

Emerging Interest in the Kallikrein Gene Family for Understanding and Diagnosing Cancer

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Kallikreins are proteolytic enzymes that constitute a subfamily of serine proteases. Novel kallikrein genes were cloned recently, and it was shown that the human kallikrein family contains 15 genes tandemly aligned on chromosomal locus 19q13.3-q13.4. Based on their altered expression in tumor cells, kallikreins may be involved in the pathogenesis and/or progression of cancer. Evidence is presented that certain kallikreins may be exploited as diagnostic cancer biomarkers. Although the function(s) of novel kallikreins is currently unknown, increasing evidence suggests that kallikreins may participate in regulatory enzymatic cascade(s). Elucidation of the function of novel kallikreins largely depends on the availability of active recombinant proteins. Here, the zymogen for kallikrein 13 was overexpressed in *Pichia pastoris* and biochemically characterized. It was shown that the kallikrein 13 zymogen displays intrinsic catalytic activity leading to autoactivation. A clipped form of kallikrein 13 was identified, indicating autocatalytic cleavage at the internal bond R¹¹⁴-S¹¹⁵. Mature kallikrein 13 displays trypsin-like activity with restricted specificity on synthetic and protein substrates. Combinatorial P1-Lys libraries of tetrapeptide fluorogenic substrates were synthesized and used for the profiling of the P2 specificity of selected kallikreins. Interestingly, it was shown that human kallikrein 13, similarly to PSA, could specifically cleave human plasminogen to generate angiostatin-like fragments, suggesting that specific kallikreins may have antiangiogenic actions. An understanding of the physiology of human kallikreins is emerging with potential clinical applications.

Key words: Kallikreins; Serine proteases; Human kallikrein 6; Human kallikrein 13; Cancer biomarkers; Angiostatin

Kallikreins are secreted serine proteases and were traditionally defined by their ability to release vasoactive peptides (kinins) from kininogen (1). Because human kallikreins exert a variety of physiological functions, kallikreins are now defined in terms of DNA and protein sequence homologies and gene structure similarities (2). Until recently, only three human kallikreins were known: KLK1 (pancreatic renal kallikrein, hK1³), KLK2 (glandular kallikrein, hK2), and KLK3 (prostate-specific antigen, PSA or hK3). Subsequently, the KLK6 gene encoding human kallikrein 6 (hK6) was identified and cloned by differential display as being overexpressed in a primary breast tumor but absent in the metastatic tumor of the same patient, and it was named protease M (3). The same gene was later cloned from an adenocarcinoma cell line and was named neurosin (4), and from brains of Alzheimer's patients and it was named zyme (5). Zyme was proposed to play a role in the pathophysiology of Alzheimer's disease (5). According to the internationally accepted nomenclature (6), protease M/zyme/neurosin was later named hK6. Similarly, the normal epithelial

cell-specific 1 gene (NES1 or hK10) was cloned by subtractive hybridization based on its absent expression in metastatic breast tumors and seems to function as a tumor suppressor in vitro and in vivo (7,8).

The progressive development of genetic tools and the large amount of sequence information available from the Human Genome Project enabled the detailed mapping of the kallikrein cluster (9–11). It was shown that it contains at least 15 genes tandemly aligned on chromosomal locus 19q13.3-q13.4 (Fig. 1). Interestingly, the human kallikrein locus contains 10 clusters of a unique minisatellite element, which is polymorphic, and it was shown that the distribution of the different alleles of these minisatellites may be associated with malignancy (12). Ten clusters of this minisatellite are distributed along the kallikrein locus. These clusters are mainly located in the promoters and enhancers of genes, in introns, and in the untranslated regions of mRNAs. PCR analysis of two clusters of these elements indicated that they are polymorphic and the distribution of the different alleles of these minisatellites may be associated with malignancy

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²Abbreviations used: hK, human kallikrein (protein); KLK, human kallikrein (gene); PSA, prostate-specific antigen; BAEE, *N*- α -benzoyl-L-arginine-ethyl-ester; TAME, *N*- α -tosyl-arginine-methyl-ester; AMC, 7-amido-4-methylcoumarin.

