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Tissue Expression and Enzymologic Characterization of Human Prostate Specific Membrane Antigen and Its Rat and Pig Orthologs

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BACKGROUND. Prostate specific membrane antigen (PSMA), also called glutamate carboxypeptidase II (GCPII), is a target enzyme for diagnosis and treatment of prostate cancer. Moreover, it is upregulated in the vasculature of most solid tumors and is therefore a potential target for the generation of novel antineoplastics. In this context, we analyze the possibility of using rat and pig as animal models for enzymologic and in vivo studies.
METHODS. We prepared the recombinant extracellular part of human, rat, and pig GCPII in S2 cell media and characterized the activity and inhibition profiles of the three orthologs by radioenzymatic assay. We performed Western blot analysis of GCPII expression in human, rat, and pig tissues using the monoclonal antibody GCP-04 and confirmed these findings by activity measurements and immunohistochemistry.
RESULTS. The three recombinant proteins show similar specific enzymatic activities and inhibition profiles. Tissue expression analysis revealed that most of the pig and human tissues show at least some GCPII-positivity, while the expression pattern in rat is more restricted. Moreover, tissues such as prostate and testes exhibit different GCPII expression levels among the species studied.
CONCLUSIONS. The rat and pig orthologs of GCPII seem to be suitable to approximate human GCPII in enzymologic studies. However, the diffuse expression pattern of GCPII in animal and human tissues could be a caveat for the potential utilization of GCPII-targeted anticancer drugs. Furthermore, variations in GCPII tissue distribution among the species studied should be considered when using rat or pig as models for antineoplastic drug discovery.
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KEY WORDS: **prostate specific membrane antigen; glutamate carboxypeptidase II; animal orthologs; prostate cancer; animal model**

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INTRODUCTION

According to estimates, prostate cancer accounted for the highest percentage (33%) of all incident cancer cases in American men in 2006. In the same year, it was the third leading cause of cancer-related death (9% of all cancer deaths) [1]. New diagnostic and therapeutic approaches for prostate cancer are therefore being actively pursued. One of the molecules thought to be relevant for both diagnosis and treatment of prostate cancer is prostate specific membrane antigen (PSMA), called also glutamate carboxypeptidase II (GCPII). Since the latter name is more general, we decided to use it throughout the whole manuscript.

GCPII is a 750-amino acid, heavily glycosylated membrane-bound metallopeptidase that utilizes two zinc ions for substrate hydrolysis [2,3]. PSMA was first identified as an antigen of the 7E11-C5.3 antibody, which was raised against membranes of LNCaP cells isolated from human prostate cancer [4]. In parallel, an exopeptidase activity of this protein was discovered in rat brain membranes, where it cleaves the peptide neurotransmitter *N*-acetyl-L-aspartyl-L-glutamate (NAAG) into *N*-acetyl-L-aspartate and L-glutamate [5]. In the prostate, several variants of this protein were found, among them so-called PSM′, which lacks the first 59 N-terminal amino acids and is dominant (on the mRNA level) in normal human prostate, while full-length PSMA is the prevalent form in primary prostate tumors [6–8]. GCPII expression is up- or down-regulated in some non-prostatic tumors [9], and its role in cancer is also supported by the fact that it is expressed in prostatic as well as non-prostatic tumor-associated neovasculature, whereas normal human vascular endothelium is GCPII negative [10–12].

Many studies have been conducted to assess the possibility of using GCPII as a diagnostic tool in prostate cancer patients. One successful discovery is ProstaScint (Capromab pendetide, CYT-356), a ^{111}In-labeled immunoconjugate of 7E11-C5.3 antibody [13] which is currently used as a radioimmunoscintigraphic imaging agent capable of detection of prostate cancer and/or its metastases [14,15] (for review see Ref. [16]).

In addition to diagnostic strategies, many therapeutic strategies based on GCPII are being evaluated at present. Most of these are based on modified components of the immune system, such as dendritic cells pulsed with PSMA-derived peptides [17–19] or anti-PSMA antibodies either conjugated to toxins [20] or radiolabeled by ^{90}Yttrium/^{177}Lutecium [21,22].

For the development of novel treatment and diagnostic approaches, an appropriate animal model is an invaluable tool. To this end, the orthologs of the human target molecule must be characterized in terms of sequence identity, tissue expression, and activity.

Rat and pig GCPII have very high amino acid sequence identity with human GCPII (85% and 91%, respectively) [23–25]. Before human recombinant GCPII expressed in insect cells became available [26], a number of enzymologic and inhibition analyses of GCPII were performed using rat brain extracts. The rat and pig orthologs of GCPII have been cloned and partially characterized [24,25], but little direct comparison of the enzymatic activities of these orthologs has been published to date. Tissue localization and expression of GCPII in various species has been studied by several laboratories both on the mRNA and protein level. However, these analyses are incomplete, and the individual studies were accomplished by different methods.

Therefore, we decided to map GCPII protein expression and activity in human, rat, and pig tissues in one consistent study. Recently, we published the preparation and analysis of a novel monoclonal antibody, GCP-04, which recognizes an epitope in the extracellular portion of the GCPII molecule [26,27]. We have shown that this antibody is more sensitive than the prototype 7E11-C5.3 antibody [27]. Twenty-one various tissues from each species were examined with the GCP-04 antibody and, in selected tissues, NAAG-hydrolyzing activity was determined to confirm the immunochemistry data. In addition, we have prepared the extracellular domains of GCPII of *Homo sapiens sapiens* (amino acids 44–750; hGCPII), *Rattus norvegicus* (amino acids 45–752; rGCPII), and *Sus scrofa* (amino acids 44–751; pGCPII) by recombinant expression in *Drosophila* S2 cells to compare their proteolytic activities. Altogether, this study analyzes the possibility of using these common species as animal models for the development of GCPII-based cancer diagnosis and therapy.

MATERIALS AND METHODS

Tissue Samples

Human samples were obtained during autopsy performed 4–8 hr postmortem (after 2 hr at room temperature, the corpses were stored at 5°C). Rat samples were collected from Lewis strain rats; pig samples originated from healthy miniature pigs of the MeLiM strain with hereditary melanoma [28].

For Western blotting and activity measurements, tissue samples were frozen immediately in liquid nitrogen and subsequently homogenized in 50 mM tris(hydroxymethyl)aminomethane (Tris; Sigma–Aldrich, St. Louis, MO), 150 mM NaCl (PENTA, Prague, Czech Republic), pH 7.5 supplemented with protease inhibitors (Complete Mini, EDTA-free, Roche, Mannheim, Germany). The samples were then sonicated (10 μm amplitude, Soniprep 150 machine, Sanyo,

San Diego, CA) three times for 10 sec on ice and Triton X-100 (Serva, Heidelberg, Germany) was added to 1% (v/v). After a 30-min incubation on ice, the suspension was centrifuged at 16,000*g* for 10 min and the supernatants were stored for further analysis at −80°C. In addition, samples used for activity measurements were dialyzed against 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS; Sigma), 20 mM NaCl, pH 7.4. Protein concentrations were determined using Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) according to the manufacturer's protocol.

For immunohistochemistry, the samples were fixed in 10% buffered formalin for a minimum of 24 hr.

