Inhibition of Serine Proteinases Plasmin, Trypsin, Subtilisin A, Cathepsin G, and Elastase by LEKTI: A Kinetic Analysis†

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ABSTRACT: The human LEKTI gene encodes a putative 15-domain serine proteinase inhibitor and has been linked to the inherited disorder known as Netherton syndrome. In this study, human recombinant LEKTI (rLEKTI) was purified using a baculovirus/insect cell expression system, and the inhibitory profile of the full-length rLEKTI protein was examined. Expression of LEKTI in Sf9 cells showed the presence of disulfide bonds, suggesting the maintenance of the tertiary protein structure. rLEKTI inhibited the serine proteinases plasmin, subtilisin A, cathepsin G, human neutrophil elastase, and trypsin, but not chymotrypsin. Moreover, rLEKTI did not inhibit the cysteine proteinase papain or cathepsin K, L, or S. Further, rLEKTI inhibitory activity was inactivated by treatment with 20 mM DTT, suggesting that disulfide bonds are important to LEKTI function. The inhibition of plasmin, subtilisin A, cathepsin G, elastase, and trypsin by rLEKTI occurred through a noncompetitive-type mechanism, with inhibitory constants (K_i) of 27 ± 5, 49 ± 3, 67 ± 6, 317 ±36, and 849 ± 55 nM, respectively. Thus, LEKTI is likely to be a major physiological inhibitor of multiple serine proteinases.

We have been employing oligonucleotide microarray analysis to identify differences in gene expression between primary head and neck squamous cell carcinomas and matched nonmalignant biopsy specimens. One of the genes identified as being downregulated in the tumors is LEKTI[1] (SPINK5), which encodes a putative 15-domain serine proteinase inhibitor (*1*, *2*) that has been linked to the autosomal recessive genetic disorder known as Netherton syndrome (*2*−*8*).

LEKTI was initially cloned following sequence identification of two polypeptides, HF6478 and HF7556, which were isolated from human blood filtrates (*1*). The polypeptides turned out to be fragments of a much larger precursor protein, LEKTI, which contains two Kazal-type domains containing six conserved cysteine residues and 13 other domains containing only four of the six cysteines. One of the isolated polypeptides, HF7556, which corresponded to domain 6, inhibited trypsin but not chymotrypsin, elastase, or plasmin (*1*, *9*). No inhibitory function could be assigned to the other polypeptide, HF6478, which corresponded to domain 1 (*10*). Because of the presence of Kazal-type domains in the translated protein and its pattern of expression in different organs, the gene encoding these polypeptides was named lympho-epithelial Kazal-type-related inhibitor (LEKTI) (*1*, *9*). More recently, a 30 kDa protein of unknown function was isolated from human epidermal keratinocytes and shown to have an N-terminal sequence corresponding to LEKTI domain 8 (*11*).

LEKTI (SPINK5) was also independently cloned by Chavanas et al. (*2*) as the genetic locus responsible for Netherton syndrome. Mutations in the LEKTI gene cause Netherton syndrome (*2*−*8*), which is characterized by congenital ichthyosis, hair shaft abnormalities, immune deficiency, elevated IgE levels, and failure to thrive.

Currently, the actual biologic function(s) of LEKTI protein is a subject of speculation. LEKTI mRNA localizes to the upper epidermis and pilosebaceous units of skin (*5*). Patients with Netherton syndrome have elevated levels of trypsin-like hydrolytic activity in their stratum corneum associated with overdesquamation of corneocytes (*5*). These findings have led to the hypothesis that LEKTI plays an important

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[1] Abbreviations: LEKTI, ; SPINK5, ; IgE, immunoglobulin E; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide electrophoresis; BSA, bovine serum albumin; DTT, dithiothreitol; RT-PCR, reverse transcriptase polymerase chain reaction; PFU, plaque-forming units; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; EDTA, ethylenediaminotetraacetic acid; TBS, Tris-buffered saline; mAb, monoclonal antibody; IgG, immunoglobulin G; MALDI-MS, matrix-assisted laser desorption ionization mass spectroscopy; IC_{50}, median inhibitory concentration; SEM, standard error of the mean.

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Table 1: Oligonucleotides Used in Cloning Procedures for Heterologous Expression of LEKTI in Sf9

oligonucleotide	sequence (5′ → 3′), restriction sites (underlined), stop codon (bold), six-histidine tag (italic)	restriction enzyme for cloning
LEKTI-1s	CggatccgACATGAAGATAGCCACAGTGTCAGTGC	*Bam*HI
LEKTI-1794as	GTTGCCGTGGATTTTCCCATCTAGACCCT	*Xba*I
LEKTI-1794s	AGGGTCTAGATGGGAAAATCCACGGCAAC	*Xba*I
LEKTI-3203as	TCTTCCTGTCATTGCTCAGACGGGGG	*Not*I[a]
LEKTI-2390s	GGAAAACTTATCTGCACTCGAGAAAGTGACCCTGTCCGGGG	*Xho*I
LEKTI-3192as	GGGCTCGAG**TCA***GTGATGGTGATGGTGATG*TTCGTCAGACGGGGGCATG	*Xho*I

[a] From the pFASTBAC1 donor plasmid.

physiologic role by inhibiting serine proteinases present in skin. Also, a tryptase inhibitory role for LEKTI has been proposed on the basis of amino acid sequence identity between it and the leech-derived tryptase inhibitor (*9*).

