGTCTCAACTTTTGGCAAAGAGA  1650
H  P  R  R  G  D  L  Q  I  H  L  I  S  P  S  G  T  K  S  Q  L  L  A  K  R   (564)

TTGCTGGATTTTTCCAATGAGGGGTTCACAAACTGGGAATTCATGACCGTCCACTGCTGGGGAGAAAAGGCTGAA  1725
L  L  D  F  S  N  E  G  F  T  N  W  E  F  M  T  V  H  C  W  G  E  K  A  E   (589)

GGGGAGTGGACTCTGGAAGTGCAGGATATACCATCGCAGGTCCGAAACCCAGAGAAACAAGGAAAGCTGAAAGAA  1800
G  E  W  T  L  E  V  Q  D  I  P  S  Q  V  R  N  P  E  K  Q  G  K  L  K  E   (614)

TGGAGCCTCATTTTATATGGCACCGCAGAGCACCCATATCGCACCTTCAGCTCCCATCAGTCTCGCTCACGGATG  1875
W  S  L  I  L  Y  G  T  A  E  H  P  Y  R  T  F  S  S  H  Q  S  R  S  R  M   (639)

CTGGAGCTCTCCGTCCCGGAGCAGGAGCCTCCCAAGGCTGCTGGACAACCACCCCAGGCAGAGACTCCAGAAGAC  1950
L  E  L  S  V  P  E  Q  E  P  P  K  A  A  G  Q  P  P  Q  A  E  T  P  E  D   (664)

GAGGAAGAGTACACAGGTGTGTGCCATCCCGAGTGTGGTGACAAAGGCTGTGATGGTCCCAATGCAGACCAGTGC  2025
E  E  E  Y  T  G  V  C  H  P  E  C  G  D  K  G  C  D  G  P  N  A  D  Q  C   (689)

TTGAACTGTGTCCACTTCAGCCTGGGGAACTCCAAGACAAACAGGAAGTGTGTGAGCGAGTGCCCCTTGGGCTAC  2100
L  N  C  V  H  F  S  L  G  N  S  K  T  N  R  K  C  V  S  E  C  P  L  G  Y   (714)

TTTGGGGATGCAGCTGCAAGACGCTGCCGTCGCTGCCATAAGGGATGTGAGACGTGCACGGGCAGGAGCCCAGCA  2175
F  G  D  A  A  A  R  R  C  R  R  C  H  K  G  C  E  T  C  T  G  R  S  P  A   (739)

CAGTGCCTGTCTTGTCGCCGTGGGTTCTATCACCACCAGGAGACGAACACGTGTGTGACCCTCTGTCCTGCCGGA  2250
Q  C  L  S  C  R  R  G  F  Y  H  H  Q  E  T  N  T  C  V  T  L  C  P  A  G   (764)

CTTTATGCTGATGAAAGTCAGAGACTCTGCCTCAGGTGCCACCCGAGCTGTCAGAAGTGCGTGGATGAACCTGAG  2325
L  Y  A  D  E  S  Q  R  L  C  L  R  C  H  P  S  C  Q  K  C  V  D  E  P  E   (789)

AAGTGCACTGTGTGTAAGGAGGGATTCAGCCTGGCACGGGGCAGCTGCATTCCGGACTGTGAACCGGGTACCTAC  2400
K  C  T  V  C  K  E  G  F  S  L  A  R  G  S  C  I  P  D  C  E  P  G  T  Y   (814)

TTCGATTCAGAGCTCGTCAAATGTGGGGAATGCCATCACACCTGCCGGACCTGCGTGGGGCCCAGCAGAGAAGAG  2475
F  D  S  E  L  V  K  C  G  E  C  H  H  T  C  R  T  C  V  G  P  S  R  E  E   (839)

TGTATTCACTGTGCAAAAAGCTTCCACTTCCAAGACTGGAAATGTGTGCCGGCCTGCGGTGAGGGCTTCTACCCG  2550
C  I  H  C  A  K  S  F  H  F  Q  D  W  K  C  V  P  A  C  G  E  G  F  Y  P   (864)

GAGGAGATGCCTGGCTTACCCCACAAAGTGTGTCGAAGATGTGAGGAGAACTGCCTGAGCTGCGAGGGCTCCAGT  2625
E  E  M  P  G  L  P  H  K  V  C  R  R  C  E  E  N  C  L  S  C  E  G  S  S   (889)

AGGAACTGCAGCAGATGTAAAGCTGGCTTCACACAGCTGGGAACCTCCTGCATCACCAACCACACGTGCAGCAAT  2700
R  N  C  S  R  C  K  A  G  F  T  Q  L  G  T  S  C  I  T  N  H  T  C  S  N   (914)

