Characterization of the Precursor of Prostate-specific Antigen

ACTIVATION BY TRYPSIN AND BY HUMAN GLANDULAR KALLIKREIN

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The precursor orzymogen form of prostate-specific antigen (pro-PSA) is composed of 244 amino acid residues including an amino-terminal propiece of 7 amino acids. Recombinant pro-PSA was expressed in Escherichia coli, isolated from inclusion bodies, refolded, and purified. Thezymogen was readily activated by trypsin at a weight ratio of 50:1 to generate PSA, a serine protease that cleaves the chromogenic chymotrypsin substrate 3-carboxyphenylpropionyl-L-arginyl-L-prolyl-L-tyrosine-p-nitroaniline-HCl (S-2586). In this activation, the amino-terminal propiece Ala-Pro-Leu-Ile-Leu-Ser-Arg was released by cleavage at the Arg-Ile peptide bond. The recombinant pro-PSA was also activated by recombinant human glandular kallikrein, another prostate-specific serine protease, as well as by a partially purified protease(s) from seminal plasma. The recombinant PSA was inhibited by α1-antichymotrypsin, forming an equimolar complex with a molecular mass of approximately 100 kDa. The recombinant PSA failed to activate single chain urokinase-type plasminogen activator, in contrast to the recombinant hK2, which readily activated single chain urokinase-type plasminogen activator. These results indicate that pro-PSA is converted to an active serine protease by minor proteolysis analogous to the activation of many of the proteases present in blood, pancreas, and other tissues. Furthermore, PSA is probably generated by a cascade system involving a series of precursor proteins. These proteins may interact in a stepwise manner similar to the generation of plasmin during fibrinolysis or thrombin during blood coagulation.

Prostate-specific antigen (PSA) was first identified in human seminal plasma in 1969 (1). Its clinical significance as a marker for prostate cancer became evident in the mid-1980s when Wang et al. (7) and Papsidero et al. (8) reported that PSA could be used as a marker for the detection of prostate cancer. In 1987, Stamey et al. (9) showed that elevated serum levels of PSA correlated with the clinical stage of prostate cancer in a large group of patients. Since then numerous studies have confirmed the value of serum PSA determinations in the management of prostate cancer (2–6).

PSA from seminal plasma has been referred to as γ-seminoprotein (10), E1 antigen (11), and p30 (12). In 1979, it was purified from prostate tissue by Wang et al. (13). PSA is a serine protease composed of a single chain polypeptide and one carbohydrate chain with a total mass of 33–34 kDa (13–18). It has chymotrypsin-like specificity toward its physiological substrates, semenogelin I and II (17), and a number of synthetic substrates (15, 16, 18). PSA has been shown to cleave several tyrosyl peptide bonds in semenogelin I and II, which are the major gel-forming proteins produced by the seminal vesicles (19, 20). PSA is believed to be involved in the lysis of the seminal clot that occurs immediately after ejaculation (20, 21). PSA isolated from seminal plasma has also been reported to activate single chain urokinase-type plasminogen activator (scuPA) (22). Thus far, three physiologic inhibitors have been shown to inhibit PSA; these include α1-antichymotrypsin (16, 23, 24), α2-macroglobulin (16), and activated protein C inhibitor (25, 26).

PSA is a member of the human tissue kallikrein family (14, 27). The three members of this gene family (28) include tissue kallikrein, human glandular kallikrein (29), and PSA. PSA has approximately 70% sequence identity with tissue kallikrein and 80% with hK2 (30). The latter two proteins, however, have trypsin-like substrate specificity (30).

PSA and hK2 have other similarities in addition to their amino acid sequence homology. For example, both are synthesized in a precursor orzymogen form and require minor proteolysis for activation. In 1987, Lundwall and Lilja (14) isolated the cDNA coding for human PSA and showed that the protein is synthesized as a zymogen of a serine protease. The zymogen has not been found in tissue or physiological fluid, however, apparently due to its rapid conversion to the active form.

In 1987, Schedlich et al. (29) isolated the cDNA coding for hK2. This protein is synthesized exclusively in the prostate. The activated form of hK2 has been isolated recently from seminal plasma (31), but its physiologic substrate(s) has not been identified.

