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# Analysis of Epitope Structure of PSP94 (Prostate Secretory Protein of 94 Amino Acids): (II) Epitope Mapping by Monoclonal Antibodies

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**Abstract** PSP94 has shown potential to be a serum biomarker for evaluating prostate cancer. Studies of the epitope structure is crucial for this endeavour. In this article, we have used 15 different monoclonal antibodies (MAb) to analyse the epitope structure of PSP94 and to compare with the results obtained from our previous work using polyclonal antibody and recombinant PSP94. Firstly, we determined the relative activities of the 15 MAb population by direct and competitive ELISA. The two predominant MAbs (MAb PSP-6 and -19) in 15 MAbs were selected for further studies of the epitope structure. By comparing the binding activities of recombinant GST-PSP94 and natural PSP94 with MAbs, and by comparing their affinity with MAbs in an in vitro denaturing experiment, PSP94 was shown to have a similar, prevalently linear epitope structure as we demonstrated by polyclonal antibody. Using recombinant GST fusion protein with PSP94 and with each half of the N- and C-terminal 47 amino acids (GST-PSP-N47/C47) in *E. coli* cells, the different epitopes recognized by 15 monoclonal antibodies were delineated and the polar distribution of the epitope structure of PSP94 was characterized. Results of direct ELISA of recombinant N47 and C47 and their competitive binding against natural PSP94 (competitive ELISA) showed that the N- and C-termini represent the immuno-dominant and immuno-recessive area separately. A majority of the monoclonal antibodies (12/15) showed preferential binding of the N-terminal sequence of the PSP94 protein. Using GST-PSP-N47 as a standard protein, an epitope map of the 15 monoclonal antibodies was obtained. The results of this study will help to define the clinical utility of PSP94. J. Cell. Biochem. 65:186–197. 

**Key words:** epitope mapping; monoclonal antibodies; linear epitope; immuno-dominant; immuno-recessive; ELISA; competitive ELISA; recombinant GST-PSP94; N-terminal and C-terminal peptides

Human prostate secretory protein of 94 amino acids (PSP94) is one of three predominant prostate secretory proteins (prostate specific antigen [PSA], prostatic acid phosphatase [PAP]). Because of the potential as a diagnostic biomarker and as a therapeutic agent [Garde et al., 1991, 1993; Mundel and Shedth 1993; Lokeshwar et al., 1993], the structure of PSP94 gene and the encoded mature protein have both been very well studied [Seidah et al., 1984; Akiyama et al., 1985; Mbikay et al., 1987; Ulvsback et al., 1989, 1991; Green et al., 1990; Nolet et al., 1991a; Liu et al., 1993; Ochiai et al., 1995] including the homologue in pig and rat [Fernlund et al., 1994, 1996]. The structure of the alternative splicing PSP94 mRNA in prostate tumor tissue [Xuan et al., 1995a] and its possible association specifically with age, sexual maturity and malignancy have been studied [Xuan et al., 1997b]. The promoter region responsible for cell-specific expression have been identified [Ochiai et al., 1995]. By studying both in prostate cancer tissues and in primate animal, alternatively initiated transcripts were characterized and its possible implication to steroid hormone regulation has been suggested [Mbikay et al., 1988; Nolet et al., 1991a; Xuan et al., 1997b]. The function of PSP94, however, is still unknown and controversial. For this reason, different names have been assigned

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depending on the various proposed functions: PIP (prostatic inhibin protein) or β-inhibin refers to the possible function as a tumor suppressor, an inhibitory factor for the secretion of β-FSH (follicle stimulating hormone) from the pituitary gland [Sheth et al., 1988; Garde et al., 1991; Lokeshwar et al., 1993; Mundel et al., 1993; Garde et al., 1993]; "β-microseminoprotein" (β-MSP) [Hara 1989; Ulvsback et al., 1989; Green et al., 1990; Ulvsback et al., 1991] and SMI (sperm motility inhibitor) [Chao et al., 1996] refers to its relation with semen and reproductive biology of both male and female [Tanaka et al., 1996]; IGBF (immunoglobulin binding factor) [Liang et al., 1991] refers to the possible function as an immunosuppressive factor in seminal plasma.

Using purified PSP94 from semen, both polyclonal and monoclonal antibodies have been developed by several research laboratories [Vaze et al., 1979; Dube et al., 1987a; von der Kammer et al., 1990; Wright Jr. et al., 1990; Vanage et al., 1992; Huang et al., 1993]. Techniques for determining PSP94 level in serum samples from prostate cancer patients have been well developed: two dimensional gel electrophoresis [Carter et al., 1982], different radio-immunoassays (RIA) [Vaze et al., 1979; Tremblay et al., 1987; Huang et al., 1992, 1993] and enzyme immunoassays [Teni et al., 1989; von der Kammer et al., 1990], or two-site immunoradiometric assay (IRMA) [Huang et al., 1992]. All the analytical methods were based on the immunological assays using antibodies, however, there have been thus far no systematical studies on the epitope structure of PSP94. In order to study the epitope structure and protein biochemistry of PSP94, we have successfully expressed human PSP94 protein in *E. coli* as a recombinant GST (glutathione S-transferase)-PSP94 fusion protein and used this system as an experimental model to manipulate and study the epitope structure [Xuan et al., 1997a]. By in vitro denaturing experiments and by comparing recombinant PSP94 with natural PSP94, we have found that the major epitope of PSP94 is of a linear dimension [Xuan et al., 1996]. By peptide mapping and competitive ELISA using polyclonal antibody we have found the N- and C-termini represent the immuno-dominant and immuno-recessive area of PSP94, respectively [Xuan et al., 1997a].

In this study, we employed 15 monoclonal antibodies to perform the experiments similar to those conducted previously using a polyclonal antibody. Our purpose is to compare the results obtained by both polyclonal and monoclonal antibodies, and to further confirm our conclusions based on our epitope structure studies using a polyclonal antibody.

