Substrate Specificity of Prorenin Converting Enzyme of Mouse Submandibular Gland

ANALYSIS USING SITE-DIRECTED MUTAGENESIS*

Kazuhisa Nakayama§, Won-Sin Kim¶, Tsutomu Nakagawa¶, Masami Nagahama¶, and Kazuo Murakami¶

From the §Institute of Biological Sciences, ¶Gene Experiment Center, and ¶Institute of Applied Biochemistry, University of Tsukuba, Tsukuba, Ibaraki 305, Japan

Renin is produced from a larger, inactive precursor, prorenin, through endoproteolytic cleavage at paired basic amino acids. Recently, we have purified and characterized an enzyme, which catalyzes the endoproteolytic process, from mouse submandibular gland. The enzyme, named prorenin converting enzyme, specifically cleaves the peptide bond on the COOH-side of the Arg residue at the Lys-Arg pair of mouse Ren 2 prorenin, but does not cleave mouse Ren 1 and human prorenins. In this study, by synthesizing a series of mutant mouse prorenins using site-directed mutagenesis and the Xenopus oocyte expression system, we have investigated the role of the basic pair as the recognition signal for cleavage by PRECE as well as the determinant of the substrate specificity. The results indicate that the basic amino acid at the COOH-side but not at the NH2-side of the basic pair of Ren 2 prorenin is essential for processing directed by prorenin converting enzyme, and that the Arg residue at the COOH-side is more preferable for processing than the Lys. The results also demonstrated that the presence of a Pro residue next to the Lys-Arg pair prevents the processing of Ren 1 prorenin.

Renin (EC 3.4.23.15) is the key enzyme of the renin-angiotensin system and controls the rate-limiting step of the formation of a potent vasoactive peptide, angiotensin II, by catalyzing the formation of angiotensin I from angiotensinogen (1, 2). It thus plays an important role in the regulation of blood pressure. In a manner similar to many other peptide hormones and neuropeptides, renin is produced from a larger, inactive precursor, prorenin, by endoproteolytic removal of the “propeptide” at paired basic amino acids during intracellular transport (3, 4).

Recently, we have purified and characterized an enzyme, which catalyzes the endoproteolytic process, from mouse submandibular gland (SMG) (5). The enzyme, named prorenin converting enzyme (PRECE), is a serine protease consisting of two polypeptide chains of 17 and 10 kDa, and, by taking its inhibitor profile and molecular properties into account, it appears to be different from previously reported endopeptidases responsible for cleavage of other prohormones at paired basic residues (6-12). It specifically cleaves the peptide bond on the COOH-side of the Arg residue at the Lys-Arg pair of mouse Ren 2 prorenin, which is produced mainly in the SMG, but does not cleave mouse Ren 1 and human prorenins, both of which are produced mainly in the kidney. However, the reason for the strict substrate specificity of PRECE has remained unclear, since the two types of mouse prorenins share more than 95% identity in the amino acid sequence, and the sequence is the same in the Lys-Arg pair of the processing site (13).

Cleavage of peptides at sites marked by paired basic amino acids is a common feature of prohormone processing (14). Genetic evidence (15-19) and studies using synthetic analogs (10, 11, 20) and site-directed mutants (21-25) of prohormones have suggested that basic pairs play a pivotal role in prohormone processing. However, the specificity of cleavage is not well understood; precursor proteins often contain basic pairs which are never cleaved, and tissue-specific use of certain pairs often is observed (14, 26). It has been reported by surveying various prohormone sequences that there is a hierarchy for the use of certain pairs of basic amino acids at the processing sites (22, 27): Lys-Arg (70%), Arg-Arg (15%), Lys-Lys (10%), and Arg-Lys (5%). The data suggest that processing enzymes may have a preference for cleavage on the COOH-side of the Arg residues. Indeed, it has been shown recently using site-directed mutagenesis and the expression system in heterologous mammalian cells that the alteration of the Lys-Lys potential cleavage site of β-endorphin in pro-opiomelanocortin to Lys-Arg elevates synthesis of its processed products (21), and the precursor of neuropeptide Y with the Lys-Arg or Arg-Arg pair at the processing site is processed more efficiently than that with Arg-Lys or Lys-Lys (25).

