Biochemical and Molecular Characterization of the Prostate-Specific Membrane Antigen (PSMA)

John Karl Troyer

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BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF
THE PROSTATE-SPECIFIC MEMBRANE ANTIGEN (PSMA)

by:
John Karl Troyer

A Dissertation Submitted to the Faculties of
Eastern Virginia Medical School
and
Old Dominion University
in Partial Fulfillment of the
Requirements for the Degree of

DOCTOR OF PHILOSOPHY

BIOMEDICAL SCIENCES

EASTERN VIRGINIA MEDICAL SCHOOL
and
OLD DOMINION UNIVERSITY
1995

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ABSTRACT

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF THE PROSTATE SPECIFIC MEMBRANE ANTIGEN (PSMA)

John K. Troyer
Old Dominion University
and
Eastern Virginia Medical School
1995
Director: Dr. George L. Wright Jr.

Prostate cancer is the most common malignancy and the second leading cause of cancer death in males in the United States. Additionally, the number of deaths attributed to prostate cancer is increasing at a rate of approximately 8% a year. Development of new diagnostic and therapy strategies are needed in order to improve the life expectancy of patients with this disease. One tool which may allow for improvements in prostate cancer diagnosis and therapy is the monoclonal antibody (MAb) 7E11-C5.3 which was first described in 1987. Since then, the antigen recognized by MAb 7E11-C5.3 has been named the prostate specific membrane antigen or PSMA. Antibody-radionuclide conjugates of 7E11-C5.3 have been successfully used to localize metastatic disease in vivo and treat human prostate tumors in nude mice suggesting that PSMA may have promise as an important new diagnostic and therapeutic tool for prostate cancer. Many questions remain to be answered however, regarding the basic biology of MAb 7E11-C5.3 and PSMA before the true potential of this new marker is known. The present study has attempted to answer these questions utilizing a comprehensive characterization of PSMA at the biochemical, physical and molecular level. PSMA was found to be localized at the inner face of the plasma membrane and within mitochondria in LNCaP cells. The MAb 7E11-C5.3 epitope was
determined to consist of only the peptide backbone of PSMA and localized to the intracellular domain with a minimal reactive peptide of 6 amino acids (MWNLLH). PSMA was detected in normal, benign and malignant prostate tissues as well as normal small intestine, brain and salivary gland indicating that this marker is not as specific as once thought. Additionally, PSMA was detected in seminal fluid but not in serum using MAb 7E11-C5.3. Finally, the promoter of the PSMA gene was cloned upstream of a reporter gene construct which was expressed in androgen and androgen receptor free conditions indicating that the regulation of PSMA is markedly different from that of the prostate specific antigen (PSA). This study has provided a solid foundation on which to build a more thorough understanding of MAb 7E11-C5.3 based imaging and therapy applications and continues to suggest that PSMA is a novel and important new prostate biomarker.
DEDICATION

This work is dedicated to my best friend, partner and wife, Jill. Without her loving support, understanding and uncanny ability to put up with me, this treatise would not be possible.
ACKNOWLEDGMENTS

The author would like to thank Dr. George L. Wright, Jr. and Dr. Kenneth D. Somers for the major roles they played in the education of a scientist. The mentorship of Dr. Wright allowed for an independent and varied education available in only a very few places. The degree of tutelage went far beyond the scientific process and included the business and political aspects of the modern day scientific community which will be a necessity for a successful scientific career in the future. Dr. Somers’ zeal and excitement for the scientific process, his ability to ask questions, and his unwavering dedication to the education of new scientists was an inspiration and is the model for a true research scientist.
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CHAPTER I
INTRODUCTION

A. REVIEW OF THE LITERATURE

Prostate cancer is the most common malignancy and the second leading cause of cancer death in males. It is estimated that 244,000 males will be diagnosed with this disease and 40,400 will die of prostate cancer in 1995 (1). In other words, every male in the United States has a 10% probability of contracting prostate cancer in his lifetime. Undoubtedly, as the population continues to age, prostate cancer will become an increasing medical problem. Prostate adenocarcinoma is unique in that it exists as two distinct clinical entities; a latent or histological cancer and a clinically evident cancer (2). Latent tumors are usually not clinically manifested nor detected in men until autopsy, meaning they die with their tumors instead of from them. In contrast, clinically diagnosed prostate adenocarcinoma is often aggressive and highly metastatic (2). One of the major goals in the study of prostate biomarkers is not to detect all prostate tumors, but just those which are clinically important. This specificity will allow clinicians to specifically treat patients with clinically significant disease and permit patients with latent cancer to continue living an otherwise healthy life. As with any malignancy, the key to managing this disease is early detection and treatment of these aggressive tumors to reduce both morbidity and mortality (3,4).
In many human tumors, the progression from the normal cellular state to benign hyperplasia to preneoplastic lesions then to carcinoma in situ can be followed by observing the set of genetic mutations which have accumulated. The classic example of this observation is the monoclonal progression of colorectal carcinoma described by Fearon and Vogelstein. In this model, an accumulation of genetic changes including mutations or loss of the APC gene, mutations of oncogenes (K-ras), loss or inactivation of tumor suppressor genes such as the DCC gene and p53, as well as DNA hypomethylation occur as tumorigenesis proceeds from benign disease to metastatic carcinoma (5,6). In the past ten years it has been recognized that many oncogenes and tumor suppressor genes are mutated or deleted in human tumors. In contrast, a clear pattern of genetic alterations has not been found in the progression of prostate pathology from the normal prostate to benign prostatic hyperplasia (BPH) to prostatic intraepithelial neoplasia (PIN) to prostate adenocarcinoma (CaP). In fact, it is unclear whether a progression exists or whether these pathologies represent independent disease processes. For example, the combined data from three independent studies showed that less than 4% of prostate samples analyzed contained Ha-ras mutations (7-9). The importance of Rb gene inactivation in prostate carcinoma has been suggested (10,11) but is not overwhelmingly significant with 11 of 41 informative tumors (27%) showing loss of heterozygosity at the Rb gene locus while the frequency of the p53 mutations is a relatively infrequent event (10-15%) when compared to many other common cancers (12,13), although bone metastatic prostate tumors may have an increased percentage of p53 mutations indicating that they are late events in the progression of prostate tumors (14-16). While it is evident that these mutations may play a role in the progression of prostate pathology, it is obvious that few of the known genetic alterations will be useful for the detection and diagnosis of this disease and of little use in differentiating latent from aggressive
tumors. This fact and the recent controversy regarding whether and when to treat prostate cancer patients (17) and which treatment modality to use, suggests that additional biomarkers need to be identified in order to more accurately differentiate benign from malignant prostate disease, to identify the clinically important prostate carcinomas, and to develop potential targets for new treatment strategies.

Several prostate biomarkers have been identified and have proven to be clinically useful. The first of these markers was prostatic acid phosphatase (PAP), identified in 1938 (18). Prostatic acid phosphatase is a secretory product of the normal prostate gland and can be measured in the serum of patients with extracapsular prostate adenocarcinoma. Since an elevated serum PAP level is indicative of metastatic disease and contraindicates a radical prostatectomy, a serum PAP assay was utilized for many years to determine if extraprostatic disease was present prior to surgery (19).

The most useful biomarker in all of cancer biology is the prostate specific antigen (PSA) (20). While at the time of its identification PSA appeared to be only expressed by the prostate gland in males, it is not nearly as prostate specific as originally thought. Several studies have recently shown PSA to be present in breast, colon, ovarian, parotid, kidney and liver tumors, normal breast, amniotic fluid and breast milk (21), as well as normal salivary gland (22) and normal endometrium (23). Like PAP, PSA is a secretory product of the prostate and is normally confined to the genitourinary tract and cannot be detected in the serum. Damage to the prostate vasculature during prostatitis or benign hyperplasia results in a moderate leakage of PSA into the blood stream (0-10 ng/ml) and extracapsular prostate adenocarcinoma causes a dramatic increase in serum PSA values (10- >1,000 ng/ml). With the advent in recent years of highly sensitive serum PSA measurements (25,26), the detection rate of prostate adenocarcinoma has been
markedly enhanced when combined with a digital rectal exam (DRE). The combined use of DRE and serum PSA measurement is now considered to be the optimum diagnostic protocol for the detection of prostate adenocarcinoma (27). An elevated serum PSA level does correlate with poor prognosis and some have suggested the use of a DRE and serum PSA values to screen males for BPH and CaP. However, approximately 30% of BPH patients give false positive PSA levels and 25-30% of prostate cancer patients have normal serum PSA concentrations (27-29). It is clear that although PSA is a very useful marker of prostate pathology, there is much room for improvement.

Various other markers of prostate disease have been reported including clinical and pathological markers such as apoptotic index (30), tumor stage (31) and volume (32); proliferative markers including PCNA (33), Ki-67 (34), and mitotic index; nuclear morphometric analysis (35); the neuroendocrine markers neuron specific enolase, chromogranins, and bombesin (36); mutations and expression of the androgen receptor (37-39); epithelial-stromal cell interactions with E-cadherin (40), type VII collagen and laminin receptors (41); blood group antigens (42); mucins such as PD-41 (43); ploidy analysis utilizing flow cytometry and image analysis techniques (44); and other markers including prostate secretory protein (PSP) (45), Turp-27 (46,47), PR92 (48), cathepsin D (49), PR-1 (50), Leu-7 (51), nucleoside diphosphate kinase (nm23) (52), fatty acid synthase (53), and bcl-2 expression (54) are being evaluated for efficacy as prostate biomarkers. Notably, PSP can be detected in seminal plasma and urine of prostate cancer patients but does not correlate with prostate cancer stage and grade as well as PSA (55,56). The PD-41 MAb which recognizes a mucin called prostate mucin antigen (PMA) appears to be the only marker identified to date which is specific for prostate carcinoma unlike the majority of the markers which react to normal, BPH and CaP. The c-erbB-2/neu oncogene is highly
overexpressed in breast adenocarcinoma and has been reported to be prevalent in human prostate cancer (57-59) but its level of expression and subcellular localization must be critically addressed before it is known whether it will have any prognostic value for prostatic carcinoma. While many of these markers have either not been fully investigated or not proven to be highly significant when analyzed alone, a panel of several of these markers analyzed concurrently may provide a useful measure of the status of prostatic disease.

The primary treatment for prostate cancer is prostatectomy and orchietomy in the form of physical or chemical castration (60) which removes the primary source of testosterone from the pituitary to gonadal axis (61). One of the most important and highly debated decisions confronted by the physician treating prostate carcinoma is whether a prostatectomy should be performed (62,63). The main parameter regarding this decision is whether there is extraprostatic disease or if the tumor remains organ confined. It is generally agreed that a radical prostatectomy is most advantageous to patients with organ confined disease but of little use, and actually to the detriment, of patients with extracapsular disease (64). Since serum PSA values are predictive of metastatic dissemination of prostate carcinoma, they are utilized to determine the extent of disease. However, as noted above, there are significant false positive and false negative results. A precise clinical tool which would definitively determine the presence or absence of extraprostatic disease may be of use in this clinical decision in cases where there is no clear evidence of micrometastatic spread. The identification of micrometastatic foci prior to surgery would spare patients with metastatic disease the risks and complications of radical prostatectomy. These patients could then be more appropriately treated with chemotherapy and radiation protocols since it is doubtful they would receive any benefit from a radical prostatectomy.
One of the significant problems which must be addressed in this and other studies of prostate biomarkers is that many prostate genes are highly regulated by androgens (65). Androgen regulated gene expression is important in normal male development and is mediated by the androgen receptor (AR) (66,67). The androgen receptor belongs to a family of steroid receptors including the glucocorticoid and progesterone receptors which are localized in the cytoplasm. The hydrophobic nature of steroid hormones allows them to traverse the phospholipid bilayer of cell membranes and interact with their receptors in the cytoplasm. Following ligand binding, the receptor-ligand complex moves to the nucleus where it is capable of binding to specific DNA sequences, or hormone responsive elements (HRE), and acts as a transcription factor (68,69).

Interestingly, several hormones can bind and activate these receptors. For example, testosterone will bind and activate the AR, but the testosterone metabolite dihydroxytestosterone (DHT) has approximately ten times greater affinity for the AR than testosterone (70,71).

Not only do androgens play an important role during male development, they are also integral in maintaining the normal structure and function of the prostate gland throughout the life of an adult (72,73). Removal of androgen hormones either physically or chemically, results in the involution of the prostate via induction of apoptosis, or programmed cell death, as indicated by the fact that expression of the prostatic cell death gene, TRPM-2, is induced by castration (61). This involution of the prostate gland forms the basis for orchiectomy as one of the primary treatments for prostate adenocarcinoma.

The prostate specific antigen is a prime example of a gene which is only expressed in the presence of androgen hormones (74-78). A dramatic decrease in serum PSA values is observed in prostate cancer patients following androgen ablation and may drop from >1,000 ng/ml to near 0 ng/ml (29). A small promoter
fragment (300 bp) from the PSA gene inserted in front of a chloramphenicol acetyl transferase (CAT) reporter gene proved that this gene is upregulated in the presence of androgen hormones (79,80).

While the prostate gland does dramatically shrink in size and tumors cease to grow following hormone ablation, the majority of prostate tumors become androgen independent and begin to proliferate again several years subsequent to orchiectomy. In many cases PSA is also expressed in the absence of androgens and can be detected in the serum. One possible cause of this renewed tumor growth and PSA expression may be point mutations in the androgen receptor which allow binding and activation by multiple steroid hormones including estrogen, progesterone and some androgen receptor antagonists (37-39). However, it is reasonable to suggest that a significant percentage of prostate tumors may begin to proliferate following androgen ablation without an increase in PSA expression. In fact, a recent report suggests that some androgen independent tumors may produce factors which suppress PSA expression (81). Such tumors would escape detection by serum PSA measurement and would not be discovered until more acute symptoms of metastatic disease become evident such as bone pain and lymph node enlargement. Additionally, an alternative pathway for PSA expression has been suggested in LNCaP cells in the absence of steroid hormones (82) which could result in an increase in serum PSA values without a concomitant increase in tumor growth. As a result, PSA measurements, while extremely useful for identifying recurrence of many hormone insensitive tumors (25,26,29), fail to demonstrate the state of the prostate from the time of initiation of therapy to recurrence since the expression of PSA is effectively altered and may give false negative or positive results in some cases. If a marker could be identified which is not down-regulated following hormone ablation therapy, the steady state of the prostate could be monitored and recurrence may be identified following therapy at

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a much earlier time which may offer an increased life expectancy for prostate carcinoma patients.

A recently discovered prostate associated biomarker designated the prostate specific membrane antigen (PSMA), recognized by the mouse monoclonal antibody (MAb) 7E11-C5, may have the properties to meet some or all of the needs described above. The murine MAb 7E11-C5 was produced against a crude membrane extract of the LNCaP prostate carcinoma cell line by Horoszewicz, et al. (83). Initial immunohistochemical analysis showed PSMA expression, as recognized by MAb 7E11-C5, to be highly restricted to normal, benign and malignant prostate epithelia and appeared to have an intracellular localization (83,84). Therefore, PSMA is similar to PSA in that it represents a potential novel prostate specific but not carcinoma specific marker. The nature of the antigen was initially identified to be a predominantly 100 kDa membrane associated glycoprotein (85-87). Recently, the cDNA for PSMA was cloned and the DNA and amino acid sequence were determined (88) which encodes a single 100 kDa transmembrane glycoprotein (89).

The initial biochemical characterization of the MAb 7E11-C5.3 epitope on PSMA demonstrated that it was susceptible to both periodate oxidation (90,91) and proteolytic digestion. Periodate cleaves vicinal hydroxyl groups on carbohydrate residues and thus, is used as a general assay for carbohydrate content in antigenic epitopes. These observations led to the initial conclusion that the epitope consisted of a glycopeptide (91,92). Lectins, which bind to specific carbohydrate residues were utilized in studies to block MAb 7E11-C5 binding to PSMA. These lectin blocking experiments indicated that the carbohydrate in the antigenic epitope consisted of galactose residues. However, further studies which attempted to more specifically determine the type of carbohydrate present in the epitope were unable to detect any carbohydrate at all (92).
Two classes of carbohydrates found in glycoproteins are well characterized based on the linkage of the sugar to the peptide backbone. These classes are O-linked and N-linked sugars which are linked to the free hydroxyl groups of serine and threonine residues and the free amino group of asparagine residues, respectively (93). As a result of the distinct differences between these two classes of linkages, it is relatively simple to distinguish the two by treatment of the antigen with sodium borohydride (94) which cleaves O-linked sugars or by incubating the cell line with tunicamycin (95) which inhibits the addition of the initial oligosaccharide to the neo-peptide. Neither of these treatments was able to abrogate 7E11-C5.3 binding which was confusing in light of the periodate sensitivity which suggested a carbohydrate was present as part of the epitope. Additionally, attempts to digest the antigen with a variety of glycosidases and blocking experiments with specific monomeric or polymeric carbohydrates and amino sugars were equally unable to inhibit 7E11-C5.3 binding. Since periodate oxidation is a highly accepted method for determining carbohydrate content in antigenic epitopes (96,97), these data added considerable confusion to the understanding of the biochemical nature of the antigen and its antigenic epitope. Several possibilities may explain the apparent conflict in these data: 1) The epitope may indeed contain a carbohydrate moiety which is neither O- nor N-linked in nature which would explain the failure of sodium borohydride and tunicamycin treatments to abrogate antibody binding; 2) the periodate treatment may affect a part of the epitope which is not a carbohydrate (i.e. the polypeptide backbone or some other post-translational modification); or 3) the specific carbohydrate in the epitope may be inaccessible or unaffected by the conditions normally utilized in the biochemical characterization assays.

Our understanding of the basic biology of PSMA and MAb 7E11-C5.3 is far behind the pursuit of approval from the Food and Drug Administration (FDA)
for use in clinical applications. Although the utility of this MAb for diagnosis and therapy has yet to be fully established, clinical trials using an $^{111}$Indium labeled conjugated form of MAb 7E11-C5.3 ($^{111}$In CYT-356) to localize metastatic prostate foci (98) and sites of prostatic carcinoma recurrence following radical prostatectomy (99,100) were found to be superior to traditional staging and imaging modalities. Phase I clinical trials have recently been initiated to evaluate the efficacy of 7E11-C5.3 radionuclide immunoconjugates for treating metastatic prostate cancer (Maguire, Cytogen Corporation, personal communication). Another possible clinical application using RT-PCR to detect metastatic cells expressing PSMA mRNA in the whole blood of patients has recently been reported (101,102).

Since this monoclonal antibody or molecular probes for PSMA may be utilized for *in vivo* clinical assays, it is imperative that the prostate specific membrane antigen recognized by MAb 7E11-C5.3 be more fully characterized. Specifically, the subcellular localization of the antigen needs to be confirmed by an in depth study since an intracellular epitope may hinder the ability of CYT-356 to localize a living tumor mass *in vivo*. Much of the biochemical studies of PSMA have been previously reported although, as noted above, several contradictions need to be resolved including the question of whether the epitope contains a peptide, a glycopeptide or a carbohydrate moiety. The regulation of the expression of the PSMA gene also needs to be addressed to determine if the radioimmunoscintigraphic assays can be utilized with patients receiving endocrine therapy. Previous reports suggest that PSMA glycoprotein expression may increase following hormone ablation therapy (87) while PSMA mRNA levels may decrease in the presence of androgen hormones (89). Clinical protocols for diagnosis and therapy will be greatly affected by the results of these regulatory studies. If the PSMA gene is down-regulated by androgen ablation similar to PSA,
then CYT-356 directed immunoscintigraphy and immunotherapy will have to be administered prior to hormone therapy. However, if the PSMA gene is not controlled by androgen hormones or is upregulated following ablation, both the diagnostic and therapeutic approaches can be done concomitant with endocrine therapy.

B. FOCUS OF THE PRESENT INVESTIGATION.

The objective of this study was to more fully understand the physical and biochemical nature of the PSMA glycoprotein recognized by the murine monoclonal antibody (MAb) 7E11-C5.3. The specific aims of this study were: (1) to determine the subcellular localization of PSMA; (2) to determine the biochemical and physical nature of the PSMA glycoprotein; and (3) to begin to understand the regulation of the PSMA gene.

1. To accomplish the first aim, the LNCaP cell line was analyzed by mechanical cellular subfractionation utilizing gradient density centrifugation and Western blot analysis to determine if PSMA is a cytoplasmic, nuclear or membrane glycoprotein. Light microscopy analysis utilizing multiple techniques including immunoperoxidase and immunofluorescence microscopy was undertaken to begin to determine the localization of PSMA within the cell. Then, immunoelectron microscopy of LNCaP cells was carried out to definitively determine the subcellular localization of PSMA.

2. The second aim was addressed by repeating previously reported experiments where necessary to confirm the initial observations. Once these results were verified, additional studies were carried out to explain the conflicting
results garnered from this biochemical characterization. Specifically, the question of why MAb 7E11-C5.3 reactivity to PSMA was abrogated by periodate oxidation while assays to determine the specific carbohydrate failed to decrease activity. To address this question, the MAb 7E11-C5.3 antigenic epitope on PSMA was mapped with synthetic peptides. The reactive peptides were then treated with periodate as described for the analysis of the native antigen to determine if they were susceptible to oxidation which would lead to the false conclusion that the epitope contains a glycopeptide.

The characteristics of PSMA expression in prostate cell lines, prostate tissues and exocrine products of the prostate gland, as well as non-prostate tissues and human serum was thoroughly examined by Western blot analysis. The physical characteristics of PSMA were also evaluated using polyacrylamide gel electrophoresis under differing conditions to determine the physical nature of the PSMA glycoprotein.

