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Serum Bound Forms of PSP94 (Prostate Secretory Protein of 94 Amino Acids) in Prostate Cancer Patients

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Abstract PSP94 (prostate secretory protein of 94 amino acids) was regarded as a possible prostate cancer marker, however, it has been controversial. All prior studies were designed to test the free form in serum using antibodies to PSP94. Results presented here demonstrate that PSP94 exists in prostate cancer patients in two forms, free and bound, and that the majority is present as serum bound complexes. This result was demonstrated by using both native and SDS-PAGE analyses of serum proteins from prostate cancer patients. Chromatographic separation of serum total proteins by a molecular sieve column generated two peaks (peak I and II), which were reactive with rabbit antiserum to human PSP94 in Western blot experiments. Peak I was eluted before the IgG fraction at a molecular weight larger than 150 kDa, and peak II appeared after serum albumin (~67 kDa) was eluted. By using a biotinylated PSP94 as an indicator of the free form of PSP94, we demonstrate that peak I contains serum PSP94-bound complexes and peak II is likely the free form of serum PSP94. Since the molecular weight of serum PSP94-bound complexes is close to IgG during molecular sieve separation, serum PSP94 complexes were further purified through two rounds of protein A column separation, followed by DEAE-ion exchange column chromatography. In vitro dissociation tests of the purified PSP94-bound complexes showed that the binding of serum PSP94-complexes is probably via disulfide bonds and is chemically stable. The results presented here indicate that serum PSP94-bound complexes must be considered in evaluating the clinical utility of PSP94 as a prostate cancer marker. J. Cell. Biochem. 76:71–83, 1999.

Key words: prostate cancer; diagnostic marker; serum binding proteins; differential test; PSP94

Prostate cancer (Pca) is potentially curable, if detected at an early stage. Prostate-specific antigen (PSA) is currently the most useful tumor marker for staging and monitoring Pca. However clinical data [Catalona et al., 1991; Chadwick et al., 1991] have indicated that up to 70–80% of favourable PSA values (4–10 ng/ml) in asymptomatic men may actually be false positive for Pca. In the setting of androgen ablation or hormone refractory Pca, PSA levels may not reflect clinical tumor burden. The diagnostic potential of PSA measurements in serum is limited by other conditions of the prostate such as benign prostatic hyperplasia (BPH) and prostatitis. Thus PSA remains a useful albeit imperfect tumor marker. An additional tumor marker would be useful to improve our diagnostic and prognostic capabilities.

PSA and PSP94 are the two most abundant secreted proteins from the prostate. Both proteins have been regarded as possible prostate tumor markers. Over the past 10 years, several research groups have evaluated PSP94 as a Pca marker [Dube et al., 1987b; Abrahamsson et al., 1988; van der Kammer et al., 1991; Garde et al., 1993; Huang et al., 1993; Hyakutake et al., 1993; Sheth et al., 1993; van der Kammer et al., 1993; Maeda et al., 1994]. Studies comparing PSP94 with PSA have shown that PSP94 has the potential to be a marker for monitoring the course of Pca [Dube et al., 1987a; Dube et al., 1987b; Teni et al., 1988; Garde et al., 1993; Huang et al., 1993; Hyakutake et al., 1993; Maeda et al., 1994], however, this conclusion was refuted [van der Kammer et al., 1993] and hence further research has been delayed.

Abbreviations used: ECL, enhanced chemiluminescence; HRP, horseradish peroxidase; IgG, immunoglobulin G; PAGE, polyacrylamide gel electrophoresis; PSA, prostate specific antigen; PSP94, prostate secretory protein of 94 amino acids; SDS, sodium dodecyl sulfate.

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Clarification of the antigenic regions of PSP94 molecule is important for evaluation of its potential as a Pca marker, by immunoassay of PSP94 epitopes in patient serum. For this purpose, we previously performed a systematic study of the PSP94 epitope structure [Xuan et al., 1996b, 1997b,c]. We established an *E. coli* model for in vitro expression of recombinant PSP94 protein using the GST (glutathione S-transferase) fusion vector pGEX-2T. By studying the kinetics of competitive binding among natural PSP94 (purified from seminal fluid) and recombinant GST-PSP94 proteins using in vitro denaturing analysis, we found that the epitopes of PSP94 proteins are of a linear dimension [Xuan et al., 1996b]. By peptide mapping, we found that the N-terminal 30 amino acid peptide is the only active antigen [Xuan et al., 1996b, 1997b]. We also prepared recombinant GST fusion proteins with N– (N47) and C- (C47) terminal peptides in *E. coli*. Using polyclonal antiserum and 15 monoclonal antibodies, we confirmed that the N47 is a stronger antigen than C47 [Xuan et al., 1997b,c]. From these studies, we concluded that the epitope structure of PSP94 is primarily linear, with multiple epitopes and an immunodominant N-terminus and immuno-recessive C-terminus.

Based on the epitope analysis and hence the super-structure of PSP94 predicted [Xuan et al., 1996b, 1997b,c], we consider that it will be difficult to detect binding proteins of PSP94 in patient serum directly by the traditional immunoassay. Previous clinical studies on the utility of PSP94 as a Pca marker had been conducted on the assumption that antibodies raised against purified PSP94 can recognize the total PSP94 in patients' serum. The results presented here, however, demonstrate that PSP94 exists in two forms, free and bound, in serum of Pca patients, with the majority in the bound form. This finding suggests that previous conclusions regarding the clinical utility of PSP94 serum levels, both positive (as in the majority of studies) and negative, need to be re-evaluated. Our results indicate that evaluating the levels of both free and bound forms of PSP94 as well as the ability of currently available antibodies to PSP94 to detect both forms will clarify the role of PSP94 as a diagnostic and prognostic marker in prostate cancer.

MATERIALS AND METHODS

Serum Samples for Biochemical Characterization

To demonstrate and estimate the relative proportions of free and bound forms of PSP94, blood (~10 ml) was collected after induction of anaesthesia from seven patients undergoing radical prostatectomy for Pca, radical cystoprostatectomy for bladder cancer, and radical nephrectomy for renal cell carcinoma. For large scale purification, larger amounts (>500 ml) of blood shed during surgery were collected prior to any transfusion. Since these latter samples may contain small amounts of other body fluids, 10 ml blood samples taken before surgery served as a control. All blood samples used for this study were taken without EDTA or heparin treatment. After clotting, samples were centrifuged briefly to separate serum.

