Clinical Chemistry 43:4
575–584 (1997)

Prostate-specific antigen: characterization of epitopes by synthetic peptide mapping and inhibition studies

Eva Corey,* Sandra K. Wegner, Michael J. Corey, and Robert L. Vessella

To improve our understanding of the prostate-specific antigen (PSA) antigenic regions, we studied the association targets of one anti-PSA polyclonal antibody and 10 anti-PSA monoclonal antibodies (mAbs). We also examined the ability of the mAbs to inhibit PSA enzymatic activity and block the association of PSA with α_1-antichymotrypsin (ACT). Linear epitope mapping with a polyclonal antibody indicated the presence of six major antigenic regions in PSA. Examination of the panel of mAbs established that three of them bind to linear epitopes. Five of the mAbs inhibited >90% of PSA enzymatic activity. However, inhibition of PSA enzymatic activity and hindrance of PSA-ACT association by mAbs cannot be used to predict whether the mAbs bind to free PSA, the PSA-ACT complex, or both. Some of the mAbs may block PSA-ACT association through peripheral occlusion of the binding site, or through induction of conformational changes in PSA.

INDEXING TERMS: linear epitope • α_1-antichymotrypsin • proteolytic activity

Despite the widespread use of prostate-specific antigen (PSA) as a clinical marker of prostate cancer *[1]*, little is known about the antigenic determinants of PSA involved in antibody recognition.[1] Information about the locations of the major epitopes on the surface of PSA might yield insight into the antigenic properties of the protein and specific details of the interactions of PSA with particular antibodies, which could in turn lead to methods of developing antibodies with desirable characteristics for use in diagnostic assays, such as higher specificity, equimolar binding to PSA and PSA-α_1-antichymotrypsin (ACT), and specific association only with free (f)-PSA.

Antigenic determinants as binding targets of antibodies can be divided into two categories: linear (sequential, continuous) and nonlinear (conformational, discontinuous). Linear epitopes consist of amino acid residues that are adjacent in the primary sequence; nonlinear epitopes consist of amino acid residues that are separated in the primary structure but are brought into proximity when the protein is in its native form. At present there is no simple way to identify conformational epitopes in the absence of three-dimensional structural information about the monoclonal antibody (mAb)-antigen complex. However, the identity of linear epitopes can be predicted by computer programs that calculate various parameters that have been found to be correlated with antigenic nature in previously studied antigens (e.g., hydrophilicity, flexibility, and surface probability *[2]*). The method postulates that (*a*) mAbs that bind to linear epitopes react with segments of 5–8 consecutive amino acid residues, and (*b*) these epitopes are on the surfaces of molecules that tend to be hydrophilic. However, computational techniques are not yet sophisticated enough to achieve the accuracy of experimental techniques. Other methods of identifying antibody binding sites involve (*a*) digestion of the antigen, (*b*) recombinant techniques, (*c*) phage display, (*d*) mass spectrometry, and (*e*) the use of synthetic peptides. Fragments from trypsin *[3]* or papain *[4]* digestion of antigens have been used to determine antibody binding targets. Numerous attempts with cyanogen bromide cleavage products have been published *[4–6]*. Van Duijnhoven et al. reported the use of recombinant DNA techniques for epitope mapping *[7]*. The phage display technique has found use in epitope mapping in determination of the antigenic regions of various proteins *[8–11]*. By using modern mass spectrometry techniques, epitopes can be determined as described by Zhao and Chait *[12]*, or

Tumor Immunology Laboratory of the Urology Department, School of Medicine of the University of Washington, Seattle, WA 98195.

*Address correspondence to this author at: Mailstop 356510, Department of Urology, University of Washington, Seattle, WA 98195. Fax 206-543-1146; e-mail ecorey@u.washington.edu.

[1] Nonstandard abbreviations: PSA, prostate-specific antigen; ACT, α_1-antichymotrypsin; f-PSA, free PSA; mAb, monoclonal antibody; EIA, enzyme immunoassay; BSA, bovine serum albumin; RT, room temperature; and HRP, horseradish peroxidase.

Received September 11, 1996; revised October 23, 1996; accepted November 7, 1996.

by Papac et al. *[13]*. Geysen et al. in 1987 *[14]* published a further method of identifying linear epitopes as association targets of antibodies by using overlapping synthetic peptides of antigens of known sequence. Given the recent progress in methods for simultaneous synthesis of large numbers of peptides, creating the peptide arrays corresponding to all possible contiguous segments of the protein of interest has become practical. The peptides are designed with substantial overlapping regions. Linear epitopes are then defined by identifying the peptides that associate most strongly with antibodies developed against the full-sized antigen. This methodology has been used with success in many cases *[15–19]*. We used the overlapping synthetic peptide method in combination with a commercially available rabbit polyclonal anti-PSA antibody and a panel of novel anti-PSA mAbs developed in our laboratory to characterize the linear epitopes of PSA.

An independent means of characterizing the binding targets of anti-PSA mAbs is by studying the effect of the mAbs on the enzymatic activity of PSA. This activity is held to be essential for formation of the PSA-ACT complex *[20, 21]*, implying that antibodies that affect the activity may be associating with epitopes proximate to the ACT binding site, and may therefore have special properties, such as the ability to distinguish f-PSA from the complex. One certainly expects to find a correlation between inhibition of PSA enzymatic activity by mAbs and inhibition of the association of PSA with ACT. Our results show that although the two inhibitory functions are strongly correlated, the presence of these inhibitory properties did not necessarily imply that the mAb was associating directly with the PSA-ACT interface.

Materials and Methods

PSA purification

PSA was purified from seminal plasma as previously described *[22]*, with the addition of chromatography on the Fractogel EMD TMAE 600 LS ion exchanger (EM Separations, Gibbstown, NJ). PSA purity was checked by sodium dodecyl sulfate gel electrophoresis under nonreducing conditions, and the concentration was determined by UV absorbance at 280 nm ($\epsilon^{0.1\%}$=1.84).

Anti-PSA monoclonal antibodies

Ten anti-PSA mAbs were developed in our laboratory by immunization of BALB mice with purified seminal plasma PSA *[23]*.

Synthetic peptides

Synthetic peptides were purchased from Zeneca (Cambridge Biochemicals, Wilmington, DE). They were synthesized on 96-well format plates by solid-phase methodology with Fmoc chemistry. Cysteine residues in peptides were in the reduced form. The amide group was chosen as the C terminus of the peptides to provide maximal resemblance to internal sequences. Peptides were cleaved from the solid support and dried. As a quality control, two peptides synthesized on the same plate were evaluated, one of an arbitrary reference sequence and one from the PSA sequence (peptide 155). These peptides were analyzed by HPLC to determine purity, and amino acid analysis was used for estimation of the average yield. Peptides used for screening were not purified; the purity was ~60%, with an average yield of ~2 mg. The PSA sequence was spanned by peptides 15 amino acid residues long with 10-residue overlaps. Biotin was added to a four-residue extension ("handle") of sequence SGSG at the N terminus to allow easy attachment to solid supports through association with immobilized streptavidin. The peptides were designated by the PSA sequence numbers of their first residues. The first peptide (1) spanned the protein sequence from position 0 (R) to position 14 (W), the second peptide (5) positions 5–19 (W-A), and so on.

