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Differential Expression of PSP94 in Rat Prostate Lobes as Demonstrated by an Antibody Against Recombinant GST-PSP94

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Abstract Prostate secretory protein (PSP94, 94 amino acids) is one of the most abundant proteins secreted from the prostate. Its biological role is unknown and still controversial, although it is assumed to have the potential to be a biomarker and a suppressor of prostate cancer. In order to establish an animal model to further elucidate its biological role, we expressed the mature form of rat PSP94 in *Escherichia coli*, using a glutathione S-transferase (GST) fusion expression vector; we generated a polyclonal rabbit antibody against the recombinant protein. The antibody specifically recognized recombinant rat PSP94 and cross-reacted only very weakly with its human homologue. Using the characterized anti-rat PSP94 antibody, we found that PSP94 was located primarily in rat prostate. Furthermore, PSP94 is present at different levels in different lobes of rat prostate, with significant levels detectable only in the lateral lobe (LP). In addition, the most abundant PSP94 expression was found in the prostate lobe secretions, and PSP94 levels in LP secretions were at least seven times higher than in secretions from the dorsal prostate (DP). The rat ventral prostate (VP) and other regions of the male accessory glands were found to be almost completely devoid of PSP94. Since most rat prostate dysplasia induced by steroid hormone treatment occurs only in dorsolateral prostate, prostate tissue-specific expression and the expression of PSP94 in dorsolateral, but not other, lobes of the prostate suggest a potential role in prostate targeting and prostate cancer development. J. Cell. Biochem. 74:406–417, 1999.

Key words: PSP94; glutathione S-transferase; lateral prostate; dorsal prostate; ventral prostate; coagulation gland; seminal vesicles

Abbreviations used: PSP94, prostate secretory protein of 94 amino acids; crude rat PSP94, crude preparation of recombinant rat PSP94; GST, glutathione S- transferase; PSA, prostatic-specific antigen; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; LP, lateral prostate; DP, dorsal prostate; VP, ventral prostate; CG, coagulation gland; SV, seminal vesicles.

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The prostatic secretory protein (PSP94, 94 amino acids), also known as β-microseminoprotein [Abrahamsson et al., 1988; Hara et al., 1989; Hyakutake et al., 1993], is one of the three most abundant secretory proteins (PSP94, prostatic acid phosphatase [PAP]; prostatic-specific antigen [PSA]) from the prostate gland [Dube et al., 1987; Abrahamsson et al., 1988; for review, see Hara et al., 1989]. PSP94 has attracted a good deal of interest, as this protein has been proposed as a useful biomarker and suppressor of prostate cancer.

PSP94 was first suggested to function as an inhibitor of follicle-stimulating hormone (β-FSH) released from the pituitary gland [Sheth et al., 1988]. At the paracrine or autocrine level, PSP94, synthesized directly by prostate tissue,

may inhibit prostatic tissue growth [Sheth et al., 1988; Garde et al., 1993; Lokeshwar et al., 1993; Mundle et al., 1993]. Studies have shown enhanced apoptosis in rat prostate exposed to increasing amounts of exogenous PSP94. Several studies [Mundle et al., 1993] have reported that PSP94 may represent a novel and potentially nontoxic form of therapy for hormone-independent prostate cancer. However, other investigators have reported that PSP94 failed to inhibit β-FSH-stimulated prostate cancer cell growth [Kohan et al., 1986; Gordon et al., 1987].

PSP94 expression in human prostate cancer tissue, demonstrated by immunohistochemistry (IHC) and in situ hybridization (ISH), showed a decreased level [Hyakutake et al., 1993; Tsurusaki et al., 1998], while levels of serum PSP94 determined by enzyme-linked immunosorbent assay (ELISA) were elevated in prostate cancer patients [Dube et al., 1987; Huang et al., 1993; Maeda et al., 1993]. Correlative studies of the expression of serum PSP94 with PSA have shown that PSP94 may be as useful as PSA as a biomarker of prostate cancer, especially when used as an androgen-independent marker in monitoring the course of hormone therapy [Abrahamsson et al., 1988; Teni et al., 1988; Garde et al., 1993]. These observations have also been questioned, however [van der Kammer et al., 1993].

PSP94 is synthesized as a precursor of 114 amino acids and is secreted in a mature form of 94 amino acids in human prostate after removal of a 20-amino acid signal peptide. Studies on tissue distribution in humans showed that PSP94 exists in most mucus secretory tissues and in high abundance (approximately 25% of the level seen in prostate) in respiratory tract secretions [Ulvsback et al., 1989; Weiber et al., 1990]. However, this finding was based primarily on studies done in human pathological samples; it has not been confirmed in pig [Fernlund et al., 1994; Tanaka et al., 1996] and rodent [Fernlund et al., 1996; Xuan et al., 1999] studies, although the presence of rat PSP94 has been reported primarily in dorsal and lateral prostate [Fernlund et al., 1996; Xuan et al., 1999].

In order to determine the tissue-specific expression of rodent PSP94 and also further elucidate on its biological function, we developed and characterized a polyclonal antibody, using the glutathione S-transferase (GST) gene fusion technique. The antibody generated was then used to study its expression and tissue distribution in rat tissues by Western blotting and immunohistochemistry. The results showed that rat PSP94 appears to be highly differentially expressed in different lobes of the rat prostate.

MATERIALS AND METHODS

Rodent Strains, Anatomy, and Dissection

Sprague-Dawley rats used in this study were male retired breeders aged 6–10 months (approximately 400 g). Male mice (strain C57BL6) were 6–8 weeks old. Rodent prostate tissue dissection was performed according to the description and definition reported by other investigators [Sugimura et al., 1986; Lee, 1987]. The urethra and vas deferens, together with the male accessory sex glands, including the prostate gland complex, coagulating gland (CG), and seminal vesicles (SV) were dissected out from the anaesthetized adult animals. The prostate gland complex was further dissected into three individual lobes: dorsal (DP), lateral (LP), and ventral (VP) prostatic lobes. Right and left parts of ventral lobes of unequal size were found. The dissected samples were frozen in liquid nitrogen immediately and then stored at −80°C.

