**Advances in Brief**

**Prostate-specific Membrane Antigen: A Novel Folate Hydrolase in Human Prostatic Carcinoma Cells**

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**Abstract**

A novel monoclonal antibody has been developed that reacts strongly with human prostatic carcinoma, especially tumors of high grade. This antibody (7E11C-5) is currently in Phase 3 trials as an imaging agent for metastatic disease. We have cloned the gene that encodes the antigen that is recognized by the 7E11C-5 monoclonal antibody and have designated this unique protein prostate-specific membrane (PSM) antigen. PSM antigen is a putative class II transmembranous glycoprotein exhibiting a molecular size of 94,000. Functionally, class II membrane proteins serve as transport or binding proteins or have hydrolytic activity. Preliminary studies have demonstrated binding of pteroylglutamate: TBS, Tris-buffered saline; PHMB, p-hydroxymercuribenzoate. (3). PSM antigen is a putative class II transmembranous glycoprotein exhibiting a molecular size of 94,000 with no homology to other characterized membrane proteins. Those developing future therapeutic strategies in the treatment of prostate cancer that utilize folate antagonists need to consider this mechanism of resistance.

**Introduction**

Recent investigations from our laboratory using the human adenocarcinoma cell line LNCaP have identified and cloned a full-length cDNA that encodes for a PSM antigen (1, 2). The message for the PSM antigen in LNCaP cells and in tumor tissue is spliced alternatively, different from that in normal tissue. Unlike tumor tissue, the mRNA expressed in normal tissue encodes for a protein that is predominantly cytosolic in location (3). PSM antigen is a putative class II transmembranous glycoprotein (M, 94,000) with no homology to other characterized integral membrane proteins except for a 150-amino acid segment that is homologous (>54%) to the transferrin receptor.
another class II membrane protein (1, 4). Functionally, class II membrane proteins serve as transport or binding proteins or have hydrolytic activity (5). Although a portion of PSM antigen exhibits modest homology with the transferrin receptor, amino acids common to the internalization sequence of the receptor are absent (6, 7), and PSM antigen probably does not operate as a transport but rather as a binding protein or protease. Using cDNA probes specific for PSM antigen, we detected strong protein with transport or hydrolytic properties characteristic of expression of a RNA from human small bowel (2). Therefore, transport but rather as a binding protein or protease. Using eDNA probes specific for PSM antigen, we detected strong expression of a RNA from human small bowel (2). Therefore, we considered the possibility that PSM antigen may represent a protein with transport or hydrolytic properties characteristic of mucosal cell membranes. The brush border of small intestine is notable for high activity of pteroyl poly-g-glutamyl carboxypeptidase (folate hydrolase; Ref. 8). Preliminary studies in our laboratory demonstrated binding of pteroylmonoglutamate (folate hydrolase: Ref. 8).

Membrane proteins serve as transport or binding proteins or have hydrolytic activity (5). Although a portion of PSM antigen is membrane bound with a molecular weight in the range of that of PSM antigen (9). In this article, we report that PSM antigen exhibits folate hydrolase activity using MTXGlu₃ and PteGlu₄ as substrates.

### Materials and Methods

**Materials.** MTXGlu₃ (4-NH₂-10-CH₃-PteGlu₄), PteGlu₄, and pABAGlu₄ were purchased from Dr. B. Schircks Laboratories (Jona, Switzerland), and samples were >98% pure when evaluated by high-performance liquid chromatography. NAAG (40 Ci/mmol) was purchased from DuPont New England Nuclear (Boston, MA). Protein A Sepharose 4 Fast Flow was purchased from Pharmacia (Piscataway, NJ). The 7E11-C5 monoclonal antibody to prostate specific membrane antigen was obtained from Cytogen Corp. (Princeton, NJ). All other reagents (PHMB, homocysteine, DTT, reduced glutathione) were of the highest purity commercially available from Sigma Chemical Co. (St. Louis, MO).

