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Cloning and Characterization of cDNA Encoding Potentially Functional Mouse Glandular Kallikrein

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Abstract : We cloned a cDNA (pPRC-1) which was comprised of 841 nucleotides from the cDNA library of a male ICR mouse submandibular gland (SMG[†]). The nucleotide sequences of pPRC-1 were identical to those of exons 2 and 3 of the mGK-21 gene, a potentially functional glandular kallikrein identified in a Balb/c mouse, except for one nucleotide residue. Although this substitution changes Ile (ATT) in pPRC-1 to Val (GTT) in mGK-21, this difference has been explained by strain polymorphism. From the amino acid sequences predicted from its cDNA, we speculated that mGK-21 gene products/pGK21 consist of 261 amino acids including the NH_2-terminal signal peptide (residues 1~17), the short propeptide (residues 17~24), and the active peptide (residues 25~261). Although we did not demonstrate the enzyme activity of pGK21, it was assumed that pGK 21 was involved in the maturation of certain bioactive polypeptide(s) in mouse SMG for the following reasons : (a) mGK-21 gene was apparently expressed in a male ICR mouse SMG; (b) the proposed active site His^{65}, Asp^{120}, and Ser^{213} residues were completely conserved in pGK21 just like other glandular kallikreins; (c) the cloned cDNA was translated to a predicted 27 kDa polypeptide chain *in vitro*; (d) the 27 kDa polypeptide chain porduced by CHO cells was produced to a putative active form by trypsin.

Key words : epidermal growth factor-binding protein, glandular kallikrein, prorenin converting enzyme, submandibular gland.

Mouse glandular kallikreins are a highly homologous subfamily of serine proteases encoded by 25 genes, mGK-1~mGK-25, all of which are located on chromosome 7 (Mason *et al.*, 1983). Of the 25 characterized genes, 14 genes have the potential to encode functional proteins and the remaining 10 genes are considered to be pseudogenes. Of these, the physiological function of only 7 genes is currently known (Clements, 1989). For example, mouse pro-nerve growth factor (NGF) is cleaved to its mature form by the arginyl esteropeptidase, γ-NGF (mGK-3) and α-NGF (mGK-4) (Ronne *et al.*, 1984; Ullrich *et al.*, 1984; Evans *et al.*, 1985; Van Leeuwen *et al.*, 1986). Mouse pro-epidermal growth factor (EGF) is potentially cleaved by three kallikreins, epidermal growth factor-binding protein (EGF-BP) type A (mGK-22), B (mGK-13), and C (mGK-9) (Anundi *et al.*, 1982; Lundgren *et al.*, 1984; Blaber *et al.*, 1987; Drinkwater *et al.*, 1987a; Isackson *et al.*, 1987). Another member of the mouse kallikrein gene family, γ-renin (mGK-16) cleaves synthetic renin substrate (Poe *et al.*, 1983; Drinkwater *et al.*, 1988) and mGK-6 is a potent kininogenase (Van Leeuwen *et al.*, 1986). Also, we have demonstrated that EGF-BP type B is a prorenin converting enzyme (PRECE) (Nakayama *et al.*, 1988, 1990; Kim *et al.*, 1990, 1991a, b). Of the remaining 7 genes, 4 genes; mGK-1, mGK-5, mGK-8 and mGK-11 have been shown to be expressed at higher levels in the male SMG, but a function has yet to be assigned for the products of these genes (Mason *et al.*, 1983; Fahnestock *et al.*, 1986; Drinkwater *et al.*, 1987b, c; Evans *et al.*, 1987). Three other genes (mGK-14, mGK-21, and mGK-24) are potentially functional but the expression data are unavailable (Evans *et al.*, 1987).

In this study, we have cloned the cDNA of mGK-21 gene from a library of male ICR mouse submandibular gland and determined the full length sequences. We have subcloned its cDNA in the mammalian expression vector, pcDNA3 and examined the function of the encoded protein using mouse *Ren*-2 prorenin as a substrate.

