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Characterization of the enzymatic activity of human kallikrein 6: autoactivation, substrate specificity, and regulation by inhibitors☆

Angeliki Magklara,[a,b] Ali A. Mellati,[a] Gregory A. Wasney,[a] Sheila P. Little,[c] Georgia Sotiropoulou,[d] Gerald W. Becker,[e] and Eleftherios P. Diamandis[a,b,*]

[a] *Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, 600 University Avenue, Toronto, Ont., Canada M5G 1X5*
[b] *Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ont., Canada M5G 1L5*
[c] *Central Nervous System Research, Lilly Research Laboratories, a Division of Eli Lilly and Company, Indianapolis, IN 46285, USA*
[d] *Department of Pharmacy, University of Patras, Patras, Greece*
[e] *Roche Diagnostics Corporation, Indianapolis, IN 46250, USA*

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Abstract

Human kallikrein 6 (hK6) is a trypsin-like serine protease, member of the human kallikrein gene family. Studies suggested a potential involvement of hK6 in the development and progression of Alzheimer's disease. The serum levels of hK6 might be used as a biomarker for ovarian cancer. To gain insights into the physiological role of this enzyme, we sought to determine its substrate specificity and its interactions with various inhibitors. We produced the proform of hK6 and showed that this enzyme was able to autoactivate, as well as proteolyse itself, leading to inactivation. Kinetic studies indicated that hK6 cleaved with much higher efficiency after Arg than Lys and with a preference for Ser or Pro in the P2 position. The efficient degradation of fibrinogen and collagen types I and IV by hK6 indicated that this kallikrein might play a role in tissue remodeling and/or tumor invasion and metastasis. We also demonstrated proteolysis of amyloid precursor protein by hK6 and determined the cleavage sites at the N-terminal end of the protein. Inhibition of hK6 was achieved via binding to different serpins, among which antithrombin III was the most efficient.

Keywords: Serine proteases; Human kallikreins; Enzymatic activity; Human kallikrein 6; Enzymatic cascade; Cancer

Tissue kallikreins are secreted serine proteases with a highly conserved gene and protein structure. They are encoded by tandemly localized genes and constitute large families in rodents and humans. The human kallikrein gene family maps to chromosome 19q13.4 and includes 15 members, most of them cloned the past 3–4 years [1]. One member, human kallikrein 3 (hK3/prostate specific antigen, PSA), is a valuable tumor marker of prostatic adenocarcinoma [2]. Recent data advocate that many other kallikreins are promising biomarkers of ovarian, prostate, testicular, and breast carcinomas [3].

Human kallikreins are synthesized as prepropeptides; upon processing, the signal peptide is removed to yield the inactive, zymogen form (prokallikrein). Conversion to the enzymatically active, mature protein occurs by limited proteolysis at the amino-terminal end of prokallikrein. For the 15 known kallikreins, activation occurs by cleavage after lysine (7 enzymes), arginine (7 enzymes), or glutamine (1 kallikrein). Most of the kallikreins are predicted to have trypsin-like activity, cleaving preferentially after a Lys or Arg. This suggests that they may be able to either autoactivate themselves

☆ *Abbreviations:* KLK, kallikrein gene; hK, kallikrein protein; PSA, prostate-specific antigen; MSP, myelencephalon-specific protease; CNS, central nervous system; ELISA, Enzyme-linked immunosorbent assay; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; AMC, 7-amino-4-methyl-coumarin; ACT, α_1-antichymotrypsin; API, α_1-protease inhibitor; AAT, α_1-antitrypsin; ATIII, antithrombin III; α_2M, α_2-macroglobulin; PCI, protein C inhibitor; α_2AP, α_2-antiplasmin; APP, amyloid precursor protein; BSA, bovine serum albumin; ECM, extracellular matrix; uPA, urokinase-type plasminogen activator; Aβ, amyloid β peptide.

* Corresponding author. Fax: +416-586-8628.
E-mail address: ediamandis@mtsinai.on.ca (E.P. Diamandis).

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or activate other prokallikreins in a cascade enzymatic pathway [4]. Once active, serine proteases are commonly regulated via binding to serpins (serine protease inhibitors), which leads to their inactivation. PSA/hK3 can bind to a number of serpins, such as α_1-antichymotrypsin (ACT) [5,6], α_1-protease inhibitor (API), α_1-antitrypsin (AAT) [6], and antithrombin III (ATIII) [7] as well as to another extracellular protease inhibitor, α_2-macroglobulin (α_2M) [8]. Human kallikrein 2 (hK2) can also form complexes with protein C inhibitor (PCI) [9], α_2-antiplasmin (α_2AP), α_2M, ACT, and ATIII [7,10–12].

