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**PROSTATE SPECIFIC MEMBRANE ANTIGEN (PSMA):
IMMUNOASSAY DEVELOPMENT AND
CHARACTERIZATION OF TRANSCRIPTIONAL REGULATION**

by

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B.S. July 1992, Zhongshan University, Guangzhou, China

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and

OLD DOMINION UNIVERSITY

May 2002

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ABSTRACT

PROSTATE SPECIFIC MEMBRANE ANTIGEN (PSMA): IMMUNOASSAY DEVELOPMENT AND CHARACTERIZATION OF TRANSCRIPTIONAL REGULATION

Zhen Xiao

Eastern Virginia Medical School and Old Dominion University, 2002

Director: Dr. George L. Wright, Jr.

Prostate cancer (PCA) is the most common cancer and the second leading cause of death among American men. The high mortality is greatly attributed to the lack of early detection tools and effective treatment for metastasis and relapses. Biomarkers that can discriminate benign from malignant tumor and signal the development of androgen independent and metastatic tumor are needed. A biomarker designated prostate specific membrane antigen (PSMA) has the potential to fulfill this need. The objective of this study is to develop a clinically useful immunoassay for quantitation of serum PSMA and to study the molecular mechanism underlying the upregulation of the PSMA gene after androgen deprivation therapy. Surface Enhanced Laser Ionization/Desorption (SELDI) ProteinChip[®] mass spectrometry was used for the first aim. A baculovirus recombinant PSMA was generated, purified and characterized. After the SELDI immunoassay conditions were optimized, a standard curve was established using rPSMA as the substitute antigen. Serum PSMA levels, as measured by SELDI immunoassay, were able to distinguish benign from malignant prostate disease. To understand the transcriptional regulation of the PSMA gene, the second aim focused on the 14 AP-1 sites in the 5' region of the PSMA gene. By Northern blot, the PSMA mRNA levels in LNCaP cells were found to be increased by the inducers of AP-1, EGF and TPA, and decreased by

androgens. The PSMA promoter activity, as analyzed by luciferase assay, was also induced by EGF and cotransfected AP-1 proteins, but suppressed by androgens. The binding of AP-1 to three putative AP-1 sites in the vicinity of the PSMA transcription initiation sites was demonstrated by gel shift assay. The DNA binding was also decreased by androgens and increased by EGF. Gel shift competition further indicated that the AP-1 binding might be inhibited by the interaction between androgen receptors and AP-1. To summarize this study, the serum PSMA level was measured by SELDI immunoassay and was shown to be a more effective biomarker than PSA for differentiating benign from malignant prostate disease. Moreover, PSMA gene transcription is activated by AP-1 and suppressed by androgens possibly through the inhibition of AP-1 binding to the PSMA promoter by androgen receptor.

With much love and many thanks

To

Baba, Mama, Hanzi

And

Dr. George L. Wright, Jr.

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LIST OF ABBREVIATIONS

7E11	Monoclonal antibody 7E11-C5.3
A. I.	Androgen independent
BPH	Benign prostate hyperplasia
DRE	Digital rectal examination
EGF	Epidermal growth factor
EMSA	Electrophoretic mobility shift assay
IMAC	Immobilized metal affinity chromatography
IMD	imidazole
IS	Internal standard
MoAb	Monoclonal antibody
MOI	Multiplicity of infection
PCA	Prostate cancer
PSA	Prostate specific antigen
PSMA	Prostate specific membrane antigen
rPSMA	Recombinant PSMA
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SELDI-TOF-MS	Surface enhanced laser desorption/ionization time-of-flight mass spectrometry
TPA	12- <i>o</i> -Tetradecanoylphorbol-13-acetate
WCL	Whole cell lysate

CHAPTER I

INTRODUCTION

A. REVIEW OF THE LITERATURE

1. Prostate Cancer and PSA Test

Prostate cancer (PCA) is the most common cancer in men in the United States with 198,100 new cases estimated to be diagnosed in 2001. PCA is second to lung cancer in the number of cancer deaths in men with 31,500 deaths estimated for 2001 (1). The high mortality rate is due mainly to the difficulty in detection and treatment of patients with the aggressive, lethal form of PCA. According to the Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute, one-third of patients who are diagnosed with PCA have already developed advanced malignancy (2,3). These patients might receive palliative treatments and eventually die with PCA. On the other hand, for those patients who have been diagnosed with early stage localized PCA, more treatment options are available, including radical prostatectomy, radiation therapy, and recently developed cryotherapy. Patients who fail these treatment strategies, or are diagnosed with extraprostatic spread, will receive systemic androgen deprivation therapy. However, androgen independent tumor and/or metastatic disease are very likely to prevail in these patients, pointing to the need for alternative therapies to effectively treat hormone refractory disease.

The introduction of the PSA test has greatly enhanced the screening and early

The model of this dissertation is Cancer Research.

detection of PCA (4). The shortcoming of the PSA test is its lack of sensitivity in discriminating PCA from benign prostate hyperplasia (BPH), especially in the PSA range of 4-10ng/ml, which is also called the 'grey zone' (Fig. 1) (5,6). The addition of the PSA-related parameters such as PSA velocity, density and free to total PSA ratios has slightly improved the specificity, reducing the number of unnecessary biopsies (4). While PSA has remained the best and most widely used tumor marker, additional biomarkers are still required to improve the early detection, diagnosis and prognosis of PCA (7-10). It is in this context that the discovery and clinical potential of prostate specific membrane antigen (PSMA) have been viewed to be very meaningful and promising.

2. Discovery of PSMA

PSMA was first identified by a murine monoclonal antibody produced against LNCaP cell membranes and designated 7E11-C5.3 (7E11) (11). The discovery process is summarized in Fig. 2. Tryptic peptide sequences of the 7E11 immunoprecipitated protein were analyzed and used to design degenerate primers, resulting in the cloning of a 2.65kb PSMA cDNA by RT-PCR (Genebank Accession #M99487) (12). SDS-PAGE and Western blotting analyses revealed PSMA to be a 100kDa glycoprotein with the ability to form dimers of 180-200kDa (13,14).

3. Biological Characteristics of PSMA

PSMA cDNA sequence analysis showed that PSMA is a 750 amino acid type II transmembrane glycoprotein (Fig. 3). It is composed of three structural domains, with

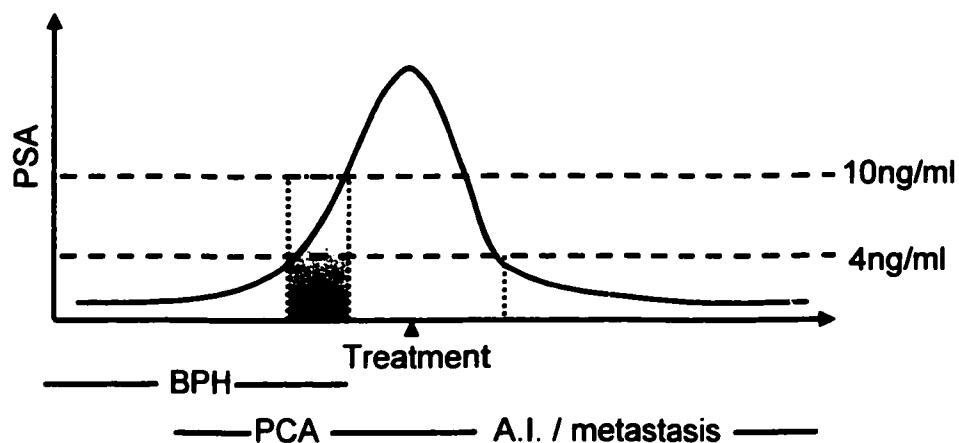


Fig.1. A schematic representation of the change of serum PSA levels during prostate cancer progression. *X* axis, the progression of prostate cancer from normal to benign prostate hyperplasia (BPH), prostate cancer (PCA), and eventually androgen independent (A.I.) and metastatic tumors. *Y* axis, serum PSA level in ng/ml. The serum PSA levels are low in normal men (<4ng/ml). When PSA levels fall in the range of 4 to 10ng/ml, it becomes difficult to discriminate BPH and PCA. The grey shaded area represents the overlap between BPH and PCA. After the treatment such as surgical removal of prostate, radiation therapy or androgen deprivation therapy, PSA levels might decline while the tumors continue to progress. In this situation, surrogate tumor biomarkers are needed for post treatment prognosis.

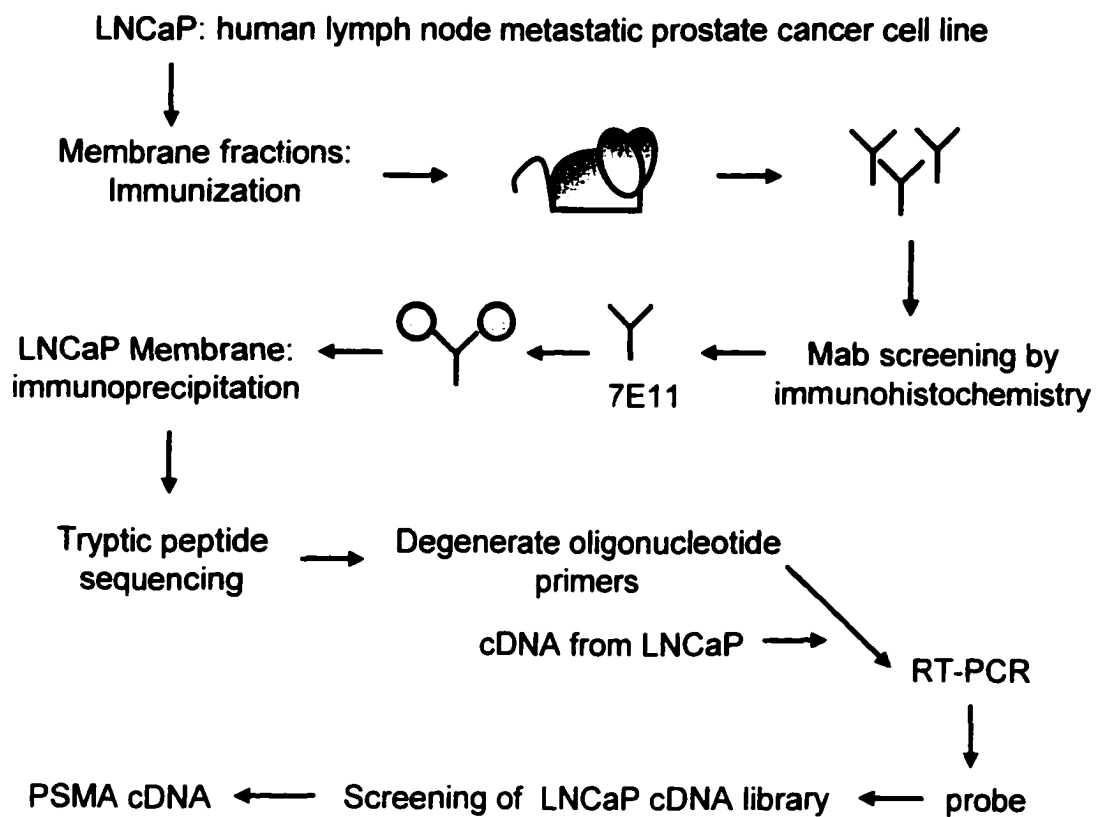


Fig. 2. The discovery process of PSMA protein and gene.

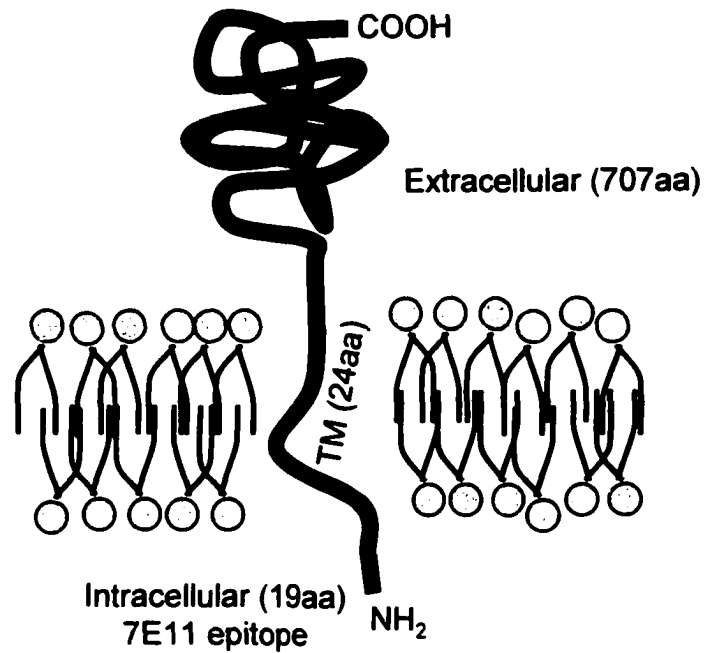


Fig. 3. PSMA is a transmembrane glycoprotein located on the cell membrane. *TM*, transmembrane domain of PSMA. The epitope for MoAb 7E11 is at the 19 amino acids at the intracellular domain. An alternatively spliced mRNA results in PSMA', a protein without the membrane-spanning and intracellular regions.

a 19 amino acid N-terminal intracellular domain, followed by a 24 amino acid transmembrane domain and a 707 amino acid extracellular domain (12). The epitope recognized by 7E11 is located intracellularly at the N-terminal 19 amino acids (15). Glycosylation analysis indicated that there are 10 N-linked glycosylation sites and little O-linked glycosylation. Carbohydrate constitutes about 25% of the total molecular weight (16).

Extensive immunohistochemical studies using 7E11 demonstrated that PSMA was expressed predominantly in normal, benign and malignant prostate epithelia, with weak reactivity in the small intestine, brain, kidney and salivary glands (14,17-19). Recently, researchers have developed a number of second generation antibodies that revealed PSMA reactivity in a variety of solid tumor-associated neovasculatures but not normal vasculature (20,21). PSMA appeared to be down regulated in benign prostatic hyperplasia (BPH) and overexpressed in poorly differentiated primary tumors. The highest expression of PSMA was found in bone and lymph node metastatic prostate carcinomas, especially in patients with recurrences after androgen deprivation therapy (19, 22-24). Besides the observations at the protein level, a similar expression profile was observed at mRNA level. In needle biopsy specimens using *in situ* hybridization with a PSMA specific RNA probe, PSMA transcripts were shown to be limited to the basal cells in the normal prostate glands, whereas they were detected at a much higher level in carcinoma cells from hormone-refractory patients than in the cells of those who showed a good response to hormonal therapy (25). The effect of androgen on PSMA level was further studied *in vitro* using LNCaP as the model. By ELISA, PSMA expression was shown to be upregulated when cells were grown in

androgen stripped medium, and decreased in cells grown in androgen supplemented medium (23). Another study using RNase protection assay also showed the downregulation of PSMA RNA by androgens (13). These results clearly indicate that the expression of PSMA, at either protein or RNA level, is closely associated with malignant transformation and development of hormone independence. Besides being expressed in prostate and other tissues, PSMA was also detected in body fluids. Western blotting showed the presence of PSMA in both serum and seminal plasma (14,26,27). In serum, the highest level of PSMA was found in patients with late-stage prostate cancer (14,27).

Type II transmembrane proteins are usually proteases, receptors or transport proteins, but the function of PSMA is not clear. Nonetheless, there are a number of speculations. A human brain enzyme called N-acetylated α -linked acidic dipeptidase (NAALADase) showed 86% homology to a region of PSMA (28). NAALADase cleaves α -linked glutamate from the N-acetylaspartylglutamate (NAAG), and NAAG is a potential neurotransmitter in brain. Another enzymatic activity that might relate to PSMA is folate hydrolase (FOLH), which removes the γ -linked glutamates from folate in a sequential manner. The FOLH in human duodenal intestine shares high sequence homology with PSMA (29). Folate has been known to be important for prostate tissue development. While *in vitro* enzymatic studies demonstrated both NAALADase and FOLH activities in PSMA-expressing LNCaP cells, the *in vivo* functional implication, particularly in prostate tissue warrants further investigation.

Based on the cloned cDNA, the entire gene for PSMA has been isolated and sequenced (Genebank accession #AF007544) (30). The gene is composed of 19 exons

spanning about 62kb of genomic DNA on the short arm of chromosome 11 (11p11.2) (31). Fluorescent *in situ* hybridization (FISH) analysis suggested the potential presence of a second related gene on the long arm of chromosome 11 (11q14), but the relevance of this second gene to PSMA and to prostate cancer development is not clear (32). Since androgen has shown the ability to change the PSMA RNA level, the PSMA promoter region was recently cloned in an attempt to study the androgen responsiveness at the transcription level (30,33). However, the data on promoter activity were ambiguous in those studies, probably due to the difficulty in transfection of LNCaP cells. Therefore, the molecular mechanism by which androgen alters the PSMA RNA level remains unknown.

4. Clinical Application of PSMA

Since its initial discovery, PSMA has received attention as a potential biomarker for detection/diagnosis, prognosis, and as a therapeutic target. The orientation of PSMA on prostate cell membranes makes it an attractive target for imaging, radiotherapy, and immunotherapy. Anti-PSMA MoAb 7E11 conjugated with ^{111}In and marketed by Cytogen Corporation as Prostatint[®], is being used for radiographic imaging, and shows to provide higher sensitivity than computed tomography (CT) scanning for detecting lymph node metastatic prostate cancer and disease outside the field of surgical exploration (34-36). ^{90}Y conjugated 7E11 has recently entered phase III trials for radiation therapy of prostate cancer recurrence and metastasis (37,38). Second generation antibodies that recognize the extracellular domains of PSMA, are in clinical trials to evaluate their utility for radiological imaging and radiotherapy

(39,40). The potential of these antibodies to develop anti-angiogenic therapies has also been suggested (41).

Since PSMA is highly specific for prostate epithelial cells and its expression is elevated with PCA progression, it was proposed as an ideal antigen to stimulate immune responses against prostate cancer cells. Following this consideration, researchers have pulsed autologous dendritic cells (DCs) with PSMA derived peptides and infused DCs into patients with metastatic hormone refractory prostate cancer, in an attempt to stimulate cytotoxic T cell killing of tumor cells (42). This approach is currently in phase II clinical trials and has provided some promising immune responses in patients with post-radical recurrences (43-47).

PSMA has also been used for molecular staging and diagnostic approaches. Nested RT-PCR using PSMA specific primers has detected hematogenous circulating prostatic cells, which showed a much higher sensitivity than the PSA-based assay (48-51). Although the finding of prostatic cells in the circulation does not necessarily imply distant metastasis, the results have presented a step forward in applying PSMA for early detection of aggressive PCA, and thereby offering the patients more options for treatments in a likely curable stage. This method is currently subject to further modification in order to improve the accuracy and reproducibility.

PSMA has been detected in serum by Western blotting, with high levels correlated with higher pathological stages and worse prognostic outcome (14,27). The inverse correlation of PSMA with androgen levels is of particular interest, making PSMA a valuable surrogate biomarker for PCA progression, especially in instances when the expression of PSA is decreased after surgical or hormone ablation. Considerable

efforts have been devoted to the development of a quantitative immunoassay for measuring PSMA in body fluids. Various combinations of antibodies to both the intracellular and extracellular domains have been used in ELISA formats in an attempt to quantitate PSMA. However, the progress has fallen behind the anticipation because a good pair of 'capture' and 'detector' antibodies has not been identified. So far the only effective method to detect serum PSMA is still by Western blotting, which is useful as a semi-quantitative analytic method for research, but unsuitable as a clinical assay.

B. FOCUS OF THE PRESENT INVESTIGATION

PSMA is a potential biomarker for improving the diagnosis and prognosis of prostate cancer. It is also an ideal target for gene and immunotherapy strategies. However, the clinical application of PSMA remains impeded by two important questions:

- 1) Can serum PSMA levels be quantitated and used as a diagnostic biomarker?
- 2) How is PSMA regulated by androgens?

Answers to both questions will certainly benefit the basic biology and the clinical utility of PSMA.

Several lines of evidence suggested that PSMA is differentially expressed in BPH and PCA (23-25,27). Therefore, PSMA might be useful as a marker, either alone or in concert with PSA, to differentiate PCA from BPH in the PSA 4.0ng/ml to 10ng/ml grey zone; thus reducing the number of unnecessary biopsies. However, in order to

critically evaluate PSMA as a biomarker, it is necessary to develop an assay format to quantitatively measure PSMA in body fluids. Although Western blot analysis has been used to detect PSMA in body fluids, our laboratory and others have found it unreliable for quantitation and unsuitable as a clinical assay (14,27,52-55). Attempts to develop an immunoassay for quantitation of PSMA have been unsuccessful due to the lack of purified PSMA protein standard and a sensitive method for quantitation. To the best of our knowledge, no test is available for measuring PSMA in sera. The clinical application necessitates the development of a simple, fast, accurate and reproducible approach that can detect and quantitate serum PSMA. Surface Enhanced Laser Ionization/Desorption (SELDI) ProteinChip[®] technology, an innovative chip based affinity mass spectrometry system was applied to address this question. The ProteinChip[®] system provides a platform for immunoassay development, with the capability for immobilizing antibody, which in turn can capture PSMA from serum and convert it to a mass signal that correlates to the quantity of the antigen present.

Besides the lack of an appropriate tool for quantitation, the clinical application of PSMA has also been hampered by the lack of understanding about its gene regulation. Although initial observation suggests that the activity of PSMA gene promoter is downregulated by androgens and upregulated in the absence of androgens (13,22,23,30,33), no androgen responsive element has been identified. This also implies the existence of other transcriptional enhancer regions that might selectively activate the PSMA gene in the absence of androgens. Therefore the objective of the second part of this study was to search for such promoter/enhancer regions and related

transcription factors to better understand the mechanism of the PSMA gene regulation by androgens.

C. PRELIMINARY STUDY: SEQUENCE ANALYSIS OF THE 5' REGION OF THE PSMA GENE

Based on the hypothesis that transcription factors other than androgen receptors are likely to be involved in the regulation of the PSMA gene, a preliminary sequence analysis was conducted to search for the possible binding sites for some of the known transcription factors, especially those that might be related to the androgen responsiveness.

The computer software MacVector was used to analyze a 5.1kb fragment starting from the 5' end of the PSMA genomic sequence (nts 1-5100). This includes the 2.4kb upstream region, the first exon and the first intron of the PSMA gene. No androgen responsive elements were identified in the entire region based on the consensus androgen responsive element (ARE) sequence. Instead, a total of 14 putative binding sites for the transcription factor AP-1 family members (c-Jun/c-Fos) were identified. Fig. 4A illustrates the physical distribution of the putative AP-1 binding sites throughout the 5' end region (nts 588-4973) of the PSMA gene. Table 1 summarizes the features of these AP-1 sites. For all these 14 AP-1 sites, at least 6 out of the 7 base pairs were matched with the sequence of the consensus AP-1 site.

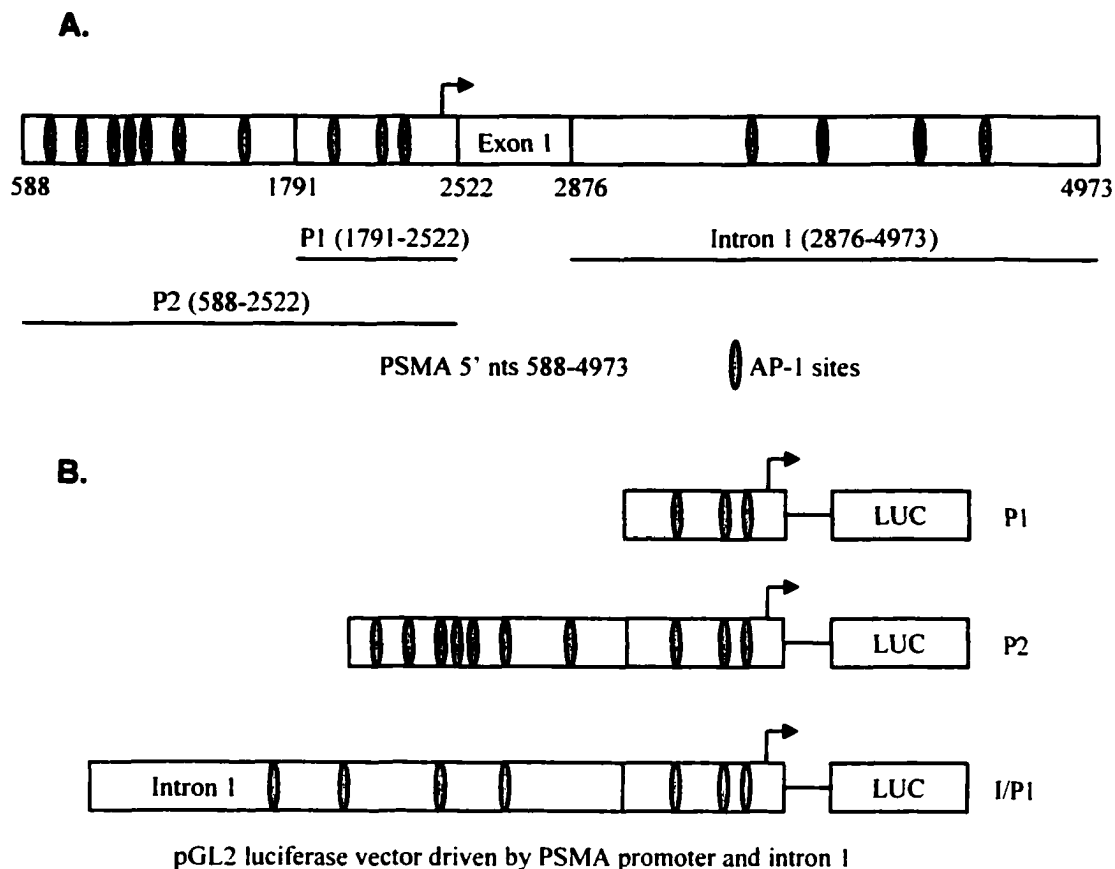


Fig. 4. AP-1 sites in the 5' region of the PSMA gene and the luciferase constructs used in this study. *A*, The distribution of putative AP-1 binding sites in the 5' end region (nts 588-4973) of PSMA. The numbers in parentheses stand for the beginning and the end nucleotides of the PSMA fragments in pGL2 constructs P1, P2 and I/P1 according to their position in PSMA genomic sequence (Genbank accession number AF007544). *B*, pGL2 luciferase constructs used in this study. The PSMA fragments P1, P2 and I/P1 were cloned into the pGL2-basic vector. I/P1 is a fragment with Intron I added to the 5' end of P1. The shaded ovals represent the putative AP-1 binding sites. The arrows stand for the transcription initiation site (nt 2488) of the PSMA gene.

Table 1 Putative transcription factor AP-1 binding sites present in the PSMA gene 5' end 4.5kb region

Number of AP-1 sites	Position	Sequence orientation	Sequence	Number of bp matched with the consensus sequence
1	649	-	TTAtTCA	6/7
2	877	-	gGAGTCA	6/7
3	1008	-	TaAGTCA	6/7
4	1093	-	TTAaTCA	6/7
5	1145	+	TTAGTCA	7/7
6	1299	-	TTtGTCA	6/7
7	1509	+	TGgGTCA	6/7
8	1964	+	TGcCTCA	6/7
9	2321	+	TTAtTCA	6/7
10	2370	-	TTAtTCA	6/7
11	3899	-	TGtGTCA	6/7
12	4217	-	TTAGTcC	6/7
13	4593	+	TGAGTCA	7/7
14	4826	-	aGAGTCA	6/7
Consensus AP-1 binding site			TTAGTCA	
			TGAG/CTCA	

The result from this sequence analysis brought the AP-1 transcription factor family to our attention, because the members of this family interact with androgen receptors and the interactions have mutual effect on both of their transactivation ability (56,57). In order to determine if AP-1 is involved in the androgenic regulation of the PSMA gene, the following specific aims were proposed: 1) To determine if the AP-1 inducers such as EGF and TPA can increase the expression of PSMA; 2) To analyze the effect of AP-1 family members on the PSMA promoter activity; and 3) To analyze the DNA binding activity of AP-1 proteins in the PSMA promoter region and the effect of androgens on the AP-1 DNA binding. The results from this part of the study would reveal the mechanism underlying the androgenic regulation of the PSMA gene expression.

CHAPTER II

MATERIALS AND METHODS

A. MATERIALS

1. Cell and Cell Culture

Sf9 cells derived from *Spodoptera frugiperda* ovarian cells and High Five[®] (Hi5) cells derived from *Trichoplusia ni* egg cell homogenates were obtained from Invitrogen (Carlsbad, CA). Both insect cell lines were grown at 27°C, either as monolayers or as suspension cultures in spinner flasks in Hink's TNM-FH medium (Grace's medium) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (Sigma, St. Louis, MO). For recombinant PSMA expression, viral infections were performed on Hi5 cell monolayers grown in Excel 405 serum free medium (JRH Biosciences, Lenexa, KS).

