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Complex formation between human kallikrein 13 and serum protease inhibitors

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Abstract

Background: The kallikrein family is a group of 15 serine protease genes clustered on chromosome 19q13.4. Human kallikrein gene 13 (*KLK13*) is a member of this family and encodes for a trypsin-like, secreted serine protease (hK13). Given that other kallikreins are sequestered by serum protease inhibitors, we hypothesized that hK13 may also interact with similar inhibitors. Our objective was to identify serum protease inhibitors that interact with human hK13. *Methods*: Recombinant hK13 produced in yeast was added to male and female sera and various biological fluids and the spiked samples were analyzed with an hK13 ELISA assay. Enzymatically active hK13 was ^{125}I-labeled and used in in vitro reactions with candidate protease inhibitors and serum samples. The mixtures were then subjected to gel filtration and SDS-PAGE analysis. Candidate inhibitors were also tested in enzymatic assays of hK13 activity. *Results*: The recovery of recombinant hK13 from male and female sera, measured by three versions of the hK13-ELISA, ranged from 5% to 10%. The same recovery was obtained when serum samples from males and females were spiked with hK13 from amniotic fluid and seminal plasma. However, when hK13 was added to other biological fluids, such as amniotic fluid and breast milk, recovery ranged from 70% to 98%. In vitro analysis indicated that enzymatically active ^{125}I-labeled hK13 forms SDS-stable complexes with α_2-antiplasmin, α_2-macroglobulin and α_1-antichymotrypsin. When added to serum, active hK13 formed stable complexes with molecular masses corresponding to hK13 and the inhibitors mentioned above. *Conclusions*: hK13 interacts and forms complexes with serum protease inhibitors, including α_2-macroglobulin, α_1-antichymotrypsin and α_2-antiplasmin.

Keywords: Human kallikreins; Human kallikrein 13; Serine proteases; Serpins; Protease inhibitors; Tumor markers; Complex formation

Abbreviations: KLK, kallikrein gene; hK, kallikrein protein; hK13, human kallikrein 13; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; BSA, bovine serum albumin; AMC, 7-amino-4-methylcoumarin; α_1-ACT, α_1-antichymotrypsin; α_2-M, α_2-macroglobulin; α_2-AP, α_2-antiplasmin.

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1. Introduction

The kallikreins are a subfamily of the serine protease family [1,2]. The human Kallikrein gene family was, until recently, thought to include only 3 members: *KLK1*, encoding for pancreatic/renal kallikrein (hK1); *KLK2*, encoding for human glandular kallikrein 2 (hK2) and KLK3, encoding for prostate-specific antigen (PSA; hK3) [3]. PSA, the best known of the three classical kallikreins, is widely used for diagnosis and management of patients with prostate cancer [4–6]. Recently, new serine proteases with high degree of homology to the three classical kallikreins were cloned. These newly identified serine proteases have now been included in the expanded human kallikrein gene family. The entire human kallikrein gene locus on chromosome 19q13.4 now includes 15 genes, designated *KLK1* to *KLK15*; their respective proteins are known as hK1 to hK15 [7].

KLK13, previously known as *KLK-L4*, is one of the newly identified kallikrein genes [8]. *KLK13* was cloned by the positional candidate approach. The gene spans approximately 10 kb of genomic DNA sequence, and has five coding exons and four introns [8]. The protein has 47% and 45% sequence identity with hK3 (PSA) and hK2, respectively [8]. Predictions made from the primary structure suggest that hK13 has trypsin-like substrate specificity [8]. However, the function of the protein is still unknown. Recently, we developed an immunofluorometric assay for hK13 and found that this protease is expressed in various tissues including esophagus, tonsil, trachea, lung, cervix and prostate. hK13 is also found in seminal plasma, amniotic fluid, follicular fluid, ascites of ovarian cancer patients, breast milk and cytosolic extracts of ovarian cancer tissues [9].

Accumulating evidence suggests that human kallikrein 13 is implicated in endocrine-related malignancies and may serve as a novel cancer biomarker [8,10,11]. Our initial findings indicated that *KLK13* is down-regulated, at the mRNA level, in breast cancer tissues and cell lines. *KLK13* expression is an independent and favorable prognostic marker for breast carcinoma [11]. The expression of this gene is regulated by steroid hormones, mainly androgens and progestins, and to a lesser extent by estrogens, in the breast cancer cell line BT-474 [11]. Taken together these data suggest that human kallikrein 13 could be involved in the pathogenesis and/or progression of some endocrine-related cancers and may find applicability as a novel cancer biomarker.

One mechanism of controlling serine protease activity is by complexation with serine protease inhibitors (serpins). Serpins are usually poor substrates that strongly inhibit the serine protease, after the protease hydrolyzes a peptide bond in the inhibitor. The specificity of these inhibitors depends on the amino acid sequence around their reactive site. Proteases captured by α_2-macroglobulin are covalently bound by a reactive thiol ester in α_2-macroglobulin, which binds mainly to lysine residues in the proteases, to form stable amide cross-links [12]. α_2-Macroglobulin also forms base-labile ester cross-links to serine, threonine, tyrosine, or carbohydrate groups in captured proteases. Given that other kallikreins, such as hK3 (PSA) and hK2 (human glandular kallikrein), are sequestered by protease inhibitors present in serum [13–22], we hypothesized that hK13 may also interact with similar inhibitors. Our objective, then, was to identify hK13 protease inhibitors in serum.

