Protein Science (1998), 7:259–269. Cambridge University Press. Printed in the USA.

Determination and analysis of antigenic epitopes of prostate specific antigen (PSA) and human glandular kallikrein 2 (hK2) using synthetic peptides and computer modeling

TIMO PIIRONEN,[1] BRUNO O. VILLOUTREIX,[2] CHARLOTTE BECKER,[2] KELLY HOLLINGSWORTH,[1] MAUNO VIHINEN,[1] DOMINIQUE BRIDON,[3] XIAOXING QIU,[3] JOHN RAPP,[3] BARRY DOWELL,[3] TIMO LÖVGREN,[1] KIM PETTERSSON,[1] AND HANS LILJA[2]

[1]Department of Biotechnology, University of Turku, Turku, Finland
[2]Department of Clinical Chemistry, Lund University, University Hospital, Malmö, Sweden
[3]Diagnostic Division, Abbott Laboratories, Abbott Park, Illinois

(RECEIVED July 16, 1997; ACCEPTED October 23, 1997)

Abstract

Prostate specific antigen (PSA) and human glandular kallikrein 2 (hK2), produced essentially by the prostate gland, are 237-amino acid monomeric proteins, with 79% identity in primary structure. Twenty-five anti-PSA monoclonal antibodies (Mabs) were studied for binding to a large array of synthetic linear peptides selected from computer models of PSA and hK2, as well as to biotinylated peptides covering the entire PSA sequence.

Sixteen of the Mabs were bound to linear peptides forming four independent binding regions (I–IV). Binding region I was localized to amino acid residues 1–13 (identical sequence for PSA and hK2), II (a and b) was localized to residues 53–64, III (a and b) was localized to residues 80–91 (= kallikrein loop), and IV was localized to residues 151–164. Mabs binding to regions I and IIa were reactive with free PSA, PSA-ACT complex, and with hK2; Mabs binding to regions IIb, IIIa, and IV were reactive with free PSA and PSA-ACT complex, but unreactive with hK2; Mabs binding to region IIIb detected free PSA only. All Mabs tested ($n = 7$) specific for free PSA reacted with kallikrein loop (binding region IIIb). The presence of Mabs interacting with binding region I did not inhibit the catalytic activity of PSA, whereas Mabs interacting with other binding regions inhibited the catalysis. Theoretical model structures of PSA, hK2, and the PSA-ACT complex were combined with the presented data to suggest an overall orientation of PSA with regard to ACT.

Keywords: alpha-1-antichymotrypsin; computer-aided molecular modeling; human glandular kallikrein; monoclonal antibodies; peptide mapping; prostate cancer; prostate specific antigen; serine protease; serpin

The incidence of prostate cancer has increased during the last decade mainly due to prolonged lifetime and increased screening. This fact underlines the need of improved diagnostic approaches and new treatments. One of the first steps for developing diagnostic tools of enhanced accuracy is to increase our fundamental knowledge about PSA, hK2, their different isoforms, serpin-complexed forms, and the way these molecules interact with monoclonal antibodies used in routine laboratory assays.

Prostate specific antigen (PSA or hK3), human glandular kallikrein 2 (hK2 or hGK-1), and tissue kallikrein (hK1) are structurally similar serine proteases comprising the three-member group of glandular kallikreins at the long arm of human chromosome 19 (Riegman et al., 1992). hK1 is expressed in nonprostatic tissues, mainly in kidney, pancreas, and salivary glands. PSA and hK2 are abundantly expressed in the prostate and the two proteins have been purified and characterized from seminal fluid (Wang et al., 1979; Deperthes et al., 1995). The mature forms of PSA (28.4 kDa) and hK2 are monomeric polypeptides of 237 amino acid residues sharing 79% identity in primary structure. Each kallikrein carries a single Asn-linked carbohydrate chain, although at different locations (Lundwall & Lilja, 1987; Schedlich et al., 1987; Bélanger et al., 1995).

PSA has been shown to possess chymotrypsin-like protease activity (Lilja et al., 1989; Christensson et al., 1990). The active single-chain form of PSA forms stable covalent complexes with several extracellular protease inhibitors, such as α_1-antichymotrypsin (ACT), α_2-macroglobulin (AMG), pregnancy-zone protein (PZP), protein C inhibitor (PCI), and α_1-antitrypsin (Christensson et al., 1990; Espanã et al., 1991; Stenman et al., 1991; Christensson & Lilja, 1994; Zhang et al., 1997). PSA-ACT complex is the major

Reprint requests to: Timo Piironen, Department of Biotechnology, University of Turku, BioCity 6th floor, Tykistökatu 6A, FIN-20520 Turku, Finland; e-mail: timo.piironen@utu.fi.

molecular form in serum, whereas a minor proportion occurs in a free noncomplexed form, despite the high concentrations of ACT and AMG in blood (Lilja et al., 1991; Stenman et al., 1991). PSA-AMG complexes are not recognized because the immunodetection of PSA is sterically shielded by the complex formation with AMG. By contrast, several independent antigenic epitopes remain exposed on the PSA moiety of the PSA-ACT complex (Pettersson et al., 1995). Elevated serum concentrations of total PSA (>4 μg/L), as determined by immunoassays, result from various prostatic diseases, especially in the cancer of the prostate (CAP). The diagnostic potential of total PSA (PSA-T) measurements is limited by the fact that PSA-T concentrations are also increased in many subjects with benign prostatic hyperplasia (BPH) (Stamey et al., 1987; Hudson et al., 1989; Catalona et al., 1991). However, recent studies have shown that the proportion of PSA-F to PSA-ACT complexes in serum is significantly higher in BPH than in CAP (Stenman et al., 1991; Christensson et al., 1993).

The physiological function of hK2 is not yet known, but the primary structure of hK2 suggests it is likely to possess a trypsin-like catalytic activity (Schedlich et al., 1987). Despite high (10–50%) mRNA levels of hK2 relative to those of PSA in prostatic tissue (Chapdelaine et al., 1988; Henttu et al., 1990; Young et al., 1992), the hK2 concentration found in seminal fluid was 0.1–1% of the PSA concentration (Deperthes et al., 1995). Correspondingly, hK2 concentrations compared to PSA were found to be less than 2% in 80% of the total 334 clinically nondefined serum samples (Piironen et al., 1996).

Purified PSA and recombinantly produced PSA and hK2 have been used for defining the antigenic epitopes recognized by monoclonal anti-PSA antibodies (Lövgren et al., 1995; Pettersson et al., 1995; Eerola et al., 1997). Antigenic epitopes accessible only on free PSA have been shown to be unique to PSA alone, i.e., they do not crossreact with hK2. By contrast, some of the anti-PSA Mabs reactive with total PSA (free and complexed PSA) also recognized hK2 with about the same affinity. The knowledge of antibody specificities has lead to the development of a highly specific dual assay for PSA-F and PSA-T (Mitrunen et al., 1995), a PSA-ACT assay (Oesterling et al., 1995; Björk et al., 1996), and an hK2 assay (Piironen et al., 1996).

It has been suggested that serpins bind to their proteinase targets via formation of a reversible complex, referred to as Michaelis complex (Lawrence, 1997). The enzymes then cleave the P1-P'1 peptide bond of the serpin reactive loop, resulting in the formation of a covalent acyl–enzyme intermediate. This cleavage is followed by the insertion of the reactive loop into the β-sheet A. Because the loop is covalently linked to the enzyme, it seems that the proteinase could move along together with the loop to a new position on the serpin molecule with respect to the expected initial Michaelis-like orientation (Stratikos & Gettins, 1997; Wilczynska et al., 1997). This stable serpin–protease molecular form is often termed stable acyl–enzyme complex.

Recently, several model structures have been presented for PSA, hK2, and the PSA-ACT complex (Vihinen, 1994; Villoutreix et al., 1994, 1996; Bridon & Dowell, 1995) using the comparative model building method (Greer, 1990). In such model structures, the overall conformation of the molecule is likely to be represented very accurately. The modeling of the PSA-ACT Michaelis-like complex (Villoutreix et al., 1996) has been challenging because no detailed experimental structural information has been reported so far. This model can therefore only be used as a crude guideline for specific residue–residue interactions.

A detailed knowledge about the anti-PSA antibody epitopes should lead to a better understanding of presently employed assays. Such data should also help in improving the standardization of this important marker of prostate cancer.

