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Characterization of prostate-specific antigen binding peptides selected by phage display technology

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Prostate-specific antigen (PSA) is an important marker for the diagnosis and management of prostate cancer. Free PSA has been shown to be more extensively cleaved in sera from benign prostatic hyperplasia patients than in sera from prostate cancer patients. Moreover, the presence of enzymatically activatable PSA was characterized previously in sera from patients with prostate cancer by the use of the specific anti-free PSA monoclonal antibody (mAb) 5D3D11. As an attempt to obtain ligands for the specific recognition of different PSA forms including active PSA, phage-displayed linear and cyclic peptide libraries were screened with PSA coated directly into microplate wells or presented by two different anti-total PSA mAbs. Four different phage clones were selected for their ability to recognize PSA and the inserted peptides were produced as synthetic peptides. These peptides were found to capture and to detect specifically free PSA, even in complex biological media such as sera or tumour cell culture supernatants. Alanine scanning of peptide sequences showed the involvement of aromatic and hydrophobic residues in the interaction of the peptides with PSA whereas Spotscan analysis of overlapping peptides covering the PSA sequence identified a peptide binding to the kallikrein loop at residues 82–87, suggesting that the peptides could recognize a non-clipped form of PSA. Moreover, the PSA-specific peptides enhance the enzymatic activity of PSA immobilized into microplate wells whereas the capture of PSA by the peptides inhibited totally its enzymatic activity while the peptide binding to PSA had no effect in solution. These PSA-specific peptides could be potential tools for the recognition of PSA forms more specifically associated to prostate cancer. Copyright © 2005 John Wiley & Sons, Ltd.

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INTRODUCTION

Prostate-specific antigen (PSA), a 237-amino acid protein (33 kDa) that belongs to the kallikrein serine-protease family (Lundwall and Lilja, 1987) is currently an important serum marker for the diagnosis and follow-up of prostate cancer. In prostate diseases like cancer, benign hyperplasia (BPH) or prostatitis, PSA which is secreted from the prostatic glandular epithelium in the seminal fluid enters into the circulation, leading to increased PSA concentrations in the serum (Stamey *et al.*, 1987). However, the presence of several molecular forms has been reported in different studies. In seminal fluid, the majority of PSA is composed of enzymatically active free PSA whereas the remaining PSA consists of either internally cleaved PSA at the C-terminal of R85, K145 and K182 or complexed to protein C inhibitor (Christensson and Lilja, 1994). In blood, the majority of PSA molecules is complexed with protease inhibitors which are present in serum including α1-antichymotrypsin (ACT) and α2-macroglobulin (A2M) whereas a part remains as a free uncomplexed form despite the presence of a large excess of inhibitors capable of forming complexes with PSA (Lilja *et al.*, 1991; Zhu *et al.*, 2003). The molecular nature of free PSA has not been yet fully explained. The presence of proforms of the protein and internal cleavages, for example at K145-K146 or K182-S183, are two commonly proposed explanations of free PSA in the circulation (Mikolajczyk *et al.*, 2001). However, 1–10% of free active PSA, in serum from prostate cancer patients, has been recently detected (Niemela *et al.*, 2002; Wu *et al.*, 2004b).

In previous studies, cleaved PSA forms have been shown to be more abundant in the sera of BPH patients than in those of prostate cancer patients (Charrier *et al.*, 1999, 2001) whereas a subfraction of free PSA called intact PSA was characterized as closely associated with prostate cancer (Steuber *et al.*, 2002). Thus, instead of measuring crude free PSA, selective determination of specific PSA subforms would be useful to discriminate benign from malignant prostatic diseases.

With the aim to define more specific tools for such discrimination, we described the properties of two anti-free PSA 5D3D11 and 6C8D8 monoclonal antibodies

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Abbreviations used: PSA, prostate-specific antigen; BPH, benign prostatic hyperplasia; ACT, α1-antichymotrypsin; A2M, α2-macroglobulin; mAb, monoclonal antibody; BSA, bovine serum albumin; OD, optical density.

(mAbs) (Michel *et al.*, 1999, 2005) and showed that the specificity of 5D3D11 was restricted to both active and activatable PSA whereas 6C8D8 recognized all free PSA including intact PSA, proforms and internally cleaved PSA. Using 5D3D11 for capturing PSA in serum of prostate cancer patients, we identified the presence of a mature inactive PSA form which can be activated into active PSA by use of high saline concentration or capture by an anti-total PSA mAb capable of enhancing PSA activity (Michel *et al.*, 2005). Moreover, according to PSA models built by comparative modelling with the crystal structure of horse prostate kallikrein described previously (Carvalho *et al.*, 2002), we assumed that PSA present in serum could correspond to mature intact PSA with closed conformation of the kallikrein loop. We also described the characteristics of 16H9A12, another anti-PSA mAb, the epitope of which was shown to be located on the kallikrein loop (Michel *et al.*, 2001a). However, other studies described cyclic peptides that bind specifically to non-clipped form of mature PSA and enhance its enzymatic activity (Wu *et al.*, 2000, 2004b). The authors suggested that these peptides could interact near the active site of the enzyme.

With the aim to further understand the regulatory mechanism of the PSA activity in serum at the molecular level, we also selected such peptides using both phage-display linear dodecapeptide library and phage-display cyclic heptapeptide library with PSA either immobilized into microplate wells or presented by two different anti-PSA monoclonal antibodies. We report here the characterization of the different selected peptides and their specificity for capturing and detecting PSA. Their effect on the PSA enzymatic activity was studied under different experimental conditions and PSA presentations and the localization of the binding site of two of them on the kallikrein loop was determined.

MATERIALS AND METHODS

PSA and anti-PSA monoclonal antibodies

Human seminal PSA (P117-7), human serum PSA-ACT (P192-3) and human serum ACT (P159-5) were supplied by SCIPAC (Sittingbourne, UK). Anti-total PSA mAbs 11E5C6, 8G8F5, 5D5A5 and 16H9A12 were produced by bioMérieux (Marcy l'Etoile, France) and were described previously (Michel *et al.*, 1999, 2001a, 2001b, 2005).

Phage display peptide libraries

The Ph.D.-12™ and Ph.D.-C7C™ phage-display peptide libraries were obtained from New England BioLabs Inc. (Beverly, MA). Ph.D.-12™ is a combinatorial library of random peptide 12-mers already described (Jolivet-Reynaud *et al.*, 2004). Ph.D.-C7C™ is a combinatorial peptide 7-mers flanked by a pair of cysteine residues and also fused to the minor coat protein (pIII) of M13 phage. Under non-reducing conditions, the cysteines will spontaneously form a disulphide cross-link, resulting in phage display of cyclized peptides. The disulphide-constrained peptide library is expressed at the N-terminus end of PIII with the cysteine preceded by alanine and the second cysteine followed by a short spacer (Gly-Gly-Gly-Ser).

