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

Received for publication, July 19, 2004, and in revised form, January 27, 2005 Published, JBC Papers in Press, February 15, 2005, DOI 10.1074/jbc.M408132200

Iacovos P. Michael†‡, Georgia Sotiropoulou†, Georgios Pampalakis‡, Angeliki Magklara§¶, Manik Ghosh†, Greg Wasney‡, and Eleftherios P. Diamandis†‡§

From the †Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario M5G 1X5, Canada, the ‡Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario M5G 1L5, Canada, and the §Department of Pharmacy, School of Health Sciences, University of Patras, Rion, 26500 Patra, Greece

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 $\alpha_1$-antiplasmin and $\alpha_1$-antitrypsin were able to inhibit hK5 with an inhibition constant ($K_i$) of $1.0 \times 10^{-2}$ and $4.2 \times 10^{-4}$ $M^{-1} min^{-1}$, respectively. No inhibition was observed with the serpins $\alpha_1$-antitrypsin and $\alpha_1$-antichymotrypsin, although $\alpha_2$-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) angioatin 4.5 from plasminogen, (b) “cystatin-like domain 3” from low molecular weight kininogen, and (c) fibrinopeptide B and peptide $\beta15$-$42$ from the $\beta$ 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 localize 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 (ACP7) (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/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...
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 69NGSD, 172LYSS, 208NISV, and 252NGSL. The activation of the enzyme has been shown to require cleavage of an arginine residue (Arg66-Ile67) (18), suggesting that a trypsin-like serine protease may be involved in this process.

Recent studies have shown that KKL5 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-Arg-AMC (PPR-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-Glu-Arg-AMC (QAR-AMC), Boc-Gln-Gly-Arg-AMC (QGR-AMC), Tos-Gly-Arg-AMC (RPR-AMC), Boc-Glu-Arg-AMC (GGR-AMC), Boc-Glu-Lys-Arg-AMC (GLR-AMC), Boc-Lys-Arg-AMC (LKR-AMC), Boc-Val-Pro-Arg-AMC (VPR-AMC), H-Pro-Phe-Arg-AMC (PFR-AMC), Boc-Leu-Glu-Arg-AMC (BEL-AMC), Boc-Leu-Lys-Arg-AMC (LKR-AMC), Boc-Glu-Arg-AMC (QAR-AMC), and Boc-Glu-Lys-Arg-AMC (GLR-AMC). The synthetic AMC substrates were purchased from Sigma. The following synthetic AMC substrates were purchased from Sigma. The following synthetic AMC substrates were purchased from Sigma.

**Purification of Recombinant hK5**—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.

**Enzymatic Activity Assays and Kinetic Constant Determination**—hK5 (12 nm) was incubated at 37 °C, in a microtitrator plate, with assay buffer (100 mm phosphate, 0.01% Tween 20, pH 8.0) and varying concentrations (0.06–3 nm) 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 360 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 non-linear 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 α2-antiplasmin, at room temperature with gentle agitation for different time points. Ten microliters of the substrate mixture were added in 24 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.

**Digestion of Biotinylated ECM Components**—Five micrograms of collagen types I, II, III, IV, laminin, and fibronectin—Each component was digested in 0.5 μM 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 2 μg of hK5 (100 ng in Me_SO) in 1.5-ml tubes and incubated for 2 h at 4 °C. The mixture was then dialyzed against 3 days in 4 liters of phosphate buffer at 4 °C with changes of buffer twice a day.

**Mass Spectrometry and NH2-terminal Sequencing**—Mass spectrometric analysis of the cell culture supernatant. Protein was induced by methanol, at 30 °C, and monitored with SDS-PAGE. PAGE analysis of the cell culture supernatant. Protein was induced by methanol, at 30 °C, and monitored with SDS-PAGE. PAGE analysis of the cell culture supernatant. Protein was induced by methanol, at 30 °C, and monitored with SDS-PAGE. PAGE analysis of the cell culture supernatant. Protein was induced by methanol, at 30 °C, and monitored with SDS-PAGE.
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. 1A). 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 (Met1–Ala29) and an activating peptide of 37 amino acids (Ala30–Arg66) (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. 1A). The NH2-terminal sequence of all four bands was found to be Ile-Ile-Aas-Gly-Ser-Asp, which corresponds to the NH2-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. 1B, lane 2), indicating that recombinant hK5 is differentially glycosylated. A duplicate of the aforementioned gel, including horseradish peroxidase glycoprotein as a positive control (Fig. 1C, lane 4) and the non-glycosylated protein soybean trypsin inhibitor as a negative control (Fig. 1C, lane 3), was subjected to glycoprotein staining. In vitro deglycosylation abolished staining of glycosylated forms of hK5 with acidic fuchsin sulfite (Fig. 1C, 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. 2A) and gelatin (Fig. 2B).

