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Analysis of Epitope Structure of PSP94 (Prostate Secretory Protein of 94 Amino Acids): (I) Immuno-Dominant and Immuno-Recessive Area

Jim W. Xuan,[1]* Dongmei Wu,[1] Yuzhen Guo,[2] Seema Garde,[2] Madhulika Baijal-Gupta,[2] and Joseph L. Chin[1]

[1]Department of Surgery, University of Western Ontario, London, Ontario, Canada
[2]Procyon Biopharma Inc., London, Ontario, Canada

Abstract PSP94 is a potential biomarker for evaluating patients with prostate carcinoma. We have systematically studied the epitope structure of PSP94 by using a polyclonal antibody against human PSP94. Results of peptide mapping and ELISA tests of dose response to rabbit antiserum against human PSP94 protein showed that only the N-terminal peptides (N30 and M23) are immunoreactive while all the synthetic peptides (C28, C10) located closer to the C-terminus are completely devoid of antigenic activity with the polyclonal antibody. These results were confirmed by analysis of reciprocal competitive binding of PSP94 polyclonal antibody by the N-terminal peptides (N30 and M23) v. either recombinant GST-PSP94 fusion protein, purified recombinant PSP94, or natural PSP94 protein. To further delineate the antigenic activity of the N- and C-termini, we have also expressed N- and C-terminal half of the whole PSP94 (each 47 peptides) using the *E. coli* GST expression system. The recombinant N47/C47 peptides were released by thrombin cleavage from the GST fusion protein and characterized by Western blotting experiments. Dose response of the recombinant GST-PSP-N47 and -C47 peptides to PSP94 polyclonal antibody showed differential binding activities. Competitive binding of these recombinant N47/C47 proteins against the GST-PSP94 protein demonstrates that the polyclonal antibody has a higher affinity for the N47 peptide than the C47 peptide. Based on the immunological studies of both synthetic peptides and recombinant PSP94- N/C terminal proteins, we propose an epitope structure of human PSP94 with an immno-dominant N-terminus and an immuno-recessive C-terminus. J. Cell. Biochem. 65:172–185. © 1997 Wiley-Liss, Inc.

Key words: epitope structure; peptide mapping; immuno-dominant; immuno-recessive; ELISA; competitive ELISA; recombinant GST-PSP94; recombinant GST-PSP N-terminal and C-terminal peptides

Human prostate secretory protein of 94 amino acids (PSP94) has been attracting interest for its potential as a diagnostic biomarker and as a therapeutic agent [Doctor et al., 1986; Dube et al., 1987b; Abrahamsson et al., 1988; Linard et al., 1988; Hara et al., 1989; Ulvsback et al., 1989; von der Kammer et al., 1990; Wright Jr. et al., 1990; Garde et al., 1991; Liang et al., 1991; Nolet et al., 1991b; Garde et al., 1993; Huang et al., 1993; Lokeshwar et al., 1993; Ochiai et al., 1995; Xuan et al., 1995a]. PSP94 was observed to inhibit in vivo secretion of β-FSH (follicle stimulating hormone) from the pituitary gland. For this reason, it has also been called β-inhibin and prostatic inhibin peptide (PIP) [Sheth et al., 1988; Garde et al., 1991, 1993; Lokeshwar et al., 1993; Mundel et al., 1993]. PSP94 protein is prominent in seminal fluid [Dube et al., 1987a] and has also been reported under the name "β-microseminoprotein" (β-MSP) [Hara et al., 1989; Ulvsback et al., 1989, 1991; Green et al., 1990].

The structure of the PSP94 gene and the encoded mature protein have both been determined by amino acid sequencing of the purified protein from human seminal plasma [Seidah et al., 1984; Akiyama et al., 1985; Kohan et al., 1986], cDNA sequencing [Mbikay et al., 1987; Ulvsback et al., 1989; Liu et al., 1993], and genomic sequencing [Green et al., 1990; Nolet

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*Correspondence to: Dr. Jim W. Xuan, Urology Research Laboratory, London Health Sciences Center, 375 South Street, London, Ontario, Canada N6A 4G5.

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et al., 1991a; Ulvsback et al., 1991; Ochiai et al., 1995]. Alternative splicing of PSP94 mRNA in prostate tumor tissue [Xuan et al., 1995a] and the promoter region responsible for cell-specific expression [Ochiai et al., 1995] have been identified. One study demonstrated apoptosis in rat prostate exposed to high levels of exogenous PSP94 [Mundel et al., 1993]. In a rat prostate adenocarcinoma model, PSP94 was found to inhibit the growth of tumor cells in vitro and also to suppress tumor growth in vivo [Garde et al., 1993; Lokeshwar et al., 1993]. The C-terminus of PSP94 was proposed to be the "active core" accounting for all its activity [Seidah et al., 1984]. This segment is basic and is predicted to be released by a post Gln-Arg cleavage [Arbatti et al., 1985]. C-terminal peptides (10mer [Lokeshwar et al., 1993] or 28mer [Arbatti et al., 1985; Mahale et al., 1993]) were shown both in vitro and in vivo to have antitumour activity. However, the function of PSP94 is still not clear, since its function as prostate inhibin peptide has been disputed [Kohan et al., 1986; Gordon et al., 1987].

PSP94 was reported to be a prostate tumor marker, by comparison of the expression of PSP94 with two established markers, prostatic acid phosphatase (PAP) and prostate specific antigen (PSA), in benign prostatic hyperplasia and neoplastic tissues [Vaze et al., 1979; Doctor et al., 1986; Dube et al., 1987a; Tremblay et al., 1987; Abrahamsson et al., 1988; Teni et al., 1989; Huang et al., 1992, 1993; Garde et al., 1993]. As with PSA and PAP, PSP94 has been localized in the prostate epithelial cells by immunohistochemistry (IHC) [Doctor et al., 1986; Dube et al., 1987a; Abrahamsson et al., 1988] and in situ hybridization [Mbikay et al., 1987; Brar et al., 1988]. PSP94 was reported to be androgen-independent, as its synthesis and secretion persist even after androgen deprivation [Sheth et al., 1988; Kharbanda et al., 1990].

