The New Kallikrein-like Gene, KLK-L2
MOLECULAR CHARACTERIZATION, MAPPING, TISSUE EXPRESSION, AND HORMONAL REGULATION*

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Since in rodents the kallikreins are represented by a large multi-gene family, the restriction of this family in humans to three genes is somewhat surprising. In an effort to identify new human kallikrein genes, we examined a genomic area of about 300 kilobases on chromosome 19q13.3-q13.4, a region that contains most of the currently known kallikreins. By using the positional candidate approach, we were able to identify a new gene named KLK-L2 (for kallikrein-like gene 2). Screening of human EST libraries allowed us to delineate the full genomic and cDNA structure of the new gene. KLK-L2 consists of 5 coding exons and 4 introns and has significant similarities to other members of the kallikrein multi-gene family. Homology studies suggest that the protein is likely secreted. KLK-L2 is expressed mainly in breast, brain, and testis and to a lesser extent in many other tissues. KLK-L2 is up-regulated by estrogens and progestins in the breast cancer cell line BT-474.

Strategies for new gene discovery are undergoing rapid evolution. Traditional procedures for new gene identification, such as CpG island mapping and cross-species hybridization will soon be replaced by methods that are based on accumulating knowledge of chromosomal localization of genes and increasing amounts of nucleotide sequence data (1). Positional candidate cloning is a new approach for gene discovery that combines the knowledge of map position with the increasingly dense human transcript maps and the available expressed sequence tags (ESTs) (2).

The kallikreins are a group of serine proteases involved in the post-translational processing of polypeptide precursors (3). This enzyme family primarily consists of plasma kallikrein and tissue or glandular kallikreins. Plasma kallikrein is encoded by a single gene that is structurally different from the genes encoding tissue kallikreins. The tissue kallikreins comprise a large multigene family of enzymes in rodents, with a highly conserved sequence and tertiary structures. In the mouse genome, at least 24 genes have been identified (4). A similar conservation to other known kallikreins, and its tissue expression pattern.

MATERIALS AND METHODS

DNA Sequence on Chromosome 19—We have obtained sequencing data of approximately 300 kb of nucleotides on chromosome 19q13.3-q13.4 from the web site of the Lawrence Livermore National Laboratory. This sequence was in the form of nine contigs of different lengths. We performed a restriction analysis study of the available sequences using the WebCutter computer program, and with the aid of the EcoRI restriction map of this area (also available from Lawrence Livermore National Laboratory) we constructed an almost contiguous stretch of genomic sequences. We identified the relative positions of the known kallikrein genes PSA (GenBank™ accession number X14810), KLK2 (GenBank™ accession number M18157), and zyme (GenBank™ accession number U60801) by using the alignment program BLAST 2 (19). New Gene Identification—We used a number of computer programs to predict the presence of putative new genes in the genomic area of interest. We initially tested these programs using the known genomic sequences of the PSA, protease M, and NES1 genes. The most reliable computer programs GeneBuilder, Grail 2, and GENEID-3 were selected for further use.

EST Searching—The predicted exons of the putative new gene were subjected to homology search using the BLASTN algorithm (19) on the National Center for Biotechnology Information web server against the human EST data base (dbEST). Clones with >95% homology were obtained from the I.M.A.G.E. consortium (20) through Research Genetics Inc., Huntsville, AL (Table I). The clones were propagated, purified, and sequenced from both directions with an automated sequencer using insert-flanking vector primers.

Tissue Expression—Total RNA isolated from 26 different human tissues was purchased from CLONTECH, Palo Alto, CA. We prepared cDNA as described below for the tissue culture experiments and used it some 19q13 (7–9), a region that is syntenic to the mouse tissue kallikrein gene family locus on chromosome 7 (10). However, in marked contrast to the large rodent kallikrein gene family, the human kallikrein gene family was, until recently, composed of three genes. From Southern blot analysis, the size of this family has been suggested to vary from just 3–4 genes (11–12) to as many as 19 genes (13). Recently, a few new putative members of the human kallikrein gene family have been discovered such as the zyme (14) (also called protease M (15) or neurosin (16)), the normal epithelial cell-specific-1 gene (NES1) (17), and neurosin (18).