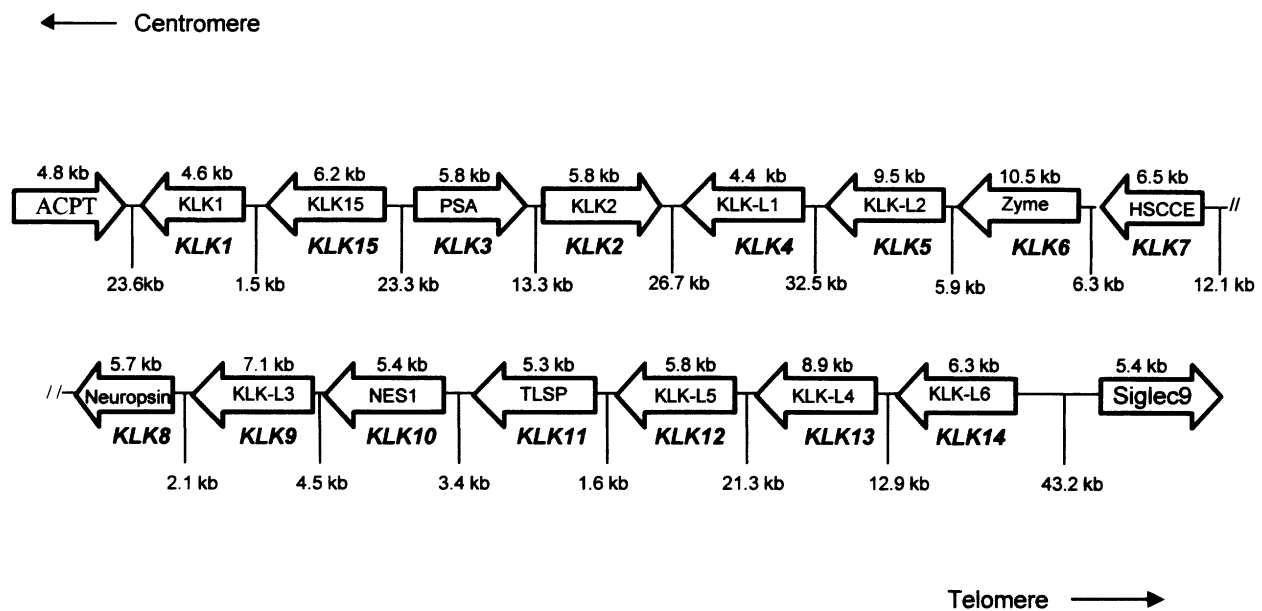


Figure 1. Schematic presentation of the human kallikrein gene cluster on chromosomal locus 19q13.3-q13.4. The original gene names are presented inside the arrows with official names indicated in bold under each arrow. Arrowheads show the direction of transcription. Gene lengths are indicated above each gene and the lengths between genes are shown to the approximate kilobase. The genes Siglec 9 and testicular acid phosphatase (ACPT) are not members of the kallikrein family. Figure is not drawn to scale.

(12). Kallikrein genes are predicted to encode serine proteases with either trypsin- or chymotrypsin-like activity. The residues of the catalytic triad of serine proteases are conserved in all human kallikreins. An aspartate residue is present in the substrate binding pocket of most kallikreins, indicating a trypsin-like specificity, while a serine residue present in PSA confers chymotrypsin-like activity. All kallikreins are synthesized as prepropeptides with a signal peptide of 17–20 amino acids (aa) at their NH₂-terminus, followed by the mature (enzymatically active) protein. Kallikreins are expressed in many tissues, including steroid hormone-producing or hormone-dependent tissues, such as the prostate, breast, ovary, and testis (2). There is growing evidence that several of the kallikrein genes are related to malignancy and may serve as tumor biomarkers, as reviewed by Yousef and Diamandis (2). PSA is the best tumor marker currently available for the diagnosis and monitoring of prostate cancer (13,14) and hK2 is used as an additional biomarker (15). Recent evidence suggests that hK6 may represent a novel circulating tumor marker for the diagnosis and monitoring of ovarian tumors (16–18). However, the physiological function(s) of PSA and hK2 and their possible role(s) in the growth and dissemination of malignant tumors are not well understood. Elucidation of the physiological role(s) of novel kallikreins largely depends on the availability of adequate amounts of active recombinant proteins, their detailed biochemical characterization, and the identification of their physiological substrates. It should be noted that the use of PSA and hK2 proteins purified from biological samples has

often led to results that are contradictory or difficult to interpret due to residual contaminants in the protein used in functional assays (14).

In this report, active recombinant hK13 was produced in *Pichia pastoris* (yeast) to a final yield of 14 mg purified protein per liter of yeast culture and characterized in terms of its mechanism of activation, enzymatic activity, and substrate specificity. Recombinant hK6 and hK15 were compared with hK13 in enzymatic activity assays using synthetic and protein substrates. All three kallikreins are encoded by genes that display aberrant expression in malignant tumor cells. The KLK6 and KLK13 genes are usually downregulated or completely inactivated in metastatic breast tumor cells (3,19), while the KLK15 gene was reported to be upregulated in prostate cancer (20). Furthermore, it was shown recently that the hK15 protein may be involved in a proteolytic cascade regulating the activation of pro-PSA (21). The large-scale production of active recombinant hK13 described in this report will be useful in investigating its putative role in cancer, and the characterization of its substrate specificity should facilitate the discovery of specific and selective substrates and potent inhibitors. Finally, it was shown here that human plasminogen could be a physiological substrate for hK13, because this enzyme could generate angiostatin-like fragments by specific proteolysis of plasminogen. Angiostatin is a circulating endogenous inhibitor of angiogenesis and metastatic growth (22,23). Recently, PSA was also shown to have antiangiogenic and antimetastatic activity that is related to its function as a serine protease and results from the pro-

duction of angiotensin from plasminogen (24,25). Our data suggest that other kallikreins should be evaluated for potential antiangiogenic actions.

MATERIALS AND METHODS

Expression and Purification of Recombinant Proteins

Recombinant hK6 was produced as described elsewhere (Tsetsenis, T., Bayés, A., Ventura, S., Avilés, F. X., Vendrell, J., Sotiropoulou, G. Human kallikrein 6: An autoregulated serine protease aberrantly expressed in tumor cells and able to generate angiotensin from plasminogen, submitted, 2002).

The cDNA encoding pre-pro-KLK13 was cloned by RT-PCR using total RNA isolated from the 76N normal mammary epithelial cell strain. The primers used for PCR amplification were: 5'-CCGAATTCGGAGCCATGTGGCCCTGG-3' (sense) and 5'-GCAGAATTCCTCAACTTTTATTGTGGGCC-3' (antisense). Restriction sites used for cloning are underlined. Total RNA was isolated from actively dividing 76N cells using the RNeasy Mini kit, and RNase-free DNase I (Qiagen) was added to remove residual DNA contamination. The OneStep RT-PCR kit (Qiagen) was used for RT-PCR. The cDNA sequence encoding pro-hK13 (S²⁰-Q²⁷⁷) was amplified with the primers: 5'-GGCTCGAGAAAAGATCCCAGGAGTCTTCC-3' (sense) and 5'-GCAGAATTCCTCAACTTTTATTGTGGGCC-3' (antisense). The PCR product was cloned into the *Pichia pastoris* expression vector pPIC9 (Invitrogen) between the 5' promoter and the 3' terminator of the AOX1 gene in-frame with the yeast α -mating factor (26). The *Pichia pastoris* strains GS115 (his4) and KM71 were stably transformed with the expression construct using the *Pichia* Expression Kit (Invitrogen). Expression of the recombinant protein was induced by methanol and monitored by SDS-PAGE analysis of the cell culture supernatant. The recombinant protein was purified by hydrophobic interaction chromatography on a Butyl-Toyopearl 650 M column (Tosoh). Mass spectrometry and NH₂-terminal sequencing were carried out as described previously (27). All DNA manipulations were carried out according to standard methods using the *E. coli* strain DH5 α (28). Restriction endonucleases and *Taq* DNA polymerase were purchased from Gibco BRL, T4 DNA ligase and *Vent* polymerase from New England Biolabs, and ultrapure 2'-deoxynucleotide, 5'-triphosphates (dNTPs) from Pharmacia. Culture media for *E. coli* were purchased from Difco and for *Pichia pastoris* from Sigma.

