Identification of the Fourth Member of the Mammalian Endoprotease Family Homologous to the Yeast Kex2 Protease

ITS TESTIS-SPECIFIC EXPRESSION

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We used the polymerase chain reaction to identify a mouse testis cDNA that represented another member of a growing class of mammalian endoproteases involved in the processing of precursor proteins. This cDNA encoded a 655-residue protein, designated PC4, containing a bacterial subtilisin-like catalytic domain closely related to those of the recently characterized precursor-processing endoproteases, furin, PC1/PC3, PC2, and Kex2. Within this domain, the amino acid sequence of PC4 was 70, 58, 55, and 45% identical with those of mouse furin, mouse PC1/PC3, mouse PC2, and yeast Kex2, respectively. Northern blot analysis indicated that the PC4 mRNA was detectable only in the testes after the 20th day of postnatal development. Moreover, this message was mainly expressed in the round spermatids. These data suggest that PC4 represents a prime candidate for a precursor-processing endoprotease in the testicular germ cells and that its gene expression is regulated during spermatogenesis.

A common step essential for maturation of many bioactive peptides and proteins is limited endopeptidolysis of larger, inactive precursors at sites marked by paired or multiple basic amino acids (1-4). Although purification and characterization of candidate processing endoproteases have been reported (5-9), little is known about the physiological functions of them in the endopeptidolytic process, except for the Kex2 protease of the yeast *Saccharomyces cerevisiae* (10-13). It is a Ca$^{2+}$-dependent serine protease with a bacterial subtilisin-like catalytic domain and is responsible for the processing of pro-factor and pro-killer toxin at the paired basic residues. Recently, three mammalian Kex2-like proteins, furin (14-17), PC2 (18, 19), and PC3 (also referred to as PC1) (20-22), have been identified by cDNA cloning. PC2 (23, 24) and PC1/PC3 (22-25) were demonstrated to direct precursor cleavages at dibasic sites, whereas furin appears to be involved in precursor cleavages at sites marked by the Arg-X-Lys/Arg-Arg consensus motif (25-28).

Since the catalytic domain sequence is highly conserved among three mammalian Kex2-like endoproteases, we used the polymerase chain reaction (PCR)* to identify and clone a new member of this growing family. We present here the primary structure of the fourth member of the mammalian endoprotease family and data on its specific expression during spermatogenesis.

**EXPERIMENTAL PROCEDURES**

**PCR Amplification of Genomic DNA**—To amplify mouse genomic DNA by PCR, two degenerate oligonucleotide primers corresponding to stretches of amino acid sequences conserved among three mammalian Kex2-like proteases were designed (Fig. 1A). DNA amplification using a GeneAmp amplification kit (Perkin Elmer Cetus Instruments) was performed in a Perkin Elmer Cetus thermal cycler for 40 cycles of denaturation (94 °C, 1 min), annealing (55 °C, 2 min), and extension (72 °C, 2 min). After electrophoresis on a polyacrylamide gel, the amplified DNA fragments of interest were extracted from the gel, blunted-end ligated with T4 DNA polymerase (GIBCO-BRL), and ligated into the Smal site of pBluescript-II (Stratagene). Sequencing of several clones revealed that a clone, designated GF-4, with a 350-bp insert DNA encoded a novel member of the Kex2 protease family (Fig. 1, B and C).

**Cloning of Mouse PC4 cDNA**—Approximately 2 × 10⁶ clones from a λgt11 cDNA library of dY mouse testis (29) were screened by hybridization with the λgt11 probe DNA fragment. Twenty-four positive clones were obtained, and the cDNA inserts were separately subcloned into the EcoRI site of pBluescript-II. Both strands of a clone with the longest cDNA insert were sequenced using a Sequenase Version 2.0 kit (United States Biochemical Corp.).

**Preparation of Spermatogenic Cells**—Spermatogenic cells were isolated from mature (60-day-old) ddY mice as described previously (30). Briefly, cell suspension from the seminiferous epithelium was prepared by sequential enzymatic dissociation of testes with collagenase and trypsin. The germ cells were separated by sedimentation velocity at unit gravity on 2-4% bovine serum albumin gradients. Fractions collected were assayed by phase contrast microscopy, and the identical cell populations were combined.