2. Materials and methods

2.1. ^{125}I-labeling of hK13

Recombinant hK13 protein, produced in yeast as previously described [9], was ^{125}I-labeled with Iodobeads®, following the protocol provided by the manufacturer (Pierce Chemical, Rockford, IL). Briefly, 1 iodobead was washed with 500 μl of reaction buffer (0.1 mol/l sodium phosphate buffer, pH 6.5), and then added to 5 μl of Na ^{125}I (100 μCi/μl) along with 500 μl of reaction buffer. This mixture was incubated for 5 min. hK13 (50 μg) was diluted in 500 μl reaction buffer and then added to the above solution and incubated for 5 min. The bead was then removed and the mixture was loaded onto a PD-10 desalting column (Amersham Biosciences, Piscataway, NJ) pre-equilibrated with reaction buffer. Twenty fractions (1 ml each) were then eluted using the reaction buffer. Two peaks of

radioactivity were detected: tubes 3–5 (radiolabeled hK13) and tubes 8–12 (free iodine). The fractions containing the labeled hK13 were pooled and purified further by gel filtration high-performance liquid chromatography.

2.2. Immunofluorometric assay for hK13

For the monoclonal/polyclonal immunoassay configuration (described in detail in Ref. [9]), white polystyrene microtiter plates were coated with mouse monoclonal 11C1 antibody specific for hK13 (100 μl of coating antibody solution containing 500 ng of antibody diluted in 50 mmol/l Tris buffer, pH 7.80 in each well) by overnight incubation. The plates were then washed six times with the washing buffer (9 g/l NaCl and 0.5 g/l Tween 20 in 10 mmol/l Tris buffer, pH 7.40). hK13 calibrators or samples were then pipetted into each well (50 μl/well along with 50 μl of the general diluent; 50 mmol/l Tris with 60 g/l BSA) and incubated for 1 h with shaking; the plates were then washed 6 times with washing buffer. Subsequently, 100 μl of rabbit anti-hK13 antiserum, diluted 1000-fold in buffer A (containing the components of the general diluent plus 25 ml/l normal mouse serum, 100 ml/l normal goat serum, and 10 g/l bovine IgG), was applied to each well and incubated for 30 min; plates were then washed as described above. Finally, 100 μl/well of alkaline phosphatase-conjugated goat anti-rabbit IgG (Fc fragment-specific), diluted 2000-fold in buffer A, was added to each well and incubated for 30 min, and plates were washed as above. Diflunisal phosphate (100 μl of a 1 mmol/l solution) in substrate buffer (0.1 mol/l Tris, pH 9.1, 0.1 mol/l NaCl, and 1 mmol/l $MgCl_2$) was added to each well and incubated for 10 min. Developing solution (100 ìl, containing 1 mol/l Tris base, 0.4 mol/l NaOH, 2 mmol/l $TbCl_3$, and 3 mmol/l EDTA) was pipetted into each well and mixed for 1 min. The fluorescence was measured with a time-resolved fluorometer, the Cyberfluor 615 Immunoanalyzer (MDS Nordion, Kanata, ON, Canada). The calibration and data reduction were performed automatically, as described elsewhere [23]. We have also used another two versions of the hK13 ELISA assay as described elsewhere [9]. In the polyclonal/polyclonal assay configuration, the capture antibody was a sheep anti-mouse polyclonal antibody, while in the monoclonal/monoclonal assay configuration, we coated directly with the 11C1 monoclonal antibody and detected with the biotinylated 2–17 monoclonal antibody. More details of these assays have been published elsewhere [9].

2.3. hK13 hybrid assays

Hybrid immunoassays were performed by utilizing the mouse monoclonal 11C1 antibody specific for hK13 as the capture antibody and replacing the rabbit polyclonal anti-hK13 detection antibody with a rabbit antibody against each of the six common serine protease inhibitors (α_2-macroglobulin, α_1-antitrypsin, α_1-antichymotrypsin, inter-α-trypsin inhibitor, α_2-antiplasmin and antithrombin III) at optimal dilutions (2000-fold dilution in buffer A).

2.4. Recovery

Recombinant hK13 was added at different concentrations to the general diluent (control), to sera from males and females and to various biological fluids and the spiked samples were measured with the monoclonal–monoclonal, monoclonal–polyclonal and polyclonal–polyclonal hK13 ELISAs. Recoveries were then calculated after subtraction of the endogenous concentrations.

2.5. Gel filtration chromatography

Gel filtration chromatography on a TSK–GEL column was used for determining the molecular mass of endogenous (or added) hK13 in biological fluids. All samples were incubated in 0.1% BSA for 2 h at 37 °C prior to gel filtration. The column (0.75×60 cm), equilibrated with 0.1 mol/l NaH_2PO_4/0.1 mol/l Na_2SO_4, pH 6.8, was run at a flow rate of 0.5 ml/min at room temperature (23 ± 2 °C) and 0.5 ml fractions were collected. The column was calibrated with molecular mass markers. The eluted fractions were analyzed for radioactivity and by the hK13 ELISAs.