Purification and Biotinylation of Human PSP94 From Semen

Human PSP94 was purified from semen samples according to the protocol reported previously [Baijal-Gupta et al., 1996] with the following modifications: only one round of ammonium sulfate precipitation from 30–70% saturation was conducted, and the use of analytical HPLC anion exchange column was eliminated. The purification of PSP94 was characterized by overloading (~ 20 µg) on a 15% SDS-PAGE without any other visible bands after Coomassie blue R-250 staining. For biotinylation and detection of the labelled free form of PSP94, a Biotin- Blot Protein Detection Kit (BioRad, Mississauga, Ontario, Canada) was used. The biotinylation reaction was initiated by adding 9.75 µl of NHS-Biotin (N-hydroxysuccinimide biotinate in dimethylformamide, 75 mM) to 1 mg PSP94 in 0.1 M sodium borate buffer pH 8.8 in a total volume of 350 µl, followed by incubation at room temperature for 4 h. Twenty µl of 1 M NH_4Cl was added and incubated at room temperature for 10 min. Biotin-labelled PSP94 was desalted by three rounds of spinning/dilution using a Centricon-3 cartridge (Amicon, Beverly, MA). For detection of biotin-PSP94, ECL (enhanced chemiluminescence)-Western blotting (ECL Western Blotting Kit, Amersham, Oakville, Ontario, Canada) was used. The transferred blot (ECL-Hybond nitrocellulose membrane, Amersham) with biotin-PSP94 was first reacted with avidin-HRP

(horseradish peroxidase) conjugate at 1:15,000 dilution in 0.5% blocking reagent (Boehringer Mannheim, Laval, Quebec, Canada)/ TBS (50 mM Tris-HCl pH 7.5, 150 mM NaCl). ECL detection was performed according to the manufacturer's instructions (Amersham). The efficiency of biotinylation labelling of PSP94 was determined by a dot blot test using the same procedure. Labelling efficiency was such that as little as 10 ng of the labelling mixture bound to ECL-Hybond membrane was detectable. However, using the HRP colour development reagent (BioRad), sensitivity of detection was reduced at least 10-fold.

SDS-PAGE (SDS-Polyacrylamide Gel Electrophoresis)

The Laemmli system [Laemmli, 1970] was used for SDS-PAGE. A 15% PAGE (30% T, 2.67% C) was prepared according to the BioRad protocol. For samples of high-density serum proteins, a high concentration of SDS (1%) and reducing agent (2.5% β-mercaptoethanol) was used to dissociate serum proteins. For the purified serum proteins, standard sample dye (Bio-Rad) at a final concentration of SDS (0.4%) and reducing agent (1% β-mercaptoethanol) were used. The bromophenol blue sample dye used contained (5×) 50% glycerol and 0.3 M Tris-HCl pH 6.8.

Native PAGE

The discontinuous buffer system of Ornstein-Davis (Tris/chloride/glycine) was used [Davis, 1964]. A Bio-Rad mini-protein II system was used to prepare the 15% polyacrylamide gel (30% T, 2.67% C). A pH 8.8 electrode buffer was used. Serum samples were mixed with non-denaturing, bromophenol blue sample dye at a final concentration of 10% glycerol, 60 mM Tris-HCl pH6.8, and loaded directly.

Molecular Sieve Column Purification

1.5 ml of serum was applied to 30 ml Sephacryl S-200HR (Pharmacia, Montreal, Quebec, Canada) packed in a 45 × 1.5 cm column (Bio-Rad Econo-column), and eluted by PBS (phosphate buffered saline). Fractions of 0.5 ml were collected at a flow rate of 0.1 ml/min. All fractions were monitored by $OD280_{nm}$. About 10 µl of sample was taken from each fraction tube for Western blotting analysis.

Protein A Affinity Column Purification

The low salt method of protein A affinity chromatography was followed [Harlow et al., 1988]. Fractions of Peak I after molecular sieve separation of serum total protein were pooled, pH was adjusted to ~8.0 by adding 1/10 volume of 1.0 M Tris-HCl (pH 8.0) and the sample was applied to a protein A column (Gibco/BRL, Burlington, Ontario, Canada). The pass-through portion (with no affinity for the protein A matrix) was collected, immediately after a void volume of buffer was passed. About five column volumes of PBS were used for washing, and the washing solution was saved. The bound IgG portion was eluted from the column by 100 mM glycine (pH 3.0) in a stepwise fashion with 500 µl per fraction of 100 mM glycine (pH 3.0) and neutralized immediately in 50 µl of 1 M Tris-HCl (pH 8.0). All IgG containing fractions were monitored by $OD280_{nm}$ and pooled. Desalting and concentrating of protein samples were performed by using Centricon Concentrators 30 (Amicon, Inc.) according to manufacturer's instruction.

DEAE Ion Exchange Column Purification

A Macro-Prep DEAE-Support (Bio-Rad) column (bed volume 25 ml) was equilibrated with 100 ml 25 mM Tris-HCl. pH 8.0. 10 ml of pooled, concentrated and desalted samples from two rounds of passing through portion of Protein A column (in 25 mM Tris-HCl pH 8.0) were applied and then washed with 50 ml 25 mM Tris-HCl. pH 8.0. Elution was conducted by a linear NaCl gradient starting from 0.02 M NaCl to 1M NaCl in 25 mM Tris-HCl pH 8.0 each of 100 ml, at a flow rate of 1 ml/min. Eluate was collected 3 ml/tube and peak area was monitored at $OD280_{nm}$. Stepwise elution was performed by washing the loaded column subsequently with 50 ml 25 mM Tris-HCl pH 8.0, 50 ml 100 mM NaCl/25 mM Tris-HCl pH 8.0, 50 ml 150 mM NaCl/25 mM Tris-HCl pH 8.0, 50 ml 300 mM NaCl/25 mM Tris-HCl pH 8.0.

Western Blotting

The ECL procedure was performed using an ECL Western Blotting Kit (Amersham) according to the protocol provided by the manufacturer. The primary antibodies were obtained by immunization of rabbits with human PSP94 purified from seminal plasma and emulsified in

Freund's adjuvant according to a standard protocol. Secondary antibodies, HRP (horseradish peroxidase) conjugated antiserum against rabbit IgG, were either from the Amersham ECL kit or purchased from Dimension Laboratories (Mississauga, Ontario, Canada). A modified second antibody, ImmunoPURE goat anti-rabbit IgG (H+L) min × Hn Sr Prot, from Pierce (Rockford, IL), was also used to minimize cross reactivity to human serum proteins. Blocking reagents used were solutions of either 5% skim milk powder. The first and second antibodies were diluted 5,000 and 1,000 times, respectively, as described previously [Xuan et al., 1996b].

