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Major Role of Human KLK14 in Seminal Clot Liquefaction*[S]

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Liquefaction of human semen involves proteolytic degradation of the seminal coagulum and release of motile spermatozoa. Several members of human kallikrein-related peptidases (KLKs) have been implicated in semen liquefaction, functioning through highly regulated proteolytic cascades. Among these, KLK3 (also known as prostate-specific antigen) is the main executor enzyme responsible for processing of the primary components of semen coagulum, semenogelins I and II. We have recently identified KLK14 as a potential activator of KLK3 and other KLKs. This study aims to elucidate the cascade-mediated role of KLK14 *ex vivo*. KLK14 expression was significantly lower ($p = 0.0252$) in individuals with clinically delayed liquefaction. Concordantly, KLK14 expression was significantly ($p = 0.0478$) lower in asthenospermic cases. Specific inhibition of KLK14 activity by the synthetic inhibitor ACT_{G9} resulted in a significant delay in semen liquefaction, a drop in the "early" (30 min postejaculation) "chymotrypsin-like" and KLK1 activity, and an increase in the "late" (90 min postejaculation) chymotrypsin-like activity. Conversely, the addition of recombinant active KLK14 facilitated the liquefaction process, augmented the early chymotrypsin-like activity, and lowered late chymotrypsin-like activity. Given that the observed chymotrypsin-like activity was almost completely attributed to KLK3 activity, KLK3 seems to be regulated bidirectionally. Accordingly, a higher level of KLK3 fragmentation was observed in KLK14-induced coagula, suggesting an inactivation mechanism via internal cleavage. Finally, semenogelins I and II were directly cleaved by KLK14. Semenogelins were also able to reverse KLK14 inhibition by Zn^{2+}, providing a novel regulatory mechanism for KLK14 activity. Our results show that KLK14 exerts a significant and dose-dependent effect in the process of semen liquefaction.

Spermatogenesis, the process of differentiation of testicular stem cells into mature spermatozoa, is initiated in the seminiferous tubules of the testes, which produce immature sperm cells (1–3). Subsequent maturation occurs during epididymal transition, where immature spermatozoa acquire motility and fertilizing capacity (4, 5). Sperm motility is particularly important at the time of fertilization, since it facilitates sperm penetration to the zona pellucida of the oocyte and fusion of the two cells (1, 2, 6).

Asthenospermia, described as impaired sperm motility, is considered as one of the main factors of male subfertility or infertility and may be caused by a number of conditions, including incomplete liquefaction, delayed liquefaction, or nonliquefaction of semen (7–9). Normally, human semen coagulates spontaneously upon mixing of its various glandular fractions in order to form a depository of spermatozoa in the rear vaginal cavity (10–12). Subsequent liquefaction of coagulum within minutes (~5–20 min after ejaculation) allows for a progressive release of motile spermatozoa (10, 13). Liquefaction is achieved through a stepwise proteolytic cleavage of the gel proteins semenogelin I and II (SgI and -II) into soluble proteins, followed by their peptidic fragmentation. These peptides are eventually degraded into their constituent amino acid residues (14–18).

Semen coagulation/liquefaction is under tight regulatory control. For instance, Sg proteins chelate with the excess of free Zn^{2+} immediately after ejaculation and undergo structural modifications, inducing aggregate complex formation (19–23). Sg degradation is mainly modulated through activation of KLK3 (kallikrein-related peptidase 3), also known as prostate-specific antigen (22, 24, 25). The enzymatic activity of KLK3 is tightly controlled through a number of endogenous inhibitors and regulatory feedback loops. For instance, along with Sg proteins, the serine protease inhibitor, protein C inhibitor, is secreted from the lumen of seminal vesicles (26). Recent evidence indicates that protein C inhibitor complexes with Sg, preventing its premature hydrolysis by active KLK3 (27). KLK3 activity is believed to be further inhibited by free Zn^{2+} in prostatic secretions (25, 28). Sg chelation with free Zn^{2+} results in an immediate drop in the available Zn^{2+}, which consequently leads to KLK3 activation. Conversely, Zn^{2+} is released gradually as Sg proteins are fragmented by KLK3. The increased level of Zn^{2+} serves as a negative feedback loop to prevent excessive proteolysis that may damage the integrity of spermatozoa (29).

Recent evidence indicates an additional level of complexity in the regulation of the proteolytic cleavage of Sg proteins. For instance, *in vitro* data suggest that other proteases, particularly other members of the kallikrein-related peptidase (KLK)[2] fam-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Fig. 1.

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[2] The abbreviations used are: KLK, human kallikrein-related peptidase; ACT, antichymotrypsin; AMC, 7-amino-4-methylcoumarin; ELISA, enzyme-linked immunosorbent assay; *p*NA, *para*-nitroanilide; Sg, semenogelin; TBS, Tris-buffered saline; Suc, succinyl; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

ily (*i.e.* KLK2 and KLK5), are directly or indirectly involved in Sg processing (30–32). In addition, emerging reports suggest a cascade-mediated protease activation mechanism, regulated by a number of positive and negative feedback loops. For example, KLK5 is suggested to autoactivate and, in turn, activate pro-KLK3 (33). Likewise, although still controversial, KLK2 has been suggested to activate pro-KLK3 (34–36). Finally, our recent work implicates KLK14 as a potential activator of pro-KLK3 as well as several other seminal pro-KLKs (*i.e.* pro-KLK1 and pro-KLK11) (37). Characteristic to classic proteolytic cascades, we have previously proposed a bidirectional regulatory mechanism of KLK3, in which KLK14-mediated activation is followed by inactivation via internal cleavage of active KLK3 at position Lys^{145} (37).

KLK14 is a trypsin-like KLK, with preference over P1-Arg (38, 39). Zinc ions have been shown to strongly inhibit KLK14 enzymatic activity (38), reinforcing the potential role of the protein in semen liquefaction. In an attempt to delineate a possible function of KLK14 in seminal plasma, this study examines the interaction between this enzyme and other potential components of the seminal proteolytic cascade involved in semen liquefaction.