In this study, we have characterized the binding properties of 25 anti-PSA Mabs for binding to a large array of synthetic linear PSA or hK2 peptides. Results were integrated with the data obtained from sandwich binding studies (Lövgren et al., 1995; Pettersson et al., 1995; Eerola et al., 1997), computer models of PSA, hK2, and the PSA-ACT Michaelis-like complex (Villoutreix et al., 1996), and studies of PSA catalytic activity in the presence of selected Mabs. The experimental results were used to evaluate a model of the stable PSA-ACT acyl–enzyme complex.

Results

Peptide inhibition of anti-PSA Mab association with PSA

The binding of 15 Mabs to purified PSA was significantly and reproducibly inhibited by eight of the selected 25 peptides listed in Table 1. Based on these results (Table 2), four separate binding regions were discerned. At binding region I, peptide P1ph inhibited Mabs 3C1, 4H5, and 5F11. At binding region II, peptides P5p and P6p inhibited Mabs H164 and 2C1. At binding region III, peptide P9p inhibited Mabs 5A10, 9B10, 6, 30, 25, 2E9, and H68; peptide P11p inhibited Mabs 9B10, 2H12, 6, 30, 19, 25, 2E9, and H68; and peptide P12p inhibited Mabs 9B10, 2H12, 6, 30, and 25. At binding region IV, peptide P19p inhibited Mab 10. These data were in agreement with Mab specificity and sandwiching characteristics.

Table 1. *List of selected linear peptides P1–P25*[a]

Peptide	Amino acid residues	Representing
P1ph	1–13	PSA and hK2
P2p	2–15	PSA
P3p	10–25	PSA
P4h	41–51	hK2
P5p	50–64	PSA
P6p	53–67	PSA
P7p	69–79	PSA
P8p	69–83	PSA
P9p	79–93	PSA
P10h	80–91	hK2
P11p	80–91	PSA
P12p	84–93	PSA
P13p	103–114	PSA
P14p	114–128	PSA
P15p	133–147	PSA
P16p	136–146	PSA
P17h	140–146	hK2
P18p	151–160	PSA
P19p	151–167	PSA
P20h	158–171	hK2
P21p	158–171	PSA
P22p	161–173	PSA
P23p	203–219	PSA
P24p	224–237	PSA
P25h	230–237	hK2

[a]p, PSA sequence; h, hK2 sequence; ph, shared PSA and hK2 sequence.

Table 2. *Inhibition (%) of anti-PSA Mab immunoreactivity by linear peptides P1–P25*[a]

Mab	Specificity of Mab	Epitope	Reg. I P1ph	Reg. II P5p	Reg. II P6p	P7p	Reg. III P9p	Reg. III P11p	Reg. III P12p	Reg. IV P19p	Baseline mean	Baseline SD
5A10	Free PSA	dc	0	−1	−1	4	**18**	−21[b]	−1	1	1.2	2.2
9B10	Free PSA	dc	−4	3	0	3	**95**	**64**	**43**	−1	−1.0	2.6
2H12	Free PSA	dc	9	1	6	**57**	2	**17**	**36**	n.d.	1.0	5.3
6	Free PSA	dc	13	5	1	−1	**33**	**33**	**23**	2	4.6	4.8
30	Free PSA	dc	1	10	5	4	**57**	**20**	**51**	−1	4.2	3.8
19	Free PSA	dc	15	10	10	16	8	**34**	10	n.d.	8.1	5.4
25	Free PSA	dc	15	19	−4	25	**75**	**80**	**62**	9	7.4	8.8
2E9	Total PSA	c	−4	2	0	−1	**98**	**68**	−2	−1	0.1	2.4
H68	Total PSA	c	−1	0	1	4	**98**	**79**	−1	−2	1.0	2.2
36	Total PSA	c	−1	−11	−1	0	−6	−1	2	0	−1.7	4.4
2H11	Total PSA	dc	−3	−1	0	−2	−12	−4	−2	−3	−3.4	5.1
7H2	Total PSA	c	−5	−17	4	−5	−11	0	−1	−2	−3.8	5.6
9E8	Total PSA	c	−5	10	0	−6	8	−5	1	1	0.1	5.2
10	Total PSA	dc	−5	2	−2	−3	4	−2	2	**22**	−0.8	3.0
H164	Total PSA	dc	10	**88**	**85**	7	5	3	−2	−1	2.7	4.0
H117	Total PSA+hK2	c	−5	0	1	−4	0	−4	2	−6	−1.9	3.7
3C1	Total PSA+hK2	c	**13**	−10	−1	4	n.d.	−5	−1	−12	−1.4	4.5
4H5	Total PSA+hK2	c	**14**	−9	−2	3	n.d.	−4	3	−5	−2.2	4.9
5F11	Total PSA+hK2	n.d.	**19**	10	n.d.	n.d.	2	n.d.	0	−3	2.8	3.8
F5	Total PSA+hK2	c	−2	1	4	−2	7	−3	−2	−19	−1.1	5.0
H50	Total PSA+hK2	dc	−7	−7	0	−3	1	−1	3	4	−3.1	3.5
2C1	Total PSA+hK2	c	2	**92**	**97**	4	n.d.	5	15	13	3.4	7.9
H179	Total PSA+hK2	dc	−9	−3	4	−8	7	2	−5	−1	−4.2	4.4

[a]Inhibition by the positive peptides (shown in bold) was greater than the mean + 3 × SD of the baseline. Peptides P2–4, P8, P10, P13–18, P20–25 showed no significant inhibition of Mabs immunoreactivities. Mabs recognizing continuous (c) or discontinuous (dc) epitopes are indicated after Mab code. n.d., not determined.

[b]Addition of peptide significantly increased the immunoreactivity.

In contrast to this, the binding of Mab 2H12, which is specific for free PSA based on its sandwiching abilities, was inhibited not only by peptides of region III, but also by peptide P7p (amino acid residues 69–79).

Binding of anti-PSA Mabs to biotinylated peptides

Thirteen europium (Eu)-labeled Mabs were found to bind to biotinylated peptides immobilized to streptavidin-coated strips (Table 3). Mab 3C1 was bound to region I; Mabs H164, 2C1, and 2H12 were bound to region II; Mabs 5A10, 9B10, 2H12, 6, 30, 25, 2E9, and H68 were bound to region III; Mabs 10 and E86 were bound to region IV. These results were in agreement with the Mab specificity toward PSA and hK2, with the sandwiching characteristics of the Mabs as well as with the results from the inhibition study (above). However, the direct binding of Mab 2H12 was found not only to region III, but also to region II (amino acid residues 50–64).

The results from the inhibition and binding studies (Tables 2 and 3) were combined in order to investigate further the binding sites of the individual Mabs. At binding region I, Mabs 5F11, 4H5, and 3C1 were localized to interact at least with residues 1–13. Binding region II was localized to residues 53–64 due to the observed interaction with Mab H164 (residues 53–64) and Mab 2C1 (residues 55–64). At binding region III, Mab 19 was localized to residues 80–91; Mabs 5A10 and 2E9 to residues 82–89; Mab H68 to residues 82–87; Mabs 9B10 and 2H12 to residues 84–89; and Mabs 6, 30, and 25 to residues 84–88. At binding region IV, Mabs 10 and E86 were localized to residues 151–164. As a summary, binding region I involved amino acid residues 1–13; region II, residues 53–64; region III, residues 80–91 (= kallikrein loop); and region IV, residues 151–164.

Mab interference with catalytic activity of PSA and complexation between PSA and ACT

When preincubating PSA with Mabs, the catalytic activity of PSA toward a small chromogenic substrate decreased significantly (activity less than 25% of the control) with 13 of the 22 Mabs investigated (Table 4). Similarly, the same 13 Mabs and also Mab H68 showed significant inhibition when PSA-Mab complexes were reacted with ACT. As a summary, Mabs binding to regions II (H164, 2C1), III (5A10, 9B10, 2H12, 6, 30, 19, 2E9, H68), and IV (10) all inhibited the catalytic activity of PSA, whereas Mabs binding to region I (3C1, 4H5, 5F11) had no effect on the enzymatic properties of PSA. Additionally, Mabs 2H11, 7H2, and H50 also inhibited the catalytic activity of PSA, but the localization of these epitopes were not evident from the peptide mapping results. According to sandwiching studies, these Mabs were partly overlapping with regions II and IV.

