

# Human Kallikrein-related Peptidase 14 (KLK14) Is a New Activator Component of the KLK Proteolytic Cascade

## POSSIBLE FUNCTION IN SEMINAL PLASMA AND SKIN\*

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Nashmil Emami and Eleftherios P. Diamandis<sup>1</sup>

From the Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario M5G-1L5, Canada and the Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario M5G 1X5, Canada

Human kallikrein-related peptidases (KLKs) are a family of 15 serine proteases mainly known for their biomarker utility in various neoplastic and non-neoplastic diseases. Despite significant progress in understanding their clinical application, little is known about the activation mechanism(s) of this important family of enzymes. Emerging evidence indicates that KLKs are activated in a stepwise manner, which is a characteristic of proteolytic cascades. Thus far, KLK cascades have been implicated in semen liquefaction and skin desquamation. Many members of the KLK family have been reported to be active in seminal plasma and/or skin, suggesting their involvement in common proteolytic cascades. KLK14, in particular, is highly active and has recently been proposed as one of the key trypsin-like proteases involved in skin desquamation. This study aims to elucidate a probable cascade-mediated role of KLK14 by 1) examining KLK14-mediated cleavage of a heptapeptide library encompassing activation sites of the 15 KLKs and 2) verifying activation of certain candidate downstream targets of KLK14 (*i.e.* pro-KLK1, -KLK3, and -KLK11). Heptapeptides encompassing activation motifs of KLK2, -3, -5, and -11 were cleaved with a high ( $\geq 85\%$ ) cleavage efficiency. Activation of these candidates was confirmed using full-length recombinant proteins. Pro-KLK11, -KLK3, and -KLK1 were rapidly activated in a concentration-dependent manner. Pro-KLK3 regulation was bidirectional because activation was followed by inactivation via internal cleavage of active KLK3. We are proposing a putative cascade model, operating through multiple KLKs. Identification of novel members of such proteolytic cascades will aid in further defining mechanisms involved in seminal/skin homeostasis.

Proteases are a major group of enzymes participating in a multitude of physiological processes, including coagulation, apoptosis, and immune responses (1). Due to the irreversible nature of proteolytic activation, proteases often remain as inactive zymogens in quiescent conditions. Activation is often triggered by an external stimulus and mediated by highly orchestrated cascades (2). Within a cascade, proteases function coordinately to ensure sequential activation of protease zymo-

gens. The result is a rapid amplification of signal, in response to a minute amount of stimulus (2).

Proteolytic cascades are tightly regulated through a series of highly orchestrated feedback loops, internal cleavages, and (auto)degradations. Also, inhibitors play a major role by targeting activated proteases (2). These multiple regulatory points are critical in preventing deleterious effects due to uncontrolled protease activation. Dysregulated protease activation has been implicated in several pathological conditions, such as amyloidogenesis in Alzheimer disease, intravascular coagulation in sepsis, and desquamation in various skin disorders as well as tumor metastasis, invasion, and angiogenesis in cancer (2–5).

KLKs<sup>2</sup> belong to a subgroup of secreted serine proteases within the S1 family of clan SA (6). So far, 15 members of the family have been identified, most of which have been reported as potential tumor biomarkers and implicated in various neoplastic events, such as tumor growth, angiogenesis, invasion, and metastasis (7, 8). Physiologically, KLKs are believed to be involved in a wide range of processes, including seminal clot liquefaction, skin desquamation, renal homeostasis, myelination, and enamelogenesis (6, 9–11). Although significant progress has been made in understanding the physiologic and pathologic functions of KLKs, activation mechanisms of this group of enzymes are largely unknown.

KLK5, -14, and -7 are postulated to participate in a proteolytic cascade in the skin (9). KLK5 and -7 were originally isolated and cloned from the stratum corneum (SC), the outermost layer of skin (12, 13). *In vitro* data suggest that KLK5 autoactivates and activates KLK7 and -14. In turn, activated KLK14 is believed to send positive feedbacks to amplify KLK5 activation. Activated KLK5, -7, and -14 function in skin desquamation through degradation of corneodesmosomal proteins (*i.e.* desmoglein 1 (DSG1), desmocollin 1 (DSC1), and corneodesmosin (CDSN)). KLK5 was shown to cleave all three components, whereas KLK7 and KLK14 were able to digest only CDSN and DSG1, respectively (14, 15). Overdesquamation in a number of skin disorders, such as Netherton syndrome, has mainly been attributed to dysregulated proteolytic activity of these KLKs (16, 17). As well, KLK5 and -7 possess antimicrobial function in skin, presum-

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<sup>1</sup> To whom correspondence should be addressed: FRCPC, Dept. of Pathology and Laboratory Medicine, Mount Sinai Hospital, 600 University Ave., Toronto, Ontario M5G 1X5, Canada. Tel.: 416-586-8443; Fax: 416-619-5521; E-mail: ediamandis@mtsinai.on.ca.

<sup>2</sup> The abbreviations used are: KLK, human kallikrein-related peptidase; AMC, 7-amino-4-methylcoumarin; FPLC, fast protein liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; PCI, protein C inhibitor; RP, reverse phase; HPLC, high performance liquid chromatography; SBzl, thiobenzyl ester; SC, stratum corneum.

ably through a cascade-mediated cleavage of the cathelicidin precursor, hCAP18 (human cationic antimicrobial protein 18), to its antimicrobial active form (LL-37) (18).

In addition to these KLKs, varying levels of KLK1, -6, -8, -10, -11, and -13 have been reported in SC (15, 19, 20). KLK1, -6, and -13 have recently been proposed to process DSG1 (15), suggesting their participation in the proteolytic cascade mentioned above. However, the activation mechanism of these KLKs remains elusive.

Additional evidence supporting proteolytic cascades of KLKs comes from the work done with KLK2, -3, and -5 in seminal plasma and *in vitro*. KLK5 has been shown to autoactivate and, in turn, activate pro-KLK3 (10). Activated KLK3 is consequently inactivated by KLK5, through a series of internal cleavages (10). Similarly, although debatable (21), active KLK2 has been reported to cleave and activate pro-KLK3 *in vitro* (22, 23). Activated KLK2 and -3 may contribute to seminal clot liquefaction through hydrolysis of seminal vesicle proteins, *i.e.* semenogelins (SgI and -II) and fibronectin (24). Semen liquefaction is under a tight regulatory control by a number of endogenous inhibitors, such the protein C inhibitor (PCI), as well as inhibitory  $Zn^{2+}$  (25–27). Several other KLKs, including KLK1, -11, and -14, are known to be expressed in varying levels in seminal plasma (20, 28, 29) and may be involved in a common activation pathway.

KLK14 has recently been characterized kinetically as a trypsin-like KLK, with substrate preference over P1-Arg (30, 31). According to a new report, KLK14 is possibly a key protease in the skin, contributing to approximately half of the total trypsin-like proteolytic activity in the SC layer (32). In addition,  $Zn^{2+}$  has been shown to strongly inhibit KLK14 enzymatic activity (30), suggesting a potential role of the protein in seminal plasma.

In an attempt to delineate the possible involvement of KLK14 in cascades, this study examines the interaction between this enzyme and other members of the family, using an unbiased library of activation motifs of the 15 KLKs, and further verifies those that are known to be expressed in skin and/or seminal plasma.

