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The presence of two types of prorenin converting enzymes in the mouse submandibular gland

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We have recently demonstrated, by protein and cDNA sequence analyses, that prorenin converting enzyme (PRECE) in the ICR mouse submandibular gland is identical to the epidermal growth factor-binding protein (EGF-BP) type B, the mGK-13 gene product identified in Balb/c mouse. However, in the course of cDNA cloning, we noticed the presence of the other cDNA type highly homologous but not identical to the PRECE cDNA. The sequence of the newly identified cDNA was identical to that of the pSGP-2 cDNA cloned from NMRI mice, which also encodes EGF-BP type B different at 9 out of 261 amino acids from the mGK-13 product. Although this difference has been explained by strain polymorphism, our results indicate that these two proteins are distinct gene products. The product of the newly identified cDNA also had a prorenin converting activity. Thus, the products of both cDNAs identified in previous and present studies are involved in maturation of two bioactive polypeptides, renin and EGF.

Prorenin converting enzyme; Epidermal growth factor-binding protein; Glandular kallikrein; Serine protease; Processing; Submandibular gland

1. INTRODUCTION

Renin, an aspartyl protease, is the key enzyme of the renin–angiotensin system, and plays a pivotal role in the regulation of blood pressure [1]. It is produced from a larger, inactive precursor, prorenin, through endoproteolysis at paired basic amino acids, Lys-Arg, during intracellular transport. Although the kidney is the major source of circulating renin, other tissues are also capable of renin synthesis. For example, the submandibular gland (SMG) of male mice produces a large amount of renin. Renins in the kidney and the SMG are encoded by separate genes, *Ren-1* and *Ren-2*, respectively [2].

Recently, we have purified and characterized an endoprotease involved in processing of *Ren-2* prorenin, named prorenin converting enzyme (PRECE), from ICR mouse SMG [3–5]. It consists of two polypeptide chains of 17 and 10 kDa linked by disulfide bonds [4]. Protein and cDNA sequence analyses [6] have revealed that PRECE is identical to the epidermal growth factor-binding protein (EGF-BP) type B, the product of the mGK-13 gene identified in Balb/c mouse [7]. EGF-BPs have been demonstrated to be members of the glandular kallikrein family [8], and to be responsible for conversion of the 9-kDa EGF processing intermediate to mature EGF [9]. Thus, PRECE is involved in the maturation of two bioactive polypeptides produced in mouse SMG, *Ren-2* renin and EGF.

By the way, during the cloning process of the PRECE cDNA [6], we noticed the presence of the other population of cDNAs hybridizing with the PRECE probe but not identical to the PRECE cDNA. In this study, we sequenced the newly identified cDNA and examined the function of the encoded protein.

Abbreviations: SMG, submandibular gland; PRECE, prorenin converting enzyme; EGF-BP, epidermal growth factor-binding protein; CHO, Chinese hamster ovary.

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2. MATERIALS AND METHODS

cDNA cloning procedures were described previously [6]. Both strands of the cloned cDNA (pPRECE-2) were sequenced using the Sequenase Version 2.0 kit (United States Biochemical Corp.).

Prorenin converting activity of the protein encoded by the pPRECE-2 cDNA was examined in a manner similar to that employed in the previous study [6]. Briefly, the cDNA insert was subcloned into the pSVD expression vecor [10], and transfected into Chinese hamster ovary (CHO) cells. A cell line stably expressing the highest level of the protein (CHO/PR2) was then selected. The conditioned medium of CHO/PR2 cells cultured in a serum-free medium was treated with trypsin. The [^{35}S]methionine-labeled medium of CHO/MRB cells (a *Ren-2* prorenin producing cell line) [10] was then incubated with the trypsin-treated or -untreated conditioned medium of CHO/PR2 cells, and subjected to immunoprecipitation with anti-*Ren-2* renin antiserum and SDS-PAGE analysis.

3. RESULTS AND DISCUSSION

In the previous study [6], we screened ~1×10^5 clones from the cDNA library of male ICR mouse SMG by hybridization with the PRECE oligonucleotide probe, and obtained ~100 positive clones. Since the analyzed