Activity Assays

The proteolytic activity of recombinant enzymes was tested in vitro using synthetic chromogenic and fluorogenic substrates. The activity against BAEE (*N*- α -benzoyl-L-arginine-ethyl-ester) was assayed in 125 mM phosphate buffer, pH 8.5, and that against TAME was assayed in 40 mM Tris-HCl, 10 mM CaCl₂, pH 8.0. The initial reaction rates were monitored with a Perkin Elmer spectrophotometer at 254 and 247 nm, respectively. Hydrolysis of the Z-Phe-Arg-AMC fluorogenic substrate or

the P2 sublibrary was monitored fluorometrically with an excitation wavelength of 380 nm. The initial rates of fluorescence emission were monitored at 460 nm on a Perkin Elmer Luminescence Spectrometer (LS50B) and all data points were collected in triplicate. The rate of substrate cleavage was measured in relative fluorescence units per minute. Stock solutions of the Z-Phe-Arg-AMC substrate were prepared in dimethyl sulfoxide (DMSO). The final concentration of DMSO was less than 2%. Assays were performed using an enzyme concentration of 12.5 nM and substrate concentrations of 2–50 μ M. Steady-state kinetic constants K_m and k_{cat} were determined from averaged data of initial reaction rates versus substrate concentration by nonlinear fitting to the Michaelis-Menten equation. All assays were carried out at 25°C and the reaction mixture was stirred. Self-hydrolysis of the substrates was negligible for the applied reaction times. Purified and TPCK-treated bovine trypsin (Worthington) was assayed for comparison. All substrates were purchased from Sigma.

Zymography

Protein samples were mixed with sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 4% SDS, and 0.01% bromophenol blue) and subjected to electrophoresis on 12% acrylamide gels containing 0.09% SDS and 0.1% copolymerized gelatin or 0.2% casein. To remove SDS, gels were incubated in 2.5% Triton X-100 for 2 h at room temperature and then in 0.1 M glycine-NaOH, pH 8.3, for 16 h at 37°C. For detection of protease activity, the gel was stained with Coomassie brilliant blue R-250 and destained in a mixture of 50% methanol and 7.5% acetic acid. Proteolytic activities were detected as clear zones in the blue background, indicating lysis of the gelatin or casein substrate.

Deglycosylation of Recombinant hK13

Recombinant hK13 (2 μ g) was deglycosylated, without previous denaturation, in a 40- μ l reaction by incubation in 50 mM Tris-HCl, pH 7.5, containing 4 units of *N*-glycosidase F (Boehringer Mannheim) for 24 h at 37°C.

Cleavage of Plasminogen

Plasminogen isolated from human plasma (Chromogenix) was incubated in the absence or presence of active hK13 at a molar ratio of 75:1 in 30 μ l of 10 mM Tris-HCl, pH 8.0, at 37°C. The reaction was stopped with reducing electrophoresis sample buffer (29). The cleavage products were resolved on a 12% polyacrylamide gel and stained with Coomassie brilliant blue R-250 or blotted onto Immobilon-P membrane (Millipore) for NH₂-terminal sequencing.

Synthesis of Sublibrary P2

The sublibrary P2 consists of 19 sub-sublibraries of the general type: Ac-[X]-[X]-[O]-Lys-AMC, where [X] is a mixture of 19 amino acids and [O] a spatially addressed amino acid for each individual sub-sublibrary.

The following 19 Fmoc-amino acids were used: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(O-*t*-Bu)-OH, Fmoc-Glu(O-*t*-Bu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-His(Boc)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Nle-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(O-*t*-Bu)-OH, Fmoc-Thr(O-*t*-Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(O-*t*-Bu)-OH, and Fmoc-Val-OH. The 4-sulfamylbutyryl AM resin was used as a solid support. The first Fmoc amino acid was attached to the resin by means of the PyBOP/DIPEA coupling reagent. The introduction of P3 position was performed by the reagent mixture method (30). Initially, the isokinetic mixture of 19 Fmoc amino acids was preactivated (DICl/HOBt) for 10 min. Subsequently, it was added to the resin, followed by agitation for 3.5 h. The resin was washed with DMF, CH₂Cl₂, and MeOH. The introduction of the P4 position was performed in the same manner as for the P3 position. After the incorporation of the last isokinetic mixture, the Fmoc-protecting group was removed as usual and the free amino group was acetylated by means of AcOH/DICl/HOBt. Subsequently, the resin was activated with iodoacetonitrile (31). Then the mixture of peptides was removed from the solid support by the reaction of activated resin with an excess of H-Lys(Boc)-AMC, which was previously prepared for this purpose. The filtrates were collected and the solvent was evaporated. The excess of H-Lys(Boc)-AMC present in the mixture was removed by treatment of the sub-sublibrary with isocyanate polystyrene resin, which in this case acts as scavenger resin (32). The filtrates were collected and evaporated to dryness under reduced pressure. The remaining protecting groups were removed by treatment of the sub-sublibrary with a mixture of TFA/H₂O/TIS (95:2.5:2.5) for 3 h (33). The solvents were removed under vacuum and the sub-sublibrary residue was dissolved in MeCN/H₂O in the presence of 0.1% AcOH and was lyophilized. To obtain the 19 sub-sublibraries for enzymatic screening, the procedure was repeated 18 times with a different Fmoc amino acid attached to the resin each time.