## MATERIALS AND METHODS

### Expression and Purification of GST-PSP94 Fusion Proteins and Peptides in *E. coli*

The mature PSP94 coding region was cloned into an *E. coli* expression vector pGEX-2T (Pharmacia, Montreal, Que) by PCR as reported previously [Xuan et al., 1996]. GST fusion plasmids with each half of the DNA sequences of mature PSP94 protein, i.e., GST-PSP-N47 and C47 were constructed as reported previously [Xuan et al., 1997a]. The *E. coli* PSP94 protein and peptides were translated in the same reading frame as the carrier protein GST [Xuan et al., 1996]. Recombinant GST-PSP94 (rGST-PSP94) and peptides were expressed in a proteinase deficient *E. coli* strain BL21 ((*lon ompT*, from Novagen, Madison, WI). *E. coli* bacterial culture incubation, the induction of recombinant protein by IPTG (isopropylthio-β-D-galactoside) and affinity column purification of the GST-PSP94 fusion protein using Glutathione Sepharose 4B (Pharmacia, Montreal, Que) were performed as reported previously [Xuan et al., 1994, 1995b, 1997a]. GST-PSP94 was purified from inclusion bodies of the bacterial lysate by 8M urea 0.1M glycine, pH 9.0, treatment as described previously [Frorath et al., 1992; Xuan et al., 1994, 1997a]. Recombinant GST-PSP-N47 and -C47 fusion proteins were purified from cytosol of the late exponential growing bacterial cells according to the protocol as reported previously by our group [Xuan et al., 1994, 1995b]. Briefly, transformed BL21 bacterial cells were grown to an $OD_{600}$ at ~1, 0.5 mM IPTG was added and incubated for 2 h to induce the recombinant GST-fusion protein expression. Cells were harvested and lysed by lysozyme and 1% Triton X-100 treatment. The supernatant of the lysate was applied to a Glutathione Sepharose 4B affinity column and eluted with substrate (glutathione) solution according to the manufacturer's protocols. Protein concentration was determined by Bradford method using a BioRad protein assay kit (BioRad, Mississauga, Ont).

### Generation of Polyclonal Antibodies

Natural PSP94 protein (nPSP94) was purified from human seminal plasma by ammonium sulphate precipitation and ion-exchange chromatography [Baijal-Gupta et al., ]. Rabbit

polyclonal antibody against nPSP94 was obtained by active immunization as reported previously [Vaze et al., 1979; Vanage et al., 1992], in which the purified PSP94 was mixed with Freund's adjuvant without denaturation.

### Generation and Purification of Monoclonal Antibodies (MAbs)

Generation and characterization of monoclonal antibodies of human PSP94 have been described previously [Wright Jr. et al., 1990; Huang et al., 1992, 1993]. Selected MAbs of the $IgG_1$ subclass were all purified from either ascites (MAb PSP-19) or the supernatants of culture media of mouse hybridoma cell lines using protein-A agarose beads (Gibco/BRL, Burlington, ON) by a high salt method [Harlow and Lang, 1988]. In brief, crude antibody preparation was adjusted to 3.3 M NaCl, 1.0 M sodium borate (pH 8.9), passed through a protein A column, and washed sequentially with 3M NaCl, 50 mM sodium borate (pH 8.9) and 3M NaCl, 10 mM sodium borate (pH 8.9). Purified MAbs were eluted from the column in a stepwise fashion with 500 µl per fraction of 100 mM glycine (pH3.0) and neutralized immediately in 50 µl of 1M Tris (pH 8.0). In the case of the samples purified from cell culture supernatant, the protein A column eluates contained a certain amount of bovine $IgG_1$. All $IgG_1$-containing fractions were monitored by $OD280_{nm}$, pooled and diluted in 1.5% BSA prior to use.

### In vitro Denaturing Test

About 1–2 µg of protein samples was lyophilized and redissolved in 10 µl of the following denaturing solutions: 8M urea 0.1M glycine (pH 9.0), 10% SDS, 1% SDS 0.01% Me-SH (β-mercaptoethanol). 10 µl double distilled water was used for the control and boiling test. Denaturing conditions was maintained for 3 min at room temperature (except for boiling test), at least 500 times of ELISA coating buffer (1.4 mM $Na_2CO_3$, 7 mM $NaHCO_3$ pH 9.2) was added immediately to all the samples to a final concentration at 25 ng/100 µl. Coating and renaturing were at 4°C overnight. Direct ELISA followed.

### Direct and Competitive ELISA (Enzyme Linked Immunosorbent Assay)

All ELISA protocols were performed as reported previously [Xuan et al., 1997a]. Briefly, protein samples in carbonate coating buffer were coated in triplicate onto 96 well Immunoplates (Nunc, Gibco/BRL, Mississauga, Ont.) at 4°C overnight. Coated plates were washed three times in PBS/T buffer (80 mM $Na_2HPO_4$, 20 mM $NaH_2PO_4$, 100 mM NaCl, 0.05% Tween 20), and blocked in 1.5% BSA (bovine serum albumin, RIA grade, Sigma, St. Louis, MO) in PBS at 37°C for 1 h. The wells were washed briefly and then incubated at room temperature with antibody diluted (1:10,000) in 1.5% BSA/PBS/T for 1 h at room temperature. This immunoreaction was terminated by washing three times with PBS/T. A secondary antibody, HRP (horseradish peroxidase) conjugated swine anti-rabbit IgG or antimouse IgG, was added and incubated at room temperature for 1 h. The plate was washed three times with PBS/T. Color reaction was performed in 0.4 mg/ml OPD (o-phenylene diamine dihydrochloride, Sigma, St. Louis, MO) 0.05% $H_2O_2$ in developing buffer (35 mM citric acid, 67 mM $Na_2HPO_4$, pH5.0). Results were measured by $OD_{492nm}$ using a Titrek Multiscan Plus microplate reader (EFLAB). Graphs were generated and statistical analyses were performed using SigmaPlot and SigmaStat software (Jandel Scientific Co., San Rafael, CA).

For competitive ELISA, PSP94 antiserum was diluted in 1.5% BSA/PBS/T and mixed with the competitor proteins or antibodies as solution phase at various concentrations. Antibody-competitor mixture was added immediately to the sample wells and incubated at room temperature for 2 h with shaking. The competition reaction was stopped by washing three times with PBS/T. HRP conjugated secondary antibody binding and colour development with OPD were performed as for the direct ELISA described above.