**Culture and Growth of Human Prostate Adenocarcinoma Cells (LNCaP, PC-3, TSUPr1, and Duke-145).** LNCaP cells were maintained in defined culture medium, RPMI 1640 supplemented with nonessential amino acids, 5 mm glutamine, and 5% heat-inactivated FCS. Duke-145, PC-3, and TSU-Pr1 cells were grown in MEM, Ham's F-12K, and MEM, respectively, containing 5% FCS. No antibiotic was included in the media. Cells (1 × 10⁶) were plated in T-75 tissue culture flasks containing 15 ml of medium and incubated at 37°C in a humidified atmosphere of 5% CO₂. Cell numbers were determined using a Model ZF Coulter Counter (Coulter Electronics). Prostate cells were harvested from plates by gentle scraping at 4°C into PBS [136.9 mm NaCl, 2.68 mm KCl, 8.10 mm Na₂HPO₄, 1.47 mm KH₂PO₄ (pH 7.34), and PBS], and centrifuged at 500 × g to obtain a cell pellet. Sedimented cells were routinely resuspended with 15-ml volumes of PBS (2).

**Transfection of PSM into PC-3 Prostate Cell Line.** The full-length 2.65-kb PSM cDNA was subcloned into a pREP7 eukaryotic expression vector (Invitrogen, San Diego, CA) as described previously (2). Plasmid DNA was purified from transfected DH5-α (Life Technologies, Inc.) using a Qiagen Maxiprep Plasmid Isolation Kit (Qiagen, Inc., Chatsworth, CA). Purified plasmid DNA (5 µg) was diluted with 300 µl of serum-free RPMI and mixed with 45 µl of lipofectamine (Life Technologies, Inc.), which was diluted previously with 300 µl of serum-free RPMI, to allow a DNA-liposome complex to form. The mixture was kept at room temperature for 30 min and then added to a 60-mm Petri dish containing 60-70% confluent PC-3 cells in 2.4 ml serum-free RPMI. The DNA-liposome complex containing serum-free media was mixed gently to ensure uniform distribution and was then incubated for 6 h at 37°C in a CO₂ incubator. After incubation, the medium containing liposome-DNA complex was aspirated and replaced with 6 ml of regular growth media (10% fetal bovine serum, 1% penicillin-streptomycin, and 1% glutamine). After 48 h, cells were trypsinized and split 1:3 into 60-mm dishes containing regular media supplemented with 200 µg/ml of hygromycin B (Calbiochem, La Jolla, CA). Cells were maintained for 2 weeks with changes of medium containing hygromycin B every third day until discrete colonies appeared. Colonies were isolated using a 6-mm cloning cylinder and were expanded in the same media. As a control, PC-3 cells were also transfected with the pREP7 vector alone.

**Immunohistochemistry.** The 7E11-C5 monoclonal antibody to prostate specific antigen was used. This antibody recognizes a portion of carbohydrate-containing peptide epitope on the amino-terminal end of PSM that is located on the inner portion of the cytosolic membrane. After permeabilization of LNCaP- and PC-3-transfected and -nontransfected cells with a mixture of acetone and methanol (1:1 v/v) and blocking with 5% BSA in 50 mM TBS pH 7.45, samples were incubated with 7E11-C5 antibody (20 µg/ml) for 1 h at room temperature. Negative controls were generated by substituting the same concentration of mouse IgG2a for the PSM antibody. Using a secondary IgG1 antimouse antibody conjugated with alkaline phosphatase, samples were reincubated for 1 h, rinsed in TBS, and stained with bromochloroindolylphosphate phosphate in 2-amino-2-methyl-1-propanol buffer. Cells expressing PSM demonstrate an intense blue staining.

