Korean J. Zool. 39:215-222, 1996

Substrate Specificity of Mouse Glandular Kallikreins, Epidermal Growth Factor-Binding Protein Type A, B, and C against Mouse *Ren 2* Prorenin

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In the previous studies, we have demonstrated that prorenin converting enzyme (PRECE) was identical to the epidermal growth factor-binding protein (EGF-BP) type B, which was a member of the mouse glandular kallikrein family. To examine whether or not EGF-BP type A and C are involved in the processing of prorenin, we have cloned the cDNAs of the EGF-BP type A and C from a library of male ICR mouse submandibular gland (SMG). And then CHO cells were transfected with the EGF-BP expression plasmids, and stable cell lines expressing a high level of the EGF-BPs precursor were obtained. The conditioned medium was then treated with trypsin, which has been known to effectively convert the EGF-BP type A and C precursor to the active forms. Subsequently, the prorenin converting activity of the trypsin-treated or untreated medium was examined. PRECE converted exactly prorenin to renin, but the prorenin converting activities of EGF-BP type A and C were not detected. From these results, it seems that only type B of these EGF-BPs is involved in processing Ren 2 prorenin in mouse SMG.

KEY WORDS: Submandibular Gland, Glandular Kallikrein, Prorenin Converting Enzyme, Epidermal Growth Factor-Binding Protein, Serine Protease

Mouse glandular kallikreins are a highly homologous subfamily of serine proteases encoded by 25 genes, all located on chromosome 7 These glandular kallikreins are involved in the posttranslational processing of polypeptide precursors to their biologically active forms. For example, mouse pro-nerve growth factor (NGF) is cleaved to its mature form by the arginyl esteropeptidase γ-NGF. Mouse pro-epidermal growth factor (EGF) is potentially cleaved by three kallikrein gene products epidermal growth factor-binding protein (EGF-BP) type A, B, and C. Another member of the mouse kallikrein gene family, γ-renin, cleaves synthetic renin substrate In the previous studies, we have purified and characterized an endoprotease involved in processing of *Ren-2* prorenin, named prorenin converting enzyme (PRECE), from ICR mouse SMG. It consists of two peptide chains of 17 and 10 kDa linked by disulfide bonds. Protein and cDNA sequence analysis have revealed that PRECE is identical to the EGF-BP type B, the product of the mGK-13 gene 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 newly identified cDNA was identical to that of the pSGP-2 cDNA cloned from NMRI mice, which encoded EGF-BP type B different at 9 out of 261 amino acids from the mGK13 product.

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Also we had been demonstrated that the products of the newly identified cDNA had a prorenin converting activity. Thus the products of both cDNAs of PRECE and pSGP-2 are involved in maturation of two bioactive polypeptides produced in mouse SMG, Ren-2 renin and EGF. Since EGF-BP type B is highly homologous to the EGF-BP type A and C, it is very interesting to study whether these EGF-BPs have a prorenin converting activity. In this study, we have cloned the cDNAs of EGF-BP type A and C and investigated whether or not the products of these cDNA are involved in the processing of Ren 2 prorenin.

Materials and Methods

Materials

Sequenase Version 2.0 DNA Sequencing Kit was obtained from United States Biochemical Corp. L-[^{35}S] Methionine(>1000 Ci/mmol) and ECL Western blotting analysis system were purchased from Amersham LIFE SCIENCE. F-12, Dulbecco's modified Eagle's medium, methionine-free Dulbecco's modified Eagle's medium, CHO-S-SFM II, Fetal Bovine Serum and dialyzed FBS were obtained from GIBCOBRL. Cellphect Transfection Kit was purchased from Pharmacia.

