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ANALYSIS OF PROSTATE SPECIFIC ANTIGEN AND α1-ANTICHYMOTRYPSIN INTERACTION USING ANTIPEPTIDE MONOCLONAL ANTIBODIES

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ABSTRACT

Purpose: The synthetic peptides E30D and D10P that correspond to prostate specific antigen (PSA) sequences 60-91 and 78-89, respectively, and contain the kallikrein loop were used to immunize mice to obtain anti-PSA monoclonal antibodies (mAbs).

Materials and Methods: Antipeptide mAb characteristics were studied using biosensor technology and enzyme-linked immunosorbent assay, and analyzing the mAb effects on PSA-α1-antichymotrypsin (ACT) complex formation and PSA enzymatic activity. Epitope mapping of these mAbs was performed using overlapping peptide synthesis on nitrocellulose membrane.

Results: Anti-E30D mAbs bound PSA coated on the solid phase only, whereas anti-D10P mAbs recognized PSA in detection as well as in capture. However, these mAbs appeared to be anti-total PSA mAbs. Anti-E30D and anti-D10P mAbs were directed against linear epitopes corresponding to residues H74-Y77 and N84-R88, respectively, of the PSA sequence. Anti-D10P mAb recognition of PSA and PSA-ACT complex was equimolar, although an existing molecular model suggested that the sequence corresponding to anti-D10P mAb epitope was involved in the interaction site of PSA with ACT. Furthermore, we were unable to inhibit the enzymatic activity of PSA as well as PSA-ACT complex formation. Finally, the epitope N84-R88 overlapped the cleavage site R85-F86 of PSA.

Conclusions: The linear anti-D10P mAb epitope is located outside of the PSA-ACT binding site. However, these mAbs may be of value for evaluating the presence of different molecular PSA forms in sera.

KEY WORDS: prostate specific antigen; alpha 1-antichymotrypsin; peptides; antibodies, monoclonal

The measurement of prostate specific antigen (PSA) in serum is the most useful tool available today for the diagnosis of early, curable adenocarcinoma of the prostate. PSA, a 237 amino acid glycoprotein (33 kD.) that belongs to the kallikrein serine protease family,[1] is produced by the glandular epithelium of the human prostate and secreted in the seminal fluid. PSA is normally found in concentrations less than 2.5 ng./ml. in male serum.[2] However, these concentrations can increase in conjunction with prostate cancer, benign prostatic hyperplasia (BPH), prostatitis and surgery of the prostate.[3,4]

In serum PSA forms stable complexes with several extracellular protease inhibitors, predominantly α1-antichymotrypsin (ACT), a serpin, and α2-macroglobulin, which causes inactivation of the chymotrypsin-like activity of PSA.[5] Current immunoassays detect total PSA composed of free and complexed forms. However, the measurement of percentage of free PSA in serum improves the specificity of prostate screening in men with serum PSA between 4.1 and 10.0 ng./ml.[6,7] The proportion of free PSA-to-PSA complexed to ACT is greater in men with BPH than in those with prostate cancer.[8]

To develop immunoassays for a differential diagnosis between prostate cancer and BPH, we previously raised anti-PSA mouse monoclonal antibodies (mAbs) and mapped the epitopes. We reported subsequently the characterization of 4 anti-total-PSA and 3 anti-free PSA mAbs, as well the partial localization of 2 conformational epitopes identified by anti-free-PSA mAbs.[9] The epitope recognized by the anti-free-PSA mAb 5D3D11 was located at the border of the PSA enzymatic site. In addition, PSA contains a particular sequence (D78-P89) called the kallikrein loop[10] which, according to the PSA molecular modeling of Villoutreix, was also located near the edge of the groove containing the active site of the molecule in the theoretical ACT binding site.

In an attempt to produce mAbs specific for free-PSA, we immunized mice with 2 synthetic peptides containing the kallikrein loop. Several mAbs were analyzed for specifity against PSA and PSA-ACT. We report the characterization of these antibodies and fine localization of the epitopes. The effects of these antibodies on the enzymatic activity of PSA and its complex with ACT provide new information about the interaction of PSA and ACT within the kallikrein loop.