One of the newly identified members of the human kallikrein gene family is kallikrein 6 (hK6), also known as protease M [13], neurosin [14], or zyme [15]. The elucidation of its genomic organization and fine mapping within the kallikrein locus was reported a couple of years later [16]. The mature form of hK6 is a 223-amino acid protein with a trypsin-like activity, but of unknown physiological role. Early studies at the mRNA level showed expression of *KLK6* in several tissues, including renal, endometrial, mammary, pancreatic, prostatic, and neuronal (brain, cerebellum, and spinal cord) tissues [13,14,16]. The development of a specific immunoassay [17] verified the predicted secretory nature of the protein by detecting it in various biological fluids, such as breast secretions (milk, nipple aspirate, and breast cyst fluid), male and female sera, seminal plasma, cerebrospinal fluid, and amniotic fluid. Immunohistochemistry has shown abundant hK6 expression in various other normal tissues and confirmed its epithelial localization [18].

The predominant expression of *KLK6* in brain cells [13,14] suggested a potential involvement of this kallikrein in the development and progression of Alzheimer's disease (AD) [15]. Little et al. showed accumulation of amyloidogenic fragments in cells co-transfected with hK6 and amyloid precursor protein (APP) and reported increased *KLK6* mRNA expression in AD brain. A subsequent study found decreased expression of *KLK6* in the brain of AD patients [19], results that were verified at the protein level by Zarghooni et al. [20]. Increased protein levels were detected in the cerebrospinal fluid and blood of AD patients [21], while a different group found decreased cerebrospinal fluid levels in AD patients when compared with healthy controls [22]. More recently, Scarisbrick et al. [23] reported presence of myelencephalon-specific protease (MSP), which is identical to hK6, in inflammatory CNS lesions and suggested that this enzyme may promote demyelination.

Equally interesting is the association of hK6 with ovarian cancer. Among many other cancer types, only ovarian cancer patients showed significantly elevated levels of hK6 in the circulation [24]. Another group reported increased expression of hK6 transcripts in ovarian tumors [25], which is in accordance with our own findings at the protein level [26]. HK6 overexpression tended to be higher in tumors from late stage disease and the tumor levels of the protein represent a new, independent, and unfavorable prognostic marker [26].

Current literature suggests a role of hK6 in the pathobiology of severe human diseases, such as neurodegeneration and cancer. To gain further insights into this enzyme, we determined its substrate specificity and studied the regulation of its activity by different inhibitors.

Materials and methods

Production and purification of recombinant prohK6 protein. Human embryonic kidney 293 cells, stably transfected with a plasmid containing the 1.4-Kb prohK6 cDNA, were grown as previously described [15]. Their supernatant was collected and concentrated 20 times by using Centricon ultafiltration devices (Millipore, Waltham, MA). Purification of prohK6 was achieved by ion-exchange chromatography (FPLC). Concentrated supernatant was diluted 1:2 with running buffer (50 mM acetate buffer, pH 5.3) and loaded onto a 5-mL CM Sepharose column (Amersham Biosciences Piscataway, NJ) at a flow rate of 0.5 mL/min. One-milliliter fractions were eluted with elution buffer (50 mM acetate buffer and 1 M NaCl, pH 5.3) at a flow rate of 2.5 mL/min and at a linear gradient of 0–1 M NaCl. Fractions were analyzed by ELISA and SDS–PAGE (NuPage 4–12% Bis–Tris gels; Invitrogen, Burlington, ON). Fractions were pooled and concentrated by ultrafiltration. The final product was analyzed by SDS–PAGE and ELISA to establish purity and concentration.

Autoactivation and autolysis of hK6. Immediately after purification, samples were stored at −20 °C or incubated at 37 °C up to 48 h. Aliquots were evaluated using SDS–PAGE and an enzymatic activity assay (see below). Polypeptides resolved with SDS–PAGE were blotted onto a transfer membrane, briefly stained with Coomassie blue and bands were cut out and subjected to N-terminal sequencing by the Edman degradation method.

