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Biochemical and Enzymatic Characterization of Human Kallikrein 5 (hK5), a Novel Serine Protease Potentially Involved in Cancer Progression*

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Human kallikrein 5 (*KLK5*) is a member of the human kallikrein gene family of serine proteases. Preliminary results indicate that the protein, hK5, may be a potential serological marker for breast and ovarian cancer. Other studies implicate hK5 with skin desquamation and skin diseases. To gain further insights on hK5 physiological functions, we studied its substrate specificity, the regulation of its activity by various inhibitors, and identified candidate physiological substrates. After producing and purifying recombinant hK5 in yeast, we determined the k_{cat}/K_m ratio of the fluorogenic substrates Gly-Pro-Arg-AMC and Gly-Pro-Lys-AMC, and showed that it has trypsin-like activity with strong preference for Arg over Lys in the P1 position. The serpins α_2-antiplasmin and antithrombin were able to inhibit hK5 with an inhibition constant (k_{+2}/K_i) of 1.0×10^{-2} and 4.2×10^{-4} M^{-1} min^{-1}, respectively. No inhibition was observed with the serpins α_1-antitrypsin and α_1-antichymotrypsin, although α_2-macroglobulin partially inhibited hK5 at high concentrations. We also demonstrated that hK5 can efficiently digest the extracellular matrix components, collagens type I, II, III, and IV, fibronectin, and laminin. Furthermore, our results suggest that hK5 can potentially release (*a*) angiostatin 4.5 from plasminogen, (*b*) "cystatin-like domain 3" from low molecular weight kininogen, and (*c*) fibrinopeptide B and peptide β15-42 from the Bβ chain of fibrinogen. hK5 could also play a role in the regulation of the binding of plasminogen activator inhibitor 1 to vitronectin. Our findings suggest that hK5 may be implicated in tumor progression, particularly in invasion and angiogenesis, and may represent a novel therapeutic target.

Serine proteases are enzymes that catalyze the hydrolysis of peptide bonds and contain a catalytic serine residue that acts as a nucleophile (1). They are the second largest family of proteases, after metalloproteases, and account for 176 of the 553 proteases of the human degradome (2). Human tissue kallikreins are 15 homologous serine protease genes that colocalize in tandem to chromosome 19q13.4 (3, 4). The human kallikrein locus is now fully characterized. Centromerically, the *KLK1* gene is in close proximity to the testicular acid phosphatase gene (*ACPT*) (5), and telomerically the *KLK14* gene resides next to the cancer-associated gene (*CAG*) (6) and *Siglec-9*, a member of the sialic acid-binding Ig-like lectin (Siglec) family (7). The direction of transcription of all kallikrein genes is from telomere to centromere with the exception of *KLK3* and *KLK2*. The association of many members of this family with different types of cancers, like prostate, breast, and ovarian, as well their diagnostic/prognostic value has been extensively studied (8, 9). Human kallikrein 3 (hK3[1]/prostate-specific antigen) is the most valuable marker for prostatic adenocarcinoma (10).

Human kallikrein genes encode for secreted serine proteases, translated as inactive preproenzymes. The signal peptide is removed upon entrance into the secretory pathway, and the additional cleavage of the inhibitory prosegment is required for the enzymes to become enzymatically active (1). For activation, 14 of the 15 kallikreins (except hK4) require cleavage after lysine (hK6, hK7, hK8, hK12, hK13, hK14, and hK15) or arginine (hK1, hK2, hK3, hK5, hK9, hK10, and hK11). As well, 12 kallikreins are predicted to have trypsin-like activity and 3 (hK3, hK7, and hK9) to have chymotrypsin-like activity. Certain kallikreins are capable of autoactivation such as hK2 (11, 12), hK6 (13), and hK13 (14) or could presumably activate each other and thus, be involved in a cascade enzymatic pathway (15). The proteolytic activity of kallikreins is regulated in several ways, including inhibition by serpins (serine protease inhibitors) (16).

hK5 (encoded by the *KLK5* gene) has been independently cloned by our group and given the name kallikrein-like gene-2 (*KLK-L2*) (17) and by Brattsand and Egelrud (18) who named it human stratum corneum tryptic enzyme (HSCTE) (18). According to the new human kallikrein gene nomenclature, the official name is *KLK5* (19). *KLK5* has been shown to be estrogen/progestin-regulated (17) and highly expressed in endocrine or hormone-responsive tissues such as testis, ovary, breast, and skin (17, 18).

The hK5 protein is predicted to have trypsin-like activity and is synthesized as a preproenzyme. It consists of a 29-amino acid

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[1] The abbreviations used are: hK, kallikrein protein; *KLK*, kallikrein gene; AMC, 7-amino-4-methyl-coumarin; AAT, α_1-antitrypsin; ACT, α_1-antichymotrypsin; AT, antithrombin; α_2M, α_2-magroclobulin; PNGase F, peptide *N*-glycosidase F; AS4.5, angiostatin 4.5; Z, benzyloxycarbonyl.

signal peptide, followed by a 37-amino acid activation peptide and 237 amino acids comprising the mature enzyme, which includes the serine protease domain, with a predicted molecular mass of 25 kDa (17, 18). hK5 has four potential glycosylation sites at positions ^{69}NGSD, ^{173}NVSS, ^{208}NISV, and ^{252}NGSL. The activation of the enzyme has been shown to require cleavage of an arginine residue (Arg^{66}-Ile^{67}) (18), suggesting that a trypsin-like serine protease may be involved in this process.

Recent studies have shown that *KLK5* is differentially regulated in a variety of hormone-dependent malignancies, including ovarian (20), breast (21), prostate (22), and testicular (23) cancers. Using an hK5-specific enzyme-linked immunosorbent assay method, we have recently shown that hK5 is a potential biomarker of ovarian and breast cancer (24, 25). In addition, the involvement of hK5 in skin desquamation (18, 26) and skin physiology (27) is relatively well established.

To gain insights into the physiology and pathobiology of this serine protease, we produced recombinant hK5 and determined its substrate specificity and interactions with plasma inhibitors. Furthermore, we examined its ability to cleave different plasma and extracellular matrix components.