LNCaP and PC-3 cell lines were obtained from the American Type Culture Collection. LNCaP cells were maintained in RPMI 1640 medium supplemented with 5% Fetal Bovine Serum (FBS). Cells between the 29th and 42nd generation were used in these experiments. PC-3 cells were maintained in RPMI 1640 medium supplemented with 10% FBS. Cells of the 18th to the 20th generation were used in the experiments.

2. Bacterial Strains and Plasmids

The pBlueScript SK (Stratagene, La Jolla, CA) and pGEMT plasmids (Promega, Madison, WI) were amplified in JM109 E. Coli strain. The baculovirus transfer vector pBlueBacHisB (Invitrogen, Carlsbad, CA) was amplified in Top10 E. Coli strain.

3. Antibodies

Anti *Xpress* antibody was purchased from Invitrogen (Carlsbad, CA). Anti-PSMA MoAb 7E11-C5 (IgG1) was purified from the cell culture medium of ATCC hybridoma line HB-10494 using Affi-Gel[®] Protein A MAPS[®] II kit (Bio-Rad, Hercules, CA). The following rabbit polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA): anti c-Jun (N) (sc-45x), anti c-Fos (4) (sc-52x), and anti AR (N-20) (sc-816x).

4. Patients and Serum Samples

The serum samples used in this study were obtained from the serum bank of the Virginia Prostate Center at Eastern Virginia Medical School, and were the same group of samples that had been used previously to analyze the serum levels of PSMA by Western blotting (27). All sera were collected from consented patients following the study protocol approved by the Institutional Review Board. The normal male population was defined by a negative DRE and a PSA <4.0ng/ml. The age range for normal males under age 50 was 27-39 (mean of 33.9), and 54-71 (mean of 60.3) for normal males over 50. Pre-treatment serum samples from patients with benign or

malignant prostate disease were used in this study. Benign prostate hyperplasia (BPH) patients were those who had bladder outlet obstructive symptoms, increased PSA levels (>4ng/ml), and confirmed to be BPH upon transrectal ultrasound biopsy. The prostatitis population consisted of patients with chronic symptom complex attributed to prostatic source and absent of infection by urine analyses and culture at the time of sample collection. Prostate cancer patients included clinical stages (T1 to T3).

B. METHODS

1. Construction of pBlueBacHis-PSMA Baculovirus Transfer Vector

Firstly, the 2.65kb full-length PSMA cDNA (GenBank accession number M99487) was amplified by RT-PCR using messenger RNA isolated from LNCaP cells as template, and cloned into pBlueScript SK vector. The insert was verified by sequence analysis. To generate a recombinant PSMA baculovirus transfer vector, primers were designed to add BamHI and HindIII restriction sites to 5' and 3' ends of pBlueScript-PSMA respectively by PCR (5' AGGATCCGATGTGGAATCTCCTT: 3' AAAGCTTCCCTCGAGTTTTTTTTT). The amplified product was cloned into pGEMT vector, restricted with BamHI/HindIII, and inserted into the pBlueBacHisB vector. The PSMA insert in pBlueBacHisB construct was verified by sequencing.

2. Generation of Recombinant His-tagged PSMA Baculovirus

Cotransfection of Sf9 cells with wild-type baculovirus DNA and the transfer vector was performed using the Bac-N-Blue Transfection Kit from Invitrogen. A

recombinant baculovirus was identified as blue and occlusion body negative (occ-) plaques by plaque assays on medium containing x-gal. The recombinant baculovirus was purified by four rounds of plaque assay. Insertion of the PSMA gene into the viral genome was verified by PCR using primers flanking the polyhedrin gene (forward: TTTACTGTTTTTCGTAACAGTTTTG; reverse:CAACAACGCACAGAATCTAGC).

3. Expression of Recombinant His-tagged PSMA

A high-titer viral stock ($>10^8$ pfu/ml) was generated from the initial infection and used for large-scale rPSMA expression. Hi5 cell monolayer cultures were infected and a time course study was performed with different multiplicities of infection (MOI). Both cell pellets and culture supernatants were harvested at different time points and analyzed by Western blotting to determine the cellular localization of recombinant protein. After optimizing the rPSMA expression, a large-scale infection was performed in Hi5 cells and the infected cells were harvested at the expression peak for rPSMA. Cell pellets were stored at -80°C for further purification.

4. Purification of Recombinant His-tagged PSMA

Whole cell lysates were prepared by solubilizing the cell pellets in lysis buffer consisting of 50mM Tris.HCl pH8.0, 500mM NaCl, 0.5% SDS, 1% Igepal (Sigma, St. Louis, MO) and the protease inhibitor cocktail (10 $\mu\text{g}/\text{ml}$ Leupeptin, 10 $\mu\text{g}/\text{ml}$ Aprotinin, 1mM PEFAbloc, 10 $\mu\text{g}/\text{ml}$ Pepstatin A). The lysates were subjected to sonication at 200watts for 20sec x3 pulses with 20sec cooling intervals in between pulses. Following a DNase I (100 $\mu\text{g}/\text{ml}$) digestion at room temperature for 30min.

lysates were centrifuged at 14,000rpm for 20min. The clarified supernatant was used as the starting material for rPSMA purification.

The Talon Metal Affinity Resin (Clontech, Palo Alto, CA) was used for protein purification. The insect cell lysate supernatant was added to the Talon Resin pre-equilibrated in lysis buffer. The mixture was agitated at room temperature for 1hr. then transferred onto a gravity column, which was washed with 10 bed volumes of lysis buffer (pH8.0). The rPSMA was eluted by adding 4 bed volumes each of the lysis buffer containing increasing concentrations of imidazole (10mM, 50mM, 100mM and 200mM). The flow-through and 0.5ml eluted fractions were collected and subjected to Western blot analysis. The PSMA containing fractions were pooled, dialyzed against lysis buffer, and concentrated using Centricon concentrators (MWCO 50kD, Amicon, Beverly, MA). The purified rPSMA protein was quantitated by the BCA protein assay (Pierce Chemical, Rockford, IL), and stored at -20°C until used.

5. Gel Electrophoresis and Western Blot Analysis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses were performed as previously described (58). Briefly, protein samples were electrophoresed on 10% SDS-PAGE and visualized by silver staining. For Western blotting, the gels were blotted onto Immobilon-P membranes (Millipore, Bedford, MA). The membranes were blocked with 1% I-block (Tropix, Bedford, MA) in PBS buffer, incubated with primary antibody, washed 3x with TBST (100mM Tris.HCl pH7.5, 150mM NaCl, 0.1%Tween-20) and then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Sigma, St. Louis, MO). Finally

the membranes were washed 3x with TBST. 1x with TBS, and the protein bands were visualized by ECL according to manufacturer's instructions (Amersham, Arlington Heights, IL).

6. Mass Spectrometry

In addition to gel electrophoresis and Western blotting, the purity of rPSMA was also determined on the ProteinChip[®] using Surface Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF-MS) (Ciphergen Biosystems, Inc., Palo Alto, CA) (59). Purified recombinant PSMA end product was applied directly on a Ni⁺⁺-coated Immobilized Metal Affinity (IMAC) ProteinChip[®]. The chip was washed with H₂O briefly for 5 seconds, and sinapinic acid (Sigma, St. Louis, MO) was added as the energy absorbing molecule. The chip was then placed in the PBS-1 SELDI mass reader, and the mass spectra were collected with a laser intensity of 60.

7. Glycosylation Analysis

Prior to enzymatic digestions, each exoglycosidase was tested to make sure it was enzymatically active and met the manufacture's specifications. Briefly, o-Nitrophenyl β -D-galactopyranoside and p-Nitrophenyl N-acetyl- β -D-glucosaminide (Sigma) were used as the substrates for β -galactosidase and β -N-acetylhexosaminidase, respectively. The production of O-Nitrophenol and p-Nitrophenol was monitored spectrometrically using wavelengths of 410nm and 330nm, respectively. The increases in absorbance indicated the enzyme activities. Bovine fetuin (Sigma) was used as the substrate to test

the activities of sialidase and endo- α -N-acetylgalactosaminidase. The deglycosylated fetuin protein products were compared to the untreated substrate by SDS-PAGE, and a shift in mobility of the substrate indicated that the enzymes were active.

For enzymatic digestions of the PSMA preparations, purified rPSMA (0.5 μ g) or 10 μ g of LNCaP membrane extract was boiled at 100°C in denaturing buffer (0.5% SDS, 1% β -mercaptoethanol) for 10min. After cooling on ice, 0.5% n-octyl β -D-glycopyranoside (Sigma, St. Louis, MO) was added, and the digestions were processed according to the manufacturers' specifications in a total volume of 20 μ l. The enzymes and concentration used were as follows: *Flavobacterium meningosepticum* PNGaseF (Boehringer Mannheim, Indianapolis, IN): 1U; *Flavobacterium meningosepticum* Endo F/PNGase F (Oxford GlycoSciences, Wakefield, MA): 5U; *Streptomyces plicatus* Endo H (NEB, Beverly, MA): 500U; *Clostridium perfringens* sialidase (Sigma): 0.12U; *Jack bean* β -galactosidase (Oxford GlycoSciences, Wakefield, MA): 0.1U; *Aspergillus oryzae* β -N-acetylhexosaminidase (Sigma): 0.2U; *Diplococcus pneumoniae* endo- α -N-acetylgalactosaminidase (Sigma): 1.5mU. A reaction without adding enzyme was prepared as a control to exclude the possibility of nonspecific protein degradation during incubation. The reactions were overlaid with mineral oil, incubated overnight at 37°C, and subjected to Western blot analysis using PSMA specific MoAb 7E11.

8. SELDI Immunoassay for PSMA

The SELDI ProteinChip[®] immunoassay for the detection and quantitation of PSMA is illustrated in Fig. 5. One μg of Protein G (Sigma, St. Louis, MO) in 50mM sodium bicarbonate pH8.0 was immobilized on an 8-spot pre-activated ProteinChip[®] array (CIPHERGEN Biosystems, Inc., Fremont, CA) by incubating 2hr at RT with agitation. Residual active sites were blocked by incubating the entire array in 1M ethanolamine, pH 8.0 for 30min with agitation. The array was subsequently washed 2x5min with 0.5% Triton X-100 (Sigma) in PBS, 1x5min with 0.1M sodium acetate, 0.5M NaCl, pH4.5, 1x5min with 0.1M Tris HCl, 0.5M NaCl, pH8.0, and 2x5min with PBS. MoAb 7E11 (1.5 μg) was added to the arrays and incubated at RT for 3hr. The unbound antibodies were washed off by incubating the array 2x5min in 0.1% Triton X-100 in PBS, and 2x5min in PBS. A 96-well bioprocessor was assembled over the arrays, creating multiple sample wells in the format of a 96-well plate. To establish the rPSMA standard curve, 1 to 90ng of rPSMA was used in this study as the working range and applied to the arrays in 30 μl of PBS containing 0.1% Triton X-100. To detect PSMA in serum, 400 μl of diluted serum samples were applied to the arrays (1/2 to 1/10 diluted in PBS with 0.5% Triton X-100 to obtain the mass signal of PSMA within the working range of the standard curve). The samples were incubated overnight at 4°C with vigorous agitation. The array was subsequently washed 2x5min with 0.1% Triton X-100 in PBS, 2x5min with PBS, and briefly with HPLC H₂O. Sinapinic acid (CIPHERGEN Biosystems) saturated in 50% (v/v) acetonitrile, 0.5% (v/v) trifluoroacetic acid and 0.05% Triton X-100 was used as the matrix solution, along

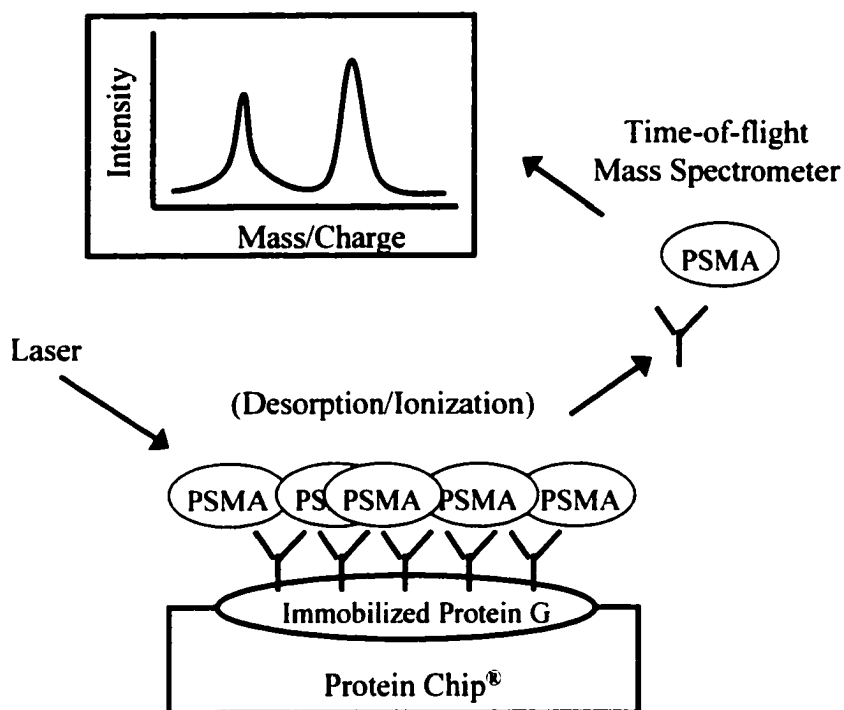


Fig. 5. Illustration of the ProteinChip® SELDI mass spectrometry immunoassay used for the quantitation of PSMA in body fluids. Protein G is used to coat a pre-activated ProteinChip to immobilize MoAb 7E11. Recombinant PSMA or body fluid is applied to the chip array. The chips are washed to remove unbound materials, an energy absorbing molecule added, and the chip subjected to SELDI-TOF-MS to produce mass spectra of the bound PSMA.

with β -galactoglobulin (MW 18,363.3 Da) included as an internal standard (IS) for normalization. The matrix solution (2x0.5 μ l) was applied to the array, and the array was air dried at RT. The detection of serum PSMA was performed using the PBS-I mass reader (Ciphergen Biosystems) with a laser intensity of 60. The data in each spectrum was averaged from 120 laser shots. The peak area of either serum PSMA or rPSMA in each spectrum was normalized against the peak area of IS. The PSMA/IS ratios were calculated and the levels of serum PSMA were determined by comparing the serum PSMA/IS peak area ratios to rPSMA/IS area ratios of the standard curve. To assess the assay performance, a total of three averaged spectra were collected from each sample. The average and standard deviations were calculated to determine the intra-assay variability. A number of serum samples were repeated on different days to assess the inter-assay variability.

9. Biostatistical Analysis

The differences of PSMA levels among the normal and patient populations were analyzed using one-way ANOVA. First, Levene's test was performed to examine the homogeneity of variances among the PSMA values of normal and patient populations. If the equality of variances was assumed (p -value >0.05), then student's t-test was performed to compare the differences of the PSMA levels. If the equality of variances could not be assumed (p -value <0.05), Tamhane's post hoc test was adopted to compare the differences of the PSMA levels. In both student's t-test and Tamhane's test, the overall significance level was set at 5%.

10. Total RNA Preparation and Northern Blot Analysis

LNCaP cells were grown as described in the section of Cells and Cell Culture. The cells were cultured in 5% charcoal/dextran-treated FBS (stripped FBS) in the presence or absence of R1881 (1, 2 or 4nM), and/or EGF (20 or 100ng/ml), and/or 12-o-Tetradecanoylphorbol-13-acetate or TPA (0.1 or 1nM) for 48 hrs. At the end of the treatment, medium was aspirated. Cells were washed with PBS, scraped and pelleted by centrifugation at 300 x g for 5 min. The supernatant was completely aspirated. The cell pellets were either stored at -80°C for later use or lysed directly in Buffer RLT in the RNeasy Kit (Qiagen, Santa Clarita, CA). Total RNA was extracted following the instructions of the kit and stored at -80°C. For Northern blot analysis, fifteen µg of total RNA was fractionated in 1.1% agarose gel by electrophoresis and blotted onto positively charged nylon membranes (Roche Molecular Biochemicals, Indianapolis, IN). The RNA was immobilized on the membrane by UV crosslink. The membrane was pre-hybridized in ULTRAhyb™ hybridization solution (Ambion, Inc., Austin, TX) prior to the addition of the digoxigenin labeled PSMA probe for hybridization. The membrane was incubated with probe overnight at 68°C. After hybridization, the blot was washed 2 x 5min in 2xSSC, 0.1% SDS and 2 x 15min in 0.1xSSC, 0.1% SDS at 68°C. Then the blot was incubated with anti-digoxigenin-AP Fab fragments (Roche, Indianapolis, IN) 1:10,000 diluted in blocking buffer and washed. To detect the PSMA mRNA band, CSPD® Ready-to-Use chemiluminescent substrate was applied to the membrane, and the membrane was exposed to X-ray film at different lengths of time.

11. Construction of Luciferase Report Vectors

The published PSMA gene sequence can be found in GenBank (accession number AF007544) (12.30). The genomic sequence also includes approximately 2.4 kb of the 5' upstream region with the transcriptional initiation site identified at nt 2488. In order to analyze the PSMA promoter, a 2kb (nts 588-2522) and a 0.8kb (nts 1791-2522) fragment of the 5' regulatory region were subcloned upstream of the pGL2 basic luciferase vector (Promega). These constructs were designated as **P1** and **P2**, respectively. Also, to analyze the potential enhancer elements in the first intron, the corresponding region (nts 2876-4973) was subcloned further upstream of the 0.8kb PSMA fragment (nts 1791-2522) in the pGL2 basic luciferase vector, and the construct designated as **I/P1**. These vectors were gratefully provided by Dr. Antonia Vlahou, EVMS. The plasmids were purified using Qiagen Endofree™ plasmid preparation kit, and the sequence identities confirmed by sequencing analysis.

12. Transient Transfection of LNCaP Cells

LNCaP cells were seeded in 6-well plates 24 hrs before transfection at 5×10^5 cells/well. The cells were approximately 70% confluent at the time of transfection. For the purpose of this study, six different transfection reagents including Transfast (Promega), Effectene (Qiagen) and Lipofectin (Gibco) Fugene 6 (Roche), Superfect (Qiagen) and LipofectAMINE (Gibco) were tested to optimize the transfection in LNCaP cells. Vector pEGFP-C1 (Clontech) is a plasmid that encodes GFP (green fluorescent protein) driven by human cytomegalovirus (CMV) immediate early promoter. It can constitutively express GFP upon entering mammalian cells, and

therefore can be used as an indicator for successful transfection. To test the transfection reagents, DNA complex mixtures were prepared by mixing 2 μ g pEGFP plasmid DNA with each of the six different transfection reagents respectively. The transfections were proceeded according to the manufacturers' instructions. The expression of GFP was examined 24 to 48 hrs after transfection. The cells that have received and internalized pEGFP vector emitted bright green light under fluorescent microscope, whereas the untransfected cells remained dim. Fig. 6 summarizes the transfections with pEGFP vector in LNCaP cells using different transfection reagents. Of the six transfection reagents, Effectene, Transfast and Lipofectin showed the highest transfection efficiency. The same results were obtained with the transfections performed in RPMI 1640 medium containing either 5% stripped FBS or 5% stripped FBS plus 2nM R1881. Transfast was chosen for the current study because of its high transfection efficiency and simple protocol.

Once the transfection conditions were perfected with pEGFP vector, the same protocol was used to introduce the luciferase reporter constructs containing PSMA promoter and/or enhancer into LNCaP cells to test their promoter/enhancer activities. LNCaP cells seeded in 6-well plates at 70% confluency were transfected with 2 μ g of P1, P2, I/P1 or pGL2-basic vectors and harvested at 48hrs after transfection. In order to examine the constructs' responsiveness to androgens, the transfections were performed in RPMI 1640 medium containing 5% stripped FBS or 5% stripped FBS plus 2nM R1881. A pGL3-PSA luciferase reporter construct carrying a 5.8kb promoter/enhancer region of PSA gene (kindly provided by Drs. E. Keller and A. Mizokami) was used as the positive control for the promoter responsiveness to

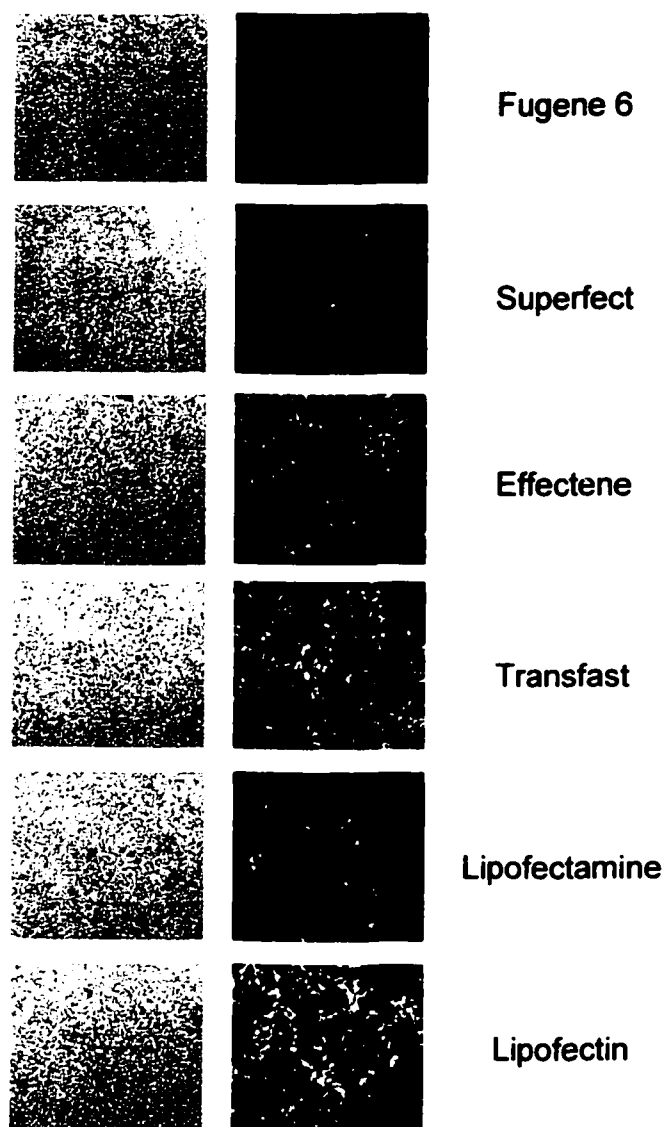


Fig. 6. Comparison of transfection reagents in LNCaP cells using GFP. GFP vector was transfected in LNCaP cells using six different transfection reagents. The cells were cultured in RPMI medium with 5% FBS and the transfection efficiency was determined under fluorescent microscope (4x) (*right panel*). The *left panel* shows the cell numbers under the normal phase (4x). Note that similar transfection efficiency was also observed in 5% charcoal stripped RPMI medium with or without 2nM R1881.

androgen. To examine the direct involvement of AP-1 proteins in the activation of PSMA promoter, the PSMA luciferase constructs were cotransfected with 2 μ g each of the pcDNA-cJun and/or pcDNA-cFos expression vector or the empty vector pcDNA3Z+ as negative control (vectors were provided by Dr. Timothy J. Bos at EVMS). The cotransfected cells were incubated in medium containing either 5% stripped FBS or 5% stripped FBS with 2nM R1881 and harvested at 48 hrs post transfection for luciferase assay. All transfections were repeated in triplicate in three independent experiments.

13. Luciferase Assay

Cells were harvested from 6-well plates 48hrs after transfection. Medium was removed by aspiration and cells washed with cold PBS. Cells were lysed in 150 μ l of 1x Reporter Lysis Buffer (RLB) (Promega, Madison, WI), then frozen at -80°C. thawed and scraped to collect the lysates. After vortexing for 30sec, the lysates were centrifuged at 4°C, 12,000rpm for 1min. The luciferase activity in 20 μ l of the supernatant was measured using TD20/20 luminometer (Turner Design) and normalized using the amount of total protein in the cell lysates. The luciferase activity of the PSMA promoter constructs was presented as the fold of induction over the pGL2-basic vector.

14. Preparation of Nuclear Extracts

LNCaP cells from one T75 flask were washed 2x briefly with cold PBS, scraped and harvested by centrifugation at 1500rpm for 5min. PBS was removed and the cells

were resuspended in cold 10mM HEPES pH7.9, 1.5mM MgCl₂, 10mM KCl, 0.5% Igepal (Sigma), 1x *Complete*[™] Proteinase Inhibitor Cocktail (Roche), 1mM PEFA (Roche), 0.5mM DTT. Cells were incubated on ice for 10min with gentle pipetting up and down 2x at 5min increments, and centrifuged at 4°C, 14,000rpm for 10min. The supernatant was discarded and the pellet was resuspended in 20mM HEPES pH 7.9, 25% glycerol, 0.42M NaCl, 1.5mM MgCl₂, 0.2mM EDTA, 1x *Complete*[™] Proteinase Inhibitor Cocktail, 1mM PEFA, 0.5mM DTT. Cells were incubated on ice for 30min with gentle pipetting up and down 2x at 5min increments, and centrifuged at 4°C, 14,000rpm for 30min. The supernatant was dialyzed against 20mM HEPES pH 7.9, 20% glycerol, 0.1M KCl, 0.2mM EDTA, 1mM PEFA, 0.5mM DTT at 4°C in porous membrane tubing (MWCO 12-14,000; flat width: 10mm; Spectra/Por) for 4 hrs with 1 change at half way point. Lysates were removed from tubing, transferred to eppendorf tubes in 25µl aliquots, quick frozen on dry ice and stored at -80°C. The protein concentration was determined by the BCA protein assay (Pierce Chemical, Rockford, IL).

15. Synthesis of DNA Probe for EMSA

Oligonucleotide probes were generated from overlapping primer sets synthesized by GibcoBRL/Life Technologies. The probes included 3 putative AP-1 binding sites from the PSMA promoter region, a consensus AP-1 site (PG32-1), 3 AP-1 like sites (PG32-1 mutants), an androgen responsive element (ARE), a mutant ARE and a CMV heterologous nucleotide. The primer sets were as follows with the core AP-1 or ARE site shown in underlined base pairs:

PSMA 1:

Forward Primer 5' TTCAAGCGATTCTCCTGCCTCAGC 3'

Reverse Primer 5' TACTCAGGAGGCTGAGGCAGG 3'

PSMA 2:

Forward Primer 5' TCTGCTCTGCTTTTATTCAGT 3'

Reverse Primer 5' TCTACAATACTCTACTGAATAA 3'

PSMA 3:

Forward Primer 5' TAGAATTTTCAGAGTTGAATAAA 3'

Reverse Primer 5' TTATGAGGAACTTTTATTCA 3'

PG32-1:

Forward Primer: 5' ACCCGGGGATCCTCTAGAATGACTCATCGG 3'

Reverse Primer: 5' CTTGCATGCCTGCAGGATCCGATGAGTCAT 3'

PG32-1 mut-1:

Forward Primer: 5' ACCCGGGGATCCTCTAGAATGACCCATCGG 3'

Reverse Primer: 5' CTTGCATGCCTGCAGGATCCGATGGGTCAT 3'

PG32-1 mut-2:

Forward Primer: 5' ACCCGGGGATCCTCTAGAATGACGCATCGG 3'

Reverse Primer: 5' CTTGCATGCCTGCAGGATCCGATGCGTCAT 3'

PG32-1 mut-3:

Forward Primer: 5' ACCCGGGGATCCTCTAGAATGACACATCGG 3'

Reverse Primer: 5' CTTGCATGCCTGCAGGATCCGATGTGTTCAT 3'

PSA ARE:

Forward Primer 5' TTGCAGAACAGC 3'

Reverse Primer 5' GAGCTAGCACTTGCTGTCT 3'

PSA mutant ARE (mARE):

Forward Primer 5' TTGCAAAAAAAGC 3'

Reverse Primer 5' GAGCTAGCACTTGCTTTTTTIG 3'

CMV Hetero-oligo:

Forward Primer: 5' TTGACGTCAATAATGACG 3'

Reverse Primer: 5' TATGGGAACATACGTCAT 3'

For each probe, the primer set was first allowed to self anneal at 48°C for 10min in the mixture of dATP (for unlabeled probe) or [α -³²P]ATP (for labeled probe) and dTTP, dCTP, dGTP. Klenow Fragment of DNA polymerase I (3' > 5' exo⁻) (NEB) was added to the mixture and the reaction was incubated at 16°C for 1 hr. The probe was phenol-extracted and then purified on a Chroma Spin TE 10 column (Clontech). The ³²P incorporation was estimated using liquid scintillation counter. The labeling efficiency was obtained at >1,000,000cpm/ μ l. The probes were stored at -20°C.