Reagents. 7-Amino-4-methylcoumarin (AMC) was purchased from Sigma (St. Louis, MO). The following synthetic peptides were purchased from BACHEM Bioscience (King of Prussia, PA): Pro–Phe–Arg-AMC (PFR-AMC), Val–Pro–Arg-AMC (VPR-AMC), Phe–Ser–Arg-AMC (FSR-AMC), Gln–Gly–Arg-AMC (QGR-AMC), Asp–Pro–Arg-AMC (DPR-AMC), Gly–Gly–Arg-AMC (GGR-AMC), Val–Leu–Lys-AMC (VLK-AMC), Glu–Lys–Lys-AMC (EKK-AMC), and Ala–Ala–Pro–Phe-AMC (AAPF-AMC). All substrates were diluted in DMSO at a final concentration of 80 mM and stored at −20 °C. The serine protease inhibitors AAT, ACT, ATIII, α_2AP, and α_2M were purchased from Calbiochem (San Diego, CA) and their purity was $\geqslant$95% as verified by SDS–PAGE. Benzamidine and soybean trypsin inhibitor were from Sigma. 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). Stock solutions were prepared and stored as per manufacturer's instructions. Casein was from Pierce Chemical (Rockford, IL).

Enzymatic activity assays. The optimum buffer system for analysis of the enzymatic activity of hK6 was a 50-mM Tris, 0.1 M NaCl, and 0.2% bovine serum albumin (BSA) solution, pH 7.3. Unless otherwise stated, 100 μL reactions were set up in microtiter wells and incubated at 37 °C. Fluorescence was measured for 20 min on a Wallac Victor fluorometer (Perkin–Elmer, Wellesley, MA) set at 355 nm for excitation and 460 nm for emission. Enzyme-free reactions were used as a negative control and background fluorescence was subtracted from each value. All experiments were done in triplicate.

Substrate specificity and kinetic constant determination. The rate of reaction of hK6 was determined by incubating 12 nM of the enzyme (or 84 nM for Val–Leu–Lys-AMC and Glu–Lys–Lys-AMC) with varying concentrations (ranging from 0.06 to 3 mM) of different fluorescent substrates. 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).

Digestion of casein, collagen type I and type IV, fibrinogen, and APP. Casein was added to prohK6 at a 10:1 molar ratio in assay buffer and the mixture was incubated at 37 °C. prohK6 (1–10 μM) was mixed with 12.5 μg of the fluorescent complexes of the collagens or fibrinogen, in reaction buffer (0.05 M Tris–HCl, 0.15 M NaCl, 0.5 mM $CaCl_2$, and 2 mM sodium azide, pH 7.6). The 200-μL reaction mixtures were incubated in microtiter plates at 37 °C for up to 8 h and fluorescence was measured. Aliquots, taken at different time points, were analyzed by SDS–PAGE and Coomassie blue staining. Two synthetic oligopeptides (SEVKMDAEFR and DAEFRHDSGYEVHHQKL) representing the N-terminal region of amyloid precursor protein (APP) were incubated with prohK6 at 37 °C for 24 h. Aliquots were analyzed by SDS–PAGE and bands were cut out and subjected to N-terminal sequencing.

Effect of protease inhibitors on hK6. prohK6 (12 nM) was pre-incubated with different concentrations of protease inhibitors at 37 °C for 15–40 min. After addition of 100 μM Phe–Ser–Arg-AMC, the residual enzyme activity was measured. To monitor the hK6–serpin complex formation, the enzyme was incubated at 1:1 and 1:5 molar ratios with the different inhibitors at 37 °C for 30 min, run on an SDS–PAGE under reducing conditions, and stained with Coomassie blue.

Results

hK6 autoactivation and autolysis

N-terminal sequencing of purified prohK6 revealed the sequence **Glu–Glu–Gln–Asn–Lys**–Leu–Val–His–Gly; the first five amino acids (in bold) correspond to the propeptide. The proform of the protein was, as expected, enzymatically inactive. After incubation of prohK6 at 37 °C for approximately 5 min, enzymatic activity was detected, as shown in Fig. 1. Prolonged incubation led to decrease of the enzymatic activity (Fig. 2A) due to autolysis, as revealed by the presence of two bands on SDS–PAGE gel (Fig 2B). N-terminal sequencing verified that the cleavage site for autolysis is between Arg^{76} and Glu^{77}.