Materials and Methods

Materials

Sequenase Version 2.0 DNA Sequencing Kit was obtained from United States Biochemical Corp. [^{35}S] Me-

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thionine (>1,000 Ci/mmol) and ECL Western blotting analysis system were purchased from Amersham Life Science. Acrylamide and low-molecular-weight protein markers were purchased from Bio-Rad Laboratories. F-12, Dulbeco's modified Eagle's medium, methionine-free Dulbecco's modified Eagle's medium, CHO-S-SFM II and fetal bovine serum were obtained from GIBCOBRL. Cellphect Transfection Kit was purchased from Pharmacia. All other chemicals were of reagent grade or higher and were purchased from Sigma Chemical Co and Fisher Scientific.

cDNA cloning

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 *Eco*RI site of the λgt 10 vector. Approximately 1×10^5 clones from the cDNA library were screened using a [^{32}P]-labeled PRECE fragment. Twenty out of ~100 positive clones were plague-purified, and the inserts were then subcloned into the pBluescript II vector (Stratagene). The sequences of both strands of a clone with the longest insert were determined by the dideoxy method (Sanger *et al.*, 1977).

Plasmid construction

For *in vitro* translation and transfection to mammalian cell lines, a 841bp Xho I-Xba I cDNA fragment covering the entire coding sequence of mGK-21 was subcloned into the downstream region of the CMV promoter of pcDNA3 mammalian expression vector (Invitrogen). The sense orientation of the mGK-21 cDNA in pcDNA3 vector (pD/GK21) was verified by restriction enzyme digests and subsequently by DNA sequencing. We designated the plasmid construct containing the pcDNA3 vector and mGK-21 cDNA as pD/GK21.

Calcium phosphate transfection

For stable transfection of CHO cells with pD/GK21 (10 μg) we used the calcium phosphate method (Chen *et al.*, 1987), as suggested by the protocol supplied in the Stratagene Eukaryotic Transfection Kit. Stable transfectants were clonally selected in 1.000 μg/ml genetin sulfate (antibiotic G418, Gibco) using cloning cylinders. One thousand cells were plated per 100 mm tissue culture plate, and approximately 10 clones were picked from each plate. Clonal cell lines were then screened for expression by Northern blot and Western blot analysis. Stable transfectants (CHO/GK21) are being maintained in cytotoxic concentrations of G418 (250 μg/ml).

Northern blot

Total RNA (20 μg) isolated from stably transfected clones of CHO cells and ICR mouse tissues were transferred to Nytran membranes by means of capillary action. The blot was prehybridized, and hybridized with [^{32}P]-labeled pPRC-1 cDNA fragment, and washed 2 times in 0.2X SSC containing 0.1% SDS at 42°C, 2 times in 0.1X SSC containing 0.1% SDS at 68°C, and 2 times 0.1X SSC at room temperature.

Western blot

The cultured medium (50 μg protein) was resolved on a 12.5% SDS-PAGE reducing gel and transferred electrophoretically to Immobilon-P membrane (Millipore), and the blot was probed with the anti-PRECE antiserum as described (Kim *et al.*, 1990). The secondary antibody consisted of a horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad). Positive photographic images were taken of each blot.

Processing activity of recombinant pGK21 *in vitro*

CHO/GK21 and CHO/PR (a prorenin converting enzyme producing cell line) cells at ~70% confluence in 60-mm dish were labeled with [^{35}S] methionine for 24h. The conditioned media of CHO/GK21 and CHO/PR cells were treated with 5 μg/ml trypsin at 4°C, and the reactions were then stopped by the addition of soybean trypsin inhibitor at a final concentration of 50 μg/ml. The conditioned medium of CHO/MRB cells (a Ren-2 prorenin producing cell line) was incubated with the trypsin-treated or -untreated conditioned medium of CHO/GK21 or CHO/PR cells (0.1 ml) in a final volume of 0.5 ml of 0.1M Tris-HCl, pH 8.0, for 4 h and then subjected to immunoprecipitation with anti-Ren-2 renin antiserum and SDS-PAGE analysis.

Results and Discussion

In order to clone the cDNAs of potentially functional mouse kallikreins (mGK-14, mGK-21, and mGK-24), we screened approximately 1×10^5 clones from the cDNA library of a male ICR mouse SMG with [^{32}P]-labeled PRECE/sty I digested 636 bp fragment and obtained about 100 positive clones. Twenty of the longest positive clones were sequenced, and then we cloned the cDNA, pPRC-1 (PRECE related cDNA), which was different from previously cloned cDNAs. The pPRC-1 contained 841 nucleotides including 14 bp 5'-, 41bp 3'-uncoding nucleotides. By comparing the nucleotide sequences of the pPRC-1 with those of exon 2 and 3 of 22 kallikrein genes, which were analyzed from a BALB/C mouse by Evans *et al.* (1987), the sequences of pPRC-1 were shown to be identical to those of mGK-21 except for one nucleotide residue, a G at site 11 of exon 3 in mGK-21 is replaced by A in pPRC-1. This substitu-