PSP94 protein has been analysed by 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]. 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]. As an antigen, PSP94 was reported to bind with two kinds of antibodies: polyclonal or monoclonal antibodies against natural PSP94 (nPSP94) purified from seminal plasma and monoclonal antibody (*CD16leu11b*) against IgG receptor FcγRIII [Liang et al., 1991, 1992]. PSP94 is a single polypeptide chain without a carbohydrate moiety, highly charged and cysteine-rich, and devoid of alanine. By using an *E. coli* recombinant GST (glutathione S-transferase)-PSP94 expression system, a similar linear epitope structure of PSP94 protein has been identified [Xuan et al., 1996b].

In this study, we employed several synthetic peptides of PSP94, and recombinant GST-PSP94 proteins or peptides to study the epitope structure. The immuno-active, dominant, and immuno-recessive areas were identified.

MATERIALS AND METHODS

Peptides Synthesized

Six peptides (Fig. 1) were synthesized by an automatic peptide synthesizer (model 9050, Milligen). All the cysteine residues in the peptides synthesized were ACM (acetamidomethyl) protected. The peptides were dialysed against water using Spectra/Por membranes (MWCO: 1,000, Spectrum, Houston, TX) for 4 days. Peptide concentration was estimated by measuring OD_{280nm} and using a solution of originally synthesized peptide (5 mg/ml) as control.

Construction of GST-PSP94 Fusion Plasmid in *E. coli* Expression Vector pGEX-2T

A PSP94 cDNA clone was isolated and sequenced as reported previously [Xuan et al., 1995a]. The mature PSP94 coding region was cloned into the *E. coli* expression vector pGEX-2T (Pharmacia, Montreal, Que) by PCR as reported previously [Xuan et al., 1996b]. The *E. coli* PSP94 protein was translated in the same reading frame as the carrier protein GST and the resultant recombinant GST-PSP94 (rGST-PSP94) contained a thrombin site to separate one from the other [Xuan et al., 1996b].

GST fusion plasmids with each half of the DNA sequences of mature PSP94 protein, i.e., GST-PSP-N47 and -C47 were constructed according to the same cloning strategy as GST-PSP94 by PCR using PSP94 cDNA as the template. Figure 2 shows the DNA sequence of the four primers used for directing PCR to generate two DNA fragments (~150 bp), each coding for

Fig. 1. **A:** Peptide mapping of human PSP94 protein. Amino acid sequence S of the six peptides covering whole PSP94 mature protein sequence used for this study are listed in the boxes. Starting and ending positions are listed above each box.

approximately half of the PSP94 terminal amino acid sequences. After ligation at the *Bam*HI sites between vector pGEX-2T and the two PCR fragments of N47 and C47, the resultant fusion protein has the same reading frame as natural PSP94. For both GST-PSP-N47 (1–47) and -C47 (48–94) fusion plasmids, the N-termini were designed to have an additional amino acid residue (Gly), which is part of the *Bam*HI site for cloning and also part of the thrombin cleavage site for separation of the fusion proteins. In both cases the Ser residue encoded by *Bam*HI restriction site was used as the first amino acid codon of the natural PSP94 protein at position 1 and 48 for N47 and C47 fusion proteins respectively. The C-terminus of PSP-N47 fusion peptide contains an *Eco* RI cloning site and the termination sequencing of the expression vector pGEX-2T. In addition to Ile at position 47, the fusion protein contains another six amino acids: Glu, Phe, Ile, Val, Thr, Asp. The C-terminus of the fusion protein GST-PSP-C47 was designed to stop at the same position as the natural PSP94. PCR was performed under previously reported conditions [Xuan et al., 1996b] using a PSP94 cDNA template. The PCR product was first purified by the spin column of an ultrafiltration membrane (Ultrafree-Mc, from Millipore, Nepean, ON), digested with *Bam*HI and *Eco*RI enzymes and ligated with pBluescript plasmid (Stratagene, CA) previously linearized by the same restriction enzymes. Recombinant clones were characterized by physical mapping and DNA sequencing. The two cloned N47 and C47 DNA fragments were finally inserted into vector pGEX-2T precut by restriction enzymes *Bam*HI and *Eco*RI. All related recombinant DNA techniques (restriction, ligation, transformation, screening, and DNA sequencing analysis) were performed using standard protocols [Sambrook et al., 1989] or as we have previously reported [Xuan et al., 1994b, 1995a,c].

Expression and Purification of Recombinant GST-PSP94 Fusion Proteins and Peptides

Recombinant GST-PSP94 was constructed as previously reported [Xuan et al., 1996b] and expressed in the *E. coli* strain BL21 (Novagen, Madison, WI), which lacks the *lon* protease and the *omp*T outer membrane protease. *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., 1994a, 1995b, 1996b; Bautista et al., 1994]. GST-PSP94 was purified from inclusion bodies of the bacterial lysate. The crude purification of recombinant PSP94 (rPSP94) protein from thrombin digestion of GST-PSP94 fusion protein was performed as described previously [Xuan et al., 1994a, 1996b]. 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., 1994a, 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 (Bio-Rad, 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., 1996]. A 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.

Western Blotting

The chemiluminescence procedure was performed using an ECL Western blotting kit (Amersham, Oakville, Ont) according to the protocol provided by the manufacturer. The primary antibody and the secondary antibody, HRP (horseradish peroxidase) conjugated swine antiserum against rabbit IgG (Dimension Laboratories, Mississauga, ON), were diluted 5,000 and 1,000 times respectively, as described elsewhere [Xuan et al., 1996b].

Direct and Competitive ELISA (Enzyme Linked Immunosorbent Assay)

All ELISA protocols were performed as reported previously [Xuan et al., 1996b]. Briefly, protein samples in carbonate coating buffer (1.4 mM Na_2CO_3, 7 mM $NaHCO_3$ pH 9.2) were coated in triplicate onto 96 well Immunoplates