In the study presented here, we produced recombinant LEKTI (rLEKTI) via a baculovirus/insect cell expression system and assessed the inhibitory activity of this protein against a panel of serine and cysteine proteinases. Our results show that full-length LEKTI inhibits both clan PA (plasmin, cathepsin G, elastase, and trypsin) and clan SB (subtilisin A) serine proteinases through a noncompetitive mechanism. However, no inhibitory activity was observed against either the serine proteinase chymotrypsin or the cysteine proteinases cathepsins K, L, and S. Kinetic analysis revealed that the inhibitory constants (K_i) for the LEKTI−plasmin, −subtilisin A, −cathepsin G, −elastase, and −trypsin interactions were on the order of $(2.7 \pm 0.5) \times 10^8$ to $(8.49 \pm 0.55) \times 10^7$ M^{-1}. These results indicate that the baculovirus/insect cell system is well-suited for future structure−function analysis of the protein.

EXPERIMENTAL PROCEDURES

Insect Cells and Reagents. The following reagents were obtained commercially as indicated: Fall Army worm cell line *Spodoptera frugiperda* (Sf9), low-melting point agarose, cellFECTIN, pFASTBAC1, pCRII-TOPO, *Escherichia coli*-competent DH10BAC, cabbage looper egg cell line *Trichoplusia ni* 5B1-4 (High Five), and ultimate serum-free insect medium from Invitrogen (Carlsbad, CA); restriction endonucleases from New England Biolabs (Beverly, MA); TALON Superflow from Clontech Laboratory (Palo Alto, CA); Insect-XPRESS medium and fetal bovine serum from BioWhittaker (Walkersville, MD); YM10 Centriplus from Millipore Corp. (Bedford, MA); precast SDS−PAGE gels, protein assay kit, SEC-250 size column, and prestained markers from Bio-Rad (Hercules, CA); BSA from Kabi Pharmacia (Franklin, OH); DTT and glycerol from Boehringer Mannheim Biochemicals (Indianapolis, IN); and penta-His mAb and six-His tagged protein ladder from QIAGEN Inc. (Valencia, CA).

Isolation and Characterization of LEKTI cDNA. Sense and antisense oligonucleotides specific for human LEKTI were synthesized by Genosys Biotechnologies Inc. (The Woodlands, TX). Total RNA was isolated from normal biopsy tissue, and relative RT-PCR was performed as described elsewhere (*12*). Essentially, the LEKTI cDNA first strand was synthesized from biopsy material representing two normal human oral mucosa tissues by using either oligo-dT and/or random primers. The cDNA was PCR amplified using Thermal Taq polymerase (Promega Corp., Madison, WI). Oligonucleotides LEKTI-1s and LEKTI-1794as and LEKTI-1794s and LEKTI-3203as (Table 1) were employed for amplification of the 5′- and 3′-halves of the LEKTI cDNA, respectively. The 1.8 kb (5′-half of LEKTI) and 1.4 kb (3′-half of LEKTI) fragments were subcloned into the pCRII-TOPO vector under conditions recommended by the manufacturer (Invitrogen). The open reading frame was subcloned as a 3.3 kb *Bam*HI−*Not*I fragment into the pFASTBAC1 donor plasmid downstream from the polyhedrin promoter. All expression plasmids were sequenced by using either vector- or cDNA-specific primers. Sequencing of the cDNA revealed six silent single-base exchanges in the open reading frame compared to LEKTI sequences (GenBank entries AJ228139 and AF086524). The deduced amino acid sequence of 1064 residues predicted an M_r of 121 234.

Generation of Recombinant Baculoviruses. To express the LEKTI with a C-terminal six-histidine affinity tag, an *Xho*I PCR fragment was amplified by using oligonucleotides LEKTI-2390s and LEKTI-3192as (Table 1) and inserted into the pFASTBAC1/LEKTI donor plasmid via the *Xho*I restriction site. The LEKTI composite viruses were generated and titered as described elsewhere (*13*).

Infection of Sf9 Cells and Miniscale Expression. To test the rLEKTI composite viruses for LEKTI expression, Sf9 cells were infected at varying multiplicities of infection with recombinant viruses, and the cell lysate and medium were collected every 24−96 h. The presence of histidine-tagged protein was confirmed by Western blot analysis using penta-His mAb directed against the six-histidine tag as per the manufacturer's recommendations. LEKTI composite viruses that displayed the highest level of expression were chosen for further experiments and spinner flasks.

Large-Scale Expression in a Spinner Flask. LEKTI was produced on a large scale by infecting spinner cultures of Sf9 cells (1.6 billion cells) in 10% serum containing Insect-XPRESS medium at a multiplicity of infection of 8 PFU. Three days after infection, the cell pellet was harvested and the rLEKTI was selectively purified from the cell lysate using Co^{2+}-charged Sepharose affinity column (TALON) followed by SEC-250 size column chromatography, as previously described (*14*). Fractions containing homogeneous LEKTI were pooled and concentrated by ultrafiltration. For long-term storage (−80 °C), glycerol was added to a final concentration of 15%. Protein was quantified using Bio-Rad Protein Assay Kit II.

