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Expression of an Allozyme of Prorenin-Converting Enzyme in the Submandibular Gland of DBA/2N Mice[1]

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A protein product of the tissue kallikrein gene family was isolated from the submandibular gland of DBA/2N mice. Amino acid sequencing showed this protein to be highly homologous to two tissue kallikreins, mK13 and mK26, also known as prorenin-converting enzymes PRECE and PRECE-2, respectively. The cDNA corresponding to the present enzyme was cloned, and its complete nucleotide sequence was determined. The cloned cDNA was different in 6 and 12 bases out of 783 nucleotides from those of mKlk-13 and mKlk-26 cDNAs, respectively, the homologies being 99.2 and 98.5% (nucleotide), or 98.3 and 96.2% (amino acid). Upon incubation with either bovine kininogens or mouse *Ren 2* prorenin, this tissue kallikrein generated bradykinin and renin, respectively, as judged by Western blotting and protein sequence analysis. Isoelectric focusing analysis of the submandibular gland tissue kallikreins suggested that the present enzyme was not expressed in CD-1 or ICR mice and that no mK13 protein was present in DBA/2N mice. These data suggest that the enzyme is an allozyme of mK13, a prorenin-converting enzyme highly expressed in the submandibular gland of DBA/2N mice. The mKlk-13 gene in mice is therefore suggested to be polymorphic, having at least two allelic forms with a high sequence homology. The designation mK13^b and mKlk-13^b for the protein and gene of this tissue kallikrein is proposed.

Key words: mK13 allozyme, prorenin-converting enzyme, tissue kallikrein.

In 1983 and 1987, Mason *et al.* (*1*) and Evans *et al.* (*2*), respectively, studied the structure of 25 mouse tissue kallikrein genes (mKlk) and showed that these genes constitute a family. The complete structure of some of these genomic DNAs has been analyzed and identified (*3-5*). The relationships between mouse tissue kallikrein genes and several of their protein products/mRNAs have recently been established (*6*); γ nerve growth factor (γNGF), α nerve growth factor (αNGF), epidermal growth factor binding protein type C (EGF-BP C), prorenin-converting enzyme (PRECE), γ renin, βNGF-endopeptidase (EGF-BP A or enzyme A), and PRECE-2 (EGF-BP B) are now designated as mK3, mK4, mK9, mK13, mK16, mK22, and mK26, respectively, and identified as the products of their respective genes, *i.e.*, mKlk-3, mKlk-4, mKlk-9, mKlk-13, mKlk-16, mKlk-22, and mKlk-26. The genomic DNA for true tissue kallikrein of the mouse (mKlk-1) was cloned from kidney tissue and sequenced over a decade ago (*3*), whereas its protein product was isolated more recently from the submandibular gland of female mice (*7*).

On the other hand, a functional linkage between the renin-angiotensin system and the kallikrein-kinin system has been established; *i.e.*, angiotensin-converting enzyme is currently known to function as a kininase (*8*). Another enzyme specified to have dual function in these systems is prorenin-converting enzyme, a member of the tissue kallikrein family (*9, 10*). Today renin, like tissue kallikrein, is classified into two groups, one for circulating and the other non-circulating tissue renin; and the physiological and pathological importance of the latter system has recently been suggested (*11*). It is therefore crucial to understand how prorenin-converting enzyme is involved in the generation of active tissue renin.

In the present study, we investigated the structure of cDNAs and protein products of members of the tissue kallikrein gene family. We analyzed the tissue kallikrein isozyme in the salivary gland of DBA/2N strain mice by isoelectric focusing and found the isozyme pattern of this inbred strain to be completely different from that seen in CD-1 (ICR) mice. Then we purified the major proteinase from the gland of DBA/2N mice and studied its enzymatic function as well as its nucleotide and amino acid sequences. The results showed that the enzyme is a tissue kallikrein

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having the ability to convert prorenin to renin, and that the enzyme is different from but highly homologous to the two known mouse prorenin-converting enzymes (PRECE and PRECE-2). The present enzyme was not expressed in CD-1 mice but was expressed in DBA/2N mice, in which strain no other PRECE protein was expressed. Since such evidence implies that the major tissue kallikrein in DBA/2N mice (tentatively referred as TK-DBA) is an allozyme of mK13, the designation of mK13^b for the present enzyme (mKlk-13^b for the gene) is proposed.

MATERIALS AND METHODS

Enzyme Assay—When tissue kallikrein isozymes of the submandibular gland were analyzed by isoelectric focusing (data shown in Fig. 1), the enzyme activity was conventionally assayed by measuring the hydrolysis of a synthetic substrate, benzoylarginine ethylester (BAEE), according to the procedure of Trautshold and Werle (*12*) with slight modifications (*13-15*). The hydrolysis of BAEE was coupled with the NAD^+-alcohol dehydrogenase system, and the rate of absorbance increase at 340 nm due to formation of NADH was measured. This assay procedure was also employed for the purification of the enzyme.

Purification of a Major Tissue Kallikrein from the Submandibular Gland of DBA/2N Mouse and Preparation of Its Antiserum—The major tissue kallikrein from the submandibular gland of DBA/2N mice (Charles River Laboratories, Kanagawa) was purified by the procedure reported previously (*15*) with slight modifications. In brief, the submandibular gland was homogenized in 20 mM sodium phosphate buffer (10%, w/v) and centrifuged at $105,000 \times g$ for 30 min. The tissue extract obtained was adjusted to pH 4.5, and insoluble material was removed by centrifugation. The acid-treated extract was neutralized with 1 M KOH solution and fractionated with acetone at 4°C; the precipitate obtained at 40-60% concentration of acetone was subjected to gel filtration by Sephadex G-75 column chromatography. The major activity peak was collected and purified further by isoelectric focusing (110-ml column; pH 7-9; 700 V; 40 h). The main peak, which had an isoelectric point (pI) of 8.7, was collected and dialyzed in 1 mM HCl at 0°C. The enzyme protein prepared in this way gave a single band by non-reducing sodium dodecyl sulfate-polyacryl amide gel electrophoresis (SDS-PAGE), and was stored at −80°C. This enzyme is tentatively referred to as TK-DBA, and will be proposed to be an allozyme of mK13 (mK13^b) later. Antiserum for TK-DBA was prepared by the procedure described previously (*15*). The enzyme thus purified was subjected to analysis of structure and enzymatic properties as described below.

