

Human Tissue Kallikrein 5 Is a Member of a Proteolytic Cascade Pathway Involved in Seminal Clot Liquefaction and Potentially in Prostate Cancer Progression*

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Human tissue kallikreins (hKs) are a family of fifteen serine proteases. Several lines of evidence suggest that hKs participate in proteolytic cascade pathways. Human kallikrein 5 (hK5) has trypsin-like activity, is able to self-activate, and is co-expressed in various tissues with other hKs. In this study, we examined the ability of hK5 to activate other hKs. By using synthetic heptapeptides that encompass the activation site of each kallikrein and recombinant pro-hKs, we demonstrated that hK5 is able to activate pro-hK2 and pro-hK3. We then showed that, following their activation, hK5 can internally cleave and deactivate hK2 and hK3. Given the predominant expression of hK2 and hK3 in the prostate, we examined the pathophysiological role of hK5 in this tissue. We studied the regulation of hK5 activity by cations (Zn^{2+} , Ca^{2+} , Mg^{2+} , Na^+ , and K^+) and citrate and showed that Zn^{2+} can efficiently inhibit hK5 activity at levels well below its normal concentration in the prostate. We also show that hK5 can degrade semenogelins I and II, the major components of the seminal clot. Semenogelins can reverse the inhibition of hK5 by Zn^{2+} , providing a novel regulatory mechanism of its serine protease activity. hK5 is also able to internally cleave insulin-like growth factor-binding proteins 1, 2, 3, 4, and 5, but not 6, suggesting that it might be involved in prostate cancer progression through growth factor regulation. Our results uncover a kallikrein proteolytic cascade pathway in the prostate that participates in seminal clot liquefaction and probably in prostate cancer progression.

Proteolytic cascade pathways are implicated in many physiological functions such as blood coagulation, fibrinolysis, apoptosis, digestion, among others (1). Proteases are usually synthesized as inactive zymogens and require limited (auto)proteolysis of their propeptide to become active (2). Activation of a zymogen by the activated form of another protease can give rise to proteolytic cascades. This allows rapid amplification of the initial signal, followed by downstream control through inhibitors or (auto)digestion. Serine proteases, the second largest family of proteases, are known to participate in proteolytic cascade pathways, e.g. factors VII, X, and XI, during the coagulation and fibrin formation cascade (1).

Human tissue kallikreins are 15 homologous serine protease genes that co-localize in tandem to chromosome 19q13.4 (3–5). The associa-

tion of multiple members of this family with many cancer types, such as prostate, breast, and ovarian, as well their diagnostic/prognostic value have been extensively studied (6–8). Human kallikrein 3 (hK3/prostate-specific antigen)² is a valuable biomarker for prostatic adenocarcinoma (9). Recently, it has been realized that human kallikreins may function through proteolytic cascades (10, 11). *KLK1* genes reside at a single locus, and many are regulated by steroids and co-expressed in various tissues and fluids and concurrently up- or down-regulated during tumor progression (5, 12). Similarly to hKs, several serine proteases involved in sequential steps during the coagulation cascade are encoded by tandemly co-localized genes, and some may share a common ancestor (1, 13, 14). Furthermore, the facts that 14 of the 15 kallikreins (except hK4) require cleavage of their propeptide after lysine (hK6, hK7, hK8, hK12, hK13, hK14, and hK15) or arginine (hK1, hK2, hK3, hK5, hK9, hK10, and hK11) by a trypsin-like enzyme, and 12 of them are predicted to have trypsin-like activity (except hK3, hK7, and hK9, which have chymotrypsin-like activity), strengthen the possibility that hKs are part of an as yet elusive proteolytic cascade.

Currently, it is known that hK2, hK4, and hK15 can activate pro-hK3 *in vitro* and that they may be involved in a proteolytic cascade in prostate tissue and seminal plasma (15–19). Other members of the family, such as hK5, hK8, hK11, hK13, and hK14, are also expressed in the prostate and are secreted in seminal plasma, so they might also participate in related cascades. Brattsand *et al.* (11) have recently suggested that a proteolytic cascade of kallikreins operates in the stratum corneum and that hK5, *in vitro*, can activate pro-hK7. In addition, some hKs, *i.e.* hK2 (20, 21), hK5 (11), hK6 (22, 23), and hK13 (24), are capable of autoactivation and may, therefore, be involved in the initiation and maintenance of a cascade, similar to factor XI of the intrinsic coagulation (25).

Human kallikrein 5 (hK5, encoded by the *KLK5* gene) is a relatively new member of the human kallikrein family of serine proteases (26, 27). Studies have shown that *KLK5* is differentially regulated in a variety of hormone-dependent malignancies, including ovarian (28), breast (29), prostate (30), and testicular (31) cancers. By using an hK5-specific enzyme-linked immunosorbent assay, we have recently shown that hK5 is a potential biomarker for ovarian and breast cancer (32, 33). We have previously shown that hK5 has trypsin-like activity with strong pref-

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² The abbreviations used are: hK, kallikrein protein; *KLK*, kallikrein gene; AMC, 7-amino-4-methyl-coumarin; pNA, p-nitroanilide; ACT, α_1 -antichymotrypsin; IGF, insulin-like growth factor; IGF-BP, insulin-like growth factor-binding protein; IGF-IR, insulin-like growth factor receptor I; MMP, metalloproteinase; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

Kallikrein 5 and Proteolytic Cascade Pathways

erence for Arg over Lys for the P1 position and that its activity is inhibited by the serpins α_2 -antiplasmin and α_1 -antithrombin (34). Furthermore, we proposed that hK5 is implicated in tumor progression by degrading components of the extracellular matrix such as collagen types I–IV, fibronectin, and laminin and by releasing angiotensin4.5 from plasminogen (34).

In this study we examined the interaction of hK5 with the remaining members of the kallikrein family and its ability to activate them. We show that hK5 is able to activate pro-hK2 and pro-hK3 and subsequently deactivate them. Our data also indicate that hK5, along with other members of the human kallikrein family, may participate in a proteolytic cascade pathway that plays a role during seminal clot liquefaction and potentially in prostate cancer progression.