Enzymes, Chromogenic Substrates, and Proteinase Assay Buffers. The following reagents were obtained commercially as indicated: human cathepsin K kindly provided by D. Bromme (The Mount Sinai School of Medicine, New York, NY); human plasmin, human cathepsin L, human cathepsin

S, human trypsin, human cathepsin G, human chymotrypsin, and human neutrophil elastase (HNE) from Athens Research & Technology, Inc. (Athens, GA); subtilisin A from Calbiochem-Novabiochem (San Diego, CA); papain from Roche Molecular Biochemicals (Indianapolis, IN); furin from New England BioLabs; succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Succ-AAPF-pNA), succinyl-Ala-Ala-Val-pNA (Succ-AAV-pNA), and D-Val-Leu-Lys-pNA (VLK-pNA) from Sigma Chemical Co. (St. Louis, MO); H-Glu-Gly-Arg-pNA (EGR-pNA) and benzyloxycarbonyl-Phe-Arg-pNA (Z-FR-pNA) from Bachem Bioscience, Inc. (King of Prussia, PA); and methoxy-Succ-Arg-Pro-Tyr-pNA (MeO-Succ-RPY-pNA) from Chromogenix Instrumentation Laboratory SpA (Milan, Italy). Proteinase assay buffers and solvents for the substrates were those described previously (*15*−*18*). PBS reaction buffer [137 mM NaCl, 27 mM KCl, and 10 mM phosphate buffer (pH 7.4)] was used with trypsin, plasmin, cathepsin G, HNE, and chymotrypsin. Cathepsin reaction buffer [0.1% CHAPS, 50 mM sodium acetate (pH 5.5), 1 mM EDTA] was used with cathepsins K, L, and S and papain. A unique reaction buffer was used with subtilisin A (PBS and 0.1% Tween 20).

Proteinase Activity and Inhibition of Proteinases. Proteinase inhibitory activity was inferred by the ability of rLEKTI to block the cleavage of small, chromogenic peptide substrates as determined by a spectroscopy technique as described previously (*19*). Inhibition of proteinase was assessed after preincubating the enzyme with rLEKTI for 2 min at 25 °C in 100 μL of assay buffer. This mixture was added to 890 or 880 μL of assay buffer in a 1 mL quartz cuvette. The proteinase activity was initiated by adding 10−20 μL of the appropriate pNA substrate. The change in absorbance at 405 nm ($A_{405} = 8.8 \times 10^{-3}$ M^{-1} cm^{-1}) was followed for as long as 10 min using a spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). The rate changes (ΔA_{405}/min) of inhibited and control reactions were determined from the velocity plots. Activities were expressed relative to control incubations from which LEKTI was excluded.

Classification of the Inhibitory Mechanism of rLEKTI. Initial velocities of the release of pNA from the peptide substrates were measured as a function of several fixed substrate concentrations (50−2080 μM) for two different rLEKTI concentrations. Nonlinear and linear regression analyses of experimental data were performed with GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA). The K_i of inhibition was calculated from the slope of inhibited reaction [$K_M/V_{max} \times (1 + [I]/K_I)$] as described previously (*20*).

SDS−*PAGE and Immunoblotting*. Proteins were mixed with 2× gel loading buffer [4% SDS, 20% glycerol, 120 mM Tris-HCl (pH 6.8), and 0.01% bromophenol blue, with or without 10% β-mercaptoethanol], heated to 95 °C for 5 min, and resolved by SDS−PAGE (10% gel) as described elsewhere (*21*). For immunodetection, electrophoretic transfer of proteins from the polyacrylamide gel onto a nitrocellulose membrane (Schleicher & Schull BioScience, Inc., Keene, NH) was achieved by using a mini-transblot electrophoretic cell (Bio-Rad) at 25 V and 4 °C for 16 h as described elsewhere (*22*). After transfer, the nitrocellulose membrane was incubated overnight at room temperature with 3% BSA in TBS to block free binding sites on the membrane. The blocked nitrocellulose membranes were incubated for 2 h at room temperature with penta-His mAb diluted 1/2000 as the primary antibody and a horseradish peroxidase-conjugated goat anti-mouse IgG (H + L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1/2500 as the secondary antibody. The immunoblot was visualized using the chemiluminescence-ECL substrate and exposed to Hyperfilm MP for 1−3 min (Amersham Bioscience Corp., Piscataway, NJ).

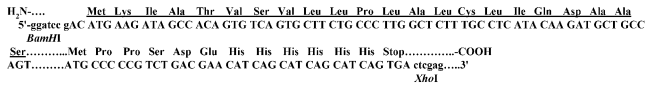

FIGURE 1: Partial sequences of the cDNA sense strand of the construct used for expression of LEKTI in Sf9 cells and its corresponding amino acid sequence. The secretory signal peptide is underlined. The six-histidine tag appended to the 1064-residue LEKTI is indicated. No secreted six-histidine-tagged LEKTI was detected by immunodetection in Sf9 cells.

Other Methods. Recombinant clones were analyzed by using the DNA sequencing core facility at The University of Texas M. D. Anderson Cancer Center. N-Terminal and internal amino acid sequence analyses of the purified rLEKTI were performed at the Baylor College of Medicine Protein Chemistry Core Laboratory (Houston, TX). The rLEKTI (280 μg) was digested with human recombinant furin (4 units) in furin reaction buffer (in a final volume of 50 μL) for 16 h at 30 °C. Proteolysis was stopped, and the components were desalted, concentrated, and separated by MALDI-MS at the Wistar Protein Microchemistry Facility (Philadelphia, PA).

RESULTS

Expression and Purification of Human LEKTI in Sf9. The previous observation that the N-terminal leader sequences, as expected, were not included in the first LEKTI domain isolated from blood filtrate suggests that this protein may involve a secretory pathway (*9*). A recombinant baculovirus expression vector was generated which encoded the entire native human LEKTI protein (including the putative signal peptide) fused in frame with a C-terminal six-histidine tag (Figure 1). When expressed in Sf9 cells, however, a secreted form of LEKTI could not be detected. The N-terminal leader sequences of proteins of higher eukaryotes often yield only small quantities of secreted proteins in yeast and insect cells. No attempts were made to improve the secretion efficiency in Sf9 by replacing or fusing the LEKTI native signal sequence with an insect cell signal sequence (e.g., melletin). Instead, we selectively purified the LEKTI precursor from cell lysates.

