

Prostasin Is a Glycosylphosphatidylinositol-anchored Active Serine Protease*

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A recombinant human prostasin serine protease was expressed in several human cell lines. Subcellular fractionation showed that this serine protease is synthesized as a membrane-bound protein while a free-form prostasin is secreted into the culture medium. Prostasin was identified in nuclear and membrane fractions. Membrane-bound prostasin can be released by phosphatidylinositol-specific phospholipase C treatment, or labeled by [³H]ethanolamine, indicating a glycosylphosphatidylinositol anchorage. A prostasin-binding protein was identified in mouse and human seminal vesicle fluid. Both the secreted and the membrane-bound prostasin were able to form a covalently linked 82-kDa complex when incubated with seminal vesicle fluid. The complex formation between prostasin and the prostasin-binding protein was inhibited by a prostasin antibody, heparin, and serine protease inhibitors. Prostasin's serine protease activity was inhibited when bound to the prostasin-binding protein in mouse seminal vesicle fluid. This study indicates that prostasin is an active serine protease in its membrane-bound form.

Prostasin is a serine protease discovered in ejaculated human semen in 1994 (1). The molecular mass of prostasin is 40 kDa when examined by SDS-polyacrylamide gel electrophoresis (PAGE)¹ under reducing conditions. Prostasin displays trypsin-like enzymatic activities by hydrolyzing peptidyl fluorogenic substrates such as D-Pro-Phe-Arg-AMC. This trypsin-like enzymatic activity can be inhibited by aprotinin, antipain, leupeptin, and benzamidin. Prostasin is present at high levels in normal human semen ($8.61 \pm 0.42 \mu\text{g/ml}$) and in the prostate gland ($143.7 \pm 15.9 \text{ ng/mg}$). Lower amounts of prostasin can also be detected in other tissues. In the prostate gland, the prostasin protein is present in the epithelial cells as well as in the secretion inside the lumen. The full-length human prosta-

sin mRNA has been deduced (2). The predicted mature prostasin peptide sequence has a potential carboxyl-terminal hydrophobic membrane anchorage domain followed by a short cytoplasmic tail. The translated amino acid residue sequence of prostasin is similar to those of human prostatic, testis, plasma kallikrein, coagulation factor XI, hepsin, plasminogen, and acrosin (2–4). A membrane-bound *Xenopus* kidney epithelial cell sodium channel-activating protease (CAP1) was found highly homologous to human prostasin, sharing 53% sequence identity at the amino acid level (5). Recently, the mouse counterpart of CAP1, mCAP1, has been cloned from a cortical collecting duct cell line (6). mCAP1 shares 77% amino acid sequence identity with human prostasin.

Serine proteases play important roles in a diverse range of the body's normal physiological processes, and they are implicated in various pathological processes such as cardiovascular disorders and cancers (7). The prostate produces a number of serine proteases such as prostate-specific antigen (8), human glandular kallikrein (9), and the most recently discovered prostatic (3). Some of these serine proteases are suspected to affect fertility or semen liquefaction (10), and others are implicated in normal prostate development or prostatic diseases (11–14). For example, both prostate-specific antigen and human glandular kallikrein have become important diagnostic and prognostic markers for prostate cancer. Serine proteases are usually regulated at the post-translational level in addition to the transcriptional regulation at their gene level. The body's own strategy of regulating the serine proteases is to bind the serine proteases with a protein inhibitor such as the inhibitors of the serpin class (15). These serpin-serine protease pairs are highly specific with regard to the two molecules involved; examples include α_1 -antitrypsin and elastase (15), kallistatin and kallikrein (16, 17), α_1 -antichymotrypsin and prostate-specific antigen (18). The mechanism of serpin inhibition of serine proteases involves the formation of a covalently linked complex at a 1:1 stoichiometry (19). Such a complex exhibits resistance to treatment with SDS or boiling (16, 17).

The physiological functions of prostasin are not fully understood. In a recent study (20) we showed that prostasin expression is significantly down-regulated in high grade prostate tumors and is lost in highly invasive human and mouse prostate cancer cell lines. Transfection of two human prostate cancer cell lines DU-145 and PC-3 with human prostasin cDNA reduced *in vitro* invasiveness of the cells, suggesting an invasion suppressor role for prostasin. This anti-invasion activity is apparently conferred by the cellular prostasin but not the secreted prostasin. In the present study, we determined that prostasin is a GPI-anchored membrane protein in addition to being a secreted protease. The subcellular localization of prostasin was investigated in cells expressing native or recombi-

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¹ The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; GPI, glycosylphosphatidylinositol; PBP, prostasin-binding protein; r-hPro, recombinant human prostasin; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid; PI-PLC, phosphatidylinositol-specific phospholipase C; ER, endoplasmic reticulum; mPBP, mouse prostasin-binding protein; AMC, 7-amino-4-methylcoumarin; AFC, 7-amino-4-trifluoromethylcoumarin.

nant prostatin. We have also identified a prostatin-binding protein (PBP), a potentially serpin class serine protease inhibitor specific for prostatin. We further demonstrated that the membrane-bound prostatin is an active serine protease. These results will provide structural and regulatory information for further investigation of the functions of prostatin in normal prostate development, prostatic diseases, as well as reproductive biology.

EXPERIMENTAL PROCEDURES

Cell Lines and Plasmid DNA Transfection—A human embryonic kidney epithelial cell line, 293-EBNA (Invitrogen, Carlsbad, CA), was maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Human prostate cancer cell lines LNCaP, DU-145, and PC-3 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The LNCaP and the DU-145 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1 mM sodium pyruvate; the PC-3 cells were maintained in F-12K medium supplemented with 10% fetal bovine serum. All cells were kept at 37 °C with 5% CO₂. All tissue culture media, sera, and supplements were purchased from Life Technologies, Inc.

A full-length human prostatin cDNA of 1,896 base pairs (including a 209-base pair 5'-untranslated region, 1,032 base pairs of the coding region, and a 655-base pair 3'-untranslated region) was generated by means of reverse transcription-polymerase chain reaction with the following two primers, 5'-AGA CCG TGC TGG TGA CTC GT-3' and 5'-TGT GCT CAA ACA TTT TAA TC-3', using the total RNA of LNCaP cells as template (2). The amplified cDNA was cloned into a mammalian expression vector, pREP-8 (Invitrogen), at its polylinker site. Transfection of the prostatin cDNA plasmid into 293-EBNA cells was carried out using electroporation. The electroporated cells were then subcultured for selection of transfectants (293/Pro) using 5 mM histidinol (Sigma) in the culture medium for 2 weeks. The pREP-8 vector plasmid was transfected into 293-EBNA cells and subjected to histidinol selection to establish the control cells (293/Vec).

The DU-145 and the PC-3 cells, which do not express prostatin (20), were also transfected using plasmids containing the full-length human prostatin cDNA. The methods for plasmid engineering and establishment of transfectants that express human prostatin have been described previously (20). The resulting cell lines that express human prostatin were designated DU-145/Pro and PC-3/Pro.

SDS-PAGE and Western Blot Analysis—These procedures were carried out for all experiments unless stated otherwise. Samples were suspended in 1 × SDS sample buffer (62.5 mM Tris-HCl at pH 6.8, 2% v/v glycerol, 2% w/v SDS, and 2% β-mercaptoethanol), boiled for 5 min, and resolved in a 10% polyacrylamide gel. The resolved proteins were then transferred to a nitrocellulose membrane. The membrane was stained with India ink for 15 min (1:1,000 in TBS-T: 20 mM Tris at pH 7.6 containing 0.137 M NaCl and 0.1% Tween 20), blocked in 5% non-fat milk for 1 h, and incubated with the primary antibody for 30 min in a tray or a Surf-blot apparatus (Idea Scientific, Inc., Minneapolis). After washing, the membrane was incubated with a secondary antibody conjugated with horseradish peroxidase (Sigma, used at a 1:10,000 dilution) for 30 min. Signals were detected using ECL (enhanced chemiluminescence) with WestPico reagents (Pierce) following the supplier's protocol. The membrane was then exposed to Kodak x-ray film. The primary antibodies used were as follows: polyclonal antibodies against prostatin (recombinant or native, used at 1:1,000), a monoclonal antibody against β₁-integrin (used at 1:1,000), and a monoclonal antibody against poly(ADP-ribose) polymerase (used at 1:500). Antibodies against β₁-integrin and poly(ADP-ribose) polymerase were from BD Transduction Laboratories (San Diego).

