

## LEKTI, a Novel 15-Domain Type of Human Serine Proteinase Inhibitor\*

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**Proteinase inhibitors are important negative regulators of proteinase action *in vivo*. We have succeeded in isolating two previously unknown polypeptides (HF6478 and HF7665) from human blood filtrate that are parts of a larger precursor protein containing two typical Kazal-type serine proteinase inhibitor motifs. The entire precursor protein, as deduced from the nucleotide sequence of the cloned cDNA, exhibits 15 potential inhibitory domains, including the Kazal-type domains, HF6478, HF7665, and 11 additional similar domains. An inhibitory effect of HF7665 on trypsin activity is demonstrated. Because all of the 13 HF6478- and HF7665-related domains share partial homology to the typical Kazal-type domain but lack one of the three conserved disulfide bonds, they may represent a novel type of serine proteinase inhibitor. The gene encoding the multi-domain proteinase inhibitor, which we have termed LEKTI, was localized on human chromosome 5q31-32. As shown by reverse transcriptase-polymerase chain reaction and Northern blot analysis, it is expressed in the thymus, vaginal epithelium, Bartholin's glands, oral mucosa, tonsils, and the parathyroid glands. From these results, we assume that LEKTI may play a role in anti-inflammatory and/or antimicrobial protection of mucous epithelia.**

Proteinases are enzymes required for nonspecific processes of digestion and intracellular protein turnover as well as specific proteolytic activation of inactive precursors of many regulatory proteins, such as enzymes and peptide hormones. In addition, they are involved in several processes of extracellular

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ228139. Scanning of this sequence against the data base resulted in identification of a related sequence with the accession number AF086524.

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matrix remodeling. Depending on the nature of their reactive center, they are subdivided into the classes of serine, cysteine, aspartate, and metalloproteinases (for review see Ref. 1). To control the action of proteinases *in vivo*, organisms produce another group of proteins, namely the proteinase inhibitors (for review see Refs. 2–4). Indeed, many pathological effects are due to the non-regulated action of endogenously produced proteinases or such proteinases encoded or synthesized by viruses, bacteria, and parasites (for review see Ref. 5). For instance, a genetically determined fault of the  $\alpha_1$ -proteinase inhibitor may lead to an enhanced proneness to lung emphysema caused by uncontrolled action of leukocyte elastase (6–8). Thus, proteinase inhibitors represent an important therapeutic tool for a large number of different disorders.

Here we report the isolation of two peptides (HF6478 and HF7665) from human blood filtrate (hemofiltrate), which may represent a novel class of proteinase inhibitor. Blood filtrate, a by-product of ultrafiltration of the blood from patients with acute renal failure, is routinely used by us as a source for the systematic as well as random isolation of novel human peptides (9). Due to the cut-off limit of the hemofilters (approximately 20,000 Da), it mainly contains peptides exhibiting a molecular mass below 20,000 Da. Nevertheless, we succeeded in isolating members of many different peptide/protein families such as hormones, cytokines, growth factors, enzymes, proteinase inhibitors, transport, and plasma proteins (10). The two isolated peptides, HF6478 and HF7665, as deduced from cDNA cloning, are part of a common putative precursor protein termed LEKTI, which contains 11 additional similar peptides plus two typical Kazal-type serine proteinase inhibitor domains (a total of 15 potential inhibitory domains). We demonstrate the ability of HF7665 to inhibit trypsin and show the expression of the corresponding gene in human thymus, vaginal epithelium, Bartholin's glands, oral mucosa, tonsils, and the parathyroid glands. From the data obtained, we assume that LEKTI may be important for the anti-inflammatory and/or antimicrobial protection of mucous epithelia.

