Journal of Neurochemistry, 2002, **80**, 477–487

Substrate specificity, inhibition and enzymological analysis of recombinant human glutamate carboxypeptidase II

Cyril Barinka,* Markéta Rinnová,* Pavel Šácha,* Camilo Rojas,† Pavel Majer,† Barbara S. Slusher† and Jan Konvalinka*

**Institute of Organic Chemistry and Biochemistry of the Academy of Science of the Czech Republic, Czech Republic*
†Guilford Pharmaceuticals Inc., Baltimore, Maryland, USA

Abstract

Glutamate carboxypeptidase II (GCPII, EC 3.4.17.21) is a membrane peptidase expressed in a number of tissues such as kidney, prostate and brain. The brain form of GCPII (also known as NAALADase) cleaves *N*-acetyl-aspartyl glutamate to yield free glutamate. Animal model experiments show that inhibition of GCPII prevents neuronal cell death during experimental ischaemia. GCPII thus represents an important target for the treatment of neuronal damage caused by excess glutamate. In this paper we report expression of an extracellular portion of human glutamate carboxypeptidase II (amino acids 44–750) in *Drosophila* Schneider's cells and its purification to homogeneity. A novel assay for hydrolytic activity of recombinant human GCPII (rhGCPII), based on fluorimetric detection of released alpha-amino groups was established, and used for its enzymological characterization. rhGCPII does not show dipeptidylpeptidase IV-like activity assigned to the native form of the enzyme previously. Using a complete set of protected dipeptides, substrate specificity of rhGCPII was elucidated. In addition to the previously described substrates, four novel compounds, Ac-Glu-Met, Ac-Asp-Met and, surprisingly, Ac-Ala-Glu and Ac-Ala-Met were identified as substrates for GCPII, and their respective kinetic constants determined. The glycosylation of rhGCPII was found indispensable for the enzymatic activity.

Keywords: enzyme glycosylation, glutamate carboxypeptidase II, NAALADase, neuroprotection, zinc metallopeptidase.
J. Neurochem. (2002) **80,** 477–487.

Membrane glutamate carboxypeptidase II (GCPII) is a metalloproteinase expressed in various tissues and organs (Berger *et al*. 1995; Chang *et al*. 1999). Its proteolytic activity was firstly recognized in brain cells, where it has been shown to be responsible for the cleavage of *N*-acetyl-L-aspartyl-L-glutamate (NAAG) yielding free glutamate in the extracellular space. Based on the initial characterization of its substrate specificity, the enzyme was termed *N*-acetylated alpha-linked-acidic-dipeptidase (NAALADase; Robinson *et al*. 1987).

NAAG is an abundant neuropeptide found in millimolar concentrations in the brain (Coyle 1997). It is a mixed agonist/antagonist at *N*-methyl-D-aspartate ionotropic receptors and an agonist at the group II metabotropic glutamate receptors (mGlu; Wroblewska *et al*. 1993, 1997). After release from pre-synaptic terminals, NAAG diffuses from the synaptic cleft and is rapidly hydrolysed by GCPII located on adjacent astrocytes. Free glutamate subsequently acts at the various glutamate receptor subtypes. Excessive receptor activation by glutamate is thought to be at least partially responsible for the neuronal injury caused by stroke (Fagg and Foster 1986). Inhibition of NAAG metabolism was suggested as a possible strategy to attenuate excitatory amino

Received July 20, 2001; revised manuscript received October 30, 2001; accepted November 11, 2001.

Address correspondence and reprint requests to J. Konvalinka, Institute of Organic Chemistry and Biochemistry of the Academy of Science of the Czech Republic, Flemingovo n. 2, 166 10 Praha 6, Czech Republic. E-mail: konval@uochb.cas.cz

Abbreviations used: Ac, acetyl group; AMC, 7-amino-4-methylcoumarine; DIEA, diisopropylethylamine; DPM, disintegrations per minute; DPP IV, dipeptidyl peptidase IV; FAB-MS, fast atom bombardment mass spectrometry; FBS, foetal bovine serum; Fmoc, 9-fluorenylmethyloxycarbonyl; MOPS, (3-[*N*-morpholino]propanesulphonic acid); NAAG, *N*-acetyl-L-aspartyl-L-glutamate; NAALADase, *N*-acetylated-alpha-linked-acidic dipeptidase; OPA, *o*-phthaldialdehyde; PBS, phosphate-buffered saline; 2-PMPA, 2-(phosphonomethyl)pentanedioic acid; PNGase F, peptide:*N*-glycosidase F; PSMA, prostate-specific membrane antigen; PSM′, cytoplasmic form of PSMA; RFU, relative fluorescence units; rhGCPII, recombinant human glutamate carboxypeptidase II; SDS–PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate.

acid transmission and to provide neuroprotection during cerebral ischaemia (Tortella *et al*. 2000). Slusher *et al*. (1999) showed that inhibition of GCPII by the specific inhibitor 2-(phosphonomethyl)pentanedioic acid (2-PMPA) robustly protects against ischaemic injury in a tissue culture model of stroke and in rats after transient middle cerebral artery occlusion. The inhibitor increases NAAG concentration and attenuates the ischaemia-induced rise in glutamate concentration. Both effects apparently contribute to neuroprotection. GCPII has been thus shown to represent a plausible target for treatment of neurological disorders in which excessive glutamate transmission is pathogenic.

The second form of carboxypeptidase activity of GCPII is that of pteroylpoly gamma-glutamate carboxypeptidase. This activity was first characterized from the brush border of intestinal mucosa where it converts pteroylpoly gamma-glutamate to the pteroylglutamate (folate) form that is available for intestinal uptake (Pinto *et al*. 1996). The activity was assigned to prostate-specific membrane antigen (PSMA), a known prostate cancer marker. The pteroylpoly gamma-glutamate carboxypeptidase activity of PSMA is responsible for the cleavage of methotrexatepolyglutamate and is thus important for its uptake and metabolism (Heston 1997).

Both NAALADase and pteroylpoly gamma-glutamate carboxypeptidase activities have been assigned to a single membrane protein, PSMA (Pinto *et al*. 1996), and the new name for the enzyme, irrespective of its localization, has been coined: membrane glutamate carboxypeptidase (Rawlings and Barrett 1997) or glutamate carboxypeptidase II (Luthi-Carter *et al*. 1998).

GCPII is a class II membrane glycoprotein with an apparent molecular mass of 94–100 kDa. Class II membrane proteins typically have a short cytoplasmic amino terminus, a single membrane-spanning domain and a large extracellular domain. Apart from the membrane-bound version of GCPII, mRNA coding for an alternatively spliced variant, PSM′, has been found in human prostatic cancer cells (Su *et al*. 1995). The protein coded by the variant is truncated at the N-terminus, lacks the signal sequence and transmembrane domain of GCPII and, as it is a cytosolic protein, should not be glycosylated (Hart 1992). Normal human prostate expresses more mRNA coding for PSM′ than mRNA for membrane-bound GCPII. This ratio is reversed upon malignancy (Su *et al*. 1995).

No direct structural data for PSMA (GCPII) are available at present. Israeli *et al*. (1993) and Mahadevan and Saldanha (1999) identified sequence similarity of PSMA to the transferrin receptor. Rawlings and Barrett (1997) proposed a model for human GCPII based on a region of similarity to the M28 peptidase family (metallopeptidases). They predict six individual domains in the PSMA molecule: a short cytoplasmic tail, a transmembrane domain and four large extracellular domains. Based on homologies to aminopeptidases from *Streptomyces griseus* and *Vibrio proteolyticus*, the region spanning amino acids 274–587 was proposed to represent the catalytic domain. These proteases have a binuclear Zn^{2+} centre at the active site in which the two zinc atoms share a bridging carboxylate ligand. Speno *et al*. (1999) used site-directed mutagenesis to analyse five putative zinc ligands in the molecule of GCPII. Substitutions of these amino acids led to severe disruption of GCPII enzymatic activity. It is consistent with the assignment of these residues and suggests that GCPII is indeed structurally similar to the other M28 peptidase family members.