Molecular Cloning of *Rattus Norvegicus* and *Sus Scrofa* GCPII

cDNAs of *R. norvegicus* (cloned in pBluescript) and *S. scrofa* GCPII (cloned in pCR®2.1-TOPO) were kind gifts from Tomasz Bzdega, Georgetown University [23], and Charles H. Halsted, University of California [25], respectively. cDNA segments encoding rGCPII (amino acids 45–752) or pGCPII (amino acids 44–751) were amplified by polymerase chain reaction (PCR) with *Pfu* polymerase (Promega, Madison, WI), using the following primers: forward 5′-AAAAGATCTAAACCCTCCAATGATTCTACTAGCAGTGTTTCC-3′ and reverse 5′-AAACTCGAGTTAGTCTACTTCTCTCAGAGTCTCTGCTGC-3′ for rGCPII, forward 5′-AAAAGATCTAAATCTCCCAATGAAGCTGCTAACATTAGTCC-3′ and reverse 5′-AAACTCGAGTTAGGCTACTTCTCTCAGAGTCCCTGCTGCGGCC-3′ for pGCPII. The resulting cDNAs were cloned into the pMT/BiP/V5-HisA vector (Invitrogen, San Diego, CA) using *Bgl*II and *Xho*I restriction sites (New England Biolabs, Beverly, MA). Verification of DNA sequence was performed with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

Transfection of Insect Cells and rGCPII/pGCPII Expression

Drosophila Schneider 2 (S2) cells in SF900II medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) were grown at 24°C. At a density of $2–4 \times 10^6$ cells/ml, the cells were co-transfected with 19 μg of the expression plasmid and 1 μg of pCoHYGRO (Invitrogen) selection vector following the calcium-phosphate-mediated transfection procedure provided by the manufacturer. After a 16-hr incubation at 24°C the cells were transferred into fresh medium and grown for 2 more days. Subsequently, the cells were selected in medium containing 300 μg/ml Hygromycin B (Invitrogen) for 3 weeks. The hygromycin-resistant cells were transferred into serum-free medium supplemented with 100× Defined Lipid Concentrate and 50×Yeastolate Ultrafiltrate (Invitrogen) and incubated at 24°C with stirring at 120 revolutions per minute (RPM). At a density of 10×10^6 cells/ml, protein expression was induced with 1 mM $CuSO_4$ (Sigma). On days 1 and 3 postinduction the medium was supplemented with L-glutamine and 20% D-glucose (Sigma) to final concentrations of 1 mM and 0.1%, respectively. The conditioned medium was harvested 5 days after induction by centrifugation at 500*g* for 10 min and was frozen at −20°C. For biochemical studies, the conditioned medium was dialyzed 3×10 hr against 20 mM MOPS, 20 mM NaCl, pH 7.4.

Determination of NAAG-Hydrolyzing Activity and Inhibition Profile

Radioenzymatic assay. Tissue homogenate, purified hGCPII [26], or conditioned medium containing rGCPII or pGCPII was diluted into reaction buffer (20 mM MOPS, 20 mM NaCl, pH 7.4) to a final volume of 180 μl. For IC_{50} measurements, 2-(phosphonomethyl)pentanedioic acid (2-PMPA) (kind gift from MGI Pharma, Baltimore, MD) of various concentrations (0–5,000 pM) was also included. After a 5-min preincubation at 37°C, the reaction was started by addition of 20 μl of a mixture of unlabeled and radiolabeled NAAG (950 nM NAAG + 50 nM ^{3}H-NAAG for IC_{50} measurements, 0.2–100 μM NAAG + 50–100 nM ^{3}H-NAAG for K_M measurements; PerkinElmer, Boston, MA). The reaction mixtures were incubated at 37°C and then GCPII activity was quenched by the addition of 200 μl of 200 mM KH_2PO_4 (Sigma–Aldrich), pH 7.4. Samples were applied onto an AG1-X8 Resin column (formate form, Bio-Rad), and released L-glutamate was eluted with 2 ml of 1 M formic acid (Lachema, Brno, Czech Republic) and quantified by liquid scintillation (after mixing with the scintillation cocktail-Rotiszint ECO Plus, Roth, Karlsruhe, Germany). For k_{cat} calculation, concentrations of rGCPII and pGCPII in the media were determined by quantification of Western blots, employing the ImageQuant TL program (Amersham Biosciences, Piscataway, NJ), using a calibration curve constructed from hGCPII of known concentrations. The concentration of hGCPII was determined by active-site titration of the purified protein by 2-PMPA using high performance liquid chromatography (HPLC) assay-see below. Data analysis was performed using GraFit (version 5.0.4, Erithacus Software Limited, Horley, UK).

HPLC assay. The active site concentration of purified hGCPII was determined by inhibition of the enzyme by 2-PMPA, assayed in a total reaction volume of 120 μl in

the reaction buffer (20 mM MOPS, 20 mM NaCl, pH 7.4). After incubation of purified hGCPII (final concentration approximately 90 nM) with 100 μM *N*-acetyl-L-aspartyl-L-methionine (a substrate of GCPII [26]) in the presence of increasing concentrations of 2-PMPA (0–1,000 nM) for 12 min at 37°C, the reaction was stopped by addition of 60 μl of 33 mM ethylenediaminetetraacetic acid (EDTA), 66 mM sodium borate (Sigma–Aldrich), pH 9.2. The released methionine was derivatized using 20 μl of AccQ-Fluor Reagent (Waters, Milford, MA) dissolved in acetonitrile (Sigma). Fifty microliter of the resulting mixture were resolved on a Luna C18(2) column 250 × 4.6 mm, 5 μm (Phenomenex, Torrance, CA) mounted to a Waters Alliance 2795 system equipped with a Waters 2475 fluorescence detector using a 10–70% linear gradient of B (56 mM sodium acetate-Lachema, 6.8 mM triethanolamine-Sigma–Aldrich, 60% acetonitrile-Sigma–Aldrich, pH 6.6) in A (140 mM sodium acetate, 17 mM triethanolamine, pH 5.05) over 15 min. Fluorescence was monitored at $\lambda_{ex}/\lambda_{em} = 250/395$ nm. Released methionine was quantified using a calibration curve constructed from methionine of known concentrations. The active site concentration was determined from the plot of v_i/v_0 (the ratio of individual reaction rate to the rate of an uninhibited reaction) against the inhibitor concentration using GraFit.

Preparation of the GCP-04 Monoclonal Antibody

The preparation of the GCP-04 antibody is described elsewhere [26,27]. Briefly, hybridomas secreting the GCP-04 monoclonal antibody (IgG1) were prepared by standard methods from mice (F1 hybrids of BALB/c and B10.A strains) immunized with the extracellular part of recombinant human GCPII (i.e., amino acid residues 44–750). After purification by Protein A affinity chromatography, the resulting IgG (stock concentration 7 mg/ml) was stored in phosphate buffered saline (PBS) containing 0.02% azide at 5°C until further use.