16. Electrophoretic Mobility Shift Assay (EMSA)

Gel mobility shift assays were based on the conditions described by Hadman *et al* (60) with slight modifications. Ten to fifteen μ g of nuclear extract and 1 μ g of poly (dI.dC) (Amersham Pharmacia) were added to the binding buffer containing 10mM HEPES pH 8.0, 17.5% glycerol (v/v), 0.1mM EDTA, 20mM NaCl, 4mM MgCl₂, 2mM Spermidine (Sigma) and 2mM DTT (Gibco). The mixture was preincubated on ice for 20min. Approximately 50,000cpm of the labeled probe was added to each

reaction and incubated on ice for 20min. The reaction was loaded onto a 6% (59:1) acrylamide gel and run with 1xTBE buffer at 20mA. The gel was dried in vacuum gel drier at 80°C for 1hr and exposed to X-ray film for autoradiography. For cold probe competition assays, unlabeled probes were added in 50- or 100- fold excess during preincubation. For antibody competition assays, 0.5 to 2µg of antibody was added during the 20min preincubation or after the addition of labeled probe followed by 15min incubation on ice.

CHAPTER III

RESULTS

A. EXPRESSION AND PURIFICATION OF RECOMBINANT PSMA

1. Construction of pBlueBacHisB-PSMA Transfer Vector

Fig. 7A shows the map of pBlueBacHisB-PSMA construct. The pBlueScript-PSMA transfer vector was constructed by RT-PCR cloning of the full length PSMA cDNA from LNCaP cell mRNA. The subsequent PCR step of adding BamHI and HindIII restriction sites to the 5' and 3' ends of PSMA fragment is shown in Fig. 7B. The amplified product was then conveyed from the pGEMT vector into the pBlueBacHisB vector linearized with the restriction enzymes BamHI and HindIII. The restriction digestion of pBlueBacHisB-PSMA construct showed the existence of 2.65kb PSMA cDNA (Fig. 7C). Partial sequencing confirmed the correct PSMA coding region with the 6x histidine tag at its amino terminus.

2. Generation and Identification of rPSMA Baculovirus

The strategy used to generate and identify rPSMA baculovirus is outlined in Fig. 8. First, insect cells were cotransfected with pBlueBacHisB-PSMA plasmid and the linearized wild-type AcMNPV DNA. Forty eight hours after transfection, the media from the transfected plates were harvested and used as initial viral stock in the plaque assay to select rPSMA baculovirus. Because the *in vivo* homologous recombination would add a promoter to drive the *Laz* gene expression in the linearized baculovirus

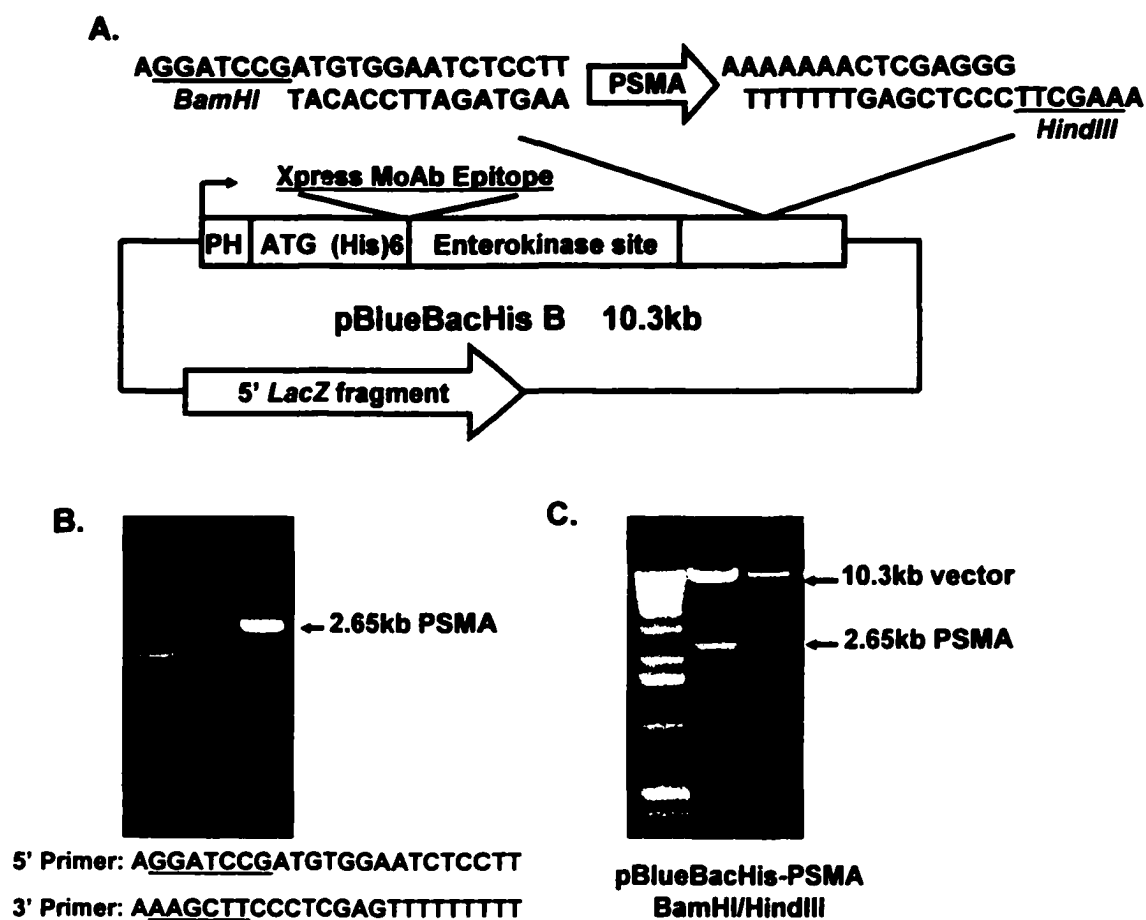


Fig. 7. Construction of pBlueBacHisB-PSMA transfer vector.

A, A map of pBlueBacHis-PSMA transfer vector. *PH*, polyhedrin gene promoter; *(His)⁶*, six histidine tag fused to 5' end of PSMA gene; *Xpress MoAb Epitope*, recognition site for the Anti-Xpress MoAb that detects *(His)⁶*-fusion protein. *B*, PCR amplification of PSMA cDNA from pBlueScript phagemid vectors. *Lane 1*, 1kb DNA ladder; *Lane 2*, amplified products from a randomly isolated pBlueScript plasmid showing nonspecific amplification; *Lane 3*, amplified products from pBlueScript-PSMA. The arrow indicates the 2.65kb PSMA amplified product. *C*, Restriction digestion of pBlueBacHis-PSMA with BamHI and HindIII. *Lane 1*, 1kb DNA ladder; *Lanes 2 and 3*, 5 μ g or 1 μ g of pBlueBacHis-PSMA plasmid digested with BamHI and HindIII. The arrows indicate the 10.3kb linearized pBlueBacHisB vector and the 2.65kb PSMA restricted fragment.

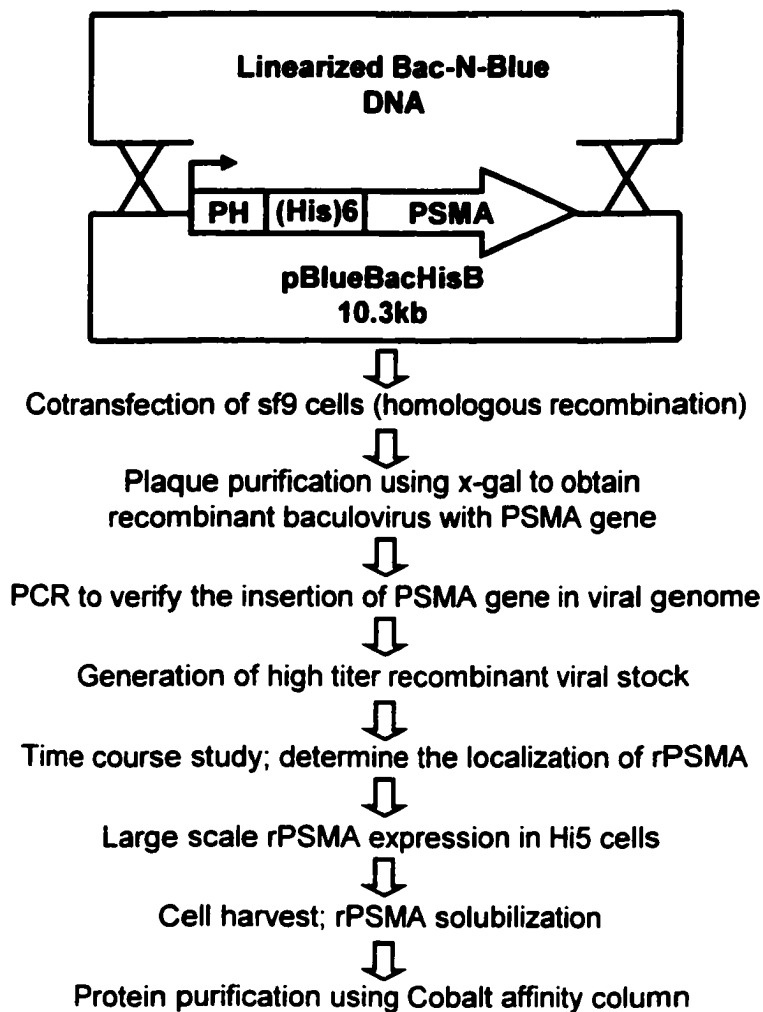


Fig. 8. A schematic diagram outlining the strategy for expression and purification of recombinant PSMA using baculovirus /insect cell system.

DNA, the active β -galactosidase would be produced along with the recombinant protein. Thus, by using the chromogenic substrate x-gal in the agarose medium, the recombinant PSMA viruses were visualized as the blue and occlusion body negative plaques (Fig. 9A). Four rounds of plaque assays were repeated to ensure the purity of the rPSMA baculovirus. To further rule out any false positives, PCR analyses were performed on putative rPSMA viral DNA (Fig. 9B). Since the primers flanked the polyhedrin gene, the wild type polyhedrin gene fragment without the rPSMA insert was amplified as a 630bp band, whereas the rPSMA insert was shown as a 3.2kb amplified fragment. Therefore, the PCR result confirmed the correct insertion of the 2.65kb PSMA gene behind the polyhedrin promoter.

The expression of rPSMA protein was monitored by Western blot analyses using antiXpress (Invitrogen, Carlsbad, CA) and PSMA specific MoAb 7E11. AntiXpress recognizes the enterokinase digestion site at the amino terminus of the Histidine-tagged proteins expressed from the pBlueBacHisB vector (Fig. 7A), whereas the epitope for 7E11 is located at the first 6 amino acids of the amino terminus of PSMA (15). Fig. 10 shows that both antibodies detected predominantly a 100kDa protein band in the recombinant virus infected cells (lane 4, Fig. 10A and B), but not in the uninfected (lane 1) or the wild type virus infected insect cells (lane 2). After being expressed, rPSMA remained located in insect cells, and was not secreted in the cell culture medium (lane 3). Like the native PSMA from LNCaP cells (14), a 200kDa dimer of rPSMA was detected in high protein concentration of insect cell lysates (lane 5, Fig. 10A). Note that the 45kDa band detected by 7E11 was likely due to the proteolytic degradation in the crude insect cell lysates or an artifact in SDS-PAGE

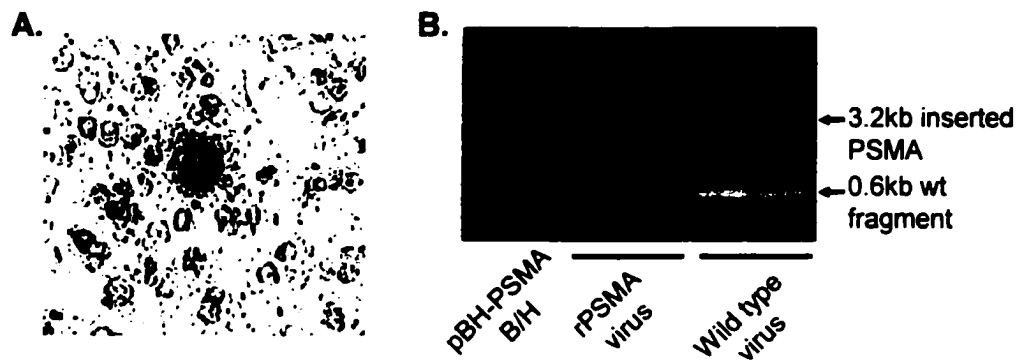


Fig. 9. Generation of recombinant PSMA baculovirus.

A. A recombinant PSMA baculovirus plaque shown in plaque assay.

B. PCR analysis verified the insertion of PSMA gene into baculovirus genome. Baculovirus DNA was isolated from the infected insect cells and subjected to PCR analysis using primers specific to the 5' and 3' ends of the polyhedrin gene. The amplified product was run on 1% agarose gel and stained with ethidium bromide. *Lane 1*, 1kb DNA ladder; *Lane 2*, pBlueBacHis-PSMA restricted by BamHI and HindIII; *Lanes 3 and 4*, amplified product from the rPSMA baculovirus DNA; *Lanes 5 and 6*, amplified product from the wild type baculovirus DNA. The 3.2kb product represents the rPSMA baculovirus and 630bp product is derived from the wild type baculovirus.

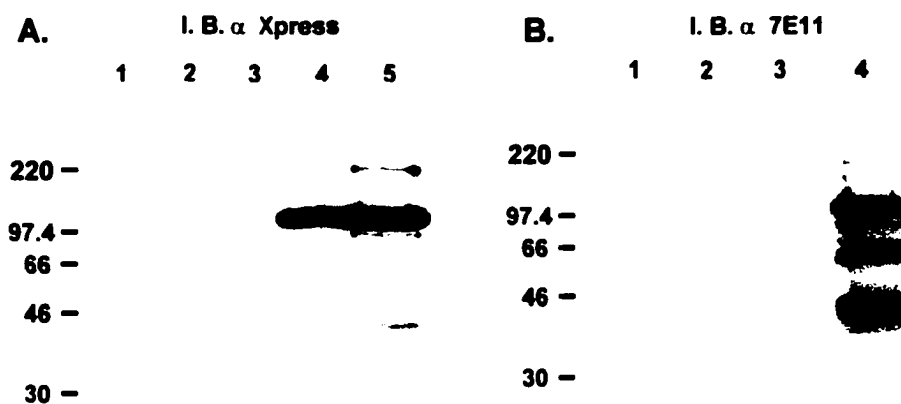


Fig. 10. Western blot analysis to detect the expression of rPSMA. Hi5 insect cells were infected with wild type or rPSMA baculovirus. PSMA expression in whole cell lysate (WCL) and culture medium was analyzed using MoAb anti-Xpress (A) or 7E11 (B). Lane 1, uninfected Hi5 cells; Lane 2, wild type virus-infected WCL; Lane 3, rPSMA virus-infected culture medium; Lane 4, rPSMA virus-infected WCL 2 μ g; Lane 5 (only with MoAb anti-Xpress), rPSMA virus-infected WCL 5 μ g.

sample preparation (61). Taken together, Western blot analyses demonstrated that rPSMA was correctly expressed in the insect cells, and also implied that the addition of six histidine amino acid residues to the amino terminus of PSMA did not mask or interfere with the epitope recognized by MoAb 7E11.

3. Optimization of rPSMA Expression

To determine the rPSMA expression conditions, time course experiments were performed on insect cells infected with recombinant baculovirus at different MOI and the amounts of rPSMA expressed were analyzed at 24-hr intervals (Fig. 11). Increased MOI from 1 to 5 did not seem to alter the expression of rPSMA, as the expression peaked at 48 hrs post infection (p.i.), regardless of the MOI. This was followed by a decrease, possibly due to the rapid degradation of rPSMA as a foreign protein in insect cells. By 96 hrs p.i. rPSMA was detected in culture medium accompanying cell lysis. Therefore, the optimal expression conditions were to infect cells at a MOI of 5 and to harvest at 48hr post infection.

4. Large Scale Expression and Purification of rPSMA

Large-scale rPSMA expression was carried out in monolayer cell culture, since it had previously been shown that the Hi5 insect cells appeared to provide better glycoprotein expression levels in monolayer cultures (62). Because PSMA is a heavily glycosylated transmembrane protein, lysis buffer containing the detergents SDS and Igepal was required for solubilization. Purification of rPSMA was performed using a cobalt affinity column and imidazole gradient elution, resulting in a rPSMA elution

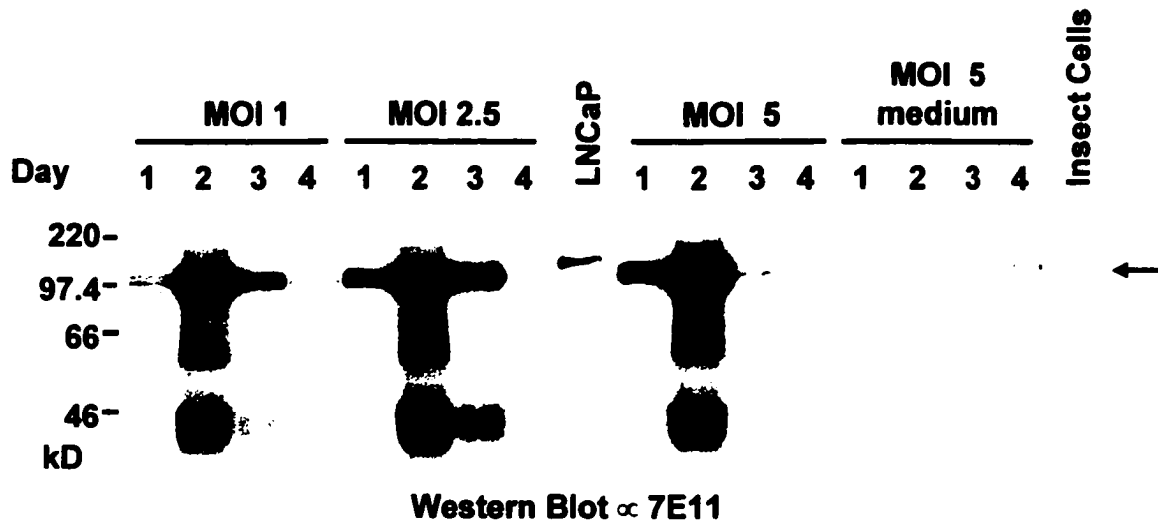


Fig. 11. Time course and cellular localization study of recombinant PSMA expression. Hi5 insect cells were infected with rPSMA viral stock at MOI 1, 2.5 and 5. Infected cells were harvested from day 1 to day 4. Both whole cell lysate and medium were subjected to Western blot analysis using 7E11. Cell membrane extract from LNCaP was used as positive control and uninfected Hi5 cells were used as negative control.

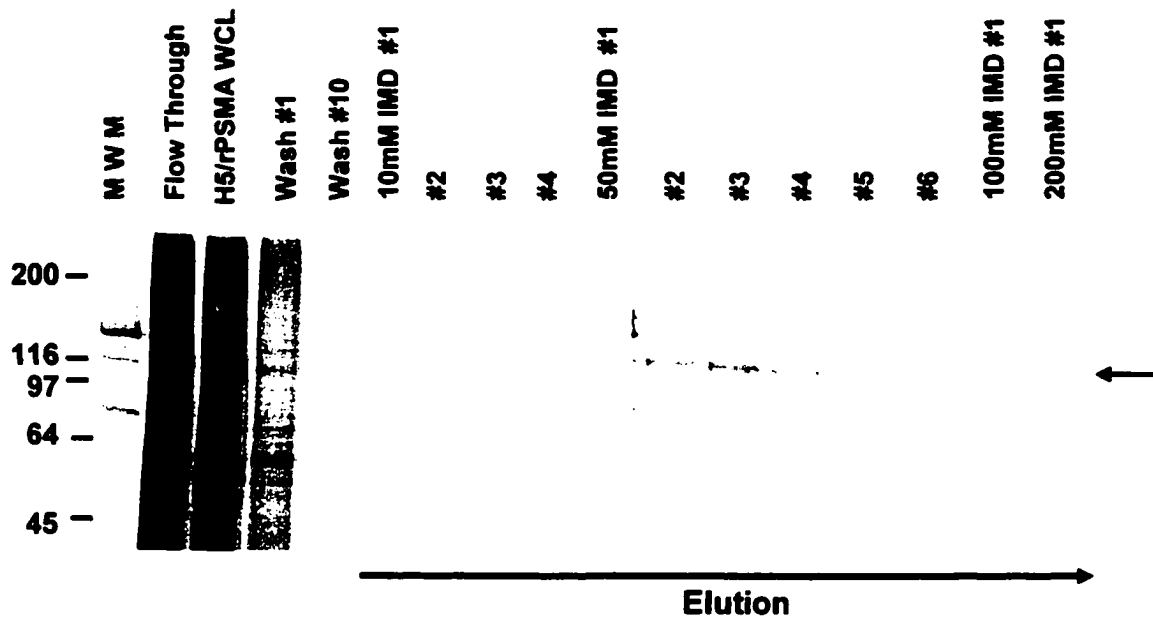


Fig. 12. Purification of recombinant PSMA using Cobalt affinity chromatography. The WCL (0.5 μ g), flow-through and eluted fractions (20 μ l) were analyzed on a 10% SDS-PAGE followed by silver staining. *IMD*, imidazole.

peak in the 50mM imidazole fractions (fraction #3 in 50mM imidazole, Fig. 12). In addition to the 100kDa rPSMA commonly shown in all eluted fractions, the 200kDa rPSMA was also readily detected by silver stain in the elution peak. The pooled rPSMA containing eluants were dialyzed to eliminate the interference of imidazole with protein quantitation in the BCA assay. Approximately 0.5 mg of rPSMA protein was obtained from 30mg of insect cell crude lysate using 1ml of the cobalt affinity resin, to give a final yield of 1.67% (i.e. the amount of rPSMA/ the amount of crude lysates).

5. Characterization of rPSMA

Purity and molecular size SDS-PAGE and Western blotting were run in parallel to analyze the purity of rPSMA in the final product (Fig. 13A and B). In addition to the major 100kDa rPSMA band (lanes 1 and 2, Fig. 13A), another closely migrating >100kDa band and a band of approximately 190kDa were also observed by silver staining when doubling the amount of rPSMA applied to the gel (lane 1, Fig. 13A). Since all the 100kDa and 190kDa species were readily detected by the 7E11 antibody (lanes 1 and 2, Fig. 13B), the two 100kDa bands were likely due to the slight differences in glycosylation (refer to the data in Fig. 14), whereas the 190kDa protein most likely represents a high molecular weight rPSMA formed at high concentration. The latter species has also been detected in native PSMA preparations (14). The purity and molecular size of rPSMA was further confirmed by mass spectrometry by applying the rPSMA directly to a Ni⁺⁺-coated IMAC ProteinChip[®]. As shown in Fig. 13C, the purified rPSMA protein product was accurately detected with an observed

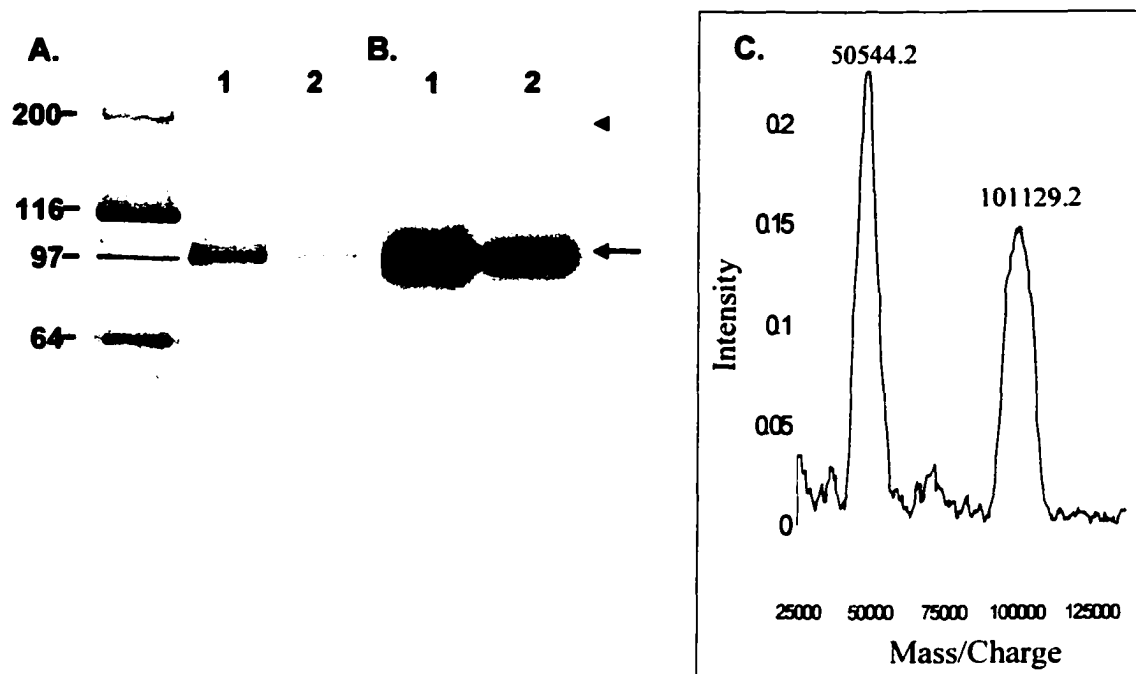


Fig. 13. The purity of rPSMA. *A*, Recombinant PSMA was detected on a 10% SDS-PAGE gel by silver staining. *Lane 1*, 0.5 μg of rPSMA; *Lane 2*, 0.2 μg of rPSMA. A single band of approximately 100 kDa was shown in low concentration, which was more pronounced as a doublet band in high concentration (*arrow*). A PSMA dimer (190 kDa) was also detected in higher protein concentration (*arrowhead*). *B*, A Western blotting using 7E11 run in parallel with silver staining showed both the 100 kDa and 190 kDa rPSMA species. The amounts of rPSMA in lanes 1 and 2 were the same as in (*A*). No other contaminants were detected. *C*, Recombinant PSMA was applied directly to a Ni⁺⁺-coated IMAC ProteinChip[®] and the chip subjected to SELDI-TOF-MS. A peak of the correct mass of (101129 Da) was observed. Also observed was a second peak, which is the doubly charged PSMA species (50544 Da).

mass of 101129.2Da. The peak at 50544.2Da represents the doubly charged rPSMA, i.e. a signal generated at half the mass/charge value of the singly charged PSMA species. Collectively the silver stained gel, immunoblotting and mass spectrometry demonstrate that the rPSMA has been purified to near homogeneity and was of the predicted molecular size.