Discussion

Sequence alignment of PSA and hK2

The overall sequence identity between PSA and hK2 is 79% and is especially high in the areas surrounding the catalytic triad and

Table 3. *Binding of Eu-labeled Mabs to biotinylated peptides*[a]

Mab	Specificity of Mab	Epitope	PSA residues 1–237[b] 15mers	Binding region	PSA residues 56–106[b] 8mers	Binding region
5A10	Free PSA	dc	75–89* and 80–94*	III	82–89*	III
9B10	Free PSA	dc	75–89*	III	82–89*	III
2H12	Free PSA	dc	50–64*** and 75–89*	II and III	n.s.b.	
6	Free PSA	dc	75–89*	III	81–88**, 82–89**, 83–90*	III
30	Free PSA	dc	75–89*** and 80–94***	III	81–88**, 82–89**, 83–90*	III
19	Free PSA	dc	n.s.b.		n.s.b.	
25	Free PSA	dc	75–89** and 80–94**	III	81–88***	III
2E9	Total PSA	c	75–89*** and 80–94***	III	82–89*	III
H68	Total PSA	c	75–89*** and 80–94*	III	80–87* and 82–89*	III
36	Total PSA	c	n.s.b.		n.s.b.	
2H11	Total PSA	dc	n.s.b.		n.s.b.	
7H2	Total PSA	c	n.s.b.		n.s.b.	
9E8	Total PSA	c	n.s.b.		n.s.b.	
10	Total PSA	dc	150–164*	IV	n.s.b.	
E86	Total PSA	dc	150–164**	IV	n.d.	
H164	Total PSA	dc	50–64***	II	56–63*	II
H117	Total PSA+hK2	c	n.s.b.		n.s.b.	
3C1	Total PSA+hK2	c	(−1)-14*	I	n.s.b.	
4H5	Total PSA+hK2	c	n.s.b.		n.s.b.	
5F11	Total PSA+hK2	n.d.	n.s.b.		n.s.b.	
F5	Total PSA+hK2	c	n.s.b.		n.s.b.	
H50	Total PSA+hK2	dc	n.s.b.		n.s.b.	
2C1	Total PSA+hK2	c	50–64*** and 55–69**	II	n.s.b.	
H179	Total PSA+hK2	dc	n.s.b.		n.s.b.	
66	Total PSA+hK2	dc	n.s.b.		n.s.b.	

[a]Binding was considered significant when signal was greater than 10 × SD of the background. Mabs recognizing continuous (c) or discontinuous (dc) epitopes are indicated after Mab code. n.d., not determined; n.s.b., no significant binding.
[b]* signal = 5,000–25,000 cps; ** signal = 25,000–100,000 cps; *** signal > 100,000 cps.

within the structurally conserved regions. The differences between PSA and hK2 are concentrated in surface loops, mainly located around the active site (Villoutreix et al., 1996). These observations suggest that some anti-PSA Mabs are likely to interact also with hK2 (cross reactivity) and that antibodies specific for PSA should interact with regions located in the loops surrounding the active site because they are rich in nonconservative amino acid substitutions when compared to hK2. The four independent binding regions found in this study are mapped onto the PSA and hK2 sequences (Fig. 1A). Furthermore, within the four binding regions, the colored residues represent the main binding sites for the Mab molecules as established in this study.

Epitope map of PSA and hK2

Results from peptide mapping, computer modeling, and previously published sandwich binding studies (Lövgren et al., 1995; Pettersson et al., 1995; Eerola et al., 1997) provided the data for detailed determination of anti-PSA Mab epitopes with PSA and hK2. Binding regions I–IV are shown in the PSA/hK2 epitope map where binding of the anti-PSA Mab is compared in relation to other Mabs by using sandwich binding studies (Fig. 1B). In total, 16 Mabs reacted with the peptides forming four independent binding regions (I–IV). Binding regions II and III were divided into two subclasses (a and b) according to Mab specificity. Mabs binding to region I and IIa recognized total PSA (= free PSA and PSA complexed to ACT or PCI) and total hK2 (= free hK2 and hK2 complexed to ACT or PCI). Mabs binding to region IIb, IIIa, and IV recognized total PSA, but not hK2. Mabs binding to region IIIb detected free PSA only. In our study, all Mabs specific for free PSA reacted with binding region IIIb.

Computer-aided analysis of PSA epitopes

The analysis was performed by comparing the results from this study to the data obtained from sandwich binding studies (above) and theoretical model structures for PSA, hK2, and PSA-ACT Michaelis-like complex (Villoutreix et al., 1996) and a preliminary model for the PSA-ACT stable acyl–enzyme complex was generated according to Wilczynska et al. (1997). In the following, we make the reasonable assumption that the stable PSA-ACT acyl–enzyme complex is the macromolecular system that reacts with the monoclonal antibodies, but comments will also be provided for the expected Michaelis-like complex structure.

Region I (Ile 1 [16][4] to Pro 13 [28])

As shown in our previous modeling study (Villoutreix et al., 1996), several residues in this area are fully accessible to the solvent, whether the protease is free or complexed to ACT within

Table 4. *Mab interference with catalytic activity of PSA (2–5-fold excess of Mabs) and complexation between PSA and ACT (10-fold excess of Mabs)*

Mab	Specificity of Mab	Catalytic activity with substrate (%)	Complexation with ACT (%)	Binding region
No Mab	—	100	100	—
5A10	Free PSA	0	0	IIIb
9B10	Free PSA	6	0	IIIb
2H12	Free PSA	0	0	IIIb
6	Free PSA	1	7	IIIb
30	Free PSA	3	9	IIIb
19	Free PSA	1	1	IIIb
25	Free PSA	n.d.	n.d.	IIIb
2E9	Total PSA	2	0	IIIa
H68	Total PSA	111*	21	IIIa
36	Total PSA	52	37	—
2H11	Total PSA	1	1	—
7H2	Total PSA	2	21	—
9E8	Total PSA	n.d.	n.d.	—
10	Total PSA	3	0	IV
E86	Total PSA	n.d.	n.d.	IV
H164	Total PSA	9	2	II
H117	Total PSA+hK2	166**	90	—
3C1	Total PSA+hK2	93	68	I
4H5	Total PSA+hK2	105	62	I
5F11	Total PSA+hK2	122	66	I
F5	Total PSA+hK2	88	88	—
H50	Total PSA+hK2	2	1	—
2C1	Total PSA+hK2	1	0	II
H179	Total PSA+hK2	44	242**	—
66	Total PSA+hK2	77	32	—

[a]*Not enough Mab to test >threefold excess; ** Mab significantly enhanced the enzymatic activity of PSA.

the stable acyl–enzyme form (Fig. 2A and B, inset, in green).[5] This also holds true if ACT was trapped into the PSA active site as shown in the Michaelis-like model structure (Fig. 2B). The most accessible residues are Gly 3 [18], Gly 4 [19], Trp 5 [20], Glu 6 [21], and a short stretch of polar and charged residues involving Glu 8 [23] to Ser 11 [26]. Gln 12 [27] is buried and hydrogen bonded mainly to the side chain of His 54 [71]. Pro 13 is also shielded from the solvent mainly by residues Ala 111 [117], Val 112 [118], Lys 113 [119], and Trp 14 [29]. Because PSA and hK2 have the same residues in region I and surrounding areas, and Ile 1 [16] plugs into the protein core, and Cys 7 [22] is bridged to Cys 149 [157], it is very likely that the two proteins here present with the same 3D conformation. This suggests that an antibody could associate with about the same affinity to PSA and hK2 and that an important part of the epitope involved some or all of the solvent-accessible residues noted above. The fact that peptide P1ph (residues 1–13) reacted with the Mabs 3C1, 4H5, and 5F11, and peptide P2p (residues 2–15) was unreactive with Mabs may result from different structural conformations of the peptides. Some peptides may adopt structures that are relatively different from the structure of the intact protein.