EXPERIMENTAL PROCEDURES

Materials—7-Amino-4-methylcoumarin (AMC) was purchased from Sigma. The following synthetic AMC substrates were purchased from Bachem Bioscience (King of Prussia, PA): Boc-Phe-Ser-Arg-AMC (FSR-AMC), Boc-Val-Pro-Arg-AMC (VPR-AMC), H-Pro-Phe-Arg-AMC (PFR-AMC), Z-Gly-Gly-Arg-AMC (GGR-AMC), Boc-Leu-Gly-Arg-AMC (LGR-AMC), Boc-Leu-Lys-Arg-AMC (LKR-AMC), Boc-Leu-Arg-Arg-AMC (LRR-AMC), Boc-Gln-Arg-Arg-AMC (QRR-AMC), Boc-Gln-Ala-Arg-AMC (QAR-AMC), Boc-Gln-Gly-Arg-AMC (QGR-AMC), Tos-Gly-Pro-Arg-AMC (GPR-AMC), Tos-Gly-Pro-Lys-AMC (GPK-AMC), Boc-Glu-Lys-Lys-AMC (EKK-AMC), Boc-Val-Leu-Lys-AMC (VLK-AMC), and Suc-Ala-Ala-Pro-Phe-AMC (AAPF-AMC). The substrate Suc-Leu-Leu-Val-Tyr-AMC (LLVY-AMC) was purchased from the Peptide Institute Inc. (Osaka, Japan). All substrates were diluted in dimethyl sulfoxide (Me_2SO) at a final concentration of 80 mM and stored at −20 °C. The protease inhibitors, α_1-antitrypsin (AAT), α_1-antichymotrypsin (ACT), antithrombin (AT), α_2-antiplasmin, and α_2-magroclobulin (α_2M) were purchased from Calbiochem and their purity was ≥95% as verified by SDS-PAGE. All inhibitors were diluted in water at a final concentration of 1 mg/ml and stored at −20 °C. Fluorescent conjugates of bovine skin collagen type I, human placenta collagen type IV, and human plasma fibrinogen were purchased from Molecular Probes (Eugene, OR). The substrates mouse sarcoma collagen type I, chicken sternal cartilage collagen type II, calf skin collagen type III, human placenta collagen type IV, human foreskin fibroblast fibronectin, human plasma vitronectin, laminin, low molecular weight kininogen and plasminogen were purchased from Sigma.

Cloning and Expression of the Pro-form of hK5 in Pichia pastoris—The cDNA encoding pro-hK5 was cloned by the OneStep™ reverse transcriptase-PCR kit (Qiagen, Valencia, CA) using total RNA isolated from the 70N normal breast cell strain. The primers used for PCR amplification were: GGCTCGAGAAAAGAGCCCGGTCGGATGAC (forward) and GTGCGGCCGCCTGGGATGACTGAGGAGTTGGCC (reverse). Restriction sites (underlined) for the endonucleases XhoI and NotI were used for cloning. The PCR product was cloned into the *P. 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. The *P. pastoris* strain KM71 was stably transformed with the expression construct using the *Pichia* Expression Kit (Invitrogen). Expression of the recombinant protein was induced by methanol, at 30 °C, and monitored with SDS-PAGE analysis of the cell culture supernatant.

Purification of Recombinant hK5—Purification of hK5 was achieved with cation-exchange fast performance liquid chromatography followed by reversed-phase high perfrmance liquid chromatography. Concentrated yeast culture supernatant (×10) diluted 1:2 with running buffer (10 mM sodium acetate, pH 5.3) was loaded onto a 5-ml CM FF-Sepharose cation exchange column (Amersham Biosciences). The column was eluted with 10 mM sodium acetate and 1 M NaCl, pH 5.3, with a linear gradient of 0–1 M NaCl. Fractions were analyzed by enzyme-linked immunosorbent assay and those containing hK5 were pooled, supplemented with 1% trifluoroacetic acid, and loaded onto a 1-ml Vydac C4 reverse phase column by using deionized H_2O, 0.1% trifluoroacetic acid. Elution was performed with acetonitrile (AcCN), 0.1% trifluoroacetic acid with a linear gradient of 0–100% AcCN. The concentration of the purified recombinant hK5 was determined by using an hK5-specific enzyme-linked immunosorbent assay and a total protein assay. The purity of the protein was verified by SDS-PAGE.

Mass Spectrometry and NH_2-terminal Sequencing—Mass spectrometric analysis for positive identification of hK5 was performed as previously described (28). NH_2-terminal sequencing was performed with the Edman degradation method. Proteins were transferred by electroblotting to a polyvinylidene difluoride membrane and visualized with Coomassie Blue stain. The bands were excised and applied to the sequencer.

Glycosylation Analysis—hK5 deglycosylation was performed as described previously (29). Briefly, denatured hK5 in 5% SDS, 10% mercaptoethanol, was incubated with peptide *N*-glycosidase F (PNGase F) (New England Biolabs, Beverly, MA) in 0.5 M sodium phosphate, pH 7.5, and 10% Nonidet P-40 surfactant. The mixture was incubated at 37 °C for 2 h. Deglycosylated hK5, as well as horseradish peroxidase and soybean trypsin inhibitor (controls), were subjected to SDS-PAGE in two separate gels. One gel was stained with SimplyBlue Safe-Stain (Invitrogen) and the other with GelCode® Glycoprotein staining kit (Pierce).

Zymography—Gelatin (Novex® 10% Zymogram, Gelatin, Invitrogen) and casein (Novex 12% Zymogram, Casein, Invitrogen) zymography were performed as described previously (30). Briefly, hK5 was mixed with Tris glycine SDS sample buffer and run for 90 min at 125 V at 4 °C. After electrophoresis, the gels were incubated in renaturing buffer for 30 min at room temperature, followed with incubation in developing buffer at 37 °C overnight. Gels were stained with SimplyBlue Safe-Stain (Invitrogen). Areas of protease activity appeared as clear bands against a dark blue background.

Enzymatic Activity Assays and Kinetic Constant Determination—hK5 (12 nM) was incubated at 37 °C, in a microtiter plate, with assay buffer (100 mM phosphate, 0.01% Tween 20, pH 8.0) and varying concentrations (0.06–3 mM) of fluorescent substrates in a final volume of 100 μl. The initial rate of AMC release was measured on a Wallac Victor fluorometer (PerkinElmer Life Sciences) set at 355 nm for excitation and 460 nm for emission. Enzyme-free reactions were used as negative controls. All experiments were done in triplicate. A standard curve with known concentrations of AMC was used to calculate the rate of product formation. The Michaelis-Menten constants were calculated by nonlinear regression analysis using the Enzyme Kinetics Module 1.1 (Sigma Plot, SSPS, Chicago, IL).