Preparation of Prorenin and Its Antiserum—A Chinese hamster ovary cell line transfected with the expression plasmid of mouse *Ren 2* preprorenin (*16*) was a generous gift from Dr. K. Nakayama (University of Tsukuba, Ibaraki). The cells were grown and prorenin was purified from the conditioned medium according to the procedure described previously (*16*).

The anti-*Ren 2* renin antiserum raised in a rabbit (*17*) was provided by Dr. H. Izumi, Tohoku University School of Dentistry, Sendai. The purification of mouse *Ren 2* renin and preparation of the antiserum were described previously (*17*).

Measurement of Kinin-Releasing Activity—Kinin-releasing activity was qualitatively determined by measuring the liberation of kinin by high-performance liquid chromatography (HPLC). The tissue kallikrein sample (40 μg) was incubated with 125 μg of either H or L kininogen (a gift from Dr. H. Kato, National Cardiovascular Center Research Institute, Osaka) in 20 mM Tris-HCl (pH 8.0) containing 150 mM NaCl (total volume, 500 μl). The reaction mixture was incubated at 37°C for 1 h, then boiled for 15 min. Samples were next ultrafiltered through a Centricon 10 (cut-off MW, 10,000; Amicon, Scientific Systems Division, Danvers, MA) and analyzed by RSiL C18 LL column chromatography ($\phi 4.6 \times 250$ mm, particle size, 5 μm; Nippon BioRad Laboratories, Tokyo) as described by Maeda (*18*). The column was operated with a solvent system comprising 17% acetonitrile, 0.25 M Na_2SO_4, 0.01 M KH_2PO_4, and 0.2% H_3PO_4 at a flow rate of 1 ml/min (Jusco 880-PU Intelligent HPLC Pump, Japan Spectroscopic, Tokyo). The peptide peak that eluted at the position of authentic bradykinin was further separated by reverse-phase HPLC (RP-300 column, $\phi 2.1 \times 220$ mm), with a linear gradient of acetonitrile (3.5-70%, or 0-70%) in 0.1% trifluoroacetic acid (TFA) at a flow rate of 200 μl/min. The peptide peaks eluted were collected and analyzed by a protein sequencer (Model 476A; Applied Biosystem, CA).

Measurement of Prorenin-Converting Activity—Aliquots of partially purified prorenin (0.2 μg; Blue Sepharose eluate following DEAE-Toyopearl chromatography) were incubated with various concentrations of purified TK-DBA for 1 h at 37°C in 20 μl of 10 mM Tris-HCl buffer (pH 8.0). The reaction was terminated by adding 5 μl of sampling buffer for SDS-PAGE, and the mixture was subjected to SDS polyacrylamide gel electrophoresis (10% acrylamide gel) under non-reducing conditions, followed by immunoblotting with antiserum specific for renin from the mouse submandibular gland (anti-*Ren 2* renin, 1,000-times diluted; Ref. *17*).

For determination of the N-terminal amino acid sequence of the protein produced by tissue kallikrein, 5 μg of purified prorenin was mixed with 2.5 μg of the purified enzyme in 100 μl of 25 mM Tris-HCl buffer, pH 8.0. The mixture containing prorenin and the enzyme was incubated for 60 min, then subjected to SDS-PAGE. The separated protein was transferred to an Immobilon-P^{SQ} filter (Millipore, Bedford, MA) and stained with 0.25% Coomassie Brilliant Blue R-250 (in 45% ethanol/10% acetic acid solution). The part of the filter containing 38-kDa protein, which was the reaction product generated from prorenin, was excised for analysis of its amino terminal amino acid sequence (Shimadzu protein sequencer, PPSQ-10, Kyoto).

Determination of the Amino Acid Sequence—A 100-μl aliquot of purified TK-DBA (10 mg/ml in 1 mM HCl) was mixed with 1 ml of 0.5 M Tris-HCl buffer containing 6 M guanidine HCl and 2 mM EDTA. Then 1460 μg of 2-mercaptoethanol (500-fold molar excess over enzyme) was added, and the solution was rotated at room temperature for 19 h under a N_2-stream. 4-Vinylpyridine (4900 μg; 2.5-fold molar excess over 2-mercaptoethanol) was then added, and the solution was incubated further for 2 h. The enzyme protein thus *S*-pyridylethylated was dialyzed against distilled water for 3 days and lyophilized. The two subunits of the enzyme were separated by reverse-phase HPLC

using a C8 column (ϕ4.6×150 mm; Japan Spectroscopic) with a linear gradient of acetonitrile (0-60% in 36 min; 1 ml/min) in 0.1% TFA. Two peaks, which eluted at retention times of 27.7 and 28.6 min, were collected. The first peak was the large subunit; and the second, the small subunit. Both subunits were subjected to restricted digestion and protein sequencing.

