Substrates of the Prostate-Specific Serine Protease Prostase/KLK4 Defined by Positional-Scanning Peptide Libraries

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BACKGROUND. Prostase/KLK4 is a member of the human kallikrein (KLK) gene family that is expressed in prostate epithelial cells under the regulation of androgenic hormones. In this study, we sought to characterize the substrate specificity of KLK4 in order to gain insight into potential physiological roles of the enzyme.
METHODS. A chimeric form of KLK4 was constructed in which the pro-region of KLK4 was replaced with the signal and propeptide sequence of trypsinogen (proT-KLK4) to create an activation site susceptible to enterokinase cleavage. proT-KLK4 was expressed in *Drosophila* S2 cells, purified, and activated with enterokinase to generate mature KLK4. The extended substrate specificity of KLK4 was defined by screening tetrapeptide positional scanning synthetic combinatorial libraries (PS-SCL).
RESULTS. The preferred P1-P4 positions as determined by PS-SCL were: P1-Arg; P2-Gln/Leu/Val; P3-Gln/Ser/Val; P4-Ile/Val. The trypsin-like specificity of KLK4 was further confirmed using synthetic chromogenic peptides. Based upon the optimal cleavage site residues, a database search for potential KLK4 substrates identified several proteins with potential roles mediating normal prostate physiology or neoplastic growth including KLK3/PSA, parathyroid hormone-related peptide (PTHrP), and members of the bone morphogenetic protein (BMP) family. Recombinant KLK4 was able to activate pro-PSA/KLK3 and degrade members of the insulin-like growth factor (IGF) binding protein (IGFBP) family.
CONCLUSIONS. These results identify potential KLK4 substrates that may serve to define the role of this protease in normal prostate physiology, and facilitate studies of the consequences of KLK4 expression in pathological conditions. *Prostate 62: 1–13, 2005.* © 2004 Wiley-Liss, Inc.

KEY WORDS: **prostate; kallikrein; PSA; IGFBP; serine protease**

INTRODUCTION

Serine proteases regulate important physiological processes involving digestion (chymotrypsin), tissue remodeling (urokinase), blood coagulation (thrombin), fertility (acrosin), inflammatory responses (elastase), and programmed cell death (granzymes). Serine proteases also appear to play important roles in the processes of neoplastic growth and metastasis (uPA) [1].

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Studies of normal and neoplastic prostate tissues have identified several serine proteases with expression profiles highly restricted to prostate epithelium [2–4]. Moreover, several of these prostate proteases including prostate specific antigen (PSA/KLK3), human glandular kallikrein 2 (KLK2), TMPRSS2, and prostase/KLK4 (aliases: PRSS17/EMSP1) are also regulated by androgenic hormones.

Prostase/KLK4 (hereafter designated KLK4) was originally cloned using a subtractive hybridization approach designed to identify transcripts specifically expressed in human prostate tissues [3]. The KLK4 gene is located on chromosome 19q13 and encodes a 254 amino acid polypeptide that contains a canonical serine protease catalytic triad, and shares 35% amino acid identity with the PSA protein. The human KLK4 cDNA also shares 84% nucleotide and 72% amino acid identity with a previously characterized porcine protease named enamel matrix serine protease 1 (EMSP1) that was identified in early stages of mammalian tooth formation [5]. In developing enamel, EMSP1 is expressed during the early maturation stage and is believed to degrade the extracellular matrix surrounding enamel crystallites, a function which allows the enamel layer to fully mineralize [6]. In the human prostate, KLK4 expression is restricted to the luminal and basal epithelium of normal glands, and transcripts encoding KLK4 are highly expressed in both primary and metastatic prostate adenocarcinomas [3]. Studies of KLK4 expression in other malignancies demonstrate KLK4 expression in cell lines derived from breast [7] and endometrial neoplasms [8]. KLK4 is overexpressed in serous epithelial-derived ovarian carcinomas [9], a finding that has been shown to indicate a poor clinical prognosis [10].

Proteases may participate in multiple processes leading to invasive and metastatic cancer growth [11,12]. The enzymatic activities of proteases, when expressed in ectopic locations such as bone, may alter the local environment to favor tumor cell proliferation, tumor cell invasion, angiogenesis, and bone remodeling. Secreted proteases may process and activate growth factors present in the extracellular space. PSA/KLK3 has been shown to activate parathyroid hormone-related peptide (PTHrP), a growth factor that increases the rate of prostate tumor growth in vivo [13]. PSA/KLK3 and KLK2 can degrade components of the extracellular matrix such as fibronectin, a process that facilitates cell invasion and migration [14]. KLK2 also activates urokinase plasminogen activator (uPA), a protease that has been strongly associated with prostate cancer invasion and metastasis [1,15]. Based upon these findings that support a role for serine proteases in carcinogenesis, we sought to characterize the KLK4 protease in terms of substrate specificity, activity, and inhibition. Thus, the aims of this study were to express active KLK4 enzyme and to determine potential physiological substrates in order to gain insights as to the specific mechanism(s) whereby KLK4 may contribute to neoplastic growth.

MATERIALS AND METHODS

General Methods and Reagents

DNA manipulations, such as plasmid isolation, plasmid purification, *E. coli* transformation, agarose gel electrophoresis, and SDS–PAGE, were performed according to standard procedures [16]. Restriction and modification enzymes were purchased from Promega Corporation (Madison, WI), Stratagene (La Jolla, CA), and Life Technologies (Rockville, MD) and used according to the manufacturer's protocols. The pRm-Ha3 and pcophyg plasmids were generous gifts from Dr. Lawrence S.B. Goldstein at the University of California, San Diego, CA and Gerald M. Rubin at the University of California, Berkely, CA, respectively.

Expression of Active Recombinant KLK4

To express a pro-form of KLK4 that could be activated using a known protease (enterokinase), a chimeric DNA sequence comprised the trypsinogen signal and propeptide sequences joined in frame with the sequence encoding pro-KLK4 (proT-KLK4) was synthesized. The full-length KLK4 cDNA previously cloned in our laboratory [3] was used as a template for 20 cycles of PCR amplification using primers PRU2: 5′-TTTGTGGCAGCTGCTCTTGCTGCCCCCTTTGATGATGATGACAAGATCATAAACGGCGAGGAC-3′ and PRD2: 5′-ATCTTTATAATCACCGTCATGGTCTTTGTAGTCGCGGCCGCCACTGGCCTGGACGGTTTT-3′, that comprised the trypsinogen signal and propeptide sequence. Five microliters of the first-round reaction product was used as template for a second round of PCR amplification using primers PRU1: 5′-ATGATGGAGCTCTTACCACCATGAATCCACTCCTGATCCTTACCTTTGTGGCAGCTGCTCTT-3′ and PRD1: 5′-CATCATGTCGACTTAATCCTTGTAGTCGATGTCATGATCTTTATAATCACCGTC-3′. After 25 cycles of amplification, the reaction product was digested with Sac I and Sal I endonucleases. The expected 850-bp PCR fragment was separated on a 1% agarose gel, purified using glass beads (Bio-101, Vista, CA) and ligated into the Sac I and Sal I site of the *Drosophila* expression plasmid pRmHa-3 [17]. Following transformation of *E. coli* DH5α cells, plasmid DNA was isolated and the construct orientation and accuracy were verified by DNA sequencing.

Drosophila melanogaster S2 cells were transfected with 10 μg of the chimeric pHmRa-3-proT-KLK4 plasmid

and 1 μg of pcophyg in serum-free insect cell medium (HyQ SFX-Insect, HyClone, Logan, UT) using a calcium phosphate-DNA coprecipitation method [18]. The pcophyg plasmid confers hygromycin resistance to S2 cells [19]. The pRmHa-3 vector expresses cloned genes under the control of a metallothionein promoter [17]. After 48 hr, the cells were transferred into serum-free medium containing hygromycin B at a final concentration of 500 μg/ml and propagated for 4 weeks to produce stable cell lines.