3. To begin to understand the regulation of the PSMA gene, a suitable model system had to be developed to study the expression of prostate specific genes. A model system was designed utilizing the PPC-1 primary prostate carcinoma cell line and which allowed for the control of both the level of androgen receptor expression and the concentration of androgen hormones in a prostate epithelial cell. The PSA promoter was cloned and utilized as a control for androgen upregulation in these experiments. In order to isolate the 5' flanking DNA from the PSMA gene, PCR primers specific for the PSMA cDNA were used to amplified probes for the PSMA gene. These molecular probes were used to screen a human cDNA library to isolate the PSMA cDNA. A restriction fragment encompassing 700 bp of the 5' end of the cDNA was then used to probe a human genomic library and several PSMA genomic clones were identified. The genomic clones were mapped and sequenced, and a fragment containing approximately
1000 bp upstream of the translational start site was cloned into a basic chloramphenicol acetyl transferase (CAT) reporter gene construct containing no endogenous enhancers or promoters. The PSMA 5' flanking DNA or the PSA control constructs were transiently transfected into the model system described above and assayed for CAT mRNA expression to definitively determine if PSMA was regulated by dihydroxytestosterone.

In summary, this work has provided important new information regarding the PSMA glycoprotein which will substantially impact ongoing clinical trials for in vivo imaging and therapeutic strategies. The biochemical nature of the MAb 7E11-C5.3 epitope was deduced and mapped to the amino terminal end of the PSMA peptide backbone in the intracellular domain. PSMA was found to be expressed in normal, benign, and malignant prostate tissue, normal small intestine, salivary gland, and brain tissue and shed into the seminal fluid but not serum of males. The PSMA glycoprotein was shown to be a 100 kDa monomer under reducing and denaturing conditions and a second species of approximately 180 kDa was observed under non-denaturing conditions which indicated PSMA may exist as a dimer or has considerable secondary and tertiary structure in its native state. The PSMA detected in tissue and seminal plasma was slightly larger (120 kDa) indicating PSMA from LNCaP may be different in the level of post-translational modifications. Finally, a model system was devised to study the promoter of PSMA which was found to be quite different from the PSA promoter in that it was not inducible by androgen hormones.
CHAPTER II

MATERIALS AND METHODS

A. MATERIALS.

1. POTENTIAL HAZARDS. Hazardous aspects of this study included the use of radioactive isotopes, toxic chemicals, potentially infected tissues, body fluids, and recombinant DNA molecules. All work with radioisotopes was carried out in designated laboratories approved with the oversight of the Radiation Safety and Biohazards Institutional Committees. Absorbent bench covers, rubber gloves, lab coats, disposable plastic labware were used to prevent and/or contain contamination. Periodic swipe tests were carried out by the investigator as well as by the Radiation Safety Office as provided for in the laboratory licensing. Toxic, corrosive or carcinogenic chemicals were limited to the laboratories where the studies were being conducted. Fume hood, gloves, plastic face shield, and lab coats were used, and fire extinguishers and Spill-Packs were at hand at all times. Excess acids or bases were neutralized prior to disposal, and all waste chemicals were handled under the direction of the Environmental Safety Committee. Similarly, all human tissues and body fluids were handled with protective clothing, gloves and masks and/or as recommended by the Biosafety Committee. Disposal of these products was by autoclaving or incineration. Recombinant DNA experiments were conducted in a Biosafety Level 2+ laboratory in accordance with
the NIH Guidelines for Research Involving Recombinant DNA Molecules and Institutional Biohazards Committee.

2. CELLS. LNCaP and PC3 cells were obtained from the American Type Culture Collection, DU145 cells were kindly provided by Don Mickey (Duke University). The PPC-1 cell line was provided by Dr. Art Brothman (University of Utah) and the PPC-1AR5 sub-line containing the androgen expression vector was provided by Dr. Michael McPhaul (University of Texas Southwestern Medical Center).

3. REAGENTS. Lectins were obtained from E.Y. Laboratories (San Mateo, CA). O-glycanase and N-glycanase enzymes were purchased from Genzyme (Boston, MA). Methyltrienalione (R1881) was purchased from New England Nuclear (Wilmington, DE), the pGEM7Zf+, pCAT basic vectors and the pGEM T cloning system were purchased from Promega (Madison, WI). Reagents for in vitro translation and RNAse protection assays were purchased from Ambion (Austin, TX). EDAC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide), OPD (0-Phenylenediamine dihydrochloride), all other enzymes, carbohydrates, carbohydrate conjugates and chemicals were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise noted.

4. MONOCLONAL AND POLYCLONAL ANTIBODIES. The monoclonal antibody 7E11-C5.3, purified by protein-A affinity chromatography from murine ascites, was provided by Cytogen Corporation (Princeton, NJ). The MAb concentration was determined using a single radial immunodiffusion system (TAGO, Burlingame, CA). Monoclonal antibodies OKT-9 (Ortho Immunology Systems, Raritan, NJ.), Hsp-70 (Sigma Immunochemicals, St. Louis, MO), PCNA
(DAKO Corporation, Carpinteria, CA), and the ployclonal antiserum PG-21 specific for the androgen receptor (Affinity Bioreagents, Neshanic Station, NJ.) were purchased and utilized according to the manufactures recommendations.

5. LNCAp XENOGRAFT TUMORS. Male athymic (nu/nu) Swiss background nude mice and Fox Chase SCID inbred mice (CB-17/ICrTac-scid DF), 4-6 weeks old (Taconic Farms, (Germantown, NY) were housed in sterilized cages with filter bonnets, and were given autoclaved laboratory rodent chow (Purina, St. Louis, MO) and filtered tap water ad libitum. Mice were given injections of 2.5 mg of cyclophosphamide i.p. one day prior to s.c. injections in the left rear flank with $1 \times 10^7$ LNCAp cells in exponential growth phase, in 0.2 ml. of sterile medium or PBS. Subsequently, LNCAp tumors were propagated by s.c. implantation of tumor fragments aseptically transferred from donor to cyclophosphamide-treated, recipient mice. The same methods were used to grow LNCAp tumors in SCID mice, except SCID mice were not treated with cyclophosphamide.

6. TISSUES AND SEMINAL PLASMA. All pathological tissue specimens were obtained from biopsy, surgery or autopsy materials which were removed only for appropriate diagnostic and therapeutic purposes. That which was not required for diagnosis and would normally be discarded was provided to this study. Similarly, samples of body fluids routinely taken for diagnosis or monitoring, no longer required for diagnosis, were also utilized. Since these tissues and body fluids were classified as discarded and the anonymity of the patient was maintained in all records and publications, such procurement qualified for NIH exemption number 4. Prostatic fluids were obtained by prostate massage and seminal plasma by masturbation from normal donors and patients with BPH, prostatitis, and active prostate adenocarcinoma. The purpose of the study, the
amount and how the sample was collected and the risks and benefits were explained fully to the patient by the attending urologist. A signed and witnessed consent form was required prior to collection of the specimen. There was little to no risk associated with these procedures. The confidentiality of the patient was maintained by assigning code numbers to the specimens which were utilized on all report forms and publications. Patient files and history forms were kept in locked file cabinets.

Following collection, the semen samples were treated as previously described (103,104) with minor modifications. Briefly, the samples were frozen prior to liquefaction and stored at -70°C until further analysis. The samples were thawed at room temperature by adding 1/2 volume dilution buffer (123mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1mM EDTA, and 37 mM Tris pH 8.0). Pefablock (Boehringer Mannheim) was added to a final concentration of 0.1 mM and 50X protease inhibitor cocktail (0.28 mM Antipain; 0.75mM Pepstatin; 60mM EDTA ) was added to a final concentration of 1X. The samples were centrifuged for 5 min. at 25,000 x g to remove cells or cellular debris and the supernatants, termed seminal plasma, were transferred to eppendorf tubes and stored at -70°C.
B. METHODS.

1. TISSUE CULTURE. All cells were grown in RPMI 1640 media supplemented with L-glutamine and 5% fetal calf serum (Gibco-BRL, Gaithersburg, MD) at 37°C and 5% carbon dioxide. The androgen expression plasmid was maintained in the PPC-1AR5 sub-line with selective media supplemented with 400 μg/ml G418 (Life Technologies, Inc., Grand Island, NY). Stripped serum media was supplemented with pure Finasteride, kindly provided for this study by Merck and Co., (West Point, PA), a well characterized inhibitor of 5 alpha-reductase activity (105-108) at a concentration of 500 nM/L for the hormone regulatory studies.

2. MECHANICAL SUBCELLULAR FRACTIONATION. The method employed here was modeled after several studies found in the literature (109-111). Briefly (see Fig. 1), cultured LNCaP cells were harvested and lysed in a hypotonic media (1mM NaHCO) then dounce homogenized with fifty up and down strokes. It was imperative that the majority of the cells were lysed since the initial centrifugation steps would pellet out unbroken cells as well as nuclei. Therefore, the homogenization was monitored by trypan blue staining and the subsequent steps were not performed until >99% of the cells are lysed. The homogenate was centrifuged at 500 x g for 5 min. at 4°C and the pellet resuspended in buffer containing 16.0% sucrose then underlayed with 20.0% sucrose and centrifuged in a swinging bucket rotor at 150,000 x g for 60 min. at 4°C. The nuclei (N) were pelleted to the bottom of the 20.0% sucrose while other membrane components remained at the interface of the two layers. The supernatant was removed by
FIGURE 1. Subcellular fractionation of LNCaP cells. The cells were fractioned into a nuclear fraction (N), heavy membrane fraction (HM), light membrane fraction (LM), and cytoplasmic fraction (C), utilizing a series of linear and gradient centrifugation steps as shown.
aspiration and the nuclear pellet resuspended in 16.0% sucrose and centrifuged again to remove any residual membranes since it has been shown to be difficult to obtain totally pure nuclei (111). The supernatant from the 500 x g spin was centrifuged at 10,000 x g for 15 minutes at 4°C. The pellet from this step represented the heavy membrane fraction (HM) containing predominately the rough endoplasmic reticulum, the Golgi apparatus and mitochondria. The supernatant was centrifuged at 150,000 x g for 60 minutes at 4°C. The pellet from this step was the light membrane fraction (LM) containing smooth endoplasmic reticulum, plasma membrane and any vesicular membranes while the supernatant represented the soluble cytoplasmic fraction (C).

The fractions obtained from this fractionation method were analyzed by Western blot and ELISA for the presence of PSMA. Control antibodies were used as markers for mitochondria (MU213 specific for HSP70 found in mitochondria), plasma membrane (OKT-9, specific for the transferrin receptor) and nucleus (α PCNA antibody specific for PCNA) were utilized to verify that the partitioning was efficient.

3. LIVING LNCAP IMMUNOFLUORESCENCE STAINING. LNCaP cells were grown to approximately 50% confluency on collagen coated chambered slides (Nunc, Naperville, IL.). The media was removed by aspiration and the cells were washed twice with PBS (136 mM NaCl, 1.7 mM KCl, 8 mM Na2HPO4, 1.5, mM KH2PO4, 0.9 mM CaCl2, 0.5 mM MgCl2, pH 7.4) warmed to 37° C. Blocking serum (10% goat serum in PBS) was added to the chambers and incubated at room temperature for 1 hr. then removed by aspiration. The primary antibody (7E11-C5.3 at 20µg/ml, OKT-9 at 15 µg/ml, PSA-5 at 1 µg/ml; or IgG1 isotype matched control at 10 µg/ml) was added to the cells and incubated at room temperature in a humid chamber for 1 hr. The cells were washed twice with PBS.
followed by the addition of secondary antibody (fluorescin isothiocyanate [FITC]
labeled goat F(ab')\(\alpha\) mouse antibody with Evan's blue counter stain, Baxter
Healthcare Corporation, West Sacramento, CA) and incubated at room
temperature for 1 hr. The cells were washed twice with PBS then visualized with a
fluorescence microscope.

4. **FIXED LNCAP CELL IMMUNOFluorescence STAINING.** LNCaP
cells were grown to approximately 50% confluency on chambered slides as
described above. The cells were fixed for 20 min. in 10% buffered formalin and
rinsed twice with PBS. The cells were permeabilized briefly in 0.1% Triton-X100
in PBS for 5 min., washed twice with PBS, then stained as described above using
7E11-C5.3; OKT-9; PSA-5; and the IgG isotype matched negative control
antibody.

5. **IMMUNOPEROXIDASE STAINING.** The immunoperoxidase staining was
carried out exactly as described above for the fixed immunofluorescence staining
except the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) was
used with a peroxidase labeled horse \(\alpha\) mouse antibody. The positive staining was
visualized by the addition of a color substrate (diaminobenzidine [DAB]) for 10
min., then rinsed twice with PBS and counter stained with Mayer's Hematoxylin
for 5 min.

6. **IMMUNOELECTRON MICROSCOPY.** To definitively determine the
localization of the PSMA glycoprotein, a method for immunoelectron microscopy
of PSMA was designed from previously reported studies in the literature (112-120)
which were utilized with 7E11-C5.3 and IgG1 isotype matched control antibodies.
LNCaP cells were grown to confluency on plastic coverslips. The coverslips were
fixed in half strength Karnovsky’s fixative (2% paraformaldehyde and 1%
glutaraldehyde in cacodylate buffer) for 30 minutes then osmicated in osmium
tetroxide following standard conditions. The coverslips were embedded in LR-
White and polymerized overnight at 4°C. Ultrathin sections were cut with a glass
knife and mounted on nickel EM grids. The grids were stained using the hanging
drop method by suspension on drops of blocking buffer (10% goat serum in
filtered PBS) for 1 hr. then primary antibody for up to 48 hours, rinsed by repeated
hanging drop incubations in wash buffer, then incubated with a secondary goat anti
mouse immunoglobulin antibody labeled with a 10 nm gold bead. Following
several rinses, the grids were counterstained by placement at the bottom of a drop
of filtered uranyl acetate for 15 minutes in the dark then rinsed 3 times by dipping
20 times for each wash in fresh sterile water. After the final rinse the grids were
placed at the bottom of a drop of lead citrate and stained for 15 seconds then rinsed
3 times as described above. The grids were air dried on filter paper then analyzed
on a JEOL transmission electron microscope.

7. SERUM STARVATION-STIMULATION. LNCaP cells were grown to
approximately 50% confluency on chambered slides as described above. The cells
were washed three times with PBS warmed to 37°, serum free RPMI 1640 media
was added to each well and the slides were incubated for an additional 48 hr. at
37°C. Following serum starvation, the serum free media was removed by
aspiration and replaced with RPMI 1640 media supplemented with 5% calf serum
and incubated at 37°C. Slides were removed at time points (0 min.; 15 min.; 30
min.; 60 min.; 2 hr.; 4 hr.; 6 hr.; 8 hr.; 10 hr.; 24 hr.; and 48 hr.) following serum
starvation and fixed for 20 min. in 10% buffered formalin and stored in PBS at 4°
C. After all of the time points were collected and fixed the cells were
permeabilized briefly in 0.1% Triton-X100 in PBS for 5 min. then washed twice
with PBS. Immunofluorescence staining was carried out as described above using MAb 7E11-C5.3 and an IgG1 isotype matched control antibody.

8. MITOCHONDRIAL PURIFICATION. Mitochondria were purified from LNCaP cells following a previously reported procedure (121). Six 162 cm² tissue culture flasks were seeded with LNCaP cells and grown to confluency. The cells were harvested then pelleted by centrifugation. The cell pellet was resuspended in 6 ml 1X Mitochondrial Isolation Buffer (MIB) (0.25 M sucrose, 40 mM Tris pH 7.0, 0.1 mM EDTA) then dounce homogenized on ice with 40 up and down strokes. The homogenized material was centrifuged in a Beckman JA20 rotor at 2,000 x g for 30 min. at 4°C the supernatant collected and centrifuged at 8,500 x g for 35 min. at 4° C. The supernatant from this step was discarded and the pellet resuspended in 6 ml 1X MIB and centrifuged again at 8,500 x g for 35 min. The supernatant was discarded and the pellet resuspended in 6 ml 1X MIB and dounce homogenized. This suspension represented the crude mitochondrial fraction. The crude mitochondrial fraction was applied to a two-step sucrose gradient composed of 10 ml of 25% sucrose in TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) and 13 ml of 42.5% sucrose in TE buffer and centrifuged at 26,000 x g for 75 min. at 4 °C. The percentage of sucrose in each solution was confirmed by a refractive index of 1.3775 and 1.4035 respectively. The mitochondria were collected at the interface of the two sucrose layers and diluted in 2 volumes TE buffer and applied to a second sucrose gradient purification. The mitochondria were again collected at the interface of the two sucrose layers, diluted in 2 volumes TE buffer then centrifuged at 22,000 x g for 20 min. at 4° C. The pellet from this step, representing the purified mitochondria was resuspended in 2 volumes PBS and stored at -20° C until needed.
9. MEMBRANE PREPARATIONS. LNCaP cells were harvested and pelleted by centrifugation at 1,000 x g. The pellet was washed once with ice cold phosphate buffered saline (136 mM NaCl, 1.7 mM KCl, 8 mM Na2HPO4, 1.5, mM KH2PO4, 0.9 mM CaCl2, 0.5 mM MgCl2, pH 7.4) and pelleted again. The pellet was resuspended in hypotonic buffer (1mM NaHCO3) containing a protease inhibitor cocktail (0.28 mM Antipain; 0.75mM Pepstatin; 60mM EDTA) and incubated on ice for 30 min. then dounce homogenized. The homogenate was centrifuged at 2,000 x g for 5 minutes in a Beckman JA20 rotor to pellet whole cells and nuclei. The supernatant was collected and centrifuged at 138,000 X g for 2 hours. The supernatant was discarded and the pellet, representing a crude membrane preparation, was resuspended in PBS and stored at -70° C.

10. PSMA AFFINITY COLUMN PURIFICATION. Membrane preparations were resuspended in solubilization buffer (30 mMTris pH 7.5, 0.5M NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 0.5 mM DTT) and protease inhibitor cocktail (as described above) and incubated for 2 hr. at 4° C. on a rotator. The solubilized material was centrifuged at 100,000 x g for 1 hr. at 4° C. to pellet non-solubilized material. The supernatant was collected, diluted 1:2 with 20 mM Tris pH 8.0 and loaded onto an Affinica Protein A-7E11-C5 affinity column constructed using the manufacturers instructions (Schleicher & Schuell, Keene, NH). The column was washed with wash buffer (20 mM Tris pH 8.0, 0.1% NP-40, 0.1 mM DTT) and eluted with 2N NH4OH, pH 11.0. The eluted fraction was placed in 3500 mw cut off dialysis tubing (Spectrum, Houston, TX) and dialyzed against 20 mM Tris HCL pH 4.0 containing 0.1 mM DTT for 2 hr. at 4° C. The eluant was then concentrated against polyethylene glycol compound (MW 15,000-20,000) to approximately 500 μL. Protein concentrations were estimated using the BCA protein assay following the manufacturers instructions (Pierce, Rockford IL).
11. SDS-PAGE PURIFICATION OF PSMA. Affinity column purified PSMA was loaded into the lanes of an SDS-PAGE mini-gel at 30 μg/lane. One lane of purified PSMA and one molecular weight marker lane were stained with coomasie blue. The remaining PSMA bands were excised using the stained lane as a guide and placed in dialysis tubing (12-14,000 mw cut-off; Spectrum, Houston, TX) containing 500 μl CAPS buffer (10 mM CAPS pH 11.0; 0.5 mM DTT) and electroeluted for 2 hr. at 12 mAmps in CAPS buffer. The acrylamide bands were removed from the dialysis tubing and the eluted protein was dialyzed against distilled deionized water for 2 hr. then removed from the dialysis tubing and dried in a Savant speed-vac concentrator.

12. PHYSICAL AND BIOCHEMICAL TREATMENT. Purified PSMA from crude LNCaP membrane preparations was boiled for 10 min. in the presence or absence of mercaptoethanol or SDS. Periodate oxidation (122) and sodium borohydride (123) treatments were carried out as previously described. Briefly, for the periodate oxidation, the antigen was incubated in 50 mM sodium acetate (pH 4.5) containing 5 mM sodium periodate for 1 hr. at room temperature in the dark. The reaction was quenched by the addition of 0.1 M glycine for 30 min. For the sodium borohydride treatments, the antigen was incubated in 0.1 M NaOH containing 2.0 M NaBH4 at room temperature for 16 hr. The reaction was quenched by the addition of 0.5 μl glacial acetic acid. The treated samples were then analyzed by RIA or western blot analysis. Proteolytic digestion was carried out by incubating the purified PSMA at 37°C for 24 hr. using 100 μl of the following protease solutions: trypsin type III (1, 10, and 100 U/ml); alpha chymotrypsin type VII (5, 50, and 500 mU/ml); Protease type XXI (2, 20, and 200 mU/ml); and Protease type XXVI (8, 80, and 800 mU/ml). The treated PSMA was then analyzed using a modified RIA assay or western blot analysis.
13. GLYCOSIDASE TREATMENT. Purified PSMA was treated with Beta-galactosidase, fucosidase, endo F, and chondroitinase ABC in Eppendorf tubes following methods previously described (19-21). For the N-glycanase digestion the antigen was boiled for 3 min. in the presence of 0.5% SDS and 0.1M mercaptoethanol then diluted in PBS containing 10 mM phenanthroline and NP-40. The N-glycanase (Genzyme, Boston, MA: 0.3 mU) was added and the reaction incubated overnight at 37°C. For the O-glycanase digestion, the antigen was first denatured in SDS and mercaptoethanol as for the N-glycanase treatment then digested with neuraminidase for 2 hr. O-glycanase (0.5 mU) was added and the reaction mixture incubated overnight at 37°C.

14. MODIFIED RADIOIMMUNOASSAY (RIA). The wells of a microtiter plate were rinsed with distilled water. The antigen was added at a concentration of 3 μg/well and dried overnight at 37°C then fixed with 0.1% gluteraldehyde for 5 min. at room temperature. The wells were rinsed twice with PBS and non-specific binding sites were blocked with 10% goat serum for 30 min. at room temperature. The blocking serum was removed by aspiration and 25 μl of the primary antibody was added to each well and incubated for 1 hr. at room temperature. Each well was rinsed 3 times with PBS followed by the addition of 100,000 counts/well of a ¹²⁵I radiolabelled goat anti mouse secondary antibody and incubated for 1 hr. at room temperature. The wells were rinsed 4 times and each well counted on a gamma counter and reported as counts per minute (CPM).