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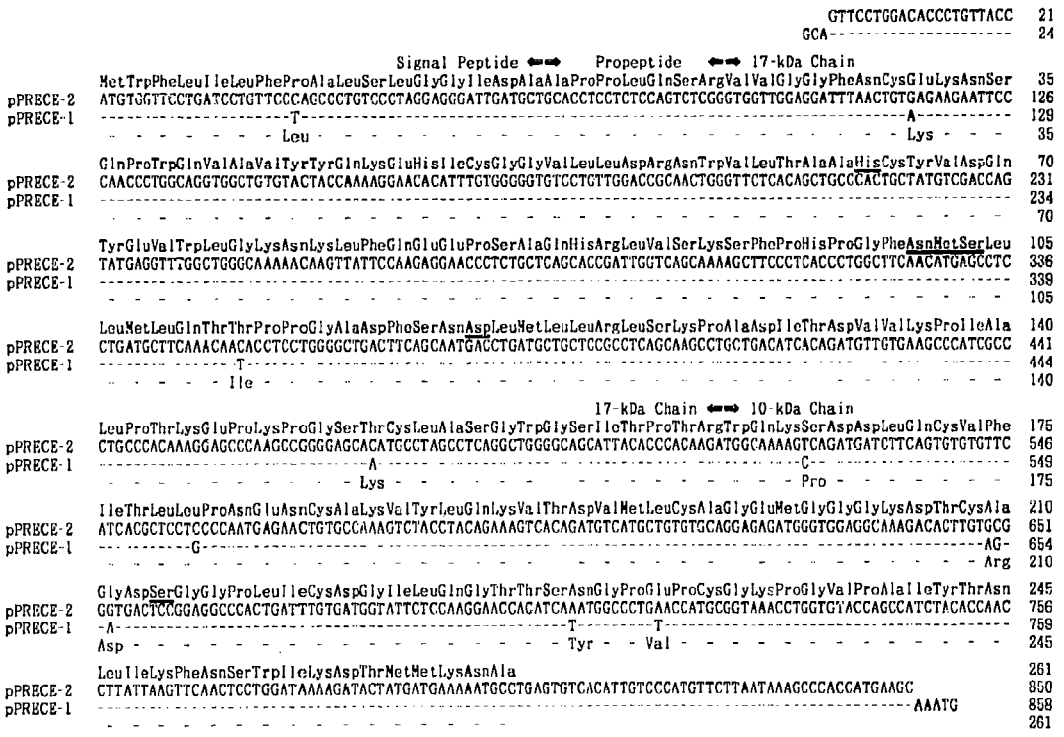

Fig. 1. Nucleotide and deduced amino acid sequences of PRECE-2 along with those of PRECE-1. Residues of PRECE-1 identical with those of PRECE-2 are indicated by hyphens. Proposed active site His, Asp and Ser residues are underlined. A consensus sequence for *N*-glycosylation is double underlined.

twelve out of the positive clones appeared to share the same restriction patterns, we sequenced the longest clone (pPRECE-1) and demonstrated that the sequence is identical to the exon sequences of the mGK-13 gene encoding EGF-BP type B previously identified in Balb/c mouse [7]. However, further analysis revealed that four out of the twelve clones showed restriction patterns somewhat different from those of the rest, i.e. the PRECE cDNA clones, when digested with some enzymes, such as *Nsp*(7524)I and *Rsa*I (data not shown). Therefore, we sequenced a clone (pPRECE-2) longest among the four clones. Fig. 1 shows the nucleotide and deduced amino acid sequences of the pPRECE-2 along with those of the pPRECE-1. The pPRECE-2 cDNA sequence was identical to that of the pSGP-2 cDNA previously cloned from NMRI mouse, which is also proposed to code for EGF-BP type B [11]. The protein encoded by the pSGP-2 is different at only 9 out of 261 amino acid residues from that encoded by the mGK-13 [7,11], and this difference has been explained by strain polymorphism [7]. However, the fact that the pPRECE-1 and pPRECE-2 are cloned from the same cDNA library of ICR mouse SMG indicate that these two proteins are the products of separate genes. Therefore, we tentatively designated the protein encoded by the pPRECE-2 as PRECE-2, and redesignated that encoded by the pPRECE-1 as PRECE-1.

Since PRECE-1 and PRECE-2 share a high degree of amino acid sequence identity as described above, it was reasonably speculated that PRECE-2 could also possess prorenin converting activity. To address this possibility, we initially transfected CHO cells with the PRECE-2 expression plasmid, and then selected a cell line expres-

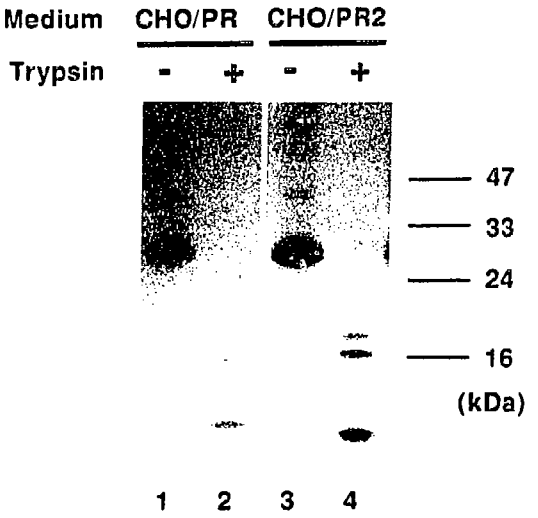

Fig. 2. Trypsin treatment of the conditioned medium of CHO/PR2 cells. The radiolabeled conditioned medium of CHO/PR (lanes 1 and 2) or CHO/PR2 (lanes 3 and 4) cells was incubated with (lanes 2 and 4) or without (lanes 1 and 3) trypsin (5 μg/ml) at 4°C for 30 min. The reaction was stopped by the addition of soybean trypsin inhibitor (50 μg/ml). The medium was then immunoprecipitated with anti-PRECE-1 antiserum [6], and analyzed by SDS-PAGE under reducing conditions followed by fluorography.