S2 cells containing pRMHa-3-proT-KLK4 were used to seed 400 ml of serum-free medium in a 2-L spinner flask. Protein expression was induced by the addition of 0.75 mM $CuSO_4$ at a cell density of 7×10^6 cells/ml. After 3 days of induction, the cells were removed by centrifugation. The 400-ml culture supernatant was concentrated to 50 ml by ultrafiltration (10 kMW-cut-off, Amicon) and passed through a 0.45-μm filter to remove particulates. The filtered supernatant was mixed with 1 ml of anti-FLAG M2 affinity gel (Sigma, St. Louis, MO), and the gel was gently rocked at 4°C overnight to capture the FLAG-tagged proT-KLK4. The gel was then transferred to a column and washed with 20 mM Tris, pH 7.4 plus 150 mM NaCl, and the proT-KLK4 was eluted with 100 mM Glycine, pH 3.5, as recommended by the manufacturer. Immediately after the elution of proT-KLK4 from the column, the pH was neutralized by adding 1/10 volume of 1 M Tris-HCl, pH 8.0. Protein-containing fractions were identified by Bradford assay [20] (Bio-Rad Laboratories, Hercules, CA). The pooled fractions were dialyzed against 20 mM Tris-HCl, pH 7.4 plus 50 mM NaCl at 4°C overnight. About 600 μg of proT-KLK4 was purified to homogeneity from a 500-ml insect cell culture.

In order to remove the trypsinogen propeptide and activate KLK4, about 10 μg of proT-KLK4 was incubated with 0.5 U of recombinant enterokinase (rEK) (Novagen, Madison, WI) in 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM $CaCl_2$ at room temperature for 16 hr. The activated enzyme was dialyzed against 20 mM sodium phosphate buffer, pH 7.4, and purified by FPLC on a Mono-Q column (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). The enzyme was eluted with a linear gradient of 0–0.7-M NaCl and active fractions were determined by enzymatic activity assays, collected, and dialyzed against PBS.

Immunoblot Analysis

SF2 cells containing pRMH-3-proT-KLK4 were induced with $CuSO_4$, pelleted by centrifugation, and lysed with SDS-sample buffer (2% (w/v) SDS, 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 25 mM DTT, and 0.05% (w/v) bromophenol blue), followed by heating at 95°C for 10 min. A portion of the spent medium was mixed with an equal volume of 2× SDS sample buffer, followed by heating at 95°C for 10 min. Both samples were separated by SDS–PAGE, and the proteins were blotted to a nitrocellulose membrane using a semi-dry transfer apparatus according to the manufacturer's protocol (Bio-Rad Laboratories). KLK4 protein was detected using 1 μg/ml of anti-FLAG M2 monoclonal antibody (Sigma) and 5 ng/ml of goat anti-mouse IgG-conjugated with horseradish peroxidase (Pierce). The signal was detected with chemiluminescent substrates (Supersignal West Pico Chemiluminescent Substrates, Pierce).

Generation of Anti-KLK4 Antibody B6

The DNA sequence encoding mature KLK4 (from residues 31 to 254) [3] was fused to the C-terminal sequence of glutathione S-transferase (GST) contained in the pGEX vector (Amersham Pharmacia Biotech, Inc.) and used to transform *E. coli* DH5α cells. Fusion protein expression was induced by the addition of 1 mM IPTG at OD = 660 nm of 1.0, and the cells were incubated for another 4 hr. The cells were harvested by centrifugation, suspended in 10 mM Tris-HCl, pH 8 and 1 mM EDTA, and disrupted by ultrasonication in ice water. The pelleted cell lysates washed at least three times with a buffer containing 0.2M NaCl, 1% deoxychlolic acid, 1% NP-40, 20 mM Tris-HCl (pH 7.7), and 2 mM EDTA, followed by further washes with 0.5% Triton X-100 and 1 mM EDTA until the insoluble protein fraction remained. The purified KLK4-GST fusion polypeptide was dissolved in 8-M urea at a protein concentration of 10 mg/ml. Tenfold dilutions of the protein were used for the immunization of mice according to institutional protocols approved by the Fred Hutchinson Cancer Research Center IRB. ELISAs were performed using the GST-KLK4 fusion protein as a positive control and GST alone as a negative control. Hybridomas were further screened against COS-7 cells expressing KLK4 protein as positive controls and COS-7 cells alone as negative controls. Subsequent studies identified one hybridoma, B6, that recognized only S2 cell lysates containing chimeric KLK4, but not native S2 cells.

Determination of KLK4 Substrate Specificity Using Positional Scanning-Synthetic Combinatorial Libraries (PS-SCL)

The substrate specificity of KLK4 was determined using two tetrapeptide positional scanning-synthetic combinatorial libraries. A detailed description of the preparation and use of these libraries was reported elsewhere [21]. The concentration of catalytically active KLK4 was determined by active-site titration with the

pseudo-suicide inhibitor 4-methylumbelliferyl *p*-guanidinobenzoate (MUGB) using a Fluoromax-2-spectrofluorimeter at 25°C [22]. The P1 specificity of KLK4 was determined using a P1-diverse library in which the P1 position was systematically held constant with one of the 20 canonical amino acids, while the P2-P4 positions were randomized (cysteine was omitted and norleucine is included). The P2-P4 specificity was determined using a P1-Arg fixed library in which the P2, P3, or P4 position was systematically held constant while the other two positions were randomized (cysteine was omitted and norleucine was substituted for methionine). The final substrate concentration was 7.3 nM of each compound per well. Proteolysis reactions were initiated by the addition of 100 μl of enzyme (final enzyme concentration of 10 nM in 50 mM Tris, pH 8.8, 50 mM NaCl, 1% DMSO (from substrates), 0.01% Tween 20). All library assays were performed in 96-well Microfluor-1 Black ''U'' bottom plates (Dynex Technologies, Chantilly, VA) on a Molecular Devices SpectraMax Gemini spectrophotometer with excitation at 380 nM and emission at 460 nM (37°C for 10 min).

Analysis of KLK4 Enzyme Activity

Chromogenic peptides were dissolved in 50-mM sodium borate buffer, pH 8.5, at the final concentration of 125 mM. One hundred microliters of substrate were mixed with 0.5 μg of protease at the final concentration of 10 μg/ml in a micro-spectrophotometer cuvette. The rate of pNA release from chromogenic peptides was monitored at 405 nm at room temperature. The specific activity was calculated using the protein concentration determined by Bradford assays [20]. Chromogenic peptide substrates for KLK4 were purchased from Sigma (St. Louis, MO): N-Acetyl-Tyr-Val-Ala-Asp-*p*-nitroanilide (A-3831), N-Benzoyl-Pro-Phe-Arg-*p*-nitroanilide-HCl (B-2133), N-Benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide-HCl (B-2291), N-Benzoyl-Val-Gly-Arg-*p*-nitroanilide-HCl (B-4758), N-Benzoyl-Phe-Val-Arg-*p*-nitroanilide-HCl (B-7632), D-Leu-Ser-Thr-Arg-^{TM}-nitroanilide (L-1391), N-Methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (M-4765), N-Methoxysuccinyl-Ala-Ala-Pro-Met-*p*-nitroanilide (M-7771), D-Pro-Phe-Arg-*p*-nitroanilide-2HCl (P7959), N-*p*-Tosyl-Gly-Pro-Arg-*p*-nitroanilide-acetate-salt (T-1637), N-*p*-Tosyl-Gly-Pro-Lys-*p*-nitroanilide-acetate-salt (T-6140), D-Val-Leu-Lys-*p*-Nitroanilide-2HCl (V-0882), and D-Val-Leu-Arg-*p*-nitroanilide (V-6258). The substrate peptide for PSA, 3-carbomethoxypropionyl-Arg-Pro-Tyr-*p*-nitroaniline-HCl (S-2586) was purchased from Chromogenix (Franklin, OH).

Kinetic studies of KLK4 activity were performed against the peptide substrate B-FVR-pNA. Rates of hydrolysis of varied concentrations (0–300 μM) of B-FVR-pNA were determined at constant enzyme concentration (5 nM) in 50 mM Tris, pH 8.8, 50 mM NaCl, 1% DMSO (from substrates), 0.01% Tween 20, at 23°C. Enzyme activity was monitored at 405 nm in a 96-well plate format using the Molecular Devices UVmax Kinetic Microplate reader. K_m and k_{cat} were determined using KALEIDAGRAPH software (Synergy Software, Reading, PA).

