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 cascade 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 (≥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 activity, 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 proteasezymogens. 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 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-
Cascade-mediated Function of KLK14

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 (Sgl and -II) and fibrinectin (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 Zn²⁺ (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, Zn²⁺ 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-Asn-C, N-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-Asn-C, N-Ala-Thr-Pro-Lys-Ile-Ile-Asp-C, N-Glu-Ser-Ser-Lys-Leu-Asn-C, N-Asp-Glu-Asn-Lys-Ile-Ile-Gly-C, and N-Asp-Glu-Asp-Lys-Leu-Val-Glu-C were purchased from Genemed Synthesis (San Francisco, CA) and were diluted in water and stored at −20 °C. The synthetic substrates, succinyl-Arg-Pro-Tyr-para-nitroanilide-HCl (RPNPA), 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 μg of heptapeptides were incubated at 37 °C with 1 μg 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 μl. Reactions were stopped at different time points by freezing the samples with liquid nitrogen. A 150-μl aliquot of each time point was diluted 2-fold with loading buffer (0.1% trifluoroacetic acid in H₂O). 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 (TOYOPEARL) and a mobile phase consisting of 0.1% trifluoroacetic acid in water (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 (MD Sciex). The HPLC was conducted using an Xterra C18 column (3.0 × 50 mm, 2.5 μm) 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™ accession number AAH05313) was PCR-amplified and cloned into the pcDNA3.1(-) (Invitrogen) mammalian expression vector at EcoRI and Xbal 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 °C and 5% CO₂ in Dulbecco’s modified Eagle’s medium culture medium (Invitrogen) supplemented with 10% fetal bovine serum. Approximately 180 × 10⁶
cells were seeded into 10 175-cm² 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-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-
Cascade-mediated Function of KLK14

Heptapeptide Screening—In an attempt to identify potential downstream targets of KLK14, a library of 15 heptapeptides representing the putative P4-P3-P2-P1-P̂1-P̂2-P̂3

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 → 531.4 (A), Hep1N at m/z 252.6 → 391.2 (B), and Hep1C at m/z 288.7 → 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.

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-P̂1-P̂2-P̂3...
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 P3 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 (≥85% digest after 5 h) to low efficiency (≤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 KLQCV (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 $\sim$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 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 $\sim$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. 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
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 g/liter in seminal plasma, whereas certain other seminal KLKs, including KLK1 and -11, have 10–10³-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,
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

![Schematic presentation of proposed kallikrein cascades in seminal plasma.](image)
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$^{2+}$/H$^{+}$ 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 KLK activators. We have begun to investigate alternative KLK1 activation mechanism(s) through possible cross-talks, using various approaches, such as activity-
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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 Zn2+ 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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