Molecular Cloning and Characterization of rKlk10, a cDNA Encoding T-Kininogenase from Rat Submandibular Gland and Kidney†

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ABSTRACT: We have cloned and determined the nucleotide sequence of a novel kallikrein-like mRNA, designated rKlk10*, from rat submandibular gland and kidney with the aid of the polymerase chain reaction (PCR). This cDNA contains 737 base pairs comprising the sequence encoding a mature protein of 235 amino acid residues, partial zymogen peptide, and 3′ noncoding sequence. Sequence comparisons showed that rKlk10 mRNA shares 87 and 88% sequence identity with rat tissue kallikrein at nucleic acid and amino acid levels, respectively. It encodes a 26 428-Da acidic protein whose derived amino acid sequence matches completely with the partial amino acid sequence of a kallikrein-like enzyme designated as T-kininogenase, K10 protein, or antigen-γ purified from rat submandibular gland [Xiong et al. (1990) *J. Biol. Chem. 265,* 2822–2827; Gutman et al. (1991) *Eur. J. Biochem. 784,* 1–5; Berg et al. (1991) *Biochem. J. 280,* 19–25]. The protein encoded by rKlk10 retains the key amino acid residues determining kallikrein cleavage specificity. Northern blot analysis with an rKlk10-specific oligonucleotide probe showed that its mRNA level in the submandibular gland is decreased dramatically by administration of the β agonist isoproterenol. Tissue-specific expression of rKlk10 was analyzed by Northern blotting and Southern blotting of PCR-amplified cDNA, which showed that rKlk10 is expressed at high levels in the submandibular gland and low levels in the kidney but not in seven other tissues including prostate, liver, heart, adrenal gland, testes, pituitary, and pancreas. rKlk10 cDNAs cloned from the kidney and submandibular gland show sequence identity. Specific expression of rKlk10 in the kidney in addition to the submandibular gland indicates that rKlk10 may be involved in the regulation of renal function. The PCR-based cloning strategy provided an efficient and reliable way to potentially identify all expressed kallikrein-related genes in rat submandibular gland.

Tissue kallikreins (EC 3.4.21.35) are a group of closely related serine proteases which are characterized by limited substrate specificity. Several mammalian enzymes in the kallikrein families, including rat tissue kallikrein, rat tonin, γ subunit of nerve growth factor (NGF)[1] of the mouse, mouse epidermal growth factor-binding proteins (EGF-BPs), and human prostate-specific antigen (PSA), have been characterized [for review, see Clements (1989)]. These enzymes are potentially involved in a plethora of physiological processes. The best characterized member in this family is true tissue kallikrein, which specifically cleaves low molecular weight kininogen at two peptide bonds to release vasoactive bradykinin or lysylbradykinin (Fiedler, 1979; Kato et al., 1985). Kinin plays a role in the regulation of local blood flow and electrolyte transport (Cuthbert & Margolius, 1982; Carretero & Scicli, 1989). Many previous studies suggested that the kallikrein–kinin system is involved in the regulation of systemic blood pressure and the development of hypertensive and diabetic diseases (Margolius et al., 1984, 1989; Carretero & Scicli, 1989; Pravenec et al., 1991). Tonin cleaves angiotensinogen to release angiotensin II and it is thought to play a role in the regulation of cardiovascular function in rat (Boucher et al., 1974). The γ subunit of NGF is known to process the precursor of the β subunit of NGF and generate its active form. Similarly, EGF-BPs cleave the precursor of EGF to yield active EGF (Bergers & Shooter, 1977). Human PSA was shown to cleave high molecular weight seminal vesicle protein and is involved in liquification of the seminal fluid clot (Watt et al., 1986).

These enzymes are encoded by a large number of closely related and tandemly arranged genes in the kallikrein gene family (Wines et al., 1991). The size of the kallikrein gene family varies among different species, with 15–20 members in the rat (Gerald et al., 1986), 23–30 members in the mouse (Evans et al., 1987), and 3–5 genes in the human (Evans et al., 1988) as estimated by Southern blot analysis. In the past several years, a great effort has been made to elucidate the number of genes in the kallikrein gene family as well as their structure, expression, and function. In the rat, for instance, 15 genes or cDNAs in the kallikrein family have been identified and sequenced. Some of the proteins encoded by these genes, such as S3 protein (rK9),[2] tonin (rK2), rk7, and rK8, have been purified and characterized (Boucher et al., 1974; Yamaguchi et al., 1991; Elmoujahed et al., 1990). In addition, several rat enzymes including proteinase B (Kato et al., 1987), T-kininogenase (Xiong et al., 1990), K10 (Gutman et al., 1991), and antigen-γ (Berg et al., 1991) have kallikrein-like NH_2-terminal structures and activities, but their corresponding genes have not been identified.

In the present study, we have developed a simple and reliable cloning procedure to clone kallikrein-like mRNAs. A novel mRNA was cloned and sequenced from rat submandibular gland and kidney, which codes for the kallikrein-like enzyme

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[1] Abbreviations: PCR, polymerase chain reaction; EGF, epidermal growth factor; EGF-BP, epidermal growth factor-binding protein; NGF, nerve growth factor; PSA, prostate-specific antigen.

[2] Nomenclature of the genes and proteins of the kallikrein gene family is according to the Discussion Group on Kallikrein Gene Nomenclature at the Kinin '91 Congress in Munich, Germany (Berg et al., 1992).

