Epitope mapping of prostate-specific antigen with monoclonal antibodies

Diane C. Jette, Fernando T. Kreutz, Bruce A. Malcolm, David S. Wishart, Antoine A. Noujaim, and Mavanur R. Suresh

Prostate-specific antigen (PSA) is a widely used marker for screening and monitoring prostate cancer. We identified and characterized the epitopes of two anti-PSA monoclonal antibodies (mAbs) designated B80 and B87. The epitopes were initially mapped as nonoverlapping by developing a sandwich immunoassay to measure PSA with the two anti-PSA mAbs. The two antibodies do not cross-react with homologous pancreatic kallikrein, but recognize epitopes unique to PSA. B80 and B87 can recognize both free and complexed PSA and hence measure total PSA. Epitope scanning and bacteriophage peptide library affinity selection procedures were used to identify and locate an epitope on PSA. A possible epitope for B80 was identified as being located on or near PSA amino acid residues 50–58 (-GRH-SLFHP-). The epitope for B87 was likely on an exposed nonlinear conformational determinant, unique to PSA, and not masked by the binding of B80 or α1-antichymotrypsin.

INDEXING TERMS: immunoassay • prostate cancer • bacteriophage peptide library

Prostate-specific antigen (PSA) is a 33-kDa single-chain glycoprotein produced by prostatic epithelium. The primary structure of PSA was first described by Watt et al. [1] and shows a high degree of homology with serine proteases of the kallikrein family [2, 3]. PSA is a major protein in seminal plasma and shows chymotrypsin-like substrate specificity [4–6]. It is normally found in low concentrations (<2.5 µg/L) in male blood plasma but can be increased in conjunction with prostate cancer, benign prostatic hyperplasia (BPH), and surgical trauma to the prostate [7–9]. Monoclonal antibody (mAb)-based RIAs and ELISAs are used to measure PSA in serum. The results are used to screen for prostate cancer and monitor patients during treatment [8, 10, 11].

The PSA gene is a member of the human tissue kallikrein family referred to as serine proteases because the mechanism of proteolytic cleavage involves a serine residue at the active site [1, 12, 13]. PSA is a 6-kb gene product of chromosome 19 in the region of q13.2–q13.4 with 4 introns and 5 exons [14]. It has a >84% nucleotide sequence homology with human glandular kallikrein (hK2), and 73% homology with human pancreatic kallikrein (hK1), indicating a common ancestral gene [3, 15–18]. Human serum kallikrein is not a member of this gene family and does not have a high degree of sequence homology to PSA. The amino acid homology with porcine pancreatic kallikrein (pK1) is 59.6%. Previously all three gene products were renamed [15, 17], as shown in Table 1.

In serum, PSA can form stable complexes with two major serum protease inhibitors, α1-macroglobulin (AMG) and α1-antichymotrypsin (ACT) [20–22]. When PSA is complexed to AMG it is thought to be completely encapsulated with no epitopes accessible for immunodetection [23]. However, when PSA is complexed to ACT, some epitopes proximal to the active site are masked while others are available for binding to anti-PSA antibodies [21]. Immunoassays involving antibodies directed against PSA epitopes that are masked by ACT will fail to detect this complex. The proportion of serum PSA complexed to ACT has been found to be significantly higher with prostate cancer than with BPH. The ratio of free to complexed PSA, therefore, may be useful in distinguishing between prostatic cancer and BPH [21–23]. It is important, therefore, to determine if antibodies used to measure PSA in serum are recognizing free PSA, PSA-ACT, or both. In addition, several authors have recently discussed the importance of standardizing PSA assays, because many of the commercially available assays yield different PSA values [24–30]. In this paper, we attempt to identify and characterize the epitopes that are recognized by two anti-PSA mAbs.

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4 Nonstandard abbreviations: PSA, prostate-specific antigen; BPH, benign prostatic hyperplasia; mAb, monoclonal antibody; hK2, human glandular kallikrein; hK1, human pancreatic kallikrein; AMG, α1-macroglobulin; ACT, α1-antichymotrypsin; HRPO, horseradish peroxidase; hsaAb, bispecific mAb; ABTS, 2,2’-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TMB, tetramethylbenzidine; PBS, phosphate-buffered saline; BSA, bovine serum albumin; pK1, porcine pancreatic kallikrein; and TBS, Tri-buffered saline.
Materials and Methods