Inhibition of KLK4 Activity

Trypsin and activated KLK4 (10 μg/ml) were incubated individually with PMSF (phenylmethylsulfonyl fluoride) (2 mM), alpha-2-macroglobulin (10 U/ml), aprotinin (40 μg/ml), leupeptin (40 μg/ml), TLCK (N-α-Tosyl-L-lysine chloromethyl ketone) (1 mM), or trypsin inhibitor (100 μg/ml) (all from Roche Molecular Biochemicals, Indianapolis, IN) at room temperature in 50 mM sodium borate buffer, pH 8.5, in a total volume of 50 μl. Residual KLK4 activity was measured by mixing the reaction with an equal volume (50 μl) of 250 mM B-FVR-pNA in 50 mM sodium borate buffer, pH 8.5. The rate of hydrolysis (release of pNA) was monitored at 405 nm as described above.

Activation of Pro-PSA by KLK4

Five micrograms of pro-PSA [23] (generously provided by Dr. Stephan D. Mikolajczyk, Beckman Coulter, San Diego, CA) were mixed with 0.5 μg of activated, purified KLK4 in 20 μl of 12.5 mM sodium borate buffer, pH 8.5 at room temperature for 20 min. One hundred microliters of 1 mM 3-carbomethoxypropionyl-Arg-Pro-Tyr-pNA (S2586), a synthetic peptide substrate for PSA, were added to the enzyme mixture, and the PSA activity was monitored at 405 nm. Control reactions were performed with the S2586 substrate combined with mature active PSA alone, pro-PSA alone or active KLK4 alone.

Degradation of Insulin-Like Growth Factor (IGF)-Binding Proteins by KLK4

Recombinant human insulin-like growth factor binding protein-3 (IGFBP-3), IGFBP-4, IGFBP-5, and IGFBP-6 were iodinated with ^{125}I-Na (Amersham) and chloramine-T as previously described [24]. KLK4 degradation of ^{125}I-IGFBPs was determined by incubating 0.05 μg of human recombinant KLK4 with 30,000–60,000 CPM of labeled IGFBP in a 10 μl volume of PBS at 37°C for 1, 2, 10, and 30 min. Controls included equivalently labeled amounts of ^{125}I-IGFBP without KLK4. The reactions were terminated by adding SDS sample buffer and boiling for 5 min. The samples were fractionated by SDS–PAGE and the dried gel was autoradiographed. Densitometry of

the intact ^{125}I-IGFBPs was determined for each time point and quantified as a percentage of the control ^{125}I-IGFBP.

RESULTS

Expression and Purification of Active KLK4 Protein

The KLK4 cDNA encodes a preproprotein of 254 amino acids [3]. The predicted KLK4 pro-peptide sequence (SCSQ) ends with Gln, a sequence not predicted to undergo proteolysis by known trypsin- or chymotrypsin-like proteases. In order to express active KLK4 in vitro, we assembled a chimeric KLK4 construct comprising the signal sequence of the trypsinogen gene [25], the sequence encoding the mature prostate protein, and a 3× FLAG tag at the C-terminus (Fig. 1A). The trypsinogen signal peptide is cleaved between Ala-15 and Ala-16, leaving the APFDDDDK activation peptide (SWISS-PROT: P07477, TRY1_HUMAN) [26] which is subsequently available for enterokinase-mediated cleavage resulting in pro-enzyme activation [27].

Drosophila melanogaster S2 cells were co-transfected with pHmRa-3 plasmid carrying the chimeric KLK4 gene and the pcophyg hygromycin resistance plasmid. After 4 weeks of culture under drug selection, stable cell lines were obtained. The expression of KLK4 was confirmed by immunoblots of cell lysates and culture supernatants with the M2 anti-FLAG antibody (Fig. 1B). The observed molecular weight of 35 kDa is slightly larger than the theoretical molecular weight of 27 kDa deduced from the predicted polypeptide sequence, a finding that may reflect glycosylation at residue Gln-169. The KLK4 protease secreted into the cell culture medium was purified to homogeneity by M2-antibody affinity chromatography. The purified pro-enzyme was activated by enterokinase (enteropeptidase). Because the experiments designed to identify KLK4 substrates could be misinterpreted as a result of residual enterokinase contamination, we initially attempted to isolate active KLK4 by anti-FLAG affinity chromatography. However, enterokinase-treated, activated KLK4 was unable to bind to the anti-FLAG affinity column. Immunoblot assays of enterokinase-treated KLK4 demonstrated reactivity with the anti-KLK4 B6 monoclonal antibody but not the anti-FLAG M2 antibody; strongly implying that the chimeric KLK4-FLAG lost the C-terminal 3× FLAG sequence during or after enterokinase treatment. We hypothesize that activated KLK4 cleaved its own 3× FLAG tag at the Arg-250 position (Fig. 1C). Subsequent experiments confirmed that Arg is a preferred P1 amino acid for KLK4 proteolytic activity. The loss of the FLAG peptide

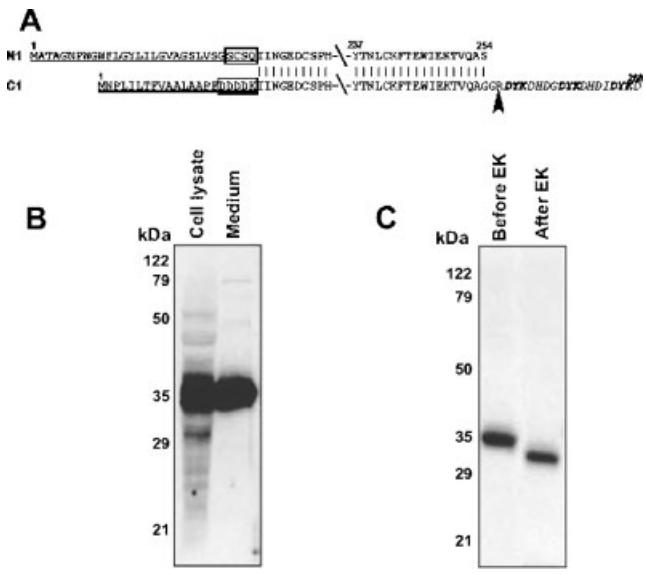

Fig. 1. Construct for expression and purification of recombinant active KLK4. **A**: Amino acid sequence of native (NI) and chimeric (CI) KLK4. The upper sequence represents the native prepro-KLK4 which includes signal (underline) and propeptide (box) sequences. The lower sequence represents the chimeric KLK4 polypeptide consisting of trypsinogen signal (underline) and propeptide (box) sequences joined with the putative mature KLK4 polypeptide. The enterokinase [46] recognition sequence is DDDDK. EK cleaves the peptide bond between the lysine (K) residue of the propeptide and isoleucine (I), the first amino acid of the mature KLK4. The C-terminal 3× FLAG tag (italic) encodes a DYK epitope sequence (bold) recognized by the M2 FLAG antibody. The amino acid residues, GGR, between the mature enzyme and 3× FLAG tag result from a *Not*I restriction site introduced for cloning. The R residue (arrowhead) is predicted to undergo proteolysis by active KLK4. **B**: Western blot analysis of the chimeric KLK4 expressed in *Drosophila* cells. The KLK4 was detected with anti-FLAG M2 antibody. The left represents KLK4 in the whole cell lysate. The right represents KLK4 secreted into the culture medium. **C**: SDS–PAGE of affinity-purified KLK4 before (**left**) and after (**right**) enterokinase treatment. The proteins were stained with Coomassie Brilliant Blue.

was also indicated by a 3 kDa molecular weight shift of activated KLK4 in SDS–PAGE, a finding that is not entirely explained by the loss of the 8 amino acid propeptide, APFDDDK (data not shown). Anti-KLK4 monoclonal antibody chromatography successfully separated the activated enzyme from residual pro-KLK4 and enterokinase. We found that the majority of enzyme (~90%) was present as the active form and about 10% remained as uncleaved pro-KLK4 (data not shown). The fractions containing the active enzyme were pooled, concentrated, and dialyzed against PBS. The enzyme thus purified was found be nearly 100% active, as determined by active site titration experiments (see below).