MATERIALS AND METHODS

Biological materials. The PSA used in this study was from seminal origin. The anti-free-PSA mAbs 6C8D8, 5D3D11 and 12E6H9, and the anti-total-PSA mAbs 5C10D9, 7F2F2, 5D5A5 and 11E5C6 were obtained by immunization of mice with purified seminal PSA as described previously.[9]

Peptide synthesis and coupling. Peptide synthesis was performed as described previously.[11] Biotinylation of peptides was performed selectively at the N-terminus end after cleavage of the last coupled amino acid 9-fluorenylmethoxycarbonyl group according to Deibel et al.[12] The peptides were conjugated to bovine serum albumin (BSA) (30 mol. peptide: 1 mol. BSA). Peptide E30D was coupled to BSA via the free amino groups of

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the peptides with 25% glutaraldehyde according to Avrameas.[13] The peptide D10PC was coupled to BSA via its added C-terminal cystein with sulfosuccinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate according to Samoszuk et al.[14]

Production and biotinylation of mAbs. BALB/c JYco female mice 4 to 6 weeks old were immunized by intraperitoneal injection with 50 μg. peptide-BSA conjugate emulsified with an equal volume of Freund's complete adjuvant. Then 2 other injections were performed using incomplete adjuvant every 2 weeks. Spleen cells were harvested 4 days after the last injection, and mAbs were obtained and purified as described previously.[9] Purified mAbs were biotinylated using D-biotinoyl-E-aminocaproic acid N-hydroxy succiniomide ester according to Gretch et al.[15]

Evaluation of mAb kinetic constants. The binding of mAbs to PSA was measured using biosensor technology and the Fab' forms of the mAbs to avoid experimental and computational problems associated with the use of bidentate ligands as described previously.[9] All experiments were performed on a BIAcore 1000† instrument with PSA or PSA-ACT immobilized via the primary amine group according to manufacturer instructions. The association (ka) and dissociation (kd) rates of each mAb were determined using BIAevaluation, Version 3.0. The affinity was calculated as, ka/kd, and expressed in $M.^{-1}$.

Indirect enzyme-linked immunosorbent assay (ELISA). A total of 100 μl. peptide, PSA or PSA-ACT complex at a concentration of 1 mg./l. in 0.05 M. carbonate buffer, pH 9.6, were coated on 96-well plates. After 1 night of incubation at room temperature, the plates were washed 3 times with phosphate buffered saline (50 mM. phosphate buffer and 150 mM. sodium chloride, PBS), pH 7.2, containing 0.05% Tween-20 and blocked for 1 hour at 37C with PBS containing 10% goat serum. After a second wash with PBS Tween-20, 100 μl. monoclonal antibody in PBS Tween-20 containing 10% goat serum were added and incubated for 2 hours at 37C. After a new wash with PBS Tween-20, 100 μl. peroxidase conjugated goat anti-mouse IgG were added at a 1:5,000 dilution in PBS Tween-20 goat serum. The plates were incubated for 1 hour at 37C, washed once more with PBS Tween-20 and developed using a commercial color kit containing ortho-phenylenediamine and hydrogen peroxide. After a 10-minute incubation, the reaction was stopped with sulfuric acid, and the plates were read at 492 nm. with an ELISA plate reader. Values are expressed as the mean optical density of triplicate.

Sandwich ELISA. ELISA plates were coated with 100 μl. 5 mg./l. antipeptide mAb in PBS for 2 hours of incubation at 37C and blocked with PBS goat serum as described previously. The plates were then washed 4 times with PBS Tween-20 before adding 100 μl. PSA or PSA-ACT (both at 1 mg./l. PSA concentration) in PBS Tween-20 goat serum. After incubation overnight at 4C, the plates were washed 4 times with PBS Tween-20 and 100 μl. of 10 mg./l. biotinylated anti-total PSA mAb in PBS Tween-20 goat serum were added and incubated for 1 hour at 37C. Following another wash with PBS Tween-20 100 μl. peroxidase conjugated streptavidin were added at a 1:5,000 dilution in PBS Tween-20 goat serum. The plates were incubated for 1 hour at 37C, washed again with PBS Tween-20, developed and read as described previously. Values are expressed as the mean optical density of triplicate.