## RESULTS

### Analysis of the Relative Activities of 15 Monoclonal Antibodies (MAb) Against Human PSP94

In order to select one or two representative MAbs in 15 MAbs to study the epitope structure of PSP94, we first tested the composition of the activities of 15 monoclonal antibody population derived from hybridoma cell lines of mice immunized with natural PSP94 (nPSP94). MAbs were purified by protein A column. All 15 purified MAbs were tested for the immuno-binding activities at a dilution of 10 k (1:10,000) to nPSP94 and the 7 MAbs with highest activities ($OD_{492} > 2$) were tested for further dilutions. As shown in Figure 1, starting from a dilution of 10 k till the dilution to 100 k, two MAbs, MAb PSP-6 and -19, retained the reactivity to nPSP94 (50 ng), and apparently these two MAbs comprise the two predominant MAbs in

the whole MAb population. The polyclonal antibody served as a control and also showed approximately the same levels of activity as MAb PSP-6 and -19. Next, in order to compare MAbs MAb PSP-6 and -19 with polyclonal antibody and determine if these two MAbs represent also the predominant antibody activities in the polyclonal antibody population, competitive ELISA of polyclonal antibody against these two MAbs was conducted. Figure 2 showed the results, in which only the competitive binding of polyclonal antibody to the coated nPSP94 was determined by the second antibody against rabbit. For competitive binding of 25 ng per well of PSP94, two predominant MAbs in 15 MAb population only reach the competition inhibition of 5–20% in a wide range of dilutions tested to polyclonal antibody (Fig. 2), indicating the multiple epitope structure of PSP94 as we demonstrate previously by using a polyclonal antibody [Xuan et al., 1997a].

### Monoclonal Antibodies Showed the Similar Binding Activities to Recombinant GST-PSP94

We have used rGST-PSP94 to demonstrate the linear epitope of PSP94 using polyclonal antibody, since recombinant GST-PSP94 is expressed in *E. coli* as a denatured inclusion body and the purification procedure is a denature-renature protocol [Frorath et al., 1992; Xuan et al., 1997a]. Similar experiments were performed using monoclonal antibodies. Two parallel, direct ELISA experiments were performed to test and compare the binding activities of 15 MAbs to both natural and GST-PSP94 coated at an equal molar (2.5 pmol). Two predominant MAbs, MAb PSP-6 and -19 (dilution 1:25 k), showed similar high activities binding to both natural PSP94 and denatured recombinant GST-PSP94, indicating that most of immunoreactivity of MAbs population can recognize a denatured epitope of PSP94 at the same level as natural protein. The remaining weaker MAbs were tested at a higher concentration (1:2,500) in order to generated higher reaction signals. Most of the other 13 MAbs also did not lose their activities binding to rGST-PSP94 due to denaturing in expression and purification. Figure 3 summarized the result by showing the ratio of the affinity of GST-PSP94/nPSP94. Most of the MAbs retained significant immunoreactive ability, i.e., at least half the binding activities to GST-PSP94 as compared with nPSP94. Some MAbs (PSP-6, 23, 21, 3, and 17) have the ratio of GST-PSP94/nPSP94 larger than 1, indicating higher affinities to denatured recombinant GST-PSP94 than nPSP94.

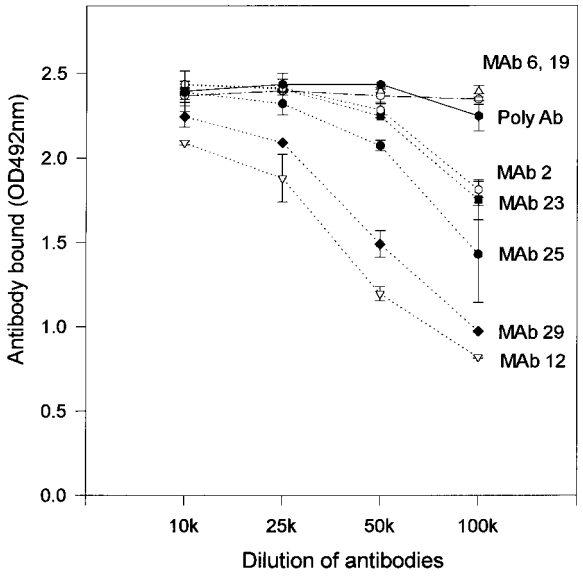


**Fig. 1.** Comparison of the affinity of monoclonal antibodies to natural PSP94. Polyclonal and monoclonal antibodies were diluted in 1.5% BSA as indicated from 1:10,000 (10 k) to 100,000 (100 k) and tested by direct ELISA for the binding activities to nPSP94 (50 ng per well). HRP-conjugated second antibodies used are against rabbit IgG (for polyclonal antibody, dilution at 1:1,000) and mouse IgG (for Mabs, dilution at 1:500). Values are means of triplicate measurements ± S.D.

### In Vitro Denatured Natural and Recombinant PSP94 Proteins Retain Most of the Binding Affinity with Monoclonal Antibodies

A series of in vitro denaturing experiments of nPSP94 were performed to test changes of the epitope activity with monoclonal antibodies. The tests were performed by different reagents: 8M urea 0.1M glycine pH 9.0, boiling, detergent SDS and reducing chemical β-mercaptoethanol (Me-SH). Plates were coated with nPSP94 at a dose of 50 ng/well, which represent the mid-log and saturation point of the dose response to PSP94 polyclonal antibody as shown previously [Xuan et al., 1996a]. Figure 4 shows the results of tests on the two predominant MAbs. As compared with control experiments, i.e., coated with nPSP94 without any denaturing treatment, both MAbs (6 and 9) showed the same binding activities to the nPSP94 by either of the above denaturing treatments, thus proving that the epitopes recognized by these two MAbs are resistant to denaturing treatments. Next, the remaining 13 MAbs were tested for their bind-

