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†

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ABSTRACT: hK4 (prostase, KLK4), a recently cloned prostate-specific serine protease and a member of the tissue kallikrein family, is a zymogen composed of 228 amino acid residues including an amino-terminal propiece, Ser-Cys-Ser-Gln-. A chimeric form of hK4 (ch-hK4) was constructed in which the propiece of hK4 was replaced by that of prostate-specific antigen (PSA) to create an activation site susceptible to trypsin-type proteases. ch-hK4 was expressed in *Escherichia coli*, isolated from inclusion bodies, refolded, and purified with an overall yield of 25%. The zymogen was readily self-activated during the refolding process to generate an active form (21 kDa) of hK4 (rhK4). rhK4 cleaved the chromogenic substrates Val-Leu-Arg-*p*NA (S-2266), Pro-Phe-Arg-*p*NA (S-2302), Ile-Glu-Gly-Arg-*p*NA (S-2222), and Val-Leu-Lys-*p*NA (S-2251), indicating that rhK4 has a trypsin-type substrate specificity. The rhK4 was inhibited by aprotinin (6 kDa), forming an equimolar 27 kDa complex. rhK4 readily activated both the precursor of PSA (pro-PSA) and single chain urokinase-type plasminogen activator (scuPA, pro-uPA). rhK4 also completely degraded prostatic acid phosphatase but failed to cleave serum albumin, another protein purified from human seminal plasma. These results indicate that hK4 may have a role in the physiologic processing of seminal plasma proteins such as pro-PSA, as well as in the pathogenesis of prostate cancer through its activation of pro-uPA.

Prostase (KLK4, KLK-L1, PRSS17), subsequently named hK4[1] (*1*), was cloned recently and demonstrated to be an androgen-regulated serine protease that is produced by the human prostate (*2*). Northern blot analysis has demonstrated its exclusive expression by the prostate (*2*), although PCR have shown a significantly lower expression by other organs (*3*). This is the third prostate-specific kallikrein discovered thus far. The detailed genomic organization (chromosome 19q13) of this new member of the human tissue kallikrein family has been described (*3*, *4*). hK4 has substantial amino acid sequence similarity to the other members of this family: 40% identity with prostate specific antigen (PSA), 40% with human glandular kallikrein-1 (hK2), and 40% with tissue kallikrein, hK1 (*5*). The cDNA of hK4 encodes a 26 amino acid residue signal peptide followed by a four amino acid propiece and an active 224 amino acid serine protease (*2*). Thus, it is produced as a zymogen or precursor form, which is possibly activated by a metalloprotease that cleaves the activation site between Gln[4] and Ile.[5] hK4 is a human ortholog to the porcine enamel matrix protease that is involved in tooth remodeling (*6*). Therefore, it is possible that hK4 is involved in tissue re-organization of the prostate.

PSA, another serine protease produced primarily by the prostate, has been used extensively as a marker for prostate cancer (*5*). It is a chymotrypsin-type protease which has a physiological function to degrade semenogelin I and II (*7*), which are the major gel-forming proteins in seminal plasma. Because PSA cleaves IGFBP3 (*8*) and PTHrp (*9*, *10*), proteins associated with cellular growth regulation and breast cancer metastasis, respectively, PSA has been implicated in growth regulation. It is synthesized as a zymogen (pro-PSA) (*11*) and was shown to be activated by hK2 (*12*−*14*), which is also a prostate-specific protein. pro-PSA was recently shown to be activated more effectively by prostin (*15*),

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[1] Abbreviations: PSA, prostate-specific antigen; hK2, human glandular kallikrein-1; pro-PSA, PSA precursor; rPSA, recombinant (active) PSA; rhK4, recombinant hK4 (prostase); pro-uPA or scuPA, single-chain urokinase-type plasminogen activator; ACT, α1-antichymotrypsin; serpin, serine protease inhibitor; PAP, prostatic acid phosphatase; STI, soybean trypsin inhibitor; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; RACE, rapid amplification of cDNA ends; S-2222, *N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-*p*-nitroaniline-HCl; S-2251, H.-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride; S-2302, H.-D-prolyl-L-phenylalany-L-arginine-*p*-nitroaniline-HCl; S-2586, 3-carbomethoxypropionyl-L-arginyl-L-prolyl-L-tyrosine-*p*-nitroaniline-HCl; S-2266, H.-D-Val-Leu-Arg-*p*-nitroaniline-HCl; S-2444, pyroglu-Gly-Arg-*p*-nitroaniline-HCl; MES, (2-[*N*-morpholino]ethanesulfonic acid) hydrate; Tris-HCl, Tris[hydroxymethyl] aminomethane-HCl; bicine-NaOH, (*N*,*N*-bis[2-Hydroxyethyl]-glycine).

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another serine protease identified in the prostate. The prostate produces a variety of other trypsin-type serine proteases (*15*) that may also be involved in the activation of pro-PSA (*14*).

Urokinase (uPA) has been strongly implicated in the development of prostate cancer (*16*, *17*) and is associated with cancer invasion and metastasis (*18*). Because uPA is synthesized as a precursor, scuPA, it requires activation by another serine protease in order to function enzymatically. We previously showed that recombinant hK2 could readily activate scuPA (*14*). Since some patients with prostate cancer have elevated hK2 levels that are discordant with PSA levels (*19*), we theorized that elevated hK2 may be uniquely involved in the pathogenesis of prostate cancer. Whether hK2 or other kallikrein(s) produced by the prostate have physiologic importance in scuPA activation is as yet unknown.