Protein Extraction and Collection of Secretion Fluids From Individual Glands

Fresh dissected or frozen samples collected after dissection were homogenized in 1 ml ice-cold 0.01 M phosphate-buffered saline (PBS) and then centrifuged at 10,000*g*. The supernatants were removed, to which 0.5% sodium dodecyl sulfate (SDS) and 1 mM phenylmethylsulfonyl fluoride (PMSF) were added, and quantified by their absorption at OD 280 nm. This clear cell extract was designated as the lysate. Lysates from whole prostate gland extract and individual dissected lobes of rodent prostates were also prepared.

For collection of secretory fluids, the freshly dissected prostatic glands were gently cut by a surgical blade, and the tissues were slightly squeezed with the blade over a sheet of aluminum foil. The fluids were then collected by a micropipette. The chopped tissues were further centrifuged at 14,000*g* for 10 min at 4°C. The supernatants were removed and pooled with the previously collected fluids. These fluids were designated as the secretory fluids from differ-

ent glands or prostate lobes. The fluids were diluted in buffer containing 0.01 M PBS, 1% SDS, and 1 mM PMSF to a final concentration of 0.1–0.8 mg/ml, as estimated by their absorption at OD 280m.

Construction of GST-PSP94 Fusion Plasmid in *E. coli* Expression Vector pGEX2T

Rat PSP94 cDNA clone was isolated and sequenced as reported previously [Fernlund et al., 1996; Xuan et al., 1999]. The mature rat PSP94 coding region was cloned into *E. coli* expression vector pGEX-2T (Pharmacia, Montreal, Que) by polymerase chain reaction (PCR). Two primers located near the N-terminus of the excision site of PSP94 precursor protein and the stop codon were synthesized (Procyon, ON). The N-terminal primer was synthesized (Fig.1A) to maintain the same reading frame as the carrier protein GST and contain a cloning site of restriction enzyme *Bam*HI. C-terminal primer comprises the 3′ end of rat PSP94 cDNA (Fig.1A) and the cloning site of restriction enzyme *Eco*RI. A PCR reaction was performed as previously reported [Xuan et al., 1995] using 10 ng of the rat PSP94 cDNA template [Fernlund et al., 1996; Xuan et al., 1999]. The PCR product was purified by the Glassmatrix method (Gibco-BRL, Burglington, ON), digested with *Bam*HI and *Eco*RI enzyme, and ligated into pBS plasmid (Stratagene, CA), linearized by the same restriction enzymes. The recombinant clone was screened and characterized by physical mapping and DNA sequencing. All recombinant DNA techniques, restriction, ligation, transformation, screening, and DNA sequencing analyses were performed according to standard protocols [Sambrook et al., 1989] or as previously reported [Xuan et al., 1996, 1999].

Expression and Purification of Recombinant GST-PSP94

The recombinant GST-rat PSP94 was expressed in the *E. coli* strain BL21 (Novagen; Madison, WI), which lacks the *lon* protease and the *omp*T out membrane protease. *E. coli* bacterial culture and the induction of recombinant protein by 0.5 mM IPTG (isopropylthio-β-D-galactoside) were performed as previously reported [Xuan et al., 1996, 1997a,b]. Recombinant GST-PSP94 fusion protein was purified by an affinity-column glutathione-Sepharose 4B (Pharmacia, Montreal, Que) as reported previously [Xuan et al., 1996, 1997a,b]. A large-scale (1.5-L LB media) preparation of the fusion protein was generated in a Biostat M Fermentor (B. Braun) at 32–34°C with agitation at 800 rpm. Recombinant rat PSP94 expression was induced by adding IPTG to a concentration of 0.5 mM, with the cell suspension reaching an OD 600 nm of 1.5. A bacterial cell pellet was collected within 2 h of IPTG treatment. Both recombinant GST-PSP94 cytosol and inclusion body fractions were purified as reported previously [Xuan et al., 1996, 1997a]. In brief, a denaturing solution (8 M urea, 0.1 M glycine pH 9.0) was employed to dissolve all the precipitated proteins in the inclusion bodies. The dissolved solution was immediately desalted by passing through a Sephadex G50 column in 0.1 M glycine buffer (pH 9.0). Renaturing of the desalted protein fractions was performed by incubating in the same buffer at 4°C overnight. Affinity-column (glutathione Sepharose 4B) purification of the dissolved *E. coli* proteins from inclusion bodies was conducted according to the manufacturer's instructions (Pharmacia). Thrombin digestion (10 U/mg protein) was performed by overnight incubation at room temperature [Xuan et al., 1996, 1997a]. After digestion, recombinant PSP94 was further purified by absorbing with GST affinity column matrix. Triton X-100 was added to the thrombin-digested GST-PSP94 mixture at a final concentration of 1%; about 10% volume of GST-glutathione Sepharose was added and incubated at 4°C for overnight. Crude preparation of rat PSP94 was collected by spinning at a microcentrifuge. Protein concentration was determined by measurements of absorbency at OD 280 and checked by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by staining with Coomassie Blue.

Generation of a Rabbit Antiserum Against Recombinant GST-PSP94

A rabbit polyclonal antibody against recombinant GST-rat PSP94 was obtained using a standard operation procedure (SOP #370–01, UWO Animal Care Committee). In brief, 1 mg of recombinant GST-rat PSP94 (0.5 ml) was emulsified in 0.5 ml of Freund's complete adjuvant (Sigma Chemical Co., St. Louis, MO) and injected intramuscularly in rabbit (approximately 1 kg). A second booster injection was performed 2 weeks later. An adequate titer of antibody activity was obtained within 1 month of the second injection.