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T-kininogenase or rK10*. Tissue-specific expression and regulation of rKlk0* gene expression were determined by Northern blot analysis and Southern blot analysis of PCR products with a K10-specific oligonucleotide probe.

EXPERIMENTAL PROCEDURES

Preparation of Rat Submandibular Gland mRNA. Total RNA was extracted from rat submandibular gland by CsCl discontinuous gradient centrifugation as described by Sambrook et al. (1989). To isolate poly(A)$^+$ RNA, total RNA was loaded onto an oligo(dT) cellulose column (Stratagene) followed by washing with high-salt and low-salt buffer sequentially to remove ribosomal and transfer RNA. The poly(A)$^+$ RNA was eluted with elution buffer according to manufacturer's instructions, and the RNA concentration was determined by A_{260}. The purity of the mRNA was monitored by agarose gel electrophoresis and the A_{260}/A_{280} ratio.

Reverse Transcription and PCR Amplification. The PCR primers were designed according to conserved sequences of rat kallikrein gene family members. With the primers, cDNAs were synthesized by reverse transcription from the poly(A)$^+$ RNA. The reverse transcription mixture containing 1 μg of rat submandibular mRNA, 100 pmol of each primer, 10 μL of 10× PCR buffer (500 mM KCl, 100 mM Tris–HCl, pH 8.3, 15 mM $MgCl_2$, 0.1% gelatin), 15 μL of 1.25 mM dNTP, and 25 units of AMV reverse transcriptase (BRL) was incubated at 42 °C for 30 min. Taq DNA polymerase (Perkin-Elmer Cetus) was added to the mixture and subjected to 30 PCR cycles (94 °C 1 min, 50 °C 2 min, and 72 °C 3 min) in a thermal cycler. The resulting products were identified as kallikrein-like by electrophoresis and Southern blotting using the rat tissue kallikrein cDNA as probe (Gerald et al., 1986).

Cloning of the PCR Products. The confirmed PCR products were eluted from agarose gels and purified by phenol–chloroform extraction. The purified PCR products were then ligated into pUC19 vector at the *Hinc*II site by blunt-end ligation. *Escherichia coli* strain JM101 was transformed and grown on plates containing isopropyl β-D-thiogalactoside (IPTG) and X-gal. White colonies were reinoculated onto duplicated master plates for positive identification.

Screening Kallikrein-Related Clones. The colonies in the master plates were transferred to 3-MM filter paper (Whatman) followed by colony hybridization with a rat kallikrein cDNA probe at low stringency [6× NET (0.9 M NaCl, 6 mM EDTA, 90 mM Tris–HCl, pH 8.0), 45 °C overnight]. The filters were washed twice in 6× SSC (0.9 M NaCl, 90 mM sodium citrate) at room temperature and once at 45 °C and then exposed to Kodak X-ray films for autoradiography. Positive clones were subjected to another cycle of colony hybridization with oligonucleotide probes specific for tissue kallikrein rKlk1* (PS) and rKlk2* (S2) to eliminate the abundant cDNA clones with these known sequences. The hybridization was carried out in 6× NET at 55 °C, followed by washing in 1× SSC twice at room temperature and twice at 55 °C. Positive colonies encoding PS or S2 were identified and eliminated by this procedure.

DNA Sequencing. Oligonucleotide primers were synthesized using the ABI (Applied Biosystems) Model 380B DNA synthesizer. The clones which hybridized to the tissue kallikrein cDNA probe but not the PS- and S2-specific oligonucleotide probes were grown in 2 mL of LB medium. Plasmid DNA was extracted and sequenced using a double-stranded DNA sequencing system (BRL) according to the protocol recommended by the manufacturer. The sequence was verified by sequencing the complementary strand of the cDNA.

Animal Treatment with Isoproterenol. Sprague–Dawley male rats (250–300 g) were placed in three experimental groups and one control group. Three groups received subcutaneous injections of isoproterenol dissolved in 0.9% NaCl at 0.1, 1.0, and 10.0 mg/kg of body weight, respectively, twice a day. The control group received 0.9% NaCl with the same regimen. The rats were sacrificed in 7 days, and the submandibular glands were homogenized in GIT buffer (4 M guanidinium thiocyanate, 0.1 M Tris–HCl, pH 7.5, 1% β-mercaptoethanol) immediately for RNA extraction.

Northern Blot Analysis. Total RNA was resolved in 1.5% agarose gels containing 0.66 M formaldehyde at 100 V for 3 h. The gel was stained with ethidium bromide, and the result was estimated under UV light. The fractionated RNA was transferred by capillary action onto a nitrocellulose filter. The filter was baked at 80 °C for 2 h followed by prehybridization in 6× SSPE (20× SSPE: 3.6 M NaCl, 0.2 M $NaPO_4$, pH 7.4, 20 mM EDTA), 0.5% SDS, 5× Denhardt's solution [1× Denhardt's solution: 0.02% Ficoll, 0.02% poly(vinylpyrrolidone), 0.02% BSA], and 100 μg/mL herring sperm DNA at 60 °C for 2 h. The end-labeled oligonucleotide probe was added to the prehybridization solution and then incubated at 60 °C overnight. The filter was washed in 2× SSC (20× SSC: 3 M NaCl, 0.3 M Na_3 citrate, pH 7.0) twice at room temperature and in 0.5× SSC twice at 55 °C. The filter was exposed to Kodak X-Omat film with intensifying screen at −70 °C.

