Submandibular Enzymatic Vasoconstrictor Messenger RNA in Rat Kidney

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Recently, we reported the isolation and identification of a potent vasoconstrictor enzyme from the rat submandibular gland, a member of the rat kallikrein gene family, which we named submandibular enzymatic vasoconstrictor (SEV). We studied whether messenger RNA (mRNA) for SEV is present in the kidney and isolated glomeruli, using the polymerase chain reaction assay with primers specific to the entire rat kallikrein family that would amplify a 430-bp fragment from their mRNA. As a probe we used a phosphorus-32–labeled oligonucleotide specific for SEV mRNA. A fragment of the predicted size was obtained on Southern blot for amplified renal RNA; however, no signal was obtained with glomerular RNA. To further confirm the presence of SEV mRNA in the kidney, polymerase chain reaction was repeated using primers specific to SEV mRNA that would amplify a 372-bp fragment from SEV mRNA alone. Again, a fragment of the predicted size was obtained on Southern blot after amplification of renal RNA but not RNA from the glomeruli. Southern blot of polymerase chain reaction–amplified RNA with primers specific to SEV mRNA that amplified a 372-bp fragment from SEV mRNA alone revealed a 430-bp fragment for both renal and glomerular RNA, indicating that glomeruli contain mRNA for a member or members of the kallikrein family other than SEV. When the Southern blots were hybridized with a 32P-labeled oligonucleotide probe specific for glandular kallikrein, a fragment of the predicted size was obtained from amplified renal RNA but not glomerular RNA. The presence of mRNA for SEV suggests that it is synthesized by the kidney. Thus, at least two members of the kallikrein family of serine proteases, glandular kallikrein and SEV, are produced by the kidney. A third member of the kallikrein family may be present in glomerular cells. Besides glandular kallikrein, SEV and the glomerular enzyme may play a role in the regulation of renal function. (Hypertension 1992;19[suppl II]:II-262–II-267)
and prostate were rapidly removed and cleaned of fat. All tissues were immediately frozen and kept in liquid nitrogen until needed. Glomeruli were isolated by a passive sieving technique.\textsuperscript{11}

**Extraction of RNA**

Total RNA was isolated from various tissues and sieved glomeruli by the cesium trifluoroacetate method.\textsuperscript{12} Briefly, frozen tissues were homogenized to a fine powder with a mortar and pestle under liquid nitrogen. Aliquots of tissue (1 g) were mixed with 18 ml of 5.5 M guanidinium thiocyanate containing 25 mM sodium citrate (pH 7.0) with 0.5% sarcosine and 0.1 M 2-mercaptoethanol and were homogenized for 60 seconds with a polytron (Brinkmann Instruments, Inc., Westbury, N.Y.). Sieved glomeruli were sonicated in the presence of the guanidinium buffer. The homogenate was centrifuged at 1,500g for 5 minutes to pellet the insoluble material, after which DNA was sheared by passing the lysate through a 16–18-gauge needle. The lysate was then layered over a cesium trifluoroacetate gradient cushion (Pharmacia LKB Biotechnology, Piscataway, N.J.) and centrifuged in a swinging-bucket rotor for 24 hours at 15°C. Under these conditions, RNA formed a pellet at the bottom of the tube, and DNA was collected in a band in the lower third of the gradient. The RNA pellet was dissolved in RNase-free water and its concentration determined by measurement of optical density at 260 nm (1 OD at 260 nm is equivalent to 30 \(\mu\)g/ml).\textsuperscript{14}

**Probes**

**Kallikrein family complementary DNA probe.** The recombinant plasmid pXP39, bearing a rat pancreatic kallikrein complementary DNA (cDNA) insert, was prepared as described previously.\textsuperscript{13} The probe sequences contained the 3' 550-bp region of rat pancreatic kallikrein mRNA, which encodes 167 amino acids at the carboxy terminal of kallikrein plus the untranslated region. For the hybridization studies, the plasmid was digested with HindIII (Boehringer Mannheim, Indianapolis, Ind.) and subjected to electrophoresis on a low melting point agarose gel, and the insert band was extracted. The kallikrein insert was radiolabeled with phosphorus-32 using the random primer method.\textsuperscript{14}

**Glandular kallikrein probe.** Ex2PSkal\textsubscript{21} (5'-TCATCATTGGTACAACTTTC-3'), represents nucleotide positions 157–177 within the second exon of the mature S3 mRNA.\textsuperscript{6} This region is highly divergent among the various members of the kallikrein gene family. The probe only recognizes regions present in the true kallikrein gene; therefore, it is specific for glandular kallikrein\textsuperscript{15,16}