Even though GCPII represents a potentially very important target for therapeutic intervention of neurological disorders, the enzyme is relatively poorly characterized. The purification of the full-length GCPII from the brain or prostate cells is difficult and yields low amounts of the enzyme (Slusher *et al*. 1990). Moreover, a reliable and high throughput enzymatic assay for the enzymatic characterization of GCPII and large scale inhibitor testing is lacking. The current assay based on the hydrolysis of radiolabeled NAAG and the detection of free labelled Glu separated by ion-exchange chromatography is troublesome, time-consuming and poorly reproducible. Very little is known about different forms of GCPII expressed in membranes of different cells and about the structure and activity of the soluble form of GCPII (PSM′), expressed in the normal human prostate.

In this paper we describe the preparation and enzymatic characterization of milligram quantities of active, recombinant human GCPII and the development of a new high-throughput assay to screen potential inhibitors. We also describe the role of glycosylation for the enzymatic activity, and analysis of the substrate specificity and inhibition of the enzyme.

Experimental procedures

Expression plasmids

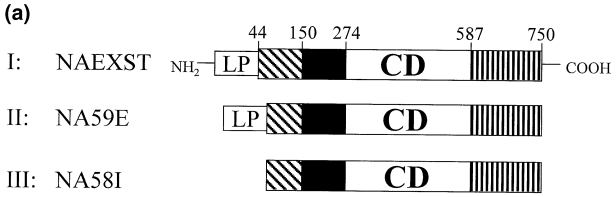
A PCR approach was used to amplify human GCPII extracellular portion (amino acids 44–750; Rawlings and Barrett 1997). Primers used were RNAEXST (*Xho*I) 5′-ATT*CTCGAG*TCATTAGGCTA CTTCACTCAAAG-3′ and FNAEXX (*Bgl*II) 5′-AA*ACTCGAGA-GATCT*AAATCCTCCAATGAAGC-3′ (newly introduced restriction sites are in italic). Template amplification (pcDNA3.1/GCPII) was performed using Pfu-polymerase (Promega, Madison, WI, USA) according to the manufacturer's protocol. A PCR reaction began with an initial denaturation step (94°C for 5 min), followed by 30 cycles of amplification (1 min at 94°C, 1 min at 58°C, and 4 min at 72°C), and ended with a final extension step (72°C for 5 min). A 2130-bp PCR fragment was purified by gel electrophoresis, digested with *Bgl*II/*Xho*I and cloned into pMT/BiP/V5-His A (Invitrogen, Carlsbad, CA, USA) previously digested with the same set of enzymes. The resulting plasmid has been designated pMNAEXST.

A similar approach was used to clone other GCPII variants. The following primer pairs were employed to amplify GCPII fragments

depicted in Fig. 1: FNA59E (*Bgl*II) 5′-AAA*AGATCT*GCATTTTT-GGATGAATTG-3′ together with RNAST (*Not*I) 5′-AAA*GCGGC-CGC*CTATTAGGCTACTTCACTCAAAG-3′ for NA59E; and FNA59I (*Kpn*I) 5′-AAA*GGTACC*AATAATGGCATTTTTGGATG AATTG-3′ together with RNAST (*Not*I) for NA59I. The NA59I fragment is expressed intracellularly and is cloned into pMT/V5-His A vector (Invitrogen), the two other fragments were cloned into pMT/BiP/V5-His A.

Identities of all sequences have been verified by dideoxynucleotide terminated sequencing using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit v2.0 (Perkin-Elmer Corporation, Norwalk, CT, USA) and an ABI Prism 310 Genetic Analyzer (Perkin-Elmer Corporation).

(a)



(b)

Lane	Fragment	Secretion	Activity
I	NAEXST	+	+
II	NA59E	+	+
III	NA59I	-	-

(c)

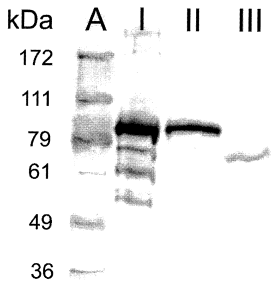


Fig. 1 Schematic representation, NAAG hydrolysing activity and expression of individual fragments of human GCPII in insect cells. (a) Putative structure of GCPII and individual domains depicted according to Rawlings and Barrett (1997). LP, leader peptide; CD, putative catalytic domain; polypeptides spanning amino acids 44–150, 151–274, and 587–750, domains of unknown function. (b) NAAG hydrolysing activity of the expressed fragments. Secretion: (+), protein secreted into medium; (–), protein expressed intracellularly. (c) Western blot analysis of the fragments expression. Lane A, molecular weight marker; lane I, conditioned medium, NAEXST fragment; lane II, conditioned medium, NA59E fragment; lane III, 10^6 cells, NA58I fragment. Proteins were resolved on 11% SDS–PAGE, electroblotted on a PVDF membrane, immunodetected by antirhGCPII mouse monoclonal antibody at 1 : 250 dilution followed by incubation with 1 : 4000 alkaline phosphatase conjugated goat antimouse antibody (Bio-Rad) and developed using NBT/BCIPmixture.

Transfection of insect cells and stable cell line generation

Schneider's S2 cells (Invitrogen) were maintained in SF900II medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Sigma) at 22–24°C. Before transfection, the cells were seeded in a 35-mm dish and grown until they reached a density of 2–4 × 10^6 cells/mL. The cells were co-transfected with 19 μg of pMNAEXST and 1 μg of a pCoHYGRO selection vector (Invitrogen) using a kit for calcium phosphate-mediated transfection (Invitrogen). The calcium phosphate solution was removed 16 h post-transfection and fresh SF900II medium supplemented with 10% FBS was added (a complete medium). The cells were grown for additional 2 days and then the medium was replaced with the complete medium containing 400 μg/mL Hygromycin B (Invitrogen). The selection medium was changed every 4 days. Extensive cell death of non-transfected cells was evident after about 1 week and cells resistant to Hygromycin B started grow out 3–4 weeks post-transfection.

rhGCPII large scale expression

The hygromycin-resistant cells were seeded in 100 mL expression medium [SF900II medium supplemented with 1 mL Chemically Defined Lipid Concentrate (Gibco) and 2 mL Yeastolate Ultrafiltrate (Gibco)] in a spinner flask (250 cm^3; Bellco Glass Inc., Vineland, NJ, USA) at density 2 × 10^6 cells/mL. The spinner was incubated at 24°C with a constant stirring rate 100 rpm until the cells reached a density of 10 × 10^6 cells/mL. At this point, the cell suspension was transferred into a larger spinner flask (3 L; Bellco), 400 mL expression medium was added, and the cells were grown to a density 8 × 10^6 cells/mL under the same conditions. Then, 500 μM $CuSO_4$ (Sigma) was used to induce rhGCPII expression. A stirring rate was increased to 130 rpm and incubation continued additional 6–8 days. Five milliliters of 20% D-(+)-glucose (Sigma) and 2 mL 200 mM L-glutamine (Sigma) were added to the cell suspension on days 2 and 5. The cells were harvested by centrifugation at 1600 *g* for 10 min followed by the second centrifugation step at 3400 *g* for 15 min. The conditioned medium was frozen and stored at − 70°C until further use.