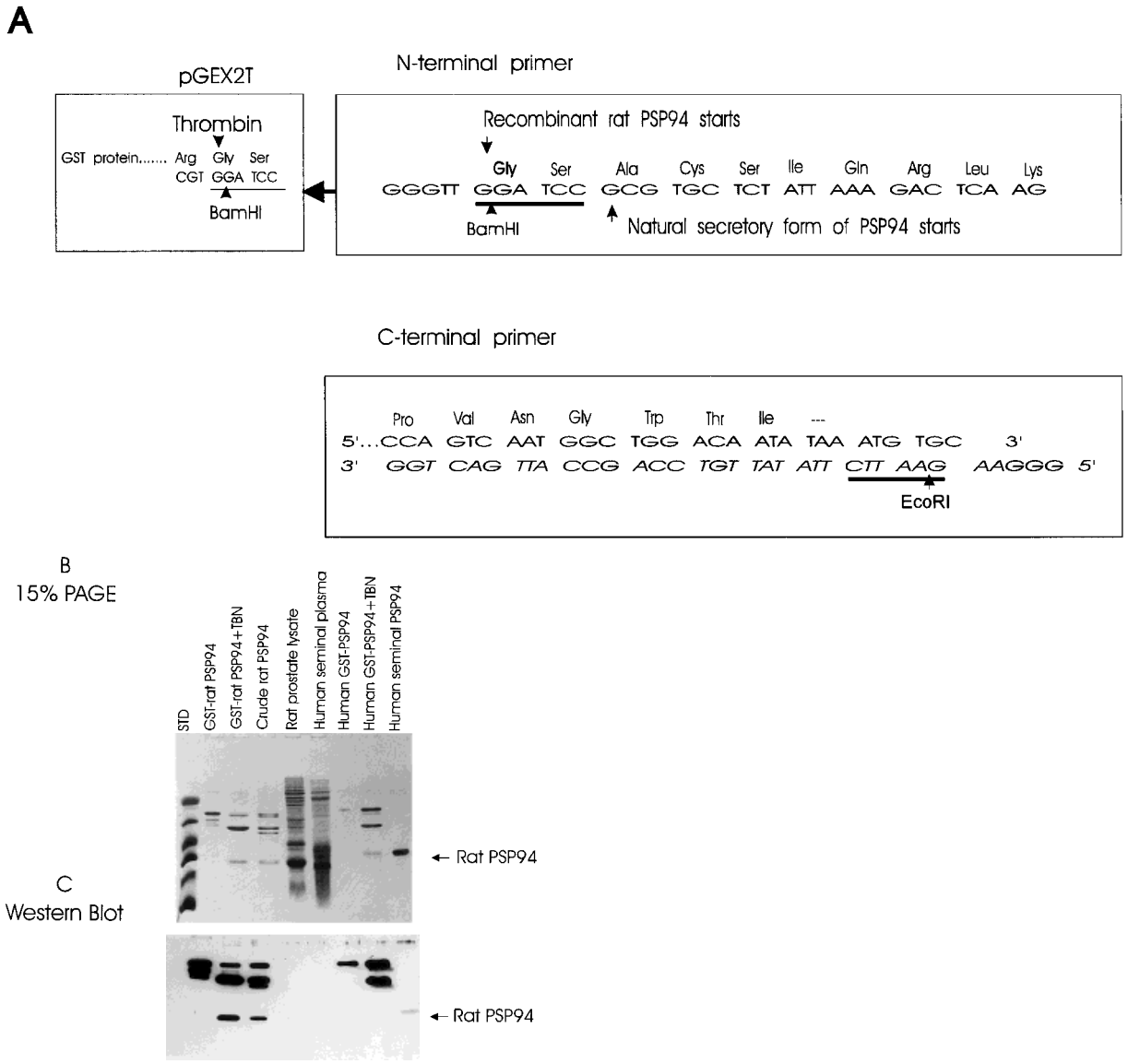

Fig. 1. Expression of recombinant glutathione S-transferase (GST)-PSP94 fusion protein in *Escherichia coli*. **A:** Strategy for polymerase chain reaction (PCR) cloning of the mature PSP94 cDNA sequence in an *E. coli* expression vector pGEX2T. The primer used in the PCR reaction in the N-terminus of the recombinant PSP94 is shown in the left box. *Bam*HI recognition sequence in both vector (right box) and N-terminal primer is underlined; arrows, digestion site. Thrombin cleavage site is indicated by arrows at the top. The extra glycine and serine residues in recombinant rat PSP94 are shown in italics. The first box shows that the *Bam*HI site in the vector and the N-terminal sequence of PSP94 are in the same reading frame. The second box shows the C-terminal of recombinant GST-rat PSP94 cloned at the *Eco*R1 site in the primer sequence. The C-terminal primer used for PCR is an antisense sequence as indicated at the second line. The first line shows sense strand and the C-terminal amino acid sequence. **B:** Analysis of expression of GST-rat PSP94 protein in the inclusion body fraction by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Crude recombinant rat PSP94 protein (crude rat PSP94) was released by thrombin (TBN) digestion, passed through a glutathione Sepharose 4B column matrix, and before loading, concentrated by centrifugation using Centricon Concentrators 30 (Amicon, MA). In this lane, thrombin is shown as a band at approximately 25 kDa (lower than GST). Recombinant GST human PSP94, human prostate tissue lysate, and seminal plasma were loaded as controls to test cross-reactivity between species. PAGE was stained with Coomassie Blue. **C:** Western blotting analysis of 15% SDS-PAGE (B). About 5% of the amount of each of the 10 samples in B were loaded and blotted (see Materials and Methods). Rabbit anti recombinant GST-rat PSP94 polyclonal antibody and secondary antibody (goat anti-rabbit IgG) were diluted 1,000 times separately. STD: protein molecular weight standards, (low range, from Gibco-BRL) (from bottom to top): 3, 6.2, 14.3, 18.4, 29, and 43 kDa.

SDS-PAGE and Western Blotting

The protein contents in the tissue lysate of whole gland or individual lobe extracts, as well as secretory fluids, were separated and analyzed by 15% SDS-PAGE (10 µg protein/lane, Coomassie Blue staining) and Western blotting (0.1–1 µg protein/lane). The chemiluminescence method was followed using an ECL Western Blotting kit (Amersham, Oakville, ON). A total of 10–50 ng of protein was boiled and loaded onto 15% polyacrylamide gels (PAGE) and electrotransferred to nitrocellulose membrane (Hybond-ECL membrane, Amersham) according to the protocol provided by manufacturer. Horseradish peroxidase (HRP)-conjugated swine anti-rabbit IgG (Dimension Laboratories, Mississauga, ON) was used as a secondary antibody.