Purification of Recombinant Human Prostatin—The 293/Pro cells were grown to a confluent monolayer in Dulbecco's modified Eagle's medium and 10% fetal bovine serum containing 5 mM histidinol. Cells were then placed in Opti-MEM I serum-free medium (Life Technologies, Inc.) for 72 h before collection of the conditioned medium. The collected medium was tested for recombinant prostatin (r-hPro) by Western blot analysis using a prostatin-specific antibody (1). For purification of the secreted prostatin, the serum-free medium was centrifuged at 10,000 rpm for 20 min to remove dead cells or debris and then passed through an aprotinin-agarose column (1.5 × 20 cm, Sigma) equilibrated with 25 mM Tris-HCl at pH 7.6 at a flow rate of 25 ml/h. After extensive washing to remove any unbound proteins, the bound prostatin was eluted with 0.1 M glycine (pH 3.0) containing 0.1 M NaCl at a flow rate of 60 ml/h. The eluted prostatin was immediately neutralized with appropriate

amounts of 1 M Tris, combined, concentrated with Centricon-10 concentrators (Amicon Inc., Beverly, MA), and stored at -20 °C before use in other assays.

Preparation of a Polyclonal Antiserum against Recombinant Prostatin—250 μg of the purified r-hPro in 0.5 ml of phosphate-buffered saline (PBS, pH 7.4) was emulsified with an equal volume of complete Freund's adjuvant (Sigma) and was injected subcutaneously into a 1.5-kg female New Zealand White rabbit (Charles River Laboratories, Wilmington, MA). Booster injections with 100 μg of r-hPro (emulsified with incomplete Freund's adjuvant, Sigma) were performed three times at 3-week intervals. Preimmune rabbit serum was collected before the initial immunization.

Immunocytochemistry—The PC-3/Pro or LNCaP cells were seeded on glass coverslips (Fisher Scientific) at a density of 5 × 10⁴/coverslip and grown for 24–36 h prior to a double immunostaining. Briefly, cells were rinsed three times in 1 × PBS, fixed in 4% paraformaldehyde, and permeabilized with 0.18% Triton X-100 in PBS for 10 min. After blocking in 10% normal goat serum (Life Technologies, Inc.) in 1 × PBS, cells were incubated with the primary antibodies for 45 min, washed, incubated with the appropriate secondary antibodies at room temperature for 30 min, and then washed three times for 10 min each in 1 × PBS. A rabbit polyclonal antibody against prostatin was used at a dilution of 1:100. A monoclonal antibody against poly(ADP-ribose) polymerase was used as a nuclear specific marker at a dilution of 1:75. A goat anti-rabbit IgG conjugated with fluorescein (1:50, Life Technologies, Inc.) and a goat anti-mouse IgG conjugated with Cy3 (1:800, Jackson Immuno-Research, West Grove, PA) were used as the secondary antibodies. The coverslips were mounted with Gel/Mount (Fisher Scientific) and analyzed on a Carl Zeiss LSM510 laser scanning microscope.

Subcellular Fractionation and Differential Extraction—Subcellular fractionation was performed as described in Krajewski *et al.* (21) and Pemberton *et al.* (22). Briefly, confluent cells in 4 × 150-cm² flasks (estimated 5–10 × 10⁷ cells/total) were washed three times with 1 × PBS and removed by mechanical force for the 293/Pro cells or trypsin treatment (0.25% with 1 mM EDTA) for the PC-3/Pro and LNCaP cells. The cells were resuspended in 7 ml of cold MES buffer (17 mM at pH 7.4, 2.5 mM EDTA, and 250 mM sucrose) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml antipain). The following steps were performed at 4 °C. Cell suspension was homogenized with a Dounce homogenizer for 60 strokes followed by centrifugation twice at 500 × g for 10 min, resulting in the crude nuclear fraction in the pellet. The supernatant was centrifuged twice at 10,000 × g for 15 min, resulting in the heavy membrane fraction in the pellet containing mitochondria, lysosomes, and peroxisomes. The supernatant from the 10,000 × g centrifugation was subjected to ultracentrifugation at 100,000 × g for 60 min, resulting in a light membrane fraction in the pellet containing the plasma membrane, microsomes, and endoplasmic reticula. The supernatant from the final centrifugation contains soluble or cytosolic proteins. The pellets from each centrifugation were washed with 2 × 10 ml of MES to eliminate carryovers.

Differential extraction of membrane fractions was carried out according to Pei *et al.* (23). Briefly, pellet/membrane fractions were divided equally into three portions and were extracted with 1% Triton X-114 in Tris buffer (10 mM Tris-HCl, pH 7.5) or high salt (350 mM NaCl in Tris buffer), or alkali (50 mM glycine/NaOH, pH 11.0) for 1 h on ice. The samples were centrifuged at 100,000 × g for 30 min. The resulting pellet was dissolved in 1 × SDS sample buffer for gel electrophoresis. The supernatant was subjected to a trichloroacetic acid precipitation to recover proteins for gel electrophoresis.

Detergent Phase Separation and Phosphatidylinositol-specific Phospholipase C (PI-PLC) Treatment—The procedure was adapted from those described by Bordier (24) and Rosenberg (25). Briefly, cells (5 × 10⁶) were lysed in 1 ml of ice-cold TBS (10 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1 mM EDTA) containing 1% Triton X-114 (Sigma) and protease inhibitors for 2 h with gentle shaking at 4 °C. The lysate was then centrifuged at 14,000 rpm for 30 min. The supernatant (500 μl, or 700–800 μg of total protein) was overlaid onto a 300-μl sucrose cushion (6% w/v sucrose in TBS containing 0.06% Triton X-114). The solution was incubated at 37 °C for 3 min and centrifuged at 300 × g for 3 min at room temperature to separate the detergent phase (pellet) and the aqueous phase. The aqueous phase was removed and extracted further with 0.5% Triton X-114 and 2% Triton X-114. The aqueous phase after the final centrifugation contains the soluble proteins. The detergent phase (pellet) from the first centrifugation was resuspended in 500 μl of ice-cold TBS, incubated at 37 °C for 3 min, and centrifuged at 300 × g for 3 min at room temperature to ensure the purity of the detergent phase.

The detergent phase was resuspended in 100 μ l of ice-cold TBS. 10 μ l of the resuspended detergent phase was subjected to PI-PLC (Sigma) digestion at 37 $^{\circ}$ C for 1 h with gentle shaking in a total volume of 100 μ l of reaction buffer (10 mM Tris-HCl at pH 7.5, 144 mM NaCl). 100 μ l of ice-cold TBS containing 2% Triton X-114 was then added to the digestion mixture and subjected to phase separation as described above. At the final step, both the aqueous and detergent phases were precipitated with 6% w/v trichloroacetic acid and 0.013% sodium deoxycholate. The precipitates were resuspended in 30 μ l of 1 \times SDS sample buffer, neutralized with ammonium hydroxide (microliter amounts), boiled, and subjected to SDS-PAGE and Western blot analysis.

Human prostates removed by radical prostatectomy performed at Orlando Regional Medical Center (Orlando, FL) were sectioned with a cryostat at 20- μ m thickness. 80 sections were collected and rinsed with PBS twice to remove prostatic fluid. The washed prostate sections were lysed in 1 ml of TBS containing 1% Triton X-114 at 4 $^{\circ}$ C overnight with rocking. The lysed prostate tissues were centrifuged and subjected to the same phase separation and PI-PLC treatment procedures as described above. Several representative prostate sections (7 μ m) cut at intervals of the 80 20- μ m sections were subjected to standard hematoxylin and eosin staining for confirmation of benign prostate morphology. The use of human tissues was approved by the Institutional Review Boards of Orlando Regional Medical Center and the University of Central Florida.

