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Table of Contents

Progress Report for DAMD17-00-1-0043: Development of a Biological Basis for PSMA Targeting in Prostate Cancer. W.D.W. Heston Ph.D. P.I.

Introduction:
PSMA is a strongly expressed protein in prostate cancer. It is a type two-membrane protein with a short 19 amino acid terminal piece inside the cell, a membrane-spanning region from amino acids 20 to 43, and the rest of the 750 amino acids residing outside of the cell. We discovered that PSMA has unique hydrolytic activity as a carboxypeptidase in releasing glutamate either from polygammaglutamated folate as a folate hydrolase or from N-acetylaspartylglutamate, NAAG, as a neuropeptidase. We observed that cells expressing PSMA were killed by polygammglutamated methotrexate while cells not expressing PSMA survived. This lead us to hypothesize those glutamated prodrugs that were substrates for PSMA may serve as therapeutic agents in the treatment of prostate cancer. Understanding the biology of PSMA function served as the basis of our application for funding.

Progress:
In aim one of our proposal we planned to characterize the structural, enzymatic, and transport activity of PSMA and PSMA like proteins for the rational development of biology based targeting strategies.

Our statement of work, was to clone enzymatically active PSMA and PSM' which could be used in x-ray diffraction: We have cloned enzymatically active PSMA. The cloned PSMA recombinant protein lacked the intracellular and transmembrane domains, but retained most of the extracellular domain and contained the folate hydrolase portion. The purpose of generating the recombinant PSMA protein was to focus on x-ray crystal diffraction. The PSM' protein that is the alternatively intracellular protein and is not glycosylated was found to lack enzymatic activity. Working with PSMA has been a major problem because the protein is extensively glycosylated and glycosylation interferes with x-ray diffraction structural determinations. To attempt to circumvent the problem, we have generated a number of mutants which have the glycosylation site mutated to see if we could identify a region in which we would have enzymatic activity without glycosylation. We have found that this is not possible. Even minor perturbations in glycosylation eliminate folate hydrolase activity. We have submitted a paper on this aspect to the journal *Prostate*, and it has been accepted for publication.

We were also to pursue an understanding of the biology of PSMA to better identify ways to approach the use of PSMA as a therapeutic target. We identified that the alternative spliced form of PSMA, PSM' lacks the transmembrane domain and is cytosolic. We initially worried about its presence because we believed that it would have activity and might interfere with prodrug activity since folate antagonists such as methotrexate are polygammaglutamated inside the cell for intracellular retention. To our surprise, we found that intracellular versions of PSMA lacked enzymatic activity. We have also discovered that the monomer form of PSMA has no enzymatic activity. It is only the dimer form of PSMA that has activity. This has major implications for PSMA

functioning as a target enzyme for prodrug activation, because one would want the tumor cells to have PSMA in the dimer form during prodrug activation. PSMA will change from one form to the other without this requiring a covalent bond as would occur with a proteins sulphydryl groups. Dimer-Monomer interchange is in a dynamic equilibrium. We do not yet understand what induces the change in forms. We will focus on identifying methods to increase the enzymatic dimer form of PSMA in prostate tumor cells.

The other internalization aspects of PSMA were also a focus of our studies. What is responsible for the internalization of the PSMA protein? We initially thought it was the dileucine motif of the short intracellular domain. It turned out that there is a unique motif, being MXXXL. This explains why the PSMA protein is found at the apical surface rather than the basolateral surface as would be predicted from the di-leucine motif. We continue to develop means to optimize protein internalization as well.

Aim two: In aim two we were to test polygammaglutamated derivatives for antitumor activity. We have been puzzled by the specificity of the hydrolytic function of PSMA. It is more restricted in enzymatic hydrolysis than we first believed, so that not all gamma-glutamated anti-folate derivatives are in fact substrates. We have examined the binding of methotrexate polygammglutamates and a number of substrates, agonists and antagonists, and they all confirm that there is almost no binding to the monomer form, and are in fact binding the dimer form.

We have tested methotrexate polygammaglutamate *in vivo* in animal models of PSMA expressing tumor cells and have observed growth inhibition and have much less toxicity of methotrexate polygammaglutamate when compared with methotrexate. We have observed growth suppression, but after three weeks of constant exposure, the animals began to develop gastro-intestinal toxicity to the methotrexate-polygammaglutamate. We are contemplating means to circumvent this toxicity with further enhancement of tumor selective delivery.

Key Research Accomplishments:

We have identified a recombinant form of PSMA that has allowed us to discover that PSMA is able to undergo spontaneous inter-conversion between a monomeric and a dimeric form that does not require a covalent modification. Of importance is that all of the enzymatic activity or binding of ligands or antagonists occur with the dimer form.

We have also observed that methotrexate polygammaglutamate is able to inhibit the growth of a PSMA expressing tumor *in vivo*, as a proof of principal of the prodrug concept, but that there is still toxicity associated with the polygammaglutamate form of methotrexate, even though the toxicity is substantially less than free methotrexate. Further study is necessary to achieve acceptable toxicity for the targeting of the prodrug we need to further increase the specificity of prodrug delivery.

Reportable Outcomes:
Ghosh, A., and Heston WDW: Effect of carbohydrate moieties on the folate hydrolysis activity of the prostate specific membrane antigen, (PSMA). *The Prostate*, accepted for publication.

O'Keefe, DS, Bacich DJ, and Heston WDW: Comparative analysis of Prostate Specific Membrane Antigen (PSMA) versus a Prostate-Specific Membrane Antigen-*Like* Gene. *The Prostate*, accepted for publication.

Conclusions: PSMA internalization requires a new internalization motif, MXXXL. This explains that the dileucine repeat is in fact not the motif functioning in PSMA's internalization and explains why PSMA is found at the cellular apical surface rather than the basal surface. We have identified that PSM' and other cytosolic versions of PSMA, are not enzymatically active and are unlikely to interfere with the pro-drug activation of PSMA. We have identified that even minimal changes in the glycosylation of PSMA eliminates enzymatic activity and thus reduces the likely hood that we will be able to develop a mutant that we will be able to determine the structure of the active site by X-ray diffraction studies.

Appendices:

Ghosh, A., and Heston WDW: Effect of carbohydrate moieties on the folate hydrolysis activity of the prostate specific membrane antigen, (PSMA). *The Prostate*, in press.

O'Keefe, DS, Bacich DJ, and Heston WDW: Comparative analysis of Prostate Specific Membrane Antigen (PSMA) versus a Prostate-Specific Membrane Antigen-*Like* Gene. *The Prostate*, in press.

Effect of carbohydrate moieties on the folate Hydrolysis activity of the Prostate Specific Membrane Antigen (PSMA)

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Running Title: Impact of N-linked glycosylation on PSMA protein

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Abstract:

Background: Prostate Specific Membrane Antigen or PSMA has been recognized as one of the important cellular marker for prostate cancer, the expression of which is enhanced many fold in prostate cancer and other tumor neovasculature. PSMA is a type II membrane glycoprotein, with a short cytoplasmic N-terminal region, a transmembrane domain and a 701 amino acid extracellular portion with 10 potential N-linked glycosylation sites. PSMA is a folate hydrolase, which cleaves terminal glutamates from poly- gamma- glutamated- folates; and NAALADase, which hydrolyses alpha-glutamate-linked dipeptide, N-acetyl-aspartyl-glutamate (NAAG) and is a glutamate carboxypeptidase.

Methods: In our study we have used various enzymes or site directed mutagenesis to remove sugar molecules from PSMA protein and studied its folate hydrolase function.

We have performed a biochemical characterization of N-linked glycosylation of the various mutant proteins.

Results: PSMA protein expressed in different prostate cancer cell lines is differentially glycosylated. Removal of sugar residues either enzymatically or by mutagenesis abolishes the enzyme activity of PSMA protein completely.

Conclusion: N-linked carbohydrate structures are important for the folate hydrolase function of the protein. Removal of sugars partially or completely causes PSMA to be enzymatically inactive, improperly folded and resulting in increased rate of degradation.

Introduction:

Prostate cancer is the second leading cause of cancer death in men (1). Tissue specific proteins serve as biomarkers of prostate cancer, have been used in screening, diagnosis and predicting disease progression. Prostate specific membrane antigen (PSMA), which was originally identified in LNCaP cells by its immunoreactivity with mAb 7E11-C5 is a membrane bound protein and has been designated as a target of imaging for prostate cancer (2). PSMA is expressed in normal prostate (2-4) as well as nearly 100% of prostate carcinomas (5). PSMA expression is found to be increased in higher-grade cancer, metastatic disease and hormone refractory prostate carcinoma (4,5). It has also been used to identify circulating prostatic tumor cells. In addition to prostate cancer, PSMA protein has been found to be strongly expressed in the tumor vascular endothelium of large number of solid tumors (6-8), while absent in the endothelium of the normal tissues. A gene for human PSMA (2.65 kB) has been cloned, sequenced and mapped to chromosome 11p11.2 (9). The DNA sequence reveals that the protein-coding region has 54% homology with human transferrin receptor (nucleotide region 1250-1700, located in the catalytic domain of the protein). The protein encoded by the PSMA gene is about 750 amino acids long, contains a short N-terminal cytoplasmic region of 19 amino acids, a 24 amino acid transmembrane domain and remaining 707 amino acid extracellular C-terminal fragment. PSMA has two important enzymatic properties: a) folate hydrolase activity, which hydrolyzes the γ-linked terminal glutamates from poly-glutamated folates. b) NAALADase or N-acetyl-aspartyl-L-glutamate carboxypeptidase activity, with hydrolysis of the terminal α-linked glutamate from important neuropeptide

NAAG. PSMA shows homology to a family of membrane carboxypeptidases, M28 (12). They contain 2 co-catalytic zinc metallopeptidase sites, wherein there are two side-by-side peptidase enzymatic "pockets" and two coordinated zinc ions. Prediction program [Predict protein (PHD) mail server utility] analysis of secondary structure predictions revealed six domains within PSMA protein (10). Domain A is the N-terminal cytoplasmic portion [including binding site of the 7E11.C5 antibody as well as dileucine repeat (internalization motif) found in membrane protein], domain B is the hydrophobic, transmembrane segment of PSMA including amino acids 20-39, domains C and D represents amino acids 40-144 and 173-248. Domain E is the catalytic domain (amino acid 275-596), domain F is of unknown function includes amino acids 597-756. There are linker regions rich in proline and glycine present between C & D (145-172) and D & E (249-273). The catalytic domain E contains two predicted zinc-coordinating sites. Asp^{387}, Glu^{425}, and His^{533} coordinate the first zinc atom. His^{377}, Asp^{387} and Asp^{453} coordinate the second zinc atom. The amino acid Asp^{387} coordinates both metal atoms, which are commonly found in cocatalytic peptidases. The remaining domain, F, has no known function, although in transferrin receptor it is the ligand-binding site. The protein has 10 potential N-linked glycosylation sites.

We have analyzed the role of sugar moieties on the enzyme activity of PSMA molecule. In this paper, we address the impact of N-linked glycosylation on the protein activity and protein stability.

Materials and Methods:

Cell lines and Reagents

PC3 cells (catalog number: CRl-1435), MDA PCa2b cells (catalog number: CRL-2422,), CWR22Rv1 (catalog number: CRl-2505) were obtained from ATCC. LNCaP C4-2 cells were obtained from Urocor Inc.

PSMA cDNA was originally cloned in our laboratory (4). The PSMA expressing construct, which has been used for this study is a pIRES –Neo construct of PSMA. Among the antibodies, J591 monoclonal antibody was obtained from Dr. Neil Bander, Cornell Weil College of Medicine, NY. The PM1T 485.5 monoclonal antibody was obtained from Hybritech Corp. ^{35}S Methionine was obtained from Amersham (#AG1094). Recombinant PSMA protein (human PSMA extracellular domain) was obtained from Progenics, Inc (Tarrytown, NY.).

Generation of glycosylation mutants by site-directed mutagenesis

Mutation of amino acid glycosylation sites was accomplished by Stratagene's QuickChange™ Site-Directed Mutagenesis Kit. Briefly, 100 ng of PSMA encoding cDNA (PSMA-pIRES-Neo construct) was taken; the plasmid was denatured at 95°C for 1 minute, the oligonucleotide primers containing the desired mutation were annealed to it. Using non-strand displacing action of *Pfu* Turbo DNA polymerase, the mutagenic primers were extended and incorporated (temperature cycling conditions: 95°C, 30 seconds; 55°C, 1 minute; 68°C, 12 minutes for 18 cycles). The nicked circular DNA, which was generated in this process, is nonmethylated, containing the desired mutation.

In the next step, the methylated, non-mutated parental template DNA was digested with Dpn1 at 37°C for 1 hour. The circular, nicked dsDNA was transformed into XL1-Blue supercompetent cells, which will repair the nicks in the mutated plasmid. The colonies, which appeared on the LB-amp plates were picked up, plasmid DNA was isolated and sequenced to check correct incorporation of the desired mutation. Following are the sequences of the primers used for making mutants. Primers were obtained from Invitrogen inc.

N336A (Asparagine at 336 position has been changed to Alanine)

5'senseN336A: CCTGGCTTTACTGGAGCCTTCTCTACACAAAAATC

3'senseN336A: GATTTTTGTGTAGAGAAGGCTCCAGTAAAGCCAGG

N459A (Asparagine at 459 position has been changed to Alanine)

5'senseN459A: CTCATCTATAGAAGGAGCCTACACTCTGAGAGTTG

3'senseN459A: CAACTCTCAGAGTGTAGGCTCCTTCTATAGATGAG

N476A (Asparagine at 476 position has been changed to Alanine)

5'senseN476A: GTACAGCTTGGTACACGCCCTAACAAAAGAGCTG

3'senseN476A: CAGCTCTTTTGTTAGGGCGTGTACCAAGCTGTAC

N638A (Asparagine at 638 position has been changed to Alanine)

5'senseN638A: CACTTTTTTCTGCAGTAAAGAATTTTACAGAAATTGCTTCC

3'senseN638A: CCAAGCAATTTCTGTAAAATTCTTTACTGCAGAAAAAAGTG

Preparation of cell membrane prep

Different prostate cancer cell lines endogenously expressing PSMA or PC3 cells transfected with different cDNAs of interest were harvested in PBS buffer. The cells (1x

10^8) were scraped off from the flasks into PBS and pelleted by centrifugation. The cell pellet was resuspended in 50 mM Tris buffer, pH 7.5. The pellet was homogenized manually with a Dounce Homogenizer and was centrifuged at 1000 X g at 4°C for 5 minutes. The supernatant was separated and ultracentrifuged at 70,000 X g for 35 minutes at 4°C. The supernatant was discarded and pellet was washed with 3 ml 20 mM Tris buffer, pH 7.5. The pellet was finally resuspended in 1.5 ml of 50 mM Tris HCl, pH 7.5, 0.1% Triton X100 and was homogenized for 30 strokes to break up the pellet. The pellet was transferred to 1.5 ml eppendorf tubes and was spun at 1000 X g for 10 minutes. The supernatant was collected as the source of triton extracted PSMA and stored at 4°C.