Peptide library screening with microplate bound PSA (Protocol 1)

The different screenings were carried out according to the instruction of the manufacturer with some modifications. Briefly, wells of Maxisorb plates (Nunc, Denmark) was coated with 150 µl of 0.1 M $NaHCO_3$ (pH 8.6) containing 15 µg of PSA and incubated overnight at 4°C. Wells were then washed six times with 50 mM Tris, 150 mM NaCl, pH 7.5 (TBS) containing 0.1% Tween-20 (TBS-T), filled with 250 µl of blocking buffer (0.1 M $NaHCO_3$ pH 8.6, 5 mg/ml BSA and 0.02% NaN_3) and incubated for 1 h at 4°C. In the first round of biopanning, 1.5×10^{11} phages from the initial library in 100 µl of TBS-T were incubated with the well-bound ligand for 45 min at room temperature under rocking condition. After repetitive washes with TBS-T, the bound phages were eluted from wells with 100 µl of elution buffer (0.2 M Glycine-HCl pH 2.2, 0.1N HCl, 1 mg/ml BSA). After neutralization with 60 µl of 1 M Tris-HCl pH 9.1, eluted phages were amplified by infecting 20 ml of a 1:100 dilution of an overnight culture of *Escherichia coli* ER2738 (recA+ strain cells). The culture was incubated for 4.5 h at 37°C with vigorous shaking. The supernatants were obtained and precipitated with PEG/NaCl as previously described (Scott and Smith, 1990).

In the second and third rounds of biopanning, 1.5×10^{11} amplified phages from the preceding round were incubated overnight at 4°C with a solution of PSA or PSA-ACT as described above. The procedures were identical to the first round except for the washing steps which were carried out with TBS-T 0.2% and TBS-T 0.5% for the second and third biopanning, respectively.

The phages from the third biopanning eluate were cloned and amplified for DNA sequencing and immunoanalysis. The nucleotide sequence of the gene III insert was determined as described previously (Jolivet-Reynaud *et al.*, 2004). The amino acid sequence of the insert was deduced from the nucleotide sequence.

Peptide library screenings with anti-PSA mAb-bound PSA (Protocol 2)

Anti-total PSA mAbs 11E5C6 and biotinylated 8G8F5 were immobilized into microwells as described previously for screening using mAbs as ligands (Jolivet-Reynaud *et al.*, 2004). Then, PSA (100 µg/ml) in 150 µl of TBS-T containing 1 mg/ml of BSA was incubated in mAb-coated wells overnight at 4°C. The subsequent steps of the screenings were as described above.

Recombinant phagemid construction

The pCANTAB 5E phagemid vector (Amersham) was modified first, by removing the E-tag sequence. The vector was then dephosphorylated before inserting either

peptide 2 or peptide 7 sequences at the 5′ end of the gene encoding PIII.

One hundred picomoles of sense and reverse 5′ phosphorylated oligonucleotides, corresponding to the two peptide sequences with added respective 5′ *Sfi*I and 3′ *Not*I cohesive ends, were hybridized according to standard procedures; 100 ng of the resulting products were ligated overnight at 16°C into 100 ng of the pCANTAB phagemid vector with T4 DNA ligase (Roche Diagnostics). After transformation in *E. coli* XL-1 blue strain competent cells by heat shock, the recombinant phagemids were then selected on plates of LB containing 100 μg/ml ampicillin and produced by miniprep (Qiagen protocol). The resulting constructs were checked by DNA sequencing analysis using the pCANTAB5-S1C primer: 5′CAACGTGAAAAAATTATTATTCGC3′.

Single alanine substitutions were introduced at the 12 amino acid positions of the inserted peptides using the QuickChange Site directed mutagenesis kit (Stratagene, Amsterdam, NL) with subsequent verification by DNA sequencing.

Expression of peptide mutants at the surface of recombinant phages

Three hundred microliters of $CaCl_2$ competent *E. coli* TG1 cells, prepared according to standard protocols, were transformed by heat shock, with 200 ng each of the different pCANTAB constructs. Following addition of one transformed cell (selected on LB Agar containing 100 μg/ml ampicillin) to 10 ml of 2 × YT medium containing 100 μg/ml ampicillin and 2% glucose (2 × YT-AG according to Amersham protocols), 1.5 ml of the transformed cell culture were grown in 50 ml of 2 × YT-AG medium with vigorous shacking at 37°C until 0.5 optical density (OD) at 600 nm was reached. Five milliliters were diluted in 50 ml with fresh medium of 2 × YT-AG and superinfected with M13K07 helper phage (4.10^{10} pfu) (New England Biolabs). After 2 h of incubation at 37°C, the medium was removed by centrifugation and the bacterial pellet was resuspended in 30 ml of 2 × YT containing 100 μg/ml ampicillin and 50 μg/ml kanamycin (2 × YT-AK according to Amersham protocols). Phage particles were obtained in the supernatant by overnight incubation at 30°C. Culture supernatants were precipitated with 20% polyethylene glycol 8000 and 2.5 M NaCl. After centrifugation, the phages were purified and the final pellets were diluted at 10^{13} phages/ml in sterilized TBS.

ELISA with supernatant phages from Protocol 1

Rows of ELISA plate wells were coated with 100 μl of PSA, PSA-ACT or ACT at the final concentration of 340 nM, in PBS. The plates were incubated overnight at 4°C, washed with PBS containing 0.05% Tween 20 (PBS-T0.05) and then blocked with PBS containing 0.5% of gelatin. After 2 h incubation at 37°C and three washes with PBS-T0.05, four-fold serial dilutions of each phage clone were added to each well of the microtiter plate in a final volume of 100 μl of PBS-T0.05 containing 0.1% of gelatin. The plates were incubated for 1 h at 37°C and were washed three times with PBS containing 0.5% Tween 20 (PBS-T0.5) as above. The bound phages were detected in a sandwich assay using a horseradish peroxidase-conjugated anti-M13 mAb (Amersham) at a 1:10 000 dilution in PBS-T0.5-gelatin 0.1%. After 1 h of incubation at 37°C and three washes with PBS-T0.5, the plates were developed using a commercial colour kit (bioMérieux) containing orthophenylene diamine and hydrogen peroxide. After 10 min of incubation, the plates were read at 492 nm with an ELISA plate reader (MRX-II, Dynex). For each phage clone dilution, the results were expressed as the difference between the value obtained with PSA or PSA-ACT complex and the value obtained with protein ACT. The results were then confirmed by testing optimal dilutions of the reactive clones in triplicate.

ELISA with supernatant phages from Protocol 2

Rows of ELISA plate wells were coated with mAbs as described above. PSA, PSA-ACT or ACT were added at the final concentration of 68 nM. The following steps were the same as described above for Protocol 1.