EXPERIMENTAL PROCEDURES

Reagents—The synthetic substrates, Suc-Arg-Pro-Tyr-*p*NA·HCl (RPY-*p*NA) and Pro-Phe-Arg-AMC (PFR-AMC)/Gln-Ala-Arg-AMC (QAR-AMC) were purchased from BACHEM (King of Prussia, PA) and Pharmacia Hepar-Chromogenix (Franklin, OH), respectively. Recombinant pro-KLK3 produced in *Escherichia coli*, was a gift from Spectral Diagnostic Inc. (Toronto, Canada). Mouse anti-KLK3 monoclonal antibody was purchased from Medix MAB (Kauniainen, Finland). Recombinant KLK1, -4, -5, -11, -12, and -14; KLK14-specific monoclonal (clone 2E9); and rabbit anti-KLK14/KLK3 polyclonal sera were produced in house, as described previously (38). Recombinant KLK2 was a gift from Hybritech Inc. (San Diego, CA). Conjugated goat anti-rabbit antibody and chemiluminescent substrate for Western blot were purchased from Jackson Immunoresearch Laboratories and Diagnostic Products Corp., respectively. *N*-Hydroxysuccinimide-activated Sepharose 4 Fast Flow beads were purchased from GE Healthcare. HUK-IgG antibody recognizing KLK1 and purified Sg proteins were kindly provided by Prof. J. Chao (Medical University of South Carolina) and Dr. J. Malm (Malmö University Hospital, Sweden), respectively. ACT_{G9}, a KLK14-specific recombinant mutant inhibitor, was developed in collaboration with Dr. D. Deperthes (Med-Discovery) by replacing the scissile bond of the reactive center loop of the α1- antichymotrypsin (ACT) inhibitor with KLK14 phage display-selected G9 (TVDYA) substrate, as described in detail elsewhere (40).

Materials—Coagulated semen, having a normal liquefaction rate at room temperature, was collected, split into three fractions, and frozen immediately after ejaculation in liquid nitrogen. Samples were stored at −80 °C until required. Liquefied semen was obtained from 95 subjects with normal and delayed liquefaction, under informed consent and approval by the Institutional Review Boards of Mount Sinai Hospital and the University Health Network. If required, semen coagula were artificially emulsified, by the addition of a small amount of chymotrypsin enzyme at 37 °C for up to 1 h.

Enzyme-linked Immunosorbent Assay (ELISA)—The expression level of KLK14 protein was measured using a sandwich type ELISA, with a mouse monoclonal/rabbit polyclonal configuration, as described previously (38, 41). Briefly, 500 ng/well of the monoclonal antibody against KLK14 (clone 2E9), diluted in coating buffer (50 mmol/liter Tris, 0.05% sodium azide (pH 7.8)), was immobilized on a 96-well white polystyrene plate overnight at room temperature. The plate was subsequently washed two times with washing buffer (50 mmol/liter Tris, 150 mmol/liter NaCl, 0.05% Tween 20 (pH 7.8)). Seminal plasma samples were diluted 1:10 in assay buffer (50 mmol/liter Tris, 6% bovine serum albumin, 10% goat IgG, 2% mouse IgG, 1% bovine IgG, 0.5 mol/liter KCl, 0.05% sodium azide, pH 7.8) and were loaded and incubated for 2 h with shaking at room temperature. The plate was then washed six times. 100 μl of rabbit anti-KLK14 polyclonal sera, diluted 1000-fold in assay buffer, were added and incubated for 1 h. The plate was washed six times and incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (3000-fold dilution) for 45 min. Finally, diflunisal phosphate (100 μl of a 1 mM solution) in substrate buffer (0.1 mol/liter Tris, pH 9.1, 0.1 mol/liter NaCl, and 1 mmol/liter $MgCl_2$) was added to each well and incubated for 10 min, followed by the addition of developing solution (100 μl, containing 1 mol/liter Tris base, 0.4 mol/liter NaOH, 2 mmol/liter $TbCl_3$, and 3 mmol/liter EDTA) for 1 min. The resultant fluorescence was measured with a time-resolved fluorometer (Envision; PerkinElmer Life Sciences). Similarly, the expression levels of KLK1, -2, -4, -5, -11, and -12 were measured using highly specific ELISAs, developed in house and described previously (41).

Measurement of Clinical Parameters of Semen—Liquefaction rate was estimated by attempting to draw the specimen into a Pasteur pipette. Complete liquefaction was achieved when all of the fluid entered the pipette. In addition, liquefaction level was evaluated visually by a phase-contrast microscope as a measure of disappearance of the gel-like coagulum structure.

Overall sperm motility (percentage) was determined using automated computer-assisted semen analysis as $(a + b/(a + b + c)) \times 100$, where a, b, and c represent the number of progressively motile sperm, sperms moving in random directions, and nonmotile sperms, respectively. Cases with a percentage of sperm motility equal to or less than 35% were considered as asthenospermic.

Cleavage of SgI and -II Proteins—500 ng of purified SgI and SgII were incubated individually with 56 ng of KLK14 in 30 μl of KLK14 optimal assay buffer (100 mM phosphate buffer, 0.01% Tween 20, pH 8.0) at 37 °C for various time points. Reactions were snap-frozen in liquid nitrogen and run on SDS-polyacrylamide gels under reducing conditions. Gels were silver-stained to visualize fragmentation.

Sg-mediated Reversal of Zn^{2+} Inhibition—To examine Sg-mediated reversal of Zn^{2+} inhibition, 12 nM KLK14 was incubated with 0 and 120 nM of Zn^{2+} (in the form of zinc acetate), at a final volume of 100 μl, for 10 min at 37 °C. Subsequently, the fluorogenic substrate QAR-AMC was added at a final concentration of 1 mM. Fluorescence release was measured on a Wallac

Victor fluorometer (PerkinElmer Life Sciences), set at 355 nm for excitation and 460 nm for emission. Fluorescence was measured for a total of 20 min. Five minutes after initiating the read, a 0.05 μM concentration each of SgI and SgII or 0.01 M EDTA was added to each well. Measurement was resumed as described above. Background fluorescence was subtracted from raw values. All experiments were performed in triplicate.

Enzyme Activity Assays—The "chymotrypsin-like" activity of seminal plasma samples (diluted 10 times) was kinetically examined, using 0.8 mM colorimetric substrate RPY-pNA in a final volume of 100 μl of KLK3-optimized assay buffer (0.1 mM Tris, 3 mM NaCl, 0.01% Tween 20, pH 7.5). Absorbance was measured on a Wallac Victor Fluorometer at 405 nm. Background absorbance was subtracted from raw values of seminal plasma alone, and samples were treated with either active recombinant KLK14 or ACT_{G9}, described above. Reactions were repeated three times.

KLK1-specific activity was measured by fluorescence release of the pulled down KLK1 protein, as previously described (37). Briefly, 200 ng of KLK1-specific polyclonal antibody (HUK-IgG) were immobilized on a 96-well white polystyrene plate overnight. The plate was washed two times prior to the addition of reaction mixtures. KLK1 activity was measured as an increase in the fluorescence of PFR-AMC substrate after 2 h of sample incubation. Reaction rates (fluorescence units/min) correspond to the slope of the fluorescence release-time plot.