In this study, human pro-PSA was expressed in Escherichia coli, refolded, and purified to homogeneity. Pro-PSA generated serine protease activity upon activation by trypsin, and the resulting enzyme was completely inhibited by α1-antichymotrypsin. It was also found that recombinant hK2 and a partially purified protease(s) from seminal plasma activated pro-PSA and may be the physiologic activators of the protein.
nyl-L-arginine-p-nitroanilide-HCl), and S-2444 (L-prolylthrombyl-glycycl-L-arginine-p-nitroanilide-HCl) were purchased from Pharma-Cephar-Chromogenix (Franklin, OH). A full-length cDNA coding for human PSA and reverse transcriptase-polymerase chain reaction-generated cDNA from human prostate tissue were kindly provided by Robert Yeadon and Liu (Department of Urology and Biotechnology, University of Washington). The expression vector pET-12a and E. coli strain BL21(DE3) were obtained from Novagen (Madison, WI), and the TA Cloning Kit came from Invitrogen (San Diego, CA). Aprotinin was a gift from Novo Industri A/S (Copenhagen, Denmark). Glutathione (reduced and oxidized), diisopropyl fluorophosphate, benzamidine HCl, and Bicine-NaOH were purchased from Sigma (St. Louis, MO). The expression vector pET-12a and the C-terminal His tagged protein sequencer model 477A, connected to a model 120A analyzer, was purchased from Amicon (Beverly, MA). Micro BCA protein assay kit was purchased from Pierce. LB broth was from Life Technologies, Inc., and Tween 20 was a product of Bio-Rad. Ampicillin was from Sigma. 8M urea, 50 mM Bicine, pH 8.0, 150 mM NaCl, 0.1 M NH₄Cl, 2 mM EDTA, 10 mM benzamidine, 1.25 mM reduced glutathione, 0.5 mM oxidized glutathione and by slowly stirring for 24 h at 4 °C. The final product showed strong amidoetric activity toward Pro-Phe-Arg-pNA (S-2302).

**Preparation of Recombinant Human Glandular Kallikrein (rhK2)—**

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The zymogen form of hK2 (pro-hK2) was expressed, refolded, and purified by essentially the same procedures as described above for pro-PSA. In contrast to pro-PSA, the pro-hK2 was completely activated during the isolation and refolding procedure. The final product showed strong amidoetric activity toward Pro-Phe-Arg-pNA (S-2302).

**Activation of Pro-PSA with Partially Purified Protease from Seminal Plasma—**

Human seminal plasma was obtained from normal male volunteers. Samples (30 ml) were pooled and centrifuged (10,000 × g for 20 min) to remove cells, followed by ammonium sulfate fractionation at 25–45, 46–65, and 65–80% saturation. Precipitates were then dissolved in 10 ml of 50 mM Bicine, pH 8.0, and dialyzed against the same buffer. Pro-PSA (5 µg) was incubated with 10 µg of each amonium sulfate fractions in a final 50 µl of 0.1 M Tris-HCl, pH 7.5, 0.1 M NaCl. The amidoetric activity (S-2586 2 µM final) of PSA was measured as described above except the activation reactions were not terminated with aprotinin. The amidoetric activity of the amonium sulfate fractions were also measured as controls.

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**Isolation and Amino Acid Analysis of the Activation Peptide (Pro-piece)—**

Pro-PSA (22.8 µg) was activated for 2 min with 0.5 µg of trypsin as described above, and the reaction was terminated with trichloroacetic acid at 5% final concentration. After centrifugation (10,000 × g for 5 min), the supernatant was applied to a C18 Bondapak column (4.6 mm inner diameter × 25 cm) connected to a Waters HPLC system and eluted by a gradient composed of 0.05% trifluoroacetic acid (solvent A) and 80% CH₃CN, 0.04% trifluoroacetic acid (solvent B) at 1.5 ml/min. The column fractions were monitored by absorbance at 214 nm, and peak fractions were collected manually for subsequent amino acid composition analysis.

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**Activation of Single Chain Urokinase-type Plasminogen Activator by rhK2—**

Recombinant scuPA (2.6 µg) was incubated with 0.25 µg of rhK2 or recombinant Kallikrein (200 ng) in 1 mM Tris-HCl, pH 7.5, 0.1 M NaCl. The activation reaction was terminated by the addition of aprotinin (3.0 µg) at various times. Fifty microliters of S-2444 (1 µM) were added, and the absorbance increase at 405 nm was monitored at room temperature at 1 min intervals for 10 min. For SDS-PAGE, the reactions were stopped by the addition of SDS (2% final) instead of aprotinin.