In our efforts to study the role of the kallikrein gene family in the initiation and/or progression of cancer, we have analyzed a 300-kb1 genomic region on chromosome 19q13.3-q13.4 and identified a new gene named KLK-L2 (for kallikrein-like gene 2). In this study, we describe the identification of the new gene, its genomic and mRNA structure, its precise location in relation to other known kallikreins, and its tissue expression pattern.

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1 The abbreviations used are: kb, kilobase(s); bp, base pair(s); KLK, kallikrein; KLK-L, kallikrein-like; RT-PCR, reverse transcriptase-polymerase chain reaction; PSA, prostate-specific antigen; dNTPs, deoxynucleoside triphosphates; EST, expressed sequence tag; EMSP1, enamel matrix serine proteinase 1; TSLP, trypsin-like serine protease; HSCCE, human stratum corneum chymotryptic enzyme; NES1, normal epithelial cell-specific-1 gene; contig, group of overlapping clones.
Molecular Characterization of the KLK-L2 Gene

**TABLE I**

| GenBank™ No. | Tissue of origin | I.M.A.G.E. ID | Homologous exons |
|--------------|-----------------|--------------|------------------|
| W73140       | Fetal heart     | 344588       | 4,5              |
| W73168       | Fetal heart     | 344588       | 3,4,5            |
| AA862032     | Squamous cell carcinoma | 1485736 | 4,5          |
| A002163      | Testis          | 1619481      | 3,4,5            |
| N80782       | Fetal lung      | 309611       | 5                |
| W85361       | Fetal heart     | 342591       | 5                |
| W85496       | Fetal heart     | 342591       | 5                |
| AA292366     | Ovarian tumor   | 725905       | 1,2              |
| AA394040     | Ovarian tumor   | 726001       | 5                |

EST clones with >95% homology to exons of KLK-L2 for PCR reactions with the primers described in Table II. Tissue cDNAs were amplified at various dilutions.

Breast Cancer Cell Line and Hormonal Stimulation Experiments—The breast cancer cell line BT-474 was purchased from the American Type Culture Collection (ATCC), Manassas, VA. Cells were cultured in RPMI media (Life Technologies, Inc.) supplemented with glutamine (200 mM/molar), bovine insulin (10 mg/ml), fetal bovine serum (10%), antibiotics, and antimitotics, in plastic flasks to near confluency. The cells were then aliquoted into 24-well tissue culture plates and cultured to 50% confluency. 24 h before the experiments, the culture media were changed into phenol red-free media containing 10% charcoal-stripped fetal bovine serum. For stimulation experiments, various steroid hormones dissolved in 100% ethanol were added into the culture media, at a final concentration of 10⁻⁹ M. Cells stimulated with 100% ethanol were included as controls. The cells were cultured for 24 h, then harvested for mRNA extraction.

**RESULTS**

Identification of the KLK-L2 Gene—Computer analysis of the genomic sequence predicted a putative new gene consisting of four exons. This gene was detected by all programs used, and all exons had high prediction scores. EST sequence homology search of the putative exons against the human EST data base (dbEST) revealed nine EST clones from different tissues with >95% identity to the putative exons of our gene (Table I). Positive clones were obtained, and the inserts were sequenced from both directions. The Blast 2 sequences program was used to compare the EST sequences with the predicted exons, and final selection of the exon-intron splice sites was done according to the EST sequences. The presence of many areas of overlap between the various EST sequences allowed us to further verify the structure of the new gene. The coding and genomic sequence of the gene has been deposited in GenBank™ accession number AF135028. The 3' end of the gene was verified by the presence of poly(A) stretches that are not present in the genomic sequence at the end of two of the sequenced ESTs. One of the sequenced ESTs revealed the presence of an additional exon at the 5' end. The nucleotide sequence of this exon matches exactly with the genomic sequence. To further identify the 5' end of the gene, 5'-Rapid amplification of cDNA ends was performed, but no additional sequence could be obtained. However, as is the case with other kallikreins, the presence of further up-stream untranslated exon(s) could not be excluded.