Glycosylation Because native PSMA is a glycoprotein (16), and rPSMA was observed to have approximately the same molecular size as the native PSMA, we compared the extent of glycosylation in the rPSMA with the PSMA derived from LNCaP cells, i.e. the native PSMA. A series of glycosidase digestions were performed on rPSMA and the digests were analyzed by Western blotting using MoAb 7E11. Fig. 14A shows the change of rPSMA molecular weight upon digestion with endoglycosidases specific for N-glycans. Recombinant PSMA was completely deglycosylated by PNGase F (PF), endo F/PNGaseF (F/PF) and at least partially deglycosylated by endo H (H), resulting in a nonglycosylated form of PSMA with an approximate size of 86kDa (lanes 2, 3 and 4, Fig. 14A). The sensitivity to endo H indicated that the N-glycans on rPSMA are mainly the high mannose type. This observation was further confirmed by the resistance of rPSMA to a variety of complex-type specific exoglycosidases such as sialidase (S), β -galactosidase (G), β -N-acetylhexosaminidase (AH) and various combinations (Fig. 14B). Note that the endo H treated rPSMA was slightly larger than the completely deglycosylated form (lane 4 compared to lanes 2 and 3, Fig. 14A). This suggests that besides being modified mainly with the high mannose type N-glycans, the rPSMA protein might also contain

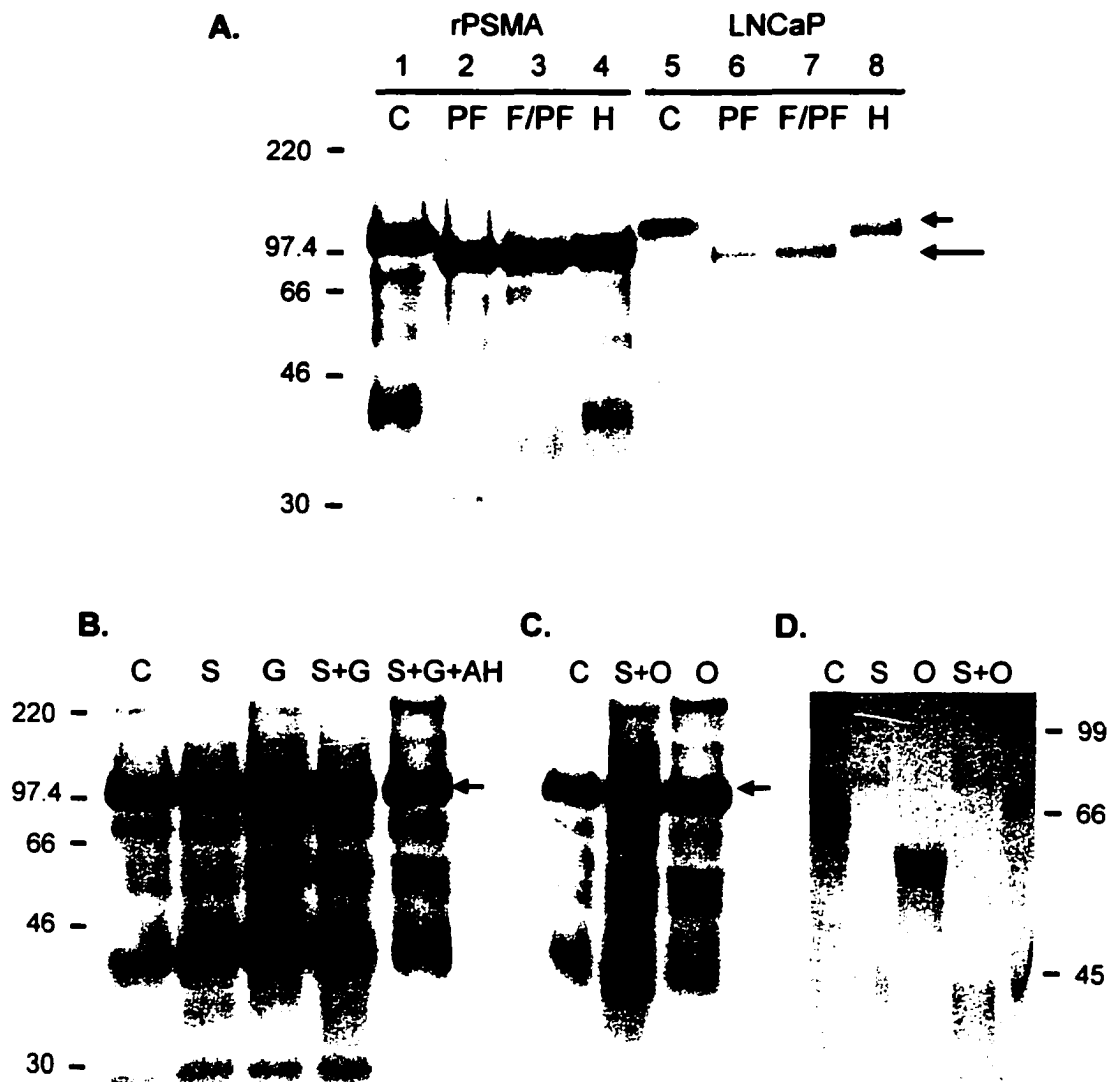


Fig. 14. Glycosylation analysis of rPSMA. Purified rPSMA or LNCaP membrane extracts were digested with endo- or exoglycosidases, and the digests were analyzed by Western blotting using MoAb 7E11. *A*, Digestion with endoglycosidases specific for N-glycans. *Lanes 1 to 4*, rPSMA; *Lanes 5 to 8*, LNCaP membrane extracts; *C*, control without enzyme; *PF*, PNGase F; *F/PF*, endo F/PNGase F; *H*, endo H. Note that PNGase F and endo F/PNGase F cleaved both recombinant and LNCaP derived PSMA, resulting in an 86kDa band (*large arrow*). Endo H cleaved most if not all the carbohydrates from rPSMA, while it only slightly cleaved LNCaP derived PSMA (*small arrow*).

Fig. 14. (Continued) *B*, Digestion of rPSMA with exoglycosidases. *C*, control without enzyme; *S*, sialidase; *G*, β -galactosidase; *S+G*, sialidase/ β -galactosidase; *S+G+AH*, sialidase/ β -galactosidase/ β -N-acetylhexosaminidase. All the exoglycosidases were proven to be active in the control tests (refer to materials and methods; data not shown). *C*, Digestion of rPSMA with endoglycosidases specific for O-glycans. *C*, control without enzyme; *S+O*, sialidase/endo- α -N-acetylgalactosaminidase; *O*, endo- α -N-acetylgalactosaminidase. Note that none of the glycosidases used in *B* and *C* were capable of stripping the carbohydrates from rPSMA or reducing the molecular size of the 100kDa rPSMA (*small arrow*). *D*, Digestion of bovine fetuin as the positive control for sialidase and O-glycanase. *C*, substrate only; *S*: sialidase; *O*, endo- α -N-acetylgalactosaminidase; *S+O*, sialidase / endo- α -N-acetylgalactosaminidase. The digests were resolved by SDS-PAGE and stained with Coomassie blue. The increases in mobility of fetuin indicated that the enzymes in the experiment were active.

Table 2 *Glycosylation of rPSMA*

Type of Glycans	Subtype	PSMA (LNCaP)	rPSMA
N-linked glycans	complex	+ ^b	
	high-mannose		+
O-linked glycans	-- ^a	- ^c	-

^a --, not available.

^b +, present.

^c -, not present.

a very small portion of the complex type N-glycans but which did not cause a significant mobility shift after cleavage by the specific exoglycosidases (Fig. 14B).

On the other hand, native PSMA from LNCaP cells was completely deglycosylated by PNGase F and endo F/PNGaseF, and showed a major, if not complete resistance to endo H (lane 8 compared to lanes 6 and 7, Fig. 14A), suggesting that the N-glycans of native PSMA are mainly composed of complex type N-glycans with possibly a minor fraction of the high mannose type. Taken together, the N-glycan profiles of rPSMA and native PSMA seem to be different, with rPSMA being composed predominantly of the high-mannose type glycans.

Since the native PSMA in LNCaP cells were shown to lack O-glycans (16), it was of interest to examine whether O-glycans were also absent in rPSMA. Digestion with endo- α -N-acetylgalactosaminidase alone (O) or in combination with sialidase (S+O) caused no molecular weight changes (lanes 2 and 3 compared to lane 1, Fig. 14C). The glycosidases used in this experiment were shown to be active using fetuin as the substrate (Fig. 14D). Therefore the result indicates that O-glycans are absent in rPSMA. The result of glycosylation analysis is summarized in Table 2.

Immunogenicity MoAbs 4G5 and 4G7 were second generation MoAbs produced in our laboratory using purified native PSMA (Wright, et al, unpublished data). MoAb J591, which recognizes an epitope on the extracellular domain of PSMA, was kindly provided by Dr. Neil Bander at Cornell University (20). These antibodies and 7E11 were used to perform Western blot analyses to determine if rPSMA could be recognized by MoAbs to both the intracellular and extracellular domains of PSMA. Fig. 15 shows that like native PSMA, rPSMA was bound by all four antibodies, with

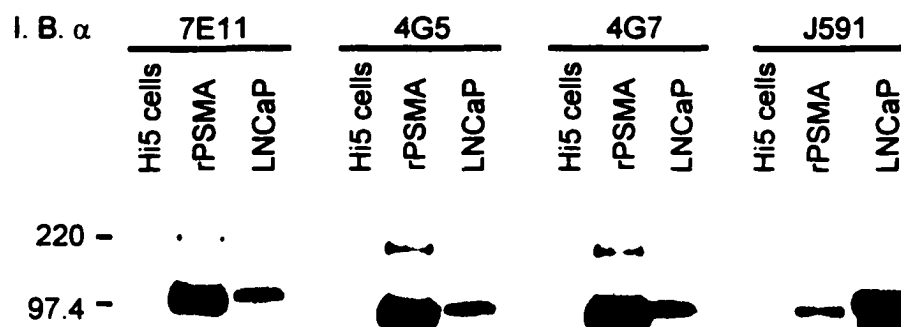


Fig. 15. Western blot analysis of rPSMA. MoAb *7E11* and two second generation MoAbs, *4G5* and *4G7*, which recognize the intracellular domains of PSMA, and *J591* which recognizes the extracellular domain were used. *Lane 1*, uninfected insect Hi5 cells; *Lane 2*, rPSMA; *Lane 3*, LNCaP membrane extracts. All four MoAbs identified the 100kDa rPSMA, whereas the 200kDa PSMA dimer was detected by *7E11*, *4G5* and *4G7*.

the major 100kDa protein detected. The minor 200kDa species was also detected by 7E11, 4G5 and 4G7 but not by J591, probably due to the different affinities to the rPSMA dimer. Since these antibodies are specific for multiple intra- and extracellular regions of the PSMA polypeptide backbone, the results indicate that the baculovirus rPSMA possesses the correct peptide sequence and similar immunoreactivity to native PSMA, despite the differences in glycosylation. Moreover, because of the similarity in immunoreactivity between the native and recombinant PSMA, rPSMA can be used as the purified substitute antigen in developing immunoassay for PSMA.

B. DEVELOPMENT OF PSMA SELDI IMMUNOASSAY

1. Optimization of Conditions for PSMA SELDI Immunoassay

ProteinChip[®] SELDI technology offers a novel approach for the development of immunoassay. Since the conditions for SELDI immunoassay have not been established, the following parameters were determined to optimize the assay:

PSMA Specific Antibody MoAb 7E11 (11), J591, and 107-1A4 (provided by Dr. R. Vessella at the University of Washington) (63), and rabbit polyclonal antibodies PSM#1 and PSM#2 (kindly provided by Dr. S. L. Su at Northwest Biotherapeutics, Inc.) are PSMA specific antibodies. Since different antibodies recognize the same PSMA protein with different specificities and affinities, these antibodies were compared in order to determine which has the highest affinity for capturing both the native PSMA and rPSMA. Out of these five antibodies, 7E11 and 107 showed higher affinities than the other three antibodies. Fig. 16 illustrates the comparison of the

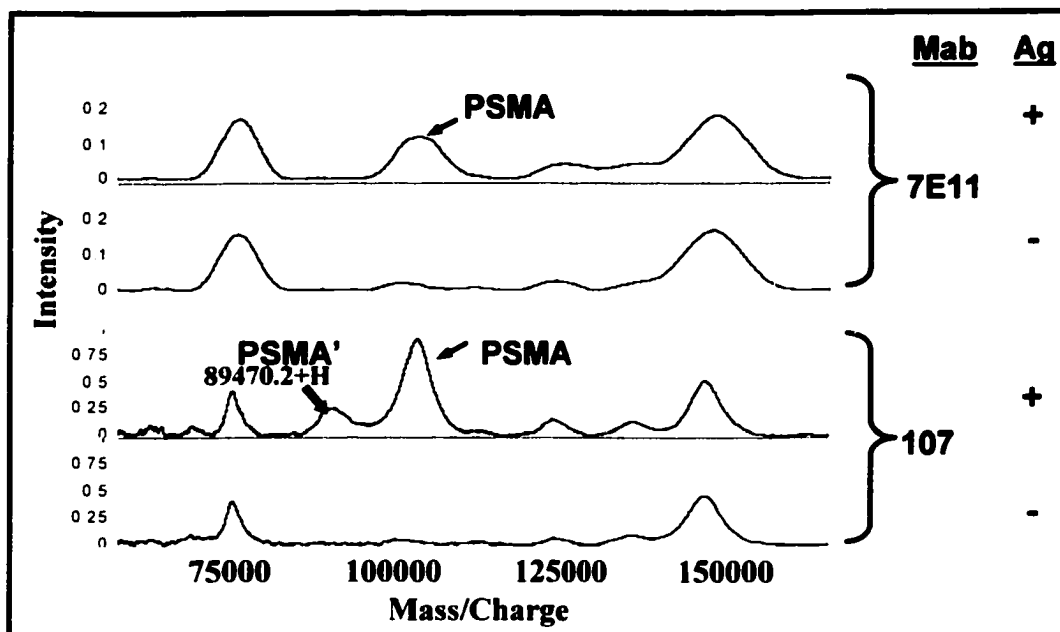


Fig. 16. Comparison of the antigen capturing efficiency of PSMA specific antibodies in SELDI immunoassay. Two antibodies (*7E11* and *107*) were immobilized on PS-1 chips. The whole cell lysate from LNCaP cells which express PSMA was used as the source of antigen (*Ag*). +, LNCaP whole cell lysate added; -, dilution buffer only (0.1% Triton X-100 in PBS). Note that beside the 101kDa PSMA captured by both antibodies (*arrows*), an 89,470Da protein was also detected by *107* (*block arrow*), which is likely to be PSMA', the splicing variant of PSMA.

PSMA peaks in the spectra obtained by 7E11 and 107. Both 7E11 and 107 were able to capture PSMA from LNCaP cell lysate. In addition to the 101kDa PSMA detected by both antibodies, an 89kDa protein was also detected using 107. Since the PSMA peak area was to be used for the quantitation of PSMA, the presence of the 89kDa peak might compromise the definition of the 101kDa peak area (Fig. 16 and the bottom panel of Fig. 17). Therefore, 7E11 was selected for further evaluation in PSMA immunoassay development.

Chip Surface Chemistry PS-1 and PS-2 are two types of preactivated protein chips manufactured by Ciphergen. Although both chips can covalently immobilize protein for the subsequent capture of another protein that has affinity to the first one, e.g. protein G and antibody, the chemistries of the two chips differ. Carbonyl diimidazole moieties were the active sites on the PS-1 chips, and the epoxy groups on the PS-2 chips. For this reason, they offer different efficiencies in protein immobilization and sensitivities in protein binding. Therefore it is necessary to compare these two chip types to maximize the coating of protein G and to obtain better sensitivity in PSMA detection. As shown in Fig. 17, when LNCaP cell lysate was serially diluted and applied to PS-1 and PS-2 chips with 7E11 or 107, the difference in peak detection was apparent. At the same protein concentration, the intensity of the 101kDa PSMA peak was much higher on the PS-1 chip (left panel) than on the PS-2 chip (right panel). Moreover, the serial dilution of antigen revealed a better linearity on the PS-1 chip. Therefore the PS-1 chip was chosen as the surface for PSMA SELDI immunoassay.

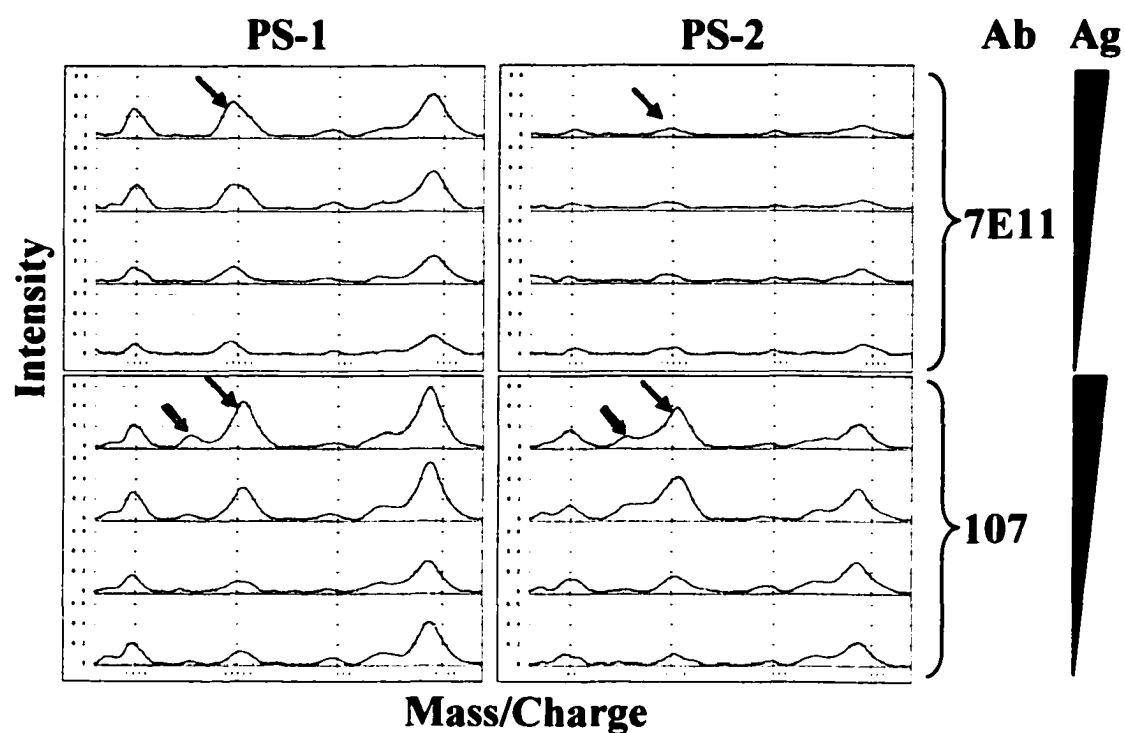


Fig. 17. Comparison of the ProteinChip[®] surface chemistries in PSMA SELDI immunoassay. Two antibodies (*7E11* and *107*) were immobilized on PS-1 or PS-2 chips. The whole cell lysate from LNCaP cells was used as the source of antigen (*Ag*) and applied to the chip in serial dilution (*wedge*, from 6, 12, 25 to 50ng/μl). Note that higher intensities were obtained on PS-1 chips than on PS-2 chips. Beside the 101kDa PSMA captured by both antibodies (*arrows*), an 89,470Da protein was also detected by 107 (*block arrow*).

Antigen Capture The next step was to determine the antigen capture capacity of the PS-1 chip. After coating the chip with protein G and MoAb 7E11, 15 to 90 ng of purified rPSMA was applied to the chip. Post-assay rPSMA samples were retrieved and analyzed along with pre-assay samples using 7E11 Western blotting. Fig. 18 showed that the 7E11 based SELDI immunoassay was capable of capturing up to 90ng of rPSMA with the saturation still not being reached. The result provided an estimation of the antigen capture capacity of the PS-1 chip array that was helpful for establishing standard curve.

Standard Curve To establish a standard curve for PSMA SLEDI immunoassay, pure rPSMA was serially diluted, immunocaptured on the PS-1 chip array, and subjected to mass analysis. Fig. 19A shows that 1 to 50ng of rPSMA was readily detected at the molecular size of 101kDa. By plotting the rPSMA concentration versus the normalized peak area ratio between PSMA and the internal control (β -galactoglobulin), a rPSMA linear standard curve was generated with R^2 of 0.985 (R^2 : the square of the correlation coefficient or the strength of the association) and CV of 8.9% (CV: the coefficient of variation; used as a gauge of the accuracy of the standard curve) (Fig. 19B).

Specificity of antigen capture from serum To further examine whether PSMA can be selectively captured and quantitated in complex body fluids, SELDI immunoassay for PSMA was performed using the serum samples from a patient with pathologically confirmed BPH and a patient diagnosed with advanced (stage T3) prostate cancer (PCA). These two serum samples were selected because they were known to contain different levels of PSMA by Western blot analysis (27). Fig. 20

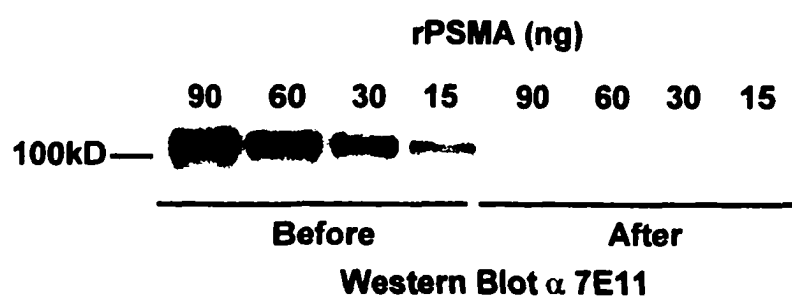


Fig. 18. The capture of rPSMA on PS-1 chip. Western blot analysis showing the amount of rPSMA added to the chip array (*Before*) and the amount of rPSMA remaining in the supernatant after incubation (*After*). This analysis shows that up to 90ng of rPSMA could be bound to the array.

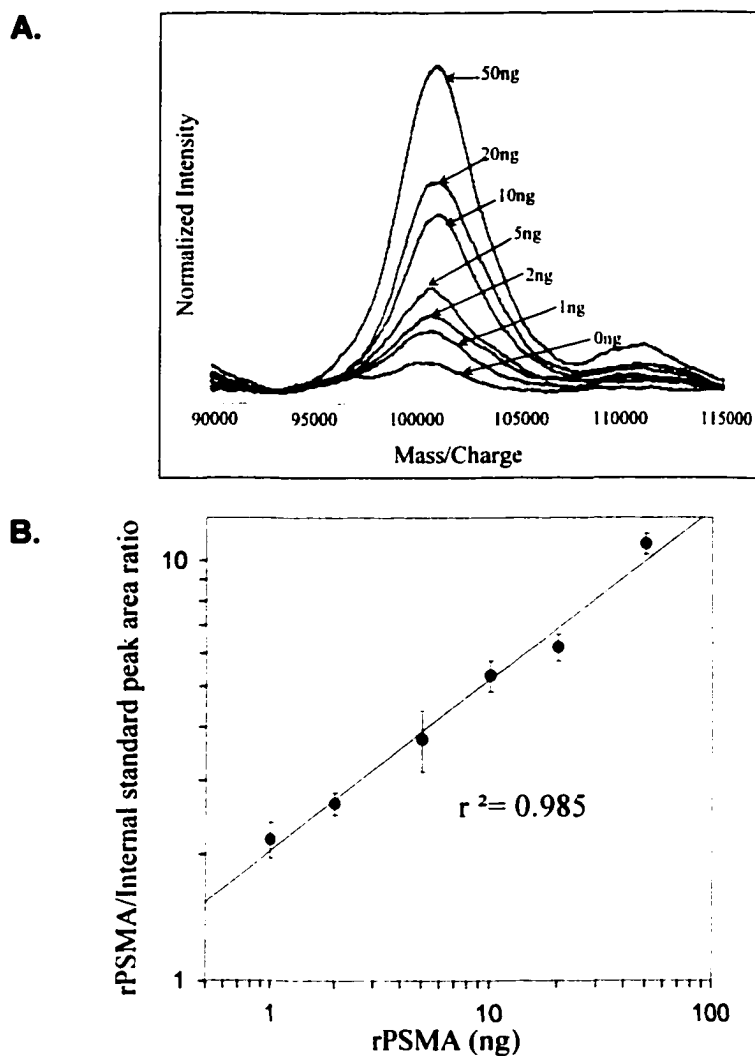


Fig. 19. The standard curve of rPSMA in SELDI immunoassay. Different concentrations of rPSMA or serum were applied to pre-activated PS1 ProteinChips[®] containing bound MoAb 7E11, the chips were washed and subjected to SELDI mass analysis. *A*, Spectra of the rPSMA (1 to 50ng) showing that both the increases in intensity and peak area correlated with the amount of antigen. *B*, Recombinant PSMA standard curve. Recombinant PSMA signal intensity of each spectrum shown in (*A*) was normalized to the internal standard (β -galactoglobulin, 25fmol/ μ l). A linear curve was generated by plotting the peak area ratio (rPSMA/internal standard) versus the amount of rPSMA. The mean and standard deviation of three separate mass spectra is shown.

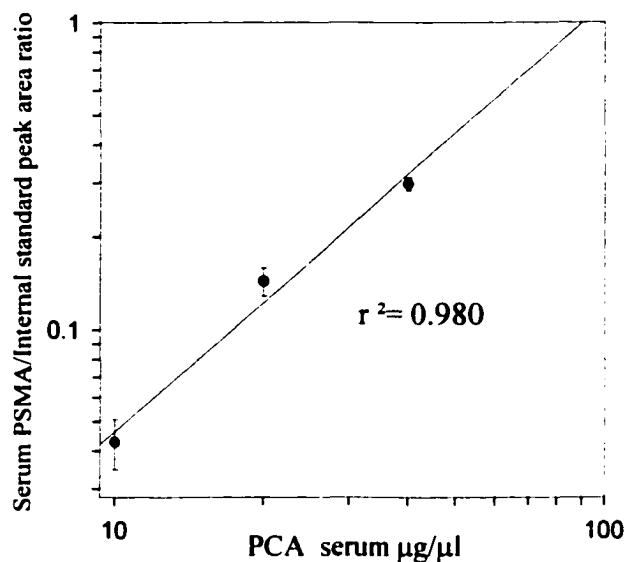


Fig. 20. PSMA detected in a serum sample from a patient diagnosed with prostate cancer (PCA). The analysis was the same as used for generating the rPSMA standard curve, except that $100\text{fmol}/\mu\text{l}$ β -galactoglobulin was spiked as the internal standard for normalization. Doubling dilutions (i.e. 10, 20, and 40 $\mu\text{g}/\mu\text{l}$) of total serum proteins show a linear regression. The mean and standard deviation of three separate mass spectra is shown.

shows the detection of PSMA from the PCA patient. Like pure rPSMA, serum PSMA was also efficiently captured, even when the total serum protein concentration was diluted as low as $10\mu\text{g}/\mu\text{l}$, which is approximately a 1:10 (v/v) dilution. The linear relationship between PSMA signals and serum protein concentrations ($R^2=0.980$, $CV=11.4\%$) suggests that the immunocapture of PSMA from a complex mixture was specific. The serum PSMA peak intensity detected by SELDI correlated with the relative integral intensity of PSMA of the same samples obtained by Western blot analysis (27), with the PCA sample having approximately two-fold greater amount of PSMA than the BPH sample. These preliminary data suggest that using the rPSMA as a standard antigen, PSMA in body fluids can be directly quantitated by the SELDI immunoassay at subnanomolar levels.

2. Quantitation of the Serum PSMA Levels

After the assay conditions were optimized, the SELDI immunoassay was used to detect and quantitate PSMA in serum from 24 normal males donors (12 age <50; 12 age >50), and from 10 BPH, 17 PCA (stage T1 to T3), and 9 prostatitis patients. The demographics of the healthy donors and patients are summarized in Table 3. Fig. 21 shows the representative mass spectra of the 101kDa PSMA peak detected in serum from a normal age-matched donor, and in sera from patients with BPH, PCA or prostatitis. The intra-assay variability of serum samples ranged from 1.2% to 20.1% with an average of 10.1%, whereas the inter-assay variability ranged from 2.9% to 18.7% with an average of 11.8%. The serum PSMA levels for the different test groups are summarized in Fig. 22. The average level of serum PSMA was 117.1ng/ml in

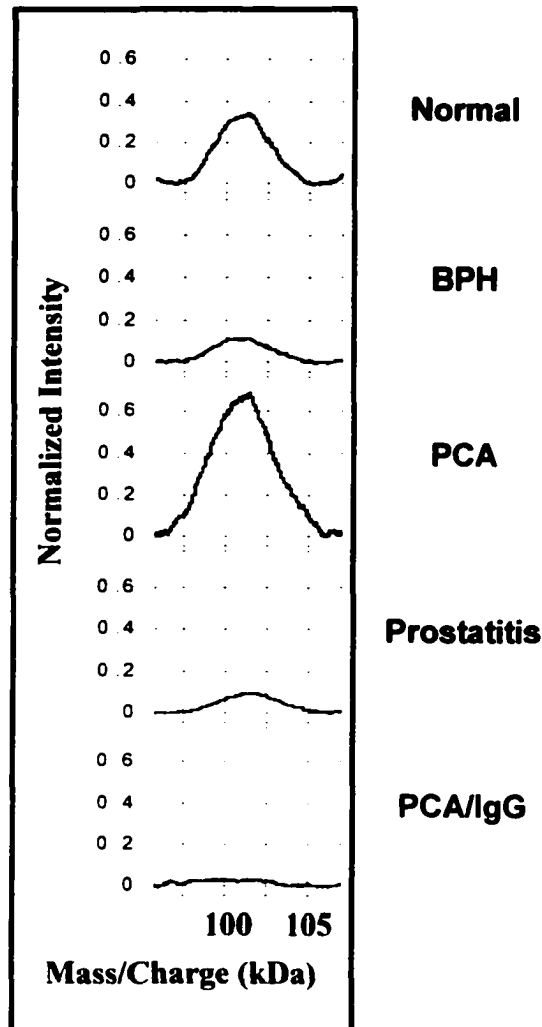


Fig. 21. Representative example of the mass spectrum obtained from the PSMA SELDI immunoassay. The 101kDa PSMA peak was captured by 7E11 and detected in the spectra of the *normal*, *BPH*, *PCA* and *prostatitis* serum samples. When mouse IgG was substituted for 7E11, no PSMA was captured from the *PCA* serum sample (*PCA/IgG*). *Normalized intensity*, the intensity of the protein peaks after normalization using β -galactoglobulin (MW 18,363.3Da) as the internal standard; *Mass/Charge (kDa)*, the protein mass (kDa). The mass range shown is from 95 kDa to 107.5 kDa.