### 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) and were diluted in water and stored at  $-20^{\circ}\text{C}$ . The synthetic substrates, succinyl-Arg-Pro-Tyr-*para*-nitroanilide-HCl (RPY-*para*-nitroanilide), Pro-Phe-Arg-AMC (PFR-AMC), and D-Val-Leu-Lys-thiobenzyl ester (VLK-SBzl), were purchased from BACHEM (King of Prussia, PA), Pharmacia Hepar-Chromogenix (Franklin, OH), and Chromogenix (Milano, Italy), respectively. Recombinant pro-KLK3 produced in *E. coli* was a

gift from Spectral Diagnostic Inc. (Toronto, Canada). Mature KLK1, produced in a baculovirus/insect cell line system, was kindly provided by Dr. M. Blaber (Florida State University). KLK14 and KLK11 were produced in house, as described previously (30). HUK-IgG, an antibody recognizing KLK1, was kindly provided by Prof. J. Chao (Medical University of South Carolina).

**Heptapeptide Library Screening**—25  $\mu\text{g}$  of heptapeptides were incubated at  $37^{\circ}\text{C}$  with 1  $\mu\text{g}$  of KLK14 at a 1,500:1 molar ratio in KLK14 assay buffer (100 mM phosphate buffer, 0.01% Tween 20, pH 8.0), in a total volume of 200  $\mu\text{l}$ . Reactions were stopped at different time points by freezing the samples with liquid nitrogen. A 150- $\mu\text{l}$  aliquot of each time point was diluted 2-fold with loading buffer (0.1% trifluoroacetic acid in  $\text{H}_2\text{O}$ ). A scrambled heptapeptide (Hep0; of random sequence) was included as a negative internal control to account for experimental variations. Probable hits (*i.e.* heptapeptides cleaved by KLK14) were identified using reverse phase high performance liquid chromatography (RP-HPLC). LC separation was carried out using an analytical C18 column (TOSOH) and a mobile phase consisting of 0.1% trifluoroacetic acid in  $\text{H}_2\text{O}$  (Buffer A) and 0.1% trifluoroacetic acid in acetonitrile (Buffer B). Samples were eluted with a linear gradient of 0–60% Buffer B at a flow rate of 0.8 ml/min. Retention times of heptapeptides were measured prior to incubation with KLK14. Absorption (214 nm) of peaks representing the remaining uncleaved heptapeptides were recorded at different incubation time points and normalized to the corresponding value of Hep0. Cleavage efficiency was calculated as a percentage height (milliabsorbance units) reduction in the absorption of the remaining uncleaved fragments. Positive hits (*i.e.* heptapeptides with cleavage efficiency of 85% or higher (within 5 h)) were selected for further verification. Cleavage sites were verified by tandem mass spectrometry. Sample separation was replicated as explained above and scanned, using an API 3000 triple quadrupole mass spectrometer (MDS Sciex). The HPLC was conducted using an Xterra C18 column (3.0  $\times$  50 mm, 2.5  $\mu\text{m}$ ) with mobile phase consisting of 50% acetonitrile containing 0.5% trifluoroacetic acid in isocratic mode. The  $m/z$  ratios corresponding to the doubly and/or singly charged daughter fragments were extracted from the total ion current scans. Collision energy of 17 V was applied to further break extracted peptides. Peptide sequences were extrapolated from extracted ion chromatograms.

**Recombinant KLK1 Production**—The full-length coding region of KLK1 protein (GenBank<sup>TM</sup> accession number AAH05313) was PCR-amplified and cloned into the pcDNA3.1(-) (Invitrogen) mammalian expression vector at EcoRI and XbaI sites. Recombinant clones were stably transfected in the human embryonic kidney cell line, HEK293. Positive clones were selected by their ability to survive serial passages in Geneticin. The clone expressing the highest amount of KLK1 was selected. Seeding density, cell number, and harvest time were optimized to maximize protein production with minimal cell death. The recombinant clone was grown in a humidified incubator at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  in Dulbecco's modified Eagle's medium culture medium (Invitrogen) supplemented with 10% fetal bovine serum. Approximately  $180 \times 10^6$

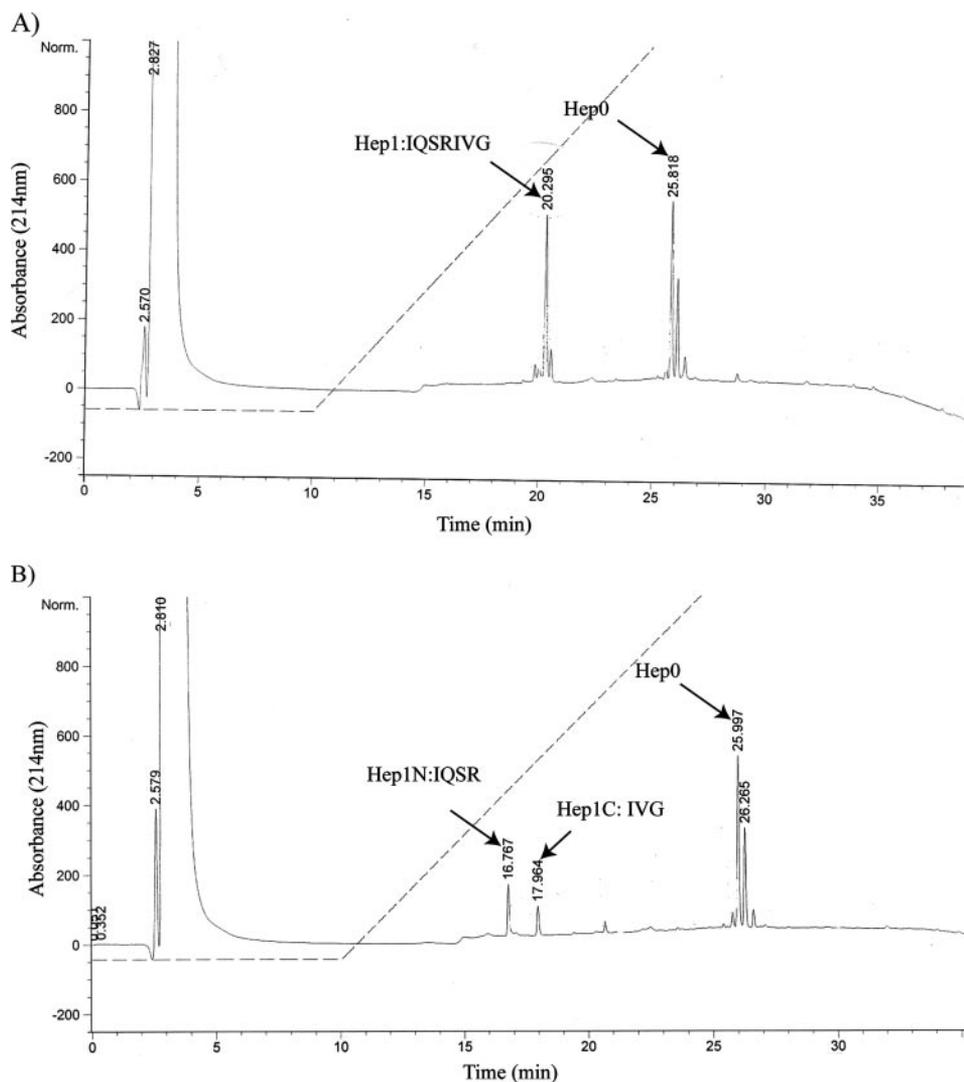
**TABLE 1**  
Relative cleavage efficiency of heptapeptides by active KLK14