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 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 Asp239, close to Ser245 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 kcat/Km 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  

Table: Steady-state kinetic analysis of hK5 with fluorogenic substrates

| Substrate                          | $K_m$ (mM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ (mM$^{-1}$ min$^{-1}$) | Activity (%) |
|------------------------------------|------------|------------------------|-------------------------------------|--------------|
| 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| 3.18                   | 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-Glu-Lys-Arg-AMC, 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-Leu-Lys-Arg-AMC, Boc-Leu-Arg-Arg-AMC, Boc-Leu-Gly-Arg-AMC) indicates hK5 prefers Leu > Glu > 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 α2-AP and the high molecular weight proteinase inhibitor α2M was examined as described under “Experimental Procedures.” The inactivation of hK5 by AT and α2-AP followed pseudo-first order kinetics when these inhibitors were in a 4–20-fold molar excess. α2-AP was a more efficient inhibitor of hK5 than AT. For example, 50% of hK5 activity was inactivated in 1.5 min while AT was 1.5 μM (Fig. 3B). No inhibition was observed by AAT and ACT. The kinetic constants for inactivation of hK5 by α2-AP and AT were derived from double-reciprocal plots of the pseudo-first order rate constant $k_{cat}$ versus inhibitor concentrations (Fig. 3, A and B, insets) and are listed in Table II. The second-order rate constants $k_{cat}/K_m$ for the reaction of hK5 with these plasma protease inhibitors revealed that the reaction of hK5 with α2-AP was 24 times faster than the reaction between hK5 and AT (Table II).

The inhibition mechanism of serpins is mediated through 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 Met358–Ser359 (AAT) (37), Leu383–Ser384 (ACT) (38), Arg383–Ser384 (AT) (39), and Arg564–Met565 (α2-AP) (40). The Arg preference for hK5 for the P1 position explains its inhibition by the serpins AT and α2-AP, and not by AAT and ACT.

The inhibition of hK5 by the high M, proteinase inhibitor α2M was monitored by using a fluorescent conjugate of fibrinogen as a substrate. In contrast to the aforementioned serpins, complex formation of serine proteases with α2M does not lead to deformation of the catalytic triad. The reaction of proteases with α2M 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
changes of α2M. Thus, α2M 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 α2M, 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 Arg14–Gly15 and Tyr686 (primary cleavage site) and Arg252–Val692 (secondary cleavage site) (42). The human serum protease trypsin, plasmin, and thrombin are “trapped” by α2M after cleavage between Arg681 and Tyr686 (primary cleavage site) and Arg696 and Val700 (secondary cleavage site) (41). Trypsin also cleaves α2M between Arg681 and Val692 (41). Because hK5 shows high specificity for Arg in the P1 position, both Arg696, Lys697 and Arg681, Val692 dipetides 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 α2-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 α2-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. 4A). Human kallikrein 3/protase-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). NH2-terminal sequencing of these fragments revealed that hK5 is able to cleave plasminogen at peptide bonds Lys75–Lys78 and Arg249-Lys550 (Table III). According to these results, the first fragment represents an angiotatin isoform, known as angiotatin 4.5 (AS4.5), which consists of plasminogen kringle 1–4 and 85% of kringle 5 (amino acids Lys78–Arg249). AS4.5 has been shown to be a potent angiogenesis inhibitor (46), significantly more effective than angiotatin K1–3 and angiotatin 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).