Determination of kinetic constants

The enzymatic activity of hK6 was examined with several 7-amino-4-methylcoumarin tripeptides ending in Arg or Lys. The results are presented in Table 1. The substrates Phe–Ser–Arg-AMC and Val–Pro–Arg-AMC were the most favorable and demonstrated the highest k_{cat} (38.3 and 22.8 min^{-1}, respectively) and k_{cat}/K_m (93.4 and 84.4 $mM^{-1}\,min^{-1}$, respectively). From the two lysine-ending substrates that were used, one (Glu–Lys–Lys-AMC) was not hydrolyzed by hK6, while the other (Val–Leu–Lys-AMC) was hydrolyzed but with very low efficiency. Failure to hydrolyze Ala–Ala–Pro–Phe-AMC, a chymotrypsin-specific substrate, confirmed that hK6 possesses no such activity.

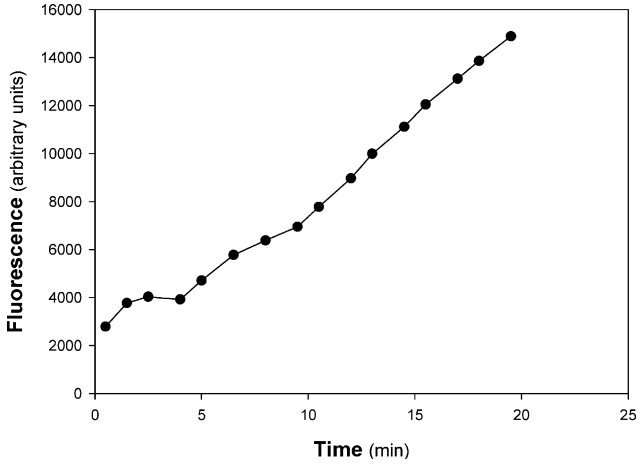

Fig. 1. Autoactivation of hK6. Purified prohK6 was incubated at 37 °C with excess of substrate (Val–Pro–Arg-AMC) and fluorescence was measured for 20 min. Auto-activation is evident after 5 min of incubation.

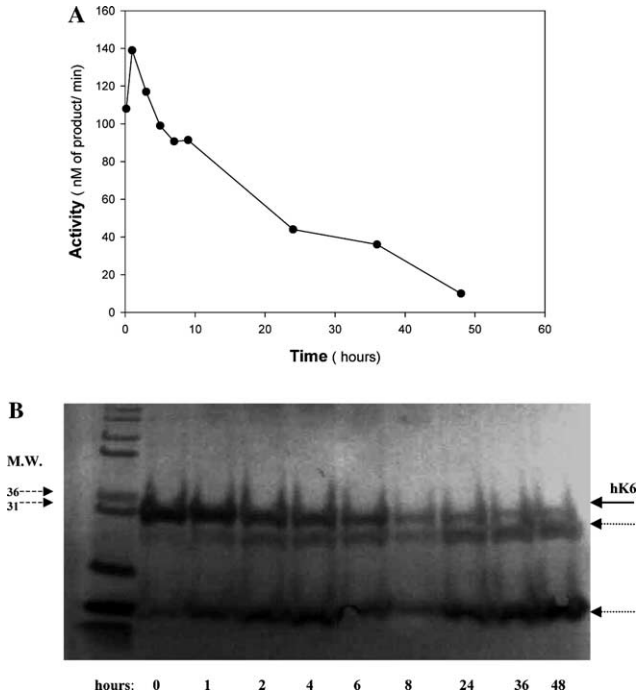

Fig. 2. Autolysis of hK6. Samples containing hK6 were incubated at 37 °C for up to 48 h. Aliquots taken at different time points were assayed for enzymatic activity or run on SDS–PAGE. (A) Decrease of the activity of hK6 with time, which is due to the progressive degradation of the protein as was seen on the gel. (B) The fragments (dashed arrows) were subjected to N-terminal sequencing which revealed that auto-cleavage occurs between Arg^{76} and Glu^{77} of the hK6 sequence. M.W., molecular weights in kilodalton.