It was not surprising that all Mabs (3C1, 4H5, and 5F11) binding to peptides from this region recognize free and ACT-complexed PSA and hK2 equally well. According to the epitope map (Fig. 1B), Mabs H117 and F5 are also located very close to Mabs 3C1, 4H5, and 5F11. One of the five disulfide bridges is located in the middle of binding region I (Cys 7 bridged to Cys 149). Because Mabs H117 and F5 were found to recognize continuous epitopes (Table 3), it is possible that both Mabs bind to

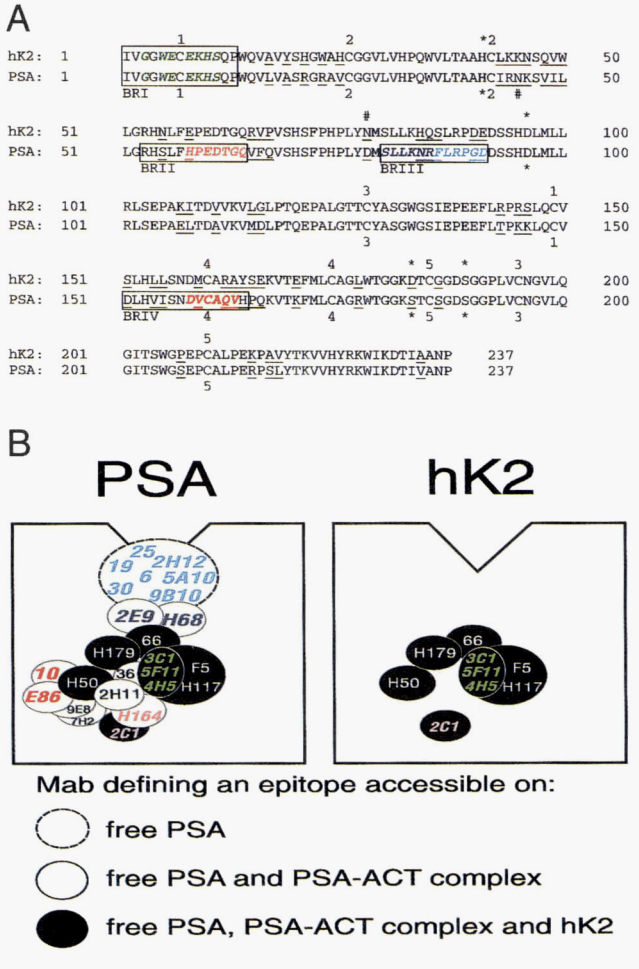


Fig. 1. A: Comparison of PSA and hK2 amino acid sequences showing four binding regions (BR). Amino acids (aa) presumably involved with the binding to Mabs are colored green in binding region I (aa 3, 5–6, 8–11), magenta in region II (aa 58–64), blue in region IIIa (aa 80–85), cyan in region IIIb (aa 86–91), and red in region IV (aa 158–163). Underlined residues are dissimilar between PSA and hK2. The * symbol indicates the catalytic triad residues (His 41 [57], Asp 96 [102], and Ser 189 [195]), as well as the residue at the bottom of the specificity pocket (Ser in PSA and Asp in hK2 183 [189]). The # symbols represent the glycosylation sites in PSA (Asp 45 [61]) and hK2 (Asp 78 [95]). Five disulfide bridges are shown with corresponding numbers at Cys residues. **B:** Epitope map of PSA and hK2 showing the relation of 25 anti-PSA Mabs, of which 15 reacted with linear peptides (colored Mabs). Overlapping circles indicate no possible sandwich formation. Touching circles indicate some interference. Separate circles indicate independent epitopes. Mabs binding to region I are shown in green; to region II in magenta; to region IIIa in blue; to region IIIb in cyan; and to region IV in red.

[4]The chymotrypsinogen nomenclature is indicated between the brackets whenever appropriate (Bode et al., 1989).

[5]In Villoutreix et al. (1996), the # symbol above Trp 5 [20] in Figure 1 is a typographic error and should be @, for solvent-accessible residue, as can be deduced easily therein from Figures 2 and 4.

a linear region in the vicinity of Cys 149 and thereby are not able to bind to PSA nor hK2 in the presence of Mabs 3C1, 4H5, and 5F11.

Region II (Arg 53 [70] to Gln 64 [81])

This area (Fig. 2A and B, in magenta) corresponds approximately to loop 3 in Villoutreix et al. (1996). Many side chains are solvent accessible and the ones that are most exposed on the surface of PSA run from His 58 [75] to Gln 64 [81]. Residues Arg 53 [70] to Phe 57 [74] are more shielded from the solvent, especially when PSA is complexed to ACT in the Michaelis-like model, whereas residues should be exposed in the stable acyl–enzyme complex (Fig. 2B, main figure and inset). Region II has some interactions with region I, in the model structure, for example, His 54 [71] with Glu 6 [21]. However, residues like Pro 59 [76] and Asp 61 [78] protrude more outside the molecule and are not involved in any direct interaction with other surrounding loops. It is possible that, due to steric hindrance, when an antibody directed against region I is present, another antibody specific for region II may not be able to interact appropriately with PSA. The residues most exposed are almost identical between PSA and hK2 and cross reactivity could be expected, unless the antibodies recognize other surrounding side chains, where PSA and hK2 differ slightly. For example, PSA displays His 58 [75] in region II and on the adjacent loop Lys 145 [153] and 146 [154], whereas hK2 has Glu 58 [75] interacting with Arg 145 [153] and Ser at position 146 [154]. When comparing the PSA and hK2 model structures, only few structural differences are expected in this area of the molecules, possibly as in the other serine protease X-ray structures that we have analyzed. Nevertheless, these small structural changes can certainly play a role for antibody recognition together with some side-chain replacements.

Two Mabs were bound to this region, of which Mab H164 recognized total PSA, and Mab 2C1 recognized total PSA and hK2. Steric hindrance between regions I and II was detected in the experimental sandwiching results when using Mab H164 in combination with Mabs from region I (touching epitopes in Fig. 1B).

Region III (Ser 80 [95b] to Asp 91 [97])

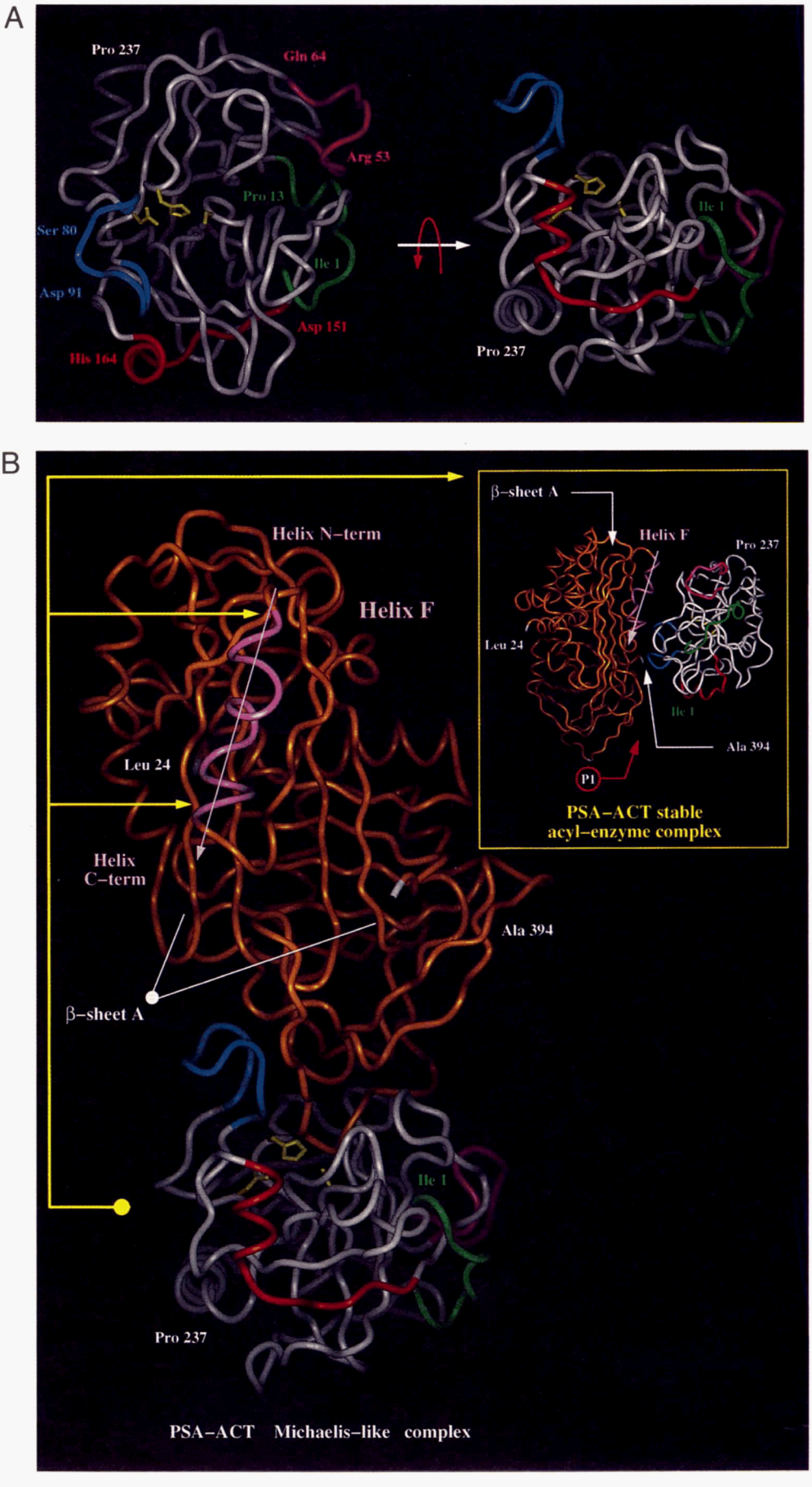
This area corresponds to loop 4 in Villoutreix et al. (1996). This loop shows an insertion of about 10 residues when compared to the serine protease family of enzymes. It is often referred to as the "kallikrein loop." In building theoretical models, large insertions tend to be difficult to predict. The conformation of this loop is thus still speculative. Most of the residues in this loop are solvent exposed (Fig. 2A, in blue), but several become shielded from the solvent after Michaelis-like complex formation with ACT (Fig. 2B, blue). In fact, in this orientation, residues Leu 87 [95i] to Asp 91 [97] pack closely against the ACT molecule (Fig. 2B). In the stable acyl–enzyme complex (Fig. 2B, inset), as well as in the Michaelis-like model, the side chain of Asn 84 [95f] or Arg 85 [95g] can be accessible to the solvent despite the presence of ACT. The overall shielding of most of this loop in the PSA-ACT model structures seems to be in good agreement with the experimental data (see below). Due to amino acid differences in this region between PSA and hK2, and because this loop protrudes significantly outside the active site area (i.e., no constraints from the direct surrounding are expected to occur and therefore part of the loop is likely to be flexible), it is expected that the two molecules possess a different conformation for the "kallikrein loop" region. The base of this loop has been suggested to form part of the binding site for the inhibitory zinc ion (Villoutreix et al., 1994). It would be interesting to investigate whether or not the antibodies specific for this region still recognize PSA in the presence of zinc. This would indicate whether some important structural changes take place upon binding of the ion. This could in turn verify the hypothesis that the base of this loop is the physiological binding site for zinc.