Mapping and Chromosomal Localization of the KLK-L2 Gene—Alignment of KLK-L2 gene and the sequences of other known kallikrein genes within the 300-kb area of interest enabled us to precisely localize all genes and to determine the direction of transcription, as shown by the arrows in Fig. 1. The PSA gene was found to be the most centromeric, separated by 12,508 base pairs (bp) from KLK2, and both genes are transcribed in the same direction (centromere to telomere). The prostate/KLK-L1 gene (GenBank™ accession number AF135023) is 26,229 bp more telomeric and transcribes in the opposite direction, followed by KLK-L2. The distance between KLK-L1 and KLK-L2 is about 35 kb; however, we could not establish it more precisely due to the presence of a gap in the genomic sequence. The zyme gene is 5,981 bp more telomeric, and the latter 3 genes are all transcribed in the same direction (Fig. 1).

Structural Characterization of the KLK-L2 Gene and Its Protein Product—The KLK-L2 gene, as presented in Fig. 2, is formed of 5 coding exons and 4 intervening introns, spanning an area of 9,349 bp of genomic sequence on chromosome 19q13.3-q13.4. The lengths of the exons are 73, 262, 257, 134, and 156 bp. The intron/exon splice sites (mGT...AGm) and their flanking sequences are closely related to the consensus splicing sites (mTGAAGT...CAm, where m is any nucleotide) (21). The presumptive protein-coding region of the KLK-L2 gene is formed of a 879-bp nucleotide sequence encoding a deduced 293-amino acid polypeptide with a predicted molecular mass of 32 kDa. There are two potential translation initiation codons (ATG) at positions 1 and 25 of the predicted first exon (numbers refer to GenBank™ accession number AF135028). We assume that the first ATG will be the initiation codon, since 1) the flanking sequence of that codon (GCGGCAATGG) matches closely with the Kozak consensus sequence for initiation of translation (GCC(A/G)CAATGG) (22) and is exactly the same as that of the homologous zyme gene. (15) At this initiation codon, the putative signal sequence at the N terminus is similar to other trypsin-like serine proteases (prostate and enamel matrix serine proteinase (EMSP) (Fig. 3). The cDNA ends with a 328-bp 3'-untranslated region containing a conserved polyadenylation signal (AAATTTAAAA) which was located 11 bp up-stream of the poly(A) tail (at a position exactly the same as that of the zyme poly(A) tail) (14).

A hydrophobicity study of the KLK-L2 gene shows a hydrophobic region in the N-terminal region of the protein (Fig. 4), suggesting that a presumed signal peptide is present. By computer analysis, a 29-amino acid signal peptide is predicted with a cleavage site at the carboxyl end of Ala29. For better characterization of the predicted structural motif of the KLK-L2 protein, it was aligned with other members of the kallikrein multigene family, (Fig. 3), and the predicted signal peptide cleavage
site was found to match with the predicted signal cleavage sites of zyme (14), KLK1 (23), KLK2 (24), and prostate/KLK-L1 (25) genes. Also, sequence alignment supports by analogy the presence of a cleavage site at the carboxyl end of Ser66, which is the exact site predicted for cleavage of the activation peptide of all the other kallikreins aligned in Fig. 3. Interestingly, the starting amino acid sequence of the mature protein IING(E/S)DC is conserved in the prostase and enamel matrix serine proteinase 1 (EMSP) genes. Thus, like other kallikreins, KLK-L2 is likely also synthesized as a pre-proenzyme that contains an N-terminal signal peptide (prezymogen) followed by an activation peptide and the enzymatic domain.

