The Biosynthesis and Secretion of Prostate-specific Antigen in LNCaP Cells

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ABSTRACT

Prostate-specific antigen (PSA) has been demonstrated to release the active form of insulin-like growth factor I in vitro (P. Cohen et al., J. Clin. Endocrinol. & Metab., 75: 1046-1053, 1992; P. Cohen et al., J. Clin. Endocrinol. & Metab., 79: 1410-1415, 1994; P. Cohen et al., Horm. Metab. Res., 26: 81-84, 1994) and has significant mitogenic activity on osteoblast cells, fibroblasts, and other cultured cells (C. S. Killian et al., Biochem. Biophys. Res. Commun., 192: 940-947, 1993). Recently, PSA has been found not only in prostate tissues but also in breast, colon, ovarian, and other tissues (E. P. Diamandis and H. Yu, J. Clin. Endocrinol. & Metab., 80: 1515-1517, 1995; E. P. Diamandis and H. Yu, Clin. Chem., 41: 204-210, 1995; A. Clements and A. Mukhtar, J. Clin. Endocrinol. & Metab., 78: 1536-1539, 1994). Therefore, PSA has been proposed as a candidate growth factor, cytokine, or growth factor regulator. In this setting, knowing how to manipulate or block the secretion of PSA by the prostate cancer cells could be a useful approach to controlling the progression of human prostate cancers. Using metabolic labeling experiments, we have studied the biosynthesis and secretion of PSA in LNCaP cells. We have further examined the effects of DTT, tunicamycin, 1-deoxy-mannojirimycin, pilocarpine, and testostereone on PSA biosynthesis and secretion. The results indicate that the secretion of PSA in LNCaP cells is constitutive instead of regulated and that the disruption of intramolecular disulfide bonds affects the transport of PSA from the endoplasmic reticulum to the Golgi apparatus. The biosynthesis of PSA is potentiated by testosterone and inhibited by brefeldin A and DTT. These results will help us understand PSA biosynthesis and secretion in human prostate cancers.

INTRODUCTION

PSA, one of the most abundant serum proteases produced by prostate epithelial cells, is a member of the kallikrein family (1) and is a glycoprotein containing approximately 7% carbohydrate (2.7% w/w) hexose, 2.8% w/w hexosamines, and 1.1% sialic acid; Ref. 2). The mature single peptide chain of PSA contains 237 amino acid residues (3), and the glycosylated form has a calculated Mr of 33,000. An N-linked carbohydrate side chain is attached to asparagine 45 (4, 5), and O-linked carbohydrate side chains, if present, are possibly attached to serine 69, threonine 70, and serine 71 (2). PSA is reported to be able to cleave the predominant seminal vesicle protein (6).

PSA has been implicated in growth regulation in vitro. PSA as low as 2.5 ng/ml has significant mitogenic activity, presumably due to activation by PSA of latent human transforming growth factor β or of other growth factors in the extracellular matrix. The release of active factors might then endow the cancerous cells with a growth advantage, leading to tumor progression. This hypothesis could also explain why prostate cancer cells tend to diffusely infiltrate the prostatic stroma rather than forming a localized and grossly visible tumor mass. Treatment targeting PSA secretion could be a useful approach for inhibiting prostate cancer cell growth. The basic knowledge of the intracellular processing and secretion of PSA in human prostate epithelial cells is still lacking. In this report, we characterized the PSA biosynthetic and secretory pathways in LNCaP cells by metabolic labeling experiments and further analyzed the roles of intramolecular disulfide bonds and glycosylation in the regulation of PSA secretion.

MATERIALS AND METHODS

Chemicals. Chemicals were all from Sigma (St. Louis, MO), except N-glycosidase F, neuraminidase, and O-glycosidase (Boehringer Mannheim).

Cells. LNCaP cells were purchased from American Type Culture Collection (Bethesda, MD). Cells were maintained in RPMI 1640 containing 2.5% or 5% FCS, penicillin, and streptomycin (5 µg/ml; Life Technologies, Inc., Grand Island, NY).