KLK3 Depletion from Seminal Plasma—1 mg of monoclonal anti-KLK3 antibody was immobilized on 1 ml of 50% *N*-Hydroxysuccinimide-activated Sepharose Fast Flow bead slurry, according to the manufacturer's protocol. Briefly, beads were equilibrated three times in 2 ml of ice-cold 1 mM HCl. They were then incubated with 1 mg of monoclonal anti-prostate-specific antigen antibody for 1 h at room temperature with end-over-end mixing. Residual active groups of beads were subsequently blocked by washing beads sequentially three times with 2 ml each of buffer A (50 mM Tris·HCl, 1 M NaCl, pH 8.0) and buffer B (0.1 M acetate, 0.5 M NaCl, pH 4.0). Beads were further washed two times with buffer A and then incubated for 15 min at room temperature. Further blocking was achieved by sequential incubation of beads three times each with buffer B, A, and B. Beads were equilibrated for protein binding in TBS (50 mM Tris, 150 mM NaCl, pH 7.5). 20 μl of seminal plasma were diluted in TBS in total volume of 1 ml and incubated with beads for 1 h at room temperature, with end-over-end mixing. The flow-through (depleted samples) was collected and further analyzed kinetically. Beads were washed five times in wash buffer (TBS with 2 M urea, pH 7.5) and eluted with 1 ml of elution buffer (0.1 M glycine with 2 M urea, pH 3.0). A mock-depleted sample was prepared in parallel, using beads alone. Percentage of depletion was estimated by measuring KLK3 in flow-through samples, using KLK3-specific ELISA. Collected flow-through samples were concentrated 10 times, using membranes with a molecular weight cut-off of 5000. 5 μl of concentrated samples were diluted in 95 μl of KLK3 optimal assay buffer. Enzymatic activity toward the tripeptide RPY-pNA substrate was measured as described above.

To ensure that the observed drop of enzymatic activity is due to exclusive depletion of KLK3, two identical reactions of 20 μl of immunodepleted and mock-depleted elutions were run on SDS-PAGE under reducing conditions. One gel was silver-stained, and the other was immunoblotted with anti-KLK3 antibody as described below.

Western Blotting for Identification of KLK3 Fragmentation in Seminal Plasma—To monitor KLK14-mediated fragmentation of KLK3 *ex vivo*, semen coagula were spiked for 1 h to various amounts of active recombinant KLK14 and were analyzed by Western blot. Similarly, KLK14-mediated fragmentation of pro-KLK3 was reconfirmed *in vitro* by incubating recombinant pro-KLK3 with active KLK14 at a 10:1 molar ratio for varying times, in a total volume of 30 μl. Recombinant and seminal proteins were resolved by SDS-PAGE, using the NuPAGE Bis-Tris, with 4–12% gradient polyacrylamide gels at 200 V for 45 min and transferred onto a Hybond-C Extra nitrocellulose membrane (GE Healthcare) at 30 V for 1 h. The membrane was subsequently blocked for 1 h with 5% milk/TBS-Tween (0.1 mol/liter Tris-HCl containing 0.15 mol/liter NaCl and 0.1% Tween 20) at 4 °C and probed using rabbit anti-KLK3 polyclonal sera (diluted 1:1000) for 1 h at room temperature. The membrane was washed three times for 15 min with TBS-Tween and treated with alkaline phosphatase-conjugated goat anti-rabbit antibody (diluted 1:8000) for 45 min at room temperature. The membrane was rewashed as above, and fluorescence was detected on x-ray film using a chemiluminescent substrate.

RESULTS

Clinical Association between KLK14 Expression and Liquefaction Rate—KLK14 concentration in seminal plasma from 95 volunteers (including 34 normal cases and 61 patients with delayed liquefaction) ranged from 0.2 to 181.2 μg/liter, with a mean of 13.2 μg/liter and a median of 6.8 μg/liter. The expression level of KLK14 had a median of 5.2 and 11.55 μg/liter and mean of 12.38 and 12.99 μg/liter in samples with delayed and normal liquefaction, respectively (Fig. 1*A*). We concluded that KLK14 levels were significantly decreased ($p = 0.0252$) in the patient group with delayed liquefaction. In addition, KLK14 expression was found to be significantly ($p = 0.0478$) lower in 70 asthenospermic patients (15 cases with undetermined or inconclusive percentage motility were excluded from the study) (Fig. 1*B*). The level of KLK14 was dropped to 9.8 μg/liter (mean) and 7.9 μg/liter (median) in asthenospermic cases, as compared with normal individuals with a mean value of 22.5 μg/liter and a median of 13.4 μg/liter.

Role of KLK14 as a Seminal Liquefying Protease—To further investigate the possible role of KLK14 in semen liquefaction, the proteolytic activity of the enzyme in seminal plasma was induced and reciprocally inhibited by using either active recombinant KLK14 or the highly specific KLK14 inhibitor ACT_{G9}, respectively. Complete liquefaction ranged from 10 to 20 min in normal samples. The addition of ACT_{G9} inhibitor to a split fraction of a normal ejaculate sample strongly delayed liquefaction (≥30 min). As expected, the progression of liquefaction was also reduced in inhibitor-treated samples, since the gel-like coagulum structure persisted longer than their untreated control counterparts (Fig. 2). Conversely, liquefaction was accelerated upon the addition of active recombinant KLK14. The gel-like structure of semen coagula seemed to be

less dense in KLK14-induced samples (Fig. 2). The coagula of normal liquefying ejaculates were dissolved too fast; for this reason, we could not determine the effect of KLK14 on liquefaction rate.

Cleavage of Sg Proteins by KLK14—Given the pronounced effect of KLK14 on semen liquefaction, we next examined whether any of the primary components of semen coagulum function as immediate downstream targets of KLK14. The ability of KLK14 to cleave purified SgI and -II proteins was tested. SgI and -II were incubated with active recombinant KLK14 in separate reactions. KLK14 was able to almost fully cleave both SgI and -II, as quickly as 12 min of incubation (Fig. 3). New fragments were generated as early as 2 min after initiation of the reaction.

Reversal of Zn^{2+} Inhibition by SgI and -II—Zn^{2+} has previously been proposed to function as a cationic protease inhibitor of KLK14 (38). As mentioned previously, SgI and -II can indirectly regulate the activity of a number of KLKs by binding to Zn^{2+} molecules, rendering them unable to inhibit KLK activity. To examine whether Sg proteins have the same effect on Zn^{2+}- mediated inhibition of KLK14, KLK14 was incubated with a 10-fold molar excess of Zn^{2+}. The enzymatic activity of KLK14 was monitored kinetically as above. The addition of SgII after 5 min of initiation of the reaction rapidly reversed the inhibition (Fig. 4), suggesting a common regulatory mechanism with several other seminal KLKs. No such effect was observed for SgI (data not shown).