**Submandibular enzymatic vasoconstrictor-specific probes.** Ex2S3\textsubscript{21} (5'-GCTGTCATTGGTACAACTTTC-3'), represents nucleotide positions 157–177 within the second exon of the mature S3 (SEV) mRNA sequence.\textsuperscript{6} Ex3bS3\textsubscript{18} (5'-GACAACGTGGTATGACC-3') represents nucleotide positions 362–379 within the third exon of the mature S3 (SEV) mRNA sequence.\textsuperscript{6} These regions are highly divergent among the various members of the kallikrein gene family. These probes will recognize only SEV and are therefore specific for SEV.\textsuperscript{15,16}

**Polymerase Chain Reaction Amplification of Submandibular Enzymatic Vasoconstrictor Messenger RNA**

**Design of polymerase chain reaction primers.** 1) Kallikrein gene family primers: a) downstream primer, designated as KALPCRU\textsubscript{4} (5'-GATGTTCAACACTGAGATC-3'), is complementary to nucleotide positions 550–570 within the fourth exon of mature rat pancreatic kallikrein mRNA; b) upstream primer, designated as KALPCRU\textsubscript{2} (5'-GAAATCCCCAACCCTGGGAGT-3'), represents nucleotide positions 141–161 within the second exon of mature rat pancreatic kallikrein mRNA.\textsuperscript{6} These regions are highly conserved among the members of the kallikrein gene family. Therefore, use of these primers will result in amplification of all the kallikrein member mRNA present in the individual tissue RNA.\textsuperscript{15,16}

2) SEV-specific primers: a) downstream primer, designated as Ex3/4S3\textsubscript{21} (5'-TTCATCTCAGAGGGGTTTGTC-3'), is complementary to nucleotide positions 507–527 within exons 3 and 4 of the reported S3 mRNA sequence; b) upstream primer, designated as Ex2S3\textsubscript{21} (5'-GCTGTCATTGGTACACTTTC-3'), represents nucleotide positions 157–177 within the second exon of S3 mRNA.\textsuperscript{6} These regions are highly divergent among kallikrein family members. These primers will recognize regions only present in SEV, and therefore will specifically amplify SEV mRNA.\textsuperscript{15,16} Amplification of kallikrein family mRNA using the kallikrein gene family primers should give a 430-bp fragment; the S3-specific primers should give a 372-bp fragment.

**Amplification Method**

cDNA was synthesized using cloned Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) according to the manufacturer's protocol. The 20-\(\mu\)l reaction mixture contained the enzyme buffer supplied by Bethesda Research, together with various RNA samples pretreated with RNase-free DNase (RQ1, Promega), 1 unit RNasin (Promega Biotech, Madison, Wis.) per microliter, 10 pmol downstream polymerase chain reaction (PCR) primer, 1 mM each (deoxynucleoside triphosphate, and 200 units reverse transcriptase. The reaction mixture was incubated for 45 minutes at 42°C, heated at 95°C for 5 minutes to kill the enzyme, and chilled on ice. It was then diluted with 80 \(\mu\)l PCR reaction buffer (50 mM KCl/10 mM Tris chloride/1.5 mM MgCl\textsubscript{2}/100 \(\mu\)g gelatin/ml, pH 8.3), followed by addition of 10 pmol of the upstream primer and 1 unit of thermostable DNA polymerase from *Thermus aquaticus* (Taq polymerase). Mineral oil (100 \(\mu\)l) was added to prevent evaporation. Control samples were processed exactly as above, except that reverse transcriptase was not included in the...
reaction mixture. The reaction was initiated by heat denaturation of RNA-cDNA hybrid (95°C) for 1 minute, annealing the primers for 2 minutes at 55°C, and then extending them for 3 minutes at 72°C. The cycle was repeated 30 times using a programmable PCR (Perkin-Elmer Cetus, Norwalk, Conn.). After the final cycle, the temperature was maintained at 72°C for 10 minutes to allow reannealing of the amplified products, which were then chilled. In all experiments, we used RNA isolated from the prostate and submandibular gland as positive controls; RNA isolated from the liver was used as a negative control, because the mRNAs for SEV and kallikrein are known to be present in the submandibular gland, and SEV mRNA is known to be present in the prostate.15,17 Kallikrein genes are not expressed in the liver.16,18