### EXPERIMENTAL PROCEDURES

*Isolation and Biochemical Characterization of the Peptides HF6478 and HF7665*—Human blood ultrafiltrate (hemofiltrate, HF) obtained from a local nephrological center (Hannoversch-Münden, Germany) was acidified with HCl to pH 3.0 and cooled to inhibit proteolysis. Following a random isolation strategy, extraction of the peptides and subsequent analysis by mass spectrometry and sequencing was performed as described for the isolation and characterization of vitronectin-derived peptides (11). Conditioned blood filtrate was loaded onto a cation exchange column (25 × 5 cm, Fractogel SP 650M, Merck, Darmstadt, Germany) and eluted with 1 M ammonium acetate, 0.5 M acetic acid, and 20% methanol at a flow rate of 50 ml/min. Aliquots of the fractions obtained were subjected to an RP-C18 column (15–20  $\mu$ m, 300 Å, 47 × 300 mm, Vydac, Hesperia, CA) and further separated at a flow rate of 42 ml/min using a gradient of 1% buffer B/min (buffer A, 0.1% trifluoroacetic acid; buffer B, 80% acetonitrile, 0.1% trifluoroacetic acid). Selected single fractions were loaded onto a cation exchange column (5  $\mu$ m, 300 Å, 10 × 50 mm Pepkat, Biotek, Östringen, Germany) and separated at a flow rate of 3 ml/min using a gradient of 1% buffer B/min (buffer A, 20 mM sodium phosphate, pH 3.0; buffer B, 20 mM sodium phosphate, pH 3.0, 1 M NaCl). To obtain highly purified homogeneous peptides, selected peak fractions were rechromatographed by analytical RP-HPLC<sup>1</sup> using a C18 column (5  $\mu$ m, 300 Å, 0.46 × 25 cm,

<sup>1</sup> The abbreviations used are: RP-HPLC, reverse phase-high performance liquid chromatography; RT-PCR, reverse transcriptase-polymerase chain reaction; RACE, rapid amplification of cDNA ends; bp, base pair(s).

Vydac, Hesperia, CA) with the same buffers as for the preparative C18 RP-HPLC described above. For the determination of intramolecular disulfide bonds, native HF7665 was cleaved by endoproteases (chymotrypsin, Glu-C, Lys-C, Asp-N; Roche Molecular Biochemicals, Basel, Switzerland), and the generated fragments were subsequently analyzed by mass spectrometry and amino acid sequencing as described (11).

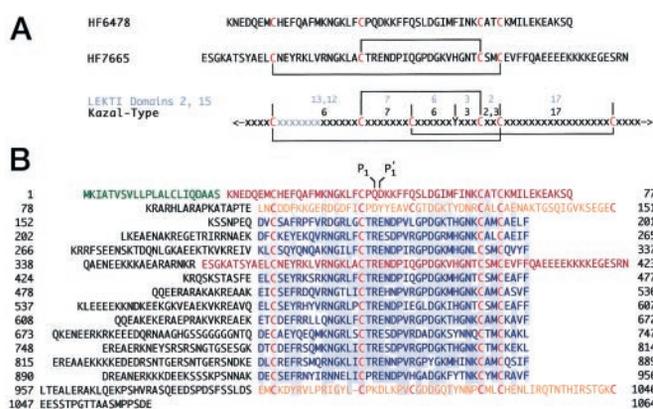
**Molecular Biological Standard Methods**—RNA extraction, cDNA first strand synthesis, polymerase chain reaction (PCR), reverse transcriptase-polymerase chain reaction (RT-PCR), RACE PCR, Northern blot hybridization, DNA sequencing, genome walking PCR, sequence analysis, and chromosomal localization of the gene by radiation hybrid mapping were performed as already described (12–14). For Northern hybridization, a blot with 20 µg of total RNA from each tissue as well as a commercially available blot (Human Immune System II, CLONTECH) with 2 µg of poly(A)<sup>+</sup> RNA from each tissue were used. As a hybridization probe, we used a 1713-bp PCR-generated and <sup>32</sup>P-labeled partial fragment of the entire cDNA mentioned below (GenBank™/EBI accession number AJ228139) which spans the region from nucleotide 679 to nucleotide 2391. PCR-based radiation hybrid mapping was performed by means of an exon-specific sense primer (PSTILP-11, TGC-CATGAATTTTCAGGCATTTATGAAAAATGG) and an intron-specific antisense primer (LEKTI-55, GAAAAATAATACATTTACCAGTTCCAG-AG) corresponding to nucleotide positions 131–162 of the cloned cDNA (EM/GenBank™ accession number AJ228139) and positions 78–51 of an intron following the nucleotide in position 252 of the cDNA, respectively. This intron was identified by the genome walking strategy using the GenomeWalker kit human (CLONTECH) (15) and cDNA-derived primers (not shown). The products obtained by the radiation hybrid mapping PCR were in the expected size range of 200 bp and could be verified as LEKTI-specific by means of direct fluorescence sequencing. Analytical RT-PCR was performed using the sense primer LEKEX-1S (CCAGTGTACGTAAGAATGAAGATCAGGAAATGTGCCATGA) in combination with the antisense primer LEKTI-53 (ATTCCTTTGCCT-GATTTTGATATTGACTGC). Both primers are exon-specific and flank the above-mentioned intron, thus allowing discrimination between amplified cDNA and genomic DNA fragments. Other molecular biological standard methods were performed according to Sambrook *et al.* (16).