Inhibition Assays—The reactions for the calculation of the second-rate constants of hK5 activity inhibition by serpins were performed under pseudo-first order conditions, as previously described (31–33). Briefly, hK5 was incubated in microtiter wells with various concentrations of the serpins AAT, ACT, AT, and α_2-antiplasmin, at room temperature with gentle agitation for different time points. Ten microliters of the above mixture were added in 240 μl of 0.1 mM VPR-AMC in assay buffer. The reaction was performed in a microtiter plate at 37 °C and the initial rate of AMC release was measured as described above. To monitor the hK5-serpin complex formation, the enzyme was incubated at 1:1, 1:5, and 1:10 molar ratios with the different inhibitors at 37 °C for 30 min, run on SDS-PAGE under reducing conditions, and stained with Coomassie Blue.

The inhibition of hK5 by the high molecular weight inhibitor α_2M was monitored by using a fluorescent conjugate of fibrinogen. hK5 (12 nM) was incubated with α_2M (12 to 1200 nM) in a microtiter plate for 30 min at 37 °C at a final volume of 100 μl, with gentle agitation. After incubation, 2.5 μl of conjugated fibrinogen (1.5 mg/ml) was applied and fluorescence was measured every 10 min for 2 h on a Wallac Victor fluorometer set at 492 nm for excitation and 535 nm for emission. Enzyme-free reactions were used as negative controls. All experiments were done in triplicate.

Biotinylation of ECM Components (Collagen Type I, II, III, IV, Laminin and Fibronectin)—Each component was diluted in 0.5 M phosphate buffer, pH 9.2, at a final concentration of 1 mg/ml and dialyzed overnight in 4 liters of the same buffer at 4 °C. Each component was mixed with 100 μl of biotin (stock solution, 1 mg/ml in Me_2SO) in 1.5-ml tubes and incubated for 2 h at 4 °C. The mixture was then dialyzed again for 3 days in 4 liters of phosphate buffer at 4 °C with changes of buffer twice a day.

Digestion of Biotinylated ECM and Plasma Components—Five micrograms of collagen types I, II, III, and IV were incubated in assay buffer (final volume 25 μl) for various time points (0.25 to 8 h) with 150 ng of hK5 at room temperature. Similarly, the substrates fibronectin (5

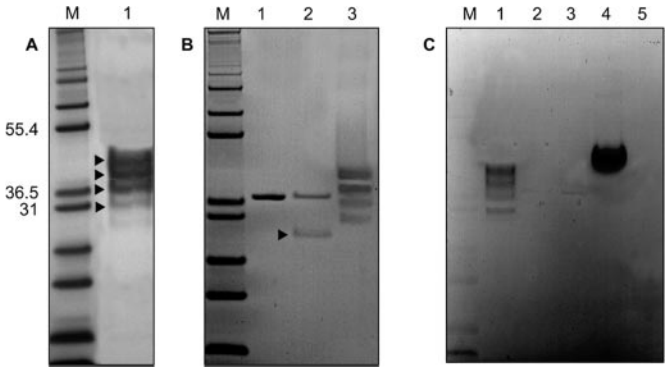

FIG. 1. **Purification, deglycosylation, and glycoprotein staining of recombinant hK5.** *A*, Coomassie Blue staining of an SDS-PAGE gel. hK5 is represented by four bands (*lane 1*) with molecular masses of ~44, 40, 35, and 30 kDa (*arrowheads*). *B*, treatment with PNGase F. *Lane 1*, PNGase F; *lane 2*, hK5 after deglycosylation with PNGase F; *lane 3*, hK5. A single band with a molecular mass of ~26 kDa was obtained after deglycosylation (*lane 2*; *arrowhead*). *C*, glycoprotein staining. *Lane 1*, hK5; *lane 2*, hK5 after treatment with PNGase F; *lane 3*, PNGase F; *lane 4*, horseradish peroxidase (a glycoprotein of ~40 kDa; positive control); *lane 5*, soybean trypsin inhibitor (an unglycosylated protein of ~21.5 kDa; negative control). After treatment with PNGase F (*lane 2*), staining is abolished. *M*, molecular mass standards.

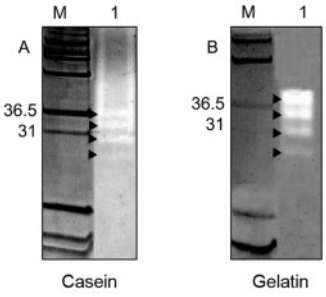

FIG. 2. **Casein and gelatin zymograms.** All four glycosylated forms of hK5 (*arrowheads*) can digest casein (*A*) and gelatin (*B*). In both cases 0.64 μg of recombinant hK5 were used per lane. *M*, molecular mass standards.

μg), laminin (5 μg), plasminogen (5 μg), fibrinogen (0.5 μg), vitronectin (0.5 μg), and kininogen (0.5 μg) were incubated with 100 ng of hK5 at 37 °C. The reactions were terminated by freezing in liquid nitrogen. Enzyme-free reactions were used as negative controls and incubated for 8 h at room temperature. Silver staining (Invitrogen) was then performed for the substrates plasminogen, kininogen, fibrinogen, and vitronectin, whereas Western blotting was performed for the biotinylated substrates as described previously (34).

Digestion of the Conjugates of Collagen I and IV, and Fibrinogen—One microgram of hK5 was incubated in microtiter wells with 2.5 μl of the conjugates of collagen types I and IV (1 mg/ml) and fibrinogen (1.5 mg/ml), in assay buffer (final volume 200 μl) at room temperature. Fluorescence was measured every 10 min for 2 h on the Victor fluorometer set at 492 nm for excitation and 535 nm for emission. Enzyme-free reactions were used as negative controls and background fluorescence was subtracted from each value. All experiments were done in triplicate.

RESULTS

Molecular Cloning, Purification, and Glycosylation Analysis of Recombinant Human Pro-hK5—The full-length cDNA encoding pro-hK5 was amplified by reverse transcriptase-PCR using total RNA isolated from the 70N normal breast cell strain, cloned into the pPIC9 vector in-frame with the yeast α-mating factor, and successfully transformed into KM71 strain of the yeast, *P. pastoris*. The secretion of hK5 into the yeast culture supernatant was monitored with SDS-PAGE; highest levels were seen after 4 days induction with methanol (data not shown). After a two-step purification procedure, we obtained recombinant hK5, with purity ≥95%, as verified by SDS-PAGE (Fig. 1*A*). The yield of recombinant protein was 1.5 mg of purified protein per liter of yeast culture, as measured with both an hK5-specific enzyme-linked immunosorbent assay and total protein assay. The identity of hK5 was verified by tandem mass spectrometry.