Heptapeptide sequence <sup>a</sup>	Pro-KLK	Percentage digestion		
		1 h	3 h	5 h
IQSR ↓ IVG	KLK1	97	99	99
IQSR ↓ IVG	KLK2	97	99	99
ILSR ↓ IVG	KLK3	87	95	97
SCSQ ↓ IIN	KLK4	0	0	0
SSSR ↓ IIN	KLK5	57	82	88
EQNK ↓ LVH	KLK6	0	0	0
QGDK ↓ IID	KLK7	41	52	64
QEDK ↓ VLG	KLK8	0	0	0
DTR ↓ AIG	KLK9	0	0	0
NDTR ↓ LDP	KLK10	0	0	0
ETR ↓ IIK	KLK11	55	77	90
ATPK ↓ IFN	KLK12	19	48	62
ESSK ↓ VLN	KLK13	0	0	0
DENK ↓ IIG	KLK14	0	0	0
DGDK ↓ LLE	KLK15	0	0	0

<sup>a</sup> Heptapeptides were designed to encompass the activation sites of each KLK (denoted by an arrow). All sequences are shown in the N → C direction and with single letter amino acid designations.

cells were seeded into 10 175-cm<sup>2</sup> tissue culture flasks and grown to 60–70% confluence. The medium was replaced with serum-free medium (Invitrogen), supplemented with 8 mM glutamine, and incubated for an additional 7 days. Cell supernatant was collected and frozen at –80 °C until further use. Purification was achieved, using anion exchange fast protein liquid chromatography (FPLC). Cell supernatant was concentrated 10 times and loaded onto a Hi-Trap DEAE-FF anion exchange column (Amersham Biosciences). The column was eluted with a linear gradient of 0–80% 20 mM Tris plus 1 M NaCl, pH 8.0 (Buffer A), at a flow rate of 3 ml/min. Fractions were analyzed by an in house enzyme-linked immunosorbent assay, and those containing KLK1 were pooled. Further purification using RP-HPLC was carried out using C8 reverse phase column, with a step gradient of 0–100% of 0.1% trifluoroacetic acid in acetonitrile, described above. The enzymatic activity of recombinant KLK1 was tested using the fluorogenic substrate PFR-AMC.

**Activation of Pro-KLK3 and Pro-KLK11 by KLK14**—Activation was monitored as an increase in the absorbance of RPY-



**FIGURE 1. Monitoring of heptapeptide (Hep) cleavage.** 25 μg of Hep1 were incubated with recombinant active KLK14 at a 1500:1 molar ratio at 0 h (A) and 1 h (B). The cleavage was monitored by RP-HPLC. Hep1N and Hep1C, the two daughter fragments generated after digestion with KLK14. The scrambled sequence Hep0 was used as an internal negative control. Cleavage sites were verified by mass spectrometry. Dashed lines, gradient profile as described under “Experimental Procedures.”

*para*-nitroanilide in KLK3-optimized assay buffer (0.1 mM Tris, 3 mM NaCl, 0.01% Tween 20, pH 7.5) and VLK-SBzl in KLK11-optimized assay buffer (50 mM Tris, 1 M NaCl, 10 mM EDTA, pH 8.5, containing 0.1 mM dinitro-5,5'-dithiodibenzoic acid), in total volume of 200 μl. Pro-KLK3 and pro-KLK11 were added to active KLK14 at various molar ratios and incubation times at 37 °C in KLK14-optimized activity assay buffer (100 mM phosphate buffer, 0.01% Tween 20, pH 8.0), total volume of 50 μl. Digestions were repeated three times. Absorbance was measured on a Wallac Victor Fluorometer (PerkinElmer Life Sciences) at 405 nm for KLK3 and 420 nm for KLK11. In the case of KLK3, the background absorbance and residual activity of KLK14 was subtracted from raw values of enzyme alone and reaction mixtures, respectively. The residual activity of pro-KLK3 was accounted for by including an additional pro-KLK3-alone reaction. In contrast, given the residual activity level of KLK11 and the very low KLK14 activity toward VLK-SBzl, the background absorbance and residual activity of KLK11 were subtracted from raw values of enzyme alone and reaction mixtures, respectively.

KLK14-mediated fragmentation of pro-KLK3 was determined by incubating pro-KLK3 with active KLK14 at a 10:1 molar ratio for vary-

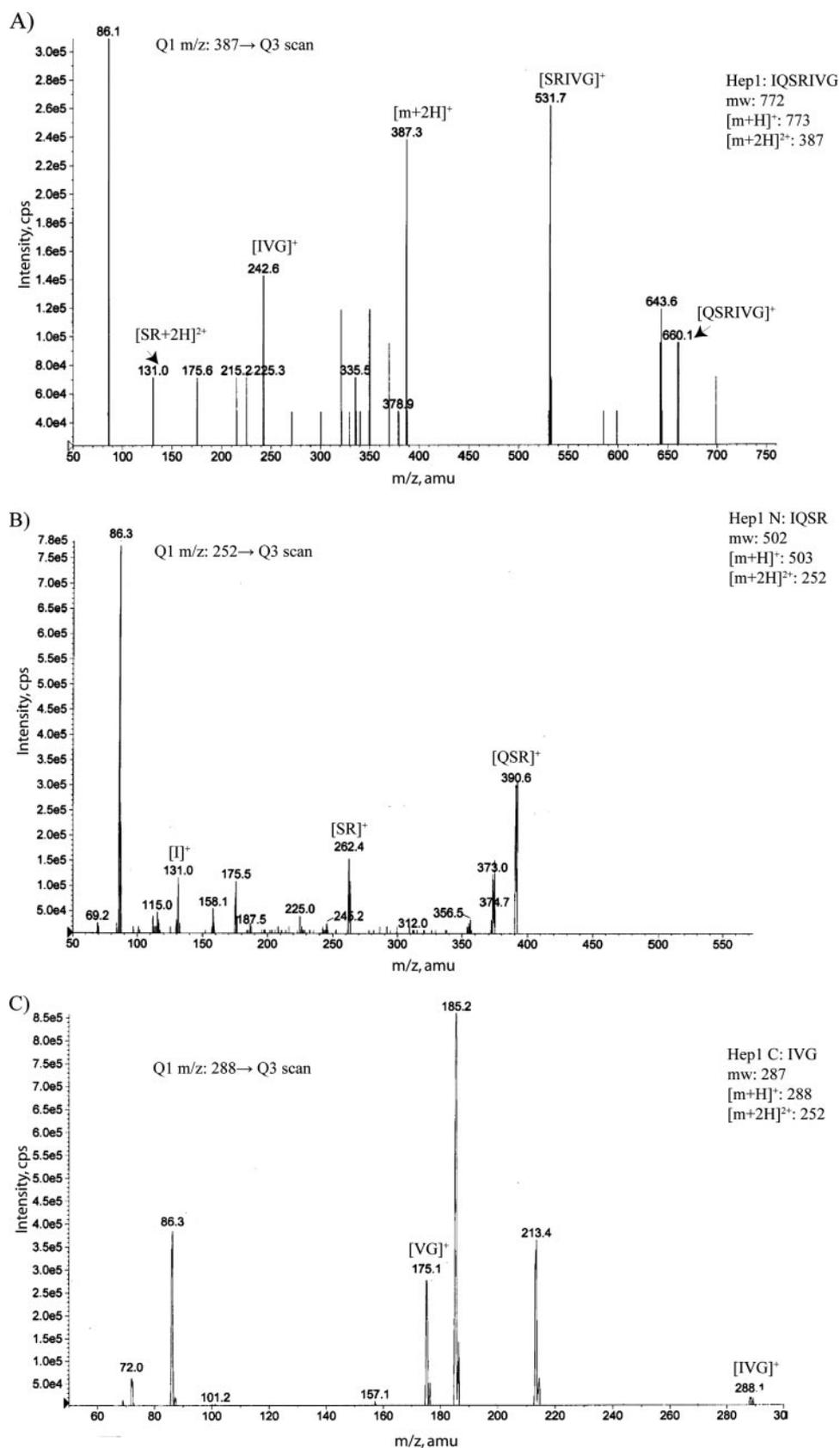


FIGURE 2. **Validating the cleavage specificity.** The cleavage site of Hep1 digested with KLK14 was determined by LC-MS/MS, using the API 3000 triple quadrupole mass spectrometer. The mass spectrometer monitored the ion transitions of Hep1 at  $m/z$  387.2  $\rightarrow$  531.4 (A), Hep1N at  $m/z$  252.6  $\rightarrow$  391.2 (B), and Hep1C at  $m/z$  288.7  $\rightarrow$  175.1 (C). The LC-MS/MS method was developed using synthetic Hep1, Hep1N, and Hep1C. The collision energy (CE) was set at 17 V.

ing times in a total volume of 50  $\mu$ l. Identical reactions were run in two separate SDS-polyacrylamide gels (1:4 ratio) under reducing conditions. One gel was silver-stained, and the gel with 4 times more sample was electroblotted to polyvinylidene difluoride membrane and stained with Coomassie Blue stain. Fragments were cut from the membrane and N-terminally sequenced.

