Expression of Pro Form of Prostate-specific Antigen by Mammalian Cells and Its Conversion to Mature, Active Form by Human Kallikrein 2

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Abstract

To study the expression, biosynthesis, and processing of prostate-specific antigen (PSA) in mammalian cells, recombinant PSA was expressed in Syrian hamster tumor cell line AV12-664 (AV12-PSA). Expression of PSA was monitored by the Tandem-MP PSA assay. PSA was secreted into the medium during the logarithmic phase of cell growth at >9 μg/ml and was stable. The PSA purified from spent medium of AV12-PSA cells did not exhibit any enzymatic activity and did not complex with the protease inhibitor, α-1-antichymotrypsin. These findings indicate that an inactive form of PSA was expressed by AV12-PSA cells. NH2-terminal sequencing confirmed the identity of the PSA purified from the spent medium of AV12-PSA cells to be pro-PSA. This demonstrates that PSA is expressed as pro-PSA by mammalian cells and suggests that pro-PSA may be present in biological fluids. Human kallikrein 2 (hK2), another member of the hK family, is also expressed predominantly in prostate epithelium. Although hK2 has been shown to exhibit trypsin-like activity, little is known about its natural substrates. Using purified proteins, we show that hK2 can convert pro-PSA to mature, enzymatically active PSA, thus establishing a physiological connection between hK2 and PSA. These findings imply that hK2 may be regulating PSA activity in vivo.

Introduction

PCa2 is the most frequently diagnosed cancer in American males. PSA has been widely used as a reliable prognostic marker in the management of patients with PCa (1). PSA, a member of the hK family of serine proteases, is a M, ~34,000 single-chain glycoprotein with one N-linked oligosaccharide attached to asparagine 45 (reviewed in Refs. 2–4). Molecular cloning of cDNA revealed that the mRNA of PSA codes for a 261-aa preproprotein, in which a hydrophilic signal sequence of 17 aa (prepro region) and a propeptide of 7 aa precede the mature protein of 237 aa (5). PSA mRNA expression is predominant in prostate epithelium (6) and is regulated by androgens (7). PSA has been shown to have chymotrypsin-like activity (8, 9), and its proposed primary biological role is to cleave the major gel-forming proteins, semenogelin I and II and fibronectin, in seminal fluid, resulting in enhanced sperm motility (10). More recently, PSA has been reported to cleave insulin-like growth factor-binding protein-3, resulting in increased availability of insulin-like growth factor, suggesting that PSA enzymatic activity may promote proliferation, migration, and metastasis of PCa cells (11). Although the above-mentioned studies emphasize the possible physiological substrates of PSA, the fundamental questions regarding its biosynthesis, activation, and regulation of activity remain unanswered.

The hK family consists of three members designated as hK1, hK2, and hK3 (PSA: Refs. 2 and 4). hK1 is primarily produced in the kidney, pancreas, and submandibular salivary gland (12). hK2, like PSA, is produced predominantly in the prostate epithelium (13), is regulated by androgens, and shares 78% aa homology with PSA (2, 4). The attributes of hK2 as a potential PCa marker have recently been reviewed (14). PSA is a chymotrypsin-like protease, whereas hK2 is a trypsin-like protease (15), indicating that the two enzymes may have different physiological roles. Little is known about the physiological role(s) of hK2. Deperthes et al. (16) have recently shown that fibronectin and semenogelins present in ejaculate were hydrolyzed by hK2. Although PSA and hK2 have been shown to be present in the same environment, their interaction with each other has not been studied.

To study biosynthesis and the regulation of the activation of PSA and to explore the physiological relationship between hK2 and PSA, it is imperative to stably express PSA and hK2 at high levels in mammalian cells. We have reported the expression and purification of hK2 in mammalian cells previously (17). Stable and high expression of PSA in mammalian cells has not yet been reported. In this study, we report the development of a mammalian cell line with high expression (>9 μg/ml) of PSA. We demonstrate that PSA is secreted into the spent medium by mammalian cells as enzymatically inactive and stable pPSA. pPSA was converted to enzymatically active PSA by hK2. This suggests a possible physiological relationship between hK2 and PSA and implies that hK2 could be regulating the PSA activity in vivo. These studies also suggest that pPSA may exist in the biological fluid and may serve as a useful marker for PCa.

Materials and Methods

Expression Vector, Cell Line, and Transfection. A 0.8-kb cDNA coding for entire pre-pPSA was cloned into the Bc/II site of pcDNA3, under the control of the GBMT promoter, using an approach similar to the one described earlier (17), resulting in the expression vector pGTD-PSA (Fig. 1). The orientation and sequence of the insert was confirmed. AV12-664 (ATCC CRL 9955) cells, cultured in DMEM (high glucose) and 10% Fetal Clone (Hyclone Laboratories, Inc., Logan, UT), were transfected with pGTD-PSA or pGTD-h using Lipofectamine (Life Technologies, Inc., Gaithersburg, MD) and single-cell clones AV12-PSA and AV12-PGTD were selected in 400 μM methotrexate (Sigma Chemical Co., St. Louis, MO).

