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13. ABSTRACT ***(Maximum 200 Words)***

PSMA is a strongly expressed protein in prostate cancer, and it is a type-two membrane protein with the bulk of the molecule and enzymatic activity outside of the cell and thus is accessible to therapeutic attack. We discovered that PSMA has activity as a unique folate hydrolase, being a glutamate carboxypeptidase. We also observed that cells expressing PSMA were sensitive to killing by polygammaglutamated methotrexate while non-PSMA expressing cells were resistant. We therefore proposed to explore the biologic potential of PSMA as a therapeutic target in terms of transport and prodrug activation. Because PSMA appeared to be a broadly active carboxypeptidase we proposed to examine polygammaglutamated derivatives of cytotoxics as potential agents that then might be transported into the cell or as prodrugs that would be activated by the enzymatic activity of PSMA releasing the drug from the polygammaglutamated-linked cytotoxic at the site of the tumor.

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Table of Contents

Revised annual report as requested for DAMD17-00-1-0043. The summary recommended a revised report and a revised statement of work. I hope what follows is in keeping with the request.

Introduction:
PSMA is a strongly expressed protein in prostate cancer, and it is a type-two membrane protein with the bulk of the molecule and enzymatic activity outside of the cell and thus is accessible to therapeutic attack. We discovered that PSMA has activity as a unique folate hydrolase, being a glutamate carboxypeptidase. We also observed that cells expressing PSMA were sensitive to killing by polygammaglutamated methotrexate while non-PSMA expressing cells were resistant. We therefore proposed to explore the biologic potential of PSMA as a therapeutic target in terms of transport and prodrug activation. Because PSMA appeared to be a broadly active carboxypeptidase we proposed to examine polygammaglutamated derivatives of cytotoxics as potential agents that then might be transported into the cell or as prodrugs that would be activated by the enzymatic activity of PSMA releasing the drug from the polygammaglutamated-linked cytotoxic at the site of the tumor.

We proposed two specific aims:
Aim one: Was to characterize the structural, enzymatic and transport activities of PSMA and PSMA like proteins for the rationale development of biology based targeting strategies.

Aim two: Was to generate a number of cytotoxic analogs of PSMA, as mitomycin, SN-38, phenylalanine mustard, vinblastine, and other depending on the outcome of specific aim one.

While that was the plan, the crux of the hypothesis was that there should be targeting ability of drugs and increased specificity for prostate cancer, based on PSMA's high expression, glutamate carboxypeptidase activity, and internalization. Our findings in aim one have dissuaded me from continuing to pursue aim two. Initial examination of polygammaglutamated analogs of methotrexate have shown activity in vitro, but have not shown activity when tested in xenografts in nude mice. When we have examined tumor grown in nude mice we have noticed substantial variability of expression of PSMA, a variability not noted in tumors growing in humans. I am collaborating with a small pharmaceutical company that has generated antagonists to the folate hydrolase activity of PSMA. Guilford Pharmaceutical Co. is studying this enzymatic activity in neural tissue. In mice they call this activity NAALADASE rather than PSMA based on a different substrate this protein that has been shown to be present in the brain. When Guilford tested the same line of prostate tumors grown in SCID mice for activity they likewise found a wide range in enzymatic activity. At a recent symposium at the annual meeting of the Neurochemistry Society, investigators using crayfish neurons, found enzymatic activity is observed only when an action potential is generated and as the action potential passes a specific point the enzymatic activity is found only at that point. This raises questions of the relationship between possible inhibitors of PSMA activity or some other factor as an inhibitor or activator being involved. So rather than having a straightforward relationship

between the presence of the protein and enzymatic activity, it winds up being more complicated. Aim one thus becomes less clear, and it is not going to be as straightforward as we originally planned to use the enzymatic activity of PSMA for designing cytotoxic drugs.

However the primary goal of the proposal was to develop a biologic basis for PSMA targeting in prostate cancer. That is still the aim of this project, so I agree with the reviewer of the submitted application that a change in statement of work is called for.

In specific aim one the original statement of work
Task one:

a. Clone enzymatically active PSMA and PSMA like protein for secretion from CHO cells and/or baculoviral infected insect cells to generate the gram amounts required for use in X-ray diffraction studies, months 1-12.

As noted, this aim has been accomplished in part. We have the primary target PSMA. The reason we planned to pursue PSMA-like gene was because we thought that it might be similar enough to PSMA that it would diminish the specificity of PSMA as the PSM-like gene is expressed in kidney and liver. Liver and Kidney are targets that we want to avoid for anti-tumor targeting. We have recently identified that this protein differs from what we originally thought it would be. That is, it is not a membrane protein, it is likely a cytosolic protein, and we have not found it to have enzymatic activity. Thus it would not interfere with drugs to be enzymatically activated by PSMA.

b. Begin x-ray diffraction characterization of the active site of PSMA and PSM-like protein. (12-24 months)

As stated in (a.), We didn't need to pursue PSM-like because it didn't have enzymatic activity. The idea of X-ray diffraction is to identify the active site and how the pocket of the active site is configured to enable for rational drug design. This also became problematic, because glycosidated proteins are difficult to use to generate X-ray diffraction profiles. When we proposed this aim, I was not aware that this was a problem. So we tried an obvious solution, which was to deglycosidate the protein. The deglycosidated protein lacked enzymatic activity, and to obtain the enzymatically active protein was the whole purpose of doing X-ray diffraction. So this has been a problem area and I don't see it moving forward unless we can find a means to obtain PSMA protein or a modified version of PSMA protein that is enzymatically active in the deglycosidated form.

c. Characterize binding enzymatic activity, internalization and toxicity of mitomycin C and SN38 polygammaglutamates months 12-24.