rhGCPII purification

The conditioned medium (0.5 L) was dialysed against 20 mM Tris–HCl, pH 6.7, for 3 days at 4°C. The dialysing buffer was changed every 24 h. QAE-Sephadex A50 (4 g, Pharmacia) was pre-equilibrated in 20 mM Tris–HCl, pH 6.7, and added to the conditioned medium. Resulting slurry was stirred for 20 min at room temperature and filtered through a sintered glass filter. Retained QAE-Sephadex was washed with 100 mL 20 mM Tris–HCl, pH 6.7. 2 × 300 mL of pooled flow-through fractions were directly applied on a Source 15S column (HR10/10, Amersham Pharmacia Biotech AB, Uppsala, Sweden) pre-equilibrated with 20 mM Tris–HCl, pH 6.7 (buffer A), at room temperature. The rhGCPII was eluted with a linear 0–0.5 M NaCl gradient in buffer A. The fractions containing rhGCPII were pooled, mixed with an equal volume of buffer C (100 mM Tris–HCl, 0.8 M NaCl, 2 mM $CaCl_2$, 2 mM $MnCl_2$, pH 7.4), and loaded on the Lentil Lectin-Sepharose (Sigma, St Louis, MO, USA) column (C10/10, Pharmacia) equilibrated with 20 mM Tris–HCl, 0,5 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, pH 7.4 (buffer D), at 10°C. The column was extensively washed with 40 mL of buffer D, and rhGCPII was eluted with 60 mL of 20 mM

Tris–HCl, 0.5 M NaCl, 0.3M Met-α-D-Man (Sigma), pH 7.4. The eluate was dialysed against 20 mM Tris–HCl, pH 8.5, overnight at 10°C, and then applied on a MonoP column (HR5/10, Pharmacia) equilibrated with 20 mM Tris–HCl, pH 8.5. The protein was eluted with Polybuffer 96 (Pharmacia) adjusted to pH 6.7 with HCl.

Preparation of rat brain synaptic membrane fraction

Brain GCPII synaptic membrane fractions from frozen rat brains were prepared as previously described (Robinson *et al*. 1987).

Monoclonal antibody

Monoclonal antibodies were prepared according to an established protocol (Langone and Van Vunakis 1986). Briefly, Balb/c mice were immunized by intraperitoneal injections of purifed rhGCPII in phosphate-buffered saline (PBS; 40 μg/100 μL) mixed with equal volume of incomplete Freund's adjuvant in 3-week intervals. Hybridomas, prepared by fusion of immune spleen cells and Sp/0 myeloma cells, were cloned by limited dilution and assayed for production of rhGCPII-specific antibodies by enzyme-linked immunosorbent assay. Positive clones were expanded and conditioned medium used at 1 : 250 dilution on immunoblots.

SDS–PAGE and western blotting

Analysed protein samples were resolved by reducing, 0.1% sodium dodecyl sulphate (SDS), 11% polyacrylamide gel electrophoresis (PAGE). Following SDS–PAGE, gels were either silver-stained or electroblotted on a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% non-fat milk in PBS for 1 h at room temperature. After two washes with 0.1% Triton X-100 in PBS, the blot was incubated with anti-rhGCPII mouse monoclonal antibody at 1 : 250 dilution in 5% non-fat milk, 0.1% Triton X-100 in PBS overnight. The membrane was washed 3 × 15 min and incubated with 1 : 4000 alkaline phosphatase-conjugated goat anti-mouse antibody (Bio-Rad Laboratories, Hercules, CA, USA) for 2 h. After three washes, the blot was developed using an NBT/BCIP mixture.

Carboxypeptidase activity

Radioenzymatic assays using ^{3}H-NAAG (radiolabelled on the terminal glutamate) were performed as described previously (Robinson *et al*. 1987), with minor modifications. Briefly, 50 mM Tris–HCl, pH 7.4 at 37°C, 1 mM $CoCl_2$, and 10 μL rhGCPII were pre-incubated for 15 min at 37°C in a final volume 450 μL. Then, 50 μL mixture of 4.95 μM 'cold' NAAG (Sigma) and 50 nM ^{3}H-NAAG (51.9 Ci/mmol; New Life Science, Boston, MA, USA) were added to each tube and incubation continued for 20 min. The reaction was stopped with 0.5 mL ice-cold 200 mM sodium phosphate, pH 7.4, cleaved glutamate was separated from the unreacted substrate by ion exchange chromatography, and measured by liquid scintillation.

Dipeptidylpeptidase IV-like activity

Dipeptidylpeptidase IV (DPP IV) activity was determined by fluorescent analysis of the Gly-Pro-AMC hydrolysis on a Perkin-Elmer LS50B fluorimeter (excitation at 380 nm, emission at 460 nm). Assays were initiated by the addition of the rhGCPII (10 μL, 60 μg/mL) to the buffered substrate solutions (12 μM Gly-Pro-AMC, 5 mM NaCl and 50 mM glycine pH 8.5 or 50 mM Tris–HCl, pH 7.7) in a total reaction volume 2 mL and followed for 15 min at 37°C. C6 rat glial cells (20 μL, 4×10^6 cells/mL in PBS) were used as a positive control (Sedo *et al*. 1998).

Synthesis of peptide libraries

The substrates of general formulae Ac-Xxx-Glu-OH or Ac-Asp-Xxx-OH were prepared by solid phase peptide synthesis on a 2-chlorotritylchloride resin (Calbiochem-Novabiochem AG, Laufelfingen, Switzerland) using Fmoc/Bu^t protection and TBTU/DIEA coupling strategy according to Barlos *et al*. (1991). N-terminal acetylation was accomplished with acetic anhydride in presence of DIEA. A mixture of 95% trifluoroacetic acid and 5% water was used for a final cleavage from the resin. The peptides were evaporated and lyophilized from acetic acid, 50% aquaeous acetonitrile and water. Dipeptides comprising Cys, Asn, Gln, His, Met or Arg were cleaved with a mixture of 90% trifluoroacetic acid, 5% water, 2.5% triisopropylsilane and 2.5% ethanedithiol, extracted with diethyl-ether, evaporated and lyophilized as described above. The peptides were analysed by HPLC and characterized by amino acid analysis and FAB-MS.

Detection of reaction products by *o*-phthaldialdehyde modification

One hundred milligrams of *o*-phthaldialdehyde (OPA; Sigma) was dissolved in 625 μL methanol and transferred into 50 mL of 0.8 M sodium borate, pH 10.0, and 50 μL mercaptoethanol (Sigma) was added. This OPA solution (250 μL) was added to an equal volume of the reaction mixtures, vortexed and left at room temperature for 10 min. Fluorescence was measured on a Perkin-Elmer LS-3B fluorimeter (excitation at 330 nm, emission at 450 nm). Hydrolysis of an Ac-Asp-Pro was determined using amino acid analysis.

Screening of potential substrates

The individual compounds were dissolved in 10% dimethylsulphoxide (DMSO; Sigma) in water. One microliter of rhGCPII (60 μg/mL) was added to the buffered substrate solutions (500 μM individual compound, 5 mM NaCl, 50 mM MOPS, pH 7.4). The reactions were allowed to proceed for 15 h at 37°C. The hydrolysis products, with the exception of proline, were determined spectrofluorometrically using derivatization with OPA (Sigma).

K_m determination

Enzyme kinetics of novel substrates were analysed by high-performance liquid chromatography (HPLC) with fluorometric detection (excitation 330 nm, emission 450 nm). The instrument used was Spectra-Physics SP8800, and reaction products were separated on a C18(2) Luna column (Phenomenex, 25 × 4.6 mm). Glutamate and methionine were measured as fluorescent derivatives after a pre-column OPA derivatization (see above). A Kratos FS 960 fluorescence detector (Krantos Analytical Instruments, Hofheim, Germany) together with an SP4400 integrator (Spectra-Physics, Mountain View, CA, USA) were used for peak quantification. Collected data were analyzed using EnzFitter curve-fitting package (Biosoft, Cambridge, UK). Typically, a nanomolar enzyme preparation in buffer 20 mM MOPS containing 10 mM NaCl, pH 7.4 was used to hydrolyse 5–2000 μmole/L concentration of the analysed substrate for 30 min at 37°C. The relative turnover of the substrate

did not exceed 20%. A total of 8–12 concentration points were used for a single determination.

rhGCPII Deglycosylation

One microliter of PNGase F (NEB, 1000 U/μL) was used to deglycosylate 10 μg of native rhGCPII in 20 mM MOPS, 20 mM Tris–HCl, pH 8.5. The deglycosylation took place at 37°C for 15 h in total volume of 100 μL.