Materials. Anti-PSA mouse IgG mAbs B80 and B87 were kindly provided by Biomira, Edmonton, AB, Canada, and the anti-PSA × anti-peroxidase (HRPO) bispecific mouse IgG mAb (bsmAb B80×HRPO) was developed in our laboratory [31]. Briefly, a quadroma or hybrid hybridoma was generated by fusing the B80-secreting hybridoma with an anti-HRPO-secreting hybridoma. The bsmAb was purified by DE-52 gradient chromatography. This bsmAb has one Fab arm with anti-PSA specificity and another Fab arm with anti-HRPO specificity engineered in one molecule. Standard media used for cell culture, RPMI-1640 supplemented with 2 mmol/L L-glutamine, 50 kilounits/L penicillin, 50 mg/L streptomycin, and 50 mL/L fetal bovine serum, were obtained from Gibco BRL, Gaithersburg, MD. Anti-mouse and anti-rabbit IgG-peroxidase conjugate, cyanogen bromide-activated Sepharose 4B, pk1, kallikrein from human serum, and 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) were obtained from Sigma, St. Louis, MO. Maxisorp™ 96-well immunoplates were obtained from Nunc, Roskilde, Denmark. PSA calibrator was obtained from Scripps Labs., San Diego, CA. ACT from human plasma and rabbit anti-ACT IgG were obtained from Calbiochem, La Jolla, CA. Molecular-mass markers for sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE), precast 4% to 15% gradient polyacrylamide gels, SDS buffer cartridges, and the Phast™ electrophoresis system were obtained from Pharmacia, Uppsala, Sweden. The Mimotope kit, used to synthesize the immobilized hexapeptides, was obtained from Cambridge Research Biochemicals, Cheshire, UK. A Vmax microplate reader and MAX Line plate washer from Molecular Devices, Menlo Park, CA, was used. Escherichia coli strain K 91 (Hfi-C thi) was used in conjuction with the bacteriophage (phase) decapetide display library [32]. Dideoxynucleotide sequencing was performed with the Sequenase 2.0 system obtained from United States Biochemicals, St. Louis, MO. One Step TMB (tetramethylbenzidine) substrate was obtained from Pierce Chemical Co., Rockford IL.

Purification of PSA. A human prostate cancer cell line (LNcap) was used as a source of PSA. The cells were grown (standard media, 275 mm² tissue culture flask) and the supernatant was collected and pooled every 3 to 5 days. The concentration of PSA in the unpurified supernatant was determined to be 0.9–1.5 mg/L by ELISA. PSA was purified from 1 L of cell supernatant by using an affinity column with immobilized anti-PSA antibodies, B80 and B87. The column was made by first washing Sepharose 4B (1 g in 3.5 mL, cyanogen bromide activated) with 2 volumes of buffer (0.5 mol/L phosphate, pH 6.8). A mixture of B80 and B87 [10 mg in 3 mL of phosphate-buffered saline (PBS), pH 7.2] was dialyzed overnight against three changes of buffer. The antibodies were added to the Sepharose gel and gently agitated at room temperature. The amount of antibody remaining in solution was determined by monitoring the reaction mixture absorbance at 280 nm. The reaction was stopped when ~90% of the antibody was bound. The column was then washed twice with PBS (10 mL), and ethanolamine (10 mL of 100 mmol/L in distilled water) was added to block any unbound reaction sites on the Sepharose (2 h at room temperature).

Before use, the mAb affinity column was washed twice (10 mL of 10 mmol/L phosphate buffer, pH 6.4). Cell supernatant containing 1.5 mg/L PSA (1 L) was cycled for 24 h through the column with a peristaltic pump (1.7 mL/min) so that the total volume of supernatant passed through the column three times. After washing the column with buffer, the PSA was eluted with glycine (100 mmol/L, pH 2.5). Column fractions (0.5 mL) were collected into tubes containing buffer (50 µL of 1 mol/L phosphate, pH 8.0) to neutralize the eluates. The protein concentration of each fraction was determined by measuring the absorbance at 280 nm and the appropriate fractions were pooled. After pooling, an ELISA was performed as described below to determine the concentration of purified PSA. The column, which can be reused, was washed (1 g/L sodium azide in 10 mmol/L phosphate) and stored at 4 °C until needed. PSA eluted from the monoclonal affinity column in fractions 3 to 5 and a total of 1.37 mg of PSA was recovered from 1.5 L of supernatant. No remaining PSA was detected in the supernatant after affinity purification.