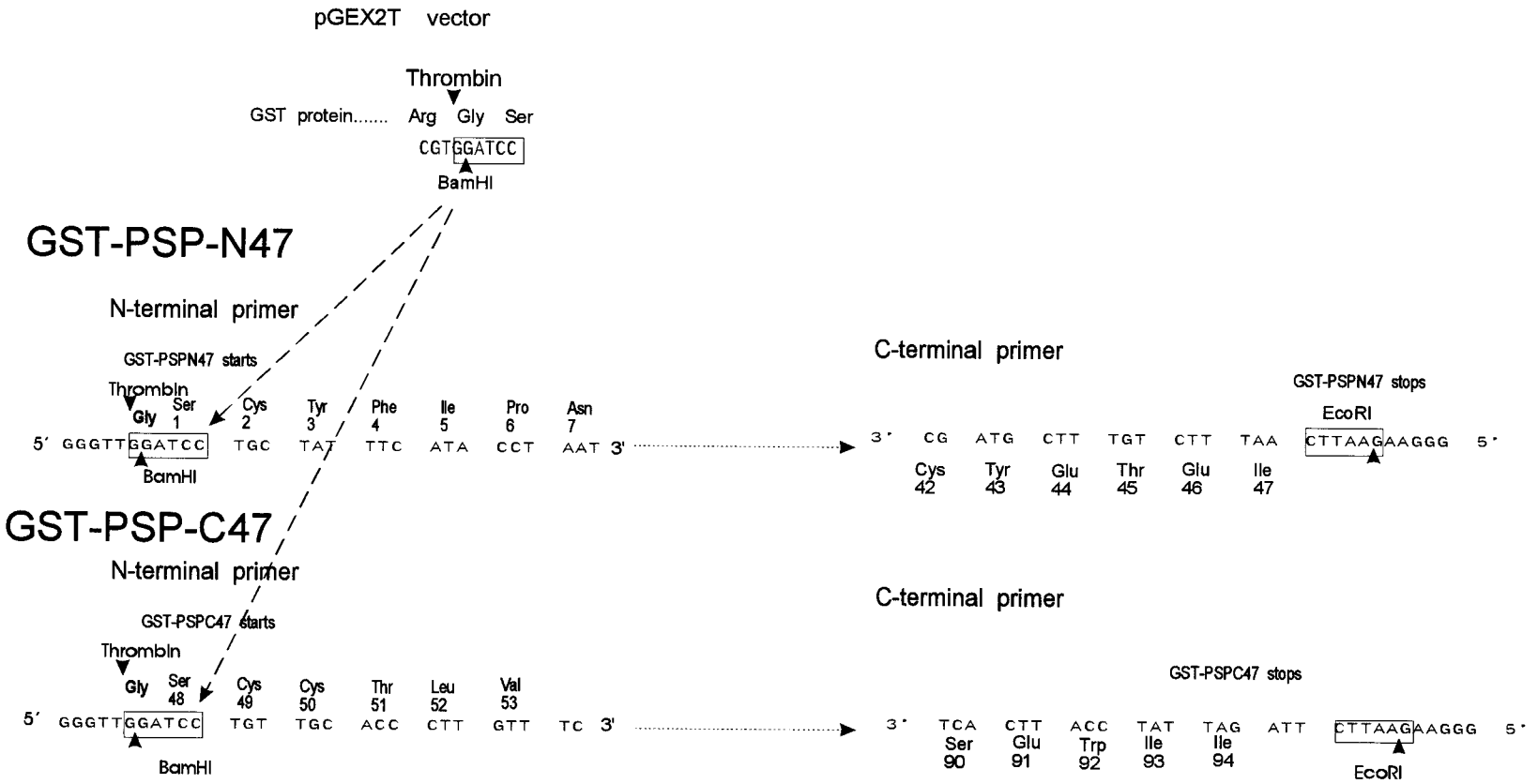

Fig. 2. Strategy for PCR-cloning of PSP-N47 and -C47 DNA sequence S into the *E. coli* expression vector pGEX-2T. DNA sequences of the four oligonucleotide primers used for PCR reaction and cloning are listed. The amino acids sequences coded by the four primers and their positions in the mature PSP94 sequence are listed above (N-terminus) or below (C-terminus). C-terminal primers are the antisense sequence. The additional Gly residues in the N-terminal end are highlighted. Arrows are the cleavage sites for thrombin and restriction enzymes *Bam*HI and *Eco*RI. Two dashed arrows indicate the insert of N-terminal sequences in the correct reading frame.

(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 conjugated swine anti-rabbit IgG) was added and incubated at room temperature for 1 h. The plate was washed three times with PBS. Colour 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 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

Peptide Mapping

In order to determine systematically the epitope structure of the PSP94 protein, six peptides covering the whole PSP94 protein sequence were synthesized (Fig. 1) and tested by direct ELISA for their ability to bind with the rabbit antiserum against human PSP94 protein purified from seminal plasma. All plastic wells were coated at the saturating levels, i.e., 1 µg (>300 pmol for a 30mer peptides) of peptide per well, and nPSP94 sample coated (10 ng/well) was used as positive control. The results of the ELISA are depicted in Figure 3A, with the N-terminal peptide 30mer (N30) showing the strongest binding ability with PSP94 polyclonal antibody. C-terminal peptides, either C10 (position 85–94) or C28 (67–94), showed the weakest affinity to the polyclonal antibody. M27 (40–66), a peptide closer to the C-terminal, was at the same low level as C28. However, M23 (21–42), a peptide located near N-terminal N30, was also highly reactive with the antibody, while the N17 peptide showed an intermediate level of affinity with polyclonal antibody between N30 and C28. To characterize the binding kinetics, the dose response of all 6 peptides to the polyclonal antibody was tested (Fig. 3B). All the N-terminal peptides (N30, M23, and N17) showed a linear response at the dose range from 10 ng to 1 µg and their affinity with polyclonal antibody was in the order: N30 > M23 > N17. The binding ability of three C-terminal peptides remained very low, even with 100-fold dose increases (10 ng–1 µg).