Western Blotting

Tissue homogenates containing 5, 20, 50, or 100 μg of total protein were separated on a reducing 10% sodium dodecylsulfate (SDS) polyacrylamide gel according to the standard protocol, and the proteins were subsequently electroblotted onto a nitrocellulose membrane. The membrane was then blocked with Casein Blocker (Pierce, Rockford, IL) for 1 hr and probed with the GCP-04 antibody (1:35,000 dilution of the 7 mg/ml stock) for 17 hr, followed by horseradish peroxidase-conjugated goat antimouse immunoglobulin (Pierce) diluted 1:25,000 (1 hr). Visualization on a MEDIX XBU film (FOMA Bohemia, Hradec Kralove, Czech Republic) was performed using SuperSignal West Femto or West Dura chemiluminiscence substrate (Pierce).

Immunohistochemistry

Tissue sample preparation for immunohistochemistry was performed as previously described [27]. Tissues fixed in 10% buffered formalin were embedded in paraffin. Four micrometer thick tissue sections were deparaffinized in xylene and rehydrated through a gradient of alcohol (decreasing ethanol:water ratio). After blocking of endogenous peroxidase activity, epitope retrieval was performed by 40-min incubation in 10 mM sodium citrate buffer solution (pH 6.0) heated to 96°C. The sections were incubated overnight at 4°C with the GCP-04 antibody (1:1,000 dilution of the 7 mg/ml stock). The antigen-antibody complexes were visualized by a biotin-streptavidin detection system (ChemMate Detection kit, DakoCytomation Co., Glostrup, Denmark) with 3,3′-diaminobenzidine (DAB, Sigma–Aldrich) as chromogen. All sections were counterstained with Harris′ hematoxylin (Sigma–Aldrich). The specificity of staining was confirmed by processing sections from the same paraffin block with omission of the GCP-04 antibody.

Statistical Analysis

All values are presented as the mean ± standard deviation.

RESULTS

Alignment of Human, Pig, and Rat GCPII Amino Acid Sequences

The alignment of human, pig, and rat GCPII amino acid sequences was made using Vector NTI Advance 10.3.0 program (Invitrogen) and the result is shown in Figure 1. All residues proposed to define the active site [3] are conserved in all three orthologs except for position 548, which is occupied by glycine in human GCPII and serine in pig and rat GCPII. Glycosylation has been shown to be indispensable for GCPII folding and activity. Human GCPII, which consists of 750 amino acids, has 10 predicted and confirmed glycosylation sites [29]. Ten potential *N*-glycosylation sites are also found in the sequence of rat GCPII (752 amino acids), while pig GCPII (751 amino acids) displays 12 potential *N*-glycosylation sites. Eight potential *N*-glycosylation sites are conserved among all three orthologs. Interestingly, glycosylation at position 140, which was shown to be essential for the NAAG-hydrolyzing activity of human GCPII [29], is not present in the rat GCPII sequence.

A linear epitope recognized by the GCP-04 antibody was mapped to amino acids 100–104 (WKEFG) of the

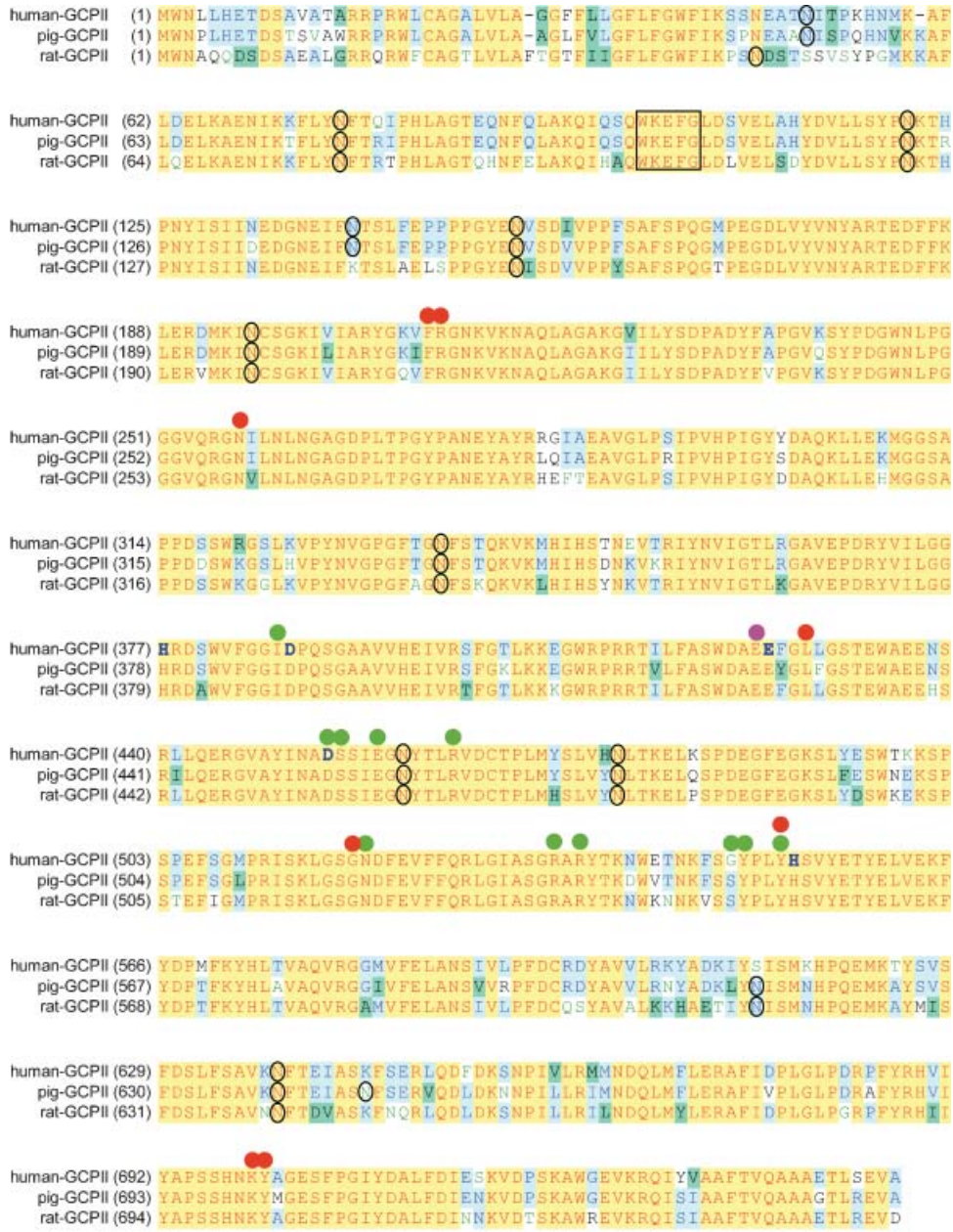

Fig. 1. Sequence alignment of the human, pig, and rat GCPII proteins. Green balls: predicted residues defining the SI pocket; red balls: residues forming the SI′ pocket; purple ball: predicted proton shuttle catalytic base; residues in blue: zinc ligands [3]; boxed "WKEFG" sequence: epitope of the GCP-04 antibody [27]; N in oval: potential *N*-glycosylation sites [29].

hGCPII sequence (see Figs. 1 and 4; Ref. [27]), and this motif is well-conserved in rat and pig GCPII. It can thus be inferred that the GCP-04 antibody will cross-react with GCPII orthologs in the animal tissues studied.