GCCGATGAGACCTTCTGCGAGATGGTGAAGTCCAATCGGCTCTGCGAACGGAAGCTCTTCATCCAGTTTTGCTGC  2775
A  D  E  T  F  C  E  M  V  K  S  N  R  L  C  E  R  K  L  F  I  Q  F  C  C   (939)

CGCACCTGCCTCCTGGCTGGGTAGGGGCACCAGCTGCCCGCAGAGGGCGGGGTCCTCCTGTCTGCCCGTTTGCCC  2850
R  T  C  L  L  A  G                                                          (946)

ATCTACCTTCCTACAGATGGTCAGCCATAGCCTGTTCCTTGGGGTAGCCCTGCATCTGACAGCTGTATCTCCCCC  2925
AGAGCTGGGTTCTACTGCAGCATCTCTGAGCACCTGAACAGGTGGAGGTGGCCCTTAAGGATATGTGGCTAAATT  3000
GACAAAAATCCCCTTGAACTCTGCTTGCTGGCTGCAGTCTAAAGTTGGACTCAAAACAGGAACAAAAAAAAAAAA  3075
```

Fig. 14. Nucleotide and deduced amino acid sequences of the partial cDNA encoding mouse PACE4A.

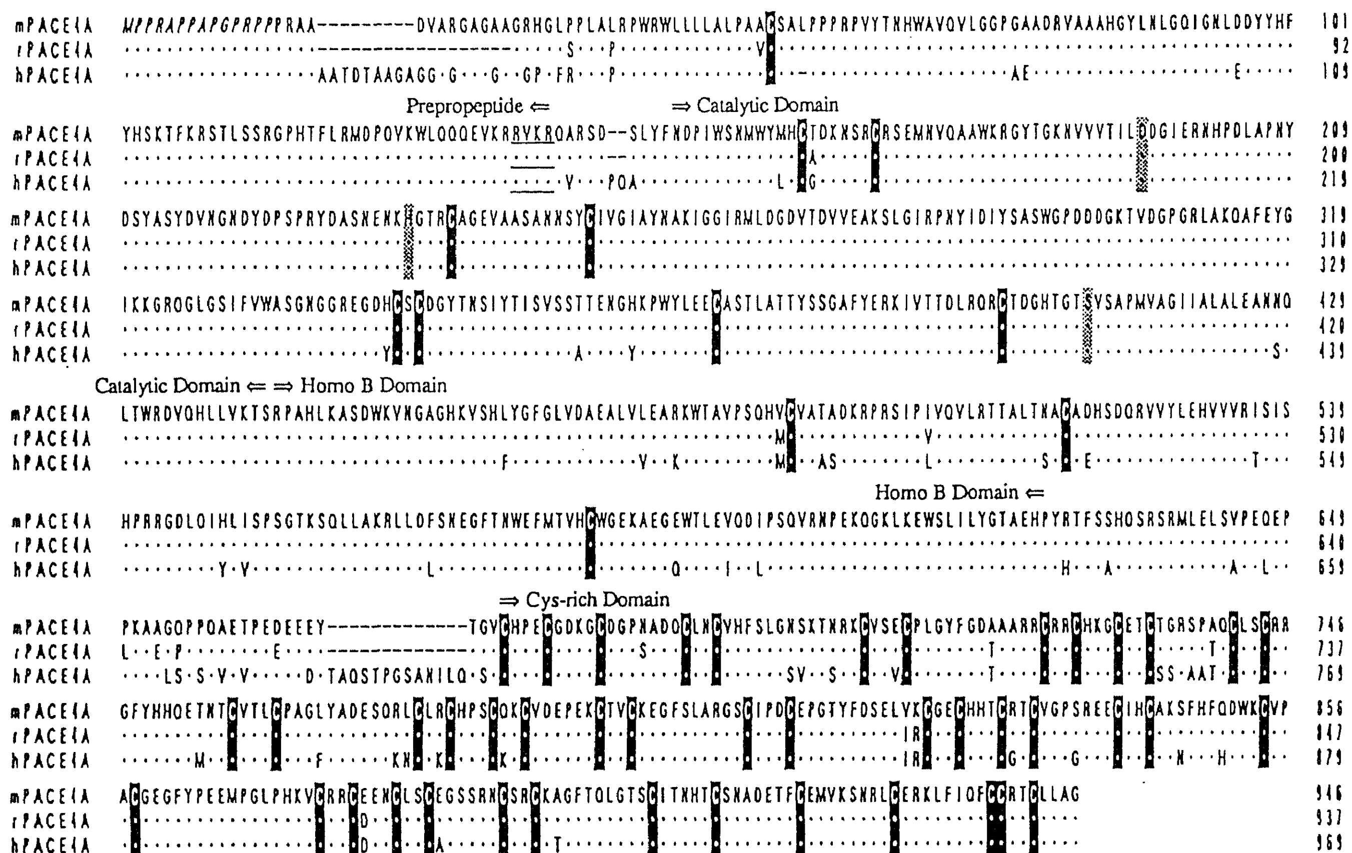

Fig.15. Amino acid sequence alignments of mouse (m), rat (r), and human (h) PACE4A. The rPACE4A and hPACE4A sequences were taken from Refs. 92 and 94, respectively, and the sequence of the NH2-terminal 14-residues of mPACE4A (shown by italics) was taken from Ref. 94. Domain boundaries are indicated by arrows. Residues of rPACE4A and hPACE4A identical with those of mPACE4A are indicated by dots, and gaps introduced into the alignment are indicated by hyphens. The catalytically important Asp, His, and Ser residues are shaded, and Cys residues are shown in dark boxes. The putative propeptide cleavage site sequence, Arg-Val-Lys-Arg, are underlined.

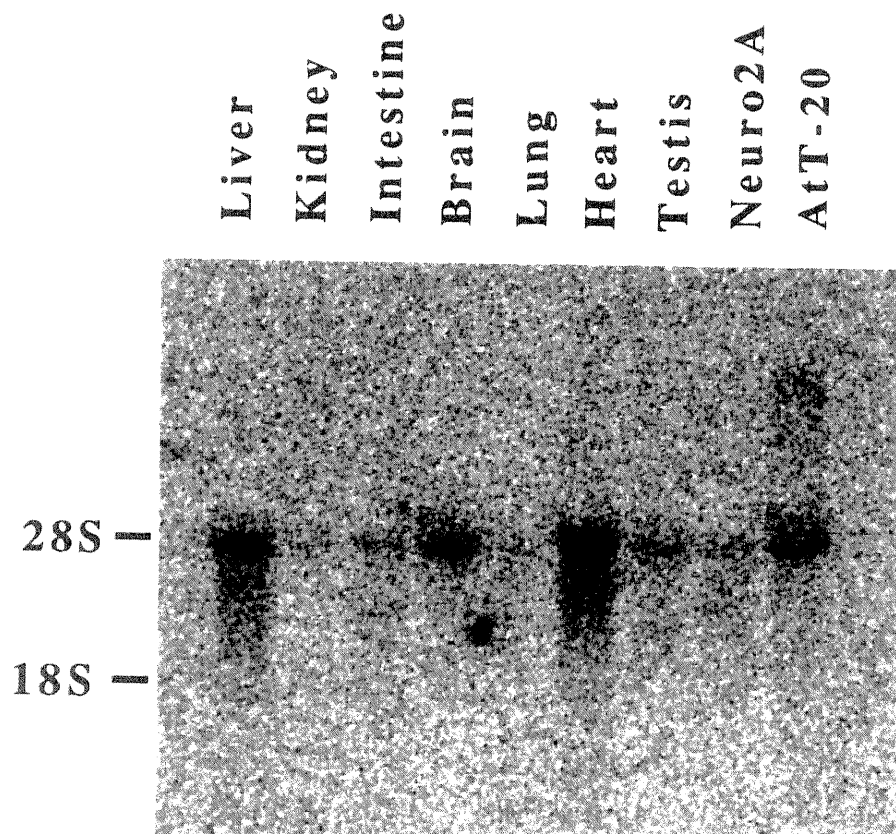

Fig. 16. Northern blot analysis of PACE4A in mouse tissues and cell lines. Experimental details are described under "EXPERIMENTAL PROCEDURES". Neuro2A, mouse neuroblastoma cell line; AtT-20, mouse corticotrophic tumor cell line.

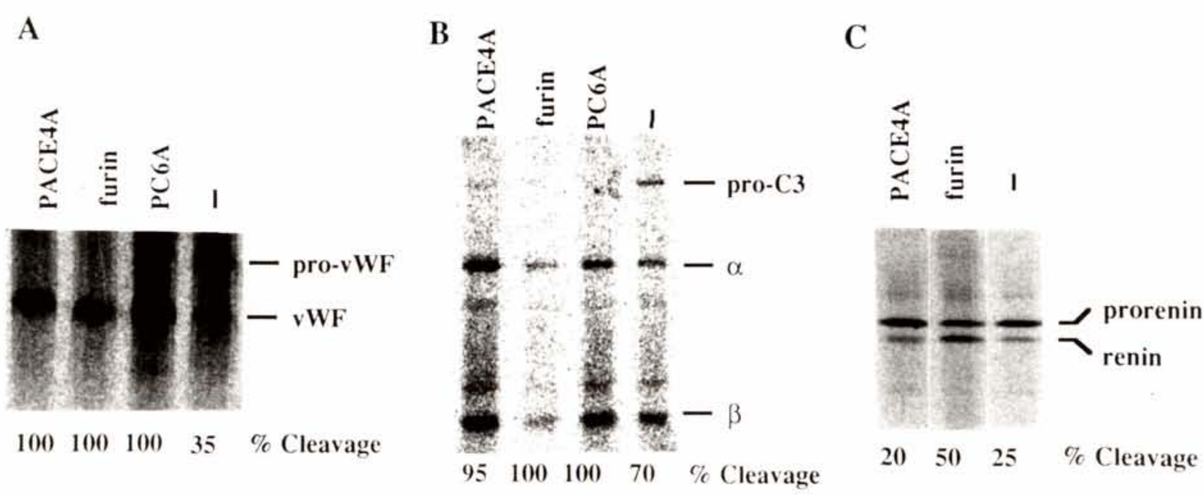

Fig. 17. Substrate specificity of human PACE4A. The data of furin and PC6A as controls are also shown. The followings were used as substrates: A, pro-vWF; B, pro-C3; C, M2R^{-4} prorenin. Experimental details are described under "EXPERIMENTAL PROCEDURES". Each cleavage efficiency is shown on the bottom of each lane.