Reciprocal, Competitive ELISA Between N-terminal Peptides With PSP94 Proteins

To further characterize the epitope structure of the N-terminus of PSP94, the N-terminal peptide (N30), for which the polyclonal antibody had the highest affinity, was used as a competitor for the recombinant GST-PSP94 fusion protein in a reciprocal competitive antibody binding assay. In the first competition assay (Fig. 4A), varying amounts of N30 peptide (10 ng to 10 µg, ~0.003 to 3 nmol) were coated as solid phase antigen and competed with three doses of the GST-PSP94 protein (50 ng, 100 ng, 500 ng, i.e., ~1.5, 3, 15 pmol) in solution phase for comparing their affinity with the PSP94 polyclonal antibody. Figure 4A showed that at all three doses (50 ng, 100 ng, 500 ng, i.e. ~1.5, 3, 15 pmol) of the competitor GST-PSP94 (solution phase) tested inhibited N30 binding with the antibody. Saturation of inhibition by GST-PSP94 (100 ng, ~3 pmol) was obtained when the dosage of solid phase N30 peptide was increased from 100 ng (~0.03 nmol) to 1 µg (~0.3 nmol). Figure 4B summarizes the saturation inhibition at different dosages of solid phase antigen N30 from 10 ng (~0.003 nmol) to 1 µg (~0.3 nmol). In the second competition assay as shown in Figure 4C, N30 (1 µg, ~0.3 nmol) was used in solution phase to compete with GST-PSP94 (from 10 ng, ~0.3 pmol to 80 ng, ~2 pmol) in the solid phase. As shown in Figure 4D, an almost equivalent degree of inhibition was obtained when solid phase GST-PSP94 protein was coated at doses from 20 ng (~0.5 pmol) to 50 ng (~1.3 pmol). When GST-PSP94 protein was coated at higher

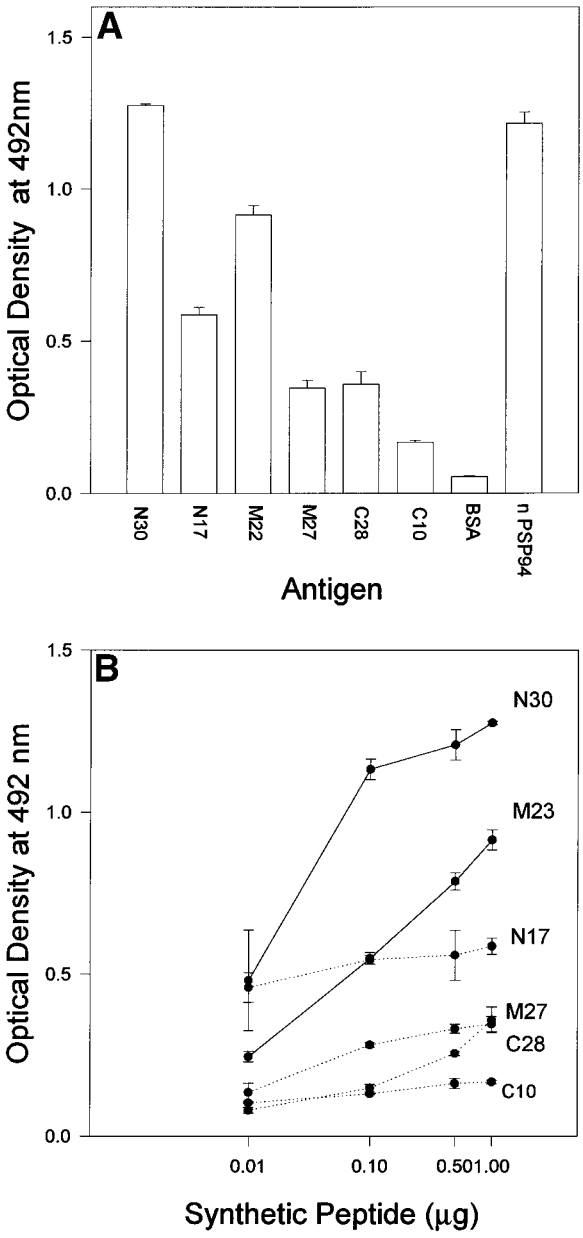

Fig. 3. **A:** Tests for the binding with rabbit against human PSP94 polyclonal antibody of the six synthetic peptides. 1 μg each peptide was coated for each well in triplicate. BSA (1.5% W/V) and nPSP94 (50 ng) were coated as negative and positive control respectively. **B:** Dose response of six peptides to the PSP94 polyclonal antibody (1:40,000 dilution of the antiserum). Approximately 10 ng, 100 ng, 500 ng, and 1 μg of peptides were coated overnight for each well and tested by direct ELISA. Values are means of triplicate measurements ± S.D.

than 1 μg, little or no competitive inhibition was exerted by N30 peptide (data not shown).

To test whether two N-terminal peptides (N30 and M23) having high affinity with the polyclonal antibody represent similar epitopes for different preparations of PSP94 protein, competitive ELISA experiments were performed. Three kinds of PSP94 protein preparations: nPSP94, rGST-PSP94 fusion protein and crude preparation of rPSP94, were used. As shown in Figure 5, three sources of PSP94 proteins at dosage of 50 ng, ~5 pmol can inhibit and compete with binding of both N-terminal peptides (N-30 and M23, 5 ng, ~1.5 pmol) with the PSP94 polyclonal antibody, but are not equally effective as competitor.

Generation and Characterization of Recombinant GST-PSP-N47 and GST-PSP-C47 Peptides

Results of reciprocal, competitive ELISA between the N-terminal peptides and PSP94 proteins indicate that there are multiple epitopes in the nPSP94 protein that act as antigen/immunogen. Because of the difficulties in identifying each epitope by peptide mapping, we decided to generate longer recombinant PSP94 fusion peptides according to the strategy as outlined in Figure 2 (see Materials and Methods). As shown in Figure 2, the coding region for the N-terminal and C-terminal 47 amino acids (each half of the PSP94 mature protein) was inserted into an *E. coli* GST-fusion vector pGEX-2T with the same reading frame and could be separated by thrombin digestion from the carrier GST protein. In contrast to the GST-PSP94 fusion protein [Xuan et al., 1996b], these two GST-fusion peptides (GST-PSP-N47 and GST-PSP-C47) were expressed in the cytosol fraction inside bacterial cells. The two affinity column-purified GST-fusion proteins were digested with thrombin and analysed by 20% high density SDS-PAGE (polyacrylamide gel electrophoresis). As shown in Figure 6A, both the cleaved recombinant N47 and C47 peptides migrated faster than the cleaved rPSP94 (at ~15 kDa), however, C47 (at ~9 kDa) migrating slower than N47 (at ~6 kDa). To qualitatively analyse the antigenic activity of the two recombinant proteins, recombinant GST fusion peptides were used directly without thrombin cleavage for Western blotting analysis, since PSP94 polyclonal antibody has no cross reactivity with the GST as demonstrated previously [Xuan et al., 1996b]. As shown in Figure 6B, similar to the cleaved recombinant C47 peptide (Fig. 4B), GST-PSP-C47 fusion protein still migrated more slowly than GST-PSP-N47. These two GST-affinity column-purified GST fusion proteins