Correlation between KLK14 and the Chymotrypsin-like Activity—Pro-KLK3 was previously proposed to function downstream of KLK14 *in vitro* (37). Unfortunately, there is no tool currently available to specifi-

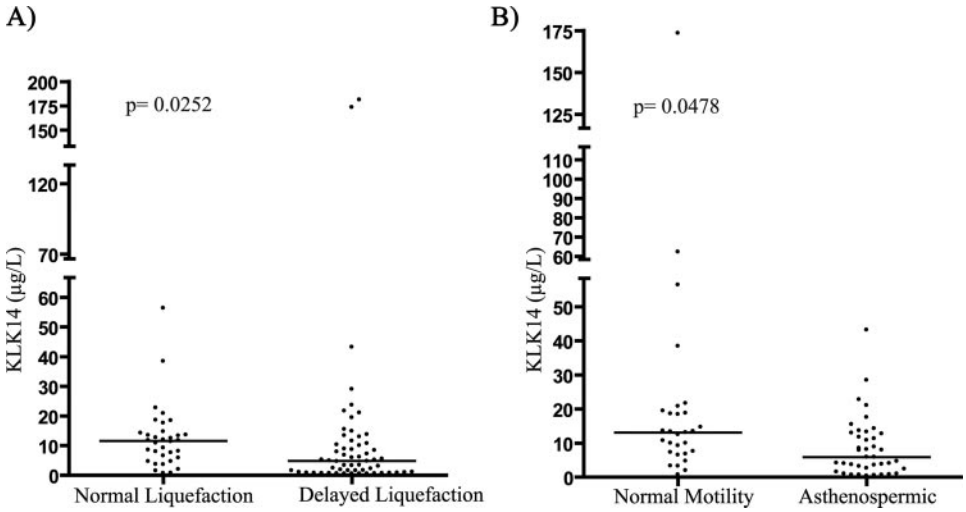

FIGURE 1. **Clinical association between KLK14 expression and liquefaction rate (*A*) and asthenospermia (*B*).** *A*, liquefaction rate. Shown is the distribution of KLK14 concentration (µg/liter) in liquefied seminal plasma of healthy males (normal) and individuals with delayed liquefaction (*i.e.* complete liquefaction did not occur naturally up to 45 min postejaculation). *B*, asthenospermia. Individuals with ≤35% sperm motility were considered as clinically asthenospermic. The *p* value was determined by the Mann-Whitney *t* test. The *horizontal lines* represent the median values.

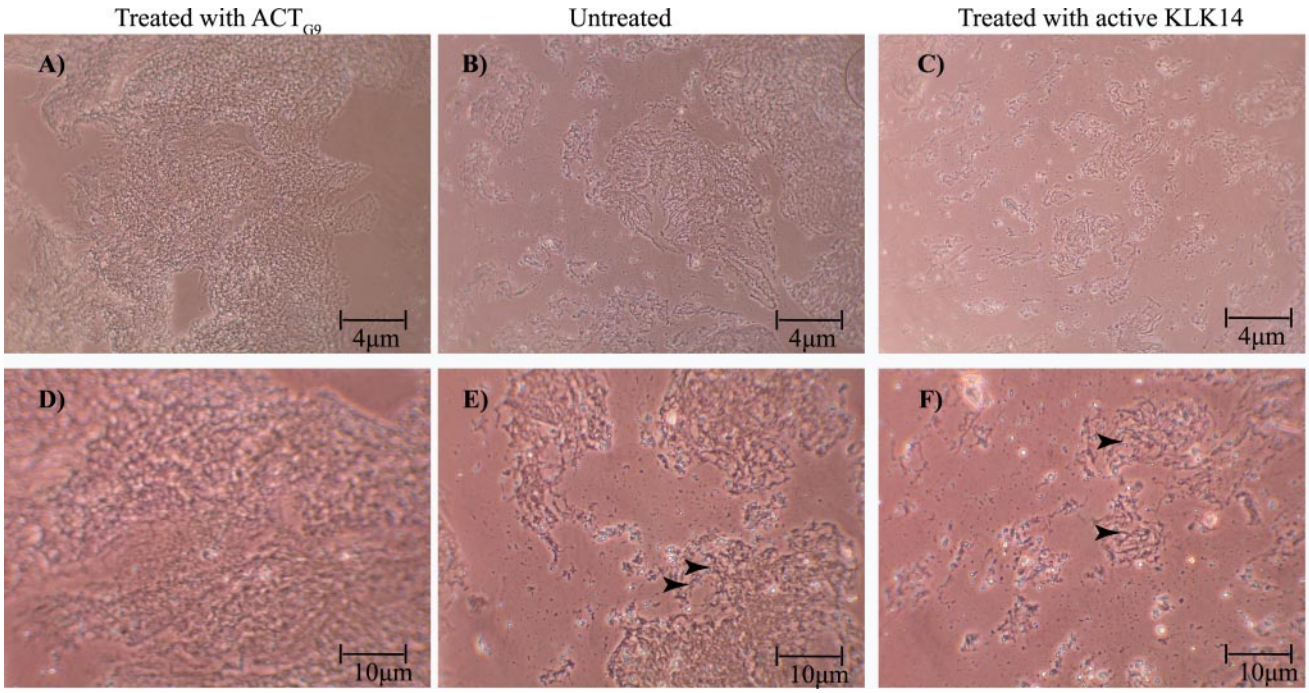

FIGURE 2. **Optical analysis of liquefaction level of semen coagulum.** The general gel-like structure of the clot is visible under a phase-contrast microscope, with sperms (*arrowheads*) entrapped in its cavities. Each of the three splits of the same ejaculate was treated with KLK14-specific inhibitor (*A* and *D*). KLK14 activity was specifically inhibited using a 0.8 µM concentration of the recombinant mutant serpin ACT_{G9}. *B* and *E*, distilled water, a control. *C* and *F*, recombinant KLK14. The sample was added to 0.8 µM recombinant active KLK14. The liquefaction level was estimated as a measure of disappearance of semen coagula. Note the gradual decrease in the intensity of the coagulum structure after 10 min of incubation at room temperature. *Scale bars*, 4 µm (*A–C*) and 10 µm (*D–F*).