ELISA and Western Blot Analyses. Murine mAbs HK1G 586.1 (17) and PSM 773 (18) have been described previously. PSM 773 is one of the components of Tandem-MP PSA assay (Hybritech Incorporated, San Diego, CA) and is specific for PSA. For ELISA analysis, serum-containing spent medium from AV12-PSA cells was collected on specified days. Total and uncomplexed (free) forms of PSA were measured using Tandem-MP PSA and Tandem-MP free PSA assays (both from Hybritech), respectively. Spent media from AV12 and AV12-PGTD cells were used as negative controls. For Western blot analysis, tissue culture flasks containing AV12-PSA cells were grown to approximately 60–70% confluency and washed with PBS, and serum-free H14 medium (JRH Biosciences, Lenexa, KS) was added. Serum-free spent medium (spent medium) harvested on specified days was concentrated using a Centricon 10 (Amicon, Inc., Beverly, MA), incubated with either...
pGTD-PSA

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\begin{align*}
\text{ATG TGG TGC CCG GTT GTC TTC CTC ACC CTG} \\
\text{Met Trp Val Pro Val Val Phe Leu Thr Leu} \\
\text{TCC GTG ACG TGG ATT GGC GCT GGG CCC CTC} \\
\text{Ser Val Thr Trp Ile Gly Ala Ala Pro Leu} \\
\text{ATC CTG CCT CGG ATT GTG GGA GGC TGG GAG} \\
\text{Ile Leu Ser Arg Ile Val Gly Gly Trp Glu}
\end{align*}
\]

Fig. 1. Schematic representation of PSA expression vector, pGTD-PSA.

To study the expression of PSA, AV12 cells were transfected with the pGTD-PSA expression vector. Cells were selected in 400 nM methotrexate for 2–3 weeks, and single-cell clones were analyzed for PSA expression using Tandem-MP PSA assay and Western blots using mAb PSM 773. Clone AV12-PSA#8 was selected for further studies, on the basis of its high expression levels (>9 µg/ml) of a PSA-immunoreactive band at \( M_r \) 34,000.

To study the PSA expression pattern in mammalian cells, spent media from AV12-PSA#8 cells were collected for 6 consecutive days and analyzed using the Tandem-MP PSA assay (Fig. 2). PSA was detected in spent media at day 1, and it accumulated to >9 µg/ml by day 6. Expression of PSA was higher during the logarithmic phase of cell growth, indicating that a stable form of PSA is secreted by the cells, as opposed to being released following cell death and lysis. When the same samples were analyzed by Tandem-MP free PSA assay, similar values were obtained (data not shown), indicating that AV12-PSA#8 cells express uncomplexed or free PSA.

To determine the identity of the protein that is secreted on day 1, the spent media from AV12-PSA#8 cells were collected and concentrated. The PSA in the media was purified by affinity chromatography using PSM 773, a PSA-specific mAb. NH₂-terminal sequencing of the purified protein revealed the sequence: APLILSRIVGG. This sequence corresponds with the sequence predicted for the NH₂-terminus of pPSA that starts with Ala⁻¹. Another species, starting at Leu⁻³, was also identified at comparable levels. Similar results were obtained when the same purification protocol was used to purify PSA from day 7 spent media of AV12-PSA#8 cells. This result indicates that PSA is secreted as pPSA by AV12-PSA#8 cells beginning on day 1 and that pPSA is stable in the spent media, even after 7 days.

To demonstrate that pPSA expressed by AV12-PSA#8 is enzymatically inactive and can be converted to enzymatically active PSA by mild trypsin treatment, a spectrophotometric assay was used, using the commercially available chromogenic substrate, methoxysuccinyl-Arg-Pro-Tyr-pNA (S-2586). A 10-fold increase in activity (from 2.38 × 10⁻⁴ to 2.27 × 10⁻³ activity units/min) was observed when spent media from AV12-PSA#8 (containing the equivalent to 14 µg of PSA, as measured by Tandem-MP PSA assay) were treated with trypsin (0.28 µg) for 60 min at 37°C. Spent media from AV12-PGTD cells (AV12 cells transfected by empty pGT-d vector) that were similarly treated with trypsin showed no detectable activity, indicating that no endogenous PSA-like proteases are secreted by AV12 cells. The positive control, PSA (14 µg), purified from seminal plasma and similarly treated with trypsin, exhibited an activity of 9.49 × 10⁻³ activity units/min.