Recently, a growing number of serine proteases have been identified in the human prostate, but their physiologic roles in prostate biology have not been well established. Prostasin (*20*) was purified from seminal plasma using a pseudo-affinity column (aprotinin-agarose). This potentially membrane-bound protein is expressed in many tissues besides the prostate. TMPRSS2, another membrane-associated serine protease, was first reported by Paoloni-Giacobino et al. (*21*). It was subsequently demonstrated to be expressed primarily in the prostate (*22*). Although in situ hybridization studies have localized this protein to the basal cells of the prostate gland, its physiological function is not known. Another membrane-bound serine protease, matriptase (*23*)/MT-SP1 (*24*), was identified in breast cancer and prostate cells, respectively, and shown to be capable of activating scuPA (*25*, *26*), protease-activated receptor 2 (PAR2) (*25*), and hepatocyte growth factor (*26*). Like prostasin, MT-SP1/matriptase is expressed by many tissues including the prostate (*25*). Most recently, prostin (*15*)/hK15 (*27*) was reported by two independent groups to be a serine protease produced by the prostate as well as the testis, kidney, and pancreas (*15*). We showed that recombinant prostin was the best activator found to date for pro-PSA (*15*). All of these serine proteases have trypsin-type substrate specificities; thus, they are potential physiologic activators of pro-PSA, and/or they may be involved in the PSA cascade.

We previously demonstrated that pro-PSA can be activated by several serine proteases including hK2 (*14*) and prostin (*15*), and theorized that pro-PSA may require a series of cascade-like proteolytic activations (PSA cascade) involving multiple serine proteases (*14*). We therefore proceeded to search for other serine proteases that are produced at significant levels within the prostate by utilizing an improved method of degenerate oligonucleotide PCR (*28*). One of the products of this search was later identified as hK4. We now report the enzymatic characterization of this new member of the tissue kallikrein family.

EXPERIMENTAL PROCEDURES

Materials. Chromogenic substrates, *N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-*p*-nitroaniline-HCl (S-2222), H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride (S-2251), H-D-prolyl-L-phenylalany-L-arginine-*p*-nitroaniline-HCl (S-2302), 3-carbomethoxypropionyl-L-arginyl-L-prolyl-L-tyrosine-*p*-nitroaniline-HCl (S-2586), H-D-Val-Leu-Arg-*p*-nitroaniline-HCl (S-2266), and pyroglu-Gly-Arg-*p*-nitroaniline-HCl (S-2444) were purchased from Pharmacia-Hepar-Chromogenix (Franklin, OH). Aprotinin was a generous gift from Novo-Nordisk (Copenhagen, Denmark). STI, soybean trypsin inhibitor, and *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, TLCK, were purchased from Sigma (St. Louis, MO). The rest of the materials were as previously described (*14*, *15*). scuPA was a generous gift from Kenji Tanaka, Welfed (Osaka, Japan).

Construction of an Expression Vector for hK4. Degenerate oligonucleotide polymerase chain reaction (PCR) techniques were employed as previously described (*15*), and a 450-bp internal sequence of hK4 from a human prostate cDNA library (CLONTECH, Palo Alto, CA) was identified. This sequence was then used to design primers for rapid amplification of cDNA ends (RACE) (*29*). The 5′ and 3′ cDNA sequences were obtained from a human prostate Marathon-ready cDNA library (a generous gift from Wen-Feng Xu of Zymogenetics, Inc., Seattle, WA) by RACE as described (*15*). PCR products were subcloned (TOPO TA cloning kit, Invitrogen, Carlsbad, CA), sequenced (*15*), and shown to match the sequence of hK4. 5′ and 3′ primers were used to generate the full-length cDNA. Because hK4 has a propiece that ends with Gln^4-, it may be activated by a metalloprotease rather than by a serine protease. To activate hK4 by limited proteolysis with a serine protease, a plasmid was constructed incorporating the seven-amino acid propiece sequence of pro-PSA at the 5′ end, instead of the native hK4 propiece. The expression vector for this chimeric-hK4 was made by PCR using the following primers: (5′ -cat atg gcg ccc ctc atc ctg tct cgg atc ata aac ggc gag gac tgc agc ccg-3′) and (5′ -gga tcc tca act ggc ctg gac ggt ttt ctc tat cca-3′), and the full-length cDNA for hK4 was used as template. The amplified product was ligated into a pET12a expression plasmid (Novagen) as described (*14*). The resultant plasmid, pET12-proPSA-hK4 chimera was sequenced to verify that the sequence was correct.

Expression, Refolding, and Purification of Recombinant hK4 (rhK4). The majority of procedures were described previously (*14*). The pET12-proPSA-hK4 chimera plasmid was constructed as above. *Escherichia coli* strain BL21(DE3) was transformed, the protein was expressed, and the inclusion bodies were isolated and solubilized by the previously published methods (*14*) with some modifications. The solubilized inclusion bodies were refolded by diluting into a buffer containing 2 M urea, 0.5 M NaCl, 0.1 M NH_4Cl, 1 mM EDTA, 10 mM benzamidine, 1.25 mM reduced glutathione, and 0.5 mM oxidized glutathione, and 50 mM Tris-HCl, at pH 8.8. The rest of the refolding procedures, including the concentration of the protein by ultrafiltration were the same as described (*14*). The concentrated protein was stored at −20 °C until use. The sample (10 μL) was incubated at room temperature with 5 μL of 1 M Tris-HCl, pH 7.5/1 M NaCl, 35 μL of water, and 50 μL of 1 mM S-2222, and the absorbance change (405 nm) was measured using a plate reader as described (*14*). The results demonstrated that the protein had been autoactivated during the refolding procedure (see Results). For purification of the refolded rhK4, the concentrated sample was dialyzed against 50 mM Bicine-NaOH, pH 8.8/20 mM NaCl, and applied to a Resource-Q column (Pharmacia) and eluted with NaCl gradient as previously described for the purification of hK2 (*14*).