Pre-pro-hK5 consists of 293 amino acids and contains a predicted signal peptide of 29 amino acids (Met^{1}–Ala^{29}) and an activation peptide of 37 amino acids (Ala^{30}–Arg^{66}) (17). Although the molecular mass of pro-hK5 inferred from the primary sequence is about 26 kDa, after purification we obtained four bands corresponding to molecular masses of ~44, ~40, ~35, and ~30 kDa (Fig. 1*A*). The NH_2-terminal sequence of all four bands was found to be Ile-Ile-Asn-Gly-Ser-Asp, which corresponds to the NH_2-terminal sequence of the active, mature form of hK5. This pointed to the possibility that hK5 may be able to autoactivate. However, enzymatically active yeast recombinant hK5 was unable to cleave the propeptide and activate mammalian recombinant pro-hK5 produced in Chinese hamster ovary cells (data not shown). It is thus likely that hK5 is activated in the supernatant by a yeast protease.

After subjecting the four forms of recombinant hK5 to *in vitro* deglycosylation by PNGase F and staining with Coomassie Blue, we observed that the four bands co-migrated as a smaller molecular mass band of ~26 kDa (Fig. 1*B*, *lane 2*), indicating that recombinant hK5 is differentially glycosylated. A duplicate of the aforementioned gel, including horseradish peroxidase glycoprotein as a positive control (Fig. 1*C*, *lane 4*) and the non-glycosylated protein soybean trypsin inhibitor as a negative control (Fig. 1*C*, *lane 5*), was subjected to glycoprotein staining. *In vitro* deglycosylation abolished staining of glycosylated forms of hK5 with acidic fuchsin sulfite (Fig. 1*C*, *lane 2*).

Zymography—To determine whether the four bands corresponding to differentially glycosylated forms of hK5 represented active forms of the enzyme, both gelatin and casein zymograms were performed. The results showed that all bands were active and able to digest efficiently both casein (Fig. 2*A*) and gelatin (Fig. 2*B*).

pH Optimum for the Enzymatic Action of hK5—The pH dependence of the hK5 enzymatic activity was checked in two buffer systems, *i.e.* 100 mM phosphate and 50 mM Tris buffer with 100 mM NaCl. The pH of 8.0 was optimal for both systems, although hK5 was 2.5 times more active in phosphate buffer. Similarly, hK5 showed 1.25 times higher activity when we used 0.01% Tween 20 instead of 0.2% bovine serum albumin as carrier in the reaction mixture. Therefore, we used the following assay buffer for further kinetic studies: 100 mM phosphate, 0.01% Tween 20, pH 8.0.

Determination of Substrate Specificity and Steady-state Kinetic Constants—Fluorogenic tripeptide substrates with the AMC leaving group were used to characterize the enzymatic activity of recombinant hK5 and determine its steady-state kinetic constants. We used 16 substrates, of which 14 were candidate substrates for trypsin-like enzymes (11 with Arg and 3 with Lys at P1 position), and two for chymotrypsin-like enzymes, with Tyr and Phe at P1 position. Results are presented in Table I. As predicted by the presence of Asp^{239}, close to Ser^{245} of the catalytic triad, hK5 was confirmed to have trypsin-like, but not chymotrypsin-like activity, because no reaction was observed for the two substrates (Suc-Ala-Ala-Pro-Phe-AMC, Suc-Leu-Leu-Val-Tyr-AMC) specific for chymotrypsin-like enzymes. Comparison of the k_{cat}/K_m for substrates Tos-Gly-Pro-Arg-AMC and Tos-Gly-Pro-Lys-AMC (Table I) indicates that hK5 exhibits a much higher preference for Arg at position P1 relative to Lys (according to the notation of Schechter and Berger

TABLE I
Steady-state kinetic analysis of hK5 with fluorogenic substrates

Substrate	K_m	k_{cat}	k_{cat}/K_m	Activity
	mM	min^{-1}	$mM^{-1}\ min^{-1}$	%
Boc-Val-Pro-Arg-AMC	0.20 ± 0.01	196.76	946.45	100
Boc-Phe-Ser-Arg-AMC	0.19 ± 0.01	169.50	877.37	92.7
H-Pro-Phe-Arg-AMC	1.77 ± 0.57	28.17	15.89	1.6
Z-Gly-Gly-Arg-AMC	0.31 ± 0.07	2.29	7.38	0.8
Boc-Leu-Gly-Arg-AMC	0.15 ± 0.01	5.18	33.00	3.5
Boc-Leu-Lys-Arg-AMC	1.01 ± 0.10	49.38	48.89	5.2
Boc-Leu-Arg-Arg-AMC	0.48 ± 0.05	18.67	38.28	4.0
Boc-Gln-Arg-Arg-AMC	1.41 ± 0.16	24.40	17.21	1.8
Boc-Gln-Ala-Arg-AMC	0.61 ± 0.03	106.97	175.33	18.5
Boc-Gln-Gly-Arg-AMC	0.57 ± 0.05	10.89	19.10	2.0
Tos-Gly-Pro-Arg-AMC	1.69 ± 0.32	20.77	12.22	1.3
Tos-Gly-Pro-Lys-AMC	NR[a]			
Boc-Glu-Lys-Lys-AMC	NR[a]			
Boc-Val-Leu-Lys-AMC	0.64 ± 0.17	4.56	7.07	0.75
Suc-Ala-Ala-Pro-Phe-AMC	NR[a]			
Suc-Leu-Leu-Val-Tyr-AMC	NR[a]			

[a] NR; no reaction.

(35)). However, cleavage of the substrate Boc-Val-Leu-Lys-AMC, indicates that hK5 could cleave after Lys but to a much lesser extent. Comparison of the k_{cat}/K_m ratio for the substrates that have the same amino acids in positions P1 and P3 (Boc-Gln-Ala-Arg-AMC, Boc-Gln-Gly-Arg-AMC, Boc-Gln-Arg-Arg-AMC and Boc-Leu-Lys-Arg-AMC, Boc-Leu-Arg-Arg-AMC, Boc-Leu-Gly-Arg-AMC) revealed that hK5 prefers Ala and Lys in the P2 position relative to Arg and Gly. Similarly, comparison of the k_{cat}/K_m for the substrates that have the same amino acids in positions P1 and P2 (Boc-Leu-Gly-Arg-AMC, Boc-Gln-Gly-Arg-AMC, Z-Gly-Gly-Arg-AMC and Boc-Leu-Arg-Arg-AMC, Boc-Gln-Arg-Arg-AMC, and Boc-Val-Pro-Arg-AMC, Tos-Gly-Pro-Arg-AMC) indicates that hK5 prefers Leu > Gln > Gly and Val > Gly in the P3 position. The best substrates for hK5 were found to be the Boc-Val-Pro-Arg-AMC, also a substrate for α-thrombin, and Boc-Phe-Ser-Arg-AMC, also a substrate for trypsin.