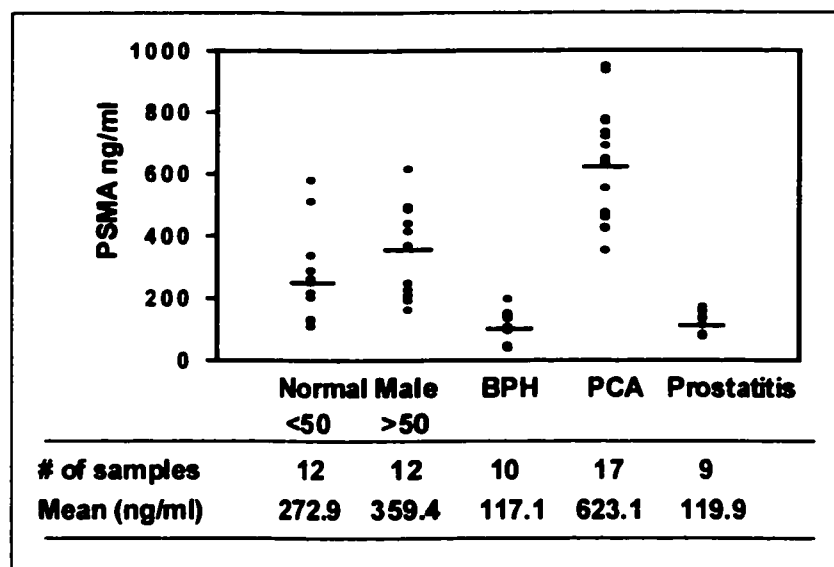


Fig. 22. Comparison of serum PSMA levels in normal male donors and patients with BPH, PCA or prostatitis. *Bars*, the mean PSMA levels in the different test groups.

Table 3 *Demographics, serum PSA and PSMA levels, and statistical data for all study groups*

	Normal Male		BPH	PCA	Prostatitis
	<50	>50			
Number of samples	12	12	10	17	9
Age					
Range (yr)	27-39	54-71	54-81	55-93	52-70
Average (yr)	33.9	60.3	68.7	68.5	62.2
SD	4.1	4.8	8.0	8.7	5.8
PSA value					
Range (ng/ml)	0.4-1.92	0.73-3.32	0.2-13.7	2.4-1230	0.5-5.6
Average (ng/ml)	0.96	1.52	4.86	107.15	1.58
SD	0.6	0.9	4.2	309.1	2.0
PSMA value					
Range (ng/ml)	106.4-576.9	159.8-611.5	35.6-193.8	349.5-946.6	73.4-168.2
Average (ng/ml)	272.9	359.4	117.1	623.1	119.9
SD	145.2	149.3	48.7	177.7	36.7
P (normal <50)^a	--	0.83	0.04	< 0.001	0.04
P (normal >50)^b	0.83	--	<0.01	<0.01	0.001
P (BPH)^c	0.04	<0.01	--	< 0.001	1.00
P (PCA)^d	<0.001	<0.01	<0.001	--	< 0.001

^a P, normal male <50 vs. each of the other groups

^b P, normal male >50 vs. each of the other groups

^c P, BPH vs. each of the other groups

^d P, PCA vs. each of the other groups

--, No comparison

patients with BPH, 119.9ng/ml with prostatitis, 272.9ng/ml and 359.4ng/ml for normal men age <50 and >50, respectively, and 623.1ng/ml for patients with PCA.

Statistical analysis of the data is summarized in Table 3. Due to the varied distribution of PSMA levels in different populations, the equality of variances was not assumed. Therefore Tamhane's post hoc test was adopted to analyze the differences of PSMA values among different populations. The average serum PSMA level in older normal males (age >50) was slightly higher than that in the younger normal males (age <50), and the difference was not significant ($p=0.83$). On the other hand, significant differences were found when comparing the average PSMA level of either the normal population versus PCA (normal<50 vs. PCA: $p<0.001$; normal>50 vs. PCA: $p<0.01$), normal versus BPH (normal<50 vs. BPH: $p=0.04$; normal>50 vs. BPH: $p<0.01$), and BPH versus PCA ($p<0.001$). In addition, the mean level of serum PSMA in prostatitis patients differed from that of the normal and PCA groups (normal<50 vs. prostatitis: $p=0.04$; normal>50 vs. prostatitis: $p=0.001$; PCA vs. prostatitis: $p<0.001$), but not from the BPH group ($p=1.00$).

PSMA serum levels in individual patients with BPH and PCA are shown in Table 4 and Table 5. These two populations were age matched (BPH: 68.6 ± 7.5 yr; PCA: 68.6 ± 8.7 yr). Within the BPH group (Table 4), the average PSA level was 4.9ng/ml, with six patients with a serum PSA of >4ng/ml, and four patients a PSA of <4ng/ml. No apparent correlation was observed between the levels of PSA and the levels of PSMA. Within the PCA group (Table 5), the average PSMA level in the low stage cancer patients (T1a to T2a, $n=11$, 668.8ng/ml) was higher than that in the high stage

Table 4 *Demographics, serum PSA and PSMA levels for BPH patients*

BPH samples	Age yr	PSA ng/ml	PSMA ng/ml
1	68	9.6	138.25
2	72	5.9	193.8
3	68	4.7	130.8
4	73	3.4	104.9
5	61	5.2	44.5
6	54	4.4	35.6
7	81	0.9	136.3
8	71	0.2	148.3
9	74	0.6	146.1
10	64	13.7	92.4
Average	68.6	4.9	117.1
Range	54-81	0.2-13.7	35.6-193.8
SD	7.5	4.2	48.7

Table 5 Demographics, serum PSA and PSMA levels for prostate cancer patients

PCA sample	Age yr	Stage	Grade ^a	Gleason Score	PSA ng/ml	PSMA ng/ml
1	70	T1c	WD	2+2	5.6	932.2
2	63	T1c	MD	2+2	3.0	455.8
3	72	T1c	PD	4+4	7.7	632.6
4	66	T2a	WD	2+3	6.4	687.6
5	64	T2a	MD	3+4	2.6	471.0
6	55	T2a	MD	2+3	2.4	946.6
7	69	T2a	MD	5	9.0	423.8
8	74	T2a	MD	NR ^b	5.5	550.2
9	67	T2a	WD	2+2	5.6	774.8
10	65	T2a	MD	3+4	7.9	765.4
11	65	T2a	MD	3+4	7.0	716.3
12	62	T2b	MD	4+5	50.2	624.2
13	82	T2b	PD	NR	9.3	730.9
14	67	T2b	MD	3+3	5.5	349.5
15	61	T2b	WD	2+2	8.2	645.0
16	71	T3	PD	4+4	1230.0	419.2
17	93	T3	MD	3+4	455.7	467.9
Average	68.6				107.2	623.1
Range	55-93				2.4-1230	349.5-946.6
SD	8.7				309.1	177.7
Low stage (T1a to T2a), 11 patients						
Average	66.4				5.7	668.8
Range	55-74				2.4-7.9	423.8-946.6
SD	5.1				2.2	181.6
High stage (T2b to T3), 6 patients						
Average	72.7				293.2	539.5 ^c
Range	61-93				5.5-1230	349.5-730.9
SD	12.5				491.5	148.8

^a WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.

^b NR, Not recorded. Score cannot be given due to limited specimen.

^c $P=0.16$ when compared to the average PSMA level in the low stage cancers.

cancers (T2b to T3, n=6, 539.5ng/ml); however, this difference was not statistically significant ($p=0.16$, student's t-test). Collectively these results demonstrate that PSMA levels in PCA were significantly higher than those observed for the BPH and the two normal male populations.

C. CHARACTERIZATION OF TRANSCRIPTONAL REGULATION OF THE PSMA GENE

1. The Effects of EGF and TPA on the PSMA mRNA level in LNCaP cells

EGF and TPA are known to be the inducers of AP-1. In order to determine the role of AP-1 proteins in androgen regulated PSMA transcription, LNCaP cells were exposed to EGF or TPA and PSMA mRNA levels were examined by Northern blot analysis. After treating LNCaP cells with the synthetic androgen R1881 for 48 hrs, the PSMA mRNA level was decreased as compared to that in the cells cultured in absence of androgens (Fig. 23). When cells were exposed to 20 or 100ng/ml of EGF, an increase was shown in the PSMA mRNA levels. This induction was suppressed when the cells were cultured in the presence of both EGF and 2nM R1881.

TPA is able to increase AP-1 transactivation via the PKC signal transduction pathway (64). As shown in Fig. 24, PSMA mRNA levels were increased in the presence of 1nM TPA and decreased in the presence of 2nM R1881. TPA at 0.1nM did not show a significant induction of the PSMA mRNA levels. This is possibly because the TPA concentration was not sufficient to cause a visible change in the

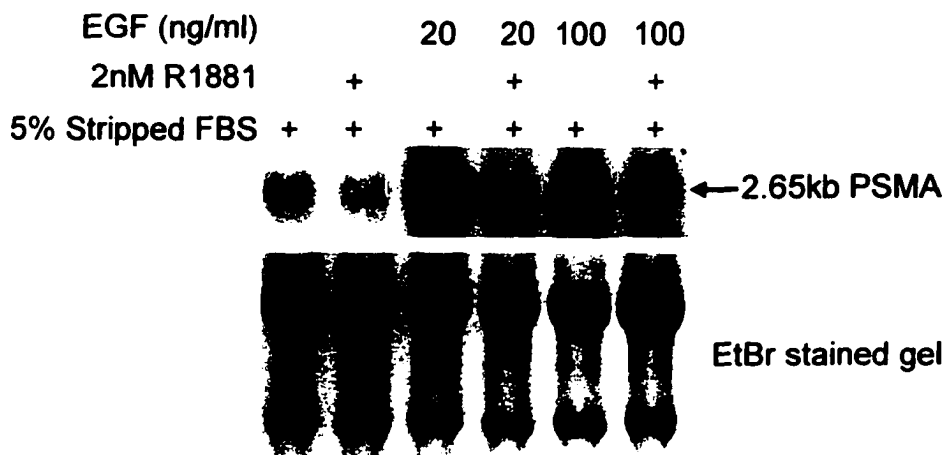


Fig. 23. The effects of androgens and EGF on the PSMA mRNA level in LNCaP cells. The cells were cultured in 5% charcoal-stripped FBS medium in the absence or presence (+) of 2nM R1881 and/or EGF (20 or 100ng/ml) for 48 hours. Fifteen μ g of total RNA were subjected to electrophoresis followed by Northern blot analysis using digoxigenin-labeled PSMA probe (*top panel*). The photograph of ethidium bromide stained gel (*bottom panel*) showing the 28S (*upper band*) and 18S (*lower band*) rRNA confirmed the equal amount and the integrity of the RNA samples.

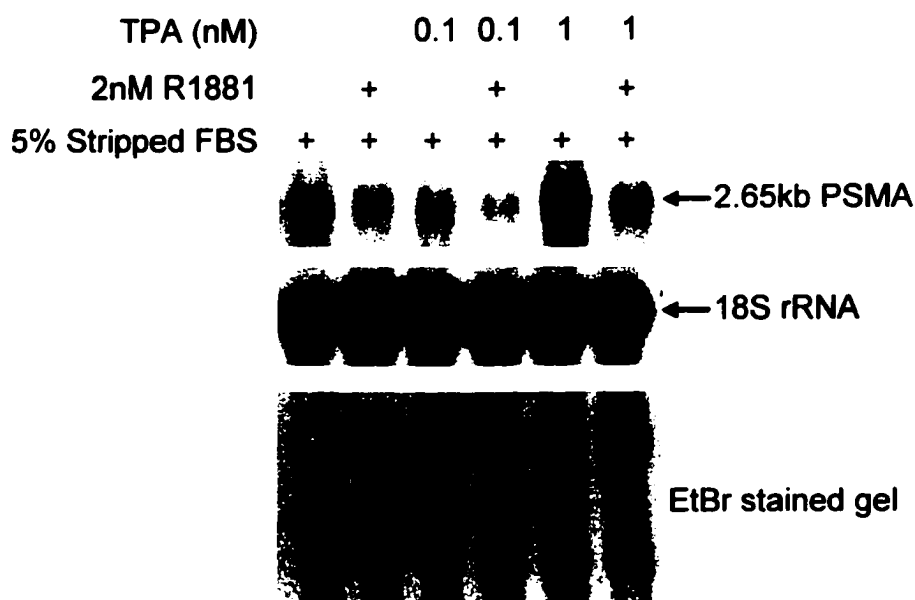


Fig. 24. The effect of TPA on the PSMA mRNA level in LNCaP cells. The cells were cultured in 5% charcoal-stripped FBS medium in the absence or presence (+) of 2nM R1881 and/or TPA (0.1 or 1nM) for 48 hours. Fifteen μ g of total RNA were subjected to electrophoresis followed by Northern blot analysis using digoxigenin-labeled PSMA probe (*top panel*). The same blot was probed with 18S rRNA to show the equal loading of RNA (*middle panel*). The photograph of the ethidium bromide stained gel (*bottom panel*) with the 28S (*upper band*) and 18S (*lower band*) rRNA also confirmed the equal amount and the integrity of the RNA samples.

PSMA mRNA levels by Northern blot analysis. Nonetheless, the increase of PSMA mRNA levels by EGF and TPA and the inhibition by androgens provided the first evidences for the possible involvement of AP-1 proteins in the regulation of the PSMA gene expression.

2. The Effects of EGF and AP-1 Proteins on the PSMA Promoter Activity

When LNCaP cells are exposed to EGF, various downstream transducers, mediators and effectors in the signaling pathway can cause changes at different levels including chromosomal remodeling, transcriptional regulation, translational regulation, post translational modification and protein stability and turnover. In order to analyze the specific effect of EGF at the level of transcription, the 5' region of the PSMA gene including a 2kb upstream region and the first intron were cloned into the luciferase reporter vector. Fig. 4 (page 13 in the Introduction section) illustrates the structures of these luciferase reporter constructs. The promoter and/or enhancer activities of these constructs were examined by transient transfection into LNCaP cells. In the first experiment, the constructs' responsiveness to androgens was analyzed. As shown in Fig. 25, construct P1, which contains the 0.8kb region immediate upstream of the PSMA transcription initiation site, showed about 40-fold activation of the PSMA promoter activity in the absence of androgens. This activity was reduced in half in the presence of 2nM R1881. P2, the construct with a 2kb upstream fragment showed the lowest promoter activity and minor suppression by androgen. This is probably due to some inhibitory elements that might exist further

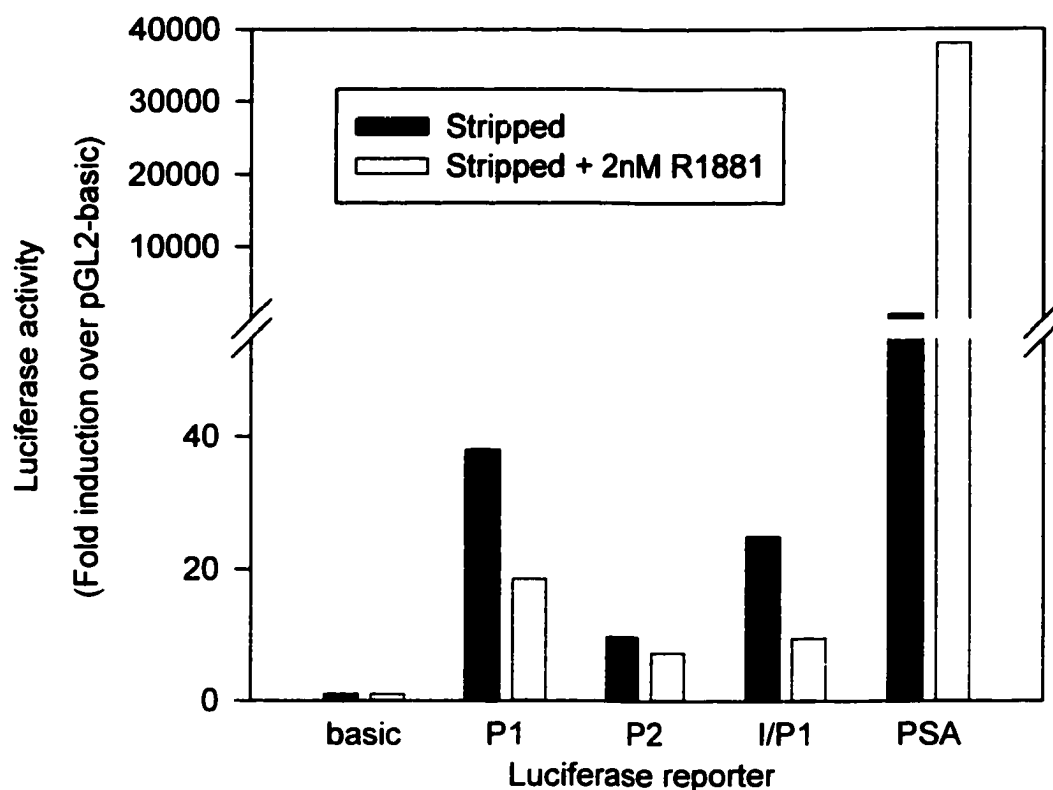


Fig. 25. The analysis of the PSMA promoter and enhancer using luciferase assay. The luciferase reporter constructs containing PSMA promoter or the first intron (*P1*, *P2*, *I/P1*) or 5.8kb promoter and enhancer region of PSA (*pGL3-PSA*) were transfected in LNCaP cells. The cells were then cultured in 5% charcoal-stripped FBS medium in the absence (*black bars*) or presence of 2nM R1881 (*grey bars*) for 48 hours and harvested for luciferase assay. The luciferase activities were determined as the fold of induction over the transfection of the pGL2-basic vector (set as 1). The luciferase activity of pGL3-PSA demonstrated the effectiveness of R1881, a synthetic androgen, in causing androgen response.

upstream of the 0.8kb fragment. The third construct I/P1 contains the first intron of the PSMA gene that was inserted further upstream of P1. This way the enhancer elements, if any, in the first intron could be analyzed. However, instead of enhancing the activity of P1, I/P1 showed less promoter activity than P1 by itself. The result suggests that the first intron could not enhance the promoter activity when being put upstream of the promoter, or at least not from this type of artificial orientation. Nonetheless, the activity of I/P1 was also decreased half in the presence of 2nM R1881. The similar suppression of P1 and I/P1 by androgens was probably due to the P1 fragment present in both P1 and I/P1 constructs. A pGL3-PSA luciferase reporter construct carrying a 5.8kb promoter/enhancer region of the PSA gene was used as the positive control for the promoter responsiveness to androgens. Fig. 25 shows that even in the absence of androgens the activity of PSA promoter was very strong, and as expected, adding androgens further induced the PSA promoter to a much higher level. This suggests that the R1881 used in the transfection experiments was effective and that androgen receptors in LNCaP cells have been functionally activate by R1881.

Next, the dose effect of androgens on these PSMA promoter and enhancer constructs were analyzed. As shown in Fig. 26, after transient transfection, LNCaP cells were cultured in the presence of 1, 2 or 4 nM R1881. As the androgen concentration increased, P1 and I/P1 showed a dose-dependent decrease in the promoter activity. The highest promoter activity was found in P1 in the stripped medium. While P2 exhibited about 10 fold induction in promoter activity, the activity did not differ much in the absence or presence of androgens. Since 2 nM R1881 was

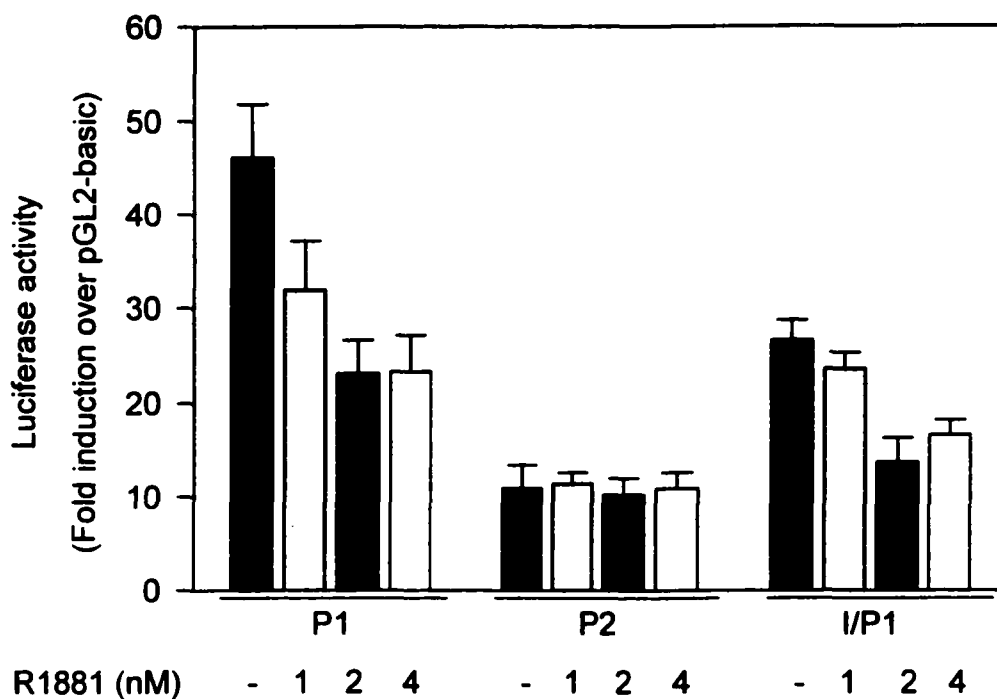


Fig. 26. The effect of androgens on the PSMA promoter. The PSMA luciferase reporter construct *P1*, *P2* or *I/P1* was transfected in LNCaP cells. The cells were then cultured in 5% charcoal-stripped FBS medium in the absence (-) or presence of 1, 2 or 4nM R1881 for 48 hours and harvested for luciferase assay. The luciferase activities were determined as the fold of induction over the transfection of the pGL2-basic vector (set as 1) and shown as the mean \pm SD of three independent experiments and triplicate transfections per experiment.

sufficient to obtain a suppressive effect on the PSMA promoter, this concentration was used in the later experiments.

The effect of EGF on the PSMA promoter and/or enhancer activity was also analyzed. LNCaP cells were transiently transfected with P1, P2 or I/P1 followed by incubation in the presence of 2nM R1881 and/or 100ng/ml EGF (Fig. 27). In P1, the promoter activity was doubled in the presence of 100ng/ml EGF as compared to the transfection in the stripped medium. The addition of R1881 showed a negative effect on the basal (in the stripped medium) or EGF induced PSMA promoter activity. The promoter activity was greatly decreased in the presence of 2nM R1881. On the other hand, P2 showed little response to either EGF or R1881. Interestingly, I/P1 could only respond negatively to androgens, but not to EGF as P1 did, suggesting that the insertion of the first intron to the 5' end of P1 might inhibit the induction of P1 by EGF.

Since the EGF induced PSMA promoter activity was best observed in P1, this 0.8kb PSMA promoter fragment became the focus in the next experiment to examine the direct effect of AP-1 proteins on PSMA promoter activity. The AP-1 expression vectors including c-Jun and c-Fos were cotransfected with P1 into LNCaP cells, then the cells were incubated in the absence or presence of 2nM R1881 (Fig. 28). Cotransfection of c-Jun or c-Fos resulted in an 8-fold or 7-fold increase in the P1 promoter activity respectively, as compared to the cotransfection of the empty expression vector pcDNA and P1. This suggested that c-Jun and c-Fos can independently induce the PSMA promoter. Moreover, the simultaneous introduction of c-Jun and c-Fos into LNCaP cells resulted in a 15-fold increase in the P1 activity.

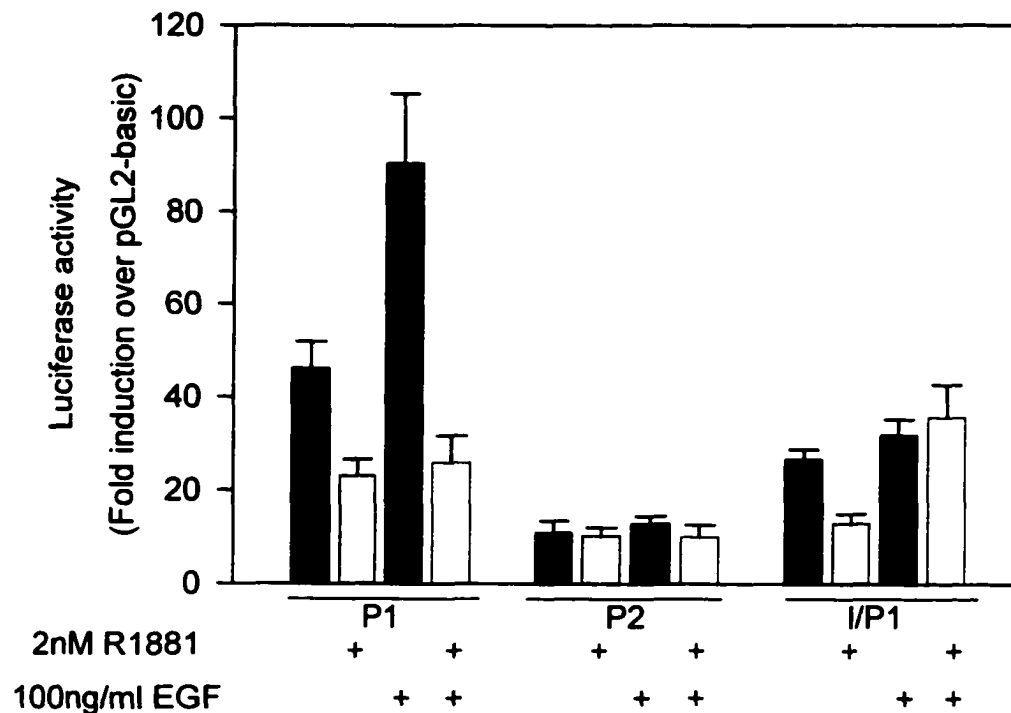


Fig. 27. The effect of EGF on the PSMA promoter. The PSMA luciferase reporter construct *P1*, *P2* or *I/P1* was transfected in LNCaP cells. The cells were then cultured in 5% charcoal-stripped FBS medium in the absence or presence (+) of 2nM R1881 and/or 100ng/ml EGF, and harvested for luciferase assay 48 hours post transfection. The luciferase activities were determined as the fold of induction over the transfection of the pGL2-basic vector (set as 1) and shown as the mean \pm SD of three independent experiments and triplicate transfections per experiment.

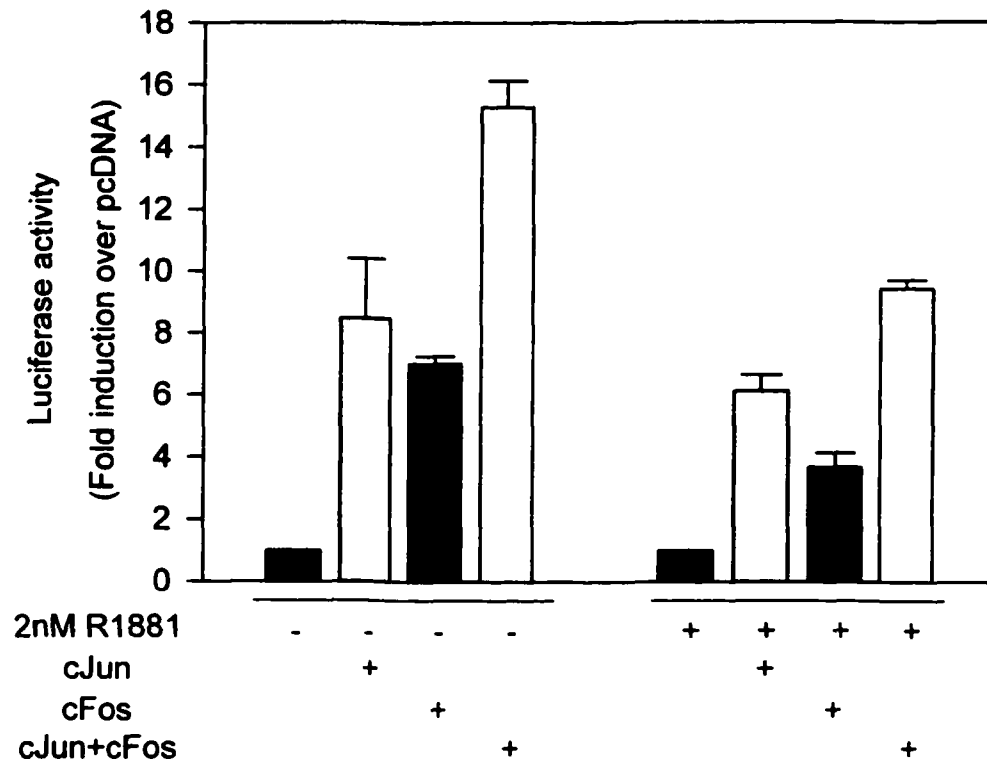


Fig. 28. The effect of AP-1 proteins on the PSMA promoter. The AP-1 expression vectors pcDNA-cJun (*cJun*), pcDNA-cFos (*cFos*) or both (*cJun+cFos*) were cotransfected with PSMA luciferase reporter construct P1 in LNCaP cells. The cells were then cultured in 5% charcoal-stripped FBS medium with (+) or without (-) 2nM R1881 for 48 hours and harvested for luciferase assay. The luciferase activities were determined as the fold of induction over the cotransfection with the empty vector pcDNA3.1/Zeo+ (set as 1) and shown as the mean \pm SD of three independent experiments and triplicate transfections per experiment.