In Vitro Dissociation of Serum Proteins

Serum samples (5 µl) were treated with the following reagents (final concentration) and conditions at room temperature for 3 min: SDS (1%), SDS (1%), and β-mercaptoethanol (2.5%), SDS (1%), and β-mercaptoethanol (2.5%) plus boiling, 1.5 M urea 50 mM Tris-HCl pH 7.5, 3 M urea 50 mM Tris-HCl pH 7.5, 1.5 M guanidine chloride and 2.5 M NaCl. For purified PSP94-bound complex preparations (60 µl), treatments were: SDS (0.4%), SDS (0.4%), and β-mercaptoethanol (1%), SDS (0.4%), and β-mercaptoethanol (1%) plus boiling, 1.5 M urea 50 mM Tris-HCl pH 7.5 , 3 M urea 50 mM Tris-HCl pH 7.5, 1.5 M guanidine chloride, and 2.5 M NaCl. The denatured samples were analysed immediately by native PAGE and Western blotting experiments.

RESULTS

Native and SDS-PAGE Analyses of Total Serum Proteins From Pca Patients

In order to differentiate various forms (free or bound forms) of PSP94 in serum, two kinds of PAGE were employed. Native, non-SDS and non-denaturing PAGE was used to separate serum proteins in their original forms. SDS-PAGE was used with serum samples treated by a reducing agent (β-mercaptoethanol) plus boiling to completely dissociate all the bound proteins in serum. Two identical sets of serum samples (10 µl each) from seven patients undergoing radical prostatectomy, radical cystoprostatectomy, and radical nephrectomy (used as non-Pca control) were analysed by Western blotting experiments to identify PSP94. Control samples were seminal plasma (~16 kDa), purified natural PSP94 (nPSP94, ~16 kDa) from seminal plasma and recombinant GST-PSP94 (~37 kDa) [Xuan et al., 1996b]. Results are shown in Figure 1. No PSP94 band corresponding to the size of the free form of purified PSP94 was found in the serum samples, when samples were run on a native, non-SDS gel (Fig. 1A). This indicates that in these sera from patients with different urological cancers, free PSP94 is present at only very low levels, likely less than 1 ng/10 µl, i.e., 100 ng/ml, based on comparison with the nPSP94 lane, where 1 ng was loaded. Figure 1B is the parallel experiment but with the samples treated with SDS, β-mercaptoethanol and boiling. A free PSP94 band was observed in all tumour serum samples, suggesting that most of the PSP94 present in serum is in a bound form and can be dissociated by detergent and reducing agents. No PSP94 was detectable in urine.

Molecular Sieve Separation of PSP94-Bound Complexes

Since native and SDS-PAGE analyses indicated the existence of serum PSP94-bound complexes, molecular sieve (Sephacryl S-200HR) column chromatography was used to separate and purify these complexes. Elution was in PBS buffer and under non-denaturing conditions. To identify bound PSP94, samples from fraction tubes were denatured by boiling with SDS-PAGE dye (Bio-Rad) and tested by SDS-PAGE and Western blotting experiments as in Figure 1. The size of free PSP94 is indicated by the positive control (PSP94 in seminal fluid). We confirmed that the rabbit antiserum against PSP94 in human seminal plasma recognized PSP94 monomer and the remaining undissociated dimers [Liang et al., 1991] in human seminal plasma (shown in first lane, Fig. 2A). Figure 2A shows two peaks of PSP94-containing fractions in serum: the first peak (peak I) was found in fractions #32–38, with higher molecular weight, and the second peak (peak II) was located at lower molecular weight in fractions #52–59 with very strong PSP94 immuno-activity in this Pca patient serum. Peak I is considered to include serum PSP94-bound complexes, since peak I proteins are larger in size than serum albumin (67 kDa in fractions #44–50), which is the most abundant component in serum (60%) and appeared as an overloaded band. These fractions eluted just before the region where IgG (~150 kDa), the second most abun-