**Activation of Pro-KLK1 by KLK14**—Pro-KLK1 was added to active KLK14 at various time points at a 1:1 molar ratio in a total volume of 50  $\mu$ l at 37  $^{\circ}$ C. Reactions were repeated in duplicate. KLK1-specific activity was measured by fluorescence release of the pulled down KLK1 protein. 200 ng of KLK1-specific polyclonal antibody (HUK-IgG) were immobilized overnight on a 96-well polystyrene plate in coating buffer (50 mmol/liter Tris, 0.05% Tween 20, pH 7.8). The plate was washed two times with washing buffer (50 mmol/liter Tris, 150 mmol/liter NaCl, 0.05% Tween 20, pH 7.8). Reaction mixtures were loaded into each well, incubated for 2 h with shaking, and washed six times with the washing buffer (above). Subsequently, 0.25 mM PFR-AMC in KLK1-optimized activity assay buffer (20 mM Tris/HCl, pH 9.0, 1 mM EDTA, 10% Me<sub>2</sub>SO, and 0.1% Triton X-100) was added to each well. The increase in fluorescence signal was measured on a Wallac Victor fluorometer, set at 355 nm for excitation and 460 nm for emission. Basal activity of both KLK1 and KLK14 were measured at time zero and subtracted from raw values. Reaction rates (fluorescence units/min) correspond to the slope of the fluorescence release-time plot.

**N-terminal Sequencing**—Sequencing was performed with the Edman degradation method.

## RESULTS

**Heptapeptide Screening**—In an attempt to identify potential downstream targets of KLK14, a library of 15 heptapeptides representing the putative P4-P3-P2-P1-P'1-P'2-P'3

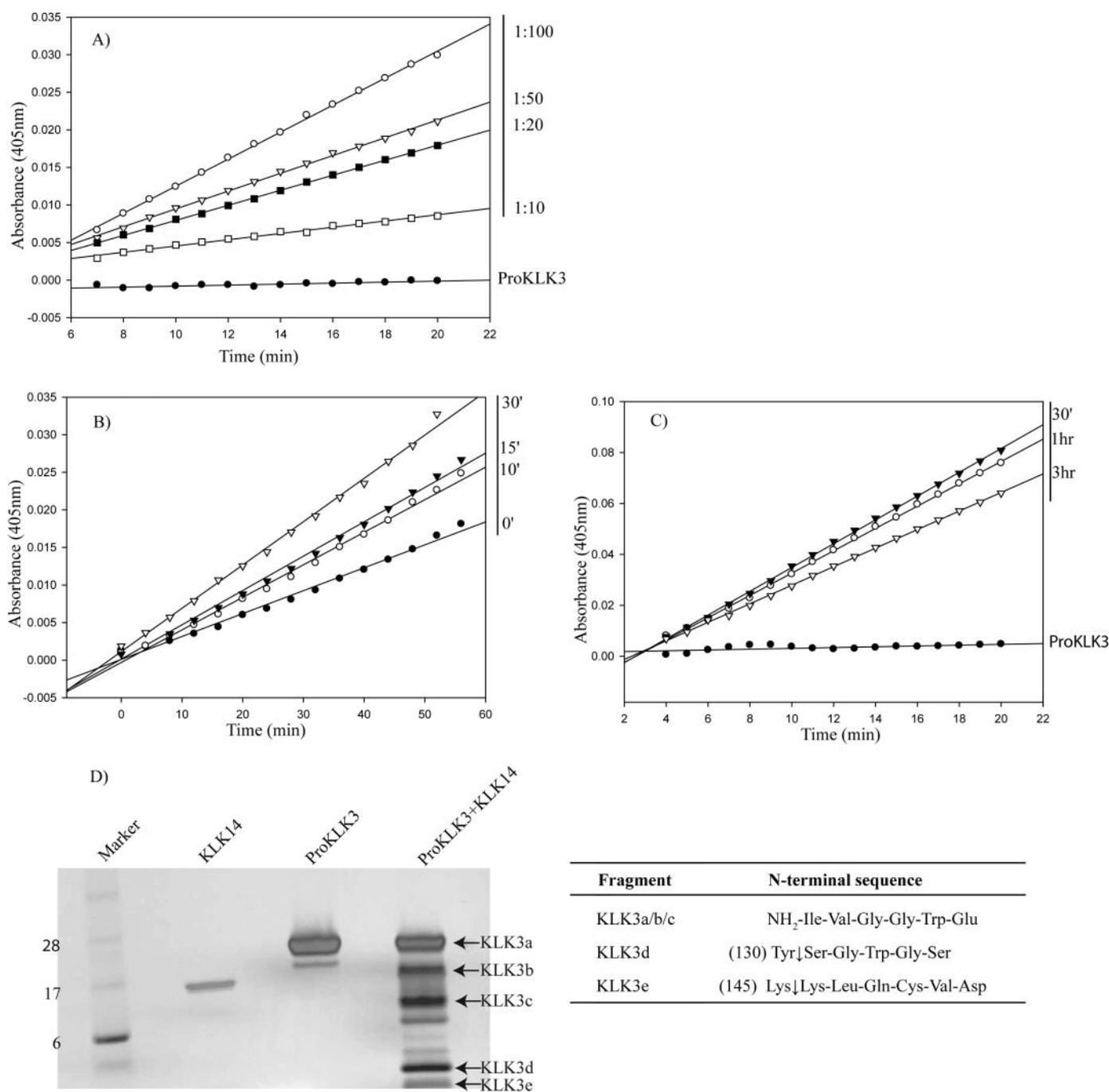


FIGURE 3. **KLK14-mediated regulation of pro-KLK3 activity.** A, molar ratio-dependent activation. Pro-KLK3 was incubated with 0.085  $\mu\text{M}$  active KLK14 in varying molar ratios of 10, 20, 50, and 100 for 15 min at 37 °C. B and C, time-dependent activation/deactivation. 0.2  $\mu\text{M}$  pro-KLK3 was incubated with KLK14 at a molar ratio of 1:10 for varying time intervals, at 37 °C. Activity was monitored through cleavage of 1 mM RPY-*para*-nitroanilide substrate. Note the gradual increase in the absorbance within the initial 30 min, followed by reduction of activity up to 3 h afterward. The *KLK3* line represents negative control (no KLK14 added). D, pro-KLK3 fragmentation by KLK14. Pro-KLK3 (0.85  $\mu\text{M}$ ) was incubated with KLK14 (0.085  $\mu\text{M}$ ) for 1 h and visualized by silver staining. The N-terminal sequence of the fragments was identified by Edman sequencing.

positions of active motifs of KLKs was designed (Table 1). Heptapeptides were incubated with the recombinant active KLK14 for various time intervals. Cleavage was monitored by RP-HPLC. Cleavage efficiency was calculated as a percentage milliabsorbance unit reduction in the peak representing the undigested peptide, normalized to that of Hep0 (Fig. 1). Cleavage specificity was determined by LC-MS/MS of the two daughter peaks, representing the P4-P1 and P'1-P'3 fragments (Fig. 2). KLK14 cleaves heptapeptides representing KLK1, KLK2,

and KLK3 propeptides with high efficiency. Heptapeptides for KLK5, -7, -11, and -12 were digested with moderate ( $\geq 85\%$  digest after 5 h) to low efficiency ( $\leq 85\%$  digest after 5 h), whereas heptapeptides for KLK4, -6, -8, -9, -10, -13, -14, and -15 were not cleaved at all (Table 1). Given the rapid nature of proteolytic cascades, we only considered screening hits with high to moderate cleavage efficiency. These results are consistent with the previously reported KLK14-mediated activation of KLK5 (9).