Linear epitope mapping

Enzyme immunoassay (EIA) plates (Costar 3590; Costar, Cambridge, MA) were coated with 100 μL of 5 mg/L streptavidin (Sigma, St. Louis, MO) in water. Plates were incubated at 37 °C until dry. After three washes with 20 mmol/L PBS, pH 7.4, containing 1 mL/L Tween 20, the plates were blocked for 1 h with 200 μL of 20 g/L bovine serum albumin (BSA) in PBS (pH 7.4) at room temperature (RT). Approximately 2 mg of peptides (~60% pure) were dissolved in 200 μL of dimethyl sulfoxide (~3 mmol/L peptide, stock solution); each peptide was diluted 1:500 in PBS:1 g/L BSA:1 g/L NaN_3 before use (~6 μmol/L peptide). These dilutions (100 μL) were incubated with streptavidin-coated wells for 1 h at RT with shaking. Each antibody (100 μL; 10 μg/mL, ~62 nmol/L), either anti-PSA mAb or rabbit anti-PSA polyclonal antibody (Dako Corp., Carpinteria, CA), were incubated with the immobilized peptides overnight at 4 °C. After three washes, the appropriate horse anti-mouse antibody (Zymed Labs., So. San Francisco, CA) or goat anti-rabbit antibody (Bio-Rad Labs., Hercules, CA), coupled with horseradish peroxidase (HRP), was added at a dilution of 1:2000 in 20 g/L BSA:PBS and incubated for 1 h at RT. Reactive peptides were detected through HRP with the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) substrate (Zymed Labs.). As a negative control, plates with immobilized synthetic peptides were treated in the same manner with primary anti-PSA antibodies omitted.

Inhibition of 60–1A2, 60–8A2, and 17–1A2 association with PSA by peptides derived from PSA

PSA was immobilized on EIA plates (750 ng/mL, ~25 nmol/L, 100 μL/well, overnight at 4 °C). 60–1A2, 60–8A2, or 17–1A2 (90 μL; 10 μg/900 μL, ~69 nmol/L) were incubated with 10 μL of peptide 155 or 160 (mAbs 60–1A2 and 60–8A2) or peptide 50 or 55 (mAb 17–1A2) (peptides at ~60 μmol/L in PBS:1 g/L BSA) for 30 min and

incubated with immobilized PSA for 20 h. The quantity of bound antibody was determined as above. Control experiments with no peptides present were run simultaneously.

INHIBITION OF PSA ENZYMATIC ACTIVITY BY MABS

Inhibition of PSA enzymatic activity against the chymotrypsin substrate S-2586 (MeO-Suc-Arg-Pro-Tyr-*p*NA; Kabi Pharmacia, Franklin, OH) by the mAbs was tested. One microgram of PSA was incubated with 1.5 or 4.5 equivalents of mAbs for 2 or 18 h in 80 μL of 100 mmol/L Tris-HCl, 100 mmol/L NaCl, pH 7.51 (PSA ~410 nmol/L, mAbs ~615 nmol/L, ~1.85 μmol/L respectively). After addition of 20 μL of 5 mmol/L S-2586, absorbance at 410 nm was read approximately every 5–10 min over 2 h. Experiments with PSA only, MOPC-21 (unrelated antibody), and ACT were run simultaneously as controls. Linear regression analysis was used to determine ΔA_{410}/min, and the degree of inhibition was calculated as the difference in these rates attributable to the presence of the antibody.

ANTIBODY INHIBITION OF ACT BINDING TO PSA

One microgram of PSA was incubated with 3 equivalents of each of the mAbs for 3 h at 37 °C in 18 μL of 100 mmol/L Tris-HCl, 100 mmol/L NaCl, pH 7.51 (~1.85 μmol/L PSA, ~5.6 μmol/L mAb). Three equivalents of ACT (Athens Scientific, Athens, GA) in 2 μL of the same buffer were then added (final concentration of ACT ~5.6 μmol/L). From these reaction solutions, 1.5 μL was removed at times 0, 1, 3, 6, and 26 h and immediately diluted into 150 μL of ice-cold PBS:10 g/L BSA. Additional dilutions of these solutions were prepared for EIA determination of PSA-mAb, mAb-PSA-ACT, and PSA-ACT complexes. The presence of these complexes was detected in three ways. For detection of the PSA-ACT and mAb-PSA-ACT complexes, we used a PSA capture process. Plates coated with the mouse anti-PSA mAb 22–8A2 (10 μg/mL, 62 nmol/L) were incubated with the diluted reaction mixtures for 2 h at RT. The ACT complexes [PSA-ACT and (or) mAb-PSA-ACT] were detected with a rabbit anti-ACT polyclonal antibody (Athens Scientific, purified in-house; 3 μg/mL, ~18.8 nmol/L) in combination with a mouse-adsorbed HRP-conjugated goat anti-rabbit antibody (Bio-Rad Labs.) at a working dilution of 1:2000 in 1 g/L BSA:PBS. MOPC-21 and PSA alone were used as controls. To confirm the detection of mAb-PSA-ACT complexes, we captured them with a horse anti-mouse antibody at 3 μg/mL (~18.8 nmol/L) (BA2080; Vector Labs., Burlingame, CA), with detection by a rabbit anti-ACT polyclonal antibody (3 μg/mL, ~18.8 nmol/L) in combination with a mouse-adsorbed HRP-conjugated goat anti-rabbit antibody (Bio-Rad Labs.). To check for anti-mouse antibody coating and capture of mAb-PSA and (or) mAb-PSA-ACT complexes in cases of negative results for ACT, we coated plates in the same way with anti-mouse antibody, but detected the presence of PSA with a rabbit anti-PSA polyclonal antibody in combination with a mouse-adsorbed HRP-conjugated goat anti-rabbit antibody (Bio-Rad Labs.). A further technique for detection of mAb-PSA-ACT was as follows. We captured the ACT complexes on EIA plates coated with the rabbit anti-ACT polyclonal antibody at 10 μg/mL (~62 nmol/L). After 2 h of incubation with the reaction mixture, the presence of the mouse anti-PSA mAb was detected by reaction with an HRP-conjugated rabbit anti-mouse antibody IgG+A+M (H+L) (Zymed Labs.) at a 1:2000 working dilution in 1 g/L BSA:PBS.