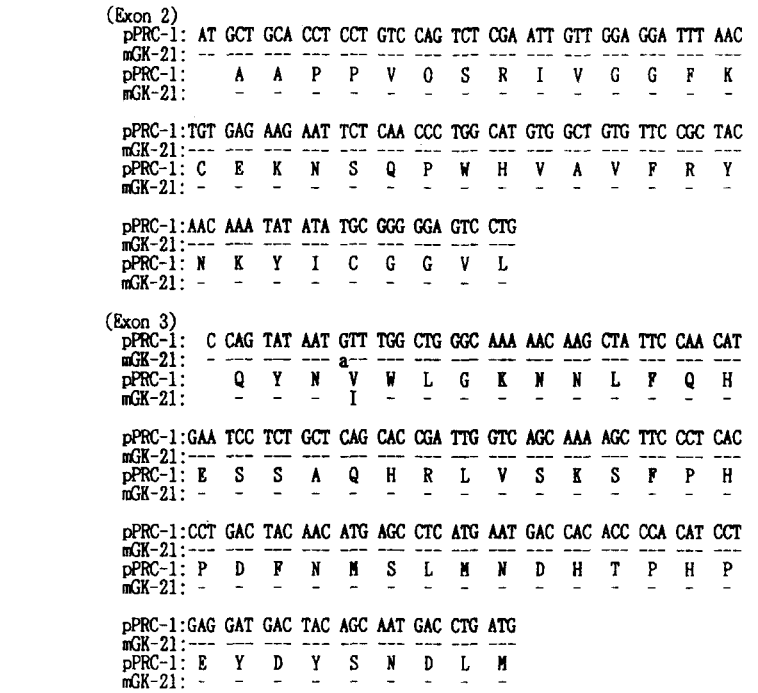

Fig. 1. Nucleotide and deduced amino acid sequences of pPRC-1 along those of the exon 2 and 3 of mGK-21. Residues of mGK-21 identical with those pPRC-1 are indicated by hyphens.

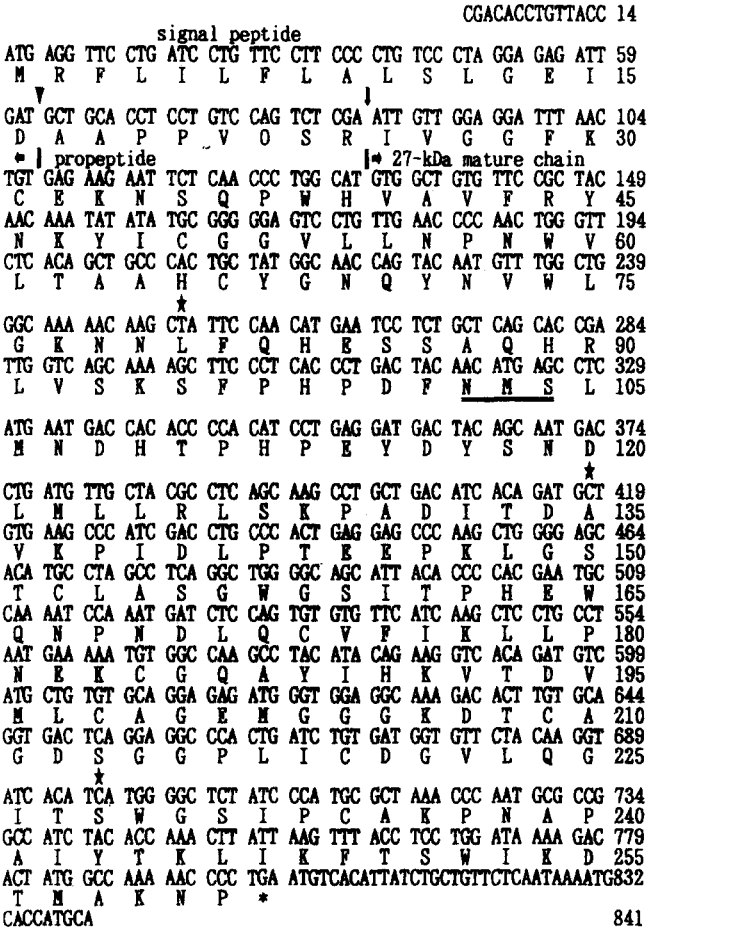

Fig. 2. Nucleotide and deduced amino acid sequences of pPRC-1. The proposed active site, His^{65}, Asp^{120}, and Ser^{213} residues are indicated by asterisk. The consensus sequences for N-glycosylation are double underlined. The arrow designates the putative autocatalytic site of active form 27 kDa polypeptide.