Regulation of hK5 Activity by Serpins—The inhibition of hK5 by the four low molecular weight plasma serpins AAT, ACT, AT, and α_2-AP and the high molecular weight proteinase inhibitor α_2M was examined as described under "Experimental Procedures." The inactivation of hK5 by AT and α_2-AP followed pseudo-first order kinetics when these inhibitors were in a 4–20-fold molar excess. α_2-AP was a more efficient inhibitor of hK5 than AT. For example, 50% of hK5 activity was inactivated by 0.8 μM α_2-AP in 0.6 min (Fig. 3*A*), whereas the same proportion of hK5 was inactivated in 6 min when AT was 1.5 μM (Fig. 3*B*). No inhibition was observed by AAT and ACT. The kinetic constants for inactivation of hK5 by α_2-AP and AT were derived from double-reciprocal plots of the pseudo-first order rate constant *k′ versus* inhibitor concentrations (Fig. 3, *A* and *B*, *insets*) and are listed in Table II. The second-order rate constants k_{+2}/K_i for the reaction of hK5 with these plasma protease inhibitors revealed that the reaction of hK5 with α_2-AP was 24 times faster than the reaction between hK5 and AT (Table II).

The inhibition mechanism of serpins is mediated through the cleavage of an exposed peptide bond in a strained loop in the reactive site region by the protease (36), which leads to the formation of a complex, deformation of the structure of the catalytic triad, and inactivation of the serine protease (16). The scissile peptide bonds in the reactive site region for the serpins are Met^{358}–Ser^{359} (AAT) (37), Leu^{383}–Ser^{384} (ACT) (38), Arg^{393}–Ser^{394} (AT) (39), and Arg^{364}–Met^{365} (α_2-AP) (40). The Arg preference of hK5 for the P1 position explains its inhibition by the serpins AT and α_2-AP, and not by AAT and ACT.

The inhibition of hK5 by the high M_r proteinase inhibitor α_2M was monitored by using a fluorescent conjugate of fibrinogen as a substrate. In contrast to the aforementioned serpins, complex formation of serine proteases with α_2M does not lead to deformation of the catalytic triad. The reaction of proteases with α_2M is mediated through the recognition and cleavage of the "bait" region (residues 666–706), an exposed peptide stretch (41). The bait region cleavage triggers conformational

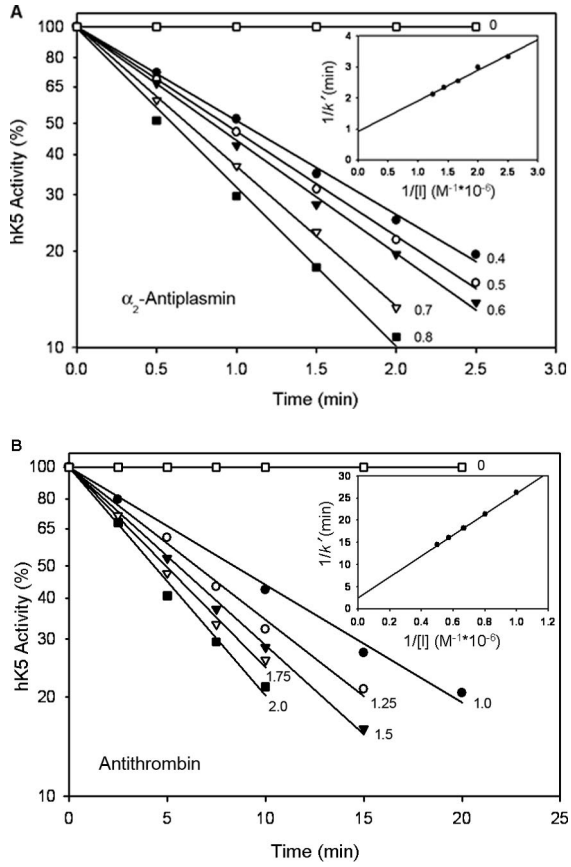

FIG. 3. **Kinetics of inactivation of hK5 by α_2-AP (*A*) and AT (*B*).** Human kallikrein 5 (final concentration, 100 nM) was incubated with various concentrations of the serpins α_2-AP and AT, and residual activity was measured at various intervals. α_2-AP inhibitor final concentration (μM): 0 (□), 0.4 (●), 0.5 (○), 0.6 (▼), 0.7 (▽), 0.8 (■). AT inhibitor final concentration (μM): 0 (□), 1.0 (●), 1.25 (○), 1.5 (▼), 1.75 (▽), 2.0 (■). The *insets* show a double-reciprocal plot of the pseudo-first order rate constant and the inhibitor concentration. The *line* drawn is a least-squares fit of the experimental points. The equation of the line for α_2-AP is $Y = 0.98X + 0.92$ ($r = 0.987$), and for AT is $Y = 23.6X + 2.6$ ($r = 0.999$). r is the Pearson correlation coefficient.

TABLE II
Inhibition of hK5 enzymatic activity by plasma inhibitors

Inhibitor	K_I	k_{+2}	k_{+2}/K_I
	μM	min^{-1}	$M^{-1}min^{-1}\ 10^{-4}$
α_2-Antiplasmin	1.07	1.09	102
Antithrombin III	9.1	0.39	4.2

changes of α_2M. Thus, α_2M functions as a "molecular trap" and protects the protease from reacting with large proteinase inhibitors and substrates, but the protease readily reacts with small substrates and inhibitors (41). Therefore, no inhibition could be observed for cleavage of small fluorogenic substrates, because they can reach the active center of the serine protease and be cleaved. By using a fluorogenic conjugate of fibrinogen, we were able to observe a 50% inhibition in the activity of hK5 by α_2M, at a molar ratio of *E*:I of 1:100. The two areas of the bait region that are mainly recognized and cleaved, located between Arg^{681} and Tyr^{686} (primary cleavage site) and Arg^{696} and Val^{700} (secondary cleavage site) (42). The human serine proteases trypsin, plasmin, and thrombin are "trapped" by α_2M after cleavage between Arg^{696} and Lys^{697} (41). Trypsin also cleaves α_2M between Arg^{681} and Val^{682} (41). Because hK5 shows high specificity for Arg in the P1 position, both Arg^{696}–Lys^{697} and Arg^{681}–Val^{682} dipeptides are strong candidates for recognition and cleavage, which leads in "trapping."