Metabolic Labeling and Pulse-Chase Experiments. Protein labeling was performed as described previously (14). Briefly, cells grown for 4 days were starved for 1 h in serum-free methionine-deficient RPMI 1640 at 37°C. The media were then changed, and cells were incubated in serum-free methionine-deficient RPMI 1640 containing Tras35S-Label (ICN, Costa Mesa, CA; 100 µCi/ml) for 10 min at 37°C. The labeling media were replaced by complete RPMI 1640 and chased for the indicated periods of time (10, 30, 60, and 120 min). Cells were stored on ice and lysed at the same time by 1% Triton X-100 in PBS (cell lysis buffer) containing the following freshly added protease inhibitors: phenylmethylsulfonyl fluoride (10 µg/ml), leupeptin (2 µg/ml), antipain (2 µg/ml), and pepstatin A (1 µg/ml). Cell lysate was incubated on ice for 10 min and centrifuged at 12,000 × g for 10 min at 4°C. Chase media were collected and centrifuged at 1,000 × g for 2 min to remove floating cells, and proteins were further precipitated by adding cold acetone to the supernatant (ratio, 7:3) at −20°C for 30 min. The precipitated proteins were resuspended in cell lysis buffer (300 µl).

Immunoprecipitation. The cell lysate and resuspended protein from chase media were precleared for 2 h in 30 µl of 10% suspension of formalin-fixed protein A (+) Staphylococcus aureus at 4°C. After centrifugation at 5,000 × g for 2 min, the supernatant was incubated for 2 h or overnight with primary antibody (rabbit antihuman PSA; DAKO) and protein A-agarose beads, which were previously incubated with 10% rabbit serum. Beads were washed three times in 0.5% Triton X-100, and the immunoprecipitated protein was eluted in 50 µl of 1% SDS and water boiled for 5 min. Samples were separated by 12% SDS-PAGE under reducing or nonreducing conditions, and fluorography was performed using the DMSO-PPO enhanced method (15). The dried gels were exposed on Kodak X-OMAT LS film for various times. Scanning densitometry was used to quantify images on a Millipore XRS Omni media scanner, and IOD values were reported (15).
Glycosidase and Alkaline Phosphatase Treatments. PSA immunoprecipitate from radiolabeled cells was treated as follows: (a) endoH, the released protein, was resuspended in citrate phosphate buffer (pH 5.75) and treated with endoH (6 milliunits) at 37°C for 12 h or overnight; (b) N-glycosidase F, the released protein, was resuspended in 0.5% NP40 (1:9) PBS and incubated for 12 h with 0.4 unit of N-glycosidase F; (c) neuraminidase, a PSA immunoprecipitate, was incubated for 6 h at 37°C with 0.1 unit of neuraminidase from Vibrio cholerae (Boehringer Mannheim); (d) O-glycosidase, the released protein, was treated as described in c for 6 h and then resuspended in Triton X-100 (1%) containing 1.5 milliunits of O-glycosidase overnight; (e) for total removal of carbohydrates, the immunoprecipitated PSA was resuspended in 50 mM sodium phosphates and incubated with a mixture of neuraminidase, O-glycosidase, and N-glycosidase as mentioned in the manufacturer’s protocol (Boehringer Mannheim); and (f) alkaline phosphatase, a PSA immunoprecipitate, was washed twice in 50 mM Tris-HCl buffer (pH 7.5) containing 120 mM NaCl and incubated with 10 mg/ml alkaline phosphatase for 6 h. The treatments were terminated by incubation with absolute ethanol at −70°C for 30 min, followed by centrifugation at 12,000 × g for 12 min and resuspension in the lysis buffer.

RESULTS AND DISCUSSION

PSA Molecule Is Susceptible to the Digestion of EndoH and N-Glycosidase F. To determine whether the attached carbohydrates on PSA molecules from LNCaP cells are O-linked as well as N-linked, we radiolabeled protein, immunoprecipitated PSA by anti-PSA antibody, and incubated the immunoprecipitated PSA with endoH, N-glycosidase F, neuraminidase, and subsequently O-glycosidase, alkaline phosphatase, and neuraminidase. In Fig. 1, PSA (shown as arrow a) is subject to the digestion of endoH (Lane 2) and N-glycosidase F (Lane 5) but not to that of O-glycosidase (Lane 4) or alkaline phosphatase (Lane 7). The treatment with the mixture of neuraminidase, N-glycosidase F, and O-glycosidase (Lane 6) did not show the further migration of digested PSA compared to Lane 5 (treated with N-glycosidase F only). This 27-kDa band of PSA is also present in Lane 1 (control), which is probably due to the mild degradation of the immunoprecipitated PSA. These findings suggest that the immunoprecipitated PSA from LNCaP cells contains N-linked but not O-linked carbohydrates. We could not rule out the presence of sialic acids in the PSA molecule from the one-dimensional SDS-PAGE (Lane 3, treated with neuraminidase).