EXPERIMENTAL PROCEDURES

Materials—The synthetic heptapeptides N-Ile-Gln-Ser-Arg-Ile-Val-Gly-C, N-Ile-Leu-Ser-Arg-Ile-Val-Gly-C, N-Ser-Cys-Ser-Gln-Ile-Ile-Asn-C, N-Ser-Ser-Ser-Arg-Ile-Ile-Asn-C, N-Glu-Gln-Asn-Lys-Leu-Val-His-C, N-Gln-Gly-Asp-Lys-Ile-Ile-Asp-C, N-Gln-Glu-Asp-Lys-Val-Leu-Gly-C, N-Asp-Thr-Arg-Ala-Ile-Gly-C, N-Asn-Asp-Thr-Arg-Leu-Asp-Pro-C, N-Glu-Thr-Arg-Ile-Ile-Lys-C, N-Ala-Thr-Pro-Lys-Ile-Phe-Asn-C, N-Glu-Ser-Ser-Lys-Val-Leu-Asn-C, N-Asp-Glu-Asn-Lys-Ile-Ile-Gly-C, and N-Asp-Gly-Asp-Lys-Leu-Leu-Glu-C were purchased from Genemed Synthesis, San Francisco, CA. The heptapeptides were diluted with water and stored at -20°C . The synthetic substrates, Val-Pro-Arg-AMC (VPR-AMC), Suc-Ala-Ala-Pro-Phe-AMC (AAPF-AMC), and MeO-Suc-Arg-Pro-Tyr-pNA-HCl (RPY-pNA), were purchased from Bachem Bioscience (King of Prussia, PA) and the latter one from Amersham Biosciences-Hepar-Chromogenix (Franklin, OH), respectively. The protease inhibitor ACT was purchased from Calbiochem; its purity was $\geq 95\%$ as verified by SDS-PAGE. A 25-cm C_{18} column, 50-Å pore size, was purchased from TOSOH, Grove City, OH. Mutated pro[Val²¹⁷]hK2 (pro-hK2^{mut}), which abolishes its ability to autoactivate, and wild-type-active hK2 (hK2^{wt}) were obtained from Hybritech Inc., San Diego, CA. Pro-hK3 was obtained from Spectra Diagnostics Inc, Toronto, Ontario, Canada. hK5 was produced in-house as has been previously described (34). Semenogelins I and II were purified from seminal plasma as has been previously described (35). Insulin-like growth factor-binding proteins 1–6 were purchased from Diagnostic System Laboratories, Webster, TX.

Cleavage of Heptapeptides by hK5—25 μg of the heptapeptides were incubated with 1 μg of hK5 (1500:1 molar ratio) in assay buffer (hK5 optimal buffer, 100 mM phosphate buffer, 0.01% Tween 20, pH 8.0), at a final volume of 150 μl . The reaction was incubated at 37°C for 0.5, 1, 2, 4, and 8 h and terminated by freezing the samples with liquid nitrogen. 100 μl from each time point was diluted 1.5-fold with loading buffer (distilled H_2O , 0.1% trifluoroacetic acid) and loaded to a C_{18} column connected to an high-performance liquid chromatography system at a flow rate of 0.8 ml/min. Elution was performed by using Buffer A (distilled H_2O , 0.1% trifluoroacetic acid) and Buffer B (acetonitrile 0.1% trifluoroacetic acid) with a linear gradient of 0–100% acetonitrile at a flow rate of 0.8 ml/min.

Activation of Pro-hK3 and Pro-hK2^{mut} by hK5—The activation of pro-hK2^{mut} and pro-hK3 was monitored by complex formation of hK2 and hK3 with the serpin ACT, an inhibitor for the aforementioned kallikreins but not for hK5. After incubating pro-hK2^{mut} and pro-hK3 with hK5 at different molar ratios and incubation times, ACT and assay buffer for hK2 and hK3 (hK2 optimal buffer, 0.1 mM Tris-HCl, 0.1 mM NaCl, 0.01% Tween 20, pH 7.5, and hK3 optimal buffer, 0.1 mM Tris-HCl, 3 mM NaCl, 0.01% Tween 20, pH 7.5) were added in a final volume 150 μl . The reaction was incubated for 4 h at 37°C and terminated by

freezing in liquid nitrogen. Positive and negative controls were included as well. 25- μl samples of each reaction were run on SDS-PAGE under reducing conditions and stained with Coomassie Blue to monitor the hK2/3·ACT complex formation.

The activation of pro-hK3 by hK5 was also monitored by the release of AMC or pNA and the increase of fluorescence or absorbance, respectively, from hK3-specific substrates, *i.e.* AAPF-AMC and RPY-pNA. Pro-hK3 was incubated with hK5, at different molar ratios and incubation times, in hK5 assay buffer at 37°C . The final volume was 50 μl , and the reaction was terminated by the addition of aprotinin (1:100 molar ratio). The activation of pro-hK3 was monitored by adding AAPF-AMC, as above, and assay buffer in a final volume of 200 μl . Reactions were set up in microtiter wells and incubated at 37°C . Fluorescence or absorbance increase was measured for 20 min on a Wallac Victor fluorometer (PerkinElmer Life Sciences) set at 355 nm for excitation and 460 nm for emission for the AMC substrate and absorbance at 405 nm for the pNA substrate. Enzyme-free reactions, for all substrate concentrations, were used as negative controls, and background fluorescence or absorbance was subtracted from each value. A reaction with hK5 alone and pro-hK3 without incubation with hK5 were used as negative controls. Duplicate reactions were run on SDS-PAGE under reducing conditions in two different gels. The first was stained with Coomassie Blue, whereas the second was electroblotted onto nitrocellulose membranes (HybondTM-C Extra). Western blots were performed by using a polyclonal hK3 as primary antibody.

Western Blotting for Detection of the hK3 Fragments in Seminal Plasma and Prostate—Seminal plasma from healthy individuals were leftovers of samples submitted for routine biochemical testing that had been stored at -80°C . The prostate extracts were the healthy samples from matched healthy cancerous prostatic tissue pairs were obtained from prostate cancer patients who had undergone radical retropubic prostatectomy for prostatic adenocarcinoma at the Charite University Hospital (Berlin, Germany). The patients had not received hormonal therapy before surgery. The tissue samples were dissected from cancerous and noncancerous (healthy) portions of the prostate immediately after surgical removal. The samples were stored in liquid N_2 until needed. To determine the malignant or benign nature of the tissue samples, a histological analysis was performed as described previously (36). These samples were approved for research purpose use by the Ethics Committee of the Charite Hospital. The samples were resolved by SDS-PAGE (NuPAGE 4–12% Bis-Tris gels, Invitrogen) and subsequently electroblotted onto nitrocellulose membranes (HybondTM-C Extra). Western blots were performed as above.

Effect of Cations (Zn^{2+} , Ca^{2+} , Mg^{2+} , Na^+ , and K^+) and Citrate on hK5 Activity—To determine the effect of the cations (solutions made from salts of ZnCl_2 , NaCl , KCl , MgCl_2 , and CaCl_2) and citrate on hK5 activity, reactions mixtures were set up as follows: purified recombinant hK5 (final concentration of 12 nM) was incubated with each cation and citrate (final concentrations of 0, 12, 60, 120, 1,200, and 12,000 nM) diluted in the assay buffer (0.1 mM Tris-HCl, 0.1 mM NaCl, 0.01% Tween 20, pH 8.0) at a final volume of 100 μl in microtubes for 10 min at 37°C with gentle agitation. After incubation, the fluorogenic substrate VPR-AMC (final concentration of 1 mM) was applied to each hK5-cation/citrate mixture separately. Reactions were set up in microtiter wells and incubated at 37°C . Fluorescence was measured for 15 min as described before. Enzyme-free reactions, for all cations/citrate and substrate, were used as negative controls, and background fluorescence was subtracted from each value. All experiments were done in triplicate.