Following infection of Sf9 with recombinant baculovirus, abundant rLEKTI was detected in cell lysates by immunoblotting with penta-His mAb. We processed the cell lysates by TALON metal-affinity and gel-filtration chromatography as previously described (*14*). The purification procedure for the cell lysate is shown in Figure 2. Although the rLEKTI protein band (≈120 kDa) was not visualized by Coomassie brilliant blue R-250 staining in total and soluble fractions (Figure 2A, lanes 3 and 4), its reactivity to penta-His mAb was apparent by Western blotting (Figure 2B, lanes 3 and 4). Two additional polypeptides (<50 kDa) in the total cell lysate (Figure 2B, lane 3) may represent partially degraded or proteolytically cleaved forms of LEKTI. Upon purifica-

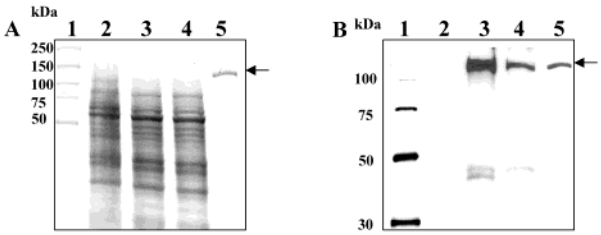

FIGURE 2: Purification of rLEKTI following expression in Sf9 cells. Cell lysates from 1 L Sf9 cell cultures (~450 mg) were subjected to metal-affinity and gel-filtration chromatography as described in Experimental Procedures. Positions of molecular mass markers are noted to the left of the gels. The location of rLEKTI (arrow) is indicated. (A) A total of 5−15 μL of each sample was resolved by 10% SDS−PAGE: lane 1, prestained molecular mass markers; lanes 2 and 3, total fraction of cell lysate from control (60 μg) and infected cells (50 μg), respectively; lane 4, soluble fraction of cell lysate from infected cells (50 μg); and lane 5, purified LEKTI (1.5 μg) after metal-affinity and gel-filtration chromatography. (B) Western blot analysis with penta-His mAb. Lanes are similar to those in panel A except for lane 1 contained six-His protein ladders. The immunodetection of minor protein bands in lanes 3 and 4 of panel B may reflect the presence of truncated LEKTI.

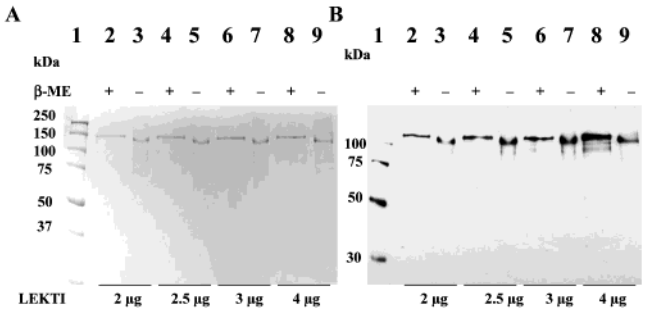

FIGURE 3: Disulfide status of rLEKTI expressed in Sf9 cells examined using SDS−PAGE (A) and Western blotting (B): lane 1, prestained molecular mass markers (A) or six-His protein ladders (B); lanes 2, 4, 6, and 8, sample prepared under reducing conditions (5% β-mercaptoethanol); and lanes 3, 5, 7, and 9, sample prepared under nonreducing conditions. Electrophoresis was performed in 10% gels at a constant voltage of 200 V for 2 h and the protein visualized by Coomassie brilliant blue R-250 staining. Positions of molecular mass markers are noted to the left of the gels. Proteins from a companion gel were transferred to nitrocellulose and immunoblotted with penta-His mAb. Due to diffusion of the reducing agent from lanes 2, 4, 6, and 8 into lanes 3, 5, 7, and 9, respectively, the band pattern was a mixed type.

tion, a sole band of ≈120 kDa was visualized by Coomassie brilliant blue R-250 staining in the pooled imidazole eluates (Figure 2A, lane 5). The ≈120 kDa protein was further suggested to be rLEKTI by its reactivity with penta-His mAb (Figure 2B, lane 5). N-Terminal sequencing of the protein band produced no amino acid sequence, which suggested that the N-terminal residue may have been blocked in vivo or modified during our sample preparation. Internal amino acid sequencing of this protein band confirmed it to be bona fide LEKTI. Approximately 0.7 mg of pure LEKTI was obtained from the Sf9 cell pellet of a 1 L culture. These results suggested that C-terminal six-histidine-tagged rLEKTI could be efficiently expressed and selectively purified from insect cells.

Disulfide Status of rLEKTI Expressed in Sf9. To evaluate whether rLEKTI contained disulfide bonds and to determine whether protein aggregates were present in our preparation, rLEKTI was analyzed by SDS−PAGE under both reducing and nonreducing conditions. In a one-dimensional SDS−PAGE gel after reducing and nonreducing sample preparation, disulfide-containing polypeptides migrate in a manner different from those of their reduced counterparts, indicating the presence of intramolecular disulfide bonds. The rLEKTI separated by SDS−PAGE under nonreducing conditions (Figure 3A, lanes 3, 5, 7, and 9) clearly migrated faster than under reducing conditions (Figure 3A, lanes 2, 4, 6, and 8), providing evidence of disulfide bonds present in the recombinant protein. In addition, no higher molecular bands representing rLEKTI aggregates are visible by Coomassie brilliant blue R-250 staining in the nonreducing SDS−PAGE, nor can they be detected by the more sensitive technique of immunoblotting (Figure 3A,B, lanes 3, 5, 7, and 9). This clearly indicates that almost all of the LEKTI expressed in insect cells contained disulfide bonds, and this Sf9-produced rLEKTI could be used to screen for inhibitory activity.