The apparent rate constants for the PSA-ACT association reaction in the presence of mAbs 16–3A2 and 22–8A2 were calculated by nonlinear regression of the data presented in Fig. 6A to Eq. 1:

$$A_{obs} = A_{PSA} + (A_c - A_{PSA})(1 - e^{-kt}) \quad (1)$$

where A_{obs} is the observed A_{410}, A_{PSA} is the fit A_{410} attributable to the f-PSA in the initial conditions (i.e., the true background), A_C is the fit A_{410} attributable to the PSA-ACT complex at completion of the reaction, k is the characteristic time constant of the reaction, and t is the reaction time. The rates were calculated merely to compare the effects of the two mAbs, and not to enable us to make estimates of the kinetic parameters of the reaction. The model assumes that the reaction rate follows a simple exponential decay related to the decrease in the remaining amount of f-PSA. While this is a slight oversimplification, since ACT was present in only threefold excess, the residuals appeared to be completely random by visual inspection, indicating that the model is not seriously flawed for treatment of these data.

Results

LINEAR EPITOPE MAPPING

From epitope mapping experiments involving the rabbit anti-PSA polyclonal antibody, we obtained a profile of the antigenicity of PSA. Such a profile depends to some extent on the polyclonal antibody selected; another polyclonal antibody might yield a slightly different picture of PSA antigenicity because of variability of the relative abundance of clones reactive with particular epitopes. The population of clones present in a polyclonal antibody depends on the form and quality of the protein preparation used for immunization, and probably on the methods of immunization as well. The six major regions to which this polyclonal exhibits the greatest affinity are sequences 10–25, 50–65, 70–85, 90–110, 130–140, and 185–195 (Fig. 1). Reactivity of synthetic peptides with the anti-rabbit antibody alone was used as a negative control; final data were corrected for this nonspecific binding.

Further evaluation of linear epitopes was performed with a set of mAbs of known specificity and sandwiching characteristics. Three of the 10 anti-PSA mAbs reacted with synthetic peptides derived from the known primary sequence of PSA. 17–1A2 exhibited binding to synthetic

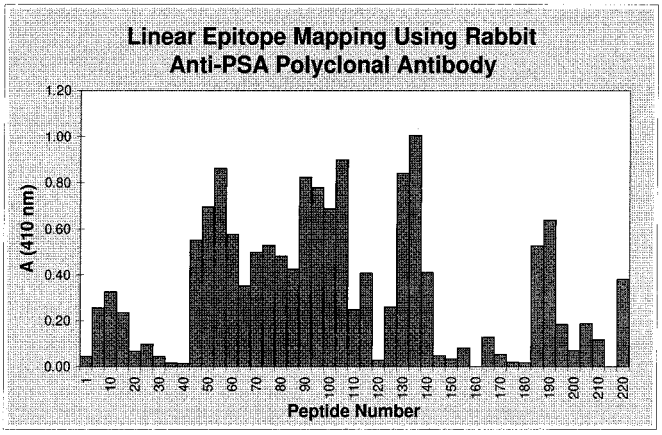

Fig. 1. Linear epitope mapping with anti-PSA polyclonal antibody.

Peptides 15 amino acid residues long with overlaps of 10 amino acid residues were tested for reactivity with a rabbit anti-PSA polyclonal antibody. Data are corrected for cross-reactivity of the anti-rabbit detection antibody with PSA peptides. Peptides are designated by the PSA sequence numbers of their first amino acid residues.

peptides 50 (sequence 50–64, LLGRHSLFHPEDTGQ) and 55 (sequence 55–69, SLFHPEDTGQVFQVS) (Fig. 2A), whereas 60–1A2 and 60–8A2 bound to synthetic peptides 155 (sequence 155–169, ISNDVCAQVHPQKVT) and 160 (sequence 160–175, CAQVHPQKVTKFML) (Figs. 3A and 4A). The binding signals of these antibodies under these

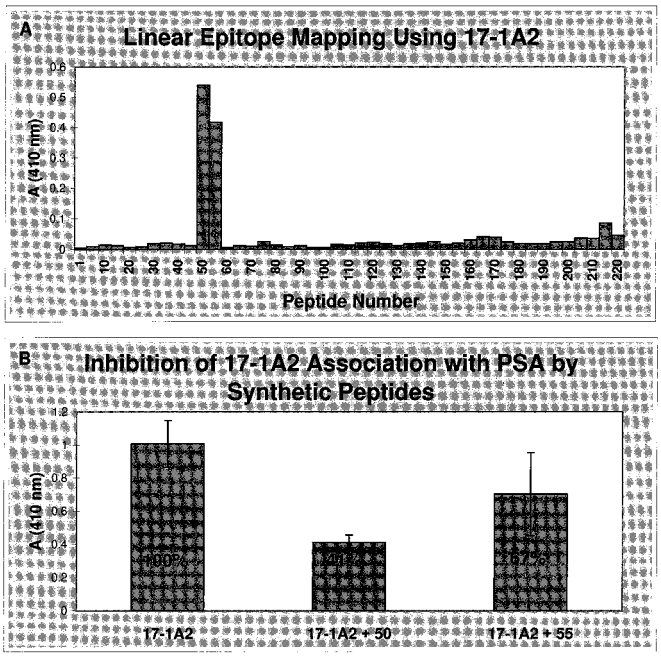

Fig. 2. (*A*) Linear epitope mapping with anti-PSA mAb 17–1A2; (*B*) inhibition of association of mAb 17–1A2 with PSA by synthetic peptides 50 and 55.

(*A*) Peptides 15 amino acid residues long with overlaps of 10 amino acid residues were tested for reactivity with the mouse anti-PSA monoclonal antibody 17–1A2. Data are corrected for cross-reactivity of the anti-mouse detection antibody with the PSA peptides. Peptides are designated by the PSA sequence numbers of their first amino acid residues. (*B*) Synthetic peptides were incubated in excess with 17–1A2 for 0.5 h, whereupon the reactivity of the mixture with immobilized PSA was determined.

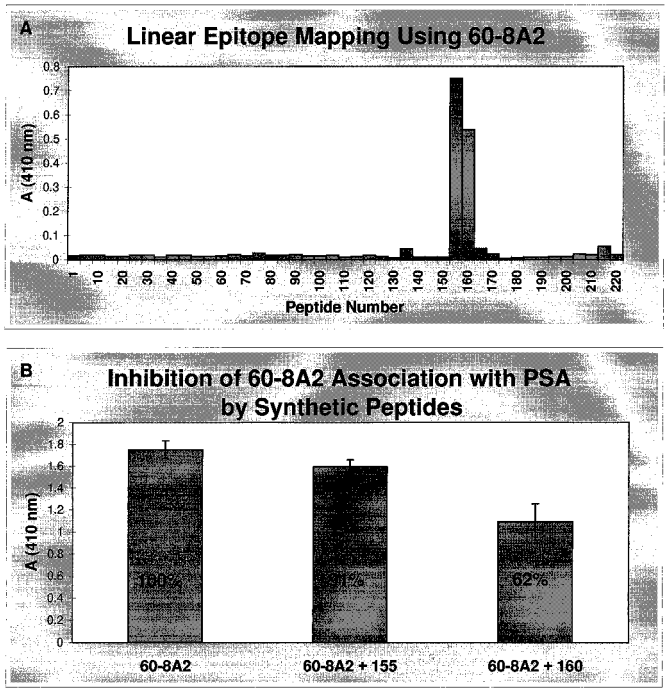

Fig. 3. (*A*) Linear epitope mapping with mAb 60–8A2; (*B*) inhibition of association of mAb 60–8A2 with PSA by synthetic peptides 155 and 160.