The stable complexes that are formed between hK5 and serpins were observed under reducing conditions with SDS-PAGE analysis. As shown in Fig. 4, complexes between the inhibitor and hK5 of about 97 kDa were observed for both AT and α_2-AP. Although hK5 occurs in four different glycosylation forms and we have shown that they are all active (zymography data), only two forms seem to form complexes with the inhibitors. Possibly, different glycosylation patterns may affect the interaction between hK5 and the inhibitors. Notably, hK5 reacts with α_2-AP through both the "inhibitory pathway" (43), via complex formation, and the "substrate pathway" (43), by cleaving the inhibitor, generating two bands with molecular masses around ~8 and ~54 kDa (Fig. 4*A*). Human kallikrein 3/prostate-specific antigen has also been shown to react through both the inhibitory and substrate pathways with the serpin ACT (44), with which it is mainly complexed (70%) in serum (45).

Digestion of Extracellular Matrix and Plasma Components—By using biotinylated components of the extracellular matrix, we were able to show that human kallikrein 5 digests them effectively and rapidly yielding a number of proteolytic fragments. Some components, including collagens I, II, and III and laminin are cleaved very quickly, revealing extensive degradation within 15 min at room temperature (Fig. 5). Fig. 6 displays the time-dependent degradation of the plasma components plasminogen, kininogen, fibrinogen, and vitronectin by hK5. Fluorescent conjugates of collagens I and IV and fibrinogen were also incubated with hK5 and a progressive increase in fluorescence emission resulting from substrate degradation was observed (data not shown).

hK5 cleaves plasminogen and generates 2 fragments, P1 and P2, with molecular masses around 50 and 30 kDa, respectively (Fig. 6). NH_2-terminal sequencing of these fragments revealed that hK5 is able to cleave plasminogen at peptide bonds Lys^{77}–Lys^{78} and Arg^{549}–Lys^{550} (Table III). According to these results, the first fragment represents an angiostatin isoform, known as angiostatin 4.5 (AS4.5), which consists of plasminogen kringles 1–4 and 85% of kringle 5 (amino acids Lys^{78}–Arg^{529}). AS4.5 has been shown to be a potent angiogenesis inhibitor (46), significantly more effective than angiostatin K1–3 and angiostatin K1–4 (47). The second fragment is similar to microplasmin, which along with AS4.5, has been shown to be generated by plasmin after plasminogen activation and consists by the remaining kringle 5 domain and the serine proteinase domain of plasmin linked together by disulfide bonds (48, 49). However, in our case, because we are using reducing conditions, this fragment is not cleaved. 30 μg of plasminogen were completely converted into these two fragments after overnight incubation with 0.3 μg of hK5. No significant change was observed when plasminogen was incubated alone (data not shown).

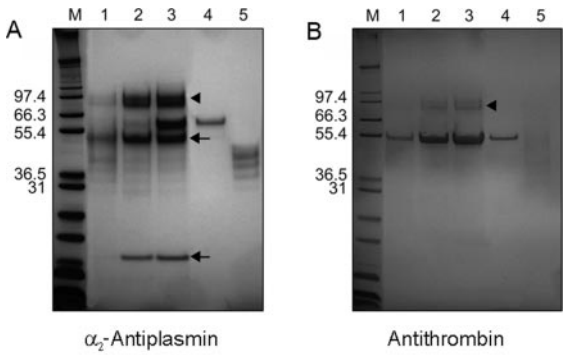

FIG. 4. **Formation of complexes between hK5 and the serpins α_2-AP (*A*) and AT (*B*).** hK5 was incubated at 1:1 (*lane 1*), 1:5 (*lane 2*), and 1:10 (*lane 3*) molar ratios with the different inhibitors at 37 °C for 30 min, the mixture was run on SDS-PAGE under reducing conditions and the gel stained with Coomassie Blue. The inhibitor alone is shown in *lane 4* and hK5 alone in *lane 5*. Two complexes were observed for both inhibitors, with molecular masses around ~97 kDa (*arrowheads*). For the reaction between hK5 and α_2-AP, two fragments with molecular masses around ~8 and ~54 kDa were also generated (*panel A*; arrows). For discussion see text. *M*, molecular mass standards.

In contrast to the classical tissue kallikrein hK1, hK5 seems unable to generate bradykinin-like fragments from low molecular weight kininogen. hK5 was able to cleave kininogen at Arg^{374}–Ile^{375} (Fig. 6, *fragment K4*; Table III), which is located near the peptide bond Arg^{371}–Ser^{372} that is cleaved by plasma and tissue kallikrein for generation of bradykinin and Lys-bradykinin, respectively. However, hK5, by cleaving at peptide bonds Arg^{222}–Ile^{223} (Fig. 6, *fragment K2*; Table III) and Arg^{252}–Asp^{253} (Fig. 6, *fragment K3*; Table III), is able to release the "cystatin-like domain 3" region from kininogen, a potent inhibitor of cysteine proteases. The second scissible peptide bond is located in a "proteinase-sensitive region" in which many proteases have been shown to cleave kininogen (50). Trypsin has been shown to cleave kininogen at the same peptide bond, *i.e.* Arg^{252}–Asp^{253} (50).

Human kallikrein 5 could rapidly digest fibrinogen by cleaving Aα and Bβ chains, whereas γ chain seems to remain intact. Four of the major generated fragments, *i.e.* F1, 2, 3, and 4 (Fig. 6), have been sequenced and revealed that hK5 cleaves the Aα chain at peptide bonds Arg^{348}–Gly^{349} and Arg^{407}–Glu^{408} (Fig. 6, *fragments F2* and *F3*; Table III), and the Bβ chain at peptide bonds Arg^{14}–Gly^{15} and Lys^{53}–Lys^{54} (Fig. 6, *fragments F4* and *F1*; Table III). Thrombin cleaves Bβ chain at the same peptide bond, Arg^{14}–Gly^{15} during the formation of fibrin, to release fibrinopeptide B and unmask the polymerization site E_B (51). Furthermore, hK5, by cleaving at Lys^{53}–Lys^{54}, could also release the peptide β15–42, a sequence that binds heparin and is significant for various biological activities of Bβ chain (51).