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 Arg272–Ile282 (Fig. 6, fragment K4, Table III), which is located near the peptide bond Arg277–Ser278 that is cleaved by plasma and tissue kallikrein for generation of bradykinin and Ly3-bradykinin, respectively. However, hK5, by cleaving at peptide bonds Arg252–Ile258 (Fig. 6, fragment K2, Table III) and Arg252–Asp253 (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 scissile 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. Arg252–Asp253 (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 Arg15–Gly16 and Arg536–Glu538 (Fig. 6, fragments F2 and F3, Table III), and the Bβ chain at peptide bonds Arg14–Gly15 and Lys83–Lys108 (Fig. 6, fragments F4 and F1, Table III). Thrombin cleaves Bβ chain at the same peptide bond, Arg14–Gly15 during the formation of fibrin, to release fibrinopeptide B and unmask the polymerization site E99 (51). Furthermore, hK5, by cleaving at Lys83–Lys108, could also release the peptide B15–42, 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. NH2-terminal sequencing of fragment V1, which is generated within 15 min, revealed that hK5 cleaves at peptide bond Arg222–Ile223 (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).
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 2-AP and AT, with second-order inhibition constants ($k_{2}/K_i$) of $1.0 \times 10^{-2}$ and $4.2 \times 10^{-4}$ M$^{-1}$ min$^{-1}$, respectively. $a_2M$, 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 thiol donor, like N-acetylcysteine (56), or the plasminogen receptor, $\alpha_2$-antiplasmin (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.

**Figure 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 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.”

**Figure 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**

| Fragment | Amino-terminal sequence |
|----------|-------------------------|
| Plasminogen |  |
| P1 Lys-Val-Tyr-Leu-Ser-Glu |
| P2 Lys-Leu-Tyr-Asp-Tyr-X^ |
| Kininogen |  |
| K1 NH$_2$-terminal blocked |
| K2 Ile-Ala-Ser-Phe-Ser-Gln |
| K3 Asp-Ile-Pro-Thr-Asn-Ser |
| K4 Ile-Gly-Glu-Ile-Lys-Glu |
| Fibrinogen (A$\alpha$ chain) |  |
| F2 Gly-Ser-Ala-Gly-X^x-X^x |
| F3 Gly-Tyr-Thr-His-Thr-Glu-Lys |
| Fibrinogen (B$\beta$ chain) |  |
| F1 Lys-Val-Glu-Ard-Lys-Ala |
| F4 Gly-His-Arg-Pro-Leu-Aasp |
| Vitronectin |  |
| V1 X^-Thr-Glu-Gly-Phe-Aasn |

a The amino acid was not determined.
Enzymatic Activity of Human Kallikrein 5

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 α2-AP, AT, and α2M. Its ability to cleave ECM (collagens type I, II, III, IV, fibronectin and laminin) and adhesion molecules (fibronectin and vitronectin) and to release angiotatin 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.