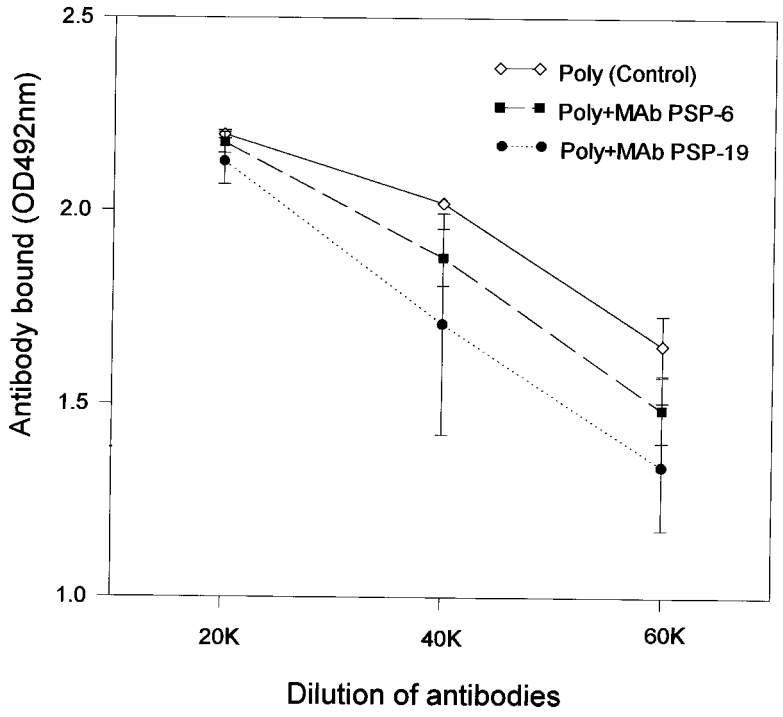


**Fig. 2.** Competitive ELISA of PSP94 polyclonal antibody vs. two predominant monoclonal antibodies MAb PSP-6 and MAb PSP-19. Natural PSP94 was coated at 25 ng per well. Polyclonal antibody was diluted from 1:20,000 (20 k) to 60,000 (60 k) as indicated, mixed with/without (control) 1:25 k of each of MAbs for competitive binding. The final results were tested for the binding of polyclonal antibody (dilution at 1:1,000) to solid phase nPSP94 by using HRP-conjugated second antibody against rabbit IgG. Error bars for each point are mean values from triplicate samples ± S.D.

ing activities to nPSP94 by treatments with boiling and 8M urea 0.1M glycine pH 9.0. The results showed that most of the MAbs retained the same level of binding and the binding activities of some MAbs (MAbs 29, 12, 23, 17, 3, 21) were improved by these treatments (data not shown), indicating straight linear epitopes recognized.

### Tests of the Affinity of GST-PSP-N47 and GST-PSP-C47 Peptides with Monoclonal Antibodies

By using a polyclonal antibody, our previous studies indicated that N- and C-termini represent immuno-dominant and immuno-recessive area of PSP94, respectively. The intention of this study is to confirm this result by using 15 monoclonal antibodies. Since results of Figure 2 suggest that there are multiple epitopes in the nPSP94 and also because of the difficulties in identifying epitope recognized by each monoclonal antibodies via peptide mapping (data not shown), we decided to generate longer recombinant PSP94 fusion peptides. Recombinant GST fusion proteins with each half of the N- and C-terminal protein sequences (N47 and C47) [Xuan et al., 1997a] were used. Analytical and quantitative ELISA experiments were conducted to compare the differential affinity of 15 monoclonal antibodies to N47/C47 fusion proteins. Two experiments were performed. The first was the direct ELISA to compare binding activities of 15 MAbs to GST-PSP-N/C47 proteins at ~100 ng/well, with nPSP94 coated (25 ng/well) as control (Fig. 5A). All the antibodies were tested at a dilution of 1:2,500 dilution. Figure 5B and C showed that all 12 MAbs tested had higher binding to GST-PSP-N47 than to GST-PSP-C47. Four MAbs with low affinity to nPSP94, i.e., PSP-33, -16, -3, and -32 (Fig. 5A) also had low affinity to both GST-PSP-N47 and GST-PSP-C47; and probably for the same reason, the difference in their ability to bind to either peptide was not significant. The remaining three MAbs (PSP-2, PSP-5, and PSP-15) did not behave as the other 12 MAbs. Next, ELISA was performed separately at two different dilutions (1:2,500 and 1:10,000) in order to optimize the concentration of these three MAbs for ELISA. PSP-19, one of the highest affinity antibodies to

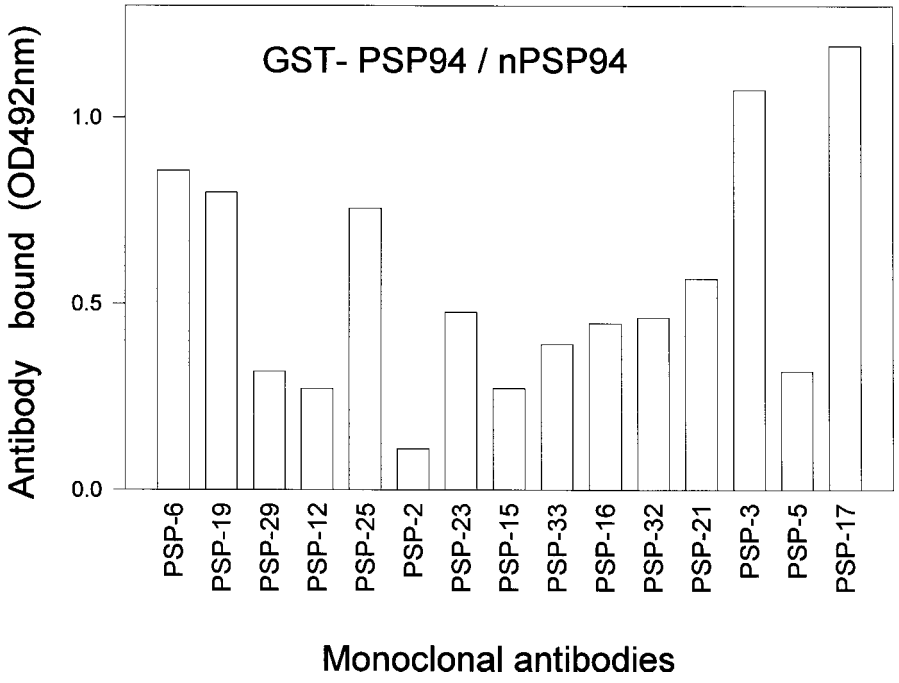


**Fig. 3.** Comparison of the affinity of 15 MAbs (dilution 1:2,500) binding to nPSP94 (coated at 25 ng, ~2.5 pmol/well) and recombinant GST-PSP94 (coated at 90 ng, ~2.5 pmol/well). Each bar stands for a ratio of $OD_{492}$ measured for antibody bound to GST-PSP94 vs. nPSP94. Values are the ratio of the mean of three measurements.