According to our modeling study, residues 75–82 could still be accessible to an antibody in the PSA-ACT Michaelis-like and acyl–enzyme complexes. However, in both cases, after residues 83–85, the loop seems to be directly in contact with the serpin. This would indicate that this part of the loop should no longer be accessible to an antibody when ACT is present. All Mabs specific for free PSA ($n = 7$) recognized peptides from the region III. Additionally, two Mabs recognizing total PSA (Mabs 2E9 and H68) were bound to the same region. However, Mabs 2E9 and H68 did not bind to peptide P12p (residues 84–93), suggesting that they could interact mainly with residues 80–83. Therefore, the region III can be separated in two subgroups according to different specificity for PSA-T (region IIIa at residues 80–83) and for PSA-F (region IIIb at residues 84–91). None of the Mabs in this region could form sandwich with PSA and another Mab from this group. In addition, none of these antibodies crossreacted with hK2, which is in agreement with the low sequence identity between PSA and hK2 in this region. The fact that two Mabs recognized region III within the PSA-ACT stable acyl–enzyme complex may imply that the serpin could adopt an overall orientation with respect to PSA similar to the one shown in the inset of Figure 2B.

Region IV (Asp 151 [159] to His 164 [172])

The end of this region reaches loop 6 (residues 164 [172]-169 [177]), which seems to have few contacts with ACT within the expected Michaelis-like complex (Villoutreix et al., 1996). Many

Fig. 2. (*facing page*) **A:** Ribbon representation of the PSA model structure. The view is down the active site (left) and rotated (right) to facilitate the reading of the figure. The catalytic triad in both diagrams involves, from left to right, Asp 96 [102], His 41 [57], and Ser 189 [195]. Region I (Ile 1 [16] to Pro 13 [28]) is shown in green, region II (Arg 53 [70] to Gln 64 [81]) in magenta, region III (Ser 80 [95b] to Asp 91 [97]) in blue, and region IV (Asp 151 [159] to His 164 [172]) in red. **B:** Ribbon representation of the PSA-ACT Michaelis-like (main figure) and stable acyl–enzyme (inset) model complexes. The orientation is similar to the one used for part A (right) for the main figure and rotated in the inset to facilitate the reading. Color code for PSA was kept as in part A and ACT is painted in orange. N-terminal and C-terminal residues of PSA and ACT are labeled as well as the serpin β-sheet A and helix F, expected to have interactions with the enzyme. According to Wilczynska et al. (1997), after formation of the covalent bond between the serpin and protease, the enzyme would re-orient on the surface of the serpin and come in direct vicinity of helix F. Their proposed orientation is reproduced in the inset. ACT is in the expected active conformation in this preliminary model (the reactive loop is exposed), whereas PSA was translated to the new position. The P1 residue of ACT is shown and the direction that the loop should follow after cleavage by protease is indicated by a red arrow.


A
Pro 237
Gln 64
Arg 53
Pro 13
Ser 80
Ile 1
Asp 91
Asp 151
His 164
Pro 237
Ile 1
B
β–sheet A
Helix F
Pro 237
Leu 24
Ile 1
Ala 394
P1
PSA–ACT stable
acyl–enzyme complex
Helix N–term
Helix F
Leu 24
Helix
C–term
Ala 394
β–sheet A
Ile 1
Pro 237
PSA–ACT Michaelis–like complex

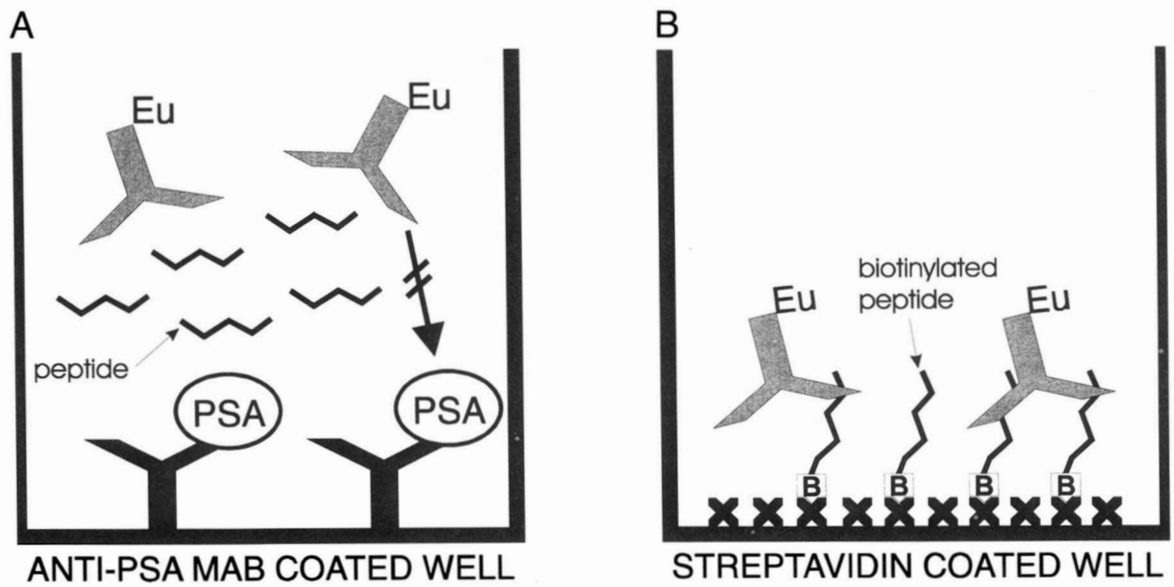


Fig. 3. Assay designs of inhibition (**A**) and direct binding protocols (**B**).

residues from region IV are solvent exposed and should be equally accessible to an antibody whether or not the molecule is free or in the stable acyl–enzyme complex with ACT (Fig. 2A and B, inset, in red). From about Asp 158 [166] to Val 163 [171], PSA adopts a helical structure held in position via a disulfide bridge (Cys 160 [168]–Cys 174 [182]). Although this region does not display insertion or deletion within the multiple sequence alignment of eight serine proteases, their 3D structures show some interesting differences (Villoutreix et al., 1996). This data stresses the importance of loop 6 for the interaction between a protease and a serpin molecule or other substrates/inhibitors. This information implies that antibody directed toward the region of loop 6 of PSA may not recognize very specifically hK2 (and vice versa). Also, the difference in sequence for the most solvent-exposed residues may play a role in the antibody discrimination of PSA from hK2.