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**Other Procedures—**

Protein concentrations were determined by the micro BCA protein assay (Pierce) using bovine serum albumin as a standard and/or by the extinction coefficient. Sequence analysis was performed according to Matsudaira (33) employing an Applied Biosistem protein sequencer model 477A, connected to a model 120A analyzer. SDS-PAGE was performed by the method of Laemmli (34) using 15% polyacrylamide gels, and protein bands were visualized utilizing Coomassie Brilliant Blue, 0.5% trichloroacetic acid, and 0.5% sodium dodecyl sulfate.

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**Extraction coefficients of 2.0 for purified recombinant pro-PSA and 3.0 for purified recombinant active hK2 were determined by A₂₈₀ and the protein concentrations measured by amino acid analysis. This corresponded well with the protein concentration as measured by the micro BCA protein.

**RESULTS**

Expression of Pro-PSA in E. coli—E. coli BL21(DE3) was transformed with the expression plasmid, pET12-pro-PSA. The cells were cultured and harvested 2 h after induction with 1 mM isopropyl-β-D-thiogalactoside. After sonication and centrifugation of the cells, essentially all of the recombinant pro-PSA was present in inclusion bodies. The inclusion bodies were then isolated and solubilized. Approximately 5 mg of unfolded pro-PSA was obtained from 1 liter of culture. The isolated pro-PSA was refolded as described under “Experimental Procedures” and purified by anion exchange and gel filtration columns. When applied to the anion exchange column (Resource Q), the
majority of the refolded pro-PSA was found in the non-adsorbed fraction, whereas the unrefolded and contaminant proteins remained bound to the column. The non-adsorbed fraction was pooled and concentrated to 5 ml and subjected to gel filtration on a Superdex G-200 column (Fig. 1). The first peak contained a contaminant that migrated at the dye front on SDS-PAGE. The second peak contained pro-PSA with a molecular mass of 31 kDa (reduced) and 28 kDa (nonreduced) (Fig. 2, lanes 2 and 5).

Prior to purification, the refolded pro-PSA sample gave several bands besides the 28-kDa band when examined by nonreduced SDS-PAGE (Fig. 2, lane 4). These bands with various mobility differences are due to conformational differences and/or differential SDS binding. Following reduction, all these bands migrated as a single band corresponding to pro-PSA (Fig. 2, lane 1), indicating that a significant portion of the soluble pro-PSA was folded incorrectly. These proteins, which bound to the Resource-Q column, generated little or no amidolytic activity upon incubation with trypsin.

Amino acid sequence analysis of the purified pro-PSA gave two amino-terminal sequences, Met-Ala-Pro-Leu-Ile-Leu-Ser-Arg-Ile-Val-Gly-Gly- and Ala-Pro-Leu-Ile-Leu-Ser-Arg-Ile-Val-Gly-Gly-, at a ratio of approximately 1:1. This indicated that the initiation Met was only partially removed in the E. coli cells. The pro-PSA was approximately 4 kDa smaller than the major band of human PSA purified from seminal plasma (spPSA) (14, 18) contributes to this mass difference. Under the above conditions, approximately 6 mg of refolded pro-PSA was routinely obtained from 25 mg of the solubilized inclusion body fraction with an overall yield of about 24%.

Activation of Pro-PSA by Trypsin—Pro-PSA was incubated with trypsin at a 50:1 weight ratio at 37 °C for various times, and the activation reaction was terminated with aprotinin. The amidase activity for pro-PSA was then determined using S-2586 as substrate. Aprotinin did not affect the amidolytic activity of PSA but completely inhibited trypsin. As shown in Fig. 3, the enzyme activity of PSA increased rapidly and reached a maximum in about 2 min followed by a slow decline. After the 2-min activation, degradation products were not seen by reduced SDS-PAGE and Western blot analyses (data not shown). Sequence analysis of the fully activated PSA gave a single new amino-terminal sequence of Ile-Val-Gly-Gly-. These results indicated that trypsin completely cleaved the Arg7-Ile8 peptide bond and did not cleave other bonds in pro-PSA under the conditions employed. This activation was exclusively caused by trypsin since recombinant PSA failed to autoactivate pro-PSA (weight ratio of 10:1) under the same conditions.