Southern Blot Analysis of PCR Products. For Southern blot analysis, one-tenth of the PCR products were directly loaded onto 1% agarose gel. After electrophoresis, the PCR products were denatured, neutralized, and transferred onto an Immobilon-N filter by capillary action in 10× SSC. The filter was subjected to hybridization with an end-labeled oligonucleotide probe. The filter was washed in 6× SSC twice at room temperature and twice at 65 °C and subjected to autoradiography.

Purification of T-Kininogenese and N-Terminal Amino Acid Sequence Analysis. T-Kininogenase was purified according to the previously described procedures with some modifications (Xiong et al., 1990). Briefly, about 15 g of rat submaxillary gland tissue was homogenized in 0.2 M sucrose, 1 mM EDTA, pH 7.0. The extract was dialyzed and separated on a DEAE-Sepharose CL-6B column equilibrated with 20 mM Tris–HCl, pH 8.0. The column was eluted with a 0–0.15 M NaCl and a 0.15–0.4 M NaCl linear gradient in 20 mM Tris–HCl, pH 8.0. The peak eluted by about 0.4 M NaCl with T-kinin-releasing activities was pooled and dialyzed in 20 mM sodium phosphate, pH 7.0. These pooled fractions were fractionated on a Mono Q HR 5/5 column, which was equilibrated in 20 mM sodium phosphate, pH 7.0. Fractions were eluted with a linear gradient of 0–0.4 M NaCl in 20 mM sodium phosphate, pH 7.0, in 30 min, at a flow rate of 1 mL/min. T-Kininogenase was eluted at about 0.18 M NaCl. After SDS–PAGE under reducing conditions, the protein was transferred to poly(vinylidene difluoride) membrane. The protein bands on the membrane were visualized by staining with 0.2% (w/v) Ponceau S in 3% trichloroacetic acid for 1 min and destained with 1 M acetic acid for 2 min. The individual bands were cut out and subjected to NH_2-terminal sequence analysis using a gas-phase protein sequencer equipped with an on-line narrow-bore PTH-amino acid analyzer (ABI Model 470A, Applied Biosystems Inc.).

RESULTS

Isolation of Kallikrein-Related cDNA Clones from Rat Submandibular Gland. A pair of PCR primers were designed based on the sequences of conserved regions within the known rat kallikrein cDNAs. The 5′ primer (TTGATGCTGCACCTCCTGG) corresponds to the sequence encoding signal peptide, and the 3′ primer (GCATGGTGGGTTTTATTGAG) is complementary to the region containing polyadenylation signal AATAAA. Reverse transcription and PCR were carried out as described in Experimental Procedures. A single species of PCR product with a length of about 700 bp was obtained which was recognized by a rat tissue kallikrein cDNA probe. It was isolated from agarose gel and ligated into pUC19 at the *Hin*cII site. After transformation of JM101 cells with the ligation material, 1000 white colonies were screened by colony hybridization, of which 84 colonies were recognized by rat tissue kallikrein cDNA. The 84 positive clones were subjected to hybridization with rKlk1 (PS) and rKlk2 (S2) specific oligoprimers which identified and eliminated 45 clones as PS and 5 as S2. The PS- and S2-specific oligonucleotide primers were synthesized according to the sequences of Ex3PS (CATATGAGGTCCTGGTTG) and Ex3S2 (GTCACGATGAGTGGGATA), whose specificities have been previously tested (Wines et al., 1989). The rest of the positive clones were sequenced. Sequencing analysis identified 11 independent clones from three independent reverse transcription and PCR reactions having identical sequences designated as rKlk10.

Nucleic Acid Sequence and Comparison with Other Members of Kallikrein Gene Family. Figure 1 shows the cDNA sequence and the translated amino acid sequence of rKlk10, which contains 737 bp, including sequences encoding the partial propeptide, a full-length mature protein of 235 amino acid residues, and a 3′ untranslated region. rKlk10 cDNA has a single six-nucleotide gap at positions 61–66 compared with rKlk1 mRNA. The derived protein of rKlk10 has the conserved key amino acid residues His57-Ser195-Asp102 (His39-Ser187-Asp94 in Figure 1) of the serine protease catalytic triad (boxed). The key residues contributing to substrate specificity (indicated by solid squares), such as aspartate-189 (Asp181 in Figure 1), glycine-216 and -226 (Gly204 and -215 in Figure 1), tyrosine-99 (Tyr91 in Figure 1), tryptophan-215 (Trp203 in Figure 1), and proline-219 (Pro207) are also retained. On the basis of the consensus sequences of glycosylation sites Asn-X-Ser/Thr and Ser/Thr-X-X-Pro (Marshall, 1974), rKlk10 protein has two potential N-linked glycosylation sites at Asn67 and Asn82 and two potential O-linked glycosylation sites of Thr117 and Ser133 (indicated by solid circles in Figure 1).

A search of the Genbank database with the FASTA program demonstrated that rKlk10 is distinct from all existing mRNAs and genes. Table I shows a comparison of rKlk10 with other kallikrein gene family members for sequence similarity which showed that rKlk10 shares 82–88% similarity at both nucleic acid and amino acid levels, suggesting that rKlk10 belongs to the kallikrein gene family.