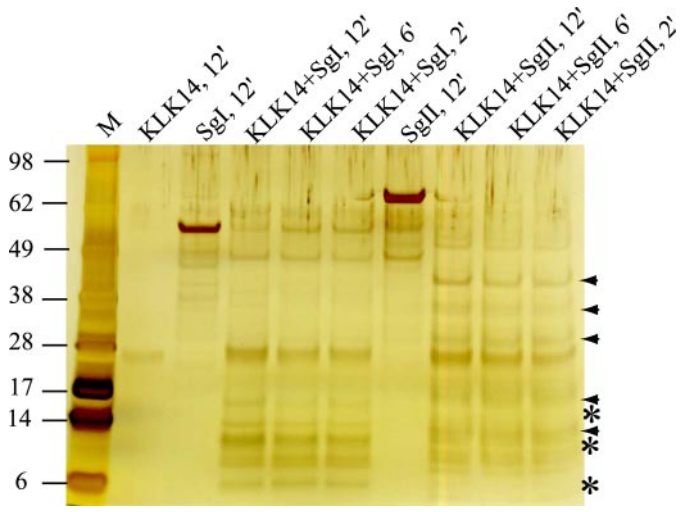

FIGURE 3. **KLK14- mediated degradation of semenogelin proteins.** KLK14 (56 ng) was incubated with 500 ng of purified SgI and SgII for varying time intervals. The mixtures were resolved by SDS-PAGE under reducing conditions, and the gel was sliver-stained. Major KLK14-generated fragments of SgI and SgII are indicated by the *stars* and *arrowheads*, respectively. *M*, molecular mass standards in kDa.

cally quantitate KLK3 enzymatic activity in complex biological samples, such as seminal plasma. However, given, KLK3 shows preference to substrates with P1-tyrosine, P2-proline, and P3-arginine. Given that KLK3 is the major chymotrypsin-like enzyme in seminal plasma and its preferential substrate recognition, we reasoned that KLK3 activity could accurately be estimated in seminal plasma by measuring the chymotrypsin activity toward the RPY-*p*NA substrate. To corroborate this assumption, a series of *ex vivo* depletion experiments were performed. Seminal plasma with ~95% depleted KLK3 exhibited almost zero activity toward the RPY tripeptide substrate, as compared with the mock-depleted control (supplemental Fig. 1*A*). In addition, eluted samples of depleted and mock controls were examined by silver stain and Western blotting against KLK3 (supplemental Fig. 1*B*). All of the proteins eluted from the immunodepleted sample were successfully identified as full-length KLK3 or KLK3 fragments by Western blotting, verifying the specificity of pull-down.

Given that KLK3 activity could confidently be assessed by measuring the chymotrypsin-like activity against the tripeptide RPY-*p*NA (referred to here as "chymotrypsin-like" for short), KLK14-mediated regulation of KLK3 was next examined. The chymotrypsin-like activity of seminal plasma was dependent upon the level of KLK14 activity, since samples treated with active recombinant KLK14 exhibited ~78% higher "early" (30 min after ejaculation) chymotrypsin-like activity, compared with those treated with the KLK14 inhibitor (Fig. 5*A*). As previously suggested, the observed increase was rapid and transient, followed by a decrease in the chymotrypsin-like activity. The reaction rate declined following longer incubation (90 min postejaculation) of seminal coagula, resulting in a reversal of the activity pattern of treated samples *versus* the untreated controls (Fig. 5*B*). The "chymotrypsin-like" activity of ACT_{G9}-treated samples increased ~10%, whereas a drop of almost 78% in was seen in samples added to active recombinant KLK14 (Fig. 5*B*).

Fragmentation of Seminal KLK3 by KLK14—Our previous *in vitro* work suggests an inactivation mechanism of KLK3 through internal cleavage of the active protein. To confirm this, we compared degraded products of KLK3 *in vitro* and in seminal plasma by Western blotting, using rabbit anti-KLK3 polyclonal sera. All major fragments identified previously by silver staining (37) were detected by our antibody (Fig. 6*A*). A very similar fragmentation pattern was observed in seminal plasma spiked with various amounts of active recombinant KLK14 (Fig. 6*B*). As expected, fragmentation was dependent on the level of KLK14 activity. Interestingly, the prominent band generated following KLK14 induction has the molecular mass of the previously identified fragment produced uniquely by KLK14, after cleavage of KLK3 at the peptide bond Lys^{145}-Lys^{146} (37).

Activation of Seminal KLK1 by KLK14—KLK1 has been proposed as one of the downstream targets of KLK14, *in vitro* (37). To evaluate a possible KLK14-mediated activation mechanism of seminal KLK1, we examined KLK1-specific activity in ACT_{G9}-treated samples as compared with an untreated split fraction of the same ejaculate. The specific activity of KLK1 was attenuated ~20% upon treatment of the ejaculate with the ACT_{G9} synthetic inhibitor against KLK14 (Fig. 7*A*). This would suggest that KLK14 could activate pro-KLK1 in seminal plasma. Given the high abundance of trypsin-like KLKs with overlapping substrate specificity, it is critical to ensure pull-down specificity of the KLK1 antibody. In order to exclude the possibility of nonspecific pull-down of physiologically relevant KLKs, protein expressions of KLK1, -2, -4, -5, -11, -12, and -14 were measured using ELISAs developed in house (supplemental Table 1). The pull-down specificity of anti-KLK1 HUK IgG was evaluated using active recombinant KLK2, -4, -5, -11, -12, and -14 in their equivalent amounts found in seminal plasma (Fig. 7*B*). Although these KLKs are highly active when soluble (data not shown), almost no enzymatic activity was observed after they were pulled down with KLK1 antibody. Based on the information provided above, a novel cascade pathway for KLK14 function in semen liquefaction was developed (Fig. 8).

DISCUSSION

Human semen coagulates spontaneously after ejaculation and consequently liquefies within 5–20 min under normal physiological conditions (11). Although the mechanism is not fully understood, the process of semen coagulation/liquefaction is believed to be regulated through a series of enzymes, mainly proteases, and inhibitory factors (14, 15).

More recently, a number of well known components of the blood coagulation and fibrinolysis systems, including protein C inhibitor, tissue and urokinase type plasminogen activator, tissue factor, tissue factor pathway inhibitor, and blood coagulation factor X, have been identified in seminal plasma and have been associated with male fertility (42–46). Given the overlapping regulatory components of the seminal and blood homeostasis, this emerging evidence suggests that analogous to fibrinolysis, semen liquefaction is regulated through highly orchestrated proteolytic cascades (7, 10, 31, 47, 48).

Classic proteolytic cascades consist of sequential activations of protease zymogens through three main phases of initiation, progression (or propagation), and execution (49). A proteolytic

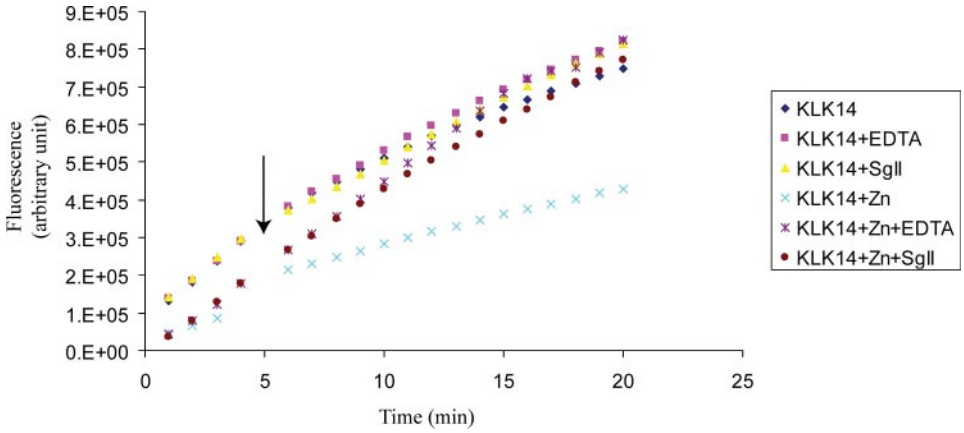

FIGURE 4. **Reversal of Zn^{2+} inhibition by semenogelin II.** Cleavage of QAR-AMC by KLK14 (12 nM) in the presence of optimal assay buffer only, 0.01 M EDTA, 0.05 μM of SgII, 120 nM Zn^{2+}, and 120 nM Zn^{2+} plus 0.01 M EDTA or 0.05 μM SgII. The *downward arrow* shows the time of addition of EDTA or SgII. Note the increase of the residual activity of Zn^{2+}-inhibited KLK14 to almost basal level after the addition of SgII.