³H]Ethanolamine Labeling, Immunoprecipitation, and Fluorography—PC-3/Pro (3 \times 10⁵) or LNCaP (1 \times 10⁶) cells were seeded in a 35-mm dish in 1 ml of Opti-MEM I serum-free medium. The next day, [1-³H]ethan-1-ol-2-amine hydrochloride (37 MBq, 1 mCi/ml, and 30.0 Ci/mmol, Amersham Pharmacia Biotech) was added to the culture medium at a concentration of 100 μ Ci/ml, and the cells were cultured for another 24 h. Cells were washed once with PBS and lysed in 0.5 ml of RIPA buffer (PBS at pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors at 4 $^{\circ}$ C for 1 h. The lysate was centrifuged at 14,000 rpm for 30 min to remove insoluble material. The supernatant was subjected to immunoprecipitation with 2 μ g/ml anti-prostatin IgG (purified using Econo-Pac protein A cartridge, Bio-Rad) and protein A-Sepharose beads (Sigma) at 4 $^{\circ}$ C overnight. The beads were washed with RIPA buffer three times, resuspended in 2 \times SDS sample buffer with β -mercaptoethanol, boiled, and analyzed by SDS-PAGE. The gel was fixed in a solution of 2-propanol: water:acetic acid (12.5: 32.5: 5) for 30 min and soaked in Amplify fluorographic reagent (Amersham Pharmacia Biotech) for another 30 min. The gel was dried, and ³H-labeled molecules were detected by exposure to an x-ray film with an intensifying screen at -80 $^{\circ}$ C for 14 days.

Prostatin Binding Assay—Seminal vesicle fluid was expressed from one pair of mouse seminal vesicles (C57BL/6 mouse, Harlan, Indianapolis) and mixed with 1 ml of 25 mM Tris-HCl, pH 7.6, and centrifuged at 14,000 rpm at 4 $^{\circ}$ C for 30 min. 5 μ l of the supernatant (50 μ g of total protein) was incubated with either 0.5 μ g of the purified recombinant human prostatin or subcellular fractions of 293/Pro and PC-3/Pro cells (prepared in the absence of serine protease inhibitors) at 37 $^{\circ}$ C for 60 min, or for various time periods for a time course study. The binding reaction was stopped by the addition of SDS sample buffer and heating at 100 $^{\circ}$ C for 5 min. Mouse tissues were homogenized in PBS (1 g of tissue/5 ml) and centrifuged in a microcentrifuge at 14,000 rpm for 30 min at 4 $^{\circ}$ C. 40 μ g of total protein for each tissue extract was used in the binding assay. 1 μ l of human or mouse plasma was also subjected to prostatin binding assay. The use of animals was approved by the IACUC of the University of Central Florida. Human seminal vesicles were obtained from radical prostatectomy performed at Orlando Regional Medical Center. No seminal vesicle metastasis from prostate cancer was found according to the pathology report. Human seminal vesicle fluid was diluted with PBS at a ratio of 1:2, mixed by vortex, and spun. 10 μ l of the diluted fluid was incubated with 0.5 μ g of purified prostatin at 37 $^{\circ}$ C for 60 min.

Membrane Overlay Zymography—The membrane overlay zymography was carried out using the protocols of Enzyme System Product (Livermore, CA) and Beals *et al.* (26). Briefly, samples were first resolved in a 10% polyacrylamide gel without SDS or β -mercaptoethanol. After electrophoresis, the gel was equilibrated in a reaction buffer (50 mM Tris-HCl, pH 9.0) for 15 min. Pre-wet acetate-cellulose membrane impregnated with the prostatin substrate D-Pro-Phe-Arg-AFC (Enzyme System Product) was then carefully laid over the gel without entrapping air bubbles. The membrane-overlaid gel was placed in a moist chamber at 37 $^{\circ}$ C for 3–5 h. The reaction was monitored using an ultraviolet lamp and photographed.

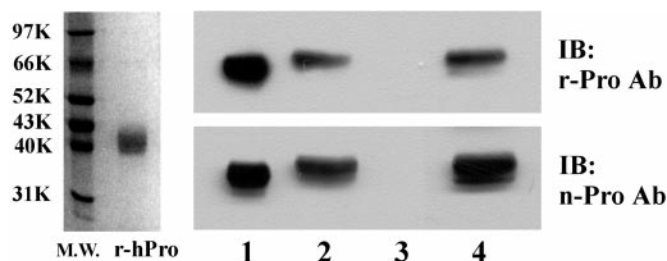


FIG. 1. SDS-PAGE and Western blot analysis. Recombinant human prostatin purified from serum-free conditioned medium of the 293/Pro cells was analyzed by 10% SDS-PAGE under reducing conditions. The purified recombinant prostatin (3 μ g) migrated at 40 kDa (left panel, r-hPro) and is recognized by an antibody made against the purified recombinant prostatin (IB: r-Pro Ab, lane 1) as well as a prostatin-specific antibody made against purified native prostatin (IB: n-Pro Ab, lane 1, Ref. 1). The quantity of purified prostatin in lanes 1 is 0.5 μ g. Samples from the 293/Pro cell lysate (lanes 2, 20 μ g), 293/Vec (lanes 3, 20 μ g) cell lysate, and human semen (lanes 4, 30 μ g) were immunoblotted with r-Pro antibody (upper right panel, 1:1,000 dilution) and n-Pro antibody (lower right panel, 1:1,000 dilution). Both antibodies recognize the recombinant prostatin as well as the native prostatin but do not have cross-reactivity with 293/Vec proteins.

RESULTS

Expression and Purification of Recombinant Human Prostatin—Serum-free conditioned medium from 293/Pro cell culture was prepared and passed through an aprotinin-agarose column for a one-step affinity-chromatographic purification of the recombinant prostatin as described under “Experimental Procedures” (see also Ref. 1). A Coomassie Blue staining of the purified recombinant prostatin is shown in Fig. 1, left panel (r-hPro). The r-hPro migrates at 40 kDa on an SDS-PAGE under reducing conditions. Because of glycosylation of the prostatin molecule (1, 2), it appeared as a rather diffused band on the gel. We prepared a polyclonal antibody (r-Pro Ab) using the purified r-hPro as an antigen. The r-Pro antibody recognized the purified recombinant prostatin (secreted form), the recombinant prostatin in 293/Pro total cell lysate (nonsecreted form), and the native prostatin in ejaculated human semen (obtained from healthy volunteers, Ref. 1) (Fig. 1, upper right panel, lanes 1, 2, and 4). The prostatin protein in the same set of samples was also recognized by a prostatin-specific antibody referenced previously (1) (Fig. 1, lower right panel). Neither antibody cross-reacts with any nonspecific protein in the control 293/Vec total cell lysate (Fig. 1, right panel, lanes 3). The results indicate that the polyclonal antibody against the recombinant prostatin is specific to prostatin and that the recombinant prostatin prepared using the amplified cDNA has an immunological reactivity similar to that of the native prostatin. The antibody against the recombinant prostatin was used in the ensuing assays conducted in this study.

Recombinant Prostatin Is a Membrane-bound Protein—A hydrophathy plot of the translated prostatin amino acid sequence indicated that the prostatin polypeptide has a potential membrane-anchorage domain at the carboxyl terminus (2). To confirm the presence of a potentially membrane-anchored form of the prostatin protein, the 293/Pro cells were subjected to subcellular fractionation by differential centrifugation. Fig. 2 shows that prostatin was present in the crude nuclear fraction (P1, 500-g pellet), heavy membranes (P2, 10,000-g pellet), and light membranes (P3, 100,000-g pellet), as determined by Western blot analysis. Prostatin was also detected in the cytosol (S). The purified r-hPro was used as positive control. Equal amounts of total protein (30 μ g) from each membrane fraction and the cytosol were applied in each lane.