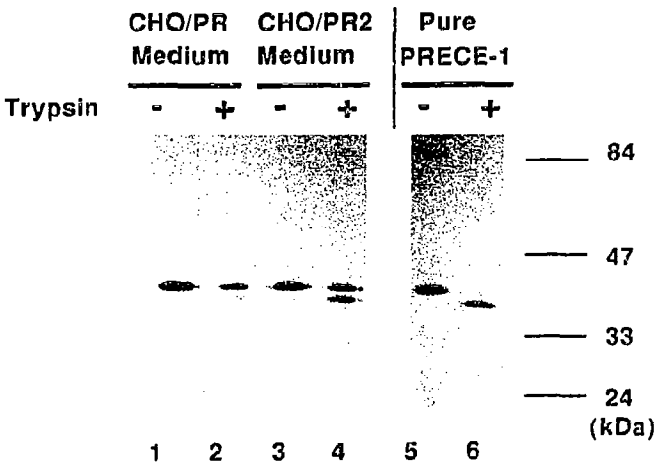

Fig. 3. Prorenin converting activity of PRECE-2. The radiolabeled conditioned medium of CHO/MRB cells (100 μl) was incubated with the trypsin-treated (lanes 2 and 4) or -untreated (lanes 1 and 3) conditioned medium of CHO/PR (lanes 1 and 2) or CHO/PR2 (lanes 3 and 4) cells (100 μl) in a final volume of 500 μl of 0.1 M Tris/HCl (pH 8.0) at 37°C for 4 h, immunoprecipitated with anti-*Ren-2* renin antiserum, and analyzed by SDS-PAGE under reducing conditions followed by fluorography. The CHO/MRB medium was also incubated with (lane 6) or without (lane 5) 100 ng of PRECE-1 purified from ICR mouse SMG [4] as a positive or negative control, respectively. The arrowhead and arrow indicate positions of prorenin and renin, respectively.

sing the highest level of the protein (CHO/PR2). As shown in Fig. 2, CHO/PR2 cells secreted a single chain form of PRECE-2 (lane 3) like CHO/PR cells expressing PRECE-1 (lane 1) [6]; the doublet SDS-PAGE bands of the PRECE-1 and PRECE-2 molecules in lanes 1 and 3, respectively, could be derived from heterogeneity in glycosylation, since both of them have a potential *N*-glycosylation site in the 17-kDa chain (Fig. 1) and the molecules treated with endoglycosidase F migrated as singlets on SDS-PAGE (data not shown). Therefore, we treated the conditioned medium of CHO/PR2 cells with trypsin, which has been demonstrated to effectively convert the PRECE-1 precursor to a two-chain, enzymatically active form (lane 2) [6]. The single chain PRECE-2 was converted to a two-chain form by the trypsin treatment (lane 4): the doublet bands at 20 and 17 kDa in lanes 2 and 4 are derived from glycosylation heterogeneity of the 17-kDa chain (data not shown). Then, the radiolabeled conditioned medium of CHO/MRB cells, which stably express *Ren-2* prorenin [10], was incubated with the trypsin-treated or -untreated CHO/PR2 medium. As shown in Fig. 3, like CHO/PR medium (lanes 1 and 2), the trypsin-treated (lane 4) but not -untreated (lane 3) medium of CHO/PR2 cells gave rise to conversion of *Ren-2* prorenin to renin. These observations indicate that the two-chain form of PRECE-2 also possesses prorenin converting activity.

Data presented here demonstrate that two isoforms of PRECE, designated PRECE-1 and PRECE-2, structurally related to each other are present in ICR mouse SMG. PRECE-1 and PRECE-2 are identical to the product of the mGK-13 gene previously cloned from Balb/c mouse [7] and that of pSGP-2 cDNA from NMRI mouse [11], respectively. Both the mGK-13 and the pSGP-2 encode EGF-BP type B. Thus, both enzymes we identified are involved in maturation of two bioactive polypeptides produced in mouse SMG, *Ren-2* renin and EGF.

Richards and his colleagues [8] have reported that the mouse glandular kallikrein gene family consists of at least 25 members, designated mGK-1 to mGK-25, including some pseudogenes, and ten of them are expressed in the SMG. Since the gene corresponding to the pSGP-2 cDNA was not isolated from the Balb/c mouse genomic library, they explained that the sequence heterogeneity between the mGK-13 and the pSGP-2 could be due to strain polymorphism [7]. However, our data unequivocally exclude this possibility. PRECE-1 and PRECE-2 are distinct gene products.

The increasing list of glandular kallikrein family members and their substrates highlights the diverse substrate specificity of this structurally related family [12]. In this context, it is very interesting whether other kallikreins, especially EGF-BP type A and type C which both are also involved in maturation of EGF, possess prorenin converting activity. To address this problem, experiments are under way in our laboratory.

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