Measurements of the Amidolytic Activity of rhK4 against Chromogenic Substrates. rhK4 (1.0 μg) was incubated at room temperature with 50 μL of 1 mM substrates, H-D-Val-Leu-Arg-*p*NA (S-2266), Pro-Phe-Arg-*p*NA (S-2302), Val-Leu-Lys-*p*NA (S-2251), pyroGlu-Gly-Arg-*p*NA (S-2444), or Arg-Pro-Tyr-*p*NA (S-2586), and 50 μL of the same buffer used for S-2222 above. The increase in absorbance (405 nm) was measured by a plate reader over 15 min. The amidolytic activities were expressed as the amounts of *p*NA generated (mg of protein)$^{-1}$ min^{-1}. Dose dependent assay was performed using increasing amounts of rhK4 with S-2222 under the same conditions as above. Optimum pH for activity was determined by using S-2266 as a substrate and changing only the buffer. The following buffers were used: 0.1 M sodium acetate−acetic acid (pH 5.0); 0.1 M MES-NaOH, (pH 5.5, 6.0, and 6.5); 0.1 M Tris-HCl, (pH 7.2, 7.6, and 8.0); 0.1 M Bicine-NaOH, (pH 8.5, 8.8, and 9.2); 0.1 M sodium borate (pH 9.7 and 10.3). Each buffer also contained 0.1 M NaCl.

Inhibition of rhK4 by Aprotinin. rhK4 (1 μg) was incubated at 37 °C for 5 min with various amounts of aprotinin in 50 μL of 0.1 M Tris-HCl, pH 7.5/0.1 M NaCl, and the residual enzyme activity was measured as described above with S-2266. After incubation, the samples were analyzed by SDS−PAGE to detect the enzyme/inhibitor complex.

Effects of other Inhibitors on Amidolytic Activity. rhK4 (1.0 μg) was incubated with each inhibitor in 50 μL of 0.1 M Tris-HCl, pH 7.5/0.1 M NaCl for 10 min at room temp. The enzyme reaction was initiated by adding 50 μL of S-2266 (0.5 mM final) to each sample. The amidolytic activity was assayed as described above. The final concentrations of the inhibitors were 1 mM, TLCK; 10 mM, benzamidine; 0.1 mg/mL, STI.

Activation of pro-PSA by rhK4 and by rhK2. Recombinant pro-PSA (5 μg) was incubated at 37 °C with purified rhK4 (0.5 μg) or rhK2 (0.5 μg) in 50 μL of 0.1 M Tris-HCl, pH 7.5, 0.1 M NaCl. At various time intervals, the reaction was stopped by the addition of 3 μg of aprotinin. 50 μL of 1 mM S-2586 (0.5 mM final) was added to the reactions, and the absorbance was monitored as described (*14*). To study the complex formation between the generated rPSA and α1-antichymotrypsin (ACT), pro-PSA (1.0 μg) was activated with rhK4 (0.1 μg) at the various time intervals, and the reaction was stopped with aprotinin (0.4 μg). The samples were incubated with ACT (4 μg) at 37 °C for 60 min and then analyzed by SDS−PAGE.

Activation of Single Chain Urokinase-Type Plasminogen Activator (scuPA) by rhK4 and rhK2. Recombinant scuPA (2.5 μg) was incubated with rhK4 (5.0 ng) or rhK2 (5.0 ng) in 50 μL of 0.1 M Tris-HCl, pH 7.5/0.1 M NaCl. The reaction was terminated by the addition of aprotinin (2.0 μg) at various time periods. Fifty microliters of 1 mM S-2444 were added, and the absorbance increase at 405 nm was monitored at room temperature at 1 min intervals for 15 min. For SDS−PAGE, the reactions were stopped by the addition of SDS (2% final) instead of aprotinin.

Cleavage of Proteins Purified from Seminal Plasma by rhK4. Human seminal plasma was obtained from normal male volunteers. Pooled samples (30 mL) were centrifuged (10000*g* × 20 min) to remove cells, followed by ammonium sulfate fractionation at 25−45 (I), 45−65 (II), and 65−80% (III) saturation. Precipitates from fraction II were dissolved in 10 mL of 50 mM Bicine-HCl, pH 8.0, and dialyzed against the same buffer. This sample was then applied to Protein-Pak DEAE (Waters) column (1 × 10 cm) connected to the Waters Advanced Protein Purification system, model 650E. The column was equilibrated, run with the same buffer at 1.0 mL/min, and eluted with NaCl gradient (0−0.6 M NaCl). The eluted fractions were analyzed by SDS−PAGE. Two homogeneous proteins were subjected to sequence analysis as described (*14*). Identified proteins, prostatic acid phosphatase (2 μg) and serum albumin (2 μg), were incubated with rhK4 (0.1 μg) in 50 μL of 0.1M Tris-HCl, pH 7.5/0.1 M NaCl for various time periods. The reactions were stopped with the addition of SDS prior to analysis by SDS−PAGE. The cleaved fragments were transferred to an Immobilon-P membrane (Millipore, Bedford, MA) and subjected to sequence analysis.