To show that the membrane-anchored prostatin is not a peculiarity in the 293 cells, we performed similar subcellular

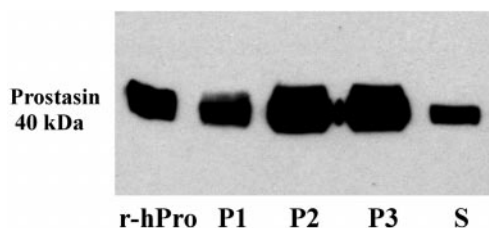


FIG. 2. Analysis of prostatin in 293/Pro cell fractions. The cells were fractionated through differential centrifugation. An equal amount of protein (30 μ g) from each centrifugation step was resolved by 10% SDS-PAGE followed by immunoblotting with a prostatin-specific polyclonal antibody (1:1,000 dilution). Prostatin (40 kDa) is detected in the nuclear fraction (P1), heavy membrane fraction (P2, including mitochondria, lysosomes, and peroxisomes), light membrane fraction (P3, including plasma membrane, microsomes, and endoplasmic reticula) as well as the cytosol (S) of 293/Pro. Purified r-hPro (0.5 μ g) was used as a positive control.

fractionation analysis on prostatin cDNA-transfected human prostate cancer cell line PC-3 (PC-3/Pro) and the human prostate cancer cell line LNCaP, which expresses endogenous prostatin (2, 20). As shown in Fig. 3A, prostatin is detected in P1, P2, and P3 fractions, but not in the cytosol (S) of PC-3/Pro. In the LNCaP cells, endogenously expressed prostatin is detected only in the P3 fraction. The membrane fractions from PC-3/Pro cells were then immunoblotted with a monoclonal antibody against a nuclear protein poly(ADP-ribose) polymerase or a monoclonal antibody against a plasma membrane-bound protein β 1-integrin to ensure the purity of each fraction. The results showed that the prostatin protein exists in a membrane-bound form in all cell lines tested. The cells transfected with the vector DNA alone (293/Vec and PC-3/Vec) were subjected to the same fractionation procedures followed by SDS-PAGE/Western blot analysis. No prostatin was detected (data not shown). We further subjected PC-3/Pro and LNCaP cells to a double immunostaining and analyzed the subcellular localization of prostatin using confocal microscopy. The confocal microscopic analysis of PC-3/Pro cells localized prostatin (green) to the ER-Golgi complex (Fig. 3B), consistent with the cell fractionation results shown in Fig. 3A. Because the nuclear membrane is practically a prominent component of the ER (27), it is not surprising that this portion of prostatin appeared in the P1 fraction. The LNCaP cells, however, did not show punctate or nuclear-ER-Golgi complex staining, again, consistent with the cell fractionation results shown in Fig. 3A.

To test if prostatin is truly a membrane-anchored protein rather than a membrane-associated protein, we subjected the P1, P2, and P3 fractions of the PC-3/Pro cells to treatment with a detergent, high salt, or alkali. As shown in Fig. 4, membrane-bound prostatin (pellet) was released into the supernatant only by the detergent treatment but not the high salt or alkali treatment. The detergent released prostatin and the membrane-bound prostatin had similar molecular weight. The results indicated that prostatin is a true membrane-anchored protein.

Membrane Prostatin Is GPI-anchored—A comparison of the potential carboxyl-terminal membrane-anchorage domain of prostatin (2) with GPI-anchored proteins (28) predicts a GPI linkage for prostatin as well (data not shown). Such a linkage may be susceptible to cleavage by PI-PLC, GPI-specific phospholipase D, or nitrous acid (25). In our studies, we first chose PI-PLC to test if prostatin is a GPI-anchored membrane protein. The 293/Pro cells were lysed in TBS containing 1% Triton X-114. After phase separation, the aqueous phase (Fig. 5, lane 1, 30 μ g of total protein) and the detergent phase (lane 2, 3 μ g of total protein) were analyzed by Western blot using the prostatin-specific antibody. The majority of the prostatin protein in

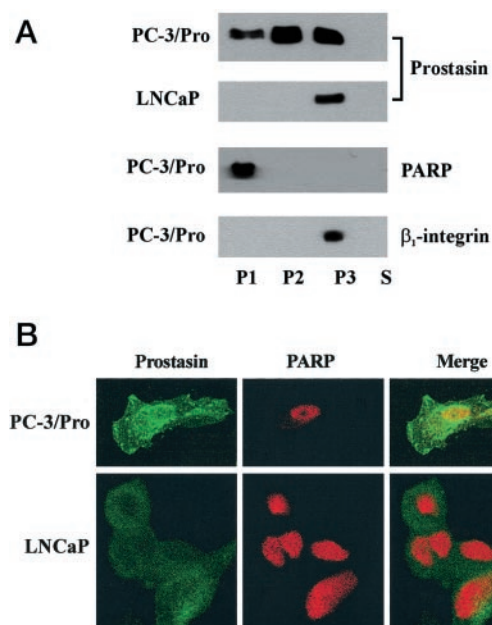


FIG. 3. Analysis of prostatin in prostate cancer cell lines. *Panel A*, Western blot analysis of membrane-bound prostatin in PC-3/Pro and LNCaP cells. The experimental procedures were the same as described in Fig. 2. Prostatin (40 kDa) is detected in the nuclear fraction (P1), heavy membrane fraction (P2), light membrane fraction (P3), but not in the cytosol (S) of PC-3/Pro. In LNCaP cell fractions, prostatin is detected only in P3. Antibodies against a nuclear protein, the poly(ADP-ribose) polymerase (PARP, 1:500, or 0.5 ng/ml), and a plasma membrane protein, β 1-integrin (1:1,000, or 0.25 ng/ml), were used as fractionation markers. *Panel B*, confocal microscopic localization of prostatin. The PC-3/Pro and LNCaP cells were fixed, permeabilized, and subjected to a double immunostaining. One focal plane for each cell type is presented to show prostatin signals (green). Prostatin is detected primarily at the nuclear-ER-Golgi complex as well as punctate regions in the PC-3/Pro cell. In the LNCaP cells, no punctate prostatin can be seen. The nuclear marker protein poly(ADP-ribose) polymerase (red) is seen in both cell types. A merge image for either cell type is presented to the right. The images were taken after subtracting background signal on a preimmune serum-stained control coverslip. Magnification, \times 400. The antibody dilution ratios are: anti-prostatin, 1:100; anti-poly(ADP-ribose) polymerase, 1:75 (or 3.3 ng/ml); goat anti-rabbit IgG conjugated with fluorescein isothiocyanate, 1:50; and goat anti-mouse IgG-Cy3, 1:800.

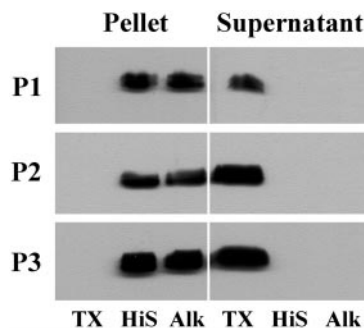


FIG. 4. Recombinant prostatin is a true membrane-bound protein. Approximately 80–100 μ g of total protein of each membrane fraction of PC-3/Pro cells (as described in legend to Fig. 3A) was subjected to detergent (TX), high salt (HiS), or alkali (Alk) treatment followed by centrifugation to separate the supernatant and the pellet for SDS-PAGE and Western blot analysis. Prostatin in all fractions can only be released from the membrane (pellet) to the supernatant (soluble protein) by the detergent treatment but not high salt or alkali treatment.