Digestion of vitronectin by hK5 (Fig. 6) generated four main fragments, two around 50 kDa and another two around 25 kDa. NH_2-terminal sequencing of fragment V1, which is generated within 15 min, revealed that hK5 cleaves at peptide bond Arg^{8}–Cys^{9} (Fig. 6, *fragment V1*; Table III), which is located in an area identical to somatomedin B, a binding site for plasminogen activator inhibitor 1 (52).

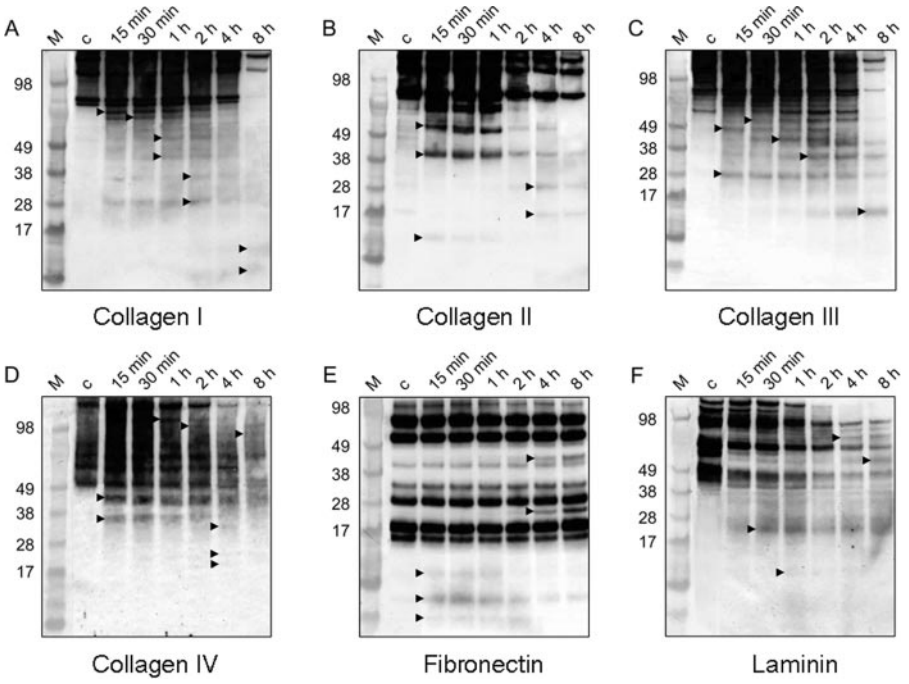

FIG. 5. **Degradation of the extracellular matrix components collagen types I (*A*), II (*B*), III (*C*), IV (*D*), fibronectin (*E*), and laminin (*F*) by hK5.** The control (*c*) represents the ECM component incubated alone at room temperature for 8 h. The different incubation times represent the duration of the incubation of the ECM component with hK5 at room temperature. Note the generation of new fragments as indicated by *arrowheads*.

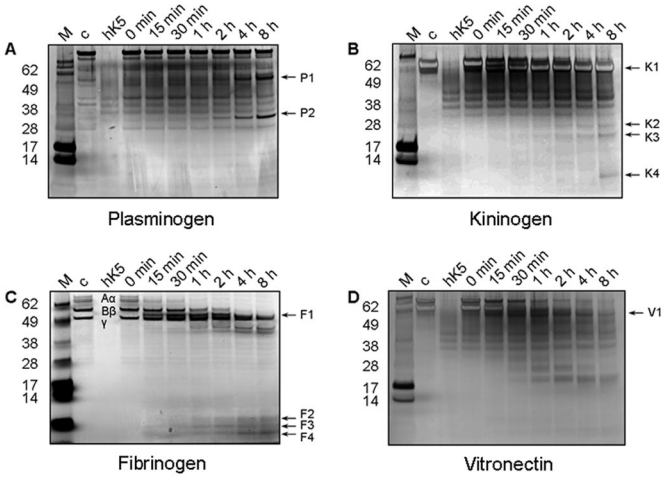

FIG. 6. **Degradation of plasminogen (*A*), kininogen (*B*), fibrinogen (*C*), and vitronectin (*D*) by hK5.** The control (*c*) represents the protein incubated alone at 37 °C for 8 h. The different incubation times represent the duration of the incubation of each protein with hK5 at 37 °C. The fragments that have been subjected to NH_2-terminal sequencing are indicated by *arrows*. For more information see Table III and "Discussion."

TABLE III
Amino-terminal sequences identified in the fragments of plasminogen, kininogen, fibrinogen, and vitronectin after proteolytic digestion with hK5

Fragment	Amino-terminal sequence
Plasminogen	
P1	^{77}(Lys) ↓ Lys-Val-Tyr-Leu-Ser-Glu
P2	^{529}(Arg) ↓ Lys-Leu-Tyr-Asp-Tyr-*X*[a]
Kininogen	
K1	NH_2-terminal blocked
K2	^{222}(Arg) ↓ Ile-Ala-Ser-Phe-Ser-Gln
K3	^{252}(Arg) ↓ Asp-Ile-Pro-Thr-Asn-Ser
K4	^{374}(Arg) ↓ Ile-Gly-Glu-Ile-Lys-Glu
Fibrinogen (Aα chain)	
F2	^{348}(Arg) ↓ Gly-Ser-Ala-Gly-*X*[a]-*X*[a]
F3	^{407}(Arg) ↓ Glu-Tyr-His-Thr-Glu-Lys
Fibrinogen (Bβ chain)	
F1	^{53}(Lys) ↓ Lys-Val-Glu-Arg-Lys-Ala
F4	^{14}(Arg) ↓ Gly-His-Arg-Pro-Leu-Asp
Vitronectin	
V1	^{8}(Arg) ↓ *X*[a]-Thr-Glu-Gly-Phe-Asn

[a] The amino acid was not determined.

DISCUSSION

The human kallikrein family of serine proteases has been implicated in many malignancies, primarily prostate, ovarian, and breast cancers (8, 9). Serum human kallikrein 5 is a candidate novel biomarker for breast and ovarian cancers (24). The involvement of hK5 in stratum corneum turnover and desquamation of epidermis has been documented (18, 26). Here, we describe the biochemical and enzymatic characterization of this novel serine protease and identify candidate physiological substrates in our efforts to understand its role in cancer progression.