Amino Acid Sequence Comparison with Other Kallikrein-Related Enzymes. The deduced amino acid sequence of rKlk10 was compared with those of tissue kallikrein (PS), tonin (rK2), S3 protein (S3), proteinase B, K10, and T-kininogenase (Figure 2). rKlk10 encodes a protein of 235 amino acid residues with an NH_2-terminal Ile. The derived protein has a single two amino acid deletion at positions 21 and 22, compared with PS. The translated amino acid sequence of rKlk10 matches completely with 61 residues of

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                                        AspAlaAlaProProGlyGlnSerArg
                                        TTGATGCTGCACCTCCTGGCCAGTCTCGG
                                              -20       -10       -1

IleValGlyGlyTyrLysCysGluLysAsnSerGlnProTrpGlnValAlaIleIleAsn
ATTGTTGGAGGATATAAATGTGAGAAGAATTCCCAACCCTGGCAAGTGGCTATCATCAAT
        10        20        30        40        50        60

GluTyrLeuCysGlyGlyValLeuIleAspProSerTrpValIleThrAlaAlaHisCys
GAATACCTATGCGGGGGTGTCCTGATAGACCCGAGCTGGGTGATCACGGCTGCCCACTGC
        70        80        90       100       110       120

TyrSerAsnTyrTyrHisValLeuLeuGlyArgAsnAsnLeuPheGluAspGluProPhe
TATAGCAACTATTACCATGTTTTGTTGGGTCGAAACAACCTATTTGAAGATGAACCCTTT
       130       140       150       160       170       180

AlaGlnTyrArgPheValAsnGlnSerProHisProAspTyrLysProPheLeuMet
GCTCAGTACCGGTTTGTCAACCAAAGCTTCCCTCACCCTGACTACAAGCCATTTCTCATG
       190       200       210       220       230       240

ArgAsnHisThrArgGlnArgGlyAspAspTyrSerAsnAspLeuMetLeuLeuHisLeu
AGGAACCACACCCGACAACGTGGAGACGACTACAGCAATGACCTGATGCTGCTCCACCTC
       250       260       270       280       290       300

SerGluProAlaAspIleThrAspGlyValLysValIleAspLeuProThrGluGluPro
AGCGAGCCTGCTGACATCACAGATGGGGTGAAGGTCATTGATCTGCCCACGGAGGAGCCC
       310       320       330       340       350       360

LysValGlySerThrCysLeuAlaSerGlyTrpGlySerThrLysProLeuAsnTrpGlu
AAGGTGGGGAGCACCTGCCTTGCCTCGGGCTGGGGCAGCACCAAACCCCTGAATTGGGAA
       370       380       390       400       410       420

LeuProAspAspLeuGlnCysValAsnIleHisLeuLeuSerAsnGluLysCysIleGlu
TTACCTGATGATCTCCAGTGTGTGAACATCCACCTACTGTCTAATGAGAAGTGCATCGAA
       430       440       450       460       470       480

AlaTyrGluGlnLysValThrAspLeuMetLeuCysAlaGlyGluMetAspGlyArgLys
GCCTACGAACAGAAGGTGACAGATCTCATGCTGTGTGCAGGAGAGATGGATGGACGCAAA
       490       500       510       520       530       540

AspThrCysLysGlyAspSerGlyGlyProLeuIleCysAspGlyValLeuGlnGlyIle
GACACTTGCAAGGGTGACTCAGGAGGCCCCCTCATCTGTGATGGTGTGCTTCAAGGCATC
       550       560       570       580       590       600

ThrSerTrpGlyAsnValProCysAlaGluProTyrAsnProGlyValTyrThrLysLeu
ACATCGTGGGGCAATGTCCCATGTGCTGAACCCTATAACCCAGGTGTCTACACTAAACTT
       610       620       630       640       650       660

IleLysPheThrSerTrpIleLysGluValMetLysGluAsnPro***
ATTAAGTTCACCTCCTGGATAAAAGAAGTTATGAAGGAAAACCCCTGAGTGTCACACTGT
       670       680       690       700       710       720

GCCCTGATCTCAATAAAACCCACCATGC 3'
       730       740
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FIGURE 1: Nucleic acid sequence and derived amino acid sequence of rKlk10. Nucleotide numbering starts at the first codon of the mature enzyme. The amino acid residues in the serine protease catalytic triad are boxed, and key residues determining substrate specificity are indicated by solid squares. The filled circles indicate potential glycosylation sites. The sequences of the PCR primers are underlined.

Table I: Sequence Similarity between rKlk10 and Members of the Rat Kallikrein Gene Family[a]

	nucleic acid level (%)	amino acid level (%)
PS	87	88
P1	87	82
S2	88	86
S3	87	82

[a] PS, rKlk1; P1, rKlk8; S2, rKlk2; S3, rKlk9.

T-kininogenase, which was recently purified and sequenced as described in Experimental Procedures. The derived amino acid sequence of rKlk10 also matches with 102 residues of the K10 protein (Gutman et al., 1991) and 67 residues of antigen-γ (Berg et al., 1991). It differs from the proteinase B sequence at residues 108 and 109 (Kato et al., 1987). The calculated molecular mass of the rKlk10 protein is 26 428 Da which is consistent with the deglycosylated molecular weight of the K10 protein (26 000 Da). Taken together, these results indicate that rKlk10 encodes the K10 protein.