GENERAL DISCUSSION

Endoproteolytic precursor cleavage is one of the key steps to yield bioactive peptides. In endocrine cells, many peptide hormones are produced from larger precursors through cleavage at pairs of basic amino acids (*e.g.* Lys-Arg and Arg-Arg) within the regulated secretory pathway (4,46,86). However, many other secretory and membrane proteins appear to be produced from precursors through cleavage at multiple, rather than paired, basic amino acids within the constitutive secretory pathway in non-endocrine cells.

Although there have been several reports on endoprotease activities capable of precursor cleavage (41,90), little is known about endoproteases physiologically involved in this process. One exception well characterized at the molecular and enzymatic levels is the Kex2 protease of the yeast *Saccharomyces cerevisiae.* It is a Ca^{2+}-dependent serine protease with a bacterial subutilisin-like catalytic domain and is involved in processing of pro-α-mating factor and pro-killer toxin at dibasic sites (24,25,34,53,54,59,60,72,73,76,77,81,87,93). Recent evidence that the Kex2 protease expressed in mammalian cells can cleave a precursor, proopiomelanocortin (POMC) (59) makes it likely that mammalian Kex2 homologous, if present, could function as prohormone processing endoproteases.

In this study, I have cloned and sequenced mouse cDNAs for mammalian Kex2 homologous,furin, PC3, and PACE4. Co-expression experiments in mammalian cells have shown that PC3 but not furin and PACE4 possesses an endoprotease activity toward dibasic sites. These observations indicate that at least one Kex2-like protein, *i.e.* PC3, is a *bona fide* prohormone processing endoprotease (CHAPTER2).

More recently, furin has been shown to cleave pro-vWF (94,98) and pro-nerve growth factor (3), and proposed to be involved in precursor cleavage at dibasic sites. This does not appear to fit my previous data (CHAPTER 1, 28) that furin cannot cleave prorenin at the dibasic site. However, the cleavage site sequence (Arg-Ser-Lys-Arg) of