15. COMPETITIVE BINDING EXPERIMENTS. The carbohydrate concentrations were adjusted to 0.05 M with PBS. The 7E11-C5.3 MAb was incubated with the carbohydrate or PBS control for 2 hours at room temperature. Fifty μl of the mixture was then used as the primary antibody for an RIA using
purified PSMA from LNCaP membrane extract as the antigen. For lectin competitive binding studies, lectins were used at a concentration of 1 mg/ml in PBS. The lectins or PBS control were added to antigen coated wells in a volume of 100 μl and incubated for 2 hours at room temperature. The wells were washed 3 times with PBS and an RIA assay was performed as described above. Data for both the carbohydrate and lectin experiments were expressed as a percentage of the control binding using the following formula:

\[
\frac{\text{CPM of treated well}}{\text{CPM of PBS control well}} \times 100\%
\]

16. TUNICAMYCIN TREATMENT. LNCaP cells were cultured in the presence of tunicamycin for 7 days as previously described (124) at Cytogen Corporation (Princeton, NJ.) and supplied for this study. The cells were harvested and membrane preparations were prepared as described above.

17. PEPTIDE SYNTHESIS. Peptides were synthesized on a Synergy Peptide Synthesizer using F-MOC chemistry (Perkin Elmer-Applied Biosystems, Foster City, CA). Following synthesis the peptides were cleaved from the column matrix by removing the entire resin from the column into a 50 ml polypropylene centrifuge tube then 50 μl thioanisole, 50 μl 1,2, ethanedithiol (EDT), and 900 μl trifluoroacetic acid (TFA) were added in a fume hood. This slurry was incubated at room temperature with shaking for one hour. Following cleavage, the peptides were extracted three times in 15 mls of t-butyl methyl ether (MTBE) decanting the ether from the peptide-resin pellet after centrifugation at 1,000 RPM for 5 min. Following the final extraction the peptide-resin pellet was resuspended in 10 ml sterile water or 30% acetonitrile in water for hydrophobic peptides then passed.
through a pasteur pipette in which a plug of glass wool had been inserted. This procedure separated the extracted peptide from the synthesis resin. The peptide was aliquoted into eppendorf tubes and dried in a Savant speed-vac overnight. The peptides were stored in the dried form at -20° C. until needed then resuspended in sterile water for use.

18. HPLC ANALYSIS OF SYNTHETIC PEPTIDES. Synthetic peptides were separated on a C18 reversed phase HPLC column (Waters Delta-Pak C18; 300A, 5 μm, 3.9x150 mm) using a Waters 650 HPLC system to test for purity. The column was equilibrated for 10 column volumes of 90% buffer A (HPLC water, 0.1% TFA) and 10% buffer B (80% acetonitrile in HPLC water, 0.09% TFA) and the baseline monitored at a wavelength of 214 nm at a flow rate of 0.2 ml/min. The percentage of TFA was titrated in both buffers such that they gave a constant baseline at a wavelength of 214 nm. A small aliquot of the peptide was diluted in 20 μl HPLC water and injected onto the column. The column was rinsed of unbound material for 5 min. at the initial buffer conditions then the percentage of buffer B was increased to 40% in a linear manner over a 50 min. time span followed by an increase to 90% buffer B in 5 min. to remove any tightly bound material then to 10% over a 10 min. period. Peptides were judged to be pure if > 80% of the peptide was contained in a single peak.

19. DIRECT BINDING PEPTIDE ASSAY. Peptides were bound to wells of microtiter plates using the procedure previously described (125) with several modifications. Briefly, 100μl of BSA (1 μg/ml) was dispensed into each well of a high binding EIA plate (Costar, Cambridge, MA) and dried overnight. The plates were rinsed twice with PBS followed by two rinses with distilled water. Various concentrations of peptides were added to the wells in a volume of 50 μl followed
by 50 μl of EDAC crosslinker (10 mg/ml) or water control and the plates incubated overnight at 4°C with shaking. The plates were washed 5 times with wash buffer (PBS containing 0.05% Tween-20). The concentration of peptides adhered to the plate was determined by protein assay of the peptide-EDAC mixture prior to and following the crosslinking incubations. A standard ELISA assay was then performed (as described below).

20. COMPETITIVE BINDING PEPTIDE ASSAY. Competition plates were made by first dispensing 100 μl BSA (1 μg/ml) into the wells of a high binding EIA plate and dried overnight. The plate was rinsed twice with PBS and twice with distilled water prior to the addition of peptides. Serial 1:5 dilutions of each peptide were made with water starting at a concentration of 1mM with a final volume of 40 μl in each well. The antibody concentration utilized was determined by finding the concentration of MAb 7E11-C5.3 which gave half maximal binding to a constant concentration of purified PSMA antigen at 200 ng per well determined to be 1 μg/ml. Forty μl of MAb 7E11-C5.3 (1 μg/ml) was added to each well and the plate was incubated at 4°C overnight with shaking.

Antigen plates were prepared by dispensing 100μl of purified PSMA (2.0 μg/ml) into the wells of a high binding EIA plate (Costar, Cambridge, MA) and incubating overnight at 4°C with shaking. The antigen plates were washed 4 times with wash buffer and blocked with blocking buffer (1% BSA in PBS containing 0.05% Tween-20) for 1 hr. at room temperature. The blocking buffer was removed and the 80 μl of the peptide-antibody mixture from the inhibition plates prepared above were transferred to the antigen plates and utilized as the primary antibody for a standard ELISA assay.
21. **STANDARD ELISA ASSAY.** Microtiter plates, activated with peptide or antigen, were blocked for 1 hr. at room temperature with blocking buffer (1% BSA in PBS containing 0.05% Tween-20) and shaking. The blocking buffer was removed and MAb 7E11-C5.3 or competition mix (as described above) was added to each well and incubated for 2 hr. at room temperature with shaking. The plates were washed 5 times in wash buffer with the final wash being removed by vacuum aspiration to ensure all of the wash buffer was removed. The secondary antibody (1 μg/ml, horseradish peroxidase labeled, horse anti-mouse antibody, Vector laboratories, Burlingame, Ca) was added at 50 μl per well and incubated for 2 hr. at room temperature with shaking. The plates were washed 5 times with wash buffer and all of the buffer was removed with the final wash. Following the final wash, 200 μl of OPD substrate was added to each well and incubated for 5 to 30 min. with shaking and read at 405 nm on an EL-340 Microplate reader (Bio-Tek Instruments, Winooski, VT).

22. **DETERMINATION OF AFFINITY CONSTANT (K_a).** The affinity of MAb 7E11-C5.3 for the native antigen and peptides was determined by a modification of a method described previously (126). MAb binding was plotted as bound/free antibody as a function of the concentration of bound antibody (M). The regression coefficient (K_a) was calculated according to the method of Scatchard (127) and student’s t test was applied to determine the significance of the results.

23. **PSMA ELISA ASSAY.** The PSMA ELISA assay was performed by incubating 100 μl each sample (diluted to 20 μg/ml) per well of high binding-flat bottom ELISA plates (Costar Corporation) overnight at 4° C with shaking. The plates were washed with wash buffer (PBS containing 0.05% Tween-20) three
times followed by two washes with distilled water. Nonspecific binding sites in the wells were blocked by incubating 100 μl blocking buffer (PBS containing 2% BSA and 0.05% Tween-20) in each well for two hours at room temperature with shaking. The blocking buffer was aspirated from the wells and the primary antibody added directly without washing in a volume of 100 μl per well and incubated at room temperature for 2 hr. The plates were washed as above and 100 μl of the secondary antibody (Vector Laboratories, horse α mouse immunoglobulin-horseradish peroxidase conjugate: 1:5000) was added and incubated for 2 hr. at room temperature followed by washing as above. The color substrate (o-phenylenediamine dihydrochloride, Sigma Chemical Company) was added at 200 μl per well and the absorbance read on a micro-plate reader (Bio-Tek Instruments) at 405 nm at intervals of 5, 10, 15, and 30 minutes.

24. WESTERN BLOT ANALYSIS. Western blot analysis for PSMA was carried out on the individual samples by loading equal protein concentrations into the lanes of 4-20% gradient SDS-PAGE gels and running under reducing and denaturing conditions (128) then electrophoretically blotted to Immobilon-P membranes (Millipore, Bedford, MA). The membranes were incubated for 1 hr. at 37°C in blocking buffer (5X Denhardt’s buffer, 1X BBS, 0.1% NP-40, 1.5% BSA). The blocking buffer was removed and the membranes incubated with MAb 7E11-C5.3 (20μg/ml) for 1 hr. at room temperature. The primary antibody was removed and the membranes washed for 10 min. x 3 with wash buffer (30 mMTris pH 7.5, 0.5M NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 0.5 mM DTT) followed by the secondary antibody incubation (horse anti-mouse horseradish peroxidase labeled secondary antibody diluted in blocking buffer at a dilution of 1:10,000; Vector Laboratories, Burlingame, CA.) for 1 hr. at room temperature. The secondary antibody was removed and the membranes were washed for 10 min. x 3 in wash
buffer. The blots were developed using the ECL method (Amersham Life Sciences, Arlington Heights, IL) according to the manufacturers instructions and exposed to X-ray film. Apparent molecular weights were calculated on multiple blots using known molecular weight markers.

25. COMPETITIVE INHIBITION WESTERN BLOT ANALYSIS. The competitive blocking studies were carried out identically to the Western blot experiments described above except the PSMA active peptide N1.19 was added in a 20 fold molar excess over the MAb 7E11-C5.3 during the primary antibody incubation.

26. TWO DIMENSIONAL (2-D) GEL ELECTROPHORESIS. 2-D experiments were done using the Investigator 2-D Electrophoresis System (Millipore, Marlborough, MA) following the manufacturers directions. Briefly, 10 μg of affinity purified antigen was loaded on top of a preparative isoelectric focusing tube gel and overlaid with sample overlay buffer. The tubes were electrophoresed for 17 hours at 1000 volts followed by 15 min. at 1500 volts. The gels were extruded onto pre-cast 4-20% gradient SDS-PAGE gels and layered with a 1% agarose sticker containing 0.1% Bromphenol blue. The second dimension was run at 16 mAmps overnight. The gel was removed from the apparatus and blotted to Immobilon-P (Millipore, Bedford, MA) for Western blot analysis. Isoelectric points were estimated by determining the pH gradient of a tube gel, run at the same time as the sample, which contained only ampholytes. The analytical tube gel was cut into 1 cm sections, incubated in 3 ml double distilled water for 2 hr. followed by pH measurement.
27. ANALYSIS OF PSMA IN SERUM. Serum samples from informed and consented normal donors were collected. Serum from patients with BPH or CaP were obtained from the tissue bank of the Virginia Prostate Center, Eastern Virginia Medical School. Four separate pools of serum were prepared using samples from 5 non-pregnant normal females, 5 normal males under the age of forty, 5 BPH patients, and 5 patients with stage D2 CaP were pooled. Twenty µg of affinity purified PSMA was used to spike 500 µl of the stage D2 pooled serum and incubated for 12 h at 37°C. An additional 500 µl aliquot of the same Stage D2 pooled serum representing 29.09 mg of total protein was incubated in solubilization buffer (as described above) for 2 h at 4°C and applied to a 7E11-C5.3 affinity column and eluted and prepared as described above for PSMA purification. Twenty five µg of membrane preparations from LNCaP cells and normal prostate tissue; 100 µg of a normal seminal plasma; 400 µg of the spiked D2 serum; 20 µg of affinity purified stage D2 serum; and 400 µg of non-spiked D2 serum, BPH serum, normal male serum, and normal female serum were loaded into the lanes of two identical 4-20% SDS-PAGE gels, electrophoresed, transferred to Immobilon-P membranes and developed for Western blot analysis as described above with either 7E11-C5.3 or an isotype matched (IgG1) control antibody.

28. ISOLATION OF GENOMIC DNA FROM LNCAP CELLS. Three 162 cm² tissue culture flasks were seeded with LNCaP cells and grown to confluency. The cells were washed twice with TBS (30 mM Tris, 150 mM NaCl pH 7.3) then removed by scraping using a rubber policeman into a volume of 0.5 ml TBS and collected in ependorf microcentrifuge tubes. The cells were pelleted at 2000 RPM in a benchtop microcentrifuge for 10 min. at 4°C, resuspended in 1 ml ice cold TBS and pelleted again. The pellet was resuspended in 4 ml TE buffer (pH 8.0)
followed by 40 ml extraction buffer (10 mM Tris pH 8.0, 0.1 M EDTA, 20 μg/ml RNAse A, 0.5% SDS) and incubated at 37°C. for 1 hr. Proteinase K was added to a final concentration of 100 μg/ml and incubated at 50°C for 3 hr. then cooled to room temperature. An equal volume of TE saturated phenol was added to the tube, mixed gently, then centrifuged at 5000 x g for 15 min. at room temperature. The aqueous phase was removed and extracted two more times with phenol. Following the third phenol extraction, 0.2 volumes of 10 M ammonium acetate was added followed by 2 volumes of ethanol at room temperature. The DNA was pelleted at 5000 x g for 5 min. at room temperature, washed with 70% ethanol, air dried then resuspended in sterile water.

29. CLONING OF THE PSA PROMOTER. All plasmid constructs were prepared using standard methods (129). The 5' flanking DNA fragment of the PSA gene was obtained by PCR amplification of 100 ng of genomic DNA from the LNCaP cell line using oligonucleotide primers derived from the published sequence of the PSA gene (130-132). The sequences for these primers were: PSA1 5'-CATTGTTTGCTGCTGCACGTTGGAT-3' and PSA2 5'-TCCGGGTGCAGGTGGTAAGCTTG-3'. The oligonucleotides were synthesized in the Core Molecular Biology laboratory at Eastern Virginia Medical School and purified by HPLC chromatography as described below. The PCR reaction mixture (with final concentrations of 200 μM dNTP; 500nM primers; 100 ng LNCaP genomic DNA; 2 mM MgCl₂; 1X PCR reaction buffer) was prepared on ice in a final volume of 99 μl without Taq polymerase, overlayed with 50 μl mineral oil then placed into the wells of a thermo cycler (Perkin Elmer). The PCR reaction conditions for the amplification of the PSA promoter were as follows: 5 min. initial denaturation at 94°C; 1 μl Taq polymerase (2.5 U/μl) was added to each reaction mixture following the denaturation and gently mixed; 30 cycles of: 45
sec denaturation at 94° C; 1 min. annealing at 61° C; and 1 min. extension at 72° C; a final extension of 6 min. at 72° C was carried out following cycling to ensure that all products were full length and the PCR fragments were purified by HPLC chromatography as described below. The peaks were collected by hand and the DNA precipitated with 2 volumes ice cold ethanol at -70 C for 1 hr, centrifuged at 14,000 RPM at 4 C then washed with 70% ice cold ethanol then resuspended in sterile water. The purified PCR products were subcloned into the pGemT vector (Promega) following the manufactures protocol. Positive clones were sequenced by double stranded automated DNA sequencing using the dideoxynucleotide chain termination method to verify the cloning of the proper DNA fragment.

30. HPLC PURIFICATION OF SYNTHETIC OLIGONUCLEOTIDES. The synthetic oligonucleotides were cleaved from the synthetic resin in 1.5 ml ammonium hydroxide at room temperature for 1 hr then placed in a screw top tube at 55° C for 5 hr. The cleaved oligonucleotides were dried in a Savant speed-vac overnight and stored at -20° C. until needed. A C18 reversed phase column (Waters Delta-Pak C18; 300A, 5μm, 3.9x150 mm) was equilibrated with 100% buffer A (100 mM Triethylammonium Acetate pH 7.0) and 0% buffer B (100 mM Triethylammonium Acetate pH 7.0, 70% acetonitrile) at 0.8 ml/min. and the baseline monitored at a wavelength of 260 nm. The oligonucleotides were resuspended in 40 μl HPLC grade water then loaded onto the column. A gradient was run from 0% buffer B to 40% buffer B over 40 min. ramped to 100% Buffer B in 5 min. then to 0% buffer B in 5 min. The predominant peak was collected by hand and dried in a Savant speed-vac and stored at -20° C until needed.
31. DNA SEQUENCE ANALYSIS. The DNA dideoxy chain termination sequencing analysis was performed using an ALF DNA Sequencer (Pharmacia, Upppsala, Sweden) using the manufacturers procedures. In brief, 5 \( \mu \)g DNA was denatured with 8 \( \mu l \) 2N NaOH in a final volume of 40 \( \mu l \) at room temperature.

7 \( \mu l \) 3M sodium acetate, 4 \( \mu l \) water and 120 \( \mu l \) cold 100% ethanol were added then incubated in a dry ice/ethanol bath for 15 min. The DNA was precipitated by centrifugation at 14,000 RPM for 15 min. at 4°C then washed with 70% cold ethanol. The pellets were air dried then resuspended in 10 \( \mu l \) water. 10 pmoles of fluorescein labeled primer (T7 Universal or Reverse primers) and 2 \( \mu l \) annealing buffer were added to the DNA and incubated at 65°C for 5 min., moved to 37°C for 10 min. then room temperature for 10 min. 3 \( \mu l \) dimethylsulfoxide (DMSO), 1 \( \mu l \) extension buffer, and 2 \( \mu l \) T7 DNA polymerase (4U/\( \mu l \)) was added to the DNA mixture and 4.5 \( \mu l \) of this solution was added to 2.5 \( \mu l \) of A,C,G,T mixes prewarmed to 37°C and incubated at 37°C for 5 min. The reactions were stopped by adding 5 \( \mu l \) stop buffer and samples stored at -20°C until needed. 6 \( \mu l \) of each sample was separated on a denaturing polyacrylamide gel and analyzed by fluorescence analysis.

32. CLONING OF THE PSA PROMOTER INTO A BASIC CAT VECTOR.

A pGemT clone which contained the PSA promoter (PSA3) was digested with Sph I and Pst I at 37°C for 3 hr. The restriction fragments were purified by anion exchange chromatography and the peak containing the 600 bp promoter fragment was precipitated and air dried. The pellet was resuspended in sterile water and stored at -20°C until needed. The PSA promoter fragment was directionally cloned into the Pst I and Sph I sites of the pCAT basic vector (Promega) which contains no promoter or enhancer sequences.
33. CLONING OF THE PSMA CDNA. A DNA probe for the PSMA cDNA was obtained by PCR amplification of a single stranded cDNA population synthesized from LNCaP poly-a RNA. The sequences for these primers were: 5'-ATGTTGCCTCTCTCTCTCGC TCGG-3' and 5'-TTGCCTTCAAAGCCTTC ATCAGGG-3'. These primers amplify an approximately 1,700 bp fragment of the PSMA cDNA between +37 and +1733 of the published cDNA sequence. The PCR amplification of the PSMA cDNA fragment was carried as described for the PSA promoter except the annealing temperature was 50°C. The PCR fragments were purified and cloned into the pGemT vector as described above and positive clones were verified by sequence analysis. The insert from one clone was purified and labeled with digoxygenin (Boehringer Mannheim, Mannheim, Germany) following the manufactures protocol. This labeled probe was used to screen a lambda phage cDNA library made from the LNCaP cell line.

34. LIBRARY SCREENING. Lambda phage were plated on LB agar plates at a density of 10,000 PFU/plate and grown overnight at 37°C or until near confluency. The plates were incubated at 4°C for at least 1 hour or overnight to cool the agar. Nylon membranes were numbered and placed on top of the plates for 2 min. and holes were poked through the membrane and agar in an asymmetric fashion with a needle. The membranes were removed from the plates and immediately denatured plaque side up on filter paper saturated with denaturing buffer (1.5 M NaCl, 0.5 M NaOH) for 2 min. then transferred to neutralization buffer (1.5 M NaCl, 1M Tris pH 8.0) for 5 min. followed by rinse solution (0.2 M Tris pH 7.5, 0.3 M NaCl, 30 mM sodium citrate) for 30 sec. The membranes were dried on 3mm paper plaque side up, UV crosslinked at 150 mJoule and stored dry until needed. The membranes were prehybridized in a shaking water bath at 65°C in prehybridization buffer (5X SSC, 1% blocking buffer, 0.1% N-lauroylsarcosine, 0.02% SDS) for 2
hr. The prehybridization buffer was removed and the diluted and boiled probed (15 ng/ml) was added and incubated overnight in a shaking water bath at 65°C. Following the overnight incubation the hybridization solution was removed and saved for reuse at a later time. The membranes were washed twice for 5 min. in 2X wash buffer (2X SSC containing 0.1% SDS) at room temperature with shaking then twice for 15 min. in 0.5X wash buffer (0.5X SSC containing 0.1% SDS) at 65°C.

The membranes were incubated in blocking buffer (2% casein, 100 mM Tris, 150 mM NaCl pH 7.5) for 30 min. at room temperature then an α digoxigenin antibody labeled with alkaline phosphatase was added at a dilution of 1:5,000 and incubated for 1 hr. The membranes were washed 3 times for 10 min. each in wash buffer (100 mM Tris, 150 mM NaCl pH 7.5) then the alkaline phosphatase enzyme was activated with activation buffer (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM MgCl₂) for 2 min. The activation buffer was removed and 2 ml of color substrate (3.37 mg/ml nitroblue tetrazolium salt, 1.75 mg/ml 5-bromo-4-chloro-3-indolyl phosphate) was added to each membrane and incubated in the dark for 5-30 min. Positive plaques were isolated by taking a core of the agar plate and isolating lambda phage in SM buffer containing 1 drop of chloroform. A secondary and tertiary screening was carried out on these isolated phage to ensure that a homogeneous lambda phage population was isolated.