2.6. Enzymatic activity of hK13

The measurements were performed with the Wallac Victor Fluorometer using a 355-nm excitation filter

and a 460-nm emission filter. The fluorescence of standard 7-amino-4-methylcoumarin (AMC) concentrations was also measured on this instrument and used for conversion of fluorescence per unit time to pmol of AMC per unit time. Unless otherwise indicated, all measurements were performed in 100 μl of the standard kinetic analysis buffer (50 mmol/l Tris, pH 7.4, 0.1 mol/l NaCl, and 0.1% BSA) at 37 °C using 8.2 nmol/l of hK13. BSA was added to the reaction mixture in order to minimize adsorption of the enzyme to the walls of the microtiter wells. The AMC substrates Val-Pro-Arg-AMC, Gln-Gly-Arg-AMC, Phe-Ser-Arg-AMC, Asp-Pro-Arg-AMC, Val-Leu-Lys-AMC and Glu-Lys-Lys-AMC were from Bachem (San Carlos, CA). Substrates were used at concentrations between 0.2 and 5 K_m. The K_m and K_i were calculated using Lineweaver–Burk plots with the Enzyme Kinetics Software provided by Sigma Plot 8 (SPSS Science, Chicago, IL).

2.7. Inhibition of hK13

The activity of hK13 (8.2 nM) was monitored using the substrate Val-Pro-Arg-AMC (250 μmol/l). Substrate hydrolysis was measured for 10 min starting either immediately or 30 min after mixing hK13 with the various inhibitors. The inhibitory effects were evaluated by comparison with inhibitor-free controls.

3. Results

3.1. Recovery of hK13 from serum

The recovery of recombinant hK13 from male and female sera, measured by 3 different versions of the hK13-ELISA, ranged from 5% to 10%. The same recovery was obtained when serum samples from males and females were spiked with hK13 endogenously present in amniotic fluid and seminal plasma. However, when hK13 was added to other biological fluids, such as amniotic fluid and breast milk, recovery ranged from 70% to 98%. This led us to hypothesize that hK13 interacts with binding proteins, more likely protease inhibitors, in serum resulting in the masking of epitopes recognized by hK13 antibodies, thus leading to low recovery. In milk and amniotic fluid, this effect was minimal.

3.2. Complex formation between hK13 and proteinase inhibitors

Twenty nanograms of ^{125}I-labeled hK13 was added to 100 μl of male or female serum samples or to a 60 g/l BSA solution (control). These mixtures were incubated for 1 h at 37 °C and then subjected to gel filtration chromatography. The eluted fractions were analyzed for radioactivity and by the hK13-specific

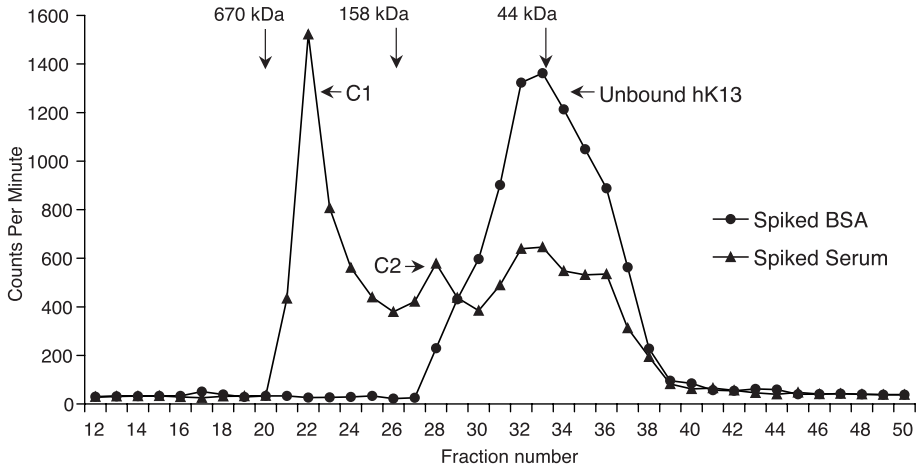

Fig. 1. Gel filtration chromatography of serum spiked with ^{125}I-labeled hK13. The presence of hK13 in the eluted fractions was monitored by radioactivity counting. In addition to the unbound hK13 detected around fractions 33, two other peaks were identified—one around fraction 22 representing complex 1 (C1) and one around fraction 28 representing complex 2 (C2). Elution times of molecular mass standards (in kDa) are also shown.