When cotransfection was followed by incubation of LNCaP cells in the presence of 2nM R1881, a similar induction pattern by c-Jun, c-Fos or both was observed, but all at a suppressed level. This result demonstrated the antagonistic effect of androgens on the AP-1 induced PSMA promoter activity. Regardless of the suppression by androgens, the data obtained in luciferase assay and Northern blot analysis strongly indicated that AP-1 proteins were directly involved in the activation of PSMA promoter.

3. Analysis of the DNA Binding Activity at the AP-1 Sites in the PSMA Promoter Region

In order to prove the direct involvement of AP-1 in the transcriptional regulation of the PSMA gene, the crucial evidence would be to show the binding of AP-1 at the putative AP-1 sites in the PSMA promoter region. According to the observations in the luciferase reporter assays, the P1 fragment demonstrated the highest promoter activity both in the presence and absence of androgens. The P1 fragment also showed the most notable responses to EGF, androgens and the overexpressed AP-1 proteins. Thus the three putative AP-1 sites in the P1 fragment were of particular interest in studying the regulation of the PSMA promoter. Moreover, given the fact that the three putative AP-1 sites are located immediately upstream of the PSMA transcription initiation site, it is reasonable to believe that these sites are more likely to have an important impact on the basic transcriptional machinery. For these reasons, the three

putative AP-1 sites in the P1 fragment were subjected to the detailed DNA binding analysis in the following gel shift assays.

The radiolabeled EMSA probes were generated for the three AP-1 sites and the flanking sequences. The probes were named PSMA1, PSMA2 and PSMA3 (Fig. 29 and Table 6). Also generated were a consensus AP-1 probe called PG32-1 and three AP-1 like probes: mut1, mut2 and mut3 (Table 6). By using these probes, the following gel shift assay experiments were performed to prove the binding of AP-1 proteins at the three putative sites:

DNA Binding Blocked by Consensus AP-1 Nuclear extracts from LNCaP cells were incubated with radiolabeled probes including consensus AP-1 (lanes 1 to 3, Fig. 30), PSMA1 (lanes 4 to 7), PSMA2 (lanes 8 to 11) and PSMA3 (lanes 12 to 15). A protein complex was formed on the consensus AP-1 site (lane 2), and a complex of similar size was also formed on the three putative AP-1 sites in PSMA promoter (lanes 5, 9 and 13; the upper shift with the large arrow). Another smaller protein complex was formed on the PSMA2 and PSMA3 sites (lanes 9 and 13; the lower shift with the small arrow), probably representing a protein complex formed by different AP-1 family members. The upper shift was consistently observed in all experiments, whereas the lower shift varied among some of the experiments. Nonetheless, the formation of both protein complexes on the PSMA AP-1 sites was inhibited by preincubation of the cold consensus AP-1 probes with LNCaP nuclear extracts (lanes 7, 11 and 15). The competition of DNA binding between the consensus AP-1 probe and PSMA probes provided the first evidence for the specificity of AP-1 binding at the three PSMA AP-1 sites.

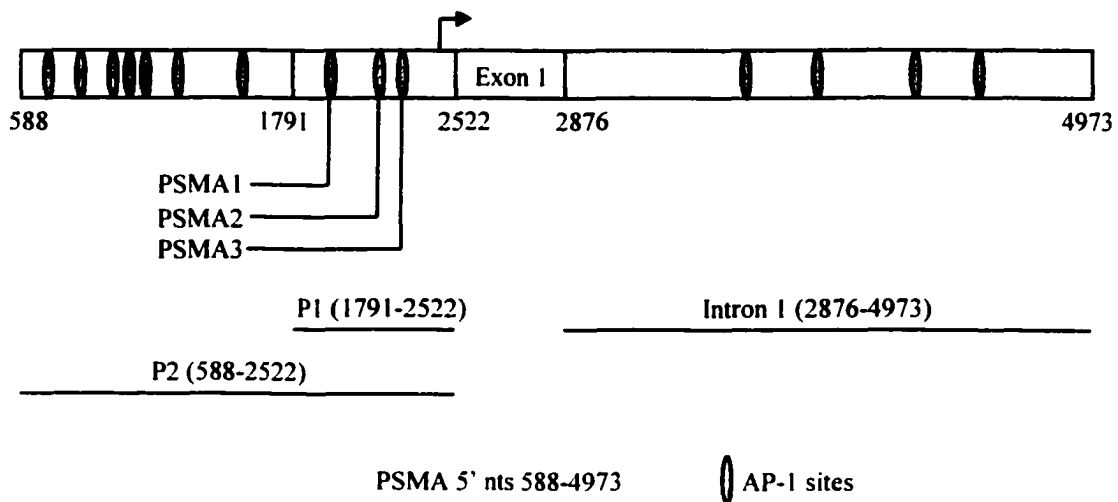


Fig. 29. The three AP-1 sites in the close vicinity of the PSMA transcription initiation site. The EMSA probes that contain these three AP-1 sites and the flanking sequences were named *PSMA1*, *PSMA2* and *PSMA3* respectively. The numbers represent the number of the nucleotides in the PSMA genomic sequence (Genbank accession number AF007544). The *shaded ovals* represent the AP-1 sites. The *arrow* stands for the transcription initiation site (nt 2488) of the PSMA gene.

Table 6 EMSA probes with AP-1 sites used in this study

Name of the Probe	Feature	AP-1 site	
PMSA1	1964-1997 ^a	TGcCTCA	
PSMA2	2309-2342	TTAtTCA	
PSMA3	2350-2383	TTAtTCA	
AP-1	PG32-1 ^b	TGAGTCA	
AP-1 like ^c	{ PG32 mut 1	TGgGTCA	
		PG32 mut 2	TGcGTCA
		PG32 mut 3	TGtGTCA

^a The numbers represent the beginning and the end nucleotides in AP-1 sites according to their positions in PSMA genomic sequence (Genebank accession number AF007544).

^b PG32-1 is a 48-bp ds oligonucleotide containing a consensus AP-1 site in the center and flanked with artificially designed nucleotides (a gift from Dr. T. Bos at EVMS).

^c The sequence of AP-1 like probes are the same as PG32-1 except for the single change at the third nucleotide in the consensus AP-1 site.

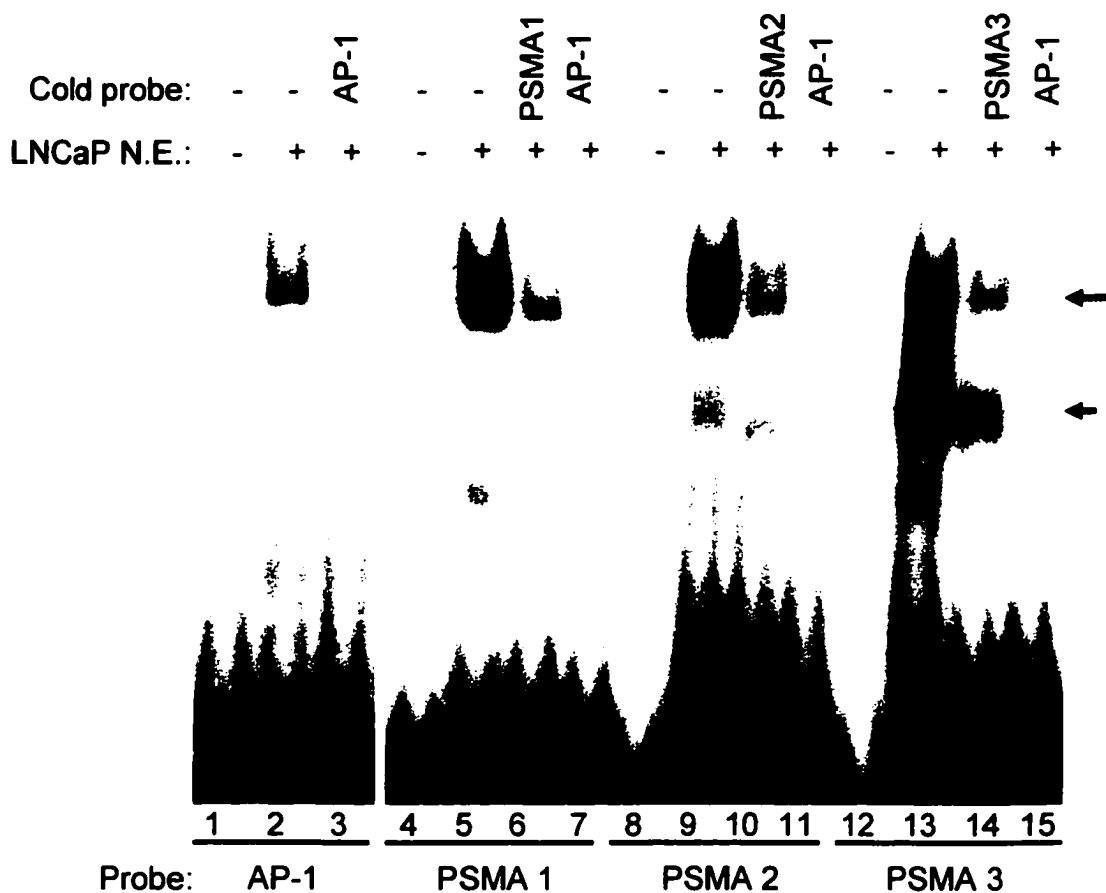


Fig. 30. Inhibition of DNA-binding activity by AP-1 probe. The LNCaP cells were cultured in 5% FBS medium for 48 hours. The nuclear extracts (*LNCaP N. E.*) were prepared and used in EMSA. The following radiolabeled probes were used: AP-1 (*lanes 1 to 3*), PSMA 1 (*lanes 4 to 7*), PSMA 2 (*lanes 8 to 11*), and PSMA 3 (*lanes 12 to 15*). In competition assays, cold probes were preincubated with the nuclear extracts prior to the addition of the labeled probes. The arrows indicate two different complexes formed on the probes.

DNA Binding Blocked by AP-1 Like Probes The consensus AP-1 site contains seven base pairs: TGAGTCA. When a single nucleotide change was introduced at the third nucleotide in the consensus AP-1 site, the new sites were called mut1, mut2 and mut3 (Table 6). Despite the single nucleotide change, the mutants still keep the DNA binding activity similar to the consensus AP-1 site when incubated with LNCaP nuclear extracts (lanes 2, 4, 6 and 8, Fig. 31A). Thus the mutants were called 'AP-1 like sites' and used as the cold probe in the competition experiments with the PSMA AP-1 sites. When the cold AP-1 like probes were preincubated with LNCaP nuclear extracts, the DNA binding activities on the PSMA1, PSMA2 and PSMA3 sites were partially inhibited (lanes 4, 5 and 6, Fig. 31B, C and D). Preincubation with a nonspecific oligonucleotide from CMV genome did not affect the DNA binding at all (lane 7, Fig. 31B, C and D). The inhibition by AP-1 like probes provided further evidence for the binding of AP-1 proteins at the three PSMA AP-1 sites.

DNA Binding Inhibited by AP-1 Specific Antibodies In order to demonstrate that AP-1 proteins were in the complexes formed on the three PSMA AP-1 sites, AP-1 specific antibodies were used in the preincubation with the LNCaP nuclear extracts before the addition of the radiolabeled PSMA AP-1 probes. As shown in Fig. 32, both c-Jun and c-Fos specific antibodies were able to block the formation of the protein complexes on the three PSMA AP-1 sites (lanes 3, 4, 8, 9, 13 and 14, Fig. 32), whereas nonspecific rabbit IgG did not affect the DNA binding activity at all (lanes 5, 10 and 15). Moreover, both the upper shift (large arrow) and lower shift (small arrow) were inhibited. The competition by c-Jun and c-Fos specific antibodies suggested that AP-1 proteins were indeed in the protein complexes formed on the three AP-1 sites,

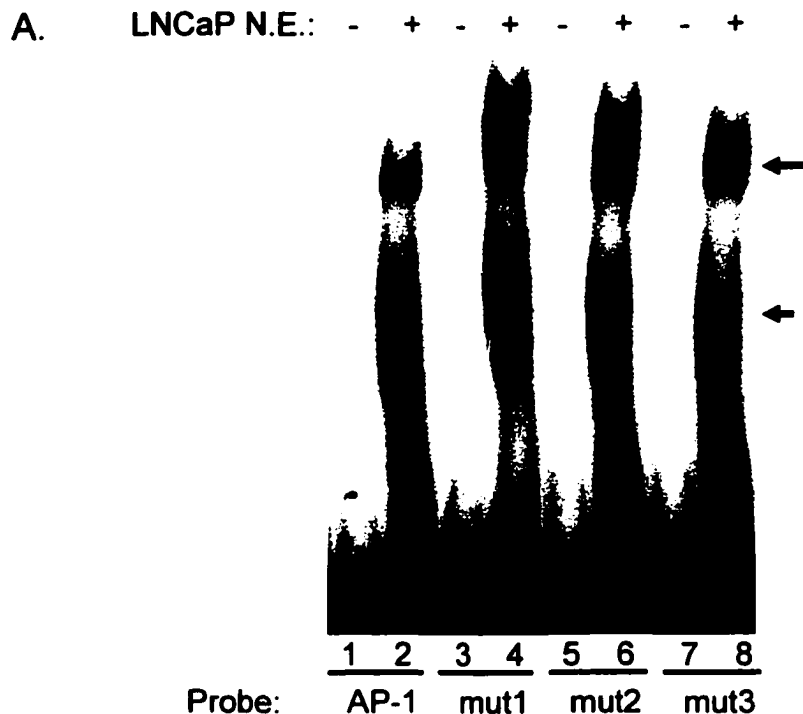
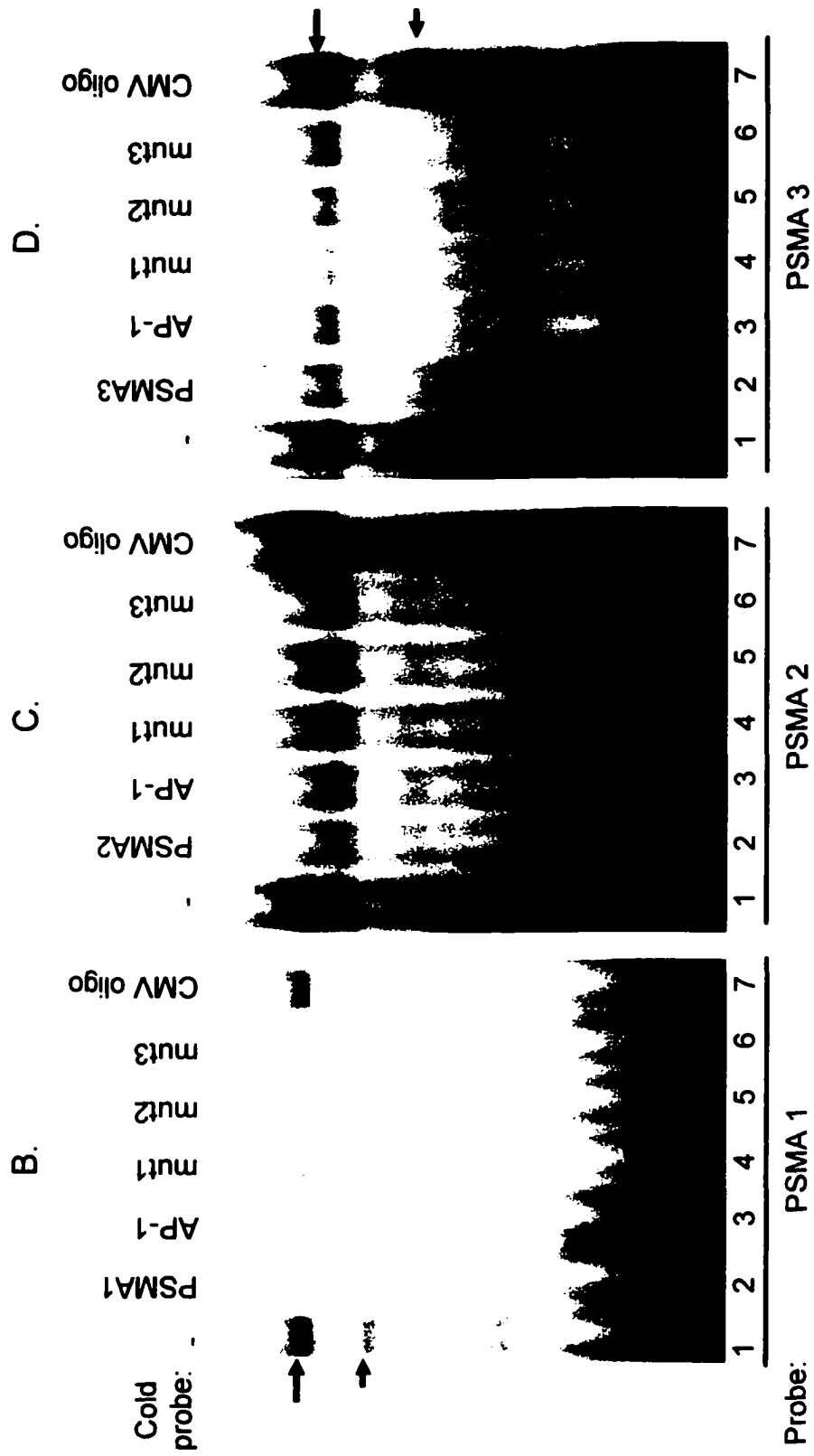


Fig. 31. Inhibition of DNA-binding activity by AP-1 like probes. The LNCaP cells were cultured in 5% FBS medium for 48 hours. The nuclear extracts (*LNCaP N. E.*) were prepared and used in EMSA. The following radiolabeled probes were used: AP-1, mut-1, mut-2 and mut-3 (A); PSMA 1 (B); PSMA 2 (C); PSMA 3 (D). In competition assays (panels B, C and D), cold probes *PSMA 1*, *PSMA 2*, *PSMA 3*, *mut-1*, *mut-2*, *mut-3* or irrelevant oligonucleotides from CMV gene (*CMV oligo*) were preincubated with the nuclear extracts prior to the addition of the labeled probes. The arrows indicate two different complexes formed on the probes.

Fig. 31. (continued)



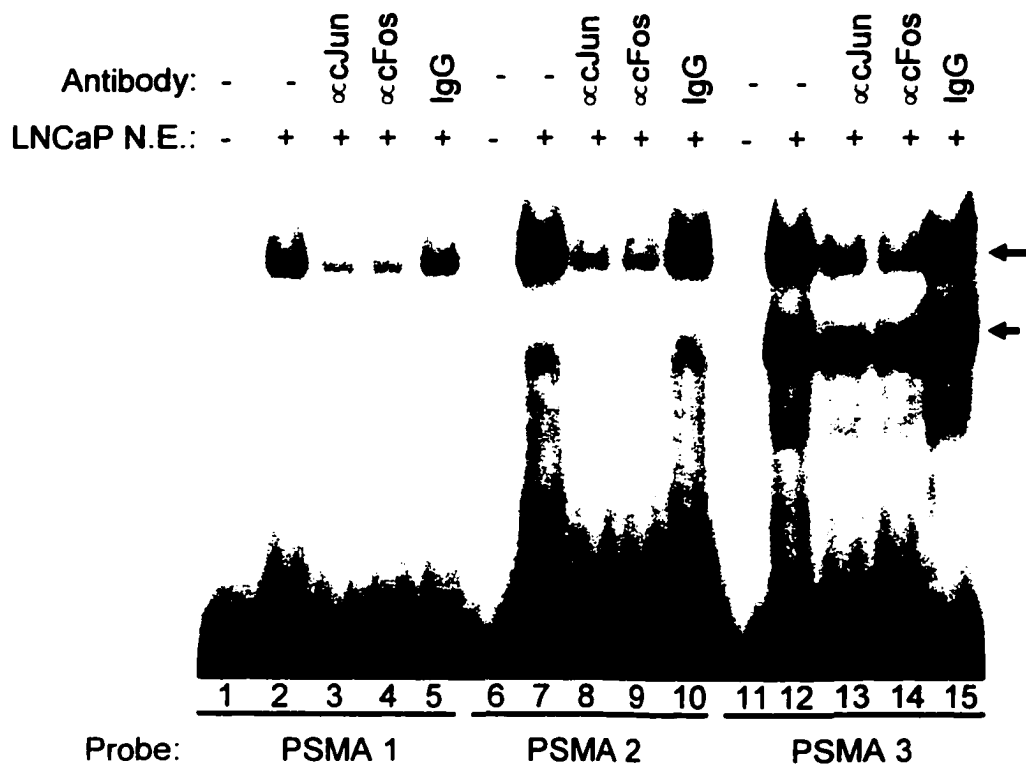


Fig. 32. Inhibition of DNA-binding activity by AP-1 antibodies. The LNCaP cells were cultured in 5% FBS medium for 48 hours. The nuclear extracts (*LNCaP N. E.*) were prepared and used in EMSA with the radiolabeled PSMA 1 (*lanes 1 to 5*), PSMA2 (*lanes 6 to 10*) or PSMA3 (*lanes 11 to 15*) as probes. For antibody competition assays, rabbit polyclonal antibody specific to cJun (*αcJun*) or cFos (*αcFos*) or nonspecific rabbit IgG (*IgG*) was incubated with the nuclear extracts prior to the addition of the labeled probe. The arrows indicate two different complexes formed on the probes.

and that the interactions between the antibodies and these AP-1 proteins interfered with their DNA binding activities.

The Binding of In Vitro Translated AP-1 Proteins Beside the above three competition experiments, a direct approach to prove the binding of AP-1 proteins at the PSMA AP-1 sites was to use purified AP-1 proteins in the incubation with the radiolabeled probes. AP-1 proteins including c-Jun and c-Fos were generated by *in vitro* translation in rabbit reticulocyte lysate system (kindly provided by Dr. T. Bos, EVMS). Equal amount of translated products were mixed to allow the formation of AP-1 dimers. The mixture was then added to the incubation with the PSMA AP-1 probes. As shown in Fig. 33, *in vitro* translated AP-1 proteins were able to form complexes on all three PSMA AP-1 sites (lanes 2, 5 and 8, large arrow). Other complexes were observed only in the LNCaP nuclear extracts (small arrow), probably because of a variety of AP-1 proteins present in the LNCaP nuclear extracts versus the single type of AP-1 dimer in the *in vitro* translated product. The size of *in vitro* AP-1 complex was similar to the upper shift observed in LNCaP nuclear extracts, suggesting that the upper shift in the LNCaP nuclear extracts is likely to be the dimer of c-Jun and c-Fos.

Taken together, the data from the above four experiments suggested that AP-1 proteins are the proteins that bind to the PSMA AP-1 sites.

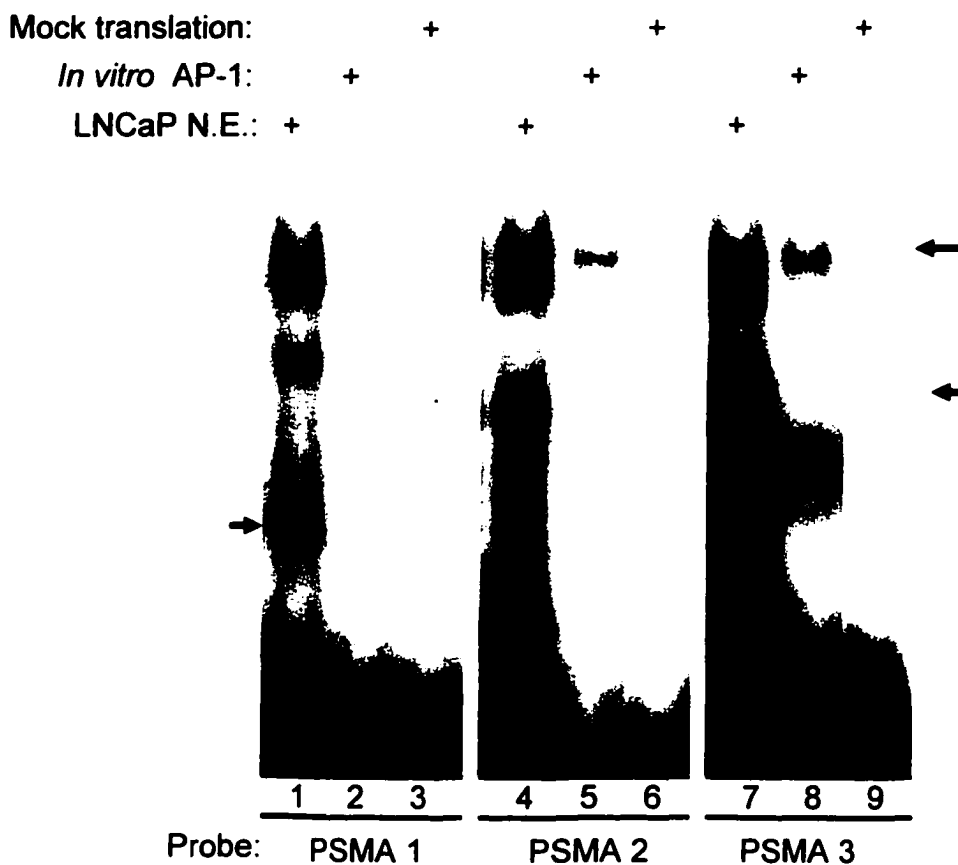


Fig. 33. The DNA-binding activity of *in vitro* translated AP-1. The LNCaP cells were cultured in 5% FBS medium for 48 hours. The nuclear extracts (*LNCaP N. E.*) were prepared and used in EMSA. *In vitro* AP-1 was the mixture of equal amount of *in vitro* translated c-Jun and c-Fos proteins from rabbit reticulocyte lysates. Product from the translation reaction without the input of c-Jun or c-Fos mRNA was used as negative control (*mock translation*). The following radiolabeled probes were used: PSMA 1 (*lanes 1 to 3*), PSMA 2 (*lanes 4 to 6*), PSMA 3 (*lanes 7 to 9*). The *large arrow* indicates the shift shared by the LNCaP nuclear extracts and *in vitro* AP-1. The *small arrow* indicates the complexes formed only with LNCaP nuclear extract.

4. Examine the Effects of Androgens and EGF on the DNA Binding at the AP-1 Sites

After the binding of AP-1 at the three putative AP-1 sites was made clear, the next logical question was if androgens and EGF have an impact on the AP-1 binding activities and how such an impact may relate to the downregulation of PSMA transcription by androgens and the upregulation of PSMA transcription by EGF. To answer this question, the effect of androgens and EGF on the AP-1 binding at the PSMA sites was examined in the following experiments:

Effect of Androgens on AP-1 Binding LNCaP cells were cultured in the absence of androgens, or in the presence of 1, 2 or 4 nM R1881. Nuclear extracts were prepared and used in the incubation with three radiolabeled PSMA AP-1 probes. As shown in Fig. 34, the maximum AP-1 binding was observed in the absence of androgens (lane 1 in panels A, B and C). As R1881 concentration increased, the AP-1 binding at all three sites was decreased (lanes 2 to 4). Both the upper shift (large arrow) and lower shift (small arrow) were decreased at the PSMA1 and PSMA2 sites (panels A and B), whereas at the PSMA3 site, only the upper shift (large arrow in panel C) was inhibited while the lower shift (small arrow in panel C) was virtually not affected by androgens. Moreover, when R1881 was added to the nuclear extracts prepared from LNCaP cells cultured in the absence of androgens, AP-1 binding activity was inhibited. On the other hand, when ethanol -the solvent for R1881- was added to the same nuclear extracts, the AP-1 binding was unaffected (lane 5 versus lane 6 in panels A, B and C). Taken together, the results suggested that androgens can inhibit the AP-1 binding at the three PSMA sites.