**Cell Membrane Preparation.** Cell lysates were prepared by sonicating approximately 6 × 10⁶ cells in 50 mM Tris pH 7.4 buffer (two 10-s pulses at 20 W) in an ice bath. Membrane fractions were obtained by centrifuging lysates at 100,000 × g for 30 min. The supernatant fractions were saved, and pelleted membranes were resuspended by gentle trituration and resedimented at 100,000 x g for 30 min through 10 ml of cold 50 mM Tris pH 7.4 buffer. Washed membrane fractions were dissolved in 50 mM Tris pH 7.4 buffer containing 0.1% Triton X-100 (Tris-Triton). Enzymatic activity and immunoprecipitation preparations were performed using this membrane preparation.

**Immunoprecipitation of PSM from Membrane.** Membrane pellets (~1 mg protein) solubilized in Tris-Triton buffer were incubated at 4°C for 1 h in the presence of 7E11-C5 antiprostate monoclonal antibody (6 µg protein; Ref. 1). Protein A Sepharose gel equilibrated in Tris-Triton buffer was added to the immunocomplex. This preparation was subsequently incubated for an additional hour at 4°C. Sepharose beads were centrifuged at 500 × g for 5 min and rinsed twice with Tris-Triton buffer at pH 7.4. Isolated beads were resuspended in 0.1
m glycine buffer (pH 3.0) and vortexed, and the supernatant fraction was assayed for hydrolase activity using MTXGlul.

**Pteroyl γ-Glutamyl Hydrolase Assay.** Hydrolase activity was determined using capillary electrophoresis (10). The standard assay mixture contained 50 μM MTXGlul, 50 mM acetate buffer (pH 4.5), and enzyme to a final volume of 100 μl. A sample preparation without enzyme was incubated concurrently with enzymatic assays, and reactions were conducted for times varying between 0 and 240 min at 37°C. Activities were also determined in standard reaction mixture at varied pHs for 60 min. Reactions were terminated in a boiling water bath for 5 min, and samples were stored frozen (−20°C) until analysis. After centrifugation (7000 X g) to remove precipitated debris, capillary separation of MTX-glutamated analogues was performed with a Spectra Phoresis 1000 instrument (Thermo Separation, San Jose, CA) with a 75-μm inside diameter × 50-cm silica capillary (Polymicro Technology, Phoenix, AZ). Separation of PteGlu derivatives is achieved with an electrolyte of 20 mM sodium borate with 15 mM SDS (pH 9.5) with +20 keV at 25°C. Samples were applied hydrodynamically for 1–2 s, and absorbance was monitored at 300 nm. Data were recorded with an IBM computer using CE-1000 software (Thermo Separation).

**Protein Determination.** Protein concentrations of isolated membrane or supernatant fractions were determined by incubating diluted aliquots with bicinchoninic acid reagent (Pierce Chemical Co., Rockford, IL) at 37°C for 30 min. The spectrophotometric quantitation of protein was conducted by determining the absorbance at 562 nm against BSA standard.

**Statistical Analysis.** Data were analyzed using the Statgraphics program (version 4.0; Statistical Graphics Corp., Rockville, MD) and where summarized are expressed as mean ± SD. Student’s unpaired t test was used to determine significance of differences.

**Results**

Membrane fractions isolated from human prostate adenocarcinoma cells (LNCaP) were incubated using primarily MTXGlul as substrate. The time course of hydrolysis of the γ-linked triglutamate derivative and the subsequent appearance of MTXGlul2, MTXGlul1, and MTX after 30, 60, 120, and 240 min of incubation are illustrated in Fig. 1A. The semipurified PSM antigen exhibits pteroyl poly-γ-glutamyl exopeptidase activity that progressively liberates all of the possible glutamates from MTXGlul with accumulation of MTX.

The PSM antigen was immunoprecipitated in the presence of 7E11-C5 antiprostata monoclonal antibody, and the PSM antigen-antibody complex was adsorbed onto a Protein A Sepharose gel column. After twice washing the sepharose beads with 2-mI volumes of buffer and resolubilization of the antigen-antibody complex by adjusting the elution pH to 3.0, the supernatant fraction was assayed for hydrolase activity. Fig. 1B shows the capillary electrophoretic separation of successively cleaved glutamyl moieties from MTXGlul after 0-, 30-, 60-, and 240-min incubations. Results similar to these in Fig. 1 were obtained using PteGlu with formation of folate (PteGlu).