nPSP94 was used as normal control in analysing these three abnormal MAbs. As shown in Figure 6, 1:2,500 and 1:10,000 dilutions of three MAbs, PSP-2, -15, and -19 both resulted in fairly constant, high levels of binding to nPSP94 in the solid phase, indicating that both concentrations could be used to test the epitopes recognized. MAb PSP-2 could not bind to either peptides N47 or C47, probably because PSP-2 recognizes an area near the disulfide bond, as identified by us previously [Wright Jr. et al., 1990]. The binding of MAb PSP-19 to GST-PSP-C47 decreased to approximately 50% of that of GST-PSP-N47 when the dilution was increased from 1:2,500 to 10,000. As with MAb PSP-19, MAb PSP-15 had higher affinity for nPSP94 and lower affinity for both N47 and C 47 peptides. At both dilutions, however, PSP-15 had slightly higher affinity for the C47 than the N47 peptide. MAb PSP-5 had weak affinity for nPSP94 and both peptides.

### Competitive ELISA of GST-PSP N/C47 against nPSP94 Using Monoclonal Antibodies

In order to confirm the results from direct ELISA (Figs. 5 and 6), competitive ELISA was performed. NPSP94 (25 ng, ~2.5 pmol per well) was coated as a solid phase and GST-PSP-N/C47 proteins were used as solution competitor partners against the binding to nPSP94 by two predominant MAb PSP-6 and -19, the two different MAbs as shown in direct ELISA (Figs. 5, 6). Results are shown in Figure 7. GST-PSP-N47 in solution phase showed significantly competitive inhibition to MAb PSP-6 binding to solid phase nPSP94. The inhibition was correlated with the dose increase (from 10 ng to 1 µg, i.e., ~0.3–30 pmol) and the maximum competition inhibition by GST-PSP-N47 was achieved at ~0.5 µg (~15 pmol) vs. 25 ng nPSP94 (~2.5 pmol) in solid phase. GST-PSP-C47 as competitor (solution phase) showed no competitive binding against nPSP94 (solid phase) with MAb PSP-6 in all the dosage tested (10 ng to 1 µg). The competition inhibition of either GST-PSP-N47 or GST-PSP-C47 vs. nPSP94 was not significant for MAb PSP-19 and showed no correlated with the dose increase (Fig. 7).

## DISCUSSION

Prostate cancer is the second leading cause of cancer death among adult males in North America and Europe [Catton, 1992]. Early detection of prostate cancer and thus improved diagnostic biomarkers are crucial in this endeavour. Over the past 10 years, there have

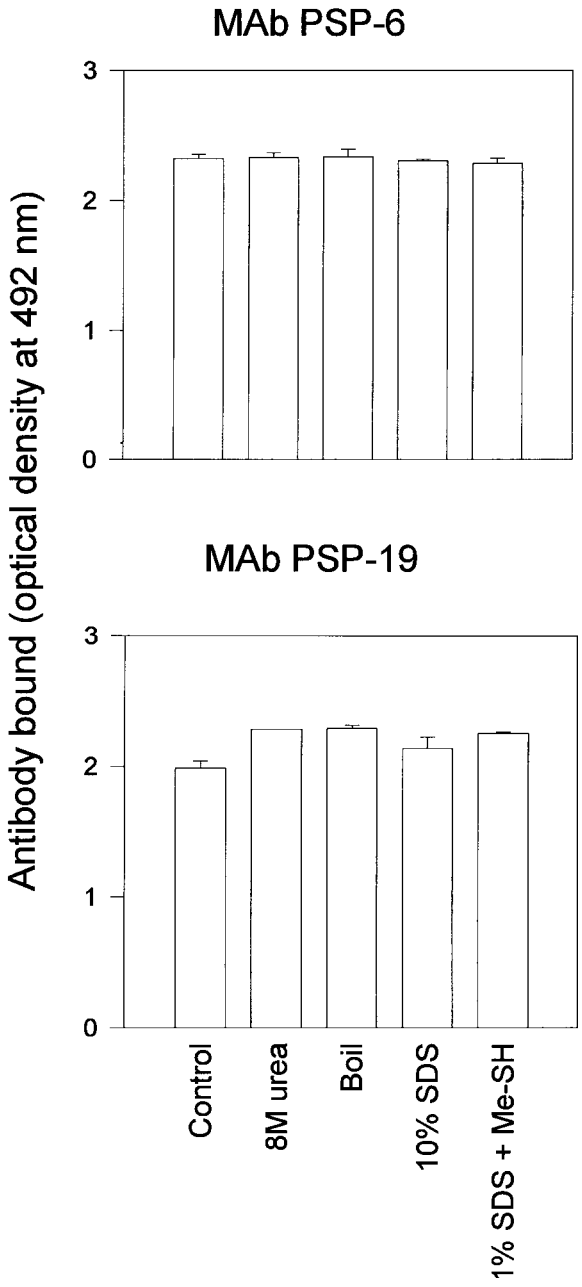


**Fig. 4.** Direct ELISA to assess two predominant MAbs (MAb PSP-6 and -19) of their ability binding to nPSP94 denatured in vitro by various conditions: 8M urea 0.1M glycine (pH 9.0), boiling, 10% SDS, and 1% SDS 0.01% β-mercaptoethanol (Me-SH). Error bars for each column are mean values from triplicate samples ± S.D.