Cloning and sequencing of the PRECE related cDNA(pPRC)

Double stranded cDNA was synthesized from the poly(A)+RNA of male ICR mouse SMG using the cDNA synthesis system (Amersham Corp.) and inserted into the EcoRI site of the λgt10 vector. Approximately 1×10^5 clones from the cDNA library were screened using a ^{32}P-labeled PRECE fragment: 636-bp StyI fragment covering the sequence highly conserved between the EGF-BPs and the synthetic oligonucleotide probes which is specific to EGF-BP type A and C, respectively; type A:5′-TAAGTCGGCCCCAGTAGGTACACT T-3′, type C:5′-ACTCAGGATGTCGGATGTGG TTTCT-3′. Twenty out of ~100 positive clones were plague-purified, and the inserts were then subcloned into the pBluescriptII vector (Stratagene). The sequences of both strands of a clone with the longest insert were determined by the dideoxy method.

Plasmid construction and transfection

A 860bp XhoI-XbaI cDNA fragment covering the entire coding sequence of EGF-BP type A or C was subcloned behind the SV40 promoter of pSVD vector (Fig. 2). The expression plasmid, pSVDE-A or pSVDE-C, was transfected into CHO (Chinese hamster ovary) cells (DXB-11 strain), which have a mutation in the dihydrofolate reductase (DHFR) gene, by calcium phosphate coprecipitation. The transfected cells were cultured in Dulbecco s modified Eagle's medium containing 11.5 mg/liter proline and 10% dialyzed fetal bovine serum for 2days at 37°C in 5% CO_2, and then stable transfectants which were resistant to methotrexate were selected. Clonal cell lines were then screened for secretion of the proEGF-BP type A and C by Western blotting of the culture media with anti-PRECE antiserum.

Processing activity of recombinant EGF-BP type A and C in vitro

Cell lines expressing the highest levels of EGF-BP type A and C were designated CHO/PEA and CHO/PEC respectively. CHO/PEA, CHO/PEC and CHO/PRECE(Prorenin converting enzyme producing cell lines; see Ref. 11) cells at ~70% confluence in 60-mm dishes were labeled with [^{35}S] methionine for 24 h. The conditioned media of CHO/PEA, CHO/PEC and CHO/PRECE cells were then treated with 5 μg/ml trypsin. The conditioned medium of CHO/MBR cells (0.1 ml) was incubated with the trypsin-treated or -untreated conditioned medium of CHO/PEA, CHO/PEC and CHO/PRECE cells (0.1 ml) in final volume of 0.5 ml of 0.1 M Tris-HCl, pH 8.0, for 4 h and then subjected to immunoprecipitation with anti-Ren-2 renin antiserum and SDS-PAGE analysis.

Results

In order to clone EGF-BP type A and C, we screened approximately 1×10^5 clones from the cDNA library of male ICR mouse SMG and