LEKTI Inhibitory Activity and IC_{50}. Because sufficient amounts of rLEKTI were available, we tested its inhibitory properties with the serine proteinases chymotrypsin, cathepsin G, trypsin, plasmin, HNE, and subtilisin A. Although rLEKTI had a strong inhibitory effect on trypsin, plasmin, cathepsin G, HNE, and subtilisin A, it had no inhibitory effect on chymotrypsin (Table 2). Moreover, rLEKTI did not inhibit the cysteine proteinase papain or cathepsins K, L, and S (Table 2). We emphasize that an immediate inhibition of the activity of these multiple serine proteinases was observed when rLEKTI is added after the addition of the enzyme and appropriate substrate. To understand whether LEKTI behaves as a slow- or fast-binding inhibitor, we measured the time course of various proteinase activities in the presence of different concentrations of rLEKTI (Figure 4A−G). As shown in Figure 4, the extent of product formation over the assay period in the absence and presence of inhibitor was linear with respect to time. The linear shapes of these inhibition curves indicate that rLEKTI is not a time-dependent inhibitor, suggesting that LEKTI binds rapidly to these proteinases and inactivates them. The assays shown in Figure 4 were repeated three times, and the data were used to calculate an apparent IC_{50} for LEKTI. On the basis of the IC_{50} values, rLEKTI appears to be a potent inhibitor of plasmin ($IC_{50} = 30 \pm 3$), cathepsin G ($IC_{50} = 66 \pm 7$), and subtilisin A ($IC_{50} = 49 \pm 5$). rLEKTI is only a moderate inhibitor of HNE ($IC_{50} = 300 \pm 15$), however, and a weak inhibitor of trypsin ($IC_{50} = 800 \pm 32$). As expected, no inhibition was observed against papain or chymotrypsin (Figure 4F, G).

Effects of Disulfide Reduction on rLEKTI Inhibitory Activity. Due to the effect of disulfide bonds on the structure of rLEKTI, the influence of a reducing agent on the rLEKTI inhibitory activity of plasmin and subtilisin A was examined. In the absence of a reducing agent, rLEKTI inhibitory activity was constant at pH 7.8 and room temperature for 1 h (Figure 5A, B). In contrast, rLEKTI inhibitory activity of plasmin and subtilisin A was readily inactivated by 20 mM dithiothreitol (DTT) during this time. In control experiments, the addition of 0.80 mM DTT alone had no effect on plasmin and subtilisin A activity (data not shown). Therefore, the reduction in rLEKTI inhibitory activity was not due to an artificial increase in proteinase activity by the presence of DTT.

Table 2: Inhibitory Profile of rLEKTI

proteinase (final concentration)	LEKTI[a] (nM)	*I*/*E*[b]	inhibition[c] (%)	substrate (final concentration)
trypsin (50 nM)	1500	30	96	EGR-pNA (1.0 mM)
plasmin (17 nM)	100	5.9	99	VLK-pNA (0.4 mM)
subtilisin A (36 nM)	300	8.3	97	Succ-AAPF-pNA (1.0 mM)
cathepsin G (50 nM)	300	6	90	Succ-AAPF-pNA (2.0 mM)
HNE (20 nM)	1000	50	92	Succ-AAV-pNA (1.0 mM)
chymotrypsin (10 nM)	500	50	9	Succ-AAPF-pNA (1.0 mM)
chymotrypsin (10 nM)	500	50	0	MeO-Succ-RPY-pNA (1.0 mM)
papain (10 nM)	500	50	5	Z-FR-pNA (1.0 mM)
cathepsin K (20 nM)	1000	50	0	Z-FR-pNA (1.0 mM)
cathepsin L (20 nM)	1000	50	0	Z-FR-pNA (1.0 mM)
cathepsin S (20 nM)	1000	50	0	Z-FR-pNA (1.0 mM)

[a] Proteinases and LEKTI were incubated for 2 min at 25 °C. Proteolysis was followed spectrophotometrically by the addition of substrate. Proteinase assay buffers are listed in Experimental Procedures. [b] *I*/*E* is the rLEKTI concentration/proteinase concentration ratio. [c] Percent inhibition = 100[1 − (velocity in the presence of inhibitor/velocity of uninhibited control)].