Preparation and analysis of PSA-ACT complex. The complex was prepared by using the method described by Lilja et al. [22]. Purified PSA (7 µg) was added to purified ACT (50 µg) in reaction buffer (50 µL of 50 mmol/L Tris-HCl, pH 7.8, with 0.1 mol/L NaCl) and incubated for 6 h at 37 °C. The degree of complex formation was analyzed by SDS-PAGE. A Phast electrophoresis system was used to analyze free and complexed PSA. A precast acrylamide gel (4% to 15% gradient) was run under standard conditions (10 mA, 250 V in SDS-barbital buffer) as per the manufacturer’s instructions. Samples containing 4 µL of purified PSA, complexed PSA, unbound ACT, and low-range molecular-mass markers were analyzed. SDS-PAGE could effectively resolve PSA at ~30 kDa, ACT at ~60 kDa, and PSA-ACT complex at ~90 kDa. A small amount of free PSA was seen on the electrophoresis gel after complex formation. The PSA-ACT complex immunobssay used in this study will not detect free PSA or ACT.

Measurement of PSA and PSA-ACT by ELISA

PSA sandwich ELISA. We previously developed two mouse mAbs, B80 and B87, using human PSA isolated from seminal plasma. These two antibodies were selected for their ability to form an efficient sandwich assay for PSA in serum. The antibodies have high affinity for PSA (Kd ~2 × 10⁻¹⁰ mol/L for both) and the assay exhibited a good correlation (r = 0.98) with

<table>
<thead>
<tr>
<th>Table 1. Nomenclature of kallikrein family proteins.*</th>
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<tbody>
<tr>
<td>Formal name</td>
</tr>
<tr>
<td>hK1</td>
</tr>
<tr>
<td>hK2</td>
</tr>
<tr>
<td>hK3</td>
</tr>
<tr>
<td>pK1</td>
</tr>
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</table>

* Modified from McCormack et al. [19].
the Hybritech PSA Tandem RIA with 154 prostate cancer serum samples (Krantz MJ and Suresh MR, manuscript in preparation). A PSA sandwich ELISA was developed with mAb B87, as the capture antibody, immobilized on plastic in a microtiter plate. The bsmAb B80×HRPO was used as the tracer antibody [31]. Microtiter wells were coated with mAb B87 (1 μg in PBS, 16 h, 4 °C). The plate was then blocked [10 g/L bovine serum albumin in PBS (BSA-PBS), 1 h, 37 °C]. Samples or calibrators (50 μL) were added to each well along with bsmAb B80×HRPO (10 ng) and HRPO (300 ng in 50 μL of PBS). The plate was incubated with shaking (30 min, room temperature). After washing [3 × 200 μL of PBS containing 0.5 mL/L Tween 20 (PBS-Tween)], ABTS substrate (100 μL of 0.5 g/L ABTS, 0.5 μL of 30% H2O2, in 0.1 mol/L citrate with 0.1 mol/L phosphate, pH 4.0) was added to each well and the amount of PSA present in the sample was determined by measuring the absorbance at 405 nm with a microplate reader. The assay was calibrated for PSA concentration with 99% pure PSA obtained commercially. The calibrators were diluted in BSA-PBS (and not serum). This assay was used to determine the PSA concentration in cell supernatant and PSA affinity-purified eluates. The dose–response curve, shown in Fig. 1, is linear from 0 to 75 μg/L PSA and is not saturated even at 250 μg/L PSA. Each point is an average of duplicate measurements and the SEM is indicated. Further optimization and the clinical utility of this assay are currently being evaluated in our laboratory [31 and work in progress]. Another experiment directly compared the quantitative recovery of fixed samples of PSA ranging from 3 to 100 μg/L with and without ACT in molar ratios of 1:0, 1:0.5, 1:1, and 1:5. The absorbance values obtained at 100 μg/L were 1.286, 1.210, 1.296, and 1.206 and at 6 μg/L were 0.107, 0.086, 0.112, and 0.097 for the respective ratios. The absorbance values similarly were within experimental error for 3, 12, 25, and 50 μg/L PSA with and without ACT in the above ratios.

**PSA-ACT ELISA.** Microtiter wells were coated with either B80 or B87 (1 μg in PBS, overnight, 4 °C) and blocked as above. PSA-ACT complex was titrated from 100 ng/well (100 μL BSA-PBS) in twofold serial dilutions. The plate was incubated (shaking, 1.5 h, room temperature) and then washed. Anti-ACT rabbit IgG polyclonal antisera was diluted (100 μL, 1:1000 dilution in BSA-PBS) and added to each well. The plate was again washed. Anti-rabbit IgG-HRPO was diluted as above and added to each well. The plate was incubated for 1 h and washed. ABTS substrate was added and the amount of immobilized PSA-ACT complex present in the sample was determined by measuring the absorbance at 405 nm.