TABLE 1
Relative efficiency of cleavage of heptapeptides by hK5

Heptapeptide sequence	Pro-hK	Cleavage efficiency
(N→C) ^a		
IQSR ↓ IVG ^b	hK1	High
IQSR ↓ IVG	hK2	High
ILSR ↓ IVG	hK3	High
SSSR ↓ IIN	hK5	Moderate
DTR ↓ AIG	hK9	Moderate
ETR ↓ IIK	hK11	Moderate
ATPK ↓ IFN	hK12	Moderate
QGDK ↓ IID	hK7	Low
QEDK ↓ VLG	hK8	Low
DGDK ↓ LLE	hK15	Low
SCSQ ↓ IIN	hK4	No cleavage
EQNK ↓ LVH	hK6	No cleavage
NDTR ↓ LDP	hK10	No cleavage
ESSK ↓ VLN	hK13	No cleavage
DENK ↓ IIG	hK14	No cleavage

^a Single letter representation.

^b Arrows indicate cleavage site. Bold letters indicate P1 and P1' sites.

Reversal of Zn²⁺ Inhibition of hK5 Activity by Semenogelin I and II—hK5 (final concentration, 10 nM) was incubated with Zn²⁺ (final concentration, 5 μM) to a final volume of 100 μl in microtubes for 10 min at 37 °C with gentle agitation. After incubation, the fluorogenic substrate VPR-AMC (final concentration of 0.8 mM) was added. Reactions were set up in microtiter wells and incubated at 37 °C. Fluorescence was measured for 20 min as described before. At certain points, during measurement, semenogelin I or II (final concentration, 0.05 μM) and EDTA (final concentration, 10 mM) were added in each microtiter well. Enzyme-free reactions were used as negative controls, and background fluorescence was subtracted from each value. All experiments were done in triplicate.

Cleavage of Semenogelins I and II by hK5—Purified semenogelins I and II (5 μg) were incubated separately with 165 ng of hK5 in 100 mM Na₂HPO₄, 0.01% Tween 20, pH 8.0, 0.2 M urea or with 165 ng of hK2 in 0.1 M Tris-HCl, 0.1 M NaCl, 0.01% Tween 20, pH 7.5, 0.2 M urea at 37 °C for 2 and 8 h. The reactions were terminated by freezing in liquid nitrogen. Subsequently, the reactions were run on SDS-PAGE under reducing conditions, and the gels were stained with silver staining.

Cleavage of IGFbps by hK5—IGFBP1–6 (450 ng) were incubated separately with 50 ng of hK5 in 100 mM Na₂HPO₄, 0.01% Tween 20, pH 8.0 at 37 °C for different time points. The reactions were terminated by freezing in liquid nitrogen. The reactions were run on SDS-PAGE under reducing conditions, and the gels were stained with silver staining.

N-terminal Sequencing—N-terminal sequencing was performed with the Edman degradation method. Proteins were transferred by electroblotting to a polyvinylidene difluoride membrane and visualized with Coomassie Blue stain.

RESULTS

Cleavage of Heptapeptides by hK5—Because fourteen out of the fifteen human kallikreins (except hK4) require cleavage after Arg (hK1, hK2, hK3, hK5, hK9, hK10, and hK11) or Lys (hK6, hK7, hK8, hK12, hK13, hK14, and hK15) for propeptide release and activation, and hK5 has trypsin-like activity (34), we designed heptapeptides encompassing the putative P1–P4 and P'1–P'3 positions of the activation site of each kallikrein. We incubated these peptides with hK5 for various time intervals, and the reactions were monitored with high-performance liquid chromatography chromatography, using a C₁₈ column. The time-dependent decrease of the height and the area under the main peak representing the intact heptapeptide and the generation of one or two new peaks representing the P1–P4 and P'1–P'3 fragments was indicative of the efficiency of hK5 to cleave each heptapeptide. hK5 was able to cleave the heptapeptides encompassing the cleavage sites for hK1, hK2, and hK3 with high efficiency; the heptapeptides for hK5, hK9, hK11, and hK12 with moderate efficiency and the heptapeptides for hK7, hK8, and hK15 with low efficiency. No cleavage was observed for the heptapeptides for hK4, hK6, hK10, hK13, and hK14. The data are summarized in Table 1.

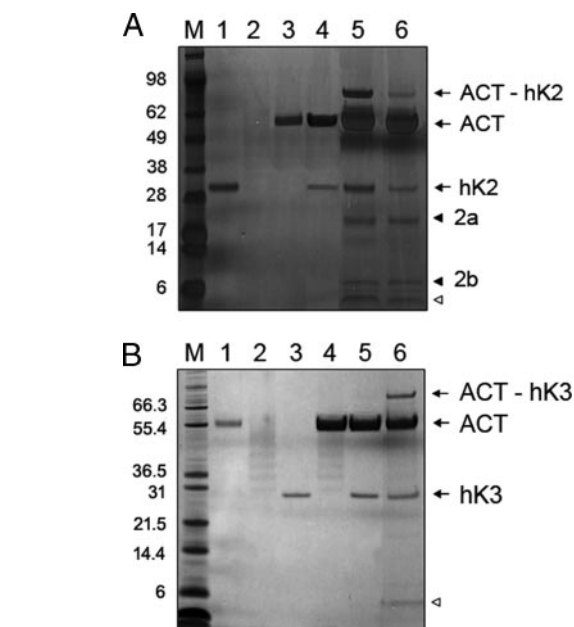


FIGURE 1. Activation of pro-hK2^{mut} (A) and pro-hK3 (B) by hK5. A, hK5 was incubated with pro-hK2 (0.5 μM) at a 1:5 molar ratio (lane 6). Subsequently, ACT at an hK2:ACT molar ratio of 1:10 was added, and the reaction was incubated for 2 h at 37 °C. The activation was monitored by formation of the ACT-hK2 complex (arrow). Active hK2 was incubated alone with ACT as a positive control (lane 5), whereas pro-hK2 was incubated with ACT (without hK5) as a negative control (lane 4). The kallikreins pro-hK2 and hK5 and the inhibitor ACT alone are shown in lanes 1–3, respectively. Filled arrowheads show the two fragments generated from hK2 as a result of autodegradation (lane 5) or internal cleavage by hK5 (lane 6). For sequencing data see Table 3. The open arrowhead represents the low molecular weight fragment cleaved from ACT after the ACT-hK2 complex formation. B, hK5 was incubated with pro-hK3 (0.5 μM) at 1:5 molar ratio for 5 min at 37 °C (lanes 6). Subsequently, ACT at an hK3:ACT molar ratio of 1:10 was added, and the reaction was incubated for 2 h at 37 °C. The activation was monitored by the ACT-hK3 complex formation (arrow). hK5 and pro-hK3 were incubated with ACT (without hK5) as negative control (lanes 4 and 5, respectively). The ACT inhibitor and the kallikreins hK5 and pro-hK3 alone are shown in lanes 1–3, respectively. The open arrowhead represents the low molecular weight fragment generated from ACT after the ACT-hK3 complex formation. M, molecular mass standards with shown masses in kilodaltons.

tapeptides encompassing the cleavage sites for hK1, hK2, and hK3 with high efficiency; the heptapeptides for hK5, hK9, hK11, and hK12 with moderate efficiency and the heptapeptides for hK7, hK8, and hK15 with low efficiency. No cleavage was observed for the heptapeptides for hK4, hK6, hK10, hK13, and hK14. The data are summarized in Table 1.

