Substrate Specificity of Prorenin Converting Enzyme of Mouse Submandibular Gland

ANALYSIS USING SITE-DIRECTED MUTAGENESIS*

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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 using these prorenins as substrates.

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-Arg pairs at the processing sites (22, 27); Lys-Arg (70%), Arg-Arg (15%), Lys-Lys (10%), and Arg-Lys (5%) have different processing efficiencies.

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) prorenins 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

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$$ The abbreviations used are: SMG, submandibular gland; PRECE, prorenin converting enzyme.
the corresponding cDNA fragment of native preprorenin with mutated fragments. For constructing chimerase 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 between two DNA types (28, 30).

Preparation of Prorenins and Assay for PRECE—Synthesis of preprorenin mRNA from the cloned cDNA, microinjection into Xenopus oocytes, and labeling of the oocytes with [35]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). PRECE was purified from SMGs of male ICR mice as described previously (5). Native and mutant prorenins synthesized by oocytes were incubated with the purified PRECE 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 indicated, the resulting renin activity was determined by the angiotensin I generating 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 PRECE, 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, Arg-Lys, Lys-Lys, Lys-Arg, Lys-Lys, and Lys-Lys instead of the Lys-Arg native pair were named M2RR, M2KK, M2RK, M2QR, and M2KQ, respectively (Fig. 1).

As shown in Fig. 2A, all the native and mutated Ren 2 prorenins, with the exception of M2KK, were processed to renin by incubation with PRECE, although with different processing efficiencies. We next examined the effect of the amino acid substitutions on the kinetics of the processing. Time course results are shown in Fig. 2B. Under conditions described in the legend for Fig. 2, native Ren 2 prorenin and M2RR, both of which have an Arg residue at the COOH-side of the basic pair, and M2QR, which has a single Arg residue instead of the basic doublet at the processing site, were processed rapidly with half-times of approximately 9, 10, and 12 min, respectively, while M2KK was processed much more slowly with a half-time of about 240 min. M2RR was processed at an intermediate rate (half-time ~ 30 min) between those of native and M2KK prorenins. M2KQ was not processed at all after 4 h of incubation. In all cases, with the exception of M2KQ, enzymatically active renin was produced in parallel with the cleavage of prorenin (Fig. 2C). These results indicate that the basic amino acid at the COOH-side but not the NH2-side of the basic pair is essential for the processing of Ren 2 prorenin by PRECE, and that the Arg residue at the COOH-side is more preferable for processing than the Lys.

Identification of the Determinant of the Substrate Specificity of PRECE—In the previous study, we demonstrated that PRECE processes mouse Ren 2 prorenin but neither mouse Ren 1 nor human prorenin to renin (6). However, the reason for the strict substrate specificity of PRECE remained unclear, 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 including the region around the processing site of Ren 2 and Ren 2 prorenin including the region around the site of Ren 1 were named M1DKM2 and M2DKM1, respectively.

The results of their incubation with PRECE are shown in Fig. 4A. Native Ren 1 prorenin due to its glycosylation near the processing site remained a possibility that PRECE may be unable to cleave M2DKM1 prorenin because of the heterogeneity of glycosylation, because 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), we used an Asn residue near the processing site, i.e. at position 5 (Fig. 3A). Because oligosaccharide-deficient prorenins, M2DKM1 prorenin being glycosylated at an Asn residue near the processing site, was processed in the presence of PRECE, these results indicate that the basic amino acid at the COOH-side but not the NH2-side of the basic pair is essential for the processing of Ren 2 prorenin by PRECE, and that the Arg residue at the COOH-side is more preferable for processing than the Lys.
processing specificity depends on the structure around the processing site of prorenin and not on its glycosylation. These indicate that the basic amino acid at the NH2-side of the basic pair is not 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 monoarginy1 cleavages of prosomatostatin (39, 40) and prostriatal natriuretic peptide (41, 42); the monoarginy1 enzymes will not cleave after basic pairs, and all the monoarginy1 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 monoarginy1 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 sequence.
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 endoproteases 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).

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