ELISA competition of mAbs. ELISA plates were coated with 100 μl. PSA at a concentration of 2 mg./l. in 0.1 M. carbonate buffer, pH 8.3. After 2 hours of incubation at 37C, the plates were washed 4 times with PBS Tween-20 and blocked for 2 hours at 37C with PBS containing 10% goat serum. Following a second wash with PBS Tween-20 100 μl. of the first native mAb at a concentration of 50 mg./l. in PBS Tween-20 goat serum were added and incubated for 2 hours at 37C. After another wash with PBS Tween-20, 100 μl. of the second biotinylated mAb diluted in PBS Tween-20 goat serum at concentrations ranging from 2 to 100 μg./l. were added and incubated for 2 hours at 37C. The subsequent steps were performed as described previously, and values are expressed as the mean optical density of triplicate.

Inhibition of PSA-ACT complex formation by antipeptide mAbs. ELISA plates were coated with 100 μl. 5 mg./l. native anti-total PSA mAb in PBS for 2 hours of incubation at 37C and blocked with PBS goat serum as described previously. The plates were then washed 4 times with PBS Tween-20 before adding 100 μl. 1 mg./l. PSA in PBS Tween-20 goat serum. After incubation overnight at 4C the plates were washed 4 times with PBS Tween-20, and 100 μl. 10 mg./l. native antipeptide mAb in PBS Tween-20 goat serum were added and incubated for 15 minutes at 37C. After the plates were washed 1 mg./l. ACT in PBS Tween-20 goat serum was added and incubated for 2 hours at 37C. The plates were washed again with PBS Tween-20, and 100 μl. of a 1:2,000 dilution of rabbit anti-human ACT in PBS Tween-20 goat serum were added. After incubation for 2 hours at 37C the plates were washed in phosphate buffered saline Tween-20, and 100 μl. of a 1:5,000 dilution of peroxidase conjugated goat anti-rabbit IgG in PBS Tween-20 goat serum were added. The plates were incubated for 1 hour at 37C, washed again with PBS Tween-20, developed and read as described previously. The values were expressed as the mean optical density of triplicate.

For each anti-total PSA mAb captured PSA the complex formation in the absence of the second mAb corresponded to a relative 100% calibrator calculated as the mean optical density obtained in the presence of ACT minus the mean optical density obtained in the absence of ACT. This formula allowed subtraction of the background attributable to the cross-reactivity between anti-rabbit and mouse mAbs. In the presence of the second mAb the residual complex formation was determined using the same calculation and expressed as a percentage of the relative 100% calibrator. The assay was performed in the presence of goat serum as diluent. The possible presence of goat-serpin complexes that could interfere with formation of the PSA human ACT complex was eliminated by verifying that the goat serum had no effect on PSA proteolytic activity.

Peptide synthesis on nitrocellulose membrane. The simultaneous synthesis of different peptide sequences was performed on a nitrocellulose membrane using 9-fluorenylmethoxycarbonyl amino acid chemistry.[16] Each peptide was generated in nmol. quantities suitable for immunological detection. Antibody reactivity to membrane bound peptides was analyzed by an indirect colorimetric immunoassay as described previously.[11] Spots corresponding to peptides with antibody reactivity produced a positive blue signal.

Determination of PSA enzymatic activity. PSA enzymatic activity was determined by hydrolysis of the substrate MeO-Suc-Arg-Pro-Tyr-pNA.hydrochloric acid at a final concentration of 5 mM. in 50 mM. tris hydrochloric acid, pH 7.8 and 0.1 M. sodium chloride.[5] Hydrolysis was measured at 405 nm. in an ultraviolet recording spectrophotometer. All reactions were performed at 37C and initiated by addition of 5 μg. PSA. Optical density was monitored for 30 minutes.