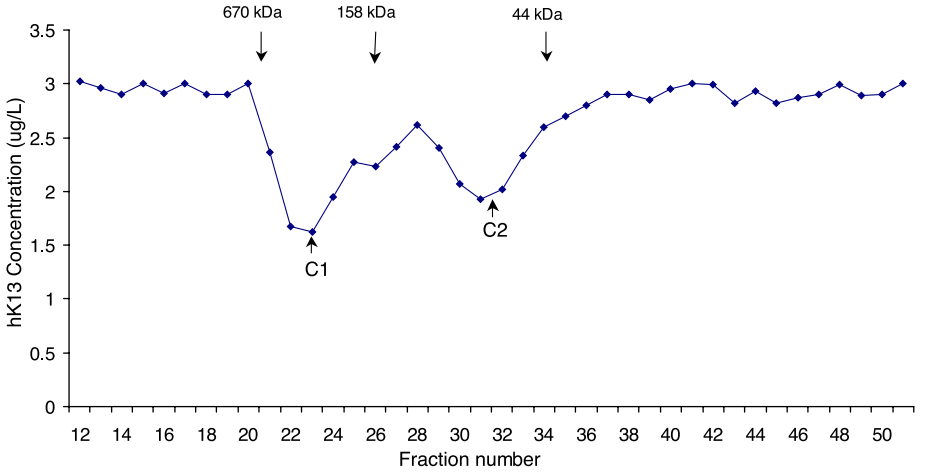

Fig. 2. Gel filtration chromatography of serum. Equal amounts of hK13 were added to each of the eluted fractions. The fractions were incubated and assayed for hK13 with the ELISA-type hK13 immunoassay. Note reduced recovery in 2 areas, around fractions 23 (C1) and 32 (C2). Areas designated C1 and C2 correspond to uncomplexed inhibitors, also depicted in their complexed form in Fig. 1. Arrows on top indicate molecular mass standards.

ELISA (Fig. 1). The radioactive hK13 mixed with the general diluent eluted as 1 broad peak around fraction 33 (~ 44 kDa molecular mass). This fraction corresponds to free, highly glycosylated hK13 as described elsewhere [9].When radioactive hK13 was mixed with serum, three peaks were detected. The first peak eluted around fraction 22 (complex 1; C1; molecular mass = 600 kDa), the second around fraction 28 (complex 2; C2; molecular mass ~ 100 kDa) and the third peak eluted around fraction 33 (unbound hK13). This led us to assume that fractions 22 and 28 contain hK13 complexed with binding proteins present in serum.

3.3. Determination of the molecular weight of hK13 binders

Two hundred microliters of serum, negative for hK13 by ELISA, was subjected to gel filtration chromatography. A portion of hK13 (1.5 ng) was then added to each of the eluted fractions and incubated at 37 °C for 2 h. The fractions were then analyzed for hK13 using the ELISA immunoassay (Fig. 2). We found a distinct reduction in immunoreactive levels of hK13 around fractions 21–24 and 29–33. Fractions 21–24 ($\geq$ 600 kDa) most likely contain the inhibitor present in complex 1 (C1; see Fig. 1) and fractions 29–33 (~ 80 kDa) likely contain the inhibitor present in complex 2 (C2; see Fig. 1). The slightly longer elution times in Fig. 2, compared to those of Fig. 1, are due to the fact that Fig. 1 depicts complexes of hK13 with inhibitors while Fig. 2 represents the elution profile of uncomplexed inhibitors.

3.4. Identification of hK13 proteinase inhibitors

Reaction mixtures were prepared, in which 10 μg of hK13 were mixed with an approximately equi-

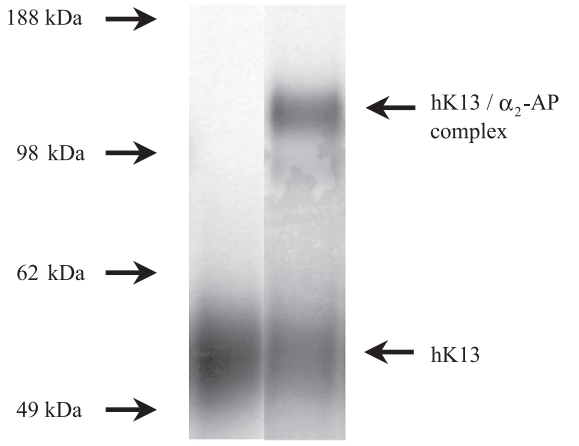

Fig. 3. Autoradiogram of an SDS-PAGE gel containing ^{125}I-labeled hK13 mixed with a general diluent (control experiment; lane 1) and α2-antiplasmin (α2-AP) (lane 2). Arrows on left indicate molecular mass standards.

molar amount of α_2-antiplasmin (70 kDa), α_1-antichymotrypsin (68 kDa), α_2-macroglobulin (600 kDa), α_1-antitrypsin (51 kDa), antithrombin III (61 kDa) and C1 esterase inhibitor (104 kDa). hK13 was also spiked in serum. The respective incubations were all performed at 37 °C for 2 h in 0.1% BSA. One set of reaction mixtures was analyzed by SDS-PAGE and another by gel filtration chromatography. SDS-stable complexes formed when hK13 was incubated with α_2-antiplasmin (Fig. 3), α_2-macroglobulin (Fig. 4B) and α_1-antichymotrypsin (Fig. 4A and B). These complexes had molecular masses corresponding to the aggregate mass of hK13 and each of the three inhibitors. These three complexes were also detected when the mixtures were subjected to gel filtration chromatography, as