Not done and no plans to do until we understand why PSMA has enzymatic activity or not and is expressed or not in our PSMA models in animals

d. Characterize binding enzymatic activity internalization and toxicity vinblastine and phenyl mustard (months 24-36)

Not done and no plans to do until we understand why PSMA has enzymatic activity or not and is expressed or not in our PSMA models in animals.

Aim one continued:

e. Generate and characterize phage display ligands with selective binding to PSMA or PSM-like protein (months 6-24).

This is still ongoing. We have generated two clones that appeared to induce internalization of PSMA. However when we synthesized the peptide of the phage and examined them for induction of internalization they did not induce internalization. So we are still searching for ligands that will be have a good affinity for PSMA with a special emphasis on ligands that will induce internalization.

Task Two:

All of the sub aims of this aim were the proposed synthesis of polygammaglutamated derivatives of known cytotoxic chemicals. None of these tasks have been started with regard to the original proposed compounds. I decided not to press ahead with this for the following reasons. When I moved from Memorial Sloan-Kettering Cancer Center, it made it more difficult to interact with Bill Bornmann. Dr. Bornmann is head of the organic synthesis core for MSKCC, which is a synthesis core for MSKCC not for outside groups. He was discouraged from writing grants or participating as an active PI on grants outside of Memorial. That only became clear after a while after I was located at CCF. That has changed in the last month, and he is now being encouraged to be a participant on grants and can be active as a co-investigator. Another complication related to the rationale for the aim itself. I initially felt based on the broad substrate activity of PSMA that the proposed compounds might have cytotoxic activity as would a methotrexate polygammaglutamate, The advantage in folate antagonists was that after the glutamate is removed, the folate is taken into the cell by a folate transporter where it is fully active. The proposed compounds will not be fully the original toxin following removal of the glutamate during PSMA enzymatic hydrolysis because PSMA does not remove the last glutamate. Thus the toxin will be a glutamated toxin, which is likely to be less active than the unmodified toxin. I initially thought that any loss of activity would be balanced by the binding to of the toxin to the site of PSMA expression. This doesn't seem to be as clear as initially considered because. When we investigated two folate antagonist analogs in additions to methotrexate. The polygammaglutamated folate analog of 1843U89 was not a substrate for PSMA. This made it more of a necessity to obtain the x-ray diffraction data on PSMA.

I am the PI so it is my responsibility to see the aims are accomplished, and I did not accomplish these aims and I accept responsibility for that. In retrospect, I believe it was a flawed aim. The data we were obtaining suggested that it was not going to be so straightforward as we initially envisioned. So in a revised version of the statement of work I would not include this aim. In the time I have left, my main focus will be on understanding the biologic control for PSMA and obtaining x-ray diffraction data of the protein structure.

Revised statement of work.

Aim one: To characterize the structural, enzymatic and transport activities of PSMA and PSMA like proteins for the rationale development of biology based targeting strategies.

a. Clone enzymatically active PSMA and PSMA like protein for secretion from CHO cells to generate the gram amounts required for use in X-ray diffraction studies, (year 3).

To obtain enzymatically active recombinant protein is accomplished. We have found that CHO cells appear to be best for production of enzymatically active protein. However we are focusing in this last year of the grant on determining whether we can identify modification of the protein that will maintain the enzymatic activity of PSMA, but reduce post-translational modification of the PSMA protein in order that it will be less difficult to use in x-ray diffraction studies. We have identified that certain mutants of the protein in which we eliminate sites for glycosidation in the protein still maintain enzymatic activity and a couple of sites that don't. This is on going.

b. Characterize binding, enzymatic, transport and toxicity of PSMA and PSM-like proteins with iodomethotrexate, methotrexate polygammaglutamate and N-acetylaspartylglutamate (years 1-3 ongoing).
This is basically accomplished. As described above, N-acetylaspartylglutamate was nontoxic and did not interfere with transport. Methotrexate trigammaglutamate was toxic to cells such as LNCaP, C4-2 and CWR-22R that express PSMA but not to PC-3 or Du-145 which do not express PSMA, following exposure in three day culture assays. Agents that are known inhibitors of PSMA such as quisqualic acid and PMPA were able to block the toxicity of methotrexate trigammaglutamate, while agents such as PMPA are selective inhibitors of PSMA at nM concentrations have no effect on the growth rate themselves. . The only aspect of this that has not been accomplished is the generation of the iodomethotrexate. Rather than iodomethotrexate that will likely have binding characteristics in the uM range, we will synthesize 2[[[4methoxy3Iodobenzylamino] methyl (hydroxyphosphinoyl) methyl] pentanedioic acid (PPMPA). This derivative is an analog of PMPA and is a potent antagonist of PSMA. This compound has binding characteristics in the nM range. It will thus provide the iodo-derivative that will help in x-ray diffraction characterization studies and will also have greatly improved binding properties in the range we would like of compounds to be synthesized as toxins.

c. Begin x-ray diffraction characterization of the active site of PSMA and PSM-like protein. (final year). This will be the main task we plan to accomplish. The need for glycosidation of the protein is a major hindrance. We are investigating what minimal sugar modifications can be generated to enable this aspect to be accomplished more readily. This I regard as the most important aspect of the proposal and will continue our efforts and focus on this aspect.

Aim two: **Generate and characterize phage display ligands with selective binding to PSMA or PSM-like protein (months 18-36).** I propose to change aim two to identification of ligands for targeting PSMA by phage display using a random cyclic peptide library. This is still ongoing. We have generated two clones that appeared to induce internalization of PSMA. However when we synthesized the peptide of the phage and examined them for induction of internalization they did not induce internalization. So we are still searching for ligands that will be have a good affinity for PSMA with a special emphasis on ligands that will induce internalization. Such ligands can be linked to toxins such as doxorubicin or be part of a targeting liposome which could target gene therapy containing DNA expression therapeutic vectors to PSMA expressing tumors. As reported last time we have identified and develop preclinical data that the PSMA promoter/enhancer can be used for gene therapy of tumors. The use of ligand containing liposomes would further enhance targeting and therapy.