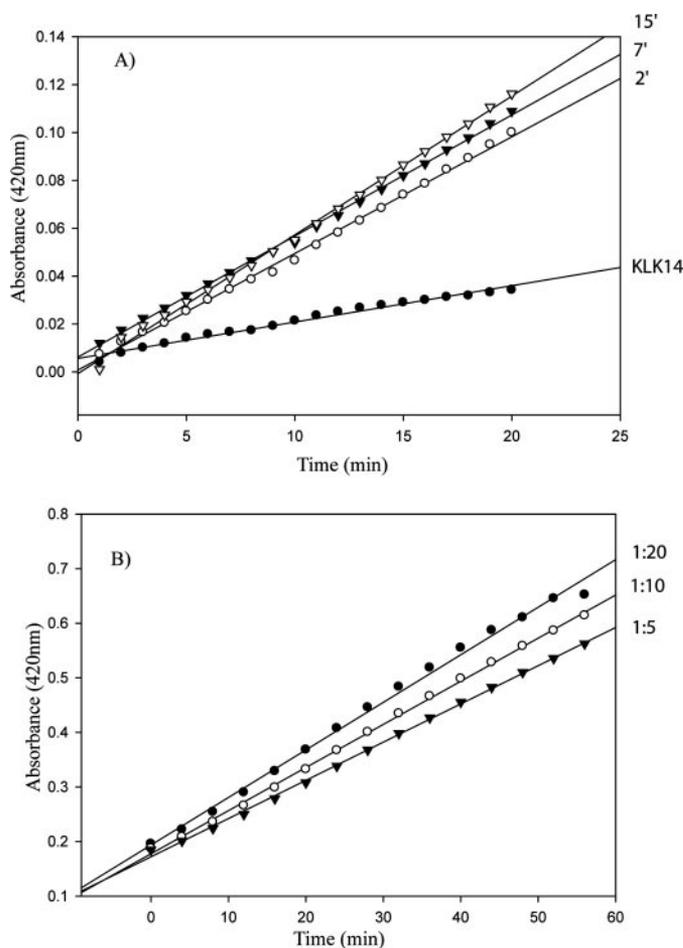
## Cascade-mediated Function of KLK14

**Activation/Deactivation of Pro-KLK3 and Pro-KLK11**—Given the high cleavage efficiency of heptapeptides representing the propeptides of pro-KLK3 and pro-KLK11, we examined whether these proteins function as immediate downstream targets of KLK14. The ability of KLK14 to activate recombinant, pro-forms of these proteins was tested. Since KLK3 exhibits specificity toward chymotrypsin-like substrates, the chromogenic synthetic tripeptide RPY-*para*-nitroanilide with chymotrypsin-like specificity was employed. KLK3 activation was dependent on the enzyme to substrate molar ratio (Fig. 3A). Characteristic of proteolytic cascades, the activation seemed to be rapid and transient; KLK14 activated pro-KLK3 within the initial 5 min. KLK3 enzymatic activity was incrementally amplified over the next 30 min (Fig. 3B). However, the reaction rate declined following longer incubation (Fig. 3C), suggesting a deactivation mechanism that may act as a negative feedback loop regulating the proteolytic activity of KLK3. This observation was confirmed by sequencing the cleaved fragments (Fig. 3D). Bands a, b, and c have the N-terminal sequence of IVG-GWE (the sequence of active KLK3), indicating KLK14-mediated activation of pro-KLK3. In addition, two bands with the sequence SGWGS (band d), cleaved after tyrosine 130, and KLQCVD (band e), cleaved after lysine 145, were detected. These bands represent internal cleavages, leading to inactivation of activated KLK3.

Similarly, KLK14-mediated activation of pro-KLK11 was determined using the VLK-SBzl substrate. KLK14 alone exhibited a low preference for the above substrate (with ~75% less absorbance after 20 min of substrate incubation) as compared with KLK11, which was activated for 15 min with KLK14. Although cloned in its pro-form, our recombinant KLK11 exhibited a low basal activity (data not shown), which was subtracted from the absorbance readings of the reaction mixtures. KLK11 was activated within 2 min of incubation with active KLK14, in a both dose- and time-dependent manner (Fig. 4, A and B, respectively).

**Cloning, Expression, and Purification of Recombinant Pro-KLK1**—As suggested by the library screening (Table 1), pro-KLK1 is a candidate target, activated by KLK14. To confirm KLK14-mediated activation of pro-KLK1, KLK1 protein was produced recombinantly. Stable HEK293 cell lines, expressing pro-KLK1, were generated. One of the clones with the highest expression was chosen for further study. The highest expression was observed at day 10 of culture in serum-free medium. Samples were FPLC-fractionated. Fractions 14–23 contained varying amounts of KLK1, as determined by a KLK1-specific enzyme-linked immunosorbent assay and silver staining. Pooled fractions were further purified by RP-HPLC. Unfortunately, pure recombinant pro-KLK1 could not be isolated, possibly due to autodegradation and/or internal cleavage of the protein in the supernatant (data not shown). The purified recombinant KLK1 was determined to be enzymatically active, with a reaction rate of ~2,000 fluorescence units/min, using 120 nM recombinant pulled down KLK1 and 1 mM PFR-AMC substrate (data not shown).