Recombinant expression of non-glycosylated rhGCPII

S2 cells stably expressing rhGCPII were grown in 60-mm tissue culture plates in SF900II medium supplemented with 10% FBS. At the density 5×10^6 cells/mL, tunicamycin (Sigma, 1 mg/mL stock solution in DMSO) was added to the final concentration 10 μg/mL, and rhGCPII expression induced by 1 mM $CuSO_4$. Cells were incubated for additional 36 h and harvested.

Preparation of cell lysates

Harvested cells were counted and centrifuged at 500 *g* at 4°C. The conditioned media were removed and frozen at −70°C until further use. The cells were resuspended in ice-cold TBS and re-spinned. The supernatants were discarded. The cell pellets were resuspended in 20 mM MOPS, 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, pH 7.4 (buffer D) to concentration 40×10^6/mL, sonicated three times for 20 s on ice, and subjected to centrifugation at 15 000 *g* for 10 min; the supernatant fraction is reffered to as the cell lysate.

Separation of glycosylated and non-glycosylated rhGCPII

Cell lysates were mixed with 100 μL of Lentil Lectin Sepharose (Sigma), the slurry was incubated for 20 min on ice and then centrifuged at 700 *g* for 5 min. The supernatant, containing non-glycosylated rhGCPII, was removed and the resin with glycosylated rhGCPII bound was washed three times with ice-cold buffer D. A total of 200 μL of 0.1 M sodium borate, pH 6.5, was used for elution of glycosylated proteins bound to the resin. NAAG hydrolysing activity and rhGCPII distribution in cell lysates, 'non-glycosylated rhGCPII' fraction and the fraction eluted with sodium borate were measured by radioenzymatic assays using ^{3}H-NAAG or western blot, respectively (see above).

Results

Expression of proteolytically active fragments

In order to obtain human glutamate carboxypeptidase II in quantities sufficient for enzymological analysis, we established an efficient source of the recombinant enzyme. When attempts to produce the active enzyme in *Escherichia coli* turned out to be unsuccessful (C. Barinka *et al.* unpublished observation), we decided to use insect cells for the heterologous expression. Specifically, *Drosophila* expression vector in Schneider's S2 cells (Invitrogen) was chosen. The DNA sequences coding two different fragments of human GCPII, designated NAEXST (fragment I in Fig. 1) and NA59E (fragment II), were PCR amplified, cloned into pMT/Bip/V5-His A *Drosophila* expression vector in an open reading frame with a BiP signal sequence that directs the secretion of the proteins into the medium. These fragments span amino acids 44–750 and 59–750, respectively, of the mature human GCPII. The third fragment, designated NA59I (amino acids 59–750, fragment III in Fig. 1) was cloned into the pMT/V5-His A vector and expressed intracellularly. Figure 1 shows the fragments used for heterologous expression together with the schematic representation of the putative domains of GCPII structure, as suggested by Rawlings and Barrett (1997). All three constructs involve the predicted proteolytic domain, homologous with the *Aeromonas proteolytica* and *Streptomyces griseus* aminopeptidases (designated CD in Fig. 1). As shown in Fig. 1, all three fragments were expressed (Fig. 1c). Only the proteins secreted to the medium show proteolytic activity (compare to Fig. 5). The protein I spanning amino acids 44–750 in the mature human GCPII sequence was used for further purification and characterization, and is referred to as recombinant human GCPII (rhGCPII) throughout the following paragraphs.

rhGCPII purification

Using the BiP signal sequence, rhGCPII was secreted into the medium, and the BiP leader peptide was efficiently cleaved off along the secretory pathway. N-terminal sequencing of the secreted rhGCPII revealed amino acids RSKSSNEAT. The first two amino acids identified were coded by *Bgl*II recognition site nucleotides, and the rest of the above sequence corresponded to amino acids 44–50 of human GCPII.

rhGCPII was purified by combination of ion-exchange chromatography, Lentil Lectin-Sepharose chromatography and chromatofocusing, as described in the Experimental Procedures section. The overall purification was 12-fold with 36% recovery, yielding 42 mg of pure rhGCPII from 1 L of medium. The enzyme purity was assessed by silver staining of reducing 11% SDS–PAGE gels. SDS–PAGE of pooled fractions at various stages in the purification is shown in Fig. 2. The final purity of the enzyme was higher than 98% as seen from the mass spectrometry analysis (data not shown). The protein purity was further documented by the fact that the enzyme readily crystallizes from concentrated preparations (unpublished observation).

Analysis of rhGCPII substrate specificity

Taking advantage of the pure recombinant enzyme, a panel of dipeptides of general formulae Ac-L-Xxx-L-Glu or Ac-L-Asp-L-Xxx (Xxx standing for any naturally occurring amino acid) was tested for the cleavage by rhGCPII. Liberation of a C-terminal amino acid residue was determined fluorometricaly exploiting OPA derivatization (Lindroth and Mopper 1979) of a liberated amino group. Identities of amino acids hydrolysed were confirmed by an amino acid analysis (data not shown).

The results of the substrate specificity analysis using a panel of dipeptides are schematically presented in Fig. 3.

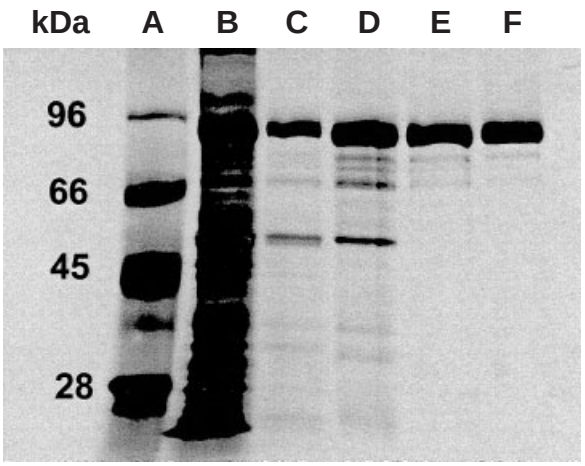


Fig. 2 SDS–PAGE analysis of pooled fractions from individual steps during the purification of rhGCPII. Lane A, molecular weight marker, lane B, conditioned medium; lane C, pooled QAE-Sephadex flow-through; lane D, pooled Source 15S; lane E, pooled Lentil-Lectin Sepharose protein; lane F, pooled chromatofocusing fractions. Protein samples were resolved on 11% SDS–PAGE and silver stained.