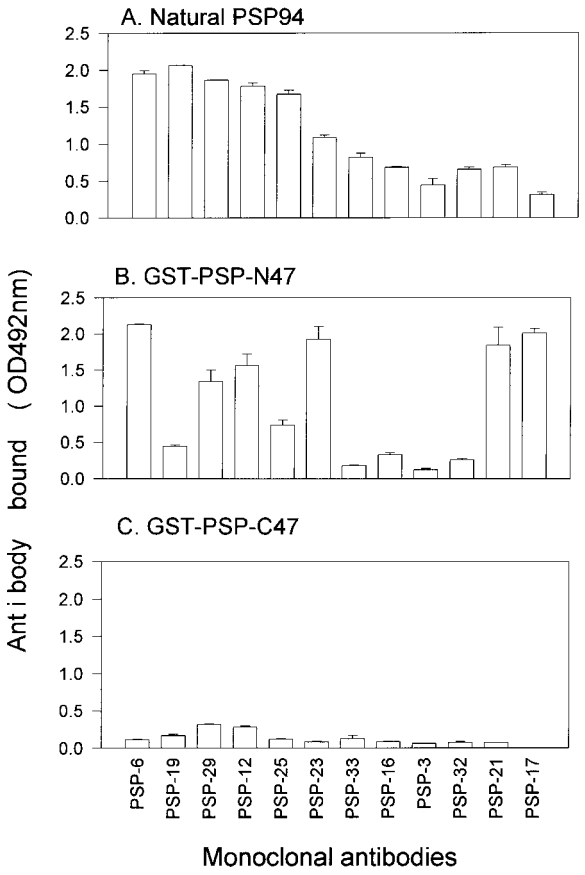


**Fig. 5.** Characterization of the epitopes recognized by 15 monoclonal antibodies. **A:** Comparison of the affinity of 12 MAbs by direct ELISA with 100 ng of natural PSP94 protein coating each well. Direct ELISA of 12 MAbs for their affinity with GST-PSP-N47 (**B**) and -C47 (**C**) was also conducted using approximately 100 ng of each fusion protein. All MAbs were tested at 1:2,500 dilution, with the exception of PSP-6, PSP-19 and PSP-17 at 1:10,000. Second antibody (HRP-conjugated rabbit against mouse IgG) was used at 1:500 dilution. Error bars are mean values from triplicate samples ± S.D.

been several research groups [Dube et al., 1987b; Tremblay et al., 1987; Ulvsback et al., 1989; Wright Jr. et al., 1990; Weiber et al., 1990; Liang et al., 1991; van der Kammer et al., 1991, 1993; Huang et al., 1992, 1993; Hyakutake et al., 1993; Lokeshwar et al., 1993; Garde et al., 1993; Sheth et al., 1993; Maeda et al., 1994] evaluating PSP94 as a prostate cancer marker. Most of the publications have indicated that PSP94 has the potential to be a prostate cancer marker; however, this conclusion has not been accepted by the scientific or the clinical communities [van der Kammer et al., 1991]. To re-evaluate the potential of PSP94 as a serum marker for prostate cancer, a systematic study of the epitope structure of PSP94 is of the paramount importance. For this purpose, we have performed studies using recombinant GST-PSP94 and peptides as tools [Xuan et al., 1997a]. We have generated 15 monoclonal antibodies (MAb) using the purified PSP94 protein from human seminal plasma as immunogen [Wright Jr. et al., 1990; Huang et al., 1992, 1993] and

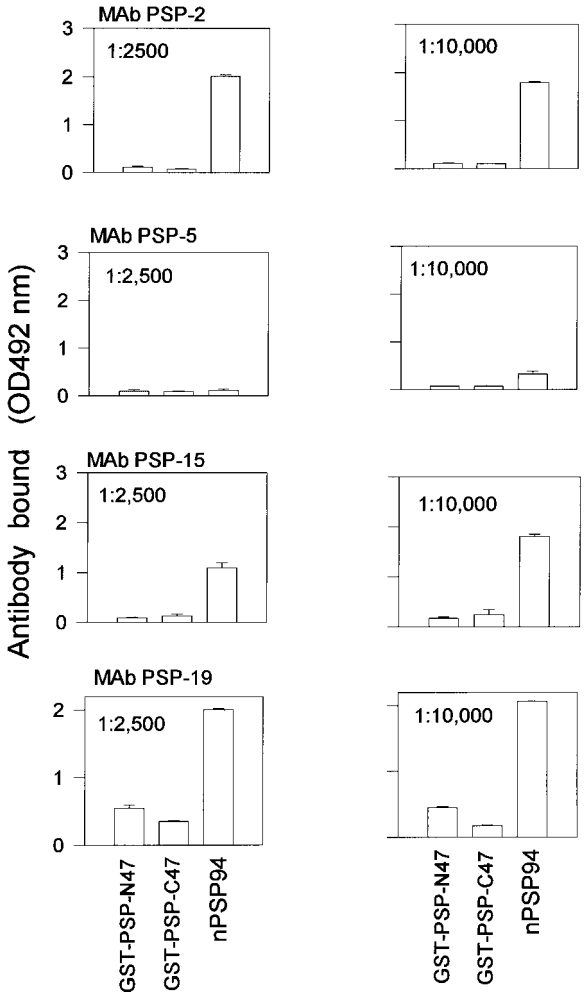


**Fig. 6.** Determination of the epitope of MAbs by GST-PSP-N/C47 fusion proteins. All three preparations of PSP94 proteins, GST-PSP-N47, -C47, and nPSP94, were coated with 100 ng per well and tested by direct ELISA for binding with different dilutions of monoclonal antibodies: MAbs PSP-2, PSP-5, PSP-15, and PSP-19. Two dilutions were tested (1:2,500 and 1:10,000) separately in parallel graphs. Error bars are mean values from triplicate samples ± S.D.