**Oligonucleotides**—Oligonucleotides were purchased as “Research-Oligos” from Perkin Elmer (Weiterstadt, Germany). Within the nucleotide sequences of the oligonucleotides mentioned, the letters in italics represent “add-on regions” of the oligonucleotides that do not correspond to coding regions of the cDNA/gene but contain recognition sequences for certain restriction endonucleases.

**Cloning of the LEKTI cDNA**—The following degenerate oligonucleotides were constructed from the amino acid sequence of the peptide HF6478 (listed in 5'→3' orientation): MEMC-1, CCTTCGAATTTCTAG-AAARAAYGARGAYCARGARATGTG (sense); CHEF-1, CAYGARTT-YCARGCNTTYATGAARAAYGG (sense); CHEF-2, ATCATYTTTRCA-NGTNGCRCAYTT (antisense). As an oligonucleotide complementary to the poly(A) tail of eukaryotic mRNA, UNIP-2 was used: CCTGAATTC-TAGAGCTCATTTTTTTTTTTTTTTT. A part of the LEKTI precursor cDNA was amplified using a two-step strategy as follows. cDNA first strand was synthesized from biopsy material representing 17 human tissues (parotid gland, pancreas, skeletal muscle, brain, stomach, liver, kidney, lung, heart, heart auricle, bladder, tonsils, corpus cavernosum penis, vaginal epithelium, colon, bone marrow, and blood). 1/300 of each cDNA first-strand reaction mixture was subjected to RT-PCR amplification under the following conditions: 94 °C, 3 min; 98 °C, 1 s, 45 °C, 30 s, 72 °C, 2 min, 30 cycles; 72 °C, 3 min, 1 cycle; cold start; primers used: MEMC-1/UNIP-2. 1/100 of each preamplification step was used as a template for a second PCR amplification: 94 °C, 3 min; 98 °C, 1 s, 50 °C, 30 s, 72 °C, 2 min, 30 cycles; 72 °C, 3 min, 1 cycle; cold start; primers used: CHEF-1/CHEF-2. Homogenous 140-bp PCR fragments from vaginal epithelium and tonsil cDNA were cloned in pGEM T-vector and sequenced. The obtained partial cDNA sequence served as a basis for further RACE PCR experiments (see above), finally resulting in cloning and sequencing of the entire LEKTI cDNA.

**Detection of Functional Protein Domains**—The potential Kazal-type proteinase inhibitor domains occurring within the LEKTI amino acid sequence derived from the cDNA sequence were identified by means of the MacPattern program (17) and the Prosite data base (18) on an Apple Power Macintosh computer.

**Production of Recombinant HF7665 (rHF7665)**—A partial cDNA fragment encoding the peptide HF7665 was PCR-amplified under standard conditions using cDNA from human vaginal epithelium and the primers VADEX-61S, GAATCTGGAAAAGCAACCTCATATGC (5'-phosphorylated), and VADEX-61AS, CCGTATGGTACCGAATTTCTTAC-TAGTTTCTTGATTGCGCTTCCTTC. The *Escherichia coli* expression



**FIG. 1. Amino acid sequences of LEKTI and the isolated domains HF6478 and HF7665 in N→C-terminal direction.** *A*, structures of HF6478 and HF7665 in comparison with one other and the typical Kazal-type domain. Cysteine residues are printed in red. Disulfide bonds are indicated by black dashes. Of all LEKTI domains, the actual pattern of disulfide bonds was only determined for HF7665 (see text). A scheme of the typical Kazal-type domain including conserved disulfide bonds is also presented and compared with the cysteine pattern of the LEKTI domains 2 and 15 (see also *B*). The numbers of amino acids spacing the cysteine residues as well as a conserved tyrosine residue (*Y*) and the flanking cysteines are given in black and gray letters for the Kazal-type domain and the LEKTI domains 2 and 15, respectively. *B*, amino acid sequence of LEKTI as deduced from the 3528-bp cDNA sequence. A typical N-terminal secretory signal sequence is printed in green. The six-cysteine Kazal-type domains are printed in orange (lines 2 and 15). The peptides HF6478 (line 1) and HF7665 (line 6) purified from human blood filtrate are printed in purple. The other 11 four-cysteine domains are printed in blue. Sequences conserved within the 15 domains are hatched in gray. The putative P<sub>1</sub> and P<sub>1</sub>' sites are indicated. Cysteine residues probably involved in the formation of disulfide bonds are printed in red.