Synthesis of the α -Aryl Lactone Inhibitor

For the synthesis of the α -aryl-lactone inhibitor {3-[4-(guanidinomethyl)phenyl]-6-methylidene-tetrahydro-2-pyranone} the method described by Rai and Katzenellenbogen (34) was modified. The enol lactone was synthesized using 4-(bromomethyl) phenyl acetic acid as the starting material. Treatment of the acid with ammonia yielded an amino acid, which was further protected as the *tert*-butyloxy carbonyl (BOC) derivative. Alkylation of this protected amino acid with 4-bromo-1-butyne (35), using 3.3 equiv of LDA and 3.3 equiv of *n*-BuLi prior to the addition of the electrophile, proceeded in moderate yield. The acetylenic acid was deprotected, and the guanidino group was introduced by refluxing the resulting amino acid with 3,5-dimethylpyrazole-1-carboxamide nitrate in the presence of diisopropylethylamine. The guanidino acid was solubilized in acetonitrile containing a trace of TFA. Cyclization to the lactone proceeded smoothly in the presence of a catalytic quantity of mercuric trifluoroacetate.

RESULTS

Production and Activation of Recombinant pro-hK13

The full-length cDNA encoding hK13 was cloned by RT-PCR using total RNA isolated from 76N normal mammary epithelial cells. The sequence encoding pro-hK13 (S²⁰-Q²⁷⁷) was amplified from the cDNA template and cloned into the pPIC9 vector in-frame with the yeast α -mating factor. The *Pichia pastoris* recognition signal (Leu-Glu-Lys-Arg-X-) designed for proteolytic processing was included at the amino-terminus. The recombinant protein was secreted and accounted for approximately 90% of the total protein in the culture supernatant. Subsequently, it was purified by hydrophobic interaction chromatography and analyzed by denaturing SDS-PAGE (Fig. 2, left lane). The yield of recombinant protein production was 14 mg of purified protein per liter of yeast culture. Pre-pro-hK13 consists of 277 aa and contains a predicted signal peptide (M¹-S¹⁶) and an activation peptide (Q²¹-K²⁵) with a predicted activation site between K²⁵-V²⁶ (19). Although the molecular mass of pro-hK13 (zymogen) inferred from the primary sequence is about 28.8 kDa, recombinant pro-hK13 migrated as a diffuse band at about 45–48 kDa in denaturing gels (Fig. 2, left lane). Nonetheless, recombinant hK13 was correctly

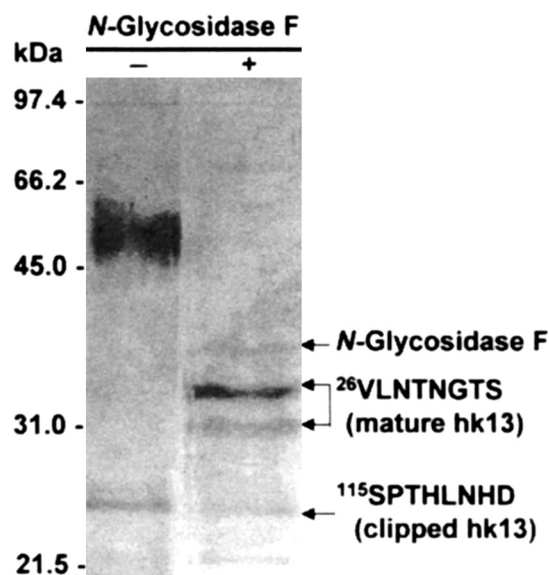


Figure 2. Overexpression of recombinant human kallikrein 13. For the production of pro-hK13 (S²⁰-Q²⁷⁷), *Pichia pastoris* was stably transformed with the expression construct pPIC9/pro-KLK13. The recombinant protein was purified by hydrophobic interaction chromatography, was incubated in the absence (left lane) or presence (right lane) of *N*-glycosidase F, and subjected to electrophoresis on a 12% polyacrylamide gel. The NH₂-terminal sequences of deglycosylated proteins were determined experimentally and are indicated on the right. The upper bands displayed the NH₂-terminal sequence of mature hK13 (26VLNTNGTS-) and the lower bands that of internally clipped hK13 (115SPHLNHD-). Molecular weight markers are shown on the left in kilodaltons.

processed but its NH₂-terminal sequence was determined to be that of mature hK13 (²⁶VLNTNGTS-), indicating that the zymogen was fully activated. This was confirmed by the fact that the activity of purified hK13 against BAEE could not be increased upon treatment with Lysil endopeptidase or trypsin (not shown). A minor band of 25 kDa was also detected in the purified protein (Fig. 2, left lane), which displayed the NH₂-terminal sequence S¹¹⁵PTHLNHD-; therefore, it corresponds to an internal fragment of hK13 (S¹¹⁵-Q²⁷⁷). It most likely results from proteolytic self-cleavage of hK13 at the internal bond R¹¹⁴-S¹¹⁵. It should be noted that the amount of the 25-kDa fragment varied between different protein preparations, being undetectable in some preparations. Clipping of mature hK13 at R¹¹⁴ is likely a process of autoinactivation, because none of the resulting fragments retain all residues of the catalytic triad (H⁷⁶, D¹²⁴, S²¹⁸) that are essential for the enzymatic activity. It should be noted that the enzymatic activity of purified hK13 against BAEE was stable when the protein was kept at 25°C for 24 h (not shown), indicating a very slow rate of autoproteolysis.