Results of further characterization of PSMA as a target. That PSMA can be utilized as a target for anti-tumor therapy is seen in these often-repeated experiments:

With a folate antagonist triglutamate incubated with the cells for three days in vitro in the presence or absence of the Specific PMSA inhibitor PMPA and then the number of cells determined by colorimetric assay. CWR-22 cells and LNCaP cells express PSMA, and Du-145 cells do not express PSMA. PC-3 cells that don't express PSMA behave in a similar fashion as Du-145 cells and PC-3 cells transfected with a PSMA expression vector behave like LNCaP cells.

Table 1. Ability of polygammaglutamated folate antagonist to inhibit cell growth in PSMA positive (CWR-22, LNCaP) and PSMA negative (Du-145) cell lines.

LY231514 (Permetrexed) triglutamate

	Concentration	Control	+PMPA (100nM)
CWR22	0	100%	101%
cells	1.0uM	63%	100%
	0.25uM	84%	101%
LNCaP	0	100%	99%
cells	1.0uM	42%	72%
	0.25uM	51%	105%
Du-145	0	100%	100%
Cells	1.0uM	95%	93%
	0.25uM	101%	96%

Methotrexate triglutamate

CWR22	0	100%	98%
Cells	1.0uM	68%	99%
	0.25uM	85%	99%
LNCaP	0	100%	100%
	1.0uM	40%	76%
	0.25uM	35%	108%
Du-145	0	100%	106%
	1.0uM	78%	84%
	0.25uM	84%	92%

So it demonstrates that in this in vitro system that these polygammaglutamated forms are active only if their hydrolytic activity to remove the glutamates is not inhibited by potent and selective inhibitor of PSMA's hydrolytic activity.

We are waiting for the folate antagonist 1843U89 triglutamate and should have that material before the end of the month. Because it did not appear to be metabolized by PSMA, I hypothesize that it will not have activity and if it does its activity will not be reversed by PMPA.

In terms of structure activity we asked, is there a relationship between the length of the glutamate linkage and cytotoxic activity and its reversal by PMPA.

Table 2. Effect of glutamate chain length o growth inhibitory activity of glutamated derivatives of methotrexate on LNCaP cell growth.

Drug	Treatment	Solvent Control
MTX-5 glutamates	88%	100%
" " + PMPA	105%	100%
MTX-4 glutamates	68%	100%
" " + PMPA	101%	100%
MTX-3 glutamates	51%	100%
" " + PMPA	99%	100%
MTX-2 glutamates	62%	100%
" " + PMPA	106%	100%
MTX-1 glutamate	40%	100%
" " + PMPA	42%	100%
MTX- 0 glutamate	102%	100%
" " + PMPA	103%	100%

The data in table 2 are those following the exposure of the drug for 72 hours to LNCaP cells (starting concentration ten thousand cells plated the day before the addition of drug). MTX stands for the folate antagonist methotrexate. All assays were done in sextuplicate and the standard deviation from the mean in each case was never greater than 10% of the mean. The number of glutamates is based on the total glutamates of the molecule thus MTX 5 glutamates is one alpha-glutamate and 4-gamma linked glutamates, two glutamates is one alpha and one gamma-linked glutamates. MTX-1 glutamate is methotrexate and MTX-0 glutamate is MTX without any glutamates. PMPA on its own has no inhibitory effect on growth when added at a final concentration of 100nM, and its Ki for inhibiting PSMA is 0.2nM. MTX and its glutamated analogs are added at a final 1uM concentration in this table.

The data show that if there are at least two total or more glutamates in the methotrexate molecule, then the polyglutamated drug is not active in inhibiting growth in the presence of the PSMA hydrolase inhibitor PMPA. Six different cells lines have been exposed to these agents in a full dose range of a 100-fold difference in concentration. Not shown here are the data that in cells that lack PSMA there is not cytotoxicity towards the cells with MTX that has two or more glutamates present. This is in keeping with the inability of these derivatives to use the folate transporter to gain access to the cell. Methotrexate without the first glutamate, is inactive and this is demonstrated in this table as well. With one glutamate that is the active form and is the well-known folate antagonist, methotrexate. Methotrexate is active in the cells lacking PSMA expression. Methotrexate is active on its own as it is readily transported as such into the cells and because it doesn't require removal of glutamate does not have its inhibitory activity blocked by PMPA. Methotexate is active against cells whether or not they express PSMA. It is the most active compound as well. This is to be expected because it does not require any metabolism to be active. The next most active agent is MTX with three glutamates. As the length increases the compound becomes less active with almost no activity as the length reached 5 glutamates. We have examined the activity of these folate antagonists in serum and folate free media. The inhibitory activity was only slightly increased in folate free media. We are currently exploring the role of in vivo administration of these compounds.

A paper characterizing the in vitro activity of polygammaglutamated analogs of folate antagonists will be completed as soon as we have the in vitro cytotoxicity results of cells exposure to the polygammaglutamated folate antagonist compound 1843U89, this will likely be accomplished within the next two months.

Key Research Accomplishments:

1. Identification of features associated with activity in structure activity relationships of PSMA requirements for metabolic activation of folate antagonists.
1. Identification of requirement for glycosidation of PSMA for enzymatic activity.
2. Generation and characterization of recombinant PSMA that is enzymatically active.
3. The identification and characterization of a PSM-like gene.

Reportable outcomes.
Characterization of PSMA-like protein, paper has been submitted to the journal PROSTATE and is in press. See attached.