Clostripain (Promega, Madison, WI), ASP-N endoproteinase (ASP-N, Sigma Chemical, St Louis, MO), and α-chymotrypsin (Wako Pure Chemicals, Osaka) were used for restricted digestion, and the peptide fragments generated were separated on C8 or C18 columns (ϕ4.6×150 mm and ϕ4.6×250 mm, respectively) with a linear gradient of acetonitrile in 0.1% TFA or with a gradient of the mixture of acetonitrile and 2-propanol (3:7) in 0.1% TFA. Most of the fragments separated were purified further by re-chromatography and analyzed by use of a Shimadzu protein sequencer, PPSQ-10.

Cloning and Sequence Analysis of a cDNA Highly Homologous to mKlk-13 cDNA—The submandibular gland was surgically removed from 8- to 10-week-old male DBA/2N mice and total RNA was prepared by the standard procedure (*19*).

Oligonucleotide primers synthesized were A1 (5′-TCCAGGACACCCTGTTACC-3′), A2 (5′-ACATGGGACAATGTGACAC-3′), A3 (5′-CCTCCTGATGCTTCAAACAA-3′), K1 (5′-TTAACCCTCACTAAAGGGAAC-3′), and K2 (5′-CGTAATACGACTCACTATAGG-3′). Primers A1 and A2 were designed based on the reported nucleotide sequence (*10*) of untranslated 5′- and 3′-regions of mKlk-13 cDNA, and A3 was a unique coding region of mKlk-13/mKlk-26 cDNAs (nucleotide numbers 6-24 for A1, 828-810 for A2, and 336-355 for A3; see also Fig. 5A). K1 and K2 were specific to T3 and T7 sites of pBluescript II KS−. The cDNAs were synthesized in a 20-μl reaction mixture containing RNase inhibitor, reverse transcriptase, submandibular total RNA (1.4 μg), oligo dT_{20} (1 μM), and other integrals. The mixture was incubated at 42°C for 30 min, and a 1-μl portion of the resulting cDNA mixture was transferred to 50 μl of amplification buffer containing 1.25 U of Ex Taq (Takara Shuzo, Otsu) and a 0.25 μM concentration of each of the primers A1 and A2. The DNA amplification by polymerase chain reaction (PCR) was carried out for 35 cycles in a DNA thermal cycler (GeneAmp PCR System 9600, Perkin-Elmer, Foster, CA); each cycle consisted of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1.5 min.

PCR-amplified DNAs (amplicons) were analyzed in a 2% low melting temperature agarose gel, the gel areas containing DNA bands were cut out, and the DNAs were purified by the standard procedure (*20*). The amplicons (1 pmol in 10 μl) were treated with T4 DNA polymerase and subcloned into pBluescript II KS− with a DNA Blunting Kit (Takara Shuzo). The reaction mixture (3 μl) was mixed with 0.05 pmol of *Sma*I-treated pBluescript II KS− (Stratagene, La Jolla, CA) in a total volume of 24 μl and incubated with ligase at 16°C for 1 h. The ligated DNA (5 μl) was transferred into a tube containing competent *E. coli* XL1-Blue suspended in 100 μl of transfection buffer, and the mixture was subjected to transfection following the standard procedure. A portion (50 μl) of cell suspension was grown overnight on an L-agar plate containing 30 μg/ml ampicillin and 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; both from Wako Pure Chemicals, Osaka) at 37°C. White colonies were selected and grown overnight in 1 ml of L broth containing ampicillin. A 10-μl portion of these cultures was subjected to heat extraction as described previously (*21*). A 3-μl portion out of 100 μl was used as a template for PCR amplification using primers K1 and K2 to select colonies carrying the insert. By the alkaline lysis method using a SpinBind Mini-Prep System (FMC BioProducts, Rockland, ME), a plasmid was isolated from the rest of the cultures that had been demonstrated to carry the insert. A total of 0.5 μg of plasmid DNA was used for cycle sequencing using a Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer Cetus) according to the manufacturer's protocol. The labeled DNA in the reaction mixture was purified by ethanol precipitation and sequenced with an ABI Prism 377 DNA Sequencer (Perkin-Elmer).

RESULTS

A Major Tissue Kallikrein from DBA/2N Mice—The isoelectric focusing pattern of the submandibular tissue kallikrein was compared between CD-1 or ICR and inbred DBA/2N mice (Fig. 1). In CD-1 mice, two and three major peaks were detected for male and female mice, respectively. Some of these peaks were further separated by a second isoelectric focusing using carrier ampholytes with a pH range of 4-6, which procedure had been established for identifying the four major submandibular gland tissue kallikreins (mK1, mK22, mK9, and mK13) (*7, 22*). Accordingly, the first peak in males was identified as mK9 *plus* mK22, whereas the second peak corresponded to mK13. In females, the third peak (identified as mK1) appeared in addition to the two peaks seen in males. On the other hand, the isoelectric focusing pattern of the gland extract from DBA/2N mice was completely different from that of CD-1 mice. There appeared at least three different tissue kallikrein isozymes in this strain, and all of their pIs were different from those of CD-1 mice. The isozyme with a pI value of 8.7 was the most abundant and was thus subjected to purification as described in "MATERIALS AND METHODS."

The purified enzyme (TK-DBA) gave a specific activity of 292 units/mg when assayed with BAEE as a substrate. It gave a single band with an approximate molecular mass of 27 kDa under non-reducing conditions. Under reducing conditions, the enzyme gave two bands with molecular weights of 16,000 and 11,000, which were very close to those determined by use of the protein sequencer (15,454.5 and 10,503; see below and Fig. 5). Immunological assessment (Ouchterlony's double diffusion) was carried out with this purified TK-DBA and other tissue kallikreins (mK22, mK9, and mK13), and antisera to these enzymes. The antiserum raised against TK-DBA strongly cross-reacted with mK13, and anti-mK13 antiserum cross-reacted with TK-DBA (data not shown).