Sequence analysis

The amino acid sequences of peptides were compared by use of the Mac Vector, Ver. 4.5 software (Kodak). Basically, the regions of highest similarity were detected with the Clustal W programme, which tentatively searches for best local identities.

Spot synthesis of nitrocellulose membrane bound peptides

The simultaneous synthesis of different peptide sequences was performed on a nitrocellulose membrane using 9-fluorenylmethoxycarbonyl amino acid chemistry (Frank and Doring, 1988). Each peptide was generated in nanomolar quantities. Biotinylated peptide reactivity to membrane-bound PSA peptides was analysed by an indirect colorimetric immunoassay as described previously (Jolivet-Reynaud *et al.*, 1998) using alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA). Spots corresponding to reactive peptides produced a positive blue signal. Intensity of spots was expressed as relative intensity on a scale ranging from 0–5.

Peptide synthesis

C-terminus biotinylated peptides were synthesized on an Applied Biosystems (Foster City, CA) automatic synthesizer (model 431A) using resin functionalized with Fmoc-Lys(Bio)OH, fluorenylmethoxycarbonyl and *t*-butyl protecting groups and trifluoroacetic acid deprotection. The peptides were purified and analyzed as described previously (Jolivet-Reynaud *et al.*, 1998).

Standard ELISA

Ninety-six well plates Maxisorp (Nunc, Denmark) were coated with 100 µl/well of PSA, PSA-ACT or ACT at a concentration of 68 nM in PBS. After an overnight incubation at 4°C, the plates were washed three times with PBS containing 0.05% Tween-20 (PBS-T) and blocked for 2 h at 37°C with either PBS containing 2 mg/ml BSA (PBS-BSA) or PBS containing 0.2% gelatin (PBS-G). Following a second wash with PBS-T, 100 µl of biotinylated peptide in PBS-T containing 1 mg/ml of BSA or PBS-T containing 0.05% of gelatin (diluting buffers PBS-T-BSA and PBS-T-G, respectively) were added. After 2 h of incubation at 37°C and a new wash with PBS-T, a 1:5000 dilution of peroxidase-conjugated streptavidin in diluting buffer (Jackson ImmunoResearch Laboratories, West Grove, PA) was added for 1 h at 37°C. The plates were washed once more with PBS-T and developed as described above. The values were expressed as the mean OD of triplicate.

Sandwich ELISA

PSA was captured by synthetic peptides as follows: briefly, biotinylated peptides (10 µg/ml in PBS) were bound to streptavidin-coated plates as described previously (Jolivet-Reynaud *et al.*, 1999). Different concentrations of PSA, PSA-ACT or ACT in diluting buffers were incubated overnight at 4°C. The plates were washed before adding 100 µl of anti-total mAb (1 µg/ml in the diluting buffer) for 2 h at 37°C. After a new wash, peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) was added (1:5000 in diluting buffer). The plates were developed as described above.

PSA-bound to mAbs was detected by synthetic peptides as follows: wells were coated for 2 h at 37°C with 100 µl of different anti-PSA mAbs (10 µg/ml in PBS). The plates were then washed and blocked as described before. Following a new wash, 100 µl of different concentrations of PSA in diluting buffer were added and incubated overnight at 4°C. The plates were washed and 100 µl of the biotinylated peptides in diluting buffers were added for 2 h at 37°C. The following steps were carried out as described above in standard ELISA.

Culture of LNCaP cells

The LNCaP cell line (American Type Culture Collection ATCC CRL-1740) was derived from human metastatic adenocarcinoma of the prostate (Horoszewicz *et al.*, 1983). The culture was carried out in 125 cm^2 flasks maintained at 37°C in a humidified atmosphere of 5% CO_2. Cells were grown in RPMI-1640 medium with phenol red supplemented with 10% foetal bovine serum, 100 U/ml penicillin/streptomycin and 2 mM glutamine until near confluency. The T-PSA concentration was measured by VIDAS.

Determination of the enzymatic activity of PSA in solution

The PSA enzymatic activity was determined by hydrolysing the fluorescent substrate morpholinocarbonyl-Lys-Gly-Ile-Ser-Ser-Gln-Tyr-7-amino-4-(trifluoromethyl) coumarin (Mu-KGISSQY-AFC, Neomps, Strasbourg, France) at the final concentration of 400 µM in either Tris-HCl 50 mM pH 7.5, NaCl 0.15 M containing 2 mg/ml BSA (TBS-BSA), or TBS-BSA containing 1.5 M NaCl (Denmeade *et al.*, 1997). 20 ng of PSA was preincubated with 200 ng of either anti-PSA peptides or mAbs for 30 min at 37°C in reaction buffer (1 mol PSA/150 mol peptide or 1 mol PSA/2 mol mAb) before being added to the substrate. The emission of fluorescence was measured with the Fluoroskan reader (Thermo Life Science, Cergy-Pontoise, France) at 37°C, every minute for 2 h, using an excitation wavelength of 390 nm and an emission wavelength of 510 nm. Enzymatic activity was determined by the slope of the kinetic, and was expressed as fluorescence units*1000/min.

Determination of the enzymatic activity of PSA-coated into microplate wells

ELISA wells of black plate (NUNC maxisorp) were coated overnight at 4°C, with 100 µl of either 2 or 10 µg/ml PSA in TBS (Tris-HCl 50 mM pH 7.5, NaCl 0.15 M) and blocked for 1 h at 37°C with TBS containing 2 mg/ml BSA (TBS-BSA). After 3 washes with TBS-Tween 0.05% (TBS-T), 100 µl of 20 or 100 µg/ml of either peptides or mAbs diluted in TBS-T-BSA were added and incubated for 1 h at 37°C. Following three washes with TBS-T, the wells were incubated for 15 min at 37°C with 100 µl of either TBS-BSA or TBS-BSA containing NaCl 1.5 M. Then, the wells were emptied and 100 µl of the PSA substrate Mu-KGISSQY-AFC, diluted at 400 µM in either TBS-BSA or TBS-BSA containing 1.5 M NaCl, were added. The hydrolysis of the substrate was measured as described above.

Determination of the enzymatic activity of captured PSA

ELISA wells of black plate (NUNC maxisorp) were coated for 2 h, at 37°C, with 100 µl of 10 µg/ml streptavidin in 0.1 M carbonate buffer pH 9.6 and blocked for 2 h at 37°C with TBS (Tris-HCl 50 mM pH 7.5, NaCl 0.15 M) containing 2 mg/ml BSA (TBS-BSA). After three washes with TBS-Tween 0.05% (TBS-T), 100 µl of 10 µg/ml of biotinylated peptides or mAbs diluted in TBS-BSA were added. After 2 h of incubation at 37°C, the wells were washed three times with TBS-T and 100 µl of PSA diluted in TBS-T-BSA were added and incubated overnight at 4°C. Following three new washes with TBS-T, the wells were incubated for 15 min at 37°C with 100 µl of either TBS-BSA or TBS-BSA containing NaCl 1.5 M. Then, the wells were emptied and 100 µl of the PSA substrate Mu-KGISSQY-AFC, diluted at 400 µM in either TBS-BSA or TBS-BSA containing 1.5 M NaCl, were added. The hydrolysis of the substrate was measured as described above.