293/Pro cells is associated with the membrane, which was retained in the detergent phase. The size difference between the soluble and the membrane-bound prostatin may be attributed to the GPI moiety that is linked to prostatin (Fig. 5, lanes

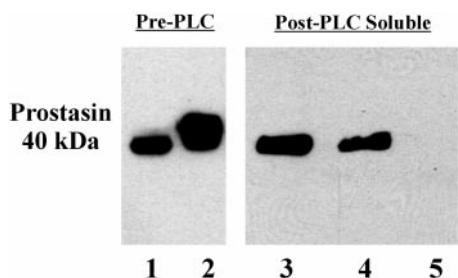


FIG. 5. Detergent phase separation of 293/Pro cells and phospholipase C treatment. The 293/Pro cells were lysed in TBS containing 1% Triton X-114 and subjected to phase separation (700–800 μ g of total protein as the starting quantity). The detergent phase containing membrane-associated proteins (equivalent to 1/10 of the total starting membrane-associated proteins) was further treated with PI-PLC followed by additional phase separation. The soluble proteins (lane 1, 30 μ g) and the detergent phase proteins (lane 2, 3 μ g) before PI-PLC treatment and the soluble proteins extracted from the detergent phase after PI-PLC treatment were subjected to SDS-PAGE and Western blot analysis using a prostatin-specific antibody. The membrane-bound prostatin is released from the membrane after PI-PLC digestion as it was detected in the post-PLC soluble phase. The amounts of PI-PLC used in the reactions are: lane 3, 0.25 unit; lane 4, 0.125 unit; and lane 5, 0 unit in a total reaction volume of 100 μ l. The results indicate that prostatin is anchored to membrane via GPI. The size difference between the membrane-bound prostatin and the soluble prostatin (lanes 1 and 2) may be attributed to the GPI moiety that is linked to prostatin.

1 and 2). The detergent phase was then subjected to PI-PLC digestion at various enzyme concentrations followed by a second phase separation. In Fig. 5, lanes 3–5 represent samples of the aqueous phases after PI-PLC digestion. The PI-PLC treatment released prostatin from the detergent phase in a dose-dependent manner (lane 3, 0.25 unit; lane 4, 0.125 unit, and lane 5, 0 units). The PI-PLC-released prostatin and the soluble prostatin are similar in molecular mass as shown in Fig. 5. The results support a GPI-anchoring mechanism for the membrane-bound prostatin.

One question that remained unclear was whether the native prostatin in the prostate tissue epithelial cells is membrane-bound by GPI anchorage as well. We selected a panel of prostate cancer cell lines and 293/Pro cells that express either recombinant or endogenous prostatin, and normal human prostate tissues in our next experiment. Cell lines that express recombinant prostatin were 293/Pro, PC-3/Pro, and DU-145/Pro. The human prostate cancer cell line LNCaP and normal human prostate tissues were used for testing native cellular prostatin. All samples (300 μ g of total protein as the starting quantity) were subjected to detergent phase separation before and after PI-PLC digestion as described under “Experimental Procedures.” PC-3 transfected with a vector plasmid (PC-3/Vec) was used as negative control. The results are presented in Fig. 6A. Without PI-PLC treatment, both the recombinant and native prostatin are mainly membrane-anchored (found in the detergent phase). Soluble prostatin is detected in 293/Pro and prostate tissues. After PI-PLC treatment, the membrane-anchored prostatin is released into the soluble fraction from 293/Pro, PC-3/Pro, and prostate tissues, but not from DU-145/Pro and LNCaP. The results indicated that the native prostatin in normal human prostate tissue is also GPI-anchored. The membrane-bound prostatin in DU-145/Pro and LNCaP is resistant to PI-PLC digestion. We further tested if prostatin can be labeled biosynthetically with [3 H]ethanolamine, which is specifically incorporated in the GPI unit of GPI-anchored proteins (29). We chose the PC-3/Pro (expressing recombinant prostatin) and LNCaP (expressing native prostatin) cells for [3 H]ethanolamine biosynthetic labeling. As shown in Fig. 6B, [3 H]ethanolamine was incorporated into either recombinant or native prostatin, demonstrating that both are truly GPI-anchored.

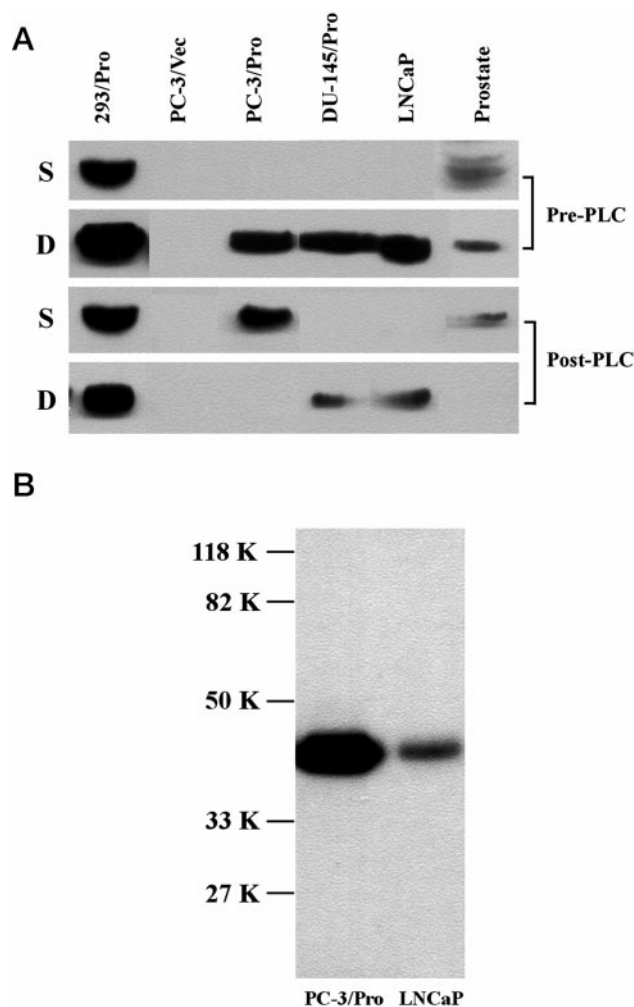


FIG. 6. Prostatin is a GPI-anchored membrane protein. Panel A, detergent phase separation and PI-PLC treatment. Human prostate tissues and cell lines that express either endogenous prostatin (LNCaP) or recombinant prostatin (293/Pro, PC-3/Pro, and DU-145/Pro) were subjected to detergent phase separation/PI-PLC digestion followed by prostatin immunoblotting (300 μ g of total protein was used for each sample as the starting quantity). Both the recombinant and native prostatin are mainly membrane-anchored (pre-PLC, detergent phase or D). Soluble prostatin is detected in 293/Pro and prostate tissues (pre-PLC, soluble phase or S). After PI-PLC treatment, the membrane-anchored prostatin is released into the soluble fraction from 293/Pro, PC-3/Pro, and prostate tissues (post-PLC, S) but not from DU-145/Pro and LNCaP (post-PLC, D). The PC-3/Vec cells showed negative results in all fractions tested. Because of a high level prostatin expression in the 293/Pro cells, a portion of prostatin remained in the detergent phase after PI-PLC digestion. Panel B, incorporation of [3 H]ethanolamine into prostatin. PC-3/Pro or LNCaP cells were incubated with 100 μ Ci of [3 H]ethanolamine in 1 ml of Opti-MEM I serum-free medium for 24 h in 5% CO₂ at 37 °C. The cell lysates were subjected to immunoprecipitation using the prostatin antibody (purified IgG fraction, 2 μ g/ml) and protein A-Sepharose beads as described under “Experimental Procedures.” After SDS-PAGE separation of the samples, the labeled protein was detected by fluorography using Amplify fluorographic reagent and exposure to an x-ray film at –80 °C with an intensifying screen for 14 days.