Characterization of a Major Tissue Kallikrein of the Submandibular Gland of DBA/2N Mice—The activity of the purified tissue kallikrein (TK-DBA) to release bradykinin was measured with L and H kininogens as substrates. Figure 2 compares the activity of the present enzyme with that of mK13. Although mK13 yielded several peptide

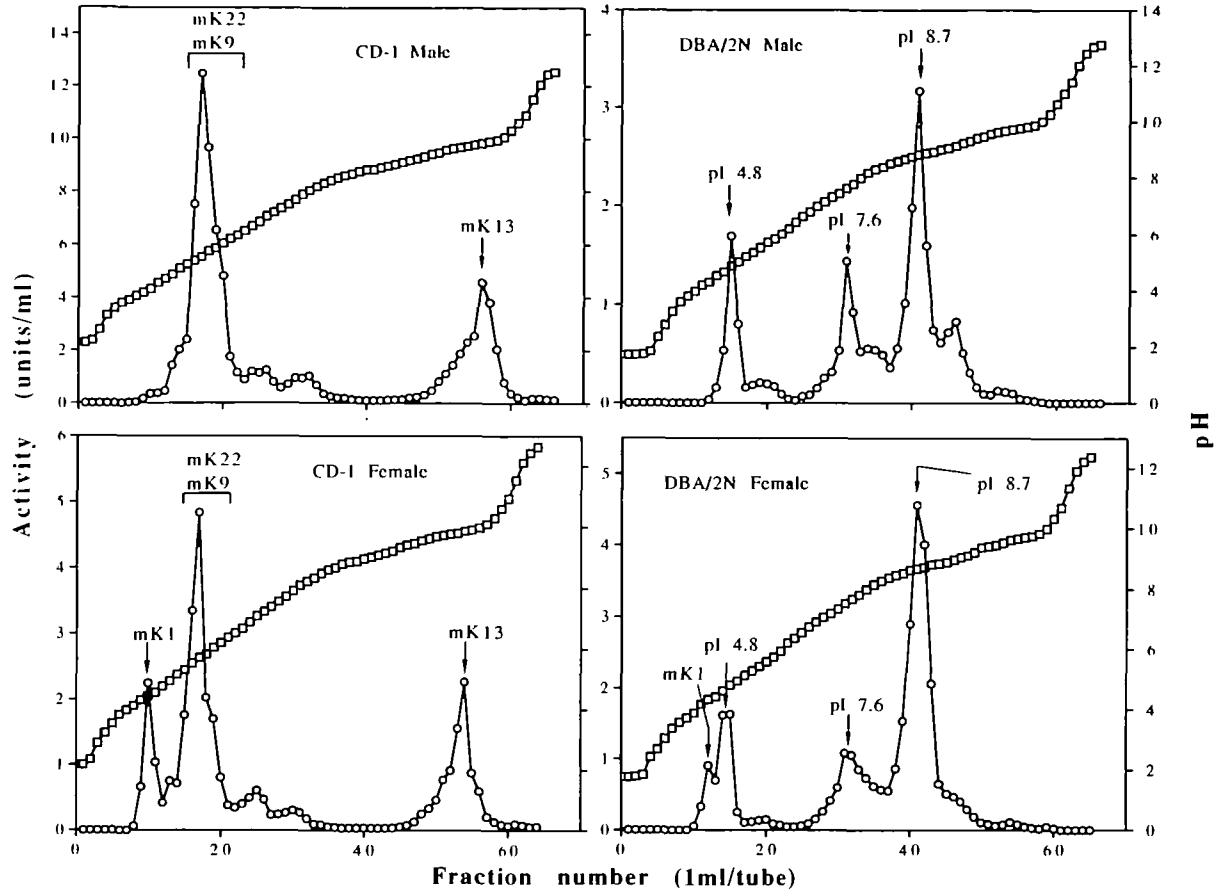

Fig. 1. **Isoelectric focusing separation of tissue kallikreins in submandibular gland extracts from CD-1 (ICR) and DBA/2N mice.** A sucrose gradient (0-50%) containing 1% Ampholine carrier ampholytes (pH 3.5-10; Pharmacia LKB Biotechnology AB, Uppsala, Sweden) was prepared in a 65-ml column. The extract of the submandibular gland was introduced into the middle of the gradient, and electrofocused for 22 h at 2 °C and 700 V. After electrofocusing, 1-ml fractions were collected, and enzyme activity and pH (at 0°C) were measured in each. Top, male; bottom, female; left, CD-1 (ICR); right, DBA/2N.

fragments from L and H kininogens, the reaction product after incubation with TK-DBA gave a distinct single peak, whose retention time agreed well with that of standard bradykinin. This peptide peak was collected and purified further by HPLC using an RP-300 column (ϕ2.1 × 220 mm column, Applied Biosystem) that was eluted with an acetonitrile gradient (0-70 or 3.5-70%, 45 min), which conditions resolved two peaks. One peak, which eluted at the acetonitrile concentration of 33.9%, was found to be ^{380}Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (bradykinin; see Ref. *23* for amino acid number) by protein sequencing, indicating that tissue kallikrein of DBA/2N mice actually has kininogenase activity. The other peak, which eluted at the acetonitrile concentration of 23.7%, was found to contain ^{187}Gln-Val-Val-Ser-Gly, which is the peptide corresponding to the sequence in the kininogen molecule that is substantially important for inhibition of thiol proteinase activity (*24*). It may be noteworthy that TK-DBA gave such a pentapeptide besides bradykinin from bovine H kininogen, though the physiological significance of such activity is obscure. The activity of TK-DBA to generate the same pentapeptide from L kininogen was not examined since the peak which may contain the peptide was small (Fig. 2).