In this study, we have prepared a series of mutated mouse prorenins using site-directed mutagenesis and the Xenopus oocyte expression system. We then investigated the role of the basic pair as the recognition signal for cleavage by PRECE as well as the determinant of the strict substrate specificity using these prorenins as substrates.

EXPERIMENTAL PROCEDURES

Plasmid Constructions and Site-directed Mutagenesis—cDNA fragments covering the sequence from the translation initiation site to the poly(A) tail of mouse ren 1 (28) and ren 2 (29) preprorenins were subcloned into the pGEM-1 vector (Promega) as described previously (5). Site-directed mutagenesis was performed using the oligonucleotide-directed in vitro mutagenesis system (Amersham). The oligodeoxyribonucleotide primers used for the mutagenesis are shown in Fig. 1. The mutant plasmids were constructed by exchanging

21027
Substrate Specificity of Prorenin Converting Enzyme

the corresponding cDNA fragment of native preprorenin with mutated fragments. For constructing chimeras of the Ren 1 and Ren 2 preprorenin cDNAs, the 115-bp DdeI-KpnI fragment, encoding the 11-amino acid COOH-terminal portion of the propeptide and 28-amino acid NH2-terminal portion of mature renin, of one type of mouse preprorenin cDNA was exchanged with the corresponding one of the other type. The DdeI and KpnI restriction sites are conserved both in two DNA types (28, 29).

Preparation of Prorenins and Assay for PROCE—Synthesis of preprorenin mRNA from the cloned cDNA, microinjection into Xenopus oocytes, and labeling of the oocytes with [35S]methionine were performed as described previously (5). Oocyte incubation medium containing labeled prorenin was used for assays. For synthesis of glycosylation-deficient prorenins, mRNA-injected oocytes were treated with tunicamycin as described by Colman et al. (30). PROCE was purified from SMGs of male ICR mice as described previously (5). Native and mutant prorenins synthesized by oocytes were incubated with the purified PROCE under previously described conditions (5). Native and mutant prorenins synthesized by oocytes were incubated with the purified PROCE under previously described conditions (5, 31), and, at the end of the incubations, the reaction products were immunoprecipitated with anti-Ren 2 renin antiserum and analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography as described previously (5, 31). When the resulting renin activity was determined by the angiotensinogen assay using hog angiotensinogen as a substrate as described previously (32).

RESULTS

Processing of Ren 2 Prorenin and Its Mutants at the Lys-Arg Pair—In order to examine the role of paired basic amino acids in the processing of mouse Ren 2 prorenin directed by PROCE, we constructed a series of Ren 2 preprorenin cDNAs mutated at the Lys-Arg cleavage site using site-directed mutagenesis, synthesized their mRNAs in vitro from the cDNAs, and microinjected them into Xenopus oocytes to produce prorenins. Mutant Ren 2 prorenins with Arg-Arg, Lys-Lys, but not at the NH2-side of the basic pair is essential for the strict substrate specificity of PROCE remained un- clear, since the two types of mouse prorenin are highly homologous (>95% identical) in the amino acid sequence and the sequence is the same in the Lys-Arg pair of the processing site. In order to identify the specificity determinant, we initially constructed chimeras of Ren 1 and Ren 2 prorenins by taking advantage of their homology as schematically shown in Fig. 3A. "Ren 1 prorenin is glycosylated at 3 selected Asn residues, i.e. at positions 5, 75, and 256, whereas Ren 2 prorenin is not glycosylated, so that M2DKM2 and M2DKM1, respectively, were named M1DKM2 and M2DKM1, respectively.