Molecular display. The tridimensional structure of PSA was obtained from the Protein Data Bank (pdb code: 1PFA.PDB), and visualization of the molecule was performed with commercial software. The putative conformational epitopes were investigated on the basis of amino acid proximity and immunological data.

RESULTS

Characterization of the peptides used as immunogens. Peptide E30D corresponded to the PSA sequence E60-D91, while

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peptide D10P corresponded to the PSA sequence D78-P89. The sequence of the peptide E30D corresponded to the sequence of peptide D10P with an extension of 18 amino acids at the N-terminal extremity and extension of 2 amino acids at the C-terminal extremity (fig. 1). D10PC corresponded to peptide D10P with a cystein added at its C-terminal extremity. The peptides E30D and D10PC were then coupled to BSA and the corresponding conjugates were used as immunogens to raise antibodies in mice. On ELISA all culture supernatants of E30D as well as D10P hybridoma cells were able to recognize PSA coated on the solid phase. From each series 2 clones were selected according their strong immunoreactivity against PSA for production of anti-E30D and anti-D10P mAbs.

Epitope mapping of antipeptide mAbs. As evidenced by ELISA mAbs 16H9A12 and 20H3C9 raised against the peptide D10PC bound peptides D10PC and E30D coated solid phase, whereas mAbs 7B11H10 and 11C5A4 raised against peptide E30D recognized E30D only (table 1). These results suggest that the 2 groups of mAbs were directed against 2 different epitopes.

Four sets of 25 overlapping octapeptides derived from the sequence of the peptide E30D, which contains the sequence of the peptide D10P, were synthesized bound on cellulose membrane. Immunoreactivity of the 2 sets of octapeptides was tested against the anti-E30D mAbs 7B11H10 and 11C5A4, whereas immunoreactivity of the second set was tested against the anti-D10P mAbs 16H9A12 and 20H3C9. As shown in figure 2, 7B11H10 and 11C5A4 recognized the same 5 continuous peptides, whereas 16H9A12 and 20H3C9 recognized the same 4 continuous peptides. The minimal sequence for the binding of 7B11H10 and 11C5A4 appeared to be HPLY (H74-Y77), whereas the epitope of 16H9A12 and 20H3C9 could be limited to the 5 amino acids NRFLR (N84 to R88). Thus, the 2 anti-E30D mAbs were identical as were the 2 anti-D10P mAbs.

Specificities of antipeptide mAbs. Both types of antipeptide mAbs recognized PSA coated solid phase. Anti-D10P mAbs also bound PSA-ACT, whereas the binding of this complex by anti-E30D mAbs was weak (table 1). Moreover, when tested for capturing antigen, 16H9A12 coated on the solid phase bound PSA in solution. However, the capture of PSA by 7B11H10 was also weak (table 2). The affinity constants of the Fab' form of mAbs 16H9A12 and 20H3C9 against PSA and PSA-ACT were measured by biosensor technology. As shown in table 3, 16H9A12 and 20H3C9 had the same affinity for the free PSA and ACT bound PSA, indicating an equimolar mAb recognition of both PSA forms. Interestingly, association and dissociation rates were of similar magnitude. The affinities of 7B11H10 and 11C5A4 for PSA or PSA-ACT also appeared to be equimolar but the values were too weak to be measured by biosensor technology in the conditions defined previously.

Competition of antipeptide mAbs with anti-PSA mAbs. As first antibody, the anti-D10P mAb 16H9A12 inhibited the binding of all mAbs, whereas the anti-E30D mAb 7B11H10 inhibited significantly only the anti-PSA mAbs 6C8D8, 5D3D11, 12E6H9, 5C10D9 and 7F2F2. As second antibody, the binding of the anti-E30D mAb 7B11H10 was significantly inhibited by the anti-D10P mAb 16H9A12, as well as the anti-total PSA mAbs 5C10D9, 5D5A5 and 11E5C6. However, binding of the anti-D10P mAb was only weakly inhibited by anti-E30D mAb, 5D5A5 and 11E5C6 (table 4).