The General Biosynthetic and Secretory Pathway of PSA in LNCaP Cells. To describe the biosynthetic and secretory pathways of PSA, we did pulse-chase experiments in LNCaP cells, the only currently characterized prostate cancer cell line known to secrete PSA.

![Fig. 1. Digestion of immunoprecipitated PSA by enzymes. Immunoprecipitated PSA was incubated with citrate phosphate buffer only (Lane 1), endoH (Lane 2), neuraminidase (Lane 3), or O-glycosidase after neuraminidase digestion (Lane 4), N-glycosidase F (Lane 5), mixture of neuraminidase, O-glycosidase, and N-glycosidase F (Lane 6), and alkaline phosphatase (Lane 7) in appropriate buffer solutions (see "Materials and Methods"). Samples were analyzed by 12% SDS-PAGE at reducing conditions, and fluorography was performed. Arrow a, undigested PSA.](https://cancerres.aacrjournals.org/)

LNCaP cells were pulsed with Tran35S-Label for 10 min and chased for 5, 30, 60, 90, 120, and 160 min. Because glycoprotein undergoes modification in the ER and Golgi apparatus, the sensitivity to endoH could be used as an analytic tool to distinguish the proteins in the ER (the endoH-sensitive or ER form) from those being processed in the medi-Golgi apparatus (the endoH-resistant or Golgi form). As shown in Fig. 2a, the Golgi form of PSA (the Mr 33,000 band indicated by arrow a in the presence of endoH) first appears between the 5- and the 30-min chase. The amount and the percentage of the Golgi form of PSA increases slowly over time. However, the ER form of PSA (the Mr 27,000 band in the presence of the endoH) still constitutes a predominate form. The secreted form of PSA, Mr 33,000 (shown as a single light band having the same position as arrow a), is detected between the 90- and the 120-min chase period in the culture media. As noted here, the Mr 27,000 band, which appears in those samples without treatment of endoH (especially for the chase times of 5 and 30 min), is most likely due to the degradation product of core-glycosylated PSA or, less likely, to the nonglycosylated product.

The kinetic data of PSA transport during the chase period are pooled from the controls with or without ethanol treatment and are shown in Fig. 2b. In cells without ethanol treatment, approximately 20% of the newly synthesized PSA was transported to the compartments of the medi-Golgi apparatus at the 30-min chase. During the next 30-min chase, approximately 32% of the newly synthesized PSA was in the Golgi form. However, there was still a substantial percentage (approximately 40–50%) of PSA retained in the ER or cis-Golgi apparatus after the 120- and 160-min chase. Cells treated with ethanol.

![Fig. 2. a, metabolic labeling of proteins and immunoprecipitation of PSA. LNCaP cells were pulsed with Tran35S-Label (100 μCi/ml) for 10 min and chased for the indicated period of time. Immunoprecipitated PSA from cell lysate was processed for endoH digestion and analyzed by 12% SDS-PAGE at reducing conditions, and fluorography was performed. Arrow a, undigested or endoH-resistant PSA. b, quantitation of the kinetic data of PSA biosynthesis and secretion. Cells were treated either with or without ethanol (final concentration at 0.2%) for 1 h before the pulse experiment (for 10 min) and during the chase period. The percentage of the endoH-resistant form was calculated from the IOD values obtained by using a Millipore XRS Omni media scanner. Vertical lines, SE for the average of three individual experiments.](https://cancerres.aacrjournals.org/)
The PSA secretion is constitutive, and there is no evidence of storage in the secretory vesicles. By definition, proteins destined for constitutive secretion bypass the storage of secretory products in secretory granules and are transported directly from the Golgi apparatus to the outside of cells by exocytosis. In contrast, proteins destined for regulated secretion are stored or concentrated in the secretory vesicles and are released in response to stimuli (16). In this setting, a secretagogue has been used to distinguish constitutive from regulated secretion. Pilocarpine is reported to be the most effective secretagogue for prostate epithelial cells (17). To test whether LNCaP cells secrete PSA in a constitutive way, LNCaP cells were treated with pilocarpine as the secretagogue. First of all, we did not see any dose-response effect of pilocarpine on PSA secretion. We did not see any endoH-resistant form at the chase time of 30 min; and (b) a higher percentage of endoH-resistant form appears after 60 min of chase.