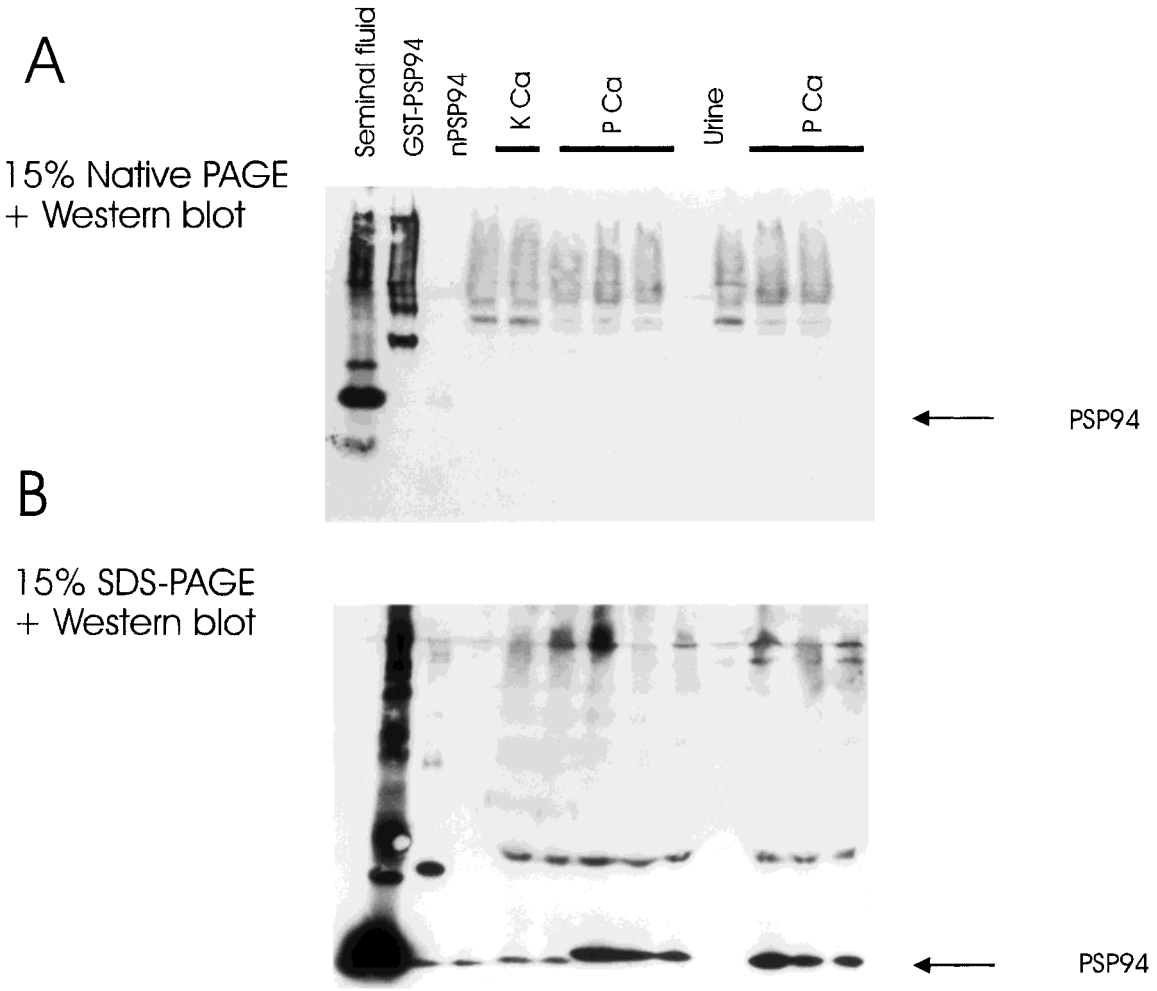

Fig. 1. Native (without SDS) and SDS-PAGE separation of total serum proteins and Western blotting analyses of free and bound PSP94 using polyclonal antibody against human PSP94 purified from seminal plasma. Serum samples from Pca (PCa) and renal cell carcinoma (KCa) patients. PSP94 was diluted with PBS and treated with 10% glycerol and dye (for native gel) and with denaturing dye containing 1% SDS 1% β-mercaptoethanol and by boiling (for SDS-PAGE). Precipitate after boiling was discarded. A urine sample was also loaded showing negative result. Samples of seminal plasma, natural PSP94 (nPSP94) and recombinant GST-PSP94 (~37 kDa) serve as controls to show the position of free PSP94 (band at ~ 16 kDa, indicated by an arrow). Natural PSP94 (10 ng loaded) in a native PAGE gel showed weaker band than SDS-PAGE as detected by Western blot.

dant (10–20%) protein in serum was eluted. The first peak has been repeatedly observed in most of the serum samples from Pca patients and as summarized in Table I. Peak II was observed as a strong signal in only one of seven patient samples, and this sample was thus selected for analysis in Figures 2 and 3. Figure 2B shows two examples of semi-quantitative test of first peak in two prostate cancer patients, in which a series of doses (1, 2, 4, and 8 ng) of standard PSP94 was loaded in the same gel. We have semi-quantitatively determined levels of these seven cancer patients at ~ 0.5–1 µg/ml (Table I).

In order to determine if peak II represents free PSP94, we used biotinylated PSP94 as an indicator to monitor molecular sieve separation of total serum proteins (Fig. 3A,B). Peak II was observed as a strong signal in one Pca sample as shown in Figure 2A, and this sample was thus selected for this experimental analysis. A large amount (15 µg) of biotin labelled PSP94 was loaded together with 1.5 ml serum onto a Sephacryl S-200HR column. Fractionation and Western blotting were performed as in Figure 2A. To differentiate free (biotinylated) PSP94 from the bound form in molecular sieve chromatography, two identical blots were assessed us-

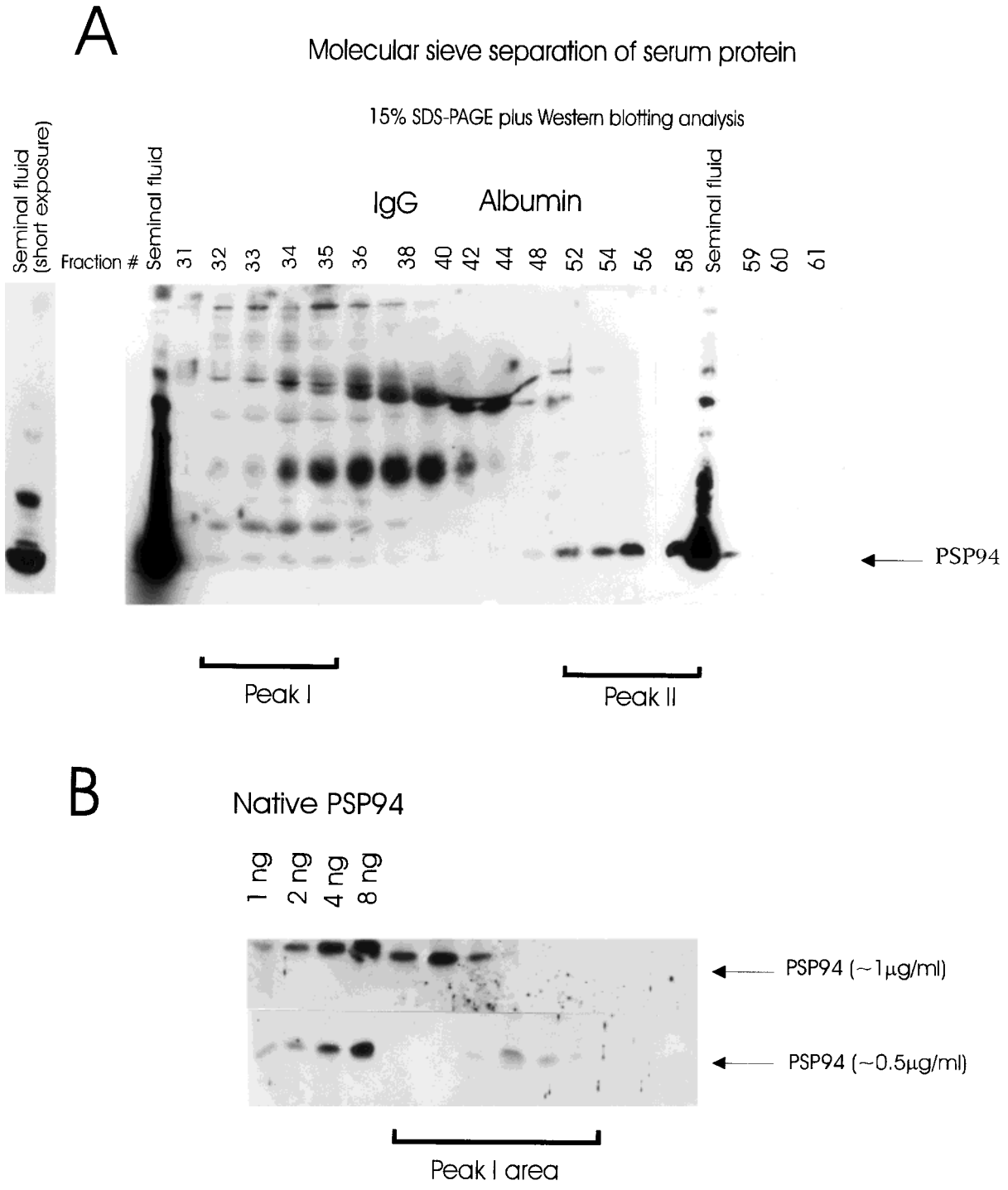

Fig. 2. Fifteen percent SDS-PAGE and Western blotting analyses of PSP94-bound complexes in serum from a Pca patient, after molecular sieve (Sephacryl S-200HR) separation of total serum proteins (**A**). All samples (~15 μl each lane) were treated with denaturing dye with 1% SDS 1% β-mercaptoethanol and boiling. Non-specific binding was not seen in the left lane (seminal fluid) following a short exposure time (first left lane) as compared with the over-exposure (second left lane). Bands with very high intensity at sizes of 25, 55, and 70 kDa were generated by the second antibody used, as demonstrated by Figure 4. **B**: Semi-quantitative estimation of serum bound PSP94 complexes in Peak I area of two Pca patients' serum. Only bands in Peak I area with identical molecular weight to PSP94 were shown. Fraction numbers of chromatographies in these two patient cases were different (not shown). The non-specific binding of IgG and albumin (not shown) was the same as in (A).