Inhibition of enzymatic activity by anti-D10P mAbs. The anti-D10P mAb 16H9A12 was tested for its ability to inhibit PSA enzymatic activity compared with the anti-free PSA mAb 6C8D8 which has been shown previously to inhibit the PSA enzymatic activity totally. As shown in table 5, when pre-incubated with PSA, 16H9A12 could not inhibit the activity of the enzyme. Under the same conditions PSA enzymatic activity was inhibited 100% by 6C8D8. The capture of PSA by anti-E30D mAbs was too weak to study the effect of these mAbs on PSA enzymatic activity.

Inhibition of PSA-ACT complex formation by anti-D10PC mAbs. The anti-D10P mAb 16H9A12 was also tested for its ability to inhibit PSA-ACT complex formation on ELISA using the 2 anti-total PSA mAbs 5D5A5 and 11E5C6 coated on the solid phase to display PSA as described previously.[9] PSA captured by these mAbs could react with ACT. As shown in table 6, 16H9A12 did not inhibit complex formation, whereas the anti-free-PSA mAb 6C8D8 totally inhibited it with PSA displayed by 5D5A5 and 11E5C6 anti-total-PSA mAbs. Although 5D5A5 and 11E5C6 should capture different amounts of PSA and affect the ability of PSA to bind to ACT, inhibition of the percentage of relative complex formation by the 2 mAbs was not significantly different. As noted previously capture of PSA by anti-E30D mAbs was too weak to study the effect of these mAbs on PSA-ACT complex formation.

Molecular display. The use of molecular display allowed visualization of the respective localizations of these epitopes on the PSA molecule. As shown in figure 3, the epitope of the anti-E30D mAb HPLY was localized just before loop 4, the kallikrein loop, on the opposite side of the active site of the molecule. This localization was outside of the ACT binding site defined by the molecular modeling of Villoutreix. In contrast, the epitope of the anti-D10P mAb NRFLR was localized in the middle of loop 4 just under the active site of PSA in the theoretical ACT binding site.

PSA	60 … 78 … 89 91
Peptide E30D	E D T G Q V F Q V S H S F P H P L Y D M S L L K N R F L R P G D
Peptide D10PC	D M S L L K N R F L R P C

FIG. 1. Sequences of peptides. Added cysteine of D10PC is underlined

TABLE 1. *ELISA immunoreactivity of antipeptide mABbs against peptides, PSA and PSA-ACT coated on solid phase*

	Anti-E30D mAb (10 ng./ml.)		Anti-D10P mAb (10 ng./ml.)		Anti-total PSA mAb (10 ng./ml. 5D5A5*
	7B11H10	11C5A4	16H9A12	20H3C9	
Solid phase coated peptide (10 μg./ml.):					
E30D	1.830†	Greater than 2.500†	1.250	1.170	0
D10PC	0	0	1.370	1.214	0
Solid phase coated antigen (1 μg./ml. PSA):					
PSA	Greater than 2.500	—	1.728	—	0.816
PSA-ACT	0.168	—	2.130	—	0.984

* Anti-total PSA mAb was used as the control.
† Diluted to 10 μg./ml.