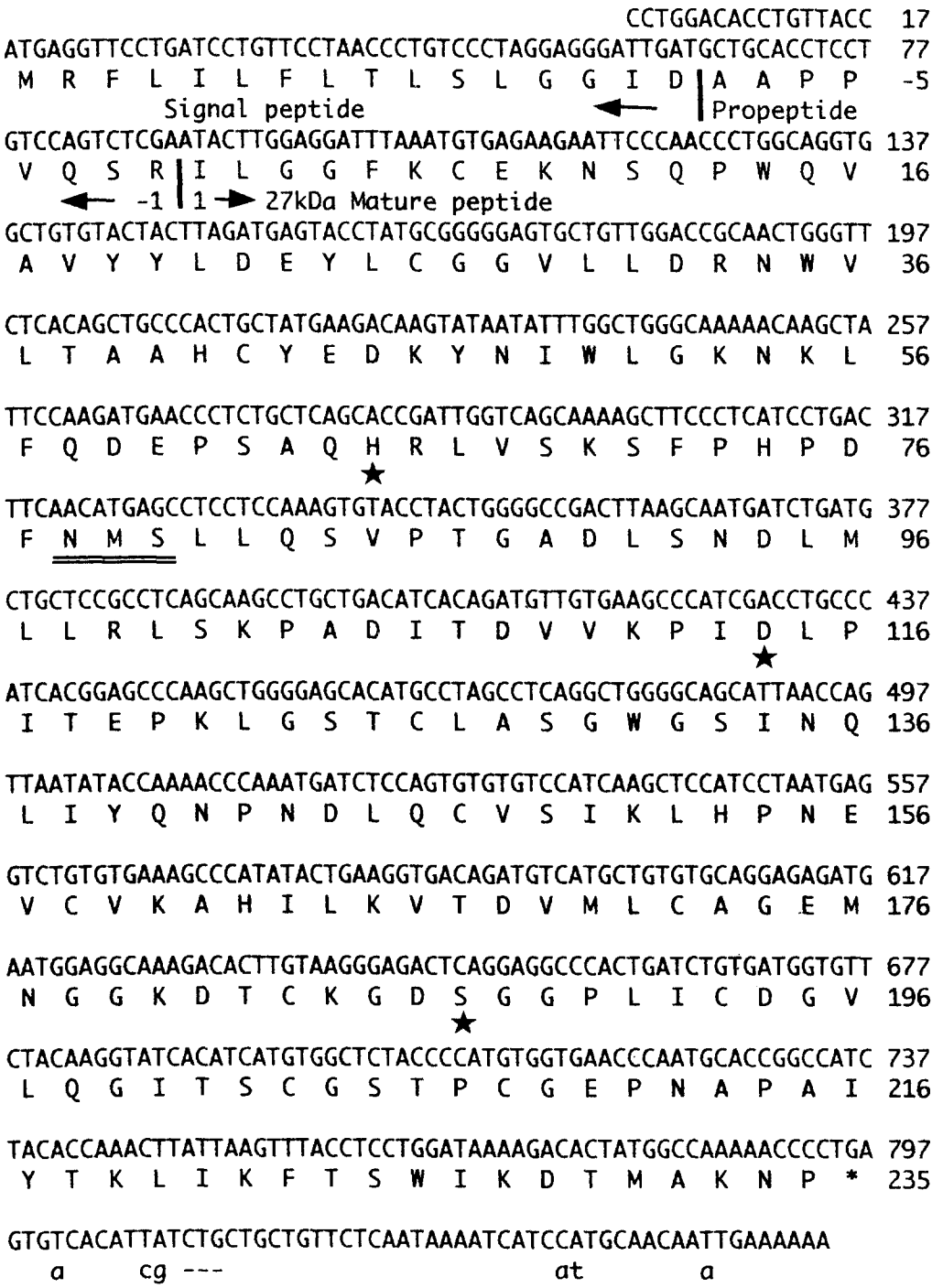

Fig. 1. Nucleotide and deduced amino acid sequences of EGF-BP type A. The proposed active site, His^{40}, Asp^{95} and Ser^{188} residues are indicated by *asterisk*. The consensus sequence for N-glycosylation is double underlined. The deleted nucleotide residues in mGK-22 gene are indicated *by hyphens*. The nucleotide residues in the mGK-22 gene different from that of the EGF-BP type A are represented by *the small letter*.