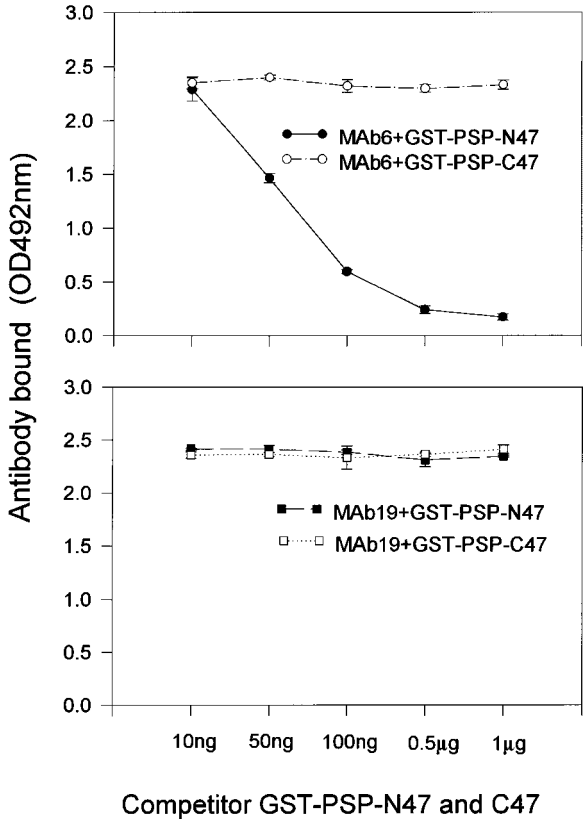


**Fig. 7.** Competitive ELISA of MAb PSP-6 and -19 binding to nPSP94 against GST-PSP-N47 and GST-PSP-C47. Natural PSP94 were coated at 25 ng (~2.5 pmol) and competed with GST-PSP-N47 and GST-PSP-C47 at different dosages (indicated on the x-axis) for binding with MAbs (dilution at 1:10,000). Each point is mean value from triplicate samples ± S.D.

developed a method of two-site immunoradiometric (IRMA) assay for measuring PSP94 in serum. By competitive binding-inhibition assay, we have found at least four different epitope groups [Wright Jr. et al., 1990; Huang et al., 1992]. We have not performed further systematic studies on the epitope structure using monoclonal antibodies. In this study we have combined both studies using either polyclonal or monoclonal antibodies and established an epitope map for PSP94. Taking all the experimental data together, we conclude that the epitope structure of PSP94 is 1) of predominantly a linear dimension, 2) of multiple epitopes, and 3) of a polar distribution of epitopes with an immuno-dominant N-terminus and immuno-recessive C-terminus.

Using recombinant GST-PSP94 as a tool for a denatured PSP94, we have previously [Xuan et al., 1996] demonstrated that nPSP94 is of a linear dimension. Since rGST-PSP94 is only partly capable of competing with nPSP94, e.g., nPSP94 still shows higher epitope activities than the denatured rGST-PSP94 [Xuan et al., 1997a], we consider PSP94 has a predominantly linear epitope structure. In the present study with similar experimental procedures using MAbs, this conclusion has been further confirmed. Most of the MAbs showed almost the same binding activities to nPSP94 by different in vitro denaturing tests (Fig. 4, and unpublished data). On the other hand, comparison of the binding activities of 15 MAbs to GST-PSP94 versus nPSP94 (Fig. 3), a fluctuation of the ratio of GST-PSP94/nPSP94 between 40–200%

in the MAb population was identified, indicating the difference of multi-epitope recognized.

The strong chemical resistance of epitope structure of PSP94 to denaturing treatment demonstrated by either polyclonal antibody [Xuan et al., 1996] or MAbs (this work), may be explained by the fact that PSP94 has a tight superstructure. PSP94 is a cysteine-rich protein, with 10 cysteine residues in a mature form of only 94 amino acids. We have compared human, monkey, baboon, and pig PSP94 cDNA sequence and found that 10 cysteine residues have been highly conserved [Xuan et al., 1997b], although PSP94 as a whole is under rapid evolution pressure [Nolet et al., 1991a,b; Xuan et al., 1997b]. Recently, Fernlund et al. found 10 cysteine residues also highly conserved in rodent PSP94 homologue, suggesting that a tightly knotted three-dimensional structure is the essential characteristic of this protein [Fernlund et al., 1996].

PSP94 is a small protein of only 94 amino acids; however, interestingly, our previous reports have indicated that PSP94 may contain multiple epitopes [Xuan et al., 1996, 1997a]. The multiple epitopes may include some non-predominant epitopes, i.e., epitopes dependent on the super structure of PSP94, since peptide mapping and reciprocal competitive ELISA indicated that synthetic peptides in the immunodominant region were not capable of completely competing with nPSP94 [Xuan et al., 1997a]. In this work, by competitive ELISA against polyclonal antibody (Fig. 2), lower competitive ability of the two predominant MAbs in 15 MAb population demonstrates again multiple epitopes of PSP94. The 15 MAbs we obtained are quite different antibodies populations from the polyclonal antibody. It might be also due to this reason that we had difficulties (data not shown) in mapping epitopes for 15 MAbs using six peptides synthesized previously [Xuan et al., 1997a]. In fact, we cannot find any two very similar MAbs in 15 MAbs in all the experiments performed in this research and previous work [Wright Jr. et al., 1990].

Our previous report by peptide (synthetic and recombinant) mapping [Xuan et al., 1996, 1997a] and the present study demonstrate that the N- and C-termini of the human PSP94 protein are the immuno-dominant and -recessive area, respectively. Testing of GST-PSP-N/C47 fusion proteins by direct ELISA (Figs. 5 and 6) showed that majority (12 over 15) of the MAbs population bind preferentially to N-terminus over the C-terminus sequences. Competitive ELISA (Fig. 7) of GST-PSP-N/C47 proteins against nPSP94 confirmed there results, suggesting that the N-terminal half of the PSP94 protein contains most of the epitopes of PSP94 protein, while the C-terminal half is immuno-recessive.