**Cross-reactivity analysis.** Human serum kallikrein and pK1 were immobilized on plastic microtiter wells. B80 or B87 was incubated with the immobilized proteins to evaluate their cross-reactivity. The binding of these antibodies to immobilized PSA, shown in Fig. 2, reaches saturation in this assay at ~12 μg/L for B80 and 50 μg/L for B87. No binding to either of the kallikreins was observed for B80 or B87 up to a concentration of 1 mg/L. Purified PSA was used as a positive control and BSA was used as a negative control. Microtiter wells were coated with either purified PSA, hK2, or pK1 (0.5 μg, PBS, overnight, 4 °C). The plates were blocked, and B80 or B87 was titrated from 1 μg/well in BSA-PBS in twofold dilutions. The plate was incubated for 3 h, then washed. Anti-mouse IgG-HRPO was diluted as above and added to each well. The plate was incubated for 1 h and washed. ABTS substrate was added and the amount of antibody binding to kallikreins or PSA was determined by measuring the absorbance at 405 nm.

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**Fig. 1.** Dose–response curve for a PSA sandwich ELISA. mAb B87, immobilized on the plate, was used as the capture antibody and bsmAb B80×HRPO was used as the tracer antibody. Pure PSA, diluted in 10 g/L BSA-PBS, was used to calibrate the assay for PSA concentration. The assay was performed as described in Materials and Methods.

**Fig. 2.** Comparison of the binding of mAbs B80 and B87 to PSA, glandular (or human serum) kallikrein, or pK1 in an immunoassay. PSA or kallikrein was immobilized on the plate. After incubating with B80 or B87, the plate was washed to remove unbound material, and anti-mouse IgG-HRPO conjugate was used as the secondary antibody. Neither B80 nor B87 cross-reacted with either kallikrein. The assay was performed as described in Materials and Methods.
SYNTHESIS AND SCREENING OF PSA HEXAPEPTIDES
The 240 overlapping hexapeptides representing the entire PSA sequence were synthesized on pins with the Mimotope kit. Standard solid-phase peptide synthesis with Fmoc-amino acid active esters was used to build each hexapeptide on aminofunctionalized pins. The peptides were acetylated at the N terminus. The pins with the immobilized synthetic peptides can be regenerated and restested several times.

A standard ELISA was used to screen the PSA-derived peptides for binding to B87 and bsmAb B80×HRPO. The pins were blocked in a 96-well microtiter plate. For screening with B87, antibody (100 ng/well, 200 μL of BSA-PBS) was added to each well of a microtiter plate and the pins incubated in this solution for 3 h and washed. Anti-mouse IgG-HRPO was diluted and added to each well of a microtiter plate. The pins were again incubated and washed. ABTS substrate was added and the amount of antibody binding to immobilized peptide was determined by measuring the absorbance at 405 nm. For screening with B80, bsmAb B80×HRPO (10 ng) and HRPO (300 ng in 200 μL of PBS) were added to each well. The pins were incubated for 1 h and subsequently washed. ABTS substrate was added and the absorbance at 405 nm was measured.

AFFINITY SELECTION OF B80 AND B87 SPECIFIC PEPTIDES FROM A RANDOM PHAGE PEPTIDE DISPLAY LIBRARY
An aliquot of a bacteriophage peptide library displaying random decapeptides [32] [4.4 × 10^11 colony-forming units in 40 μL of BSA-Tris-buffered saline, pH 7.2 (BSA-TBS)] was added to 40 μg of immobilized B80 and B87 (10 μL of a 50% slurry of antibody-linked Sepharose 4B, described above). The sample was gently mixed (room temperature, 1.5 h) and the beads were collected by centrifugation (30 s, 4000g). The supernatant was removed and the beads resuspended, washed with 500 μL of BSA-TBS (3 min), and recovered by centrifugation. This wash was repeated twice with BSA-TBS, three times with 0.5 mL/L Tween 20 in TBS (TBS-Tween), and once with BSA-TBS. The washed beads were then incubated with PSA (1 h, room temperature with shaking, 3 μg in 50 μL of BSA-TBS). The supernatant, designated as ligand eluate, was collected and the beads washed as before with 500 μL of 10 mmol/L citrate buffer, pH 2.0. After 5 min, the supernatant, designated as the acid eluate, was collected. Both the ligand and acid eluates were amplified by infecting E. coli cells with isolated phage, following procedures described in Christian et al. [32]. A second round of selection was performed as above.