To investigate the possibility that the increase in molecular weight is due to glycosylation, hK13 was treated with *N*-glycosidase F, an amidase that removes *N*-linked oligosaccharides from glycoproteins by cleaving the *N*-glycosidic bond between Asn and the first hexoseamine. A shift of the 45–48-kDa band was observed, yielding two bands of approximately 33 and 29 kDa. Both resulting bands appeared sharper than the 45–48-kDa species, which migrated as a diffuse band due to the presence of carbohydrates (Fig. 2, right lane vs. left lane). Similarly, the 25-kDa band was shifted to about 20 kDa, although at least half of the protein remained glycosylated. The NH₂-terminal sequence of both upper bands corresponded to mature hK13 (V²⁶LNTNGTS-). Therefore, the 33-kDa band represents the partially deglycosylated mature hK13. The NH₂-terminal sequence of both lower bands corresponded to clipped hK13. This result indicates that recombinant hK13 is glycosylated probably at both asparagine residues N³⁰ and N²²⁵, predicted as sites for *N*-linked glycosylation (19).

Given that the hK13 zymogen is entirely converted to mature hK13, it is most likely that this occurs via an autoactivation mechanism. The possibility that contaminating yeast protease(s) could be copurified with pro-hK13 recombinant protein was excluded, because no contaminating proteases were detected upon overloading recombinant hK13 on nonreducing substrate gels (zymograms) containing either 0.2% casein or 0.1% gelatin (Fig. 3). Bovine trypsin was tested as a positive control. Similarly to pro-hK13, upon overexpression of pro-hK6 in *Pichia pastoris*, it was found that the zymogen could be autoactivated to an active but unstable mature enzyme, yielding the self-cleavage product D⁸¹-K²⁴⁴ that is inactive (Tsetsenis, T., Bayés, A., Ventura, S., Avilés, F. X., Vendrell, J., Sotiropoulou, G. Human kallikrein 6: An autoregulated serine protease aberrantly expressed in tumor cells and able to generate angiostatin from plasminogen, submitted, 2002). This indicated autoproteolytic cleavage at the internal bond R⁸⁰-E⁸¹. Recently,

Bernett et al. (36) made the same observation. However, Bernett et al. have hypothesized that an autoinactivation is not likely to occur in pro-hK6, because it displays a low k_{cat}/K_m value for lysine in the P1 position (36). By engineering wild-type hK6, Tsetsenis et al. (Tsetsenis, T., Bayés, A., Ventura, S., Avilés, F. X., Vendrell, J., Sotiropoulou, G. Human kallikrein 6: An autoregulated serine protease aberrantly expressed in tumor cells and able to generate angiostatin from plasminogen, submitted, 2002) have shown that the enzymatic activity of hK6 is regulated by an autoactivation/autoinactivation mechanism. Autoactivation of the hK6 zymogen requires cleavage at K²¹. Based on the x-ray crystal structure of pro-hK6, which was described recently (37), the zymogen displays a completely closed specificity pocket and a unique conformation of the regions involved in structural rearrangements upon proteolytic cleavage activation, which allows postulating a novel (auto)activation mechanism for pro-hK6 (37). It should be noted that a single substitution at R⁸⁰ → Q is sufficient to stabilize the activity of mature hK6 (Tsetsenis, T., Bayés, A., Ventura, S., Avilés, F. X., Vendrell, J., Sotiropoulou, G. Human kallikrein 6: An autoregulated serine protease aberrantly expressed in tumor cells and able to generate angiostatin from plasminogen, submitted, 2002). Fully autoactivated mature and stabilized hK6 (R80Q) was used in all activity assays described here.

Enzymatic Activity and Substrate Specificity

On the basis of their primary sequences and the presence of the acidic amino acid D¹⁹¹ in their substrate binding pocket, mature hK6 and hK13 are both predicted to cleave their target substrate(s) with specificity for the basic amino acids R or K (38). Consistent with a trypsin-like activity, mature hK13 and hK6 (R80Q) could both cleave the BAEE chromogenic substrate. However, both enzymes were poorer catalysts than trypsin, because the activity of hK6 against BAEE corresponded to 64% and that of hK13 to 56% of the activity of an equimolar amount of trypsin for the same assay conditions. Interestingly, neither hK6 nor hK13 could hydrolyze the TAME substrate (*N*- α -tosyl-arginine-methyl-ester) that is typical for trypsin, suggesting that both enzymes display more restricted substrate specificity than trypsin, while no activity was observed against chymotrypsin-, plasmin-, or elastase-specific substrates (not shown). Zymography revealed a limited hydrolysis of gelatin by active hK13, while casein was not cleaved (Fig. 3). Gelatin was degraded by mature hK6, although with markedly lower efficiency when compared with trypsin (Fig. 3), while casein was barely cleaved. These results are consistent with the hypothesis that hK6 and hK13 display restricted specificity on protein substrates.

Determination of Steady-State Kinetic Constants

The steady-state kinetic constants were determined for the hydrolysis of the Z-Phe-Arg-AMC fluorogenic substrate by active hK6 and hK13. The enzyme concentration used was 12.5 nM. Both enzymes exhibited characteristic Michaelis-Menten kinetics with the Z-Phe-Arg-AMC substrate. For hK6, a K_m of 83 μ M and a k_{cat} /