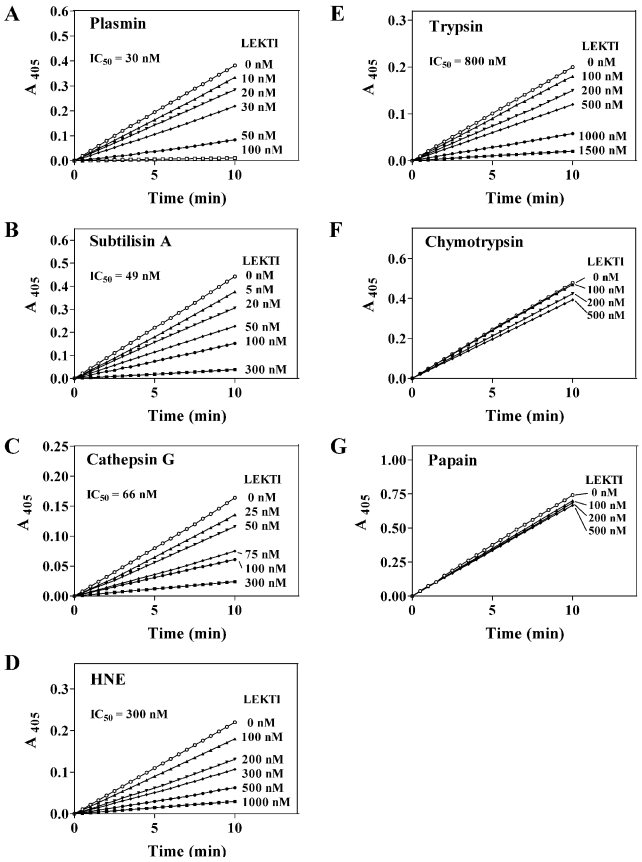

FIGURE 4: Time course of inactivation of proteinases by rLEKTI. Proteinase activity was measured as described in Experimental Procedures. The rate of release of pNA at 405 nm was plotted against reaction time. The proteinases, LEKTI concentrations, and corresponding IC_{50} values are listed in the figure: (A) VLK-pNA (0.4 mM), (B) Succ-AAPF-pNA (1.0 mM), (C) Succ-AAPF-pNA (2.0 mM), (D) Succ-AAV-pNA (1.0 mM), (E) EGR-pNA (1.0 mM), (F) Succ-AAPF-pNA (1.0 mM), and (G) Z-FR-pNA (1.0 mM) as the substrates.

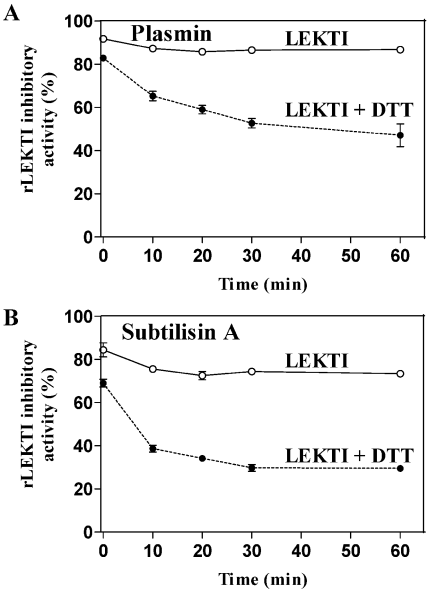

FIGURE 5: Inactivation of rLEKTI by 20 mM DTT. Reactions were carried out in 20 mM Tris-HCl (pH 7.8) and 0.25 M NaCl containing 7.7 μM rLEKTI and started by addition of DTT. At the indicated times, samples were withdrawn and diluted 77- or 26-fold in PBS (pH 7.4) containing 17 nM plasmin or 36 nM subtilisin A. The residual plasmin and subtilisin A activity was determined using VLK-pNA (0.4 mM) or Succ-AAPF-pNA (1.0 mM), respectively, as the substrate. In the absence of a reducing agent, the rLEKTI inhibitory activity of plasmin and subtilisin A remained nearly constant. rLEKTI inhibitory values are reported as an averaged value ± SEM. In control experiments, the addition of 0.80 mM DTT alone had no effect on plasmin and subtilisin A activity (data not shown).

rLEKTI Is a Noncompetitive Inhibitor. To classify the type of inhibition, the kinetic constants (K_m and V_{max}) of plasmin, trypsin, subtilisin A, cathepsin G, and HNE were determined for their respective chromogenic peptides in the presence of two rLEKTI concentrations and are shown in Table 3. The corresponding Eadie−Hofstee plots (V/[S] vs V) (Figure 6A−E) show that the apparent K_m values (=the slopes of the plots) remained fairly constant as the LEKTI concentration increased, and the apparent V_{max} values decreased markedly as the LEKTI concentration increased. This kinetic analysis indicates that the inhibition was strictly noncompetitive, suggesting the existence of a binding site for LEKTI distinct from the catalytic center. This type of inhibition is distinguished from competitive inhibition, which alters only the apparent K_m, and from uncompetitive inhibition, which alters both the apparent K_m and the apparent V_{max}. On the basis of the apparent K_i values (Table 3), LEKTI is a potent noncompetitive inhibitor of plasmin (27 ± 5 nM), cathepsin G (67 ± 6 nM), and subtilisin A (49 ± 3 nM), a moderate noncompetitive inhibitor of HNE (317 ± 36 nM), and a weak noncompetitive inhibitor of trypsin (849 ± 55 nM).

In Vitro Cleavage of rLEKTI by Furin. R-Xn-KR ↓ is the most potent consensus sequence for furin, which is known

Table 3: Effects of rLEKTI on Reaction Kinetics of Proteinases

proteinase	LEKTI (nM)	K_M[a] (μM)	V_{max}[a] (μmol mg^{-1} min^{-1})	n	K_i[b] (nM)	r^2
plasmin (17 nM)	0	257 ± 19	4.0 ± 0.15	3	−	0.9979
plasmin (17 nM)	30	277 ± 19	2.0 ± 0.07	3	27 ± 5	0.9974
plasmin (17 nM)	60	248 ± 24	1.2 ± 0.06	3	26 ± 4	0.9923
cathepsin G (50 nM)	0	1238 ± 31	2.8 ± 0.03	3	−	0.9992
cathepsin G (50 nM)	40	1288 ± 54	1.8 ± 0.04	3	67 ± 6	0.9993
cathepsin G (50 nM)	75	1214 ± 92	1.3 ± 0.05	3	66 ± 2	0.9938
HNE (20 nM)	0	369 ± 11	6.3 ± 0.08	3	−	0.9991
HNE (20 nM)	120	368 ± 32	4.3 ± 0.16	3	268 ± 36	0.9866
HNE (20 nM)	400	383 ± 43	2.9 ± 0.14	3	317 ± 36	0.9825
trypsin (50 nM)	0	485 ± 29	2.7 ± 0.07	3	−	0.9965
trypsin (50 nM)	120	480 ± 17	2.3 ± 0.04	3	731 ± 72	0.9992
trypsin (50 nM)	500	472 ± 34	1.6 ± 0.05	3	849 ± 55	0.9957
subtilisin A (36 nM)	0	329 ± 10	6.8 ± 0.08	3	−	0.9995
subtilisin A (36 nM)	50	348 ± 29	3.4 ± 0.12	3	47 ± 10	0.9878
subtilisin A (36 nM)	100	413 ± 63	2.8 ± 0.19	3	49 ± 3	0.9768