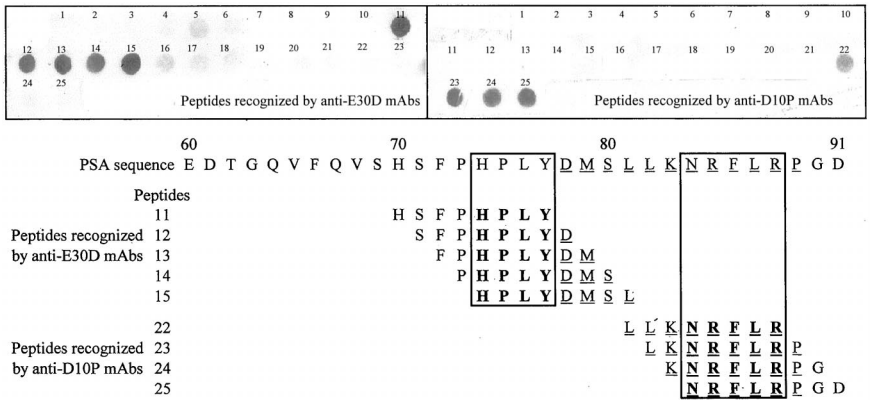

FIG. 2. Localization of antipeptide mAb epitopes on peptide sequence. PSA sequence E60-D91 corresponds to peptide E30D, containing peptide D10P (D78-P89, underlined). Four sets of 25 overlapping octapeptides covering this PSA sequence were synthesized bound on cellulose membrane. Numbers refer to respective overlapping peptides as peptide 1 corresponds to PSA sequence E60-Q67, peptide 2 to PSA sequence D61-V68 and so forth until peptide 25 which corresponds to PSA sequence N84-D91. Each set of peptides was immunoanalyzed with 1 of 4 antipeptide mAbs. Recognition of peptides by mAbs is shown by black dot.

TABLE 2. *ELISA immunoreactivity of antipeptide mAbs used to capture PSA and PSA-ACT complex*

Solid Phase Coated Antipeptide	Captured Antigen (2 μg./ml. PSA)*	
	PSA	PSA-ACT
Anti-E30D 7B11H10	0.188	0.227
Anti-D10P 16H9A12	Greater than 2.500	Greater than 2.500

* The detection mAb was the anti-total-PSA mAb 5D5A5 diluted to 1 μg./ml.

TABLE 3. *Antipeptide mAbs affinity constants against PSA and PSA-ACT complex*

	Association Rate ($Ms.^{-1}$)	Dissociation Rate (s^{-1})	Affinity Constant ($M.^{-1}$)
Anti-D10P 16H9A12 mAb:			
PSA	1.2×10^5	2.6×10^{-3}	4.8×10^7
PSA-ACT	1.3×10^5	2.7×10^{-3}	4.3×10^7
Anti-D10P 20H3C9 mAb:			
PSA	2.7×10^5	4.6×10^{-3}	5.9×10^7
PSA-ACT	3.3×10^5	5.5×10^{-3}	5.9×10^7

The values for anti-E30D 7B11H10 mAb and anti-E30D 11C5A4 mAb were too small to be detected and measured.

DISCUSSION

Numerous synthetic peptides coupled with carriers and generally administered with adjuvants have been shown to be capable of inducing immune responses against various antigens and even protective immunity against pathogens.[17] Kokolus et al previously reported production of anti-PSA antibodies using peptides defined by a motif of 2 hydrophobic and 1 hydrophilic regions according to predictive methods.[18] We attempted to raise anti-free PSA mAbs by starting from the molecular modeling of PSA and PSA-ACT complex published by Villoutreix et al,[10,19] as well as localization of the 2 anti-free-PSA mAb epitopes that we described previously[9] to define 2 peptides potentially accessible to antibody recognition and located in the theoretical ACT binding site. These peptides were chemically synthesized and used as immunogens to immunize mice.

Interestingly, the anti-D10P mAb was able to capture PSA in solution and detect PSA coated on the solid phase. These results showed that the epitope was easily accessible on the surface of the PSA molecule. On the contrary, the anti-E30D mAb recognized mainly the PSA coated on the solid phase, indicating that the anti-E30D epitope was not accessible and that PSA coating on the solid phase induced a change in PSA conformation that unmasked the anti-E30D epitope. However, all of the antipeptide mAbs recognized PSA and PSA-ACT complex and, consequently, our attempt to produce anti-free PSA antibodies failed. Moreover, recognition by the anti-D10P mAb was equimolar.