Endoglycosidase digestion

For enzymatic analysis, 2 μg of membrane preparations from different cell lines or 0.0002 μg of recombinant PSMA was incubated with Endo H (6mU/sample) or increasing concentrations of Endo F1 (16 mU, 32 mU and 48 mU/sample). Different samples were incubated overnight at 37°C and folate hydrolase assay performed.

Protein Analysis: Twenty μg protein equivalent membrane preparations from different prostate carcinoma cells lines endogenously expressing PSMA protein or PC3 cells transiently transfected with cDNAs encoding WtPSMA or its various mutants were harvested in lysis buffer (50 mM Tris.HCl, pH 7.5, 0.5% Triton X100) and subjected to treatment with a panel of glycosidases. They are either buffer treated control WtPSMA or treated with Endoglycosidase H (6 mU/sample), PNGaseF (5U/sample), glucosaminidase (8 mU/sample), β-(1-4)galactosidase (3 mU/sample), Endo-o-glycosidase (2.5 mU/sample), Sialidase A (5 mU/sample). The protein samples were treated with different glycosidases, overnight, at 37°C, analyzed on SDS-PAGE, western blotted onto PVDF

membranes and detected with PM1T 485.5 antibody (primary), followed by goat-anti-mouse HRP (secondary) and detected by ECL. Different glycosylated and deglycosylated PSMA bands have been shown by arrows.

Estimation of protein concentration and western blot analysis

The protein concentration of the whole cell extract or the membrane preparations were estimated by BCA assay. For Western blot analysis, about 50 µg of protein was analyzed on SDS-PAGE and western transferred onto a PVDF Immobilon-P membrane (Millipore). The blots were incubated in blocking buffer (3% fatty acid free BSA and 5% goat serum) for 1 hr followed by incubation with J591(8) in 3% fatty acid free BSA in Tris buffered saline containing 0.5% Tween 20 (TBST) at 1:1000 dilution. The Blots were washed thrice in TBST for 5 mins each followed by incubation with peroxidase conjugated-goat anti-mouse secondary antibody [Immunopure goat anti mouse IgG (H+L)] 1:5000 dilution in 3% BSA-TBST. The blot was developed with ECL Chemiluminescent detection reagent (Amersham Pharmacia Biotech).

Folate Hydrolase activity assay

2µg of protein (in 50 mM Tris-HCL, pH 7.5, 0.5% Triton X100) from membrane preparations were incubated in the presence of 50 µM 4-Amino-10 methylpteroyl-di-3-glutamic acid (MTXglu2) (Schircks Laboratories, Jona, Switzerland) 50 mM Tris.Cl, pH 7.5 for total volume of 100 µl for 1 hour at 37°C. Reactions were quenched with 100 µl of Na_2HPO_4 (0.5M). The reaction mixtures (50µl) is separated by Reversed-Phase HPLC with PRISM RP (5µM, 50 x 4.6 mm) (Thermo-Hypersil Keystone Scientific Operations, Bellefonte, PA) with a mobile phase of 85% 0.05M KH_2PO_4, pH 7.0 and 15% methanol.

The UV absorbance was detected at 313 nm. The peak area was calculated by ESA CoulArray Software (ESA Inc., Chelmsford, MA) for $MTXglu_2$ and MTX. The Folate hydrolase activity is reported as the average of three reactions per sample and is expressed as (nmoles of glu released)/mg of protein/hr.

NAALADase activity assay

10 μg equivalent protein from PC3 cells transfected with cDNA encoding WtPSMA or its different mutants were taken for NAALADase assay. The samples were harvested in the lysis buffer containing 50 mM Tris-HCl, pH 7.5, 0.5% Triton X-100 and the protein was quantitated using BCA protein assay reagent (Pierce, Rockford, IL). 10 μg equivalemt protein was incubated at 37°C for 1 hrs in a total volume of lysis buffer containing 4 μM N-acetyl-L-aspartyl-L- (glutamate-3, 4 - 3^H) (Perkin Elmer life sciences, Boston, MA). The reaction stopped by the addition of an equal volume of 0.5M NA_2HPO_4. The liberated glutamate was separated using ion-exchange chromatography as previously described (11). Briefly, half the reaction was layered over poly-prep prefilled chromatography column packed with AG-1-X8 formate resin, 200-400 mesh (Biorad, Hercules, CA) that had been pre-wet with distilled water. The column was washed with 2 ml of 1N formic acid, and the free-glutamate eluted in 2.5 ml of 1N formic acid followed by measurement using scintillation spectrometry.

Immunofluorescence analysis by Confocal microscopy

For immunofluorescence analysis, the PC3 cells were seeded onto glass coverslips at the density of 1×10^4 cells and were transiently transfected with cDNAs encoding WtPSMA or its various mutants. At 48 hours post transfection, the cells were washed twice with

PBS, fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature. The cells were washed with PBS twice. The cells were permeabilized with PBS containing 0.2% Triton X-100, for 10 minutes at room temperature and washed with PBS thrice. The coverslips were washed with PBS three times and blocked with 3% BSA, 5% goat serum in TBST (0.05 M Tris.HCl, pH 7.6; 0.138 M NaCl, KCl 0.0027 M), followed by incubation with primary antibody PM1T 485.5 in 3% BSA in TBST (Tris buffered saline with 0.05% Tween 20). The coverslips were visualized with secondary antibodies, biotinylated-goat-anti-mouse antibody and streptavidine-FITC subsequently. The coverslips were mounted on vectashield and visualized under confocal microscope.

Pulse labeling analysis

Briefly, 10 x 10^6 PC3 cells were seeded onto different wells of 6 well - dish and transfected with cDNAs encoding WtPSMA or its different glycosylation mutants with lipofectamine 2000 reagent (Invitrogen Inc.) At 48 hrs post-transfection, the cells were starved with methionine minus RPMI 1640 medium (Sigma, R7513) for 30 minutes, followed by labeling with 50 μCi of ^{35}S methionine supplemented labeling media for 1 hr, followed by chasing with cold methionine supplemented media (with 2% dialyzed fetal calf serum) for different periods of time 0, 2, 4, 8, 24, 48, 72 hrs respectively. The cells were harvested at the end of different chasing period with RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxy-cholate, 0.1% SDS, 50 mM Tris.HCl, pH 8.0). 100 μg equivalent protein from each time point was immunoprecipitated with PM1T485.5 antibody and analyzed by SDS-PAGE followed by western blotting onto PVDF membrane (Millipore). The blots were developed with primary antibody,

PM1T485.5 antibody (Hybritech) at 1:1000 dilution, followed by secondary antibody goat-anti-mouse HRP (1:1000) dilution and developed with ECL reagent (Amersham).

Results:

Significance of carbohydrate structures on the enzymatic activity of PSMA protein

PSMA is a type II globular membrane glycoprotein and has 10 potential glycosylation sites (Fig. 1) and they are distributed among various domains. Asn^{51}, Asn^{76}, Asn^{121}, Asn^{140} are located in domain C; the fifth residue Asn^{153} is located in the linker region between domains C and D. The sixth residue Asn^{195} is located in domain D; 7^{th} (Asn^{336}), 8^{th} (Asn^{459}) and 9^{th} (Asn^{476}) are located in the catalytic domain. The 10^{th} sugar attachment site (Asn^{638}) is located in domain F. To test the effect of glycosylation on the enzyme activity, we have pursued a strategy to deglycosylate the protein by Endoglycosidase H and F1. These enzymes cleave between GlcNAc of N-linked chitobiose units of high-mannose and hybrid type of glycans. In addition, EndoF1 can not efficiently hydrolyze fucose containing hybrid oligosaccharides (12). Following the treatment we tested the folate hydrolase activity of the protein. We have found that the overnight digestion with Endo H removes the sugar residues from the protein with the result of PSMA becoming enzymatically inactive (Fig 2B). The western blot analysis of the overnight Endo H (6 mU/20μg of protein) treated PSMA protein (membrane preparation of C4-2 cells endogenously expressing PSMA) showed a band with faster mobility compared to control (incubated with Endo H buffer) (Fig 2A), whereas the Endo F1digestion did not remove the sugar residues either partially or completely (no change in the band mobility of PSMA, Fig 2A) even with increasing enzyme concentrations (16mU, 32 mU and 48 mU respectively) indicating that PSMA expressed in C4-2 cells is composed of fucosylated oligosaccharides. Endo F1 treatment did not alter the folate hydrolase

activity of the protein (Fig 2B) significantly. This indicates that PSMA protein expressed in C4-2 cells is Endo H sensitive but Endo F1 resistant.

We have also tested the Endo H and Endo F1 cleavability of the recombinant PSMA protein expressed in Chinese hamster ovary cells (CHO) (Fig 3). As we observed before, the recombinant PSMA protein was Endo H sensitive, but Endo F1 resistant (Fig 3A). Endo H treatment reduces the folate hydrolase activity of the protein (Fig 3B), but exhibits an increase in enzyme activity following treatment with increasing concentration of Endo F1 (16mU, 32 mU and 48 mU/2μg of protein). Treatment of protein with 48 mU of endoF1 showed an increase of about 25% of folate hydrolase activity compared to untreated control.

Role of Glycosylation inhibitor on the biosynthesis and enzyme activity of PSMA

To further establish that glycosylation is important for the enzymatic activity of the protein, we have expressed the protein in presence of specific glycosylation inhibitors. The PSMA protein encoding cDNA was transiently transfected into PC3 cells and synthesized in presence of tunicamycin (10μg/ml), which potently inhibits the first step in the N-glycosylation pathway. As seen in the western blot (Fig 4B, lane 2), the apparent molecular weight of PSMA expressed in the Tunicamycin treated PC3 cells was lower than that of the protein produced without the inhibitor. The various deglycosylated forms produced by the tunicamycin treated PC3 cells showed that the N-glycosylation inhibition was not complete, but those various PSMA-specific immuno-reactant protein species present in the whole cell extract did not have any enzyme activity (Fig 4B, "Tun" activity profile), nor did they go to the cell surface as the membrane preparations of

tunicamycin treated cells did not show any immuno-reactant PSMA band as compared to untreated PSMA control (Fig 4A, lane 2) which again proves that deglycosylated protein does not translocate to the membrane.

Sugar composition of PSMA protein varies in different prostate carcinoma cell lines

Once we determined that sugar structure associated with PSMA protein is important for its enzymatic activity, it was important to characterize the nature of carbohydrate structures associated with PSMA protein obtained form different prostate carcinoma cells lines. To test this, the native form of PSMA protein (endogenous PSMA from membrane preparations of C4-2, CWR22Rv1, MDA PCa2b cells) was treated with a panel of endo and exo-glycosidases: Endo H, PNGase F (cleaves between Asn and the innermost GlcNAc of glycan), β-glucosaminidase (cleaves all non-reducing terminal β-linked N-acetylglucosamine, β-(1-4) galactosidase (cleaves terminal Gal β1→ 6>4>>3 GlcNAc residues), Endo-o-glycosidase (cleaves ser/thr-linked unsubstituted Gal β(1-3) GalNAc-α-disaccharides, Sialidase (Neuraminidase, cleaves only non-reducing terminal unbranched alpha (2-3) sialic acid. This revealed a pattern, which changes with different cell types (Fig 5). PSMA/C4-2 and PSMA/CWR22Rv1 showed sensitivity to EndoH digestion (Fig 5, lanes A2 and C2) as well as PNGase F digestion (Fig 5, lanes A3 and C3), whereas PSMA/MDA PCa2b showed resistance to EndoH digestion (lane B2). PSMA/C4-2 showed a band mobility shift with glucosaminidase (lane A4) indicating that it is sensitive this enzyme, but not with β (1-4) galactosidase, endo-o-glycosidase, sialidase (lanes A5, 6 and 7). PSMA/MDA PCa2b was sensitive to sialidase (lane B7),

but resistant to other three enzymes (glucosaminidase, β (1-4) galactosidase, endo-o-glycosidase).

Generation of PSMA N-glycosylation mutants (Asn→Ala)

PSMA protein has 10 potential glycosylation sites; in order to determine which sugar attachment site is important to the enzymatic activity of the protein, we choose to mutate the putative residues in the catalytic domain of the protein (Asn^{336}, Asn^{459} and Asn^{476}) and Asn^{638} residing in the domain F of the protein. By site-directed mutagenesis, we changed the asparagines to alanines and expressed them following transfection of PC3 cells. PC3 cells were chosen because they do not express endogenous PSMA protein. The western blot of the whole cell extracts of the PC3 cells expressing different mutant proteins showed difference in mobility of the different mutant proteins (Fig 6A) indicating that they have different sugar attachment or modifications associated with them. Moreover, the mutants N336A, N459A and N476A could be detected with J591, but not the mutant N476A indicating that epitope for J591 could be the carbohydrate structure associated with the N476A. However all four mutants could be detected with PM1T 485.5 (Hybritech) which recognizes the peptide backbone and thus detection of mutant proteins have been accomplished with PM1T 485.5 antibody. Two μg protein equivalent cell extract was used for the folate hydrolase assay demonstrates complete lack of enzyme activity of the mutants (Fig 6B) as compared to control. Similar observation was made with NAALADase activity of the mutant proteins as well (Fig 6C).

Cellular localization of PSMA and its various mutant proteins analyzed by confocal microscopy

WtPSMA exists as a cell surface glycoprotein in various cell lines. The expression of WtPSMA and its various mutants were investigated by confocal microscopy using J591 antibody (Fig 7). We have confirmed that PC3 cells were negative with this antibody (data not shown). WtPSMA and glycosylation mutant N459A showed cell surface distribution. N476A and N638A showed localization to intracellular organelles as well. The mutant N336A was observed to be mostly intracellular with very little or barely detectable membrane expression.

Nature of carbohydrate structures associated with PSMA and its mutant protein in PC3 cells

In order to determine the nature of sugar residues associated with PSMA and its various glycosylation mutants, we have performed Endo H digestion of different proteins (expressed in PC3 cells) overnight and analyzed by western blot analysis and detected by PM1T 485.5 antibody. We have found that WtPSMA, N476A and N638A were resistant to Endo H treatment, whereas, N336A and N459A were sensitive to the Endo H digestion (Fig 8). WtPSMA protein when expressed in PC3 cells revealed two specific closely migrating bands indicating different glycosylated forms of the protein, and upon Endo H treatment, the band pattern did not change. This indicates that the WtPSMA in PC3 cells is resistant to Endo H and composed of complex higher order sugar structures. The mutants N476A and N638A also showed two bands and which remained unchanged upon Endo H treatment indicating these two mutant proteins are also like their WtPSMA

counterpart composed of complex higher order sugar structures. However N476A undigested control band profile is different from that of WtPSMA and N638A, in having the upper glycosylated band as the predominant species, which remains unchanged upon Endo H treatment. N336A showed a single species, which upon digestion with Endo H showed a lower molecular weight band indicating that it is sensitive to Endo H treatment and composed of high mannose type of sugars. N459A showed two bands, which migrated as a low molecular weight species following Endo H digestion indicating that this mutant protein is also composed of mostly high mannose type of sugars.