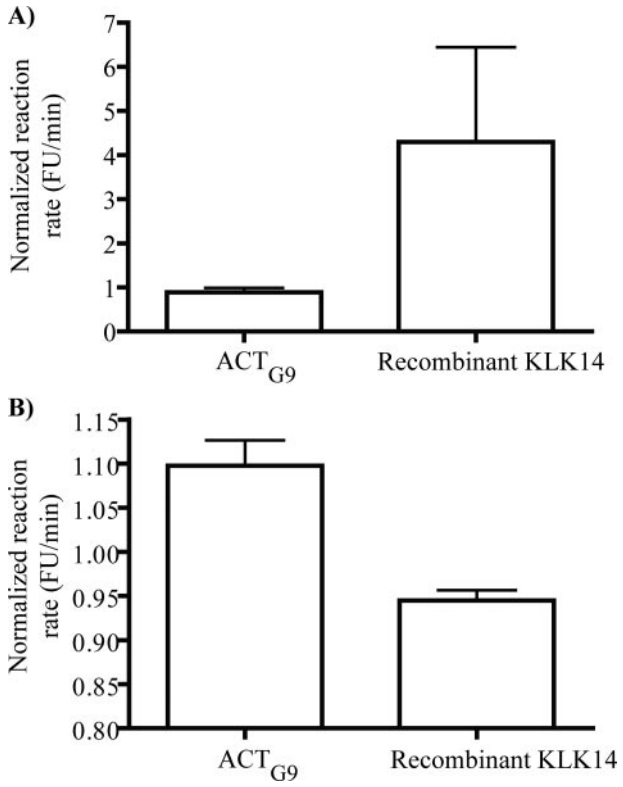

FIGURE 5. **Regulation of total chymotrypsin activity by KLK14.** Ejaculate splits were incubated alone or were individually treated with 0.8 μM active recombinant KLK14 or KLK14 inhibitor ACT_{G9}, prior to the incubation. Treated and control samples were incubated at room temperature for early total chymotrypsin activity (30 min) (*A*) and late total chymotrypsin activity (90 min) (*B*). Total chymotrypsin activity was monitored by cleavage of the RPY-*p*NA substrate(0.8 μM). Residual reaction rates of the treated samples were normalized to the basal reaction rate of the untreated sample. Note the increase of chymotrypsin activity at 30 min, after the addition of KLK14, and the subsequent decrease 90 min after treatment. The chymotrypsin activity of the sample treated with ACT_{G9} inhibitor was slightly elevated at 90 min.

cascade is often initiated by an external stimulus, which in turn triggers the initiating protease or "initiator" to self-activate. Subsequently, active initiator converts downstream propagator proteases into their active form by limited proteolysis. Finally, the active propagator activates executor enzymes during the execution phase. Furthermore, active proteases often activate more of their initiator(s) via positive feedback mechanisms, which would result in a rapid amplification of proteolytic activity. To prevent unwanted protein degradation, a typical proteolytic cascade contains multiple regulation points, including inhibitors, autodegradation, and internal cleavage mechanisms.

Accumulating evidence suggests that several members of the KLK family participate in the seminal proteolytic cascade and are involved in the process of degradation of the semen coagulum (31). *In vitro* data by our group and others suggest that KLK14 might function as a key factor in the proteolytic cascade in seminal plasma, regulating major seminal KLKs, including KLK1, KLK3, and KLK11 (37, 50). Furthermore, the enzymatic activity of KLK14 has recently been shown to be inhibited by Zn^{2+} (38), strengthening the proposed function of the enzyme in seminal plasma and prostatic tissue. Here, for the first time, we propose a cascade-mediated role for KLK14 in seminal plasma, as one of the key trypsin-like regulatory proteases involved in liquefaction of the seminal coagulum.

Trypsin-like proteases are of main importance, since they can function as activators of KLKs that are unable to self-activate (32). A prime example of KLKs lacking autoproteolytic ability is the chymotrypsin-like enzyme KLK3. As mentioned previously, KLK3 has extensively been studied as a main executor KLK in seminal plasma, functioning through cleavage of gel-like proteins and initiating semen liquefaction (22, 28). However, surprisingly, no significant difference was found in KLK3 expression level between normal and delayed liquefaction (51), suggesting possible aberration at the regulatory level of the protein, due to insufficient activation. Previously, we reported KLK14 as an activator of pro-KLK3. Interestingly, our clinical data indicate that there is a significant correlation between abnormal liquefaction and asthenospermia and the expression level of seminal KLK14. The physical constraint of retained coagula seems to adversely affect sperm motility, since we observed an ~70% drop in number of motile sperms in samples with delayed liquefaction (data not shown). Whether the observed reduced level of KLK14 is due to its abrogated expression in the prostate or its partially obstructed secretion to seminal plasma remains to be determined.

In addition, using targeted inhibition and reciprocal overactivation of KLK14 in seminal plasma, we demonstrated that KLK14 is vital for complete liquefaction of the seminal clot. The mutant inhibitor ACT_{G9} used in this study is highly potent and selective toward KLK14 (40). ACT_{G9} contains mutations at the reactive center loop of the biological inhibitor ACT, converting the natural reactive center loop to the phage display-selected KLK14 substrate G9 (40). This would confer an excellent inhibitory specificity toward KLK14; other major seminal KLKs,