35. CLONING OF THE PSMA 5' FLANKING DNA. A restriction fragment from the 5' end of the PSMA cDNA (700 bp) was isolated by digesting the PSMA1 cDNA clone with Hpa I followed by gel purification of the 750 bp fragment. The 750 bp fragment was labeled with digoxigenin as described above and used to screen a human genomic library made from a Sau II partial digestion of placental genomic DNA (Clontech Laboratories Inc., Palo Alto, CA). The
isolated genomic clones were characterized by restriction mapping and southern blot analysis using both the original PSMA probe or the oligonucliotide primer which corresponds to the very 5' end of the cDNA. By following this method, clones which had a high probability of containing the 5' flanking regions were identified. Fragments which hybridized to the probe were subcloned into a pGem7Z+ vector (Promega) and sequenced by the dideoxy-chain termination method.

36. PREPARATION OF COMPETENT CELLS FOR ELECTROPORATION. A glycerol stock culture of the E. coli strain JM109 was inoculated into 5 ml LB broth and grown overnight at 37° C. The overnight culture was added to 1 L of fresh LB broth and incubated until the OD_{600} was between 0.5 and 1.0. The cells were chilled on ice for 30 min. then transferred to four sterile 250 ml centrifuge bottles and centrifuged at 3,000 RPM at 4° C for 20 min. The supernatant was decanted and the pellets were resuspended in 10 ml cold sterile water then diluted to 250 ml and centrifuged again at 3,000 RPM at 4° C for 20 min. The supernatant was decanted and the cells were resuspended as above then 2 bottles were combined, diluted to 250 ml and centrifuged again. Following this final centrifugation the pellets were resuspended in 10 ml cold sterile 10% glycerol in a 50 ml centrifuge tube and centrifuged again. The pellet was resuspended in 3 ml cold sterile 10% glycerol and snap frozen in 80 µl aliquots in a dry ice/ethanol bath and stored at -70° C.

37. SOUTHERN BLOT ANALYSIS. The DNA was electrophoresed in a 0.8% agarose gel in TBE buffer and visualized by ethidium bromide staining. The gel was rinsed in water for 15 min. at room temperature with shaking then soaked in 0.25M HCL for 15 min. at room temperature followed by 60 min. in denaturing
buffer (1.5 M NaCl, 0.5 M NaOH) then 60 min. in neutralizing buffer (1 M Tris pH 8.0; 1.5 M NaCl). The gel was rinsed in 20X SSC (3 M NaCl, 300 mM sodium citrate, pH 7.0) and transferred using 20 X SSC overnight to positively charged nitrocellulose membranes under the pressure of weights. Following Southern transfer, the membranes were air dried and the DNA fixed by UV crosslinking at 150 mJoule and stored in air tight containers at room temperature until needed.

38. HYBRIDIZATION OF SOUTHERN TRANSFERS. Southern transfer membranes were prehybridized in a shaking water bath at 65°C in prehybridization buffer (5X SSC, 1% blocking buffer, 0.1% N-lauroylsarcosine, 0.02% SDS) for 2 hr. The prehybridization buffer was removed and the diluted and boiled probe (10 ng/ml) added then incubated in a shaking water bath at 65°C overnight. Following hybridization, the hybridization solution was removed and the membranes were washed twice for 5 min. each in 2X wash buffer (2X SSC containing 0.1% SDS) at room temperature with shaking then twice for 15 min. each in 0.5X wash buffer (0.5X SSC containing 0.1% SDS) at 65°C.

The membranes were incubated in blocking buffer (2% casein, 100 mM Tris, 150 mM NaCl pH 7.5) for 30 min. at room temperature then an αdigoxygenin antibody labeled with alkaline phosphatase was added at a dilution of 1:5,000 and incubated for 1 hr. The membranes were washed for 10 min. 3 times in wash buffer (100 mM Tris, 150 mM NaCl pH 7.5) then the alkaline phosphatase enzyme was activated with activation buffer (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM MgCl2) for 2 min. The activation buffer was removed and approximately 2 ml of lumiphos reagent (Boeringer Mannheim) was added to the membranes for 1 min. The membranes were placed between two sheets of acetate film and loaded into an X-Ray film cassette. X-Ray film was placed on the membrane in a dark room and exposed for various lengths of time then developed in an automatic film developer.
39. STRIPPING OF STEROID HORMONES FROM CALF SERUM.
Steroid hormones were removed from fetal calf serum utilizing previously published methods (133,134). Briefly, the serum used to supplement the media was stripped by incubation with activated charcoal and dextran at 55°C for one hour with stirring. This stripping was repeated several times to ensure the removal of the steroid hormones. The serum was filter sterilized and hormone levels assayed at the endocrinology laboratory located in the Howard and Georgianna Jones Institute for Reproductive Medicine

40. TRANSIENT TRANSFECTIONS. The method utilized here is an adaptation of methods previously reported (135,136). Either the parental PPC-1 cells or the PPC-1AR5 cell line containing the androgen receptor expression plasmid were seeded into 100 mm plates and grown for 48 hrs in RPMI 1640 supplemented with 5% calf serum. The media was removed by aspiration and the cells were washed twice with Tris buffered saline (TBS) (30 mM Tris, 150 mM NaCl pH 7.3) warmed to 37° C. For each transfection to be performed, 5 μg of the promoter-CAT construct DNA was mixed with 1 ml TBS and DEAE dextran (50 μg/ml). One ml of this mixture was added to the plate and incubated for 30 min. at 37° C with occasional rocking then overlayed with 10 ml RPMI 1640 containing 100 μm chloroquine phosphate and incubated for 3 hr at 37° C. The media was removed by aspiration and the plates washed twice with 5 ml Tris buffered saline at 37° C then overlayed with RPMI 1640 supplemented with 5% stripped serum or 5% stripped serum plus 1 nM methyltrienalone (R1881). After 48 Hr, the cells were collected by scraping with a rubber policeman pelleted by centrifugation and stored at -70° C until needed. All treatments were prepared in triplicate.
41. RNA EXTRACTION. Cells collected following transient transfections were lysed in 2 ml lysis buffer (10 mM EDTA pH 8.0, 0.5% SDS) then extracted with 4 ml phenol and centrifuged at 5,000 RPM for 10 min. at 4°C. The aqueous phase was transferred to a fresh tube containing 440 µl cold 30 mM Tris-Cl (pH 8.0) and 180 µl 5M NaCl. Two volumes of ice cold ethanol was added and incubated at -70°C for 1 hr. then centrifuged at 5,000 RPM for 10 min. at 4°C and air dried. The RNA was redissolved in 200 µl ice cold TE buffer and transferred to a microcentrifuge tube and precipitated with 4 µl 5M NaCl and 500 µl ice cold ethanol followed by centrifugation at 12,000 x g for 5 min. at 4°C in a microcentrifuge. The ethanol was removed, the pellets air dried then resuspended in 30 µl sterile water.

42. CHLORAMPHENICOL ACETYLCAPRASE (CAT) RNASE PROTECTION ASSAY. A high specific activity ribonuclease probe for the CAT gene was made by *in vitro* transcription of a linearized CAT plasmid using the MAXIscript kit (Ambion Inc., Austin, TX) in the presence of [α-32P] UTP, 800 Ci/mmol followed by gel purification to eliminate prematurely terminated transcription products. For each sample, 1 X 10^5 cpm of the labeled probe was added to 50 µg of total RNA and ethanol precipitated with 2 volumes ice cold ethanol and ammonium hydroxide to 0.5 M for 15 min. at -70°C. The RNA was pelleted at 14,000 RPM at 4°C and resuspended in hybridization buffer and vortexed. The tubes were incubated at 90°C for 4 min. then vortexed and centrifuged briefly and hybridized overnight at 45°C. RNase digestion buffer was then added to the tubes and incubated at 37°C for 30 min. then precipitated for 1 hr at -70°C, pelleted and stored at -20°C until needed. The digested fragments were boiled for 4 min. then separated on a denaturing polyacrylamide gel and visualized with a Phospholmager (Molecular Dynamics).
CHAPTER III
RESULTS

A. DETERMINATION OF THE SUBCELLULAR LOCALIZATION OF
THE PROSTATE SPECIFIC MEMBRANE ANTIGEN

1. LNCaP CELLULAR SUBFRACTIONATION. One method of
determining the relative subcellular localization of a molecule of interest is to
separate cells into different components and analyze the fractions. In the present
study, a subcellular subfractionation was carried out on cultured LNCaP cells to
give a rough estimate of the localization of PSMA. Utilizing a series of linear and
sucrose gradient centrifugation steps, LNCaP cells were separated into four
fractions including the nuclear fraction (N); the heavy membrane fraction (HM)
containing predominantly the rough endoplasmic reticulum and mitochondria; the
light membrane fraction (LM) composed of the plasma membrane, smooth
endoplasmic reticulum, and small vesicular membranes; and the cytoplasmic
fraction (C) containing the soluble cytoplasmic components. To ensure that this
mechanical fractionation efficiently separated the cellular components, a panel of
control antibodies was utilized to monitor the efficiency of the fractionation.
Figure 2 demonstrates that the mitochondrial HSP70 heat shock protein
(recognized by MAb MU213) was segregated into the HM fraction while the
transferrin receptor was only detected in the LM component. The prostate specific
antigen (PSA) was detected in the LM fraction with a small amount being detected in the nuclear fraction and the proliferative cell nuclear antigen (PCNA) was detected only in the nucleus. None of the markers utilized were found in the cytoplasmic fraction. These data suggest that the fractionation was efficient except for some contamination of the nuclear fraction which has been reported by many investigators.

The PSMA glycoprotein was detected in both the HM and the LM fractions (Fig. 2) indicating that two pools of this molecule exist in LNCaP cells. A small amount of reactivity was also seen in the nuclear fraction, but this may be contamination. Western blot analysis of these fractions using MAb 7E11-C5.3 was also carried out to verify the results garnered from the RIA assay as well as to determine if the PSMA found in the LM and HM fractions was similar in molecular size. Figure 3 shows that the majority of the PSMA was found in the HM and LM fractions by Western blot analysis and that the PSMA in both fractions was at approximately 100 kDa; however, the HM fraction also contained a higher molecular weight band which was barely detectable in the LM fraction.

2. DETERMINATION OF THE LOCALIZATION OF PSMA BY LIGHT MICROSCOPY. In order to determine if the MAb 7E11-C5.3 antigenic epitope on PSMA was intracellular or extracellular, LNCaP cells were grown on tissue culture slides then stained without fixation or permeabilization prior to immunofluorescence staining with MAb 7E11-C5.3 or a control antibody (OKT-9) specific for the extracellular domain of the transferrin receptor. The hypothesis for this experiment was that monoclonal antibodies would not be able to cross a living cell membrane. As a result, if the epitope was localized inside the cell there would be no staining, whereas an extracellular epitope would result in a ringed staining around the circumference of the cell. The transferrin receptor antibody was able to
FIGURE 2. Control RIA assay to determine the efficiency of the cellular subfractionation. The transferrin receptor (OKT-9) was detected predominantly in the light membrane (LM) fraction, PCNA was detected only in the nuclear fraction (N), the mitochondrial Hsp70 (MU213) was detected in the heavy membrane fraction (HM), and PSA was detected in the LM and the N fractions. PSMA was detected in both the HM and LM fractions indicating that two pools of this molecule exist in LNCaP cells. The data represents the average of 3 independent experiments ± SE.
FIGURE 3. Western blot analysis of cellular fractions. The majority of the PSMA was found in the light membrane (LM) and heavy membrane (HM) fractions with very little in the nuclear (N) and cytoplasmic (C) fractions.
bind its epitope on the extracellular face of the LNCaP cells resulting in a ring of staining around the cells (Fig. 4 panel A) as expected for an extracellular epitope. On the other hand, MAb 7E11-C5.3 was unable to bind to its epitope (Fig. 4 panel B) indicating its epitope was intracellular.

To more definitively determine the localization of PSMA within LNCaP, cells were grown on tissue culture slides to approximately 50% confluency then fixed with buffered formalin and permeabilized briefly in a weak detergent solution prior to staining. This permeabilization step was carried out to assure the antibody probes could cross the plasma membrane and stain any intracellular antigens. The fixed and permeabilized LNCaP cells were stained with antibodies specific for PSA (PSA-5), the transferrin receptor (OKT-9), or an isotype matched (IgG1) control antibody which does not react with human prostate cells as controls for intracellular, plasma membrane, and negative staining respectively. The FITC labeled secondary antibody utilized in this study was diluted in a buffer containing Evan’s blue which acts as a counter stain and allows the entire cell to be visualized under ultraviolet light by a red fluorescence. The isotype matched control antibody did not stain the LNCaP cells (Fig. 5, panel A) and all that was visible was the red counter staining of the Evan’s blue. Note the ability to differentiate the nucleus from the cytoplasm of the cells by the red fluorescence of the Evan’s blue. Staining with PSA-5 showed a remarkable vesicular staining of the LNCaP cells (Fig. 5, panel B) suggesting a localization in secretory vesicles. As expected, the transferrin receptor appeared to be predominantly localized to the plasma membrane (Fig. 5, panel C) as demonstrated by the circumscription of the cell by the yellow fluorescence. The staining for PSMA with MAb 7E11-C5.3 however, appeared to be cytoplasmic and not localized at the plasma membrane or the nucleus (Fig. 5, panel D). There was no circumscription of the cells as seen with the transferrin receptor and no vesicular staining similar to PSA.
FIGURE 4. LNCaP cells were grown on tissue culture slides to approximately 50% confluency. The slides were rinsed with PBS and stained for immunofluorescent microscopy without fixation or permeabilization. (A) Positive staining of LNCaP cells with the OKT-9 antibody specific for an extracellular domain on the transferrin receptor. (B) LNCaP cells stained with MAb 7E11-C5.3 with no positive signal indicating that the MAb 7E11-C5.3 epitope is intracellular.
FIGURE 5. Light microscopy of fixed LNCaP cells grown on cultured slides. The FITC labelled secondary antibody was diluted in Evans' blue which results in a red fluorescent counter staining of the cells. The cells were stained with an IgG1 isotype matched control (A), PSA-5 (B), OKT-9 specific for the transferrin receptor (C), and 7E11-C5.3. The isotype matched control was negative while PSA-5 gave a very vesicular staining. OKT-9 staining was typical of membrane proteins in that the staining was in a form of a ring around the cells. 7E11-C5.3 staining appeared to be predominantly cytoplasmic and not localized to the nucleus or solely to the plasma membrane.
A similar set of experiments was carried out using immunoperoxidase staining methods and visible light microscopy with identical results. The staining pattern for PSMA was observed to be cytoplasmic at low power (fig 6, panel A) and was more evident at 100 X (Fig. 6, panel B).

3. IMMUNOELECTRON MICROSCOPY OF LNCaP CELLS. A series of immunoelectron microscopy studies was then performed to definitively determine the intracellular localization of PSMA within the cytoplasm of LNCaP cells. The first set of experiments utilized a chromagen (DAB) to visualize PSMA staining. This method was similar to the standard immunoperoxidase staining shown above except it was done at the electron microscopic level where the deposits of DAB were visualized as electron dense particles. Figure 7 shows the positive staining of MAb 7E11-C5.3 at the cytoplasmic face of the cell membrane (CM) and microvilli (MV) (panels B and C) and within several mitochondria (M) (panel B and E). The isotype matched negative control antibody exhibited no staining at the cell membrane or mitochondria (Fig. 7, panels A and D).

The DAB staining methods can be relatively non-specific at the electron microscopy level. Therefore, to more precisely demonstrate the localization of PSMA, a secondary monoclonal antibody directly labeled with a 10 nm gold bead was used in place of chromagen staining. Immunogold staining with MAb 7E11-C5.3 also resulted in positive staining with a concentration of gold beads at the cytoplasmic face of the CM (see arrows) and near a gap junction (GJ) between two LNCaP cells (Fig. 8, panel A) and concentrated in and around mitochondria (panel B).
FIGURE 6. LNCaP cells were grown in a monolayer on tissue culture slides. The cells were fixed and stained with 7E11-C5.3 using a horseradish peroxidase method and visualized with DAB and counter stained with Mayer's hematoxalin. Positive cytoplasmic staining is evident at 40X (A) and is obvious at 100X (B).
FIGURE 7. Immunoelectronmicroscopy utilizing DAB staining.
(A) The IgG1 control showed negative staining at the cell membrane at 36,000 X. (B) Arrows pointing to positive 7E11-C5.3 staining at the cell membrane (CM) and within mitochondria (M) at 28,000 X. (C) Arrows pointing to positive 7E11-C5.3 staining at the CM and microcilli (MV) and mitochondria at 81,000 X. (D) IgG1 control negative staining of M at 81,000 X. (E) Positive 7E11-C5.3 staining of mitochondria at 13,000 X.

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FIGURE 8. Immunoelectron microscopy of LNCaP cells. The grids were stained with 7E11-C5.3 followed by a secondary antibody labelled with a 10 nm gold bead. (A) A cluster of gold beads at the inner face of the cell membrane (CM) and near a gap junction (GJ) between two LNCaP cells at 97,000 X. (B) Gold beads clustered in and around a mitochondria (M) at 97,000 X.
4. MITOCHONDRIAL PURIFICATION. Since two separate experiments suggested that PSMA was, in part, localized to the mitochondria of LNCaP cells, mitochondria were purified from whole cell lysates and analyzed for the presence of PSMA. Based on the immunolocalization experiments, mitochondria purified from LNCaP cells would be expected to contain the PSMA glycoprotein. In fact, the 100 kDa PSMA glycoprotein was present in the LNCaP lysate (Fig. 9, lane 1), crude mitochondrial preparation (Fig. 9, lane 2) and purified mitochondria following several sucrose gradient centrifugation steps (Fig. 9 lane 3) adding further evidence that PSMA is associated with the mitochondria of LNCaP cells.

5. EFFECTS OF SERUM STARVATION AND STIMULATION ON PSMA LOCALIZATION. LNCaP cells were grown to approximately 50% confluency on tissue culture slides. The normal media was removed and replaced with serum free media and the cells were serum starved at 37° C for 48 hours. The serum free media was then replaced with normal media and slides were collected at time points ranging from 0 to 48 hours after serum stimulation and the cells analyzed for PSMA by immunofluorescence microscopy as described above. The typical cytoplasmic staining pattern of PSMA was observed in all time points with little variation following serum stimulation suggesting that the localization of PSMA was not dependent on extracellular signals (data not shown).
FIGURE 9. Western blot analysis of crude and purified mitochondria. The mitochondria were purified by a series of centrifugation steps and sucrose gradient centrifugation. The LNCaP cell lysate contained a significant amount of the 100 kDa PSMA glycoprotein (lane 1) as did the crude mitochondrial preparation (lane 2) and pure mitochondria following two sucrose gradient centrifugation purifications (lane 3) indicating that PSMA is found in the mitochondria of LNCaP cells.

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B. BIOCHEMICAL CHARACTERIZATION OF THE 7E11-C5.3 EPITOPE ON THE PROSTATE SPECIFIC MEMBRANE ANTIGEN.

1. BASIC BIOCHEMICAL CHARACTERIZATION OF THE PSMA EPITOPE. The initial characterization of the MAb 7E11-C5.3 epitope on PSMA previously reported (92) utilized standard physical and biochemical techniques on purified material. These experiments were repeated here as a result of the apparent conflicts in the data as discussed in the Review of the Literature. However, the present study confirmed the previous results. The MAb 7E11-C5.3 epitope was stable after incubations at 100°C and after reduction or denaturation in mercaptoethanol and SDS (Fig. 10). The epitope was susceptible to oxidation of viccynyl hydroxyl groups by periodic acid suggesting a carbohydrate component of the epitope, although sodium borohydride and tunicamycin treatments (data not shown) were unable to inhibit binding. The increased binding of MAb 7E11-C5.3 to PSMA following sodium borohydride treatment most likely results from the release of O-linked carbohydrates which may mask the antigenic determinant.

Since the periodate and buffers utilized above were relatively harsh reagents, there was a possibility it was the ability of PSMA to adhere to the plastic wells of the RIA plate which was being affected and not the antigenic epitope on PSMA. To control for this possibility, untreated LNCaP lysate (Fig. 10B, lane 1), LNCaP lysate treated in water (lane 2), LNCaP lysate treated in acetate buffer (lane 3), and LNCaP lysate treated with acetate buffer and periodate (lane 4) were analyzed by immunoblot analysis. This experiment clearly demonstrates the 7E11-C5.3 epitope on PSMA is destroyed by the periodate treatment alone.

The recognition of the PSMA epitope by MAb 7E11-C5.3 under reducing and denaturing conditions indicated the integrity of the epitope was not dependent
FIGURE 10. Basic biochemical analysis of the MAb 7E11-C5.3 epitope.
A) Purified PSMA from LNCaP cells was treated with heat (boiling for 10 min.), sodium borohydride (NaBH), periodate, sodium dodecyl sulfate (SDS), and beta mercaptoethanol (2Me). The antigenic epitope was recognized in a denatured and reduced form and was susceptible to periodate oxidation but not sodium borohydride treatment. B) Western blot analysis of PSMA without treatment (lane 1), treated for 1 hr in the dark with 50 ul of water (lane 2), 50 µl acetate buffer (lane 3), and periodate in acetate buffer (lane 4). The periodate treatment alone and not the acetate buffer is affecting the MAb 7E11-C5.3 epitope on the PSMA glycoprotein. The bar graph represents the average of 3 independent experiments +/- SE.
on secondary or tertiary structure of the native molecule. In order to determine if the primary amino acid chain was important in the epitope, the PSMA glycoprotein was treated with a variety of proteases having differing specificities. These digestions revealed the epitope to be highly susceptible to proteolysis (Fig. 11) indicating the epitope was dependent on an intact peptide backbone.