Direct Comparison of the Exopeptidase Activities of Recombinant GCPII From *Homo Sapiens Sapiens*, *Rattus Norvegicus*, and *Sus Scrofa*

Recombinant hGCPII was expressed and purified as previously described [26]. rGCPII and pGCPII were expressed in Drosophila S2 cells with yields of approximately 3 mg/L and 10 mg/L, respectively. To relate the hydrolytic activities of rGCPII and pGCPII with respect to their human counterpart, the conditioned media containing rGCPII and pGCPII were assayed for their ability to cleave radiolabeled NAAG. The amount of p/rGCPII present in the conditioned media was determined by quantification of pGCPII and rGCPII levels from Western blots developed with GCP-04 antibody, with purified hGCPII as a standard (data not shown). The resulting kinetic parameters (K_M and

k_{cat}) are compared to those of purified hGCPII in Table I.

The K_M values for rGCPII and pGCPII are seven and threefold lower, respectively, than that of hGCPII. The tighter binding of the substrate is compensated for by turnover number (k_{cat}) values that are five and twofold lower, respectively, than the corresponding values for hGCPII, leading to a similar k_{cat}/K_M ratio for all the variants. Thus, the catalytic efficiencies of both rGCPII and pGCPII are comparable to the catalytic efficiency for NAAG-hydrolysis by the human ortholog.

Inhibition of hGCPII, rGCPII, and pGCPII Hydrolyzing Activity by 2-(phosphonomethyl) pentanedioic Acid (2-PMPA)

In order to analyze the inhibition profile of the rat and pig orthologs of human GCPII, we measured inhibition of NAAG-hydrolyzing activity of purified hGCPII and of rGCPII and pGCPII in conditioned media by 2-(phosphonomethyl)pentanedioic acid (2-PMPA), a prototype inhibitor of hGCPII [30]. Enzyme concentration as well as substrate concentration were held at identical levels. The results are summarized in Table II. 2-PMPA proved to be a very potent inhibitor of all three GCPII variants, with IC_{50} values in the picomolar range. rGCPII and pGCPII display very similar IC_{50} values; the IC_{50} value for hGCPII is slightly higher compared to the other two orthologs.

Analysis of GCPII Expression in Tissues by Western Blotting

GCPII tissue expression patterns were examined using the GCP-04 antibody, which recognizes human, rat, and pig GCPII with similar sensitivity (data not shown).

We performed Western blotting analysis of a set of 21 human, rat, and pig tissue homogenates including brain, spinal cord, spleen, lung, mammary gland, heart, stomach, liver, pancreas, kidney, jejunum, ileum, colon, urinary bladder, ovary, uterus, prostate, testes, skeletal muscle, skin, and fat (Fig. 2). Overall, GCPII tissue localization is diverse in these species. Only brain and kidney display pronounced GCPII expression in all three species. Moderate to high GCPII levels were found in human spinal cord, spleen, lung, liver, jejunum, ileum, ovary, and prostate; in rat spinal cord and testes; and in pig spinal cord and spleen, lung, mammary gland, heart, stomach, jejunum, ileum, large intestine, urinary bladder, ovary, uterus, testes, skeletal muscle, and fat. Only slight to negligible GCPII expression was observed in human mammary gland, stomach, large intestine, urinary bladder, uterus, testes, and fat; rat spleen, liver, urinary bladder, ovary, uterus, prostate; and pig liver, pancreas, prostate, and skin. The remaining tissues exhibited no GCPII expression detectable by the GCP-04 antibody. Thus, GCPII is expressed most abundantly in pig tissues, while many tissues that are GCPII positive in human and/or pig do not express GCPII in rat, with jejunum and ileum representing the most striking difference.

Contradictory results were obtained for prostate; while human prostate seems to express high levels of GCPII, rat and pig prostates show very low GCPII expression levels. The opposite situation was observed for testes, which are GCPII-rich in pig and rat and nearly free of GCPII in human.

The specificity of immunostaining was confirmed by Western blot analysis of 24 (eight from each species) selected tissues using two additional mouse monoclonal antibodies raised against human GCPII (antibodies GCP-01 and GCP-02; for preparation see Ref. [26]). These analyses provided similar results as Western blots probed with GCP-04 antibody (data not shown).

NAAG-Hydrolyzing Activity in Selected Tissues

In order to confirm the expression of GCPII in individual human and animal tissues, NAAG hydrolysis by homogenates of human, rat, and pig brain, prostate, urinary bladder, skin, heart, and skeletal muscle; and of human and pig fat; and of pig jejunum, kidney, and testes was analyzed (Table III). To verify the specificity of NAAG cleavage, we incubated the substrate with the tissue homogenates of pig jejunum, kidney, and testes in the presence of 50 μM 2-PMPA, a

TABLE I. NAAG-Hydrolyzing Activity of hGCPII, rGCPII, and pGCPII

Recombinant protein	K_M (nM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\times 10^5\, s^{-1}\, M^{-1}$)
hGCPII[a]	1,200 ± 500	1.1 ± 0.2	9.3 ± 4.9
rGCPII[b]	180 ± 50	0.2 ± 0.1	10.0 ± 3.8
pGCPII[b]	370 ± 80	0.5 ± 0.1	13.3 ± 2.9

[a]Purified protein; the values are from [48].
[b]Conditioned media containing the corresponding recombinant protein.

TABLE II. Inhibition of hGCPII, rGCPII, and pGCPII by 2-(phosphonomethyl)pentanedioic Acid (2-PMPA)

Recombinant protein	IC_{50} (pM)
hGCPII[a]	840 ± 130
rGCPII[b]	210 ± 110
pGCPII[b]	370 ± 40

[a]Purified protein.
[b]Conditioned media containing the corresponding recombinant protein.

specific inhibitor of GCPII activity. In all cases, 2-PMPA decreased the substrate turnover almost to background level.

For direct comparison, the activities measured were normalized for total protein concentration in the particular homogenate. The brain samples of all the three species and the sample from human prostate, pig testes, and pig kidney exhibit the highest NAAG-hydrolyzing activity, which corresponds to the strong signal observed for these tissues on Western blots. The remaining tissues display at least eightfold lower activity, including the poorly active rat and pig prostates. Human skin, skeletal muscle, fat, and heart; rat skin, skeletal muscle, heart, and urinary bladder; and pig skeletal muscle were nearly inactive, which again correlates with weak or no GCPII positivity on the corresponding Western blots.

Immunohistochemical Detection of GCPII in Human, Pig and Rat Prostate, Spinal Cord, and Kidney

Immunohistochemical detection using the GCP-04 antibody was performed in human, pig, and rat prostate, spinal cord, and kidney to confirm the results of Western blotting analysis (Fig. 3). The epithelium of the prostate glands as well as the endothelium of a proportion of prostatic capillaries were stained with GCP-04 antibody in all three species. The intensity of staining in rat prostate varied considerably and was less intense than in the prostatic tissue of human and pig. Moreover, the apical accumulation of the GCP-04 antibody was less pronounced in the rat prostate gland epithelium compared to the other species. In spinal cord, the immunohistochemistry analysis resulted in positive staining of astrocytes and glial cell processes in all three species. Moreover, the membranes of a proportion of the anterior horn motor neurons were also positive in rat. In kidney, the epithelia of both the proximal and distal tubules and some capillary endothelia in glomeruli are positively stained in all three species.