tion changed Val (GTT) in mGK-21 to Ile (ATT) in pPRC-1 but it is still an aliphatic amino acid (Fig. 1). This difference has been explained by strain polymorphism (Drinkwater *et al.*, 1987a). Although the possibility of mutation in exons 1, 4 and 5 was not excluded, mGK-21 gene was considered to be a potentially functional gene, because there were no gross exon/intron rearrangements or in-phase termination codons in the exon 2 and 3 (Evans *et al.*, 1987). Thus, we designated the pPRC-1 or mGK-21 gene products as pGK21. From the amino acid sequence predicted from its cDNA, we speculated that pGK21 consists of 261 amino acids including the NH_2-terminal signal peptide (residues 1~17), the short propeptide (residues 17~24), and the active peptide (residues 25~261) (Fig. 2). Generally, mouse glandular kallikreins have one or more autocatalytic sites in their amino acid sequences. For example, cleavages between the propeptide and the 17 kDa chain, and between the 17 and 10 kDa chains occured after Arg residue, Arg^{24} and Arg^{164}, respectively in PRECE (Kim *et al.*, 1990). The first autocatalytic site, Arg^{24} residue was conserved in pGK21. Since Arg^{164} residue in PRECE was replaced by Glu^{164} in pGK21, its active form was assumed to be approximately 27 kDa single polypeptide chain (Fig. 2).

RNAs isolated from various tissues were analyzed by blot-hybridization techniques with the following [^{32}P]-labeled pPRC-1 cDNA fragment in order to investigate the expression of the pGK21. The data shows that the mRNAs of pGK21 were present at readily detectable levels only in the SMG, but not present in other tissues analyzed (Fig. 3). This data is compatible with the our previous Western blot analysis that PRECE is detectable only in the SMG. Fig. 4 shows a comparison of the predicted amino acid sequences of the pGK21 with those of the PRECE. The pGK21 showed extensive amino acid sequence homology (PRECE: 76 %, γ-NGF, EGF-BP type A and C: 75~79%: data not shown) including active site amino acid residues, His^{65}, Asp^{120}, and Ser^{213}, primarily responsible for determining cleavage at basic amino acid. Thus, pGK21 may possess a serine protease activity. To demonstrate this enzyme activity of pGK21, the cDNA was subcloned into the pcDNA3 vector containing T7 and SP6 RNA polymerase promoters. The pcDNAGK21 and pcDNAPR plasmid (PRECE expression plasmid) was initially transcripted and translated

by using the TNT T7 Quick Coupled Transcription/Translation Systems (Promega). *In vitro* translation of the cDNA yielded a polypeptide with an apparent molecular mass of approximately 27 kDa which exhibited the same migration as PRECE band and the band was immunoprecipitatable by rabbit polyclonal anti-PRECE antibody (data not shown). This data indicates that pGK21 shares epitopes with PRECE.

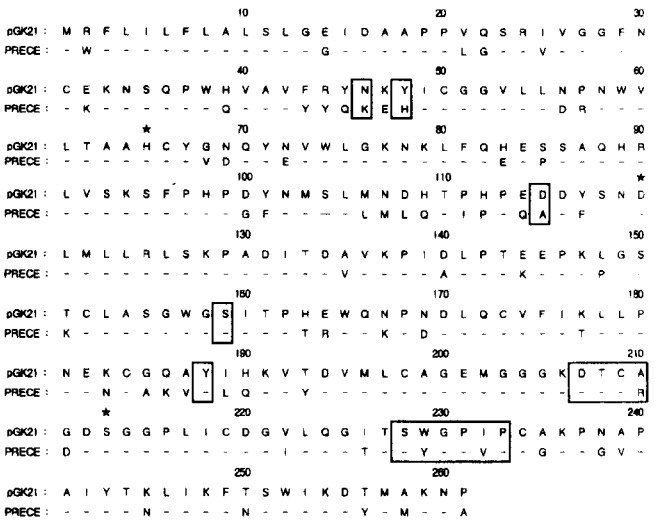

Fig. 3. Amino acid composition of the pGK21 and PRECE. The amino acid sequence of the pGK21 was aligned to the sequences of the PRECE (Kim *et al.*, 1991). Residues of PRECE identical to those of pGK21 are indicated by hyphens. Residues believed to line the substrate-binding pocket are boxed. The proposed active site amino acid residues, His^{65}, Asp^{120}, and Ser^{213} are indicated by asterisks.