Biological stability of PSMA and its N-glycosylation mutants in PC3 cells

In order to study the effect of N-glycosylation on protein stability, the half life of the PSMA mutants was determined by pulse-chase experiments. Transfected PC3 cells were pulse-labeled for 1 hr with [^{35}S] methionine, then chased with an excess of unlabeled Met for various times. Following termination of the reaction, wild-type and mutant PSMA were immunoprecipated. The immunoprecipitants were resolved by SDS/PAGE and detected and quantitated by phosphorimaging. As shown in Fig 9, WtPSMA is stable and having a half-life of 55 hrs. In contrast, the biological stabilities of glycosylaton mutants were dramatically reduced, but to different extents. N336A showed average half-life of 2 hrs; N459A, 4 hrs; N476A, 10 hrs and N638A, 55 hrs.

Discussion:

PSMA protein which has been identified and cloned a decade ago (4,13) is a type II membrane glycoprotein, contains 10 potential N-linked glycosylation sites. PSMA has two important enzymatic functions, Folate hydrolase activity and NAALAdase activity (14). An alternatively spliced cytosolic form of PSMA lacking N-terminal cytoplasmic and transmembrane regions has also been identified in normal prostatic tissue and termed as PSM' (15,16). It has been shown by both RNA and protein analysis that PSMA expression increases several fold cancerous tissue when compared with that in normal, tumor or benign hyperplasia (17-19). PSMA is also present in tumor associated neo-vasculature. The reason for several fold increase in expression of membrane- bound glycosylated form over cytosolic form in advanced prostate cancer tissues is not known. PSMA is a target for therapy (based on the location of the protein in prostate cancer and tumor associated neovasculature of other solid tumors). In this context it is very important to study the importance of sugar structures associated with PSMA protein, its relationship to the folate hydrolase function of protein and its role in progression of prostate cancer. In our present study we have focused on the folate hydrolase function of PSMA and the role of sugar moieties on the novel enzymatic functions of this protein. We have used the native form of PSMA protein as it is expressed endogenously in various prostate carcinoma cell lines, ectopically expressed PSMA in PC3 cells (PC3 cells normally do not express PSMA) as well as recombinant PSMA protein (44-750 amino acids) expressed in CHO cells as our protein source. Endoglycosidase H sensitivity/ resistance of PSMA protein revealed that PSMA is mostly composed of N-linked sugars, which are of high mannose type or fucosylated hybrid mannose type.

Apparent Endo F1 resistance of PSMA protein indicates that the sugar structures are mostly the fucosylated hybrid mannose type of linkage. Endo H can hydrolyze fucosylated hybrid mannose type of sugar with equal efficiency as high mannose type. However core -linked fucose reduces the hydrolysis rate of Endo F1 by over 50 fold relative to high mannose structures (12). This shows that PSMA in C4-2 cells is composed of fucosylated hybrid mannose type of glycans. Removal of sugar residues abolishes PSMA's enzymatic activity completely, whereas the control untreated protein incubated with Endo H or Endo F1 buffer did not inhibit enzyme activity (Fig. 2). The same observation was true for the recombinant secretory form of PSMA protein, which showed that in CHO cells, PSMA protein is composed of fucosylated hybrid mannose type of sugars (Fig. 3). The reason for the apparent 25% increase in the folate hydrolase activity of the recombinant protein treated with 48mU of Endo F1 as compared to control buffer treated protein is not known. Endo F1 (at high concentration) has probably cleaved the fucosylated recombinant PSMA Protein partially (a slightly faster mobility of the protein band compared to control) resulting in opening the substrate pocket. The sensitivity of PSMA protein from other prostate carcinoma cells lines to the treatment with different glycosidases was found to be different. found to be different. For example, PSMA/C4-2, PSMA/CWR22Rv1 was found to be sensitive to EndoH indicating that it could be composed of either high mannose type or fucosylated hybrid mannose type sugars (Fig. 5). Unlike PSMA/C4-2, PSMA/MDA PCa2b (Fig. 5), PSMA expressed in PC3 (Fig. 8) cells were composed of complex higher order structures (based on their resistance to EndoH). PSMA/MDA PCa2b cells are sensitive to sialidase indicating terminal sialic acid structures are present. Lack of cleavage by endo-o-glycosidase

indicates that o-linked structures are not present. Resistance to β (1-4) galactosidase indicates that polylactosamine structures are not present. PSMA protein from all cell types that we have tested so far, are sensitive to PNGaseF leading to complete deglycosylation of the protein leaving a 85 kD protein band which is equivalent to the predicted deglycosylated protein form of PSMA indicating the bulk of the sugar attachment is N-linked. In addition, these cells also have other sugar modifications as well. We have found that J591 antibody (6) could not detect the completely deglycosylated form of the protein (complete removal of sugar by PNGase F digestion), indicating that epitope for J591 could be the sugar structure associated with the protein. The deglycosylated protein could be detected with PM1T 485.5 antibody. For all our protein analysis we have used the PM1T 485.5 antibody, which recognizes the peptide backbone of the protien. It will be important to analyze whether prostate cancer cells are glycosylating PSMA differently than that of normal prostate or endothelial cells expressing PSMA in other solid tumors.

N-linked sugars are important for the folate hydrolase activity of the PSMA is further established by synthesizing the protein in presence of N-linked glycosylation inhibitor, tunicamycin, which showed that protein synthesized are defective in glycosylation [95 kD (minor) and 85 kD (major)] are completely inactive in enzymatic function. Moreover such defective glycosylated forms of PSMA do not make it to the cell surface.

Once we established that PSMA is composed of N-linked sugars and that the sugar residues are important for its enzymatic activity, and that different prostate carcinoma cell lines composed of different type of sugar structures, it was pertinent to

study the structure-function of PSMA protein in terms of its glycosylation. We also wanted to determine which sugar attachment sites are important for the enzymatic function of protein. With this goal in mind we have mutated the Asn residues to ala at the catalytic site of the protein (Asn^{336}, Asn^{459} and Asn^{476}) and found that the mutation of any of them abolishes the enzymatic activity of the protein completely (both folate hydrolase and NAALADase activities). N459D mutant has been reported to change in migration pattern and specific activity of the protein compared to Wt control, (20). We have mutated another potential glycosylation site N638A and found that this mutation also abolishes the folate hydrolase and NAALADAse activities of the protein. Asn^{638} is located in a domain adjacent to the catalytic domain of the protein (domain F), which probably changed the conformation of the protein making it enzymatically inactive.

Further biochemical analysis of mutant proteins revealed more dramatic changes on the physiological properties and the function of the proteins. The mutations in sugar attachment sites dramatically alter their biological stability (Fig. 9). Previously, biological stability of PSMA had not been determined. We have expressed WtPSMA or the mutant proteins into PC3 cells by transient transfection and found that absence of only one of the N-glycosylation in the catalytic site was sufficient to reduce the stability of the protein. Wt protein has a half-life of 55 hrs whereas the mutant proteins have dramatic reduction in their half-lives (2 hr for N336A; 4 hrs for N459A and 10 hrs for N476A) except for the domain F glycosylation mutant N638A. Although N638A mutant showed lack of enzymatic activity, the biological stability of this protein was comparable to that of Wt protein (55hrs). Apart from lack of enzymatic activity, N638A protein showed all characteristics of Wt protein, in terms of stability, Endo H resistance, cellular

localization apart from perturbing the specific conformation of active-site, other structural parameters of this protein remained same like WtPSMA. Mutant proteins also showed variation in terms of their EndoH sensitivity, N336A and N459A are retained in the ER, whereas N476A and N638A are resistant to EndoH, indicating that they have complex carbohydrate structures.

Impact of N-glycosylation site mutations on the stability of proteins have been reported several instances e.g., mannose-6-phosphate receptor (21), transferrin receptor (22) and acetylcholinesterase (23) and Marpin A (24). It could be possible that oligosaccharide side chains protect the protein against protease-mediated degradation [(e.g., Interferon-γ, glycans at Asn^{25} is important for protease resistance, (25)]. Another possible explanation could be that the mutations affect the folding and oligomerization process, resulting in prolonged stay in ER and an increased proteolytic breakdown in this compartment which could explain the dramatic reduction in the stability of PSMA mutant proteins.

Analysis of the carbohydrate structures of PSMA protein from LNCaP cells and in vivo specimens have been reported by Holmes et al (16) where it has been shown by endo- and exoglycosidase analysis that PSMA is mainly composed of high mannose kind of sugars. We have extended these observations and analyzed in terms of enzymatic activity of protein and found that sugars are important for the enzymatic activity of the protein. Our analysis also shows that unglycosylated or partially glycosylated versions of PSMA proteins are not enzymatically active (lower molecular weight forms produced in tunicamycin treated cells), which also makes a possibility that alternatively spliced isoform of PSMA protein, PSM' is enzymatically inactive. Barinka et al (26) has

expressed truncated cytosolic version PSMA protein and found it to be enzymatically inactive. Likewise detailed analysis of carbohydrate structures associated with PSMA protein in different cancer tissues, tumor neovasculature will help us understand the biology of prostate cancer better as it progresses into metastasis and will pave the way for developing better targeting agents.

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Figure Legends:

FIG. 1: Schematic diagram of PSMA protein showing different potential sugar attachment sites.

PSMA is a globular protein with 10 potential glycosylation sites, which have been denoted here by arrows and numbers. Based on the model proposed by Rawlings and Barrett (1997), the PSMA has a region of similarity to M28 peptidase family of metalloproteases and according to the prediction, there are 6 individual domains in the PSMA protein denoted by A, B, C, D, E and F. Domain A is the intracellular cytoplasmic domain, domain B is the transmembrane domain, domains C,D, E and F are extracellular domains. Domain E is the catalytic domain for coordinating Zn^{2+} ions. Different glycosylation sites are distributed across different domains of PSMA protein. They are as follows: 1: Asn^{51}, 2: Asn^{76}, 3: Asn^{121}, 4: Asn^{140}, 5: Asn^{153}, 6: Asn^{196}, 7: Asn^{336}, 8: Asn^{459}, 9: Asn^{476}, 10: Asn^{638}.

FIG. 2: Sugar residues are important for the enzymatic activity of the PSMA protein.

A. showing the western analysis; B, showing the folate hydrolase assay of different samples treated with Endo H and Endo F1 (panels marked with Endo H and Endo F1 respectively) along with the buffer treated control. 20 μg membrane preparations of C4-2 cells were taken as the source of protein and and treated with EndoH (2 mU/sample) and EndoF1 (1), (2) and (3) are 16mU, 32mU and 48 mU of enzyme/sample respectively, overnight at 37°C. The protein were analyzed on SDS-PAGE, western blotted onto PVDF membrane and detected with J591 antibody.

B. Folate Hydrolase assay: 2 μg protein equivalent membrane prep was used for folate hydrolase assay. The samples were separated on reverse phase HPLC and data has been plotted using ESA Coularray Software.

FIG. 3: Sugar attachments are important for the enzymatic activity of recombinant PSMA.

The western analysis (A) and folate hydrolase assay (B) of Endo H and different concentrations of Endo F1 (1, 2, 3 are for 16 mU, 32 mU and 48 mUenzyme/0.0002 μg of protein respectively) treated samples are shown

FIG. 4: Folate hydrolase assay of different membrane preparations glycosylated and deglycosylated PSMA proteins.

The PC3 cells were transfected with cDNAs encoding PSMA. After 24 hours post-transfection, the cells were grown in presence of different N-glycosylation inhibitors, Tunicamycin (10 μg /ml) for 12 hours. The cells were harvested after 36 hours post-transfection. 20 μg protein equivalent whole cell lysate or membrane preparations were analyzed on SDS-PAGE and western blotted onto PVDF membrane. 2 μg protein equivalent (membrane preparations) or 20 μg protein equivalent (for whole cell extract) were taken for folate hydrolase assay. A: showing the protein analysis and the folate hydroalse activity profile of the membrane preparations from control PC3 cells expressing WtPSMA (lane 1 for Protein expression and "control" for enzyme activity), Tunicamycin (10 μg /ml) treated PC3 cells expressing PSMA (lane 2, for protein

expression and "Tun" for enzyme activity). B: showing the western blot analysis and folate hydrolase analysis with the whole cell extracts of the same as mentioned above.

FIG. 5: Analysis of sugar composition of PSMA proteins from different prostate carcinoma cell lines.

Membrane preparations from different cell types have been used which are: C4-2 (A), MDA PCa2b (B) and CWR22Rv1 (C). Different lanes showing treatment of different membrane preparations with panel of glycosidases. They are control undigested (lane 1); Endoglycosidase H, 6mU treated (lane 2); PNGaseF , 5U (lane 3); glucosaminidase (lane 4); β- (1-4) galactosidase, 3 mU/ sample (lane 5); Endo-o-glycosidase, 2.5 mU/sample (lane 6); Sialidase A, 5 mU/sample (lane 7). The protein samples were treated with different glycosidases, overnight, at 37°C, analyzed on SDS-PAGE, western blotted onto PVDF membranes and detected with PM1T 485.5 antibody. Different glycosylated and deglycosylated PSMA bands have been shown by arrows.

FIG. 6: Expression and analysis of different glycosylation mutant proteins.

A: PC3 cells were transiently transfected with WtPSMA and its various mutant proteins and detected by western analysis with PM1T 485.5 antibody. Different lanes showing different proteins, lanes1: WtPSMA, 2: N336A, 3: N459A, 4: N476A, 5: N638A. B: Folate hydrolase profile of different mutant proteins compared to control WtPSMA and expressed in terms of (nmoles of glu released/mg of protein)/hr. C: NAALADase profile of WtPSMA and different mutant proteins.

FIG. 7: Nature of carbohydrate structures of different glycosylation mutants based on their sensitivity to Endo H.

50 μg protein equivalent of whole extract of PC3 cells expressing WtPSMA or its different mutant proteins were treated with 6mU of Endo H overnight, at 37°C, and analyzed on SDS-PAGE, detected by western blotting with PM1T485.5 antibody. The different glycosylated and unglycosylated forms of PSMA are indicated by arrows. The control untreated PSMA or its mutant proteins were indicated by "-" sign and the Endo H treated proteins were denoted by "+" sign.

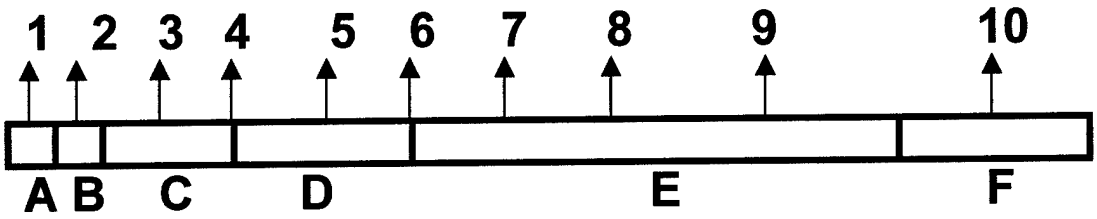
FIG. 8: Biological stability of PSMA and its different mutants.