DISCUSSION

GCPII is an important pharmaceutical target for a number of diverse pathological conditions of the nervous system caused by glutamate excitotoxicity (brain stroke, diabetic neuropathy [31,32]). Due to its expression in malignant prostate tissue, it is also an attractive target for the development of diagnostic and therapeutic tools for identifying and targeting cancer cells. Moreover, since GCPII emerges in the vasculature of most solid tumors and has been implicated in the integrin signaling critical for neoangiogenesis [33], it is a putative target for a new generation of antineoplastics that specifically block the vascularization of solid tumors and their metastases.

In order to test these diagnostic and therapeutic possibilities experimentally, it is necessary to identify and study appropriate animal models. For the correct choice of animal model, several questions must be

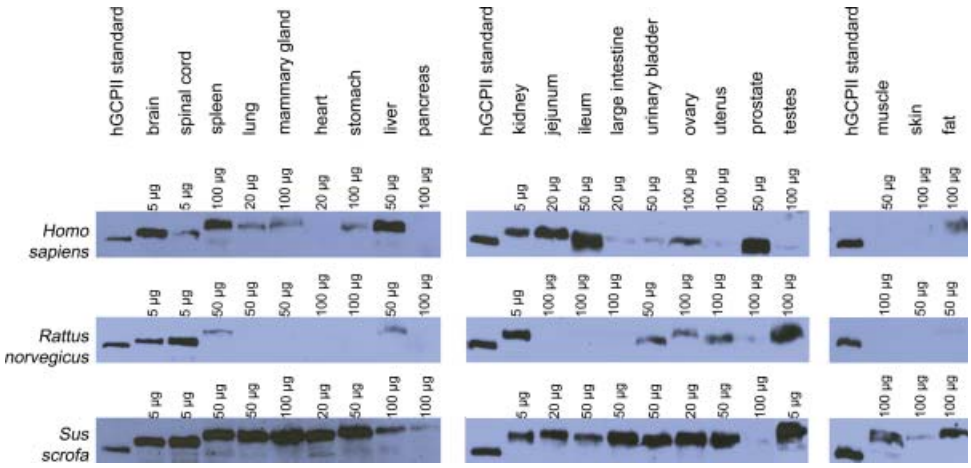

Fig. 2. Western blot analysis of GCPII expression in selected human, rat, and pig tissues using the GCP-04 antibody. The numbers indicate the amount of total protein from a particular sample applied to the gel. Five-hundred picogram of hGCPII was used as a standard on each blot. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE III. NAAG-Hydrolyzing Activity in Selected Tissue Homogenates

Activity > 50 nmol $\mu g^{-1} s^{-1} 10^{-9}$	Activity = 1–10 nmol $\mu g^{-1} s^{-1} 10^{-9}$	Activity < 1 nmol $\mu g^{-1} s^{-1} 10^{-9}$
Pig testes	Pig jejunum	Rat skin
Rat brain	Pig urinary bladder	Pig skeletal muscle
Human prostate	Pig heart	Human skin
Pig brain	Rat prostate	Rat skeletal muscle
Pig kidney	Human urinary bladder	Rat heart
Human brain	Pig skin	Human skeletal muscle
	Pig fat	Rat urinary bladder
	Pig prostate	Human fat
		Human heart

The activity is expressed as the number of nanomoles of cleaved substrate per 1 μg of total protein per second. Tissues in columns are listed in order of activity from the most active to the least active.

addressed: (i) What is the activity of the target enzyme in the animal model compared with the human enzyme? (ii) What is the inhibition profile of the animal orthologs, that is, could they be used for the screening of potential inhibitors of the human enzyme? (iii) What is the tissue distribution of the target enzyme in the animal organism and how does it correlate with human? In this paper we set out to address all of these questions for human GCPII and its orthologs from pig and rat.

While rat is a well-established animal model for basic biological studies including cancer and oncotherapy, pig has been used less frequently. However, numerous physiological and anatomical similarities with human have drawn attention to the pig as a valuable biomedical model, and its utilization in this area has been rapidly increasing during recent years. To reduce expenses and to simplify animal breeding and handling, several strains of miniature pigs have been developed for laboratory purposes as healthy (e.g., Minnesota in the USA, Goettingen in Germany) and cancerous (with hereditary melanoma-Sinclair in the USA, MeLiM in the Czech Republic) animal models. In the MeLiM strain, multiple skin melanoma appears in about 60% of all piglets [28].

As seen in Figure 1, human, rat, and pig GCPII are highly homologous. Not surprisingly, our results show comparable kinetic parameters for NAAG hydrolysis by these three enzymes and for their inhibition by 2-PMPA (see Tables I and II). The K_M value obtained for rat GCPII is in good agreement with a previous report which presented a K_M value of 140 nM for rat GCPII purified from rat brain membranes [34]. In the case of human GCPII, only data acquired using cell lysates or tissue homogenates are available in the literature; hence, the correlation with our results is less tight, although with respect to different experimental set-ups still acceptable (lysates of PC3 cells transfected with human GCPII display a K_M value of 150 nM [35]).

The slightly lower K_M and IC_{50} values for 2-PMPA observed for the rat and pig orthologs compared to the human ortholog are difficult to explain in structural terms without the 3D structures of the proteins. The only candidate sequence substitution in the vicinity of the active site is position 548, which is occupied by Gly in human GCPII and by Ser in both pig GCPII and rat GCPII (Fig. 1; Ref. [3]). However, according to our unpublished data, Gly 548 is not directly involved in inhibitor binding [36]. In any case, the differences in the presented kinetic parameters are not striking, and pig and rat GCPII therefore seem to represent good approximations for human GCPII for enzymologic and inhibition studies.

Most of the information about the tissue distribution of GCPII is derived from studies of rats and mice, performed both on the mRNA and protein expression level. Strong expression of GCPII in the prostate has been documented in humans [4,37,38]. In contrast, very low GCPII expression was recently found in the prostate of mouse, dog, and monkey [39,40]. Human as well as rat brain and kidney were confirmed to be the principal sites of GCPII expression [5,37], together with human prostate and small intestine [41]. Moderate to negligible GCPII expression was detected in human testes, seminal vesicles, heart, liver, lung, spleen, thyroid gland, small intestine, colon, breast, ovary, skin, salivary gland, urinary bladder, stomach, esophagus, and adrenal gland [9,10,41–43]. Except for brain and kidney, no rat tissue exhibited considerable GCPII expression; very low GCPII levels were found in rat adrenal gland, lung, heart, liver small intestine, pancreas, testes, and skeletal muscle [5]. In pig, only five tissues were tested, and GCPII was identified mainly in the jejunum and duodenum. Low GCPII expression was also found in pig kidney [25].