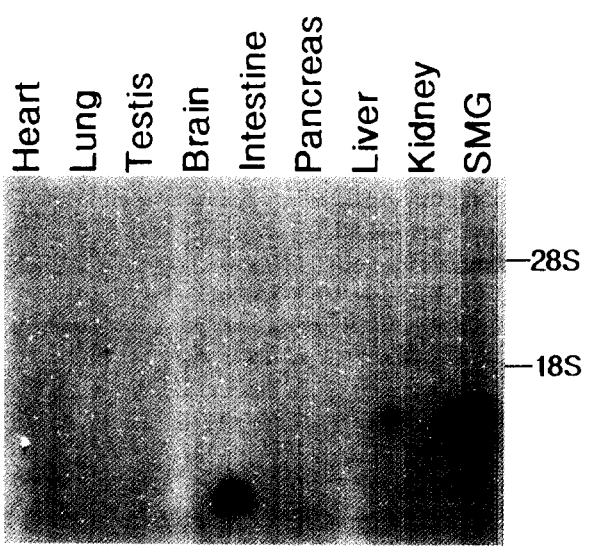

Fig. 4. Northern blot analysis. Total RNAs (20 μg) isolated from the ICR mouse tissues were electrophorsed in an agarose gel and blotted onto a membrane and then the blot was analyzed using the pPRC-1 cDNA probe as described under "Materials and Methods".

Since pGK21 is highly homologous to PRECE (Fig. 3), we examined the prorenin converting activity to demonstrate the function of pGK21 as a serine proteaes. Therefore. CHO cells were transfected with the pcDNA3, pcDNAGK21 and pcDNAPR expression plasmids, and the stable cell line (CHO/DNA3, CHO/GK21 and CHO/PR) expressing a high level of the pGK21, and PRECE was obtained by Northern and Western blot anlysis (data not shown). pGK21 and PRECE were synthesized as inactive precursors in these transfected cells for lack