Other Procedures. Protein concentrations were determined by micro BCA protein assay (Pierce, Rockford, IL) using bovine serum albumin as a standard. Protein sequence analysis and SDS−PAGE were performed as described (*14*). The remaining materials were as described previously (*14*, *15*).

RESULTS

Expression and Purification of Recombinant hK4 (rhK4). To search for novel serine proteases, degenerate oligonucleotide PCR amplification of a human prostate cDNA library was performed. The PCR produced the expected 450-bp size fragments (*15*) with the highly conserved sequences flanking the active site His and Ser residues of serine proteases. The fragments were ligated into a TA vector, and sequence analysis identified 15 different serine proteases (*15*). At the time of the initial analysis, four of the 15 serine proteases had not been reported. Subsequently, two appeared in the literature, prostase/hK4 (*2*) and testisin (*30*). Oligonucleotide primers were designed for subsequent 5′ and 3′ RACE of a human prostate cDNA library. The entire sequence of the 5′ end including the start codon could not be obtained from the Clontech cDNA library. Therefore, 5′ RACE was performed using a human prostate cDNA library from Zymogenetics, Inc. A composite sequence was thus generated from the overlapping sequences of the 450 bp middle segment and the fragments from the 5′ and 3′ RACE reactions. Subsequent PCR amplification using primers complementary to the start and stop codon regions generated the entire sequence of hK4, and the sequence was confirmed for both strands.

Because the hK4 propiece ends with Gln^4-, a chimeric hK4 was designed with a propiece that could be cleaved through limited proteolysis by a serine protease. Therefore, a plasmid was constructed with the propiece sequence of pro-PSA followed by the mature hK4 sequence. This plasmid, pET12-proPSA-hK4, was used to transform *E. coli* (BL21DE3) cells. The expressed recombinant hK4 chimera (ch-hK4) was refolded as described in Experimental Procedures. During the refolding process, ch-hK4 became autoactivated to form active recombinant hK4 (rhK4) as evidenced by the generation of amidolytic activity against S-2222 (data not shown) and the detection of the expected N-terminal sequence of the active protease. The refolded rhK4 was purified utilizing a Resource-Q column (Figure 1). When the pass-through and eluted fractions were assayed for the amidolytic activity, the last peak eluted from the column exhibited the highest

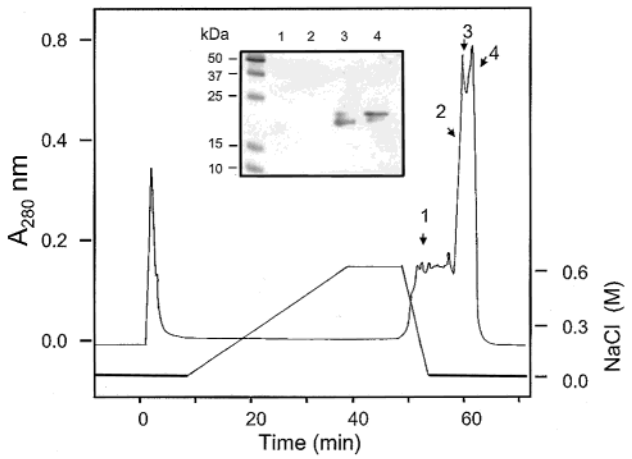

FIGURE 1: Purification of rhK4 by Resource-Q column. The last major peak (4, arrow) contained purified and active rhK4. Inset, SDS−PAGE analysis of fractions 1, 2, 3, 4 (5 μL each lane), nonreduced.

activity. Neither the pass-through fractions nor the early elution "plateau" fractions (Figure 1) demonstrated any activity. The fraction from the last peak (0.5 mg/mL) was stored at −20 °C for subsequent assays. SDS−PAGE analysis of this purified rhK4 showed a single band with an apparent molecular mass of ∼21 kDa (Figure 1, inset) and 25 kDa under reduced conditions (data not shown). Under the above conditions, 1.5 mg of soluble purified rhK4 was routinely obtained from ∼6 mg of inclusion bodies with an overall yield of ∼25%, which is similar to the yield obtained from the refolding of pro-PSA (*14*).

Amidolytic Activity against Chromogenic Substrates. The enzyme activity of rhK4 was measured by using several commercially available chromogenic substrates, and the results were compared with the activity of the previously studied rhK2 (Table 1). The enzyme activities were determined as described in Experimental Procedures. rhK4 (1.0 μg) or rhK2 (1.0 μg) was incubated with each substrate (0.5 mM, final) in pH 7.5 buffer. rhK4 had high activity toward S-2266 and S-2302. Because hK4 is a member of the human kallikrein family, substantial activity against these kallikrein substrates was an expected finding. rhK4 showed weak activity toward S-2251 and had no activity against S-2444 despite the presence of an Arg residue at the cleavage site. hK4 was also able to cleave the Lys-*p*NA bond (S-2251). This is consistent with the finding that hK4 activates scuPA, which requires the cleavage of the Lys^{158}−Ile^{159} bond (see below). rhK4 had no chymotrypsin-like activity (S-2586). These data indicate clearly that both hK4 and hK2 are trypsin-type proteases. Furthermore, hK4 has substantially higher catalytic activity than does hK2. rhK2 had weak activity toward S-2302 only and no activity toward the other substrates tested (Table 1).

pH Optimum for rhK4 Amidolytic Activity. The enzyme activity was measured at pH values ranging from 5.0 to 10.3 using S-2266 as substrate. The activity was higher under neutral conditions, with the optimum pH of 7.5. rhK4 was able to retain 40% of maximal activity at pH 10.3, while the activity dropped to 32% at pH 5.5 and to 7% at pH 5.0. Because the pH of seminal plasma ranges typically between 7.5 and 8.5, these data suggest that hK4 can function physiologically in seminal plasma.