Tissue kallikrein TK-DBA was immunologically very similar to mK13 as indicated above. This suggested that the enzyme is structurally related to prorenin-converting enzymes PRECE and PRECE-2 (mK13 and mK26, respectively), and this was verified by sequence analysis (see below and Fig. 5 for complete sequence). Therefore, we tested whether the present enzyme really has the activity of converting prorenin to renin (Fig. 3). Prorenin was incubated with the purified TK-DBA, and the reaction product was subjected to Western blotting. The protein band visualized with anti-renin antiserum shifted from 42 to 38 kDa. The sequence of amino terminal amino acid of the 38-kDa protein was determined to be Ser-Ser-Leu-Thr-Asp-Leu-Ile-Ser-Pro-Val-Val, which concurred with the amino-terminal amino acid sequence of mature *Ren 2* renin (*25*). The site of cleavage of prorenin by TK-DBA was therefore indicated to be between ^{63}Arg and ^{64}Ser, which is the same position for hydrolysis by PRECE (*9*). This result indicates that the present enzyme is a prorenin-converting enzyme specifically producing active renin from its precursor prorenin.

Sequences of TK-DBA Protein and Its cDNA—Based on the fact that the major submandibular tissue kallikrein of DBA/2N mice (TK-DBA) has prorenin-converting activity and is immunologically very similar to mK13, nucleotide primers A1 and A2 complementary to a part of the noncoding region of the mKlk-13 cDNA sequence were first

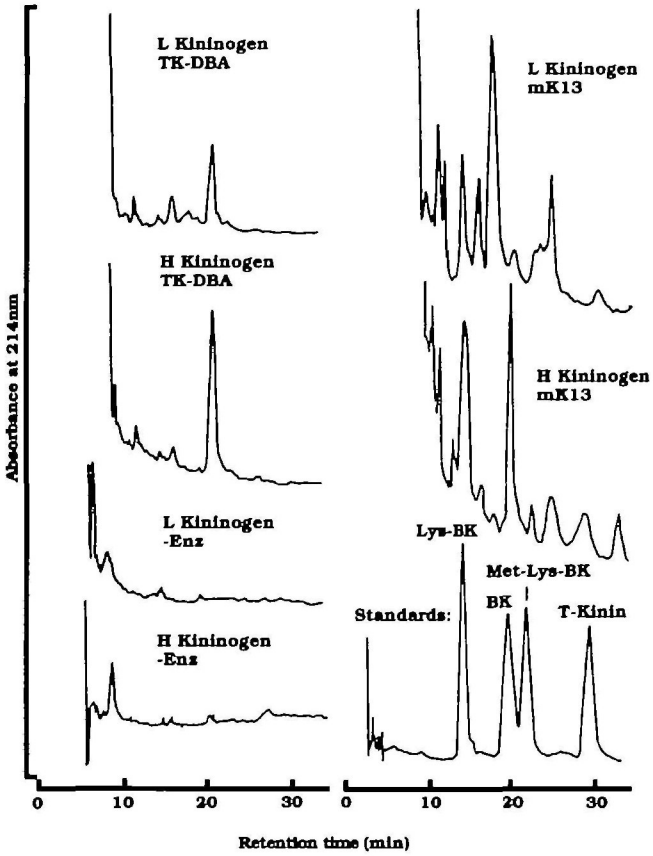

Fig. 2. **HPLC analysis of the reaction products generated from bovine H and L kininogens by TK-DBA and mK13.** The enzyme sample (40 μg) was incubated with 125 μg of either H or L kininogen in 20 mM Tris-HCl (pH 8.0) containing 150 mM NaCl (total volume, 500 μl). The reaction mixture was incubated at 37°C for 1 h, then boiled for 15 min. Samples were next ultrafiltered through a Centricon 10 (cut-off MW, 10,000; Amicon Corporation, Scientific Systems Division, Danvers, MA) and analyzed on an RSiL C18 LL column (250 mm × ϕ 4.6 mm, particle size, 5 μm; Nippon BioRad Laboratories, Tokyo). The column was operated with a solvent system comprising 17% acetonitrile, 0.25 M Na_2SO_4, 0.01 M KH_2PO_4, and 0.2% H_3PO_4 at a flow rate of 1 ml/min (Jusco 880-PU Intelligent HPLC Pump, Japan Spectroscopic, Tokyo). Peptides eluted were detected at 214 nm in a Jusco UVIDEC-1200 VI UV Spectrophotometer (Japan Spectroscopic). TK-DBA, a major tissue kallikrein purified from the submandibular gland of DBA/2N mice.

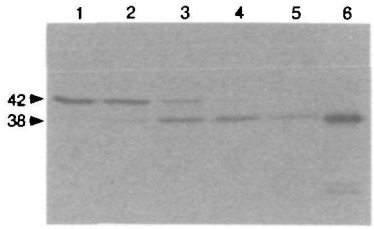

Fig. 3. **Conversion of prorenin to renin by TK-DBA, a submandibular tissue kallikrein of DBA/2N mice.** Aliquots of partially purified prorenin (0.2 μg) were incubated with various concentrations of purified TK-DBA for 1 h at 37°C in 20 μl of 10 mM Tris-HCl buffer (pH 8.0). The reaction was terminated by adding 5 μl of sampling buffer for SDS-PAGE, and the reaction product was subjected to SDS-PAGE (10% acrylamide gel) under non-reducing conditions, followed by immunoblotting with antiserum specific for *Ren 2* renin (1,000-times diluted). Lane 1, no additive; lanes 2-5, purified TK-DBA added. The amount of the enzyme added was 0.8 ng for lane 2; 8 ng for lane 3; 80 ng for lane 4; 800 ng for lane 5. Lane 6, 2 μg of extract of male mouse submandibular gland.