PC3 cells transiently transfected with different cDNAs encoding WtPSMA or its different mutants were pulsed with ^{35}S methionine containing medium for 1 hour, followed by chase with medium supplemented with cold methionine and harvested after different periods of time, namely 0, 2, 4, 8, 24, 48 hrs respectively. The samples were immuno-precipitated with PM1T485.5 antibody and ran on SDS-PAGE and analyzed by phosphoimager analysis. Different band intensities were measured determine the half-lives of different proteins (Table 1).

FIG. 9: Immunofluorescence microscopy of different mutants expressed in PC3 cells.

The PC3 cells (grown on coverslips) transiently transfected with PSMA and its various mutants were fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature, followed by permeabilizing with PBS containing 0.2% Triton X-100 for 10 minutes at room temperature. The coverslips were washed with PBS three times and

blocked with 3% BSA, 5% goat serum in TBST (0.05 M Tris.HCl, pH 7.6; 0.138 M NaCl, KCl 0.0027 M), followed by incubation with primary antibody PM1T 485.5 in 3% BSA in TBST (Tris buffered saline with 0.05% Tween 20). The coverslips were visualized with secondary antibodies, biotinylated-goat-anti-mouse antibody and streptavidin-FITC subsequently. The coverslips were mounted on vectashield and visualized under confocal microscope.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A
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D
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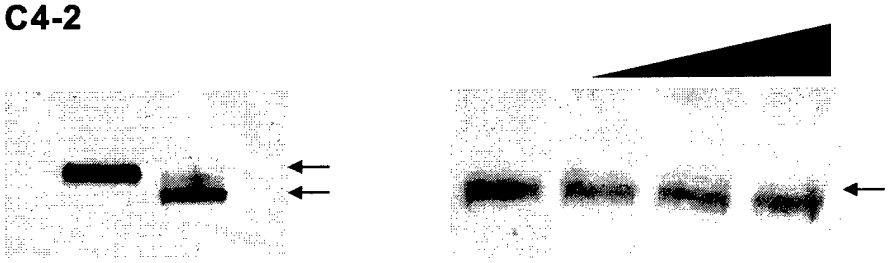
A.

C4-2



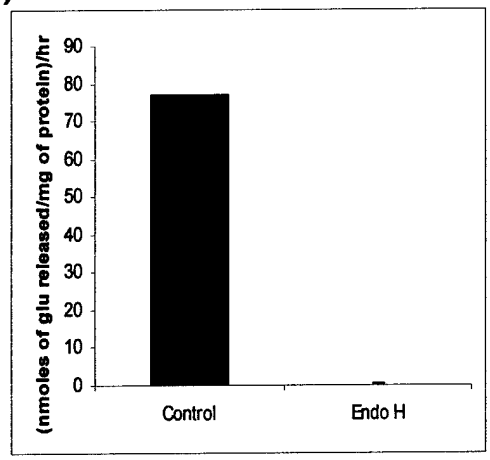
EndoH - +

EndoF1 - + + +

Detection by J591

B.

a)



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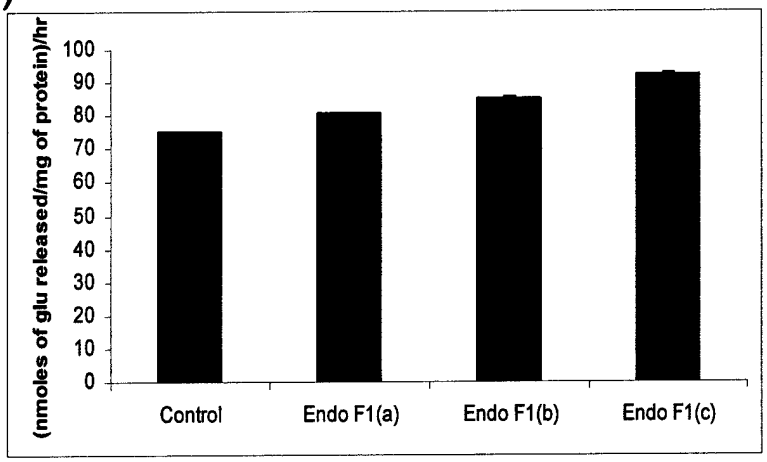

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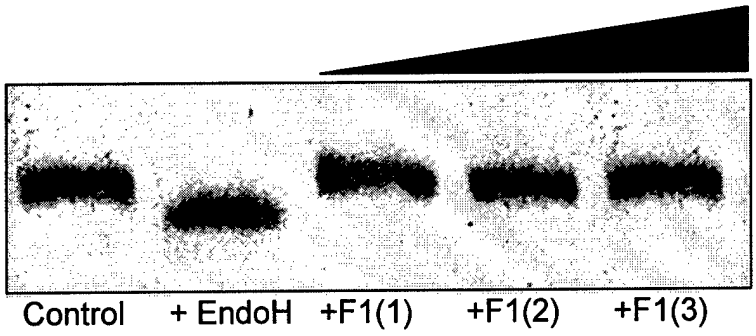
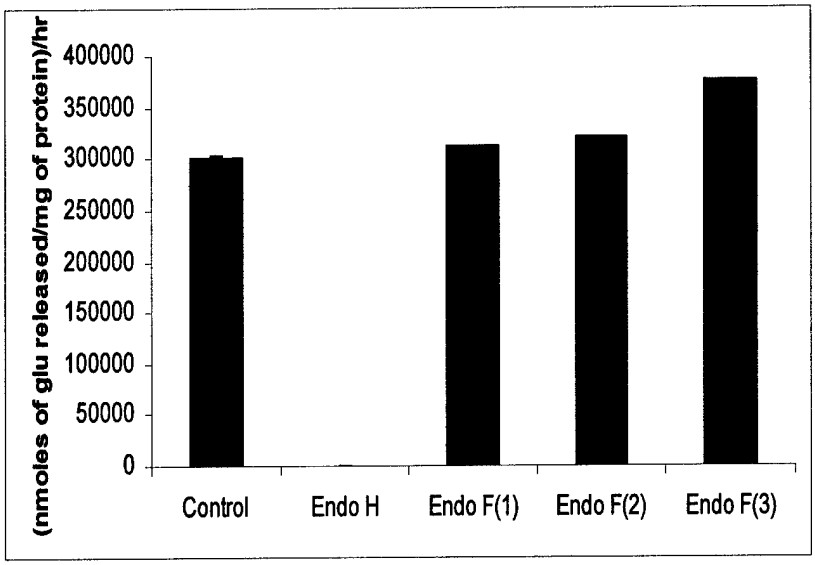

Recombinant PSMA
Control
+ EndoH
+F1(1)
+F1(2)
+F1(3)

B.


(nmoles of glu released/mg of protein)/hr
400000
350000
300000
250000
200000
150000
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50000
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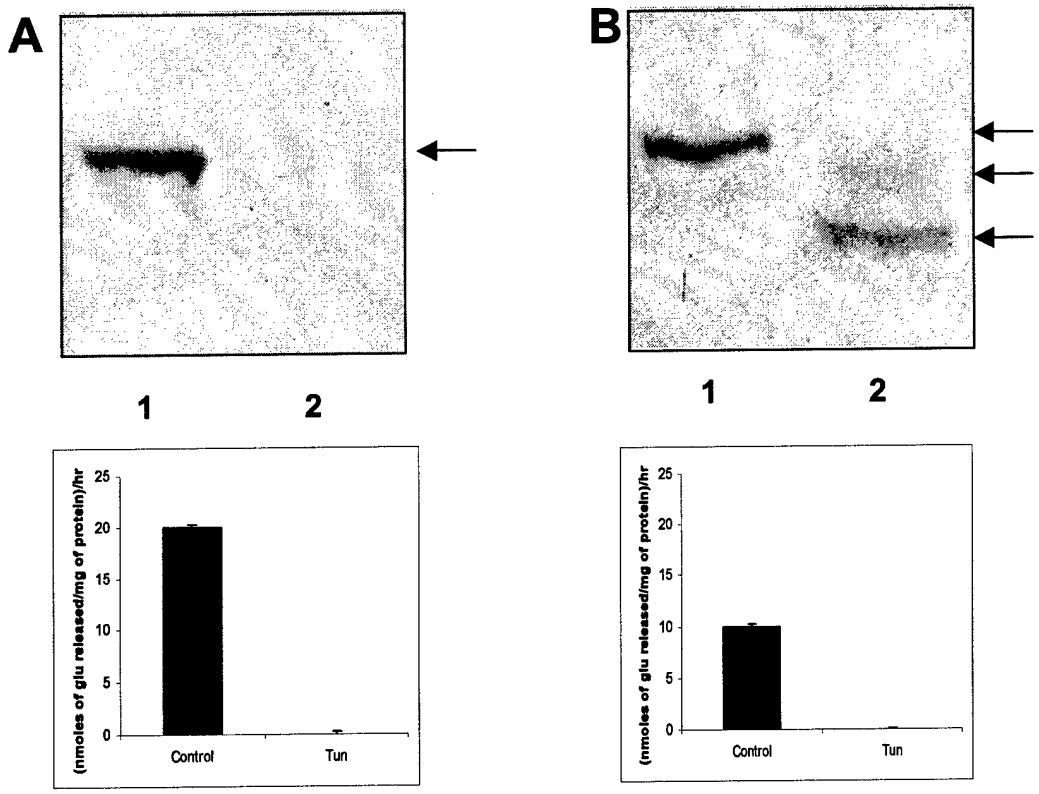
Control
Endo H
Endo F(1)
Endo F(2)
Endo F(3)


A
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(nmoles of glu released/mg of protein)/hr
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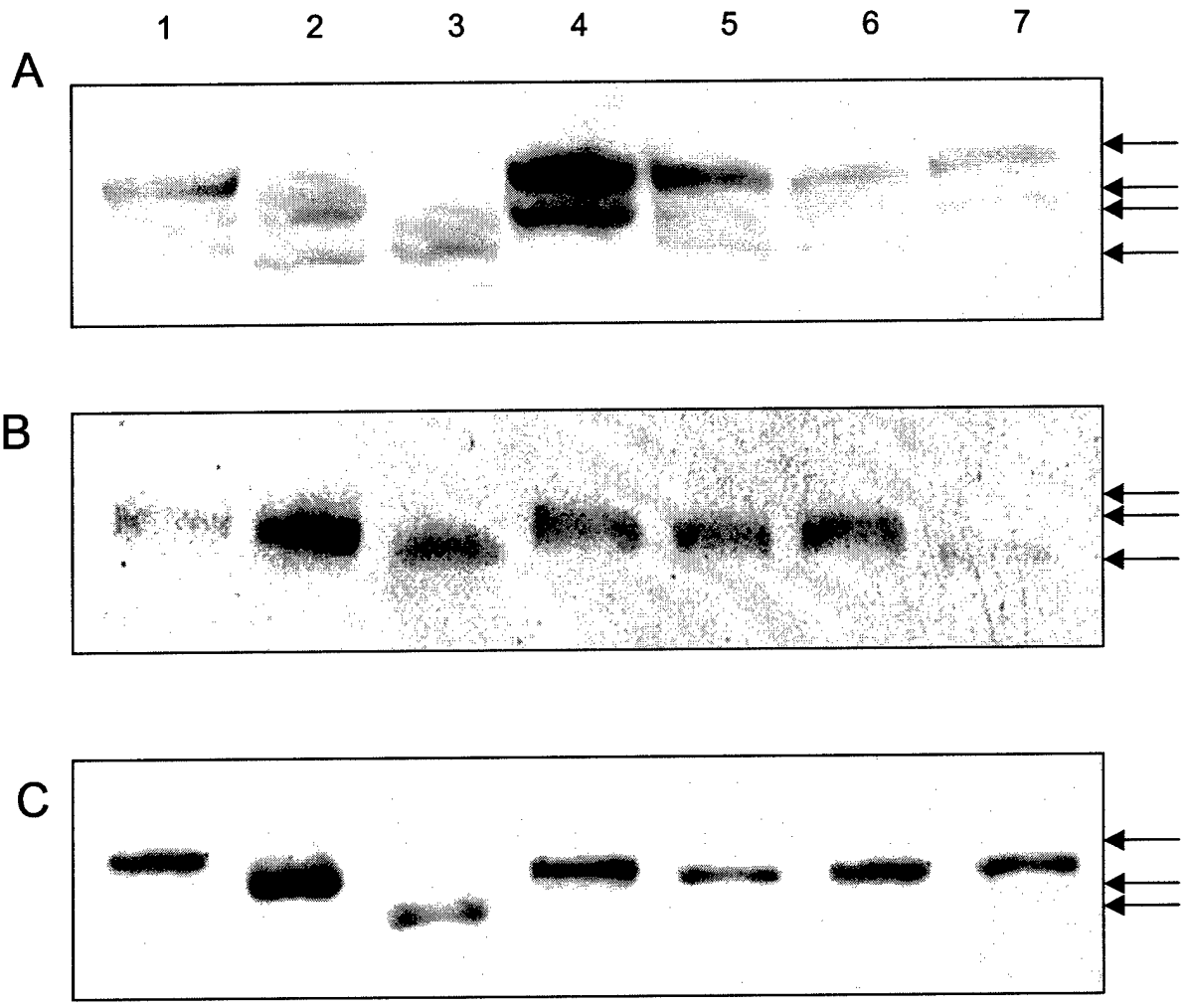
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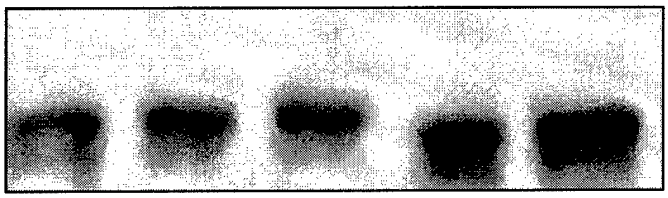
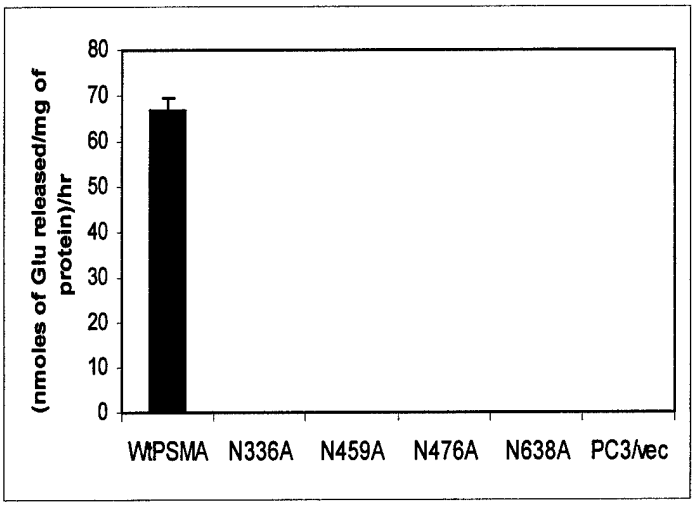
A