Identification of a Prostatin-binding Protein—An incubation of the purified r-hPro with mouse or human seminal vesicle fluid yielded a higher molecular weight form of prostatin-containing band as analyzed by SDS-PAGE and immunoblotting. The result presented in Fig. 7A indicated that the purified r-hPro formed an 82-kDa complex with a mouse seminal vesicle protein (named as the mouse prostatin-binding protein, or mPBP). The complex was apparently covalently linked and not via a disulfide bond because it was SDS- and heat-stable and

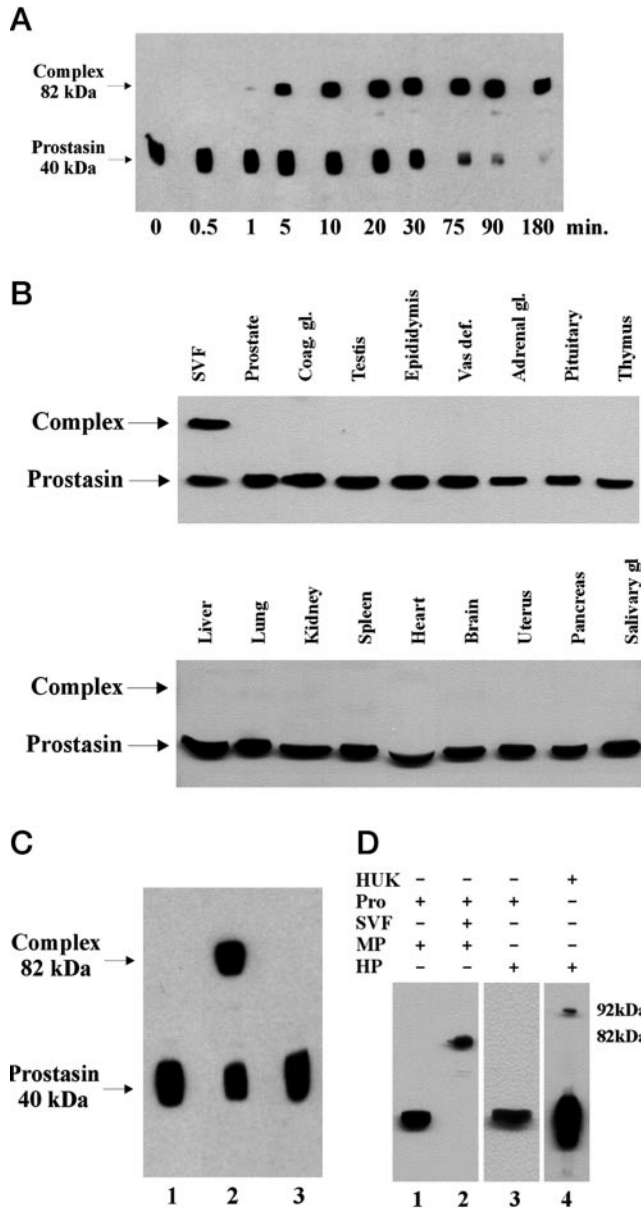


FIG. 7. Complex formation between prostasin and its binding protein. *Panel A*, 0.5 μ g of purified recombinant prostasin was incubated with mouse seminal vesicle fluid at 37 °C for various time periods as indicated. The samples were subjected to SDS-PAGE under reducing conditions followed by immunoblotting with a prostasin-specific antibody. Prostasin forms an 82-kDa complex (upper arrow) with mPBP in mouse seminal vesicle fluid. The complex formation can be detected as early as 1 min postincubation. Excess unbound prostasin is indicated by the lower arrow. Prostasin alone without incubation with mouse seminal vesicle fluid is labeled as 0 min. *Panel B*, various mouse tissue extracts were analyzed in a prostasin binding assay and prostasin immunoblotting as described. SVF, mouse seminal vesicle fluid; Coag. gl., coagulating gland; Vas def., vas deferens; Adrenal gl., adrenal gland; Salivary gl., salivary glands. *Panel C*, 0.5 μ g of purified recombinant prostasin was incubated with human seminal vesicle fluid at 37 °C for 60 min. Similarly, prostasin forms an 82-kDa complex with the hPBP in human seminal vesicle fluid (lane 2), and the complex formation was inhibited by 1 unit of heparin (lane 3). Purified recombinant prostasin alone was used as a control (lane 1). *Panel D*, human or mouse plasma was tested in a prostasin binding assay. HUK, human urinary (tissue) kallikrein (0.5 μ g); Pro, purified recombinant prostasin (0.5 μ g); SVF, mouse seminal vesicle fluid (5 μ l); MP, mouse plasma (1 μ l); HP, human plasma (1 μ l). Lanes 1–3, probed with prostasin antibody (1:1,000); lane 4, probed with a human kallikrein antibody (1:1,000).

resistant to β -mercaptoethanol. The complex formation was detected at as early as 1 min postincubation with a $t_{1/2}$ of ~5 min and reached a plateau at ~20 min. Densitometry meas-

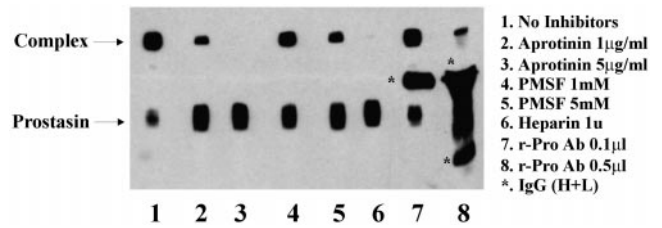


FIG. 8. Inhibition of complex formation between prostasin and mPBP. 0.5 μ g of Purified recombinant prostasin was incubated with 5 μ l of mouse seminal vesicle fluid in the presence of aprotinin (lanes 2 and 3), phenylmethylsulfonyl fluoride (PMSF; lanes 4 and 5), heparin (lane 6), prostasin antibody (lanes 7 and 8) at 37 °C for 1 h. All samples were subjected to SDS-PAGE under reducing conditions followed by immunoblotting with a prostasin-specific antibody. The complex formation (upper arrow) between prostasin and mPBP in mouse seminal vesicle fluid is inhibited by aprotinin, phenylmethylsulfonyl fluoride, heparin, and the antibody against prostasin. The asterisk (*) indicates the IgG heavy chain and light chain recognized by the goat anti-rabbit secondary antibody used in the Western blot analysis. Excess unbound prostasin is indicated by the lower arrow. Complex formation between r-hPro and mPBP in mouse seminal vesicle fluid without any other reagent was used as positive control (lane 1).

urements of the complex bands in different lanes were performed using the LabWork 3.0 software (Ultra-Violet Products, Upland, CA) (data not shown). Mouse plasma and various tissue extracts including the prostate, coagulating gland, testis, epididymis, vas deferens, adrenal gland, pituitary, thymus, liver, lung, kidney, spleen, heart, brain, uterus, pancreas, and salivary glands were subjected to the same prostasin-binding assay procedures. No SDS- and heat-stable complex was detected under the same experimental conditions (Fig. 7B). A PBP in human seminal vesicle fluid was also identified. As shown in Fig. 7C, lane 2, a higher molecular mass complex (82 kDa) was detected after an incubation of r-hPro with the human seminal vesicle fluid. The complex formation between prostasin and the human seminal vesicle PBP was inhibited by heparin (Fig. 7C, lane 3). Incubation of mouse or human plasma with r-hPro did not result in formation of any SDS- and heat-stable complex (Fig. 7D, lane 1, mouse plasma; lane 3, human plasma). In control assays, mouse plasma was incubated with prostasin for 30 min before seminal vesicle fluid was added for another 30 min of incubation to demonstrate the binding activity of the prostasin being tested in the presence of plasma (Fig. 7D, lane 2). Or, human plasma was incubated with purified human tissue kallikrein and subjected to a Western blot analysis using a human kallikrein-specific antibody (30) (Fig. 7D, lane 4). The control binding assay showed a 92-kDa kallikrein-kallistatin complex as described previously (16), demonstrating the quality of the human plasma being tested.