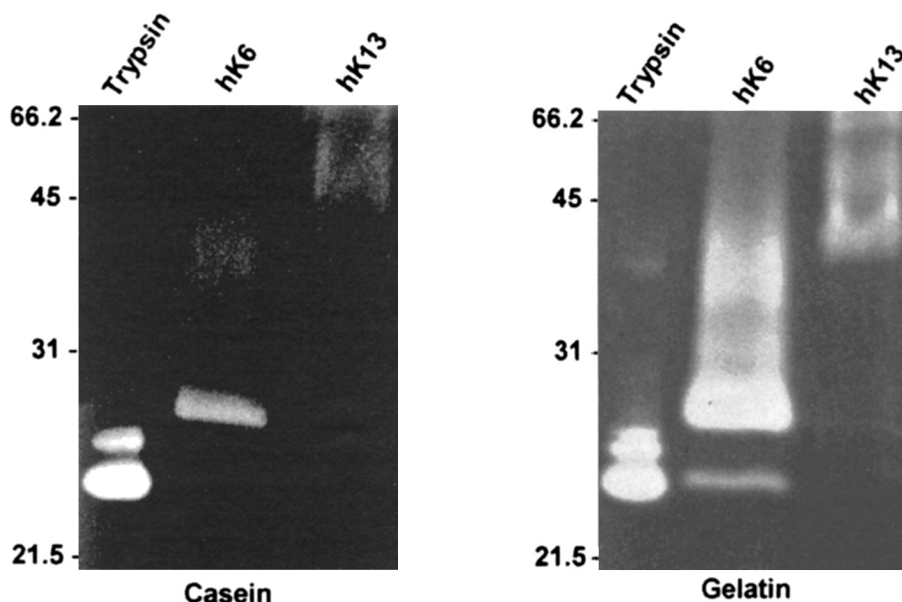


Figure 3. Activity of purified recombinant kallikreins on protein substrates. Casein and gelatin zymograms. To test whether hK6 and hK13 could cleave protein substrates, 1 μg of active recombinant hK6 (middle lane) and 1 μg of active recombinant hK13 (right lane) were analyzed on 12% polyacrylamide gels containing 0.09% SDS and either 0.1% copolymerized gelatin (right) or 0.2% casein (left). For comparison, 10 ng of bovine pancreatic trypsin was loaded (left lane). Subsequently, SDS was removed and gels were processed as described in Materials and Methods. Proteolytic activities were detected as clear zones in the blue background, indicating lysis of the gelatin or casein substrate. Molecular weight markers are shown on the left in kilodaltons.

K_m of $670 \text{ M}^{-1} \text{ s}^{-1}$ were determined. Under the same assay conditions, a K_m of $87 \mu\text{M}$ and a k_{cat}/K_m of $318 \text{ M}^{-1} \text{ s}^{-1}$ were determined for hK13, indicating that both enzymes can cleave this substrate efficiently.

P2 Substrate Specificity

Substrate specificity (the ability to discriminate among many potential substrates) is central to the function of proteases. Synthetic substrates are typically used to define substrate specificity but often result in a limited substrate specificity profile, especially for proteases with specificity beyond P1 [according to the Schechter and Berger nomenclature (39)]. In this case, combinatorial approaches are more efficient in profiling the substrate specificity (40,41). Because it was shown that both hK6 and hK13 zymogens display intrinsic catalytic activity leading to efficient autoactivation, it was postulated that both enzymes display amidolytic activity for K-X bonds because proteolytic cleavage of a K-X bond is required for their autoactivation. Therefore, the extended P4-P2 substrate specificity of active hK6 and hK13 was investigated using P1-Lys combinatorial libraries of tetrapeptide substrates that contained the fluorogenic leaving group 7-amino-4-methylcoumarin (AMC). Nineteen soluble P1 Lys and P2-diverse positional scanning protease substrate sublibraries were synthesized. The general type of the library was: Ac-[X]-[X]-[O]-Lys-AMC, where the

P1 position contains a Lys residue, [O] at P2 contains a spatially addressed amino acid for each individual sublibrary, and [X] at the P3 and P4 positions contains an equimolar mixture of 19 amino acids [Cys and Met excluded, Nle (n) included]. A total of 6859 peptides possessing amino acid diversity at the P4-P3-P2 positions were assayed and results are presented in Table 1. The rate of substrate cleavage was measured from the rate of fluorescence emission. None of the 19 sublibraries could be cleaved by active recombinant hK6. Similarly, none of the libraries was hydrolyzed by active hK13. As described above, both enzymes were active against the Z-Phe-Arg-AMC substrate. In contrast, all sublibraries were cleaved by trypsin to varying extents. Interestingly, hK15 was active against some of the sublibraries and, similarly to trypsin, it displayed the highest preference for Ala and second preference for Ser at the P2 position. Therefore, hK15 resembles trypsin and likely functions as a digestive protease, while the specificity of hK6 and hK13 might be restricted by the presence of specific amino acids in the P1' and P2' positions.

Cleavage of Plasminogen

Human plasminogen was efficiently hydrolyzed by mature hK13. As shown in Figure 4, purified hK13 caused hydrolysis of the 98-kDa pre-pro-plasminogen precursor and yielded three bands of approximately 44, 40.5, and

Table 1. Screening for the P2 Specificity of Novel Kallikreins Using Combinatorial Libraries

#	Sublibrary	% Enzymatic Activity*			
		hK6	hK13	hK15†	Trypsin
Y1	Ac-[X]-[X]-Lys-Lys-AMC	—	—	—	9
Y2	Ac-[X]-[X]-Ala-Lys-AMC	—	—	100	100
Y3	Ac-[X]-[X]-Val-Lys-AMC	—	—	+/-	26
Y4	Ac-[X]-[X]-Ser-Lys-AMC	—	—	78	85
Y5	Ac-[X]-[X]-Phe-Lys-AMC	—	—	—	14
Y6	Ac-[X]-[X]-Leu-Lys-AMC	—	—	—	14
Y7	Ac-[X]-[X]-Thr-Lys-AMC	—	—	55‡	10
Y8	Ac-[X]-[X]-Trp-Lys-AMC	—	—	—	10
Y9	Ac-[X]-[X]-Gln-Lys-AMC	—	—	—	22
Y10	Ac-[X]-[X]-Asp-Lys-AMC	—	—	—	13
Y11	Ac-[X]-[X]-Asn-Lys-AMC	—	—	+/-‡	55
Y12	Ac-[X]-[X]-Glu-Lys-AMC	—	—	—	18
Y13	Ac-[X]-[X]-His-Lys-AMC	—	—	+/-‡	63
Y14	Ac-[X]-[X]-Ile-Lys-AMC	—	—	—	10
Y15	Ac-[X]-[X]-Nle-Lys-AMC	—	—	73	76
Y16	Ac-[X]-[X]-Tyr-Lys-AMC	—	—	—	11
Y17	Ac-[X]-[X]-Gly-Lys-AMC	—	—	+/-	26
Y18	Ac-[X]-[X]-Arg-Lys-AMC	—	—	—	6
Y19	Ac-[X]-[X]-Pro-Lys-AMC	—	—	40‡	5