The results of their incubation with PROCE are shown in Fig. 4A. "Native Ren 1 prorenin was not processed as described previously (5). In the case of chimeric prorenins, M1DKM2 but not M2DKM1 was processed. The multiple bands of renin molecules observed in lanes 3-8 in Fig. 4A were probably derived from the heterogeneity of glycosylation, because glycosylation-deficient prorenins synthesized by oocytes treated with tunicamycin migrated as singlets in the sodium dodecyl sulfate-polyacrylamide gel (Fig. 4B)."

We next examined the effect of glycosylation of prorenins on processing. Ren 1 prorenin is glycosylated at 3 selected Asn residues, i.e. at positions 5, 75, and 256, whereas Ren 2 prorenin is not glycosylated, so that M2DKM1 prorenin is glycosylated at an Asn residue near the processing site, i.e. at position 5 (Fig. 3A). Because oligosaccharide chains of many glycoproteins are known to protect them from proteolytic degradation (33) and it has been argued that the presence of O-linked oligosaccharides near the Arg-Lys site in pro-opiomelanocortin prevents the cleavage at Arg-Lys (34), it remained a possibility that PROCE may be unable to cleave M2DKM1 prorenin due to its glycosylation near the processing site. However, as shown in Fig. 4B, the results obtained when glycosylation-deficient prorenins synthesized by tumi-
processing specificity depends on the structure around the to those of glycosylated prorenins. These indicate that the camcin-treated oocytes were used as substrates were similar to those of the O-Arg cleavage site.

In this study, we have initially examined the sequence preference of PRECE in the processing of Ren 2 prorenin, using a series of site-directed mutants at the Lys-Arg processing site as substrates. Mutants with Arg-Arg (M2RR) and Gln-Arg (M2QR) instead of the Lys-Arg pair were processed with efficiencies approximating that of native Ren 2 prorenin. The Lys-Lys mutant (M2KK) was processed much more slowly than native prorenin, and the Arg-Lys mutant (M2RK) was processed at an intermediate rate between those of native and M2KK prorenins. The Lys-Gln mutant (M2KQ) was not processed at all. These results and the data from our previous study (5) indicate that PRECE requires a basic residue at the COOH-side of the basic pair for the Ren 2 prorenin processing and that it has a preference for cleavage on the COOH-side of the Arg residue. The sequence preference, among all four combinations of pairs of basic residues, of PRECE for Ren 2 prorenin (Lys-Arg ≥ Arg-Arg > Arg-Lys > Lys-Lys) is similar to that of a processing enzyme, which is not currently characterized, of mouse pituitary AT-20 cells for proneuropeptide Y (25). In this case, it is noteworthy that the precursor with Arg-Lys at the processing site is cleaved in the middle of the 2 basic residues while those with Lys-Arg and Arg-Arg are cleaved at the COOH terminus of the pair (25). Therefore, it is also possible that M2RK prorenin may be cleaved in the middle of Arg and Lys residues. This problem remains to be elucidated.

In addition, the results also indicate that the basic amino acid at the NH2-side of the basic pair is not necessary for Ren 2 prorenin processing directed by PRECE. This was unexpected because genetic evidence (17) and studies using mutated prohormones (20, 24) have indicated that mutant prohormones with X-Arg (X, His, Thr, or Nle) at the processing site are not cleaved by their processing enzymes. However, it has become clear within the last some years that several hormones are produced from their precursors through cleavage at monoarginyl sites (36), and it has been suggested that higher orders of protein structure, especially β-turn, around processing sites are involved in recognition by processing enzymes (27, 36–38). Therefore, in the case of Ren 2 prorenin, the residue at the NH2-side of the basic pair may also be necessary for maintaining a correct conformation recognized by PRECE. However, the unconventional cleavage of M2QR prorenin by PRECE appears not to fit the properties proposed for the monoarginyl cleavages of prosomatostatin (39, 40) and proatrial natriuretic peptide (41, 42); the monoarginyl enzymes will not cleave after basic pairs, and all the monoarginyl sites have additional Arg residues at position −3 or −5 and 1 or more Ala or Leu residues within ±2 residues of the cleavage. The differences could be explained by the fact that PRECE is different from the monoarginyl enzymes in its molecular and enzymatic properties.