Inhibition of rhK4 by Serine Protease Inhibitors. Since rhK4 showed kallikrein-like activity, inhibition studies were performed with aprotinin, a potent inhibitor of plasma kallikrein. The enzyme was incubated for 5 min with varying amounts of the inhibitor, and the enzyme activity was measured by the amidolytic assay using S-2266. The enzyme activity decreased with increasing amounts of the inhibitor, and complete inactivation was achieved with a molar ratio (inhibitor to enzyme) of about 1.2:1 (Figure 2).

To determine whether the enzyme can form a stable complex with aprotinin, rhK4 was incubated with a 4-fold molar excess of aprotinin, and the samples were analyzed by SDS−PAGE (Figure 2, inset). Incubation of rhK4 with aprotinin resulted in the formation of a complex of 27 kDa (Figure 2, lane 3−5), which is the sum (1:1 complex) of rhK4 (21 kDa) and aprotinin (6 kDa). Other serine protease inhibitors also readily inhibited rhK4: 85% inhibition by 1 mM TLCK, 93% with 10 mM benzamidine, and 99% inhibition by 1 mg/mL of STI.

Activation of pro-PSA by rhK4 and by rhK2. Because pro-PSA is physiologically activated almost immediately after ejaculation, rapid activator(s) of pro-PSA was searched from seminal plasma. Previously, rhK2 was shown to activate pro-PSA. However, the reaction required over 30 min for substantial activation and was significantly slower than activation by trypsin (*14*). Furthermore, another as-yet-unidentified, partially purified serine protease was also shown to activate pro-PSA (*14*). These findings suggest the presence of other serine proteases in seminal plasma that could also be physiologic activators of pro-PSA.

To test for serine proteases that might have higher rates of pro-PSA activation, pro-PSA activation by rhK4 was examined. Pro-PSA was incubated with rhK4 and the reaction was stopped with aprotinin as described above. Aliquots were analyzed for PSA activity employing S-2586 as substrate (Figure 3A). rhK4 had no activity toward S-2586 (Table 1). A rapid activation of pro-PSA by rhK4 was observed within 2 min (Figure 3A, closed circles). hK2 was a significantly weaker activator of pro-PSA under the same conditions (Figure 3A, open circles). The rPSA that was activated by rhK4 formed a complex with ACT (Figure 3B), whereas pro-PSA did not form a complex. The amount of ACT:rPSA complex that formed correlated with the level of activation by rhK4. These findings indicated that rhK4 was able to activate pro-PSA to form rPSA, similar to the activation of pro-PSA by trypsin as previously demonstrated (*14*).

Activation of Single-Chain Urokinase-Type Plasminogen Activator (scuPA) by rhK4 and by rhK2. It was previously shown that rhK2 readily activates scuPA in a time-dependent manner (*14*). Therefore, hK4, another prostate-specific kallikrein could also activated scuPA. When scuPA was incubated at 37 °C with recombinant rhK4 at a 500:1 weight ratio, scuPA was readily activated, as measured by the hydrolysis of S-2444 (Figure 4A, closed circles). In these experiments, the rhK4 was inhibited by aprotinin prior to the assay of urokinase. rhK4 had no amidolytic activity toward S-2444, and therefore this substrate only measured the activity of uPA. SDS−PAGE of the reaction mixture under reducing conditions clearly demonstrated the formation of heavy (∼34 kDa) and light chains (∼21 kDa) of urokinase as the activation reaction proceeded (Figure 4B). The ∼34

Table 1: Amidolytic Activity of rhK4 against Chromogenic Substrates[a]

substrates (chromogenic)	formula	applications	hK4 specific activity (μmol mg^{-1} min^{-1})	hK2 specific activity (μmol mg^{-1} min^{-1})
S-2266	Val-Leu-Arg-pNA	trypsin, glandular kallikrein	1.42	0.00
S-2302	Pro-Phe-Arg-pNA	plasma kallikrein	1.12	0.09
S-2222	Ile-Glu-Gly-Arg-pNA	trypsin, factor Xa	0.67	0.00
S-2251	Val-Leu-Lys-pNA	plasmin	0.31	0.00
S-2444	Glu-Gly-Arg-pNA	trypsin, urokinase	0.00	0.00
S-2586	Arg-Pro-Tyr-pNA	chymotrypsin, PSA	0.00	0.00

[a] rhK4 (1.0 μg) or hK2 (1.0 μg) was incubated with 100 μL of various chromogenic substrates (0.5 mM final). Activities are expressed as the amounts of pNA generated/mg protein/min.