IMMUNOLOGICAL SCREENING OF AFFINITY-SELECTED PHAGE
Affinity-selected phage particles were screened in an in situ immunological assay with phage-infected colonies [32]. Phage clones from the eluates were used to infect fresh cells. The cells were then grown in selective media (15 × 150 mm plates, LB medium with 20 mg/L tetracycline) at a density of 200 to 500 colonies/plate and transferred to nitrocellulose membrane filters. For screening with B80, the filters were washed (30 min, TBS-Tween), blocked (30 min, 30 mL/L BSA-PBS), and then incubated with bsmAb B80×HRPO (20 μg, with 1 mg of HRPO). The filters were washed again and incubated with TMB substrate for 1 h. The filters were then washed with distilled water and allowed to air dry overnight. Alternatively, filters with colonies from a second set of plates were incubated with B87 (1 mg in 100 mL of BSA-PBS) for 2 h at room temperature. After washing, the B87 filters were incubated with anti-mouse–HRPO (1:1000 dilution in BSA-PBS, 1 h, room temperature). The filters were washed three times and incubated with TMB substrate. After 1 h the filters were washed with distilled water and allowed to air dry overnight. Positive clones were picked from the plates and amplified for sequencing. Dideoxynucleotide sequencing was performed by using the Sequenase 2.0 system according to the manufacturer’s instructions.

MODELING OF PSA
A homology model of PSA was generated with INSIGHT II molecular modeling package (Biosym Technologies, San Diego, CA). The model was prepared by initially aligning the human PSA sequence against those of both porcine kallikrein (59% sequence identity) and rat tonin (54% sequence identity) by using structural alignment routines in the SEQSEE software [33] package (Biotools, Edmonton, AB, Canada). On the basis of these two alignments and on the quality of the available structural data, the x-ray structure of rat tonin (Brookhaven Protein Databank accession code: ITON) was selected as the three-dimensional template for homology modeling the PSA structure. By using the sequence alignment produced by SEQSEE, the amino acid sequence of PSA was then substituted into that of tonin by using the Biopolymer module of INSIGHT II. The model was subjected to a brief period of conjugate gradient energy minimization to reduce unfavorable van der Waals contacts and torsional strain.

Results
ANALYSIS OF PSA–ACT COMPLEX
An immunoassay, shown in Fig. 3, that is specific for the PSA–ACT complex was developed by using mAbs B80 or B87 immobilized on microtiter plates and rabbit anti-ACT polyclonal antibody. Goat anti-rabbit antibody conjugated with HRPO was used as the signal-generating antibody. Free PSA or ACT was not detected in this assay format. Goat anti-rabbit-HRPO conjugate and rabbit anti-ACT antibody did not bind to immobilized mAbs B80 or B87 (data not shown). Twofold dilutions of PSA-ACT from 500 μg/L PSA were made (the concentration of analyte is expressed in μg/L complexed PSA). The dose–response curves were linear up to 50 μg/L and saturated at ~500 μg/L PSA-ACT complex. As little as 20 μg/L of complexed PSA could be detected in this unoptimized assay. Because both antibodies recognize the complex, we can conclude that both epitopes are not on or near the region that binds to ACT. Another experiment directly compared a fixed sample of PSA with and without ACT in ratios of 1:0.5, 1:1, and 1:5. Here again no differences were observed in the PSA values obtained, further confirming the PSA specificity of the mAbs.
EPITOPE SCANNING WITH PSA-DERIVED HEXAPEPTIDES

To further identify the PSA epitopes bound by the two mAbs, we synthesized all the overlapping hexapeptides derived from the linear amino acid sequence on PSA. Fig. 4 shows the results of the screening of PSA sequence-derived hexapeptides with bsmAb B80×HRPO. The PSA peptide number represents the position of the first amino acid in the hexapeptide relative to the PSA amino acid sequence. The data shown was not background subtracted and illustrates the low background signal from the hexapeptides, which do not bind to the bsmAb. In contrast, the monospecific B80 antibody binding measured by a goat antimouse-HRPO or biotinyl B80 along with streptavidin-HRPO gave high backgrounds and numerous peptides identified as (false) positives due to the several complex secondary polyclonal reagents used. Four potential epitopes, exhibiting strong bsmAb binding, were identified by this method: peptide 21–27 (RGRAVC), peptide 30–36 (VLVPQ), peptide 53–59 (RHSLFH), and peptide 195–201 (PLVCNG). A few other peptides show moderate reactivity. No significant binding to PSA-derived hexapeptides was seen for B87 in this assay.