vector pTrcHis B (Invitrogen, Carlsbad, CA) was cleaved with *Bam*HI and subsequently treated with S1 single-strand nuclease (Amersham Pharmacia Biotech, Freiburg, Germany) to generate blunt ends. The vector and the generated cDNA PCR fragment were then cleaved with *Eco*RI, and the latter was cloned site-directed into the blunt-ended *Bam*HI and the *Eco*RI site of the vector. Transformation of *E. coli* TOP10 cells, induction of rHF7665 expression by isopropyl-1-thio-β-D-galactopyranoside, and removal of the His tag by cleavage with enterokinase were accomplished according to the manufacturer's instructions using the pTrcHis Xpress kit and EnterokinaseMax (both from Invitrogen, Carlsbad, CA). Pure rHF7665 was finally isolated by means of RP-HPLC, and its identity to native HF7665 could be demonstrated by mass spectrometry and amino acid sequencing as described above.

**Inhibition Assays**—Inhibitory effects of HF7665 and recombinant HF7665 (rHF7665) on trypsin (Roche Molecular Biochemicals) were examined in 50 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl, and 0.01% (v/v) Triton X-100. *N*<sup>α</sup>-benzoyl-L-arginine *p*-nitroanilide (Sigma, Deisenhofen, Germany; final concentration of 220 µM) was used as substrate, and its hydrolysis was monitored by the change in absorbance at 405 nm. Various inhibitor and trypsin (bovine, Roche Molecular Biochemicals) concentrations were added to the reaction mixtures, and the residual activity of the proteinase was measured in a quartz cuvette thermostatically controlled at 25 °C. Bovine albumin (fraction V; Sigma, Deisenhofen, Germany) served as a negative control. Inhibition assays with other serine proteinases (chymotrypsin, leukocyte elastase, thrombin, tissue plasminogen activator, trypstatin, plasmin, tissue kallikrein, factor Xa, plasma kallikrein, and urokinase) were performed in a similar way but using different appropriate chromogenic substrates.

## RESULTS AND DISCUSSION

The peptide isolation procedure described above resulted in purification of two as yet unknown peptides. According to their source (human hemofiltrate) and their molecular mass, they were designated as HF6478 and HF7665 (hemofiltrate peptides with molecular masses of 6478 and 7665 Da, respectively). Both peptides exhibit deviations in their amino acid sequences and molecular masses but show an identical four-cysteine pattern (Fig. 1A).

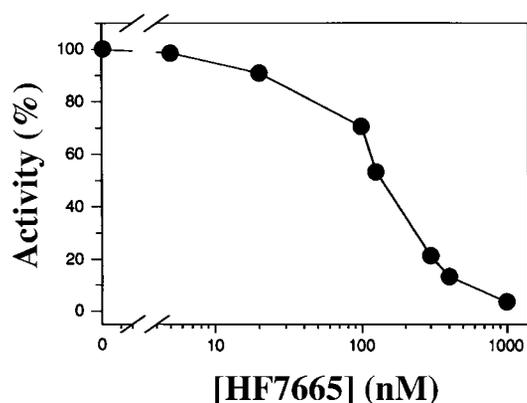


FIG. 2. Inhibitory effect of HF7665 on trypsin (concentration 6.25  $\mu\text{g/ml}$ ). Under the conditions chosen (see text), HF7665 exhibits an apparent  $\text{IC}_{50}$  of approximately 150 nM.