[a] K_M and V_{max} were calculated from data shown in Figure 4 by nonlinear regression analysis as described in Experimental Procedures. Values are reported as average values ± SEM. [b] K_i of LEKTI was calculated from the slope $[K_M/V_{max} \times (1 + [I]/K_I)]$ of inhibited reactions. Values are reported as average values ± SEM.

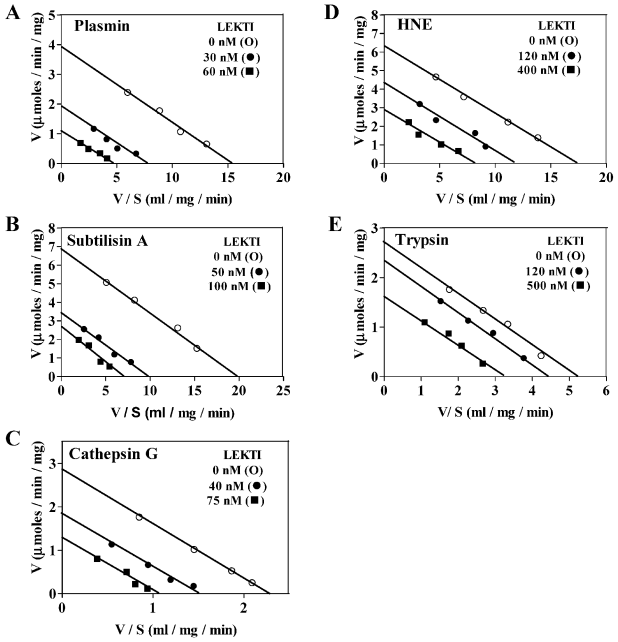

FIGURE 6: Eadie−Hofstee (V_o vs V_o/[S]) analysis of hydrolysis of the chromogenic peptide by proteinase in the presence of LEKTI. The solid lines in the Eadie−Hofstee plots represent the linear regression fits. The plot is diagnostic for competitive, noncompetitive, and uncompetitive types of inhibition: (A) VLK-pNA (0.05−0.4 mM), (B) Succ-AAPF-pNA (0.1−1.0 mM), (C) Succ-AAPF-pNA (0.14−2.08 mM), (D) Succ-AAV-pNA (0.1−1.0 mM), and (E) EGR-pNA (0.1−1.0 mM) as the substrates.

to be expressed in the stratum granulosum of the skin (*5*). A putative cleavage site is not found between the second and third domains, and thus, the LEKTI proprotein is suggested to be cleaved into at least 14 polypeptides ranging in size from 6050 to 15 180 Da. To identify the presence of these fragments, rLEKTI was incubated with furin for 16 h at 30 °C and the resulting cleavage fragments were resolved by SDS−PAGE (Figure 7A) and MALDI-MS (Figure 7B). SDS−PAGE analysis showed that furin cleaved LEKTI into six discrete polypeptides ranging in molecular mass from ~10 to ~40 kDa. The faint band around 55 kDa may represent the exogenous furin added to the reaction. However, the MALDI-MS analysis for polypetides in the range

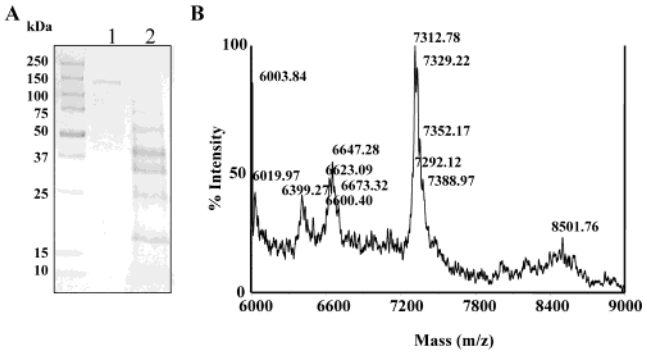

FIGURE 7: SDS−PAGE and MALDI-MS analyses of LEKTI after furin digestion. rLEKTI (280 μg) was digested with human recombinant furin (4 units) in furin reaction buffer (in a final volume of 50 μL) for 16 h at 30 °C. Proteolysis was stopped, and an aliquot (5 μg) was heated at 95 °C for 5 min in 2% SDS loading buffer with β-mercaptoethanol, The aliquots were separated by SDS−PAGE (A). The remaining protein mixtures and the components were desalted, concentrated, and analyzed by MALDI-MS (B) for peptide fragments in the range of 6−9 kDa. Positions of molecular mass markers are noted to the left of the gel.

of 6000−9000 Da detected the presence of 13 major peptide peaks. To address whether processed domains of the full-length LEKTI molecule would also have inhibitory activity, we expressed and purified a partial fragment of LEKTI containing domains 6−9. This partial LEKTI fragment inhibited subtilisin A, with an apparent K_i of 10 nM (manuscript under preparation). Collectively, these data suggest that furin could potentially cleave LEKTI preproprotein in vivo into discrete fragments with specific proteinase inhibitory function.