The propiece (activation peptide) released by trypsin cleavage of pro-PSA was identified in the supernatant of trichloroacetic acid precipitation of the reaction mixture. The supernatant was applied to a reverse phase HPLC C18 column, and several peaks were collected for amino acid analysis (Fig. 4). Only peaks A and B contained significant amounts of amino acids in their hydrolysates with the following compositions: peak A, Ala (1.0), Pro (0.9), Leu (1.8), Ile (0.9), Ser (0.7), and Arg (0.7); and peak B, Met (0.6), Ala (1.0), Pro (0.8), Leu (1.5), Ile (0.7), Ser (0.6), and Arg (0.6). These compositions are in complete agreement with the propiece sequence of Ala-Pro-Leu-Ile-Leu-Ser-Arg encoded by the PSA cDNA (14).

The specific activity of rPSA (5 μg/100 μl) generated by trypsin activation was 133 nmol of p-nitroaniline/mg protein/min toward S-2586 (1 mM final). This activity was more than 2-fold greater than that obtained by an equivalent amount of spPSA. The lower specific activity of the spPSA was likely due, in part, to the presence of contaminating proteins in this preparation, as observed by SDS-PAGE (Fig. 2, lanes 3 and 6).

Activation of Pro-PSA by Recombinant Human Glandular Kallikrein (rhK2) and Seminal Plasma Fractions—To test for potential physiological activator(s) of pro-PSA, the zymogen was examined for activation by rhK2, a proteolytic enzyme that is also synthesized in the prostate gland. The rhK2 was prepared in E. coli under conditions essentially identical to pro-PSA employing the plasmid pET12-pro-hK2. The inclusion bodies were isolated and solubilized, and the rhK2 was refolded as described for pro-PSA. Purification of the rhK2 was carried out on a Resource Q anionic exchange column eluted with a NaCl gradient (0–0.8 M) (Fig. 5). The first two peaks labeled A and C migrated as sharp single bands on SDS-PAGE with a molecular mass of about 27 kDa (Fig. 5, inset). Furthermore, both contained an amino-terminal sequence of Ile-Val-Gly-Gly-, which is characteristic of the activated form of the protein. Peak A containing rhK2 also showed significant amidase activity toward Pro-Phe-Arg-pNA (S-2302) but no activity toward Arg-Pro-Tyr-pNA (S-2586). Furthermore, rPSA, in contrast to rhK2, has no activity toward S-2302. Peak C may contain incompletely refolded protein. Pro-PSA was then incubated with the rhK2 at a molar ratio of 15:1. About 80% activation of
the zymogen was observed within 1 h when aliquots were analyzed for PSA activity against S-2586 (Fig. 6A). In these experiments, the activation of pro-PSA was stopped by the addition of aprotinin prior to the PSA assay. In contrast to trypsin, a substantially greater amount of aprotinin (10 ×) was required to completely inhibit rhK2. Amino-terminal sequence of the PSA activated by hK2 was Ile-Val-Gly-Gly-. The level of this sequence increased in parallel to the generation of amidolytic activity in the time course experiment.

Crude seminal plasma failed to activate pro-PSA in contrast to an ammonium sulfate fraction (45–65% saturation) that was active (Fig. 6B). The activating protease from seminal plasma was subject to additional purification using a gel filtration column, where the activator activity was found in the 60–120-kDa range. Accordingly, this protease appears to be much larger than hK2. These results indicated that another activating enzyme(s) may also be present in seminal fluid. Another
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possibility would be that hK2 may have been bound to a separate protein with a resultant higher total molecular mass. A detailed characterization of this latter activating enzyme(s), however, will require substantial purification.

A number of other proteolytic enzymes were also tested for their potential to activate pro-PSA. These included plasmin (substrate:enzyme weight ratio, 50:1), thrombin (10:1), factor IXa (10:1), and factor Xa (10:1), in the presence or absence of 5 mM CaCl2 (for factors IXa and Xa only). None of these enzymes activated pro-PSA under the present assay conditions.