The effects of TM and deoxymannojirimycin on PSA biosynthesis and secretion. Glycosylation often plays a role in protein stability and secretion (22). TM is an inhibitor of N-glycosylation (23) and can be used to evaluate the role of glycosylation in glycoprotein secretion. Cells were treated with TM (1—10 μg/ml and 0.2% ethanol at final concentration) for 1 h before the pulse and during the chase period. As shown in Table 2, the percentages of the endoH-resistant form at the 120-min chase period are 66.1% (5 μg/ml) and 56.1% (10 μg/ml), whereas that of the control is 54.0% (treated with 0.2% ethanol). The secreted PSA is detected in media from both TM-treated and control cells at the 120-min chase period. Thus, PSA biosynthesis is relatively resistant to the effect of TM.

Deoxymannojirimycin, which inhibits mannosidase IA/B and causes the accumulation of high-mannose oligosaccharides of the secretory vesicles, was used to determine whether the secreted PSA is glycosylated. It has been demonstrated that deoxymannojirimycin accumulates high-mannose oligosaccharides in a variety of cell types (24). The percentage of endoH-resistant form in the deoxymannojirimycin-treated cells is markedly decreased compared to that of controls over the chase period.

Table 1 The percentage of PSA release in LNCaP cells after 1-h treatment of pilocarpine

<table>
<thead>
<tr>
<th>Experiment</th>
<th>PSA media (ng/ml)</th>
<th>Cell-associated PSA/mg protein</th>
<th>Secretion index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 28</td>
<td>154</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>10^{-7} M</td>
<td>26</td>
<td>155</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>155</td>
<td>17</td>
</tr>
<tr>
<td>B. Experiment II (metabolic labeling for 10 h)</td>
<td>IOD</td>
<td>IOD</td>
<td></td>
</tr>
<tr>
<td>Control 0.3</td>
<td>15.2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>10^{-7} M</td>
<td>0.01</td>
<td>14.1</td>
</tr>
</tbody>
</table>

(0.2%) have a similar profile to cells without any treatment, except: (a) only 13% of endoH-resistant form occurs at the chase time of 30 min; and (b) a higher percentage of endoH-resistant form appears after 60 min of chase.
Man8–9 (GlcNAc)2 structure (23), is used to evaluate the secretion of unprocessed PSA in LNCaP cells. We treated LNCaP cells with deoxymannojirimycin at the concentration of 1 mM for 1 h before the pulse experiment and during the chase period. The results reveal that most of the immunoprecipitated PSA is in the endoH-sensitive form, which contains high-mannose oligosaccharides. The percentage of the endoH-resistant form is 13.7% at the 120-mm chase period, compared to that of the control (43.9%; see Table 2). However, the secreted form of PSA is also detected in the media as the control. These findings substantiate the conclusion that carbohydrate processing is not required for PSA secretion in LNCaP cells.

**DTT Impairs the Transport of PSA from the ER to the Golgi Transiently and Reversibly.** DTT has been shown to create a reducing environment within the ER that interferes with the formation of disulfide bonds required for the correct folding in the early processing of many proteins (24). Secretion of proteins having no disulfide bonds, such as α-1-antitrypsin, is minimally affected by DTT. The primary structure of PSA has been presumed to hold a geometry similar to that of other serine proteases, such as trypsin, chymotrypsin, and γ-nerve growth factor (9, 11), based on the high amino acid sequence homology to the other serine proteases. If this is correct, PSA theoretically would have 5 disulfide bonds, because PSA has 10 cysteine residues (9). Because the proper tertiary and quaternary structures are important factors to ensure the export of proteins from the ER (24–26), we examined the effects of DTT and evaluated the role of intramolecular disulfide bonds on PSA transport and secretion. When LNCaP cells were treated with DTT (1 mM) for 100 min during the chase period, the percentage of the Golgi (endoH-resistant) form drops from 60 (in control) to 41% (Fig. 4a), and the amount of the newly synthesized PSA is dramatically decreased compared to that of the controls. The secreted PSA is not detectable in the 100-min chase media of the DTT-treated cells. As noted here, the band indicated by an arrow in Fig. 4a is higher in position than the 33-kDa bands, which is most likely due to the aggregation of a large amount of nondenatured and/or partially denatured PSA molecules.