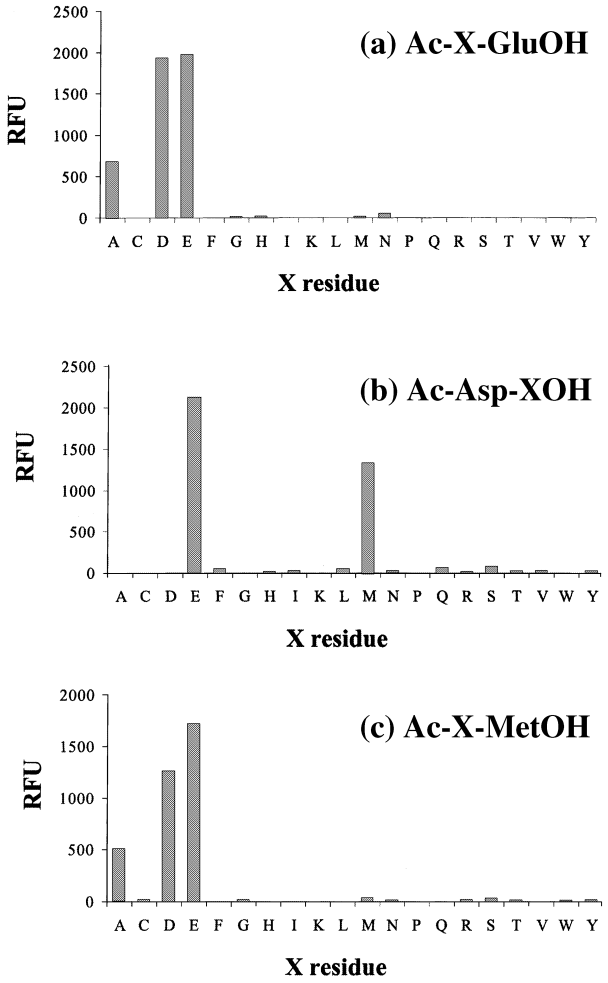


Fig. 3 (a–c) Hydrolysis of *N*-acetylated dipeptides of general formulae Ac-X-GluOH, Ac-Asp-XOH, and Ac-X-MetOH by the rhGCPII.1 μL rhGCPII (60 μg/mL) was added to the buffered substrate solutions (500 μM individual compound, 5 mM NaCl, 50 mM MOPS, pH 7.4). The reactions were allowed to proceed for 15 h at 37°C. The hydrolysis products were determined spectrofluorometrically exploiting derivatization with *o*-phthaldialdehyde. *x*-axis: an amino acid at X position; *y*-axis: relative fluorescence units (RFU).

Ac-Asp-Glu (NAAG, a naturally occurring substrate in the brain), Ac-Glu-Glu and Ac-Ala-Glu, were identified as substrates of rhGCPII in the first screen using set of dipeptides of general formula Ac-Xxx-Glu. To analyse the requirement for the amino acid at the C-terminus of the substrate, we synthesized the second panel of protected dipeptides with the general formula Ac-Asp-XxxOH. In this screen, Ac-Asp-Glu and, surprisingly, Ac-Asp-Met were found to be cleaved by the enzyme (Fig. 3b). To verify that the rhGCPII was indeed responsible for hydrolysis of the substrates, we performed control analyses in the presence of the GCPII inhibitors EDTA, PO_4 and 2-PMPA. No substrate hydrolysis could be detected with the inhibitors added (data not shown). In the light of these results, the third set of potential substrates of general formula Ac-Xxx-Met was synthesized and evaluated. In this set of dipeptides, we identified Ac-Glu-Met, Ac-Asp-Met and Ac-Ala-Met as effective substrates of rhGCPII (Fig. 3c). While the cleavage of Ac-Asp-Met merely confirms the finding from the previous experiment, Ac-Glu-Met and, especially, Ac-Ala-Met represent novel substrates of GCPII.

The results shown in Fig. 3 do not provide direct comparison of the kinetics of cleavage of these substrates by the protease since the endpoint of the analysis was well beyond the linear part of the enzymatic reaction.

Kinetic analysis of newly identified substrates of rhGCPII

The rate of cleavage of newly identified substrates were analysed by HPLC using fluorometric detection after a pre-column OPA derivatisation. The data are summarized in Table 2. We have not been able to determine the kinetic constant for Ac-Ala-Glu because the initial velocities do not follow simple Michaelis–Menten kinetics, apparently due to the substrate inhibition. As the other substrates are concerned, the Met-containing dipeptides show two to three orders of magnitude decrease of the K_m, while the k_{cat} is less affected. Not surprisingly, the enzyme shows the lowest activity towards Ac-Ala-Met (a four-order of magnitude drop of k_{cat}/K_m in comparison to the originally described Ac-Asp-Glu, compare Table 1), a protected dipeptide lacking any negatively charged residue in the side chains.

Table 1 Kinetic characterization of rhGCPII peptide substrates

Substrate	K_m [μM]	k_{cat} [s^{-1}]	k_{cat} K_m [s^{-1}/$mmol^{-1}$]
Ac-Asp-Glu[a]	0.427 ± 0.051	0.59 ± 0.16	1381
Ac-Glu-Glu	< 5.0[b]	0.78 ± 0.08	ND
Ac-Ala-Glu	ND	ND	ND
Ac-Glu-Met	53.0 ± 5.9	0.29 ± 0.01	5.5
Ac-Asp-Met	24.8 ± 2.9	0.070 ± 0.002	2.9
Ac-Ala-Met	303 ± 41	0.010 ± 0.001	0.04

The kinetic values were determined using the detection of reaction products by OPA as described in Experimental Procedures. [a]These values were determined by the radioenzymatic assay using ^{3}H-NAAG as described (Tiffany *et al.* 1999). [b]K_m value was below the detection limit of the OPA method.

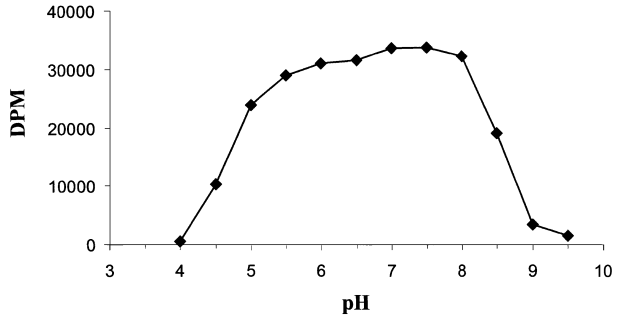


Fig. 4 pH dependence of NAAG cleavage by rhGCPII. The following buffers, together with 10 mM NaCl, were used in 50-mM concentrations spanning a pH range of 4–9.5: acetate, pH 4.0–5.0; 2-(*N*-morpholino)-ethanesulphonic acid, pH 5.5–6.5; MOPS, pH 7.0–8.0; Tris–HCl, pH 8.5–9.5. The substrate cleavage was followed by scintillation spectrometry measuring released ^{3}H-Glu separated by ion-exchange chromatography (see Experimental procedures). Typical substrate conversion did not exceed 20%.

The pH dependence of peptide cleavage by rhGCPII

To analyse the pH dependence, the cleavage of Ac-Asp-Glu was followed in pH range 4.0–9.5 by radiometric assay as well as by the fluorescence measurement using Ac-Asp-Glu as a substrate and OPA detection of the liberated C-terminal amino acid (Fig. 4). The activity shown in Fig. 4 corresponds to less than 20% turnover of the substrate. The resulting activity curve is bell-shaped with a relatively broad maximum between pH 6.0 and 8.0. The activity sharply drops at pH above 8.5 and also the activity in the pH range below 4.5 is negligible.

Dipeptidylpeptidase IV-like activity of rhGCPII

Pangalos *et al.* (1999) reported a DPP-IV-like activity in homogenates from COS cells transiently transfected with GCPII and GCPII-like cDNAs. To analyse possible aminopeptidase activity of rhGCPII we measured hydrolysis of a typical fluorogenic substrate of DPP-IV, the Gly-Pro-AMC (Kato *et al.* 1978), by the rhGCPII. Under the conditions described in the Experimental Procedures section, we have not been able to detect any DPP-IV-like activity using excessive amounts of pure rhGCPII, while C6 rat glial cells, used as a positive control (Sedo *et al.* 1998), hydrolysed Gly-Pro-AMC quite efficiently (data not shown).

Testing of specific inhibitors and comparison with the native enzyme

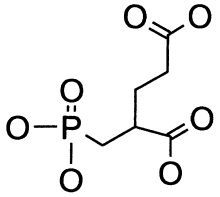
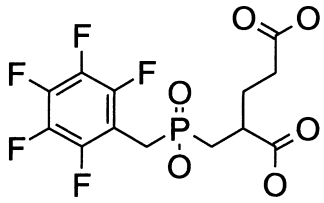
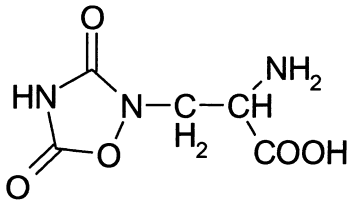
The availability of a pure, recombinant form of GCPII and the development of a fluorescent activity assay is especially advantageous for the analyses of the structure–activity relationships and for large-scale inhibitor testing. To document the usefulness of our recombinant protein and the assay, we tested the inhibitory activity of a panel of potent GCPII inhibitors, 2-PMPA (Jackson *et al.* 1996) and other compounds, summarized in Table 2. To determine whether rhGCPII correctly represents pharmacological activity of individual forms of GCPII described in rat brain, in membrane fractions of LNCaP, human prostate or transiently transfected COS cells, a set of experiments was carried out. We found that the K_i values determined for the rhGCPII are comparable to those determined using GCPII isolated from the rat brain. The data are summarized in Table 2. When using 2-PMPA, a very potent and specific inhibitor, the IC_{50} values show no significant difference. The IC_{50} values for the less specific inhibitors, e.g. quisqualate and phosphate, are shifted towards lower values, i.e. the inhibitors are apparently more potent when purified enzyme is used for the assay, which could be explained by non-specific binding of these inhibitors to the other proteinaceous components of the synaptosomal membrane preparations.