Since these previous studies cover only a few animal tissues and the data were obtained by different

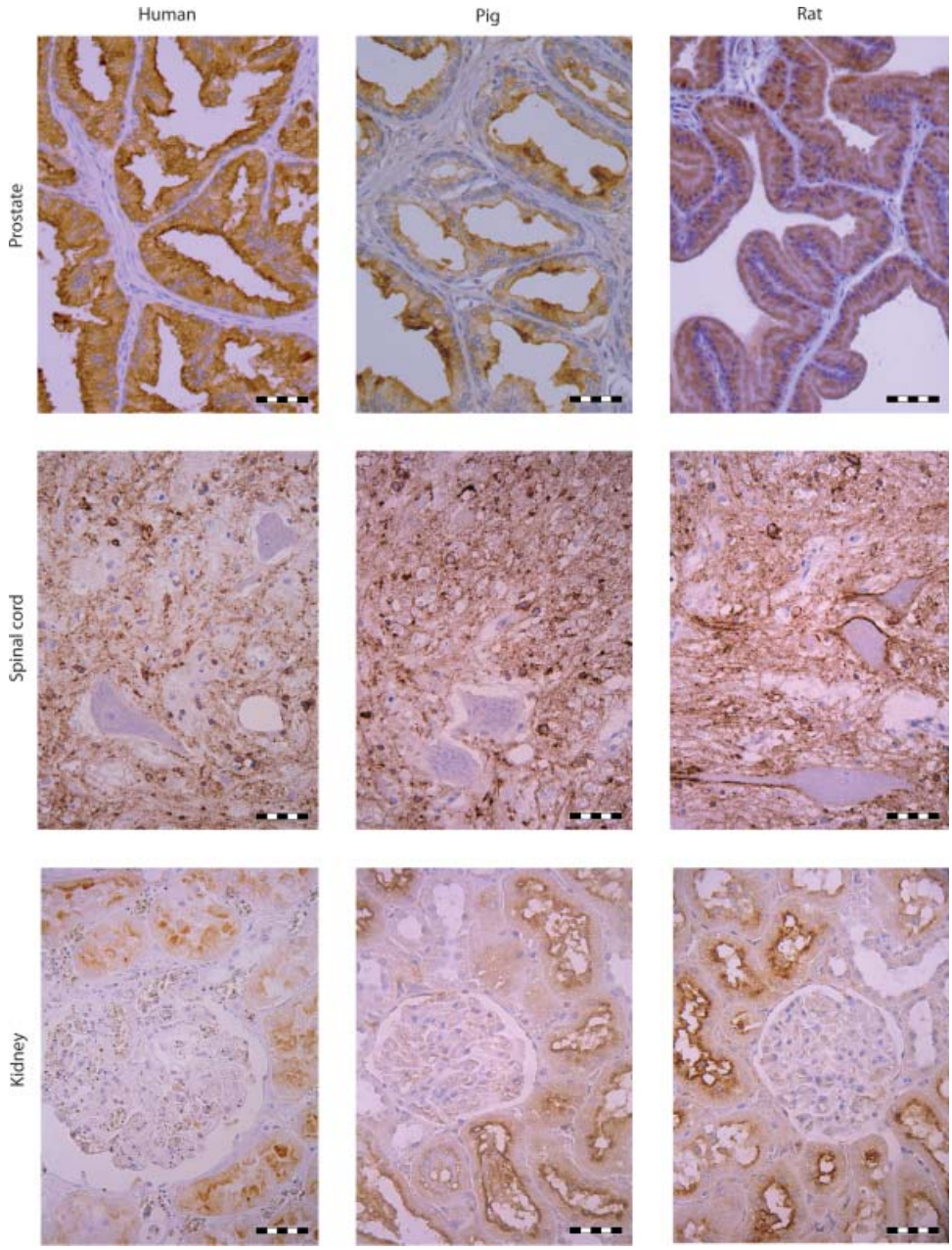

Fig. 3. Immunohistochemical detection of GCPII by GCP-04 antibody in human, pig, and rat prostate, spinal cord, and kidney (immunoperoxidase method, counterstained slightly with Harris' hematoxylin, scale bar = 50 μm). In all species, the epithelium of prostatic glands stained intensively with GCP-04 antibody, accentuated to the apical cell membrane. In spinal cord, the immunohistochemistry analysis resulted in positive staining of astrocytes and glial cell processes in all three species. Moreover, the membranes of a proportion of the anterior horn motor neurons were also positive in rat. In kidney, the epithelia of both the proximal and distal tubules and some capillary endothelia in glomeruli are positively stained in all three species.

methods, they are difficult to relate. Therefore, we set out to perform a systematic analysis of GCPII expression and enzymatic activity in individual human, rat, and pig tissues. The results of our Western blot analyses, summarized in Figure 2, show that the major sites of GCPII expression in all three species are the brain and kidney. This result is confirmed by the very high specific NAAG-hydrolyzing activity of the brain tissue homogenates from all three species and homogenate from pig kidney. Somewhat surprisingly, only human prostate was found to express significant amounts of GCPII, while little protein and significantly lower specific activity were found in pig and rat prostates. A similar result has recently been published by Aggarwal et al. [40] for mouse, dog, and macaque prostate. Aggarwal et al. also suggest that the lack of

GCPII expression in simian prostate might be responsible for the fact that prostate cancer has not been described in monkeys. A similar situation could be true for pig prostate, since there are no reports in the literature describing prostate cancer in this species. On the other hand, several rat spontaneous prostate cancer models have been described [44].

From a more general point of view, GCPII is expressed in the majority of tested human and pig tissues, while several tissues that are GCPII positive in human and/or pig do not express GCPII in rat. Surprisingly, we have not been able to detect any GCPII expression in rat jejunum and ileum. This finding is in agreement with the observation of Robinson et al. [5], who found very low GCPII activity in rat small intestine. The absence of GCPII in rat jejunum and ileum could be surprising with respect to the fact that GCPII is implicated in the transport of folates in the brush border of jejunum, but Shafizadeh has recently published that in rat small intestine, γ-glutamyl hydrolase, not GCPII, hydrolyzes dietary folate [45]. Another contradictory result was obtained for testes, in which rich GCPII expression was observed in pig and rat and very little in human. Since no function is attributed to GCPII in testes, it is difficult to speculate about the functional consequences of this observation.

The Western blot analysis of GCPII in tissues shows signals of slightly different molecular sizes. Since a consistent pattern was observed using two different monoclonal antibodies on a panel of selected tissues (data not shown), the signal cannot be attributed to non-GCPII proteins. The phenomenon may be caused by a differential GCPII *N*-glycosylation pattern in the tested tissues or by the presence of other forms (e.g., alternatively spliced) of GCPII that carry epitopes of these monoclonal antibodies.