The presence of aspartate (D) in position 239 suggests that KLK-L2 will possess a trypsin-like cleavage pattern like most of the other kallikreins (e.g. KLK1, KLK2, trypsin-like serine protease (TLSP), neuropsin, zyme, prostase, and EMSP) but different from PSA, which has a serine (S) residue in the corresponding position and is known to have a chymotrypsin-like activity (Fig. 3) (3). The dotted region in Fig. 3 indicates an 11-amino acid loop characteristic of the classical kallikreins (PSA, KLK1, and KLK2) but not found in KLK-L2 or other members of the kallikrein-like gene family (14).

Homology with the Kallikrein Multi-gene Family—We aligned the mature 227-amino acid sequence of the predicted protein against the GenBank™ database and the known kallikreins using the BLASTP and BLAST 2 sequence programs. KLK-L2 is found to have 54% amino acid sequence identity and 68% similarity with the EMSP1 gene, 50% identity with both TLSP and neuropsin genes, and 47, 46, and 42% identity with trypsinogen, zyme, and PSA genes, respectively. Multiple alignment study shows that the typical catalytic triad of serine proteases is conserved in the KLK-L2 gene (His108, Asp153, and Ser245), and as is the case with all other kallikreins, a well-conserved peptide motif is found around the amino acid residues of the catalytic triad (i.e. histidine (WLLTAAH), serine-(GDSSGGP), and aspartate(DMLI)) (14, 16).

Twelve cysteine residues are present in the putative mature KLK-L2 protein; 10 of them are conserved in all the serine proteases that are aligned in Fig. 3 and would be expected to form disulfide bridges. The other two cysteines (Cys178 and Cys279) are not found in PSA, KLK1, KLK2 or trypsinogen; however, they are found in similar positions in prostase, EMSP1, zyme, neuropsin, and TLSP genes and are expected to form an additional disulfide bond. Twenty-nine “invariant” amino acids surrounding the active site of serine proteases

| Gene      | Primer name | Sequencea | Product size (base pairs) |
|-----------|-------------|-----------|--------------------------|
| KLK-L2    | KS          | GGATCTTTAACCAGCAGAAGA   | 342                      |
|           | KAS         | GTCTGGAGAGTACAGATTCT    |                          |
| pS2       | PS2S        | GTGATGTGGCCCTGCTGCCT    | 328                      |
| PSA       | PSAS        | AGGTGCAGTGAGGAGGTCGAG   | 754                      |
| Actin     | ACTINS      | TGGGAACATCTGCAAGCTTTTC  | 372                      |
| KLK-L2    | R1          | CCAGAGGCAGCTGCAAGCTTTTC |              |
|           | R2          | TGAAACATCTGCAAGCTTTTC  |              |

All nucleotide sequences are given in the 5'-→3' orientation.

Fig. 1. An approximate 300-kb region of almost contiguous genomic sequence around chromosome 19q13.3- q13.4. Genes are represented by horizontal arrows denoting the direction of the coding sequence. Distances between genes are mentioned in base pairs. The figure is not drawn to scale.

Fig. 2. Genomic organization and partial genomic sequence of the KLK-L2 gene. Intronic sequences are not shown for the splice junctions. Introns are shown with lowercase letters, and exons are shown with capital letters. For the full sequence, see GenBank™ accession number AF135028. The start and stop codons are circled, and the exon-intron junctions are boxed. The translated amino acids of the coding region are shown underneath a single-letter abbreviation. The catalytic residues are inside triangles. The putative polyadenylation signal is underlined.
have been described (26). Of these, 26 are conserved in KLK-L2. One of the nonconserved amino acids (Ser210 instead of Pro) is also found in prostase and EMSP1 genes, the second (Leu103 instead of Val) is also found in TLSP gene, and the third (Val174 instead of Leu) is also not conserved in prostase or EMSP1 genes. According to protein evolution studies, each of these amino acid changes represents a conserved evolutionary substitution to a protein of the same group (26, 27).