The complex formation between prostasin and mPBP was investigated further by incubating the purified r-hPro with serine protease inhibitors (Fig. 8, lanes 2–5) or the prostasin antibody (lanes 7 and 8) for 15 min at room temperature before an incubation with mouse seminal vesicle fluid for another 60 min at 37 °C. Or, mouse seminal vesicle fluid was first incubated with heparin before the addition of prostasin (Fig. 8, lane 6). The complex formation between prostasin and mPBP was inhibited by serine protease inhibitors such as aprotinin at dosages of 1 μ g/ml and 5 μ g/ml, phenylmethylsulfonyl fluoride at dosages of 1 mM and 5 mM, and prostasin antibody at 0.1 μ l and 0.5 μ l. The amount of the complex was either reduced or absent in the corresponding lanes of Fig. 8. Heparin (1 unit, lane 6) inhibited complex formation. Complex formation between prostasin and mPBP without additional reagents was used as the binding reaction control (Fig. 8, lane 1). The results suggested that mPBP interacts with prostasin at the serine

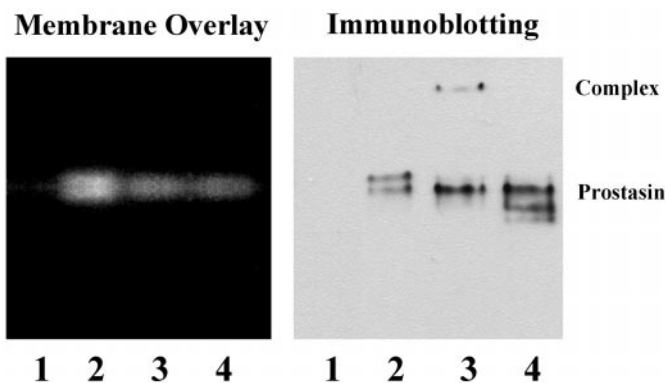


FIG. 9. Membrane overlay zymography. Samples from a prostatin binding assay were resolved on a 10% native acrylamide gel without SDS/boiling or β -mercaptoethanol. The gel was then either overlaid with a membrane impregnated with a prostatin substrate (D-Pro-Phe-Arg-AFC) (*left panel*) or transferred for prostatin immunoblotting (*right panel*). Lane 1, 5 μ l of mouse seminal vesicle fluid alone; lane 2, 0.5 μ g of purified r-hPro alone; lane 3, mixture of r-hPro and mouse seminal vesicle fluid; lane 4, same as lane 3 except r-hPro was preincubated with 5 μ g/ml aprotinin for 15 min before the addition of mouse seminal vesicle fluid. The fluorogenic substrate impregnated in the membrane was hydrolyzed by prostatin in the gel, and isoprostatin patterns in the membrane appear as fluorescent bands. The results suggest that mBPB not only binds to prostatin at the serine active site but also inhibits prostatin's serine protease activity *in vitro*.

active site and that heparin may alter mBPB binding property. The properties displayed by mBPB are shared by the serpin class serine protease inhibitors. We have observed similar properties for the serpin, kallistatin (16, 17). The predicated molecular mass of PBP (mouse or human) is estimated at \sim 47 kDa, given the 40-kDa apparent molecular mass of prostatin and considering the fact that serpin molecules lose a carboxyl-terminal fragment of \sim 5 kDa when complexed with a serine protease (31).

mBPB Inhibits the Serine Protease Activity of Prostatin—We performed a membrane overlay zymography analysis to test if mBPB inhibits prostatin activity *in vitro*. The prostatin binding assay was carried out by incubating the purified r-hPro with mouse seminal vesicle fluid in the absence of the serine protease inhibitor aprotinin (Fig. 9, lane 3) or in the presence of aprotinin (lane 4). Each sample was then divided into two equal portions and subjected to a native PAGE analysis (*i.e.*, SDS and β -mercaptoethanol were not included in the gel solution or the samples, and the samples were not heated before loading) followed by membrane overlay zymography (*left panel*) or Western blot analysis using the prostatin antibody (*right panel*). Mouse seminal vesicle fluid proteins alone (Fig. 9, lane 1) displayed no enzymatic activities toward the synthetic substrate D-Pro-Phe-Arg-AFC (*left panel*) nor cross-reactivity with the prostatin antibody (*right panel*). The purified r-hPro alone (lane 2) demonstrated enzymatic activity toward D-Pro-Phe-Arg-AFC (*left panel*) and was recognized by the prostatin antibody (*right panel*). When prostatin formed a complex with mBPB in the mouse seminal vesicle fluid, it no longer cleaves D-Pro-Phe-Arg-AFC because no fluorescence is present at the complex band location in lane 3 of the *left panel*, whereas the complex is identified by the prostatin antibody as the *upper band* in lane 3 of the *right panel*. The remaining unbound prostatin yielded, expectedly, lesser fluorescence (*left panel*, lower band in lane 3 compared with lane 2) and was recognized by the prostatin antibody (*right panel*, lane 3). When the purified r-hPro was preincubated with the serine protease inhibitor aprotinin before incubation with the mouse seminal vesicle fluid, no complex was detected (*right panel*, lane 4). A reduced level of fluorescence appeared at the prostatin band in lane 4 of

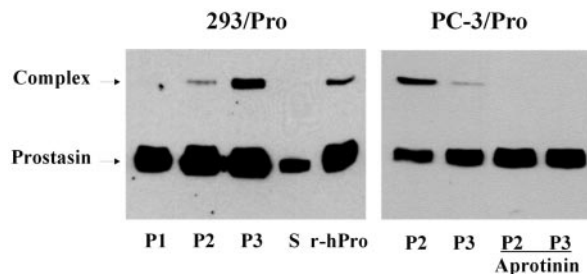


FIG. 10. Membrane-bound prostatin forms a complex with mBPB. Membrane fractions of 293/Pro and PC-3/Pro cells (30–40 μ g of total protein) were incubated with 5 μ l of mouse seminal vesicle fluid at 37 $^{\circ}$ C for 1 h. *Right panel*, samples where indicated, were preincubated with aprotinin before the addition of mouse seminal vesicle fluid. All sample mixtures were analyzed on an SDS-PAGE under reducing conditions followed by prostatin immunoblotting. P1, nucleus fraction; P2, heavy membranes; P3, light membranes; S, cytosolic proteins; and r-hPro, purified recombinant prostatin. The membrane-bound prostatin in heavy or light membrane fractions formed a complex (*upper arrow*) when incubated with mouse seminal vesicle fluid, whereas prostatin in P1 and cytosolic fractions showed no complex formation. r-hPro was used as a positive control in the *in vitro* binding assay. Excess unbound prostatin is indicated by the *lower arrow*. The addition of aprotinin at 5 μ g/ml inhibited complex formation as shown in the *right panel*.

the *left panel* because of the presence of aprotinin. Because the binding of aprotinin to prostatin was reversible while proteins were being resolved in the gel, the inhibition of prostatin activity seen in lane 4 was not complete. The results suggested that mBPB not only binds to prostatin at the serine active site but also inhibits the serine protease activity of prostatin *in vitro*. Two bands were observed in the prostatin alone sample in the immunoblot (Fig. 9, *right panel*, lane 2), the differential mobility may be caused by differential glycosylation (1). Aprotinin binding to prostatin changes the charge/mass ratio of the protein; therefore, migration of the aprotinin-bound prostatin in a native PAGE could change as well, potentially causing the multiple banding pattern seen in lane 4 of the immunoblot (Fig. 9, *right panel*).

Membrane Prostatin Binds to mBPB—To test if the membrane-bound prostatin has binding activity toward mBPB, the 293/Pro and PC-3/Pro cells were subjected to differential centrifugation as described under “Experimental Procedures” except that no protease inhibitors were added during membrane fractionation. Immediately after centrifugation, an aliquot of each membrane fraction (30–40 μ g of total protein) was incubated with an aliquot of mouse seminal vesicle fluid (5 μ l) at 37 $^{\circ}$ C for 1 h. The binding mixture was then analyzed by Western blot analysis using a prostatin-specific antibody. In Fig. 10, the *left panel* shows that the membrane-bound prostatin in 293/Pro cells (P2 and P3 fractions) formed an 82-kDa complex when incubated with mouse seminal vesicle fluid. The prostatin protein in the crude nuclear fraction (P1) and the cytosol (S) did not form any detectable complex. The purified r-hPro from the conditioned medium (*i.e.* the secreted prostatin) was used as positive control for the binding assay. In Fig. 10, *right panel*, we show that the membrane-bound prostatin in PC-3/Pro cells also formed a complex with mBPB, and this reaction was inhibited by the serine protease inhibitor aprotinin.

DISCUSSION

The prostatin serine protease is predominantly synthesized in the prostate in human (1). Recently our laboratory demonstrated that prostatin expression is significantly down-regulated in high grade prostate tumors and absent in invasive human and mouse prostate cancer cell lines. We have also shown, in an *in vitro* Matrigel invasion assay, that cellular prostatin may be an invasion suppressor of prostate cancer

(20). In the present study, we intended to investigate the intracellular distribution of prostatin and to determine whether the cellular prostatin is an active serine protease, to provide clues to the potential mechanisms of prostatin's cellular function.