AFFINITY SELECTION OF B80- AND B87-SPECIFIC PEPTIDES FROM A PHAGE PEPTIDE DISPLAY LIBRARY

A phage decapeptide display library [32] was used (4.4 × 10^11 particles in round 1) to identify peptides that would specifically bind to B80 or B87 and mimic PSA epitopes. The Sepharose beads with immobilized antibody were used for the affinity selection, and the elution conditions were the same as those used for the affinity purification of PSA. This column, with 133 pmol of immobilized antibody, was able to bind 40 pmol of PSA without saturating (molar ratio of antibody to PSA of 3.3 to 1). The molar ratio of antibody to phage was ~70 to 1. The phage was eluted by incubating resin-bound phage with PSA and acid and separated by centrifugation, providing a yield of 3.1 × 10^5 and 5 × 10^4 particles, respectively. The molar ratio of the immobilized antibody to the PSA used to elute the phage was ~2.7 to 1. Functionally, this ratio would be much closer, since not all antibody combining sites would be available for PSA binding because CNBr-based coupling is a random linkage of macromolecules to the solid phase. In addition, the affinity of the mAbs for PSA is high (i.e., Kd ~2 × 10^-10 mol/L). The molar ratio of PSA used to elute phage was 25 to 1. The ligand eluate was then amplified and reselected (2 × 10^9 particles as...
input in round 2) with ligand and acid to generate $2.3 \times 10^4$ and $7 \times 10^2$ particles, respectively. We chose to elute the phage particles from immobilized antibody with ligand (PSA) and acid to select for PSA-specific sequences [32]. After the second round, the yield increased by 140-fold for the ligand elution, whereas a threefold increase was seen with the acid elution, suggesting that antibody-binding phage had been selected. The ligand eluate gave the highest enrichment, indicating that acid eluate was less specific. Enrichment was calculated as the total yield of phage from the affinity selection elution from round 2 divided by total yield of phage from the affinity selection elution from round 1.

To select sequences that were specific to B80 or B87, an immunoblotting assay was performed on nitrocellulose lifts of colonies of cells infected with affinity-selected phage. The fraction obtained by eluting with acid in both rounds of selection did not yield any antibody-specific phage clones. B80-positive clones were seen for both eluants with ligand in the first round. B87-positive clones were only found in the eluant, which was eluted with acid in the first round and ligand in the second round.

**Table 2. Number of positive clones identified by immunoblotting of phage-infected colonies.**

<table>
<thead>
<tr>
<th>Eluate</th>
<th>Round 1</th>
<th>Round 2</th>
<th>B80-positive colonies</th>
<th>B87-positive colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no./total</td>
<td>%</td>
<td>no./total</td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>0/1500</td>
<td>0</td>
<td>49/1500</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>0/1500</td>
<td>0</td>
<td>28/1508</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>238/983</td>
<td>24</td>
<td>7/209</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>0/1500</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Four eluates are generated after two rounds of panning.

**Table 3. B80- and B87-specific peptide insert sequences identified by affinity selection of a bacteriophage peptide display library.**

<table>
<thead>
<tr>
<th>B80 sequences</th>
<th>B87 sequences</th>
<th>Cross-reactive sequences</th>
<th>PSA sequence 45-60</th>
</tr>
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<tbody>
<tr>
<td>WLGRPSRSGG (3)*</td>
<td>SWWGAASSPW (3)</td>
<td>AFHTGRIAA (3)</td>
<td>NKSVLLGRHSLFHPE</td>
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<tr>
<td>TLGRPSNLGG (2)</td>
<td>SRWRRSSSGW (4)</td>
<td>DVRAFWWGR (5)</td>
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<tr>
<td>TLGRPSNLGG (1)</td>
<td>SRWRRSSNPR (4)</td>
<td>ADEAFHTGR (4)</td>
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<tr>
<td>ANHTRGRPLV (3)</td>
<td>SQWRRRSSGT (4)</td>
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<tr>
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<td>SQVRRRSSPG (4)</td>
<td>DypsFWWGR (3)</td>
<td></td>
</tr>
<tr>
<td>SCLFYNTCV (3)</td>
<td>SOWVRRSSPG (5)</td>
<td>LVHAFHTGA (3)</td>
<td></td>
</tr>
<tr>
<td>NEWSDEENGQ (2)</td>
<td>RFRQRATIAF (1)</td>
<td>SWWGAASSPW (1)</td>
<td></td>
</tr>
</tbody>
</table>

* Frequency of appearance of each sequence from among those sequenced.