Mabs 10 and E86 were bound to this region recognizing total PSA but not hK2. This is in agreement with our analysis of several serine protease X-ray structures, which shows that these enzymes present with a local conformational change in this area. This is certainly the case when comparing PSA and hK2.

Anomalous data

Two antibodies presented with unexpected binding properties. Mab 2H12, specific for free PSA, was bound to binding region III, but additionally also to two distinct peptides, at residues 50–64 (Mab 2H12 bound to the biotinylated peptide) and at residues 69–79 (Mab 2H12 inhibited by the peptide P7p). According to the PSA model, parts of the residues 69–79 (peptide P7p) and binding region III (peptides P11-12p, residues 80–91) are in close vicinity within the 3D structure, thus possibly explaining the observed inhibition, whereas residues 50–64 (binding region II) and residues 80–91 (binding region III) are very distant. Therefore, in the latter case, it could be possible that these peptides, located far away, could represent the so-called mimotopes, epitopes sharing similar "footprints" recognized by the Mab 2H12 paratope. Furthermore, Mab 2H12 may also consist of two clones, one binding to region II and the other one binding to region III. Another anomalous result was seen with Mab 5A10, also specific for free PSA. The addition of peptide P11p (binding region III) significantly increased the immunoreactivity of Mab 5A10 in the inhibition study (Table 2).

Antigenic determinants for antibodies can be divided into continuous (linear) and discontinuous (nonlinear) epitopes. Discontinuous epitopes may also consist of two or more continuous parts brought into proximity when the protein is in the native form. It is possible that all protein epitopes are discontinuous to various degrees. The overall binding affinity can result either from the contribution of numerous contacts (set of 15–20 or more interacting residues) or is primarily a function of a few critical amino acid side chains. It is likely that both possibilities occur. Binding domains that depend on the 3D structure can hardly be displayed by the relatively short synthetic peptides. However, it might be expected that peptides that represent one continuous part of the contact region would also bind to the anti-PSA antibody, although less strongly than the intact PSA. Because the continuous/discontinuous epitope data for Mabs were available for 24 of the 25 Mabs studied through western blotting studies with SDS-PAGE and native PAGE-treated PSA and PSA-ACT samples (Pettersson et al., 1995; Nilsson et al., 1997), the comparison between continuous and discontinuous epitopes revealed that 5 (Mabs 2E9, H68, 3C1, 4H5, and 2C1) of the total 10 Mabs determined to bind to continuous epitopes were bound to peptides in this study (Tables 2 and 3). On the other hand, 10 Mabs (5A10, 9B10, 2H12, 6, 30, 10, E86, and H164) of the total 14 Mabs determined to bind to discontinuous epitopes were bound to the peptides. Apparently, Mabs that did not react with peptides, but were bound to continuous epitopes according to western blotting results, recognized linear structures not presented correctly by the collection of selected peptides and biotinylated 15-mers used in this study.

Mab interference with catalytic activity of PSA and complexation between PSA and ACT

In order to probe further the PSA-Mab interaction, the catalytic activity of PSA toward a small substrate within the PSA-Mab complex as well as PSA-Mab interference in complexation studies with ACT were analyzed (Table 4). Results from these independent activity studies were similar, i.e., if Mab inhibited the catalytic activity, it also interfered with the complexation between PSA and ACT.

Region I

Mabs (3C1, 4H5, 5F11) interacting with the binding region I did not inhibit the enzymatic activity of PSA. This is consistent with the PSA model structure shown in Figure 2A. The antibodies most likely approach PSA in such a way that the catalytic cleft is still fully accessible to a small substrate or the serpin molecule. Interestingly, Mab H117, bound close to region I, seems to increase significantly the catalytic activity of PSA. This increased activity can be the result of either (or both) an improved binding of the substrate in the catalytic cleft or of subtle changes within the active site region leading to a difference in the activation energy (lower) for the reaction when compared to free PSA alone. In any case, the increased catalytic activity of PSA suggests that this region of the protein is important for the function.

Region II

Mabs 2C1 and H164 of binding region II both inhibited the enzymatic activity of PSA. According to the PSA model, it would be expected that a small substrate could still be cleaved by the enzyme because region II is relatively distant from the active site triad. This is further expected because these two antibodies recognize the PSA-ACT acyl–enzyme complex. According to Figure 2B inset, the Mabs should approach PSA from the side and let the active site free because the serpin is already present there. A possible explanation here is that, in absence of ACT, the antibody gets tilted toward the catalytic triad area, disturbing the binding of the small substrate and serpin molecule. This hypothesis is supported by the fact that Mab 2C1 totally inhibits complex formation with ACT.

Region III

Mabs 2E9 and H68 interacting with the binding region IIIa inhibited the catalytic activity of PSA, although high (5–10-fold) molar excess of Mabs was needed for efficient inhibition. Threefold excess (highest amount tested with the small substrate) of Mab H68 did not show inhibition of PSA activity toward the small substrate. Similarly, twofold excess of Mab H68 was not enough for efficient inhibition of the PSA-ACT complex formation (data not shown). Only 10-fold molar excess of Mab H68 significantly blocked the complex formation. This suggests that these antibodies are partially covering the active site. This is in line with the fact that these Mabs recognize also the PSA-ACT complex, but with an approximately 10-fold lower affinity compared to free PSA (Pettersson et al., 1995 and unpubl. data). Mabs 5A10, 9B10, 2H12, 6, 30, and 19, interacting with the binding region IIIb, recognized only free PSA. They all inhibited the catalytic activity of PSA. Therefore, these antibodies most likely cover the active site of PSA.

Region IV

Mab 10 interacting with the binding region III also inhibited the catalytic activity of PSA. Due to the location of the epitope on the PSA surface (Fig. 2A), this result is somewhat surprising because it seems likely that the antibody would bind to PSA from a direction that would leave the active site free. Thus, PSA should still present amidolytic activity toward a small substrate. This would be further expected because Mab 10 recognizes total PSA. Possibly, the conformation of the active site cleft, with regard to the catalytic activity, is altered.

Overall orientation of the PSA-ACT complex

The above data allow us to suggest an overall orientation of ACT within the active site of PSA in the Michaelis-like complex and for the stable acyl–enzyme form. For instance, it has been shown that Mab 2E9 (interacting with region III, kallikrein loop) has reduced affinity for the stable acyl–enzyme PSA-ACT complex when compared to free PSA alone. Most likely, the kallikrein loop of PSA has some interaction with ACT, thus limiting the access of the Mabs to PSA. In addition, because H68 does not disturb the catalytic activity of PSA and that it does not inhibit totally the PSA-ACT complex formation, it seems possible that the serpin plugs into the active site of PSA in a fashion that resembles the model shown in Figure 2B (main figure). Results from Mabs 5A10 and 9B10, taken together with the data of Mabs 2E9 and H68, are consistent with the orientation of Figure 2B, main figure, and of Figure 2B, inset, as proposed by Wilczynska et al. (1997). Our results indicate that the kallikrein loop of PSA should play an important role in the interaction with ACT.

The location and characterization of the four binding regions found in this study have revealed some common aspects of the immune response at the molecular level and could be important in designing novel anti-PSA or anti-hK2 antibodies. As a summary, the precise location of PSA or hK2 epitopes was determined for a majority of the Mabs through peptide mapping. Four binding regions were found to react with 16 of the 25 Mabs analyzed, representing specificities for PSA-F, PSA-T, and PSA-T+hK2. This detailed information of antigenic determinants may enable the design of PSA and hK2 immunoassays with well understood and predefined specificities. These data can be used also in future studies, including site-directed mutagenesis of residues that influence the enzymatic activity of PSA. Additional work is needed in order to closely determine the overall orientation of PSA–serpin complexes, but a few key experiments can be designed based on the present investigation to probe further the very complicated serpin–proteinase set of interactions.