Accessibility of PSA residues

The three-dimensional structure of the PSA molecule was obtained from the Protein Data Bank (pdb code:

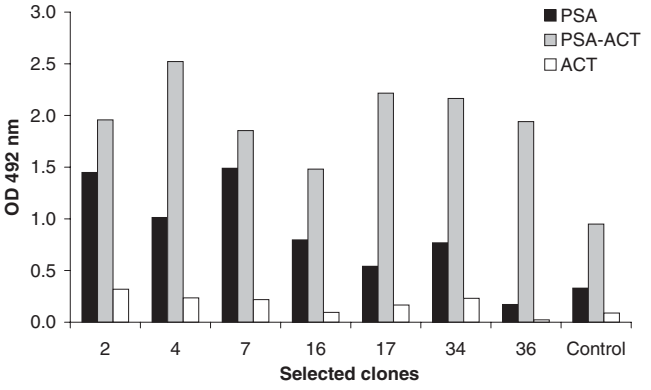

Figure 1. Reactivity of the phage-displayed peptides selected with PSA coated into the microplate wells. The reactivity against PSA, PSA-ACT and ACT was determined by ELISA with 2.5×10^{10} phages/well.

1PFA.PDB). Solvent-accessible surface areas were calculated with a probe radius of 1.4 Å and the estimation of static accessibility was performed according to the method of Lee and Richards (Lee and Richards, 1971). Based on these data, PSA residues were classified into three groups: weak, middle range and high accessibility.

RESULTS

Screening of phage display 12 mer-peptide library with PSA-coated into microplate wells

The dodecapeptide library was screened with PSA coated directly into microtiter wells. After three biopannings, 36 clones were randomly isolated and their DNA was sequenced. The deduced amino acid sequences of the corresponding inserts identified 22 different sequences. When tested in an ELISA test for their reactivity, seven clones gave a positive signal with PSA, PSA-ACT but not with ACT, indicating that the recognition was directed against a region of PSA not covered by ACT (Fig. 1). Interestingly, as shown in Table 1, the peptides contained consensus amino acids.

In order to analyse the contribution of the different residues of the selected phage displayed peptides for binding to PSA, the sequences of peptides 2 and 7 were cloned in

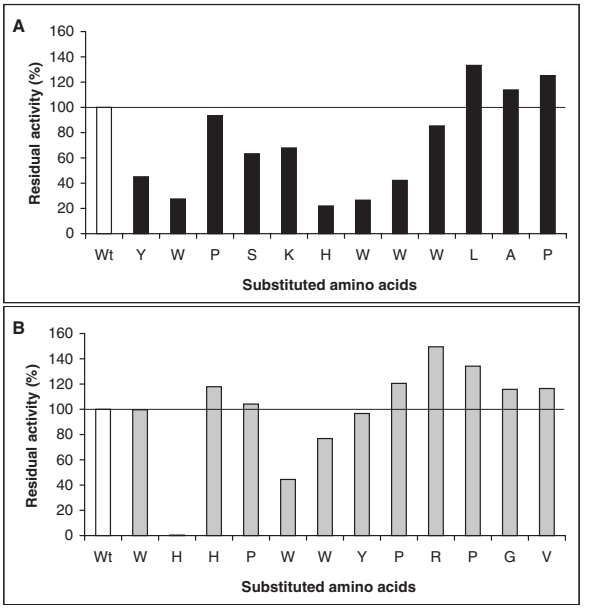

Figure 2. Analysis of critical residues on the phage-displayed peptides by alanine scanning. The different alanine mutants of clone 2 (A) and clone 7 (B), respectively, were tested by ELISA as described in Materials and Methods with 68 nM PSA and 5×10^8 phages/well. The different analogues were indicated with the residue which has been substituted by alanine (except alanine of clone 2 which was substituted by valine). Values are given as the percentage of reactivity of each alanine mutant compared to the reactivity of non-mutated phage clones 2 and 7.

the pCANTAB 5E phagemid vector and single alanine substitutions were introduced at 12 positions of the two peptides. After transformation into *E. coli* TG1 cells, the different analogues were expressed at the surface of the filamentous phage M13. As shown in Figure 2A, the substitution of Y1, W2, H6, W7 and W8 of the peptide 2 by alanine strongly reduced the binding of peptide 2 to PSA, whereas the substitution of S4 and K5 on one hand, and P3 and W9 on the other hand, had moderate or insignificant effects. Interestingly, the substitution of the last three amino acids by alanine increased the reactivities of the mutated peptides. In Figure 2B, the substitution of H2 of peptide 7 abolished totally the reactivity of the mutated peptide to PSA, whereas the substitution of W5 and W6 have only moderate effects. On the contrary, the substitution of R9 and P10 increased considerably the binding of clone 7 to PSA.

Table 1. Amino acids identities between the different reactive motifs selected with PSA coated on the microplate wells

Clones	Sequence														
36					H	**H**	**W**	T	Y	W	L	S	G	T	V	A
34					F	**H**	**W**	P	R	S	W	V	T	W	Q	S
17					S	**H**	**W**	W	**W**	W	D	A	R	G	Y	D
16					W	**H**	**W**	Q	**W**	T	P	W	S	I	Q	P
7					W	**H**	H	P	**W**	W	Y	P	R	P	G	V
4					W	**H**	**W**	H	P	L	S	W	R	Y	S	T
2	Y	W	P	S	K	**H**	**W**	W	**W**	L	A	P				

Identities are indicated in bold and highlighted with grey areas.

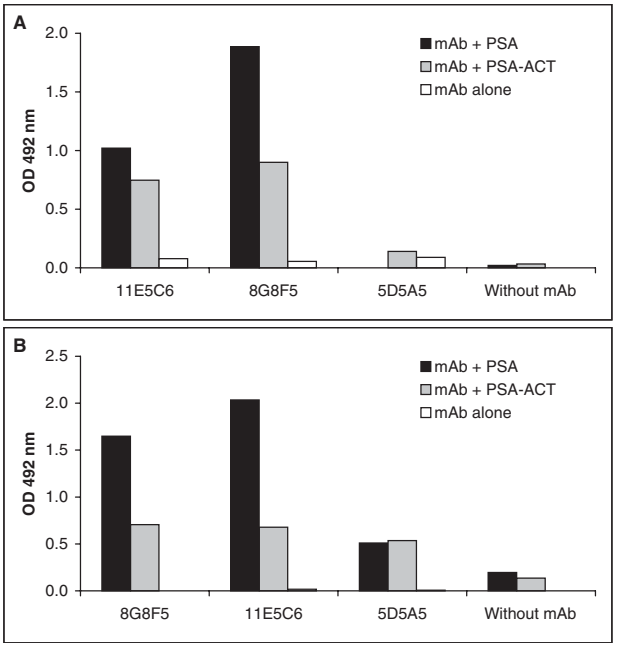

Figure 3. Reactivities of phage clones QYL (A) and PLL (B) against PSA and PSA-ACT (68 nM) each bound to either 11E5C6, 8G8F5 or 5D5A5. The reactivities were determined by ELISA with 5×10^9 phages/well and the reactivities against mAbs alone, PSA or PSA-ACT directly coated into wells were used as negative controls.