Table 1
Kinetic analysis of hK6 with synthetic 7-amino-4-methylcoumarin substrates

Substrate	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
Phe–Ser–Arg-AMC	0.410 ± 0.04	38.3	93.4
Gly–Gly–Arg-AMC	0.455 ± 0.03	6.5	14.3
Asp–Pro–Arg-AMC	0.335 ± 0.06	11.7	34.9
Gln–Gly–Arg-AMC	0.758 ± 0.08	6.25	8.2
Pro–Phe–Arg-AMC	0.625 ± 0.05	4.6	7.4
Val–Pro–Arg-AMC	0.271 ± 0.03	22.8	84.4
Val–Leu–Lys-AMC	1.72 ± 0.42	0.22	0.13
Glu–Lys–Lys-AMC	NR[a]		
Ala–Ala–Pro–Phe-AMC	NR[a]		

[a] No reaction.

Digestion of protein substrates by hK6

Casein, a universal proteinase substrate, was extensively and rapidly degraded by hK6, yielding a number of fragments (Fig. 3A). Both types of collagen and fibrinogen were cleaved by hK6 as indicated by the increase of the emitted fluorescence (Fig. 4). Collagen type I (Fig. 4A) was the best substrate for hK6, being degraded rapidly even by low enzyme concentration (1 μM); collagen type IV (Fig. 4B) required longer incubation times and higher hK6 concentrations. Fig. 3B displays the degradation of fibrinogen by hK6 with time. First, hK6 cleaves the Aα chain of the protein; this follows the pattern of plasmin digestion of fibrinogen, where the Aα carboxy-domains are rapidly cleaved, generating a 40-kDa fragment, which is subsequently degraded into smaller pieces [27]. At the same time, extensive digestion of chain Bβ takes place, while the γ chain appears intact. Synthetic peptides representing the N-terminal region of APP were cleaved at three sites by hK6 (Fig. 5). The first site (K↓M) is one amino acid before the β-secretase site (M↓D), the second one is five amino acids after the N-terminal end of the Aβ peptide (R↓H), and the third is identical to the α-secretase site (K↓L).

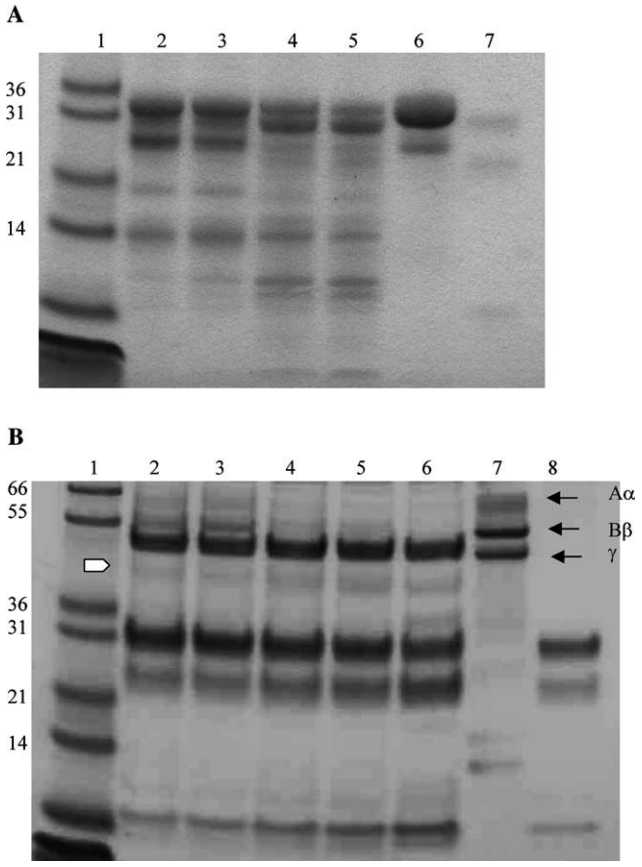

Fig. 3. (A) Degradation of casein by hK6. Lane 1, molecular weight marker (in kilodalton). Incubation for: lane 2, 30 min; lane 3, 1 h; lane 4, 6 h; lane 5, 8 h; lane 6 control, with no added hK6; and lane 7, hK6. (B) Degradation of fibrinogen by hK6. Lane 1, molecular weight marker (in kilodalton). Incubation for: lane 2, 15 min; lane 3, 30 min; lane 4, 90 min; lane 5, 2 h; lane 6, 4 h; lane 7, control, with no added hK6; and lane 8, hK6. The arrows denote the fibrinogen chains Aα, Bβ, and γ and the pentagon the 40-KDa fragment. For more discussion see text.