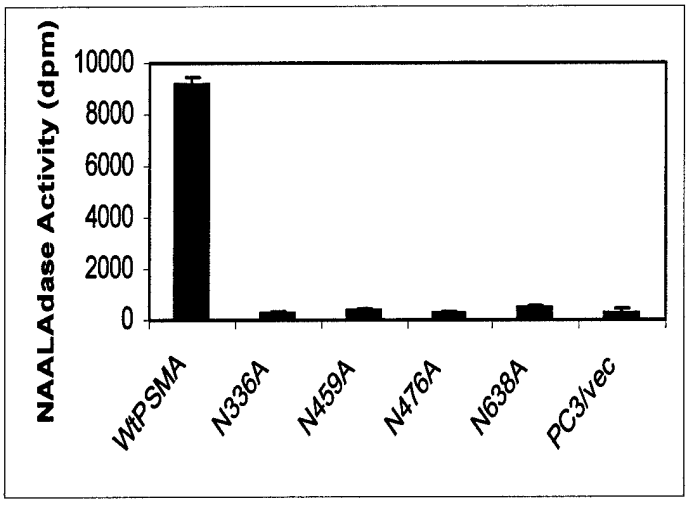


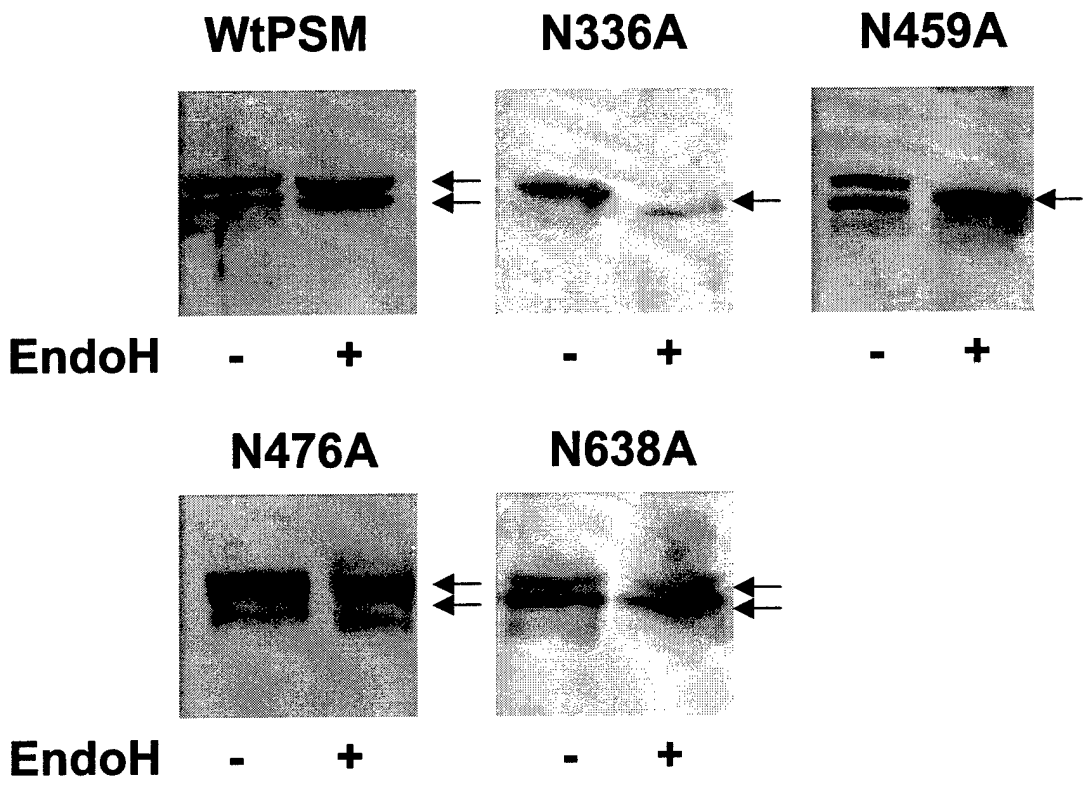
1 2 3 4 5

B


(nmoles of Glu released/mg of protein)/hr
80
70
60
50
40
30
20
10
0
WtPSMA
N336A
N459A
N476A
N638A
PC3/vec

C


NAALAdase Activity (dpm)
10000
8000
6000
4000
2000
0
WtPSMA
N336A
N459A
N476A
N638A
PC3/vec


WtPSM
N336A
N459A
EndoH - + - + - +
N476A
N638A
EndoH - + - +

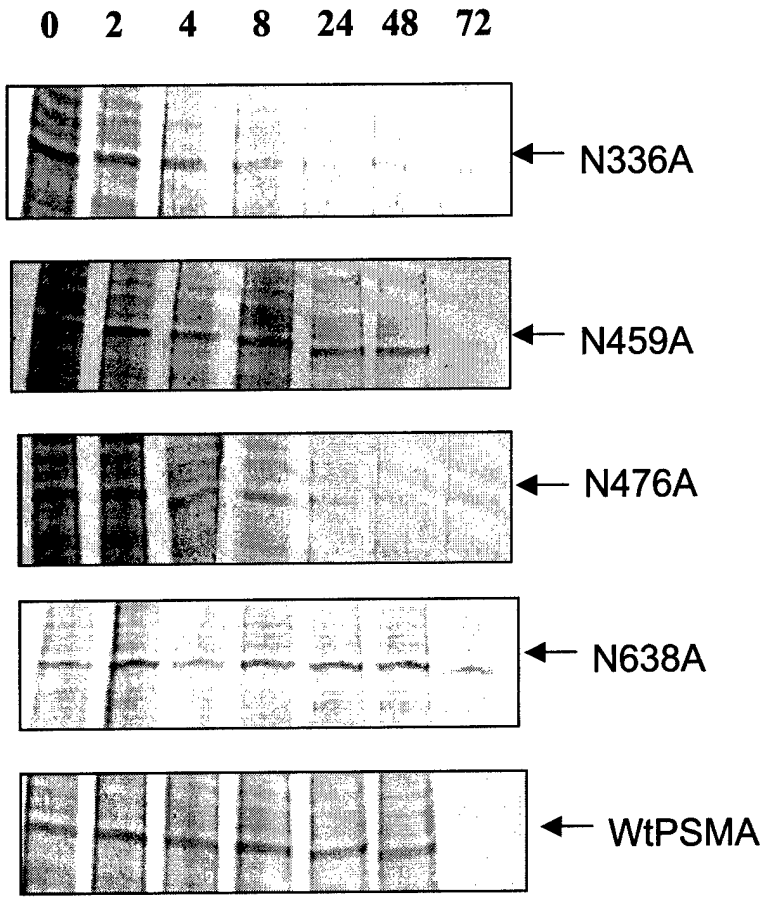
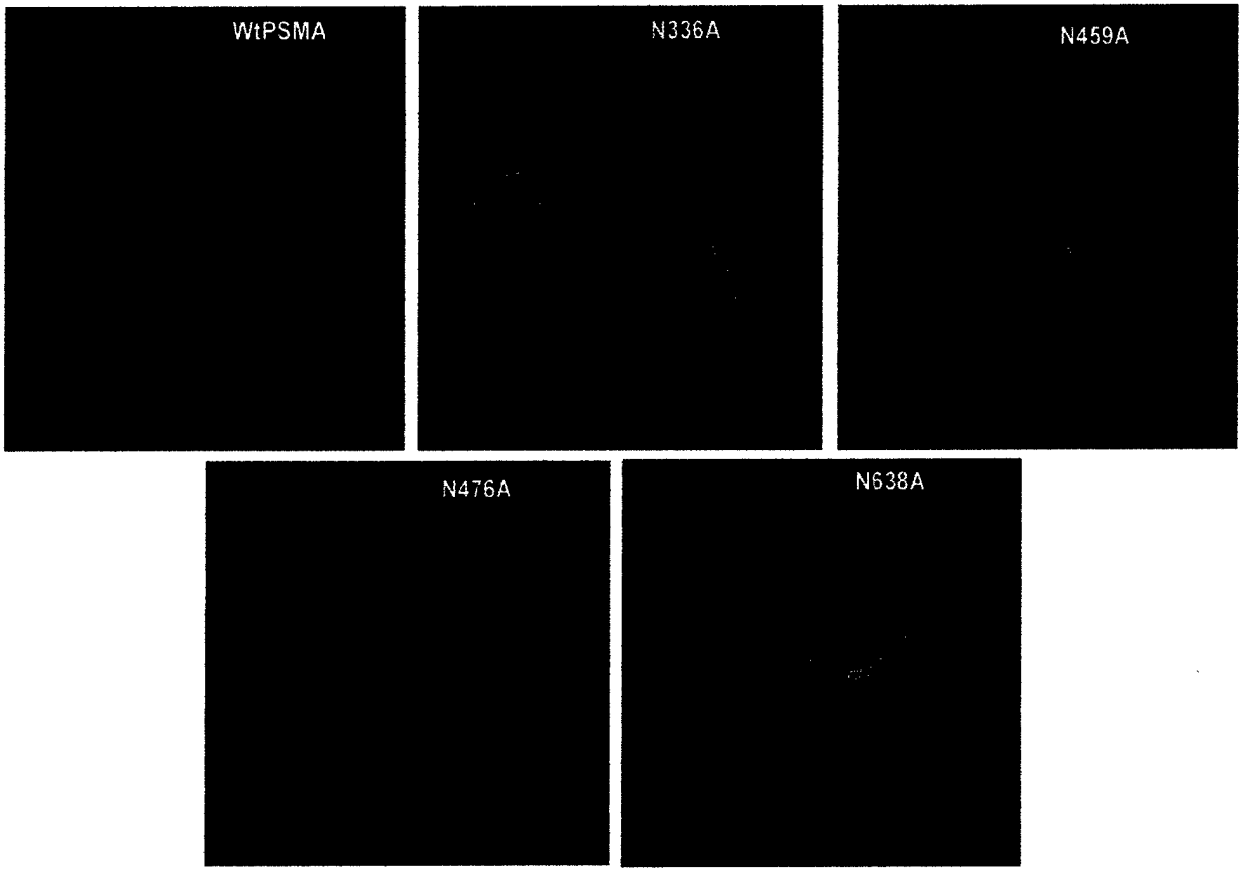


Table1:

Protein	Half life
WtPSMA	55 hrs
N336A	2 hrs
N459A	4 hrs
N476A	10 hrs
N638A	55 hrs


WtPSMA
N336A
N459A
N476A
N638A

Comparative Analysis of Prostate-Specific Membrane Antigen (PSMA) versus a Prostate-Specific Membrane Antigen-*Like* Gene

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ABSTRACT

BACKGROUND: Currently PSMA is showing promise both as an imaging and therapeutic target for occult prostate cancer metastases. First generation antibodies against PSMA are used for the FDA approved Prostascint™ monoclonal antibody scan and second generation antibodies are being developed for therapeutic targeting as well as imaging (1). However, there have been reports describing PSMA expression in non-prostatic tissues including kidney, liver and brain. As we had previously showed the existence of a human PSMA homolog, we set out to determine if this non-prostatic expression was due to expression of the PSMA or another gene. METHODS: The PSMA homolog (PSMA-Like) cDNA was cloned by screening a liver cDNA library. mRNA expression of the PSMA and PSMA-Like genes was determined via northern analyses using two different probes and protein expression confirmed in some tissues via western analysis. Transcriptional regulation of the two genes was examined using reporter constructs driving luciferase expression. RESULTS: The PSMA-Like gene possesses 98% identity to the PSMA gene at the nucleotide level and is expressed in kidney and liver under the control of a different promoter to the PSMA gene. The PSMA gene is expressed in several human tissues and is most abundant in the nervous system and the prostate. CONCLUSION: The non-prostatic expression of PSMA should be taken into consideration when designing clinical strategies targeting PSMA.

Key Words: Cancer, tumor-vasculature, folate hydrolase, therapeutics, naaladase

INTRODUCTION

Prostate-Specific Membrane Antigen (PSMA), also known as Folate Hydrolase 1 (FOLH1) is an ideal potential target for use in determining patient management and therapeutic strategies against prostate cancer. It is highly expressed in both localized and metastatic prostate cancer (2-4). Furthermore, PSMA is a type II membrane protein, with the majority of the protein located outside the cell readily available for therapeutic targeting or clinical imaging or other diagnostic-type assays (5). In addition, it now seems that therapeutic targeting of the PSMA molecule may have additional advantages; PSMA expression has been found in the endothelial cells of tumor vasculature of almost all types of tumors examined to date, including bladder, renal, breast and lung carcinomas (4, 6, 7). No PSMA expression has been reported in normal established vasculature. As such, a therapeutic approach targeted at PSMA could have broad implications for the treatment of many types of solid tumors. Accordingly, several groups are now attempting to utilize PSMA as a clinical and treatment target (1, 8-10). Clinical trials using radiolabeled antibodies to the external domain of PSMA have shown excellent results for imaging primary tumors and distant metastases not previously detected by conventional methods (10). However, although PSMA is very highly expressed in normal and cancerous prostate, there are other tissues in the body that express low levels of PSMA or a similar mRNA including kidney, liver and brain (11, 12). In order to use PSMA as a target, we wanted to know if this non-prostatic expression was in fact from the PSMA gene, and if it was not, what gene was expressed in these tissues. We recently mapped the PSMA gene to human chromosome 11p11.2, and a PSMA-*like* gene to chromosome 11q (13). Both genes

are the result of a genetic duplication that occurred 14 million years ago (13, 14). In order to determine where each of these two genes are expressed, we have cloned the PSMA-*Like* gene and demonstrated methods to distinguish the two genes at the DNA, mRNA and protein levels, which will aid in evaluating diagnostic and therapeutic strategies targeting PSMA.

Materials and Methods

Fine Mapping of the PSMA-Like gene

Primers that specifically amplify the PSMA-Like gene were used to screen the Genebridge 4 Radiation hybrid panel (Research Genetics). The primers were: 5' gccttcattttcagaacatctcatgcat 3' and 5' gtccatataaactttcaagaatgtg 3' ; the primer sequences were based on PCR sequencing/comparison of intronic regions of the PSMA-Like gene by amplification of somatic cell hybrids as described below. Conditions were 35 cycles of 94°C 30", 60°C 30", 72°C 1'. The results were analyzed using the server at http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl.fordfay.

Genomic Sequence of the PSMA-Like Gene

Sixteen sets of primers were designed based on the genomic sequence of the PSMA gene (accession AF007544) as shown in Table 1. Briefly, 100ng of DNA from a somatic cell hybrid containing the distal portion of human chromosome 11q (NA11936 from Coriell Cell Repositories, Camden, NJ) was amplified for 35 cycles using 100ng each of the primer pairs described in Table 1. Conditions were: 95°C 30", the annealing temperature indicated in Table 1 for 30" followed by extension for 1' at 72°C. In some cases as indicated, it was necessary to add DMSO to the reaction. Reactions were carried in a total volume of 50μl out using 1 U of Expand High-fidelity Taq DNA polymerase in Buffer 2 supplied by the manufacturer (Roche, Indianapolis, IN), 0.2mM of each dNTP and 150ng of each primer. PCR products were purified using the Wizard PCR Purification kit (Promega, Madison, WI) and directly sequenced on an ABI prism 3100 Genetic Analyzer.