(*A*) Peptides 15 amino acid residues long with overlaps of 10 amino acid residues were tested for reactivity with the mouse anti-PSA mAb 60–8A2. Data are corrected for cross-reactivity of the anti-mouse detection antibody with the PSA peptides. Peptides are designated by the PSA sequence numbers of their first amino acid residues. (*B*) Synthetic peptides were incubated in excess with 60–8A2 for 0.5 h, whereupon the reactivity of the mixture with immobilized PSA was determined.

conditions vary greatly in intensity: The signal from 60–8A2 was strong after 5–10 min, whereas those for 60–1A2 and 17–1A2 were measured after 60 min. This characteristic is seen to be directly related to the relative affinities of particular mAbs for native PSA *[23].* To confirm the epitopic nature of these peptides we performed competition experiments with PSA. Peptides 50 and 55 both competed with PSA for association with 17–1A2. Peptide 50 exhibited stronger inhibition of mAb-PSA binding (59%), whereas peptide 55 showed weaker inhibition (33%). Peptides 155 and 160 both competed with PSA for association with 60–8A2; however, the inhibition of mAb-PSA binding by peptide 155 was only ~10%. Peptide 160 inhibited 38% of mAb-PSA association. Synthetic peptides 155 and 160 did not inhibit the association of 60–1A2 with PSA. The other seven antibodies did not exhibit significant binding to PSA-derived synthetic peptides (data not shown). Experiments were repeated at least twice; within each experiment triplicate data were used for analysis. Negative controls without primary antibody were performed simultaneously and used as controls for reactivity of the peptides with the secondary antibody.

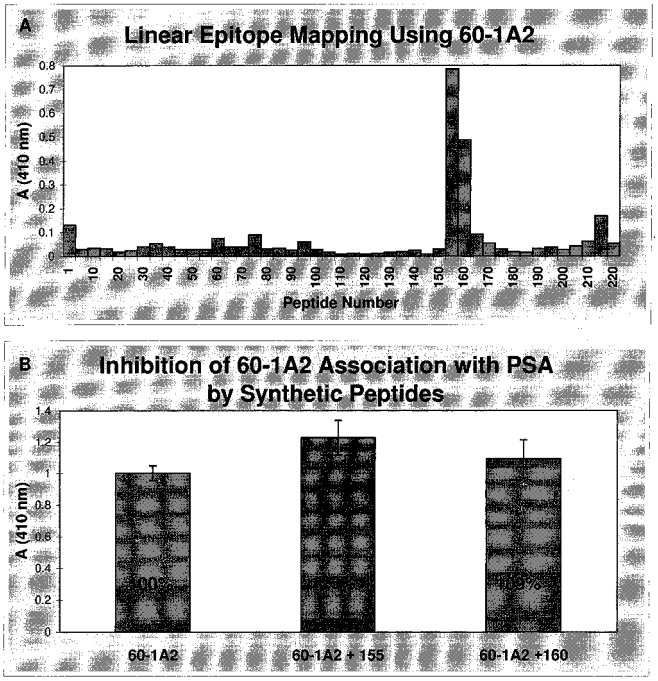

Fig. 4. (*A*) Linear epitope mapping with mAb 60–1A2; (*B*) inhibition of association of mAb 60–1A2 with PSA by synthetic peptides 155 and 160.

(*A*) Peptides 15 amino acid residues long with overlaps of 10 amino acid residues were tested for reactivity with the mouse anti-PSA mAb 60–1A2. Data are corrected for cross-reactivity of the anti-mouse detection antibody with the PSA peptides. Peptides are designated by the PSA sequence numbers of their first amino acid residues. (*B*) Synthetic peptides were incubated in excess with 60–1A2 for 0.5 h, whereupon the reactivity of the mixture with immobilized PSA was determined.

Table 1. Inhibition of PSA enzymatic activity by anti-PSA mAbs.

	1.5 Equivalents of protein		4.5 Equivalents of protein	
	$10^{-4} \cdot \Delta A_{410}$/min ± SE	% Inhibition	$10^{-4} \cdot \Delta A_{410}$/min ± SE	% Inhibition
Blank	0.3 ± 0.2		0.7 ± 0.2	
PSA	19.7 ± 0.3	0.0	20.6 ± 0.9	0.0
MOPC-21	19.4 ± 0.3	1.5	20.2 ± 0.1	1.9
ACT	13.1 ± 0.2	33.5	0.7 ± 0.1	96.6
Antibody				
15-19A2	−0.7 ± 0.3	103.4	1.6 ± 0.3	92.2
16-3A2[a]	28.6 ± 0.4	−45.2[a]	26.6 ± 0.6	−30.6
17-1A2	2.1 ± 0.2	89.2	2.8 ± 0.3	86.4
22-1A2[a]	24.5 ± 0.1	−24.5[a]	26.8 ± 0.5	−30.1
22-2A3	16.1 ± 0.2	18.1	15.7 ± 0.3	23.8
22-8A2	8.7 ± 0.4	55.8	12.2 ± 0.2	40.8
60-1A2	0.7 ± 0.5	96.4	1.2 ± 0.2	94.2
60-6A2	1.1 ± 0.2	94.5	0.8 ± 0.3	96.1
60-8A2	18.1 ± 0.1	8.2	21.2 ± 0.1	−2.9
60-9A2	0.4 ± 0.3	98.0	0.5 ± 0.2	97.6

[a] These mAbs increased PSA enzymatic activity.

PSA was incubated with 1.5 and 4.5 molar equivalents of mAbs for 2 h, whereupon the enzymatic activity of PSA was measured with substrate S-2586. Data were reduced by linear regression. ΔA_{410}/min is not corrected for the blank rate. Reaction with PSA only (no added antibody) is considered 100% of PSA enzymatic activity.

ANTIBODY INHIBITION OF PSA ENZYMATIC ACTIVITY

The influence of the mAbs on PSA enzymatic activity was determined by measuring the ability of PSA to cleave the S-2586 chymotrypsin substrate after interaction with the mAbs. Steady-state rates were measured by monitoring A_{410} over ~2 h. These data were reduced by linear regression analysis to yield ΔA_{410}/min with correlation coefficients >0.98, except in those cases when the mAbs reduced the rate by >80%. The results for 2-h incubations with 1.5 and 4.5 molar equivalents of mAbs (relative to PSA) are shown in Table 1. The mAbs can be classified in this regard as follows: (*a*) mAbs 15–19A2, 17–1A2, 60–1A2, 60–6A2, and 60–9A2 inhibited >86% of PSA enzymatic activity; (*b*) mAb 22–8A2 showed partial inhibition (~60%); (*c*) mAb 22–1A2 and 16–3A2 actually promoted PSA enzymatic activity (~120–140%), and (*d*) mAbs 22–2A3 and 60–8A2 had minimal effects on PSA enzymatic activity. Two further sets of conditions, 1.5 and 4.5 molar equivalents of the mAbs with 18-h incubations, were tested to determine the effect of incubation time on this inhibition. These experiments did not give significantly different results, except that ACT (1.5 equivalents) inhibited PSA more strongly after 18 h (64.6%) than after 2 h (33.5%).