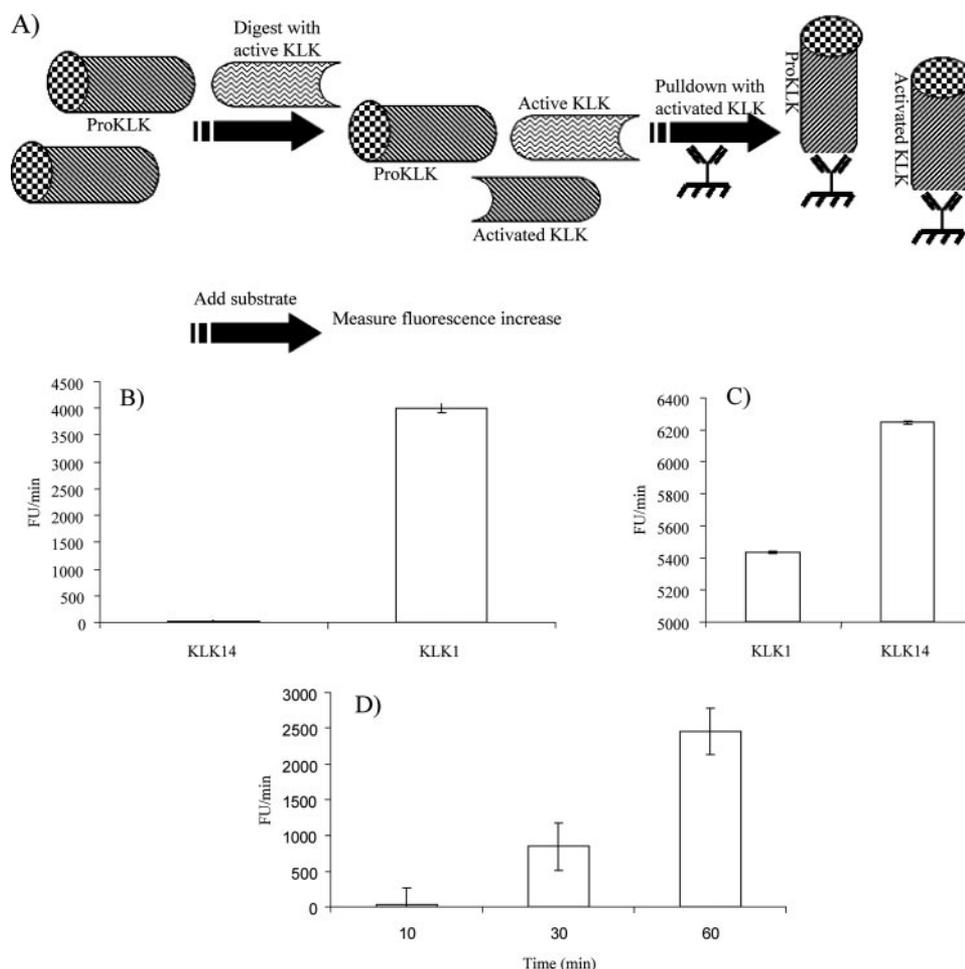
**Activation of KLK1 by KLK14**—One of the hurdles in kallikrein research has been the lack of specific activity assays, due to overlapping substrate specificities of the majority of KLKs.



**FIGURE 4. Activation of pro-KLK11 by KLK14.** A, time-dependent activation. 0.2  $\mu\text{M}$  pro-KLK11 was incubated with 0.02  $\mu\text{M}$  KLK14 for varying time intervals at 37  $^{\circ}\text{C}$ . B, molar ratio-dependent activation. 10 nM KLK14 was incubated with pro-KLK11 in varying molar ratios of 5, 10, and 20 for 5 min, at 37  $^{\circ}\text{C}$ . Activity was monitored by cleavage of a 1 mM concentration of the VLK-SBzl substrate. Note the increase in absorbance, indicative of increased enzymatic activity of KLK11. The line representing KLK14 was obtained without pro-KLK11 addition. The basal activity of KLK11 alone has been subtracted.

Here, we developed a “sandwich-type assay” (Fig. 5A) to measure specific enzymatic activity of several trypsin-like KLKs, including KLK1, with a detection limit as low as 30 nM (data not shown). In this assay, the desired KLK is pulled down in microtiter plates. The activity can then be measured using a nonspecific substrate. Given the high protein similarity between KLKs, it is important to avoid nonspecific pull-down due to antibody cross-reactivity. Fig. 5B shows the pull-down specificity of the KLK1 antibody HUK-IgG, using recombinant mature KLK1 and -14. Although soluble (nonimmobilized) KLK14 exhibited an even higher reaction rate toward the PFR-AMC substrate (Fig. 5C), almost no enzymatic activity was observed for the pulled down KLK14 on KLK1 antibody-coated plates, using the same amount of substrate. Using this assay, we confirmed that KLK1 was activated by KLK14 in a time-dependent manner (Fig. 5D).

Based on the information provided above, a cascade model for seminal plasma and skin (Figs. 6 and 7, respectively) was developed. For seminal plasma, the cascade is based on six KLKs that have already been found at appreciable amounts in this fluid. For skin, the cascade is based on five KLKs, known to



**FIGURE 5. Activation of pro-KLK1 by KLK14.** *A*, schematic presentation of the activity assay. Pro-KLK is activated by an active KLK. The activated KLK is pulled down using a specific monoclonal or polyclonal antibody. Background due to nonspecific binding of mature KLK is reduced through a series of stringent washes. The activity of the activated KLK is measured by monitoring the fluorescence release of a nonspecific substrate and normalized to the background signal. *B*, specificity of the KLK1 sandwich pull-down assay. 200 ng of anti-KLK1 antibody were immobilized overnight on a microtiter plate. A 120 nM concentration of either mature KLK1 or -14 was added to coated wells for 2 h. Enzymatic activity of pulled down KLKs were measured, using 0.25 mM PFR-AMC. The KLK14 reaction rate was almost zero when pulled down with anti-KLK1 antibody, ensuring assay specificity. *C*, to ensure that the mature form of both KLKs had a comparable enzymatic activity prior to the pull down, reaction rates of soluble (noncoated) 12 nM mature KLK1 and KLK14 were measured, using 0.25 mM PFR-AMC. *D*, time-dependent activation. 0.2  $\mu$ M KLK1 was incubated with 0.2  $\mu$ M KLK14 for 0, 10, 30, and 60 min at 37 °C. KLK1 was pulled down in 96-well microtiter plates, as described in *A*. Activity of the pulled down KLK1 was monitored by cleavage of a 0.25 mM concentration of the PFR-AMC substrate. The basal activity of KLK1 alone was subtracted, prior to calculating reaction rates. FU, fluorescence units.

be expressed in this tissue. It is conceivable that other KLKs and/or other classes of enzymes, as well as additional inhibitors, may also participate in such pathways.

**DISCUSSION**

Proteolytic cascades function to transduce signals through sequential activation of protease zymogens, enabling cells to respond to environmental cues (2). The concept of proteolytic cascade was initially proposed through the extensive work on blood coagulation mechanisms about 40 years ago (33, 34). Since then, other proteolytic cascades have been recognized in processes, such as apoptosis and matrix remodeling (35–37).

Generally speaking, proteolytic cascades consist of three main consecutive phases of initiation, propagation, and execution (2). The “initiator” zymogens are often self-activated

through autocatalysis. Active initiators then convert downstream “propagator” proteases, which consequently activate “executor” zymogens. The result is a rapid and highly controlled amplification of active executors in response to minute amounts of initiator enzymes. Therefore, the amount of active enzyme is expected to increase from the initiation to the final execution phase (2).

The idea of proteolytic cascades in KLKs came into prominence only recently, with accumulating evidence indicating their stepwise activation mechanism. For instance, with the exception of KLK4, KLKs are activated by cleavage after lysine or arginine (38) (Table 1), which are preferred trypsin-like cleavage sites. However, some of the KLKs are chymotrypsin-like (39) and thus require other trypsin-like proteases for their activation. As mentioned previously, the chymotrypsin-like enzymes KLK3 and KLK7 were shown experimentally to be activated by the trypsin-like KLK5.

Moreover, in tissues, KLKs are often expressed in groups at varying levels (20). Assuming that enzymatic activity is proportional to the expression level of each KLK, such co-expression patterns may further indicate hierarchical activation networks, consisting of initiators, progressors, and executors. For example, KLK14 is expressed at an average concentration of 5  $\mu$ g/liter in seminal plasma, whereas certain other seminal KLKs, including KLK1 and -11, have 10–10<sup>3</sup>-fold

higher expression levels (20). Moreover, consistent with the proposed hierarchical model of cascades, seminal KLK3 is expressed at the staggering rate of 10 g/liter (20) and functions as the key executor of semenogelin hydrolysis during clot liquefaction (25, 40, 41). Similarly, KLK11 expression in skin is ~9 times higher, compared with KLK14 (20), further suggesting the notion of activation networks and sequential zymogen activation.