DNA from a somatic cell hybrid containing human chromosome 11p (NA11944) was used as a positive control.

RT-PCR

RNA was either made from cell lines using Trizol (Invitrogen, Carlsbad, CA), or obtained from Clontech (BD Biosciences, Palo Alto, CA). The bone marrow endothelial cell line (BMEC) was a kind gift from Dr. Malcolm Moore, Sloan-Kettering Institute for Cancer Research, NY,NY). RT-PCR was carried out using the Superscript Preamplification Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. PCR to differentiate the PSMA and PSMA-Like genes was carried out using 2ul of the cDNA reaction as template. The primers used were: 5' acagatatgtcattctgggaggtc 3' and 5' actgtgatacagtggatagccgct 3'. Initial denaturation was for 4 mins at 95°C followed by 35 cycles of 95°C 30", 60°C 30", 72°C 1'. Ten μl of PCR product was digested with 10 U of *EcoRI* (New England Biolabs, Beverly, MA) in a total volume of 15μl at 37°C for 2 hours. Products were resolved on a 1.5% agarose gel.

Cloning of the PSMA-Like cDNA

A liver cDNA plasmid (bacterial) library (Invitrogen, Carlsbad, CA) was screened as previously described (15) using a probe generated via PCR using the following primers : 5' gtttataaaatcctccaatgaagc 3' and 5' gagcttctgtgcatcatagta 3' (exons 2-7 of PSMA) or we used a probe spanning exons 10-16, generated by the same primers as used for the RT-PCR described above. Three prime RACE was carried out using the 3' RACE system (Life Technologies, Gaithersburg, MD), with the primer 5' ttgaggtgttcttccaacgac 3' and a PSMA-Like specific primer 5' gacaaaagcaacccaatattg 3'. The cDNA sequence has been deposited in Genbank under accession number AF261715.

Northern Analyses

Multiple Tissue Northern blots were obtained from Clontech. Hybridization with the hPSM-350 riboprobe (16) was carried out overnight at 56°C, followed by washing for one hour in 0.1 x SSC, 0.1% SDS at 65°C as previously described (15). Exposure was carried out for 5 hours at –80°C. The PSMA-Like (Not1/Sal1 digest of the original clone in pSPORT) and β-actin probes were prepared using random-hexamer labeling (Invitrogen, Carlsbad, CA). Hybridization with the PSMA-Like probe or the β-actin probe (Clontech) was carried out overnight at 42°C in 50% formamide (Hybrisol I, Intergen, Purchase NY), followed by washing at 42°C for 15 minutes in 0.2 x SSC, 0.1% SDS. Quantitation of the relative amounts of PSMA expression in various tissues was carried out using the Image-J program with the gel analyzer plugin available from the NIH website http://rsb.info.nih.gov/ij/.

Regulation of the PSMA-Like Gene

The region of the PSMA-Like gene that corresponds to the PSMA enhancer was cloned and sequenced using PCR with the following primers that incorporate artificial *BamHI* restriction sites (underlined) to amplify NA11936 DNA: 5' cgcggatccgccttctaaaatgagttggg 3' and 5' cgcggatccggctactacataagtataagtc 3' which produces a product of 1648 bp. The PCR product was cloned into the *BamHI* site of the pGL3-B-PSM luciferase reporter vector containing the PSMA promoter and activity of the enhancer determined as we have previously described (17), with the addition that MDA PCa2b cells (ATCC, Rockville, MD) were maintained BRFF-HPCI Catalog # SF-30 (Biological Research Faculty Facility, Ijamsville, MD) supplemented with 15% fetal calf serum (Invitrogen). The

sequence of the PSMA-Like enhancer region has been deposited in Genbank, accession number AF480875.

Immunoblotting

Western blotting for PSMA was carried out by transferring 25ug of protein to PVDF membrane as described elsewhere (15). The blot was probed with the Cyt-351 antibody (a gift from Cytogen corporation, Princeton, NJ) or PM2M-440 (a gift from Hybritech Incorporated, a wholly owned subsidiary of Beckman Coulter, Inc., San Diego, CA) and exposed using the ECL system (Amersham Biosciences, Piscataway, NJ). Other antibodies used in studies described here are J591 (a kind gift from Dr. Neil Bander, Weill Medical College of Cornell University, NY, NY) or PEQ-226 and PM1T-485 (kindly supplied by Hybritech Incorporated, San Diego, CA).

Results

Fine Mapping of the PSMA-Like gene

PSMA-Like specific primers were designed based on PCR amplification and sequencing of a region of the PSMA-Like gene that is homologous to part of the first intron of the PSMA gene. These primers were then used to screen the Genebridge 4 radiation hybrid panel, placing the PSMA-Like gene 8.99 cR from CHLC.GATA45H10 and 0.40 cR from WI-6090 (LOD>3).

Genomic and cDNA Sequence of the PSMA-Like Gene

We were unable to screen a cDNA library to obtain the PSMA-Like cDNA clone, as we were unsure which tissue(s) it was expressed in, or indeed if it was expressed. Subsequently, we designed 16 sets of primers to the gene, the sequence of each set based on the intronic regions flanking the exons of the homologous PSMA gene. Using DNA from somatic cell hybrids retaining human chromosome 11q, we were able to generate products comprising each exon of the gene using the primers described in Table 1, except for exon 1. All exons sequenced conformed to the GT-AG intron-exon boundary rule. The differences that we were able to determine at the genomic level are summarised in Table 2.

In exon 12, a g→t change alters an *EcoRI* restriction enzyme site that is present in the PSMA gene so that it is no longer cleavable in the PSMA-Like gene. To confirm this finding, we amplified exon 12 from the DNA of 18 unrelated people and somatic cell hybrid DNA containing either 11p or 11q. All individuals exhibited three bands after

digestion, indicating the presence of one non-cleavable and one cleavable gene, confirming the g→t difference was not a polymorphism in the 11q gene (data not shown). To determine which tissues the PSMA-Like gene might be expressed in, we carried out RT-PCR using primers spanning exons 10-16, followed by restriction enzyme digestion with *EcoRI* (Fig. 1).

Using this *EcoRI* non-cleavable site as a "sequence tag" for the PSMA-Like gene, we were able to determine that liver and kidney showed a restriction enzyme banding pattern that corresponded to expression of *both* the PSMA and the PSMA-Like genes, while the other positive tissues tested showed expression only of the PSMA gene. Next, we screened a 2.3 x 10^6 colony forming units from a liver cDNA library using a probe to exons 2-7 of the PSMA cDNA sequence. Subsequent clones were digested with *EcoRI* in order to exclude PSMA clones from further analysis. Only two PSMA-Like clones could be identified this way although we found 26 full-length PSMA clones and two partial PSMA clones (beginning in exon 2 and 3 respectively). Both PSMA-Like clones that we isolated began in a region corresponding to intron 5 and exon six of the PSMA gene. We next screened the library again, this time using a probe generated from the PCR product spanning exons 10-16 of the PSMA gene. This generated a further 12 PSMA-Like clones (and no more PSMA clones), the most 5' sequences of which corresponded to the same intron 5 of the PSMA gene. We confirmed the 3' end of the gene using 3' RACE and specific primers based on the PSMA-Like gene sequence. The three longest clones from the library were sequenced, and the complete nucleotide and deduced amino acid sequence compared to that of the PSMA gene (Fig. 2). The sequence has been deposited in Genbank, accession

AF261715. The longest open reading frame of the PSMA-Like gene is homologous to the reading frame of PSMA. In addition, *in vitro* translation of the longest clone yielded the expected 46kD protein (data not shown). At the mRNA level PSMA-Like is 98% homologous to PSMA, and the protein shows 97% identity and 98% similarity to PSMA in the translated region. It should be noted however, that the expected size of PSMA-Like *in vivo* is 46kD, while PSMA is 100-120kD after glycosylation of its 84kD core.

Expression Pattern of the PSMA and PSMA-Like Genes

To determine what tissues express PSMA while avoiding detection of the PSMA-Like gene, we used a probe from the first three exons of PSMA which are not found in the PSMA-Like cDNA sequence (probe p350). Northern analysis confirmed expression of PSMA in the prostate, brain, kidney, small intestine, liver and spleen (Figs. 3A and 3B). After prostate, the next five highest expressing regions were all from the brain, and the other tissues were all at levels less than 10% of that of PSMA in normal prostate (Table 3). Similarly, western analysis using the Cyt-351 and PM2M-440 antibodies showed protein expression in the hippocampus and amygdala compared to that seen in the prostate cancer cell line MDA PCa2b (Figs. 4a and b). The Cyt-351 antibody binds to the intracellular region of PSMA that is deleted from the PSMA-Like gene (18), while PM2M-440 binds to a region within amino-acids 135 and 173 of PSMA (pers. communication with Harry Rittenhouse, Hybritech Incorporated), which is not found in the PSMA-Like cDNA.

PSMA mRNA expression was either neglible or not detected in thymus, testis, ovary, colon, leukocytes, heart, placenta, lung, muscle and pancreas. In addition, we cloned and sequenced PSMA expressed in tumor neo-vasculature and confirmed that it was not PSMA-Like. However the clone sequenced did contain two changes at nt 1784 (G→A; Gly→Asp) and nt 1817 (A→G; Asn→ Ser). To determine the relative expression of the PSMA-Like gene, we next probed with its cDNA in entirety (Fig. 5a-d). This would detect both PSMA (2.7kb) and PSMA-Like (2.0kb) mRNA. PSMA-Like was expressed at similar levels to the PSMA gene in adult kidney and liver, but not in any other tissues including fetal kidney, liver, brain and lung. Interestingly, PSMA is expressed in fetal liver and kidney, and also in adult trachea and spinal cord.

Regulation of the PSMA-Like Gene

We cloned a prostate-specific enhancer from the third intron of the PSMA gene (17, 20). As we had shown that the PSMA-Like gene was not expressed in the prostate (Fig. 1 and data not shown), we were interested to compare the sequence of the two genes "enhancer" regions. PCR using primers homologous to the PSMA enhancer were used to amplify DNA for sequencing from the 11q-containing hybrid. Surprisingly, the sequence of the two intronic regions is 99.3% identical. Because the minor differences between the two regions might alter novel prostate-specific transcription factor binding sites, we tested the PSMA-Like enhancer region for its ability to drive luciferase reporter gene expression in combination with the PSMA promoter (Fig. 6).

The PSMA-Like enhancer was able to drive luciferase expression equally as well as the PSMA enhancer in C4-2 prostate cancer cells. In addition, like the PSMA enhancer, there was no activity in the breast cancer derived cell line MCF-7. The PSMA-Like enhancer showed approximately equal activity in both the C4-2 and MDA PCa2b prostate cancer cell lines, however the PSMA enhancer repeatedly showed a 2.5 fold increase in activity in the MDA PCa2b cell line over its expression in the C4-2 cell line.

As the enhancer of the PSMA-Like gene was able to activate prostate-specific gene expression, we wanted to examine the region of the PSMA-Like gene that corresponded to the PSMA promoter, but had been unable to amplify any region of it or exon 1. A BLAST comparison with the high throughput genomic sequence database revealed homology of the PSMA-Like gene with a contig of 27 unordered pieces (Genbank accession AC024234). BLAST analysis of this contig against the promoter region and exon one and two of the PSMA gene in an attempt to identify a PSMA-Like promoter showed a deletion in AC024234 in the region corresponding to approximately 500nt upstream of the PSMA transcription start site, the entire first exon, and the first 371nt of intron one. To confirm this result, we designed primers to either side of the deletion, and used them to amplify DNA from five unrelated people. As the primers could amplify both PSMA and PSMA-Like, we expected to see approximately a 330bp product generated from all the DNA samples if the deletion had occurred, and a 1.5kb band from the PSMA gene. All five DNA samples produced a 330bp band, while the 11p hybrid produced a 1.5kb band (from the PSMA gene) and the 11q hybrid a band of approximately 330bp, verifying that deletion of the region corresponding to the promoter and exon one of PSMA had been

deleted in the PSMA-Like gene (Fig. 7). No 1.5kb band from the PSMA gene is visible in the human DNA lanes, probably due to preferential amplification of the smaller product.

To see if there is a promoter close to the transcription start site of the PSMA-Like gene, we analyzed 550 nt of sequence upstream using the promoter prediction program at http://www.fruitfly.org/seq_tools/promoter.html, which uses a neural networking method to predict promoter regions. A promoter was predicted 150 nt upstream of the 5' end of the longest clone obtained. In addition, there is a CCAAT box 161 nt and a TATA box 28 nt from the transcription start site.

Discussion

In order to resolve the origin of the so called "non-prostatic" expression of PSMA, we cloned the PSMA-Like gene and determined its expression pattern. The PSMA and PSMA-Like genes arose from a duplication event of the original gene approximately 14 million years ago (14, 20). The site of the original gene was most likely 11q14.3, as this region has conserved synteny with the location of the single murine PSMA homolog, folh1 at 7D1-2 (21). The finding that the PSMA-Like gene maps to the schizophrenia disorder type II locus is particularly interesting as a disruption in the NAALADase activity of PSMA has been implicated in the pathogenesis of schizophrenia (22).

Non-prostatic expression of the PSMA gene

The aim of this study was to analyze the possible expression of PSMA in non-prostatic tissues. We have shown here that PSMA is in fact expressed in a number of non-prostatic tissues. This expression is not due to other hypothetical or known homologs of PSMA as described in the EMBL database, as our p350 probe would either not detect these mRNAs, or would bind to an mRNA of a significantly different size to PSMA (23, 24) and observations from EMBL database (http://www.ensembl.org/Homo_sapiens). In addition, it should be noted that although there are two "homologs" of PSMA shown on chromosome 2 in the EMBL database, we have tested the possible existence of these using PCR of somatic cell hybrids containing human chromosome 2, and concluded that they are the result of artifact and in fact do not exist (data not shown).

Regulation of expression of the PSMA and PSMA-Like genes

Presumably the loss of the genomic DNA segment in the PSMA-Like gene that corresponds to the promoter and exon one of PSMA occurred subsequent to the duplication event. The similarity between the PSMA and PSMA-Like genes is remarkable, yet the very few differences between them allows us to learn more about the biology of PSMA. Despite the fact that the "enhancer" regions of the two genes are 99.3% identical, the PSMA enhancer is more than twice as active than the PSMA-Like enhancer combined with the PSMA promoter in MDA PCa2b cells. However, both enhancers work equally well in C4-2 cells. This suggests that some of the few sequence differences correspond to important enhancer factor binding sites for factors that are present only in MDA PCa2b cells. It has previously been reported that a 330 bp core region contributes most of the activity of the PSMA enhancer (19). Comparison of the two enhancer sequences reveals that two of the differences abolish binding sites for ATF/CREB and CEBP although there could be other sites as yet unrecognized that are also altered. Dissection of the factors controlling PSMA expression is important as we and others have been utilizing the PSMA enhancer to develop gene therapy strategies (17, 25).