TABLE I. Separation and Characterization of Various Forms (free and bound) of Serum PSP94 in Prostate and Bladder Cancer Patients*

Patient #	Operation (cancer)	Peak I	Peak II
1	Prostatectomy (Pca)	+++	++++
2	Prostatectomy (Pca)	++	+
3	Prostatectomy (Pca)	++	+/−
4	Prostatectomy (Pca)	++	+/−
5	Prostatectomy (Pca)	+++	−
6	Prostatectomy (Pca)	−	−
7	Cystoprostatectomy (bladder cancer)	++	−

*For peak I, high (+++) or medium (++) levels of serum PSP94 were determined semi-quantitatively (see Fig. 2, Materials and Methods) by the intensity of free PSP94 bands in Western blotting experiment after molecular sieve (Sephacryl S-200HR) column purification with the standard PSP94 protein loaded in the same gel. Concentrations of +++, ++, +, and − represent serum levels of PSP94 at >5, ~1, 0.5, and <0.05 μg/ml respectively. For peak II, serum PSP94 at low (+), very low (+/−) or not detectable levels (−) were determined by Western blotting analysis of fractions after Sephacryl S-200HR chromatography of serum samples.

ing either polyclonal anti-PSP94 antiserum (Fig. 3A) or HRP-avidin followed by ECL reaction (Fig. 3B). Two peaks of PSP94 bands showed high (peak II) and low (peak I) immunoreactivity to PSP94 polyclonal antiserum as in Figure 2A, however, the intensity of signals of peak II in Figure 3A is higher than in Figure 2A, indicating that this peak contains both free forms of natural and biotinylated PSP94. This result is confirmed by the result of the blot (Fig. 3B) reacted with HRP-avidin and ECL analysis of Biotin-PSP94. Only one peak (peak I) was detected in this blot. Thus mostly likely peak I represents PSP94-bound complexes. The intensity of peak I area in Figures 2A and 3A was compared and no significant changes were found, indicating that serum PSP94 and its binding protein can not form complexes immediately after mixing together.

Protein A Affinity Column Purification of Serum PSP94-Bound Complexes

As shown in Figure 2A, fractions of peak I, the proposed PSP94-bound complexes, eluted with or near IgG fractions in molecular sieve column chromatography. In order to purify and separate serum PSP94-bound protein complexes from IgG, peak I fractions from molecular sieve column separation were pooled and further purified by a protein A column. The results of the analysis after one round column purification are shown in Figure 4. Figure 4A shows Coomassie blue staining of PAGE analysis of this experiment. The two strong bands above PSP94 at ~ 25 and 55 kDa are likely to be IgG light and heavy chains, which have been dissociated by reducing agents plus boiling treatments. After protein A column chromatography, most of the IgG in peak I (before column) was in the eluate portion, and IgG remaining on the protein A column portion was low. Western blotting analysis (Fig. 4B) of this PAGE shows that most of the PSP94 signal was in the pass-through column portion, as compared with the PSP94 bands shown before column purification. This experiment implies that most of the serum IgG does not bind to serum PSP94 and most of serum PSP94-bound complexes showed no affinity with a protein A column.

As shown in Figure 2, Western blot analysis of serum proteins had high background of non-specific signals. In these blots, multiple positive bands at ~25, 55, 70 kDa were repeatedly observed at higher intensity than PSP94 bands. None of the peak areas of these positive bands overlapped completely with the PSP94 peak I. From the intensity of these bands, and also from their position during fractionation, we speculate that they represented albumin and the two chains of immunoglobulin (IgG), since these two most abundant components showed overloading bands in PAGE analysis. The results of protein A purification (Fig. 4A,B) also suggested that these non-specific signals might be due to the cross-reaction of two chains of IgG with polyclonal antibodies used in Western blotting experiments, since most of the IgG bands remain unchanged in the eluate and were decreased in the pass-through and wash portions from the protein A column. To determine whether the first or second polyclonal antibodies were responsible for this non-specific binding, signals of Western blotting experiment of Figure 4B were completely stripped and reacted with only second antibody (HRP-conjugated swine against rabbit IgG). The result of this control experiment is shown in Figure 4C. Since two experiments have similarly high background signals except for the PSP94 band, we conclude the second antibody (provided by the Western blotting kit) cross-reacts with human IgG. This cross-reactivity was found in three commercially available second antibodies: swine