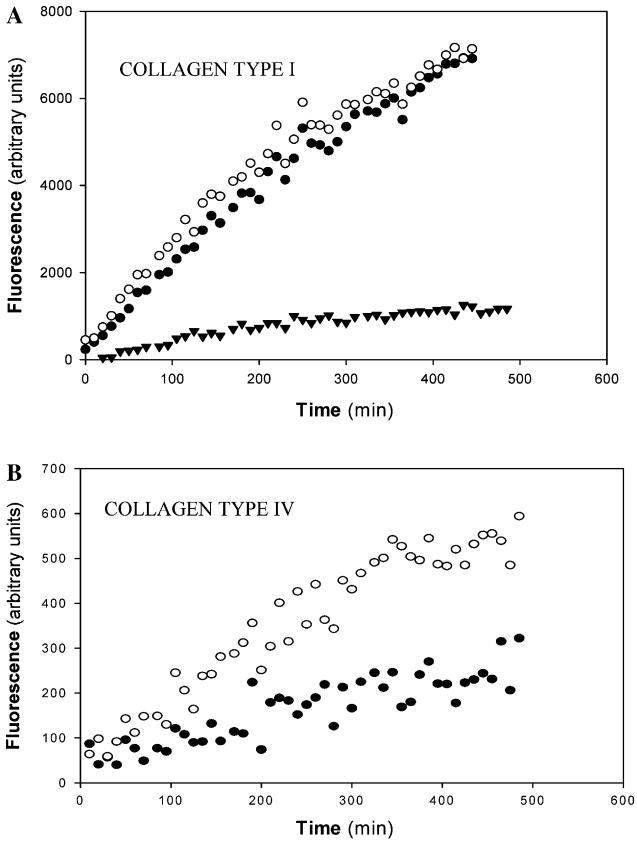

Fig. 4. Fluorescence change during the degradation of fluorescent collagen type I (A) and type IV (B) by hK6. Fluorescent complexes of the two types of collagen were incubated at 37 °C with 1 μM (▼), 5 μM (●), or 10 μM (○) of prohK6. Background fluorescence was subtracted from each value.

Effect of different inhibitors on hK6 activity

The effect of several protease inhibitors on the hydrolytic activity of hK6 is shown in Table 2. Soybean, a

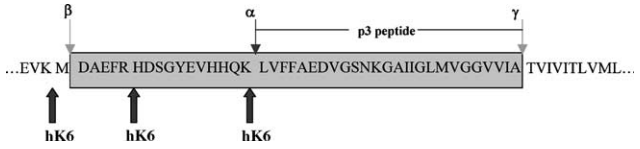

Fig. 5. Processing of the precursor of the Aβ amyloid peptide. Aβ amyloid (in frame) is cleaved from a larger precursor, amyloid precursor protein (APP). The endopeptidase α-secretase cleaves within the Aβ region, resulting in the secretion of the extracellular domain of APP; hence, the cleavage does not produce the Aβ peptide. In contrast, the β- and γ-secretase cleavages do result in production of the peptide. Block arrows indicate the cleavage points by hK6. For more discussion see text.

Table 2
Effect of protease inhibitors on hK6 activity

Inhibitor	Final concentration (μM)	Inhibition (%)
Benzamidine	63	20
Soybean	0.016	82
α_1-Antitrypsin	0.36	12
α_1-Antichymotrypsin	0.12	13
α_2-Antiplasmin	0.15	28
Antithrombin III	0.12	53

trypsin inhibitor, strongly inhibited hK6 even when used at equimolar concentrations. Benzamidine partly inhibited hK6 at relatively high concentrations. Several extracellular serine protease inhibitors were incubated with hK6 at molar ratios from 1:1 to 10:1 (Table 2). From the serpins tested, ATIII was the strongest inhibitor, since it could reduce enzyme activity down to ~50%, when it was used at a concentration 10-fold higher than that of the enzyme, followed by α_2AP. Unexpectedly, ACT inhibited hK6 more effectively than AAT, both required at concentrations 10-fold or higher than hK6. α_2M did not have any inhibitory effect at the concentrations tested. In accordance with these data, SDS–PAGE analysis showed that ATIII and α_2AP could form stable complexes with hK6 even at a 1:1 molar ratio (Fig. 6).