employed for amplification of the cDNA for TK-DBA (Fig. 4A). The total RNA prepared from the submandibular gland of male DBA/2N mice was subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) using these primers. In our initial attempt, cDNAs amplified were intended to be sequenced directly. However, restriction digestion of the RT-PCR product with *Fok*I (a single restriction site of this enzyme is present in mKlk-13 cDNA, and no site is present in mKlk-26 cDNA) showed multiple DNA bands by agarose gel electrophoresis (data not shown), indicating that the product of RT-PCR was heterogeneous. Therefore the products of RT-PCR were ligated to pBluescript II KS− using a DNA Blunting Kit (see Fig. 4B and "MATERIALS AND METHODS"). Competent *Escherichia coli* cells were transfected with the plasmid having the insert, and cultured on agar plates containing X-Gal. The 23 white colonies that appeared were examined for the presence of a plasmid with the mKlk-13 cDNA or related DNA molecule insert. The heat extracts of these colonies were subjected to PCR using primers K1/K2, A1/A2, and A3/A2, where the A3 primer is complementary to mKlk-13 and mKlk-26 cDNA (Fig. 4A), and its homologies to other tissue kallikrein mRNAs were low, being 55, 45, 60, 55, and 60% to mKlk-22, mKlk-9, mKlk-1, mKlk-3, and mKlk-4 mRNAs, respectively (numbering of nucleotides followed that in Ref. *10*). Fourteen out of the 23 colonies gave DNAs with sizes of 823, 493, and 989 bp by PCRs using A1/A2, A3/A2, and K1/K2 primers, respectively (data not shown). The facts suggest that these 14 clones contained mKlk-13 cDNA or a related DNA with high sequence homology.

From isoelectric focusing data, however, expression of mK13 protein in the submandibular gland of DBA/2N mice was not considered likely, implying the possibility that these clones contained cDNAs highly homologous but not identical to mKlk-13 cDNA. Therefore, we randomly selected 3 of the 14 colonies and extracted the plasmids. These were subjected to PCR for sequencing using fluorescence-labeled dideoxynucleotides and AmpliTaq (see Fig. 4B). The sequenced fragments from all three clones had an open reading frame of 783 base pairs, which was identical to that of other PRECE nucleotide sequences. The amino acid sequence deduced completely concurred with the sequence determined by protein sequencing, thus demonstrating that the cDNA cloned was that for TK-DBA.

Both cDNA and amino acid sequences determined for TK-DBA are presented in Fig. 5. There was a sequence, ^{78}Asn-Met-Ser, which is a consensus sequence for the *N*-glycosylation site. Indeed, no PTH-amino acid was recovered at amino acid number 78 (although PTH-Met was recovered in the subsequent cycle) by protein sequencing, indicating that ^{78}Asn is actually modified. From the data presented, the present tissue kallikrein has been proved to be a prorenin-converting enzyme and shown to be highly homologous to the protein/cDNA sequences for mK13/mKlk-13. The enzyme was expressed in DBA/2N mice, in which no mK13 was expressed, and it was not expressed in ICR mice, in which mK13 is strongly expressed. Since these data imply that TK-DBA, a major tissue kallikrein of

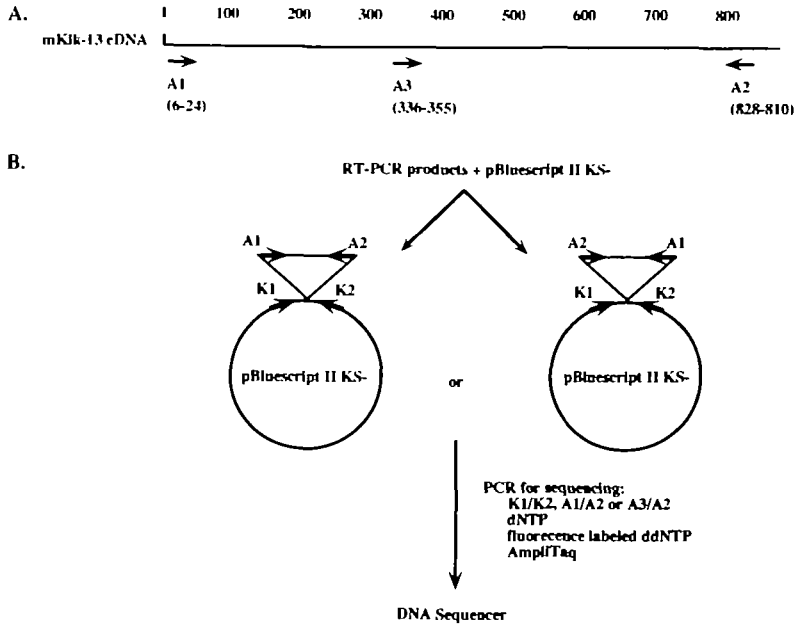

Fig. 4. **Schematic presentation of the system employed for cDNA cloning and sequencing.** A: Regions in mKlk-13 cDNA showing the positions of sequence regions complementary to primers A1, A2, and A3 were synthesized. B: Procedure for PCR-cloning and sequencing of the cDNA homologous to mKlk-13 cDNA. The sequence of K1 and K2 are respectively those of T3 and T7 sites in pBluescript II KS−.