**Northern Blot Analysis**—Total cellular RNAs of mammalian cell lines were isolated by the method of Gough (31). Total RNAs of mouse tissues and purified spermatogenic cells were isolated by the method of Chirgwin et al. (32). The RNAs were denatured with glyoxal, separated on a polyacrylamide gel, and blotted onto a GeneScreen Plus membrane (Du Pont-New England Nuclear). The blot was hybridized with the 3⁵₂P-labeled cDNA probe specific for either PC4 (the 727-bp ScaI-KpnI fragment) or furin (the 924-bp BamHI-BamHI fragment, see Ref. 17) and then washed under the previously described conditions (17).

*Abbreviations used are: PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase(s).
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Fig. 1. Identification of a mouse genomic DNA fragment encoding a new member of the mammalian Kex2 family. A, nucleotide sequences of the pertinent region of mouse furin, mouse PC2, and mouse PC3, and primers used for PCR amplification. The deduced amino acid sequences are shown over the nucleotide sequences. Amino acid residues conserved among furin, PC2, and PC3 are underlined. B, nucleotide and deduced amino acid sequences of the intron-exon junctions were deduced from those of human furin gene (15, 45). C, comparison of the deduced amino acid sequence of GF-4 with those of the corresponding region of mouse furin, mouse PC2, and mouse PC3. Gaps introduced into the alignment are indicated by hyphens.

RESULTS AND DISCUSSION

To identify a new member of the mammalian Kex2-like endoprotease family, we designed degenerate PCR primers corresponding to the stretches of the amino acid sequences conserved among furin, PC2, and PC1/PC3 (Fig. 1A). These primers covered two regions; one region was adjacent to the active site His residue, and the other was ~50 amino acids COOH-terminal to the active site region. After PCR amplification of mouse genomic DNA, a mixture of the amplified DNA fragments with lengths between 200 and 1,000 bp was subcloned into pBluescript-11. Sequence analysis revealed that the amino acid sequence deduced from a clone with a ~350-bp insert (designated GF-4, Fig. 1B) was highly homologous to those of the corresponding regions of furin, PC2, and PC1/PC3. However, it was clearly distinct from these three endoproteases (Fig. 1C).

Preliminary Northern blot analysis indicated that an mRNA transcript, which was hybridized with the GF-4 DNA fragment, was abundant in the testis. Therefore, we screened a mouse testis cDNA library using the GF-4 fragment. Fig. 2 shows the 2,495-nucleotide sequence of the cloned cDNA and the deduced 655-amino acid sequence of the encoded protein, designated PC4. The PC4 polypeptide contains a putative subtilisin-like catalytic domain (residues 116-402) homologous to those of furin, PC2, PC1/PC3, and Kex2 (Fig. 3). Within this region, PC4 shows 70, 58, 55, and 45% amino acid identities with mouse furin, mouse PC1/PC3, mouse PC2, and yeast Kex2, respectively. In addition, the regions NH2- and COOH-terminal to the catalytic domain, previously referred to as homo A and homo B domains, respectively (22), are moderately conserved between PC4 and other Kex2 family members; however, in the homo A domain, PC4 has no significant homology with Kex2. The region COOH-terminal to the homo B domain has no significant sequence similarity among them. The hydropathy profile of PC4 revealed only one hydrophobic segment as a putative signal peptide (data not shown). Thus, PC4, like PC2 and PC1/PC3, and unlike furin and Kex2, lacks a hydrophobic COOH-terminal transmembrane anchor. The NH2 terminus of the mature PC4 protein is assumed to begin at the Ser111 behind an Arg-Val-Lys-Arg sequence, which fits the Arg-X-Lys/Arg-Arg consensus motif as a signal for precursor cleavage catalyzed by furin (25), since the motif is conserved at the corresponding positions in all members of the mammalian Kex2 family (22). There is another notable structural feature; the Arg-Gly-Asp sequence, which is a receptor recognition signal of extracellular matrix proteins (33), is conserved at the corresponding positions in all mammalian members (Fig. 2; see Ref. 22). However, it is presently unknown if this sequence is functional.
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It is of note that the ratio of the 3.0- to 3.5-kb transcripts in prepuberal mouse appears to differ from that in mature mouse; this may suggest the differential expression of the two transcripts at different times of development. On the other hand, the furin mRNA was found in all developmental stages. Since the spermatocytes begin to undergo meiosis and differentiate into the haploid, round spermatids at the 18-day stage after birth (35), it is likely that the PC4 mRNA is expressed stage specifically in the germ cells. When Northern blot analysis of total RNAs from purified populations of pachytene spermatocytes, round spermatids, and a mixture of residual bodies and elongating spermatids (residual body fraction) was carried out, the ∼3.0- and ∼3.5-kb PC4 mRNA transcripts were abundant in the round spermatids (Fig. 5B). Low levels of the transcripts were also found in the pachytene spermatocyte and residual body fractions. These observations indicate that the PC4 mRNA is expressed mainly in the early stages of spermiogenesis.