ANTIBODY INHIBITION OF ACT BINDING TO PSA

To look for a correlation with the results of the enzyme-inhibition studies and learn more about the antigenic targets of the mAbs, we examined the ability of the mAbs to block the association of PSA with ACT. Five mAbs with various PSA-association characteristics were tested in these experiments: 15–19A2 and 60–6A2, which inhibit PSA enzymatic activity but bind to both f-PSA and PSA-ACT; 17–1A2, which inhibits PSA enzymatic activity but exhibits a strong binding preference for f-PSA; 22–8A2, which has a moderate effect on PSA enzymatic activity and binds to both f-PSA and PSA-ACT; and 16–3A2, which binds to both f-PSA and PSA-ACT and increases PSA enzymatic activity. Aliquots of reaction mixtures were removed over 26-h incubations to evaluate the extent of PSA-ACT association. Fig. 5 shows the quantities of mAb-PSA-ACT complex and (or) PSA-ACT complex detected by a rabbit anti-ACT polyclonal antibody after being captured by the mouse anti-PSA mAb 22–8A2; the other four mAbs were evaluated as potential inhibitors of complex formation in these experiments. The signal is normalized to the signal of PSA-ACT seen in the reaction mixture in the presence of the unrelated antibody MOPC-21, which does not associate with either PSA or ACT and is not expected to influence PSA-ACT association. Two of the mAbs (15–19A2 and 60–6A2) appeared to inhibit the association of ACT with PSA completely. The other two mAbs behaved very differently. In the reaction with mAb 16–3A2, the concentrations of ACT-containing complexes increased throughout the incubation period.

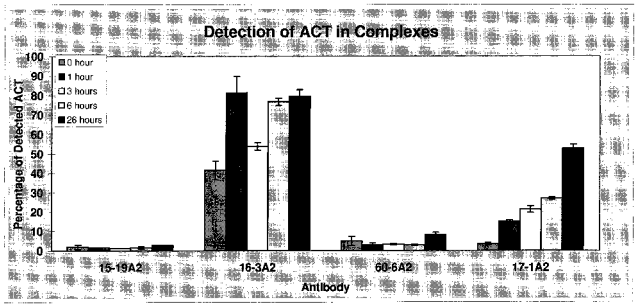

Fig. 5. Detection of ACT in complexes [mAb-PSA-ACT and (or) PSA-ACT] by anti-ACT polyclonal antibody after capture by anti-PSA mAb 22–8A2. Signal is normalized to the PSA-ACT content of the MOPCI-21 control reaction, which is treated as 100%.

The half-life of the increase appeared to be identical to the half-life of the control MOPC-21 (uninhibited) reaction, but the asymptote was smaller. mAb 17–1A2 had a dramatically different effect. This mAb increased the rate of formation of ACT-containing complexes relative to the control reaction.

To distinguish between the formation of PSA-ACT and mAb-PSA-ACT in these experiments, we captured mAb-PSA-ACT complexes by means of an anti-mouse antibody and detected the presence of ACT. Results are shown in Fig. 6A. mAbs 15–19A2, 17–1A2, and 60–6A2 evidently did not form mAb-PSA-ACT complexes. To ensure that the absence of the mAb-PSA-ACT signal is not due to failure of the capture by the anti-mouse antibody, we successfully used the same experimental setup for detection of mAb-PSA complexes with an anti-PSA polyclonal antibody (Fig. 6B). PSA was detected in complexes with all of these antibodies, with the exception of 17–1A2, which is known to be a weaker antibody. For this antibody, we performed the same experiment with a fivefold higher concentration of the mAb-PSA complex solution in the capture reaction. Under these conditions we were able to detect the signal of PSA in the mAb-PSA complex, but no mAb-PSA-ACT was detected (see Fig. 7).

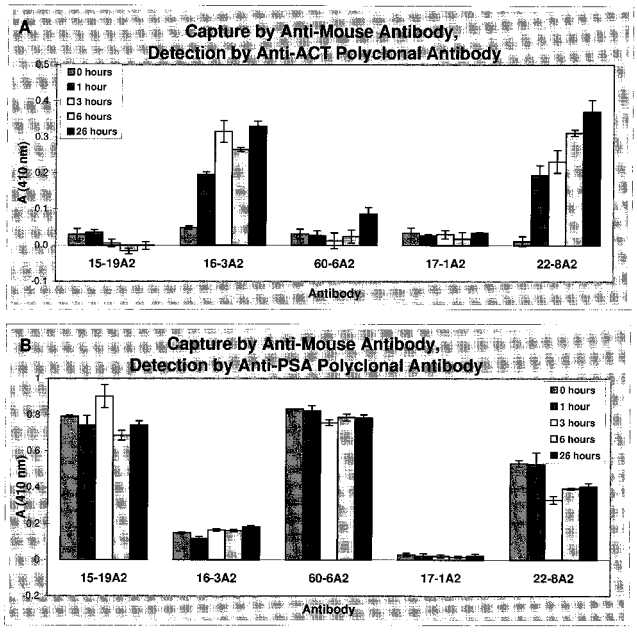

Fig. 6. (*A*) Detection of mAb-PSA-ACT complexes by anti-ACT polyclonal antibody after capture by anti-mouse antibody; (*B*) detection of mAb-PSA and (or) mAb-PSA-ACT by anti-PSA polyclonal antibody after capture by anti-mouse antibody.

These results were confirmed by experiments in which an anti-ACT polyclonal antibody was used for capture. mAb-PSA-ACT complexes were detected by anti-mouse antibodies (Fig. 8). mAb-PSA-ACT complexes were detected after PSA incubation with mAbs 16–1A2 and 22–8A2, but not with 60–6A2, 15–19A2, or 17–1A2.

Apparent rate constants were calculated for the PSA-ACT association reaction in the presence of mAbs 16–3A2 and 22–8A2, with nonlinear regression of the data presented in Fig. 6A to Eq. 1 (*Materials and Methods*). The calculated rate constants of the reaction in the presence of 16–3A2 and 22–8A2 were $0.92 \pm 0.15\ h^{-1}$ and $0.56 \pm 0.13\ h^{-1}$, respectively.