2. CARBOHYDRATE ANALYSIS. The periodate oxidation of the 7E11-C5.3 epitope argued that a carbohydrate played a significant role in antigenicity. To determine which carbohydrates were present in the antigenic epitope, lectin competitive inhibition studies were carried out. These experiments demonstrated SBA, PNA and MPA lectins (Fig. 12), which are specific for D-galactose in either a monomeric form or a polymeric form, were able to reduce MAb 7E11-C5.3 binding while lectins which bound to other carbohydrates such as mannose, glucose, fucose, neuraminic acid, and N-acetyl-glucosamine had no effect (Fig. 12). To prove the specificity of these lectin experiments, PSMA was digested with both general and specific glycosidases prior to analysis by RIA using 7E11-C5. Neither O-glycosidase nor N-glycosidase, used to cleave O-linked and N-linked oligosaccharides, respectively were able to reduce MAb 7E11-C5.3 binding (Fig. 13). Digestion with specific enzymes, in particular β-galactosidase, also did not inhibit antibody binding (Fig. 13). Similarly, MAb 7E11-C5.3 binding was not inhibited in competitive blocking experiments using specific carbohydrates or aminosugars (Fig. 14). In particular, D-galactose (D-gal) and N-acetyl galactosamine (galNAc) were unable to inhibit binding in spite of the fact that lectins specific for these sugars (SBA, PNA, and MPA) were able to abrogate MAb 7E11-C5.3 binding. The surprising inability of these glycosidases and sugars
FIGURE 11. Proteolytic digestion of PSMA. Purified PSMA was subjected to proteolytic digestion with a variety of proteases with different amino acid specificities. The PSMA from LNCaP cells was highly susceptible to trypsin (U/ml), chymotrypsin (mU/ml), protease type XXI (mU/ml), and protease type XXVI (mU/ml). The data represent the average of 3 independent experiments +/- SE.
FIGURE 12. Competitive lectin binding analysis. Purified PSMA was incubated with the indicated lectins prior to a standard RIA assay to attempt to block the antigenic epitope of 7E11-C5.3. The lectin specificities are as follows: LPA (NeuNAc, D-galNAc, and D-GlcNAc), PHA (oligosaccharides), UEA (L-fuc), WGA (D-glcNAc, NeuNAc), GSII (D-glcNAc), ConA (D-man, D-glc), SBA (D-galNAc, D-gal), PNA (D-gal-B-(1-3)-D-galNAc), and MPA (D-gal). Lectins with a specificity for galactose residues were able to partially block the MAb 7E11-C5.3 epitope. The data represents the average of 3 independent experiments +/- SE.
FIGURE 13. Digestion of PSMA carbohydrates with glycosidases.
Purified PSMA was digested with neuraminidase (NM), beta-galactosidase (B-Gal), fucosidase (Fuc), O-glycanase (O-Gly), N-glycanase (N-Gly), Endo F, and chondroitinase ABC (Chon). None of the enzymes used were able to inhibit MAb 7E11-C5.3 binding which was surprising in light of the results from the lectin binding experiments. The data represents the average of 3 independent experiments +/- SE.
FIGURE 14. Competitive blocking of the MAb 7E11-C5.3 epitope with specific carbohydrates. The MAb 7E11-C5.3 was incubated with the indicated carbohydrates in an attempt to block reactivity to purified PSMA. None of the specific carbohydrates or amino sugars were able to block the activity. Specifically, monomeric D-galactose and polymers of D-galactose (D-raffinose and gal-galNAc) were not able to compete although the lectin studies indicated a galactose component of PSMA was important in the antigenic epitope. The data represents the average of 3 independent experiments +/- SE.
to abrogate binding indicates the lectin competitive inhibition may have been non-specific.

3. DETERMINATION OF ACTIVE PSMA PEPTIDES. The amino acid sequence of PSMA has been previously reported (88). Computer modeling of this sequence (Fig. 15) indicates that there is a 19 amino acid intracellular domain followed by a 23 amino acid type II transmembrane domain while the remainder of the PSMA glycoprotein is extracellular. Since the immunolocalization experiments described above suggested the 7E11-C5.3 epitope on PSMA epitope was intracellular, several synthetic peptides were synthesized which corresponded to the 19 amino acids of the proposed intracellular domain of PSMA. Peptide N1.19 consisted of the entire 19 amino acid intracellular domain (MWNLLHETDSAVATARRPR) while peptides N1.6 (MWNLLH), N7.12 (ETDSAV), and N13.19 (ATARRPR) were synthesized to effectively separate this domain into three segments. The synthetic peptides utilized for this study were sufficiently pure as judged by the fact that all peptides gave a single predominant peak when analyzed by reverse phase chromatography on a Waters 650 HPLC system (Fig. 16). The N1.19 and the amino terminal N1.6 peptide were recognized by MAb 7E11-C5.3 in a dose dependent manner although N1.6 had approximately 30% less activity than N1.19 while N7.12, N13.19 and N669 (MFLERAA), a negative control peptide, were inactive (Fig. 17). The fact that these peptides were recognized by MAb 7E11-C5.3 indicated that the PSMA antigenic epitope was predominately composed of the core peptide structure and was indeed located at the amino terminal end of the glycoprotein.

4. PSMA PEPTIDE EPITOPE MAPPING. To further characterize the peptide portion of the PSMA epitope, the intracellular domain was completely
FIGURE 15. Molecular domains of the proposed PSMA molecule. The cytoplasmic domain is made up of the first nineteen amino terminal amino acids; a transmembrane domain is localized at amino acids 20-43; the remaining portion of the molecule is extracellular. A limited degree of homology (54%) to the transferrin receptor (TR) is located at amino acids 417-567.
FIGURE 16. A representative HPLC analysis of the PSMA synthetic peptides. The peptides were separated on a reverse phase column with an acetonitrile gradient and monitored at a wavelength of 214 nm. All of the PSMA peptides showed a single predominant peak with very little contamination.
FIGURE 17. Direct binding PSMA peptide assay with 50 μl of each molar concentration of peptide. Peptides were bound to EIA plates with BSA and EDAC chemical crosslinker and used for a standard ELISA assay with MAb 7E11-C5.3. The concentration of MAb 7E11-C5.3 used (1μg/ml) was analytically determined from the half maximal binding to the purified antigen. Peptides N1.19 (■) and N1.6 (△) were active in a dose dependent manner while peptides N7.12 (○), N13-19 (▲), and the control peptide N669 (Ο) were negative. The data represents the average of 3 independent experiments ± SE.
mapped with synthetic peptides. Table 1 shows the orientation of the synthetic peptides from the amino terminal end of the PSMA amino acid sequence beginning with the complete N1.19 intracellular reactive peptide. Equimolar concentrations of each peptide were assayed in a direct binding assay in which the small PSMA peptides were first conjugated to BSA adsorbed to an ELISA plate using the chemical crosslinker 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC). Baseline activity due to BSA and EDAC was measured in wells containing no peptide (Fig. 18 lanes 1-3). Peptides containing the first seven

Table 1. Amino acid sequences of synthetic PSMA peptides

<table>
<thead>
<tr>
<th>Name&lt;sup&gt;a&lt;/sup&gt;</th>
<th>N-Amino Acid Sequence</th>
<th>-C</th>
</tr>
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<tbody>
<tr>
<td>N1.19</td>
<td>MWNLLHETDSA VATARRPR</td>
<td></td>
</tr>
<tr>
<td>N1.12</td>
<td>MWNLLHETDSAV</td>
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</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>N7.12</td>
<td></td>
<td>ETDASV</td>
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<tr>
<td>N13.19</td>
<td></td>
<td>ATARRPR</td>
</tr>
<tr>
<td>N1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CWNLLH</td>
<td></td>
</tr>
<tr>
<td>N1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>MWNLLY</td>
<td></td>
</tr>
<tr>
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<td>MHIHST</td>
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</tr>
<tr>
<td>N470</td>
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</tr>
<tr>
<td>N664</td>
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<td></td>
</tr>
<tr>
<td>N669</td>
<td>MFLERA</td>
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</tr>
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<sup>a</sup>Pepsides named according to the distance from the amino terminal end of the PSMA amino acid sequence. amino acids: (M) methionine, (W) tryptophan, (N) asparagine, (L) leucine, (H) histidine, (E) glutamic acid, (T) threonine, (D) aspartic acid, (S) serine, (A) alanine, (V) valine, (R) arginine, (P) proline.
FIGURE 18. PSMA peptide mapping using 50 µl of each peptide at a concentration of 1 µmol/L. Baseline levels were defined by control wells containing only BSA or 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDAC), or both. All peptides containing at least the first seven amino acids were equally active, with N1.6 activity dropping by approximately one third. The methionine-containing negative control peptide (N669) was below baseline, as were several additional peptides from the PSMA extracellular domain which have a similar motif to N1.6. The data represents the average of 3 independent experiments +/- SE.
N-terminal amino acids had equal activity while N1.6 retained approximately two thirds the activity and N1.5 was below baseline levels (Fig. 18) indicating that the minimal reactive peptide was a 6mer composed of the first six amino terminal amino acids (MWNLLH), although the glutamic acid (E) at position 7 must also contribute to the epitope structure or stability of the epitope since there was a decrease in reactivity between the 6mer and 7mer peptides.

An intracellular epitope would seem to hinder the ability of MAb 7E11-C5.3 to image tumors in vivo. Therefore, several additional peptides within the extracellular domain of the proposed PSMA sequence which had a similar amino acid motif to N1.6 including N344, N470, N583, and N664 were synthesized and tested for MAb 7E11-C5.3 reactivity. None of these peptides were recognized (Fig. 18) indicating that there is only one MAb 7E11-C5.3 antigenic epitope on PSMA.

To demonstrate the specificity of the synthetic peptides and to utilize an alternative method which did not require binding of these small polypeptide molecules to microtiter plates, an indirect assay was utilized to competitively block MAb 7E11-C5.3 binding to LNCaP cell lysates. Peptides N1.19 and N1.6 were able to inhibit binding of MAb 7E11-C5.3 to PSMA in a dose dependent manner while control peptides and additional peptides present in the PSMA sequence were unable compete for MAb binding (Fig. 19).

5. AFFINITY OF MAb 7E11-C5.3 FOR PSMA AND PSMA PEPTIDES. The affinity of MAb 7E11-C5.3 was determined for the native PSMA glycoprotein and the N1.19 and N1.6 active peptides. The affinity for the native PSMA glycoprotein ($K_a \ 1.16 \times 10^{10} M^{-1}$) was approximately 6.8 fold higher than the affinity for the N1.19 peptide ($K_a \ 1.7 \times 10^9 M^{-1}$) and about 50
FIGURE 19. PSMA peptide inhibition assay. Forty µl of each peptide dilution was incubated with 40 µl of the MAb 7E11-C5.3 and then used as the primary antiserum for an ELISA assay with purified PSMA from LNCaP cells as the antigen. Peptides N1.19 (•) and N1.6 (○) competed in a dose dependent manner while the negative control peptide N669 (□) and peptides N334 (▲), N470 (▼), N583 (■) and N664 (△) were unable to block MAb 7E11-C5.3. The data represents the average of 3 independent experiments ± SE.
fold higher than the N1.6 peptide ($K_a = 2.3 \times 10^8 \text{M}^{-1}$) although the affinity for N1.6 was only 7.4 fold lower than the affinity for N1.19.

6. BIOCHEMICAL ANALYSIS OF THE PSMA ANTIGENIC PEPTIDES. The synthetic peptide mapping clearly demonstrated MAb 7E11-C5.3 has a high affinity for the primary amino acid chain of PSMA. As a result, the ability of periodate to abrogate recognition of PSMA by 7E11-C5.3 (Fig. 10) was even more confusing. To determine if this decrease in antibody binding following periodate treatment of the purified PSMA resulted from the oxidation of the peptide backbone and not a carbohydrate, the synthetic peptides were treated with periodate prior to binding to the EIA plate for the direct binding assay. Periodate treatment of the active peptides N1.19 and N1.6 did result in a loss of MAb 7E11-C5.3 binding (Fig. 20, panel A). Additionally, to mimic the effects of periodate oxidation of the polypeptide chain, deletions and substitution of some key amino acids were synthesized. Peptide N2.6 (WNLLH) with a deletion of the methionine residue or NΔ1.6 (CWNLLH) with a substitution of cysteine for the methionine and peptide N1.5 (MWNLL) with a deletion of the histidine residue or N1.6Δ (MWNLLY) with a substitution of tyrosine for the histidine residue were assayed in a direct binding assay. The deletion of either of these amino acids resulted in a loss of activity (Fig. 20, panel A) suggesting that they or at least the amino acids present in that position are important for the structure of the epitope. Substitution of either the methionine at position 1 with cysteine or the histidine at position 6 with tyrosine also resulted in a loss of activity (Fig 20, panel A) indicating that the amino terminal methionine and the carboxy terminal histidine are required for structural integrity of the PSMA epitope and are essential for MAb 7E11-C5.3 activity which, if lost by oxidation, would result in an abrogation of MAb 7E11-C5.3 binding.
FIGURE 20. Analysis of the MAb 7E11-C5.3 epitope. (A) Peptides N1.19 and N1.6 were treated with periodate as described for the native PSMA antigen. The activity of both peptides was reduced to background levels of the negative control (N669). Deletion of the methionine (N2.6) or histidine (N1.5) and substitution of the methionine with cysteine (NΔ1.6) or substitution of the histidine with tyrosine (N1.6 Δ) resulted in a similar loss of activity. (B) A MAPS N1.19 peptide was treated with 1) no treatment (lane 1), in the dark for 1 hr. in 50 μl water (lane 2), acetate buffer (lane 3), and periodate in acetate buffer (lane 4). The N1.19 peptide was affected by only periodate treatments identically to the native PSMA molecule. The data represents the average of 3 independent experiments +/- SE.
It was possible that periodate treatment of these small polypeptides may have been interfering with the ELISA assay and not oxidizing the amino acids. To control for this possibility, a N1.19 multiple-antigen-peptide-system (MAPS) peptide containing 4 linear peptide chains per molecule was analyzed by Western blot analysis following treatment with periodate (Fig. 20, panel B). The reactivity of MAb 7E11-C5.3 to this N1.19 MAPS peptide (lane 1) was not reduced by incubations in water (lane 2) or acetate buffer (lane 3) but was abrogated by treatment with acetate buffer and periodate (lane 4). Therefore, the periodate and not the reaction buffers was responsible for the loss of activity.
C. PHYSICAL CHARACTERIZATION OF THE PROSTATE SPECIFIC MEMBRANE ANTIGEN GLYCOPROTEIN.

1. PSMA EXPRESSION IN PROSTATE CELL LINES. The PSMA glycoprotein was detected predominantly as a 100 kDa band following western blot analysis of LNCaP xenograft tumor and cultured cell line membrane extracts. Occasionally, several additional bands were observed (Fig. 21) of approximately 180 and 160 kDa. There was no difference in the expression of PSMA between the cultured cells and nude mouse tumors indicating that the use of cultured LNCaP provides a valid model to study PSMA. As is the case with other supposed prostate specific markers such as PSA, PSMA was not detected in any of the other prostate cell lines tested (Fig. 21). The 160 kDa band, as well as an occasional 70 kDa band, were transient and most likely represented breakdown products of the two major species.

2. TWO DIMENSIONAL GEL ELECTROPHORETIC ANALYSIS OF PSMA.
To further characterize the PSMA expressed by the LNCaP cell line, two dimensional gel electrophoresis was performed using purified PSMA from in vitro cultured LNCaP cell membrane preparations. Surprisingly, identical isoelectric points for both species were observed with the major spots at isoelectric points of approximately 5.6, 5.7, and 5.8 for both the 100 and 180 kDa bands (Fig. 22). The tailing is consistent with the characteristics of integral membrane proteins. These results suggest the two species are very similar in their biochemical nature.

3. COMPARISON OF 100 AND 180 KDA BANDS. The fact that very similar isoelectric points were observed indicated that PSMA may exist in a dimer form. To look at this relationship more closely, LNCaP lysates were analyzed under a variety of denaturing and reducing conditions. The 180 kDa band was the
FIGURE 21. Western blot analysis of 50 μg of membrane preparations from LNCaP nude mouse tumors (lane 1), cultured LNCaP cells (lane 2), PC3 cells (lane 3), DU145 cells (lane 4), and PPC-1 cells (lane 5). The 100 kDa and 180 kDa PSMA bands are present in the LNCaP extracts but are absent in the other prostate cell lines. 

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FIGURE 22. Two dimensional gel electrophoresis of affinity purified PSMA. Ten ug of purified PSMA was analyzed according to Materials and Methods. Arrows indicate the isoelectric points of 5.6, 5.7, and 5.8 showing identical spots for both the 100 kDa and 180 kDa bands.
predominant species when LNCaP lysates were analyzed by Western blot without SDS or reducing agent (2-mercaptoethanol) in the sample buffer (Fig. 23 lane 1). The addition of SDS (lane 2) but not reducing agent (lane 3) was able to restore the migration of PSMA to 100 kDa. Next, affinity column purified PSMA was separated by SDS-PAGE and stained with coomassie blue. The two molecular weight species of 100 and 180 kDa are clearly evident (Fig. 24, lane 1). The two bands were excised, electroeluted from the gel then reapplied to an SDS-PAGE gel under reducing and denaturing conditions then immunoblotted with MAb 7E11-C5.3. The majority of the gel-purified 100 kDa band remained at the appropriate molecular weight (Fig. 24 lane 2). However, a minority of this band migrated at the higher molecular weight of 180 kDa. At the same time, the majority of the gel purified 180 kDa protein migrated at 100 kDa with a minority at 180 kDa (Fig. 24 lane 3) which suggests that the two bands are identical.

To confirm the homology of the 100 kDa and 180 kDa species, partial tryptic digestion of the two bands followed by electrophoresis on an SDS-PAGE gel and silver staining resulted in identical banding patterns. This analysis indicated that the two bands consisted of the same molecule (figure 25).

4. COPURIFICATION OF A MITOCHONDRIAL PROTEIN WITH PSMA. A third major band of an approximate molecular weight of 40 kDa was co-purified with PSMA on a MAb 7E11-C5.3 affinity column when low detergent wash buffers were used on the column (Fig. 26 lane 1) but was not immunoreactive (lane 2). This band was blotted to nitrocellulose and subjected to amino acid sequence analysis. The amino acid sequence of the amino terminal end of this band was completely identical to the precursor form of the mitochondrial isoenzyme of aspartate aminotransferase (m-AST) also known as S-glutamic-oxalacetic transaminase (SGOT).
FIGURE 23. Western blot analysis of LNCaP cell lysates under different reducing and denaturing conditions. 50 µg of an LNCaP cell lysate was prepared in sample buffer containing: 0% w/v SDS and 0% v/v 2Me (lane 1); 8% SDS and 0% 2Me (lane 2); 0% SDS and 20% 2Me (lane 3); and 8% SDS and 20% 2Me (lane 4) prior to boiling and loading on a 4-20% gradient SDS gel and run under denaturing conditions. The PSMA glycoprotein was only seen at 100 kDa when the sample buffer contained SDS while 2Me was able to reduce approximately half of the 180 kDa band to 100 kDa.
FIGURE 24. Purified PSMA was separated on an SDS-PAGE gel and stained with coomassie blue (lane 1) showing the predominant bands of 100 and 180 kDa. The individual bands were excised and purified by electroelution from the gel slices. The gel purified 100 kDa (lane 2) and 180 kDa (lane 3) bands were then analyzed by Western blot showing the majority of both species migrating at 100 kDa indicating the 180 kDa band may be a dimer form of the 100 kDa species or that PSMA has a considerable amount of secondary and tertiary structure which is not easily reduced to the 100 kDa band.
FIGURE 25  Gel purified PSMA 100 kDa (lane 1) and 180 kDa (lane 2) bands were partially digested with trypsin then applied to an SDS-PAGE gel and run under reducing and denaturing conditions. The gel was silver stained to visualize the bands. Both species gave identical peptide bands indicating they are homologous.

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FIGURE 26. PSMA was purified from LNCaP by affinity chromatography and analyzed by gel electrophoresis and staining with coomassie blue (lane 1) and immunoblotting with MAb 7E11-C5.3 (lane 2). The 100 kDa band is highly reactive while the 180 kDa band is less reactive. The 40 kDa band which co-purified with PSMA was not immunoreactive. This 40 kDa band was sequenced and was found to be 100% homologous with the mitochondrial subunit of SGOT-AST.

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5. DETECTION OF PSMA IN PROSTATE TISSUE. To investigate the expression of PSMA in prostate tissues, membrane extracts were examined by western blot analysis. Membrane preparations of normal, benign and malignant prostate tissues showed a Western blot banding pattern similar to that seen in LNCaP cell preparations except the migration of both the 100 and 180 kDa species had a slightly slower mobility of 120 and 200 kDa, respectively (Fig. 27A and 27B, see arrows). This slower mobility of both bands was observed in all prostate tissue extracts analyzed with none showing the 100 and 180 kDa banding pattern.

The level of PSMA expression and the banding pattern in normal and primary CaP tissue extracts were similar with the predominant band being the 120 kDa species (Fig. 27A). In most normal prostate tissue extracts, PSMA expression was consistent showing the 120 kDa species with a small amount of the 200 kDa band. Although some CaP tissues seemed to overexpress PSMA compared to normal tissues (fig 27A, lane 13), there did not appear to be a significant overexpression in the primary tumors. A difference in the level of PSMA expression and the banding pattern were observed in BPH tissue extracts (Fig. 27A lanes 6-9) compared to the normal and CaP tissues. BPH tissues expressed less PSMA and a more heterogeneous banding pattern with several tissues expressing only the larger 200 kDa species (Fig. 27B lanes 4 and 5). Figure 27B shows several of the tissue extracts which show a more heterogeneous expression of PSMA and a greater amount of the 200 kDa species. All prostate tissues examined expressed either the 120 kDa species, the 200 kDa species, or both. Little variation in the size of PSMA was noted between the three prostate tissue types except for some occasional smaller molecular weight species. For example, the smaller molecular weight bands in Figure 27B, lanes 2,3, and 4 may represent a degradation of the 120 and 200 kDa species, respectively.
FIGURE 27. Expression of PSMA in prostate tissue. (A) Western blot of 50 μg of LNCaP membrane extract (lane 1), normal prostate membrane extracts (lanes 2-5), BPH membrane extracts (lane 6-9), and CaP membrane extracts (lanes 10-13). (B) Western blot analysis of prostate tissue membrane extracts which show some of the heterogeneity seen in some samples. Fifty ug of LNCaP membrane extract (lane 1), normal prostate extracts (lanes 2,3), BPH tissue extracts (lanes 4,5), and CaP tissue extracts (lanes 6,7). Arrows indicate the shift in the size of the bands from the 100 and 180 kDa species observed in LNCaP to approximately 120 and 200 kDa in prostate tissue.
6. PSMA EXPRESSION IN NON-PROSTATE TISSUES. A panel of normal and malignant non-prostate tissue extracts was examined by Western blot analysis for PSMA antigen expression. The MAb 7E11-C5.3 was reactive with normal small intestine membrane extracts (Fig. 28, lane 2 and 3). Only a smear was seen when 50 μg was loaded (lane 2) but when less protein was analyzed (lane 3) several bands were observed, although the pattern was unlike that in cultured LNCaP cells (100 and 180 kDa) or prostatic tissues (120 and 200 kDa). The vertical smearing of the lane is indicative of a heavily glycosylated protein. Additionally, a low yet significant expression of PSMA was observed in cerebral cortex and salivary gland (Fig. 28, lanes 4 and 5). The size of PSMA expressed by the cerebral cortex was similar to that seen in LNCaP cell line extracts (approximately 100 kDa) while the size of PSMA detected in the salivary gland was similar to that seen in prostate tissue extracts (approximately 120 kDa.) The PSMA glycoprotein was not expressed in the majority of non-prostate tissues examined including normal skeletal and cardiac muscle, colon, breast, lung, ovary, kidney, and liver tissues (Fig. 28, lanes 5-12) or a variety of non-prostate malignancies including colon, lung, bladder, liver and breast adenocarcinomas (Fig. 29, lanes 2-6).