Conclusions: The degree of polygammaglutamation affects the activity of the polygammaglutamated folate antagonist with optimal activation seen with a total of 2 gamma-linked glutamates and decreasing with further increases in length. Enzymatic activity is dependent on the PSMA protein being glycosidated. Recombinant PSMA has further demonstrated that the dimeric form of the enzyme has a higher enzymatic activity at lower levels of drug than the monomeric form. We have also demonstrated that a highly homologous gene, the PSM-like gene, is expressed in the liver and kidney. Because it is not glycosidated and is not a membrane protein, it is not enzymatically active with the proposed glutamated substrates and will not interfere with therapies, which emphasize the role of PSMA as a folate hydrolase.

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

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2. To whom reprint requests should be addressed.

ABSTRACT

Prostate cancer is the leading cause of cancer and second leading cause of cancer death among American males. Although localized prostate tumors are slow growing and can be treated by radiotherapy or surgery, once the tumor is hormone refractory and has metastasized there are few options for the patient. Currently PSMA is showing promise both as an effective imaging agent and therapeutic target for metastases. First generation antibodies against PSMA are used for the FDA approved Prostascint™ monoclonal antibody scan. In addition, second generation antibodies are being developed for therapeutic targeting as well as imaging (1). However, there have been reports describing RNA expression of PSMA or a very similar gene in non-prostatic tissues including kidney, liver and brain. As we had previously showed that the PSMA gene had been subject to a duplication several million years ago, we set out to determine if this non-prostatic expression was due to expression of the PSMA or another gene. Here we describe the cloning and characterization of the duplicate gene which we have called the PSMA-Like gene. The PSMA-Like gene maps to the Schizophrenia disorder type II locus on 11q14.3, and possesses 98% identity to the PSMA gene at the nucleotide level. The PSMA-Like gene is expressed in kidney and liver and utilizes a different promoter to the PSMA gene.

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 the following primers. 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.

Transient Transfections and Enzyme Activity Analysis

The PSMA-Like gene was excised from pCMV·SPORT using *EcoRI and BamHI* and subcloned into the same sites in the pIRES-neo (Clontech) vector. The PSMA/PSMA-Like hybrid was made by excising 1211 nt from PSMA-neo with *XcmI* and *EcoRV* and subcloning the fragment into the same sites in PSMA-Like-neo. PC3 prostate cancer cells were plated in six-well dishes so that they would be 90% confluent the following day. The cells were then transfected with 4.5 ug of either PSMA-Like-neo, PSMA-neo or PSMA/PSMA-Like hybrid-neo and 900ng of pAdVantage (Promega) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The samples were harvested 30 hours post transfection using 120ul of lysis buffer per well (50mM Tris-HCl ph 7.5, 0.5% triton X-100) and the protein quantitated using BCA Protein Assay Reagent (Pierce, Rockford, IL). Ten ug of protein was incubated at 37°C for 1 or 20 hours in a total volume of 100ul of lysis buffer containing 4μM N-acetyl-L-aspartyl-L- (glutamate-3,4-3^H) (Perkin Elmer Life Sciences, Boston, MA). The reaction was stopped by the addition of an equal volume of 0.5M NA_2HPO_4. Liberated glutamate was separated using ion-exchange chromatography as previously described (18). Briefly, half the reaction was layered over a Poly-Prep prefilled chromatography column packed with AG-1-X8 formate resin, 200-400 mesh (Biorad, Hercules, CA) that had been washed with two volumes of distilled water. The column was washed with 2mls of 1N formic acid, and the free-glutamate eluted in 2.5mls of 1N formic acid followed by measurement using scintillation spectrometry.

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 in 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 (19), 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. Suprisingly, 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 in all individuals if the deletion had occurred, and a 1.5kb band from the PSMA gene. All five DNAs 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.

Enzymatic Activity of the PSMA-Like Gene

PC3 cells were transfected with PSMA or the PSMA-Like gene and assayed for NAALADase activity. Despite the few amino acid differences between the two genes and the fact that the PSMA-Like gene possess the enzymatic domain of PSMA (21), the PSMA-Like gene appeared to have no activity. However, we were unable to confirm PSMA-Like protein expression. This might have been because either the anti-PSMA antibodies we were using (J591, PM1T-485 and PEQ-226) did not bind to PSMA-Like or due to low expression/protein stability. Repeatedly in transient transfections the majority of the PSMA-Like transfected cells were dead after 24 hours, although all other transfections only suffered up to 20% cell death. So that we could be sure that we could detect PSMA-Like expression, and because the lack of enzyme activity might be due to loss of the protease-associated domain that is found in a number of diverse proteases and as such might be structurally or otherwise important (a.a. 160-280 of PSMA), we made a hybrid PSMA/PSMA-Like construct. The first 307 amino-acids of the hybrid protein are the first 307 a.a. of the PSMA protein, while the rest of the protein is PSMA-Like in its

entirety. As the Cyt-351 antibody binds the internal domain of PSMA (19), it detects expression of the PSMA/PSMA-Like hybrid protein. Suprisingly, this construct did not have enzymatic activity in PC3 cells, although the transfection with PSMA did (Figs. 8 a, b and c).

Discussion

In order to determine the origin of the so called "non-prostatic" expression of PSMA, we cloned and characterized the expression pattern of the PSMA-Like gene. The PSMA and PSMA-Like genes arose from a duplication event of the original gene approximately 14 million years ago (14, 22). 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 (23). 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 (24).

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 (20). 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 able to drive expression of the PSMA promoter, and the PSMA enhancer is able to drive expression of minimal heterologous promoters such as TK (20), we might expect to see prostatic activation of the native PSMA-Like promoter. However, this clearly does not occur as we do not see expression of PSMA-Like in the prostate. One possible explanation for this is that an insulator region exists between the enhancer and the PSMA-Like promoter, blocking it from becoming active in prostate-derived tissue (26). In addition, 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.