Screening of phage-display 12-mer peptide library with PSA bound to anti-PSA mAb 11E5C6

The anti-total PSA mAb 11E5C6 did not have any effect on the enzymatic activity of PSA, indicating that the conformation of PSA was not modified by its binding to 11E5C6 (Michel *et al.*, 2005). Thus, the dodecapeptide library was screened with PSA immobilized into microplate wells coated with 11E5C6. After three biopannings, 36 clones were randomly isolated and their DNA was sequenced. The deduced amino acid sequences of the corresponding inserts identified 22 different sequences. When tested in ELISA, these different clones did not recognize PSA or PSA-ACT coated directly in the wells (data not shown). However, one clone (QYLSPLVTQWEW) obtained five times recognized specifically either PSA or PSA-ACT presented by either 11E5C6 or 8G8F5 but not 5D5A5 (Fig. 3A).

Screening of phages-display 12-mer peptide library with PSA bound to anti-PSA mAb 8G8F5

The binding of the anti-total PSA mAb 8F8G5 to PSA was found to enhance the enzymatic activity of the bound PSA (Michel *et al.*, 2005). The dodecapeptide library was therefore also screened with PSA immobilized into microtiter wells coated with 8G8F5 in order to get active PSA-specific peptides. Since no reactive clones could be obtained with this library, the same protocol was used for screening the Ph.D.-C7C^{TM} phage-display peptide library, a constrained 7-mer peptide library. Twenty-seven different sequences were obtained, but only one clone, obtained nine times, gave a strong positive signal with PSA presented by 8G8F5. As shown in Figure 3B, this clone (CPLLYSWWC) did not react with either PSA, PSA-ACT or 8G8F5 alone. Moreover, CPLLYSWWC reacted also strongly with PSA presented by 11E5C6 whereas its binding to PSA presented by anti-total PSA mAb 5D5A5 was moderate.

Capture of PSA by specific synthetic peptides

The peptides from the Ph.D.12 and Ph.D.C7C libraries are expressed at the N-terminus end of the minor coat protein pIII followed by a short spacer GGGS. In addition, peptides from the Ph.D.C7C library are preceded with an alanine residue. Thus, the different selected mimotopes were produced as synthetic peptides with GGG and biotinylated at the C-terminus end via a lysine. Moreover, both cyclic and linear forms of the PLL peptide were synthesized with alanine at the N-terminus end (Table 2). In preliminary experiments, different anti-PSA mAbs were tested for their ability to detect PSA captured by the peptides. Among the different anti-total PSA mAbs tested, both 8G8F5 and 5D5A5 gave the highest signals of detection whereas the signal given by 11E5C6 was moderate. The anti-free PSA mAb 6C8D8 did not detect any peptide-bound PSA (data not shown). Then, different sandwich ELISA, using each of the selected peptides for capturing PSA, and 5D5A5 or 8G8F5 as detecting antibody, were performed. As shown in Figure 4, the synthetic peptides were all able to capture PSA, but PSA-ACT was not significantly captured by any of the peptides. For each peptide, a threshold of specific detection (mean OD of triplicate with the peptide alone + three standard deviations) allowed to evaluate the limits of PSA concentrations captured by the peptides 2, 7, QYL, PLL_l and PLL_c as 68, 34, 34, 17 and 6.8 nM, respectively.

Table 2. Sequences of the biotinylated synthetic peptides

Name	Sequence															
Peptide 2	Y	W	P	S	K	H	W	W	W	L	A	P	G	G	G	K-biotin
Peptide 7	W	H	H	P	W	W	Y	P	R	P	G	V	G	G	G	K-biotin
Peptide QYL	Q	Y	L	S	P	L	V	T	Q	W	E	W	G	G	G	K-biotin
Peptide PLL_l	A	C	P	L	L	Y	S	W	W	C	G	G	G	K-biotin		
Peptide PLL_c	A	C	P	L	L	Y	S	W	W	C	G	G	G	K-biotin		

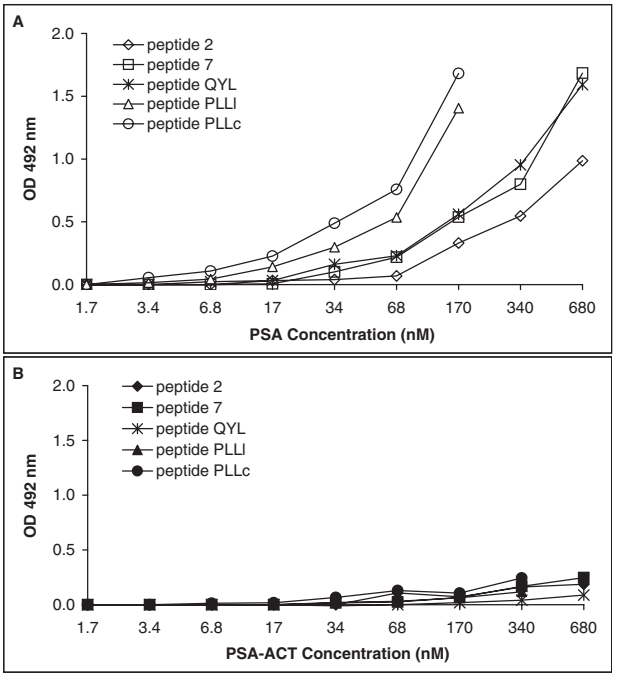

Figure 4. Capture of PSA and PSA-ACT by the synthetic peptides. The capture of different concentrations of PSA (A) and PSA-ACT (B) by the different peptides was measured by ELISA with either 8G8F5 (peptides 2, 7, PLL_l and PLL_c) or 5D5A5 (peptide QYL), as detecting mAb. BSA was added in blocking and diluting buffers for peptides 2 and 7 and gelatin for peptides QYL, PLL_l and PLL_C. For each value, the OD obtained with the buffer alone was subtracted.