To determine the amino acid sequence of its precursor protein and to investigate the expression pattern of the corresponding gene, we cloned and sequenced the HF6478-specific cDNA as described above. The entire cDNA sequence (GenBank<sup>TM</sup>/EBI accession number AJ228139) comprises 3528 bp excluding the poly(A) tail. It exhibits an open reading frame of 3192 bp encoding a putative 1064-amino acid precursor protein which includes the purified peptide HF6478 and, surprisingly, also the peptide HF7665 (Fig. 1B). Thus, HF6478 and HF7665 represent fragments of the same precursor protein. In addition, the precursor protein contains 11 motifs exhibiting a high degree of sequence identity to HF6478 and HF7665 and an absolutely identical four-cysteine pattern. Using the MacPattern program (17) and the Prosite data base (18), a typical Kazal-type motif (19, 20) was identified occurring C-terminally behind HF6478. A second related domain is located at the C terminus of the entire precursor protein. Both domains match the six-cysteine pattern of the "classical" Kazal-type inhibitors (spacing between the six cysteine residues: 6-7-10-2, 3-17) almost exactly. As the only deviation, the spacing between the first two cysteines is 13 (first Kazal domain) and 12 (second Kazal domain) instead of six amino acids. Moreover, the putative reactive center of the first Kazal-type-related domain exhibits a significant sequence identity to the pancreatic secretory trypsin inhibitor (Kazal-type, data not shown) enabling determination of potential  $\text{P}_1$  and  $\text{P}_1'$  sites as shown in Fig. 1B. This finding confirms the supposed function of the protein as a serine proteinase inhibitor.

Comparison of the 15 domains with one another revealed that their cysteine patterns are identical, but that the 13 non-Kazal-type domains lack cysteines three and six of the Kazal-type domain. However, as demonstrated for HF7665 by proteolytic digests with subsequent mass spectrometric analysis and sequencing (data not shown), the remaining four cysteines exhibit a 1-4/2-3 disulfide pattern which is in agreement with the 1-5/2-4/3-6 disulfide pattern of the six cysteines of the Kazal-type domain.

Because sufficient amounts of native HF7665 were available from human blood filtrate (concentration > 100 pM), we tested its inhibitory properties with the serine proteinases chymotrypsin, leukocyte elastase, thrombin, tissue plasminogen activator, tryptase, trypsin, plasmin, tissue kallikrein, factor Xa, plasma kallikrein, and urokinase. Indeed, we obtained a significant but temporary inhibitory effect on trypsin with an apparent  $K_i$  of approximately 150 nM (Fig. 2). To avoid errors caused by contamination with non-detectable inhibitory components of the preparation, we repeated the assays with HF7665 biotechnologically produced in *E. coli*. In this case, we obtained identical results, verifying the inhibitory effect of

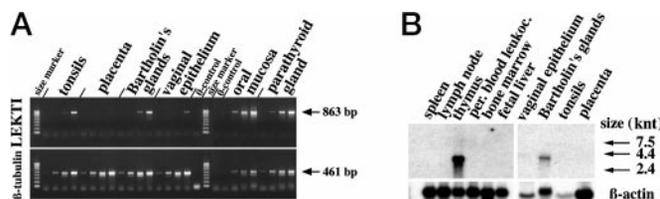


FIG. 3. Expression pattern of the *LEKTI* gene as determined by semiquantitative RT-PCR and Northern hybridization. A, RT-PCR, cDNA first strands were synthesized using 5  $\mu\text{g}$  of total RNA of each tissue. 1/300 of each first strand was subjected to RT-PCR under standard conditions using *LEKTI*- and  $\beta$ -tubulin-specific primers. After 25, 30, 35, and 40 cycles (from left to right) of RT-PCR, samples were taken and analyzed by means of agarose gel electrophoresis. PCR products in the size range expected for *LEKTI* were obtained from oral mucosa, parathyroid glands, Bartholin's gland, tonsils, and vaginal epithelium (in order of descending intensity). B, Northern blots, one commercially available Northern blot containing 2  $\mu\text{g}$  of poly(A)<sup>+</sup> RNA of each tissue (left) and one Northern blot containing 20  $\mu\text{g}$  total RNA of each tissue (right) were hybridized with *LEKTI*- and  $\beta$ -actin-specific cDNA probes under highly stringent conditions. Thymus and the Bartholin's glands show strong *LEKTI*-specific signals.

native HF7665. The amount of purified native HF6478 was only sufficient for mass spectrometry and sequence analysis. Thus, the recombinant production of all 15 domains, which should enable further structural and functional analysis of the single domains as well as the entire protein, is presently in progress.