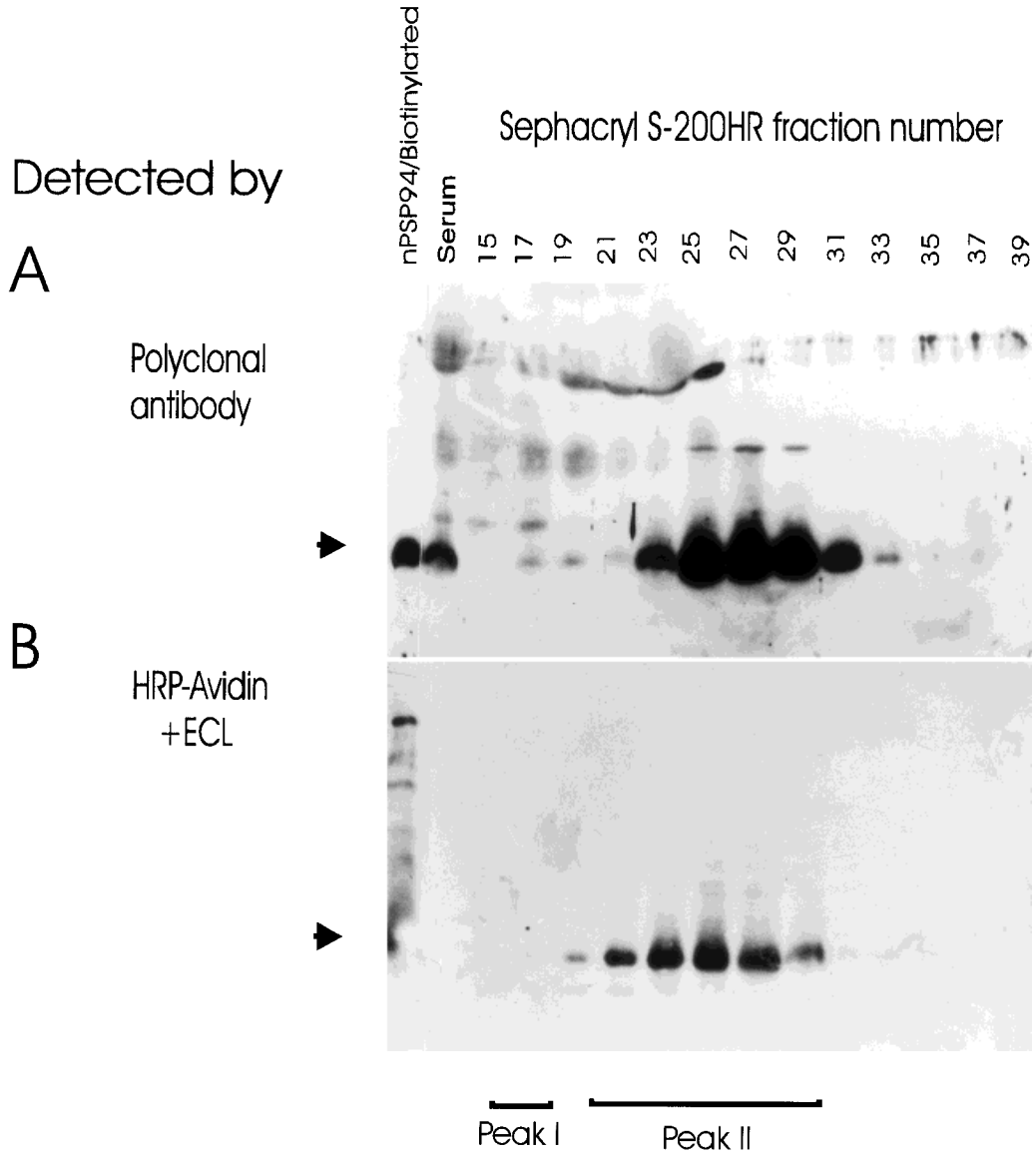

Fig. 3. Molecular sieve separation (Sephacryl S-200HR) of PSP94-serum bound complexes from the free form of PSP94 as monitored by biotinylated PSP94. Patient's serum analysed was the same as Figure 2A. Two identical Western blots were tested by polyclonal antibody (**A**) and HRP-Avidin (**B**) separately followed by ECL reaction. All samples (~15 µl each lane) were treated with denaturing dye with 1% SDS 1% β- mercaptoethanol and boiling. Arrow indicates the position of free PSP94 band (nPSP94/biotinylated).

anti-rabbit, goat anti-rabbit, a modified goat against rabbit second antibody (from Pierce) with minimized cross reactivities to human serum proteins, consistent with high levels of conservation of the IgG gene in evolution among mammals including human.

Further Purification by Ion-Exchange Chromatography

In order to further purify and confirm the existence of serum bound complexes of PSP94 and eliminate background signals in Western blot experiments, the crude preparation of PSP94 complexes (after molecular sieve separation and two rounds of passing through Protein A column) were purified by a DEAE ion exchange column with a linear NaCl gradient (0.02–1 M) elution. Figure 5A shows the chromatographic separation, in which two major protein peaks eluted at 0.03 M and 0.2 M NaCl were found. The existence of PSP94 was identified by Western blot experiment after β-mercap-

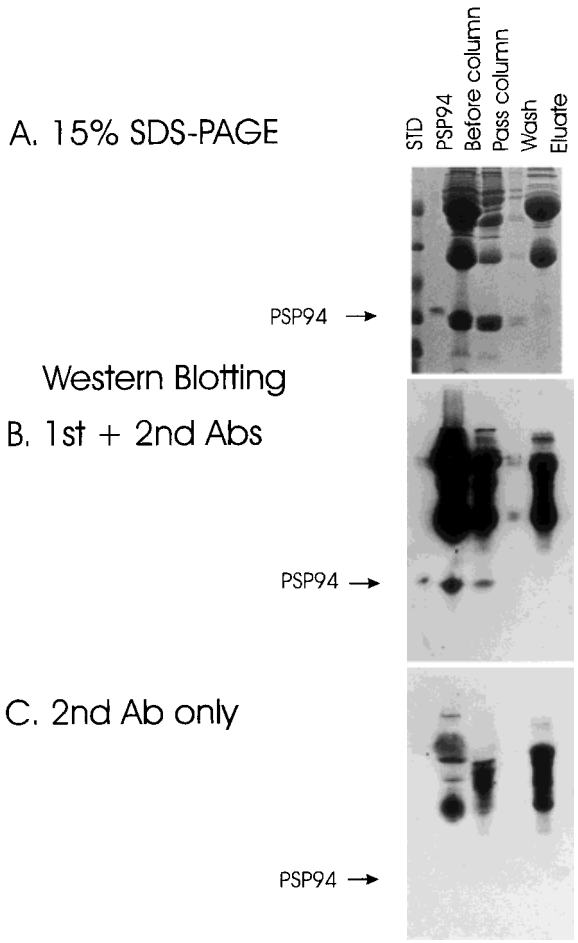

Fig. 4. Further purification of serum PSP94-bound complexes by protein A affinity column. **A**: Fifteen percent SDS-PAGE analysis of fractions of Protein A column purification. Crude preparation of PSP94-bound complexes was from peak I of the molecular sieve separation of serum from a Pca patient (Fig. 2A). Samples taken from portions before column purification, after passing through the column (not absorbed by protein A), the wash after sample loading, and the eluate, were treated with denaturing dye of 0.4% SDS 1% β-mercaptoethanol and boiling. PSP94 lane contains ~1 μg of purified natural protein from human seminal plasma. **B**: Western blot experiment of the top gel using both primary and second antibody. About 1/10 of samples of A were loaded. **C**: Western blot analysis with second antibody (anti rabbit IgG) only. Arrows indicate the position of natural PSP94 (nPSP94). Molecular weight standard (STD) proteins used are (from top to bottom): 43, 29, 18.4, 14.3, 6.2, 3 (kDa).