Activation and Deactivation of Pro-hK3 and Pro-hK2^{mut} by hK5—Given the above findings (Table 1) we examined the ability of hK5 to activate recombinant pro-hK2 and pro-hK3. After incubating both pro-hK2^{mut} and pro-hK3 with hK5 we monitored their activation through binding to the serpin ACT. Both pro-hK2^{mut} and pro-hK3, after incubation with hK5, became active and formed a complex with ACT (Fig. 1, A and B).

Because hK3 has chymotrypsin-like activity, we monitored its activation by hK5, by adding a chymotrypsin-like fluorogenic or chromogenic substrate. The activation was dependent on the pro-hK3:hK5 molar ratio and the time of incubation (Fig. 2, A and B). At a pro-hK3:hK5 molar ratio of 10:1, the reaction rate decreased in a time-dependent manner (Fig. 2C). By SDS-PAGE and Coomassie Blue staining we observed generation of degradation products of hK3 (Fig. 3A). The bands 1, 2, and 5 had N-terminal sequence of IVGGWE due to activation of pro-hK3 by hK5 (cleavage of the peptide bond at the activation site between Arg⁻¹ and Ile¹), bands 3 and 4 had a sequence of FLRPGDD due to internal cleavage of hK3 between Arg⁸⁵ and Phe⁸⁶, whereas band 6 had a sequence of STCSGDS due to internal cleavage of hK3 between Lys¹⁸² and Ser¹⁸³. These results indicate that hK5 is able to activate and subsequently deactivate hK3 by internal cleavage.

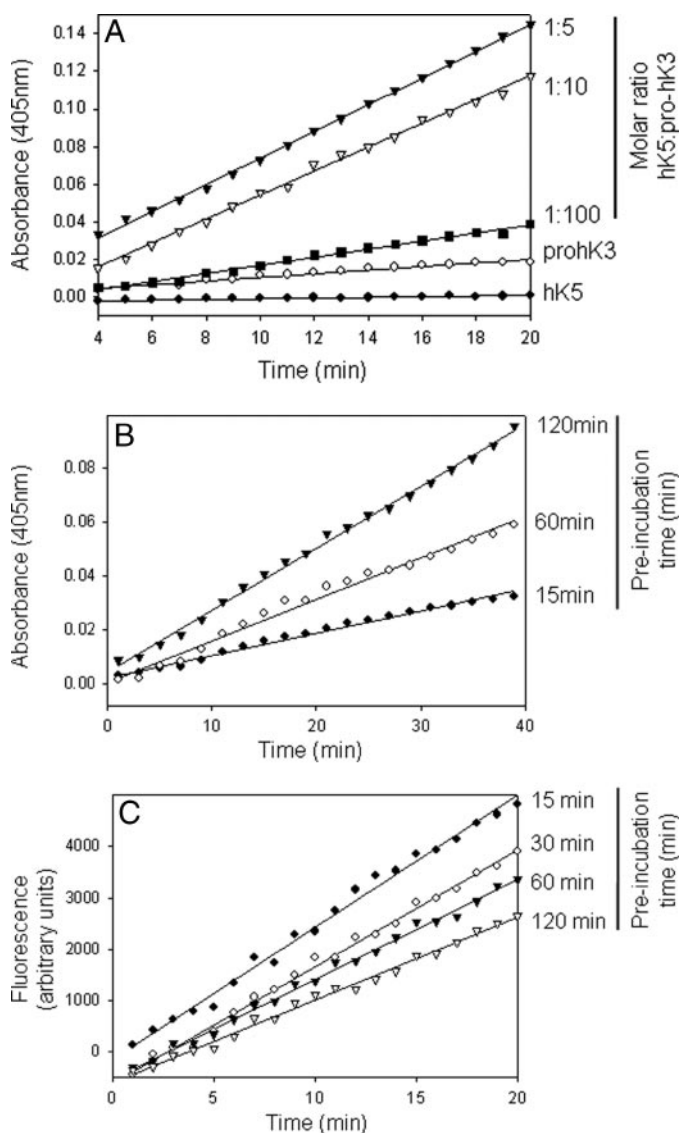


FIGURE 2. Activation (A and B) and deactivation (C) of pro-hK3 by hK5. A, molar ratio-dependent activation. hK5 (0.17, 0.085, and 0.0085 μM) was preincubated with pro-hK3 (0.85 μM) at 1:5, 1:10, and 1:100 molar ratio for 10 min, at 37 °C. B, time-dependent activation. hK5 (0.85 nM) was preincubated with pro-hK3 (0.85 μM) at a 1:1000 molar ratio for 15, 60, and 120 min, at 37 °C. C, time-dependent deactivation. hK5 was preincubated with pro-hK3 (0.85 μM) at 1:10 molar ratio for 15, 30, 60, and 120 min, at 37 °C. All reactions were terminated by the addition of aprotinin (1:100 molar ratio), and hK3 activity was monitored by the cleavage of the substrate RPY-pNA (A and B) or AAPF-AMC (C).

Clipped forms of the PSA/hK3 in seminal plasma have been previously described. Typically, 20–30% of hK3 in this fluid is clipped between residues Arg⁸⁵ and Phe⁸⁶, Lys¹⁴⁵ and Lys¹⁴⁶, and Lys¹⁸² and Ser¹⁸³ (37–39). By using a polyclonal antibody against hK3, we performed Western blots of seminal plasma, prostate extracts and at different time points of the activation of pro-hK3 by hK5. We were able to detect four of the five predicted fragments generated by the incubation of pro-hK3 with hK5 (one fragment is probably not recognized by our antibodies) (Fig. 3B). In seminal plasma, we detected all four bands generated by the cleavage of pro-hK3 by hK5 at the peptide bonds Arg⁸⁵–Phe⁸⁶ and Lys¹⁸²–Ser¹⁸³ plus two bands generated by the cleavage at the peptide bond Lys¹⁴⁵–Lys¹⁴⁶ (Fig. 3C). Interestingly, we were able to detect the same fragments in prostate tissue extracts (Fig. 3D), indicating that the deactivation of hK3 may initiate within the prostate, after its secretion.