7. SPECIFICITY OF THE MAb 7E11-C5.3 REACTIVE BANDS.

To determine if the multiple bands observed in prostate tissue extracts were specific or background artifacts, the N1.19 active PSMA peptide was again used to competitively inhibit MAb 7E11-C5.3 reactivity in immunoblot assays. The reactivity to LNCaP cell line extracts, prostate tissue extracts and seminal plasma (Fig. 30a) could be totally eliminated by incubating the antibody with a twenty fold molar excess of the N1.19 peptide (Fig. 30b).
FIGURE 28. Expression of PSMA in normal non-prostate membrane extracts. Immunoblot of membrane preparations from LNCaP cells (lane 1), small intestine (lanes 2 and 3) cerebral cortex (lane 4), salivary gland (lane 5), skeletal muscle (lane 6), cardiac muscle (lane 7), colon (lane 8), breast (lane 9), lung (lane 10), ovary (lane 11), kidney (lane 12), and liver (lane 13). All of the samples were analyzed at 50 µg per lane except for the small intestine sample in lane 3 which was 25 µg. The small intestine shows a smearing with no detectable bands similar to that seen in prostate tissue. The PSMA in the cerebral cortex (lane 4) is similar in size to the PSMA of LNCaP while the PSMA in the salivary gland (lane 5) is similar to that seen in prostate tissue.
FIGURE 29. Expression of PSMA in non-prostate malignancies. Western blot analysis of 50 ug of membrane preparations from LNCaP cells (lane 1), colon adenocarcinoma (lane 2), lung carcinoma (lane 3), bladder carcinoma (lane 4), liver adenocarcinoma (lane 5), and breast adenocarcinoma (lane 6) showing the typical 100 kDa and 180 kDa bands in LNCaP cells but no expression in other malignant tissues.
FIGURE 30. An immunoblot competition of MAb 7E11-C5.3 with the active PSMA peptides N1.19. (A) Membrane extracts from LNCaP cells (lane 1), PC3 (lane 2), normal prostate (lane 3), BPH (lane 4), CaP (lane 5), and a seminal plasma from a prostate carcinoma patient probed with MAb 7E11-C5.3. (B) an identical blot probed with MAb 7E11-C5.3 plus a 20 molar excess of PSMA N1.19 peptide. The N1.19 peptide was clearly able to block MAb 7E11-C5.3 binding to its target antigen in prostate cell lines, tissues and seminal plasma.
8. PSMA WAS NOT DETECTED IN THE SERUM OF PROSTATE CARCINOMA PATIENTS. Since other prostate markers are detected in significant concentrations in serum, I wanted to determine if PSMA could also be detected in serum by Western blot analysis. Figure 31 shows the analysis of serum by Western blotting with MAb 7E11-C5.3. When these blots were exposed for the standard amount of time (1-2 min.) no bands were evident but when the blots were exposed for greater than 10 min. the pattern in figure 31A was observed. Lanes 1-3 represent LNCaP, normal prostate tissue membrane extracts and a normal seminal plasma, respectively. Note again the difference in the size of the PSMA expressed in LNCaP (Lane 1) as compared to the tissue and seminal plasma (Lanes 2 and 3). The size of PSMA shed into serum would be expected to be similar to the size of PSMA observed in tissue extracts and seminal plasma. Serum from 5 stage D2 CaP patients were pooled and analyzed in several ways. First, the serum was spiked with affinity purified LNCaP PSMA and incubated for 12 h at 37°C prior to analysis by Western blot (Lane 4). The PSMA in the spiked serum was at the expected molecular weights of 100 and 180 kDa identical to the PSMA seen in LNCaP extracts along with a larger band of unknown composition. Secondly, 29 mg of the pooled stage D2 serum was solubilized and passed through a MAb 7E11-C5.3 affinity column, washed and eluted from the column to remove the large concentrations of albumin and other proteins which make Western blot analysis of serum difficult. No PSMA was detected in the affinity column eluant although a significant amount of serum proteins remained, particularly bands migrating at the expected molecular weights of the heavy and light immunoglobulin chains which may have adhered to the protein-A used to construct the affinity column (Lane 5). Thirdly, 400 µg of the same stage D2 serum and 400 µg of a pooled BPH, a pooled normal male, and a pooled normal female serum (Lanes 6-9) were analyzed. Several bands were present following
FIGURE 31. Detection of PSMA in serum. (A) Western blot, using MAb 7E11-C5.3 of 25 µg of LNCaP (lane 1) and normal prostate (lane 2) membrane extracts, 100 µg of a normal seminal plasma (lane 3), 400 µg of pooled serum from patients with stage D2 CaP spiked with purified PSMA (lane 4), 20 µg affinity purified pooled D2 serum (lane 5), 400 µg of the pooled D2 serum neat (lane 6), BPH pooled serum (lane 7), normal male pooled serum (lane 8), and normal female pooled serum (lane 9). (B) Western blot of 25 µg of LNCaP membrane extract (lane 1), 400 µg pooled D2 serum (lane 2) and 800 µg pooled D2 serum blotted with MAb 7E11-C5.3. (C) An identical blot probed with MAb 7E11-C5.3 plus N1.19 PSMA peptide. No PSMA was detected by affinity chromatography of the pooled D2 serum (panel A, lane 5) and while bands are present in the pooled serum, the same bands are present in the normal male and female serum. The bands in the LNCaP membrane extract were eliminated by competition with the N1.19 peptide while the bands in the pooled serum remained indicating that they are non-specific.
overexposure of the blots in all the sera including the normal female serum but none at the expected molecular weight of 120 kDa.

The bands present in the pooled stage D2 serum did appear to be overexpressed as compared to the BPH and normal sera. The presence of identical bands in the pooled normal female serum suggested that the bands seen on this blot were non-specific. To determine if this were the case competitive blocking experiments were carried out. The D2 pooled serum was subjected to SDS-PAGE gel electrophoresis in duplicate along with an LNCaP membrane extract. One of the blots was probed with MAb 7E11-C5.3 and the duplicate blot was probed with MAb 7E11-C5.3 containing the N1.19 PSMA peptide. Figure 31B shows the banding pattern for the LNCaP extract (lane 1) and 400 and 800 µg of the pooled D2 serum (lanes 2 and 3, respectively). There is a reactive band at the approximate molecular weight of 100 kDa in this pooled serum which could be interpreted to be PSMA. However, while all of the reactive bands seen in the LNCaP membrane extract were eliminated by the N1-19 peptide competition (Fig. 31C, lane 1) none of the bands in the serum were competed out (lanes 2 and 3). Additionally, an identical pattern could also be produced if the pooled serum blot was probed with only the secondary antibody or with an isotype matched IgG1 control antibody in place of MAb 7E11-C5.3 (data not shown).

14. DETECTION OF PSMA IN SEMINAL PLASMA. Since PSMA is a product of the prostate glandular epithelium, it was of interest to determine whether it could be detected in seminal plasma. Western blot analysis of seminal plasma obtained from normal (NSP), BPH (BSP) and prostate carcinoma patients (CaSP) showed PSMA was readily detectable in seminal plasma with expression of the 120 kDa band and occasionally the 200 kDa band similar to that seen in tissue extracts (Fig. 32). Normal seminal plasma showed the most consistent
FIGURE 32. Expression of PSMA in seminal plasma. Western blot analysis of 50 µg LNCaP membrane preparation (lane 1), and equal protein concentrations (200 µg/lane) of normal seminal plasma (lane 2-5), BPH seminal plasma (lanes 6-9), and CaP seminal plasma (lanes 10-13). Lanes 1-9 represent an exposure time 2 minutes while lanes 10-13 represent an exposure of 5 minutes. Note the slightly larger molecular weight of PSMA in seminal plasma compared to LNCaP. The concentration of PSMA in normal seminal plasma (NSP) is consistent while it is quite variable in BPH seminal plasma (BSP). The PSMA concentration in CaP seminal plasma (CaSP) appears to be lower than NSP but this likely results from the much higher total protein levels in CaSP as compared to both NSP and BSP resulting in a dilution of PSMA.
expression of PSMA, with little variation from sample to sample, demonstrating the 120 kDa band and often an 80 kDa band (Fig. 32, Lanes 2-5). BPH seminal plasma, similar to the BPH tissue extracts, exhibited variable expression of PSMA ranging from very low to overexpression (Fig. 32, Lanes 6-9). The majority of the CaP seminal plasma samples were similar in banding pattern to normal seminal plasma, although one sample showed only the 80 kDa band. While it appears that the concentration of PSMA in CaSP is lower than NSP, the total protein of these samples was significantly higher than NSP or BSP. In fact, when equal volumes of samples instead of equal protein concentrations were used for the immunoblots, the intensity of the CaP seminal plasma bands were comparable to those seen in the normal samples (data not shown).
D. REGULATION OF THE PSMA GENE.

1. A MODEL FOR STUDYING STEROID HORMONE REGULATION. While several model systems are available to study the basic biology of the prostate, no suitable model has been described to study the expression of prostate specific genes. Therefore, a model system was designed in the present study using a prostate epithelial cell line which allowed for control of both the level of androgen receptor expression and the level of androgen hormone in the culture media (Fig. 33). The PPC-1 cell line which lacks endogenous androgen receptor expression (137) and PPC-1AR5, a subline of PPC-1 containing an androgen receptor expression plasmid, were utilized as the basis of this prostate epithelial cell model. The level of androgen receptor expression in the PPC-1 was below the detectable limits of Western blot analysis (Fig. 34, lane 2) while the PPC1-AR5 cells overexpressed the androgen receptor (lane 3) compared to the LNCaP cell line (lane 1). A breast adenocarcinoma cell line, BT-474, was utilized as a negative control (lane 4). Androgen hormones were removed from all tissue culture media by stripping the fetal calf serum of steroid hormones with charcoal dextran. The level of steroid hormones in this media was below the limits of detection of an RIA assay for testosterone and other androgen hormones (data not shown). To ensure that the PPC-1 cells could not synthesize DHT de novo from other hormones present in the media, finasteride was added to all media to inhibit the activity of 5α-reductase. Hormone levels were then reconstituted with a stable synthetic analog of DHT, R1881, at a concentration of 1 nM which has been shown to give maximal expression of the prostate specific antigen (PSA) gene under experimental conditions (130). The level of prostate gene expression could then be carefully analyzed in controlled conditions in a native prostate epithelial
FIGURE 33. A model system for the study of prostate specific gene expression. The PPC-1 and PPC-1AR5 cell lines were used to control for the level of androgen receptor expression. To control for the level of androgen hormones, the tissue culture media was supplemented with calf serum depleted of steroid hormones then R1881 was selectively added to the media to reconstitute the level of DHT.
FIGURE 34. Western blot analysis of androgen receptor (AR) expression in cell lines. 50 μg of whole cell lysates from LNCaP (lane 1), PPC-1 (lane 2), PPC-1AR5 (lane 3), and BT474 (lane 4) were immunoblotted with an anti-androgen receptor antibody. The normal level of AR expression was observed in the LNCaP cell line while it was undetectable in both the PPC-1 and BT474 cell lines. The AR was overexpressed in the PPC-1AR5 cell line containing the AR expression plasmid.
cell environment under the four conditions possible with these two variables (Fig. 33).

2. CLONING OF THE PSA PROMOTER. To ensure that the model system described above functioned as hypothesized, the 5' flanking region (655 bp) of the PSA gene was cloned as a control from human genomic DNA by PCR amplification and inserted into a pGEM-T vector. The cloned fragment was sequenced to verify its homology with the reported PSA sequence and was found to be 100% homologous. The fragment was subcloned into a basic CAT reporter gene vector containing no endogenous promoter activity.

3. CLONING OF THE 5' FLANKING REGION OF THE PSMA GENE. A 700 bp probe from the 5' end of the PSMA cDNA was used to screen a human genomic library for clones containing the promoter region of the PSMA gene. One clone with an insert of approximately 15 kb (PSMG4.22) was isolated and analyzed by restriction digestion and Southern blot analysis using a probe designed from the 5' untranslated region of the PSMA cDNA to identify fragments with a high probability of containing the 5' flanking region of the PSMA gene. A fragment of approximately 1 kb showed a strong hybridization with this 5' probe (Fig. 35, lane 4) and was subcloned and partially sequenced. The 3' end of this fragment contained 121 base pairs of the PSMA cDNA with the remaining sequence being upstream of the transcription start site (Fig. 36). The 5' fragment contained no GC rich region and did not have a consensus TATA box but an A-T rich region was present at -30 to -60 (Fig. 36). As shown in figure 37, the entire 1 kb fragment was subcloned into the basic CAT reporter gene vector (pCATbasic) in a forward (PSMG4.22CAT) and reverse (PSMG4.22RCAT) orientation and was
FIGURE 35. Cloning of the PSMA 5' flanking region. Southern blot analysis of restriction fragments from the PSMG4.22 genomic clone showing the PSMA cDNA digested with Xho I and Pst I (lane 1), pGEM7Zf+ plasmid DNA (lane 2), PSMG4.22.5 5 kB Bam HI fragment subcloned into pGEM7Zf+ (lane 3), the 1 kB PSMG4.22 5' flanking DNA fragment (lane 4), and undigested parental PSMG4.22 genomic clone. The blot was probed with a probe from the 5' end of the PSMA cDNA.
FIGURE 36. Restriction analysis of the 1 kb PSMG 4.22 5' flanking DNA fragment. The flanking DNA does not have a consensus TATA box or G-C rich region but does contain a very A-T rich region between -30 and -60. A glucocorticoid responsive element (GRE) is also present upstream of the A-T rich region. 121 bp of the flanking DNA is homologous with the 5' untranslated region of the PSMA cDNA.
FIGURE 37. Promoter-CAT reporter gene constructs. The reporter gene constructs utilized for transient transfections of PPC-1 and PPC-1AR5 cells including the pCAT basic plasmid vector (pCATbasic), the PSA promoter fragment (PSA3CAT), the PSMA 5' flanking DNA fragment cloned in a forward direction (PSMG4.22CAT) and the same fragment cloned in a reverse orientation (PSMG4.22RCAT).
analyzed along with the PSA promoter construct (PSA3CAT) for promoter activity.

4. ANALYSIS OF PROMOTER ACTIVITY. To study the regulation of this PSMA 5' flanking region, transient transfections of pCATbasic, PSA3CAT, PSMG4.22CAT, and PSMG4.22RCAT were carried out in PPC-1 and PPC-1AR5 cells and grown under the four conditions described in figure 33 for 48 hr. Total RNA isolated from pools of three separate transfections were analyzed by an RNAse protection assay for the CAT mRNA and the protected fragments were separated on an 8% denaturing polyacrylamide gel. There was no detectable CAT mRNA following transfection of the CAT vector (Fig. 38, lanes 1-4) or the PSMG4.22RCAT construct (Fig. 38, lanes 13-16). The PSA3CAT construct showed a low level of transcription when at least one of the variables was absent (Fig. 38, lanes 5-7) and showed a greatly enhanced expression when both the AR and R1881 were present (Fig. 38, lane 8) indicating that the model system was valid. The PSMG4.22CAT construct showed a moderate level of expression with little difference between the four conditions.
FIGURE 38. Promoter activity assay. The constructs shown in figure 37 were transiently transfected into PPC-1 cells (AR-) or PPC-1 AR5 cells (AR+) in the presence or absence of the synthetic androgen R1881. The promoter activity of each construct was determined by an RNase protection assay specific for CAT mRNA. The results are shown as a graph of the relative intensity above background levels (A) and in the form of the original autoradiograph (B).

The vector control (pCATbasic) showed no promoter activity while the PSA promoter (PSA3CAT) showed maximal activity in the presence of both the AR and R1881 indicating that the model system functioned as theorized. The PSMA 5' fragment (PSMG4.22CAT) did have activity and was equally active in all four experimental conditions. As a control, the PSMA 5' fragment cloned in the reverse orientation (PSMG4.22RCAT) showed no promoter activity indicating that the activity of PSMG4.22CAT was real and not due to background expression. The data represents the average of 3 independent experiments +/- SE.
CHAPTER IV

CONCLUSIONS AND DISCUSSION

Prostate cancer is the most commonly diagnosed adenocarcinoma and the second most common cause of cancer deaths in men in the United States (1). The number of deaths attributed to prostate cancer is increasing at a rate of approximately 8% a year (1) which means that between 1995 and the year 2000, 2 million males will be diagnosed and roughly 300,000 men will have died in just a five year time span. Therapy options for prostate cancer have not improved over the past decade and remain limited with few model systems in existence which allow for the study of novel therapeutic modalities. In order to advance the treatment options for prostate cancer, novel strategies for diagnosis and therapy are needed to improve the life expectancy of patients diagnosed with this disease.

The use of monoclonal antibody directed imaging and therapy applications have shown great promise for improving the survival of prostate cancer patients. However, improvements such as increased sensitivity and specificity must be realized before many of these models prove efficacious enough to be utilized for clinical applications. These improvements are entirely dependent on an in depth understanding of the monoclonal antibody, the antigenic epitope recognized by the antibody, and the molecule which carries the epitope. With this knowledge in hand, informed modifications may be made to improve existing applications. The present study has attempted to provide a foundational knowledge of the MAb
7E11-C5.3 and the PSMA glycoprotein so that improvements in MAb 7E11-C5.3 mediated diagnostic and therapeutic strategies may be realized.

The MAb 7E11-C5.3 was first described in 1987 (83). Since then, the antigen recognized by MAb 7E11-C5.3 has been described in some detail (84-92) and has been named the prostate specific membrane antigen or PSMA (88). PSMA is a new prostate biomarker which appears to be overexpressed in poorly differentiated and metastatic prostate carcinomas (138). Antibody-radiouclide conjugates have been successfully used to localize metastatic disease in vivo (99,100) and treat human prostate tumors in nude mice (139). These reports suggest PSMA may have promise as an important new diagnostic and therapeutic tool for prostate cancer. Many questions about PSMA remain to be answered however, such as: (1) the exact localization and biochemical nature of the epitope; (2) whether PSMA can be detected in tissues and body fluids, and if so what molecular forms are present; (3) the exact physical characteristics of the PSMA glycoprotein; (4) and the regulation of the expression of the PSMA gene. The answers to these questions may allow for dramatic improvements in the in vivo diagnostic and therapeutic protocols. For example, based on the characterization of the MAb 7E11-C5.3 epitope, the sensitivity of these assays may be increased by generating new antibodies directed against different domains of PSMA.

The present study has attempted to address these key questions by providing: 1) an in depth analysis of the localization of the MAb 7E11-C5.3 epitope on PSMA; 2) definitively determining the composition of the antigenic epitope; 3) describing in detail the expression of PSMA in prostate cell lines, prostate and non-prostate tissues and body fluids; and 4) to understand the regulation of the PSMA gene at the molecular level.
The localization of the MAb 7E11-C5.3 epitope on PSMA was definitively shown to be intracellular by immunofluorescence microscopy of living LNCaP cells. This finding was surprising in light of the ability of \(^{111}\)indium labeled MAb 7E11-C5.3 (CYT-356) to image occult tumors \textit{in vivo}. It was clear however, the only mechanism which allowed for staining of LNCaP cells with this MAb was permeabilization of the cell membrane while the OKT-9 MAb, which recognizes a well characterized transferrin receptor epitope on the extracellular face of the plasma membrane (140), readily stained LNCaP cells without fixation or permeabilization. This fact strongly argues for an intracellular 7E11-C5.3 epitope. How then is CYT-356 able to image occult tumors? While there is no definite answer to this question, it is doubtful CYT-356 is able to cross the plasma membrane since 7E11-C5.3 was not able to do so \textit{in vitro}. Due to the propensity of prostate cells to enter apoptosis (141), the most probable answer is that a significant number of cells within a tumor may be lysed following cell death thereby uncovering the antigenic epitope. The number of apoptotic cells within a tumor may be substantially increased if the patient is on hormone ablation therapy since withdrawal of androgen hormones induces apoptosis (142) which may lead to an increase in the number of epitopes available to be recognized by CYT-356. Alternatively, as a tumor grows, a significant portion of the tumor volume may become necrotic (143) allowing for access to the intracellular epitope. Both of these mechanisms would result in CYT-356 “seeing” only a small percentage of the cells within a given tumor mass resulting in a significantly low sensitivity. As a result, specific second generation antibodies generated against the extracellular domain of PSMA may dramatically increase the sensitivity of imaging assays by increasing the number of cells which can be recognized by the antibody. This increased sensitivity may result in the detection of smaller metastatic foci containing a large percentage of intact cells which would not be discovered by
MAb 7E11-C5. It must be stated in any discussion of PSMA second generation antibodies however, that the MAb 7E11-C5.3 may be the most selective antibody for prostate tissue. It is highly likely that many of the monoclonal antibodies generated against the extracellular domain of PSMA may be crossreactive with other cellular antigens. Therefore, it may not be possible to greatly improve the sensitivity of PSMA imaging over that which is obtained using 7E11-C5.3

The staining of fixed and permeabilized LNCaP cells with MAb 7E11-C5.3 resulted in a diffuse cytoplasmic pattern unlike that of integral membrane proteins (transferrin receptor) or secretory proteins (PSA). This cytoplasmic staining pattern was reproducible utilizing both immunofluorescent and immunoperoxidase methods and, therefore, was not an artifact of the staining technique. It may be argued that the weak detergent permeabilization of the cells prior to staining caused the cell membrane to dissipate resulting in the cytoplasmic staining pattern. However, cells stained with the OKT-9 MAb following an identical detergent treatment retained a symmetrical staining around the circumference of the cells indicating the detergent extraction was not sufficient to cause diffusion of the membrane components.