DISCUSSION

The primary focus of this study was to identify proteinase targets of LEKTI by using the full-length human recombinant protein, purified from a baculovirus/insect cell expression system. We have succeeded in purifying a fully functional C-terminal six-histidine-tagged full-length LEKTI to homogeneity. Using this purified rLEKTI, we have unequivocally demonstrated that the precursor protein is a cross-clan inhibitor of members of the serine proteinase family (*23*). To our knowledge, this is the first report describing production of a recombinant LEKTI and characterizing its inhibitory properties.

rLEKTI was found to be a noncompetitive inhibitor of the serine proteinases plasmin, elastase, cathepsin G, subtilisin A, and trypsin, but not of chymotrypsin. Previously, native LEKTI domain 6 was found to inhibit trypsin with an apparent IC_{50} of 150 nM (*1*, *24*). In comparison, full-length rLEKTI inhibited trypsin with an apparent IC_{50} of 800 ± 32 nM. On the basis of the IC_{50} values, it appears that the endogenously cleaved native peptide is a more potent trypsin inhibitor. Since LEKTI domain 6 is unable to inhibit plasmin and elastase (*1*), this suggests that other domains in the LEKTI protein are responsible for these inhibitory activities.

Our results show that rLEKTI is a fast-binding inhibitor in the presence of substrate, and we used the general Michaelis−Menten equation for kinetic analysis. The evidence for noncompetitive inhibition of plasmin, elastase,

cathepsin G, subtilisin A, and trypsin by rLEKTI is apparent in the Eadie−Hofstee plots. The apparent K_i values of r LEKTI for plasmin, elastase, cathepsin G, subtilisin A, and trypsin determined for two different LEKTI concentrations were within 20% of each other. Moreover, the inhibitory potencies of the full-length LEKTI against plasmin (K_i = 27 ± 5 nM), cathepsin G (K_i = 67 ± 6 nM), and subtilisin A (K_i = 49 ± 3 nM) were significantly higher than those against trypsin (K_i = 849 ± 55 nM) and HNE (K_i = 317 ± 36 nM). The K_i values of LEKTI for plasmin, cathepsin G, and subtilisin A are comparable to that of the well-characterized antileukoprotease present in the epidermis which inhibits the stratum corneum chymotryptic enzyme with an apparent K_i of 60 nM (*25*). Very few noncompetitive inhibitors for trypsin have been described in the literature. Rat pancreatic secretory trypsin inhibitor (61 kDa) has been shown to inhibit trypsin with an IC_{50} of 50 μg/mL (*26*). Our results with rLEKTI demonstrate an IC_{50} of 100 μg/mL for trypsin, which is roughly equivalent to the value reported for the rat pancreatic secretory trypsin inhibitor when differences in molecular mass are taken into account.

Comparison of the trypsin inhibitory activity of full-length LEKTI with that previously reported for domain 6 suggests that processed domains are more active. On the basis of these results, it is tempting to speculate that the inhibitory potency of processed LEKTI domains against specific proteinases would be significantly higher than that of the precursor LEKTI. In preliminary experiments, we have found that a rLEKTI protein containing domains 6−9 (33 kDa) inhibited trypsin with a K_i 4-fold lower than that of the full-length rLEKTI.

Recently, Komatsu et al. (*5*) proposed a model in which the full-length LEKTI protein contains inactive domains that are cleaved by subtilisin-like proprotein convertases (SPCs), resulting in conversion to multiple active inhibitory domains. Support for this model is evidenced by colocalization of LEKTI mRNA and various SPCs in epidermis and the existence of multiple SPC cleavage recognition sites within the LEKTI protein. Consistent with this model, cleavage of rLEKTI with furin (an SPC localized in the epidermis) resulted in generation of 13 polypeptides (ranging from 6.0 to 8.5 kDa), as analyzed by MALDI-MS. An interesting finding of our study is that precursor rLEKTI by itself possesses inhibitory activity against several serine proteinases.

The precursor rLEKTI used in our studies is identical to the native precursor LEKTI, except for the six-histidine C-terminal extension. This modification is unlikely to alter the function of such a large protein. Moreover, the presence of disulfide bonds, the lack of detectable aggregates, and functional activity are consistent with a properly folded protein. Indeed, rLEKTI activity was readily inactivated by the treatment of LEKTI with DTT, providing strong evidence that disulfide bonds are very important for LEKTI activity. Furthermore, rLEKTI purified from Sf9 cell lysate inhibited multiple serine proteinases, suggesting that the active structure of LEKTI was indeed formed.

The proteinase targets inhibited strongly by rLEKTI have been implicated in numerous homeostatic and disease processes (*27*−*33*). At least three processed LEKTI polypeptides have been identified in human blood filtrates (*24*). Although HF7556 (i.e., LEKTI domain 6) does not inhibit plasmin, it is conceivable, on the basis of our results with full-length LEKTI, that either of the other two LEKTI polypeptides could. Alternatively, additional processed forms of LEKTI with plasmin inhibitory activity may exist in the blood.

In conclusion, we have purified a full-length rLEKTI protein and used it to identify multiple proteinase targets, including plasmin, cathepsin G, and subtilisin A. Importantly, the proteolytic competence of the human LEKTI expressed in insect cells is essentially identical to that of partial LEKTI domain 6 purified from human blood filtrate, providing evidence that the recombinant protein effectively resembles native LEKTI. These proteinases and their human homologues are involved in many biologic functions and diseases. Successful production of biologically active LEKTI using the baculovirus/insect cell expression system should help in future structure−function analysis of this proteinase inhibitor and should facilitate our understanding of the various homeostatic and disease processes that may involve LEKTI.

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