The optimum pH activity profiles of the immunoprecipitated PSM hydrolase from LNCaP cells and of the membrane fractions from PC-3 PSM-transfected and -nontransfected (vector alone) cells are shown in Fig. 2. The reaction was monitored as a function of pH from 2 to 10 after a 1-h incubation with MTXGlul. The extent of reaction was expressed as the concentration of MTXGlul formed per mg protein. Although all reaction products were detectable as illustrated in Fig. 1, MTXGlul was the predominant hydrolyzed species at incubation times ranging from 10 to 60 min. The pH profile of membrane fractions isolated from both LNCaP and PC-3 PSM-transfected cells are identical and exhibit two maxima of PSM hydrolase activity at pH 5 and 8 with no measurable activity above pH 10.

To determine whether non-PSM antigen-expressing human adenocarcinoma cell lines (PC-3, TSU-Pr1, and Duke-145) exhibit folate hydrolase activity, isolated membrane preparations from these cell lines were analyzed (Fig. 3). The less-differentiated...
tiated, hormone-refractory prostate cell lines (PC-3, TSU-Pr1, and Duke-145) exhibit no appreciable activity after 2-h incubations. These results are in agreement with previous findings that demonstrate neither a presence of a mRNA for PSM nor antigen immunoreactivity with 7E11-C5 in these cells.

In additional studies in which the cDNA for PSM was transfected into non-PSM antigen-expressing PC-3 cells, we observed a close correlation between PSM antigen immunoreactivity and hydrolase activity with MTXGlu₂ in membranes of LNCaP and PC-3 PSM-transfected cells (Figs. 4 and 5). Immunohistochemical analyses of LNCaP (Fig. 4A) and PSM antigen-expressing PC-3 (Fig. 4B) cells revealed distinct positive staining with 7E11-C5 anti-prostate monoclonal antibody. Fig. 4C illustrates no immunoreactivity in PC-3 cells expressing the pREP7 hygromycin vector alone. In preparations of negative controls, all three cell lines were reacted with IgG2aK rather than with 7E11-C5 antibody. No background staining resulted with the secondary antibody conjugated with alkaline phosphatase.

To compare PSM hydrolase activity with that of other γ-glutamyl hydrolases that either reside within the lysosome or are secreted as observed in several neoplastic cells (11), we examined its reactivity in the presence of thiol-containing reducing agents, namely, reduced glutathione, homocysteine, and DTT, and the thiol reagent, PHMB, at concentrations ranging from 0.05 to 0.5 mM. Of the reduced sulfhydryl derivatives, we found that only DTT (0.2 mM) was slightly inhibitory (86 ± 3% of control). Unlike γ-linked peptide hydrolase retained within the lysosome, PSM hydrolase activity was maintained in the presence of 0.5 mM PHMB.

The reactivity of PSM hydrolase against an α-glutamate dipeptide, NAAG, has been investigated (12). We observed that the PSM enzyme from either LNCaP- or PSM-transfected PC-3 cell membranes hydrolyzes NAAG producing N-acetylaspartate and glutamate. Furthermore, MTXGlu₂, PteGlu₂, and pABAGlu₂ were potent inhibitors of the PSM-mediated NAAG hydrolysis (data not shown).

Fig. 2 Effects of pH on γ-glutamyl hydrolase (PSM hydrolase) activity in LNCaP, PC-3 PSM-transfected [PC-3(+)], and PSM-non-transfected [PC-3(−)] cells. Enzymic activity is reported as μM MTXGlu₂ formed/mg protein. Each value represents the mean of three reactions containing 50–60 μg/ml protein. The following buffers were used in 50 mM concentrations spanning a pH range of 2–10: glycine-HCl, pH 2.2–3.6; acetate, pH 3.6–5.6; 2-(N-morpholino)ethanesulfonic acid, pH 5.6–6.8; Tris(hydroxymethyl)aminomethane, pH 7–8.5; and glycine-NaOH, pH 8.6–10.0.