We produced and purified hK5 in a yeast expression system and found that it is enzymatically active and has trypsin-like activity with a strong preference for Arg over Lys in the P1 position. Furthermore, we found that hK5 can be inactivated by α_2-AP and AT, with second-order inhibition constants (k_{+2}/K_i) of 1.0×10^{-2} and 4.2×10^{-4} M^{-1} min^{-1}, respectively. α_2M, at 100-fold molar excess, was also able to inhibit hK5 activity by about 50%.

Cleavage of plasminogen and subsequent release of angiostatin-like fragments has been reported for hK3 (53), hK6 (54), and hK13 (14). Fortier *et al.* (55) have recently shown that hK3 inhibits angiogenesis *in vitro* and *in vivo*, by blocking endothelial cell responses to vascular endothelial growth factor and fibroblast growth factor-2 (55). Our data indicates that hK5 can cleave plasminogen and potentially generate AS4.5, a potent inhibitor of angiogenesis (46). Plasmin has been shown to generate AS4.5 and microplasmin by a two-stage mechanism, involving preactivation of plasminogen by the urokinase-type plasminogen activator, in the presence of a small molecule sulfhydryl donor, like *N*-acetylcysteine (56), or the plasminogen receptor, β-actin (57). However, several studies have shown that a serine protease other than plasmin is responsible for the generation of AS4.5 in the prostate carcinoma cell lines PC-3, DU-145, and LNCaP, and the fibrosarcoma cell line HT1080 (58, 59). Stathakis *et al.* (59) were able to purify from HT1080-conditioned medium three serine proteases with apparent molecular masses of 70, 50, and 39 kDa. Depletion from the

medium reduces the angiostatin-generating activity to 34% of control (59). Because the mammalian recombinant hK5 produced in house has a molecular mass ~40 kDa, and hK5 presents in fluids in which AS4.5 has been identified (supernatants of the prostate cancer cell line PC-3 and the breast cancer cell line MCF-7, as well in ascites fluid of ovarian cancer patients), it is reasonable to speculate that hK5 might be one of the serine proteases responsible for AS4.5 generation.

In contrast to hK1, hK5 is unlikely to be able to release bioactive bradykinin from low molecular weight kininogen. Our results indicate that hK5 cleaves low molecular weight kininogen at peptide bond Arg^{374}–Ile^{375}, which is located downstream from peptide bond Arg^{371}–Ser^{372} cleaved by tissue and plasma kallikrein. However, it is possible that hK5 first cleaves at Arg^{371}–Ser^{372} and then at Arg^{374}–Ile^{375}. In this case, cleavage by another protease at Lys^{362}–Arg^{363} or Met^{361}–Lys^{362} will be required for the release of bradykinin and Lys-bradykinin, respectively. On the other hand, the ability of hK5 to cleave low molecular weight kininogen at Arg^{374}-Ile^{375} and Arg^{252}–Asp^{253} or Arg^{222}–Ile^{223} can lead to the release of the fragment cystatin-like domain 3, an inhibitor of cysteine proteinases, like calpains (50). This could be a mechanism through which a protease from one clan can control the activity of a protease of a different clan. These domains have been identified in plasma in pathological conditions such as disseminated intravascular coagulation and polytrauma, suggesting that such cleavages also occur *in vivo* (50).

Human kallikrein 5 rapidly cleaves fibrinogen, within the Bβ chain generating three fragments, fibrinopeptide B (β1–14, consisting of the amino acids 1 to 14), β15-53 (consisting of the amino acids 15 to 53), and the remaining carboxyl-terminal of the Bβ chain. Release of fibrinopeptide B is required during fibrin formation so that polymerization site E_B becomes unmasked (51). Thrombin is the classical enzyme with an ability to release fibrinopeptide B, although at a slower rate than the release of fibrinopeptide A from the Aα chain (51). Release of the sequence β15-53 can, subsequently, lead to the release of peptide β15-42. The latter binds heparin and participates in cell-matrix interactions (60, 61). Moreover, that peptide has been shown to mediate platelet spreading (62), fibroblast proliferation (63), endothelial cell spreading, proliferation and capillary tube formation (61, 62, 64, 65), and release of von Willebrand factor (66). Binding of β15-42 to endothelial cells is mediated by the endothelial cell receptor, VE-cadherin (65). Odrljin *et al.* (60) have shown that fibrinogen lacking β15-53 is not able to bind heparin and human umbilical vein endothelial cells (61). To our knowledge, hK5 is the first human protease shown to be able to release this fragment.

Vitronectin is an adhesive glycoprotein with multiple functions associated with hemostasis and pericellular proteolysis (52), and it is found in blood (67) (as single-chain and as a clipped form composed of two chains held together by a disulfide bridge) and in the extracellular matrix (68). Its biological functions can be modulated by proteolytic enzymes like thrombin, elastase, and plasmin, through cleavage at its cluster of basic amino acids, leading to the dissociation of plasminogen activator inhibitor 1. This transforms vitronectin from antifibrinolytic to a profibrinolytic protein (69). hK5 cleaves the NH_2-terminal of vitronectin, within the somatomedin B domain, a second area associated with plasminogen activator inhibitor 1 binding (70, 71). This cleavage will lead in the release of the first cysteine, an amino acid that has been shown, by alanine scanning mutagenesis, to be critical for binding of plasminogen activator inhibitor 1 in the somatomedin B domain (72). Thus hK5, like plasmin, elastase, and thrombin, may be involved in the regulation of plasminogen activator activity.

Recently, it has become apparent that several members of the human kallikrein family, including hK2, hK3, hK6, and hK13 can also digest ECM components (13, 30, 34, 73–75). The ability of hK5 to degrade ECM components collagen types I, II, III, and IV, as well as fibronectin and laminin, is shown here for the first time. Thus, hK5 may be implicated in cancer progression, through degradation of extracellular matrix and basement membrane components and through release of bioactive fragments participating in angiogenesis. Further studies are required in this direction.

In this study, we have shown that hK5 has trypsin-like activity that is regulated by the inhibitors α_2-AP, AT, and α_2M. Its ability to cleave ECM (collagens type I, II, III, IV, fibronectin and laminin) and adhesion molecules (fibrinogen and vitronectin) and to release angiostatin 4.5 suggests that this enzyme may play a role in invasion, metastasis, and angiogenesis. Its established role in skin desquamation and tissue remodeling is in accord with these proposals.

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