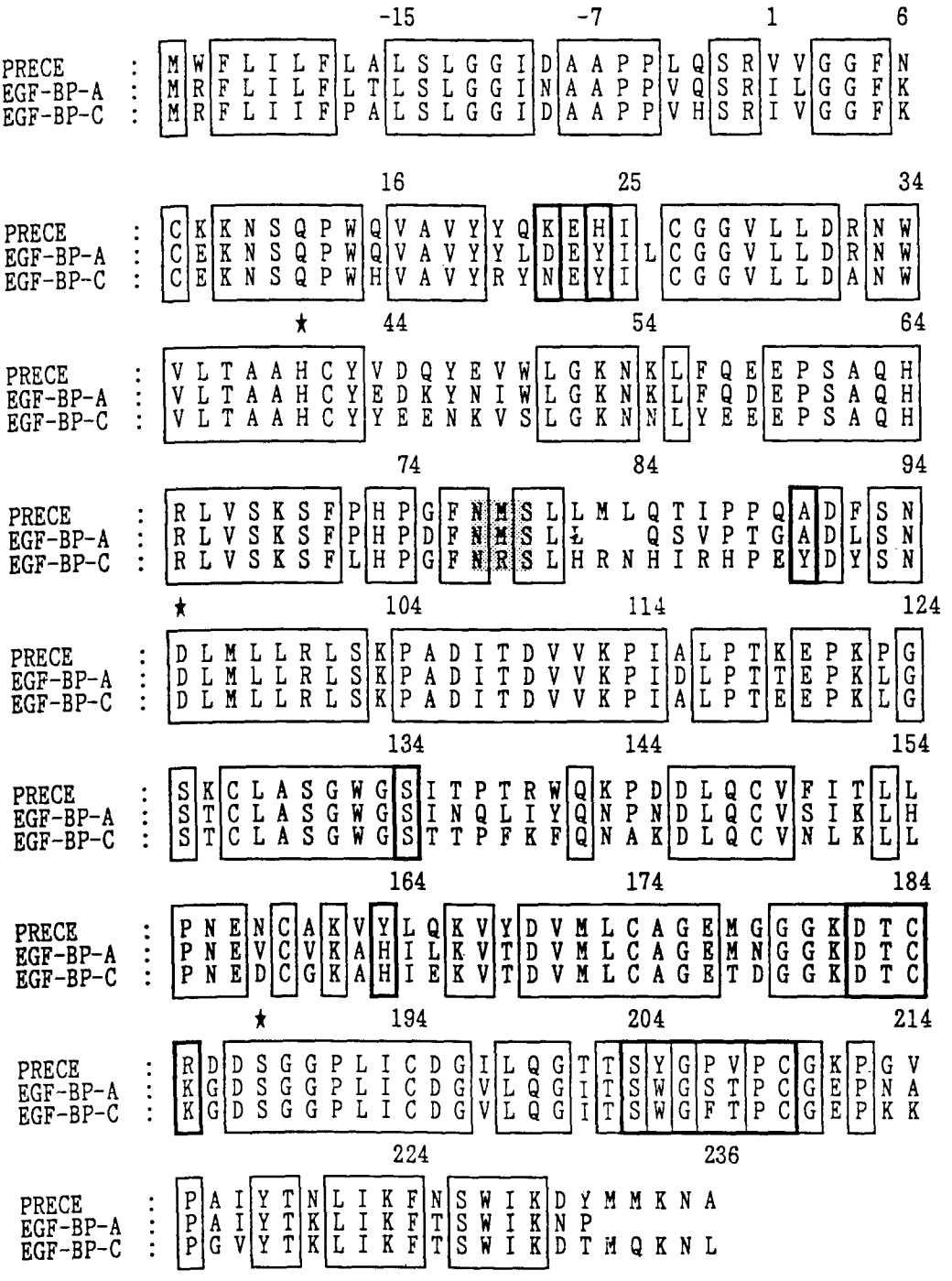

Fig. 2. Alignment of the PRECE to the related EGF-BPs. The amino acid sequence of the PRECE was aligned to the sequences of the EGF-BP type A and C. The boxed areas donate regions of the sequences which have identical residues. The residues belived to line the substrate-binding pocket are *heavily boxed*. The consensus sequence for N-glycosylation is shadowed. The proposed active site, His^{40}, Asp^{95} and Ser^{188} residues are indicated *by asterisks*.