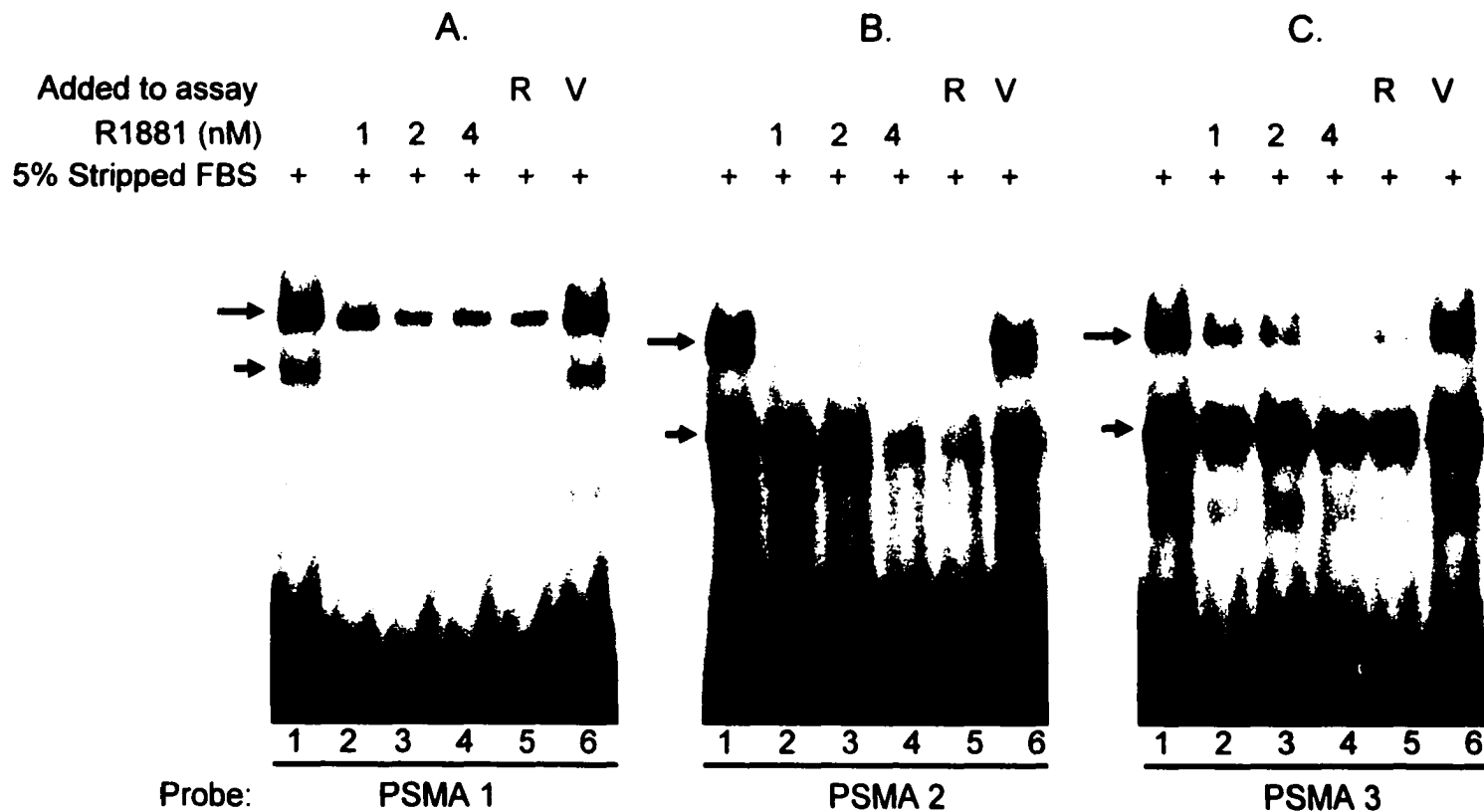


Fig. 34. Inhibition of AP-1 DNA-binding activity by androgens. The LNCaP cells were cultured in 5% charcoal-stripped FBS medium in the absence or presence of 1, 2 or 4 nM R1881 for 48 hours. The nuclear extracts were prepared and used in EMSA with the radiolabeled PSMA 1 (A), PSMA 2 (B) or PSMA 3 (C) as probes. EMSA was also performed by adding 2nM R1881 (R) or the same amount of vehicle (V) to the incubation with the nuclear extracts isolated from LNCaP cells cultured in 5% stripped FBS medium. The arrows indicate two different complexes formed on the probes.

The Effect of EGF on AP-1 Binding Similar to the approach used to study the effect of androgens, LNCaP cells were also cultured in the stripped medium in the presence or absence of R1881 and/or EGF. Nuclear extracts were prepared and used in the EMSA with the three PSMA AP-1 probes (Fig. 35). At all three sites (Fig. 35A, B and C), the AP-1 binding was increased in the absence of androgens as compared to in the presence of androgen (lane 2 versus lanes 1 and 3; lane 5 versus lane 6). Moreover, the AP-1 binding was increased by EGF, especially at a higher concentration (lane 2 versus lanes 4 and 5). This increase was also shown in the presence of androgens (lane 3 versus lane 6). The EGF induced AP-1 binding was slightly suppressed by androgens (lane 5 versus lane 6). At all three sites, the formation of the large protein complex (large arrow) was affected by EGF and androgens, whereas at the PSMA3 sites, the small protein complex (small arrow in Fig. 35C) remained virtually unaffected. This suggests that the AP-1 protein components in the complexes were different at the three sites, with some complexes more sensitive to the changes in androgens and EGF than the others and other present more consistently. Cold probes containing the three PSMA AP-1 sites or the consensus AP-1 site were included in the competition experiments run in parallel to show the specificity of the AP-1 binding (lanes 7 and 8). The results indicated that EGF could induce the AP-1 binding and androgen was able to act in an antagonistic manner to inhibit the EGF induced AP-1 binding.

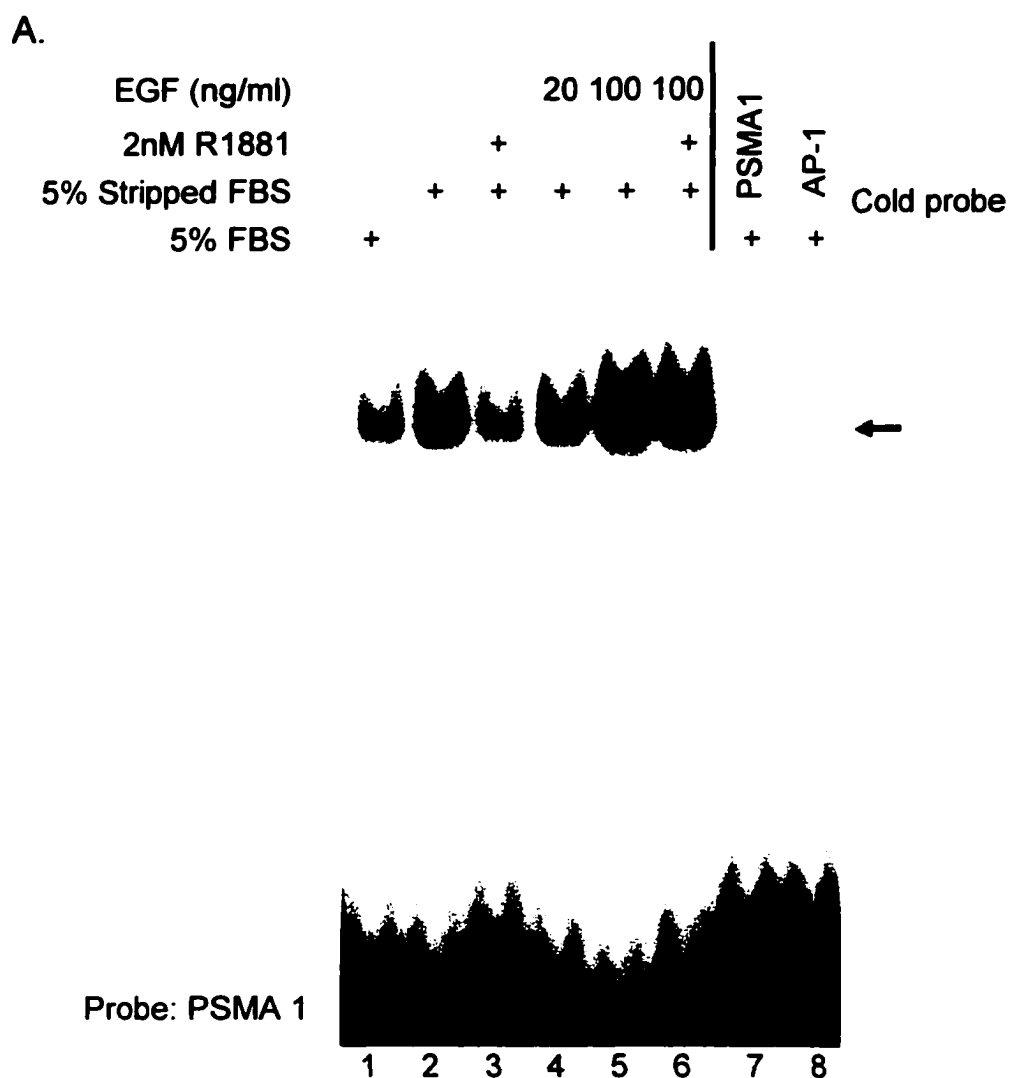


Fig. 35. Increased AP-1 DNA-binding activity with EGF. The LNCaP cells were cultured for 48 hours in 5% charcoal-stripped FBS medium in the presence or absence of 2nM R1881 and/or EGF (20 or 100ng/ml) (*lanes 1 to 6*) or 5% FBS medium (*lanes 7 and 8*). The nuclear extracts were prepared and used in EMSA. The following radiolabeled probes were used: PSMA 1 (*A*), PSMA 2 (*B*), and PSMA 3 (*C*). The specificity of AP-1 binding was shown in competition assays where the cold probe (*PSMA1*, *PSMA 2*, *PSMA 3* or *AP-1*) was preincubated with the nuclear extracts prior to the addition of the labeled probe. The arrows indicate two different complexes formed on the probes.

Fig. 35. (continued)

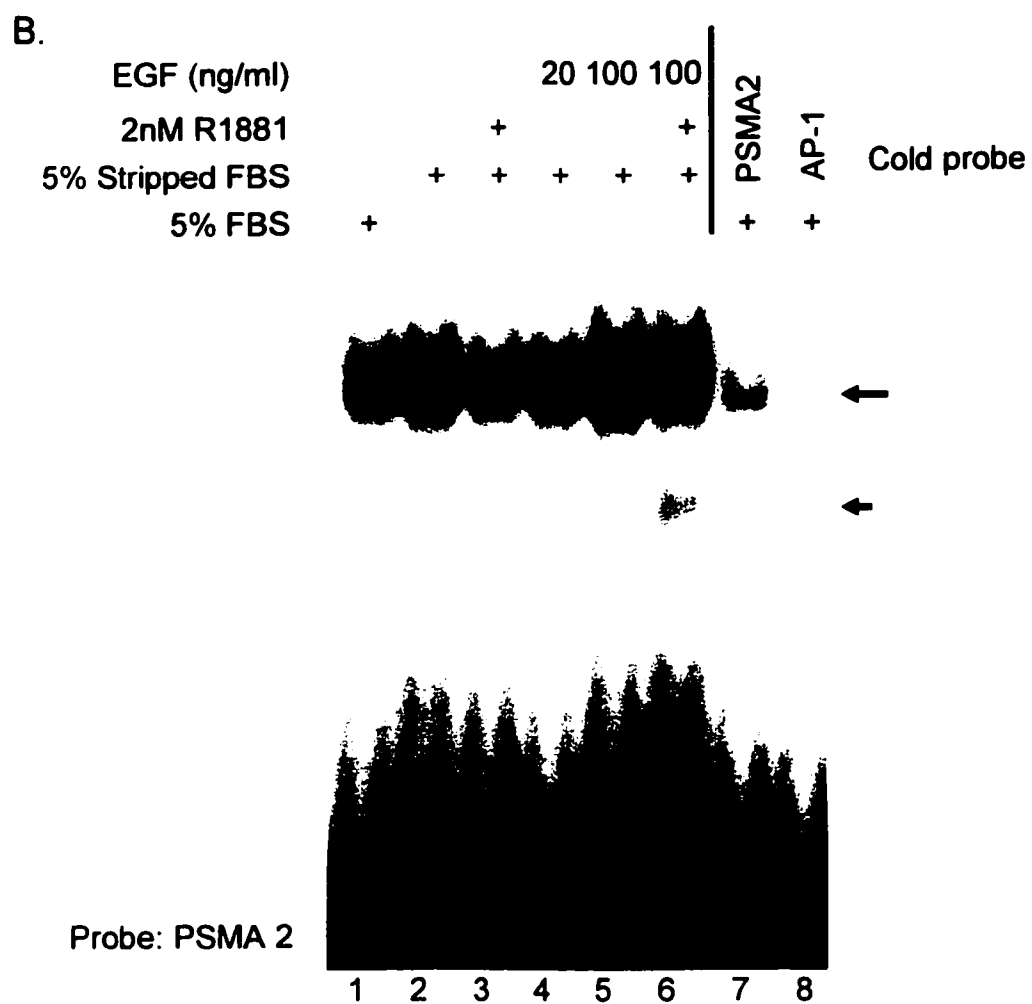
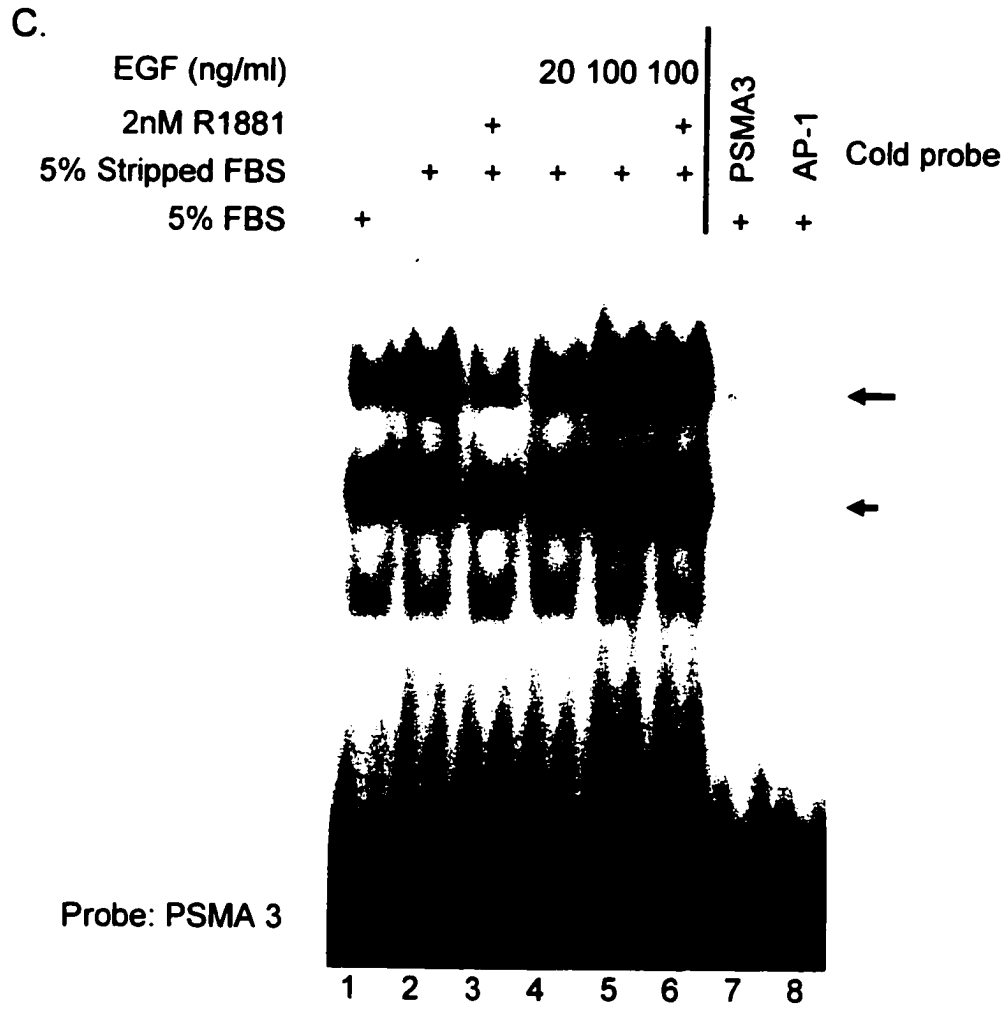


Fig. 35. (continued)

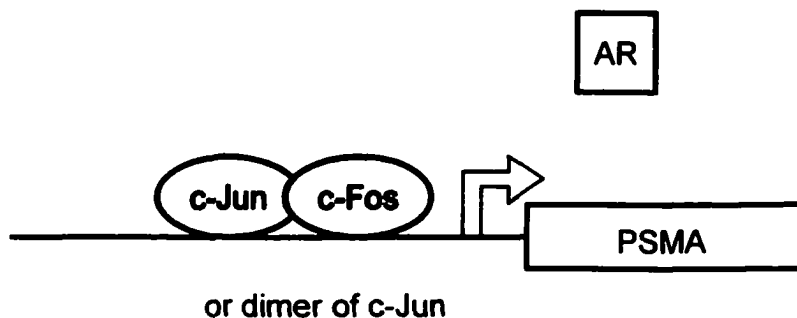


5. Probe the Interaction between the AP-1 Proteins and Androgen Receptors

Previous studies have shown the interaction between the AP-1 transcription factor family members and androgen receptors had mutual effect on both of their DNA binding and transactivation abilities (56,57). For instance, the induction of PSA gene by androgens was suppressed by the interaction between androgen receptor and AP-1 family members (56), suggesting an antagonistic effect between AP-1 and androgen receptor. Therefore it is possible that by a similar antagonistic effect, androgens might interfere with the AP-1 binding to the putative AP-1 sites in the PSMA promoter, and indirectly represses the transcription of PSMA. Based on this rationale and the data observed in the above experiments, a model was proposed to explain the androgenic inhibition of AP-1 DNA binding at the PSMA AP-1 sites and the induction of AP-1 binding in the absence of androgen (Fig. 36). In the absence of androgen, androgen receptors (AR) are inactivated and do not interact with AP-1 family members, so the AP-1 family members are free to bind to the putative sites in PSMA promoter and induce the transcription. On the other hand, when androgen is present, androgen receptors are activated and can interact with AP-1. The interaction can either interfere with the binding of AP-1 to the sites in PSMA promoter or inhibit the interaction of the AP-1 complexes with the nearby basic transcriptional machinery. Either way, the result can lead to the suppression of PSMA transcription.

Since in a previous study, the interactions between AR and AP-1 in LNCaP cells have been demonstrated by co-immunoprecipitation (56), the focus here was to demonstrate the interaction between AR and AP-1 in the protein complexes formed at the three putative AP-1 sites in the PSMA promoter.

In the absence of androgen



In the presence of androgen ▽

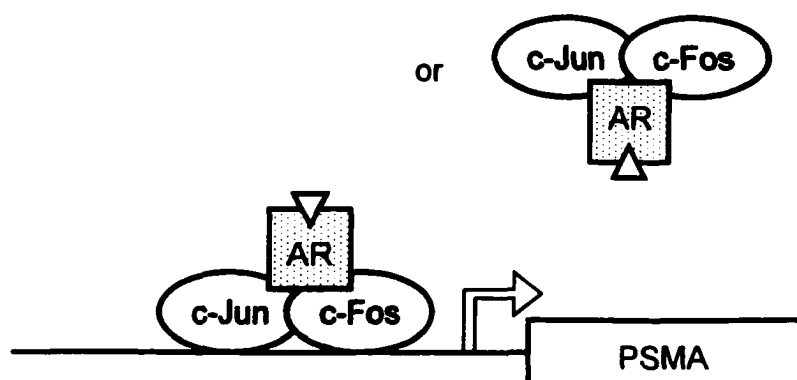


Fig. 36. Proposed mechanism for the regulation of the PSMA gene transcription by AP-1 and androgens. Note that the AR in *blank square* stands for the inactivated AR in the absence of androgen. In the presence of androgen (*triangle*), AR is activated (*dotted square*). The size of the *arrow* indicates the level of the gene transcription.

Inhibition of AP-1 Binding by AR Antibody In order to examine if AR is involved in the AP-1 complexes, an antibody specific to AR was used in the incubation with the radiolabeled PSMA AP-1 probes and LNCaP nuclear extracts (Fig. 37). When the AR antibody was added to the preincubation with LNCaP nuclear extracts before adding the radiolabeled probes, the formation of the AP-1 protein complexes was inhibited at all three AP-1 binding sites (large arrow, lanes 1, 5 and 9 versus lanes 3, 7 and 11). On the other hand, when the AR antibody was added after the radiolabeled probes, there was no inhibition of the AP-1 protein complexes at the AP-1 sites (large arrow in lanes 4, 8 and 12). Nonspecific rabbit IgG did not show any inhibitory effect on the AP-1 complexes (lanes 2, 6 and 10). The binding of AR at its own ARE site was inhibited by the AR antibody, but not by rabbit IgG, confirming the specificity of the AR antibody in recognition of AR (lanes 13, 14 and 15). The inhibition by AR antibody suggests that AR is involved in the AP-1 complex formed at the PSMA AP-1 sites. The recognition site for AR antibody appeared to interfere with the DNA binding of the complex, therefore the interaction between AR and its antibody blocked the AR/AP-1 complex from binding to the PSMA AP-1 sites. For the same reason, if the antibody was added after the AR/AP-1 complexes were formed at the AP-1 sites, the recognition site for the antibody was blocked and no inhibition was shown.

Inhibition of AP-1 Binding by ARE Androgen responsive element (ARE) is the consensus DNA element to which the activated AR can bind. To further investigate the interaction between AR and AP-1, cold ARE probe was used as a competitor in EMSA experiment (Fig. 38). When preincubated with LNCaP nuclear extracts before the radiolabeled PSMA AP-1 probes were added, cold ARE probe was able to

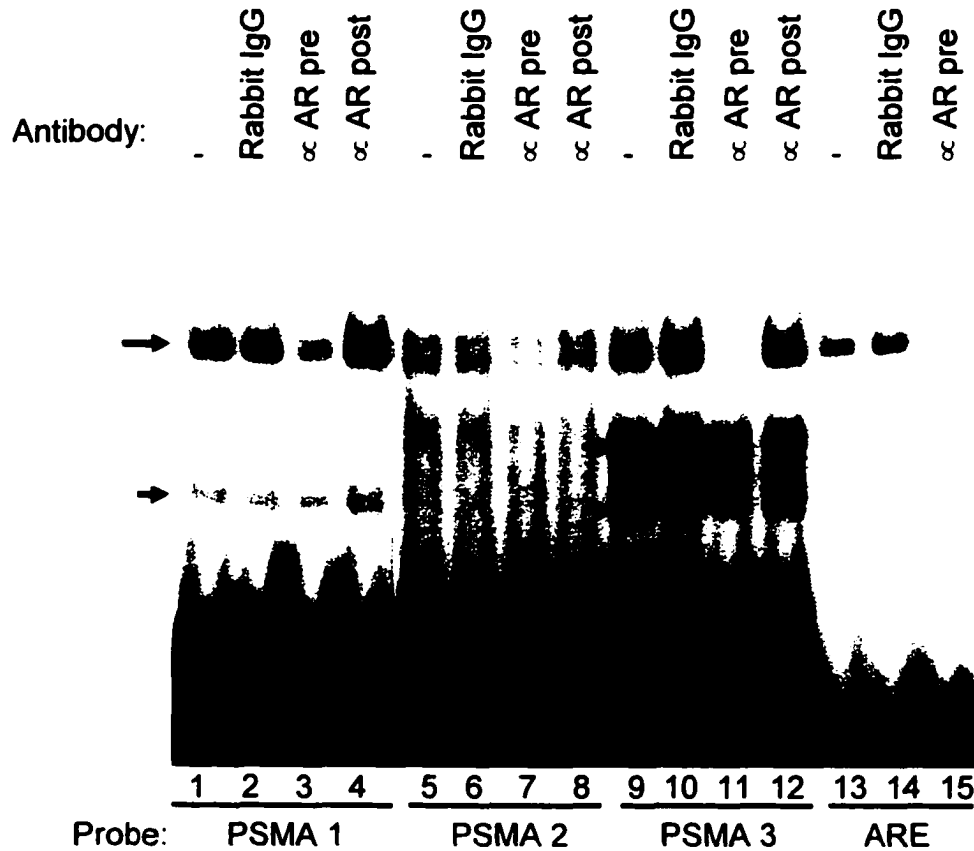


Fig. 37. Inhibition of AP-1 DNA-binding activity by androgen receptor antibody. The LNCaP cells were cultured in 5% FBS medium for 48 hours. The nuclear extracts were prepared and used in EMSA with the radiolabeled PSMA 1 (lanes 1 to 4), PSMA2 (lanes 5 to 8), PSMA3 (lanes 9 to 12) or ARE (lanes 13 to 15) as probe. For antibody competition assays, rabbit polyclonal antibody specific to androgen receptor (α AR) was incubated with nuclear extract prior to (*pre-*) or after (*post-*) the addition of the labeled probe. Nonspecific rabbit IgG (*rabbit IgG*) was used as the negative control and added in the preincubation before the addition of the labeled probe. The *large arrow* indicates the shift competed by AR antibody. The *small arrows* indicate other putative AP-1 complexes formed on the probes.

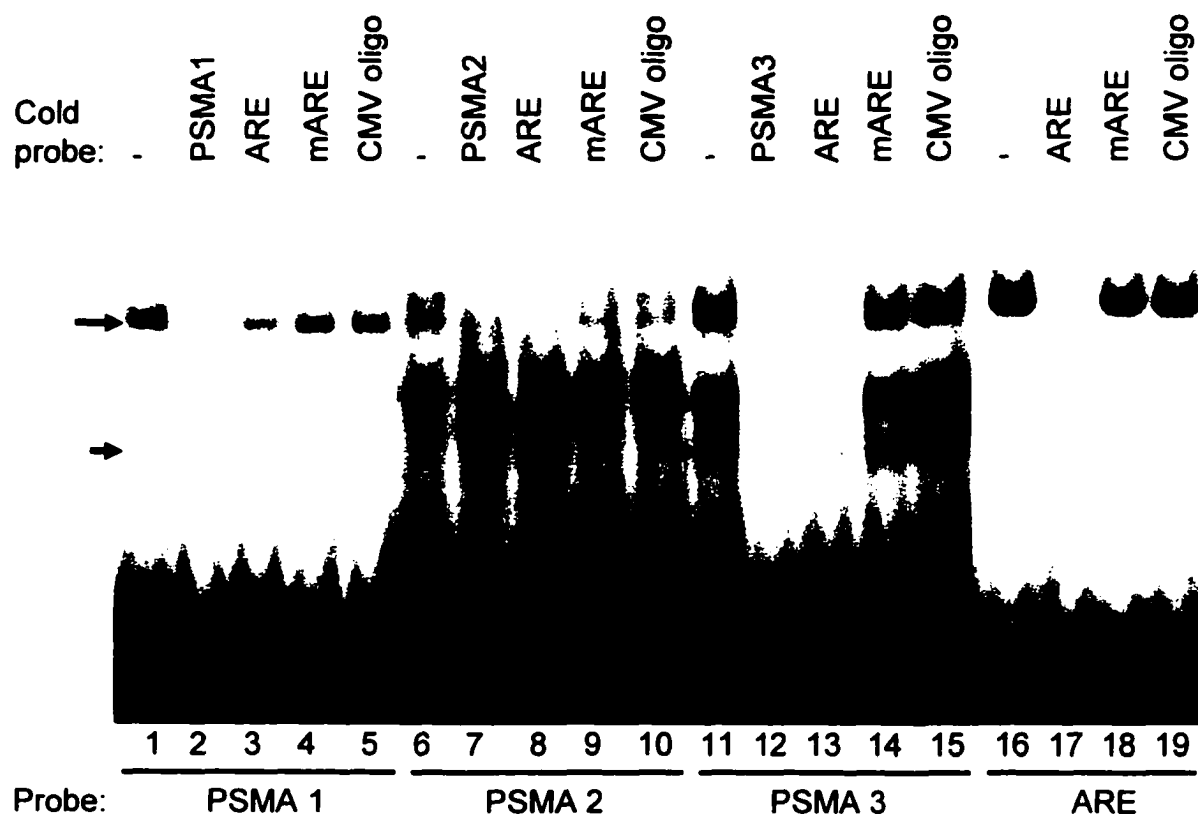


Fig. 38. Inhibition of AP-1 DNA-binding activity by androgen responsive element probe. The LNCaP cells were cultured in 5% FBS medium for 48 hours. The nuclear extracts were prepared and used in EMSA with the following radiolabeled probes: PSMA 1 (lanes 1 to 5), PSMA 2 (lanes 6 to 10), PSMA 3 (lanes 11 to 15) and ARE (lanes 16 to 19). In competition assays, cold probes (PSMA 1, PSMA 2, PSMA 3, ARE, mutant ARE or irrelevant oligonucleotides from CMV gene) were preincubated with the nuclear extracts prior to the addition of the labeled probe. The large arrow indicates the shift competed by ARE. The small arrows indicate other putative AP-1 complexes that might also be affected by ARE.

compete the binding of AP-1 complexes to all three PSMA AP-1 sites (lanes 3, 8 and 13 versus lanes 1, 6 and 11). On the other hand, cold mutant ARE (mARE) probe or nonspecific oligonucleotides from CMV genome (CMV oligo) did not have competitive effect on the AP-1 complex formation (lanes 4, 5, 9, 10, 14 and 15). Moreover, cold ARE also competed the AR binding at ARE (lane 16 versus lane 17), whereas the competition was absent with cold mARE or CMV oligo (lanes 18 and 19). All these experiments confirmed the specific competition of AP-1 binding by ARE. Besides the inhibition of the large AP-1 complex (large arrow), the small complexes (small arrows) were also competed by ARE (small arrows in lanes 3 and 13). Along with the previous experiment, the inhibition by ARE and AR antibody strongly suggested the presence of AR in the AP-1 complex formed at the PSMA AP-1 sites.