In the case of seminal plasma, additional evidence reinforcing the idea of proteolytic cascades comes from the striking overlap between regulatory components of blood and seminal homeostasis (10, 42–45). More recently, a number of well known components of the blood coagulation and fibrinolysis systems, including PCI, tissue type and urokinase type plasminogen activator, tissue factor, tissue factor pathway inhibitor,

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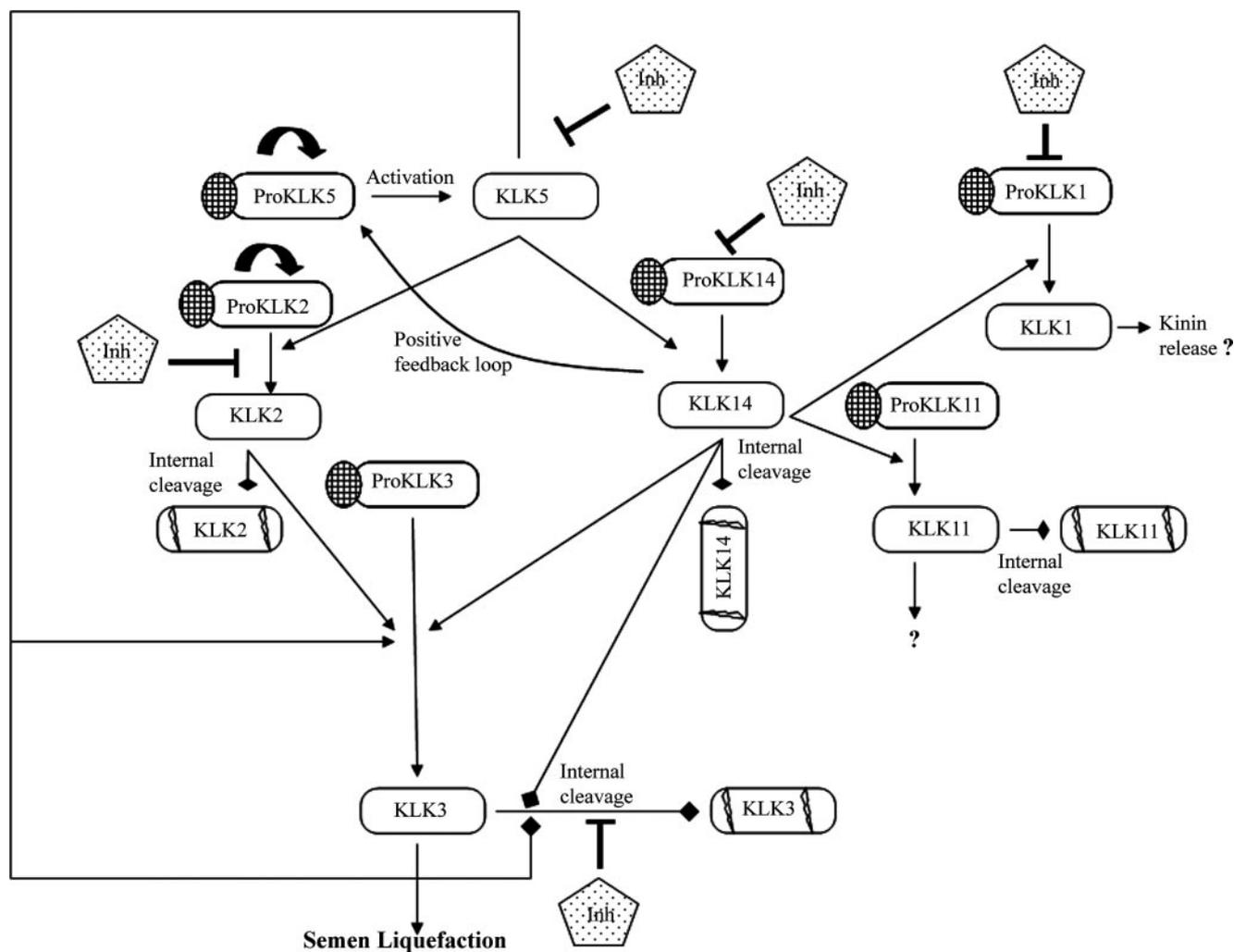


FIGURE 6. **Schematic presentation of proposed kallikrein cascades in seminal plasma.** KLK2 and -5 autoactivate and, along with active KLK14, activate pro-KLK3. KLK5 could also activate pro-KLK2 (10) and pro-KLK14 (9), as shown previously. Activated KLK3 acts as an executor protease in the liquefaction of seminal clot and release of spermatozoa through proteolytic processing of Sgl/Sgll. The cascade is regulated by a number of endogenous inhibitors (PCI, ATIII, ACT, and  $\alpha_2$ -M),  $Zn^{2+}$ , collectively shown as *Inh*, as well as by (auto)degradation of active KLKs (28). Active KLK14 can further activate pro-KLK11 and -KLK1, functions of which remain to be further elucidated.  $Zn^{2+}$  binds to active KLKs and inhibits their activity. Sgl and -Ii along with fibronectin form the semen coagulum at the time of ejaculation, entrapping motile spermatozoa. Immediately after ejaculation, Sgs chelate  $Zn^{2+}$ , rendering KLKs active. Active KLKs, in turn, engage in the above proteolytic cascades, rapidly amplifying active KLK executors. Sgs and fibronectin are subsequently degraded by the executor KLKs, resulting in semen liquefaction and release of motile sperm cells. The question mark indicates unknown function.

and blood coagulation factor X, have been identified in seminal plasma (46–50), raising the possibility of a similar proteolytic cascade in this fluid.

Here, for the first time, we propose a potential cascade-mediated role of KLK14 upstream of multiple KLK members. KLK14 is considered as the key trypsin-like protease in the SC of skin, involved in corneocyte shedding (32). Although its downstream targets in skin are not fully understood, previous reports have implicated KLK14 in skin proteolytic cascades. Moreover, given the significant overlap between proteins expressed in skin and seminal plasma, KLK14 could be a strong candidate regulatory protease in seminal plasma.

Our *in vitro* data indicate that KLK1, -3, and -11 are regulated by KLK14. Activation of pro-KLK3 is of particular interest due to its restricted expression and functional importance in seminal plasma. KLK3 is activated by cleavage after arginine at position 7 (51). Given its chymotrypsin-like substrate preference, this would exclude the possibility of autoactivation.

Thus far, several trypsin-like KLKs have been reported as potential activators of pro-KLK3. KLK2 was initially reported as the main activator of pro-KLK3 (22, 52, 53). However, subsequent reports implied that active KLK2 is unable to cleave the propeptide sequence of KLK3 (21), calling into question the previous finding. Additional prostatic KLKs, including KLK4 and -5 have also been identified as potential pro-KLK3 activators (10, 53).