REFERENCES

1. Khan, A. R., and James, M. N. (1998) Protein Sci. 7, 815–836
2. Puente, X. S., Sanchez, L. M., Overall, C. M., and Lopez-Otin, C. (2003) Nat. Rev. Genet. 4, 544–558
3. Yost, G. M., and Diamandis, E. P. (2001) Endo. Rev. 22, 184–204
4. Borgono, C. A., Michael, I. P., and Diamandis, E. P. (2004) Mol. Cancer Res. 2, 257–280
5. Yusef, G. M., Diamandis, M., Jung, K., and Diamandis, E. P. (2001) Genomics 74, 385–395
6. Yusef, G. M., Borgono, C. A., Michael, I. P., Davidian, C., Stephan, C., Jung, K., and Diamandis, E. P. (2004) Tumor Biol. 25, 122–133
7. Foussias, G., Yusef, G. M., and Diamandis, E. P. (2000) Genomics 67, 171–178
8. Diamandis, E. P., Yusef, G. M., Luo, L. Y., Magklara, A., and Obisek, C. V. (2000) Trends Endocrinol. Metab. 11, 54–60
9. Diamandis, E. P., and Yusef, G. M. (2002) Clin. Chem. 48, 1198–1205
10. Diamandis, E. P. (1996) Trends Endocrinol. Metab. 9, 310–316
11. Denneade, S. R., Lovgren, J., Khan, S. R., Lilja, H., and Isacs, J. T. (2001) Prostate 48, 122–126
12. Lovgren, J., Tian, S., Lundwall, A., Karp, M., and Lilja, H. (1999) Eur. J. Biochem. 266, 1050–1055
13. Magklara, A., Mellati, A. A., Wasney, G. A., Little, S. P., Sotiropoulou, G., Becker, G. W., and Diamandis, E. P. (2005) Biochem. Biophys. Res. Commun. 307, 948–955
14. Sotiropoulou, G., Rogakos, V., Tsetsenis, T., Pampalakis, G., Zafiropoulos, N., Similides, G., Yiotakis, A., and Diamandis, E. P. (2003) Oncol. Res. 13, 381–391
15. Yusef, G. M., and Diamandis, E. P. (2002) Biochem. Biophys. Res. Commun. 383, 1045–1057
16. Silverman, G. A., Bird, P. I., Carrell, R. W., Church, F. C., Coughlin, P. B., Granger, P. G., Lenz, A. J., Lucas, C. J., Mayer, K. W., Pemberton, P. A., Remold-O’donnell, E., Salvesen, G. S., Travis, J., and Whistock, J. C. (2001) J. Biol. Chem. 276, 33293–33296
17. Yusef, G. M., and Diamandis, E. P. (1999) J. Biol. Chem. 274, 35117–35118
18. Brattsand, M., and Egelrud, T. (1999) J. Biol. Chem. 274, 30023–30040
19. Diamandis, E. P., Yusef, G. M., and Egelrud, T. (1999) J. Biol. Chem. 274, 35117–35118
20. Kim, H., Scirilas, A., Katsaros, D., Yusef, G. M., Massobrio, M., Fracchioni, S., Piccinno, R., Gordini, G., and Diamandis, E. P. (2001) Br. J. Cancer 84, 643–650
21. Yusef, G. M., Scirilas, A., Kiryakoulou, L. G., Rendl, L., Diamandis, M., Ponzzone, R., Biglia, N., Gai, M., Roagna, R., Simondi, P., and Diamandis, E. P. (2002) Clin. Chem. 48, 1241–1250
22. Yusef, G. M., Scirilas, A., Chang, A., Rendl, L., Diamandis, M., Jung, K., and Diamandis, E. P. (2002) Prostate 51, 126–132
23. Yusef, G. M., Obusek, C. V., Jung, K., Stephan, C., Scirilas, A., and Diamandis, E. P. (2002) Urol. 60, 714–718
24. Yusef, G. M., Polymeris, M., Krueger, M., Grass, L., Scirilas, A., Channing, P. C., Scirilas, A., Borgono, C., Harbeck, N., Schmalfeldt, B., Dorn, J., Schmitt, M., and Diamandis, E. P. (2003) Cancer Res. 63, 3958–3965
25. Diamandis, E. P., Borgono, C., Scirilas, A., Yusef, G. M., Harbeck, N., Dorn, J., Schmalfeldt, B., and Schmitt, M. (2003) Tumor Biol. 24, 299–309
26. Ekholm, I. E., Brattsand, M., and Egelrud, T. (2000) J. Invest. Dermatol. 114, 56–63
27. Komatsu, T., Nakata, M., Otsuki, N., Toyama, T., Oku, R., Takehara, K., and Saji, S. (2003) J. Invest. Dermatol. 121, 542–549
28. Luo, L. Y., Grase, L., Howarth, D. J., Thibault, P., Ong, H., and Diamandis, E. P. (2001) Clin. Chem. 47, 243–246
29. Borgono, C. A., Grass, L., Scirilas, A., Yusef, G. M., Petracik, C. D., Howarth, D. H., Fracchioni, S., Katsaros, D., and Diamandis, E. P. (2003) Cancer Res. 63, 9032–9041
30. Ghosh, M. C., Grass, L., Scirilas, A., Sotiropoulou, G., and Diamandis, E. P. (2004) Tumor Biol. 25, 193–199
31. Kitz, R., and Wilson, J. B. (1992) J. Biol. Chem. 267, 3345–3349
32. Schapira, M., Scott, C. F., and Colman, R. W. (1981) Biochemistry 20, 2738–2743
33. Schapira, M., Scott, C. F., James, A., Silver, L. D., Kueppers, P., James, H. L., and Colman, R. W. (1982) Biochemistry 21, 567–572