Materials and methods

Assay reagents and instrumentation

Microtitration wells coated with anti-PSA MAb H117, anti-PSA Mab 2E9, or streptavidin, as well as DELFIA® Eu/Sm-Labeling kit, DELFIA 1234 Plate Fluorometer, DELFIA Assay Buffer, DELFIA Wash Solution, and DELFIA Enhancement Solution for the immunofluorometric assays were from Wallac Oy (Turku, Finland).

Purified proteins

Purified PSA from seminal plasma (containing ~85% of the catalytically active single-chain form and ~15% of the inactive two-chain form was generated, purified, and stored as reported previously

(Christensson et al., 1990; Pettersson et al., 1995). Recombinant baculovirus PSA and hK2 were produced as reported previously (Rajakoski et al., 1997). ACT was purified from human blood plasma as described previously (Christensson et al., 1990).

Anti-PSA monoclonal antibodies

Purified preparations of Mabs 5A10, 2E9, and 2H11 were provided by Wallac Oy. The development and characterization of our in-house Mabs 9B10, 2H12, 7H2, 9E8, 3C1, 4H5, 5F11, and 2C1 have been described earlier (Lilja et al., 1991; Pettersson et al., 1995). Mabs H68, H164, H117, F5, H50, and H179 were provided by Abbott Laboratories (Abbott Park, Illinois, USA). Mabs 6, 30, 19, 25, 36, 10, and 66 were kindly donated by Dr. Olle Nilsson (CanAg, Göteborg, Sweden) and characterized recently (Nilsson et al., 1997). Mab E86 was kindly donated by Dr. Elisabeth Paus (Central Laboratory, The Norwegian Radium Hospital, Oslo, Norway). The reactivities of anti-PSA MAbs with PSA and hK2 have been described earlier (Lövgren et al., 1995; Eerola et al., 1997). MAbs 5A10, 9B10, 2H12, 6, 30, 19, and 25 recognize only free PSA, showing no crossreactivity with hK2; MAbs 2E9, H68, 36, 2H11, 7H2, 9E8, 10, and H164 recognize free PSA and PSA-ACT complexes, showing no crossreactivity with hK2; MAbs H117, 3C1, 4H5, 5F11, F5, H50, 2C1, and H179 recognize free PSA, free hK2, PSA-ACT, and hK2-ACT complexes (Fig. 1B). MAbs were labeled with 4–7 molecules of Eu/IgG according to the instructions of the DELFIA Eu-labeling kit.

Synthetic peptides

The potential immunoreactive peptides were designed based on a molecular model of PSA and amino acid sequence alignment of PSA and hK2 (Vihinen, 1994; Bridon & Dowell, 1995). Generally, according to the models, designed peptide sequences were surface accessible on the PSA molecule. Peptides P1, P4, P6–7, P10–11, P16–18, P20–21, and P24–25 were from Department of Clinical Chemistry, Lund University, University Hospital, Malmö, Sweden. Peptides P2-3, P5, P8–9, P12–15, P19, and P22–23 were from Abbott Laboratories. Selected peptide sequences are shown in Table 1.

Selected peptides were synthesized by the stepwise solid-phase method using standard Fmoc chemistry. Peptides were cleaved, deprotected, and finally purified by reverse-phase HPLC. The homogeneity and identity of the purified peptides were confirmed by electrospray mass spectrometry. All peptides were determined to be at least 95% pure.

Overlapping biotinylated peptides were purchased from Zeneca/Cambridge Research Biochemicals (Wilmington, Delaware). They were synthesized on 96-well format plates by solid-phase methodology using Fmoc chemistry. Biotin was added to a four-residue spacer arm of sequence SGSG at the N-terminus to allow easy attachment to immobilized streptavidin. Two sets of biotinylated overlapping peptides were produced. (1) The entire mature PSA sequence was covered by peptides of 15 amino acid residues in length (15-mers) with 10-residue overlaps. The first peptide spanned from residues (−11) to 4, the second one from (−6) to 9, the third one from (−1) to 14, the fourth one from 5 to 19, and so on, until last peptide (13-mer), spanning from residues 225 to 237. (2) The amino acid residues 56–106 in PSA were studied by peptides of 8 amino acid residues in length (8-mers) with 7-residue overlaps. All peptides were determined to be at least 95% pure.

Peptide inhibition of anti-PSA Mab association with PSA

Two-site combinations of capture antibody and Eu-labeled antibody were used to determine the inhibition of 23 Eu-labeled Mabs (Mabs 66 and E86 not determined) binding to PSA by the synthetic peptides (Fig. 3A). Purified PSA (0.05 pmol/well/100 μL in assay buffer) was immobilized on H117 and 2E9 coated plates by incubating for 1 h. After a wash step (2×), 300,000-fold molar excess of peptides (30 nmol/well/25 μL in assay buffer) and Eu-labeled Mab (0.1 pmol/well/100 μL in assay buffer) were incubated with immobilized PSA for 1 h. Control experiments with no peptides added were run simultaneously. Another washing step (4×) was followed by the addition of 200 μL/well of enhancement solution. After 5 min incubation, the fluorescence was measured for 1 s in a plate fluorometer. All samples were run in duplicates and continuously shaken during the incubations. Various peptide concentrations were studied with peptides P5p, P9p, and P12p, revealing that the degree of inhibition was dependent on the peptide concentration (data not shown). Therefore, high molar excess of peptides (300,000-fold) were used throughout the study. Inhibition was considered significant if inhibition was greater than 3 × SD of the background and initial positive inhibition results were always repeated.

Binding of anti-PSA Mabs to biotinylated peptides

Biotinylated peptides (~3 pmol/well/100 μL in assay buffer) were immobilized on streptavidin-coated plates by incubating for 1 h (Fig. 3B). After a wash step (2×), each of the 25 Eu-labeled Mabs (100 ng/well/100 μL in assay buffer) were incubated with immobilized peptides for 2 h. As a negative control, wells without immobilized peptide were incubated with each Eu-labeled Mab. After another wash step (4×), the assay was continued as above. All samples were run in duplicates and continuously shaken during the incubations. Binding was considered significant if signal was greater than 10 × SD of the background.

Mab interference with catalytic activity of PSA and complexation between PSA and ACT

Purified PSA (1.4 μmol/L) was incubated separately with two- to fivefold molar excess of 22 anti-PSA Mabs for 1 h at 37 °C in 0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.15 mol/L NaCl (Table 4). Subsequently, the hydrolysis rate of 0.5 mmol/L S-2586 (MeO-Suc-Arg-Pro-Tyr-pNA) was measured at 405 nm during 10 min after the addition of the substrate. As a control, only purified PSA in buffer was incubated for 1 h at 37 °C prior to the addition of S-2586. The absorbance rate of the control was compared to the rate in the presence of different Mabs.

Complexation with ACT was studied by an immunofluorometric assay measuring the amount of PSA-ACT complex with capture anti-PSA Mab H117, 2E9, or H50 (depending on which capture Mab epitope was not overlapping with the investigated Mab) combined with Eu-labeled anti-ACT detection Mab 241 (Abbott Laboratories). Purified free PSA (3.3 nmol/L) was incubated with 10-fold molar excess of the same Mabs as above for 1 h at 37 °C in 0.1 mol/L phosphate buffer, pH 7.0, containing 0.5 mol/L NaCl. PSA-Mab complexes were then incubated for 4 h with 50-fold molar excess of purified ACT. As a control, PSA was incubated without Mabs, resulting in 39% PSA-ACT complex (61% remained free PSA). The concentration of PSA-ACT in the control was compared to the concentration of PSA-ACT in the presence of the different Mabs.

Acknowledgments

This work was supported by grants from the European Union Biomed 2 program, Area 4.1.7. (contract number BMH4-CT96-0453), the Swedish Medical Research Council (project number 7903), the Swedish Cancer Society (project number 3555), the Faculty of Medicine at Lund University, the Research Fund and the Cancer Research Fund at the University Hospital, the Crafoord Foundation, the Gunnar, Arvid, and Elisabeth Nilsson Foundation, and the Fundacion Frederico S.A.

References

Bélanger A, van-Halbeek H, Graves HC, Grandbois K, Stamey TA, Huang L, Poppe I, Labrie F. 1995. Molecular mass and carbohydrate structure of prostate specific antigen: Studies for establishment of an international PSA standard. *Prostate 27*:187–197.

Björk T, Piironen T, Pettersson K, Lövgren T, Stenman UH, Oesterling JE, Abrahamsson PA, Lilja H. 1996. A comparison of the analysis of the different prostate-specific antigen forms in serum for the detection of clinically localized prostate cancer. *Urology 48*:882–888.