The tissue-specific expression pattern of the corresponding gene was determined with the sensitive RT-PCR method. In addition to the 17 cDNA samples mentioned above, we used cDNA from the placenta, Bartholin's glands, oral mucosa, and the parathyroid gland. Of all tissues analyzed, an expression of the gene was detectable in the oral mucosa, parathyroid gland, Bartholin's glands, tonsils, and vaginal epithelium (Fig. 3A) and at very low levels also in the lung, kidney, and prostate (data not shown). To verify the cloning of an almost full-length cDNA and to detect further loci of gene expression, we performed less sensitive Northern blot hybridizations with 20  $\mu\text{g}$  of total RNA of each of the following human tissues: Bartholin's glands, vaginal epithelium, tonsils, and placenta. Of these tissues, only the Bartholin's glands gave a signal (Fig. 3B). In addition, we used a commercially available blot containing 2  $\mu\text{g}$  of poly(A)<sup>+</sup> RNA of each of the following human tissues: spleen, lymph node, thymus, peripheral blood leukocytes, bone marrow, and fetal liver. In this case, we obtained a strong hybridization signal from the thymus (Fig. 3B), indicating this organ as a locus of high level gene expression. As calculated by means of logarithmic regression, the size of the hybridizing mRNA is in the range of 3750 nucleotides. Taking into consideration the fact that eukaryotic mRNA usually contains a poly(A) tail which is not included in the cDNA sequence, this finding is in good agreement with the cDNA size of 3528 bp. From the results obtained, we termed the precursor protein "LEKTI" (lympho-epithelial Kazal-type-related inhibitor).

The chromosomal localization of the *LEKTI* gene was determined by means of the PCR-based radiation hybrid mapping (see above). The evaluation of the PCR results revealed the positioning of the *LEKTI* gene between the markers WI-4870 and D5S413, which correlates to human chromosome 5q31-32 (data not shown). This is in agreement with the typical Kazal-type inhibitor pancreatic secretory trypsin inhibitor whose gene is also located on chromosome 5 (21).

In summarizing, 13 of the 15 *LEKTI* domains exhibit a Kazal-type-derived four-cysteine residue pattern which represents a novel protein module of serine proteinase inhibitor, possibly responsible for temporary fine tuning of proteinase action. Together with the extraordinarily high number of po-

tential inhibitory domains, which is even higher than in the case of the seven-domain ovoinhibitor (22), these findings indicate that LEKTI may represent an as yet unknown type of human multidomain serine proteinase inhibitor. The isolation of the peptides HF6478 and HF7665 indicates a mechanism of specific endoproteolytic cleavage of the precursor protein *in vivo*, which may also result in the occurrence of the other 13 domains as single peptides or as parts of fragments of the precursor protein containing only lower numbers of domains. Because these domains have not yet been isolated, further attempts for their isolation from sources such as hemofiltrate or LEKTI gene-expressing tissues are planned. However, because the high degree of similarity of most of the domains will complicate their specific identification, for example by means of non-cross-reacting antibodies, the realization of this intention may be difficult.

As yet, the biological function of LEKTI is unclear. Antileukoprotease/secretory leukocyte protease inhibitor (ALP, SLPI), another serine proteinase inhibitor occurring in various external secretions, is described to exhibit antibacterial and antiviral properties (23, 24). Thus, a main role of LEKTI in antimicrobial and/or anti-inflammatory protection of mucous epithelia is conceivable. On the other hand, LEKTI shares some common structural features with agrin, a 200-kDa extracellular matrix protein (for review see Ref. 25). Like LEKTI, agrin also possesses a high number of Kazal-type-related domains, being able to inhibit serine proteinases such as trypsin and chymotrypsin (26). However, agrin seems to serve mainly as a differentiation factor, being important for the generation of neuromuscular junctions (25). Because the LEKTI gene is highly expressed in the thymus and probably in lower amounts also in the parathyroid gland (Fig. 3), the possibility of a comparable function in the regulation of differentiation processes, for example the maturation of T-lymphocytes, must be taken into consideration.

Further investigations are now necessary to determine the main target proteinases of LEKTI, its function within the thymus and parathyroid gland, the inhibitory potency of the other

12 non-Kazal-type domains in comparison to HF7665 and the Kazal-type domains, and to clarify a possible systemic as well as the pathophysiological role of LEKTI.

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**COMMUNICATION:**  
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