Discussion

Development of improved assays for PSA detection depends on the availability of specific, high-affinity mAbs,

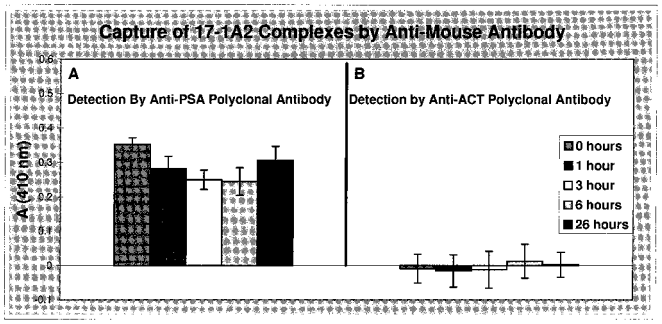

Fig. 7. (*A*) Detection of the mAb(17–1A2)-PSA-ACT complex by anti-PSA polyclonal antibody after capture by anti-mouse antibody; (*B*) detection of the mAb(17–1A2)-PSA-ACT complex by anti-ACT polyclonal antibody after capture by anti-mouse antibody.

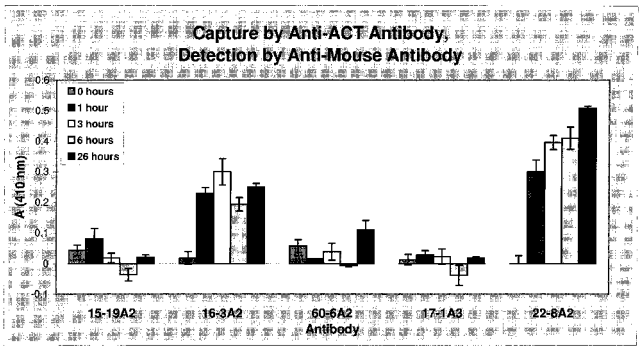

Fig. 8. Detection of mAb-PSA-ACT by anti-mouse antibody after capture by anti-ACT polyclonal antibody.

especially those capable of binding in equimolar fashion to the immunoreactive forms of PSA found in serum or distinguishing between them. Identification of strongly reactive linear epitopes and characterization of their nature and locations could be useful for classification of mAbs and prediction of their sandwiching capabilities and specificities. Various groups working with anti-PSA antibodies have reported the existence of three to six antigenic regions in PSA. These evaluations were based on the sandwiching capabilities of the mAbs, and in some cases on the f-PSA and PSA-ACT specificities *[1, 24, 25]*. The recent work of Pettersson et al. *[24]* reports the presence of six antigenic regions in the PSA molecule: Four of these are exposed for mAb association in f-PSA as well as PSA-ACT; one antigenic region is not accessible in PSA-ACT, and mAbs binding to this region are f-PSA specific; and one region associates with antibodies that show a strong preference for f-PSA. However, there are as yet no published data about the physical locations of the PSA epitopes.

There is conflict in the literature about the utility and validity of various methods of epitope characterization *[26, 27]*. There is general agreement that the most precise method is x-ray crystallographic analysis of the three-dimensional structure of antibody–antigen complexes. However, only a few of these structures have been solved to date *[28–32]*. Data from these studies indicate that a large surface of the antigen is usually involved in antibody association. On average 16–22 contact residues contribute to the energy of binding, but at widely varying levels. Laver et al. *[26]* in their review assert that the widely held view that epitopes in native proteins consist of segments of about six amino acid residues, and that they can therefore be mapped by utilizing synthetic peptides of similar length, is a misconception, since epitopes of this size often result from immune induction by denatured protein molecules. However, the work of Geysen et al. *[14]*, with synthetic peptides, involved the study of various lengths of peptides, and their results indicated that octapeptides are the longest needed for detection of linear epitopes. Moreover, in their amino acid residue replacement study they observed no epitopes in which more than five residues were in contact with the antibody. Continuing reports of the successful use of the short peptides produced by phage display or by chemical synthesis in determination of binding sequences of various antibodies are evidence for the utility of small peptides in linear epitope mapping *[6–11, 14–18]*. Data from x-ray studies also indicate that 3–5 residues generally contribute most of the free energy of association *[26, 27]*. The residues are not necessarily adjacent to each other in the primary structure; however, the antibodies in these studies were associating with nonlinear epitopes, whereas the most important contact residues of linear epitopes are likely to be proximate in the primary structure. A compromise hypothesis subsuming all of these studies might hold that in linear epitopes there are 3–6 adjacent residues that contribute the greatest part of the energy of interaction, but that there exist further contacts that stabilize the interaction, involving antigen residues that cannot be identified by short-peptide mapping.

After evaluation of the various available methods for determining the locations of the epitopes, we decided to use linear epitope mapping with synthetic overlapping peptides because of the volume of useful information provided and the high spatial resolution of the technique. Since only 5–10% of mAbs raised against native antigens bind to linear epitopes, we decided to maximize the probability of incorporating both such linear epitopes and any epitopes that might depend on local secondary structure of the protein by using peptides 15 amino acid residues long, which may be partially folded in solution. Linear epitope mapping with a rabbit polyclonal antibody indicated the presence of six major antigenic regions in PSA, in agreement with the report by Pettersson et al. *[24]*. The apparent concurrence between the six antigenic regions seen in Pettersson's sandwich assays and the number determined by our linear epitope mapping can be either real or coincidental. However, we chose a polyclonal antibody for these experiments specifically because the multiplicity of clones present greatly increases the likelihood of detecting any given antigenic region. Signals seen in our procedure probably do not arise only from clones that associate with linear epitopes. Studies *[33]* indicate that part of the signal is due in some instances to the presence of clones that bind strongly to nonlinear epitopes in the native protein, and also associate less strongly with the portions of these nonlinear epitopes that are represented in the synthetic peptide library we studied.

On the basis only of linear epitope mapping with polyclonal sera, we cannot determine which if any of the highly antigenic regions is specific for f-PSA. Additional experiments, including immunization with peptides from these parts of the protein, could help to identify such regions. Using a set of anti-PSA mAbs that we previously characterized *[23]*, we looked for a correlation between their sandwiching capabilities and the physical locations of their binding targets. The mAbs were divided into five groups on the basis of their sandwiching capabilities: (*a*) 15–19A2, 60–1A2, 60–6A2, and 60–9A2; (*b*) 16–3A2; (*c*) 17–1A2; (*d*) 22–1A1, 22–2A3, and 22–8A2; and (*e*) 60–8A2. All of the mAbs recognize both f-PSA and PSA-ACT, although 17–1A2 has a marked preference for f-PSA. 60–8A2 was most probably raised against denatured PSA; it does not bind to PSA in solution. Of this set, three mAbs bound to synthetic peptides. mAbs 60–1A2 and 60–8A2 both bind to sequence 155–174 of PSA. However, judging by the inhibition and competition data, these two mAbs appear to bind to different epitopes within this sequence. Synthetic peptides 155 and 160 competed with immobilized PSA for association with 60–8A2, but not with 60–1A2. Frequently antibodies that bind to linear epitopes still exhibit binding preference for the native

antigen, either because of the presence of additional interactions with the protein or because the secondary structure of the epitope is imperfectly modeled by the isolated peptide. Evidently in the case of mAb 60–1A2 the preference for PSA is strong enough to prevent the peptide from competing effectively with the native epitope. Peptide 160 competes more strongly with PSA for association with 60–8A2 than does peptide 155, indicating that this epitope is located in the C-terminal portion of sequence 155–174. mAb 17–1A2 associates with PSA sequence 50–69, represented by peptides 50 and 55. Peptide 50 competes more strongly than peptide 55, suggesting that the binding target is in the N-terminal portion of sequence 50–69. The other seven antibodies did not exhibit significant binding to the synthetic peptides, implying that their targets are nonlinear epitopes.