Underlined sequences are consensus sequences; these indicate motifs that the selected sequences have in common.
software [33]. Any homologous pattern less than three amino acids was considered insignificant. Multiple alignments were performed with the input sequences by using SEQSEE with the rbo.wt matrix with gap penalties of 10 and extension penalties of 2. In the case of the B80 binding peptides, those sequences that clustered together as being >40% identical (i.e., the top four sequences in Table 3) were aligned separately with SEQSEE. Those residues displaying >70% sequence conservation from this set were used to form a “consensus” sequence. Thus for the B80 set, the consensus sequence would be TLGRPS**G (where * is any amino acid). In the B87 set, a 70% sequence identity threshold was also used. These criteria identified the consensus sequence S*W**RRS. For the sake of simplification, only conserved contiguous residues were underlined in Table 3. A series of alignments was performed with the eight sequences in the B87 column (Table 3) against the PSA sequence (PIR accession A26757) with SEQSEE. The same default scoring matrix and gap penalties were used. The only sequence that yielded any reasonable score was at the N terminus (WECKHSQPW) of PSA. This particular sequence still required the introduction of two gaps and only two of the most highly conserved residues were matched. By any criteria, this is a poor match. Typically over a stretch of 10 residues, one should expect to find better than 60% sequence identity (with no gaps or breaks) if the match is significant. The nonspecific or cross-reactive sequences (Table 3) appear to be reactive to both mouse antibodies B80 and B87 and may be motifs capable of binding the constant domains of IgG1. This, however, requires further study.

**COMPUTER MODEL OF PSA**

A structural model of the molecule, if not available from x-ray crystallography, can be generated by using proteins of known structure that have a high degree of homology with the antigen. The model can assist in locating highly exposed surface regions and illustrate the spatial relations between various binding or catalytic sites and antibody epitopes.

Figure 5 shows the computer-generated homology model of PSA. The catalytic triad for this serine protease-like molecule (His-41, Asp-96, and Ser-71) is shown in white and the location of the ACT binding site, based on what is known about the binding of ACT to chymotrypsin, is indicated by the shaded residues. The B80 epitope identified by hexapeptide scanning and phage affinity selection, residues 53–60, is indicated in yellow. It is seen to be on an exposed region of the molecule well away from the ACT binding site. An epitope for B87 was not identified in this study and is likely in an exposed region that is not masked by the binding of ACT or B80 to PSA.

Computer models of PSA have been described by Vijnen [34] and Bridon and Dowell [35]. The three-dimensional structure of PSA and glandular kallikrein were modeled on the basis of pH1. Loops unique to PSA were modeled by searching from a database and the structure was refined by energy minimization and molecular dynamics. The resulting model closely resembles the PSA model generated in this study (Fig. 5). From the location of the binding site of ACT it is apparent that most of the molecule is still exposed and available for binding to antibodies when ACT is complexed to PSA. The B80 epitope (shown in yellow in Fig. 5), located in this study, is on a highly exposed region near the N terminus, well away from the ACT binding site. The exact location of the B87 epitope was not determined but the possible epitopes could be limited to exposed regions that are unique to PSA and that do not overlap with the B80 epitope or the ACT binding site.

**Discussion**

In this study we attempt to address the issue of immunospecificity of anti-PSA antibodies at the molecular level by characterizing the epitopes recognized by two new mAbs. At least five PSA isomers have been identified with pls ranging from 6.8 to 7.5. This heterogeneity is believed to be caused by differing sialic acid content of the carbohydrate side chains and not by changes in the amino acid sequence [9]. All of this variability means that antibodies directed against specific epitopes may recognize some forms of PSA and not others. There may be epitopes on PSA that are especially sensitive to the conformational changes that occur when the enzyme is converted from precursor to zymogen to enzyme to inactive enzyme. Antibodies directed against conformationally sensitive epitopes may only recognize one of these forms of PSA.

Two anti-PSA mAbs designated B80 and B87 were used in this study. The B80 hybridoma cell line was fused in our laboratory with an anti-HRPO cell line to produce a hybrid hybridoma or quadroma secreting a bispecific mAb with one binding site directed against PSA and the other against HRPO [31]. This useful reagent eliminates the need for biotinylated or secondary antibody-based detection methods and provides us with a probe potentially having the highest specific activity of HRPO-generated signal. Further, this bispecific mAb probe eliminates the high background often created by the secondary polyclonal antibody–enzyme conjugates used in conventional immunoassays.
B80 and B87 were used to measure PSA in a sandwich immunoassay, indicating that they recognize two distinct and nonoverlapping epitopes. The PSA used to calibrate this assay was obtained commercially as 99% pure protein in BSA buffer to ensure that PSA-ACT complex was not present in this calibrator. The ability of these antibodies to bind to PSA simultaneously indicates that the epitopes are nonoverlapping and far enough apart to avoid steric hindrance. Both of these antibodies fail to form a homosandwich, indicating that each antibody recognizes one unique and nonrepeating epitope. This result is not unexpected considering the relatively small size of PSA. Another recent study [36] also demonstrates by Western blot analysis that the mAb B80 binds most consistently compared with other commercially available monoclonals studied not only to free PSA and PSA-ACT but also to PSA complexed with AMG.