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                -20                             -10                              1
ATGTGGTTCCTGATCCTGTTCCCAGCCCTGTCCCTAGGAGGGATTGATGCTGCACCTCCTCTCCAGTCTCGGGTGGTTGGAGGATTTAAC
MetTrpPheLeuIleLeuPheProAlaLeuSerLeuGlyGlyIleAspAlaAlaProProLeuGlnSerArgValValGlyGlyPheAsn

          10                              20                              30
TGTGAGAAGAATTCCCAACCCTGGCAGGTGGCTGTGTACTACCAAAAGGAACACATTTGTGGGGGTGTCCTGTTGGACCGCAACTGGGTT
CysGluLysAsnSerGlnProTrpGlnValAlaValTyrTyrGlnLysGluHisIleCysGlyGlyValLeuLeuAspArgAsnTrpVal

          40                              50                              60
CTCACAGCTGCCCACTGCTATGTCGACCAGTATGAGGTTTGGCTGGGCAAAAACAAGTTATTCCAAGAGGAACCCTCTGCTCAGCACCGA
LeuThrAlaAlaHisCysTyrValAspGlnTyrGluValTrpLeuGlyLysAsnLysLeuPheGlnGluGluProSerAlaGlnHisArg

          70                              80                              90
TTGGTCAGCAAAAGCTTCCCTCACCCTGGCTACAACATGAGCCTCCTGATGCTTCAAACAATACCTCCTGGGGCTGACTTCAGCAATGAC
LeuValSerLysSerPheProHisProGlyTyrAsnMetSerLeuLeuMetLeuGlnThrIleProProGlyAlaAspPheSerAsnAsp

          100                             110                             120
CTGATGCTGCTCCGCCTCAGCAAGCCTGCTGACATCACAGATGTTGTGAAGCCCATCGCCCTGCCCACAAAGGAGCCCAAGCCGGGGAGC
LeuMetLeuLeuArgLeuSerLysProAlaAspIleThrAspValValLysProIleAlaLeuProThrLysGluProLysProGlySer

          130                             140                             150
AAATGCCTAGCCTCAGGCTGGGGCAGCATTACACCCACAAGATGGCAAAAGCCAGATGATCTTCAGTGTGTGTTCATCACGCTCCTGCCC
LysCysLeuAlaSerGlyTrpGlySerIleThrProThrArgTrpGlnLysProAspAspLeuGlnCysValPheIleThrLeuLeuPro

          160                             170                             180
AATGAGAACTGTGCCAAAGTCTACCTACAGAAAGTCACAGATGTCATGCTGTGTGCAGGAGAGATGGGTGGAGGCAAAGACACTTGTAGG
AsnGluAsnCysAlaLysValTyrLeuGlnLysValThrAspValMetLeuCysAlaGlyGluMetGlyGlyGlyLysAspThrCysArg

          190                             200                             210
GATGACTCTGGAGGCCCACTGATTTGTGATGGTATTCTCCAAGGAACCACATCATATGGCCCTACACCATGCGGTAAACCTGGTGTACCA
AspAspSerGlyGlyProLeuIleCysAspGlyIleLeuGlnGlyThrThrSerTyrGlyProThrProCysGlyLysProGlyValPro

          220                             230
GCCATCTACACCAACCTTATTAAGTTCAACTCCTGGATAAAAGATACTATGATGAAAAATGCCTGA
AlaIleTyrThrAsnLeuIleLysPheAsnSerTrpIleLysAspThrMetMetLysAsnAla***
```

Fig. 5. **Nucleotide and deduced amino acid sequence of TK-DBA.** The nucleotide sequence and its deduced amino acid sequence of TK-DBA are shown. Residues of the present enzyme determined with an amino acid sequencer are underlined. No PTH-amino acid was recovered at amino acid number 78, indicating that ^{78}Asn is glycosylated. Triple asterisks (• • •) indicate that the corresponding nucleotide sequence (TGA) is the termination codon.

```
          -20       -10         1        10        20        30
mK13^b  MWFLILFPALSLGGIDAAPPLQSRVVGGFNCEKNSQPWQVAVYIQKEHICGGVLLDRNWV
mK13    .......L.....................K.........................
mK26    ..........................................................

          40        50        60        70        80        90
mK13^b  LTAA▓CYVDQYEVWLGKNKLFQEEPSAQHRLVSKSFPHPGYNMSLLMLQTIPPGADFSN▓
mK13    ....................................F.....................
mK26    ....................................F.........T.........

         100       110       120       130       140       150
mK13^b  LMLLRLSKPADITDVVKPIALPTKEPKPGSKCLASGWGSITPTRWQKPDDLQCVFITLLP
mK13    ..........................................................
mK26    ............................T.............S.............

         160       170       180       190       200       210
mK13^b  NENCAKVYLQKVTDVMLCAGEMGGGKDTCRDD▓GGPLICDGILQGTTSYGPTPCGKPGVP
mK13    ..................................................V.........
mK26    ............................AG................N..E.........