Sequence 155–174 involved in 60–1A2 and 60–8A2 binding did not exhibit strong antigenicity in experiments with the polyclonal antibody. We hypothesize that this is due to differences in procedures used for antibody production and the quality and purity of the PSA used for immunization. The sequence 50–70, the binding target of 17–1A2, had strong antigenicity in experiments with the polyclonal antibody. Since this mAb exhibits binding preference for f-PSA, and its target region appears to have substantial antigenicity, this region may correspond to the antigenic region observed by Petterson et al., which was preferential or specific for f-PSA *[24]*. In that report, determination of f-PSA specificity was based on the sandwiching capabilities of the antibodies; according to the Western-blot data, all antibodies specific for f-PSA bound to nonlinear epitopes. However, 17–1A2 is also specific for f-PSA in sandwiching experiments with 16–3A2, 22–8A2, and 60–6A2, but this antibody reacts with PSA-ACT on Western blots and in EIA experiments in which PSA-ACT is captured by anti-PSA and anti-ACT polyclonal antibodies, while retaining a strong preference for f-PSA.

Analysis of the effects of mAb association on PSA enzymatic activity is an independent way of inferring additional information about the binding targets. Our data show that mAbs may differ in their specific binding targets, even when sandwich assays have shown that they bind to the same antigenic region. In a particularly interesting example, mAb 22–1A2 actually increases PSA enzymatic activity to >120% of that of the uncomplexed enzyme. This mAb does not sandwich with 22–2A3 or 22–8A2, of which 22–2A3 inhibits only ~20% of the PSA enzymatic activity and 22–8A2 inhibits some 60% of the activity. These results were replicated with various concentrations of mAb and incubation times, indicating that they were not due to artifacts related to incorrect concentrations of mAbs or to the presence of inactive mAb molecules in the preparation. All of these mAbs likely bind in proximity to each other near the active site, but the effects of their binding on protein function are dramatically different. mAb 22–8A2 could be sterically hindering the ACT binding site or occluding the binding pocket, or association of the mAb could be inducing slight changes in PSA tertiary structure that displace the groups necessary for its enzymatic activity.

mAbs that bind only to f-PSA might be expected to inhibit the association of ACT with PSA. However, our data show that inhibition of this association does not imply that the mAb is specific for f-PSA. Five mAbs from the panel inhibit PSA enzymatic activity; of these, four (15–19A2, 60–1A2, 60–6A2, 60–9A2) bind to f-PSA as well as to PSA-ACT. Two of these four (15–19A2, 60–6A2) were tested for the ability to interfere with PSA-ACT association, and both of them did so. Because PSA enzymatic activity is necessary for ACT binding, this phenomenon could be due either to steric hindrance of the ACT binding pocket or to conformational changes of PSA brought about by the interaction that interfere with the catalytic machinery. Thus association of certain mAbs with PSA abolishes ACT binding, yet these mAbs are capable of binding to the PSA-ACT complex. This confirms that the requirements for ACT association, which involves proper functioning of the PSA active site, are much more strict than for binding of mAbs.

The other two mAbs, 16–3A2 and 22–8A2, evaluated for inhibition of PSA-ACT association had different effects on PSA enzymatic activity. 16–3A2 actually enhanced the PSA hydrolytic rate by ~30–40%, whereas 22–8A2 decreased the activity by ~60%. A similar difference was seen in the results of the PSA-ACT formation experiments (see Fig. 6A), where the observed rate constant for the appearance of the PSA-ACT complex was ~60% higher in the presence of mAb 16–3A2 than in the reaction containing 22–8A2. This enhancement of PSA enzymatic activity is therefore seen in two independent types of reactions. The effect is probably not exerted on the product-off step, since what is being measured in the case of PSA-ACT complex formation is the appearance of the enzyme–ACT complex, a process that could not be hastened by increasing the dissociation rate. Our data are insufficient to determine whether 16–3A2 exerts its enhancement effect on the encounter step or on the chemical processes of the enzyme.

mAb 17–1A2 binds preferentially to f-PSA, and it inhibits >86% of PSA enzymatic activity. This mAb probably binds to a region of PSA that is near the site of ACT association. In studying the effect of 17–1A2 on ACT binding, we found no (17–1A2)-PSA-ACT complex, but the presence of the mAb was seen to retard the course of formation of PSA-ACT. The most likely explanation is that 17–1A2 inhibits the association of PSA with ACT, but because of the relatively weak interaction between 17–1A2 and PSA, ACT gradually replaces the mAb in the complex with PSA.

This is the first description of the specific regions of PSA involved in antibody association. Epitope mapping experiments with an anti-PSA polyclonal antibody have iden-

tified six strongly antigenic regions of PSA; these results are in agreement with published data. Inhibition of PSA enzymatic activity and (or) ACT association by anti-PSA mAbs cannot be used to determine whether the mAbs are specific for f-PSA. However, these data could serve as a starting point for a high-resolution study of the epitopes.

We hope that the localization and characterization of the association targets of anti-PSA antibodies will aid in classification and evaluation of new mAbs, and will lead to further structural insights into PSA-mAb complexes, as well as eventual development of more sensitive and specific assays for prostate cancer.

We thank James Stray for PSA purification. This work was funded by the Richard M. Lucas Foundation, a George M. O'Brien Center Award from NIDDK (1 P50 DK/CA47656–03), and the Department of Veterans Affairs. Abbott Laboratories kindly provided the synthetic peptides. We thank Paul Lange, principal investigator of the George M. O'Brien Award and chairman of the Urology Department, for his support.