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                1         10        20        30        40        50
PS              VVGGYNCEMNSQPWQVAVYYFGEYLCGGVLIDPSWVITAAHCATDNYQVL
S2              IVGGYKCEKNSQPWQVAVIN..EYLCGGVLIDPSWVITAAHCYSNNYQVL
S3              VVGGYNCETNSQPWQVAVI..GTTFCGGVLIDPSWVITAAHCYSKNYRVL
rK10            IVGGYKCEKNSQPWQVAIIN..EYLCGGVLIDPSWVITAAHCYSNYYHVL
TKGNASE         IVGGYKCEKNSQPWQVAIIN..EYL
K10             IVGGYKCEKNSQPWQVAIIN..EYL
Ag-gamma        IVGGYKxEKNSQPWQVAIIN..EYLxGGVLIDPSxVITAA
ProtB           IVGGYKCEKNSQPWQVAIIN..EYLCGGVLIDPSWVITAAHCYSNYYHVL

                51        60        70        80        90        100
PS              LGRNNLYEDEPFAQHRLVSQSFPHPGFNQDLIWNHTRQPGDDYSNDLMLL
S2              LGRNNLFKDEPFAQRRLVRQSFRHPDYIPLIVTNDTEQPVHDHSNDLMLL
S3              LGRNNLVKDEPFAQRRLVSQSFQHPDYIPVFMRNHTRQRAYDHNNDLMLL
rK10            LGRNNLFEDEPFAQYRFVNQSFPHPDYKPFLMRNHTRQRGDDYSNDLMLL
TKGNSE                                                  GDDYSNDLMLL
K10                                                   QRGDDYSNDLMLL
Ag-gamma                                                GDDYSNDLMLL
ProtB           LGRN                                    GDDYSNDLMLL

                101     110       120       130       140       150
PS              HLSQPADITDGVKVIDLPIEEPKVGSTCLASGWGSITPDGLELSDDLQCV
S2              HLSEPADITGGVKVIDLPTKEPKVGSTCLASGWGSTNPSEMVVSHDLQCV
S3              HLSKPADITGGVKVIDLPTEEPKVGSICLASGWGMTNPSEMKLSHDLQCV
rK10            HLSEPADITDGVKVIDLPTEEPKVGSTCLASGWGSTKPLNWELPDDLQCV
TKGNSE          HLSEPADITDGVKVIDLPTEEPKVGS
K10             HLSEPADITDGVKVIDL
Ag-gamma        HLSEPADITDGVKVIDLPTEE
ProtB           HLSEPADDSD

                151       160       170       180       190       200
PS              NIDLLSNEKCVEAHKEEVTDLMLCAGEMDGGKDTCKGDSGGPLICNGVLQ
S2              NIHLLSNEKCIETYKDNVTDVMLCAGEMEGGKDTCAGDSGGPLICDGVLQ
S3              NIHLLSNEKCIETYKNIETDVTLCAGEMDGGKDTCTGDSGGPLICDGVLQ
rK10            NIHLLSNEKCIEAYEQKVTDLMLCAGEMDGRKDTCKGDSGGPLICDGVLQ
TKGNSE
K10                                   LCAGEMDGRKDTCKGDSGGPLICDGVLQ
Ag-gamma
ProtB

                201       210       220       230     237
PS              GITSWGFNPCGEPKKPGIYTKLIKFTPWIKEVMKENP
S2              GITSGGATPCAKPKTPAIYAKLIKFTSWIKKVMKENP
S3              GLTSGGATPCAKPKTPAIYAKLIKFTSWIKKVMKENP
rK10            GITSWGNVPCAEPYNPGVYTKLIKFTSWIKEVMKENP
TKGNSE
K10             GITSxGNVPCAEPYNPGVYxKLIKF
Ag-gamma
ProtB
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FIGURE 2: Amino acid sequence comparison. The amino acid sequences of rK1 (PS), rK2 (S2), rK9 (S3), and rK10 and the partial sequences of T-kininogenase (TKGNSE), K10, antigen-γ (Ag-γ), and proteinase B (ProtB) are aligned. The first amino acid in the mature protein is taken as 1. The gap is introduced for the best alignment. Boxes indicate residue differences between rK10 and proteinase B.

Regulation of *rKlk10 Expression by Isoproterenol.* We designed an rKlk10-specific oligonucleotide primer, based on the most divergent region of the cDNA sequence, that could distinguish it from other known kallikrein-like genes. Primer rK10sp (ACCTTCTGTTCGTAGGCTTCGATGC), complementary to the mRNA sequence of rKlk10, was end-labeled with ^{32}P and used as a probe for Northern blot analysis. Its specificity to rKlk10 cDNA was tested. Under the same conditions for Northern blot analysis, this oligonucleotide probe, rK10sp, only hybridized to rKlk10 cDNA but not to seven other cDNAs (rKlk1, rKlk2, rKlk9, rKlk6, rKlk7, rKlk3, rKlk8) in the rat kallikrein gene family (Figure 3, upper panel).

Northern blot analysis of the regulation of rKlk10 expression by isoproterenol with the K10-specific probe is shown in Figure 3, lower panel. Each lane contains 15 μg of total RNA from the submandibular glands of the rats treated with 0.1, 1.0, and 10.0 mg of isoproterenol/kg, respectively. The rKlk10 mRNA levels in the submandibular gland were decreased by the isoproterenol treatment in a dose-dependent manner as compared with that of the control rats. As measured by densitometry, rKlk10 mRNA levels in the submandibular gland of the control rats are 22-fold higher than that in the rats treated with 10 mg/kg isoproterenol.