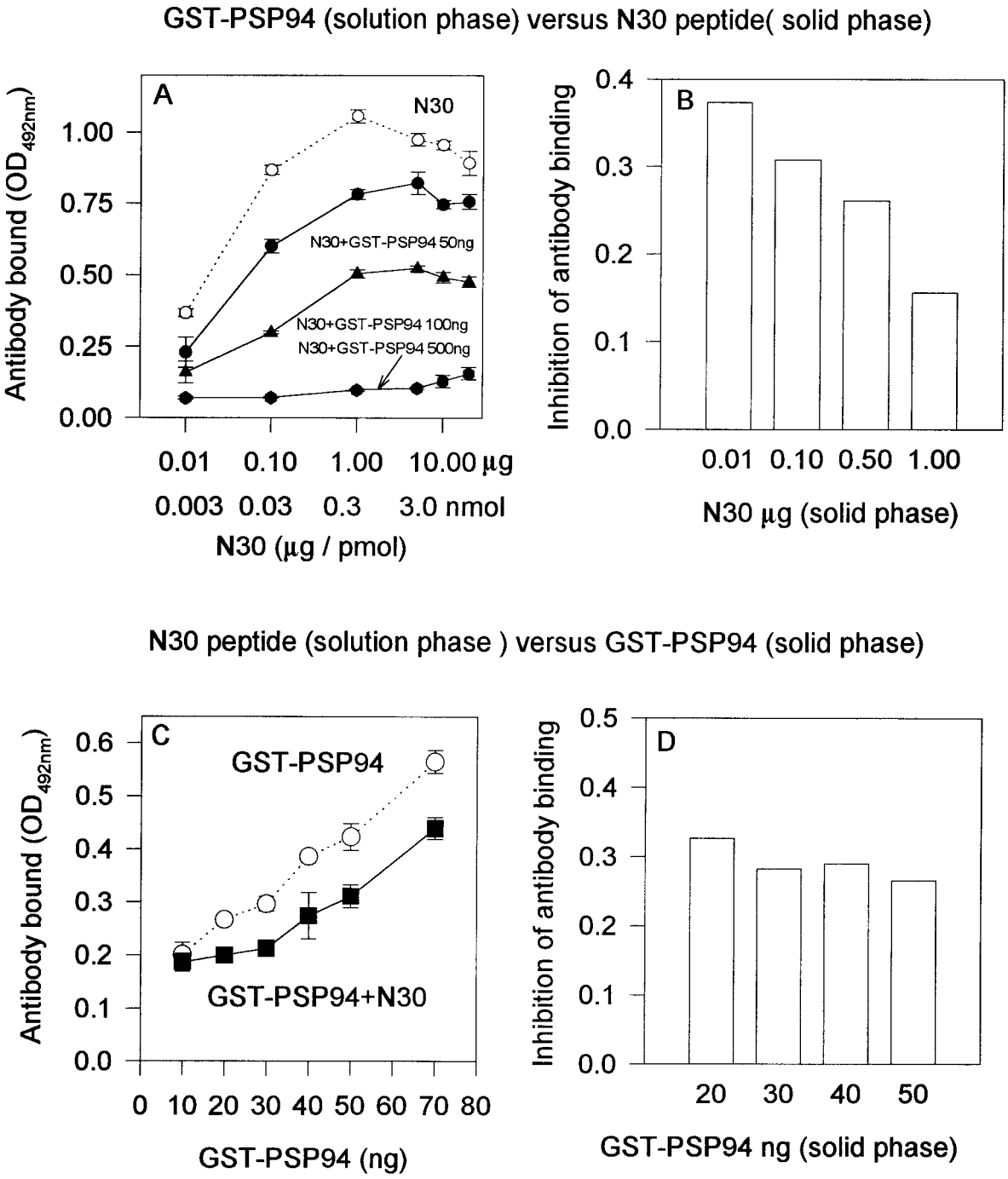

Fig. 4. Reciprocal, competitive ELISA between the N-terminal peptide (N30) and recombinant GST-PSP94. **A:** N30 peptide (dosage range as indicated on X axis) was coated as solid phase and competed with GST-PSP94 protein (solution phase) for binding with polyclonal antibody (1:40,000 dilution of antiserum). N30 dose response was used as no competition control (dashed line). **B:** Comparison of inhibition of antibody binding by GST-PSP94 protein (100 ng, ~3 pmol) against N30 peptides (dose as indicated in the plot). The degree of inhibition of antibody binding was calculated as the mean values of the triplicate samples tested as $1\text{-}OD_{492nm}$ of competition/OD_{492nm} of control. **C:** GST-PSP94 protein (dose range as indicated on X axis) was coated as solid phase and competed with N30 (1 µg, ~300 pmol per well) for binding with polyclonal antibody (1:60,000 dilution of the antiserum). **D:** Comparison of inhibition of antibody binding by N30 (1 µg, ~300 pmol) against GST-PSP94 protein (dose as indicated in the plot). Error bars for each point are mean values from triplicate samples ± S.D.