The ability of the peptides to recognize specifically PSA in complex biological media was tested by diluting different amounts of PSA in human women sera and LNCaP culture supernatants. In the case of women sera, most of the added PSA was complexed with PSA inhibitors present in human sera. However, a positive signal corresponding to 6.8 nM of captured PSA could be obtained with PLL_l when seminal PSA was added at the final concentration of 340 nM (data not shown).

Different amounts of PSA (2, 5 and 10 μg/ml) were also added to the supernatant of a 1-week culture of LNCaP at final concentrations of 68, 170 and 340 nM, respectively. The amount of secreted PSA in the supernatant (0.5 μg/ml) was not taken into account and was assimilated to the background signal of the supernatant without addition of PSA. As shown in Figure 5, significant signals could be obtained with peptides 2 and 7 and 340 nM of added PSA. However, the signals obtained with PLL_l (PLL_c was not tested) were much higher, even with 68 nM of added PSA.

Detection of PSA with synthetic peptides

Peptides 2, 7 and PLL_l recognized PSA coated directly into the microplate wells whereas the recognition of PSA-ACT was similar to the non-specific recognition of ACT (Fig. 6A). Moreover, peptides QYL and PLL_c did not detect any of the immobilized molecules.

Detection of PSA bound to either 11E5C6, 8G8F5 or 5D5A5 by the different peptides was hampered by background signals due to unspecific binding to the different mAbs alone. With peptides 2 and 7, the background signal was too high to detect a specific recognition of PSA bound to mAbs. On the contrary, PLL_c did not recognize the mAbs but the detection of PSA was not significant (Fig. 6B). However, PSA presented by 11E5C6 and 8G8F5 and detected by PLL_l gave a signal significantly higher than the signal given by the mAbs alone. QYL detected PSA presented by 8G8F5 only.

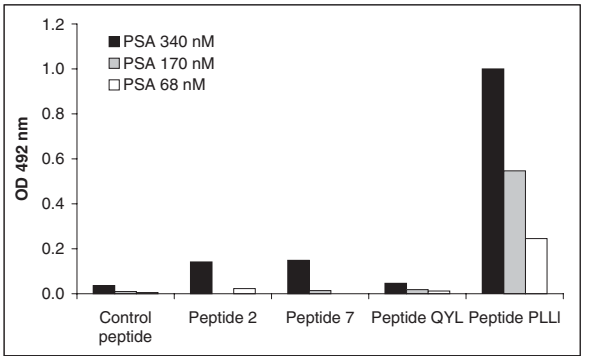

Figure 5. Capture of PSA by the synthetic peptides in LNCaP culture supernatant. PSA was measured by ELISA with 10 μg/ml of biotinylated peptide immobilized into streptavidin-coated wells and 5D5A5 as detecting mAb. Gelatin was added in blocking and diluting buffers. For each value, the OD obtained with the culture supernatant alone was subtracted.

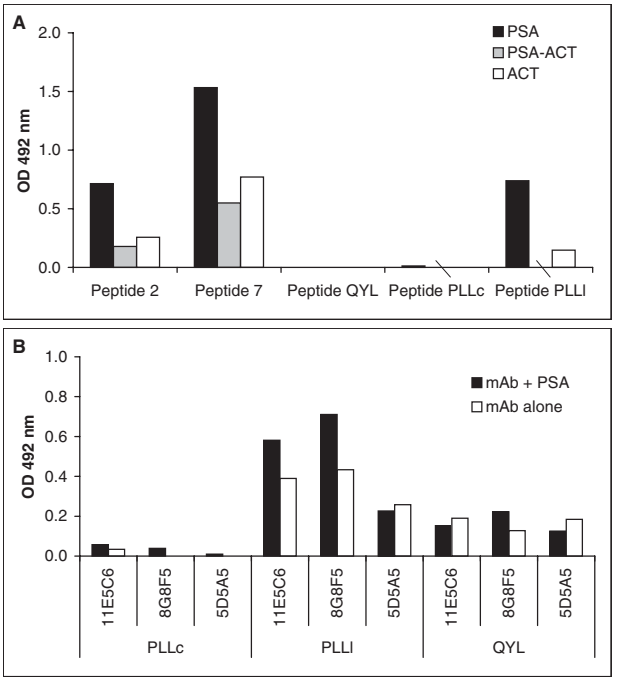

Figure 6. Detection of PSA by the synthetic peptides. (A), PSA, PSA-ACT and ACT coated into microplate wells were detected by peptides 2 and 7 at the final concentration of 50 ng/ml and by peptides QYL, PLL_c and PLL_l at 500 ng/ml. (B), PLL peptides (0.5 μg/ml) detected PSA added to mAbs 11E5C6-, 8G8F5- and 5D5A5-coated wells at the final concentration of 68 nM. Peptide QYL (10 μg/ml) detected PSA added to mAb-coated wells at the final concentration of 340 nM. For each value, the OD obtained with the buffer alone was subtracted. \ for 'not determined'.

Table 3. Comparison of the PSA peptides recognized by peptides 2 and 7

Peptide n°	PSA aa	Sequence													Spot intensity
17	49–57				I	**L**	L	G	*R*	*H*	*S*	*L*	**F**		4
27	79–87		**M**	S	*L*	**L**	**K**	**N**	**R**	**F**	**L**				5
28	82–90					**L**	**K**	**N**	**R**	**F**	**L**	**R**	**P**	**G**	5
32	94–102	*S*	*H*	D	L	M	L	L	*R*	*L*					4

The reactivity of PSA peptides with 20 μg/ml of biotinylated synthetic peptides 2 and 7 was tested as described in Materials and Methods. Amino acid identities and similarities are highlighted with dark and light grey areas, respectively. Bold, italic and normal characters correspond to strong, middle range and weak residue accessibility, respectively.

Localization of the binding site of PSA-specific peptides

In order to localize the binding region of the different PSA-specific peptides on the PSA molecule, overlapping non-peptides offset by three residues and covering the PSA sequence were synthesized on nitrocellulose membrane and tested with the five biotinylated peptides. Peptides QYL, PLL_l and PLL_c did not react with any of the different PSA overlapping peptides. However, peptides 2 and 7 reacted strongly with PSA-peptides 17, 27, 28 and 32, corresponding to PSA amino acids 49–57, 79–87, 82–90 and 94–102, respectively. The other peptides were not recognized significantly by either peptide 2 or peptide 7 (Table 3). PSA-peptides 27 and 28 defined the overlapping minimal sequence LKNRFL (PSA 82–87) composed of amino acids identified as very accessible residues located on the kallikrein loop. In view of the poor or non-existing accessibility of peptides 17 and 32 on the PSA molecule, the reactivity of these two peptides could represent non-specific cross-reactivity caused by partial similarities with overlapping sequence peptides 27 and 28. In order to verify this hypothesis, different mAbs recognizing known epitopes were tested for their ability to inhibit the bindings of peptides 2 and 7 to PSA. As shown in Figure 7, mAb 16H9A12 was the only mAb able to inhibit significantly the binding of the peptides to PSA. This result supports the localization of the PSA-specific peptide binding site on the kallikrein loop since the 16H9A12 epitope has been located on the kallikrein loop at the PSA amino acids 84–88 (Michel *et al.*, 2001a).