Activities of GCPII identified in normal and cancer cells: role of glycosylation

It has been shown that GCPII exists in two different forms – as a glycosylated type II membrane protein and a soluble cytosolic protein (Grauer *et al.* 1998; Tiffany *et al.* 1999). The two forms result from alternative splicing of mRNA (Su *et al.* 1995), and the N-terminal amino acid of the soluble form is believed to be Ala^{60} of the 'full-length' transmembrane protein (Grauer *et al.* 1998). As it is a cytosolic protein, the shorter form should not be glycosylated (Hart 1992). To test the influence of glycosylation on the rhGCPII carboxypeptidase activity, we deglycosylated the rhGCPII using PNGase F. Interestingly, deglycosylation of the native protein under conditions described in the Experimental Procedures section completely abolished its carboxypeptidase activity (Fig. 5, line 6).

To verify this surprising observation, we expressed a 'cytoplasmic' variant of GCPII (PSM′; Su *et al.* 1995; Tiffany *et al.* 1999), lacking the signal sequence and the

Table 2 Pharmacological comparison of rhGCPII with the native enzyme

Compound	Molecular structure	IC_{50} (rat synaptosomal membrane)	IC_{50} (rhGCPII)
2-PMPA		300 pM	200 pM
GPI 5232		300 nM	40 nM
Phosphate	Na_2HPO_4	200 μM	10 μM
Quisqualate		10 μM	1 μM

The IC_{50} values were determined by the radioenzymatic assay using ^{3}H-NAAG as described.

transmembrane domain. This construct, except for the N-terminal methionine, thus corresponds to the protein that has been identified in the cytosol of normal human prostatic cells. Upon transfection into insect cells, we were able to detect expression of the corresponding protein product using mouse monoclonal antibodies, but no activity was observed (Fig. 5, lane 5).

To further strengthen the claim that glycosylation is essential for rhGCPII NAAG hydrolysing activity, we expressed rhGCPII in presence of tunicamycin, which potently inhibits the first step in *N*-glycosylation pathway. As seen on western blot (Fig. 5), the apparent molecular weight of rhGCPII expressed in medium suplemented with tunicamycin (10 μg/mL) was lower than that of the protein produced in medium without the inhibitor, and apparently identical with rhGCPII deglycosylated by PNGase F *in vitro* (Fig. 5, compare lanes 2 and 6). Residual activity detected in lysates from cells cultured in the presence of tunicamycin suggests that *N*-glycosylation inhibition was not complete. Indeed, after the separation of non-glycosylated and glycosylated proteins by affinity chromatography on Lentil-Lectin Sepharose (see Experimental procedures), the NAAG hydrolysing activity could only be detected in the fraction bound to, and subsequently eluted from, the resin (Fig. 5, lane 4). As Lentil-Lectin specifically binds to mannose residues in sugar chains present on glycoproteins, all the NAAG hydrolysing activity could thus be attributed to the glycosylated protein.

Discussion

The first task in enzymological analysis of any medically relevant enzyme is to establish a reliable source of active, pure protein for further investigation. GCPII was identified as an important regulatory enzyme in the late 1980s (Robinson *et al*. 1987). Slusher *et al*. (1990) published the purification and partial enzymological characterization of rat brain GCPII in 1990. The enzyme has been identified in multiple prostate cell lines and in prostate tissues of eight mammalian species (Tiffany *et al*. 1999) and partial pharmacological and kinetic characterization of the enzymic cleavage of *N*-acetyl-Asp-Glu has been provided. Tiffany *et al*. (1999) reported virtual identity of GCPII from the rat brain and from the prostate (documented by the sensitivity towards inhibitors and similar kinetic values for the substrate cleavage). The activity of the soluble, cytosolic form of prostate GCPII (PSM′) was significant and the kinetics of substrate cleavage suggested two catalytic sites of this form of the enzyme. Recently,

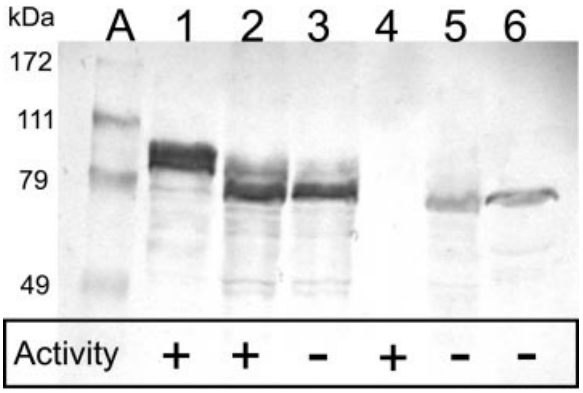


Fig. 5 NAAG hydrolysing activities of glycosylated vs. deglycosylated GCPII fragments. Proteins were expressed by stably transfected Schneider's cells grown in medium with or without tunicamycin (10 μg/mL). Cells were harvested 36 h postinduction and work out as described in Experimental Procedures. A total of 10^6 cells in denaturing buffer were loaded in a single lane, proteins resolved by 9% SDS–PAGE and electroblotted on PVDF membrane and immunostained using anti-GCP antibody as described in Experimental Procedures. (a) Molecular weight marker; 1, cells expressing rhGCPII grown in medium without tunicamycin (control); 2, cells expressing rhGCPII grown in medium supplemented with tunicamycin (10 μg/mL); 3, cell lysate after treatment with Lentil-Lectin Sepharose; 4, fraction bound to Lentil-Lectin Sepharose; 5, cells expressing NA59I fragment (intracellular); 6, 100 ng of purified rhGCPII deglycosylated with PNGase F.

Lodge *et al*. (1999) reported expression of the human GCPII in insect cells using a baculovirus vector. The system was apparently capable of producing enough recombinant protein for the production of monoclonal antibodies. However, no further enzymological or structural characterization of the protein was provided.

In an attempt to provide a reliable source of a highly pure recombinant protein for enzymological characterization, inhibitor testing and structural analysis, we set out to express the extracellular domain of human brain GCPII in bacterial and insect cells expression systems. In *E. coli*, massive amounts of recombinant protein of the correct molecular weight were produced. However, all the recombinant protein was present in insoluble inclusion bodies. No refolding conditions yielded any significant proteolytic activity, suggesting that bacterial cells do not provide the post-translational machinery necessary for correct folding and activity. As the second choice, we employed a relatively novel insect cell expression system using *Drosophila* Schneider's S2 cells. Altogether, three GCPII variants were cloned and transfected into S2 cells. Both variants secreted into medium showed marked NAAG hydrolysing activity, while the NA59I construct, expressed intracellularly, was completely inactive.