these precursors matches with the RXK/RR motif which I have proposed as a cleavage signal recognized by the furin within the constitutive secretory pathway (CHAPTER 3, 30). Taken together with the fact that furin mRNA is present in all examined tissues and cell lines including those unable to cleave prohormones at dibasic sites (CHAPTER 1, 28), I conclude that PC3 and furin are involved in precursor cleavages at dibasic sites within the regulated secretory pathway and at RXK/RR sites within the constitutive secretory pathway, respectively.

In CHAPTER 4, I described the isolation and characterization of a mouse cDNA for an isoform of PACE4. The PACE4 mRNA was detected in all examined tissues and cell lines like furin mRNA. Co-expression experiments in non-endocrine cells of PACE4 with pro-vWF, complement pro-C3, or a prorenin mutant ($M2R^{-4}$) indicate that although PACE4 can cleave precursors at sites marked by the Arg-X-Lys/Arg-Arg consensus motif within constitutive secretory pathway, its specificity is somewhat different from that of furin. It is of interest that the expression of PACE4 did not affect processing of $M2R^{-4}$ prorenin while it was capable of cleaving pro-vWF and pro-C3. Taken together with the data of Wasley *et al.* (95) showing that PACE4 cannot cleave pro-factor IX at the RXKR site, my data suggest that PACE4 has similar but not identical substrate specificity to furin. Residues outside the RXK/RR motif and/or higher orders of protein structure around the cleavage site may be responsible for discrimination of substrates for different processing endoproteases. In view of the fact that furin and PACE4 are expressed ubiquitously, these processing endoproteases may play different physiological roles in the same tissues and cells.

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