Loss of the region corresponding to exon one of the PSMA gene from the PSMA-Like gene might also explain the lack of enzymatic activity of PSMA-Like. Exon one of PSMA encodes for the single transmembrane domain. As the PSMA-Like cDNA sequence lacks a transmembrane domain, we expect it to be located within the cytosol, and therefore not subject to the glycosylation that is undergone by the PSMA protein (27). Barinka et al. recently reported that glycosylation of PSMA is indispensable for enzymatic activity (28). However, although analysis of expression of the chimeric PSMA/PSMA-Like protein via western blotting appeared to produce a correctly glycosidated protein, it was still enyzmatically inactive. The PSMA/PSMA-Like chimera only has three amino acid differences to PSMA that are not conserved in other species homologs of PSMA that are

enzymatically active. One of these differences is found at the carboxyterminal of the protein, presumably away from the catalytic region a.a. 728 (E→D) (21). However the other two of these differences (a.a. 398 I→T and a.a. 437 E→D based on the PSMA a.a. sequence) correspond to the catalytic region of PSMA, close to the amino acids responsible for zinc ligand binding and catalysis (a.a. 424 and 425). Site-directed mutagenesis of residues in the vicinity of the zinc ligands has been shown to dramatically reduce the activity of PSMA (29). Therefore it is likely that one or both of these amino acid changes found in the PSMA-Like gene alters the local structure of the enzyme, rendering it inactive and as such these residues might be good candidates for targeting by small-molecule inhibitors. Moreover, Devlin et al. reported that a polymorphism exists in the PSMA gene which alters a single amino-acid (H475Y) resulting in a 50% reduction in the folate hydrolase activity of PSMA (30). While this polymorphism is present in PSMA at an allele frequency of 0.04, it is present in 100% of PSMA-like alleles we have examined, and perhaps in combination with the other amino acid changes results in a protein with no enzymatic activity even when glycosidated correctly.

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 (31, 32) 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 artefact and in fact do not exist (data not shown). Also, we and others have mapped PSMA using BAC arrays and somatic cell hybrids to 11p11.2, but the EMBL database maps PSMA to the 11q side of the centromere (13, 14). As such caution should be excercised when using the genome database as a resource.

The reasonably high 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. (33)). 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 than found in liver or brain as determined quantitatively by RIA (12). Most importantly extensive imaging studies (34) and phase one trials using cytotoxic radiolabeled, humanized antibodies against PSMA (35) 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 (36). 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 (37).

Finally, 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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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).

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 Cyt-351 and b) antibody PM2M-440. 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.

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.

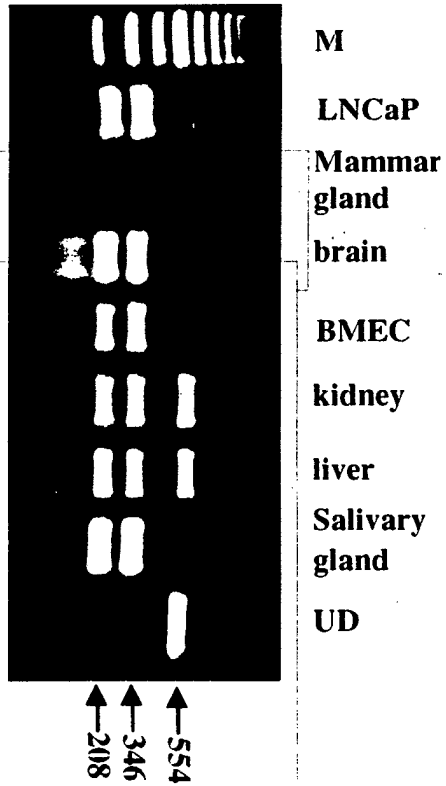
Figure 6: Comparison of the PSMA and PSMA-Like "enhancer" regions. Potential of the PSMA-Like enhancer to drive a luciferase reporter gene in combination with the PSMA promoter is reported in relative light units after adjusting for transfection efficiency. The PSMA-Like sequence can operate as a tissue-specific 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.