Determination of KLK4 Substrate Specificity by PS-SCL Screening

In order to rapidly determine the primary and extended substrate specificity of KLK4, we used an approach termed PS-SCL screening [21,28]. Two tetrapeptide combinatorial libraries were screened with recombinant active KLK4. The P1 protease specificity was determined using a P1-diverse library in which the P1 position was systematically held constant with each one of the 20 canonical amino acids (excluding cysteine and including norleucine), while the P2-P4 positions were randomized. KLK4 demonstrated a clear specificity for Arg and Lys at the P1 position (Fig. 2). The P2-P4 specificity was determined using a P1-Arg fixed library in which the P2, P3, or P4 positions were systematically held constant while the other two positions were randomized (including norleucine and excluding cysteine and methionine). The final substrate concentration was 7.3 nM of each compound per well. At the P2 site, the preference is for Gln > Leu, Val > Thr, Pro > Phe, Ile, norLeu. While none of the extended sites showed specificity as clearly defined as the P1 site, trends did emerge. KLK4 favored the amide (polar, uncharged) Gln strongly at both P2 and P3. This may be a true preference, but also could be an issue of proper register of the tetrapeptide within the substrate binding site of the enzyme. It is also possible that Gln may be required at either P2 or P3 but not both concurrently. At the P3 position, KLK4 favored Gln > Ser, Val > Ala, Phe, Thr, norLeu, Tyr. The overall trend seems to be for longer polar amino acids followed by aliphatic amino acids at both P2 and P3. In both cases (P2 and P3), the only clear trend exhibited was a preference for Gln. At P4, the specificities were rather broad but showed a trend for preference toward aliphatic and aromatic amino acids over polar/charged amino acids (Ile, Val > Tyr, Phe, Trp > norLeu, Leu). Acidic amino acids were never favored at any position. The overall trend in specificity is P1-Arg, P2-Gln/Leu,Val (aliphatic), P3-Gln/Ser/Val, P4-Ile/Val/Phe,Tyr,Trp (aromatic).

The predicted cleavage specificities KLK4 were confirmed using individual synthetic peptides by measuring the liberation of para-nitroanalide (monitored at OD = 405 nm) from these substrates. KLK4 exhibited a preference for Arg at the P1 position (Fig. 3A,B), and had essentially no activity to peptides with a hydrophobic amino acid at P1 (see AAPV and AAPM). As expected, pro-KLK4 did not manifest activity toward any peptide substrates tested. Enterokinase alone did not liberate para-nitroanilide from the peptides shown in Figure 3. In keeping with the PS-SCL results, hydrophobic residues, such as Val (see FVR), Leu (see VLR), and Phe (PFR), are preferred by KLK4 at the

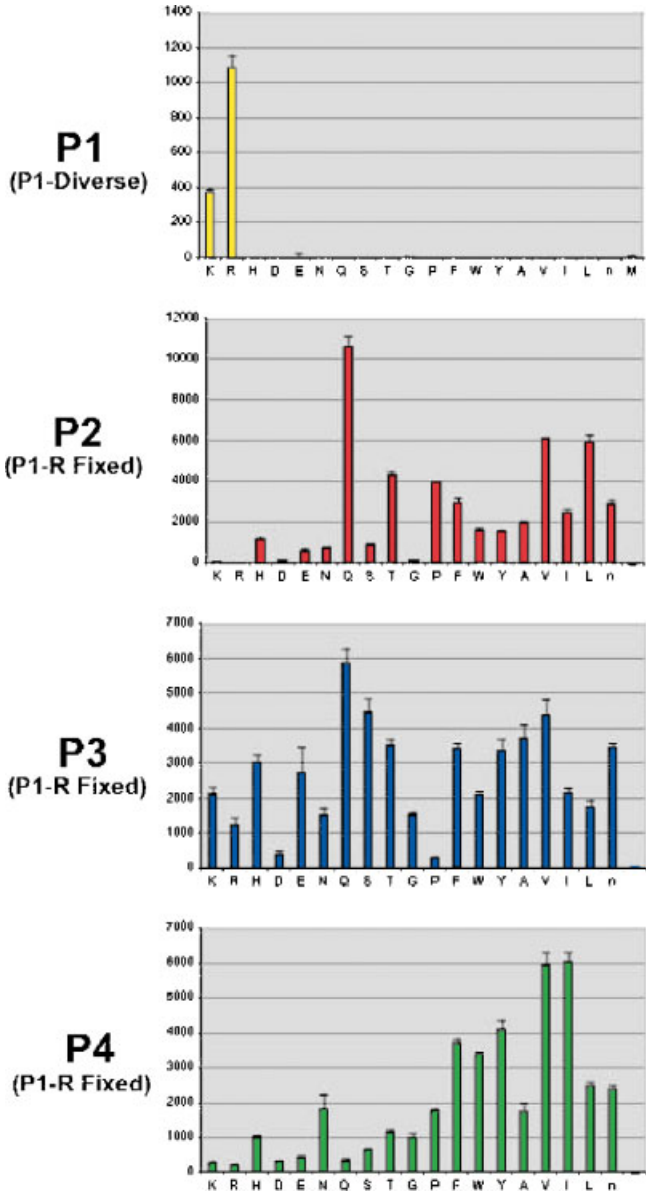

Fig. 2. Extended substrate specificity of KLK4. Results from the PI-diverse and PI-arginine-fixed positional scanning libraries are shown. The y-axis represents the rate of substrate cleavage in relative fluorescence units per second and the x-axis represents the amino acid in the PI, P2, P3, or P4 positions. For the PI-diverse library (**top**), the P2, P3, and P4 positions contain an equimolar mixture of 19 amino acids for a total of 6,859 substrates/well. In the PI-Arg-fixed libraries used for assessing P2, P3, and P4, the two positions that are not held constant contain an equimolar mixture of 19 amino acids for a total of 361 substrates/well. (For PI-diverse libraries, Cys is excluded and Nle (n) is included; for PI-fixed libraries, Met and Cys are excluded and Nle is included.) [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

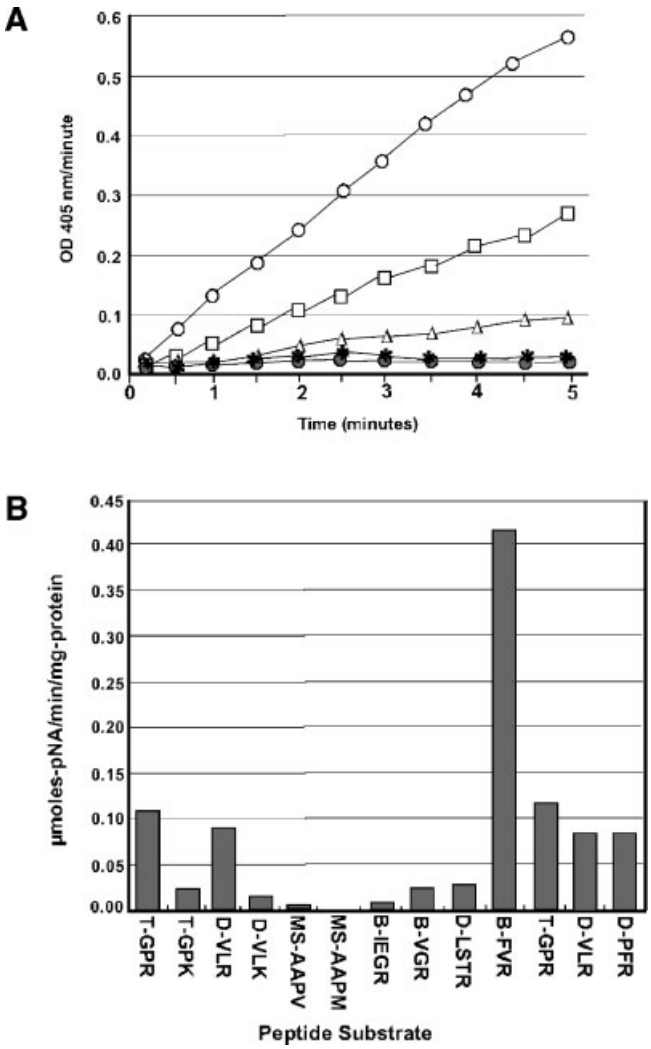

Fig. 3. Amidolytic activity of KLK4 against specific chromogenic substrates. **A**: Time course of substrate cleavage by activated KLK4, enterokinase, and pro-KLK4. Amidolytic activity of pro-KLK4 activated by preincubation with enterokinase against B-FVR-pNA (○), D-VLR-pNA (□), and B-VGR-pNA (△). Pro-KLK4 alone (asterisk) and enterokinase alone (●) were incubated with B-FVR-pNA as controls. The rate of pNA release from the chromogenic peptide was monitored at 405 nm. **B**: Substrate specificity of activated KLK4 to various chromogenic peptides. The y-axis represents the rate of peptide cleavage expressed as amount of pNA generated per mg of activated KLK4 per minute.