Tissue-Specific Expression. In order to determine the tissue distribution of rKlk10 mRNA, we performed Northern blot analysis and PCR amplification followed by Southern blot analysis using the probe rK10sp. In Northern blot analysis, 100 μg of total RNA from rat kidney, prostate, liver, heart, adrenal gland, testes, pituitary, and pancreas and 15 μg of total RNA from rat submandibular gland were hybridized with the rK10sp probe after agarose gel electrophoresis (Figure 4A). A high level of rKlk10 mRNA was found in the submandibular gland, while no detectable rKlk10 mRNA was found in 100 μg of RNA from the other eight tissues analyzed. The result indicated that rKlk10 is expressed at high levels in the rat submandibular gland.

To increase sensitivity of the analysis, we have used a PCR method to identify the tissues expressing the rKlk10 gene.

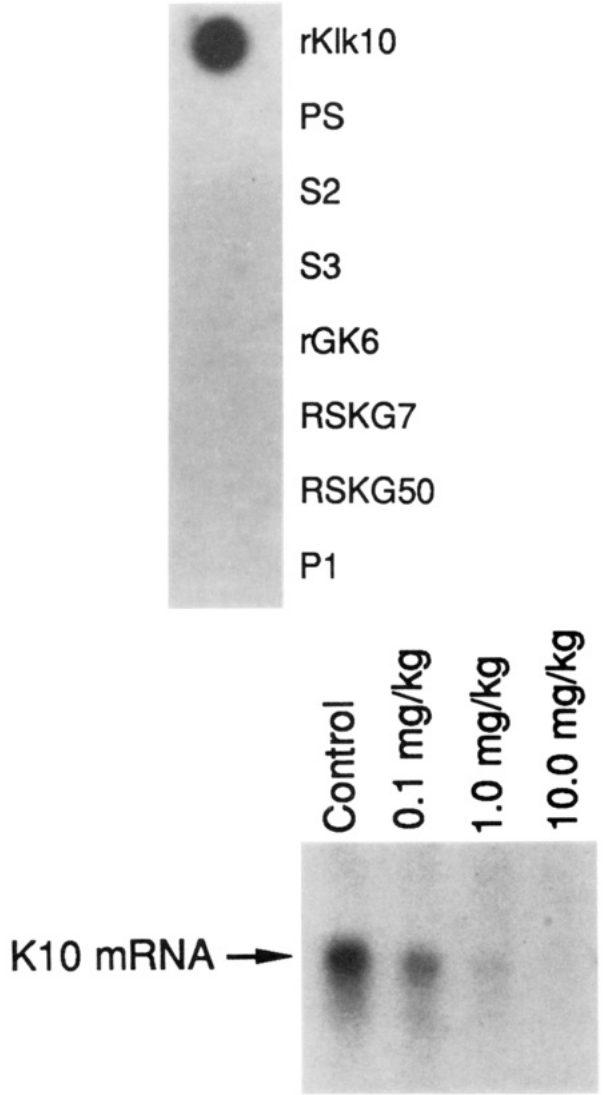

FIGURE 3: Effect of isoproterenol on rKlk10 mRNA level in rat submandibular gland. (Upper panel) Dot blot analysis to test the specificity of the rKlk10-specific oligonucleotide probe, rK10sp. Each dot contains 1 μg of cDNA of rKlk10, PS (rKlk1), S2 (rKlk2), S3 (rKlk9), rGK6 (rKlk6), RSKG7 (rKlk7), RSKG50 (rKlk3), and P1 (rKlk8), respectively. (Lower panel) Northern blot analysis with rK10sp probe. Each line contains 15 μg of submandibular RNA from the control group and from the rats treated with 0.1, 1.0, and 10.0 mg of isopropterenol/kg of body weight, respectively.

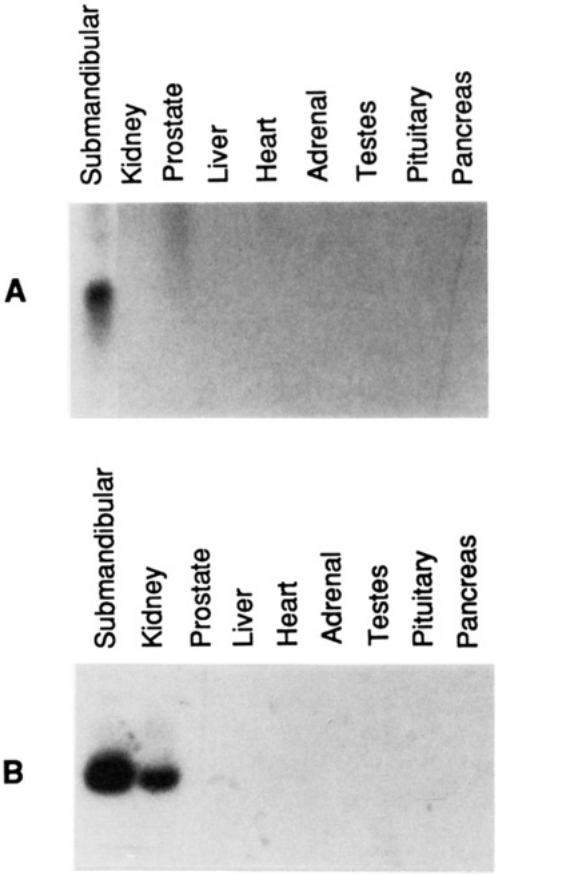

FIGURE 4: Tissue-specific expression of rKlk10 mRNA. (A) Northern blot analysis. Each line contains 15 μg of total RNA from rat submandibular gland or 100 μg of total RNA from rat kidney, prostate, liver, heart, adrenal gland, testes, pituitary gland, and pancreas. (B) Southern blot analysis of PCR products of total RNA from nine tissues. rK10sp was used as the probe in both Northern blot and Southern blot analyses.