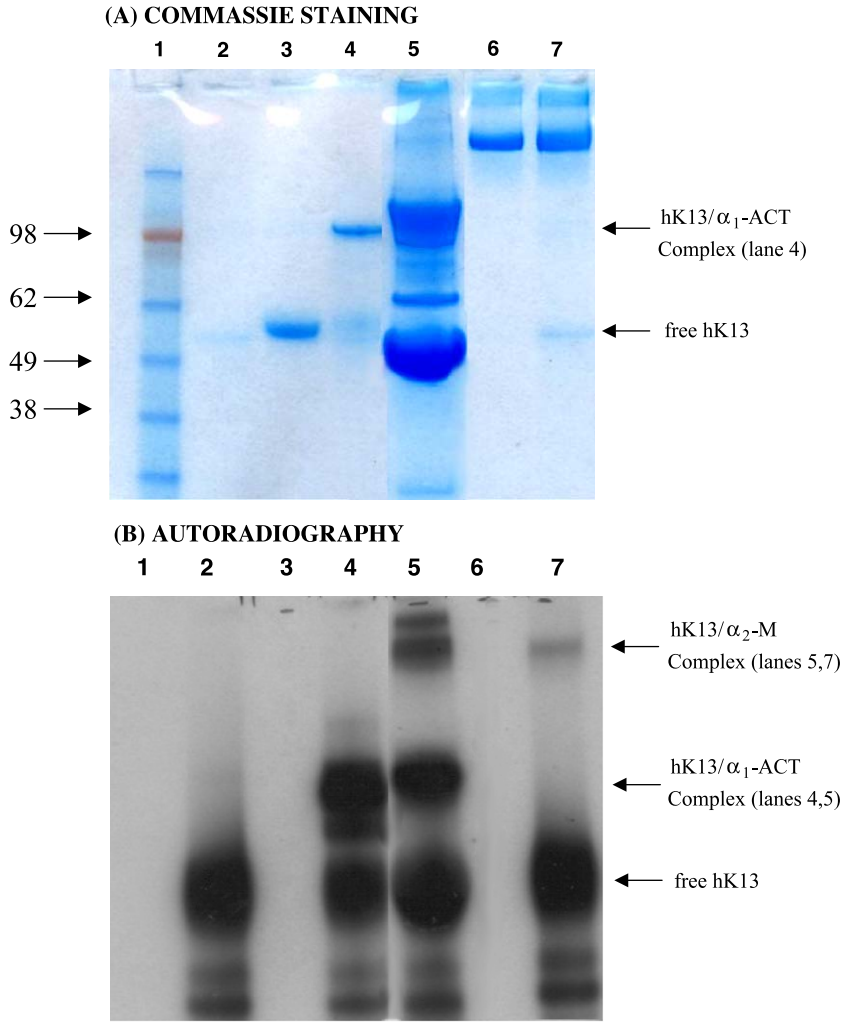

Fig. 4. (A) Commassie-stained SDS-PAGE gel. Lane 1: molecular mass standards. Lane 2: ^{125}I-labeled hK13. Lane 3: α1-antichymotrypsin (α1-ACT). Lane 4: ^{125}I-labeled hK13 incubated with α1-ACT. Lane 5: ^{125}I-labeled hK13 incubated with serum. Lane 6: α2-macroglobulin (α2-M). Lane 7: ^{125}I-labeled hK13 incubated with α2-M. (B) Autoradiogram of the gel shown in panel A. Lane 2: ^{125}I-labeled hK13. Lane 4: ^{125}I-labeled hK13 incubated with α1-ACT; note the shift of radiolabeled hK13 due to complex formation with α1-ACT. Lane 5: ^{125}I-labeled hK13 incubated with serum; note the shift of radiolabeled hK13 due to complex with α1-ACT and α2-M. Lane 7: ^{125}I-labeled hK13 incubated with α2-M; note the shift of radiolabeled hK13 due to binding to α2-M.

seen in Fig. 5B–D. No complexes were detected when hK13 was incubated with α_1-antitrypsin, antithrombin III and C1 esterase inhibitor, as judged by SDS-PAGE gel electrophoresis and gel filtration chromatography (data not shown).

3.5. Identification of hK13 inhibitors in ascites fluid

Ascites fluid samples from ovarian cancer patients, positive for hK13 by ELISA analysis, were subjected to gel filtration chromatography. The presence of hK13 in the fractions was then assessed with the hK13-specific ELISA. hK13 eluted in two peaks as shown in Fig. 6. The first peak was around fraction 32 (~ 100 kDa; hK13 complex) and the second peak was around fraction 40 (~ 35 kDa; unbound hK13). These same fractions were then analyzed by hybrid assays as described under methods. By using these assays, we confirmed that the complex detected by the hK13-specific ELISA was hK13 bound to α_1-antichymotrypsin (Fig. 6). Hybrid assays with detection antibodies other than that of α_1-antichymotrypsin gave no appreciable signal above the baseline level of fluorescence counts.