Evolution of the KLK-L2 Gene—To predict the phylogenetic relatedness of the KLK-L2 gene with other serine proteases, the amino acid sequences of the kallikrein genes were aligned together using the Clustal X multiple alignment program, and a distance matrix tree was predicted using the Neighbor-joining/UPGMA method (Fig. 4). Phylogenetic analysis separated the classical kallikreins (KLK1, KLK2, and PSA) and grouped the KLK-L2 with prostase/KLK-L1, EMSP1, and TLSP, in consistence with previously published studies (25, 28).

Tissue Expression of the KLK-L2 Gene—As shown in Table III and Fig. 5, the KLK-L2 gene is primarily expressed in the brain, mammary gland, and testis, but lower levels of expression are found in many other tissues. To verify the RT-PCR specificity, the PCR products were cloned and sequenced.

Hormonal Regulation of the KLK-L2 Gene—A steroid hormone receptor positive breast cancer cell line (BT-474) was used as a model to verify whether the KLK-L2 gene is under steroid hormone regulation. PSA was used as an estrogen up-regulated control. Our results indicate that KLK-L2 is up-regulated by estrogens and progestins (Fig. 6).

DISCUSSION

Kallikreins are a subgroup of serine proteases that play important roles in diverse physiological processes (3). Recently, some of the kallikrein genes were found to be linked to the development and/or progression of different types of malignancies. Human kallikrein 3 (KLK3/PSA) is the best diagnostic and prognostic marker for prostate cancer to date (29, 30). Recombinant KLK2 has been shown to activate PSA in vitro (31), and the combination of KLK2 and free PSA has recently been found to increase the discrimination between prostate cancer and benign prostatic hyperplasia in patients with moderately elevated total PSA levels (32).

NES1 appears to be a tumor suppressor gene (33), and zyme (protease M/neurosin) may be important in the establishment of breast and ovarian tumors and may function later in progression as a potential metastatic inhibitor (15). At least three kallikrein genes (PSA, NES1, and zyme) are down-regulated in breast cancer.

In search for new genes that may be involved in malignant diseases, we investigated the possible existence of new kallikreins. Based on mapping of the rodent kallikrein genes and the documented strong conservation between the human chro-
mosome 19q13.1-q13.4 region and the 17 loci in a 20-centimorgan proximal part of mouse chromosome 7 (10, 34), we identified a putative “critical region.” With the aid of computer programs for gene prediction and the available EST data base, we were able to identify a new gene, named KLK-L2 (for kallikrein-like gene 2). The 3′ end of the gene was verified by the presence of poly(A) stretches in the sequenced ESTs that were not found in the genomic sequence, and the start of translation was identified by the presence of a start codon in a well-conserved consensus Kozak sequence.

As is the case with other kallikreins, the KLK-L2 gene is composed of 5 coding exons and 4 intervening introns and, except for the second coding exon, the exon lengths are comparable to those of other members of the kallikrein gene family (Fig. 7). The exon-intron splice junctions were identified by comparing the genomic sequence with the EST sequence.

As is the case with most other kallikreins, except PSA and HSCCE, KLK-L2 is more functionally related to trypsin than to chymotrypsin (3). The wide range of tissue expression of KLK-L2 should not be surprising since by using the more sensitive RT-PCR technique instead of Northern blot analysis (36), many kallikrein genes were found to be expressed in a wide variety of tissues including salivary gland, kidney, pancreas, brain, and tissues of the reproductive system (uterus, mammary gland, ovary, and testis) (3). KLK-L2 is highly expressed in the brain and has been shown to have important roles in neural plasticity in mice (18). Also, the zyme gene is highly expressed in the brain and appears to have amyloid-
Molecular Characterization of the KLK-L2 Gene

In conclusion, we characterized a new member of the human kallikrein gene family, KLK-L2. This gene is hormonally regulated, and it is mostly expressed in the brain, mammary gland, and testis. Based on our experience with other kallikreins that are already used as valuable tumor markers (PSA, hk2), we speculate that KLK-L2 may also have utility in similar applications. This possibility, as well as the physiological function of the protein, need further investigation.

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Note Added in Proof—We have now identified an untranslated exon of the KLK-L2 gene; this is described in our updated GenBank™ submission (AF135028).

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