Bode W, Mayr I, Baumann U, Huber R, Stone SR, Hofsteenge J. 1989. The refined 1.9 Å crystal structure of human a-thrombin: Interactions with D-Phe-Pro-Arg chloromethylketone and significance of the Tyr-Pro-Pro-Trp insertion segment. *EMBO J 8*:3467–3475.

Bridon DP, Dowell BL. 1995. Structural comparison of prostate-specific antigen and human glandular kallikrein using molecular modeling. *Urology 45*:801–806.

Catalona WJ, Smith DS, Ratliff TL, Dodds KM, Coplen DE, Yuan JJ, Petros JA, Andriole GL. 1991. Measurement of prostate-specific antigen in serum as a screening test for prostate cancer. *N Engl J Med 324*:1156–1161.

Chapdelaine P, Paradis G, Tremblay RR, Dubé JY. 1988. High level of expression in the prostate of a human glandular kallikrein mRNA related to prostate-specific antigen. *FEBS Lett 236*:205–208.

Christensson A, Bjork T, Nilsson O, Dahlen U, Matikainen MT, Cockett AT, Abrahamsson PA, Lilja H. 1993. Serum prostate specific antigen complexed to alpha 1-antichymotrypsin as an indicator of prostate cancer. *J Urol 150*:100–105.

Christensson A, Laurell CB, Lilja H. 1990. Enzymatic activity of prostate-specific antigen and its reactions with extracellular serine proteinase inhibitors. *Eur J Biochem 194*:755–763.

Christensson A, Lilja H. 1994. Complex formation between protein C inhibitor and prostate-specific antigen in vitro and in human semen. *Eur J Biochem 220*:45–53.

Deperthes D, Chapdelaine P, Tremblay RR, Brunet C, Berton J, Hebert J, Lazure C, Dube JY. 1995. Isolation of prostatic kallikrein hK2, also known as hGK-1, in human seminal plasma. *Biochim Biophys Acta 1245*:311–316.

Eerola R, Piironen T, Pettersson K, Lövgren J, Vehniäinen M, Lilja H, Dowell B, Lövgren T, Karp M. 1997. Immunoreactivity of recombinant human glandular kallikrein using monoclonal antibodies raised against prostate specific antigen. *Prostate 31*:84–90.

Espana F, Gilabert J, Estelles A, Romeu A, Aznar J, Cabo A. 1991. Functionally active protein C inhibitor/plasminogen activator inhibitor-3 (PCI/PAI-3) is secreted in seminal vesicles occurs at high concentrations in human seminal plasma and complexes with prostate-specific antigen. *Thromb Res 64*:309–320.

Greer J. 1990. Comparative modeling methods: Application to the family of the mammalian serine proteases. *Proteins Struct Funct Genet 7*:317–334.

Henttu P, Lukkarinen O, Vihko P. 1990. Expression of the gene coding for human prostate-specific antigen and related hGK-1 in benign and malignant tumors of the human prostate. *Int J Cancer 45*:654–660.

Hudson MA, Bahnson RR, Catalona WJ. 1989. Clinical use of prostate specific antigen in patients with prostate cancer. *J Urol 142*:1011–1017.

Lawrence DA. 1997. The serpin–proteinase complex revealed. *Nature Struct Biol 4*:339–341.

Lilja H, Abrahamsson PA, Lundwall Å. 1989. Semenogelin the predominant protein in human semen. Primary structure and identification of closely related proteins in the male accessory sex glands and on the spermatozoa. *J Biol Chem 264*:1894–1900.

Lilja H, Christensson A, Dahlen U, Matikainen MT, Nilsson O, Pettersson K, Lövgren T. 1991. Prostate-specific antigen in serum occurs predominantly in complex with alpha 1-antichymotrypsin. *Clin Chem 37*:1618–1625.

Lövgren J, Piironen T, Overmo C, Dowell B, Karp M, Pettersson K, Lilja H, Lundwall Å. 1995. Production of recombinant PSA and HK2 and analysis of their immunologic cross-reactivity. *Biochem Biophys Res Commun 213*:888–895.

Lundwall Å, Lilja H. 1987. Molecular cloning of human prostate specific antigen cDNA. *FEBS Lett 214*:317–322.

Mitrunen K, Pettersson K, Piironen T, Bjork T, Lilja H, Lövgren T. 1995. Dual-label one-step immunoassay for simultaneous measurement of free and total prostate-specific antigen concentrations and ratios in serum. *Clin Chem 41*:1115–1120.

Nilsson O, Peter A, Andersson I, Nilsson K, Grundström B, Karlsson B. 1997. Antigenic determinants of prostate-specific antigen (PSA) and development of assays specific for different forms of PSA. *Br J Cancer 75*:789–797.

Oesterling JE, Jacobsen SJ, Klee GG, Pettersson K, Piironen T, Abrahamsson PA, Stenman UH, Dowell B, Lövgren T, Lilja H. 1995. Free complexed and total serum prostate specific antigen: The establishment of appropriate reference ranges for their concentrations and ratios. *J Urol 154*:1090–1095.

Pettersson K, Piironen T, Seppala M, Liukkonen L, Christensson A, Matikainen MT, Suonpaa M, Lövgren T, Lilja H. 1995. 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-alpha 1-antichymotrypsin complex. *Clin Chem 41*:1480–1488.

Piironen T, Lövgren J, Karp M, Eerola R, Lundwall Å, Dowell B, Lövgren T, Lilja H, Pettersson K. 1996. Immunofluorometric assay for sensitive and specific measurement of human prostatic glandular kallikrein (hK2) in serum. *Clin Chem 42*:1034–1041.

Rajakoski K, Piironen T, Pettersson K, Lövgren J, Karp M. 1997. Epitope mapping of human prostate specific antigen and glandular kallikrein expressed in insect cells. *Prostate Cancer and Prostatic Diseases 1*:16–20.

Riegman PHJ, Vliestra RJ, Suurmeijer J, Cleutjens CBJM, Trapman J. 1992. Characterization of the human kallikrein locus. *Genomics 14*:6–11.

Schedlich LJ, Bennetts BH, Morris BJ. 1987. Primary structure of a human glandular kallikrein gene. *DNA 6*:429–437.

Stamey TA, Yang N, Hay AR, McNeal JE, Freiha FS, Redwine E. 1987. Prostate-specific antigen as a serum marker for adenocarcinoma of the prostate. *N Engl J Med 317*:909–916.

Stenman UH, Leinonen J, Alfthan H, Rannikko S, Tuhkanen K, Alfthan O. 1991. A complex between prostate-specific antigen and alpha 1-antichymotrypsin is the major form of prostate-specific antigen in serum of patients with prostatic cancer: Assay of the complex improves clinical sensitivity for cancer. *Cancer Res 51*:222–226.

Stratikos E, Gettins PGW. 1997. Major proteinase movement upon stable serpin–proteinase complex formation. *Proc Natl Acad Sci USA 94*:453–458.

Vihinen M. 1994. Modeling of prostate specific antigen and human glandular kallikrein structures. *Biochem Biophys Res Commun 204*:1251–1256.

Villoutreix BO, Getzoff ED, Griffin JH. 1994. A structural model for the prostate disease marker human prostate-specific antigen. *Protein Sci 3*:2033–2044.

Villoutreix BO, Lilja H, Pettersson K, Lövgren T, Teleman O. 1996. Structural investigation of the alpha-1-antichymotrypsin: Prostate-specific antigen complex by comparative model building. *Protein Sci 5*:836–851.

Wang MC, Valenzuela LA, Murphy GP, Chu TM. 1979. Purification of human prostate specific antigen. *Invest Urol 17*:159–163.

Wilczynska M, Fa M, Karolin J, Ohlsson PI, Johansson LBÅ, Ny T. 1997. Structural insights into serpin–protease complexes reveal the inhibitory mechanism of serpins. *Nature Struct Biol 4*:354–357.

Young CY, Andrews PE, Montgomery BT, Tindall DJ. 1992. Tissue-specific and hormonal regulation of human prostate-specific glandular kallikrein. *Biochemistry 31*:818–824.

Zhang WM, Leinonen J, Kalkkinen N, Stenman UH. 1997. Prostate specific antigen forms a complex with and cleaves alpha-1-protease inhibitor in vitro. *Prostate 33*:87–96.