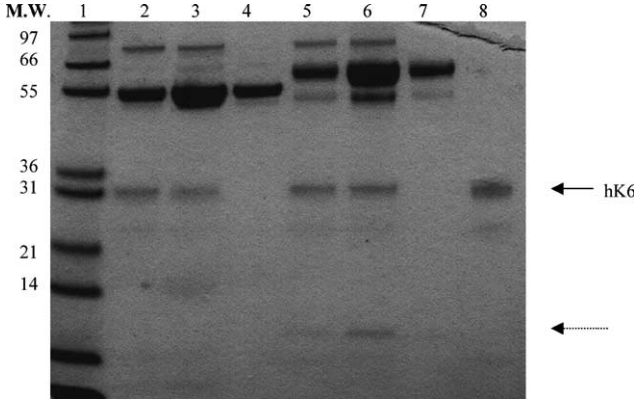

Fig. 6. Formation of complexes between hK6 and serpins. Lane 1, molecular weight marker (in kilodalton); lane 2, prohK6 and ATIII, 1:1 ratio; lane 3, prohK6 and ATIII, 1:5 ratio; lane 4, ATIII; lane 5, prohK6 and α_2AP 1:1 ratio; lane 6, prohK6 and α_2AP 1:5 ratio; lane 7, α_2AP; and lane 8, prohK6. The dashed arrow depicts a ~10-kDa fragment that is produced during the interaction of hK6 with α_2AP. SDS–PAGE was performed under reducing conditions. Note the formation of higher molecular weight complexes in lanes 2, 3, 5, and 6.

Discussion

The crystal structures of the pro- and the mature forms of hK6 were recently resolved [28,29]. The first study suggested that prohK6 is activated in vivo by limited proteolysis between Lys^{15} and Leu^{16}, either autolytically or by another, yet unknown, protease. Both studies identified an autolysis-sensitive point, located at Arg^{76}–Glu^{77} which, when processed, led to an inactive species.

We produced the proform of hK6, which, after purification and concentration, was auto-activated by a short incubation at 37 °C (Fig. 1). Prolonged incubation led to auto-degradation and decrease of its enzymatic activity. N-terminal sequencing confirmed that autolysis occurs between Arg^{76} and Glu^{77}, as previously suggested by others [28,29]. Based on the structural analysis that had predicted the trypsin-like activity of hK6 [14], we set out to experimentally determine its substrate specificity for positions P1, P2, and P3. We used a number of fluorogenic tripeptides that had either Arg or Lys at position P1. We found that hK6 could cleave all the Arg-ending substrates, while it showed very low or no activity for the Lys-ending substrates. This is in accordance with the crystallographic data from Bernett et al. [29], who reported that the structural features of hK6 suggested a generally optimized fit for a P1 guanidino group within the active site that translates into a much higher catalytic efficiency toward substrates with an Arg versus Lys residue in this position. The chymotrypsin substrate AAPF-AMC was not cleaved at all, suggesting no chymotrypsin-like activity for this enzyme. Among the synthetic substrates tested, Phe–Ser–Arg-AMC and Val–Pro–Arg-AMC were the best ones, with the highest k_{cat} and k_{cat}/K_m values. These two substrates were also the best ones for mouse neuropsin [30], a brain-related serine protease that plays an important role in neural plasticity [31]. Our preliminary studies show that human kallikrein 8 (hK8), the human homologue of mouse neuropsin [32], can also cleave Val–Pro–Arg-AMC with high efficiency, indicating that these two human kallikreins may have similar substrate specificity. Interestingly, *KLK8* displays high expression in the brain [22] and has also been associated, like *KLK6*, with ovarian cancer [33,34]. Another serine protease, thrombin, has a preference for small and hydrophobic side chains at P2 (especially Pro) and the Arg to Lys replacement at P1 in chromogenic substrates reduces the value of k_{cat}/K_m by approximately 10-fold [35].

Strong evidence suggests that invasion and metastasis in solid tumors require the action of tumor-associated proteases, which promote dissolution of the surrounding matrix and basement membranes [36]. A well-studied pathway is the urokinase-type plasminogen activator (uPA) system, which, upon activation, leads to degradation of components of the tumor stroma [37]. In this study we provide evidence that hK6 can degrade in vitro fibrinogen and collagen type I, basic constituents of the extracellular matrix (ECM), as well as collagen type IV, a major part of the basement membrane. Others have demonstrated that hK6 can also digest laminin and fibronectin [29]. These findings raise the possibility that hK6 may be an important factor in pericellular proteolysis and tumor invasion. Lysis of certain components of the ECM disrupts its dynamic interaction with the cells and is linked to altered regulation of cell proliferation that leads to tumor cell growth and malignant transformation. Such a role for hK6 is more likely in epithelial ovarian cancer, where its levels are elevated and are associated with unfavorable prognosis [24,26]; similarly, uPA is increased in breast cancer, where it is used as a clinical marker [38].