Analysis of Polymerase Chain Reaction–Amplified Products by Southern Blot

Southern blotting was performed as described previously.19 PCR-amplified fractions from the kidney (20 µl), glomeruli (50 µl), prostate (5 µl), submandibular gland (5 µl), and liver (50 µl) were applied to a 1.5% agarose gel, and electrophoresis was performed at 100 V for 4 hours. The gel was briefly rinsed in deionized water, soaked in 1.0 M NaCl/0.5 M NaOH for 2×15 minutes with shaking, and then neutralized by soaking in 0.5 M Tris (pH 7.4)/1.5 M NaCl for 2×15 minutes. The gel was transferred to a Nytran membrane by capillary blotting, after which the membrane was baked at 80°C for 2 hours, UV cross-linked for 2 minutes, and prehybridized at 42°C for 2 hours in hybridization solution (50% formamide, 5× SSC [1× SSC is 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.4], 50 mM sodium phosphate [pH 7.0], 0.1% sodium dodecyl sulfate, 0.1% sodium pyrophosphate, 5× Denhardt’s solution [0.2% bovine serum albumin, Ficoll, and polyvinyl pyrrolidone], and 100 µg/ml denatured herring sperm DNA) before hybridization at 55°C for 24 hours with the kallikrein cDNA probe. For hybridization with the oligonucleotide probes, the membrane was prehybridized for 2 hours at 42°C in the same solution used for hybridization of the cDNA probe (except that formamide was eliminated) and then hybridized at 37°C for 24–36 hours with the oligonucleotide probe end-labeled with 32P by the terminal transferase method.20 The blots were washed four to five times for 15 minutes each in 0.1× SSC and 0.1% sodium dodecyl sulfate at 65°C for the cDNA probe and 37°C for the oligonucleotide probes. The filters were air-dried and then exposed to Kodak X-AR film (Eastman Kodak Co., Rochester, N.Y.) with a Cornex Lighting Plus intensifying screen (Du Pont Co., Wilmington, Del.) at −70°C. Magnitude of the signals was compared with an ultrascan II laser densitometer (Pharmacia LKB).

Results

Preliminary attempts to detect SEV mRNA in the kidney using Northern blots were negative, suggesting that SEV mRNA was either nonabundant or absent. Glandular kallikrein was readily detectable in the kidney by Northern blot (data not shown). We used the PCR assay to determine whether mRNA for SEV is present in total RNA isolated from the kidney and glomeruli. Southern blots of PCR-amplified samples from the kidney, prostate, and submandibular gland were obtained using the kallikrein gene family primers and were hybridized with the SEV-specific probe Ex2S318. They revealed an amplified 430-bp fragment, which is the expected size. No amplification was observed with mRNA from either liver or isolated sieved glomeruli (Figure 1).

To further confirm the presence of SEV mRNA in the kidney, samples were PCR-amplified using two different SEV mRNA-specific primers. If SEV mRNA was present, we should see a 372-bp fragment. Southern blots of PCR-amplified samples from the kidney, prostate, and submandibular gland hybridized to the SEV-specific probe Ex3bS318 revealed an amplified 372-bp fragment, whereas no hybridization was observed with RNA from either liver or glomeruli (Figure 2). In different experiments, kidney and glomerular RNA were amplified using the kallikrein gene family primers. When Southern blots of the PCR-amplified RNAs were hybridized using a 32P-labeled kallikrein cDNA probe that recognizes the entire kallikrein gene family, a hybridization signal of 430 bp was obtained for both kidney and glomeruli (Figure 3). To ascertain whether the member of the kallikrein gene family found in the glomeruli was glandular kallikrein, the Southern blot filter of these PCR-amplified samples was hybridized with

Figure 1. Hybridization of submandibular enzymatic vasoconstrictor (SEV)–specific probe Ex2S318 to a Southern blot of products obtained by polymerase chain reaction (PCR) amplification using the kallikrein gene family primers from 1 µg of each tissue RNA (+) and their controls (−), that is, without reverse transcriptase added to the reaction mixture. Size of the PCR-amplified fragments was estimated to be 430 bp using a DNA ladder (PUC8 plasmid cut with restriction enzyme Ded I). An identical hybridization pattern was obtained when the Southern blot filter was rehybridized with the S3-specific probe Ex3bS318. SMG, submandibular gland.
glandular kallikrein-specific probe Ex2PSkal18 (Figure 4). Although a hybridization signal was readily obtained with total renal RNA, no signal was obtained with glomerular RNA.