Inhibition of rPSA by α1-Antichymotrypsin Inhibitor (ACT)—To test the effect of ACT on rPSA, the enzyme was incubated for 2 h with varying amounts of the inhibitor, and the remaining enzyme activity was measured by the amidolytic assay with S-2586. The enzyme activity decreased with increasing amounts of the inhibitor with complete inactivation achieved with a molar ratio of inhibitor to enzyme of about 2:1 (Fig. 7). These results were similar to those previously published for PSA isolated from seminal plasma (16). rPSA, human PSA from seminal plasma, or pro-PSA was then incubated with 2-fold molar amount of ACT, and the samples were analyzed by SDS-PAGE (Fig. 7, inset). Incubation of rPSA with ACT resulted in the formation of a complex of approximately 100 kDa (lane 6). This molecular mass corresponds to a 1:1 complex of rPSA (31 kDa) and ACT (70 kDa). PSA derived from seminal plasma also formed a 1:1 complex with ACT (lane 3), whereas pro-PSA did not interact with the inhibitor (lane 5).

Effect of PSA and rhK2 on Single Chain Urokinase-type Plasminogen Activator—When scuPA was incubated with recombinant PSA at a 1:1 weight ratio at 37 °C, no detectable activation of the plasminogen activator, measured by the hydrolysis of S-2444, could be detected (Fig. 8A, open circles). In contrast, scuPA was readily activated by rhK2 at a 1:1 weight ratio (Fig. 8A, closed circles). In these experiments, the rhK2 was inhibited by aprotinin prior to the assay of the plasminogen activator. SDS-PAGE of the reaction mixture under reducing conditions clearly demonstrated the formation of the heavy chain (~34kDa) and the weakly staining light chain (~21 kDa) of urokinase as the activation reaction proceeded (Fig. 8B).

DISCUSSION

Although PSA has been well studied as a tumor marker for prostate cancer, the physiologic role(s) and the activation mechanism of PSA has not been extensively examined. Besides its ability to cleave seminal vesicle proteins involved in seminal clot formation, other potential enzymatic functions of PSA have recently been described. First, PSA has been reported to cleave insulin-like growth factor binding protein 3 and could lead to the release of insulin-like growth factor from this regulatory protein (35). It is not known if PSA is directly involved in growth regulation by this mechanism. Second, PSA has also been reported to cleave parathyroid hormone-related protein (36, 37). Because parathyroid hormone-related protein has mitogenic effects and is found in both prostate epithelial cells and seminal plasma, PSA may be involved in a separate growth regulatory mechanism. Third, PSA has been reported to have proteolytic properties against pro-epidermal growth factor (38). If PSA indeed regulates growth mediators as reported above, the activation of pro-PSA should have significant physiological consequences besides seminal clot resolution.

The activation mechanism of pro-PSA may be complex and involve several enzymes. hK2, a potential physiological pro-PSA activator, is synthesized as a precursor. The activator that was partially purified from seminal plasma may also be synthesized as a precursor. Accordingly, these precursors will require proteolytic activation prior to the activation of pro-PSA. Thus, it seems likely that a “cascade mechanism” such as that found in fibrinolysis or blood coagulation (39) may exist in both prostate epithelial cells and seminal plasma.

To study the activation mechanism for pro-PSA, methods were developed to prepare recombinant PSA in a precursor form. This is because conventional means of purifying PSA from seminal plasma result in complete activation of the precursor. More importantly, the use of recombinant pro-enzymes avoids the problem of contaminating enzymes in other preparations (see below). Pro-PSA was expressed by pET112 system in E. coli, refolded, and purified with a relatively high yield. It has been shown that the efficiency of protein refolding is dependent upon the purity of the starting material (40–42). We were able to achieve >90% purity of pro-PSA in the solubilized inclusion bodies, mainly because of a high level of expression of the protein (5–10 mg of protein/liter of culture). For the refolding of PSA, denaturants and redox reagents were selected based on standard conditions (40–44).
Optimization of the refolding buffer, however, was necessary. For example, it was important to remove urea by dialyzing directly against a low ionic strength buffer. Stepwise removal of urea by dialysis against a buffer containing 1–3 M urea resulted in partial precipitation of the protein and incomplete refolding. In the present method, approximately a quarter of the soluble protein was refolded, making it possible to obtain pure pro-PSA with a final yield of 24% of the total inclusion body proteins. The evidence that pro-PSA was refolded correctly included the following: 1) efficient and complete activation by trypsin, 2) strong enzymatic activity of activated pro-PSA against S-2586, 3) inhibition by its physiologic inhibitor ACT, which forms an equimolar complex with pro-PSA, and 4) immunoreactivity with monoclonal anti-PSA antibody. 2