Fig. 3 Comparison of pteroyl hydrolase activity in membranes isolated from adenocarcinoma cell lines LNCaP, PC-3, Duke-145, and TSU-Pr1. Membranes were isolated as described in "Materials and Methods." Each value represents the mean of triplicate reactions normalized to 1 mg/ml protein. Bars, SD.
Fig. 4 Immunohistochemical analysis of LNCaP and PC-3 PSM-transfected and -nontransfected cells. A 2.65-kb PSM cDNA containing a hygromycin selection vector was cloned into non-PSM antigen-expressing PC-3 cells and maintained in regular media supplemented with hygromycin B. As a control, PC-3 cells were also transfected with the pREP7 vector alone (PC-3 PSM-nontransfected cells). Cells were permeabilized in acetone-methanol (1:1 v/v) mixture and blocked with 5% BSA/TBS, and the 7E11-C5 monoclonal PSM antibody was added to the cells. A secondary antimouse IgG1 antibody conjugated with alkaline phosphatase was added, and PSM-positive cell staining was performed with bromochloroindolylphenol phosphate. A. intense immunoreactivity associated with LNCaP cells using the monoclonal PSM antibody. B. comparable staining occurs in PC-3 cells transfected with PSM expression vector. C. PC-3 cells expressing pREP7 hygromycin vector alone.

Discussion

Expression of PSM antigen is increased in prostate cancer and is represented strongly in metastatic disease (1). A monoclonal antibody, 7E11-C5, that recognizes PSM antigen was generated initially against LNCaP membranes (13), and a modified version of the antibody for use as an imaging agent is currently in Phase 3 clinical trials for detection of metastatic prostate disease, especially that involving lymph nodes and bone (14).

We report herein that membrane-bound PSM antigen has pteroyl poly-γ-glutamyl carboxypeptidase (folate hydrolase) activity. γ-glutamyl hydrolase activity is also present in lysosomes of cells, and these enzymes may be responsible for regulating the length of exogenous and endogenous folyl polyglutamate chain lengths. A characteristic difference between these two hydrolases is that the PSM enzyme exhibits substantial activity at pH values 7.5–8.0 in addition to having an acidic pH of 4.5–5 optimum. Moderate levels of hydrolase activity are present within the LNCaP cytosolic compartment and may represent the short intracellular fragment of this class II enzyme. This reflects an interesting situation in these cells in which the majority of RNA codes for the membrane-bound enzyme that is localized extracellularly. An explanation of this finding may be the alternative splicing of the PSM gene that occurs at a high rate in both LNCaP cells and prostate cancer specimens (3). The ratio of the mRNAs in these samples that code for the class II membrane and the cytosolic proteins is 10:1. In normal prostate tissue, the mRNA coding for the membrane protein is only 1/10 that of the cytosolic form (3).

Given the high expression of pteroyl hydrolase activity associated with the PSM antigen, consideration must be given to its role in the cellular physiology of the prostate. Retention of intracellular folates and reactions of the folate requiring enzymes are controlled by several processes, namely, the intracellular concentration of binding proteins, the ratio of oxidized and reduced state of folates, and notably, their degree of polyglutamation (15). It would be expected that a pteroyl hydrolase would reduce the level of polyglutamated folates and, as folyl polyglutamate synthetase is usually found present in limited amounts in most tissues, pteroyl hydrolase could play a role in decreasing the extent of glutamation of folates. Investigations are in progress into the extent of polyglutamation as well as the number...
and type of folate species in normal, benign, and malignant prostatic tissues.