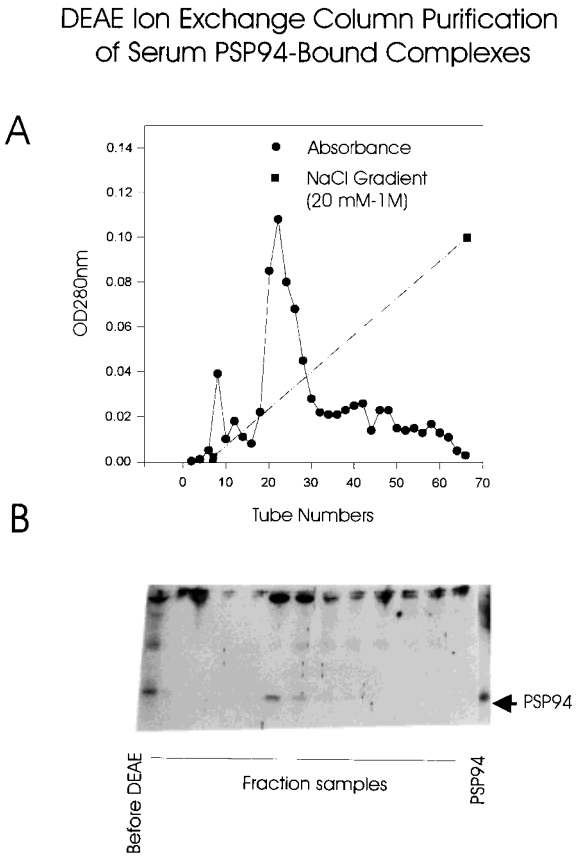

Fig. 5. DEAE ion exchange column purification of serum PSP94-bound complexes purified from Figure 4 after molecular sieve chromatographic separation, and twice passing through a protein A column. The pooled passing through fractions after protein A chromatography were used as input of DEAE ion exchange column. **A**: DEAE ion exchange (Bio-Rad, Macro-Prep DEAE Support) chromatography. PSP94 containing peaks of elution were identified by $OD280_{nm}$ and was eluted by ~150 mM NaCl. **B**: Western blotting analysis of PSP94 containing peak area eluted by a linear NaCl gradient of DEAE ion exchange separation of serum PSP94-bound complexes. An equal amount (~15 μl each lane) of all samples was loaded with denaturing dye and boiling treatment. Before DEAE: twice protein A column passing through fractions (see Fig. 4) pooled for subsequent DEAE ion exchange purification. PSP94: natural PSP94 purified from human seminal plasma.

toethanol and boiling treatment of samples from each elution tube. PSP94-bound complexes peak area was located at the second peak in fraction tubes eluted at ~ 0.15 M NaCl (Fig. 5B). Samples applied for DEAE column purification (before DEAE, Fig. 5B) were the pooled portions after two rounds of passing through Protein A column. These samples showed lower background signals than those passing through Protein A only once in Western blotting analysis (Fig. 4B). Fractions eluted by ~ 0.15 M NaCl, diluted by ~20 times of loaded samples of before DEAE during elution process (see Materials and Methods), revealed strong immunoreactive signals to PSP94 specific antibody. Stepwise elution with 0.15 M NaCl (see Materials and Methods) and the subsequent Western blotting experiment confirmed this result. A strong band was observed on top of the PAGE gel in Figure 5B, which is unknown and may be due

to partial dissociation of PSP94 complexes (the same as Figs. 2A and 3A). No background signals of IgG and albumin as in Figures 1 and 2 were detectable in 0.15 M NaCl fraction (#20) at this purification step.

In Vitro Dissociation Tests of the Purified Serum PSP94-Bound Complexes

To test the nature of the binding of PSP94 with serum proteins, crude preparations of serum PSP94-bound complexes, purified by molecular sieve and two rounds of protein A column purification (shown in Fig. 6), were treated by several denaturing chemical regents: 1% SDS, 1% SDS 1% β-mercaptoethanol, 1% SDS 1% β-mercaptoethanol plus boiling, high concentrations of urea (1.5 to 3 M), 1.5 M guanidine hydrochloride (GHCL), and 2.5 M NaCl. Figure 6 shows that the binding of PSP94-bound complexes is very stable, with chemical resistance to most of these denaturing treatments. Only strong reducing conditions (1% SDS, 1% β-mercaptoethanol plus boiling) effectively dissociated the bound complexes, indicating the binding is likely through disulfide bond. Similar results (data not shown) were also obtained using serum samples from two Pca cancer patients. No significant background signal was detected in all these tests as shown in native PAGE and Western blotting analyses.

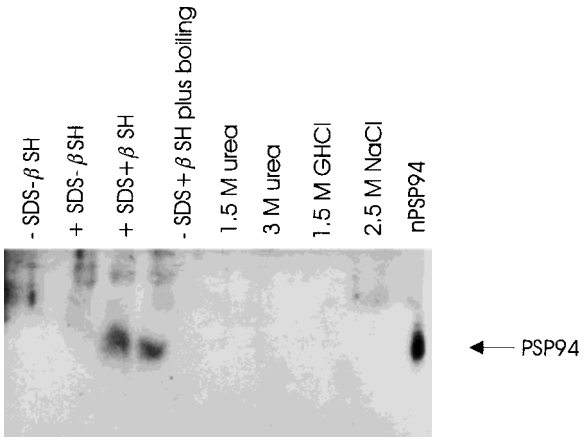

Fig. 6. Fifteen percent native PAGE and Western blotting analysis of different conditions to dissociate PSP94 from crude preparations of serum PSP94-bound complexes after molecular sieve and two rounds of protein A column purification. For experimental details see Materials and Methods. β-SH, β-mercaptoethanol; GHCl, guanidine hydrochloride; nPSP94, natural PSP94. Native gel shows low background signals with lower sensitivity. Due to effects of the presence of higher salt in denatured samples, gel is somewhat distorted. Arrows indicate free PSP94 band.

DISCUSSION

PSA and PSP94 are similar in various respects: both are predominant secretory proteins of the prostate, and hence named as γ- and β-microseminoprotein respectively [Hara et al., 1989]; both PSA and PSP94 were initially believed to be prostate specific, but later found to be present in a number of secretory tissues and fluids [Abrahamsson et al., 1989; Ulvsback et al., 1989; Weiber et al., 1990; Sheth et al., 1993; Fernlund et al., 1994; Yu et al., 1995a,b; Xuan et al., 1997a]. In particular, both have been detected in other steroid hormone-responsive epithelial tissues, such as female breast and breast tumor tissues [Nolet et al., 1991a; Riegman et al., 1992; Garde et al., 1993; Yu et al., 1995b; Xuan et al., 1997a]. Both proteins are detectable in serum by ELISA. In addition, both PSA [Christenssson et al., 1994; Stenman et al., 1994] and PSP94 (this study) have been shown to exist in serum in both free and bound forms and the binding is SDS-stable. By identifying serum bound form of PSP94, we sought to clarify the controversial issue of the clinical utility of PSP94 as compared with PSA, since all the previous researchers have analysed only free form of serum PSP94.