It is of particular interest what precursor(s) is the physiological substrate(s) for PC4. Peptides produced locally within the testis have been implicated as potential paracrine and autocrine factors mediating interactions between testicular cells (for review, see Ref. 36). However, most of them are produced in the non-germ cells, i.e. Leydig and Sertoli cells (36). Indeed, coexpression of PC4 and a precursor with a dibasic cleavage site, prorenin, which is produced in Leydig cells (37, 38), in culture cell lines did not result in processing of the precursor (data not shown), although similar procedures have been employed to demonstrate precursor-processing activities of other Kex2-like endoproteases (22-28). One of the possible candidate substrates is proenkephalin, since it is produced in the germ cells during meiotic prophase (spermatocytes) and following meiosis (spermatids) (39,40). However, it is of note that the only detectable proenkephalin-derived peptide in the germ cells is [Met]enkephalin-Arg-Phe (40); [Met]enkephalin, which is abundant in the brain and other tissues, is barely detectable. Therefore, PC4 expressed specifically in the germ cells may have a dibasic site preference different from those of other Kex2-like endoproteases.

Considering the presence of at least four Kex2-like endoproteases, i.e. furin, PC2, PC1/PC3, and PC4, with different tissue distributions, precursor cleavages in mammals appear to be regulated in a very complicated manner. For example, it has been reported that PC1/PC3 and PC2 are abundant in both the anterior and intermediate lobes, and only in the intermediate lobe, respectively, of the pituitary (19, 21), and are capable of cleaving the same precursor, proopiomelanocortin, with different dibasic site preferences (23, 24); the data

FIG. 3. Schematic representation of the structures of PC4 and other Kex2 family members. The percentage amino acid identity in each region of each protease with PC4 estimated using the SCD-GENETYX HOMOAPP program (Software Development Co., Ltd.) is shown. SP, signal peptide; C-rich, Cys-rich domain; TM, transmembrane domain; S/T-rich, Ser/Thr-rich domain.

FIG. 4. Distribution of PC4 mRNA. Total RNAs (10 µg each) from various mouse tissues and cell lines were analyzed using the PC4 cDNA probe as described under "Experimental Procedures." GH, and GH,C, rat pituitary cell lines; NB-1, a human neuroblastoma cell line; CHO, a Chinese hamster ovary cell line; BSC-40, an African green monkey kidney cell line; HepG2, a human hepatoma cell line; PC12, a rat pheochromocytoma cell line; SMG, submandibular gland.

FIG. 5. Expression of PC4 mRNA in mouse testicular cells. A, developmental changes of PC4 mRNA accumulation in mouse testis. Total RNAs (20 µg each) from the testes of indicated ages of mice were analyzed using the PC4 or furin cDNA probe as described under "Experimental Procedures." B, PC4 mRNA expression in purified spermatogenic cells. Total RNAs (5 µg each) from pachytene spermatocytes (PS), round spermatids (RS), and a mixture of residual bodies and elongating spermatids (RB) were analyzed using the PC4 cDNA probe as described under "Experimental Procedures."
could account for the difference in the processing pattern of proopiomelanocortin between these two lobes (3). Furthermore, Hutton and his colleagues (41, 42) have demonstrated that two distinct Kex2-like proteases in insulinoma secretory granules, although neither has yet been identified, cleave proinsulin at two distinct dibasic sites. These data make it tempting to conclude that these processing endoproteases require not only the basic pair but also other structural feature(s) for precursor cleavage, as previously proposed (43, 44). Therefore, further characterization of their cleavage specificities will be necessary to clarify the mechanisms of regulation of precursor processing. Indeed, we have recently demonstrated that furin requires an Arg residue at the fourth residue upstream of the cleavage site besides the basic pair for precursor cleavage and have suggested that it is involved in maturation of a wide variety of secretory and membrane proteins, such as growth factors, serum proteases, receptors, and viral envelope glycoproteins, in nonneuroendocrine cells rather than peptide hormones and neuropeptides in neuroendocrine cells (25). Finally, even though PC4 is highly homologous to other Kex2-like endoproteases, its role as a precursor-processing endoprotease must await experimental proof. Further studies are under way to settle this problem.

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