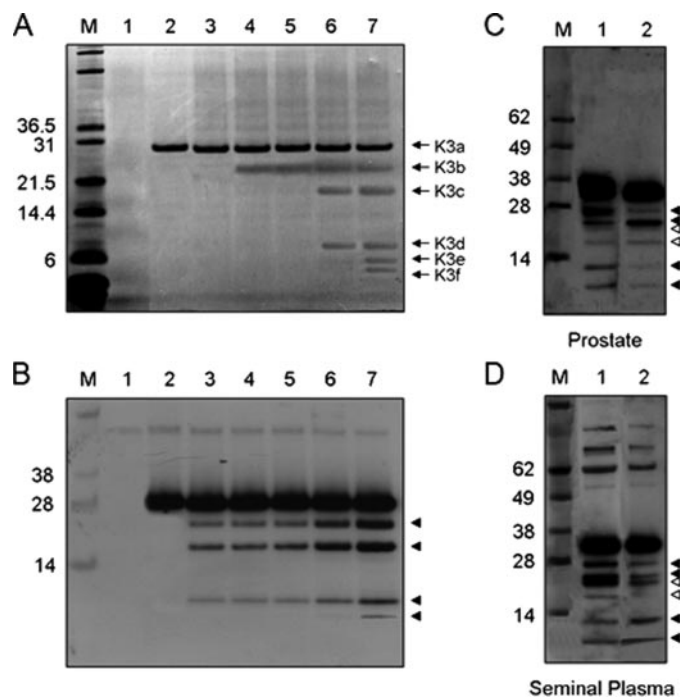


FIGURE 3. Deactivation of hK3 by hK5 (A and B) and detection of hK3 fragments *in vivo* (C and D). Pro-hK3 was incubated with hK5 at a 1:10 molar ratio for 5, 15, 30, 60, and 120 min at 37 °C (lanes 3, 4, 5, 6, and 7, respectively) visualized by Coomassie Blue (A) and Western blotting (B). The kallikreins hK5 and pro-hK3 alone are shown in lanes 1 and 2, respectively. The fragments that have been subjected to NH₂-terminal sequencing are indicated by arrows. The hK3 fragments generated by hK5 can be detected by Western blots in tissue extracts and seminal plasma (closed arrowheads; panels C and D, respectively). The open arrowheads represent hK3 fragments generated by unknown protease(s). For more information see Table 2 and “Discussion.”

During the activation of pro-hK2^{mut} by hK5 we also observed the generation of two fragments (Fig. 1A, lane 6). The N-terminal sequence of *band 1* was IVGGWE due to activation of pro-hK2^{mut} by hK5 (cleavage of the peptide bond at the activation site between Arg⁻¹ and Ile¹). The N-terminal sequence of *band 2* was SLQCVSL due to internal cleavage of hK2^{mut} between Arg¹⁴⁵ and Ser¹⁴⁶. hK2 has been shown to internally cleave itself after autoactivation (40). However, the overall catalytic efficiency of the mutated hK2 form, *i.e.* hK2^{mut}, used in this study, is <0.01% of wild-type hK2 (41). In the same study, it has also been shown that hK2^{mut} has a slightly altered P1 from Arg to Tyr. These data led us to conclude that hK2^{mut} fragmentation is due to hK5 activity and not to autolysis.

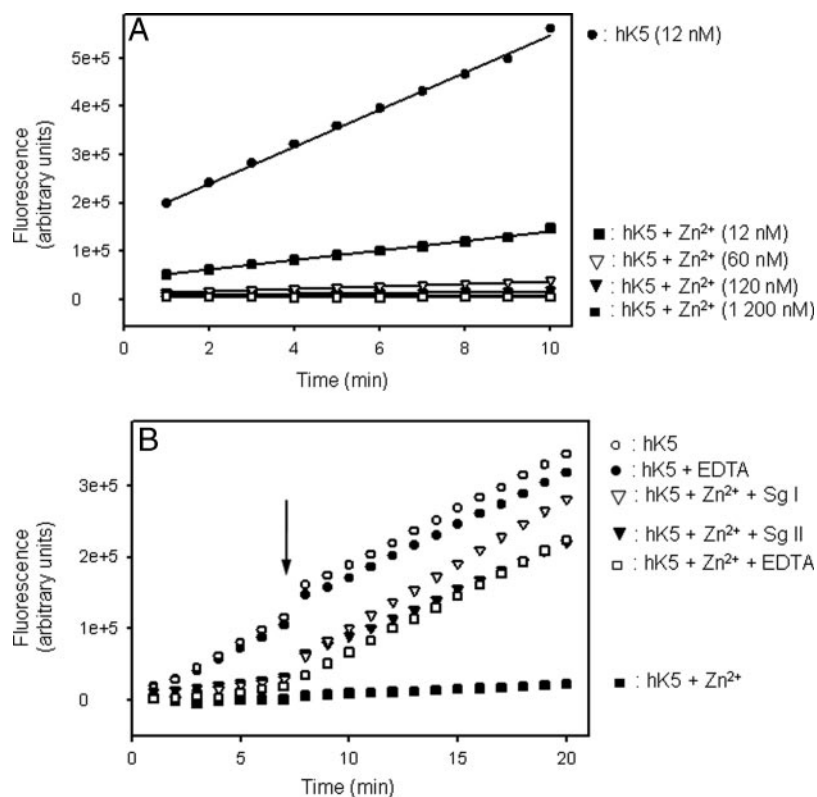
Effect of Cations and Citrate on hK5 Activity—The cations Zn²⁺, Ca²⁺, Mg²⁺, Na⁺, and K⁺ and the anion citrate are present at high levels in seminal plasma and prostatic fluid and play an important role in the regulation of protease activity (42). After incubating each of the ions with hK5, we added the fluorogenic substrate VPR-AMC to monitor residual enzyme activity. Citrate seems to enhance hK5 activity at relatively high amounts (Table 2). From the cations tested, Zn²⁺ showed strong inhibition of hK5 activity (Fig. 4A and Table 2). At a 1:10 molar ratio (hK5: Zn²⁺) the inhibition was 97.5%, indicating that the high levels of Zn²⁺ in seminal plasma and prostatic fluid could regulate hK5 activity. The ability of Zn²⁺ to efficiently inhibit hK2 (20) and hK3 (43, 44) has been previously investigated. Sodium and other tested cations had no effect on hK5 activity (Table 2), in contrast to hK3 whose activity increases with increasing Na⁺ (44).