It has previously been shown that PSA complexes with ACT in
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serum. We inferred that enzymatically inactive pPSA would not complex with ACT. To test whether the trypsin converted PSA would complex with ACT, ACT was added to the spent media of AV12-PSA#8 cells following preincubation with trypsin. After 4 h, the reaction was stopped by the addition of SDS-PAGE sample buffer and β-mercaptoethanol and boiling. The samples were then electrophoresed, electroblotted, and probed with PSA-specific mAb PSM 773 mAb (Fig. 3). A M₉₄,400 complex comigrating with PSA-ACT (Lane 1) was detected in trypsin-treated spent media of AV12-PSA#8 cells (Lane 6). In contrast, PSA-ACT complex was not observed when untreated AV12-PSA#8 spent media were incubated with ACT (Lane 4). No immunoreactive band was observed when trypsin-preincubated AV12-PSA#8 spent media were incubated with ACT (Lane 8). Although hK₂ and PSA share ~78% aa similarity, hK₂ was not immunodetected by PSM 773 mAb (Lane 9), confirming the PSA specificity of this mAb. These results indicate that hK₂ can convert enzymatically inactive pPSA to an active form of PSA, which can form a complex with ACT.

To confirm the above results, affinity-purified preparations of hK₂ (15, 17) and pPSA were mixed and the conversion of pPSA to mature PSA was analyzed by HIC (Fig. 4) and aa sequencing. Despite their similarities, hK₂, pPSA, and mature PSA have distinctly different retention times. Our results confirmed that pPSA in the day 7 spent media of AV12-PSA#8 cells was enzymatically inactive and did not complex with ACT. These results also indicated that, upon mild trypsin treatment, this pPSA was converted to an enzymatically active form that could covalently complex with ACT. It appears that Arg₋⁴ of pPSA is the major site for trypsin reactivity because no PSA degradation products were detected by Western blot using PF1D 215 mAb (data not shown). PF1D 215 mAb has previously been shown to detect PSA degradation products present in seminal fluid (20).

hK₂, another member of hK family, is predominantly expressed in the prostate epithelium, the site where PSA is also abundantly expressed. hK₂ exhibits arginine-restricted trypsin-like activity (15). To test whether pPSA can be converted to PSA by hK₂, purified hK₂ was added to the spent media of AV12-PSA#8 cells. After 60 min of incubation at 37°C, ACT was added to these samples. After another 4 h of incubation at 37°C, the samples were electrophoresed, electroblotted, and probed with PSM773 (Fig. 3). A M₉₋₉₄,000 band comigrating with purified PSA-ACT (Lane 1) was detected in hK₂-treated spent media (Lane 8). Although hK₂ and PSA share ~78% aa similarity, hK₂ was not immunodetected by PSM 773 mAb (Lane 9), confirming the PSA specificity of this mAb. These results indicate that hK₂ can convert enzymatically inactive pPSA to an active form of PSA, which can form a complex with ACT.

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PSA enzymatic activity. Control sample of purified pPSA incubated without hK2 showed no conversion to PSA (data not shown).

Discussion

Like other serine proteases, PSA is translated as an inactive pro-precursor. After passage through the secretory pathway, the signal peptide is cleaved, yielding the pro form of the protein. Here, we describe the development of a stable mammalian cell line (AV12-PSA#8) with high expression (>9 μg/ml) of PSA. The following four lines of evidence presented here unequivocally demonstrate that PSA is secreted as pPSA from mammalian cells: spent media of AV12-PSA#8 cells had no PSA-like enzymatic activity; the PSA form in the spent media did not complex with ACT, as demonstrated by Western blot analysis; trypsin treatment of the spent media resulted in mature complex with ACT (Fig. 3). This result suggests a possible physiological relationship between hK2 and PSA, as well as ACT and protein C inhibitor. PSA also exists as a PSA-a2-macroglobulin complex and as free PSA. Our data suggest that pPSA may exist in biological fluids, implying that some of the free PSA in the serum and seminal fluid may be pPSA. This suggests that pPSA may be a useful marker for prostatic diseases, a hypothesis currently under investigation.

Thus far, the protease(s) responsible for cleaving the propeptide from pPSA to form the active molecule have not been identified. hK2, another member of the hK family, exhibits arginine-restricted, trypsin-like activity (15), and like PSA, it is predominantly expressed in the prostate epithelium (13). hK2 is also secreted as prohK2 by mammalian cells (17), which is converted to enzymatically active mature hK2 extracellularly. hK2 has autocatalytic activity because it can cleave prohK2 at Arg−1, releasing the propeptide (21). Here, our data clearly show that mature hK2 clips the propeptide from the pPSA, converting it to enzymatically active mature PSA (Fig. 4), which can then complex with ACT (Fig. 3). This result suggests a possible physiological regulatory relationship between hK2 and PSA. Studies are in progress to discover if hK2 can also cleave mature PSA, further regulating PSA activity and complex formation with protease inhibitors. Similarly, PSA may also be regulating hK2 activity by clipping hK2 at chymotrypsin-sensitive sites. The reagents described here plus the hK2 expressed in mammalian cells (17) should facilitate these experiments.

The above results collectively indicate that PSA is expressed as pro form in mammalian cells and can be converted to the enzymatically active mature form extracellularly by hK2. These results also suggest that pPSA may be present in biological fluids and, therefore, could be a useful marker for prostatic diseases. The cell lines described here constitute a valuable source of pPSA to be used as an immunogen and to study the biological roles of pPSA and PSA in detail.

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References

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