The fine epitope mapping performed with overlapping peptides showed that along the E30D sequence the epitope recognized by the anti-E30D mAbs was located outside of the theoretical ACT binding site, whereas the anti-D10P mAb epitope was within this region. Interestingly, recognition of the PSA-ACT complex coated on the solid phase by anti-E30D mAbs was weak. This finding suggests that when coated on the solid phase PSA-ACT interaction did not allow the anti-E30D mAb epitope to be unmasked. The absence of anti-E30D mAbs directed against epitopes in the kallikrein loop can be explained by the coupling of this peptide with

TABLE 4. *Competition of antipeptide mAbs with anti-PSA mAbs*

First mAb	% Inhibition of Binding of Second Biotinylated mAb*								
	Anti-E30D 7B11H10	Anti-D10P 16H9A12	6C8D8	5D3D11	12E6H9	5C10D9	7F2F2	5D5A5	11E5C6
7B11H10	81	24	56	49	47	36	37	18	11
16H9A12	60	76	66	79	62	67	62	50	44
6C8D8[9]	14	7	68	5	62	12	20	0	6
5D3D11[9]	15	3	0	68	0	0	0	13	3
12E6H9[9]	11	1	83	75	64	17	48	0	0
5C10D9[9]	41	0	0	0	63	90	73	19	11
7F2F2[9]	14	0	89	0	84	51	78	0	9
5D5A5[9]	74	39	0	0	0	96	0	87	74
11E5C6[9]	56	29	0	0	0	0	0	32	87

* The inhibition percentage was calculated as, (1 − [absorbance (mAb1 + mAb2) − absorbance (mAb1 + control buffer)/absorbance (control buffer + mAb2) − absorbance (mAb1 + control buffer)]) × 100.

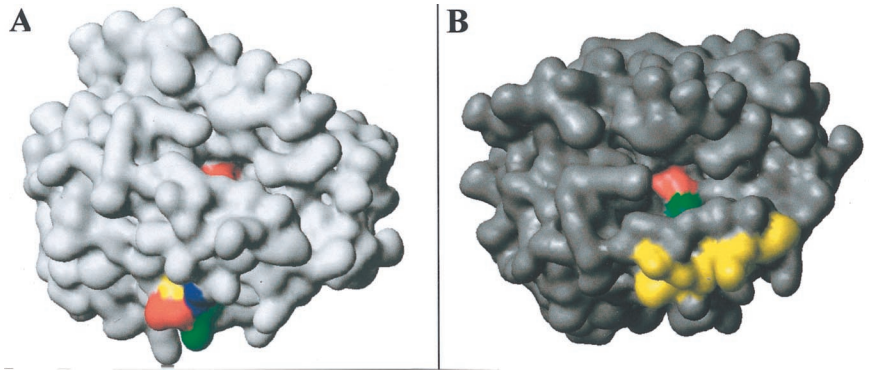

FIG. 3. Localization of antipeptide mAb epitopes on molecular model of PSA molecule. Catalytic triad, necessary for serine protease activity, is composed of H41 (green), D96 (not shown) and S189 (orange). *A*, anti-E30D mAb epitope residues, HPLY, include H74 (yellow), P75 (orange), L76 (green) and Y77 (blue). *B*, anti-D10P mAb epitope NRFLR (yellow).

TABLE 5. *Inhibition of the enzymatic activity of PSA by antipeptide D10PC mAb*

Anti-PSA mAb	Mean Absorbance Changes/Min. × 1,000 ± SD	Mean % Inhibition ± SD
PSA	29.73 ± 0.01	0.0
Control mAb	30.85 ± 0.09	−3.8 ± 0.3
Anti-D10P 16H9A12	32.76 ± 0.04	−10.2 ± 0.1
Anti-free-PSA 6C8D8	0.24 ± 0.08	99.2 ± 0.3

PSA was pre-incubated with either mAb or buffer as control for 30 minutes at 37C (1 mol. PSA/1 mol. mAb) before being added to 5 mM. substrate S-2586 at a final concentration of 0.4 μM. for PSA and the mAb. The reaction was monitored by measurement of the absorbance at 405 nm. for 30 minutes. PSA pre-incubated in the presence of buffer alone represented 100% PSA activity. PSA was also pre-incubated with a control mAb, which did not bind to PSA, as a control procedure.