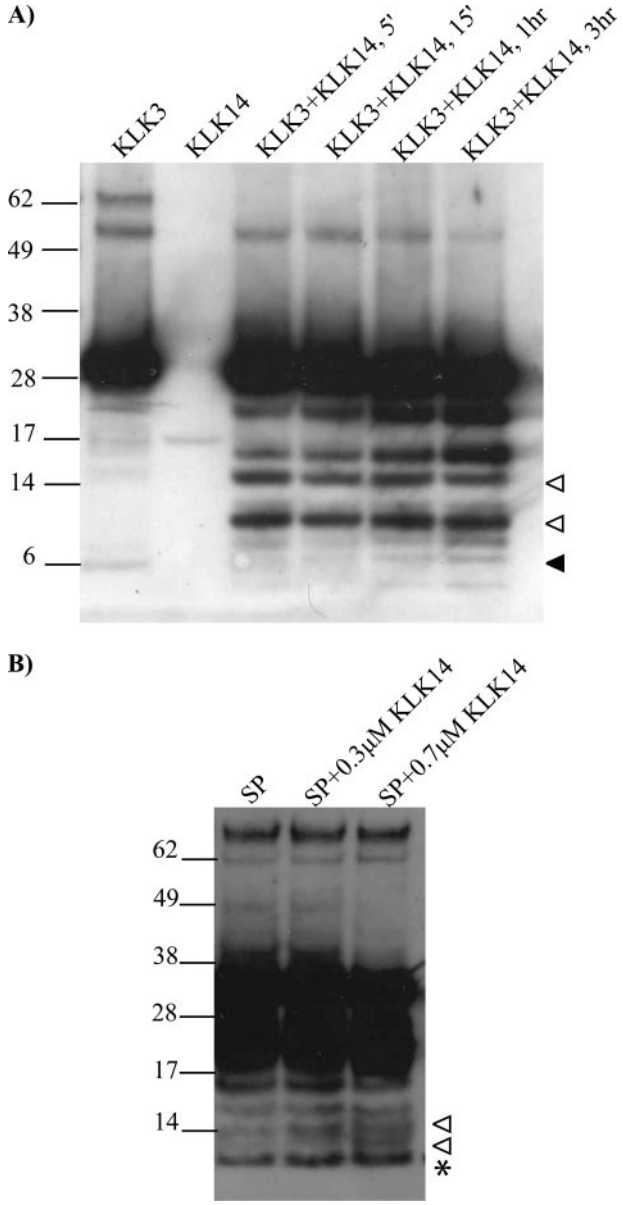

FIGURE 6. **KLK14-mediated internal cleavage of recombinant KLK3 (*A*) and seminal KLK3 *ex vivo* (*B*).** *A*, recombinant KLK3. Pro-KLK3 was incubated with KLK14 at a 1:10 molar ratio for varying time intervals, at 37 °C. *B*, seminal KLK3 *ex vivo*. 2 μl of seminal plasma, containing ~22 nM total KLK3, were diluted 15 times in PBS and treated with either 0.3 μM (1:70 molar ratio) or 0.7 μM (1:30 molar ratio) of KLK14. Reaction mixtures were incubated for 1 h at room temperature. KLK3 fragments were immunodetected, using a rabbit polyclonal KLK3 antibody (1:1000). The *filled arrowhead* represents fragments generated from the recombinant KLK3 but not detected in *B*. The *open arrowheads* illustrate common fragments to the recombinant and seminal KLK3. The *asterisk* shows a dose-dependent increase in the intensity of one of the KLK3 fragments in seminal plasma. *SP*, seminal plasma.

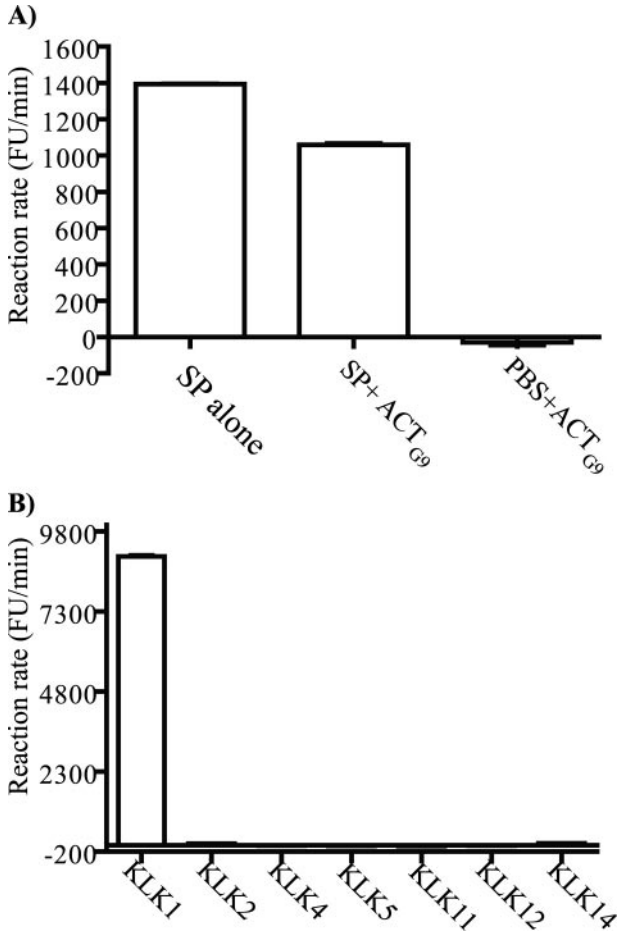

FIGURE 7. **KLK14-mediated activation of seminal KLK1.** *A*, *ex vivo* activation. Split ejaculates were treated with 0.8 μM ACT_{G9} inhibitor or incubated alone. Samples were incubated at 37 °C for 10 min. KLK1 was pulled down in 96-microtiter plates, coated with anti-KLK1 antibody, as follows. 200 ng of anti-KLK1 antibody were immobilized overnight on a microtiter plate. 100 μl of each of the treated and untreated samples were loaded to each well in triplicates and incubated at room temperature for 2 h. Activity of the pulled-down KLK1 was monitored by cleavage of 0.5 mM of the PFR-AMC substrate. *B*, specificity of the KLK1 sandwich pull-down assay. Recombinant active KLK1, 2, 4, 5, 11, 12, and 14 were loaded on a KLK1 antibody-coated microtiter plate, at their physiologic level listed in supplemental Table 1. Enzymatic activity of pulled-down enzymes were measured as described in *A*. The reaction rate of all of the KLKs except KLK1 was almost zero.

including KLK2, -3, -4, -5, and -12, were not inhibited by this protein (40).[3]

Although KLK14 inhibition considerably delayed semen liquefaction, it did not completely block the process. This suggests functional redundancy in activator components of the seminal proteolytic cascade, compensating for KLK14 function. The physiological relevance of other candidate activators of the cascade, such as KLK5 and KLK2, needs to be further investigated.

As mentioned previously, Sgs are the main effector components of the semen liquefaction cascade. Our *in vitro* data suggest that KLK14 cleaves Sg proteins with high efficiency. In addition, our previous studies have implicated KLK14 in the processing of fibronectin, another key component of the semen coagulum (22). Furthermore, Sg proteins play an instrumental role in seminal clot liquefaction through sequestration of Zn^{2+} from active executors, thus modulating their proteolytic activity (29). Such a reversal effect of Sg has been shown for several members of the KLK family, including KLK3 and KLK5 (28, 31). Our results suggest a similar regulatory mechanism for KLK14 in seminal plasma, at the physiologically relevant molar ratio of 10-fold excess Zn^{2+} (28) to SgII protein.