obtained about 100 positive clones. Twenty out of the longest positive clones were sequenced and then we have cloned the cDNAs of the EGF-BP type A and C. The cloned cDNA of EGF-BP type A comprises 854 nucleotides included 17 bp 5′-, 57 bp 3′- non coding nucleotides which is identical to the mGK-22 gene exons, exception for 9 nucleotide residues in the 3′-untranslated region (Fig. 1). Also the nucleotide and deduced amino acid sequences of EGF-BP type C were identical with those of mGK-9 gene exons[6](data not shown). Fig. 2 compares the predicted amino acid sequences of the EGF-BP type A and C with those of the PRECE (type B). Not only all these proteases share extensive amino acid sequence homology (EGF-BP type A; 79%, type C; 75%) but also the amino acid sequences near the active sites (His^{40}, Asp^{95}, and Ser^{187}) are highly conserved. But the EGF-BP type A, C and PRECE show less homology in the residues Lys^{22}, His^{24}, Ala^{90}, Ser^{134}, Tyr^{163}, Asp^{182}, Thr^{183}, Cys^{184}, Arg^{185}, Ser^{204}, Tyr^{205}, Gly^{206}, Pro^{207}, Val^{208}, Pro^{209}, and Cys^{210} that are believed to line the substrate-binding pocket than in the rest of the sequence. Especially, the residues Ala^{90}, Ser^{134}, and Tyr^{163} are distinctly less homologous. In order to demonstrate prorenin converting activity of the EGF-BP type A and C encoded by the cloned cDNAs, we investigated the activity *in vitro*. To produce a large amount of EGF-BP type A and C precursor, CHO cells were transfected with the expression plasmids, and a stable cell line expressing a high level of the EGF-BP type A and C precursor (CHO/PEA and CHO/PEC) were obtained. As shown in Fig. 4, CHO/PEA and CHO/PEC cells secreted a single major chain form pEGF-A, pEGF-C; the doublet SDS-PAGE bands of the lane 1, 3, and 5 could be derived from heterogeneity in glycosylation of 27-kDa chain. Therefore, we treated the conditioned medium of CHO/PEA and CHO/PEC cells with trypsin, which has been demonstrated to effectively convert the proEGA, proEGF-C, and PRECE to a single or two chains, enzymatically active forms (lane 2, 4, and 6). Then, the radiolabeled conditioned medium of CHO/MRB cells, which stably express Ren-2 prorenin, was incubated with the trypsin-treated CHO/PEA, CHO/PEC or CHO/PRECE. As shown in Fig. 5, unlike CHO/PRECE trypsin-treated medium(lane 6), the CHO/PEA and CHO/PEC trypsin-treated

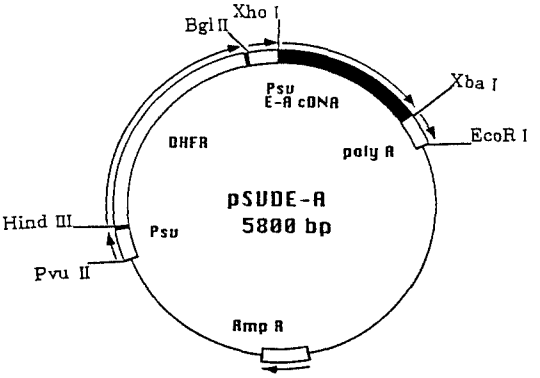

Fig. 3. Diagram of the expression plasmid of EGF-BP type A or C. Psv, SV40 early promotor; E-A cDNA, mouse EGF-BP type A or C cDNA; poly A, the polyadenylation signal from bovine growth hormone gene; Amp, β-lactamase gene from pBR322; DHFR, mouse dihydrofolate reductase gene.