**Discussion**

Using the Northern blot technique, Ashley and MacDonald\(^9\) have reported the presence of SEV mRNA in the rat submandibular gland and prostate but not elsewhere. In this study, we used the PCR assay, an extremely sensitive technique compared with the Northern blot, to search for SEV mRNA in the kidney and isolated glomeruli. We demonstrated SEV mRNA in the kidney; however, neither SEV nor glandular kallikrein mRNA was found in the glomeruli. This was not due to degradation of mRNA, because Southern blots of PCR-amplified glomerular RNA revealed a 430-bp band when hybridized with the kallikrein cDNA probe that recognizes all kallikrein family mRNA. These results indicate that a member or members other than glandular kallikrein or SEV are probably present in the glomeruli, thereby agreeing with data reported by Chao's group\(^10\) using in situ hybridization in the rat kidney. We do not know where in the glomeruli the unknown kallikrein member is located, or its corresponding enzyme. It has been reported that mRNA for kallikrein genes other than glandular kallikrein is found in the rat kidney.\(^{15,21}\) If the unknown kallikrein member releases vasoactive peptides from accessible substrates, it may modify glomerular function by influencing either vascular resistance or mesangial contractility. At least two members of the kallikrein family of proteases release vasoactive peptides: kallikrein releases kinin, and tonin releases angiotensin II. As demonstrated by the properties of SEV, some of these enzymes also have direct actions. Identification and characterization of the glomerular protein encoded by the unknown mRNA may be needed to understand its possible functions.

The number of kallikrein genes in the rat is between 11 and 20.\(^{16}\) A number of them have been sequenced.\(^{6,9}\) As far as we can determine, the oligonucleotides we used are specific, as they do not recognize other known kallikrein genes. Because the sequences of some members of the kallikrein gene family are still not completely identified, we cannot overlook the possibility that oligonucleotide probes also recognize some unknown members of the family. However, the fact that there was no difference in the results obtained with two different SEV oligonucle-
otide probes (Figure 1) or with three oligonucleotides (Figure 2) suggests this is highly unlikely.

The presence of mRNA for SEV but not glandular kallikrein in the prostate indicates that SEV and glandular kallikrein have a different tissue expression pattern. We needed PCR to reveal the presence of SEV mRNA. Thus, only a small proportion of the total kidney RNA is SEV mRNA. Low concentration of a particular mRNA in RNA extracted from a whole organ may be due to 1) low expression of the gene, 2) expression in a discrete cell population that is diluted by RNA from other cells, or 3) high mRNA turnover. The PCR technique in the manner performed here is not quantitative; however, the different probes had similar specific activity. Thus, differences in the signals may reflect differences in the mRNA concentration. Using a densitometer to compare the density and size of the autoradiographic signals, we found that SEV mRNA was approximately 10% of the glandular kallikrein mRNA.

The fact that SEV mRNA is present in the kidney but not the glomeruli indicates that SEV is located in extraglomerular structures. The present findings allow us to predict that SEV will be found in the kidney. They establish a base from which further studies on the possible role of SEV and glomerular kallikrein members in renal function can proceed. To understand the possible role of SEV better, it will be necessary to determine the renal cell type that synthesizes it. Kallikrein is released into the lumen of the distal nephron, and a number of studies have identified the connecting cells as the cells that contain kallikrein. Kallikrein activity with different characteristics from that of true kallikrein was shown in the basolateral membrane–enriched kidney fractions. In addition, it has been reported that immunoreactive material is found in the basolateral membrane. These studies were made with polyclonal antibodies raised against urinary kallikrein. It is unlikely that polyclonal antibodies differentiate between proteases with more than 80% homology in their amino acid sequences, such as most members of the kallikrein family. We found that antibodies against kallikrein precipitate SEV, and, therefore, studies with polyclonal antikallikrein would be unlikely to discriminate between kallikrein and SEV. Thus, it is possible that connecting cells synthesize both kallikrein and SEV. Immunohistochemical studies have also suggested the presence of a member or members of the kallikrein family other than glandular kallikrein in the kidney.

We do not have information about the effects of SEV in the kidney, but on release, it might contribute to regulation of renal function. SEV induces contractions of isolated vascular tissue without need of previous incubation with a plasma-derived substrate. Thus, SEV may represent a novel mechanism whereby a proteolytic enzyme can influence function within or near the cells that produce it by interacting directly with a putative substrate that is probably present on the target cell ectomembrane.

In summary, we found that the mRNA for a vasoconstrictor protease named SEV is present in the kidney, suggesting SEV synthesis. The mRNA for SEV was not found in the glomeruli, indicating synthesis by extraglomerular structures. A member of the kallikrein family of proteases other than SEV or glandular kallikrein is present in the glomeruli. The precise role of renal SEV or the glomerular kallikrein-like enzyme remains to be determined.

References


Key Words • polymerase chain reaction • kallikrein • kidney • submandibular gland • messenger RNA
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