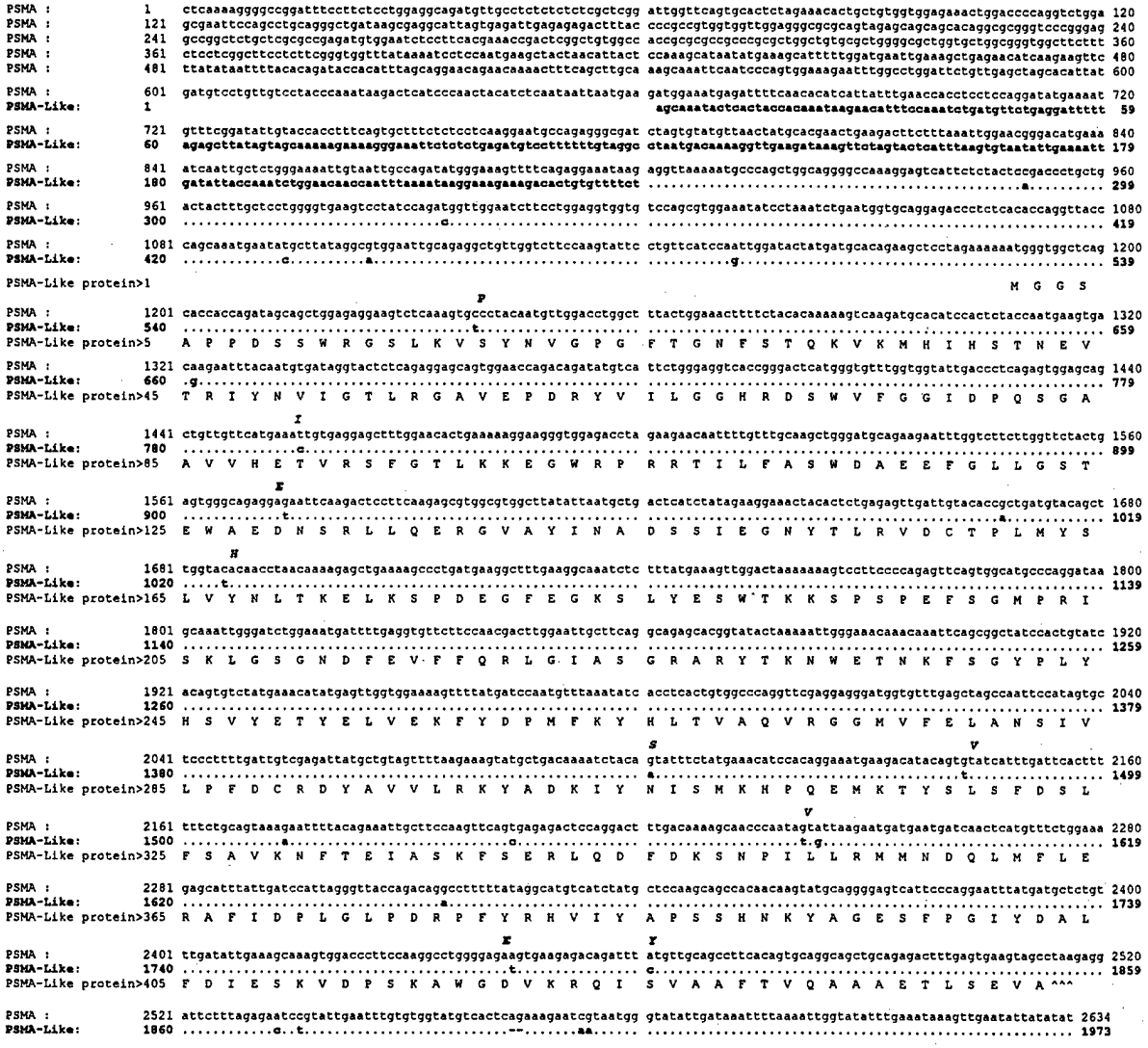
Figure 7: Deletion of the original PSMA-Like promoter, exon one and part of intron one. PCR analysis was performed using primers based outside the region predicted to be deleted in the PSMA-Like gene. Amplification of the predicted 300 bp band if a deletion had in fact occurred is seen in all five human 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.

Figure 8: Enzymatic analysis of PSMA-Like. a) Depiction of PSMA and PSMA prime versus PSMA-Like and the hybrid protein. The hybrid protein consists of the first 307 a.a. of PSMA, followed by PSMA-Like in its entirety. int=internal domain, TM=transmembrane region. b) Immunoblot analysis of PSMA or PSMA/PSMA-Like hybrid expression in transiently transfected PC-3 cells c) NAALADase activity of the transfected cell lines expressed in disintegrations per minute (DPM). Vector alone and hybrid transfected cells have no detectable NAALADase activity. All values have been adjusted for background by subtracting the DPM value of a reaction with no protein added.

Figure 1



O'Keefe et al. Figure



O'Keefe et al. Figure 2

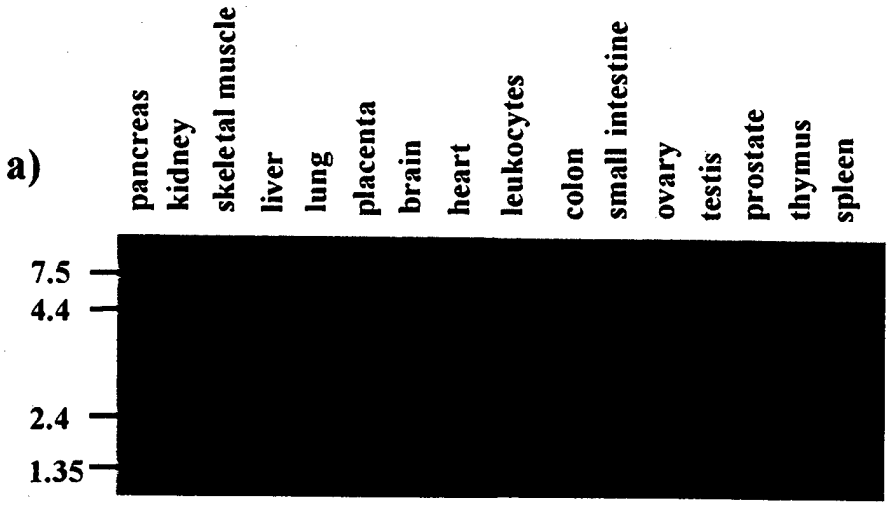
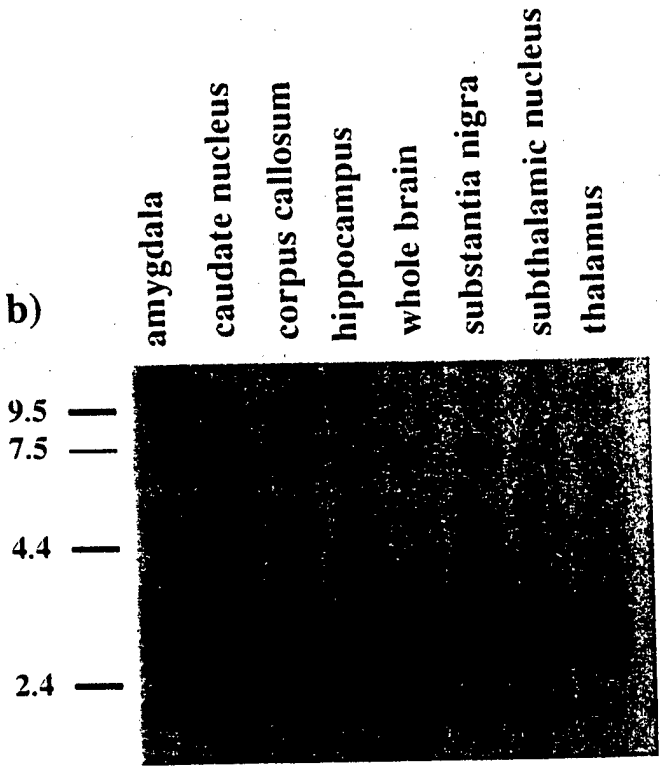

a)
pancreas
kidney
skeletal muscle
liver
lung
placenta
brain
heart
leukocytes
colon
small intestine
ovary
testis
prostate
thymus
spleen
7.5
4.4
2.4
1.35