We next investigated the determinant of the substrate specificity of PRECE. In the previous study (5), we have demonstrated that PRECE processes SMG-type mouse Ren 2 prorenin but does not process kidney-type mouse Ren 1 and human prorenins. However, the reason for the strict substrate specificity of PRECE remained unclear, since the two types of mouse prorenins are highly homologous in the amino acid.
Substrate Specificity of Prorenin Converting Enzyme

**FIG. 3.** A, schematic representation of the structure of Ren 1 and Ren 2 prorenins and their chimeras. The regions derived from Ren 2 and Ren 1 prorenins are represented by open and shadowed boxes, respectively, CHO, oligosaccharide chain; a.a., amino acids. B, amino acid sequence of the exchanged region of Ren 2 and Ren 1 prorenins. The residues different from each other are indicated by asterisks.

**FIG. 4.** Processing of native and chimeric mouse prorenins. Prorenins synthesized by oocytes incubated in the absence (A) or presence (B) of tunicamycin were used as substrates. Oocyte medium containing native Ren 2 (lanes 1 and 2), M2DKM1 (lanes 3 and 4), M1DKM2 (lanes 5 and 6), or native Ren 1 (lanes 7 and 8) prorenin was incubated with (lanes 2, 4, 6, and 8) or without (lanes 1, 3, 5, and 7) PRECE under conditions described in the legend for Fig. 2A.

**FIG. 5.** Processing of native Ren 1 and Ren 2 prorenins and their mutants at position 1. Oocyte medium containing native Ren 1 (lanes 1 and 2), M1S (lanes 3 and 4), M2P (lanes 5 and 6), or native Ren 2 (lanes 7 and 8) prorenin was incubated with (lanes 2, 4, 6, and 8) or without (lanes 1, 3, 5, and 7) PRECE under conditions described in the legend for Fig. 2A.

sequence and the sequence is the same in the Lys-Arg pair of the processing site. The results obtained using chimeric and site-directed mutants of mouse prorenins demonstrate that the Pro residue next to the Lys-Arg pair disturbs the processing of Ren 1 prorenin by PRECE. These findings were expected to some extent on the basis of the fact that some postarginyl endopeptidases cannot cleave Arg-Pro peptide bonds. However, these do not explain why PRECE does not process human prorenin which has a Leu residue next to the Lys-Arg pair. Higher order structures around the processing site of human prorenin may be not recognized by PRECE, since the amino acid sequence around the site is not well conserved between mouse and human prorenins.

Even so, data from our present and previous (5) studies suggest that prorenins in the SMG and the kidney may be processed by different enzymes, supported by the data of us...
and other researchers: Ren 2 prorenin is processed by the extract of the SMG but not by that of the kidney (31); in the SMG, prorenin is completely processed intracellularly to renin in a short time (approximately 15 min) (3); in the kidney, only a small fraction of prorenin is processed intracellularly (4); a processing enzyme for human prorenin recently purified from human kidney is a cathepsin B-like cysteine protease and is different from PRECE in its molecular and enzymatic properties (43).

Acknowledgments—We thank Dr. K. Yanagisawa for encouragement, Dr. T. Shinagawa for useful discussion, and N. Foster for critical reading of the manuscript.

REFERENCES
Substrate specificity of prorenin converting enzyme of mouse submandibular gland. Analysis using site-directed mutagenesis.
K Nakayama, W S Kim, T Nakagawa, M Nagahama and K Murakami


Access the most updated version of this article at http://www.jbc.org/content/265/34/21027

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/34/21027.full.html#ref-list-1