P2 position over peptides with Gly and Thr at the P2 position (see IEGR, VGR, and LSTR).

Kinetic Analysis of KLK4 Cleavage of the Peptide Substrate B-FVR-pNA

Active KLK4 exhibited the greatest activity toward the B-FVR-pNA peptide relative to all peptide substrates tested (Fig. 3B), and this peptide was used for further kinetic studies of KLK4 activity. Enzyme concentrations were determined by active-site titration with MUGB. KLK4 proteolytic activity was monitored by measuring pNA release (absorbance at 405 nm) from B-FVR-pNA upon hydrolysis of the scissile bond (between P1-Arg and the paranitroanilide group). The data were fitted to the Michaelis Menen equation to yield the final measurements of: $k_{cat} = 1.28 \pm 0.025\ sec^{-1}$, $K_m = 48.3 \pm 2.8\ \mu M$, $k_{cat}/K_m = 2.64 \times 10^4\ M^{-1}\ sec^{-1} \pm 0.11 \times 10^4\ M^{-1}\ sec^{-1}$.

Inhibition of KLK4 Activity by Serine Protease Inhibitors

Since KLK4 showed serine protease-like activity, we tested various inhibitors to evaluate their inhibitory activity toward KLK4. The recombinant active enzyme was incubated for 10 min with various protease inhibitors and the remaining enzyme activity was measured using B-FVR-pNA as a substrate. The serine protease trypsin was used as a positive control. As shown in Figure 4, all inhibitors tested except trypsin inhibitor exhibited very similar inhibitory effects toward KLK4 and trypsin. Trypsin inhibitor from chicken egg white inhibited trypsin more effectively than KLK4. These results suggest that the overall three-dimensional structure and active site configuration of KLK4 may be similar to trypsin.

Identification of Potential Physiological KLK4 Substrates

Using the substrate sequence specificity information obtained through PS-SCL and experiments with specific synthetic peptides, we developed a scoring system to identify potential physiological KLK4 substrates. Amino acids located in each position (P1-P4) were

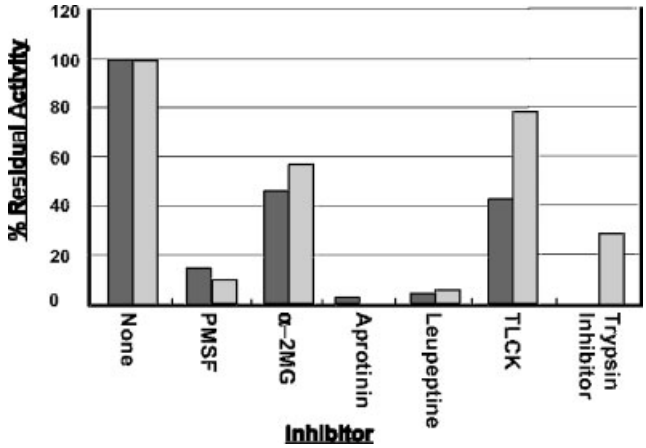

Fig. 4. Effect of protease inhibitors on KLK4 activity. KLK4 (dark shade) and trypsin (light shade) were incubated with the indicated protease inhibitors and the remaining enzymatic activity was measured using B-FVR-pNA as substrate.

given a numerical value reflecting their relative positional cleavage activity. Residue position scores were determined by dividing the fluorescence intensity of each individual amino acid at a specific position by the total fluorescence values of all amino acids at that position and multiplying this result by 100. The calculated values were rounded to the nearest whole number. The calculated score for amino acids at each position are: P1, K=26 and R = 74; at P2, H = 2, E = 1, N = 2, Q = 22, S = 2, T = 9, P = 8, F = 6, W = 3, Y = 3, A = 4, V = 13, I = 5, L = 12, M = 6, C = 2; at P3, K = 4, R=2, H = 6, D=1, E = 5, N = 3, Q = 11, S = 9, T = 7, G = 3, P = 1, F = 7, W = 4, Y = 7, A = 7, V = 9, I = 4, L = 3, M = 7, C = 9; at P4, K = 1, R = 1, H = 3, D = 1, E = 1, N = 5, Q = 1, S = 2, T = 3, G = 3, P = 5, F = 10, W = 9, Y = 11, A = 5, V = 15, I = 15, L = 6, M = 6, C = 2. Motif scores were determined by adding the individual amino acid scores for the P1-P4 positions, and used for searches of protein databases to identify potential KLK4 substrates. Due to high reactivity, the position scanning peptide libraries did not contain methionine or cysteine residues. For database comparisons, scores for the structurally similar norleucine and serine amino acids were substituted for methionine and cysteine, respectively.

Protein sequences from the Protein Information Resource-Protein Sequence Database (PIR-PSD; http://www-nbrf.georgetown.edu/pirwww/pirhome.shtml) were acquired. This database has about 260,000 protein entries derived from a wide variety of species. We created a sub-database comprised only human proteins (about 11,000 entries), and selected only those annotated as containing a pro-peptide or activation peptide sequence(s) (148 entries). Each protein was then given a score based upon the P1-P4 values predicted to be most susceptible to KLK4 activity, and the proteins were sorted in descending order (Table I). The P1 position was given a special status because of the strong bias shown by trypsin-like proteases for substrates with R or K at this location. Thus, only proteins that have a propeptide or activation peptide with R or K at P1 were considered as candidate proteins. Among the potential KLK4 substrates of interest in the context of prostate pathophysiology are precursor forms of PSA, PTHrP, and members of the bone morphogenetic protein (BMP) family.

Activation of Pro-PSA by KLK4

PSA is a protease expressed highly and almost exclusively in the prostate gland with important normal physiological roles in seminal fluid coagulation. The restricted expression of PSA and KLK4 to the prostate suggested that these proteases could participate in a protease activation cascade within seminal fluid. In order to determine if KLK4 is able to activate pro-PSA, recombinant pro-PSA was incubated with KLK4 and its activity was monitored using 3-carbomethoxypropionyl-Arg-Pro-Tyr-p-nitroaniline, a synthetic substrate for PSA (Fig. 5). Pro-PSA alone and active KLK4 alone were each unable to cleave the synthetic PSA substrate. Combining pro-PSA with KLK4 resulted in the liberation of pNA indicating that pro-KLK4 was converted to enzymatically active PSA. This result supports the prediction that KLK4 is able to cleave the ILSR PSA propeptide leading to enzyme activation.

Degradation of IGF-Binding Proteins by KLK4

In addition to mediating the activation of specific pro-enzymes, serine proteases may also be involved in the inactivation or degradation of proteins. Of those proteins of relevance for the development or progression of prostate carcinoma, IGF and the IGFBPs appear to play important roles [29]. In order to determine if KLK4 was capable of degrading IGFBPs, we incubated recombinant I-131-labeled IGFBP-3,-4,-5, and -6 with recombinant active KLK4 and measured their rates of degradation. All four IGFBPs were either partially or completely degraded by KLK4. IGFBP-5 was the most actively degraded with more than 80% of the starting material eliminated within 2 min of the incubation (Fig. 6). IGFBP-4 was nearly completely digested within 30 min. In contrast, IGFBP-3 and -6 showed slow and incomplete degradation during the same time interval. Incubation of active KLK4 with albumin did not result in any appreciable degradation over 60 min as assessed by SDS–PAGE [33].