References

1. McCormack RT, Rittenhouse HG, Finlay JA, Sokoloff RL, et al. Molecular forms of prostate-specific antigen and human kallikrein gene family: a new era. Urology 1995;45:729–44.
2. Hopp TP, Woods KR. A computer program for predicting protein antigenic determinants. Mol Immunol 1983;20:483–9.
3. Zanelli IM, Rafferty B, Apostolou B. Large scale screening program for selection of antisera for radioimmunoassay of human parathyroid hormone. J Immunoassay 1983;4:175–206.
4. Rawling EG, Martin NL, Hancock RE. Epitope mapping of the *Pseudomonas aeruginosa* major outer membrane porin protein OprF. Infect Immun 1995;63:38–42.
5. Kessler MM, Zhelkovsky AM, Skvorak A, Moore CL. Monoclonal antibodies to yeast poly(A) polymerase (PAP) provide evidence for association of PAP with cleavage factor I. Biochemistry 1995;34:1750–9.
6. Kelleher M, Curtis JM, Sacks DL, Handman E, Bacic A. Epitope mapping of monoclonal antibodies directed against lipophosphoglycan of *Leishmania major* promastigotes. Mol Biol Parasitol 1994;66:187–200.
7. Van Duijnhoven HPL, Verschuren MCM, Timmer EDJ, Vissers PMAM, Groeneveld A, Ayoubi TAY, Van de Ven WJM. Application of recombinant DNA technology in epitope mapping and targeting. J Immunol Methods 1991;142:187–8.
8. Wang LF, Du-Plessis DH, White JR, Hyatt AD, Eaton BT. Use of a gene-targeted phage display random epitope library to map an antigenic determinant on the bluetongue virus outer capsid protein VP5. J Immunol Methods 1995;178:1–12.
9. Schllekens GA, Lasonder E, Feijlbrief M, Koedijk DG, Drijfhout JM, Scheffer AJ, et al. Identification of the core residue of the epitope of a monoclonal antibody raised against glycoprotein D of herpes simplex virus type 1 by screening of a random peptide library. Eur J Immunol 1994;24:3188–94.
10. Sioud M, Dybwad A, Jespersen L, Suleyman S, Natvig JB, Forre O. Characterization of naturally occurring autoantibodies against tumor necrosis factor-alpha (TNF-alpha): in vitro function and precise epitope mapping by phage epitope library. Clin Exp Immunol 1994;98:520–5.
11. Jorieux S, Gaucher C, Pietu G, Cherel G, Meyer D, Mazurier C. Fine epitope mapping of monoclonal antibodies to the NH2-terminal part of von Willebrand factor (vWF) by using recombinant and synthetic peptides: interest for the localization of the factor VIII binding domain. Br J Haematol 1994;87:113–8.
12. Zhao Y, Chait BT. Protein epitope mapping by mass spectrometry. Anal Chem 1994;66:3723–6.
13. Papac DI, Hoyes J, Tomer KB. Epitope mapping of the gastrin-releasing peptide/anti-bombesin monoclonal antibody complex by proteolysis followed by matrix-assisted laser desorption ionization mass spectrometry. Protein Sci 1994;3:1485–92.
14. Geysen HM, Rodda SJ, Mason TJ, Tribbick G, Schoofs PG. Strategies for epitope analysis using peptide synthesis. J Immunol Methods 1987;102:259–73.
15. Becker S, Armbruster, Muller B, Echner H, Kapurnotu A, Livaniou E, et al. Theoretical and experimental epitope mapping of thymosin beta-4. J Immunol Methods 1994;177:131–7.
16. Pollock JM, Douglas AJ, Mackie DP, Neill SD. Peptide mapping of bovine T-cell epitope for the 38 kDa tuberculosis antigen. Scand J Immunol 1995;41:85–93.
17. Stemmer C, Briand JP, Muller S. Mapping of linear epitopes of human histone H1 recognized by rabbit anti-H1/H5 antisera from autoimmune patients. Mol Immunol 1994;31:1037–46.
18. Kolesanova EF, Kozin SA, Lemeshko AO, Archakov AI. Epitope mapping of cytochrome P450 2B4 by peptide scanning. Biochem Mol Biol Int 1994;32:465–73.
19. Ricchiuti V, Briand JP, Meyer O, Isenberg DA, Pruijn G, Muller S. Epitope mapping with synthetic peptides of 52-kDa SSa/Ro protein reveals heterogeneous antibody profiles in human autoimmune sera. Clin Exp Immunol 1994;95:397–407.
20. Lilja H, Christensson A, Dahlén U, Matikainen MT, Nilsson O, Pettersson K, Lövgren T. Prostate-specific antigen in serum occurs predominantly in complex with α_1-antichymotrypsin. Clin Chem 1991;37:1618–25.
21. Christensson A, Bjorg T, Nilsson O, Dahlén U, Matikainen MT, Cockett AT, et al. Serum prostate-specific antigen complexed to α_1-antichymotrypsin as an indicator of prostate cancer. J Urol 1993;150:100–5.
22. Sensabaugh GF, Blake ET. Seminal plasma protein p30: simplified purification and evidence for identity with prostate specific antigen. J Urol 1990;144:1523–6.
23. Corey E, Wegner SK, Stray JE, Corey MJ, Arfman E, Lange PH, Vessella RL. Characterization of ten new monoclonal antibodies against prostate-specific antigen by analysis of affinity, specificity and function in sandwich assays. Int J Cancer 1997; in press.
24. Pettersson K, Piironen T, Seppala M, Liukkonen L, Christensson A, Matikainen MT, et al. Free and complexed prostate-specific antigen (PSA): in vitro stability, epitope map, and development of immunofluorometric assays for specific and sensitive detection of free PSA and PSA–α_1-antichymotrypsin complex. Clin Chem 1995;41:1480–8.
25. Frankel A, Rouse RV, Wang MC, Chu TM, Herzenberg LA. Monoclonal antibodies to a human prostate antigen. Cancer Res 1982;42:3714–8.
26. Laver WG, Air GM, Webster RG, Smith-Gill SJ. Epitopes on protein antigens: misconceptions and realities. Cell 1990;61:553–6.
27. Smith-Gill SJ. Protein epitopes: functional vs. structural definitions. Res Immunol 1994;145:67–70.
28. Padlan EA, Silverton EW, Sheriff S, Cohen GH, Smith-Gill SJ, Davies DR. Structure of an antibody–antigen complex: crystal structure of the HyHEL-10 Fab-lysozyme complex. Proc Natl Acad Sci U S A 1989;86:5938–42.
29. Braden BC, Fields BA, Ysern X, Goldbaum FA, Dall'Acqua W, Swarz

FP, et al. Crystal structure of the variable domain of antibody D1.3 and turkey egg white lysozyme: a novel conformational change in antibody CDR-L3 selects for antigen. J Mol Biol 1996;257:889–94.

30. Bossart-Whitaker P, Chang CY, Novotny J, Benjamin DC, Sheriff S. The crystal structure of the antibody N10-staphylococcal nuclease complex at 2.9A resolution. J Mol Biol 1995;253:559–75.

31. Malby RL, Tulip WR, Harley VR, McKimm-Breschkin JL, Laver WG, Webster RG, Colman PM. The structure of a complex between the NC10 antibody and influenza virus neuraminidase and comparison with the overlapping site of the NC41 antibody. Structure 1994;2:733–46.

32. Bentley GA, Boulot G, Riottot MM, Poljak RJ. Three-dimensional structure of an idiotope–anti-idiotope complex. Nature 1990;348:254–7.

33. Pinpoints: mimotopes & peptide technology. Victoria, Australia: Newsletter of Chiron Mimotopes, September 1993.