As the PSMA-Like enhancer is capable of activity when combined with the PSMA promoter, we might expect to see prostatic activation of the native PSMA-Like promoter via the PSMA-Like enhancer. However, this clearly does not occur as we do not see expression of the PSMA-Like gene in the prostate. One possible explanation for this is that an insulator region exists between the PSMA-Like enhancer and promoter, blocking expression of the PSMA-Like gene in prostate-derived tissue (26). Similarly, although the

region corresponding to the PSMA-Like promoter in the PSMA gene is almost identical, it is not activated by the PSMA enhancer or we would expect to see a 2.0kb mRNA band in prostate tissue via northern analysis. This supports the idea of an insulator region because previously the PSMA enhancer has been shown to be capable of activating even the most minimal promoters such as the TK gene promoter (19).

Clinical targeting of PSMA

Exon one of the PSMA gene encodes for the single transmembrane domain, however this region is lost from the PSMA-Like gene. As the PSMA-Like cDNA sequence lacks a transmembrane domain, we expect the PSMA-Like protein to be located within the cytosol, and therefore not subject to the glycosylation that is undergone by the PSMA protein (27). Barinka et al. and our own results have demonstrated that glycosylation of PSMA is indispensable for enzymatic activity (28,29). Based on these findings, we would predict that expression of the PSMA-Like protein would not result in enzymatic activity that could affect prodrug therapies targeted at PSMA. In addition, due to the hypothesized intracellular localization of the PSMA-Like protein, cells expressing it would not be targeted by antibodies or other extracellular agents directed against PSMA.

Even though our studies use normal prostate RNA for comparison with other normal tissues, it should be remembered that prostate cancer is reported to contain a 10 fold increase in PSMA mRNA expression. Nevertheless, the expression of PSMA mRNA in the various regions of the brain, the spinal cord, liver and kidney is of significant concern and needs to be addressed when designing therapeutic strategies utilizing PSMA as a target.

However the brain and spinal cord are protected by the blood-brain barrier. In addition, there is no immunohistological evidence of PSMA protein expression in the brain or liver, despite several studies of these tissues (reviewed in Tasch et al. (29)). The same studies have shown positive staining for PSMA in the kidney, where it is expressed weakly in a subset of proximal tubule cells. It is possible that PSMA protein expression is regulated post-transcriptionally, so that the amount of mRNA present is not an indicator of actual protein. Prostate cancers contain over 1000 fold greater levels of PSMA protein than found in liver or brain as determined quantitatively by RIA (12). Most importantly extensive imaging studies (31) and phase one trials using cytotoxic radiolabeled, humanized antibodies against PSMA (32) have shown specificity for prostate, prostate cancer, and the neovasculature of other solid tumors including renal cell carcinoma. With both treatments there was a low frequency of side-effects, and in addition it seems the treatment with the antibody conjugated to β-emitters might be in the therapeutic range, as some of the patients with prostate cancer had as much as an 85% reduction in PSA. Consistent with a blood-brain barrier effect, immunotargeted antibody approaches with radiolabeled antibodies against PSMA used for imaging do not show localization in the brain (33). It is also heartening that there have been no reports of tissue injury in phase II trials stimulating the immune system against PSMA. Indeed, the trials have shown little if any toxicity, but a positive response rate in 30% of the patients (34).

Conclusions

We have cloned and characterized a gene that is highly homologous to PSMA, and determined ways to distinguish the two genes at the DNA, mRNA and protein levels. PSMA can be a useful clinical target for prostate cancer, however both the presence of the PSMA-Like gene and expression of PSMA in other tissues should be taken into consideration when designing diagnostic and therapeutic strategies. In addition, the small but significant differences between the evolutionary twins, PSMA and PSMA-Like, allows us to learn more about the function and regulation of PSMA.

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Figure Legends

Figure 1: Expression of the PSMA and PSMA-Like Genes. RT-PCR followed by gene-specific restriction enzyme digestion was used to differentiate expression of the PSMA and PSMA-Like genes in various tissues. The PSMA gene yields two bands of 346 and 208 bps following digestion with *EcoRI*, while the PSMA-Like gene remains uncut at 554 bps. M=100 bp ladder (Gibco), LNCaP=prostate cancer cell line, BMEC=bone marrow endothelial cells, UD=undigested.

Figure 2: Nucleotide and deduced amino acid sequence alignment of the PSMA-Like gene compared to the PSMA gene. Differences in nucleotide sequence are indicated in bold lower-case lettering, while amino acids found in PSMA and not PSMA-Like are indicated in bold capitals above the sequence. The Genbank accession numbers for PSMA-Like and PSMA are AF261715 and M99487 respectively.

Figure 3: Expression of PSMA as determined by northern analysis using a probe that will not detect the PSMA-Like gene. a) Expression of the 2.6 kb PSMA transcript is clearly strongest in the normal prostate, although expression of PSMA can also been seen in other tissues. b) Expression of PSMA is also found in the brain, although the levels vary depending on the region examined. RNA marker sizes are indicated in kb.

Figure 4: PSMA protein expression via immunoblot analysis. a) Using antibody PM2M-440 and b) antibody Cyt-351. Both antibodies are specific for PSMA because they bind to regions of the protein that is missing in the PSMA-Like protein. Densitometry of this blot reveals approximately 40-fold less PSMA protein in the brain tissues amygdala and hippocampus than is seen in the prostate-cancer cell line MDA-PCa2b. The size of the proteins is around 130kD, although it seems that the brain might glycosidate PSMA differently to the prostate. A slightly larger band appears only with the PM2M-440 antibody in the bladder sample; we assume this is non-specific as it is not detected with the Cyt-351 antibody and northern analysis showed no expression of PSMA RNA in the bladder.

Figure 5: PSMA and PSMA-Like mRNA expression in various tissues as seen via northern analysis. Expression of PSMA vs PSMA-Like was determined by utilizing a probe that detects a 2.7kb band corresponding to PSMA, and a 2.0kb band corresponding to PSMA-Like. a) and b) PSMA and PSMA-Like expression in normal human tissues, c) PSMA expression in brain tissues and d) PSMA expression in fetal kidney and liver. β-actin was used as a loading control.

Figure 6: Comparison of the PSMA and PSMA-Like "enhancer" regions. Potential of the PSMA-Like enhancer to drive a luciferase reporter gene in combination with the PSMA promoter is reported in relative light units after adjusting for transfection efficiency. The PSMA-Like sequence can operate as a tissue-specific enhancer, as evidenced by reporter gene expression in the prostate cancer cell lines MDA PCa2b and C4-2, but not the breast cancer line MCF-7. Interestingly the enhancer has the same

activity as the PSMA enhancer in C4-2 cells, while in MDA PCa2b the PSMA enhancer induces more than twice as much reporter gene expression than the PSMA-Like enhancer clones. The black bar indicates the PSMA promoter/enhancer construct, the wavy bars are two individual clones of the PSMA promoter/PSMA-Like enhancer construct, while the white bar is the PSMA promoter alone. The standard deviation of triplicate experiments is shown.

Figure 7: Deletion of the original PSMA-Like promoter, exon one and part of intron one. PCR analysis was performed using primers based outside the region predicted to be deleted in the PSMA-Like gene. Amplification of the predicted 300 bp band if a deletion had in fact occurred is seen in all five human DNAs (hum 1-5) and DNA from the 11q human-hamster hybrid (11q). Amplification of the 1500 bp band in the homologous region of the PSMA gene is generated from the 11p hybrid (11p). "Ham" indicates the parental hamster DNA of the hybrids.

Exon	Bases	Sense Primer	Sense Primer Sequence	Anti-sense Primer	Antisense Primer sequence	PCR product size expected
1	2488-2863	2529	tctctctctcgctcggattgg	2863	cgaagaggaagccgaggag	335 n/a
2	4994-5099	4341	tgtttctggccgcctatgcg	5254	agtatagtcctcctcagatg	914*
3	10726-10912	10630	caaagtacttttgtgtaactctgc	11082	cataggaaagtagttgacacgg	452#
4	18275-18376	18157	cctgaaggattcattcaccctc	18457	gacccttaattatcggctgaaca	300##
5-6	24400-25500	24323	atgtccaacagtccccatgcag	25593	gacatgcttagtccattgtacc	1270##
7	27927-28020	27871	gaaccgtttgaatgaaactgag	28058	ttacccaaatagccatccatgg	187*
8-9	35216-36281	35127	gcagatgctcaataagtgaatcc	36334	ccagcacataacagttacttgatc	1207#
10	37697-37816	37619	tagatgctattgagtcgtttgc	37867	aaactgagactcagataggctg	248#
11	39896-39978	39825	ctgggcttggtagtgtcctggg	40045	gcttggcaaacaagtcctggctac	220**
12	41911-41974	41792	tgtcgttaatatgggtcagctc	42035	ttaactagactgctgctcctag	243*
13	46402-46469	46317	tggtaggaatttagcagtggtc	46687	gatgctactaatgggctacctc	370**
14	53129-53220	53053	cttctggttaatggacatctag	53264	caatcccacactgaattcagtg	211♦
15	54364-54454	54278	agaatggggtttagtttaatgg	54536	tgagtcacttttggagtcag	258*
16-17	56661-57307	56614	ttgtaagctatccctataagag	57393	agttcagcaacagtcatgttag	779♦
18	62423-62515	62305	gggtggtcctgaaaccaatccc	62553	gtgatattacagaaaggagtc	248**
19	64209-64518	64127	atccaggaattgcagagtgctc	64586	ttcagttttaatccatagggag	459**

Table 1: Primer sequences and PCR conditions used to amplify the PSMA-Like gene. All sets of primers generated the indicated size product, except for exon 1 which was not able to be amplifed. Annealing temperatures are indicated; *55°C, **57°C, #58°C, ##60°C. ♦ indicates an annealing temperature of 55°C in the presence of 5% DMSO.

Exon # in PSMA	Nucleotide changes PSMA⇒PSMA-like	Amino-acid change PSMA⇒PSMA-like
1	Not present	n/a
2	No change	No change
3	nt. 630 t→a nt. 584 t→c nt. 594 a→t	Threonine→Threonine Valine→Alanine Alanine→Alanine
4	nt. 739 c→t	Proline→Serine
5	nt. 777 c→t nt. 787 t→c nt. 877 g→a	Glycine→Glycine Tyrosine→Histidine Glycine→Arginine
6	nt. 948 c→a nt. 993 t→c nt. 1023 g→t	Serine→Serine Aspartic acid→Aspartic acid Glutamine→Histidine
7	nt. 1092 t→c nt. 1103 g→a nt. 1150 a→g	Tyrosine→Tyrosine Arginine→Glutamine Isoleucine→Valine
8	nt. 1237 c→t	Proline→Serine
9	nt. 1320 a→g	Threonine→Threonine
10	nt. 1454 t→c	Isoleucine→Threonine
11	No Changes	No Changes
12	nt. 1572 g→t	Glutamic acid→Aspartic acid
13	nt. 1665 g→a nt. 1684 c→t	Proline→Proline Histidine→Tyrosine
14	No Changes	No Changes
15	No Changes	No Changes
16	nt. 2099 g→a nt. 2140 g→t	Serine→Asparagine Valine→Leucine
17	nt. 2172 g→a nt. 2202 t→c	Lysine→Lysine Serine→Serine
18	nt. 2239 g→t and nt. 2241 a→g nt. 2314 g→a	Valine→Leucine Arginine→Arginine
19	nt. 2442 a→t nt. 2459 a→c nt. 2531 a→c nt. 2534 c→t nt. 2562 AG is deleted in PSMA-like nt. 2571 c→a nt. 2572 g→a	Glutamic Acid→Aspartic Acid Tyrosine→Serine 3' UTR 3' UTR 3' UTR 3' UTR 3' UTR 3' UTR

Table 2: Nucleotide and inferred amino acid sequence changes relative to the PSMA gene deduced from genomic sequencing of the PSMA-Like gene.

Tissue	Expression (relative to normal prostate)
prostate	100
corpus callosum	59
substantia nigra	35
caudate nucleus	20
hippocampus	17
subthalamic nucleus	12
whole brain	9
kidney	9
thalamus	7
small intestine	6
liver	5
amygdala	4
spleen	2

Table 3: Relative amounts of PSMA in various tissues. The amount of PSMA in various tissues was determined using densitometry of the northern analyses shown in Fig. 5. Expression of PSMA in prostate was arbitarily set at 100. The next five highest expressing tissues are all from the brain, while the next highest expressing organ, kidney, has less than 10% of the amount of PSMA that is seen in the normal prostate. All values were adjusted for β-actin expression (data not shown).

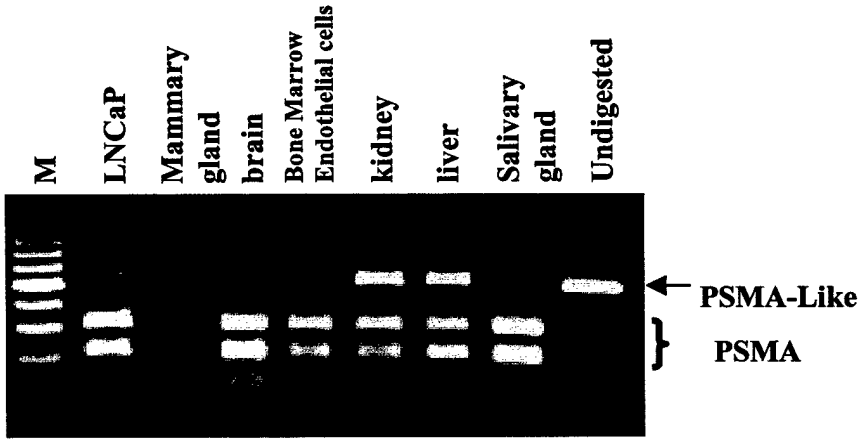
M
LNCaP
Mammary gland
brain
Bone Marrow Endothelial cells
kidney
liver
Salivary gland
Undigested
PSMA-Like
PSMA