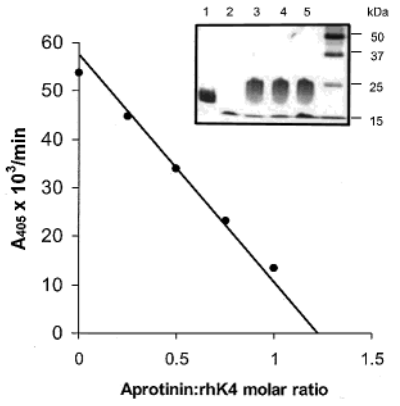

FIGURE 2: Inhibition of rhK4 by aprotinin. rhK4 (1.0 μg) was incubated with various amounts of aprotinin, and the remaining enzyme activity was measured with S-2266 (0.5 mM final). Inset, SDS−PAGE analysis of rhK4 (2.5 μg) and aprotinin. Lane 1, rhK4; lane 2, aprotinin (5 μg); lane 3, rhK4 and aprotinin (2.4 μg); lane 4, rhK4 and aprotinin (4.8 μg); lane 5, rhK4 and aprotinin (9.6 μg).

kDa band was transferred to a PVDF membrane and subjected to sequence analysis; this showed the expected N-terminal sequence of the heavy chain: IIGGEFTTIEN-QPWFA. In comparison to rhK4, rhK2 had very little scuPA-activational activity under the same conditions (Figure 4A, open circles) and required a much higher amount (10:1 weight ratio of scuPA:hK2) to generate fully active uPA (data not shown). Under the same conditions, plasminogen was not activated by rhK4 (data not shown). These results indicate that hK4 is a potent activator of scuPA and may be a physiologic activator of scuPA in the prostate.

Purification of Seminal Plasma Proteins and Cleavage of Prostatic Acid Phosphatase by rhK4. Because hK4 is produced exclusively by the prostate, the potential physiologic substrates are likely to be present in seminal plasma. As described in Experimental Procedures, human seminal plasma samples were pooled, centrifuged, and fractionated by ammonium sulfate precipitation. The sample from 45 to 65% saturation was then applied to a DEAE column and eluted with NaCl (Figure 5). Samples from the eluted fractions were analyzed by SDS−PAGE (Figure 5, inset). Two of the three peaks on the elution profile corresponded to two separate bands on SDS−PAGE: peak 2, ~52 kDa and peak 3, ~54 kDa. N-terminal sequences of the two bands demonstrated that the peak 2 sample was prostatic acid phosphatase (PAP), and peak 3 was serum albumin. The sequence of peak 1 was not obtained, probably because of a blockage of the N-terminal residue.

To determine whether any of these two proteins were substrates/target proteins for rhK4, each sample was incu-

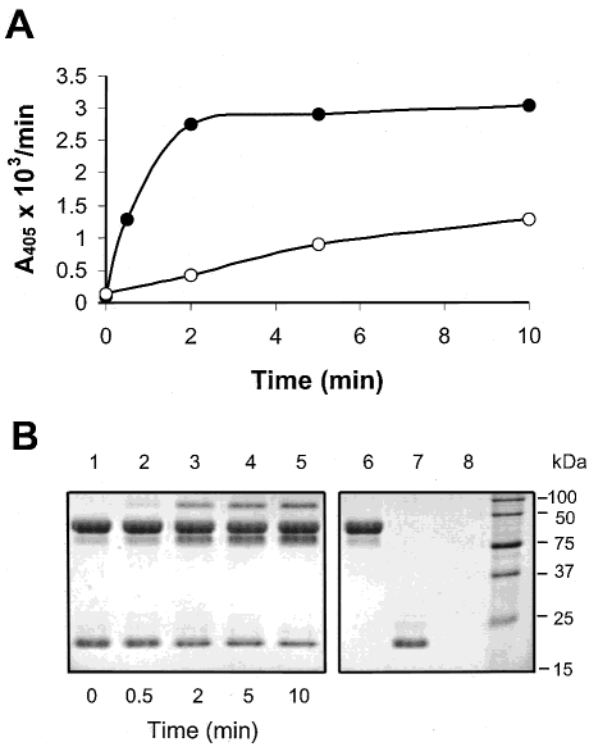

FIGURE 3: Time course of pro-PSA activation by rhK4 or rhK2 and formation of the α1-antichymotrypsin (ACT)/rPSA complex. (A) Amidolytic activity analysis of rPSA. Pro-PSA (5 μg) was activated with rhK4 (0.5 μg) (closed circles) or rhK2 (0.5 μg) (open circles) to generate rPSA, and the reaction was terminated with aprotinin (3 μg). Amidolytic activity against S-2586 (1.0 mM final) was monitored. (B) SDS−PAGE analysis of ACT/rPSA complex formation after pro-PSA activation by rhK4. pro-PSA (1.0 μg) was activated with rhK4 (0.1 μg), the reaction was terminated with aprotinin (0.4 μg), then incubated with ACT (4 μg). Lane 1−5, pro-PSA was activated for 0−10 min as indicated; lane 6, ACT; lane 7, pro-PSA; lane 8, rhK4 and aprotinin.

bated with rhK4 at a 20:1 weight ratio for various times and analyzed by SDS−PAGE. PAP (peak 2) was the only protein that was cleaved by rhK4 during the 1-h incubation (Figure 6). Samples taken at different time points of during the incubation, showed progressive loss of the ~52 kDa band and an initial increase in a ~33 kDa fragment (Figure 6, lane 3) and subsequent loss in intensities of all bands. The rate of cleavage was also dependent on the amount of rhK4 (data not shown). The N-terminal sequence of the ~33 kDa fragment showed the primary cleavage site was Arg^{73}-Ser^{74} bond: QVYIR^{73}-S^{74}TDVDRTL of PAP, to form 73- and 256-amino acid residue fragments. rhK4 selectively cleaved PAP without cleaving serum albumin, validating the substrate specificity of this serine protease. These results suggest that hK4 may be involved in the physiologic clearance of PAP.