A key feature of some tumors is that those expressing excessive pteroyl hydrolase activity are resistant to MTX chemotherapy (16). When MTX is polyglutamated, it is retained intracellularly and antagonizes folate-dependent enzymes (17). Either a diminished folyl polyglutamate synthetase or an enhanced pteroyl hydrolase activity will result in decreased levels of polyglutamated MTX. Each circumstance will contribute to forms of acquired and intrinsic resistance of tumor cells to this chemotherapeutic agent. Enhanced activity of a lysosomal γ-glutamyl hydrolase in human sarcoma cells confers resistance to MTX (18). The relationship between this form of resistance and PSMA hydrolase activity is currently under investigation in LNCaP and PC-3 PSMA antigen-transfected cells. It is important to note that enhanced folate hydrolase activity in prostate cancer cells overexpressing PSMA may not be the only mechanism for MTX resistance. PSMA-negative prostate cancers may display determinants of MTX resistance through other mechanisms that can involve diminished membrane transport of MTX, overproduction of target enzymes (e.g., dihydrofolate reductase), or decreased binding affinity of dihydrofolate reductase for MTX (16–18). To date, clinical trials have not displayed appreciable activity with MTX as a single agent in the treatment of prostate cancer (19).

The presence of a γ-glutamyl carboxypeptidase has been described in rodent testes that prefers β-citryl glutamate as a substrate (20). These studies compared the activity of the rodent enzyme with that of brain carboxypeptidase that hydrolyzes NAAG, a neurotransmitter (20–22). It was observed that NAAG was not a substrate for the enzyme derived from rat testes (20). Because β-citryl glutamate is not commercially available, we investigated whether PSMA hydrolase cleaves the α-linked glutamyl moiety from NAAG. By contrast to rodent carboxypeptidase, the PSM enzyme hydrolyzes the α-linked glutamyl peptide. However, MTXGlu3, PteGlu3, and pABAGlu3 are potent inhibitors of the NAAG reaction, and thus deglutamation of γ-linked folates may be the preferred reaction.

In a fashion similar to rat brain NAAG carboxypeptidase, PSMA hydrolase is present in the extracellular environment and thus may play a role as a "neuronal" peptidase. Indeed, the prostate epithelium express many neuron-like features. In the normal prostate gland, the presence of neuroendocrine cells are hypothesized to represent an end stage of differentiation (23), and prostate cancer cells often migrate along neural tracts (24). How, and whether, PSMA hydrolase plays a role in the neuroendocrine-like features of prostate cancer remains to be determined. Adequate folate levels and neural function are closely associated, and PSMA hydrolase could represent one area for interaction. We have observed that polyglutamated folates are potent inhibitors of the PSMA-mediated NAAG reaction. Although polyglutamated folates are not typically extracellular substances, PSMA hydrolase may degrade polyglutamated folates that compete for NAAG binding in neuronal tissue. In this regard, it is of interest that immediately after chemotherapeutic rescue with high doses of folate derivatives, seizure activity has been reported, thus suggesting a competitive relationship between these substances (25).

In summary, we have identified a function for PSMA antigen as a pteroyl poly-γ-glutamyl carboxypeptidase (folate hydrolase). Carcinoma cells that express mRNA for PSMA antigen or have been transfected with cDNA for PSMA exhibit folate hydrolase activity. PSMA hydrolase exhibits exopeptidase activity and is expressed strongly in prostate cancer, especially in tumors that have metastasized to bone and lymph tissue. Cancers cells that express this enzyme demonstrate resistance to methotrexate therapy. Those developing future therapeutic strategies in the treatment of prostate cancer that utilize folate antagonists need to consider this mechanism of resistance. To optimize use of PSMA hydrolase as a therapeutic target, we plan to further determine its substrate specificities and selectivity and discern its primary cellular function. Future investigations on this enzyme
will be facilitated by utilizing PC-3 cells that were transfected successfully with the 2.65-kb cDNA for PSM hydrolase. Information derived from this study of the highly expressed pteroyl hydrolase should provide further understanding of the unique biology of the prostate.

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References


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