3.6. Hydrolysis of various peptide substrates by hK13

Substrates ending at either arginine or lysine were tested. The kinetic constants for hydrolysis of these substrates by hK13 are shown in Table 1. The best substrate was Val-Pro-Arg-AMC having the highest K_{cat} and K_{cat}/K_m values. Phe-Ser-Arg-AMC was also cleaved efficiently, having a relatively high K_{cat} value

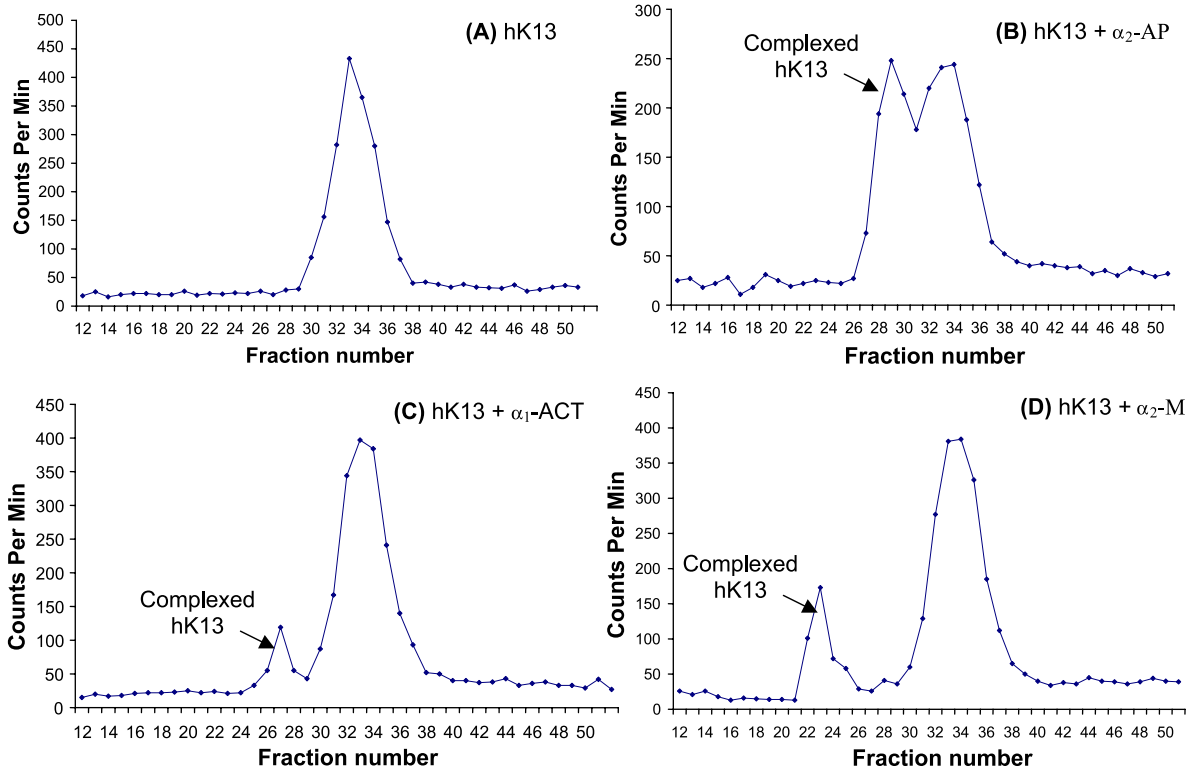

Fig. 5. (A) Gel filtration chromatography of ^{125}I-labeled hK13 in the general diluent. (B) ^{125}I-labeled hK13 mixed with α2-antiplasmin (α2-AP). (C) ^{125}I-labeled hK13 mixed with α_1-antichymotrypsin (α_1-ACT). (D) ^{125}I-labeled hK13 mixed with α2-macroglobulin (α2-M). Note the presence of bound forms of hK13 in panels B, C and D.

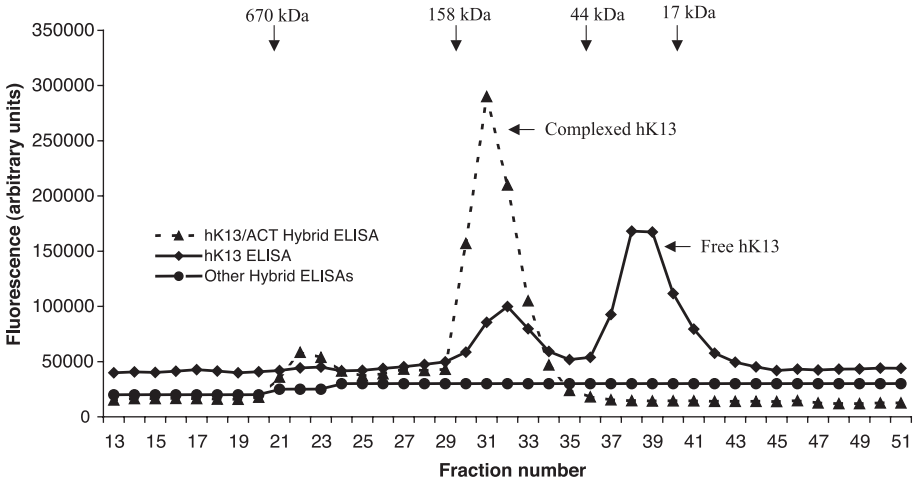

Fig. 6. Gel filtration chromatography of ascites fluid from an ovarian cancer patient. The fractions were analyzed for hK13 with the hK13-specific ELISA. The first peak corresponds to complexed hK13 and the second peak corresponds to unbound (free) hK13. The fractions were then analyzed with the hK13/α1-ACT, hK13/α2-AP, hK13/α1-AT, hK13/antithrombinIII, hK13/inter-α-trypsin inhibitor, and hK13/α2-M hybrid assays as described in Materials and methods. The complexed peak represents hK13 complexed with α1-ACT. Arrows on top show molecular mass standards.