Reversal of Zn²⁺ Inhibition by Semenogelins I and II—Semenogelins I and II are secreted by the seminal vesicles and are highly abundant proteins in human seminal plasma (35, 45). Jonsson *et al.* (46) have previously shown that semenogelins I and II have the ability to bind zinc

TABLE 2
Regulation of hK5 activity by ions

Ion	Concentration tested	Molar ratio (hK5:ion)	Residual activity	Ion concentration (42)	
				Seminal plasma	Prostatic fluid
	<i>mM</i>		%		<i>mM</i>
Citrate	12	1:1	105	19–48	7–208
	120	1:10	117		
	1200	1:1000	132		
Zn ²⁺	12	1:1	25	1–4	1–20
	60	1:5	6.5		
	120	1:10	2.5		
	1200	1:100	0.5		
Ca ²⁺	1200	1:100	100	5–13	7–39
Mg ²⁺	1200	1:100	100	2–7	6–32
Na ⁺	1200	1:100	100	103–129	110–327
K ⁺	1200	1:100	100	18–39	28–157

FIGURE 4. Inhibition of hK5 activity by Zn²⁺ (A) and reversion by semenogelins I and II (B). A, cleavage of VPR-AMC (0.2 mM) by hK5 (12 nM); with only hK5 (○), with Zn²⁺ at 12 (■), 60 (▽), 120 (□), and 1200 (▼) nM. B, cleavage of VPR-AMC (0.8 mM) by hK5 (10 nM); with only hK5 (○), hK5 and 0.01 M EDTA (■), hK5 and 5 μM Zn²⁺ (□), and with hK5 and 5 μM Zn²⁺ before and after the addition of 0.05 μM SgI (▽), 0.05 μM SgII (▼), and 0.01 M EDTA (□). The arrow indicates the time of addition of semenogelins and EDTA.



and regulate the activity of hK3. In this study, after incubating hK5 with Zn²⁺, and accessing hK5 residual activity with the fluorogenic substrate VPR-AMC, we demonstrated that addition of semenogelins I and II was able to rapidly reverse the inhibition, likely by sequestration of Zn²⁺ by semenogelins (Fig. 4B).

Cleavage of Semenogelins I and II—Semenogelins have the tendency to aggregate, therefore we used urea (0.2 M final concentration) to keep them in solution. hK2 and hK3 are known to cleave semenogelins and play a crucial role in the processes that leads to the liquefaction of the seminal clot after the ejaculation (20, 43, 47). In this experiment the same amounts of hK5 and hK2^{wt} (internal control) were incubated in separate reactions with semenogelins I and II. After 8-h incubation, hK5 was able to fully digest semenogelin I and II (Fig. 5, A and B). The generation of fragments was observed within 5 min after initiation of the reaction (data not shown).

Cleavage of IGFBP1–6 by hK5—IGFBPs comprise a family of six soluble proteins with a primary physiological role to interact and regulate the bioavailability of insulin-like growth factors (IGF-I and IGF-II) by forming complexes and sequestering IGFs away from their receptors,

i.e. IGF-IR (48, 49). Numerous studies have shown that their ability to bind IGFs is due to their N and C termini, which are highly conserved among them (50). The middle (or linker) domain is the least conserved region of this protein family and is the target of many proteases, *e.g.* human kallikrein 3, plasmin, thrombin, MMP1–3, and cathepsin L (48, 51). Cleavage within this domain will result in the release of the two termini and abolishment of the ability of IGFBPs to bind IGFs. We examined if hK5 is also able to cleave IGFBPs *in vitro*. Indeed, hK5 was able to cleave all IGFBPs (Fig. 6) except IGFBP6 (data not shown). All major cleavage sites were located within the linker domain (Table 3). Two of these sites, *i.e.* IG3c and IG5c, were also cleavage sites for thrombin (52, 53).

DISCUSSION

Human tissue kallikreins represent the largest group of serine proteases in the human genome. Members of this family are primarily known for their clinical applicability as cancer biomarkers (6–8). Their involvement in pathological (*e.g.* cancer progression, neurodegeneration, and skin diseases) and physiological processes (*e.g.* seminal plasma liquefaction and skin desquamation) is recently becoming apparent and

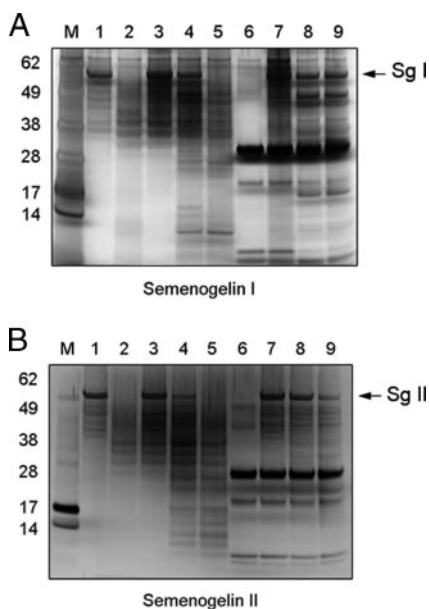


FIGURE 5. Degradation of semenogelins (Sg I (A) and II (B)). hK5 (165 ng, lanes 3–5) and hK2 (165 ng, lanes 7–9) were incubated with 500 ng of semenogelins I and II for 0, 1, and 4 h at 37 °C. Semenogelins I and II, incubated for 4 h alone at 37 °C, are shown in lane 1, in A and B, respectively. hK5 and -2, incubated for 4 h alone at 37 °C, are shown in lanes 2 and 6, respectively, in each panel. Intact semenogelins are indicated by arrows.

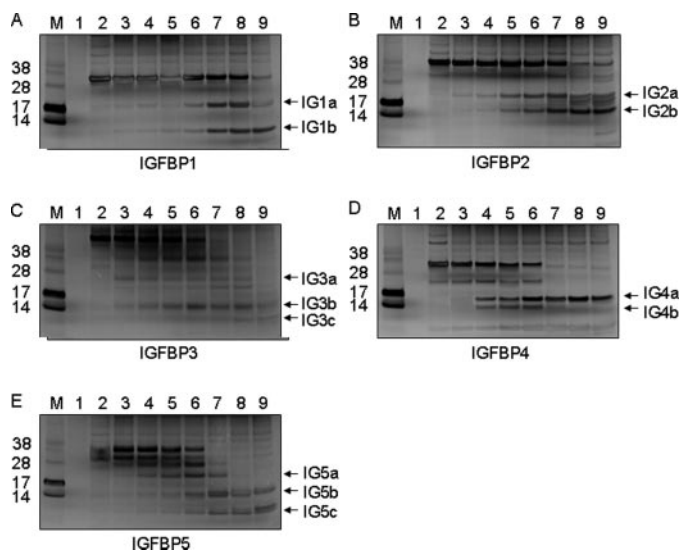


FIGURE 6. Degradation of insulin-like growth factor-binding proteins (IGFBPs) 1 to 5 (A–E, respectively) by hK5. hK5 (25 ng) was incubated with IGFBPs (0.5 μg) for 0, 0.25, 0.5, 1, 2, 4, and 8 h at 37 °C. hK5 and IGFBPs, incubated for 8 h alone at 37 °C, are shown in lanes 1 and 2 of each panel, respectively. The fragments that have been subjected to NH₂-terminal sequencing are indicated by arrows. For more information see Table 2 and “Discussion.”

represents a challenging area of investigation (3, 4). Ample evidence suggests the existence of cross-talk among members of the human kallikrein family (10). Here, we examine the involvement of human kallikrein 5 in a kallikrein proteolytic cascade and the role of this pathway during physiological and pathological conditions in the prostate.