DISCUSSION

In this study, we have expressed recombinant active KLK4 protease and determined the extended substrate specificity of this enzyme using positional scanning combinatorial tetrapeptide substrate libraries: P1-Arg, P2-Gln/Leu,Val (aliphatic), P3-Gln/Ser/Val, P4-Ile/Val/Phe,Tyr,Trp (aromatic). Although the physiological role of KLK4 has yet to be determined in vivo, the results from substrate screening experiments indicated that the PSA pro-enzyme would be a likely substrate for KLK4 activity in the prostate gland. In contrast to KLK4, the enzymatic substrate specificity of PSA has been extensively characterized, and the physiological function involves proteolysis of the seminal fluid protein semenogelin [30], a reaction that results in the liquefaction of seminal fluid leading to enhanced sperm motility. In this context, other prostate proteases are hypothesized to participate in a proteolytic cascade culminating in PSA cleavage and activation [31]; a process that would parallel the cascade of serine protease activation involved in blood coagulation and

TABLE I. Protein Database Entries With Predicted KLK4 Cleavage/Activation Sites

Accession number	Cleavage site	Score	Description
S65464	RQQR	108	Pregnancy-associated plasma protein A precursor
EKHUX	VVPR	106	Protein-glutamine gamma-glutamyltransferase plasma
PSHU	ISPR	106	Phospholipase A2 IB precursor
IYHU2	RYQR	104	Inter-alpha-trypsin inhibitor heavy chain 2 precursor
B26823	YVTR	103	Pancreatic elastase II A precursor
KQHU	IQSR	102	Tissue kallikrein precursor
BMHU7	SKQR	102	Bone morphogenetic protein 7 precursor
ICHU1A	IKPR	101	Interleukin-1 alpha precursor
RIHUS1	ELQR	100	Somatostatin I precursor
KXHU	VDPR	98	Protein C (activated) precursor
BMHU5	RSVR	97	Bone morphogenetic protein 5 precursor
ABHUS	VFRR	96	Serum albumin precursor
JC2466	IHRR	95	Inhibin beta-C chain precursor
HYHUMB	AQIR	95	Meprin A beta chain precursor
TBHU	FNPR	95	Thrombin precursor
A32297	ILSR	94	PSA/KLK3/semenogelase precursor
S68826	LSAR	93	Pancreatic elastase isoform 2 precursor
S68825	LSAR	93	Pancreatic elastase isoform 1 precursor
PTHU	VKKR	93	Parathyraid hormone precursor
C8HUB	RQMR	92	Complement C8 beta chain precursor
A44454	SQAR	91	Defensin alpha-5 precursor
EXHU	NLTR	91	Coagulation factor Xa precursor
A42332	SRVR	91	Carboxypeptidase B precursor, pancreatic
BMHU6	RTTR	91	Bone morphogenetic protein 6 precursor
KFHU	DFTR	91	Coagulation factor IXa precursor
B29934	PSSR	90	Pancreatic elastase IIIB precursor
S29127	FRSR	88	Carboxypeptidase A CPA1 precursor
JC2476	WEGR	88	Cathepsin K precursor
A29934	SSSR	87	Pancreatic elastase IIIA precursor
OYHUCR	RQER	87	Natriuretic peptide receptor C precursor
IJHUCE	RQKR	86	Cadherin 1 precursor
A39967	ATGR	86	Inter-alpha-trypsin inhibitor heavy chain 1 precursor
B38992	RQKR	86	Cadherin 13 precursor
IJHUCN	RQKR	86	Cadherin 2 precursor
REHUK	PMKR	86	Renin precursor
A59090	RLPR	86	Aspartic proteinase BACE precursor
IJHUC5	RQKR	86	Cadherin 5 precursor
IJHUG1	RQKR	86	Desmoglein 1 precursor
IJHUG3	RQKR	86	Desmoglein 3 precursor
S69207	LNSR	85	Vascular endothelial growth factor C precursor
A40304	RVRR	84	Brain-derived neurotrophic factor precursor
A42687	RSRR	84	Neurotrophin-4 precursor
C8HUA	RVRR	84	Complement C8 alpha chain precursor
C26823	DMSR	84	Pancreatic elastase II B precursor
FNHU	KSKR	84	Fibronectin precursor
KXHUC1	RSKR	84	Proprotein convertase 1 precursor
JC2549	RVGR	84	Apolipoprotein F precursor
TCHU	RSKR	84	Calcitonin precursor
B48149	LGRR	83	Epithelial glycoprotein antigen GA733-2 precursor
SPHUB	RIAR	83	Neurokinin 1 precursor, beta splice form
S30350	LGKR	83	Inter-alpha-trypsin inhibitor heavy chain 3 precursor
TGHU	QTKR	82	Beta-thromboglobulin precursor
IJHUDB	RAKR	82	Desmocollin 3b precursor
IJHUDA	RAKR	82	Desmocollin 3a precursor

(*Continued*)

TABLE I. (*Continued*)

Accession number	Cleavage site	Score	Description
A24248	RARR	82	Inhibin alpha chain precursor
A56171	NRRR	81	Carboxypeptidase A2 precursor
HYHUMA	QKSR	81	Meprin A alpha chain precursor
IJHUCP	RHKR	81	Cadherin 3 precursor
WFHU2	RHRR	81	Transforming growth factor beta-1 precursor
S66518	SKGR	80	Proteinase-activated receptor 2 precursor
C38992	GLRR	80	Cadherin 4 precursor
KXHUZ	RWKR	79	Plasma protein Z precursor
A31249	RKKR	79	Transforming growth factor beta-2 precursor, short form
KXHUC2	RKKR	79	Proprotein convertase 2 precursor
B31249	RKKR	79	Transorming growth factor beta-2 precursor, long form
KXHUS	RKRR	79	Plasma protein S precursor
PTH2UL	RLKR	78	Parathyroid hormone-related peptide precursor
B37499	RLKR	78	Glial cell line-derived neurotrophic factor precursor
C40304	RRKR	77	Neurotrophin-3 precursor
B24248	RRRR	77	Inhibin beta-A chain precursor
PFHUG1	RRKR	77	Platelet-derived growth factor chain A precursor

fibrinolysis [32]. Takayama et al. have recently demonstrated that recombinant chimeric KLK4 protease expressed in a bacterial system is capable of activating PSA [33]. The experiments reported here utilizing a different recombinant KLK4 enzyme confirm the ability of KLK4 to proteolyze pro-PSA to an enzymatically active form. These results suggest that additional proteases capable of activating pro-KLK4 may also be expressed in the prostate.

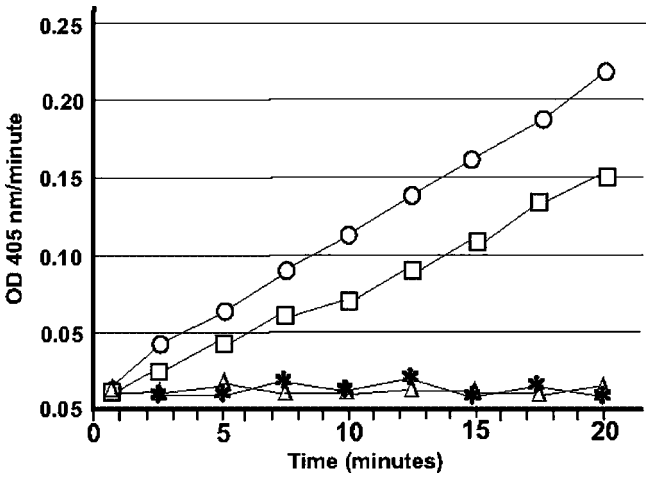

Fig. 5. Time course of pro-PSA activation by KLK4. Enzymatic amidolytic activity against 3-carbomethoxypropionyl-Arg-Pro-Tyr-pNA (S2586), a peptide substrate for PSA, was determined for active PSA (circle), pro-PSA after the treatment with active KLK4 (square), pro-PSA alone (triangle), and active KLK4 alone (asterisk).