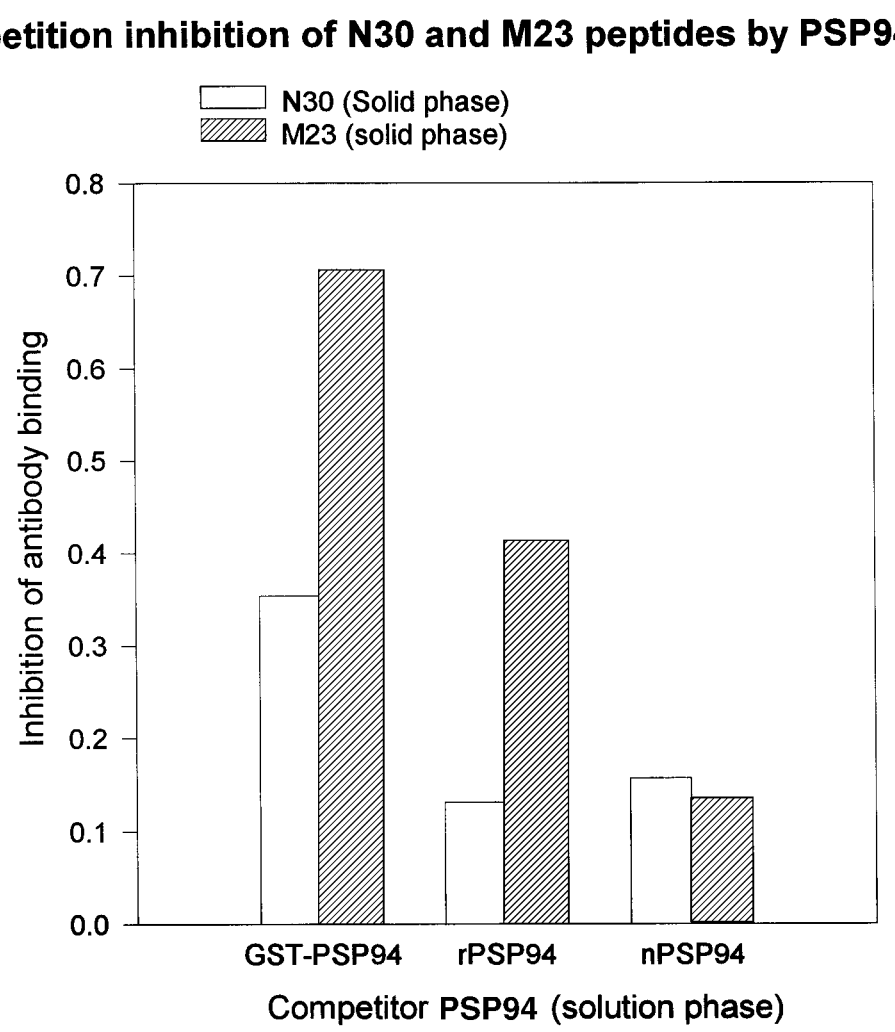

Fig. 5. Comparison of the affinity of two N-terminal peptides (N30 and M23) with three kinds of PSP94 proteins. About 10 pmol each of GST-PSP94 fusion protein (350 ng), purified recombinant PSP94 (100 ng) and nPSP94 (100 ng) were coated as solid phase and competed with 0.5 μg i.e., ~130 pmol of the peptides in the solution phase for binding with PSP94 polyclonal antiserum (dilution 1:40,000). The degree of inhibition of antibody binding was calculated as the mean value of the triplicate samples tested as 1-OD_{492nm} of competition/OD_{492nm} of control.

also showed some truncated forms similar to what we have previously seen in other GST-fusion proteins [Xuan et al., 1994a, 1995b, 1996b; Bautista et al., 1994]. The gel was Western blotted and reacted with PSP94 polyclonal antibody. The results are shown in Figure 6C. Both GST-PSP-N47 and -C47 fusion proteins bound strongly with the PSP94 polyclonal antibody as did the positive control GST-PSP94, indicating that these two proteins were recombinant PSP94 proteins with correct antigenic activity. Multiple bands were seen in Western blotting test in all the recombinant GST-PSP94 proteins. Carrier protein GST still reacted negatively with PSP94 antiserum.

Tests of the Affinity of GST-PSP-N47 and GST-PSP-C47 Peptides With PSP94 Polyclonal Antibody

Next, analytical and quantitative ELISA experiments were conducted to compare the N47/C47 fusion proteins for their differences in affinity to the PSP94 polyclonal antibody. Two experiments were performed. First, dose response (from 10 ng to 1 μg) of the two fusion proteins to PSP94 polyclonal antibody was compared and the results are shown in Figure 7A. In the direct ELISA, GST-PSP-C47 consistently generated nearly half the value of OD_{492nm} as those of the GST-PSP-N47, indicating a lower

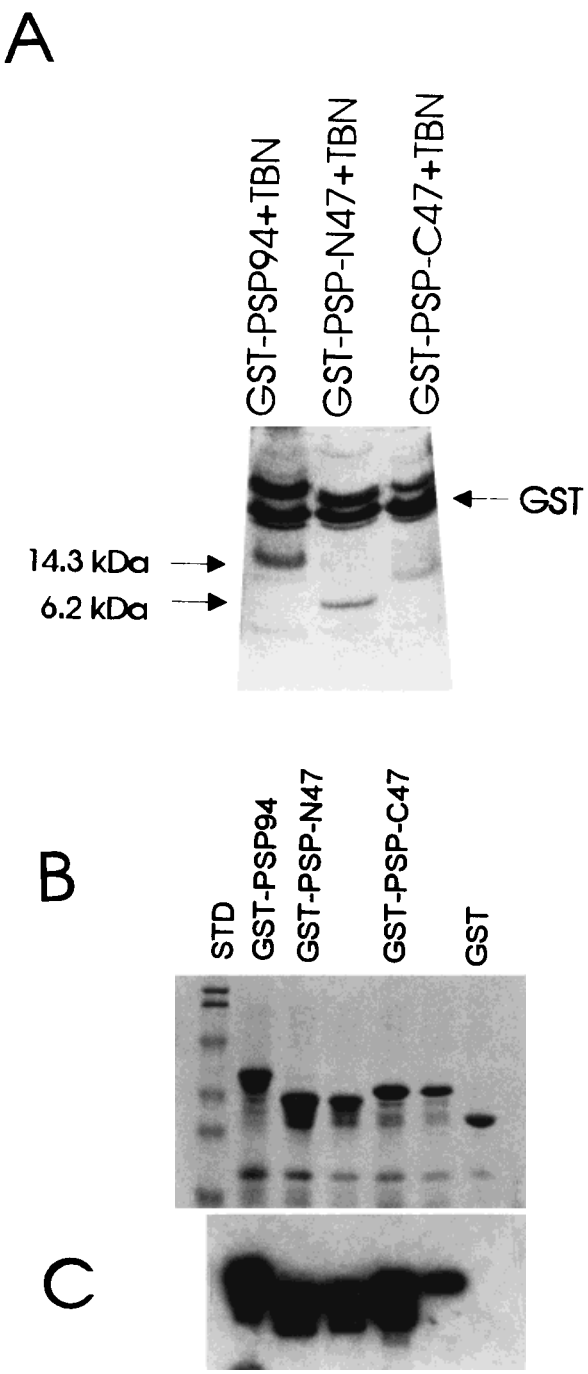

Fig. 6. Expression of GST fusion proteins with half of the N- and C-terminal peptide sequence of PSP94. **A:** Twenty percent SDS-PAGE (High density, Pharmacia PhastGel) analysis of two GST-PSP-N47/C47 fusion proteins using GST-PSP94 fusion protein as control. Molecular weight was measured by using low molecular weight protein standard from Gibco/BRL. **B:** Fifteen percent SDS-PAGE analysis of GST-PSP94 (20 µg) and -N/C47 fusion proteins. GST-PSP-N/C47 were loaded with 10 µg and 20 µg separately for each. Protein molecular weight standard (low range, from BioRad) used are from bottom: 14.4, 21.5, 31, 42.7, 66.2, 97.4 kDa. **C:** Western blotting analysis of 15% PAGE of (B) using polyclonal antibody against human PSP94.