Both the 5′ primer (TATAGCAACTATTACCAT) and 3′ primer (AGTGTAGACACCTGGGTTATAG) were rKlk10-specific. Ten micrograms of total RNA from rat submandibular gland, kidney, prostate, liver, heart, adrenal gland, testes, pituitary, and pancreas was reverse transcribed, followed by 30 cycles of PCR amplification. One-tenth of the PCR products was subjected to Southern blot analysis using rK10sp as the probe. A 500-bp fragment of rKlk10 cDNA was identified in the PCR product of the kidney in addition to the submandibular gland (Figure 4B). The rKlk10 mRNA level in the kidney is lower than that in the submandibular gland. In contrast, no rKlk10 mRNA was detected by this method in seven other tissues.

Isolation and Sequence Analysis of rKlk10 mRNA in Rat Kidney. Even though both PCR primers in the tissue distribution study were rKlk10-specific and the specificity of the rK10sp probe was tested against the known members in the kallikrein gene family, there is a possibility that the PCR product was generated from an unknown mRNA other than rKlk10. To confirm the authenticity of rKlk10 expression in rat kidney, the PCR product from rat kidney was cloned into the pUC19 vector and sequenced. The cloned PCR product of 531 bp (corresponding to 121–654 of nucleotide numbering in Figure 1) from the kidney showed a sequence identical to rKlk10 cDNA cloned from the submandibular gland, as shown in Figure 1, demonstrating that rKlk10 is specifically expressed in the kidney in addition to the submandibular gland.

DISCUSSION

In the present studies, we have cloned and sequenced rKlk10 mRNA from rat submandibular gland and kidney using a PCR-based cloning strategy. It contains 737 nucleotides and codes for a mature protein of 235 amino acid residues and partial propeptide. The translated NH_2-terminal amino acid in the rK10 enzyme is Ile, which is identical to tonin but different from true tissue kallikrein (Val). It is evident that this mRNA belongs to the kallikrein gene family since it shares 82–88% identity with other members of the gene family at both nucleic acid and amino acid levels. Sequence comparison indicated that rKlk10 encodes T-kininogenase (Xiong et al., 1990), K10 (Gutman et al., 1991), or antigen-γ (Berg et al., 1991) purified from rat submandibular gland. Northern blot and Southern blot analyses of PCR-amplified rKlk10 cDNA using an rKlk10-specific oligonucleotide probe showed that the rKlk10 gene is expressed at a high level in the submandibular gland and a low level in the kidney.

The catalytic triad characteristic of serine protease and the key amino acid residues accounting for the tissue kallikrein

substrate specificity are conserved in the protein encoded by rKlk10. The enzymes in the kallikrein family have similar primary and tertiary structures (Bode et al., 1983; Wines et al. 1991). In spite of the structural similarity, these enzymes show very divergent substrate specificity at both primary and extended residues on both sides of the scissile bond (Chretien et al., 1980; Fiedler & Leysath, 1979; Seidah et al., 1979; Wang et al., 1992; Chagas et al., 1992). Some residues in these enzymes are known to be critical for the cleavage preference. Data from X-ray diffraction studies have indicated that Tyr99 and Trp215 form a hydrophobic sandwich which determines the P2 specificity toward a bulky hydrophobic side chain (Bode et al., 1983; Chen & Bode, 1983). In tonin, these two residues are substituted by His99 and Gly215, which might account for its distinct cleavage specificity (Wang et al., 1992). Asp189 is located at the bottom of the substrate-binding pocket and is responsible for binding with the P1 side chain of a substrate. Gly216 and Gly226 are located in the opening of the pocket that is involved in distinguishing Lys from Arg side chains (Craik et al., 1985). The insertion of Pro219 enlarges the substrate-binding pocket in kallikrein as compared with that of trypsin. In protein rK10, all these residues, Tyr99 (Tyr91 in Figure 1), Trp215 (Trp203 in Figure 1), Asp189 (Asp181 in Figure 1), Gly216 (Gly204 in Figure 1), Gly226 (Gly215 in Figure 1), and Pro219 (Pro207 in Figure 1) (chymotrypsin numbering system), are conserved. The deletion of tissue kallikrein Phe36 (F21 in Figure 2) and Gly38 (G22 in Figure 2) in the rK10 protein, however, may cause a P3 affinity change since fragment 34–38 is thought to contribute to P3 specificity in kallikrein (Chen & Bode, 1983). Gutman et al. (1991) demonstrated that the K10 protein has substrate specificity different from that of true tissue kallikrein. K10 can accommodate polar or nonpolar residues such as Val, Ser, and Phe at the P2 position, which is similar to tonin. Recent studies by Chagas et al. (1992) showed that the enzyme subsites S2–S3′ are important determinants for the substrate specificity of tissue kallikrein and T-kininogenase.