Direct and Competitive ELISA

Direct ELISA. We followed previously reported protocols [Xuan et al., 1996, 1997a,b]. In brief, protein samples were coated in triplicate in the wells of a 96-well Immunoplate (Nunc, Gibco-BRL) at 4°C overnight. The coated plate was washed in PBS and blocked in 1.5% bovine serum albumin (BSA)/PBS/T (RIA grade; Sigma). The first antibody reaction was carried out at room temperature and was stopped by washing three times with PBS/T. The secondary antibody, HRP-conjugated swine antiserum against rabbit IgG, was diluted in PBS and incubated in the wells at room temperature for 1 h. Wells were then washed three times with PBS. Colormetric detection of bound antibody was performed by adding 0.4 mg/ml OPD (o-phenylene diamine dihydrochloride; Sigma) and 0.05% H_2O_2 and incubating at room temperature for 20 min. OD 492 nm was measured on a microplate reader (Titrek Multiscan Plus, EFLAB). Graph generation and statistical analysis were performed using SigmaPlot and SigmaStat computer software programs (Jandal Co., San Rafael, CA).

Competitive ELISA. Coating and blocking of the sample wells, HRP-conjugated secondary antibody reaction, and color development with OPD were performed as for the direct ELISA. PSP94 antiserum was diluted in 1.5% BSA/PBS/T at a ratio of 1:500 and mixed with the competitor proteins at various concentrations. Antibody-competitor mixture was added immediately to the sample wells in triplicate and incubated at room temperature for 2 h with shaking. The competition reaction was stopped by washing three times with PBS/T.

Immmunohistochemistry (IHC)

Prostatic tissues were fixed in Carnoy's fixative and embedded in paraffin. Sections, 4 µm thick, were cut, deparaffinized, and rehydrated. The hydrated sections were treated in 0.5% H_2O_2 in absolute methanol for 15 min to remove endogenous peroxidase activity. After rinsing in PBS for 20 min, sections were blocked in 1% BSA in PBS (blocking solution) for 15 min. After draining off the blocking solution, the sections were rinsed twice in PBS. The sections were then incubated overnight with the recombinant GST-rat PSP94 antiserum and diluted 1:100 in blocking solution, at 4°C in a humid chamber. After washing with PBS, the sections were incubated with a biotinylated goat anti-rabbit IgG secondary antibody (Jackson Laboratories, PA; diluted 1:200 in blocking solution) for 1 h at room temperature. The antibody binding sites were then demonstrated by the avidin-biotin-peroxidase complex (ABC) procedure for 1 h at room temperature. After incubation with ABC solution, the sections were rinsed in PBS for three changes. The peroxidase activity was then visualized by a glucose oxidase-diaminobenzidine (DAB)-nickel intensifying procedure [Chan et al., 1995]. The sections were lightly counterstained with Mayer's hematoxylin. Control sections were performed by using the blocking solution without primary antibody.

RESULTS

Expression of Mature Form of Rat PSP94 in *E. coli*

In order to study PSP94 expression in rodent tissues, we used a bacterial carrier protein GST (approximately 26 kDa) to express the mature form of rat PSP94 (93 amino acids) in *E. coli*. The same carrier protein fused with rat PSP94 was used to immunize rabbits to generate an antiserum to rat PSP94. As shown in Figure 1, the recombinant rat PSP94 (95 amino acids) contains only two additional amino acid residues (Gly, Ser) in the N-terminal region of mature protein within the thrombin recognition site. Analysis of lysates from *E. coli* recipient transformed with this recombinant construct indicates that the expression of GST-rat PSP94 was mostly in inclusion bodies (data not shown),

as reported previously [Xuan et al., 1994, 1996, 1997a]. Similar to our previous reports, truncated forms of GST-rat PSP94 was also observed (Fig.1B, lane 2). Recombinant rat PSP94 can be released from GST by digestion with thrombin. Its apparent molecular weight is about 12 kDa, as estimated in a 15% SDS-PAGE (Fig. 1B). The digestion mixture was purified by GST affinity column matrix (glutathione-Sepharose 4B); at least 60% of the carrier protein was removed (Fig. 1B) in this crude preparation of recombinant rat PSP94 (crude rat PSP94).

Characterization of a Polyclonal Antibody Against Recombinant GST Rat PSP94

The recombinant GST-PSP94 was used to immunize rabbit, and the antiserum obtained was tested for specificity to rat PSP94 by Western blot (Fig.1 B). The rabbit antiserum recognized both GST and recombinant rat PSP94, as either a fused protein or a released free form (Fig. 1C). It had very weak cross-reaction with the human analogue of PSP94 only when it was overloaded on gels (Fig.1C) and showed no cross-reactivity with proteins in human prostate lysate, seminal plasma proteins,and recombinant human PSP94 (except carrier GST protein).

Quantitative analysis was performed to characterize the binding of rabbit antiserum to recombinant GST-rat PSP94. Since rat PSP94 (MW approximately 10 kDa) comprises only about 27% of the whole GST fusion protein (approximately 37 kDa), we expected that GST and rat PSP94 would comprise 73% and 27% of the antibody activity, respectively. Figure 2A shows the result of an ELISA to determine the effect of diluting antibody on its capacity to bind to GST and its derived proteins: GST, GST-rat PSP94, and a crude preparation of recombinant rat PSP94 (crude rat PSP94). As expected, GST-rat PSP94 (100 ng/well) produced a stronger signal than GST alone (100 ng/well), and the signal generated using a crude preparation of recombinant rat PSP94 from 100 ng of GST-rat PSP94 was weaker. In all the dilutions tested, the rabbit antiserum to rat PSP94 showed lower activity in ELISA (Fig. 2A) than the antibody to human PSP94 [Xuan et al., 1997a]. A dose response of binding of GST-rat PSP94 and crude rat PSP94 with rabbit antiserum at a dilution of 1:500 was obtained. The crude rat PSP94 again showed lower