         220       230
mK13^b  AIYTNLIKFNSWIKDTMKKNA
mK13    .....................
mK26    .....................
```

Fig. 6. **Amino acid sequence of mouse mK13^b (TK-DBA) as deduced from its cDNA and the alignment with the sequences of mK13 and mK26.** Residues identical to those of mK13^b are indicated by dots. Proposed active sites (His, Asp, and Ser) are shadowed. A consensus sequence for *N*-glycosylation is double-underlined.

DBA/2N mouse submandibular gland, is an allozyme of mK13, the enzyme will be designated as "mK13^b" hereafter.

The cDNA sequence was compared with those of mKlk-13 and mKlk-26. By analogy with other PRECEs, the protein sequence of the present enzyme deduced from its cDNA had a hydrophobic signal sequence (−24 to −8 numbering relative to ^{1}Val of the mature protein) followed by a seven-residue zymogen peptide. The amino acid sequence directly determined or deduced from cDNA cloned is shown in Fig. 6, along with the sequences of mK13 and mK26 for comparison. The sequence homology of the present enzyme to enzymes mK13 and mK26 is 98.3 and 96.6%, respectively, with no alteration in sequence around the active site and *N*-glycosylation site.

DISCUSSION

Earlier, Lundgren *et al.* (*26*) reported the complete sequence of a pSGP-2 cloned from NMRI mice, and showed that its deduced amino acid sequence concurred with the sequence of EGF-BP type B, the partial structure of which had been reported by Anundi (*27*). From BALB/c mice, Drinkwater *et al.* (*4*) identified and sequenced the coding region of three distinct tissue kallikrein genes (mKlk-22, mKlk-13, and mKlk-9) that encoded the three EGF-BPs previously reported (EGF-BP A, EGF-BP B, and EGF-BP C) (*26-28*). There was only a single unmatched amino acid between the sequences deduced from the nucleotide sequences of mKlk-22 and EGF-BP type A, whereas 11 unmatched amino acids were seen between the sequences deduced from mKlk-13 and EGF-BP type B genes. The sequence of mKlk-9 was virtually identical with a cDNA clone for EGF-BP type C (*29*), with four nucleotide changes in the untranslated region. These differences were explained as a result of a polymorphic strain difference (*4*).

Kim *et al.* (*9, 10*) purified an endoprotease, designated PRECE (a prorenin-converting enzyme) from the submandibular gland of ICR mice (CD-1 mice), and showed that the cDNA sequence of this serine protease completely concurred with the amino acid sequence deduced from mKlk-13. On the other hand, the amino acid sequence deduced from a cDNA encoding the other prorenin-converting enzyme, PRECE-2, was found in the same CD-1 strain, and its sequence completely agreed with that of pSGP-2 (*30*). Although a small change between mKlk-13 and pSGP-2 had been noted and been explained as a result of strain polymorphism (BALB/c *vs.* NMRI) (*4*), Kim *et al.* claimed that the change was not due to strain polymorphism (*30*), because mRNAs encoding PRECE (mKlk-13 gene product) and PRECE-2 (pSGP-2 gene product) were found in the same CD-1 strain.

In the present study we purified a tissue kallikrein from the submandibular gland of DBA/2N mice. The protein sequence of this enzyme was found to be very similar to that of PRECE and/or PRECE-2, and has not been reported previously. The enzyme had the ability to produce bradykinin as well as to convert prorenin to renin, thus clearly indicating it to be a prorenin-converting enzyme. Of the three mouse PRECEs, two of them (PRECE and PRECE-2) are expressed in CD-1, whereas the third enzyme (present enzyme) is expressed in DBA/2N. The protein sequence of the prorenin-converting enzyme in the DBA/2N mice (TK-DBA) was closer to the mK13 sequence than to the mK26 one. The isoelectric focusing data (Fig. 1) implied that no TK-DBA (mK13^b) was present in CD-1 and that no mK13 (PRECE) was detected in DBA/2N mice. Although it is not yet clear whether a complete copy of the gene for the present enzyme (mKlk-13^b) is present in CD-1, or whether complete copies of the mKlk-13 and mKlk-26 genes are present in the DBA/2N strain, our data support the idea that TK-DBA is an allele (allozyme) of mK13, suggesting the designation of mK13^b for this tissue kallikrein. Also, the presence of tissue kallikrein allozyme has not been described before, and this is the first report that implies such a possibility. We are now planning to confirm this

possibility by analyzing the genomic DNA of various strains of the mouse including the present two. Since mK13 and mK13^{b} hydrolyzed kininogens very differently (Fig. 2), suggesting different substrate specificities of the allozymes, it is also important to investigate their enzymatic properties in order to elucidate the biological significance of the presence of allozymes for prorenin-converting enzyme.

On the other hand, the mouse renal renin gene, designated as *Ren 1*, has two common alleles (*Ren 1*^{c} and *Ren 1*^{d}); whereas there is only a single allele for the extra-renal renin gene, designated *Ren 2*, which product has recently been termed tissue renin. The tissue renin is claimed to be important for the regulation of local blood pressure; it is expressed in tissues such as the kidney, adrenal gland, brain, lung, liver, pituitary gland, submandibular gland, testis, and ovary (*31*). It is reported that prorenin is secreted by a constitutive pathway, whereas renin is secreted by a regulated pathway in AtT-20 cells transfected with cDNA for preprorenin (*32-35*); and renin-binding protein is considered to function in the secretory process (*36, 37*). Prorenin is generated from preprorenin and is converted to active two-chain renin *via* one-chain renin. The enzyme that produces one-chain renin from prorenin was first reported by Kim *et al.* and designated as prorenin-converting enzyme (PRECE) (*9*). PRECE is, therefore, implied to function in the latter pathway in tissues that express renin. PRECE is not detectable by Western blot analysis in several tissues, but its strong expression has been demonstrated in the submandibular gland (*10*). However, a prorenin-converting enzyme, though at a low level, ought to be expressed in at least tissues where tissue renin is expressed and secreted by the regulated pathway. We are now planning to employ RT-PCR/Southern, a high-performance technique for analyzing extremely low levels of mRNA, for the detection of mRNA for prorenin-converting enzyme, in these tissues.

A high level of prorenin is detected in normal human serum and in the serum from nephroblastoma patients (*38, 39*), implying that prorenin is also extensively secreted from the tissues. Restricted proteolysis yielding active tissue renin would, therefore, be indispensable for tissue renin to function properly at such sites as cardiovascular tissues where prorenin is taken up (*40*). Again, it is possible that a prorenin-converting enzyme is synthesized and expressed in the tissues mentioned above.

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