To date, the only assay available for the screening of the activity of GCPII has been a radioenzymatic assay using tritiated Ac-Asp-[^{3}H]Glu (Robinson *et al*. 1987). It is a very sensitive assay, enabling determinations of nanomolar concentrations of cleaved glutamate. On the other hand, the assay is not useful for high-throughput testing of large inhibitor series. Moreover, the handling and disposing radiolabelled substrate represents an additional burden for the experimenter. We therefore designed a simple assay based on the chemical modification of the newly liberated amino acid of the N-terminally blocked substrate by a fluorophor and subsequent fluorometric determination. Our system exhibits an approximately 100-fold lower sensitivity in comparison with the radioenzymatic one. However, because of the availability of highly active, pure recombinant GCPII, the sensitivity does not represent a major concern. Moreover, the new assay is less labourious, more reproducible and possible to automate for the high-throughput screening and analysis.

To determine whether rhGCPII correctly represents the pharmacological activity of different forms of GCPII described in individual tissues (rat brain, membrane fractions of LNCaP, human prostate or transiently transfected COS cells), a set of inhibition values was determined for the rhGCPII and found to be comparable with the values determined using GCPII isolated from the rat brain (see Table 2). For 2-PMPA, a very potent and specific inhibitor of GCPII, the IC_{50} values show no significant difference. The assay using purified recombinant enzyme yields lower IC_{50} values for the less specific inhibitors, such as quisqualate and phosphate. We speculate that phosphate and quisqualate probably bind to other proteins in the synaptosomal membranes and thus higher concentrations are needed to reach 50% inhibition of the enzyme.

The usefulness of the system was further documented by testing a series of peptide substrates of GCPII, and determination of the pH optimum of the enzymatic cleavage. Using libraries of all possible dipeptides of general formulae Ac-Asp-Xxx, Ac-Xxx-Glu and Ac-Xxx-Met we identified four new effective substrates of GCPII: Ac-Glu-Met, Ac-Asp-Met, Ac-Ala-Glu and Ac-Ala-Met. Interestingly, the Met-containing dipeptides are the first examples of a substrate without C-terminal Glu for this enzyme. Moreover, the Ac-Ala-Met substrate surprisingly lacks the carboxyl moiety in the side chain. In order to rule out possible oxidation of the side chain sulphur atom of the methionine, we analysed the presence of a sulphonyl group by mass spectrometry. No significant oxidation of the Met residue was observed suggesting that negative charge at the side chain of the C-terminal amino acid is not a necessary prerequisite for the substrate to be hydrolysed. This finding might be important also for the design of specific inhibitors of GCPII.

Detailed enzymologic analysis of newly identified substrates reveals at least two orders of magnitude higher K_m values and thus weaker binding for the compounds containing Met as a C-terminal residue, while the corresponding k_{cat} values are less affected. The specific activity of rhGCPII

(k_{cat}/K_m) against these substrates decreases in the sequence Ac-Glu-Met, Ac-Asp-Met and Ac-Ala-Met, reflecting thus the requirement for the negatively charged residue in the substrate for the productive binding to the enzyme binding cleft. There are several amino acids with basic side chain residues that might be positioned in the vicinity of the substrate binding site of GCPII and form thus favourable electrostatic interactions with negatively charged residues of the substrate (D. Mahadevan *et al.*, manuscript in preparation). Speno *et al*. (1999) mutated several residues thought to be important for catalysis and ligand binding. Mutation of residues R463, R536 and K545 increased the K_m values for Ac-Asp-Glu several times, showing thus the importance of electrostatic interactions for the proper substrate binding. The surprising finding that substrates containing Met at their C-terminus could be cleaved by the enzyme, albeit less efficiently, will have to be analysed in structural terms once a three-dimiensional structure of the enzyme becomes available.

The pH dependence of GCPII seems to be similar to other carboxypeptidases, namely the carboxypeptidase A and glutamate carboxypeptidase G1 from *Pseudomonas*. The sharp fall of the activity in the pH range above 8.0 seems to be caused by ionization of the critical residues binding catalytic Zn^{2+} ion.

Recently, Pangalos *et al*. (1999) reported a novel variant of human GCPII called NAALADase like peptidase, mapped to chromosome 11 and expressed within discrete brain areas. The authors analysed the activity of this novel variant and compared it to the previously described GCPII from rat brain (denominated NAALADase I) and NAALADase-like enzyme from human ileum (NAALADase L). They report that the novel enzyme as well as 'conventional' GCPII exhibits dipeptidyl peptidase IV activity, as shown by analysing lysates of COS cells transiently transfected by the corresponding cDNAs. In an attempt to confirm this surprising observation, we tested the activity of rhGCPII with a model substrate Gly-Pro-AMC. No significant hydrolysis of the substrate was observed with pure recombinant human GCPII. Abbott *et al*. (2000) point out that a DPP IV-like activity of a zinc-containing carboxypeptidase is rather surprising. Because Pangalos *et al*. (1999) used cleared lysates of transiently transfected COS cells, the apparent DPP IV activity might be attributed to the up-regulation of an endogenous protease, perhaps fibroblast-activating protein (Wesley *et al*. 1999).

Several authors have shown the presence of mRNA corresponding to the cytosolic, truncated form of GCPII in prostate cells. Indeed, the mRNA coding for cytosolic form of GCPII, PSM′, is more prevalent in the normal prostate than the mRNA for membrane-bound form (Su *et al*. 1995). These authors showed the presence of an alternatively spliced GCPII mRNA that lacks 266 nucleotides at the 5′-end (including ATG and the transmembrane domain) and should thus correspond to a cytosolic protein (Heston 1997).

While there is solid evidence for alternatively spliced mRNA corresponding to the PSM′ in the cytosol of prostate cells, the existence and activity of the corresponding truncated cytosolic protein has not been consecutively proven. Grauer *et al*. (1998) isolated PSM′ from the lysate of LNCaP cells using monoclonal antibodies against various domains of PSMA. The cells were fractionated and PSM′ localized to the cytoplasm. The protein was purified but no analysis of the activity was provided. Tiffany *et al*. (1999) separated cytosolic and membrane-bound forms of GCPII from prostate LNCaP cells and using fractionated cell lysates showed proteolytic activity of both forms.

In an attempt to analyse the activity of a truncated, intracellular version of GCPII (corresponding to PSM′) we have attempted to express the DNA coding for the corresponding fragment lacking the signal sequence and transmembrane domain of GCPII (see Fig. 1). This truncated mutant should have Ala^{60} as the amino acid following the start methionine. The NA59E, one of two variants of GCPII that provided active protein upon expression in *Drosophila* cells, is of the same length as the putative PSM′ variant. However, intracellular expression of this DNA construct in insect cells yields no detectable NAAG-hydrolysing activity.

It could be argued that a cytosolic form of GCPII would probably not be glycosylated (Hart 1992; Lodge *et al*. 1999) and might thus differ in both structure and activity from the full-size, membrane-anchored form of the enzyme. It is interesting to note in this context that intracellular concentration of phosphate, an effective inhibitor of GCPII, is approximately 1 mM, i.e. almost one or two orders of magnitude above the corresponding K_i value.

In an *in vitro* experiment, we show that the deglycosylation of the rhGCPII *in vitro* using a peptide : *N*-glycosidase F *in trans* renders it completely inactive (see Fig. 5). In order to confirm this observation, we expressed rhGCPII in insect cells as a non-glycosylated protein, blocking N-linked protein glycosylation with tunicamycin. The majority of the rhGCPII produced in the presence of tunicamycin was not glycosylated (as shown on affinity chromatography using Lentil-Lectin) and was completely inactive (Fig. 5, lane 3). Taken together, these data seem to suggest that the putative truncated, soluble cytosolic form of GCPII might not be a proteolytically active species.

In conclusion, the recombinant expression system here described enables production of an active recombinant protein and its enzymological characterization that might pave the way to the structural characterization of this important pharmaceutical target and development of new potent inhibitors.

Acknowledgements

The authors wish to thank Jaroslav Zbrozek for expert amino acid analyses, Alexi Sedo for the access to Perkin Elmer spectrofluo-

rimeter and Ivan Hilgert for anti-GCPII antibodies. This work (performed under the research project Z4055 905) was supported by an International Research Scholar's Award from the Howard Hughes Medical Institute (HHMI 75195-54081) to J.K., grant IAA5055108 from the Grant Agency of the Academy of Science of the Czech Republic and by a research support from Guilford Pharmaceuticals.