To begin to determine the intracellular localization of PSMA, LNCaP cells were partitioned into nuclear, cytoplasmic, and membrane components and examined for the presence of PSMA. The cellular subfractionation of LNCaP cells clearly demonstrated two pools of PSMA; one pool in the LM fraction the other in the HM fraction with very little in either the cytoplasmic or nuclear fractions. One of these pools may be localized to the plasma membrane since the LM fraction contained a significant concentration of PSMA by both ELISA and western blot analysis. The other pool of PSMA was most likely inside intracellular organelles which are partitioned in the HM fraction. The rough endoplasmic reticulum (ER) may contain a significant number of plasma membrane proteins as they transit
from the ER to the membrane. The concentration of these plasma membrane components in the ER may be enhanced if there is a defect in trafficking of molecules through this pathway (144). Alternatively, PSMA may be found in the mitochondria of LNCaP cells since the mitochondria make up a significant portion of the HM fraction as judged by the detection of the mitochondrial specific Hsp60.

Immunoelectron microscopy of LNCaP cells showed that PSMA is indeed found at two locations. The MAb 7E11-C5.3 epitope on PSMA could routinely be found localized at the cytoplasmic face of the cell membrane substantiating the conclusion of an intracellular epitope. The only distinct positive staining in addition to the plasma membrane was found to be surrounding and within the outer mitochondrial membrane. A localization solely at the cytoplasmic face of the cell membrane would result in a ringed staining identical to that of an extracellular epitope. However, the additional localization to mitochondria would explain the cytoplasmic appearance of the staining at the light microscopy level. Positive staining for PSMA at both the cell membrane and mitochondria was observed utilizing both an indirect chromagen staining and a direct staining with a gold labeled secondary antibody. These identical results using multiple immunoelectron microscopy techniques as well as the negative staining by an isotype matched control antibody suggests the staining was specific.

While the controls for the immunolocalization experiments did suggest the conclusions were valid, it was necessary to prove that PSMA was found in the mitochondria of LNCaP cells. PSMA was detected by western blot in mitochondria purified utilizing a multiple step sucrose gradient purification which was proven to yield high purity mitochondria (121). While it is difficult to judge the purity of this mitochondrial preparation short of electron microscopic examination, the mitochondria were applied to the sucrose gradient twice to ensure adequate purification. It would seem unlikely that a small amount of
contamination with other cellular membranes would give such a strong signal upon immunoblotting with 7E11-C5.3.

These data strongly suggest the PSMA glycoprotein is localized at the plasma membrane with the epitope facing the cytoplasm and within mitochondria. While one would not expect a relatively prostate specific glycoprotein to be localized to an organelle which is considered to be rather generic in its constitution, there is precedent for the localization of plasma membrane glycoproteins to the mitochondria. The Her2/neu oncogene product has also been shown to be localized at the plasma membrane and mitochondria (145). Interestingly, like PSMA, a large percentage of Her2/neu staining in prostate and breast carcinoma cells appears to be cytoplasmic in nature (58,59) and not localized solely at the plasma membrane. This observation would seem to suggest there is a significant amount of communication between the extracellular environment and intracellular organelles. It is likely that a monitoring mechanism exists which may modify the metabolic activity of the organelle and that a significant number of plasma membrane receptors, including Her2/neu and PSMA, may be involved in this process. While such a mechanism may not have been proposed ten years ago, a similar communication has been described between the mitochondria, peroxisomes and the nucleus (146) and during starvation, myocardial cells appear to have an adaptive interaction between the intracellular and extracellular environments (147). Additionally, many studies have been carried out in recent years on the organization of the extracellular matrix which have come to the conclusion that cells seem to be able to sense the macromolecular composition of the extracellular matrix and to modify their production of matrix components accordingly (148). These studies indicate that a considerable amount of communication occurs between the different cellular components. Therefore,
the suggestion that PSMA may be involved in subcellular communication is not without precedent.

Additional evidence of the interaction between PSMA and the mitochondria may be derived from the 40 kDa protein which was co-purified on a MAb 7E11-C5.2 affinity column. This protein was only co-purified when no detergent was added to the wash buffers on the affinity column which suggests that it was associated with PSMA but disassociated by detergent washes. This 40 kDa protein was not immunologically active by western blot analysis. When purified and sequenced by amino-terminal sequencing (performed at the University of Virginia protein sequencing laboratory), it was found to be 100% identical to the precursor form of the mitochondrial aspartate aminotransferase (m-AST) enzyme (149). The precursor form contains a short signal sequence at the amino terminus for recognition and transport to mitochondria which is cleaved off after transport to the mitochondria (150). Since only the precursor form was present, it may be possible that one role of PSMA involves the transport of mAST to the mitochondria.

The co-purification of the m-AST precursor molecule in addition to the localization studies suggested it may be possible that the transport of m-AST to mitochondria was dependent on a signaling event at the plasma membrane. It was theorized that serum starvation of LNCaP cells may result in the localization of PSMA solely at the cell membrane with serum stimulation causing PSMA to move from the cell membrane to the cytoplasm. However, serum starvation or stimulation of LNCaP cells failed to alter the staining pattern of PSMA. It is just as plausible that a signal at the cell membrane results in a sequestration of PSMA at the membrane and if the ligand for PSMA is not present in the tissue culture media PSMA would remain spread throughout the cell. In fact, a detailed examination of MAb 7E11-C5.3 staining of prostate tissue showed some ductal
epithelial cells to exhibit staining confined to the plasma membrane with no cytoplasmic staining (138). This staining pattern may reflect the presence or absence of a possible PSMA ligand which has not been duplicated in the in vitro studies.

The basic physical and biochemical analysis of the 7E11-C5.3 epitope on PSMA revealed that it was recognized in both a reduced and denatured form based on the activity of the antigen in mercaptoethanol and SDS respectively. The PSMA epitope was also highly susceptible to proteolytic digestion by a variety of proteases and susceptible to oxidation by periodate. These observations led to the initial conclusion that the MAb 7E11-C5.3 epitope on PSMA consisted of a glycopeptide (92). Treatment of antigens with periodic acid has been utilized successfully in the past to indicate the presence of carbohydrates in antigenic epitopes (95). In an attempt to determine the type of carbohydrate linkage in the 7E11-C5.3 epitope, the antigen was treated with sodium borohydride to cleave O-linked oligosaccharides and LNCaP cells were grown in the presence of tunicamycin prior to antigen preparation to inhibit the addition of N-linked polysaccharides to the nascent peptide chain in the endoplasmic reticulum. Neither of these more specific treatments had any effect on MAb 7E11-C5.3 activity which contradicts the original interpretation of the periodate results.

One possible explanation of these contradicting results was that the periodate or the acetate buffer utilized in the treatments was affecting the ability of the antigen to adhere to the assay plate resulting in falsely reduced measurements. To ensure that the periodate was actually affecting the integrity of the PSMA epitope, purified PSMA was treated with the acetate buffer or acetate buffer containing periodate prior to western blot analysis (Fig. 10, panel B). This analysis proved that periodate was able to directly inhibit binding of MAb 7E11-C5.3 to PSMA while the assay conditions and buffers had no effect at all.
Lectin binding experiments also indicated that carbohydrates were present in the epitope and that this carbohydrate moiety contained D-galactose in some form since only lectins which were specific for either monomeric or polymeric galactose were able to block MAb 7E11-C5.3 binding. But, as observed with the basic biochemical analysis, it was impossible to demonstrate specificity by either glycosidic digestion of PSMA or by competitive inhibition experiments using specific monomeric or polymeric carbohydrates or aminosugars. Therefore, significant contradictions remained in the biochemical analysis of the MAb 7E11-C5.3 epitope with some evidence suggesting a glycopeptide epitope and others indicating that there was no carbohydrate component.

While much of the biochemical characterization of the epitope remained ambiguous, it was clear that the peptide chain contributed substantially since proteolytic digestion abolished 7E11-C5.3 activity. Peptide epitope mapping was therefore attempted to establish the identity of the 7E11-C5.3 epitope. Evidence existed which suggested that the antigenic epitope was intracellular (151). Since there were only 19 amino acids in the intracellular domain, peptides were synthesized to span this domain. Importantly, all of the peptides utilized in this study were at least 85% pure as judged by reverse phase high pressure liquid chromatography (HPLC) analysis. The direct and competitive inhibition assays clearly demonstrated the MAb 7E11-C5.3 epitope was located within this intracellular domain. Complete mapping of the peptide epitope with peptides synthesized with sequential deletions from the C-terminal end proved the minimal reactive peptide consisted of the first six N-terminal amino acids (MWNLLH). There was a possibility the small peptides were not bound to the microtiter plates as efficiently as larger peptides. However, the competitive inhibition assay (Fig.
19) did not require the peptides to be bound and was an important control experiment to verify the observations in the direct binding assay.

The affinity of MAb 7E11-C5.3 for N1.19 ($K_a$ $1.7 \times 10^9$ M$^{-1}$) was approximately 6.8 fold lower than the affinity for the native PSMA glycoprotein ($K_a$ $1.16 \times 10^{10}$ M$^{-1}$) while the affinity of MAb 7E11-C5.3 for N1.6 ($K_a$ $2.3 \times 10^8$ M$^{-1}$) was 7.4 fold lower than the affinity for N1.19. The direct assays used in this study were a convenient means of assaying MAb binding to peptides, which would otherwise be unable to adhere to the plastic of the assay plates. However, the peptides were covalently linked to BSA which may significantly alter their conformation as compared to the native PSMA. It is not surprising nor unprecedented then that the affinity constants for the peptides and the native antigen are slightly different (152). This difference may be explained not only by the effects of crosslinking of the peptides to BSA as discussed above but also in differences between the linear synthetic peptides and the native PSMA molecule which may have a significantly different conformation and carbohydrate content which may act to stabilize the epitope. Additionally, modifications of the amino acids in the native protein, such as acetylation or other post-translational changes, might further stabilize the antigenic determinant of the native PSMA. Although the affinity of the N1.6 peptide may be lower than N1.19 and the native PSMA molecule for the same reasons as discussed above, the competitive inhibition studies suggested the smaller peptide does indeed have a lower affinity which most likely results from a destabilization of the antigenic determinant. Nevertheless, the epitope mapping studies clearly demonstrated that the epitope recognized by MAb 7E11-C5.3 is composed of the primary peptide chain in the intracellular domain of PSMA and that the minimal reactive peptide consists of the first six amino acids.
(MWNLLH). This reactive peptide is predicted to be located at the intracellular face of the plasma membrane (88) supporting the immunolocalization data. Epitope mapping data suggesting the epitope consisted of only the core peptide and did not contain a carbohydrate moiety would explain the failure of the sodium borohydride, tunicamycin, glycosidase treatments and specific carbohydrates to block the MAb 7E11-C5.3 activity. However, the periodate oxidation and lectin competitive binding experiments were still contradicting if the MAb 7E11-C5.3 only binds to the peptide chain. Although periodate oxidation has been successfully utilized to identify carbohydrate epitopes, oxidation of amino acids may also occur (96). The treatments utilized in this study (5 mM periodate for 1 hr. at 25°C) should have been mild enough to preclude damage to the polypeptide chain while non-reducing sugars and pyranosidically linked hexoses within the oligosaccharide chains would have been oxidized (97). Harsher conditions including longer incubations, higher temperatures, or higher periodate concentrations could have resulted in the destruction of the peptide chain in addition to any carbohydrate. Since methionine residues are particularly susceptible to periodate oxidation (96) the possibility existed that the PSMA peptide epitope was particularly sensitive to these treatments. This was particularly possible since all of the reactive peptides began with the very N-terminal methionine of PSMA making it even more subject to oxidative attack since there is no protection on the amino terminal end of this amino acid. Treatment of the reactive PSMA peptides (N1.19 and N1.6) with periodate did, in fact, abrogate MAb 7E11-C5.3 binding in an ELISA assay and by western blot analysis of a large molecular weight MAPS (multiple-antigen peptide system) N1.19 polymer indicating that the peptide chain itself was being oxidized.

The sensitivity of the reactive peptides to oxidation was further confirmed by either substitution or alteration of the methionine or the histidine residues.
resulting in a loss of antibody binding. Clearly, both the methionine and the histidine residues were essential for activity of these peptides and were affected by periodate supporting the conclusion that the MAb 7E11-C5.3 epitope on PSMA consists solely of the peptide backbone and does not contain a carbohydrate moiety.

Based on the peptide mapping experiments, an alternative mechanism for CYT-356 localization of prostate tumors in vivo may be that a second low affinity peptide epitope exists within the extracellular domain of PSMA. Several peptides containing a similar motif to the N1.6 minimal reactive peptide were identified (N470 [MYSLVH]; N344 [MHIHST]; N583 [MVFELA]; AND N664 [MNDQLM]), synthesized and tested for reactivity with MAb 7E11-C5.3. Although these peptides were not active by either a direct binding or a competitive binding assay, it is possible that CYT-356 may have a low affinity for any one of these peptides which might result in a detectable signal upon immunoscintigraphy. Clearly, more studies are needed to definitively determine how CYT-356 is able to image solid tumors.

While the peptide mapping experiments explained one of the conflicting results garnered from the biochemical characterization, the ability of lectins to inhibit binding of MAb 7E11-C5.3 to PSMA remained confusing. The ability of some lectins to block the MAb 7E11-C5.3 binding may have occurred as a result of non-specific steric hindrance since the native PSMA does contain a substantial amount of carbohydrate with the deglycosylated polypeptide having a Mr of 84,000 and the fully glycosylated molecule having an Mr of 100,000 (89). Additionally, only lectins with a specificity for D-galactose were able to block MAb 7E11-C5.3 suggesting that there may be a significant amount of galactose present on the native PSMA glycoprotein although not necessarily in the antigenic epitope. If a number of D-galactose containing oligosaccharides were in close proximity to the epitope, it would be possible for these carbohydrates to sterically hinder the binding of MAb 7E11-C5.3.
proximity but not directly in the antigenic epitope, the binding of lectins to these carbohydrate residues may mask the MAb 7E11-C5.3 epitope.

The expression of PSMA in the LNCaP cell line indicates that it remains the only prostate adenocarcinoma model which expresses any of the known prostate specific markers. None of the other prostate cell lines expressed PSMA or PSA and since there was no difference in the expression of PSMA between nude mouse tumors and cultured cell line extracts, in vitro cultured LNCaP cells represent a valid model for the study of PSMA. The 160 kDa band shown in figure 21 was rarely observed, but the 180 kDa band was frequently present in addition to the principal 100 kDa band. Two dimensional gel electrophoresis demonstrated that the two major species are very similar based on nearly identical isoelectric points and tailing reminiscent of membrane glycoproteins. Analysis of purified PSMA under different denaturing and reducing conditions suggested that the 180 kDa species may be either a dimer or a form which retains a significant amount of secondary and tertiary protein structure. The fact that SDS alone in the sample buffer resulted in only the 100 kDa band being detected suggests that the 180 kDa band represents an incompletely denatured form of PSMA. This non-denatured form may be a homodimer of two 100 kDa molecules, a heterodimer of one 100 kDa species and another unknown molecule (possibly m-AST) or simply a form with a significant amount of tertiary structure causing it to migrate at a higher than predicted molecular weight. Reducing agents were not sufficient to reduce the 180 kDa species to 100 kDa suggesting that there is not a significant amount of disulfide bonding in the native PSMA molecule. Polyacrylamide gel purification of both the 100 and 180 kDa bands followed by analysis on an additional western blot again demonstrated that the two species were identical since the purified 100 kDa band showed both the 100 and 180 kDa species while the majority of the purified 180 kDa band migrated at the lower molecular weight.
The fact that some of the 100 kDa band migrated at 180 following re-analysis argues against the possibility that the 180 kDa species is a heterodimer since the 100 kDa band was gel-purified prior to re-analysis. Therefore, the 180 kDa band most likely represents a dimer of two 100 kDa species or is a monomer with significant secondary and tertiary structure. This assertion was supported by the tryptic digestion of the purified bands followed by silver staining which showed identical banding patterns. While the 180 kDa could be eliminated from western blots if the SDS concentration in the sample buffer was at least 8%, this band was observed when the amount of protein loaded on a gel was high which argues that the 180 kDa band represents an incompletely denatured form of the predominant 100 kDa band. The possibility that the 100 kDa band represented a breakdown product of the 180 kDa species does not stand up in light of the facts that some of the 100 kDa band migrated at the higher molecular weight after re-analysis and that the PSMA cDNA encodes for a 100 kDa glycoprotein (89).

A predominant 120 kDa PSMA band was found to be present in prostate tissue extracts with an occasional observation of a second band of 200 kDa. Similar to the observations of PSMA in LNCaP cell extract, the 200 kDa species was most often seen when there was a large amount of the 120 kDa band suggesting a strong relationship between the low and high molecular weight components. The finding of a 120 kDa and 200 kDa bands was in contrast to the 100 kDa and 180 kDa bands found in crude membrane extracts and purified preparations from the LNCaP prostate carcinoma cell line. This slower mobility of PSMA in prostate tissue extracts may have resulted from a post-translational modification or an alternative splice variant. The fact that normal, BPH and CAP tissue extracts exhibited the same size components, either the 120 kDa band, the 200 kDa band or both, indicates the PSMA glycoprotein recognized by MAb 7E11-C5.3 is synthesized identically in normal and pathological prostate tissues.
Interestingly, a splicing variant of the PSMA mRNA has been reported. However, the smaller splice variant would theoretically result in a truncated protein lacking both the intracellular 19 amino acids and the transmembrane domain. Based on the epitope mapping discussed above, this smaller splicing variant cannot be detected with MAb 7E11-C5.3 since its epitope is found within the intracellular domain. It is interesting that the PSMA gene may be producing products which cannot be currently detected. Comparisons between the level of PSMA mRNA and the PSMA glycoprotein are made difficult and suggest that additional probes are needed to differentiate the full length PSMA and the theoretical truncated form of this glycoprotein. Since only one splicing variant has been found, the slightly different size of PSMA as detected by 7E11-C5.3 in prostate tissue and LNCaP cells most likely results from differences in post-translational modifications.

There were no apparent differences in the expression of PSMA between normal and malignant prostate tissues. The BPH tissues however, showed a lower level of the 100 kDa band and was variable from sample to sample. This variation is consistent with the observed reduction in PSMA mRNA (89) and PSMA antigen expression (138) in BPH specimens. The often low and variable expression of the 120 kDa band in the BPH specimens may have resulted from a greater expression of the smaller splice variant or post-translational modifications.

PSMA was reported to be detected in frozen sections of proximal tubules of normal kidneys (83), and Lopes et al. (84) described PSMA expression in cardiac and skeletal muscle by immunoperoxidase staining. The same immunostaining reactivity was observed in formalin-fixed paraffin embedded sections of these normal tissues (154). However, in the present study, PSMA was not detected in extracts of these same normal tissues by western blot analysis which suggests the immunoperoxidase staining was non-specific. This conclusion is consistent with the fact that 7E11-C5.3 antibody-isotope conjugates did not localize to skeletal
muscle in mice (8) or in monkeys (155). Immunoblot analysis did detect PSMA in extracts of normal cerebral cortex of the brain, normal salivary gland and normal small intestine. These latter results correlate with the finding of PSMA mRNA in brain and salivary gland and no PSMA mRNA in other normal tissues, including skeletal muscle (89). It is of interest to note that PSA has recently been found to be present in breast, colon, ovarian, parotid, kidney and liver tumors, normal breast, amniotic fluid and breast milk (21), as well as normal salivary gland (22) and normal endometrium (23). These results suggest PSA can no longer be regarded as being a specific biomarker of the prostate gland. In spite of the detection of PSMA by western blot analysis, PSMA has not been detected by immunostaining of frozen sections (83,84) or paraffin embedded sections (154) of normal brain, salivary gland and small intestine. This disparity may be the result of masked antigenic epitopes in the tissue sections or that the expression was below the detection limits of the immunohistochemistry assay. By using more sensitive assays, PSMA, like PSA, may eventually be found to be present in a variety of human tissues. The possibility that PSA may have a broader function than its association with semen liquefaction (21) points to the importance of determining the function of PSMA.

The finding that different species of PSMA were detected in brain (100 kDa) and salivary gland (120 kDa) support the hypothesis that the differences seen between PSMA in LNCaP cells and prostate tissue extracts may indeed result from a change in post-translational modification and is not an artifact of in vitro cell culture. The high molecular weight smearing and multiple banding pattern on the immunoblot of small intestine was a pattern markedly different from that which was observed in LNCaP cell extracts and prostate and non-prostate tissue extracts. The banding pattern was suggestive of a heavily glycosylated glycoprotein or mucin (156). It is possible that MAb 7E11-C5.3 binds to an epitope other than
PSMA in the small intestine or the smearing may be due to non-specific binding of the antibody. Since the secondary antibody (used without the primary MAb 7E11-C5.3) did not bind blots of small intestine, it appears likely that MAb 7E11-C5.3 specifically bound to a component in this extract. Additionally, PSMA may be glycosylated differently in the intestine resulting in the different banding pattern.