The specificity of the antibodies was determined by measuring the degree of cross-reactivity with pK1, which shows a 59.6% amino acid sequence homology with PSA. The fact that neither B80 nor B87 cross-reacts with this related protease shows that both antibodies recognize sequences on the molecule that are not common to pK1 and PSA. Since we can also assume that the epitope resides on an exposed surface structure and is not hidden in the molecule, the number of possible epitopes is greatly reduced. Further studies on cross-reactivity with hK2 (84% homology to PSA) is needed to establish the unique specificity of these mAbs.

Sequential or linear epitopes are continuous strands of residues present on the surface of a protein. Discontinuous or nonlinear epitopes are sequences brought into proximity on the surface of the molecule. Antibodies to discontinuous epitopes may react weakly to subregions of the epitope made up of a few residues in a linear sequence. Linear epitopes can be identified and located by epitope scanning, a technique first introduced by Geyson et al. [37]. It is based on the principle that linear epitopes can be mimicked by peptides with as few as six residues. Some of these peptides represent actual sequences found on the antigen and indicate the location of the epitope on the protein molecule. Others mimic the conformation and binding characteristics of the epitope and can bind specifically to the antibody. These peptides are referred to as mimotopes. Hexapeptides representing all possible overlapping amino acid sequences of PSA were synthesized on a solid-phase pin and tested for binding to B80 and B87 in an ELISA format. The hexapeptide scan identified four possible locations of the B80 epitope. Two of these peptides at residues 30–36 and 195–201 are found in the interior of the molecule (on the basis of a computer-generated model of PSA [34]), and are hence not likely candidates for the epitope. The remaining two sequences at 21–27 and 53–59 are adjacent to one another on the surface of the molecule. This technique of epitope scanning, which can identify linear epitopes that can be defined by a short linear peptide, failed to locate the B87 epitope. It is likely that B87 recognizes a conformational epitope that cannot be mimicked by a short peptide based on the linear sequence of PSA. It is also possible that the epitope for B87 may be a linear epitope that is >6 residues. A short peptide representing a part of this epitope may not be able to bind to the antibody paratope. Further, differences in conformation caused by interactions with neighboring residues that are not directly involved in binding may be critical to binding. A short peptide will not be able to mimic this conformation and thus may not bind to the antibody.

Bacteriophage peptide display libraries can be used to affinity-select peptides that specifically bind to a given antigen or antibody [38–40]. The major advantage of using recombinant peptide libraries is that a huge diversity of conformational structures can be created and screened within a single library. By this approach, a bacteriophage display library [32] was used to identify sequences that bind specifically to B80 or B87.

Among the phage sequences identified (Table 3), one consensus sequence is homologous to a linear sequence seen on PSA and identified as a possible epitope by epitope scanning. The fact that two different techniques independently identified the same region on PSA as the B80 epitope is strong evidence that the epitope involves residues 51–54 (GRHS). The other antibody-specific sequences (Table 3) may represent mimotopes, peptides that mimic the conformation of the epitope. The other remaining sequences listed in the third column of Table 3 are peptides that were cross-reactive with B80 or B87. This consensus sequence has been observed in other unrelated affinity selection experiments and may represent cross-reactive binding to constant regions of antibodies. The other potential PSA mimotopes identified by this method are currently being evaluated.

In conclusion, the epitopes of two high-affinity anti-PSA mAbs, B80 and B87, were studied and well characterized. They bind both free PSA and PSA complexed with ACT, and do not cross-react with pK1 or hK2. To locate the epitopes, overlapping heptapeptides representing the entire PSA amino acid sequence were synthesized and screened in an immunoassay for binding with the two mAbs. B80 showed specific binding to several peptides. B87 did not show binding to a specific peptide, suggesting that the B87 epitope may be nonlinear. A bacteriophage peptide display library was used to select for peptide sequences that bind specifically to B80 and B87. Several consensus sequences were identified with this technique. One peptide sequence was homologous to a linear sequence on PSA, which was also identified as a possible B80 epitope by PSA hexapeptide scanning.

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References