Several close homologs of GCPII have been identified in human and rat, the closest of which is GCPIII [46,47]. Because of its high sequence similarity to GCPII, there is a possibility of crossreaction of anti-GCPII antibodies with these homologs. This can cause a high level of discrepancy among GCPII expression studies that utilize different monoclonal antibodies. Our GCP-04 monoclonal antibody could be expected to recognize GCPIII because there is only a single amino acid substitution in the GCP-04 epitope region of human and rat GCPIII compared to human GCPII (Fig. 4). Indeed, we have already shown that GCP-04 crossreacts with recombinant extracellular domain of human GCPIII (amino acids 36–740; hGCPIII), with an approximately two-order-of-magnitude lower sensitivity than for hGCPII [27,48]. In analogy, the NAAG-hydrolyzing activity that we determine for individual tissue extracts might actually represent the superposition of activities of various GCPII homologs. We have recently shown that human GCPIII activity is approximately three times lower than human GCPII activity under the conditions used in this study [48].

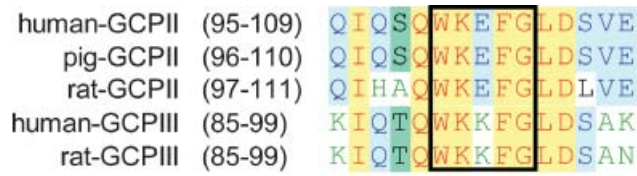

Fig. 4. Comparison of the GCP-04 antibody epitopes in various GCPII and GCPIII variants. The epitope recognized by the GCP-04 antibody is framed. The numbers in parentheses indicate the position of the epitope in the protein sequence.

The data presented in this work are relevant to the selection of an appropriate animal model for drug development and testing. The similar kinetic parameters of rGCPII and pGCPII suggest that they could be suitable to approximate human GCPII in enzymologic studies; on the other hand, different tissue localization must be taken into account when using these species for in vivo studies. Although anti-GCPII antibodies have been shown to be relatively selective for binding to bone and soft tissue metastases of prostate cancer in several imaging studies [21,22], the diffuse expression pattern of GCPII in animal and human tissues might be a potential caveat for the utility of GCPII-targeted compounds for cancer treatment.

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REFERENCES

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, Thun MJ. Cancer statistics, 2006. CA Cancer J Clin 2006;56:106–130.
2. Davis MI, Bennet MJ, Thomas LM, Bjorkman PJ. Crystal structure of prostate-specific membrane antigen, a tumor marker and peptidase. Proc Natl Acad Sci USA 2005;102:5981–5986.
3. Mesters JR, Barinka C, Li W, Tsukamoto T, Majer P, Slusher BS, Konvalinka J, Hilgenfeld R. Structure of glutamate carboxypeptidase II, a drug target in neuronal damage and prostate cancer. EMBO J 2006;25:1375–1384.
4. Horoszewicz JS, Kawinski E, Murphy GP. Monoclonal antibodies to a new antigenic marker in epithelial prostatic cells and

serum of prostatic cancer patients. Anticancer Res 1987;7:927–935.

5. Robinson MB, Blakely RD, Couto R, Coyle JT. Hydrolysis of the brain dipeptide *N*-acetyl-L-aspartyl-L-glutamate. Identification and characterization of a novel *N*-acetylated-alpha-linked acidic dipeptidase activity from rat brain. J Biol Chem 1987;262:14498–14506.
6. Su SL, Huang IP, Fair WR, Powell CT, Heston WD. Alternatively spliced variants of prostate-specific membrane antigen RNA: Ratio of expression as a potential measurement of progression. Cancer Res 1995;55:1441–1443.
7. Schmittgen TD, Teske S, Vessela RL, True LD, Zakrajsek BA. Expression of prostate specific membrane antigen and three alternatively spliced variants of PSMA in prostate cancer patients. Int J Cancer 2003;107:323–329.
8. Grauer LS, Lawler KD, Marignac JL, Kumar A, Goel AS, Wolfert RL. Identification, purification and subcellular localization of prostate-specific membrane antigen PSM' protein in the LNCaP prostatic carcinoma cell line. Cancer Res 1998;58:4787–4789.
9. Kinoshita Y, Kuratsukuri K, Landas S, Imaida K, Rovito PM Jr, Wang CY, Haas GP. Expression of prostate-specific membrane antigen in normal and malignant human tissues. World J Surg 2006;30:628–636.
10. Chang SS, Reuter VE, Heston WD, Bander NH, Grauer LS, Gaudin PB. Five different anti-prostate-specific membrane antigen (PSMA) antibodies confirm PSMA expression in tumor-associated neovasculature. Cancer Res 1999;59:3192–3198.
11. Chang SS, ÓKeefe DS, Bacich DJ, Reuter VE, Heston WD, Gaudin PB. Prostate-specific membrane antigen is produced in tumor-associated neovasculature. Clin Cancer Res 1999;5:2674–2681.
12. Liu H, Moy P, Kim S, Xia Y, Rajasekaran A, Navarro V, Knudsen B, Bander NH. Monoclonal antibodies to the extracellular domain of prostate-specific membrane antigen also react with tumor vascular endothelium. Cancer Res 1997;57:3629–3634.
13. Lopes AD, Davis WL, Rosenstraus MJ, Uveges AJ, Gilman SC. Immunohistochemical and pharmacokinetic characterization of the site-specific immunoconjugate CYT-356 derived from anti-prostate monoclonal antibody 7 E11-C5. Cancer Res 1990;50:6423–6429.
14. Wynant GE, Murphy GP, Horoszewicz JS, Neal CE, Collier BD, Mitchell E, Purnell G, Tyson I, Heal A, Abdel-Nabi H, Winzelberg G. Immunoscintigraphy of prostatic cancer: Preliminary results with 111In-labeled monoclonal antibody 7E11- C5.3 (CYT-356). Prostate 1991;18:229–241.
15. Elgamal AA, Troychak MJ, Murphy GP. ProstaScint scan may enhance identification of prostate cancer recurrences after prostatectomy, radiation, or hormone therapy: Analysis of 136 scans of 100 patients. Prostate 1998;37:261–269.
16. Bander NH. Technology insight: Monoclonal antibody imaging of prostate cancer. Nat Clin Pract Urol 2006;3:216–225.
17. Tjoa B, Boynton A, Kenny G, Ragde H, Misrock SL, Murphy GP. Presentation of prostate tumor antigens by dendritic cells stimulates T-cell proliferation and cytotoxicity. Prostate 1996;28:65–69.
18. Murphy GP, Tjoa B, Ragde H, Kenny G, Boynton A. Phase I clinical trial: T-cell therapy for prostate cancer using autologous dendritic cells pulsed with HLA-A0201-specific peptides from prostate-specific membrane antigen. Prostate 1996;29:371–380.
19. Tjoa BA, Lodge PA, Salgaller ML, Boynton AL, Murphy GP. Dendritic cell-based immunotherapy for prostate cancer. CA Cancer J Clin 1999;49:117–128.
20. Fracasso G, Bellisola G, Cingarlini S, Castelletti D, Prayer-Galetti T, Pagano F, Tridente G, Colombatti M. Anti-tumor effects of toxins targeted to the prostate specific membrane antigen. Prostate 2002;53:9–23.
21. Milowsky MI, Nanus DM, Kostakoglu L, Vallabhajosula S, Goldsmith SJ, Bander NH. Phase I trial of yttrium-90-labeled anti-prostate-specific membrane antigen monoclonal antibody J591 for androgen-independent prostate cancer. J Clin Oncol 2004;22:2522–2531.
22. Bander NH, Milowsky MI, Nanus DM, Kostakoglu L, Vallabhajosula S, Goldsmith SJ. Phase I trial of 177lutetium-labeled J591, a monoclonal antibody to prostate-specific membrane antigen, in patients with androgen-independent prostate cancer. J Clin Oncol 2005;23:4591–4601.
23. Bzdega T, Turi T, Wroblewska B, She D, Chung HS, Kim H, Neale JH. Molecular cloning of a peptidase against *N*-acetylaspartylglutamate from a rat hippocampal cDNA library. J Neurochem 1997;69:2270–2277.
24. Luthi-Carter R, Berger UV, Barczak AK, Enna M, Coyle JT. Isolation and expression of a rat brain cDNA encoding glutamate carboxypeptidase II. Proc Natl Acad Sci USA 1998;95:3215–3220.
25. Halsted CH, Ling E, Luthi-Carter R, Villanueva JA, Gardner J, Coyle JT. Folylpoly-gamma-glutamate carboxypeptidase from pig jejunum. Molecular characterization and relation to glutamate carboxypeptidase II. J Biol Chem 1998;273:20417–20424.
26. Barinka C, Rinnova M, Sacha P, Rojas C, Majer P, Slusher BS, Konvalinka J. Substrate specificity, inhibition and enzymological analysis of recombinant human glutamate carboxypeptidase II. J Neurochem 2002;80:477–487.
27. Sacha P, Zamecnik J, Barinka C, Hlouchova K, Vicha A, Mlcochova P, Hilgert I, Eckschlager T, Konvalinka J. Expression of glutamate carboxypeptidase II in human brain. Neuroscience 2007;144:1361–1372.
28. Horak V, Fortyn K, Hruban V, Klaudy J. Hereditary melanoblastoma in miniature pigs and its successful therapy by devitalization technique. Cell Mol Biol 1999;45:1119–1129.
29. Barinka C, Sacha P, Sklenar J, Man P, Bezouska K, Slusher BS, Konvalinka J. Identification of the *N*-glycosylation sites on glutamate carboxypeptidase II necessary for proteolytic activity. Protein Sci 2004;13:1627–1635.
30. Jackson PF, Cole DC, Slusher BS, Stetz SL, Ross LE, Donzanti BA, Trainor DA. Design, synthesis, and biological activity of a potent inhibitor of the neuropeptidase *N*-acetylated alpha-linked acidic dipeptidase. J Med Chem 1996;39:619–622.
31. Slusher BS, Vornov JJ, Thomas AG, Hurn PD, Harukuni I, Bhardwaj A, Traystman RJ, Robinson MB, Britton P, Lu XC, Tortella FC, Wozniak KM, Yudkoff M, Potter BM, Jackson PF. Selective inhibition of NAALADase, which converts NAAG to glutamate, reduces ischemic brain injury. Nat Med 1999;5:1396–1402.
32. Zhang W, Slusher BS, Murakawa Y, Wozniak KM, Tsukamoto T, Jackson PF, Sima AA. GCPII (NAALADase) inhibition prevents long-term diabetic neuropathy in type 1 diabetic BB/Wor rats. J Neurol Sci 2002;194:21–28.
33. Conway RE, Petrovic N, Li Z, Heston W, Wu D, Shapiro LH. Prostate-specific membrane antigen regulates angiogenesis by modulating integrin signal transduction. Mol Cell Biol 2006;26:5310–5324.
34. Slusher BS, Robinson MB, Guochuan T, Simmons ML, Richards SS, Coyle JT. Rat brain *N*-acetylated α-linked acidic dipeptidase activity. Purification and immunologic characterization. J Biol Chem 1990;265:21297–22301.
35. Luthi-Carter R, Barczak AK, Speno H, Coyle JT. Molecular characterization of human brain *N*-acetylated alpha-linked