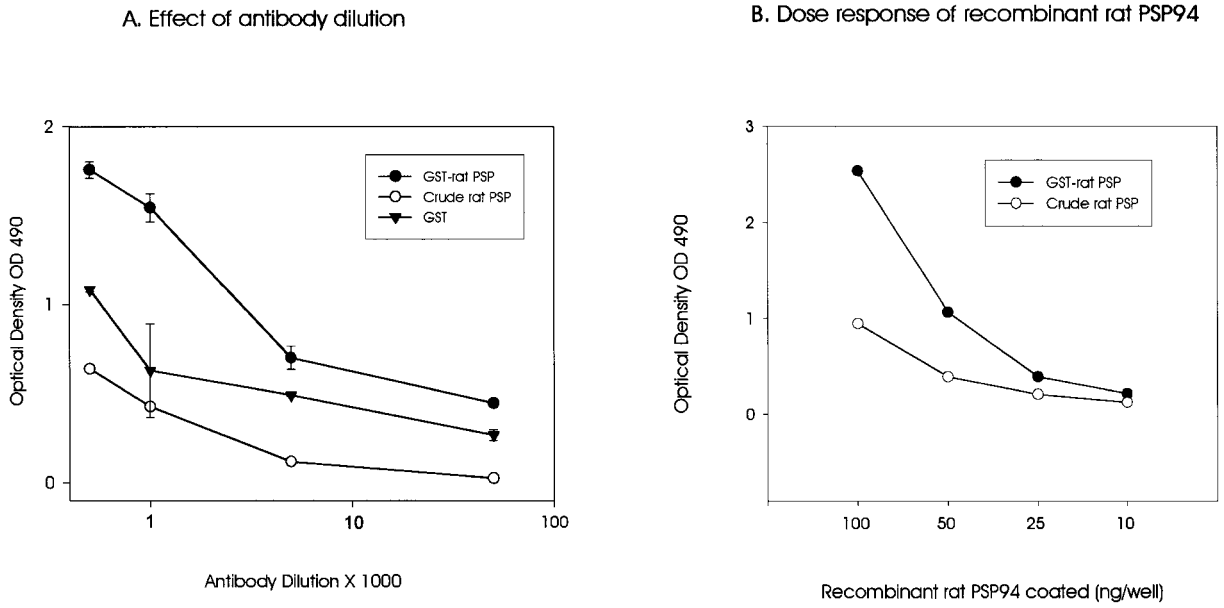

Fig. 2. Enzyme-linked immunosorbent assay (ELISA) of the immunoreactivity of the rabbit antiserum against recombinant glutathione S-transferase (GST)-rat PSP94. **A:** Test of antibody dilution (sequentially diluted) with three recombinant proteins: GST, GST-rat PSP94, and crude rat recombinant PSP94 (crude rPSP94). All three proteins were used to coat wells (100 ng/well). Quantity (ng) of the crude rPSP94 refers to the amount of GST-rat PSP94 assessed before purification. **B:** Test of dose response of recombinant rat PSP94s coated at different amount indicated in the x-axis in the solid phase with the polyclonal antibody at 1:500 dilution Error bars of the points are mean values from triple samples ±S.D.

(30–50%) binding activity than the original GST fusion protein.

Quantitative analysis of the rabbit antiserum to recombinant GST-rat PSP94 was also performed using competitive ELISA. Figure 3A shows the result of GST-rat PSP94 coated in the solid phase competing with GST and crude preparation of rat PSP94 in the solution phase, at an antibody dilution of 1:500. Crude rat PSP94 comprises about one-fourth to three-fourths the ability of GST to compete binding with polyclonal antibody with GST-rat PSP94 in the solid phase. Figure 3B shows the results of an inverse competitive ELISA, in which the crude preparation of recombinant rat PSP94 was in solid phase, and competed with GST in the solution phase. In this situation, GST protein competed 80–90% less effectively, indicating that different epitope were recognized by the antibody in these two different assays.

Characterization of the Prostate Lobe-Specific Expression of PSP94 by Western Blot Experiments

Rat PSP94 expression was determined first by testing rat prostate lobe samples dissected according to established definitions [Sugimura et al., 1986; Lee, 1987]. Homogenized tissue lysates were prepared and analyzed by 15% SDS-PAGE. A differential display of protein patterns in tissue lysates from three lobes of prostate glands and other two male sex accessory glands (coagulating gland and seminal vesicles) was demonstrated (Fig. 4A). As compared with total rat prostate lysates, this display in different lobes of prostate was repeatedly shown in two rats tested (Fig. 4A). Western blot analysis of Figure 4A was conducted and results (Fig. 4B) showed only one strongly reactive band to rabbit antiserum to recombinant GST-rat PSP94. This protein band was highly lateral lobe specific. PSP94 expression was not detectable in any other rat tissues. Tissue samples from large number of rats ($n > 15$) were tested; a representative Western blot of four of these LP samples is shown in Figure 4C. A positive signal was apparent in all rat lateral prostate. Mouse prostate and the sex accessory glands were dissected and tested as well, and none of samples from three mice shown in Figure 4C yielded positive signal.