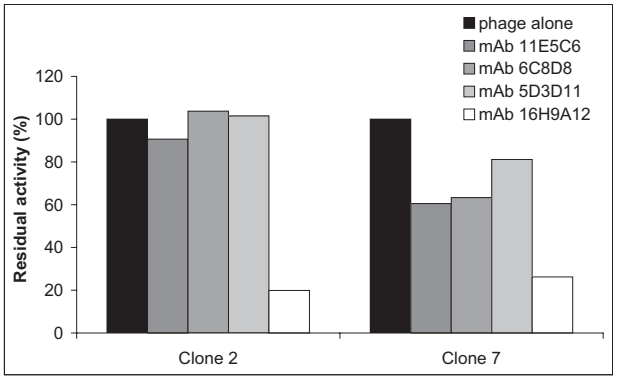

Figure 7. Inhibition of phage clone reactivity by different anti-PSA mAbs. 100 μl of mAb solutions at the final concentration of 10 μg/ml were incubated for 10 min at 37°C with PSA-coated into wells plates, before adding 5×10^9 phages/well.

Modulation of the PSA enzymatic activity

The effect of the different peptides on the PSA enzymatic activity was tested with PSA and compared to the respective effect of mAbs 8G8F5, 6C8D8 and 16H9A12 on PSA under the same conditions. Moreover, different saline conditions (0.15 M and 1.5 M NaCl) were used since the use of high salt concentration has been shown previously to lead to a change of the PSA conformation to an active open form (Michel *et al.*, 2005). When incubated with PSA in solution, the different peptides as well as mAb 16H9A12 did not modify the enzymatic activity of PSA whereas 6C8D8 and 8G8F5 inhibited or enhanced, respectively, the PSA activity as reported before (Michel *et al.*, 1999; Michel *et al.*, 2005) (Fig. 8A).

With PSA immobilized into the microplate wells, the enzymatic activity of 0.2 μg of coated PSA was enhanced by PLL_l only. However, with 1 μg of coated PSA, the enzymatic activity of PSA was significantly enhanced by

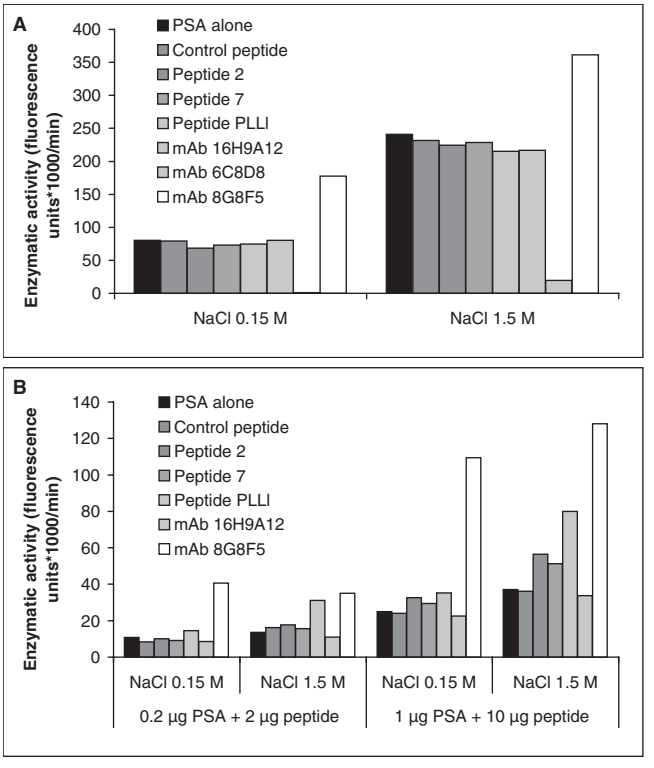

Figure 8. Effect of the synthetic peptides on the enzymatic activity of PSA in solution (A) or coated into microplate wells (B).

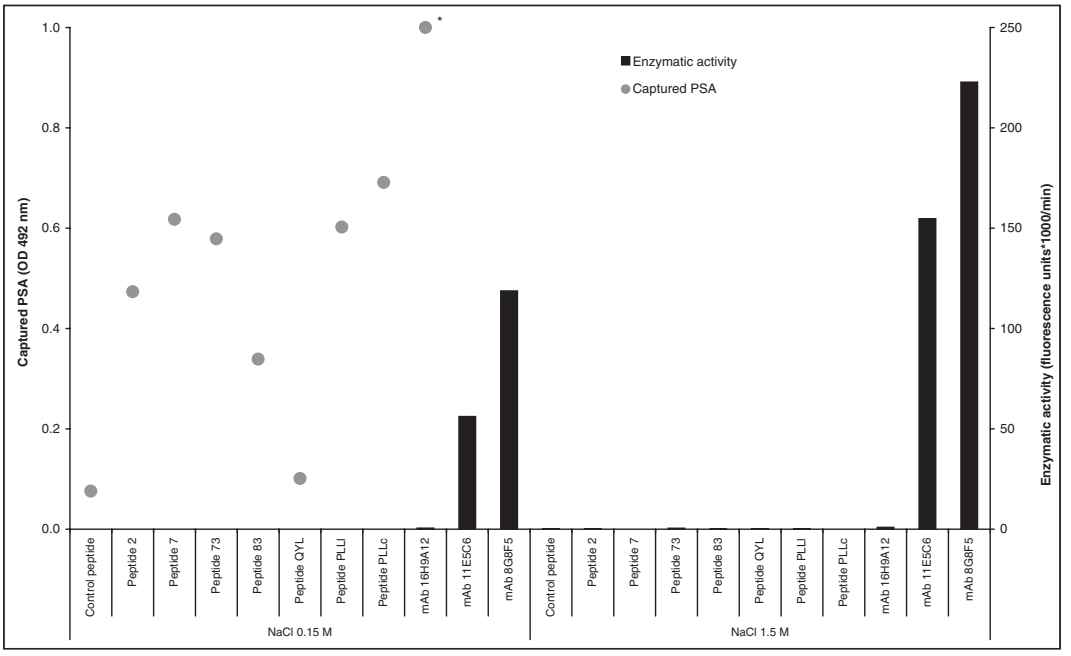

Figure 9. Enzymatic activity of PSA-bound to synthetic peptides or anti-PSA mAbs. The amounts of PSA bound to peptides or mAbs were quantified by ELISA performed under the same experimental conditions, and using 5D5A5 as detecting mAb. *evaluated OD superior to 2.5.

peptides 2, 7 and PLL_l. As expected, the PSA enzymatic activity was strongly enhanced by the binding to 8G8F5. However, mAb 16H9A12 did not have any detectable effect on the PSA activity (Fig. 8B).