acidic dipeptidase (NAALADase). J Pharmacol Exp Ther 1998; 286:1020–1025.

36. Bařinka C, Rovenská M, Mlčochová P, Hlouchová K, Plechanovová A, Majer P, Tsukamoto T, Slusher BS, Konvalinka J, Lubkowski J. Structural insight into the pharmacophore pocket of human glutamate carboxypeptidase II. J Med Chem 2007;50: 3267–3273.
37. Israeli RS, Powell CT, Corr JG, Fair WR, Heston WD. Expression of the prostate-specific membrane antigen. Cancer Res 1994; 54:1807–1811.
38. Cunha AC, Weigle B, Kiessling A, Bachmann M, Rieber EP. Tissue-specificity of prostate specific antigens: Comparative analysis of transcript levels in prostate and non-prostatic tissues. Cancer Lett 2006;236:229–238.
39. Bacich DJ, Pinto JT, Tong WP, Heston WD. Cloning, expression, genomic localization and enzymatic activities of the mouse homologue of prostate-specific membrane antigen/NAALADase/folate hydrolase. Mamm Genome 2001;12:117–123.
40. Aggarwal S, Ricklis RM, Williams SA, Denmeade SR. Comparative study of PSMA expression in the prostate of mouse, dog, monkey, and human. Prostate 2006;66:903–910.
41. Troyer JK, Beckett ML, Wright GL Jr. Detection and characterization of the prostate-specific membrane antigen (PSMA) in tissue extracts and body fluids. Int J Cancer 1995;62:552–558.
42. Sokoloff RL, Norton KC, Gasior CL, Marker KM, Grauer LS. A dual-monoclonal sandwich assay for prostate-specific membrane antigen: Levels in tissues, seminal fluid and urine. Prostate 2000;43:150–157.
43. Renneberg H, Friedetzky A, Konrad L, Kurek R, Weingartner K, Wennemuth G, Tunn UW, Aumuller G. Prostate specific membrane antigen (PSM) is expressed in various human tissues: Implication for the use of PSM reverse transcription polymerase chain reaction to detect hematogenous prostate cancer speed. Urol Res 1999;27:23–27.
44. Lucia MS, Bostwick DG, Bosland M, Cockett ATK, Knapp DW, Leav I, Pollard M, Rinker-Schaeffer C, Shirai T, Watkins BA. Workgroup I: Rodent models of prostate cancer. Prostate 1998; 36:49–55.
45. Shafizadeh TB, Halsted CH. γ-glutamyl hydrolase, not glutamate carboxypeptidase II, hydrolyzes dietary folate in rat small intestine. J Nutr 2007;137:1149–1153.
46. Pangalos MN, Neefs JM, Somers M, Verhasselt P, Bekkers M, van der Helm L, Fraiponts E, Ashton D, Gordon RD. Isolation and expression of novel human glutamate carboxypeptidases with *N*-acetylated alpha-linked acidic dipeptidase and dipeptidyl peptidase IV activity. J Biol Chem 1999;274:8470–8483.
47. Lambert LA, Mitchell SL. Molecular evolution of the transferrin receptor/glutamate carboxypeptidase II family. J Mol Evol 2007; 64:113–128.
48. Hlouchova K, Barinka C, Klusak V, Sacha P, Mlcochova P, Majer P, Rulisek L, Konvalinka J. Biochemical characterization of human glutamate carboxypeptidase III. J Neurochem 2007;101: 682–696.