affinity of GST-PSP-C47 with the polyclonal antibody. Secondly, competitive binding of the two PSP-N/C47 peptides with the PSP94 polyclonal antibody was performed to differentiate their ability to bind with that of the rGST-PSP94 protein. As shown in Figure 7B, GST-C47 in solution phase as competitor showed no competitive binding with GST-PSP94 (solid phase) with the PSP94 polyclonal antibody at all competing doses tested, from 10 ng to 1 µg, i.e., ~2 pmol to 200 pmol. On the other hand, GST-PSP-N47 showed competitive inhibition binding with the polyclonal antibody. The inhibition was significant and correlated with the dose increase (from 10 ng to 1 µg, i.e., ~2 pmol to 200 pmol) of GST-PSP-N47 competitor (solution phase).

DISCUSSION

We have established an in vitro expression system for human PSP94 by using an *E. coli* GST vector (pGEX 2T) and we have demonstrated that the bacterially expressed protein have the similar antigenic activity as natural PSP94 [Xuan et al., 1996b]. By using the GST-PSP94 fusion protein, and also reciprocal, competitive ELISA and in vitro denaturing tests, we have found that natural PSP94 has a linear epitope structure, at least as a prevalent epitope structure [Xuan et al., 1996b].

Our previous report [Xuan et al., 1996b] indicated that the N-terminus of the human PSP94 protein is probably the immuno-dominant area. In the same report, we also indicated that the C-terminus (C-28) may represent an immunorecessive area [Xuan et al., 1996b]. It is unlikely that our observation is due to host specific epitope selection, since our results were consistent with a report from another group [Dube et al., 1987a]. Two research groups had repeatedly and independently raised rabbit antisera and obtained similar results. In order to systematically study the epitope structure, complete peptide mapping was performed in this study. Another peptide located in the N-terminus, M23 (position 21–43, Fig. 1) also was found to be antigenic. N17 is a weaker antigen, indicating that dominant epitopes are located after N17, which is consistent with the results derived from tests of antigenic activity of β-Gal fused protein with PSP94 with the truncated N-terminus in *E. coli* [Linard et al., 1988]. All three peptides (C28, M27, and C10) located at or near the C-terminus showed no or very weak