The glandular ducts of the prostate usually provide efficient barriers to prevent the escape of high concentrations of prostate secretory proteins into the general circulation. Both PSA and PSP94 can leak out from the prostate glandular ducts into the blood circulation at a detectable concentration. The abnormal levels in serum in Pca patients indicate irregular or erratic control of the secretion of prostate cancer cells, as well as possibly defective or less efficient tissue barriers. We hypothesize that the similar, wide tissue distribution of PSA and PSP94 in secretory tissues and their similar means of escape from prostate secretory ducts into the general circulation suggest a similar control mechanism of secretion of these two proteins. We also propose that PSA and PSP94 may have a similar use as serum markers for prostatic cancer, and that the utility of free and bound proteins must be considered.

PSA has been found to form complexes in serum with antichymotrypsin (ACT), α_2- macroglobulin, protein C inhibitor, and pregnancy zone protein [Christenssson et al., 1994; Stenman et al., 1994]. The ratio of free PSA to total PSA was lower in controls than in patients with

cancer. Using this ratio, 38–60% of the false-positive results in the range 2.5–25 μg/L, were eliminated without loss of sensitivity [Stenman et al., 1994; Catalona et al., 1995; Prestigiacomo et al., 1997], however, the clinical utility in staging of Pca is still questioned [Bangma et al., 1997].

In the present study, we have demonstrated that, as with PSA, serum PSP94 exists in two forms, free and bound, with the majority being in the bound form. This conclusion is supported by our native and SDS-PAGE analyses of serum samples from Pca patients. It is also confirmed by our semi-quantitative tests on a number of prostate cancer patients (Table I). By using the same method, we have analysed total serum proteins separated by molecular sieve chromatography as monitored by a biotin labelled free form of PSP94. As shown in Figures 2 and 3, we could eliminate, at least qualitatively, the possibility of loss of PSP94 in the separation of serum proteins due to SEC (size exclusion chromatography), since we have demonstrated that the peak II of smaller molecular weight is free form of serum PSP94. Identification and characterization of the PSP94 binding protein is currently underway in our laboratory.

We have also determined that the explanation for consistently high background signals using a Western blotting kit to analyse serum PSP94-bound complexes, is due to cross-reactivity of the second antibodies (swine/goat against rabbit IgG) used, either from swine or goat against rabbit. As shown in Figure 2A, background signals appeared in Western blots only when testing serum samples but not in testing seminal plasma, where the antigen of human PSP94 was purified and the polyclonal antibody was then generated [Xuan et al., 1997b]. We have eliminated these background signals either by pre-absorption of the second antibody with normal swine/goat serum (data not shown), or by testing the purified preparation of serum bound PSP94 complexes in the reaction. Background signals of non-specific binding shown in Western blotting experiments were decreased in native PAGE (Figs. 1A, 6), probably due to band diffusion. By further purification of serum PSP94 complexes under non-denaturing conditions in two rounds of Protein A column and DEAE ion exchange chromatographies, non-specific binding in the Western blotting experiments was completely eliminated (Fig. 5B).

From our epitope analysis, we have previously shown that most of the antigenicity of PSP94 is in the N-terminus and that the C-terminus of PSP94 is an immuno-recessive area [Xuan et al., 1996b, 1997b,c]. We predicted that the C-terminus is buried inside while only part of the N-terminus is exposed outside [Xuan et al., 1996b]. It is conceivable to assume that the serum binding protein of PSP94 will bind to the N-terminal area. It will be difficult to identify serum PSP94-bound forms using the limited epitopes of PSP94 itself by the traditional ELISA directly. Consistent with this suggestion, our results have indicated that neither PSP94 polyclonal antibody (Fig.1) nor 15 monoclonal antibodies (data not shown) could recognize the PSP94-bound protein complex in a native PAGE-Western blotting experiment. We have also tried to use trypsin and hydroxylamine to digest PSP94 from serum bound complexes, with negative results (unpublished data).

PSP94 has been described as immunoglobulin binding factor (IGBF) and was thought to bind to the Fc portion of IgG [Liang et al., 1991]. This might explain our finding that serum PSP94-bound complexes cannot bind to protein A, because of competition binding to Fc region. However, PSP94's ability to bind IgG was not confirmed by our experiments [Xuan et al., 1996a]. PSP94 is a small, non-glycosylated peptide consisting of 94 amino acids in its mature form. As shown by comparative evolution studies of PSP94 from the rat [Fernlund et al., 1996], mouse [Xuan et al., 1999], pig [Fernlund et al., 1994], monkey [Nolet et al., 1991b], baboon [Xuan et al., 1997a], and human [Xuan et al., 1995, 1997a], PSP94 is a cysteine-rich protein, with $>$ 10 % of amino acids being cysteine, and the cysteine residues are highly conserved between species [Nolet et al., 1991b; Xuan et al., 1995, 1997a, 1999; Fernlund et al., 1996]. This is consistent with our finding that the epitope structure of PSP94 [Xuan et al., 1996b,c] and serum PSP94-bound complexes (this study) are resistant to treatments by various denaturing reagents. This structural property may also indicate that PSP94 is able to bind via disulfide bonds with unknown functional consequences.

As we have demonstrated by in vitro denaturing experiments of human serum, binding of PSP94 with serum bound proteins is very stable and likely strengthened by disulfide bonds. It might be due to the partial dissociation of -SH

bonds of PSP94 that different motilities of PSP94 bands in PAGE were observed, for example, in Figure 1A and B. This binding is specific, as we have tested if the re-association will be achieved autonomously and rapidly. Biotinylated PSP94 was added to a serum sample in the presence of 1% SDS 1% β-mercaptoethanol plus boiling, the mixture was quickly desalted in a Centricon 3 spin column, and tested after 1 h of self reassociation by molecular sieve chromatograph (as in Fig. 3, data not shown), no biotinylated PSP94 was found in higher molecular weight peak I area, indicating the binding is very specific and may involve other factors.

Although PSP94 is similar to PSA with regard to being a prostate secretory protein and its association with Pca as a serum marker, it may have different clinical utilities. PSP94 gene expression has been reported to be androgen independent [Kharbanda et al., 1990; Garde et al., 1993; Hurkadli et al., 1994], and this has been supported recently by a clinical study on the prognostic significance of PSP94/β-microseminoprotein as novel indicator for progression under endocrine therapy of prostate cancer [Sakai et al., 1999]. Thus PSP94 may have additional clinical utility as an androgen-independent marker, since in the setting of androgen ablation or hormone refractory Pca, PSA levels being androgen dependent are unreliable. Additional prognostic variables would be useful when considering other additional treatments (i.e., neo-adjuvant or adjuvant systemic agents). Differential tests of PSP94 (bound to free) provide a possible way to complement PSA testing as further refinement of diagnostic and prognostic techniques is important for Pca.

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