One of the most important differences between the recombinant preparations of PSA and hK2 compared with these enzymes purified from seminal plasma may be the presence of contaminating enzymes in the latter preparations. For example, this was one of the reasons why rPSA exhibited more than a 2-fold higher amidolytic activity relative to PSA derived from seminal plasma (spPSA). The lower activity of spPSA was due in part to protein contamination in the preparation (Fig. 2, lanes 3 and 6). Furthermore, Western blot analysis of spPSA performed under reducing conditions showed the presence of several bands of lower molecular weight, indicating that some degradation of PSA may have occurred during and/or prior to purification. Christensson et al. (16) also reported degradation in their spPSA preparation. By active-site titration of spPSA with 4-nitrophenyl guanidobenzoate, their preparation contained approximately 35% inactive protein (16). These authors have also suggested that some spPSA preparations have contaminating proteases such as trypsin. By utilizing aprotinin-Sepharose chromatography, they were able to completely eliminate kallikrein-like activity in their spPSA preparation (16).

Another possibility would be the presence of contaminating chymotrypsin-like enzymes in spPSA preparations. Akiyama et al. (15) reported that their spPSA cleaved denatured lysozyme and insulin A and B chains at several sites. The bonds between Leu-Ser, Phe-Glu, and Leu-Cys(SO3H) were rapidly hydrolyzed by their spPSA preparation. In our preliminary studies, synthetic peptides with sequences based on the reported cleavage sites in the B chain of insulin was readily cleaved by spPSA in contrast to rPSA, which was inactive. 2 The rPSA cleaved the Tyr-pNA bond in the substrate S-2586 as observed with other PSA preparations but did not cleave the leucyl or phenylalanyl peptide bonds in the synthetic peptide (B chain of insulin). This result indicated that spPSA preparations often are contaminated with other chymotrypsin-like enzymes(s).

To characterize the activation system for pro-PSA and to test a candidate activator in seminal plasma, we expressed and refolded pro-hK2. After refolding and purification, the recombinant hK2 was found exclusively in the active form as shown by both its activity toward S-2302 and its amino-terminal sequence. This spontaneous generation of the active form from the pro-hK2 was probably due to auto-activation. Recently, Dube et al. (31) isolated hK2 from seminal plasma by immunoaffinity column chromatography. However, the physiologic role of this protein has not been described. Prostasin, another serine protease found in seminal plasma, has also been shown to have kallikrein-like activity against synthetic peptides (45). Although this protein is not specific to the prostate, its potential role in activating precursors of other serine proteases in seminal plasma such as pro-PSA, pro-hK2, or scuPA has not been described.

Urokinase-type plasminogen activator is associated with prostate cancer invasion and metastasis (46–49). This protein is synthesized in a scuPA that requires activation by another enzyme to acquire biological function. Once activated, urokinase-type plasminogen activator mediates other proteolytic events through the generation of plasmin from plasminogen. For example, plasmin has been shown to activate latent growth factors (50) as well as latent collagenase (51, 52). Consequently, plasmin may promote tumor growth and invasion. The mechanisms involved in regulating urokinase-type plasminogen activator activity may therefore have a significant role in prostate cancer.

The physiologic activator of scuPA in the prostate has not been fully characterized. Recently, Yoshida et al. (22) reported that spPSA could activate scuPA. However, PSA has chymotrypsin-like specificity and is unlikely to cleave the activation site of scuPA which is the Lys150–H Gly159 bond. Since PSA has 80% sequence homology with hK2 it seems likely that hK2 may be co- purified with PSA (16). Also, hK2 has trypsin-like activity and is more likely to activate scuPA. Our study demonstrates that, indeed, the recombinant PSA is unable to activate scuPA, whereas the recombinant hK2 activated scuPA in a time-dependent manner. Recent preliminary studies with hK2 immunoassays suggest that some patients with prostate cancer have elevated hK2 levels that are discordant with PSA levels. These findings suggest that hK2 may be uniquely involved in prostate cancer metastasis by activating scuPA at the tissue level. Whether hK2 activity against scuPA has physiologic importance requires further investigation.

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Addendum—Soon after submission of this article, the activation of pro-PSA by hK2 was reported independently by another group (53).

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