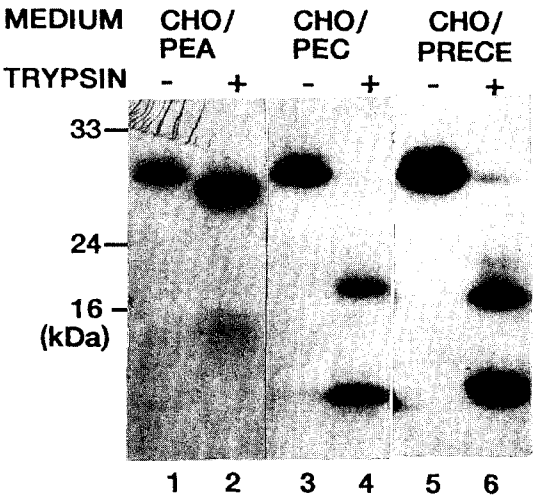

Fig. 4. Trypsin treatment of the conditioned medium of CHO/PEA, CHO/PEC and CHO/PRECE cells. The radiolabeled conditioned medium of CHO/PEA (lane 1, 2), CHO/PEC (lane 3, 4) and CHO/PRECE (lane 5, 6) was incubated with (lane 2, 4 and 6) or without (lane 1,3 and 5) trypsin (5 μg/ml) at 4°C for 30 min.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

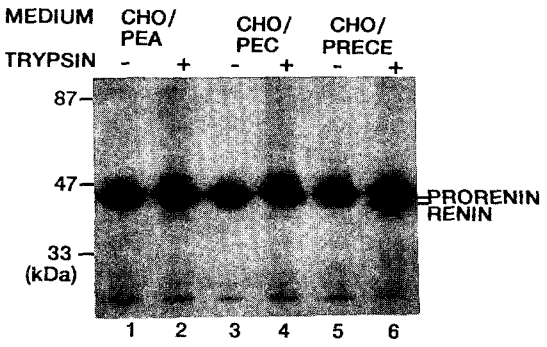

Fig. 5. Prorenin converting activities of EGF-BP type A and C. The radiolabeled conditioned medium of CHO/MRB cells (100 μl) was incubated with the trypsin-treated (lane 2, 4 and 6) or -untreated (lane 1, 3 and 5) conditioned medium of CHO/PEA (lane 1, 2), CHO/PEC (lane 3, 4) and CHO/PRECE (lane 5, 6) cells (100 μl) in a final volume of 500 μl of o.1M Tris/HCl (pH8.0) at 37°C for 4h, immunoprecipitated with anti-Ren-2 renin antiserum, and analyzed by SDS-PAGE under reducing conditions followed by fluorography.

medium did not give rise to conversion of Ren-2 prorenin to renin (lane 2 and 4). This results indicate that the recombinant EGF-BP type A and C produced from pSVDE-A, and pSVDE-C do not possess prorenin converting activity.