and a low K_m, resulting in a 3-fold lower K_{cat}/K_m value than that obtained for the best substrate. Lysine containing substrates were not cleaved as efficiently (Table 1).

3.7. Comparison of hydrolysis of two substrates by trypsin and hK13

Trypsin and hK13 hydrolysis of Val-Pro-Arg-AMC and Val-Leu-Lys-AMC are shown in Table 2. Trypsin's $K_{cat}/K_m$for Val-Pro-Arg-AMC was approximately 2.5-fold higher than that of hK13. Clearly, trypsin was far more efficient in cleaving substrates ending at Lys than hK13 (Table 2).

3.8. Effects of protease inhibitors on hK13 activity

The effect of several protease inhibitors on the hydrolytic activity of hK13 is shown in Table 3. α_2-Antiplasmin inhibited hK13 in a dose-dependent manner; its K_i was determined to be 9.9 nmol/l. This K_i is close to that determined for activated protein C (a serine protease) and protein C inhibitor [24]. α_1-Antichymotrypsin also inhibited hK13 enzymatic activity but not as effectively as α_2-antiplasmin and with no apparent dose response. α_2-Macroglobulin, in vitro, was found to be the least effective inhibitor of the three, as shown in Table 3. α_1-Antitrypsin,

Table 1
Hydrolysis of 7-amino-4-methylcoumarin (AMC)-linked substrates by human kallikrein 13

Substrate	K_{cat} (min^{-1})	K_m (mmol/l)	K_{cat}/K_m (min^{-1} $mmol/l^{-1}$)
VPR–AMC[a]	75	0.213	353
QGR–AMC	6.3	0.378	17
FSR–AMC	20.8	0.186	112
DPR–AMC	18.1	0.305	60
VLK–AMC	10.5	3	3.5
EKK–AMC	NR[b]	NR	NR

[a] Amino acids are represented with single letter abbreviation.
[b] NR, not reactive.

Table 2
Comparison of substrate hydrolysis by hK13 and trypsin

Enzyme	Substrate	K_{cat} (min^{-1})	K_m (mmol/l)	K_{cat}/K_m (min^{-1} $mmol/l^{-1}$)
Trypsin	VPR–AMC	123	0.15	821
	VLK–AMC	159	0.12	1331
hK13	VPR–AMC	75	0.21	353
	VLK–AMC	10.5	3	3.5

Table 3
Effect of protease inhibitors on hK13 enzymatic activity[a]

Inhibitor	hK13/inhibitor ratio	% Inhibition
α_2-antiplasmin	1:5	17
	1:10	25
	1:20	53
α_2-macroglobulin	1:3	7
	1:6	10
	1:600	12
α_1-antichymortrypsin	1:8	12
	1:16	29
	1:32	30
	1:28	39
α_1-antitrypsin	1:5 to 1:25	0
Antithbomvin III	1:5 to 1:25	0
Cl esterase in inhibitor	1:5 to 1:25	0
Benzamidine	1:310	3
	1:7768	15
	1:31,000	25
Soybean trypsin inhibitor	1:6	29
	1:12	49
	1:24	55
	1:48	62

[a] Substrate used was VPR–AMC.

antithrombin III and C1 esterase inhibitor had no effect on hK13 enzymatic activity. Inhibition was further seen with benzamidine and soybean trypsin inhibitor.

4. Discussion

It is already well known that 2 kallikreins, hK2 (human glandular kallikrein 2) and hK3 (PSA) form complexes with many proteinase inhibitors in serum [13–22]. The identification of all possible protease inhibitors that bind to different kallikreins represents an important step towards our understanding of the biological role of kallikreins. In addition, kallikrein/protease inhibitor complexes may have clinical applications [15]. For example, the proportion of the PSA/α_1-antichymotrypsin complex was shown to be higher in patients with prostatic cancer than in those with benign hyperplasia. Measurement of the free or the complexed fraction of PSA has higher specificity for cancer than the assay of total PSA immunoreactivity [14,15]. Our hK13 ELISAs revealed that the recovery of recombinant hK13 from serum was incomplete, with values ranging from 5% to 10%. We did not observe any differences between the monoclonal–monoclonal, monoclonal–polyclonal and polyclonal–polyclonal immunoassay configurations. We utilized three different ELISA assays because we wanted to see if the complexed hK13 could be detected using different antibodies that would recognize different epitopes on hK13 that may not be masked by the inhibitor. These data led us to hypothesize that serum contains proteinase inhibitors such as α_2-macroglobulin and members of the serine protease inhibitor family (serpins) that may be sequestering hK13.