T-Kininogenase, K10, antigen-γ and proteinase B were purified from rat submandibular gland in four laboratories independently and they all showed similar activities and NH_2-terminal sequences (Kato et al., 1987; Xiong et al., 1990; Gutman et al., 1991; Berg et al., 1991). As shown in Figure 2, the amino acid sequence deduced from rKlk10 mRNA matches with the known 102 amino acid residues of the K10 protein, 67 residues of antigen-γ, and 61 residues of the amino acid sequence of T-kininogenase. Furthermore, the rK10 protein showed the same molecular mass as deglycosylated K10 protein. However, the calculated pI value of rK10 protein is 4.81 while the K10 protein showed pI = 4.4, a difference which may be attributed to glycosylation. These results indicate that rKlk10 is the cDNA encoding protein K10, T-kininogenase, and antigen-γ. T-Kininogenase reported previously by Xiong et al. (1990) showed two additional amino acid residues at positions 21 and 22, which was confirmed by an X-ray diffraction study (unpublished results). Recently, we have purified and sequenced T-kininogenase from the rat submandibular gland again and its N-terminal amino acid sequence matches completely with the amino acid sequence derived from rKlk10 mRNA. It is possible that the difference in the amino acid sequence of T-kininogenase represents genetic polymorphisms of rats or sequencing errors. The amino acid sequences of rK10 and proteinase B are identical except for residues 108 and 109. It cannot be concluded whether proteinase B is encoded by the rKlk10 gene or by another gene with a similar sequence.

Most genes in the kallikrein gene family are expressed in a tissue-specific manner. In the rat tissue kallikrein gene family, most genes are expressed in the salivary gland while only PS (rKlk1), S3 (rKlk9), and RSKG7 (rKlk7) have been previously demonstrated to be expressed in the kidney (Chen et al., 1988; Saed et al., 1992). Our results demonstrate that the rKlk10 gene is expressed at a high level in the submandibular gland and at a relatively low level in the kidney. Southern blot analysis of PCR products did not detect rK10 gene expression in prostate, liver, pancreas, heart, testes, adrenal, and pituitary gland. Kallikrein-related enzymes in the kidney are thought to play a role in the regulation of renal blood flow, water, and sodium excretion. They may be also involved in blood pressure regulation (Margolius, 1984, 1989; Carretero & Scicli, 1989). The cellular origin and physiological significance of rK10 in renal function remain to be studied.

The expression of the rat kallikrein genes is regulated by multiple factors including hormones, salt intake, and parasympathetic and sympathetic stimulation [see reviews, Murray et al. (1990) and Clements (1989)]. Several hormonal responsive elements and cAMP-binding sites were found in the 5′ regions of the tissue kallikrein gene (Murray et al., 1990). Salivary gland kallikrein level is known to be under β-adrenergic control since the administration of the β agonist isoproterenol dramatically decreased tissue kallikrein levels in rat submandibular gland and saliva. It was postulated that the effect of isoproterenol is due to decreased protein synthesis or secretion (Bedi, 1991). Northern blot analysis using a K10-specific oligonucleotide probe shows that rKlk10 mRNA levels in rat submandibular gland are decreased by administration of isoproterenol in a dose-dependent manner (Figure 3, lower panel). The result indicates that rKlk10 gene expression is negatively regulated by β-adrenergic stimulation and that the regulation is at the transcriptional level. The effects of β-adrenergic stimulation are known to be mediated by increased levels of cAMP (Bhalla et al., 1972) which may bind to cAMP-binding sites in the 5′ flanking region of the tissue kallikrein gene (Murray et al., 1990). The cAMP-binding site identified in the kallikrein gene may serve as a negative regulatory element of transcription. The mechanism of the regulation of rKlk10 expression remains to be further investigated.

During the screening of kallikrein-related cDNA clones, we found that PS (rKlk1) cDNA clones were about 10-fold as frequent as other kallikrein-like cDNAs such as S2 (rKlk2) and S3 (rKlk9) in the rat submandibular gland, which was correlated with the finding that PS is the most abundant mRNA in the salivary gland (Clements et al., 1990). rKlk10 mRNA showed a frequency similar to S2 and S3 cDNA. This frequency difference may reflect differences in expression levels of individual genes in the rat submandibular gland. On the basis of the fact that most kallikrein-like enzymes are expressed in the rat submandibular gland, we used mRNA from the submandibular gland as the template to isolate novel kallikrein-like cDNA species. Kallikrein genes share a high sequence similarity, and they contain several highly conserved regions where the sequences are identical among most kallikrein genes. These highly conserved regions make it possible to design a pair of PCR primers to amplify many potential cDNAs in the kallikrein gene family and can thereby be used to identify new members of the gene family.

Reverse transcription and PCR (RT-PCR) have been widely used in cloning cDNAs and detecting gene expression (Haribabu & Dottin, 1991; Hess et al., 1992; Konig et al.,

1992). Reverse transcriptase and Taq DNA polymerase lack proof-reading activity and could potentially introduce mismatches. In our study, however, we found this kind of error to happen rarely if at all. We have sequenced a great number of clones with known sequences, such as S3 (rKlk9), RSKG7 (rKlk7), P1 (rKlk8), S2 (rKlk2), and PS (rKlk1) and no mismatch has been found. Although the 11 rKlk10 cDNA clones were from three independent reverse transcription and PCR reactions, they all showed identical sequences without any errors, proving that PCR-based cloning and sequencing are reliable. The PCR-based cloning method thus provided a useful technique for cloning kallikrein-like genes because it is more efficient than the conventional screening due to the high magnitude of amplification by PCR. In the analysis of kallikrein-like clones, we found that almost all known kallikrein-like cDNAs were amplified by PCR, including PS (rKlk1), S2 (rKlk2), S3 (rKlk9), RSKG7 (rKlk7), P1 (rKlk8), and RSKG50 (rKlk3) (Shai et al., 1989) (data not shown). Therefore, this approach has the potential to clone all other kallikrein-like genes expressed in the rat salivary gland and is also suitable for cloning other genes belonging to multiple gene families.

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