Figure 3(a)



O'Keefe et al. Figure 3(b)

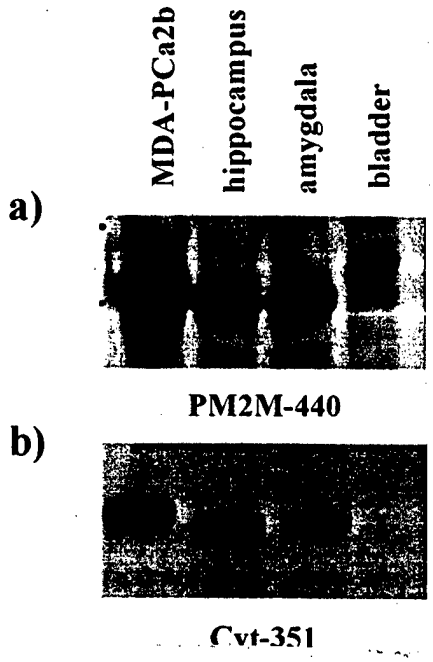


Figure 4

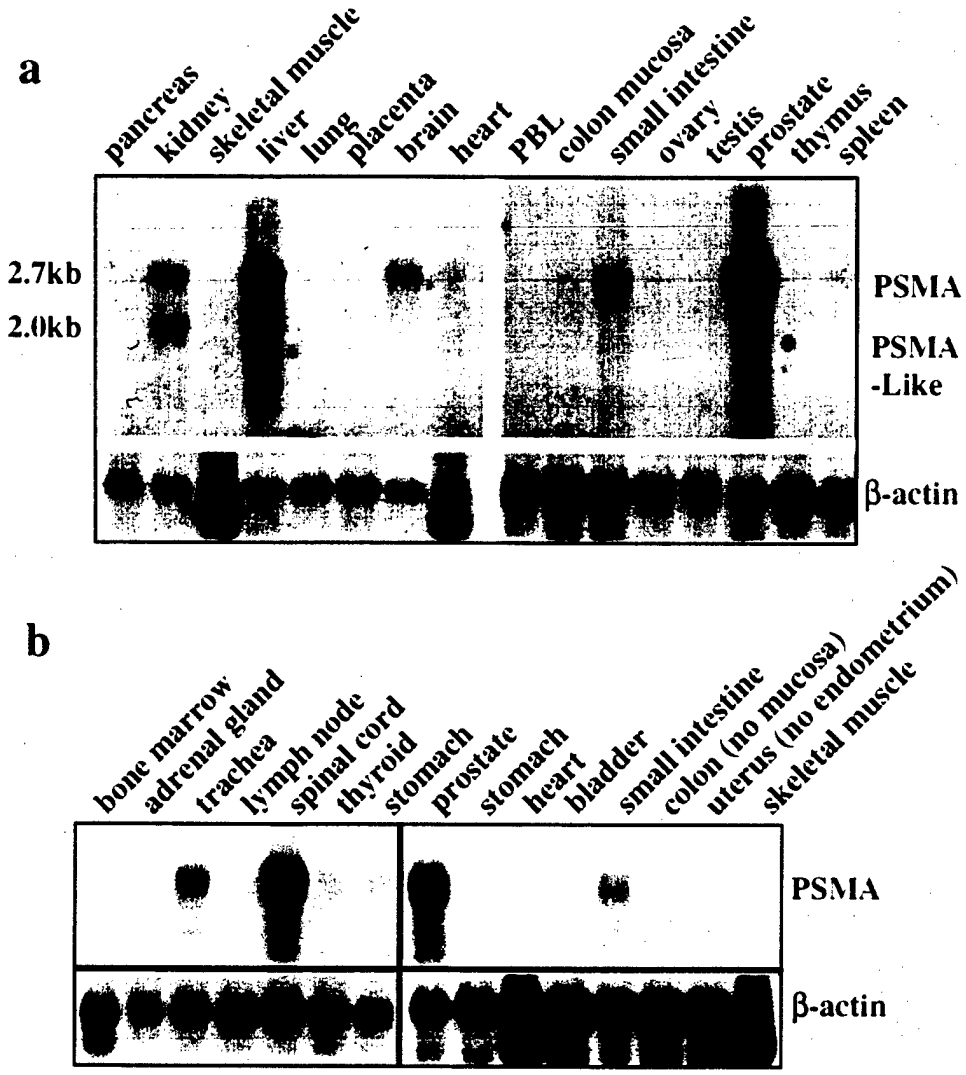
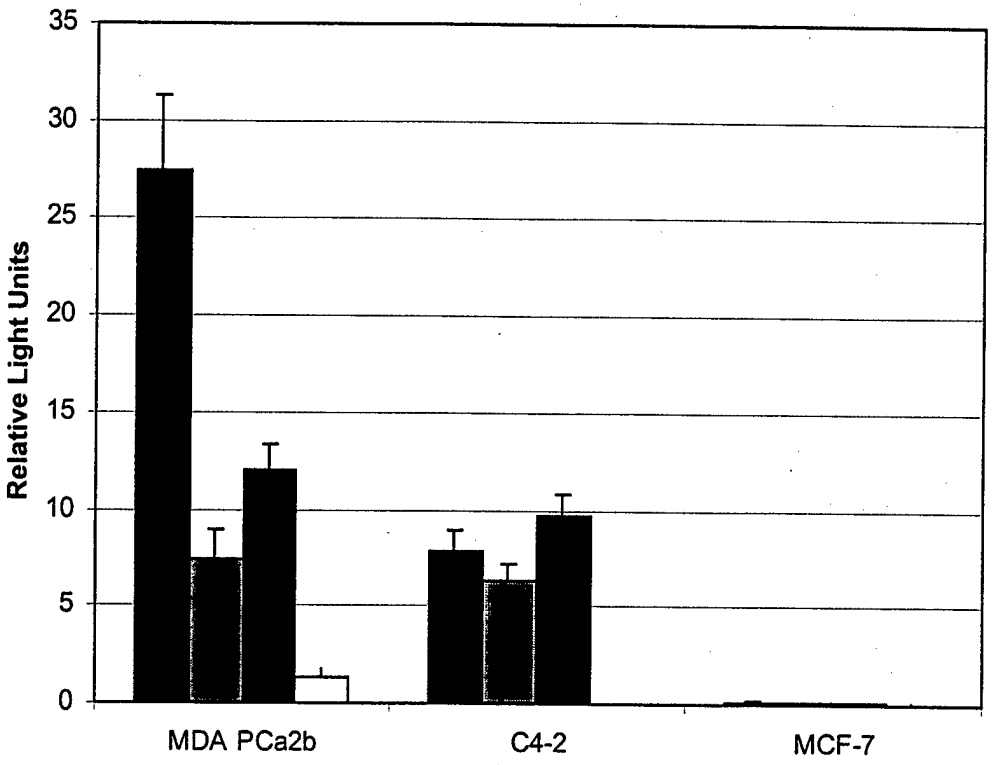
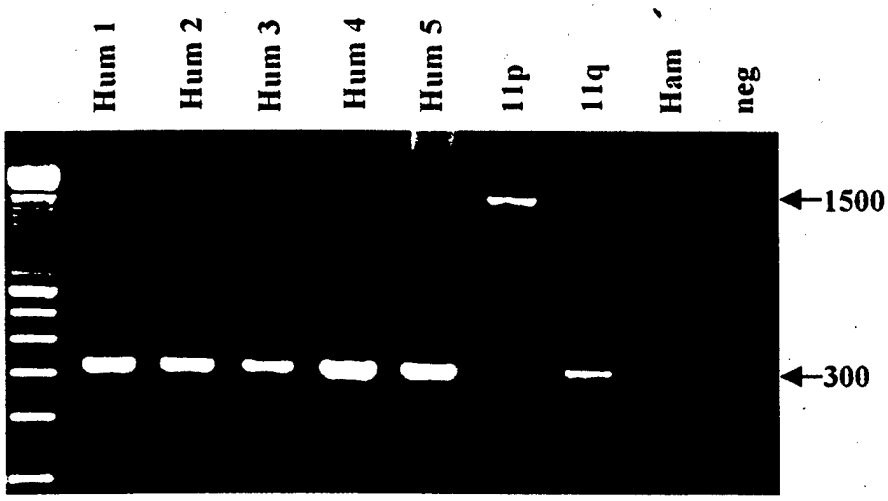