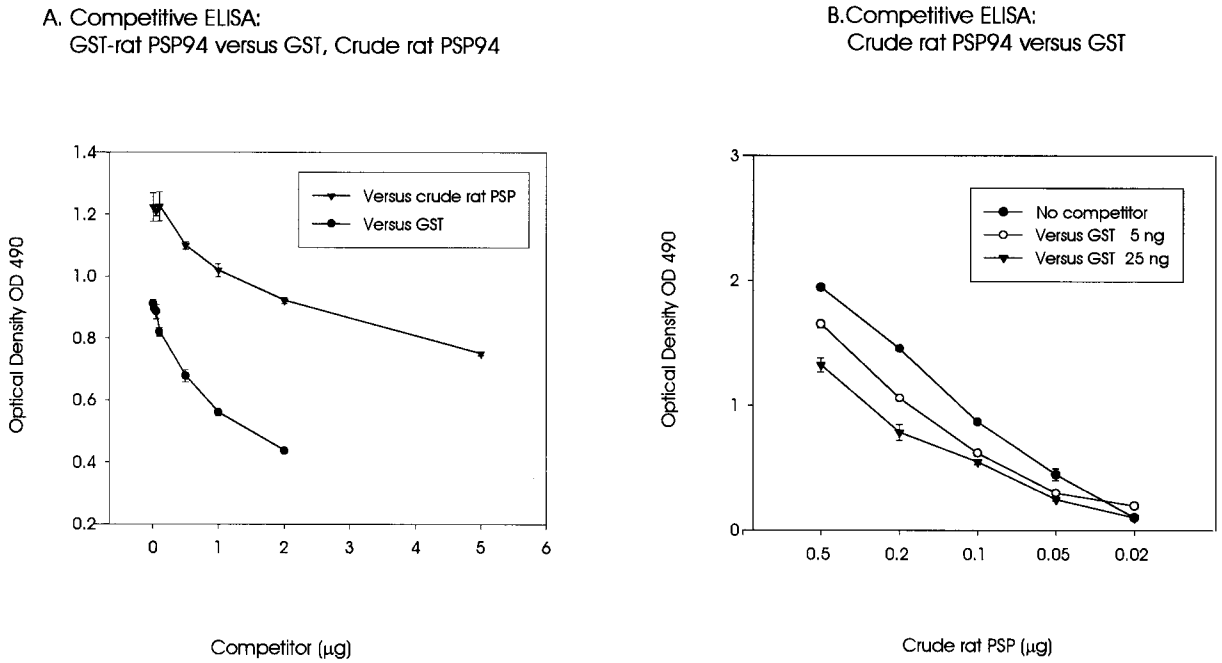

Fig. 3. Competitive enzyme-linked immunosprbent assay (ELISA) among three recombinant proteins: glutathione S-transferase (GST), GST-rat PSP94, and crude preparation of recombinant rat PSP94 (crude rat PSP94). **A:** Competition of the binding with the rabbit antiserum between GST-rat PSP94 (coated in the solid phase 100 ng/well) and competitors in the solution phase: GST and crude rPSP94 (refers to the amount used for purification). Antibody dilution was at 1:500. **B:** Competition of the binding with the rabbit antiserum between crude rPSP94 (coated in the solid-phase 100 ng/well, amount of ng refers to the amount used for purification) and competitors in the solution phase was GST. Antibody dilution was at 1:500. Error bars of the points are mean values from triple samples ±S.D.

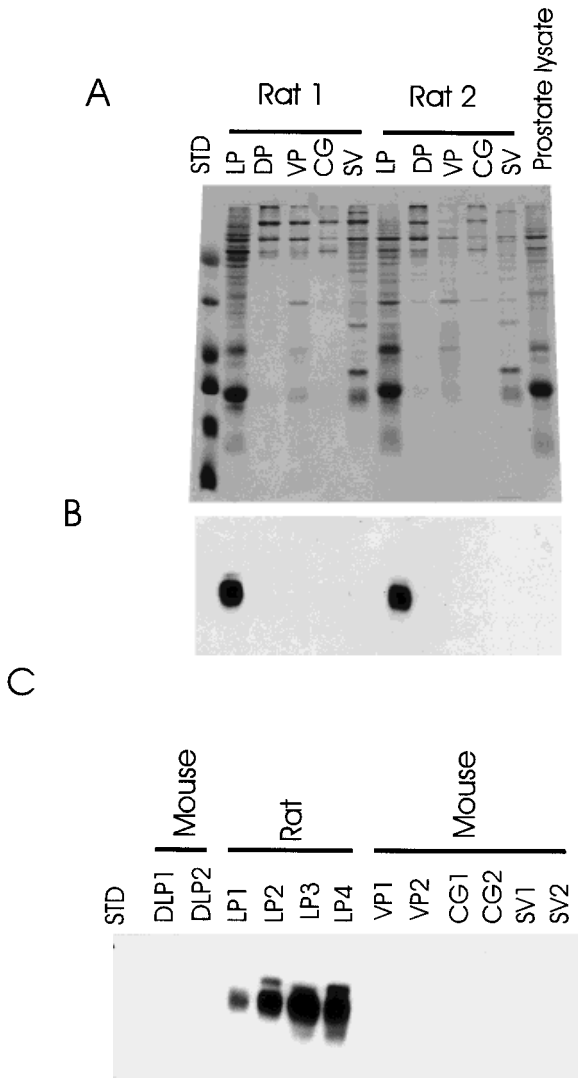

Fig. 4. **A:** Differential display of protein extracts from rat prostate lobes and male rat sex accessory glands. Protein samples (10 µg/lane) were separated in 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Blue. Standard prestained protein markers (low range, from Gibco-BRL) used are (from bottom to top): 3, 6.2, 14.3, 18.4, 29, and 43 kDa. **B:** Western blotting experiments of gel A. Rabbit antiserum used were diluted at 1:10,000. **C:** Western blot analysis of rodent tissue lysate of prostate lobes and the other sex accessory glands. Only results from four rats and two mice were shown.

Differential Expression of PSP94 in Rat Prostatic Secretory Fluids

Because the secretion of prostatic fluid is the major biological function of the prostate, we tested the hypothesis that differential expression of PSP94 in rat prostatic fluid is, as in the tissue themselves, prostate lobe specific as well. We tested an equal amount of fluid proteins from three lobes (LP, DP, VP) and two accessory glands (SV, CG) by Western blot experiments, using rabbit antiserum against recombinant rat PSP94. In order to obtain a semiquantitative estimation, different amounts of protein were loaded on to a 15% SDS-PAGE (Fig. 5A). After Western blotting using the anti-GST-rat PSP94 antibody (Fig.5B), a short exposure time (1 s) showed a visible band in 0.75 µg of total LP secretory fluid protein. 5–15 µg of proteins from two other prostate lobes (DP, VP) and accessory glands (SV and CG) showed no delectable signal, indicating that PSP94 present in LP secretory fluids is at least seven times higher than any of the other four lobes and glands. Overex-