The activity of PSA captured by the different peptides bound to microplate wells was also measured and compared with the activity of PSA captured by either mAbs 11E5C6, 16H9A12 or 8G8F5. As shown in Figure 9, no enzymatic activity could be detected with PSA bound to any of the different peptides as well as to mAb 16H9A12 whereas the activity of 8G8F5 was enhanced. However, the dosage of captured PSA by ELISA showed that except for QYL, the other peptides, as well as 16H9A12, captured sufficient amounts of PSA to show enzymatic activity. These results suggest that the capture of PSA by immobilized peptides and mAb 16H9A12 inhibited the enzymatic activity of PSA.

DISCUSSION

The selection of PSA-specific peptides has already been reported by Wu *et al.* using a cyclic peptide library (Wu *et al.*, 2000; Pakkala *et al.*, 2004; Wu *et al.*, 2004a; Wu *et al.*, 2004b). In the present study, using both linear and cyclic libraries, we also selected PSA-specific peptides and showed that the cyclization of the peptide was not essential to obtain modulation of the PSA enzymatic activity. Although the strongest activation was given with the cyclic PLL peptide, linear peptides also had a modulating effect. There was a little difference in the ability of linear and cyclic peptides to capture PSA. PLL_l was the only peptide able to detect PSA whereas PLL_c did not detect PSA either coated onto the plate or bound to mAb.

In previous studies of the immune response to infectious or auto-immune diseases, mimotopes selected from phage-display peptide libraries using mAbs (Jolivet-Reynaud *et al.*, 2001; Jolivet-Reynaud *et al.*, 2004) or cerebrospinal fluid IgG (Jolivet-Reynaud *et al.*, 1999) could be produced as synthetic peptides with the same characteristics of specificity and reactivity. In this study, different selected phage clones recognized both free PSA and PSA-ACT complex. However, the specificity of the corresponding synthetic peptides was restricted to free PSA only. This finding suggests that the interaction of peptides with a specific protein sequence was different from the peptide–paratope interaction.

Using peptides 2, 7, QYL, PLL_l and PLL_c in sandwich assays for capturing PSA, the lowest concentrations of detectable PSA by the anti-PSA 5D5A5 mAb were 2, 1, 1, 0.5 and 0.2 µg/ml of PSA solution, respectively, whereas QYL and PLL detected 10 and 2 µg/ml of PSA captured by anti-PSA mAb 8G8F5, respectively. These two kinds of sandwich assays are not sensitive enough to allow PSA to be quantified in sera from patients with prostatic diseases where the concentration of PSA was superior to 2–4 ng/ml. However, the capture of PSA added in complex media such as sera of women or supernatant of LNCaP culture showed that the recognition of PSA was specific. Moreover, these peptides appeared not to capture or detect hK11, another kallikrein also overexpressed in sera from BPH and prostate cancer patients (unpublished data).

Pakkala *et al.* (2004) have proposed that interactions of their cyclic peptides with PSA were based on the aromatic and hydrophobic features of the amino acid side chains. In this study, a consensus motif, composed of HW-W could be shown between the sequences of the different phage clones selected with PSA coated into plates. Moreover, we also identified hydrophobic and aromatic residues to be critical for the binding of both peptides 2 and 7 to PSA, particularly Y1, W2, H6, W7, W8 for peptide 2 and H2, W5, W6 for

peptide 7. The position of such amino acids on the peptide sequence was also critical since alanine scanning showed that the replacement of W9, in peptide 2 or W1, H3, Y7, in peptide 7, did not modify the respective peptide bindings to PSA. These results suggest the binding of the peptides 2 and 7 via two short stretches of amino acids (YW–HWW and H–WW, respectively). PLL peptides, which contain also aromatic and hydrophobic residues Y–WW, could also interact with PSA via these residues.

Using overlapping peptides covering the PSA sequence, the binding site of peptides 2 and 7 to PSA was located in the middle of the kallikrein loop (L82-L87) at residues shown to be very accessible on the surface of the PSA molecule. The inhibitory effect of mAb 16H9A12 on the peptide binding to PSA is in agreement with this localization since the 16H9A12 epitope was also located on the kallikrein loop. Indeed, the epitope was localized at residues N84-R88 (Michel *et al.*, 2001) and subsequently restricted to the minimal sequence F86-R88 (unpublished data). Without structural studies of the PSA–peptide complex, any hypothesis on the interaction of peptides with PSA at the molecular level is very speculative. However, the substitution of the positively charged R9 by alanine increased the reactivity of the corresponding analogue to 140% of the reactivity of the original peptide 7. This suggests the involvement of at least one of the positively charged residues K, N, R of the kallikrein loop.

The most interesting property of the PSA-specific peptides concerns their modulating effect on the PSA enzymatic activity. Indeed, the peptides did not have any detectable effect in solution. However, they were able to activate the enzymatic activity of PSA coated into microplate, whereas the capture of PSA by the peptides inhibited its enzymatic activity. By comparison, the anti-total PSA mAb 16H9A12 did not activate the enzymatic activity of PSA in solution or coated into microplate. Moreover, the enzymatic activity of 16H9A12-bound PSA was totally inhibited. On the contrary, mAb 8G8F5, an anti-total PSA mAb shown previously to activate the PSA enzymatic activity, was able to activate the activity of PSA under three different experimental conditions (in solution, coated or captured by mAb 8G8F5). Taken together, these results suggest that the binding of either mAb 16H9A12 or peptides to PSA immobilized into microplate wells decrease the flexibility of the kallikrein loop, thereby hampering the accessibility to the enzymatic site of PSA. However, 16H9A12 is an anti-total PSA mAb whereas the reactivity of the peptides is restricted to free PSA. The absence of PSA activation by 16H9A12 suggests that the peptide binding site is closer to the active site of PSA than the 16H9A12 epitope and that it involves amino acids that are critical for the regulatory properties of the kallikrein loop.

The activity of the anti-free PSA mAb 5D3D11 was reported to be restricted to both active and activatable PSA (Michel *et al.*, 2005) in agreement with the previous localization of its epitope on the side of the groove containing the PSA enzymatic site (Michel *et al.*, 1999). Moreover, the PSA-specific peptides identified by Wu *et al.* (2004a) bind only to the non-clipped form of PSA. Since the binding sites of peptides 2 and 7 were found in the present study to overlap the cleavage site R85-F86 of PSA, these peptides should also bind to the intact form of PSA. It therefore seems that these peptides are good candidates for detecting PSA forms specifically associated to prostate cancer. Whether or not these peptides are specific of active PSA is currently under investigation.

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