*Activities were normalized to the maximum activity observed for the library of highest preference. The concentration of the substrate was 50 μ M. Enzymes were used at concentrations of 10–20 nM. All recombinant proteins were active against the Z-Phe-Arg-AMC substrate. †Recombinant pro-hK15 was produced in *Pichia pastoris* and activated with immobilized trypsin (Sotiropoulou et al., unpublished data). ‡Marked differences in the substrate preference between mature hK15 and trypsin.

38 kDa (Fig. 4, lane 2). Small amounts of the 44- and 40.5-kDa species were observed when plasminogen was incubated at 37°C in the absence of hK13 (Fig. 4, lane 1) and are likely products of plasminogen autoproteolysis, although the extent of proteolysis by hK13 was more pronounced. Based on its size, the 38-kDa band corresponds to an angiostatin-like fragment (42,43), although its NH₂-terminal sequence remains to be determined. The 38-kDa band was specifically produced by the hK13 proteolytic activity, because its production was prevented by the α -aryl lactone inhibitor (Fig. 4, lane 3), which inhibits the hK13 activity (not shown). Also, PSA has been shown to produce biologically active angiostatin by cleaving pre-pro-plasminogen (24). Our results indicate that hK13 and possibly other kallikreins should be evaluated for antiangiogenic function.

DISCUSSION

In the present study, recombinant pro-hK13 was produced and characterized. It was shown that the hK13 zymogen displays intrinsic catalytic activity leading to full activation to the corresponding mature/active enzyme. Active hK13 displays trypsin-like activity with restricted substrate specificity shown by the fact that gelatin, casein, and synthetic peptide substrates were not efficiently hydrolyzed. PSA was shown to display chy-

motrypsin-like activity (44), also with restricted specificity on physiological substrates (45). The observed efficient autoactivation of hK13 and hK6 zymogens shows that both enzymes display amidolytic activity for K-X bonds. Screening of combinatorial P1-Lys P2-scanning tetrapeptide sublibraries showed that neither hK6 nor hK13 could cleave any of the 6859 substrates, although their enzymatic activity was confirmed using the Z-Phe-Arg-AMC fluorogenic substrate. This suggests that the specificity of hK6 and hK13 may, as well, be restricted by the presence of specific amino acids in the P1' and P2' positions. On the contrary, hK15 seems to be a digestive type of enzyme with similarities to trypsin, although some differences in its P2 preference were observed when compared with trypsin. Detailed characterization of the substrate specificity of hK6 and hK13 will require the application of substrate phage display. Knowledge of the substrate specificity of kallikreins may not only give valuable insights into their physiological substrates, it will also provide the basis for the design of specific and selective substrates and potent inhibitors.

Kallikreins as Cancer Biomarkers

There is strong but circumstantial evidence linking kallikreins and cancer. PSA (hK3) and hK2 are widely

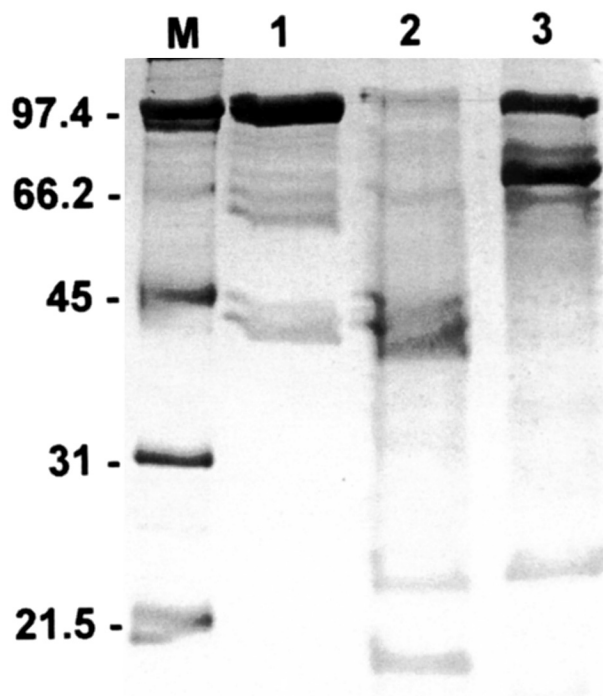


Figure 4. Hydrolysis of plasminogen by hK13. Plasminogen isolated from human plasma was incubated in the absence (lane 1) or presence (lane 2) of active hK13 at a molar ratio of 75:1. The cleavage products were resolved on a 12% polyacrylamide gel and stained with Coomassie brilliant blue R-250. The specificity of plasminogen cleavage was assessed in the presence of a synthetic α -aryl-lactone inhibitor of the enzymatic activity of hK13 (lane 3). Molecular weight markers are shown on the left (M) in kilodaltons.

used as tumor markers for the diagnosis and monitoring of prostate cancer (13,14), and PSA was proposed as a marker for the prognosis of breast cancer (46,47). Accumulating evidence suggests that other kallikreins could also be related to hormonal malignancies (breast, prostate, testicular, ovarian). For example, KLK8 (neuropsin, TADG-14) and KLK7 are differentially expressed in ovarian cancer (48,49). In addition, KLK4 and KLK5 are indicators of poor prognosis (50–52), while KLK9 has been shown to be a marker of favorable prognosis in ovarian cancer (53). Table 2 summarizes all the available data on measurements of kallikrein proteins in serum and tumor tissue extracts for the purpose of disease diagnosis, monitoring, prognosis, or subclassification. Based on these data, hK6 (16–18), hK10 (54,55), and hK11 (56) are emerging serum biomarkers for ovarian cancer. In addition to their diagnostic/prognostic potential, kallikreins may also constitute attractive targets for the development of therapeutics (2,14).