Based on our in vitro experiments, we identified three protease inhibitors that can bind to hK13. These inhibitors are α_2-antiplasmin, α_2-macroglobulin and α_1-antichymotrypsin. Identification of α_1-antichymotrypsin as an hK13 inhibitor was somewhat surprising since hK13 is predicted to have trypsin-like activity [8]. However, previous studies have shown that α_1-antichymotrypsin can also bind hK2 and hK6, two other trypsin-like kallikreins [19, 25]. The in vitro formation of a complex between hK13 and α_2-macroglobulin resulted in the loss of hK13 immunoreactivity, as revealed by our hK13 ELISA assays. hK13 migrates together with α_2-macroglobulin in gel filtration chromatography experiments using ^{125}I-labeled hK13, as well as by SDS-PAGE (Figs. 1, 2, 4 and 5). The observed loss of hK13 immunoreactivity is consistent with the known ability of α_2-macroglobulin to enclose the proteinase, due to its large size [12–14]. Confirmation of complex formation between hK13 and α_2-antiplasmin is provided in Figs. 3 and 5 and between hK13 and α_1-antichymotrypsin in (Figs. 1, 2, 1, 2, 4, 5 and 6. We verified that the hK13/α_1-antichymotrypsin complex is present in vivo, in the ascites fluid from an ovarian cancer patient (Fig. 6). In serum, levels of immunoreactive hK13 are too low and are not usually measurable by our ELISA assays [9]. We hypothesize that hK13 is not measurable in serum due to sequestration by α_2-macroglobulin and other inhibitors (Fig. 1).

Despite avid binding of hK13 to serum α_2-macroglobulin (Figs. 1 and 4), our in vitro experiments showed a relatively weak complexation (Figs. 4 and 5). We speculate that this is due to the presence of biologically inactive α_2-macroglobulin in the commercially available preparations (Dr. D. Deperthes, personal communication).

We examined the substrate specificity of hK13. Four of the 6 tested peptide–AMC substrates were

hydrolyzed by hK13 and all of them had arginine in P_1. From these substrates, Val-Pro-Arg-AMC, which is also cleaved by Kex2, thrombin and trypsin, was the best, with the highest K_{cat} and K_{cat}/K_m values. Phe-Ser-Arg-AMC was also cleaved efficiently. Substrates with lysine in P_1 were either hydrolyzed very slowly or not at all (Table 1). We also examined hK13 enzymatic activity in the presence of the six serpins, α_2-macroglobulin, benzamidine and soybean trypsin inhibitor (the last two being controls). From the serpins, α_2-antiplasmin decreased hK13 enzymatic activity by 50% when the inhibitor was used at a 20-fold molar excess. Upon increasing the concentration of α_2-antiplasmin, hK13 activity decreased even further. We determined its K_i to be 9.9 nmol/l. α_1-Antichymotrypsin was not as effective as α_2-antiplasmin in inhibiting hK13 enzymatic activity. In order to decrease hK13 activity by 40%, a 120-fold molar excess of α_1-antichymotrypsin was used. α_2-Macroglobulin was the least effective of the three inhibitors. This is not surprising since α_2-macroglobulin is known to enclose the protease in such a way that the enzyme is unable to hydrolyze macromolecules but is still active against low-molecular mass synthetic substrates [12]. This has already been shown for PSA, where there was no inhibition of hydrolytic activity against low-molecular-mass substrates when α_2-macroglobulin complexed the protease [13]. Benzamidine, a synthetic inhibitor for trypsin-like enzymes, decreased but did not abolish hK13 enzymatic activity when used at high concentrations. Similar comments apply to soybean trypsin inhibitor.

In recent years, aberrant expression of serine proteases such as plasminogen activator has been shown to correlate positively with the invasiveness and metastatic potential of tumor cells [26]. Specific inhibitors for many proteolytic enzymes have been identified and it has been contemplated that these inhibitors inhibit extracellular degradation, which, in turn, prevents tumor cell invasion. For example, plasminogen-activator–inhibitor 1 is suggested to protect the tumor stroma from ongoing urokinase-plasminogen-activator-mediated proteolysis in many human tumors [27]. The proteolytic activity associated with tumors is probably a highly regulated cascade and the interplay between proteases and their inhibitors may play a specific role in tumor development and progression. It has already been demonstrated that multiple kallikreins are overexpressed in ovarian carcinoma [28]. The possible coordinated expression between kallikreins and their inhibitors in malignancy has been reported [29]. Now that more inhibitors are beginning to be identified for many kallikreins, it will be worthwhile to examine the expression of all these molecules in cancer tissues, in order to understand the role of this complicated system in cancer invasion and metastasis.

In conclusion, we present here the first evidence that hK13, which has been shown to be overexpressed in ovarian carcinoma forms complexes with α_2-macroglobulin and two members of the serpin family (α_2-antiplasmin and α_1-antichymotrypsin). These data will contribute to improve our understanding of the role of this enzyme and its regulation in health and disease states.

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