The effects of DTT on PSA transport and secretion are reversible. LNCaP cells were treated with DTT (1 mM) for 2 min immediately after the 10-min pulse and recultured with serum-free complete media for the following chase period. PSA was immunoprecipitated by a mixture of monoclonal and polyclonal antibodies (DAKO) and analyzed by 12% SDS-PAGE under nonreducing conditions. At the 30-min chase, we observed a 30% decrease in the percentage of the endoH-resistant (Golgi) form compared to that of the control (Fig. 4b). At the 60- and 120-min chases, the percentage of the Golgi form approached almost the same level as that of the control, and the secreted form of PSA was found in both culture media at the 120-min chase. These findings suggest that the effect of DTT on the PSA molecule is both transient and reversible.

In this study, the decreasing amount of detectable and immunoprecipitated PSA found in the DTT-treated cells might be due to failed recognition of the unfolded PSA protein by the antibody during the immunoprecipitation procedure. A mixture of monoclonal and polyclonal antibodies was used in the immunoprecipitation procedure in the repeated DTT-treated experiments, which show results similar to those in Fig. 4a, although this does not rule out the possibility that failed recognition of the antibody to the unfolded PSA results in a decreased amount of immunoprecipitable PSA. On the other hand, PSA with disrupted intramolecular disulfide bonds might undergo degradation (shown in Fig. 4a) in the ER; thus, the PSA available for immunoprecipitation is decreasing.

**Dihydrotestosterone Facilitates the Biosynthesis and Secretion of PSA.** Dihydrotestosterone has been reported to increase the differentiation and production of PSA in LNCaP cells (27–29). We asked whether there was an effect of testosterone on PSA biosynthesis and transport between the ER and Golgi apparatus. We treated LNCaP cells with testosterone (10^{-8} M) for 12 h, followed by a 10-min pulse, and chased for the indicated period of time. As shown in Fig. 5, the testosterone-treated cells produced more cell-associated and secreted PSA when compared to the controls. We observed that the testosterone-treated LNCaP cells (for 12 h) have a cell proliferation index similar to that of the controls; therefore, the increased PSA production
is not caused by an increase in cell proliferation. The percentage of the endoH-resistant form at the 120-min chase period is 48%, compared to 54% for the control (Table 2). These results suggest that testosterone one treatment potentiates PSA biosynthesis and facilitates its transport from the ER toward the Golgi apparatus and secretion.

The Effect of Saponin on PSA Biosynthesis and Secretion. Saponins have been known to have direct cytotoxic and growth-inhibitory effects against human colon cancer cell line HCT-15 with dose response and could increase the number of vesicles (30). We were interested in knowing whether saponins could alter the secretory pathway of prostate tumor cells by increasing the number of vesicles, thereby increasing the storage of PSA in the cytoplasm. However, as shown in Table 2, no effect of saponin was noted.

In conclusion, we have defined the biosynthetic and secretory pathways of PSA in LNCaP cells and demonstrated that PSA secretion in LNCaP cells is constitutive. The biosynthesis of PSA is potentiated by testosterone and inhibited by BFA and DTT. The study with DTT treatment has demonstrated the presence of intramolecular disulfide bond(s) in PSA molecules, suggesting that the three-dimensional structure of PSA affects PSA transport from the ER toward the Golgi apparatus and secretion. Other tumors besides those of the prostate have been found to express PSA, as mentioned previously. PSA is encoded by locus containing three genes encoding the tissue kallikrein, human glandular kallikrein-1, and PSA (32). The established function of PSA is to dissolve seminal vesicle protein (6). It is not clear whether those tumors expressing PSA specifically turn on the gene encoding PSA or generally turn on all three genes or other combinations. The level of PSA expression has been found to be considerably higher in prostate tissue than in the other sources (11). Therefore, one might expect that the unregulated secretion of PSA observed in LNCaP would be more pronounced and play a larger role in the prostate than in other tissues that secrete PSA.

Future work will be focused on illustration of the autocrine and/or paracrine effects of PSA. Understanding the biological effects of PSA and other kallikreins, combined with the information obtained from our study, may provide a strategy to prevent cancer cells from progression.

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