In AD patients, amyloid plaques containing aggregated β-amyloid peptide (Aβ), generated from the proteolytic cleavage of APP, appear in specific regions of the brain and are linked to the pathophysiology of the disease [39]. Western blots of cells co-transfected with hK6 and APP had shown increased accumulation of the α-secretase fragment [15]. Immunoprecipitation experiments with supernatants of the cells showed drastic reduction of the p3 fragment and production of a truncated form of the Aβ peptide. Using synthetic peptides corresponding to the N-terminal sequence of the Aβ peptide, we showed that hK6 could cleave them in vitro at three different sites (Fig. 5). One site was identical to the one assigned to α-secretase, therefore accumulation of this fragment can be due to direct cleaving action of APP by hK6. The truncated form could be generated by hK6 cleavage between the Arg and His residues close to the N-terminal end of the Aβ peptide (Fig. 5). Insoluble pools of the Aβ peptide in brains of AD patients exhibit considerable N- and C-terminal heterogeneity. Mounting evidence suggests that both C-terminal extensions and N-terminal truncations help precipitation and amyloid plaque formation. While mechanisms underlying the increased generation of C-terminally extended peptides have been extensively studied, relatively little is known about the cellular mechanisms underlying production of N-terminally truncated Aβ [40]. HK6 may be involved in this process, contributing to the downstream effects of amyloid accumulation in AD. However, it is not clear yet whether the truncated form that is generated by the action of hK6 participates in the formation of the plaques. If not, then, the role of hK6 in the brain may be protective rather than harmful, as it has been suggested for another serine protease, plasmin. Plasmin was demonstrated to enhance α-cleavage and to degrade Aβ peptide [41] and its down-regulation in AD brains led the authors to conclude that it may have a protective role in the disease. Alternative actions of hK6 in the brain, and especially in neurodegeneration, could involve inflammatory responses, as reported by Scarisbrick et al. [23].

It is well known that the balance of serine protease activity and inhibitors is critical to physiological events and pathology. Thus, it is important to know which serpins are effective inhibitors of hK6. A hallmark of serpin–serine protease interaction is the formation of a complex that is not easily dissociated by heating or incubation in SDS [42]. This stability indicates the presence of a covalent bond between the enzyme and inhibitor, which arises through stabilization of a covalent intermediate, analogous to that formed during peptide bond hydrolysis [43]. Also, a stoichiometry of 1:1 is typical for serpins reacting with their target proteinases [44]. From the four serpins tested (AAT, ACT, α_2AP, and ATIII) only the latter two could form such complexes with hK6 (Fig. 6), indicating that they are selective inhibitors of this kallikrein. This is not surprising since both ATIII and α_2AP have Arg residues at the P1 reactive center which can be efficiently cleaved by hK6, while AAT has Met and ACT has Leu [45,46].

With the discovery of many novel human kallikreins, new questions are raised regarding regulation and mode of action [1–4]. In this study, we focused on the characterization of the enzymatic activity of hK6. Its trypsin-like proteolytic activity is regulated by three distinct mechanisms: first, by its expression as a zymogen, which can be subjected to auto-activation or activation by other proteases, second, by its complexation with serpins, and third via its autolysis or proteolysis by other enzymes that leads to inactivation. The substrate specificity of hK6 is similar to those of thrombin and plasmin, as was shown here by using synthetic and physiological substrates, as well as by the fact that it can bind efficiently to their respective serpins. The importance of this finding is not clear yet, however, it is tempting to speculate that there may also be a functional analogy between these proteases. For example, they may be sharing, in vivo, some physiological substrates, like fibrinogen; or receptors, belonging to the protease-activated receptor (PAR) family, may be mediating hK6's action in the brain or other tissues, as is the case for thrombin [47]. The ability to cleave ECM proteins, along with its overexpression in ovarian cancer, classifies hK6 in the spectrum of serine proteases that may play an active role in tumor metastasis. In vivo studies toward these directions will clarify the functional role of this kallikrein in health and disease.

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