We show, for first time, that hK5 is able to efficiently activate both pro-hK2 and pro-hK3. Previous reports have demonstrated that hK2 (15–17), hK4 (18), and hK15 (19) are also able to activate pro-hK3. However, the activation by hK2 seems to be significantly lower than that of hK4 and hK15 (18, 19). Human kallikrein 5, along with hK4, are the most efficient potential activators of pro-hK3, because both are able to activate it within 5 min at a pro-hK3:hK4/5 molar ratio of 10:1; with hK5

TABLE 3
N-terminal sequences identified in the fragments of hK2, hK3, and IGFBP1–5 after proteolytic digestion with hK5

Fragment	N-terminal sequence ^a
hK2	
K2a	NH ₂ -Ile-Val-Gly-Gly-Trp-Glu
K2b	(Lys ¹³⁷) ↓ Ala-Leu-His-Val-Thr-Asn
hK3	
K3a/b/e	NH ₂ -Ile-Val-Gly-Gly-Trp-Glu
K3c/d	(Arg ⁸⁵) ↓ Phe-Leu-Arg-Pro-Gly-Asp
K3f	(Lys ¹⁸²) ↓ Ser-Thr-Cys-Ser-Gly-Asp
IGFBP-1	
IG1a	NH ₂ -Ala-Pro-Trp-Gln-Gly-Ala
IG1b	(Lys ¹³⁷) ↓ Ala-Leu-His-Val-Thr-Asn
IGFBP-2	
IG2a	NH ₂ -Glu-Val-Leu-Phe-Arg
IG2b	(Arg ¹⁶⁴) ↓ Gln-Met-Gly-Lys-Gly-Leu
IGFBP-3	
IG3a	NH ₂ -Gly-Ala-Ser-Asp-Gly
IG3b	(Arg ¹⁵⁸) ↓ Tyr-Lys-Val-Asp-Tyr-Glu
IG3c	(Arg ²⁰⁶) ↓ Gly-Val-His-Ile-Pro-Asn
IGFBP-4	
IG4a	NH ₂ -Asp-Glu-Ala-Ile-His
IG4b	(Arg ¹⁷⁰) ↓ Thr-His-Glu-Asp-Leu-Tyr
IGFBP-5	
IG5a	NH ₂ -Glu-Pro-Cys-Asp-Glu-Lys
IG5b	(Arg ¹²³) ↓ Ile-Ser-Glu-Leu-Lys-Ala
IG5c	(Arg ²¹²) ↓ Ala-Val-Tyr-Leu-Pro-Asn

^a Arrow indicates internal cleavage site.

being able to completely convert pro-hK3 to its mature form. Takayama *et al.* (17), after fractionating seminal plasma with gel filtration, were unable to activate pro-hK3 with the fraction predicted to contain hK2. Instead, the activator was in the fraction of the range 60–120 kDa, indicating that the physiological activator has a higher molecular mass. Human kallikrein 5 is a strong candidate, because recombinant hK5 has a molecular mass of ~50 kDa when produced in a mammalian system. Previous studies have shown that hK2 is able to become autoactivated (21, 40). In addition, we here show that hK2 is also activated by hK5.

Interestingly, hK5 had the ability to also deactivate both hK3 and hK2 by internal cleavage. 20–30% of human kallikrein 3 isolated from seminal plasma is known to be clipped between residues Arg⁸⁵ and Phe⁸⁶, Lys¹⁴⁵ and Lys¹⁴⁶, and Lys¹⁸² and Ser¹⁸³ (54–56). Until now, the enzyme that was responsible for its cleavage and inactivation was unknown. Here we showed that hK5 is able to internally cleave and inactivate hK3 between residues Arg⁸⁵ and Phe⁸⁶, and Lys¹⁸² and Ser¹⁸³ (55). Furthermore, we show that these clipped forms, in addition to seminal plasma (54–56), also exist in prostate tissue extracts. Human kallikrein 2, once autoactivated, is then autodeactivated by internal cleavage between residues Arg¹⁴⁵ and Ser¹⁴⁶, and Arg²²⁶ and Lys²²⁷ (40). Our results suggest that hK5 can also internally cleave between residues Arg¹⁴⁵ and Ser¹⁴⁶ and deactivate hK2^{mut}, which abolishes its ability to autodeactivate (41). Deactivation by internal cleavage seems to be a common way of regulation of human kallikrein activity. hK6, -7, -13, and -14 have been shown to autodeactivate (23, 24, 57, 58), hK11 to be internally cleaved, probably by plasmin,³ and hK5 to exist in clipped forms *in vivo* (27).

One of the main characteristics of the prostate epithelial cells is their ability to accumulate cellular levels of zinc that are 3- to 10-fold higher than other mammalian cells (59). Previous studies have shown that zinc has the ability to inhibit the activity of hK2 (20) and hK3 (43, 44). Our results suggest that zinc efficiently inhibits hK5 activity as well. This inhibition is reversible, and for the first time we showed that semenogelins I and II are able to revert it. Jonsson *et al.* (46) extensively studied

³ I. P. Michael, G. Pampalakis, S. D. Mikolajczyk, J. Malm, G. Sotiropoulou, and E. P. Diamandis, unpublished data.