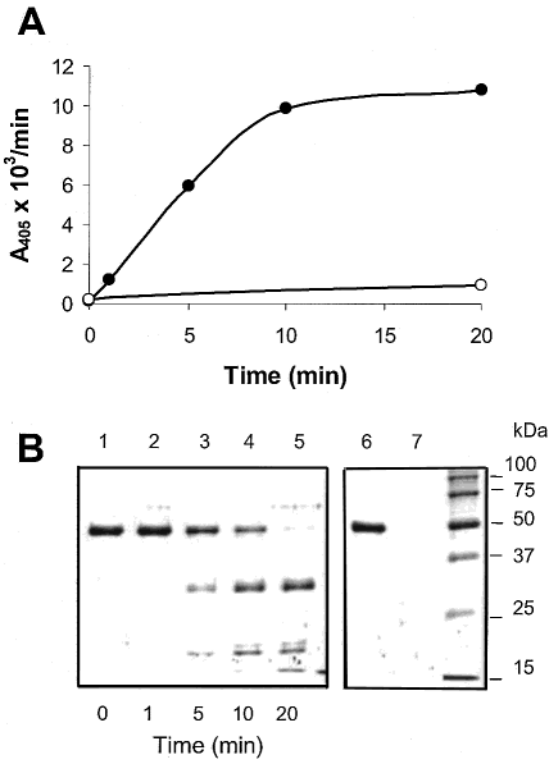

FIGURE 4: Time course of scuPA activation by rhK4 and by rhK2. (A) Amidolytic activity of scuPA activated by rhK4 or rhK2. scuPA (2.5 μg) was activated with rhK4 (5.0 ng) (closed circles) or with rhK2 (5.0 ng) (open circles). The reaction was terminated at various times with aprotinin (2 μg). Enzyme activity was measured with S-2444 (0.5 mM final). (B) SDS−PAGE analysis of urokinase formation (heavy and light chains) during scuPA activation by rhK4. Lanes 1−5, scuPA (2.5 μg) activated by rhK4 (5 ng) at 0, 1, 5, 10, and 20 min, respectively. Lane 6, scuPA (2.5 μg); lane 7, rhK4 (5 ng); all samples were reduced.

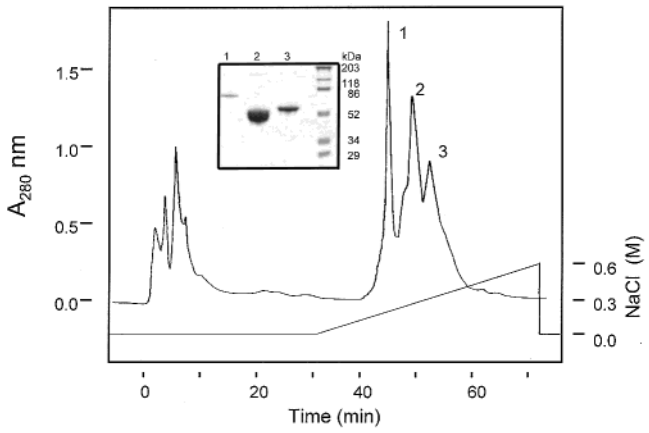

FIGURE 5: Purification of prostatic acid phosphatase (PAP) and serum albumin on a DEAE column. Ammonium sulfate fraction (45−65% saturation) of human seminal plasma was applied to the column as described under Experimental Procedures. Peak 2 contained PAP, and peak 3 contained serum albumin.

DISCUSSION

We characterized the biochemical properties of rhK4 by measuring its specific amidolytic activities, its activity against potential physiologic substrates, and its interaction with various serine protease inhibitors. As expected, the refolding of the recombinant serine proteases in this study required substantial experimental rigor. The resultant refolded proteins were extremely pure and fully active. The differences in

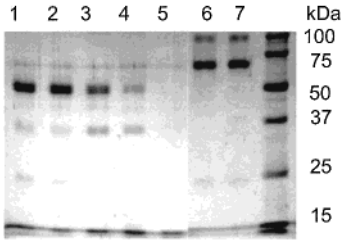

FIGURE 6: SDS−PAGE analysis of prostatic acid phosphatase (PAP), serum albumin, and rhK4. Lanes 1−5, PAP (2 μg) cleaved by rhK4 (0.1 μg) at 0, 1, 10, 30, and 60 min, respectively. Lane 6−7, serum albumin (2 μg) incubated with rhK4 (0.1 μg) at 0, 60 min, respectively.

specificity of rhK4 against the chromogenic substrates that were tested suggest optimal peptide substrate sequences. For example, in the P1 site, hK4 prefers Arg- instead of Lys- (S-2266 > S-2302 ≫ S-2222 ≫ S-2251). From the observation that S-2266 was the best chromogenic substrate tested, it appears that hK4 has very similar specificity to that of glandular kallikrein and trypsin. The cleavage sites of pro-PSA, scuPA, and PAP were ILSR-∧-IVGG, PRFK-∧-IIGG, and VYIR-∧-STDV, respectively. Most of these have hydrophobic residues in the P4 to P2 positions. The partial inhibition of rhK4 by TLCK further supports the preference of hK4 for Arg- instead of Lys- at the P1 site. Both STI and aprotinin were potent inhibitors of rhK4. In the future, studies with mutant serpins may determine the exact sequences that are important in the enzyme:inhibitor interaction.