TABLE 6. *Inhibition of PSA-ACT complex formation by anti-PSA mAbs*

Second mAb (anti-PSA)	First mAb (anti-total-PSA) Mean % Complex Formation ± SD	
	5D5A5	11E5C6
PBS + goat serum*	100	100
Control mAb†	99.2 ± 3.9	99.7 ± 2.5
Anti-D10P 16H9A12†	101.8 ± 9.5	115.0 ± 5.2
Anti-free-PSA 6C8D8†	0.3 ± 0.5	0.1 ± 1.2

* For each anti-total PSA mAb captured PSA the complex formation in the absence of the second mAb corresponds to a relative 100% calibrator calculated as mean absorbance obtained in the presence of ACT − mean absorbance obtained in the absence of ACT.

† In the presence of the second mAb residual complex formation was determined using the same calculation and expressed as a percentage of the relative 100% calibrator ± 1 SD.

BSA using glutaraldehyde that probably occurred via the free amino group of residue K24, which is contained in the D10P epitope. For this reason the D10P was coupled to BSA via the C-terminal end added cysteine.

The structure of the PSA-ACT complex has been investigated by Villoutreix et al using the 3-dimensional modeled structures of the uncleaved ACT molecule that were developed based on the x-ray structure of uncleaved antithrombin.[10] When building the PSA-ACT complex, loop 4 of the PSA was opened manually to allow insertion of the ACT molecule. According to this modeling, the authors noted that the original conformation of loop 4 was not optimal and/or that this loop opened upon binding to a serpin. Villoutreix et al suggested that in loop 4 PSA/hK2 residues Leu 95c [81] and Leu 95d [82] may interact with the Phe 280 of ACT.[10] Lys 95e [83] in PSA/hK2 interacts with Glu 195 of ACT. Depending on the structure of loop 4, Arg 95g [85] could interact with Glu 195 of ACT, whereas in the model of Villoutreix et al this Arg residue points outward. Arg 95j [88] in PSA and hK2 forms a salt bridge with Glu 109 of ACT. The authors also indicated the possibility that some of the charged residues in PSA and hK2 interact with backbone atoms in these areas of ACT instead of with the aforementioned side chains.

In another study anti-free-PSA epitopes were localized within region 80-91 by testing inhibition of the immunoreactivity of different PSA mAbs by synthetic peptides covering the PSA sequence.[21] However, most anti-PSA mAb epitopes are conformational. The binding inhibition by peptides has been proved to be an indirect approach. Moreover, these peptides were 8 to 16 amino acids long. Our data showed experimentally that these residues were involved in the interaction of PSA with the antipeptide D10P mAbs. However, the mAb equimolar recognition of free PSA and PSA complexed with ACT indicated that the 16H9A12 epitope (residues N84 to R88) could not be part of the PSA-ACT interaction. The discrepancy between our results and the complex molecular modeling of Villoutreix highlights the difficulty of modeling protein-to-protein interactions. Moreover, some important changes may occur upon ACT binding which cannot be investigated by molecular dynamic modulation.

In addition, Watt[22] and Zhang[23] et al characterized different molecular forms of PSA in human seminal fluid. They identified 3 different amino-terminal sequences that corresponded, respectively, to a major PSA cleavage at residues K145-K146 and 2 minor cleavages at K182-S183 and R85-F86. The anti-D10P mAb epitope (N84 to R88) was located at this third cleavage site. Thus, it is unlikely that the anti-D10P is still functional in the cleaved PSA form. However, the minor cleaved site R85-F86 was not detected on PSA in sera from cancer or BPH.[8,24]

CONCLUSIONS

The mAbs that we studied previously were directed against conformational epitopes. The detection of cleaved PSA forms by these mAbs could be hampered by conformational PSA change upon cleavage. On the contrary, the antipeptide mAbs described in the present study recognized linear epitopes. Thus, the binding to cleaved PSA in sera should not be affected. Recently, Charrier et al reported a higher proportion of cleaved PSA forms in serum from BPH than in serum from prostate cancer.[25] Also, recent studies have shown new alternative transcripts coding for variant proteins of PSA.[26,27] These mAbs could be of value for evaluating the presence of such molecules in sera.

Xavier Lacoux performed the peptide synthesis, and Geneviève Sibaï performed mAb purifications and peptide conjugates.

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