Our data suggest that KLK14 regulates the activity of KLK3 bidirectionally. Activation occurs within a few minutes and continues up to 30 min. Subsequent deactivation possibly occurs through internal cleavage of active KLK3. Internal cleavage and subsequent degradation is one of the key mechanisms responsible for KLK3 inactivation. Purified KLK3 from seminal plasma contains fragments cleaved between residues Arg<sup>85</sup> and Phe<sup>86</sup>, Lys<sup>145</sup> and Lys<sup>146</sup>, and Lys<sup>182</sup> and Ser<sup>183</sup> (54–57). Our previous work identified KLK5-mediated fragmentation at positions 85 and 182 (10). However, the enzyme

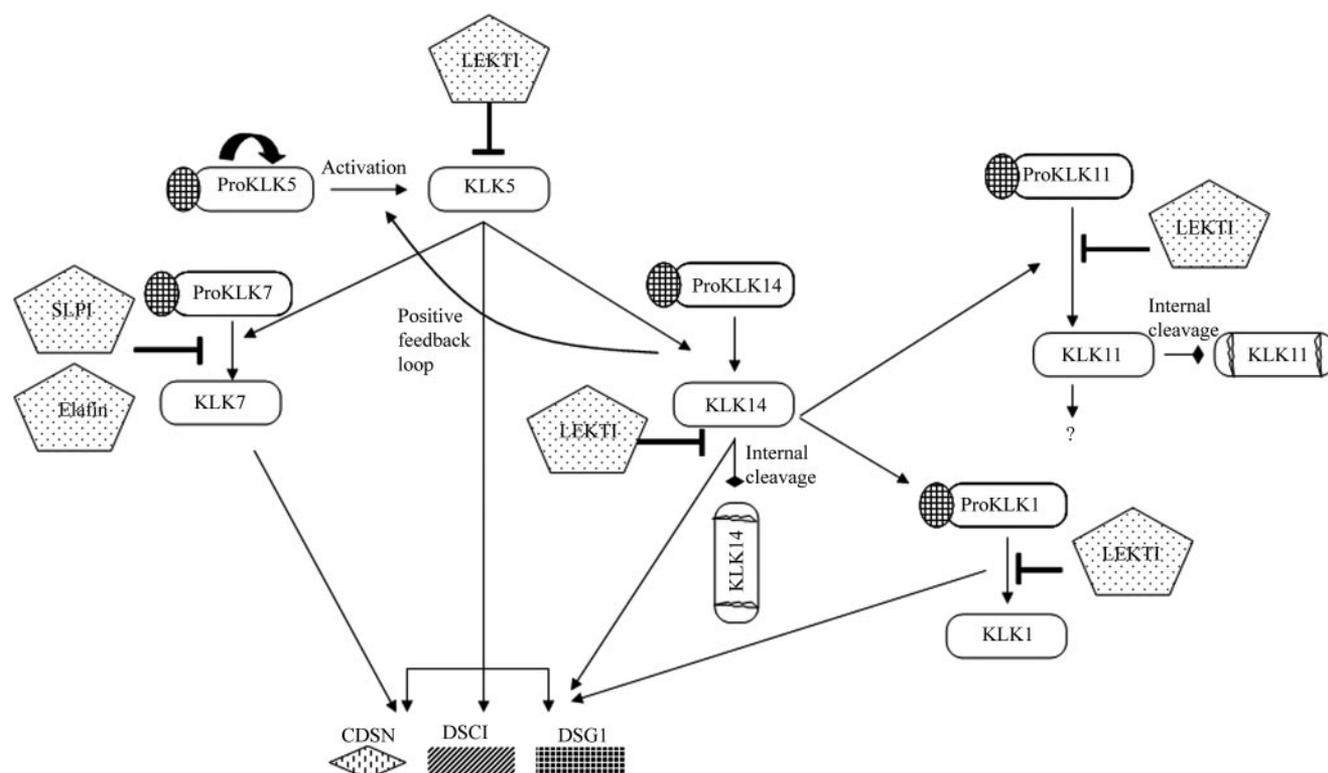


FIGURE 7. Schematic presentation of proposed kallikrein cascades in skin. KLK5 autoactivates and activates KLK14 and -7. Activated KLK14 activates pro-KLK1 and -11. Cascades are postulated to be triggered by SC acidification of the superficial layer of skin. Executor KLKs function in skin desquamation through degradation of the corneodesmosomal proteins (*i.e.* DSG1, DSC1, and CDSN). Desquamation is regulated by various serine protease inhibitors, such as SLPI, elafin, and certain LEKTI domains, positive feedback loops, and internal cleavages. The *question mark* indicates unknown function.

responsible for cleavage after lysine 145 was unknown until now. Here, we have shown that KLK14 catalyzes the cleavage and inactivation of KLK3 at this site.

In addition, we demonstrated that KLK14 is able to activate pro-KLK11. Although not fully characterized, KLK11 is one of the two most highly expressed KLKs in the SC of skin (19, 20). Similarly, the concentration of KLK11 in seminal plasma ranks third after KLK3 and KLK2 (20, 28). Seminal plasma depleted from KLK11 has previously been shown to retain its ability to cleave and activate pro-KLK11 *in vitro* (28), suggesting that seminal plasma contains KLK11 activator enzyme(s). Despite the fact that KLK2 and plasmin had initially been identified as candidate activators of this enzyme, further experiments ruled out activation via KLK2 (28). Here, we identified KLK14 as an upstream activator of pro-KLK11, functioning as quickly as 2 min, at the physiologically relevant molar ratio of 1:10.

Despite the fact that KLK1 has been known for over 50 years, its physiological activating enzyme remains elusive. However, emerging evidence points to a possible cascade-mediated function of the protein. For instance, in skin, KLK1 has been implicated in SC desquamation, through cleavage of DSG1 (15). Given the substrate overlap between KLK1 and other KLKs of the skin proteolytic cascade, it is conceivable that KLK1 functions through a common proteolytic network. In seminal plasma, along with other seminal KLKs, KLK1 is secreted from the prostate gland and is known to complex with PCI (58, 59). PCI has been shown to complex with several other seminal KLKs, including the two prominent members of the proteolytic cascade, KLK2 and -3 (59, 60). Clinically, prostatic KLK1 has

been associated with insufficient sperm motility, underlying a male subfertility condition described as asthenospermia (61). Sperm motility is reportedly improved in these patients upon KLK1 administration (62). At present, kinin is the only recognized terminal inducer of sperm motility (63). Active kinins are released from seminal kininogens through limited proteolysis by a number of kininogenases, including KLK1 (64, 65). Sperm motility is believed to be mediated through the B2R (B2 subtype of bradykinin) receptor and subsequent release of intracellular  $Ca^{2+}$  in testicular peritubular cells (62, 66). However, kinin antagonists failed to completely inhibit sperm motility (67), suggesting an alternative mechanism whereby sperm motility is stimulated independent of the kinin signaling pathway. Impaired sperm motility may be caused by a number of other conditions, including incomplete or delayed liquefaction or nonliquefaction of semen (42, 68, 69). Whether KLK1 partially affects sperm motility through the liquefaction cascade needs to be further explored.

Here, we propose for the first time that KLK14 is a potential candidate activator of KLK1. However, the recombinant pro-KLK1 produced in the mammalian expression system was partially active in the absence of KLK14, suggesting its autoactivation or proteolytic activation by other proteases. Since the KLK1-stably transfected HEK293 cell line is devoid of any other KLK expression, additional protease families may be involved. As mentioned previously, proteases with trypsin-like activity can potentially function as KLK1 activators. We have begun to investigate alternative KLK1 activation mechanism(s) through possible cross-talks, using various approaches, such as activity-

based protein profiling and multidimensional protein identification technologies (70, 71).

In conclusion, the data presented here strongly suggest an additional level of complexity to the modeled proteolytic cascades in skin and seminal plasma (Figs. 6 and 7). Although trigger factors of these cascades remain to be fully elucidated, skin desquamation may be stimulated by SC acidification and subsequent release of active initiator KLK5 (72). In seminal plasma, cascade activation is more likely triggered at the time of semen ejaculation due to an immediate drop in the available  $Zn^{2+}$ , since this ion is spontaneously chelated by Sg proteins (41, 73–76). Given the pathological relevance of uncontrolled proteolytic activity, further understanding of KLK activation mechanisms is essential for future drug development.

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