Serine proteases with trypsin-type specificity are often involved in biological processes that require limited proteolysis. For example, in the blood coagulation system, the clotting factors activate specific downstream proenzymes in a "cascade-like" manner, such that a minute amount of upstream activation can result in an amplification that leads to rapid clot formation (*31*). In the human prostate, a similar cascade-like process may well be operational (*14*). During fertilization, a seminal clot forms almost instantaneously; however, it dissolves within minutes to allow the release of motile sperm. Such processes likely involves precursor or zymogen activation. Every zymogen of prostate-associated serine proteases discovered to date, including hK2, hK4, TMPRSS2, matriptase/MT-SP1, prostasin, and prostin, requires limited proteolysis for its activation. Because they are trypsin-type serine proteases, they are all potential activators of PSA. Of these proteases, only hK2, hK4, and prostin have been tested so far for their ability to activate pro-PSA. Both hK4 and prostin have now been shown to be better activators of pro-PSA than hK2. Because prostin has not yet been completely purified and characterized, it is not yet clear whether hK4 or prostin is the more potent activator of pro-PSA. Nevertheless, because rhK4 rapidly activated pro-PSA in 2 min at a 1:10 ratio (Figure 3A), hK4 may well be the physiologic activator of pro-PSA. Further studies with purified proteins and analyses of their temporal production and activations in seminal plasma will be necessary to determine the precise mechanisms of pro-PSA activation. The interactions between these serine proteases will continue to be greatly clarified by the production and characterization of recombinant proteins.

Studies with purified or recombinant serine proteases have suggested many physiologic roles of these enzymes in

prostatic diseases. For example, PSA has been shown to cleave IGFBP3 (*8*) and PTHrp (*8*), which may lead to abnormal growth of prostate cells. The ultimate consequence may be the development of benign prostatic enlargement or even cancer. Therefore, determining the mechanisms involved in the activation of pro-PSA may help elucidate the regulation of both normal and abnormal prostate growth. One of the strongest pieces of evidence for the roles of serine proteases in prostate cancer is the fact that the cancer cells overexpress urokinase-type plasminogen activator (uPA) (*16*, *17*). uPA activates plasminogen to generate plasmin (*32*), which in turn activates metalloproteases including procollagenase (*33*, *34*). Therefore, prostate cancer cells may overproduce proteolytic enzymes that aid their invasion through adjacent connective tissue. We demonstrated that recombinant hK2 and rhK4 can both activate pro-uPA (scuPA) to generate urokinase (*14*). This points to the likelihood that interactions between these prostate-specific proteases and uPA may be involved in malignant processes of the prostate. Both hK2 and hK4 may therefore become future targets for anticancer drugs. Because both hK4 and hK2 are produced almost exclusively by the human prostate, specific inhibitors may be developed that would control the level of hK4- or hK2-mediated activation of scuPA. The finding that rhK4 can activate scuPA even at 1:500 ratio suggests potential physiologic importance, especially since other known scuPA activators such as plasmin and plasma kallikrein have not been found at significant levels in the prostate.

Another potential role of serine proteases in prostate biology is the regulation of prostatic acid phosphatase (PAP). We demonstrated that recombinant hK4 readily degrades PAP in a dose-dependent manner. PAP has been shown to dephosphorylate c-ErbB-2, thereby reducing the phosphotyrosine level of ErbB-2 in prostate cells (*35*). Transient expression of PAP furthermore decreased the growth rate of C81-LNCaP, an androgen-unresponsive prostate cancer cell line that lacks endogenous expression of PAP (*35*). These data, reported by Zhang et al., suggest a potentially important role of PAP in androgen-mediated regulation of prostate epithelial cell growth. Consequently, degradation of PAP by hK4 may be deleterious to the cellular growth regulation and lead to the development of prostate cancer.

One of the potentially important clinical uses of these serine proteases will be in the development of better tumor markers. For example, in prostate cancer management, the serum PSA test has been an invaluable tool (*5*). However, PSA is not a perfect tumor marker because a significant number of patients with prostate cancer have normal levels of PSA. Furthermore, even in cases where PSA levels are elevated, approximately 70% of patients do not have prostate cancer. It is intriguing that newer tests, including the measurements of the ratio between "free" PSA and PSA complexed with ACT, have improved the detection of cancer. Patients with prostate cancer have a higher ratio of PSA-ACT complex to free-PSA as compared to normal men (*5*). Because PSA must be in its cleaved, active form before it can form a stable complex with ACT (*14*), an increased level of the pro-PSA activator may be responsible for this phenomenon. With the increasing evidence for multiple roles of serine proteases in cancer progression, such as those described above, it would be worthwhile to further study not only the utility of these proteins as tumor markers, but also their potential as targets for the treatment of prostate cancer.

Future studies of hK4 and other prostate-associated serine proteases will better elucidate the possible biological roles of these proteins in prostatic diseases. The production of antibodies directed against peptide epitopes or purified recombinant proteins will improve estimates of their expression levels in prostate cancer as well as in benign prostatic hyperplasia and prostatitis. Improved methods of producing recombinant serine proteases may also facilitate the discovery of other physiologic substrates, and lead to the identification of their physiological activator(s). The ultimate goal of these studies is the development of better tools for the diagnosis and management of prostatic diseases.

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