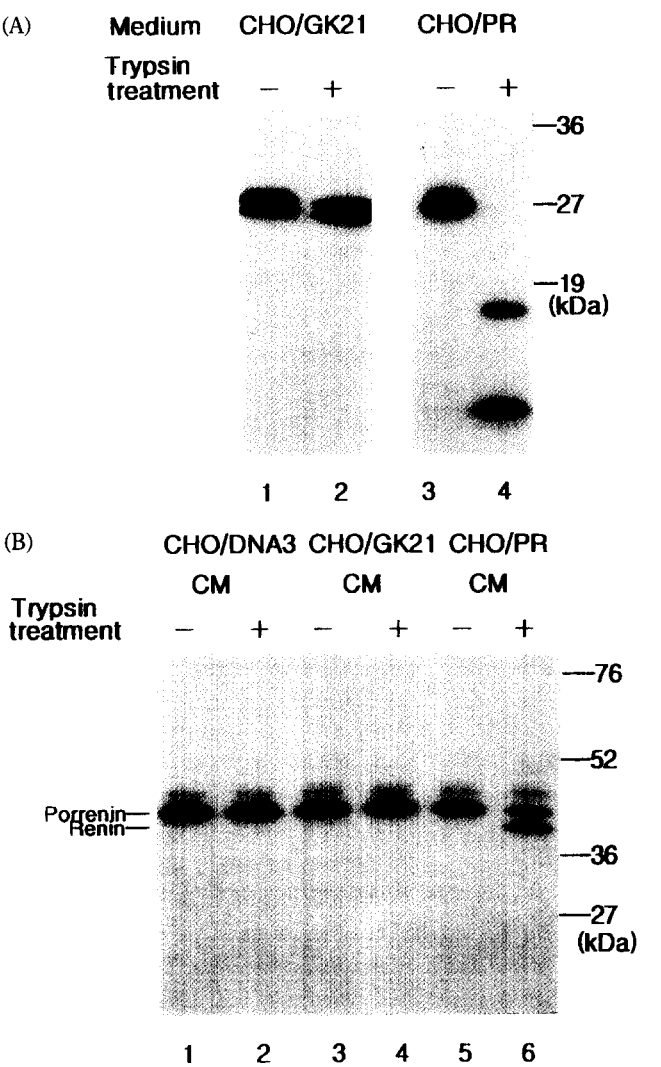

Fig. 5. Prorenin converting activity of recombinant pGK21 produced in CHO cells. A. the conditioned medium (CM) of CHO/GK21 (lanes 1 and 2) and CHO/PR (lanes 3 and 4) cells were treated with trypsin for 2 h. immunoprecipitated with anti PRECE antiserum. and electrophoresed in the SDS-polyacrylamide gel under reducing condition. B. the conditioned medium of CHO/MRB cells was incubated with the conditioned medium of CHO/DNA3 (lanes 1 and 2), CHO/GK21 (lanes 3 and 4) or CHO/PR (lanes 5 and 6) cells treated with (lanes 2, 4. and 6) or without (lanes 1. 3. and 5) trypsin for 2 h. and analyzed as described under "*Materials and Methods*".

of an activating enzyme which may be in the SMG (Kim *et al.*, 1991a). The conditioned medium from CHO/DNA 3, CHO/GK21 and CHO/PR were then treated with trypsin, since trypsin activated proPRECE effectively (Kim *et al.*, 1991a, b). As shown in Fig. 5A, in a similar fashion to the activation of proPRECE, trypsin effectively converted the 27 kDa precursor form of pGK21 to an active form which may be cleaved at putative autocatalytic site, Arg^{24} (lane 2). The doublet SDS-PAGE bands in Fig. 5A could be explained by heterogeneity in glycosylation, since the deduced amino acid sequence has a potential N-glycosylation site (see Fig. 2), and these molecules treated with endoglycosidase F migrated as a single band on SDS-polyacrylamide gel (Kim *et al.*, 1991a). Subsequently, the trypsin-treated or -untreated conditioned medium of CHO/DNA3, CHO/ GK21 and CHO/PR were incubated with ^{35}S-labeled conditioned medium from CHO/MBR cells, which stably express *Ren*-2 prorenin (Hatsuzawa *et al.*, 1990). As shown in Fig. 5B, trypsin-treated conditioned medium (lane 6) of CHO/PR cells caused conversion of prorenin to renin, but the untreated one (lane 5) and trypsin-treated (lane 4) or -untreated (lane 3) conditioned medium of CHO/GK21 cells did not. The conditioned medium of CHO/DNA3 cells (cells transfected with the control plasmid lacking the cDNA insert) had no prorenin converting activity. This observation indicated that the protein encoded by mGK-21 gene does not possess prorenin converting activity.

In this study, we failed to demonstrate prorenin converting activity of pGK21. However, it may be nothing strange that pGK21 did not possess prorenin converting activity, since the substrate specificities of some kallikreins appear to be very strict. For example, it was reported that γ-NGF cannot cleave proEGF (Frey *et al.*, 1979) and proposed that EGF-BP cannot cleave pro-NGF (Blaber *et al.*, 1989). Although pGK21 and PRECE share a high degree of amino acid sequence identity, 6 residues (Lys^{46}, His^{48}, Ala^{115}, Arg^{210}, Tyr^{229} and Val^{232}) among the 15 amino acid residues (Lys^{46}, His^{48}, Ala^{115}, Ser^{159}, Tyr^{188}, Asp^{207}, Thr^{208}, Cys^{209}, Arg^{210}, Ser^{228}, Tyr^{229}, Gly^{230}, Pro^{231}, Val^{232} and Pro^{233} in PRECE) that are believed to line the substrate-binding pocket, were replaced by Asn^{46}, Tyr^{48}, Asp^{115}, Ala^{210}, Trp^{229} and Ile^{232} in pGK21, repectively (Mason *et al.*, 1983; Fig. 3). Thus, it was assumed that the substrate specificity of pGK21 appears to be as strict as other glandular kallikreins. Although we did not demonstrate the enzyme activity of pGK21, it was assumed that pGK21 was involved in the maturation of certain bioactive polypeptide(s) in a mouse SMG for the following reasons: (a) mGK-21 gene was apparently expressed in male ICR mouse SMG; (b) the proposed active site His^{65}, Asp^{120}, and Ser^{213} residues were completely conserved in pGK21 just like other glandular kallikreins; (c) the cloned cDNA was translated to a predicted 27 kDa polypeptide chain *in vitro* and *in vivo*; (d) the 27 kDa polypeptide chain porduced by CHO čells was processed to a putative active form by trypsin. However, it was not clear what its substrate is. To address this question experments are under way in our laboratory.

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