We first established a mammalian expression system to produce a recombinant human prostatin. The purified secreted recombinant prostatin displayed biochemical characteristics similar to those of the native prostatin (purified from human semen, Ref. 1), such as the molecular mass on SDS-PAGE, immunological reactivity (Fig. 1), enzymatic activity toward the synthetic substrate D-Pro-Phe-Arg-AFC (Fig. 9), and responsiveness to serine protease inhibitors (Figs. 8 and 9). We then used a polyclonal antibody specific for human prostatin to determine whether prostatin can exist in a membrane-bound form because its predicted structure suggested this possibility (2). By means of sequential centrifugation of the 293/Pro and PC-3/Pro cell components (as shown in Figs. 2 and 3A), we were able to identify prostatin in various subcellular compartments such as the crude nuclei, heavy membranes (including mitochondria, lysosomes, and peroxisomes), and light membranes (including plasma membrane, microsomes, and endoplasmic reticula). A confocal microscopy analysis of the PC-3/Pro cells (Fig. 3B) revealed prostatin's subcellular localization to be primarily at the nuclear-ER-Golgi complex (27). The immunofluorescently localized prostatin at the nuclear-ER-Golgi complex is believed to be that identified in the Western blot analysis of the nuclear fraction P1. The endogenously expressed prostatin in the LNCaP cells, however, was only detected in the light membrane fraction P3 (Fig. 3A) not at the nuclear-ER-Golgi complex (Fig. 3B). The different subcellular localization of prostatin between the recombinant expression system and the endogenous expression system may be caused by expression level differences. Cells expressing recombinant prostatin produced high amounts of prostatin with the 293/Pro being the highest followed by PC-3/Pro and DU-145/Pro. The prostatin expression level in the LNCaP cells was considerably lower than that in these transfected cell lines. The expression levels were determined by a semiquantitative Western blot analysis (data not shown). Alternatively, different cell lines may have different protein sorting mechanisms, leading to different subcellular localization patterns (32). On the other hand, GPI-anchored prostatin might be associated with sterols and therefore can be found in many compartments of the cell including the plasma membrane, the Golgi apparatus, ER, nucleus, lysosomes and mitochondria, and in lipid particles (33). Prostatin in the nuclear fraction (P1) did not show binding activity to PBP (Fig. 10). It is presently unclear why prostatin in this fraction was unable to form a complex with mPBP. The functional significance of prostatin in the nuclear-ER-Golgi complex is also unclear at present and will be investigated in the future.

Despite the apparently different subcellular localization of prostatin in overexpressing cells *versus* endogenously expressing cells, prostatin is found in an membrane-bound form in all cell lines tested as well as in normal human prostate tissues (Fig. 6A). The membrane-bound prostatin was released when extracted with a detergent but remained membrane-bound when treated with high salt or alkali (Fig. 4), ruling out the possibility that prostatin is associated with another membrane-bound protein via noncovalent linkages. We also demonstrated that the membrane-bound and the detergent-released prostatin have similar molecular mass (Fig. 4), ruling out the possibility that prostatin is covalently linked to another membrane-bound protein.

The native prostatin in normal prostate tissue and the re-

combinant prostatin in 293/Pro and PC-3/Pro cells were easily released from the membrane with PI-PLC treatment (Figs. 5 and 6A), suggesting that prostatin is bound to the membrane via a GPI anchor rather than through a true transmembrane domain. The membrane-bound prostatin in LNCaP cells (native) or DU-145/Pro cells (recombinant), however, was resistant to PI-PLC treatment (Fig. 6A). As reported in Englund (34) and Hiroshi *et al.* (35), not all GPI-anchored proteins are susceptible to PI-PLC digestion. The membrane-anchored prostatin in LNCaP and DU-145/Pro could potentially be susceptible to other phospholipases such as GPI-phospholipase D (34, 35). Our results from the [³H]ethanolamine biosynthetic labeling experiment with PC-3/Pro and LNCaP cells, however, provided direct evidence that in the prostate epithelial cells recombinant or native prostatin is GPI-anchored, regardless of its sensitivity to PI-PLC treatment (Fig. 6B).

Among all four human cell lines that express either recombinant or native prostatin, as well as normal human prostate tissue, prostatin exists mainly as a membrane-bound protein (Fig. 6A). A small portion of prostatin in the 293/Pro cells is in the cytosolic fraction. This cytosolic prostatin could be a misprocessed or misfolded form that was exported from the ER before GPI anchor attachment, a mechanism documented previously (36). The presumably misfolded prostatin in the cytosol had no binding activities when it was incubated with mouse seminal vesicle fluid, possibly because of the misfolding. The secreted recombinant prostatin, when purified from the 293/Pro culture medium, however, is enzymatically active and able to form a complex with mPBP (Figs. 9 and 10), indicating that the cytosolic prostatin is not the source of secreted prostatin. The soluble fraction of prostatin seen in the human prostate tissues (Fig. 6A) before PI-PLC treatment may also be a misfolded form by the same mechanism described above or may be attributed to residual prostatic fluid caused by possible incomplete washing before tissue lysis.

We identified a PBP in mouse and human seminal vesicles (Fig. 7). Prostatin forms an 82-kDa, SDS- and heat-stable complex when incubated with seminal vesicle fluid as determined by SDS-PAGE under reducing conditions followed by prostatin immunoblotting. This complex is apparently covalently formed between prostatin and PBP and not via a disulfide linkage. We have chosen to use mouse seminal vesicles for an in-depth analysis of PBP because of easier availability. Complex formation between prostatin and mPBP was inhibited by the polyclonal prostatin antibody, heparin, and serine protease inhibitors. In a membrane overlay zymography analysis (Fig. 9), the prostatin-mPBP complex showed no activities to a synthetic substrate D-Pro-Phe-Arg-AFC, whereas unbound prostatin was active. These results suggest that mPBP may be a serpin class serine protease inhibitor. The true nature of the mechanism of prostatin inhibition by mPBP will be investigated upon purification and sequence analysis of this protein. An incubation of mouse or human plasma with r-hPro did not result in formation of any covalently-bound complex. This result would rule out the possibility of mPBP being one of the known members of the serpin family present normally in the blood, such as α_1 -antitrypsin, α_1 -antichymotrypsin, kallistatin, plasminogen activator inhibitor, and protein C inhibitor. At present, the functional significance of PBP with respect to prostate biology is unclear. Future studies will be aimed at determining the prostatin binding site in PBP, which could potentially reveal clues on prostatin's natural protein substrate.

One of our goals for the present study was to determine whether the membrane-anchored prostatin is an active serine protease. To accomplish this, we needed a prostatin-specific

enzymatic activity assay that is applicable for membrane-bound prostaticin because this form of prostaticin exists in a complex mixture. The membrane overlay zymography assay was not applicable for the membrane-anchored prostaticin because lipid-associated proteins cannot be well resolved in non-denaturing native gel electrophoresis. The identification of mPBP offered us an indirect but prostaticin-specific assay to address this question. As presented in Fig. 10, the membrane-bound human prostaticin also displayed binding activity to mPBP, and the binding is inhibited by a serine protease inhibitor (aprotinin) that competes for the serine active site, suggesting that the membrane-bound prostaticin is likely an active serine protease. Demonstration of membrane-bound prostaticin being an active serine protease will provide clues for investigating the signal transduction pathway(s) involved in the anti-invasion activity of prostaticin because this anti-invasion activity is conferred by the cellular prostaticin but not the secreted prostaticin (20).

Prostaticin is made in the prostate and secreted as an active serine protease (1), whereas PBP is made in the seminal vesicles. The fact that prostaticin forms a complex with PBP suggests that the two proteins interact with each other when semen is ejaculated, thereby implicating a role for both proteins in semen coagulation and liquefaction. Prostaticin and PBP in male reproductive tracts may serve together in a partnership to affect fertility. Investigating the prostaticin-PBP partnership could also lead to a better understanding of the various factors affecting fertility or, causing infertility. Overall, prostaticin, as a GPI-anchored or a secreted active serine protease, may have multiple physiological functions, depending on the localization of the prostaticin protein, whether it is membrane-bound or secreted.

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