Comparison of AP-1 DNA Binding in LNCaP and PC-3 Cells To further confirm that it was AR - not the other factors - that interact with AP-1 in the protein complexes, the AP-1 binding activity was compared between LNCaP - an AR expressing cell line and PC-3 - a prostate cancer cell line that does not express AR (65). As shown in Fig. 39, when the nuclear extracts from LNCaP (L) were incubated with the radiolabeled PSMA AP-1 probes, strong DNA binding activities were detected at all three sites (lanes 1, 5, and 9). On the other hand, when the nuclear extracts from PC-3 (P) were used in EMSA, little AP-1 binding activities were detected at the three sites (lanes 2, 6 and 10). Although the protein complex formation was competed by cold PSMA AP-1 probes (lanes 3, 7 and 11), cold ARE probe did not have any competitive effect on these protein complexes (lanes 4, 8 and 12). The result suggested that when PC-3 derived nuclear extracts were used, much less AP-1

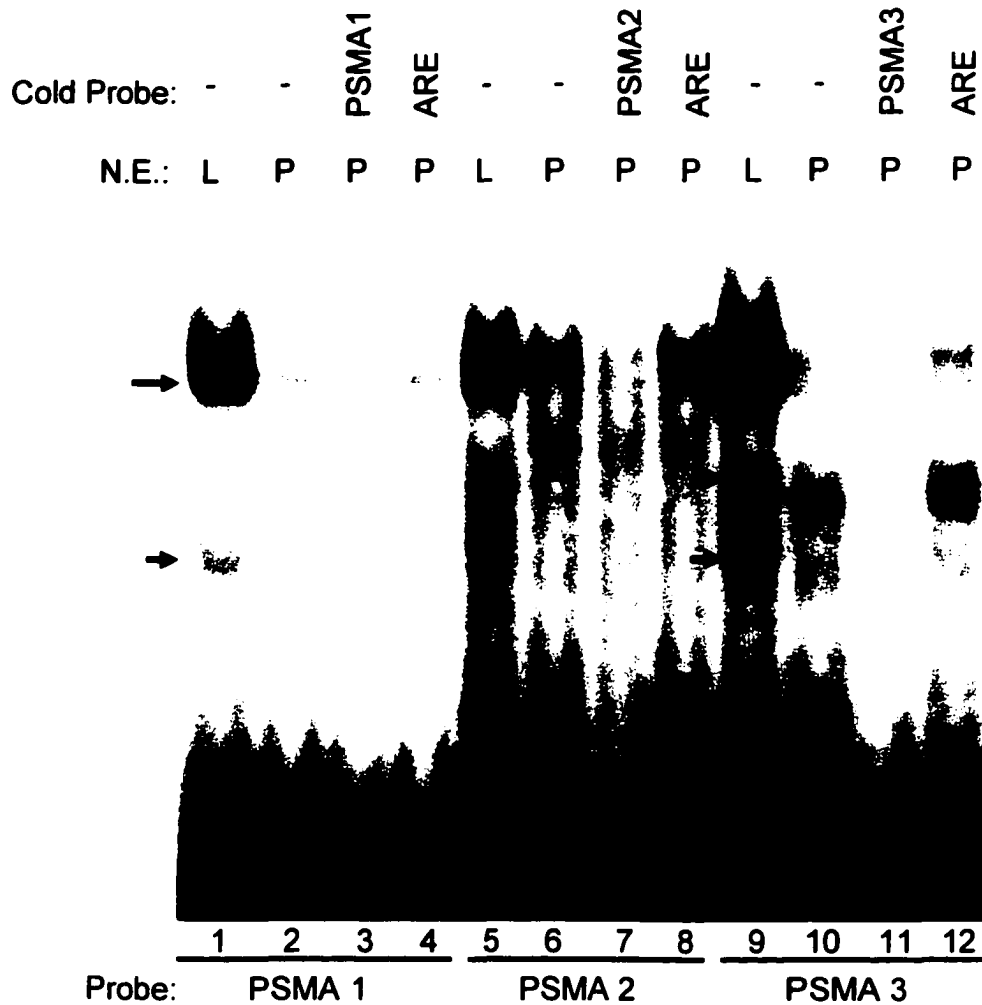


Fig. 39. Comparison of the AP-1 DNA-binding activity in LNCaP and PC-3 cells. The LNCaP cells (*L*) were cultured in 5% FBS medium and PC-3 cells (*P*) were cultured in 10% FBS medium. The nuclear extracts (*N. E.*) were prepared after 48 hours and used in EMSA with the following radiolabeled probes: PSMA 1 (*lanes 1 to 4*), PSMA 2 (*lanes 5 to 8*), PSMA 3 (*lanes 9 to 12*). In competition assays, cold probes (*PSMA 1*, *PSMA 2*, *PSMA 3* or *ARE*) were preincubated with the nuclear extracts from PC-3 cells prior to the addition of the labeled probe. The *large arrow* indicates the common AP-1 complex in two cell lines. The *small arrows* indicate other putative AP-1 complexes present in LNCaP cells.

protein complexes were formed on the three PSMA AP-1 sites as compared to the LNCaP nuclear extracts. This is understandable because PSMA was found to be expressed only in LNCaP cells, but not in PC-3 cells (13). Moreover, PC-3 cells do not express AR; therefore AR was not involved in these protein complexes in PC-3 cells. Therefore the inhibition of AP-1 DNA binding on the PSMA AP-1 sites by ARE was specific to the AR expressing cells.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

A. GENERATION OF RECOMBINANT PSMA

1. Technical Concerns

The successful expression and purification of recombinant PSMA in the baculovirus/insect cell system was accomplished in this study. Full length PSMA cDNA was constructed into a 6xHistidine-tag containing expression vector to generate the recombinant virus and the recombinant PSMA was expressed in virus-infected insect cells. Since PSMA is known as a transmembrane protein (12), the following five problems were circumvented in establishing the rPSMA expression and purification procedures:

First, the insect cell lines vary in terms of their virus replication efficiency and foreign protein productivity (66). For example, not all insect cell lines express transmembrane proteins at the same level. Therefore, both the Sf9 and Hi5 insect cells were tested in order to obtain a high level expression of rPSMA. While Sf9, the first insect host evaluated, was capable of accommodating virus replication and generating high titer rPSMA virus stock, its rPSMA transcription and translation efficiency was somehow low compared to that of Hi5 cells. Thus the Hi5 insect cell line was chosen as the host for the large-scale rPSMA expression experiments.

Second, the original serum-supplemented medium was replaced with serum-free medium. Having no obvious influence on the Hi5 cell growth and rPSMA expression efficiency, this modification has eliminated the possible contamination of serum proteins, making the purification process easier.

Third, the rPSMA expression level was also compared in the suspension versus monolayer cell cultures, with a higher level of rPSMA expression obtained in the monolayer culture. This difference was not surprising considering some of the unfavorable factors in the suspension culture, such as shear stress that might affect the expression of glycoproteins destined to be presented on the cell surface (66).

Fourth, unlike other secretory proteins that can be easily purified from culture supernatant, protein solubilization is a major concern especially when purifying a transmembrane glycoprotein. In the case of native PSMA, detergents such as SDS and Triton-X were required to solubilize and purify PSMA from LNCaP cells (14). However, because Triton-X would interfere with the binding of the histidine tag in the rPSMA to the metal chelating affinity resin, another detergent called Igepal was applied together with SDS to solubilize the rPSMA in insect cells.

Fifth, two different metal chelating affinity resins have been compared for the rPSMA purification efficiency. Because the Nickel affinity resin was found to bind non-specific proteins more than the Cobalt affinity resin, the Cobalt affinity resin was selected to purify the rPSMA.

2. Characteristics of rPSMA

Western blot analyses indicated that the rPSMA consisted of two molecular species, a 100kDa and 200kDa protein. These molecular species agreed with the molecular weight observed previously for native PSMA (14). Furthermore, rPSMA was shown to react with multiple MoAbs specific for PSMA including 7E11, which recognizes the first 6 amino acids of the amino terminus located at the inner surface of the cell membrane (15), and J591 which binds to the extracellular domain (20). These results strongly suggest that the baculovirus rPSMA mimics the native PSMA with respect to protein conformation and immunoreactivity. This observation is supported by a recent paper by Lodge et al. who described the generation of a rPSMA in a baculovirus expression system and showed that PSMA specific T cells could be induced *in vitro* when antigen presenting cells were pulsed with rPSMA. These experiments demonstrated that the rPSMA was immunologically recognized similar to the native PSMA (67).

Although the native and recombinant PSMA did not show apparent differences in molecular weight, their glycosylation profiles were different. The glycans present on rPSMA were primarily N-linked high mannose type, with a small amount of the complex type. On the contrary, the high mannose type N-glycans were rare in LNCaP derived native PSMA, with the complex type N-glycans being the major glycans (16). Thus the glycosylation analysis suggests that there might be two subpopulations of N-glycans in the PSMA glycoprotein molecule, and that there might be significant differences in terms of the amount and ratios of these N-glycans present in the native and rPSMA. Although the differences in glycosylation between the native and

baculovirus recombinant proteins have been reported previously (66), it is unclear what caused the production of the high mannose type N-glycans in the rPSMA when compared to the native PSMA. A possible explanation is that different insect cell lines might have different capacities to process N-glycans; some are unable to fully process N-glycans from the high mannose type to the complex type (66,68). For example, a previous study on the N-glycosylation pathway in baculovirus-infected insect cells showed that the N-glycans on gp64, a major structural glycoprotein in AcMNPV were unable to be processed to the complex type (69). The variable patterns in glycosylation might also explain the two closely migrating 100kDa rPSMA species observed on SDS-PAGE, even though their differences in size were so little that by mass spectrometry they were detected as a single but relatively broad 101kDa peak. Nonetheless, the complexity of glycosylation did not appear to affect the immunoreactivity of the rPSMA.

N-glycans in LNCaP derived PSMA were found to be mainly of the complex type. This observation is not in agreement with a previous study by Holmes et al. who reported that the N-glycans on LNCaP derived PSMA are the endo H sensitive high mannose type, and that this might result from a possible defect in the glycosylation pathway in LNCaP cells (16). However, it is notable that in Holmes' study (16) only a small amount of PSMA in the LNCaP cell lysates was hydrolyzed by endo H, whereas the majority of PSMA remained intact as the 100kDa species. This suggests that in contrast to their conclusion, the majority of the N-glycans on LNCaP derived PSMA were actually endo H resistant, and thus are the complex type. The partial deglycosylation of PSMA by endo H observed in Holmes' study (16) might be due to

the different LNCaP cell extracts used for the glycosidase experiments: *whole cell lysates* were used by Holmes, in contrast to the *membrane extracts* used in our study. Whole cell lysates are likely to contain various premature proteins resulting from different steps in the post-translational modification cascade, while most glycoproteins present on cell membranes and secretory proteins are of the mature complex type glycans. This could explain why the N-glycans on native PSMA were shown to be of the high-mannose type or multiple glycosylated forms in Holmes' study, compared to the complex type found in the present study. This consideration was actually proven in the Holmes study (16) based on their observation that the N-glycans on PSMA in prostate cancer patient's serum (i.e. a secreted form of PSMA) were exclusively of the complex type. Another possible explanation is the difference in cell culture and passage. If the LNCaP cells were not synchronized in culture, it could generate artifact products during glycosidase digestion. Overall it seems more persuasive that a defect in the glycosylation pathway is unlikely to exist in LNCaP cells, so that the N-glycans on PSMA can be processed to the complex type.

Recombinant PSMA will have many potential applications in clinical and basic science research for prostate cancer. For example, rPSMA can be applied as 1) a standard reference for the development of serodiagnostic assays; 2) an antigen substitute in phage display library screening and for PSMA specific recombinant MoAb generation; 3) a pharmaceutical material in preparing therapeutic strategies, such as the introduction of immunocompetent T cell or dendritic cell based immune responses; or 4) a vaccine for prostate cancer prevention or treatment.

The successful production and purification of a full length glycosylated rPSMA protein will provide a renewable source of this unique prostate cancer-associated antigen, and promote the study and application of this potential tumor biomarker for the diagnosis, prognosis and therapy of prostate cancer.

B. PSMA SELDI IMMUNOASSAY

One of the purposes of this study was to develop a quantitative immunoassay to measure PSMA in serum. Prior to this study, the only successful way to detect PSMA in body fluids was by Western blotting, an approach that is useful for preclinical research, but not easily converted to a reliable quantitative clinical assay (26,27,53). By using the purified rPSMA, a quantitative PSMA immunoassay using the innovative ProteinChip[®] SELDI-MS technology was successfully developed. The concept and potential application of the mass spectrometry immunoassay has been described in a number of previous studies (59,70,71). In this report, the ProteinChip[®] array was used as the assay platform. The PSMA protein was captured on the antibody-coated preactivated array. This was followed by mass spectrometry to detect and quantitate the antigen. The initial results, described herein, demonstrated that the 101kDa PSMA was correctly identified by its mass and could readily be quantitated in serum.

Based on these results, a group study was further conducted to quantitate PSMA in serum from normal male donors and patients diagnosed with BPH, PCA and prostatitis. The ability of PSMA as a potential biomarker of disease progression was assessed. The highest PSMA levels were found in sera from patients with prostate

cancer, and the PSMA values also differed significantly from the normal and benign groups. These results confirmed a similar trend of difference among the normal, BPH and PCA samples observed by Western blot analysis in our previous study (27), and therefore validate the sensitivity, specificity and feasibility of SELDI ProteinChip[®] technology as an immunoassay format for measuring serum PSMA.

Most noteworthy was the observation that PSMA levels significantly discriminated BPH from the PCA samples that had PSA values between 4-10ng/ml. PSA has been considered the standard tumor biomarker for prostate cancer diagnosis, but its clinical utility is hampered by the sensitivity and specificity issues that have been raised by many investigators (4,7). When a PSA cutoff of 4ng/ml is used as the parameter for diagnosis, a substantial overlap will be present between BPH and PCA (4). For instance, approximately 25% of patients who are diagnosed with PCA have a PSA value less than 4ng/ml (72), whereas an equal percent of BPH patients have PSA >4ng/ml. Therefore the sensitivity and specificity of a cutoff at 4ng/ml is problematic. If the PSA cutoff for recommending a prostate biopsy is lowered to improve sensitivity, it will trigger a significant number of additional and unnecessary biopsies (73). Free to total PSA ratio or percentage of free PSA has been employed to improve the operating characteristics, however 5-10% of PCA still remained undetected (4,7). Using the SELDI PSMA immunoassay described in this study, all samples with a PSA between 4-10ng/ml were correctly “diagnosed” as either BPH or PCA. It is in this context that serum PSMA will be of most interest for clinical application, because the observation suggests that PSMA may be a clinically valuable biomarker, either used

alone or in combination with PSA, for the early and accurate diagnosis of PCA, i.e. discriminating BPH and PCA.

Despite the success in the differentiation of BPH and PCA, the serum PSMA levels were not able to distinguish low stage from high stage prostate cancers, or Gleason grades less than 6 from 7-10. Nonetheless, due to the small sample size, this observation is still inconclusive and certainly requires further studies with larger numbers of samples to evaluate correlations with grade and stage, and the prognostic utility of serum PSMA.

This is the first report describing, not only the quantitation of PSMA in serum, but the use of an unconventional immunoassay format. One of the major differences from the conventional immunoassay platforms is that SELDI requires only small quantities of primary antibody and does not need a second PSMA antibody or labels as required for ELISA and other similar immunoassay formats to detect and quantitate antigens. For this reason, the conventional assays usually detect antigen indirectly based on the colorimetric or fluorometric changes in solution, whereas SELDI immunoassay can quantitate antigen directly according to the mass signal. This enables SELDI to bypass the problem of cross reactivity of antibody with other molecules similar to the specific antigen, while the same problem has been shown to affect the performance of conventional assays and led to the overestimation of antigen. PSMA SELDI immunoassay described in this study was based on the first generation MoAb 7E11. Although five PSMA specific antibodies have been tested herein, more PSMA antibodies that recognize either the intracellular or extracellular domains of the PSMA

glycoprotein should also be evaluated in the future to assess if these antibodies can further enhance the assay sensitivity.

Another advantage of the SELDI ProteinChip[®] immunoassay system is that multiple biomarkers can be measured simultaneously in a multiplex format. In a previous study, we described the successful measurement of both PSMA and PSA (both the free- and antichymotrypsin complexed PSA) in seminal plasma and sera (59). The multiplex format would have a major benefit as it could simultaneously measure a combination of PSMA with the various PSA forms that have proven to have an advantage over PSMA or PSA alone in discriminating BPH from PCA. Therefore plans will be made in the future study to extend the SELDI immunoassay to a multiplex version.

C. REGULATION OF THE PSMA TRANSCRIPTION BY AP-1

1. Androgen Independent Expression of PSMA

Previous studies have shown that the expression of PSMA was downregulated in the presence of androgen and upregulated after androgen deprivation therapy (19,22-24). While the expression of prostate tissue related genes such as PSA and human glandular kallikrein (hK2) is normally induced by androgens (74-79), androgen independent gene expression was not unseen (80,81). The development of prostate organ and the progression of prostate cancer are androgen dependent initially, but very often, prostate cancer cells become androgen independent during tumor progression

and metastasis. Growth factors and cytokines such as EGF, IGF and IL-6 were shown to be involved in the gene regulation in the androgen independent stages (82-86). As observed in this study, the increased transcription of PSMA gene when LNCaP cells were cultured with EGF - especially when androgens were absent in the medium - suggested that EGF is a growth factor responsible for the androgen independent expression of PSMA. EGF exerts action through binding to the corresponding EGF receptors on the cell membrane and initiates an intracellular phosphorylation cascade, leading to the activation of mitogen-activated protein kinases (MAPKs), p42/extracellular signal-regulated kinase (ERK)2, which is a key kinase in mediation of growth factor-induced mitogenesis in prostate cancer cells. This cascade can finally lead to the recruitment of transcription factors such as AP-1 (87).

2. The Binding of AP-1 at the PSMA Promoter Region

The sequence analysis in the 5' region of the PSMA gene revealed 14 putative AP-1 binding sites. EMSA examined the DNA binding at three of the AP-1 sites in the close vicinity of the PSMA transcription initiation site. The results showed that AP-1 transcription factors are indeed bound to these sites. The specificity of the binding was confirmed by the competition of the consensus AP-1 or AP-1 like binding sites, the inhibition by the c-Jun and c-Fos specific antibodies and the binding of *in vitro* translated AP-1 proteins. AP-1 is a transcription factor family members consist of homodimers and heterodimers of Jun, Fos, or activating transcription factor (88-90). The stability of these homo- and heterodimers varies among the different members.

The several protein complexes observed at the three PSMA AP-1 sites were probably due to the formation of different dimers among the AP-1 family members. The most frequently observed complex was the large complex, which was with the same size as the complex formed by the *in vitro* translated c-Jun and c-Fos and was competed by c-Jun and c-Fos specific antibodies. For this reason, this large complex was most likely the dimer of c-Jun and c-Fos. Moreover, the large complex was also the primary complex of which the formation was affected by androgens and EGF.

3. The Interaction between Androgen Receptors and AP-1

The AP-1 binding of AP-1 at the three PSMA sites was decreased by androgen and increased by EGF. In order to explain this result along with the observation that the transcription of PSMA was upregulated in the absence of androgen and downregulated by androgen, a model was proposed and shown in Fig. 36. According to this model, in the absence of androgens, AP-1 proteins, free from the interaction with AR, are able to bind to the AP-1 sites and induce a high level of PSMA transcription. This induction was further enhanced by EGF and TPA, the inducers of AP-1. On the other hand, in the presence of androgens, AR is activated and by interacting with AP-1, AR can exert the inhibitory effect on the AP-1 DNA binding activity to the PSMA promoter or the transactivation of the PSMA gene. Either of these effects can result in the suppression of PSMA transcription. Besides gaining support from the data in the present study, this model was also in agreement with the previous observation that the interaction between AR and AP-1 can mutually suppress their DNA binding and transactivation

activities (56,57). Since the interaction between AR and AP-1 has been demonstrated by coimmunoprecipitation (56, 91), similar coimmunoprecipitation experiments were not repeated in this study. In stead, the focus of this study was to examine specifically the presence of AR in the AP-1 complexes at the three PSMA sites. The EMSA competition experiments with the ARE probe and AR specific antibody indicated that AR is indeed present in the AP-1 complexes at the three AP-1 sites. Furthermore, the competition by ARE was only seen in the nuclear extracts from LNCaP cells, but not in the nuclear extracts from PC-3 cells. Since PC-3 is a cell line that does not express AR, the results confirmed that the competition was specific to the AR expressing cell line LNCaP. All these results provided a clue to the interaction between AR and AP-1 at AP-1 sites in the PSMA promoter.

Androgens are the most important steroid hormone in male reproductive organ development (92,93). Androgens were also related to the development of prostate cancer. The effect of androgens is mediated by AR. AR belongs to the nuclear receptor superfamily which includes receptors for steroids (such as estrogen, progesterone, glucocorticoid and mineralocorticoid), retinoid, thyroid hormone and Vitamin D. Before its association with androgens, the unliganded AR is associated with heat-shock proteins (HSPs) and is inactivated. Upon binding to androgens, the AR dissociates from the HSPs, forms homodimer and binds to specific DNA elements called androgen responsive elements (AREs) in the target gene promoter or enhancer region. The formation of AR/ARE complexes and the subsequent recruitment of other cofactors result in the positive or negative regulation of the target gene (94-97). Beside the direct involvement in the gene regulation, ARs can also regulate gene expression

in an indirect format (98). In most cases, the indirect effects of androgens/AR are mediated by other transcription factors such AP-1 family members. Crosstalk between the AR and AP-1 may be the mechanism contributed to the inhibition of the PSMA transcription by androgens. The interaction of AR and AP-1 (c-Jun/c-Fos) has been related to both the inhibitory and stimulatory effects of androgens. These interactions probably involved the DNA-binding domain and the hinge region of the AR and the leucine zipper region of c-Jun. The ultimate impact of the interaction on gene regulation may depend not only on the genomic structure of the target gene studied but also on the experimental conditions. The stimulatory effects of c-Jun have been reported, which acts as a coactivator for the AR and mediates AR-induced transactivation (91). On the other hand, inhibitory effects have also been described in the regulation of PSA gene where the interactions of c-Jun and AR mutually inhibit binding to their respective responsive element (56). Following the same line, a similar inhibitory effect was proposed in this study for the androgenic regulation of the PSMA gene. The results from this study suggested that the interaction between AR and AP-1 may prevent AP-1 from binding to the respective sites in the PSMA promoter and therefore lead to the inhibition of the PSMA transcription. Although the present study was conducted in LNCaP cells - an *in vitro* system, the *in vivo* observation of the induction of PSMA expression in the absence of androgens apparently has confirmed the proposed model in this study (19,22-24).

D. CONCLUSIONS

The two aims of this study were 1) to quantitate serum PSMA using ProteinChip[®] SELDI immunoassay; and 2) to characterize the transcriptional regulation of PSMA gene by AP-1. Both of these aims were successfully achieved in the study.

To accomplish the first aim, the baculovirus/insect cell system was used to express and purify a rPSMA. A recombinant baculovirus containing a 6x histidine-tagged PSMA gene was generated, from which rPSMA was expressed and purified using cobalt-chelating affinity chromatography. The purity and correct molecular size of rPSMA were demonstrated by gel electrophoresis and mass spectrometry. Glycosidase digestion showed that the oligosaccharides on rPSMA are primarily N-linked high-mannose type. Although the glycosylation of rPSMA is different from the native PSMA, it did not affect the immunoreactivity of rPSMA to antibodies specific for either the intra- or the extracellular domains of PSMA. The purified rPSMA was successfully used to develop a SELDI ProteinChip[®] immunoassay for quantitation of PSMA in serum. PSMA was captured from serum by anti-PSMA antibody bound to the ProteinChip arrays. The captured PSMA was detected by mass spectrometry and quantitated by comparing the mass signal integrals to a standard curve established using purified rPSMA. The levels of serum PSMA from prostate cancer patients was significantly different from the levels in sera from patients with benign prostate hyperplasia, making it possible to discriminate BPH from PCA, especially in the PSA 4-10ng/ml “grey zone”. This is, to the best of our knowledge, the first successful study

to quantitate PSMA in serum and to demonstrate the potential of PSMA as a clinically useful diagnostic biomarker for improving the specificity in differentiating prostate cancer from BPH. These results warrant further studies to evaluate the clinical utility of the PSMA SELDI immunoassay.

For the second aim, sequence analysis predicted 14 putative AP-1 family transcription factors (c-Jun/c-Fos) binding sites in the upstream region and the first intron of the PSMA gene. The effect of EGF and TPA (both inducers of the AP-1 family transcription factors) on PSMA RNA levels was examined by Northern blot analyses. The two inducers were found to increase the PSMA RNA levels. However, in the presence of androgens this induction was inhibited. The PSMA promoter/enhancer activity was analyzed by the luciferase reporter assay. A 0.8kb and 2kb fragment upstream of the PSMA coding region and the first intron were cloned into a pGL2 luciferase reporter vector, and the constructs designated P1, P2 and I/P1 respectively. The effect of androgens and EGF on the promoter/enhancer activity was examined by transfecting the reporter constructs individually into LNCaP cells, which express endogenous PSMA. The promoter activity of P1, P2 and I/P1 fragments was shown to increase in the absence of androgens, with the highest activity found with the P1 construct. In agreement with the Northern blot data, EGF induced the PSMA promoter/enhancer activity while androgens acted antagonistically on the EGF induction. The cotransfection of the AP-1 expression vectors (pcDNA-cJun/cFos) with P1 increased the luciferase activity, indicating the direct involvement of AP-1 in the PSMA promoter activation. Electrophoretic mobility shift assays (EMSA) demonstrated the binding of AP-1 to the three AP-1 sites in P1, which are in the close

vicinity of the transcription initiation site. The specificity of this binding was confirmed by 1) the competition of the unlabelled consensus AP-1 and AP-1 like binding sites; 2) the inhibition by the cJun and cFos specific antibodies; and 3) the binding of *in vitro* translated AP-1 (cJun/cFos) proteins. Furthermore, the binding of AP-1 was reduced in the presence of androgens and increased significantly by EGF. Additionally, the AP-1 binding was competed by either the unlabelled androgen response element (ARE) or the androgen receptor (AR) specific antibody, indicating that the AP-1 binding to the PSMA promoter might be inhibited by the interaction between androgen receptors and AP-1.

Overall the results suggest the direct involvement of AP-1 (cJun/cFos) in the transcriptional activation of the PSMA gene and the antagonistic effect of androgen on the AP-1 induced PSMA promoter activity. We propose the following model to explain the regulation of the PSMA gene: in the presence of androgens, the binding of AP-1 to the PSMA promoter is decreased, possibly by the interaction between AP-1 and androgen receptors, resulting in the suppression of PSMA transcription; whereas in the absence of androgen, the binding of AP-1 to the PSMA promoter is increased, leading to the upregulation of PSMA gene transcription. In summary, the study in the second aim demonstrates for the first time, that AP-1 family members are involved in the upregulation of the PSMA gene transcription in the absence of androgen.

In conclusion, by developing a clinically useful SELDI immunoassay for quantitation of serum PSMA and characterizing the molecular mechanism underlying the transcriptional regulation of the PSMA gene, the results from this study will

facilitate both the basic understanding and the clinical utility of PSMA in prostate cancer diagnosis and treatment.

CHAPTER V

REFERENCES

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PUBLICATIONS

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AWARDS

- 2001 Scholar-in-Training Award from the American Association for Cancer Research
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- 1998 First Place Award for presentation on the 1998 EVMS Research Day
- 1992 Award for Excellence in Honor Thesis Research
- 1988-1992 Outstanding College Student Scholarship