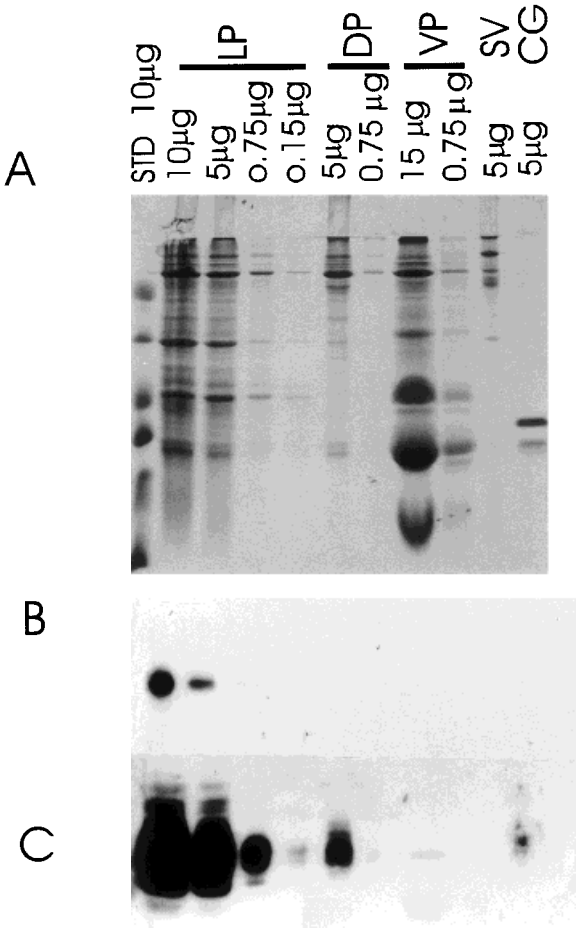

Fig. 5. **A.** Differential display of total proteins from secretory fluids from three dissected lobes of rat prostate and two other male rat sex accessory glands. Protein samples (µg as indicated per lane) were separated in 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Blue. Standard prestained protein marker used (see legend of Fig. 4). **B:** Western blotting experiments of gel A. Rabbit antiserum used were diluted at 1:10,000. Exposure time 1 s. **C:** Overexposure of Western blot B, exposure time 10 s.

posed film of Figure 5B shows positive signal from DP loaded 5 µg of fluid protein. The result also confirms that the level of differential expression of PSP94 in LP is at least seven times higher than DP.

Expression of PSP94 in Different Rat Tissues

PSP94 has been reported to be expressed at low levels, compared with prostate, in human and primate stomach, trachea and bronchus. We tested those tissues as well as others (bladder, liver, and kidney) in rat for PSP94 expression and found that none had significant levels of PSP94 (Fig. 6). One low-molecular-weight (approximately 5-kDa) weakly positive band in rat trachea tissue lysate was visible in Western blotting test (Fig. 6B). This band did not have migration properties consistent with PSP94.

Immunohistochemical Detection of PSP94 in Rat Prostate Gland

In order to confirm the results of Western blotting experiments, immunohistochmistry analysis was performed to locate PSP94 expression in prostate cells in situ. Staining for PSP94 was positive in lateral lobe, but negative in ventral lobe (Fig. 7A). Weak staining was found in dorsal lobe (not shown). The positive signal was localized to the apical borders and the cytoplasm of the epithelial cells in both lateral and dorsal prostates. The luminal secretions were also positively stained. However, smooth muscle cells were negative to PSP94 staining (Fig. 7B).

DISCUSSION

Our studies of tissue distribution of PSP94 in rodents demonstrated that its expression is highly specific to the prostate, as shown by RT-PCR plus Southern blots, as well as by in situ hybridization analysis [Xuan et al., 1999]. Expression of rodent PSP94 gene was found highly specific to only one prostate lobe, i.e., the

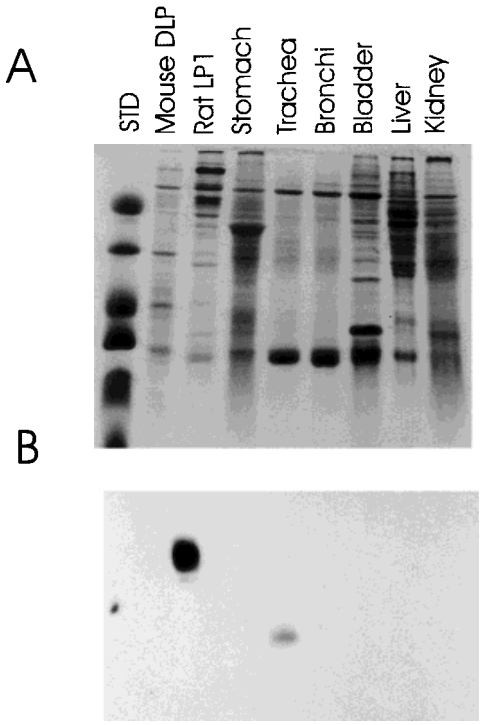

Fig. 6. Prostate-specific expression of rat PSP94 as demonstrated by tissue distribution studies. **A:** Total proteins (10 µg per lane) from lysate of different rat tissues were separated in 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Blue. Standard prestained protein marker used (see legend of Fig. 4). **B:** Western blotting experiments of gel A. Rabbit antiserum used were diluted at 1:10,000. Exposure time 3 s.

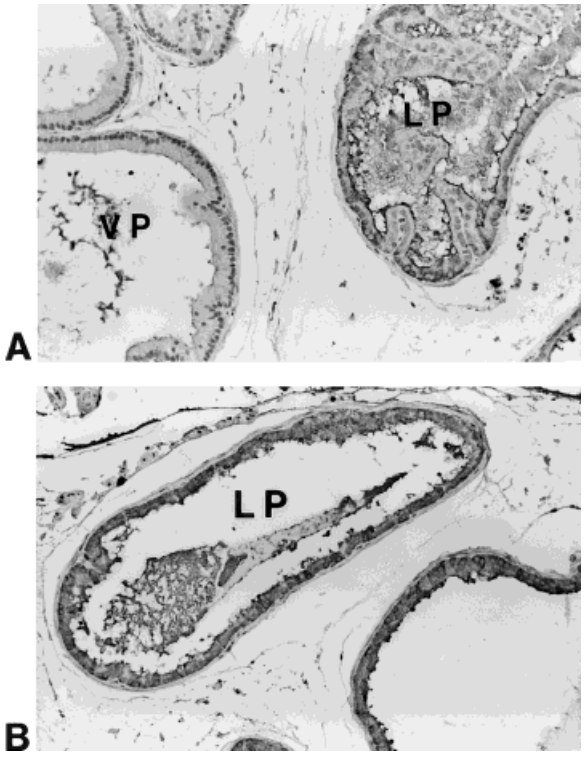

Fig. 7. Immunohistochemical detection of rat PSP94 expression in normal rat lateral and ventral prostate. **A:** Positive signal is seen over the apical cytoplasm of the secretory epithelial cells and the luminal secretions in the lateral lobe (LP) , whereas no immunoreaction is seen over the secretory epithelium of the acini in ventral lobe (VP). ×130. **B:** Micrograph showing the immunostaining of rat PSP94 in the lateral prostate. Similar to A, positive reaction is seen over the apical cytoplasm of the secretory epithelium and the luminal secretory contents in the acini of LP. The fibromuscular stroma is unreacted. ×130.