Discussion

Richards and his colleagues 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. These kallikreins demonstrate a high degree of sequence homology; correspondingly, they exhibit similar molecular properties such as molecular weight, amino acid composition, and isoelectric point. Also, they often cross-react immunologically and may be copurified with one another. It has been reported that γ-NGF could not cleave proEGF and it has been proposed that EGF-BP could not cleave proNGF, although these two kallikreins shared a high degree of amino acid sequence identity. In addition, kidney kallikrein could not cleave Ren-2 prorenin. Thus, the substrate specificities of some kallikreins appear to be very strict. In recent years, Fahnestock *et al.* demonstrated that contamination of high molecular weight (HMW) EGF precipitations with β-NGF-endopeptidase erroneously led to earlier designation of the product of mGK-22 as an EGF-BP type A and EGF forms a high molecular weight complex with EGF-BP type C. Thus, it is perplexing that a particular kallikrein is involved in processing of only one given precursor. In this context, it is of particular interest whether other kallikreins, especially EGF-BP type A and C. In this study, to examine whether or not EGF-BP type A and C are involved in the processing of prorenin, we have cloned the cDNA of the EGF-BP type A and C from a library of male ICR mouse submandibular gland. However, as shown in Fig. 5, the prorenin converting activities of EGF-BP type A and C were not detected so as PRECE converted exactly prorenin to renin (lane 2, 4, and 6). In serine protease, serine residue of active site forms a triad with histidine and aspartic acid residues which in the correct spatial configuration constitutes the catalytic triad necessary for serine protease activity. As shown in Fig. 2, active site amino acid, His40, Asp95, and Ser187 residues are highly conserved and the amino acid residue Asp186, thought to be primarily responsible for determining cleavage at basic amino acid, is also conserved. Thus they may possess a serine protease activity. Actually, EGF-BP type A has a β-NGF-endopeptidase activity and EGF-BP type C is involved in EGF processing. However, the EGF-BP type A, C, and PRECE show less homology in the residues Lys^{22}, His^{24}, Ala^{90}, Ser^{134}, Tyr^{163}, Asp^{182}, Thr^{183}, Cys^{184}, Arg^{185}, Ser^{204}, Tyr^{205}, Gly^{206}, Pro^{207}, Val^{208}, Pro^{209}, and Cys^{210} that are believed to line the substrate-binding pocket than in the rest of the sequence. Especially, the residues Ala^{90}, Ser^{134}, and Tyr^{163} are distinctly less homologous. A variant residue Lys^{214} which may be important for specific binding EGF, is replaced by alanine and valine in EGF-BP type A and B, repectively (Fig. 2). EGF-BP type C residues considered important in strict substrate specificity (Arg^{78}, His^{84}, His^{87},and Lys^{214}) are replaced by (Met^{78}, Gln^{84}, Pro^{87},and Ala^{214}) in EGF-BP type A, (Met^{78}, Gln^{84}, Pro^{87}, and Val^{214}) in EGF-BP type C, respectively. It is therefore

apparent that each of these EGF-BPs exhibits a different substrate specificity. In this study, even though the recombinant EGF-BP type A and C do not show a prorenin converting activity, it is apparent that they are involved in processing of β-NGF and EGF, respectively. Therefore, although these EGF-BPs share a high degree of amino acid sequence homology, their substrate specificities appear to be very strict. However, there still remains a possiblity that PRECE could be involved in other substrate processing such as β-NGF and EGF in mouse SMG.

Acknowledgements

We would like to thank Dr. K. Nakayama for kindly providing CHO cells and anti-renin antiserum. We also thank Dr. K. Murakami for encouragement.This work was supported by the Genetic Engineering Research Program (1994), Ministry of Education, Korea.

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(Accepted March 11, 1996)

생쥐 선상칼리크레인(상피세포증식인자 결합단백질 Type A, B, 그리고 C)의 *Ren 2* Prorenin에 대한 기질특이성

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생쥐 악하선의 prorenin변환효소(PRECE, Prorenin converting enzyme)가 생쥐 선상 kallikrein family중의 하나인 상피세포증식인자 결합단백질(EGF-BP, Epidermal growth factor-binding protein) type B와 동일한 것으로 밝혀짐에 따라 EGF-BP type A와 C가 prorenin의 수식에 관여하는지를 조사하기 위하여 EGF-BP type A와 C에 대한 cDNA를 ICR male 생쥐 악하선 cDNA library로부터 각각 cloning하여 mammalian cell line인 CHO(Chinese hamster ovary)세포주에서 발현시켜 전구체형의 EGF-BP들을 가장 강하게 발현하고 있는 안정된 세포주를 선택하였다. 이 안정된 세포주에서 분비되는 불활성형인 전구체형의 EGF-BP는 trypsin으로 처리하여 활성형으로 전환시켜서 prorenin에 대한 기질특이성을 조사하였다. 그 결과 prorenin변환효소가 생쥐 Ren-2 prorenin을 활성형의 renin으로 수식하는 것과는 다르게 trypsin 처리와 비처리에 관계없이 EGF-BP type A와 C에서는 prorenin변환활성을 확인할 수 없었다. 따라서 EGF-BP들중에서 오로지 type B만이 생쥐 악하선에서 Ren-2 prorenin의 수식에 관여하는 것으로 추정된다.