Kallikreins May Participate in a Novel Regulatory Enzymatic Cascade

The coexpression of multiple kallikreins in tissues indicates that they may participate in cascade enzymatic pathways. Consistent with this hypothesis, the differential expression of certain kallikreins in diverse malignancies follows a parallel pattern (2). Interactions between serine proteases are common, and substrates of serine proteases are usually other serine proteases that are activated from an inactive precursor (57). The coordinated action of serine proteases in cascade pathways underlies important regulatory functions and is manifested in the apoptosis pathway, the blood coagulation cascade ensuring a rapid and amplified response to trauma, and the plasminogen–plasmin pathway, which regulates blood clotting. The hypothesis that specific kallikreins are involved in a cascade enzymatic pathway(s) is supported by recent reports showing the ability of kallikreins to activate other kallikrein zymogens (20,59–61). For ex-

ample, PSA (hK3) is secreted as an inactive and stable zymogen, which is converted to active PSA extracellularly by the action of hK2 (58), while hK15 and prostase (hK4) can activate pro-PSA more efficiently when compared with hK2 (20,59,60). In addition, hK5 is predicted to activate pro-hK7 in the skin (62). Bhoola et al. have recently provided strong evidence for the involvement of a “kallikrein cascade” in initiating and maintaining systemic inflammatory responses and immune-modulated disorders (63). The participation of kallikreins in cascade reactions involving other nonkallikrein serine proteases is also possible, because there is experimental evidence that hK2 and hK4 can activate the proform of urokinase-type plasminogen activator (pro-uPA) (59,64). In addition, PSA can inactivate the amino-terminal fragment of the parathyroid hormone-related protein (PTHrP) (65) and was reported to cleave the insulin-like growth factor-binding protein-3 (IGFBP-3) (66). The fact that the hK13 zymogen possesses intrinsic catalytic activity, as shown here, indicates that it may act as an upstream activator in an enzymatic cascade.

Physiological Function of Kallikreins

Secreted serine proteases are expected to act on extracellular targets, such as components of the extracellular matrix, growth factors, or surface receptors. It is well documented that normal cell behavior is regulated by the interplay of positive and negative factors (67). Proteases are usually overexpressed in metastatic tumor cells and are considered to promote tumor invasion and metastasis by degrading components of the basement membrane and extracellular matrix. However, certain kallikrein genes, including PSA (KLK3) (14), KLK6 (3), KLK13 (14), and NES1 (KLK10) (7), exhibit a reduced or absent expression in breast and prostate metastatic tumor cells and may have a tumor suppressor function. In particular, NES1 has been suggested to be a tumor suppressor in breast cancer, because when transfected into MDA-MB-231 metastatic breast tumor cells, it com-

Table 2. Kallikrein Proteins as Cancer Biomarkers

Kallikrein	Sample Type	Method	Application	Ref.
hK2	Serum and tissue	Immunoassay; immunohistochemistry	Diagnosis, prognosis, and monitoring of prostate and breast cancer	15
hK3 (PSA)	Serum and tissue	Immunoassay; immunohistochemistry	Diagnosis, prognosis, and monitoring of prostate and breast cancer	15,46
hK6	Serum	Immunoassay	Diagnosis, prognosis, and monitoring of ovarian cancer	17,18
	Breast tumor cytosols	Immunoassay	Prognosis: association with hormone receptors	Diamandis et al., unpublished data
hK10	Serum	Immunoassay	Diagnosis and monitoring of ovarian cancer	55
	Ovarian tumor cytosols	Immunoassay	Prognosis; high levels associated with poor prognosis	54
hK11	Serum	Immunoassay	Diagnosis and prognosis of ovarian and prostate cancer	56

pletely suppressed anchorage-independent growth in soft agar and the growth of tumors implanted in nude mice (8). Interestingly, a recent study has shown that hK6 may be associated with the induction of differentiation of human head and neck squamous carcinoma cells (SCC25) to basal keratinocytes by the vitamin D₃ analog EB1089 (68). Expression profiling by oligonucleotide microarrays revealed that EB1089 reverses the malignant phenotype of SCC25 cells by inducing the expression of genes that are reduced or eliminated in cancer cells, with KLK6 (protease M) being one of the two most strongly induced genes (68). On the other hand, breast tumors expressing high levels of PSA had a better prognosis and overall survival than patients whose tumors expressed lower or no PSA levels (69). Recently, PSA was shown to inhibit angiogenesis by generating biologically active angiostatin from plasminogen (24,25). Angiostatin is a potent endogenous inhibitor of tumor angiogenesis (22,23) and is currently evaluated as an anticancer treatment. The mechanism(s) providing angiostatin *in vivo* is not known, although several proteolytic enzymes, such as specific matrix metalloproteinases, have been associated with the production of angiostatin *in vitro* (70–73). It should be noted, however, that these enzymes are usually secreted as inactive zymogens that need activation in order to exert their functions, and are produced by tumor-activated stromal cells, while angiostatin is thought to be produced by tumor cells (74). Therefore, tumor proteases are likely to be involved in its generation. We have shown here that human plasminogen was preferentially hydrolyzed by mature hK13 to generate angiostatin-like fragments. Therefore, inactivation of the KLK13 gene expression in metastatic breast tumor cells may contribute to tumor angiogenesis and metastasis.

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