lateral prostate (LP) [Fernlund et al., 1996; Xuan et al., 1999]. In this study, we have extended this finding to the protein expression level by immunological detection using an antibody raised against recombinant GST-rat PSP94. Differential expression was found in two major lobes of rat prostate gland: the dorsolateral prostate (DLP) and the ventral prostate (VP). These two anatomically well-defined regions of the rat prostate are known to exhibit marked cytological, biochemical and functional differences [Sugimura et al., 1986; Lee, 1987; Waakes et al., 1988]. For example, high levels of zinc are sequestered in the DLP. The DLP is sensitive to sex hormone-induced cancer [Leav et al., 1988; Ho et al., 1993], while the VP is uniquely susceptible to Cd-induced cancer [Waakes et al., 1988].

It has been suggested that protracted stimulation of this tissue by sex hormone steroid may play an important role in the carcinogenic process. Simultaneous exposure of intact rats to testosterone (T) and estradiol-17β (E2) consistently induces intraductal dysplasia exclusively in the DLP [Leav et al., 1988; Ho et al., 1993], which closely resembles the lesion in the human gland. The presence of invasive carcinoma in the DLP after long term (52 weeks) exposure to $T + E_2$ suggests that dysplasia may progress to adenocarcinoma [Leav et al., 1988]. Dysplasia in DLP was accompanied by increase in the number of moderate affinity, high capacity, estrogen-binding site (type II sites) found exclusively in DLP of these animals. By contrast, a proliferative response and type II sites were not observed in the VP [Ho et al., 1993]. In this study, we have found exclusive expression of PSP94 in LP and DP, with the majority of PSP94 in LP. It will be important to correlate its potential role as a prostate cancer suppressor with the enhanced expression of PSP94 in the DLP/DP as demonstrated in this study. Studies on prostatic-specific expression and differentiation expression in different prostate regions suggests that PSP94 should be investigated for involvement in events contributing to tumor initiation and malignancy.

We have examined the expression of PSP94 in fetal, prepubertal and adult human prostates by in situ hybridization and immunohistochemistry [Chan et al., 1999]. A differential distribution pattern of PSP94 expression is found specifically in the peripheral zone, but it is variable in the central and transition zones and in fetal prostates at 6–7 months gestation. This finding is in contrast to those of two other major prostatic secretory proteins, PAP and PSA, which are relatively invariable. Since most human and rodent prostate tumors originate in the peripheral zone or lateral/dorsal lobe of the prostate, it will be important to explore the potential involvement of PSP94.

We have characterized the polyclonal antibody against recombinant GST-rat PSP94 both qualitatively (by Western blotting, Fig. 1) and quantitatively (by direct and competitive ELISA; Figs. 2, 3). Surprisingly, we found that this antibody reacts strongly with natural rat PSP94, as shown by Western blotting experiments conducted for either tissue lysate (Fig. 4) and prostatic fluids from prostate lobes (Fig. 5). We have also found that this antibody is less useful in ELISA and IHC than in Western blotting experiments. In order to obtain a reliable, reproducible reaction signal in ELISA and IHC experiments, antibody was used at a dilution of 1: 100–500, while in Western blots a dilution of 1:5, 000–10,000 was appropriate. Although definitive experiments to determine the reasons for this concentration differences have not been performed, we hypothesize that the antibody raised against the GST-rat PSP94 fusion protein reacts mostly with antigenic sites in the denatured form of proteins. After tissue fixation, many of these antigenic sites are presumably masked and so could evade detection by the antibody. PSP94 denatured in SDS and β-mercaptoethanol (Bio-Rad sample buffer) before Western blotting would, on the other hand, be open to antibody recognition.

We have performed a systematic study of the epitope structure of human PSP94. By peptide mapping and studies of the recombinant PSP94 peptides located at both ends (N-47 and C-47) of the protein, we found that the N- and C-termini of human PSP94 are immunodominant and recessive regions, respectively [Xuan et al., 1996, 1997a,b]. In a previous study [Xuan et al., 1999], we showed that the amino acid sequence of the N-terminus is more divergent among species than the sequence of the C-terminus using PSP94 protein sequences from humans, primates, pigs, and rodents for analysis and comparison. PSP94 protein sequences in two rodents (mouse and rat) are particularly divergent [Xuan et al., 1999]. Consistent with this observation, we have found that antibody against

recombinant GST-rat PSP94 showed no cross-reactivity with mouse PSP94 (Fig. 4C).

Studies on the prostatic-specific and differential expression in different prostate regions can lead to a new strategy for gene therapy and immunotherapy for prostate cancer. Currently, a transgenic mouse model [Greenberg et al., 1995] has been established using the promoter region of the probasin gene, a prostatic (DLP)-specific gene [Greenberg et al., 1994]. Several targeting vectors have been used successfully to express heterologous genes to the prostate epithelium of transgenic mice [for review, see Green et al., 1998]. These include regulatory elements derived from the rat C3 (1) prostate steroid-binding protein gene, rat probasin gene, human prostate-specific antigen, and mouse mammary tumor virus (MMTV), long terminal repeat (LTR). Of greatest interest is the transgenic adenocarcinoma mouse prostate (TRAMP) model [Greenberg et al., 1995], which is a transgenic mice harboring a rat probasin promoter driving prostate-specific epithelial expression of the SV40 large T antigen [Greenberg et al., 1995]. Interestingly, target tissues in transgenic mice are mostly to DLP [Greenberg et al., 1995], the same distribution as that of natural probasin in prostate lobe tissues [Spence et al., 1989]. The important finding of the present research on the differential expression of PSP94 in LP will shed light on the potential role of PSP94 as a targeting gene for gene therapy and immunotherapy, as PSP94 is one of the most abundant proteins secreted from prostate.

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