By competitive radioimmunoassays (IRMA), four epitope groups have been characterized previously [Wright Jr. et al., 1990; Huang et al., 1992]: MAb PSP-17 (group A), PSP-6 (epitope group B), PSP-19 (group C), and PSP-29 (group BC). As shown in Figure 5, the aforementioned four MAbs are also mostly the strongest MAbs against the nPSP94 protein. MAb PSP-6 is the strongest antibody against GST-PSP-N47 fusion protein in the four MAbs, and also the weakest against GST-PSP-C47 peptide. MAb PSP-19 had the weakest binding activity to both GST-PSP-N/C47 proteins (Figs. 5 and 6), and for this reason neither of these recombinant peptides can compete binding against natural PSP94 with MAb PSP-19 (Fig. 7). By using the affinity to the N-terminal peptide as a standard for epitope mapping, MAbs PSP-6 and -19 appear to recognize different epitopes. If this is indeed the case, it is consistent with the results of our previous competitive binding-inhibition assay [Wright Jr. et al., 1990] that MAbs PSP-6 and -19 belong to epitope groups B and C. Since MAb PSP-19 showed slightly higher affinity to the GST-PSP-N47 protein in two dilutions of MAbs (Figs. 5, 6), it may recognize an epitope in the junction area of N47 and C47. MAb PSP-29, similar to PSP-12, showed intermediate binding ability to both N47 and C47, and the difference between the two MAbs are not the greatest among the 15 MAbs tested. We suggest that MAb-29 and -12 recognize two close or overlapping epitopes, possibly located near the end of N47 sequence. This would be consistent with our previous reports that MAb-29 and -12 belong to epitope group BC and B [Wright Jr. et al., 1990; Huang et al., 1992]. In all 15 MAbs, MAbs PSP-6, -17, and -21 showed strongest and most preferential binding to GST-PSP-N47 as opposed to -C47 (Fig. 5), indicating that they recognized the epitopes nearest to the N-terminus.

Figure 8 summarizes the results of epitope mapping of 15 MAbs using recombinant GST-PSP-N/C 47 peptides. In the mapping analysis, we have compared MAb affinities with GST-

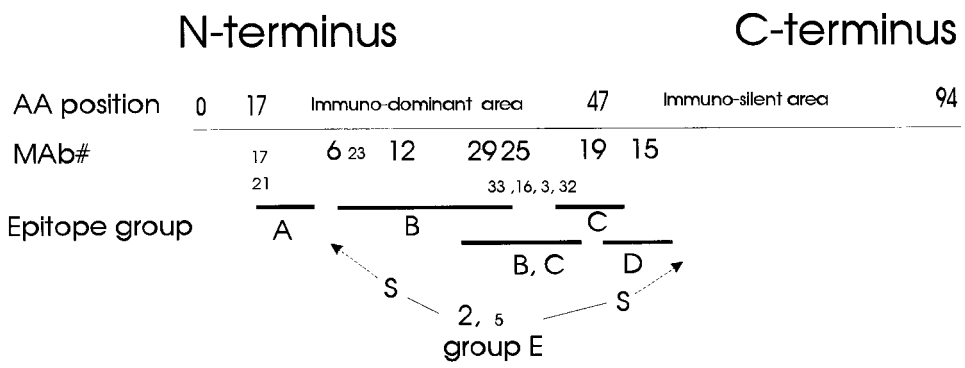


**Fig. 8.** Epitope mapping of 15 monoclonal antibodies. Amino acid (AA) position is indicated on the top. Six epitope groups are listed under the bold bar and numbers of MAbs are listed on the top. Strong and weak MAbs against the natural PSP94 are listed by large and small fonts. Dashed lines are the unidentified disulfide bonds recognized by MAbs.

PSP-N47, the difference in the affinity between GST-PSP-N/C47, and the affinity with nPSP94 (Figs. 5–7). This epitope map is consistent with our previous epitope analysis by using two-site IRMA and competitive binding-inhibition assays [Huang et al., 1992, 1993]. In the assay, we selected MAb PSP-19, one of the strongest MAb against natural PSP94 as a catcher to increase the sensitivity and another one PSP-6 as a tracer [Huang et al., 1992, 1993]. To facilitate the simultaneous binding by two antibodies, the epitopes recognized by both are well separated as demonstrated in this study. Two other epitope groups, D and E, are listed in Figure 8. MAb PSP-15 (group D) has an intermediate level of binding activity to nPSP94 (Fig. 6) and its binding affinity to nPSP94 also showed fairly resistant to denaturing treatment (Fig. 3). MAb PSP-15 is the only one of 15 MAbs tested that showed slightly high affinity to C47 peptides and thus recognizes the area farthest from the N-terminus of the PSP94 sequence. However, it has low affinity for both N47 and C47 peptides, indicating the epitope recognized is near the junction area of these two peptides. As we have demonstrated previously [Wright Jr. et al., 1990], MAb PSP-2 (group E) recognized an epitope containing a disulfide bond, possibly located between N47 and C47, since MAb PSP-22 has no binding activity to either peptide. MAb PSP-5 (group E) is the weakest antibody (Figs. 5, 6), possibly since recognition of an epitope requires a very fragile conformation of the natural protein. The four weaker MAbs, PSP-33, -16, -3, and -32, were mapped between epitopes B and C, since they have weaker affinity to both GST-PSP -N/C47 proteins.

Two cardinal factors are considered in epitope selection in the immune system: host specificity and accessibility of the antigen. As demonstrated in this work and our previous study [Xuan et al., 1996], both rabbit and mouse host immune systems react with the immunogen of the human nPSP94 in a strikingly similar manner in that N- and C-termini represent immunodominant and -recessive regions. Our experimental data were repeatedly observed using independently raised rabbit antisera (data not shown) and were also reported previously by other researchers [Dube et al., 1987a]. Thus it is highly unlikely that this observation is due to host factors in the specific epitope selection. Selection of epitopes is also dependent on exposure of the epitope structure to the aqueous environment and therefore to the immune apparatus. Virtually any region on the exposed surface of a protein may serve as an epitope. However, hydrophilicity of the local regions within the protein has predictive value. Computer analysis [Xuan et al., 1996] of the predicted secondary structure of PSP94 showed three high points of hydrophilicity, two of which are located in the C-terminus: 79–85 (EKKDPKKK), 69–74 (KKEDCK), and one in the N-terminal end (12–17, GDSTRK). Our systematical studies indicate a polar distribution of the epitopes and that the C-terminus is deleted for antigenic/immunogenic activity. Based on this observations, we have hypothesized that the highly charged and basic C-terminus has been buried in the cross linked, correctly folded protein [Xuan et al., 1996]. To confirm this hypothesis, it will be necessary to perform computer

modelling, NMR or X-Ray crystallographic study.

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