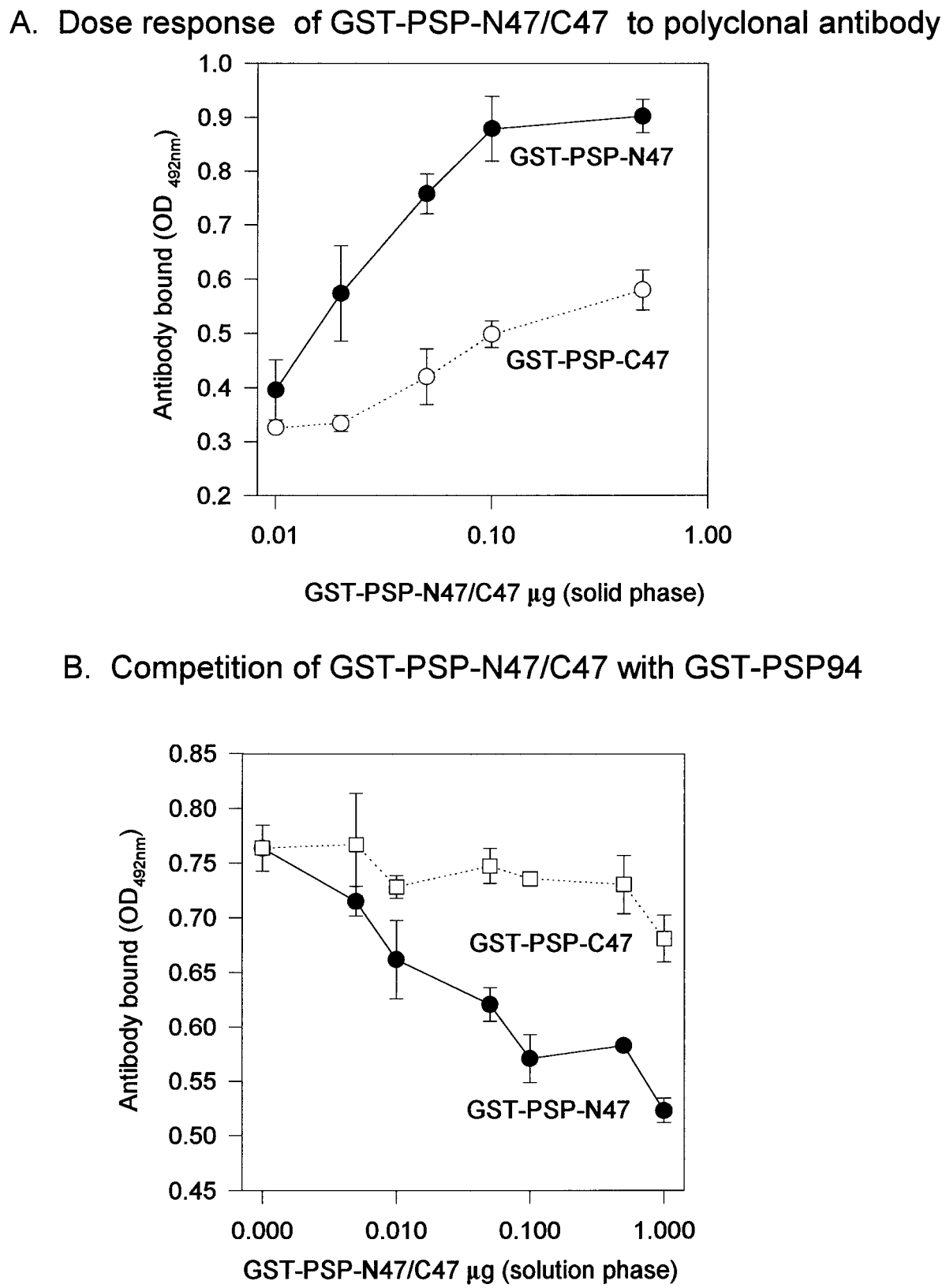

Fig. 7. Comparison of the affinity of two recombinant GST-PSP-N/C47 fusion proteins with PSP94 polyclonal antibody. **A:** Dose response (10 ng–1 µg, ~0.3–300 pmol) of GST-PSP-N47 and GST-PSP-C47 to polyclonal antibody. **B:** Competitive ELISA. GST-PSP94 were coated at 25 ng (~0.7 pmol) and competed with GST-PSP-N47 and GST-PSP-C47 at different dosages (indicated on the X axis) for binding with the polyclonal antibody. Each point is mean value from triplicate samples ± S.D.

affinity with the polyclonal antibody (Fig. 3A and B). In order to assess the antigenic activities of the two N-terminal peptides in the whole epitope structure, reciprocal, competitive ELISA were performed to compare the affinity of three sources of GST-PSP94, nPSP94 and rPSP94. As shown in Figures 4 and 5, PSP94 protein sequences, for example GST-PSP94, apparently have higher affinity for PSP94 antibody than any N-terminal peptides, as 100 ng, ~3 pmol of competitor GST-PSP94 (in solution phase) can exert 20–30% competitive inhibition against N30 peptides from 0.1 μg to 10 μg, i.e., ~30 to 3,000 pmol (Fig. 4B). 500 ng, ~15 pmol of GST-PSP94 can achieve complete inhibition (Fig. 4A). As shown in Figure 5, similar competitive inhibition was obtained using different preparations of PSP94 proteins, confirming our previous conclusion that the recombinant PSP94 has epitope structures similar to nPSP94 [Xuan et al., 1996b]. Figure 5 shows that GST-PSP94 inhibits M23 binding with antibody more effectively than it inhibits N30 binding. Figure 5 also shows that nPSP94 had less ability to compete with both N-terminal peptides than rGST-PSP94 when tested at some concentrations of the PSP94 antiserum. Similar results were obtained previously [Xuan et al., 1996b] that in a competitive ELISA, rGST-PSP94 competed more effectively for binding with PSP94 polyclonal antibody than nPSP94. These results may be due to the fact that nPSP94 binds more effectively with PSP94 antibody than recombinant PSP94 proteins with polyclonal antibody in the competitive ELISA. Taken together, these results indicate that nPSP94 and rGST-PSP94 differ in epitope structure, although these differences are not prevalent in the whole epitope structure. On the other hand, N30 peptide (1 μg, ~300 pmol) as a competitor in the solution phase can only achieve 30% inhibition of GST-PSP94 binding at the range of 10–70 ng, i.e., ~0.5–2 pmol. The difference in the reciprocal, competitive ELISA as shown in Figure 4 implies that PSP94 possesses multiple epitopes and one or two peptides cannot compete against the whole PSP94 protein at equal molar concentration, with some concentrations of PSP94 polyclonal antibody.

PSP94 contains multiple epitopes and peptide mapping to locate each epitope may be difficult. Therefore, N47 and C47 peptides, i.e. each half of the protein, were expressed in *E. coli* as GST fusion proteins. DNA sequence analysis of the terminator region of pGEX-2T used for the construction of fusion plasmid (see Materials and Methods), indicated that the C-terminus of GST-PSP-N47 contains six additional amino acids in the C-terminal end. These extra sequences apparently do not increase any affinity with PSP94 polyclonal antibody, since all six amino acid residues are hydrophobic, non-antigenic, and show no homology with the PSP94 sequence. Results of PAGE analysis of either fusion or cleaved C47/N47 protein showed different relative migration rates in high density PAGE (Fig. 6A and B), which is most likely due to the highly positively charged (basic) C-terminus (see Fig. 1). Our experimental data [Xuan et al., 1996b] and reports by others [Carter et al., 1982; Dube et al., 1987a; Wright Jr. et al., 1990; Liang et al., 1992] have demonstrated that both natural and recombinant PSP94 proteins (10 kDa) showed lower than expected relative mobility (~15 kDa) in all forms of PAGE. As shown in Figure 6A, the cleaved PSP-N47 has a relative mobility (about 6 kDa), which is closer to its real molecular weight, while C47 peptide showed retarded mobility as the same as whole PSP94 protein. We may conclude that the highly positively charged C-terminus caused the C47 peptide and PSP94 protein to migrate slower than standard protein in SDS-PAGE. Testing of GST-PSP-N/C47 fusion proteins by direct ELISA and competitive ELISA against the GST-PSP94 fusion protein (Fig. 7) with PSP94 polyclonal antibody confirmed the results derived by our peptide mapping and reciprocal competitive ELISA. Only GST-PSP-N47, and not GST-PSP-C47, showed the capability of competition-inhibition against PSP94 protein, 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.

PSP94 has been measured in blood samples from prostate cancer patients and has been found to be a potential tumor marker by several researchers [Vaze et al., 1979; Doctor et al., 1986; Dube et al., 1987a; Tremblay et al., 1987; Abrahamsson et al., 1988; Teni et al., 1989; Huang et al., 1992, 1993]. Since PSP94 is high in concentration in seminal plasma (1320 ± 183 μg/ml) [Dube et al., 1987a], its functions with respect to reproductive biology has been proposed: either as a sperm coating antigen [Johansson et al., 1984; Akiyama et al., 1985; Hara et al., 1989] or as an immunosuppressive factor

(immunoglobulin binding factor, IgBF) [Liang et al., 1991, 1992; Maeda et al., 1993; Xuan et al., 1996a]. The expression of sperm coating antigens is central to the consideration of sperm immunogenicity in the reproductive tract. As an IgBF, PSP94 has the ability to bind with the CD16 series monoclonal antibodies against type III IgG receptor and may protect spermatozoa from attack by the immunosurveillance system of the female reproductive tract. These proposed functions raise the question of the antigenic structure of PSP94. By delineating the epitope structure of PSP94, we hope to improve our understanding of this potential tumor marker for prostate cancer and ultimately define its clinical utility.

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