References

Abbott C. A., Yu D., McCaughan G. W. and Gorrell M. D. (2000) Post-proline-cleaving peptidases having DP IV like enzyme activity. Post-proline peptidases. *Adv. Exp. Med. Biol.* **477**, 103–109.

Barlos K., Chatzi O., Gatos D. and Stavropoulos G. (1991) 2-Chlorotrityl chloride resin. Studies on anchoring of Fmoc-amino acids and peptide cleavage. *Int. J. Pept. Protein Res.* **37**, 513–520.

Berger U. V., Carter R. E., McKee M. and Coyle J. T. (1995) N-acetylated alpha-linked acidic dipeptidase is expressed by non- myelinating Schwann cells in the peripheral nervous system. *J. Neurocytol.* **24**, 99–109.

Chang S. S., Reuter V. E., Heston W. D., Bander N. H., Grauer L. S. and Gaudin P. B. (1999) Five different anti-prostate-specific membrane antigen (PSMA) antibodies confirm PSMA expression in tumor-associated neovasculature. *Cancer Res.* **59**, 3192–3198.

Coyle J. T. (1997) The nagging question of the function of N-acetylaspartylglutamate. *Neurobiol. Dis.* **4**, 231–238.

Fagg G. E. and Foster A. C. (1986) Excitatory amino acid synaptic mechanisms and neurological function. *Trends Pharmacol. Sci.* **7**, 357–363.

Grauer L. S., Lawler K. D., Marignac J. L., Kumar A., Goel A. S. and Wolfert R. L. (1998) Identification, purification, and subcellular localization of prostate-specific membrane antigen PSM′ protein in the LNCaP prostatic carcinoma cell line. *Cancer Res.* **58**, 4787–4789.

Hart G. W. (1992) Glycosylation. *Curr. Opin. Cell Biol.* **4**, 1017–1023.

Heston W. D. (1997) Characterization and glutamyl preferring carboxypeptidase function of prostate-specific membrane antigen: a novel folate hydrolase. *Urology* **49**, 104–112.

Israeli R. S., Powell C. T., Fair W. R. and Heston W. D. (1993) Molecular cloning of a complementary DNA encoding a prostate-specific membrane antigen. *Cancer Res.* **53**, 227–230.

Jackson P. F., Cole D. C., Slusher B. S., Stetz S. L., Ross L. E., Donzanti B. A. and Trainor D. A. (1996) Design, synthesis, and biological activity of a potent inhibitor of the neuropeptidase N-acetylated alpha-linked acidic dipeptidase. *J. Med. Chem.* **39**, 619–622.

Kato T., Nagatsu T., Kimura T. and Sakakibara S. (1978) Fluorescence assay of x-prolyl dipeptidyl-aminopeptidase activity with a new fluorogenic substrate. *Biochem. Med.* **19**, 351–359.

Langone J. J. and Van Vunakis H., eds. (1986) Immunmological techniques. Part I: Hybridoma technology and monoclonal antibodies. *Methods Enzymol.* **121**, 1–947.

Lindroth P. and Mopper K. (1979) High performance liquid chromatographic determination of subpicomole amounts of amino acids by pre-column fluorescence derivatization with *o*-phthaldialdehyde. *Anal. Chem.* **51**, 1667–1674.

Lodge P. A., Childs R. A., Monahan S. J., McLean J. G., Sehgal A., Boynton A. L., Salgaller M. L. and Murphy G. P. (1999) Expression and purification of prostate-specific membrane antigen in the baculovirus expression system and recognition by prostate-specific membrane antigen-specific T cells. *J. Immunother.* **22**, 346–355.

Luthi-Carter R., Berger U. V., Barczak A. K., Enna M. and Coyle J. T. (1998) Isolation and expression of a rat brain cDNA encoding glutamate carboxypeptidase II. *Proc. Natl Acad. Sci. USA* **95**, 3215–3220.

Mahadevan D. and Saldanha J. W. (1999) The extracellular regions of PSMA and the transferrin receptor contain an aminopeptidase domain: implications for drug design. *Protein Sci.* **8**, 2546–2549.

Pangalos M. N., Neefs J. M., Somers M., Verhasselt P., Bekkers M., van der Helm L., Fraiponts E., Ashton D. and Gordon R. D. (1999) Isolation and expression of novel human glutamate carboxypeptidases with N-acetylated alpha-linked acidic dipeptidase and dipeptidyl peptidase IV activity. *J. Biol. Chem.* **274**, 8470–8483.

Pinto J. T., Suffoletto B. P., Berzin T. M., Qiao C. H., Lin S., Tong W. P., May F., Mukherjee B. and Heston W. D. (1996) Prostate-specific membrane antigen: a novel folate hydrolase in human prostatic carcinoma cells. *Clin. Cancer Res.* **2**, 1445–1451.

Rawlings N. D. and Barrett A. J. (1997) Structure of membrane glutamate carboxypeptidase. *Biochim. Biophys. Acta* **1339**, 247–252.

Robinson M. B., Blakely R. D., Couto R. and Coyle J. T. (1987) 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.* **262**, 14498–14506.

Sedo A., Malik R. and Krepela E. (1998) Dipeptidyl peptidase IV in C6 rat glioma cell line differentiation. *Biol. Chem.* **379**, 39–44.

Slusher B. S., Robinson M. B., Tsai G., Simmons M. L., Richards S. S. and Coyle J. T. (1990) Rat brain N-acetylated alpha-linked acidic dipeptidase activity. Purification and immunologic characterization. *J. Biol. Chem.* **265**, 21297–21301.

Slusher B. S., Vornov J. J., Thomas A. G., Hurn P. D., Harukuni I., Bhardwaj A., Traystman R. J., Robinson M. B., Britton P., Lu X. C., Tortella F. C., Wozniak K. M., Yudkoff M., Potter B. M. and Jackson P. F. (1999) Selective inhibition of NAALADase, which converts NAAG to glutamate, reduces ischemic brain injury. *Nat. Med.* **5**, 1396–1402.

Speno H. S., Luthi-Carter R., Macias W. L., Valentine S. L., Joshi A. R. and Coyle J. T. (1999) Site-directed mutagenesis of predicted active site residues in glutamate carboxypeptidase II. *Mol. Pharmacol.* **55**, 179–185.

Su S. L., Huang I. P., Fair W. R., Powell C. T. and Heston W. D. (1995) Alternatively spliced variants of prostate-specific membrane antigen RNA: ratio of expression as a potential measurement of progression. *Cancer Res.* **55**, 1441–1443.

Tiffany C. W., Lapidus R. G., Merion A., Calvin D. C. and Slusher B. S. (1999) Characterization of the enzymatic activity of PSM: comparison with brain NAALADase. *Prostate* **39**, 28–35.

Tortella F. C., Lin Y., Ved H., Slusher B. S. and Dave J. R. (2000) Neuroprotection produced by the NAALADase inhibitor 2-PMPA in rat cerebellar neurons. *Eur J. Pharmacol.* **402**, 31–37.

Wesley U. V., Albino A. P., Tiwari S. and Houghton A. N. (1999) A role for dipeptidyl peptidase IV in suppressing the malignant phenotype of melanocytic cells. *J. Exp. Med.* **190**, 311–322.

Wroblewska B., Wroblewski J. T., Saab O. H. and Neale J. H. (1993) N-acetylaspartylglutamate inhibits forskolin-stimulated cyclic AMP levels via a metabotropic glutamate receptor in cultured cerebellar granule cells. *J. Neurochem.* **61**, 943–948.

Wroblewska B., Wroblewski J. T., Pshenichkin S., Surin A., Sullivan S. E. and Neale J. H. (1997) N-acetylaspartylglutamate selectively activates mGluR3 receptors in transfected cells. *J. Neurochem.* **69**, 174–181.