In addition to PSA, searches of protein databases for polypeptides containing predicted sequences susceptible to KLK4 cleavage identified several candidates that may have physiological relevance in the context of

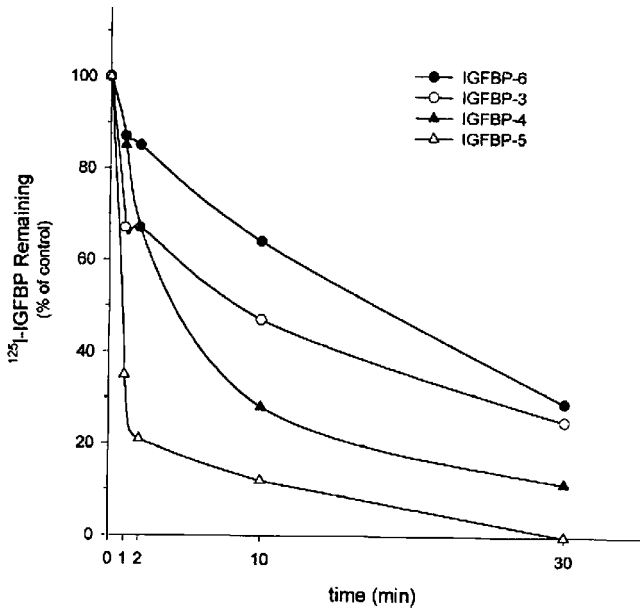

Fig. 6. KLK4-mediated proteolysis of IGF-binding proteins. Time course of I125-labeled IGF-binding protein (IGFBP) degradation following exposure to active KLK4. Following IGFBP incubation with KLK4 for the indicated times, samples were fractionated by SDS–PAGE, exposed to radiographic film and quantitated by densitometry. Values are expressed as the amount of IGFBP remaining relative to control experiments run without KLK4 treatment (% control).

neoplastic prostate tumor growth and metastasis. Proteins potentially activated by KLK4 proteolysis include PTHrP, vascular endothelial growth factor C (VEGFC), and members of the BMP family. PTHrP is a widely expressed protein that functions as a polyhormone with intracrine, juxtracrine, paracrine, and possibly endocrine effects [34]. Several biologically active PTHrP peptides are produced as a result of alternative splicing and endoproteolysis [34], and the PTHrP [1–37] form has been recognized as a primary pathogenic agent responsible for the hypercalcemia syndrome associated with malignant neoplasms [35]. Studies of prostate carcinoma demonstrate that PTHrP is able to induce cellular proliferation and may act locally in an autocrine fashion to stimulate cancer growth [36]. VEGFC is a member of the platelet-derived growth factor/vascular endothelial growth factor (PDGF/VEGF) family that promotes angiogenesis [37]. The specificity of VEGFC signaling leading to endothelial cell proliferation, angiogenesis, and lymphangiogenesis is mediated by VEGFC proteolytic processing and subsequent interactions with the VEGFR-2 and -3 tyrosine kinase receptors [38]. The expression level of VEGFC has been shown to correlate with lymph node metastasis of human prostate cancers [39]. BMPs are bone-inducing factors in the TGF-beta superfamily that are synthesized as functionally inactive precursor proteins. After secretion, pro-BMPs are proteolytically cleaved to yield biologically active proteins. Members of the BMP family have been shown to modulate prostate cell growth under different hormonal conditions [38], and may play a role in the osteoinductive activity of prostate metastases [40]. Several BMP family members have sites predicted to be activated by KLK4 proteolysis. While these and other putative KLK4 substrates have yet to be experimentally characterized, they represent potential mediators through which KLK4 may influence tissue remodeling during disease pathogenesis.

While protease enzymatic activity may serve to activate a specific cohort of proteins, proteolytic cleavage may also degrade and inactivate protein substrates. Of relevance for prostate pathology, the enzymatic activities of hK2 and PSA/hK3 have been shown to cleave and consequently inactivate members of the IGFBP family [41]. Insulin-like growth factors (IGFs) are mitogenic regulators of normal and neoplastic prostate cell growth [29]. IGF action is regulated in part by IGFBPs which modulate the bioavailability of IGF. High levels of circulating IGF correlate with the development of prostate carcinoma [42]. Prostatic hK3/PSA has been shown to cleave IGFBP-3 and -4 but not IGFBP-2 and -5, whereas KLK2 is able to cleave all IGFBPs [41]. In the studies reported here, we have shown that KLK4 is also able to cleave and degrade IGFBPs with rapid proteolysis of IGFBP-5 and -4. Proteolysis of IGFBP-6 and -3 was also observed, though to a lesser extent. These results suggest that in addition to KLK2 and KLK3, KLK4 may influence the growth of prostate cells through the degradation of IGFBPs leading to increased tissue access to IGF peptides.

The capacity to functionally characterize novel proteins currently lags far behind the current rate of discovery. The rapid determination of extended substrate specificities using positional scanning approaches overcomes a major hurdle in the study of newly identified proteolytic enzymes. Including KLK4, the KLK family alone now has at least 15 members that encode serine proteases. Of these, the substrate specificities for only KLK1, KLK2, and KLK3/PSA have been extensively evaluated. Several members of the kallikrein family have now been implicated in carcinogenesis including KLK4, KLK6/protease M, KLK9, and KLK10 [9,43–45]. The results of substrate screening analyses should assist in efforts to define the similarities and differences between these kallikrein family members and identify physiological substrates important for their activity in normal and pathological conditions. Exploiting these results could potentially allow for the synthesis of sensitive substrates suitable for monitoring protease activity and assist in the design of selective inhibitors to modulate protease activity for therapeutic benefit.

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REFERENCES

1. Achbarou A, Kaiser S, Tremblay G, Ste-Marie LG, Brodt P, Goltzman D, Rabbani SA. Urokinase overproduction results in increased skeletal metastasis by prostate cancer cells in vivo. Cancer Res 1994;54:2372–2377.
2. Rittenhouse HG, Finlay JA, Mikolajczyk SD, Partin AW. Human Kallikrein 2 (hK2) and prostate-specific antigen (PSA): Two closely related, but distinct, kallikreins in the prostate. Crit Rev Clin Lab Sci 1998;35:275–368.
3. Nelson PS, Gan L, Ferguson C, Moss P, Gelinas R, Hood L, Wang K. Molecular cloning and characterization of prostase, an androgen-regulated serine protease with prostate-restricted expression. Proc Natl Acad Sci USA 1999;96:3114–3119.