a
pancreas
kidney
skeletal muscle
liver
lung
placenta
brain
heart
PBL
colon mucosa
small intestine
ovary
testis
prostate
thymus
spleen
2.7kb
2.0kb
PSMA
PSMA
-Like
β-actin
b
bone marrow
adrenal gland
trachea
lymph node
spinal cord
thyroid
stomach
prostate
stomach
heart
bladder
small intestine
colon (no 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



O'Keefe et al. Figure 6



O'Keefe et al.

Figure 7

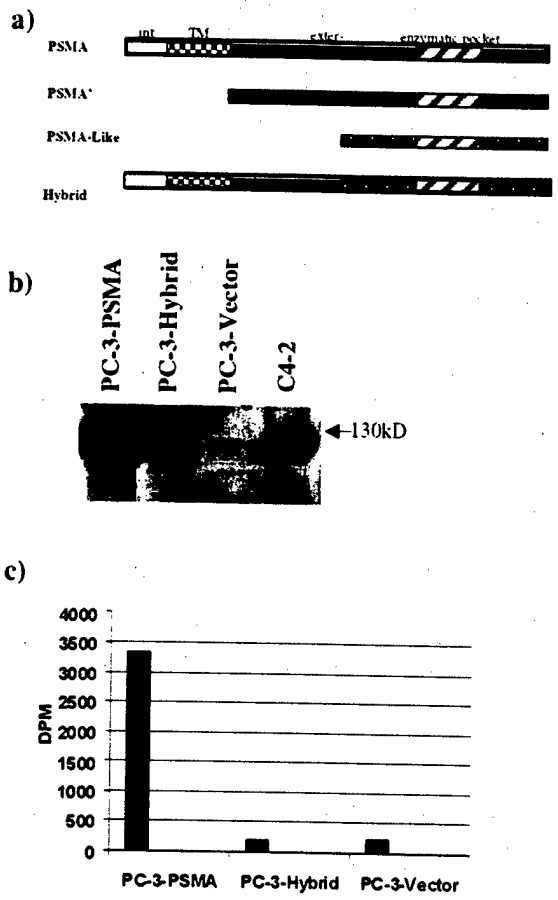

a)
PSMA
PSMA'
PSMA-Like
Hybrid
b)
PC-3-PSMA
PC-3-Hybrid
PC-3-Vector
C4-2
130kD
c)
DPM
4000
3500
3000
2500
2000
1500
1000
500
0
PC-3-PSMA
PC-3-Hybrid
PC-3-Vector

Figure 8