Moreover, we previously demonstrated that KLK14 is able to regulate pro-KLK3 *in vitro*. At the astounding expression level of 10 mg/ml, KLK3 is the most abundant chymotrypsin-like enzyme in seminal plasma (41, 52). However, the majority of

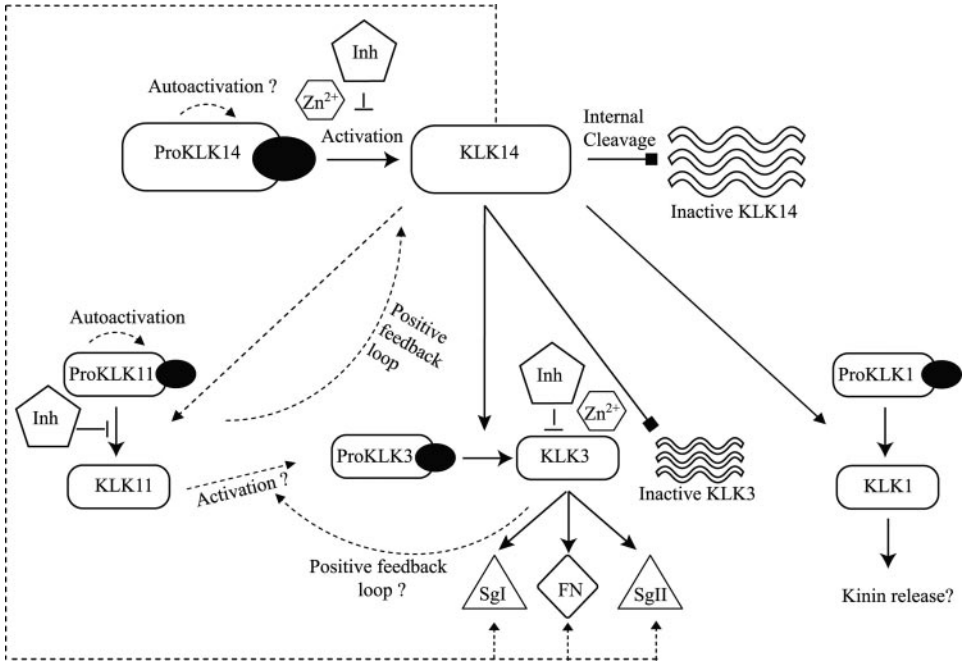

FIGURE 8. **Schematic presentation of proposed KLK cascade in seminal plasma.** KLK14 activates pro-KLK3 as well as pro-KLK11 and KLK1 (38). Activated KLK3 acts as the main executor protease in the liquefaction of semen coagulum through proteolytic fragmentation of SgI/SgII and fibronectin (*FN*). Activated KLK11 may also activate pro-KLK3 (50), functioning at the propagation level. Moreover, active KLK14 can directly cleave gel-like proteins (*i.e.* SgI/SgII and fibronectin). Signal amplification is achieved mainly through positive feedback loops. The cascade is regulated by a number of endogenous inhibitors (*Inh*) as well as Zn^{2+} and internal cleavage of active KLKs. The *solid lines* specify interactions that were confirmed *ex vivo* in this study. The *dotted lines* represent those that have been shown *in vitro*, using full-length recombinant proteins (38, 50). The *question marks* indicate possible interactions suggested *in vitro*, using fusion recombinant proteins that contain only the active motifs of each KLK (50).

active KLK3 is complexed with seminal inhibitors, such as protein C inhibitor and α_2-macroglobulin (27, 53, 54), rendering it inactive. Although a number of chromatographic and immunologic approaches have previously been proposed to measure active KLK3 (55–57), their low recovery rate limits their use as a sensitive comparative means in complex biological samples. Due to this technical limitation and given the substrate preference of KLK3 for the RPY tripeptide substrate, we examined the "chymotrypsin-like" activity of seminal plasma as a measure of KLK3 activity. To ensure that the majority of observed enzymatic activity against this substrate is due to KLK3 activity, we compared samples depleted from KLK3 with mock controls. As expected, upon 95% depletion of KLK3, almost no enzymatic activity was observed. Eluted proteins were identified as KLK3 or KLK3 fragments, excluding the possibility of simultaneous depletion of other chymotrypsin-like enzymes.

KLK14-mediated regulation of KLK3 activity seems to be bidirectional, since we observed a reversal in the correlation pattern between KLK14 and the "chymotrypsin-like" activity following longer incubation. Given the importance of chymotryptic proteolysis of Sg proteins during semen liquefaction, activation of KLK3 is most likely triggered within seconds postejaculation and continues until complete fragmentation of gel-like proteins. Aberrant proteolysis due to prolonged protease activity is prevented by subsequent inactivation of executor chymotryptic enzyme(s). This finding is in agreement with our *in vitro* observation of sequential activation and deactivation of pro-KLK3 by KLK14 (37). We have previously found that deactivation is achieved mainly through internal cleavage of active KLK3 (37). Here we have shown that exogenous KLK14 could fragment KLK3 *ex vivo* in a dose-dependent manner, with a pattern similar to the one observed *in vitro*.

Similarly, consistent with our previous *in vitro* data, KLK14 seems to activate seminal KLK1. Likewise, KLK2 has recently been identified as another putative activator of pro-KLK1 (50), reinforcing the link between KLK1 and the seminal KLK cascade. Although not fully understood, KLK1 has clinically been shown to enhance sperm motility in asthenospermic patients (58, 59). As mentioned previously, semen liquefaction is one of the main postejaculatory determinants of sperm motility. Whether KLK1 functions through regulating coagulation/liquefaction of semen needs to be further explored.

In summary, the present study provides strong evidence for the crucial cascade-mediated function of KLK14 in regulating the coagulation and liquefaction of human semen (Fig. 8). Cascade activation is more likely triggered at the time of semen ejaculation, as a result of mixing of different components of seminal plasma and subsequent redistribution of Zn^{2+} to Sg proteins. It is conceivable that additional members of the KLK family and/or other proteases participate in this proteolytic cascade. In addition, the complex interplay between proteases and their regulatory checkpoints needs to be further elucidated.

Understanding KLK-mediated proteolytic events in seminal plasma can shed light not only on the physiological role of this important family of enzymes but also on some of the causes of abnormal sperm motility. Accordingly, therapeutic induction of the seminal proteolytic cascade can be utilized to supplement the current clinical treatment of male subfertility. Conversely, targeted inhibition of key components of the cascade may have potential pharmaceutical utility as a novel topical contraceptive strategy.

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