FIGURE 1

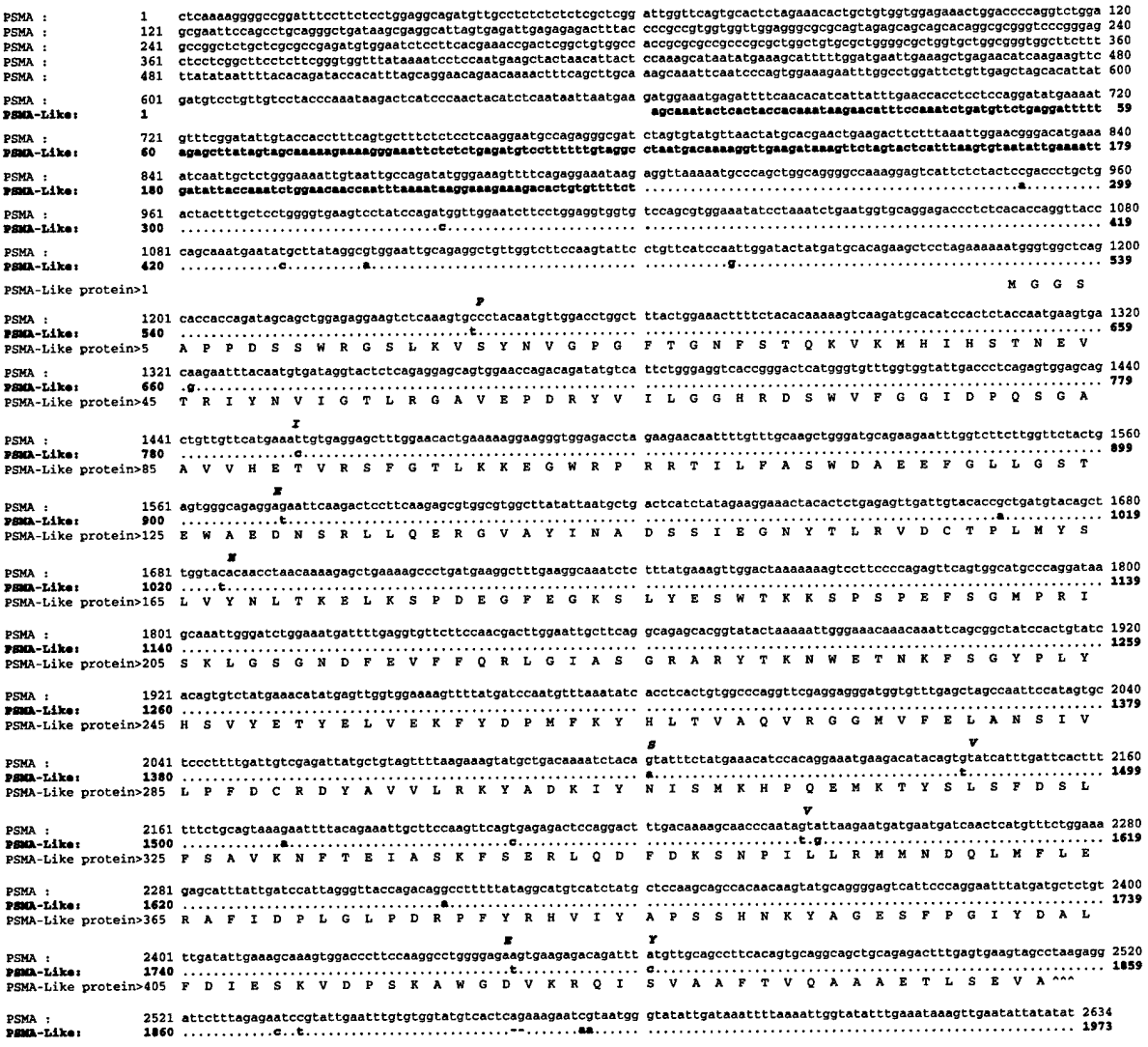

FIGURE 2

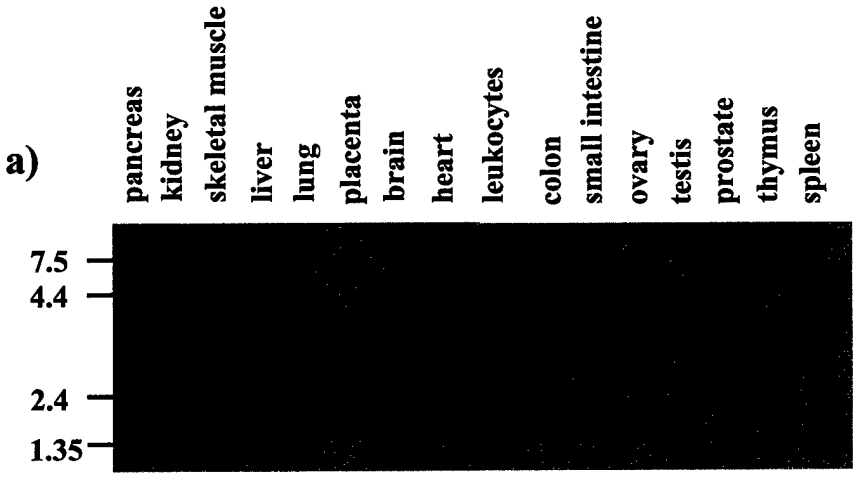
a)
pancreas
kidney
skeletal muscle
liver
lung
placenta
brain
heart
leukocytes
colon
small intestine
ovary
testis
prostate
thymus
spleen
7.5
4.4
2.4
1.35

FIGURE 3-A

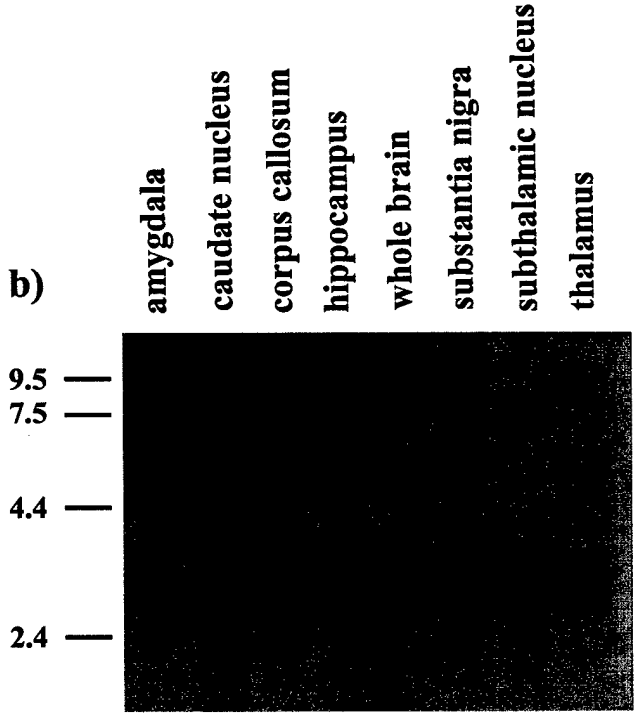
b)
amygdala
caudate nucleus
corpus callosum
hippocampus
whole brain
substantia nigra
subthalamic nucleus
thalamus
9.5
7.5
4.4
2.4

FIGURE 3-B

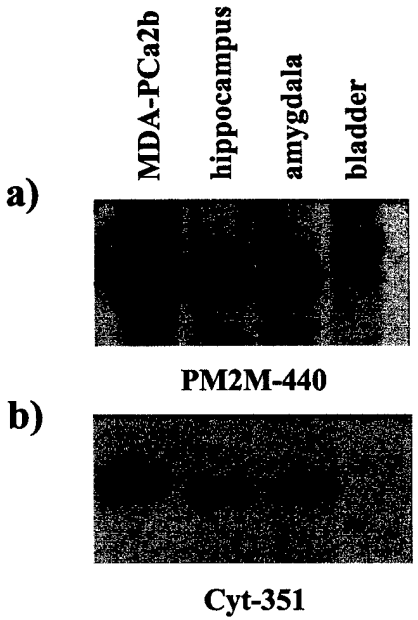

FIGURE 4

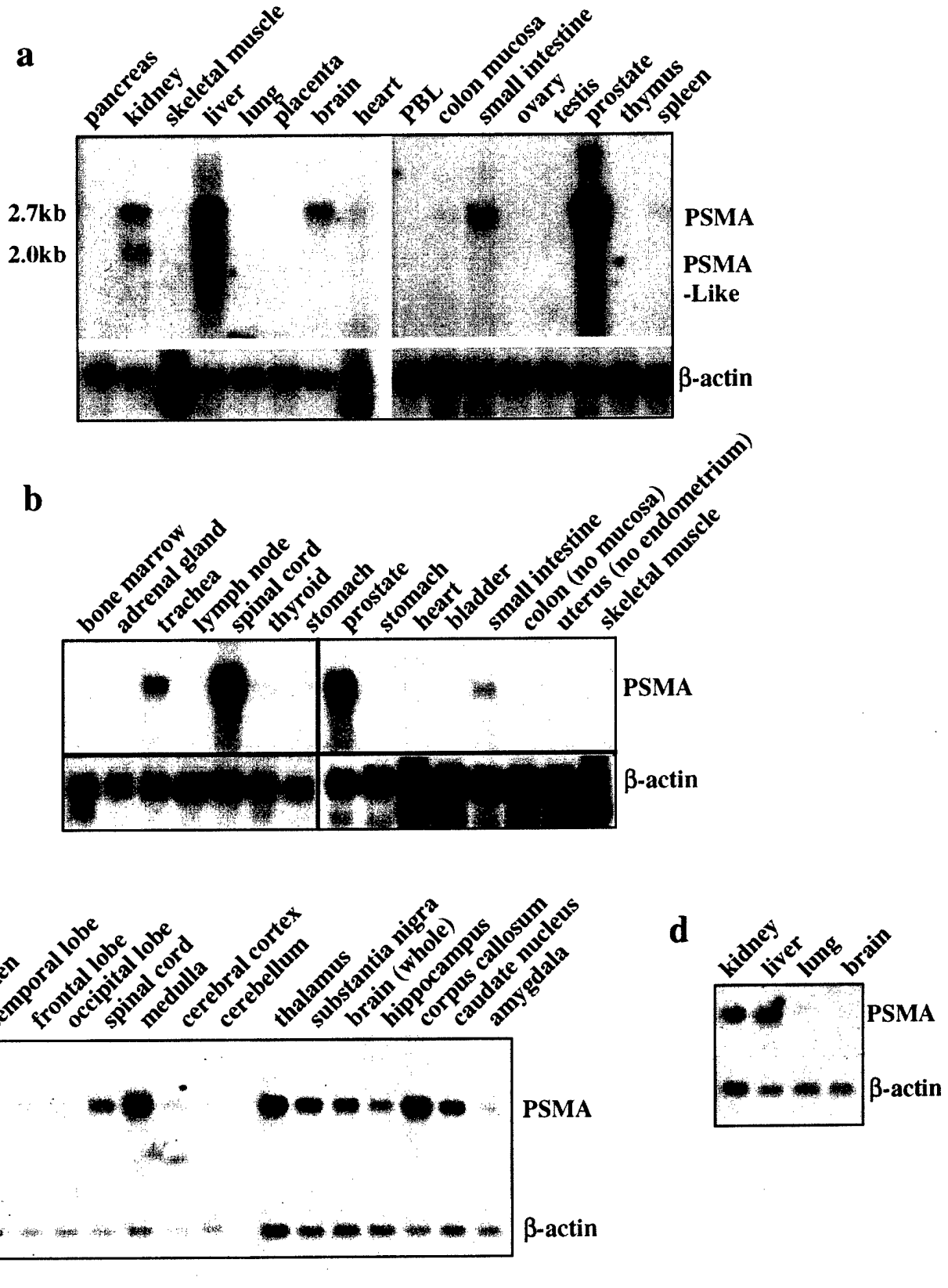
a
pancreas
kidney
skeletal muscle
liver
lung
placenta
brain
heart
PBL
colon mucosa
small intestine
ovary
testis
prostate
thymus
spleen
2.7kb
2.0kb
PSMA
PSMA
-Like
β-actin
b
bone marrow
adrenal gland
trachea
lymph node
spinal cord
thyroid
stomach
prostate
stomach
heart
bladder
small intestine
colon (no mucosa)
uterus (no endometrium)
skeletal muscle
PSMA
β-actin
c
putamen
temporal lobe
frontal lobe
occipital lobe
spinal cord
medulla
cerebral cortex
cerebellum
thalamus
substantia nigra
brain (whole)
hippocampus
corpus callosum
caudate nucleus
amygdala
PSMA
β-actin
d
kidney
liver
lung
brain
PSMA
β-actin

FIGURE 5

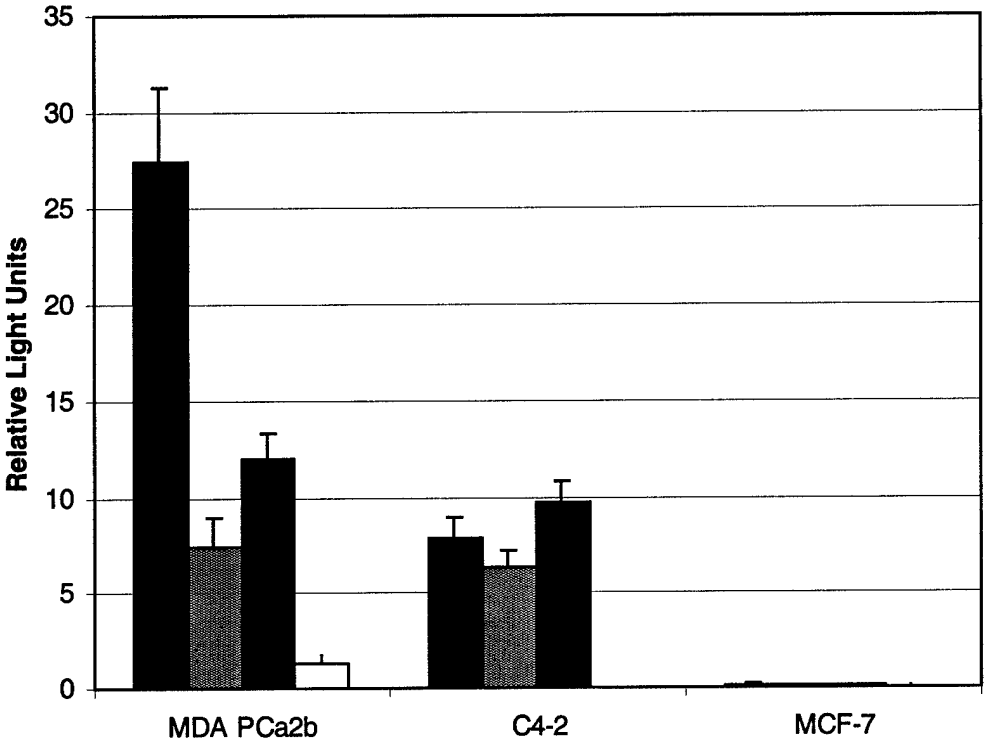

FIGURE 6

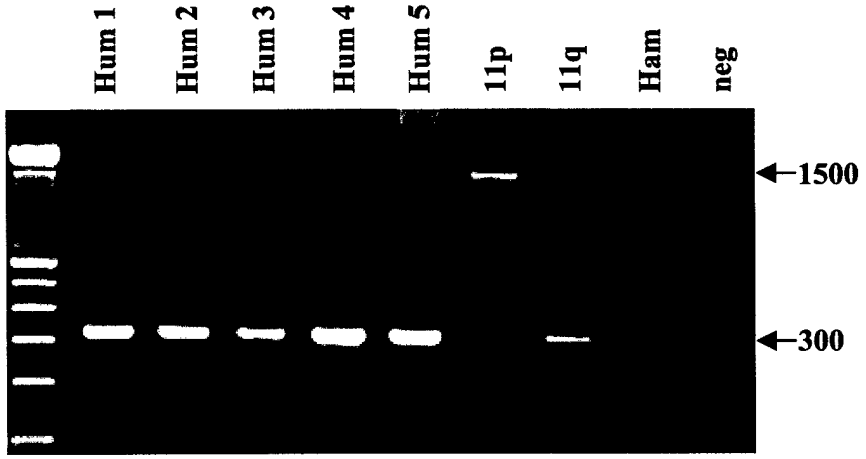

FIGURE 7