4. Lin B, Ferguson C, White JT, Wang S, Vessella R, True LD, Hood L, Nelson PS. Prostate-localized and androgen-regulated expression of the membrane-bound serine protease TMPRSS2. Cancer Res 1999;59:4180–4184.
5. Simmer JP, Fukae M, Tanabe T, Yamakoshi Y, Uchida T, Xue J, Margolis HC, Shimizu M, DeHart BC, Hu CC, Bartlett JD. Purification, characterization, and cloning of enamel matrix serine proteinase 1. J Dent Res 1998;77:377–386.
6. Hu JC, Zhang C, Sun X, Yang Y, Cao X, Ryu O, Simmer JP. Characterization of the mouse and human PRSS17 genes, their relationship to other serine proteases, and the expression of PRSS17 in developing mouse incisors. Gene 2000;251:1–8.
7. Yousef GM, Obiezu CV, Luo LY, Black MH, Diamandis EP. Prostase/KLK-L1 is a new member of the human kallikrein gene family, is expressed in prostate and breast tissues, and is hormonally regulated. Cancer Res 1999;59:4252–4256.
8. Stephenson SA, Verity K, Ashworth LK, Clements JA. Localization of a new prostate-specific antigen-related serine protease gene, KLK4, is evidence for an expanded human kallikrein gene family cluster on chromosome 19q13.3-13.4. J Biol Chem 1999; 274:23210–23214.
9. Dong Y, Kaushal A, Bui L, Chu S, Fuller PJ, Nicklin J, Samaratunga H, Clements JA. Human kallikrein 4 (KLK4) is highly expressed in serous ovarian carcinomas. Clin Cancer Res 2001;7:2363–2371.
10. Obiezu CV, Scorilas A, Katsaros D, Massobrio M, Yousef GM, Fracchioli S, Rigault de la Longrais IA, Arisio R, Diamandis EP. Higher human kallikrein gene 4 (KLK4) expression indicates poor prognosis of ovarian cancer patients. Clin Cancer Res 2001;7:2380–2386.
11. Del Rosso M, Fibbi G, Pucci M, D'Alessio S, Del Rosso A, Magnelli L, Chiarugi V. Multiple pathways of cell invasion are regulated by multiple families of serine proteases. Clin Exp Metastasis 2002;19:193–207.
12. Stetler-Stevenson WG, Yu AE. Proteases in invasion: Matrix metalloproteinases. Semin Cancer Biol 2001;11:143–152.
13. Cramer SD, Chen Z, Peehl DM. Prostate specific antigen cleaves parathyroid hormone-related protein in the PTH-like domain: Inactivation of PTHrP-stimulated cAMP accumulation in mouse osteoblasts. J Urol 1996;156:526–531.
14. Webber MM, Waghray A, Bello D. Prostate-specific antigen, a serine protease, facilitates human prostate cancer cell invasion [In Process Citation]. Clin Cancer Res 1995;1:1089–1094.
15. Frenette G, Tremblay RR, Lazure C, Dube JY. Prostatic kallikrein hK2, but not prostate-specific antigen (hK3), activates single-chain urokinase-type plasminogen activator. Int J Cancer 1997; 71:897–899.
16. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: A laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989.
17. Bunch TA, Grinblat Y, Goldstein LS. Characterization and use of the *Drosophila* metallothionein promoter in cultured *Drosophila melanogaster* cells. Nucleic Acids Res 1988;16:1043–1061.
18. Cherbas L, Moss R, Cherbas P. Transformation techniques for *Drosophila* cell lines. Methods in cell biology, Vol. 44. NY: Academic Press, Inc.; 1994. pp 161–179.
19. Rio DC, Laski FA, Rubin GM. Identification and immunochemical analysis of biologically active *Drosophila* P element transposase. Cell 1986;44:21–32.
20. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–254.
21. Harris JL, Backes BJ, Leonetti F, Mahrus S, Ellman JA, Craik CS. Rapid and general profiling of protease specificity by using combinatorial fluorogenic substrate libraries. Proc Natl Acad Sci USA 2000;97:7754–7759.
22. Jameson GW, Roberts DV, Adams RW, Kyle WS, Elmore DT. Determination of the operational molarity of solutions of bovine alpha-chymotrypsin, trypsin, thrombin and factor Xa by spectrofluorimetric titration. Biochem J 1973;131:107–117.
23. Kumar A, Mikolajczyk SD, Goel AS, Millar LS, Saedi MS. Expression of pro form of prostate-specific antigen by mammalian cells and its conversion to mature, active form by human kallikrein 2. Cancer Res 1997;57:3111–3114.
24. Andress DL, Birnbaum RS. Human osteoblast-derived insulin-like growth factor (IGF) binding protein-5 stimulates osteoblast mitogenesis and potentiates IGF action. J Biol Chem 1992;267: 22467–22472.
25. Emi M, Nakamura Y, Ogawa M, Yamamoto T, Nishide T, Mori T, Matsubara K. Cloning, characterization and nucleotide sequences of two cDNAs encoding human pancreatic trypsinogens. Gene 1986;41:305–310.
26. Kimland M, Russick C, Marks WH, Borgstrom A. Immunoreactive anionic and cationic trypsin in human serum. Clin Chim Acta 1989;184:31–46.
27. Kitamoto Y, Yuan X, Wu Q, McCourt DW, Sadler JE. Enterokinase, the initiator of intestinal digestion, is a mosaic protease composed of a distinctive assortment of domains. Proc Natl Acad Sci USA 1994;91:7588–7592.
28. Backes BJ, Harris JL, Leonetti F, Craik CS, Ellman JA. Synthesis of positional-scanning libraries of fluorogenic peptide substrates to define the extended substrate specificity of plasmin and thrombin. Nat Biotechnol 2000;18:187–193.
29. Yu H, Rohan T. Role of the insulin-like growth factor family in cancer development and progression. J Natl Cancer Inst 2000;92: 1472–1489.
30. Malm J, Hellman J, Hogg P, Lilja H. Enzymatic action of prostate-specific antigen (PSA or hK3): Substrate specificity and regulation by Zn(2+), a tight-binding inhibitor. Prostate 2000;45: 132–139.
31. Takayama TK, Fujikawa K, Davie EW. Characterization of the precursor of prostate-specific antigen. Activation by trypsin and by human glandular kallikrein. J Biol Chem 1997;272:21582–21588.
32. Davie EW, Fujikawa K, Kisiel W. The coagulation cascade: Initiation, maintenance, and regulation. Biochemistry 1991;30: 10363–10370.
33. Takayama TK, McMullen BA, Nelson PS, Matsumura M, Fujikawa K. Characterization of hK4 (prostase), a prostate-specific serine protease: Activation of the precursor of prostate specific antigen (pro-PSA) and single-chain urokinase-type plasminogen activator and degradation of prostatic acid phosphatase. Biochemistry 2001;40:15341–15348.
34. Strewler GJ. The parathyroid hormone-related protein. Endocrinol Metab Clin North Am 2000;29:629–645.
35. Broadus AE, Mangin M, Ikeda K, Insogna KL, Weir EC, Burtis WJ, Stewart AF. Humoral hypercalcemia of cancer. Identification of a novel parathyroid hormone-like peptide. N Engl J Med 1988;319:556–563.
36. Iwamura M, Abrahamsson PA, Foss KA, Wu G, Cockett AT, Deftos LJ. Parathyroid hormone-related protein: A potential autocrine growth regulator in human prostate cancer cell lines. Urology 1994;43:675–679.

37. Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N, Alitalo K. A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J 1996;15: 1751.
38. Joukov V, Sorsa T, Kumar V, Jeltsch M, Claesson-Welsh L, Cao Y, Saksela O, Kalkkinen N, Alitalo K. Proteolytic processing regulates receptor specificity and activity of VEGF-C. EMBO J 1997;16:3898–3911.
39. Tsurusaki T, Kanda S, Sakai H, Kanetake H, Saito Y, Alitalo K, Koji T. Vascular endothelial growth factor-C expression in human prostatic carcinoma and its relationship to lymph node metastasis. Br J Cancer 1999;80:309–313.
40. Bentley H, Hamdy FC, Hart KA, Seid JM, Williams JL, Johnstone D, Russell RG. Expression of bone morphogenetic proteins in human prostatic adenocarcinoma and benign prostatic hyperplasia. Br J Cancer 1992;66:1159–1163.
41. Rehault S, Monget P, Mazerbourg S, Tremblay R, Gutman N, Gauthier F, Moreau T. Insulin-like growth factor binding proteins (IGFBPs) as potential physiological substrates for human kallikreins hK2 and hK3. Eur J Biochem 2001;268:2960–2968.
42. Wolk A, Mantzoros CS, Andersson SO, Bergstrom R, Signorello LB, Lagiou P, Adami HO, Trichopoulos D. Insulin-like growth factor 1 and prostate cancer risk: A population-based, case-control study [see comments]. J Natl Cancer Inst 1998;90:911–915.
43. Anisowicz A, Sotiropoulou G, Stenman G, Mok SC, Sager R. A novel protease homolog differentially expressed in breast and ovarian cancer. Mol Med 1996;2:624–636.
44. Yousef GM, Kyriakopoulou LG, Scorilas A, Fracchioli S, Ghiringhello B, Zarghooni M, Chang A, Diamandis M, Giardina G, Hartwick WJ, Richiardi G, Massobrio M, Diamandis EP, Katsaros D. Quantitative expression of the human kallikrein gene 9 (KLK9) in ovarian cancer: A new independent and favorable prognostic marker. Cancer Res 2001;61:7811–7818.
45. Goyal J, Smith KM, Cowan JM, Wazer DE, Lee SW, Band V. The role for NES1 serine protease as a novel tumor suppressor. Cancer Res 1998;58:4782–4786.
46. Ek S, Hogerkorp CM, Dictor M, Ehinger M, Borrebaeck CA. Mantle cell lymphomas express a distinct genetic signature affecting lymphocyte trafficking and growth regulation as compared with subpopulations of normal human B cells. Cancer Res 2002;62:4398–4405.