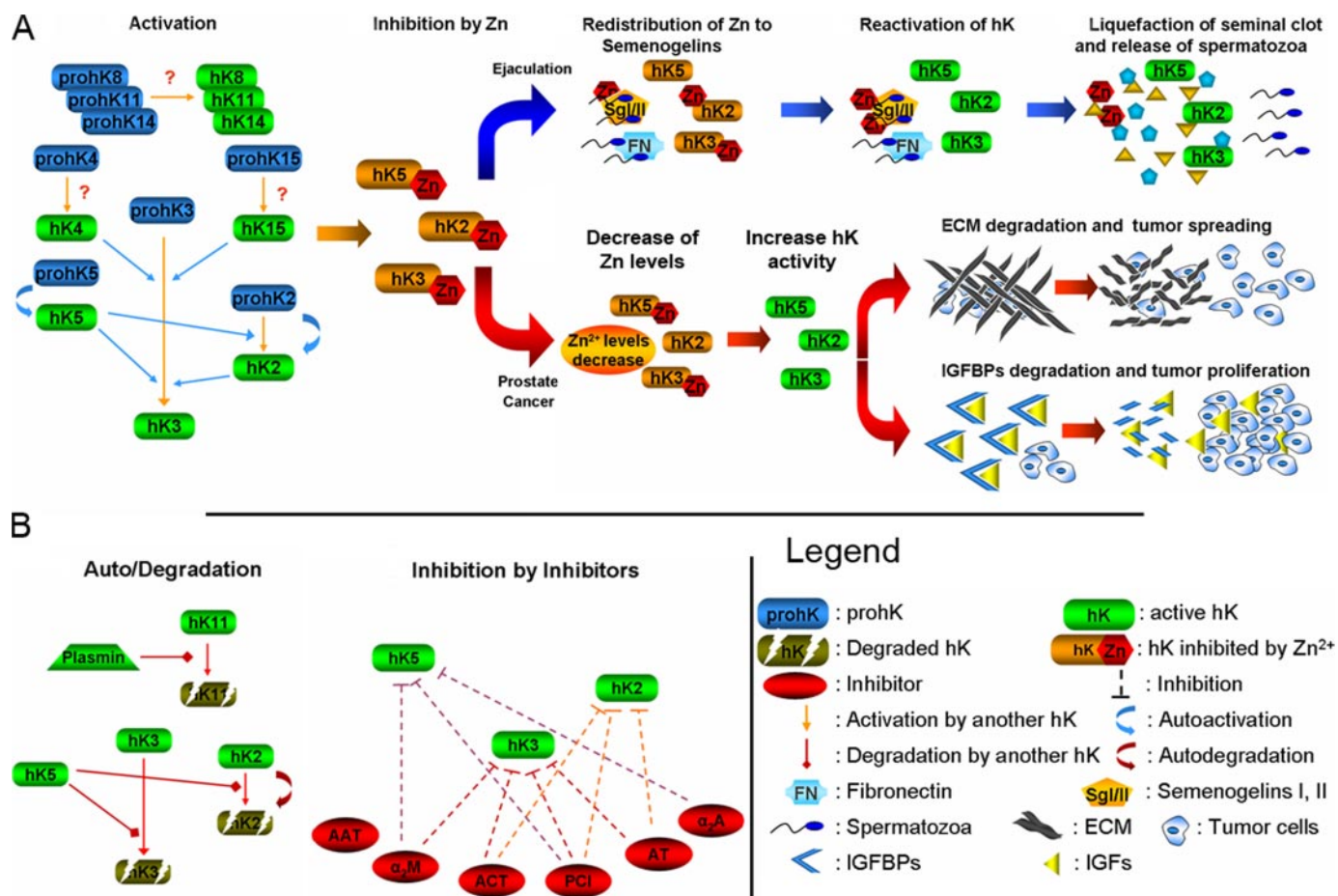


FIGURE 7. Human kallikrein physiology and pathobiology in the prostate. A, role of the proteolytic cascade pathway after ejaculation and during prostate cancer. At ejaculation, the sperm-rich epididymal fluid mixes with prostatic fluid (including hK2, -3, -4, -5, -8, -11, and -15) along with the secretions of the seminal vesicles, (*i.e.* semenogelins I and II, and fibronectin), constituting the seminal plasma. The seminal vesicle secretion constitutes ~60% and the prostatic secretion ~30% of the ejaculated volume. Semenogelins I and II along with fibronectin are the predominant structural proteins of seminal plasma aggregate together to form a gelatinous mass. Semenogelins have the capacity to capture Zn²⁺, leading to hKs activation and subsequent degradation of the semenogelins and fibronectin, resulting in seminal clot liquefaction. During prostate cancer both the facts that zinc levels decrease, primarily due to down-regulation of zinc transporters, and hK levels increase, leading to increased serine protease activity and subsequent degradation of extracellular matrix components and IGFBPs. B, regulation of the proteolytic cascade pathway by autodegradation of hKs and by inhibition of hKs by serpins. This model was developed based on data presented here and previous literature reports. For more details refer to the "Discussion."

the ability of semenogelins to bind zinc and regulate hK3 activity. The redistribution of zinc to semenogelins from human kallikreins may have an important physiological role during ejaculation and liquefaction of the seminal clot, as discussed below. Furthermore, zinc seems to also have a major role in the pathogenesis of prostate cancer (59). Its levels decrease 10 and 20 times in the prostate tissue and prostatic fluid, respectively (60, 61). This is primarily due to the down-regulation of the zinc-accumulating apparatus (ZIP1 and ZIP2), which are responsible for the uptake and accumulation of zinc in prostate cells, in the malignant loci as compared with the adjacent normal tissue (62). The decrease of zinc levels during prostate cancer initiation will lead to higher human kallikrein activity, which could contribute in prostate cancer progression.

Semenogelins I and II are dominant proteins of human seminal plasma, which, together with fibronectin, give rise to the gel-like coagulum of newly ejaculated semen (35, 45, 63). Various studies have shown that both hK2 (20) and hK3 (43, 47) are able to degrade semenogelins I and II. Here, we show that hK5 is also able to efficiently degrade semenogelins. Given that hK5 is able to also degrade fibronectin (34), it is possible that hK5, along with other human kallikreins, contributes in the liquefaction of the seminal plasma shortly after ejaculation.

Our results also suggest that hK5, similar to other proteases (*e.g.* the serine proteases trypsin, thrombin, and plasmin, the metalloproteinases

MMP-1, -2, and -3, and cathepsins D and L) and other members of the human kallikrein family (*i.e.* hK1, -2, -3, and -4) (64–66), is also able to degrade IGFBP1, -2, -3, -4, and -5. This will lead to an increase of bioavailable IGFs, allowing them to bind and activate type 1 IGF receptor (48, 51, 67) and indirectly modulate cell survival, mitogenesis, and differentiation. By this way, hK5 might play a role in tumorigenesis (67, 68) and particularly in prostate cancer progression (69).

Overall, the results of this study point to the existence of a proteolytic cascade pathway in the prostate, in which human kallikreins play a predominant role (Fig. 7). Under physiological conditions, hKs are activated in the prostate but are "silenced" by an allosteric reversible inhibition by Zn²⁺. After ejaculation, hKs are reactivated due to Zn²⁺ redistribution to semenogelins, and liquefy the seminal clot, leading to the release of motile spermatozoa (70). On the other hand, during prostate cancer initiation and progression, the decrease of Zn²⁺ levels may result in an increase in hK activity and increased degradation of extracellular matrix components and insulin-like growth factor-binding proteins. These events could enhance prostate cancer progression and metastatic potential. In addition to Zn²⁺ regulation, this pathway seems to be regulated by two additional mechanisms: (auto)degradation by internal cleavage and inhibition by serpins (4).

The predominant role of serine proteases in many enzymatic cascade pathways is well established. Here, we propose the involvement of

Kallikrein 5 and Proteolytic Cascade Pathways

human kallikreins in an enzymatic cascade pathway that leads to the liquefaction of seminal plasma. The same cascade may play a major role in prostate cancer progression. Further, studies are necessary to further characterize this pathway and all of its participating components.

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