The significant detection of PSMA in the brain, salivary gland and small intestine by MAb 7E11-C5.3 raises obvious concerns regarding the efficacy and safety of using MAb 7E11-C5.3 immunoconjugates for diagnostic imaging and especially antibody targeted therapy. These cross-reactivities, however, should not dampen efforts to fully understand the function and clinical potential this novel biomarker may have in the diagnosis and therapy of prostate cancer.

PSMA was not detected in serum by Western blot analysis. This observation was surprising since previous reports have suggested that PSMA could be detected in serum by an immunoassay (83) and by western blot analysis (157). Immunoblot analysis of serum in the present study resulted in multiple bands similar to those reported previously by Rochon, et al. but only after the blots were overexposed to the X-ray film. They identified a band of 116 kDa as the PSMA component. In the present study, a band between 100-120 kDa was also observed. Importantly, although it did appear that this band was slightly overexpressed in pooled stage D2 serum compared to normal and BPH serum, the same banding pattern was observed in both normal female and male serum, suggesting these bands resulted from non-specific binding with either the primary or secondary antibody or that PSMA is present and shed into both male and female serum from common PSMA expressing organs.

The PSMA glycoprotein is relatively stable in serum since purified PSMA spiked into the pooled D2 serum was detected after 12 hours at 37° C at the correct molecular weight of 100 kDa. Therefore, the failure to detect PSMA does not
appear to result from degradation. Additionally, PSMA was not detected following the enrichment of serum over 200 fold by affinity chromatography suggesting that the failure to detect PSMA was not a result of a low concentration. When MAb 7E11-C5.3 was mixed with the specific PSMA peptide (N1.19) then used as a probe, the PSMA bands in the LNCaP cell extract were eliminated while all the bands in the serum samples remained. In fact, an identical banding pattern was observed when only the secondary antibody was used to probe the serum immunoblot. These results clearly suggest that the bands observed in serum are non-specific, probably the result of non-specific binding by the secondary antibody. Since Rochon, et al., did not compete the reactivity with purified PSMA or show results using only the secondary antibody, the 116 kDa band they reported as being PSMA is most likely a serum protein reacting with the secondary antibody. These results and conclusions do not negate the possibility that PSMA is shed into serum however. The failure to detect PSMA by western blotting may have been the result of degradation or masking of the antigenic epitope over long term exposure to the blood stream. Alternatively, PSMA may be shed into the serum by cleavage at the cell membrane leaving the transmembrane and intracellular domains which carries the MAb 7E11-C5.3 epitope in the cell which would negate its detection with 7E11-C5.3.

Since PSMA could not be detected in serum it was encouraging to note the detection of PSMA in seminal plasma which indicated that clinical assays for PSMA may be possible. The 120 kDa, the 200 kDa band or both PSMA components were found to be present in seminal plasma from normal donors and from patients with benign or malignant prostate tumors. The PSMA detected in seminal plasma from normal males and prostate carcinoma patients showed a consistent banding pattern while the banding pattern in seminal plasma from BPH patients varied greatly. A thorough statistical evaluation of PSMA detection in
seminal plasma will be needed to determine if any correlation exists between PSMA concentration and prostate pathology.

Thus far, this study has shown that PSMA exists in tissues and seminal plasma as a predominant 120 kDa band; in contrast, PSMA was found as a 100 kDa glycoprotein in extracts of LNCaP cells. Occasionally, a dimer form of PSMA having a molecular weight of 180 kDa (LNCaP cell extracts) or 200 kDa (tissue extracts, seminal plasma) was observed. The finding of PSMA in seminal plasma coupled with the overexpression of PSMA observed in poorly differentiated and metastatic prostate tissues (138), suggests that measurement of PSMA concentrations in seminal plasma might be used to predict and monitor tumor progression. Although Western blot analysis failed to detect PSMA in serum, further studies will be required to determine if any form of PSMA is shed in serum. The production of second generation antibodies against different antigenic epitopes other than the one recognized by MAb 7E11-C5.3 may be required to develop a highly sensitive immunoassay in order to determine if PSMA is or is not present in serum.

Model cell lines for the study of adenocarcinoma of the prostate are rare in contrast to the prevalence of cell lines from other malignancies such as breast (158-160) and colon (161-163) adenocarcinomas. Only a few well described immortalized prostate cell lines exist such as LNCaP (164), DU145 (165,166), and PC3 (167,168). Of these cell lines, only LNCaP retains the expression of the prostate specific biomarkers PSA and PSMA (169,170). Additionally, the slow growth of LNCaP cells is similar to the large majority of primary prostate tumors (171) making it the best in vitro prostate cancer model. The other prostate cell lines noted above all contain gross karyotypic changes, do not express prostate specific markers and have growth characteristics more similar to highly metastatic tumors than primary adenocarcinomas. While the Dunning rat ventral prostate
models offer an additional system to study prostate cancer, questions concerning
the validity of utilizing a model derived from an animal which rarely gets prostate
adenocarcinoma must be addressed. As a result, the LNCaP cell line represents
the best model for the study of prostate cancer. Unfortunately, the LNCaP cell can
not be utilized for the study of androgen regulated genes.

The level of androgen receptor expression in LNCaP cells is normal,
however, the use of LNCaP cells for the study of androgen regulated gene
expression is not an option since the receptor contains a point mutation which has
been shown to confer much different specificities and binding kinetics to the
receptor (177). Therefore, a model to study the expression of prostate specific
genes needed to be constructed which would allow for the control of the level of
wild type androgen receptor and the level of androgen hormones while keeping the
prostate epithelial cell environment intact.

In the present study, the PPC-1 cell line (178-180) was utilized as the basis
for the model system. Since this cell line did not appear to express the androgen
receptor (137) a subline of PPC-1 was acquired which contained an androgen
receptor expression plasmid. This subline (PPC-1AR5) overexpressed the wild
type androgen receptor as compared to LNCaP cells. The hypothesis was that
PPC-1 cells could be utilized to study the expression of genes in the absence of
androgen receptor while PPC-1AR5 could be used to study expression in the
presence of the androgen receptor. The assumption made in this model was that
neither the transfection of the androgen expression plasmid nor the selective media
altered the PPC-1 cells in any manner except to induce the expression of the
androgen receptor. Any changes to the cells which result from the androgen
receptor would be expected and desirable and should not interfere with the
interpretation of these data since the control of the expression of prostate specific
genes by the androgen receptor may be mediated in both a direct and indirect

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fashion. Therefore, it would be desirable to imitate as many of these androgen receptor mediated cellular changes as possible in PPC-1AR5.

Of considerable concern however, was the fact that the parental PPC-1 cells do not express the androgen receptor but grow quite well in tissue culture and in nude mice (179). Alternative pathways for the expression of prostate genes have been shown to exist (81,82) which may result in expression of some genes under conditions where they would not be expected to be expressed. Since the PPC-1 cells do not express PSA or PSMA, this concern may not be a valid one. However, these alternative pathways were taken into consideration during the development of the experimental design and were controlled for by utilizing the PSA promoter to ensure that androgen regulated genes were only expressed at a high level when both the androgen hormone and androgen receptor were present.

The level of androgen hormones in this model system were manipulated by first removing all of the steroid hormones from the media components by activated-charcoal dextran stripping. The stripping was carried out three times to ensure the majority of the hormones were removed and the level of androgen hormones in the media was below the detectable limits of an RIA assay. Nevertheless, very low molar concentrations of these hormones may have a significant impact on the expression of regulated genes (181). In the proposed model, if a small amount of these hormones were present and able to influence the expression of androgen receptor regulated genes, the PSA promoter would also be expressed and would indicate whether the model system was functioning as theorized. An additional concern was the fact that DHT, the active form of testosterone could be synthesized \textit{de novo} from a large number of testicular as well as adrenal hormones by a number of different pathways (182). The common step in all of these pathways is the final conversion of testosterone to DHT by the enzyme 5α reductase. To inhibit \textit{de novo} synthesis, Finasteride, a powerful
inhibitor of 5α reductase activity (183-186) was added to all of the media components to ensure that no DHT was synthesized within the model system.

Reconstitution of the androgen hormone levels was achieved by supplementing the media with the synthetic androgen R1881. This synthetic form of DHT has virtually the same kinetics of binding to the androgen receptor as DHT (187-190) but is much more stable. Therefore, the direct effects of androgen action could be studied without concern of the breakdown of DHT or the effects of the catabolites of DHT degradation on expression.

The model system designed for this study allowed for an elegant examination of the expression of androgen regulated genes by permitting the transfection of control and experimental constructs in all four possible variations (AR- DHT-; AR+ DHT-; AR- DHT+; AR+ DHT+) giving precise control of both the androgen receptor expression and the androgen hormone levels. The PSA promoter-CAT construct (PSA3CAT) was an integral part of this model system since use of this control allowed for the determination that the model system was working as hypothesized. If any or all of the possible problems as described above were manifested in the model, the PSA3CAT construct would have been transcribed at a high level under conditions other than when transfected PPC-1AR5 cells were treated with R1881.

The 1 kb 5’ fragment isolated from the human placental genomic library was clearly hybridized with a probe designed from the 5’ end of the PSMA cDNA. This 5’-flanking DNA fragment was found at the very end of the parental genomic clone and therefore, this study could not address any enhancer or other regulatory elements further upstream than -879. Partial DNA sequencing of the PSMA 5’ fragment showed that it did not contain a consensus TATA box, was not GC rich but did have an A-T rich region present at -30 to -60. Many TATA-less eukaryotic genes have been described in the literature (191-194). The overriding feature of
these TATA-less promoters is that the majority contain G-C rich regions and all have recognition sites for transcription factors. Although the 5' fragment isolated in the present study did not have a G-C rich region, the expression of PSMA mRNA has been shown to be stimulated or inhibited by a number of cytokines and peptide growth factors (195,196) arguing that the gene must contain recognition elements for transcription factors such as NFκB, AP1, and AP2. Many of the well characterized transcription factors are able to function without a TATA box in an unknown mechanism which is independent of an association with the TATA binding protein (TBP). Interestingly, the p53 tumor suppressor gene has recently been shown to upregulate both the typical TATA containing promoters and TATA-less promoters (197).

Reporter gene assays have been successfully utilized in the past to study promoter activity (198). Several different methods are commonly used but the most frequent assay is the CAT assay (199-202) which allows for the measurement of chloramphenicol acetyltransferase (CAT) activity following transcription and translation of the construct under the control of the experimental promoter. Since this assay usually measures the level of CAT enzyme activity, the conclusions regarding the promoter activity are based on indirect evidence since the promoter must first be transcribed, the transcript must be translated, and the translated protein must be enzymatically active. Standard CAT activity assays may yield significantly false results due to the relatively long half life of the CAT protein. As a result, the true transcriptional activity of the promoter may not be measured since these assays function at the post-translational level and when studying the hormonal regulation of a promoter, changes in transcription may be rapid and would be missed if only enzyme activity was measured. Other methods of measuring the expression of the reporter gene are to measure the CAT protein by western blot analysis or by directly measuring CAT mRNA levels. The
measurement of mRNA levels would allow for a specific and sensitive
determination of CAT expression at any given time point as a result of the rapid
turnover of mRNA compared to the CAT protein and the true level of promoter
activity can be directly measured. In the present study, a ribonuclease protection
assay designed to specifically measure CAT mRNA was employed to study the
regulation of the PSMA gene.

Transient transfections of the pCATbasic vector, the PSA3CAT,
PSMG4.22CAT and PSMG4.22RCAT constructs were carried out under all four
experimental conditions. The results from these experiments suggest that the
model system developed for this study functioned as hypothesized. The
pCATbasic vector transfections showed no expression of CAT mRNA while the
PSA3CAT construct was highly induced when both the androgen receptor and
R1881 were present. The low levels of PSA3CAT expression in the first three
conditions (Fig. 38, lanes 5-7) may have resulted from several different
possibilities. A basal level of expression may be present in the absence of
androgenic stimulation resulting from a leakage of the promoter which may be
endogenous or caused by the removal of PSA gene elements during construction of
the promoter fragment into the pCAT vector which are necessary for proper
transcriptional control. Alternatively, a very low level of androgen receptor
expression may exist in PPC-1 cells that was not detected by western blot analysis
which could have resulted in a low level of PSA3CAT expression. A low level
expression of the receptor, coupled by minute concentrations of steroid hormones
remaining in the stripped media could account for the level of expression seen in
this experiment. Importantly, the approximately 6 fold increase in CAT mRNA
expression in the presence of both the androgen receptor and androgen was very
similar to previously reported studies on the PSA promoter (132). Therefore, it
can be concluded that this model system functioned as intended and could be utilized to draw conclusions from experimental PSMA constructs.

The PSMG4.22CAT construct showed a low level of promoter activity and was not affected by changes in either the concentration of androgen hormone or androgen receptor levels. Importantly, when the PSMA fragment was cloned in a reverse orientation (PSMG4.22RCAT) there was no transcription of CAT mRNA. This pattern of PSMG4.22CAT transcription was unexpected since the PSMA mRNA in LNCaP cells has been reported to be downregulated in the presence of androgen hormones (89) and the PSMA glycoprotein may be slightly overexpressed in patients undergoing androgen ablation treatment (154), although definitive studies have yet to be carried out to substantiate both of these observations. However, since the PSMA gene does have a responsive element for androgen hormones (GRE), a differential expression of the PSMG4.22CAT construct was expected.

One explanation of this observation may simply be that there are additional elements within the PSMA gene necessary for proper transcription to occur. This possibility is likely in light of the fact that a 641 bp fragment cloned upstream of the CAT gene was incapable of transcribing the CAT mRNA (203) but when additional enhancers were added to the construct, CAT activity was detected (203). This observation argues that there are enhancer sequences between the 5' end of this construct (-565) and the 5' end of the fragment used in the present study (-879). Other enhancers either further upstream of -879 or within intron sequences of the PSMA gene may be required to mimic the in vivo transcription of the PSMA gene. Alternatively, additional GRE elements may be necessary since the synergistic effects of two GRE elements has been reported for other promoters (204).
An alternative explanation for the observations of PSMG4.22CAT transcription may be that androgen hormones are able to regulate the PSMA gene in an indirect fashion. This hypothesis is probable since direct androgen stimulation of responsive genes has only been reported to result in transcriptional upregulation and not inhibition (66-69) although indirect negative regulation has been described for TGF-β2 expression in LNCaP cells (205). A mechanism for indirect androgen suppression of transcription of PSMA may be that DHT upregulates the expression of other hormones which may negatively feedback on PSMA expression. The observation that the PSMA gene does not contain a consensus TATA box or G-C rich region makes it likely that recognition sequences for transcription factors are present within the promoter sequence. Many of the known transcription factors are highly responsive to cytokine and growth factor signals and may lead to the downregulation of PSMA following androgen stimulation. In fact, PSMA mRNA expression has been reported to be sensitive to several growth factors and cytokines (206). Alternatively, the lack of responsiveness to androgen hormones, even in an indirect manner, may be a result of the PPC-1 model. The PPC-1 cells utilized for the model system may lack the expression of the growth factors or the transcription factors necessary to properly regulate the PSMA gene. Alternatively, the responsive elements for these factors may not be present in the 5’ fragment utilized in this study. But, since all of the previous studious have been carried out in LNCaP cells, the results garnered in the present study may reflect the true transcription of the PSMA gene while the use of the LNCaP cell line may have resulted in aberrant conclusions since alternative pathways for upregulation (82) and downregulation (81) of the expression of androgen regulated genes has been described in LNCaP cells.

In spite of the incongruencies between the previously reported results of PSMA mRNA expression and the present study, the promoter activity of the
PSMA gene has been shown to be quite different from the simple regulation of the PSA gene. This observation suggests that the PSMA gene may be highly regulated by several different direct and indirect mechanisms. Such a high level of regulation of the PSMA promoter may make the use of this promoter for gene therapy applications difficult.

In conclusion, this study has added significant knowledge to the understanding of both the 7E11-C5.3 monoclonal antibody and PSMA. Furthermore, the results of this study support the concept that this glycoprotein is a unique prostate biomarker which may have promise as a target for novel diagnostic and therapeutic strategies. The impact of this study may be seen in the development of new second generation antibodies directed against the extracellular domain of PSMA in order to both enhance the \textit{in vivo} imaging and therapy approaches and determine if PSMA is actually shed into the serum. Although a serum assay for PSMA may add yet another tool for the screening and diagnosis of prostate cancer, it is unclear whether any improvements in detection could be achieved over the PSA assays currently available. Additionally, a more thorough understanding of the hormonal regulation of PSMA may allow for \textit{in vivo} manipulation of PSMA expression prior to monoclonal antibody directed therapy. Additional studies will be required to determine the function of PSMA. Nevertheless, this study has provided a solid foundation on which to build a more thorough understanding of MAb 7E11-C5.3 based imaging and therapy applications as well as functional studies of the PSMA glycoprotein.
CHAPTER V
SUMMARY

The objective of this study was to more fully understand the physical and biochemical nature of the PSMA glycoprotein recognized by the murine monoclonal antibody (MAb) 7E11-C5.3. The specific aims of this study were: (1) to determine the subcellular localization of PSMA; (2) to determine the biochemical and physical nature of the PSMA glycoprotein; and (3) to begin to understand the regulation of the PSMA gene.

1. To accomplish the first aim, the LNCaP cell line was analyzed by mechanical cellular subfractionation utilizing gradient density centrifugation and Western blot analysis to determine if PSMA is a cytoplasmic, nuclear or membrane glycoprotein. Light microscopy analysis utilizing multiple techniques including immunoperoxidase and immunofluorescence microscopy was undertaken to begin to determine the localization of PSMA within the cell. Then, immunoelectron microscopy of LNCaP cells was carried out to definitively determine the subcellular localization of PSMA.

2. The second aim was addressed by repeating previously reported experiments where necessary to confirm the initial observations. Once these results were verified, additional studies were carried out to explain the conflicting results garnered from this biochemical characterization. Specifically, the question of why MAb 7E11-C5.3 reactivity to PSMA was abrogated by periodate oxidation while assays to determine the specific carbohydrate failed to decrease activity. To address this question, the MAb 7E11-C5.3 antigenic epitope on PSMA was
mapped with synthetic peptides. The reactive peptides were then treated with periodate as described for the analysis of the native antigen to determine if they were susceptible to oxidation which would lead to the false conclusion that the epitope contains a glycopeptide.

The characteristics of PSMA expression in prostate cell lines, prostate tissues and exocrine products of the prostate gland, as well as non-prostate tissues and human serum was thoroughly examined by Western blot analysis. The physical characteristics of PSMA were also evaluated using polyacrylamide gel electrophoresis under differing conditions to determine the physical nature of the PSMA glycoprotein.

3. To begin to understand the regulation of the PSMA gene, a suitable model system had to be developed to study the expression of prostate specific genes. A model system was designed utilizing the PPC-1 primary prostate carcinoma cell line and which allowed for the control of both the level of androgen receptor expression and the concentration of androgen hormones in a prostate epithelial cell. The PSA promoter was cloned and utilized as a control for androgen upregulation in these experiments. In order to isolate the 5' flanking DNA from the PSMA gene, PCR primers specific for the PSMA cDNA were used to amplified probes for the PSMA gene. These molecular probes were used to screen a human cDNA library to isolate the PSMA cDNA. A restriction fragment encompassing 700 bp of the 5' end of the cDNA was then used to probe a human genomic library and several PSMA genomic clones were identified. The genomic clones were mapped and sequenced, and a fragment containing approximately 1000 bp upstream of the translational start site was cloned into a basic chloramphenicol acetyl transferase (CAT) reporter gene construct containing no endogenous enhancers or promoters. The PSMA 5' flanking DNA or the PSA control constructs were transiently transfected into the model system described.
above and assayed for CAT mRNA expression to definitively determine if PSMA was regulated by dihydroxytestosterone.

PSMA was found to be localized at the inner face of the plasma membrane and within mitochondria in LNCaP cells. The MAb 7E11-C5.3 epitope was determined to consist of only the peptide backbone of PSMA and localized to the intracellular domain with a minimal reactive peptide of 6 amino acids (MWNLLH). PSMA was detected in normal, benign and malignant prostate tissues as well as normal small intestine, brain and salivary gland indicating that this marker is not as specific as once thought. Additionally, PSMA was detected in seminal fluid but not in serum using MAb 7E11-C5.3. Finally, the promoter of the PSMA gene was cloned upstream of a reporter gene construct which was expressed in androgen and androgen receptor free conditions indicating that the regulation of PSMA is markedly different from that of the prostate specific antigen (PSA). This study has provided a solid foundation on which to build a more thorough understanding of MAb 7E11-C5.3 based imaging and therapy applications and continues to suggest that PSMA is a novel and important new prostate biomarker.
CHAPTER VI
REFERENCES CITED


45. Dube, J.Y., Frenette, G., Paquin, R., Chapdelaine, P., Tremblay, J.,
Tremblay, R.R., Lazure, C., Seidah, N., and Chretien, M. Isolation from
human seminal plasma of an abundant 16-kDa protein originating from the
prostate, its identification with a 94-residue peptide originally described as

46. Starling, J.J., Sieg, S.M., Beckett, M.L., Wirth, P.R., Wahab, Z.,
Schellhammer, P.F., Ladaga, L.E., Poleskin, S., and Wright Jr., G.L.
Human prostate tissue antigens defined by murine monoclonal antibodies.

47. Wright Jr., G.L., Beckett, M.L., Lipford, G.B., Haley, C.L., and
Schellhammer, P.F. A novel prostate carcinoma-associated glycoprotein
complex (PAC) recognized by monoclonal antibody Turp-27. *Int. J.

with restricted specificity for tumor-associated antigen of prostate and

49. Makar, R., Mason, A., Kittelson, J.M., Bowden, G.T., Cress, G.T., and
Nagle, R.B. Immunohistochemical analysis of cathepsin D in prostate


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137. Brothman, A. University of Utah. Personal communication.


155. Lopes, D., Cytogen Corporation, Princetown, NJ. Personal communication.


