Eur. J. Biochem. **271**, 2782–2790 (2004) © FEBS 2004 doi:10.1111/j.1432-1033.2004.04209.x

Amino acids at the N- and C-termini of human glutamate carboxypeptidase II are required for enzymatic activity and proper folding

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Human glutamate carboxypeptidase II (GCPII) is a co-catalytic metallopeptidase and its putative catalytic domain is homologous to the aminopeptidases from *Vibrio proteolyticus* and *Streptomyces griseus*. In humans, the enzyme is expressed predominantly in the nervous system and the prostate. The prostate form, termed prostate-specific membrane antigen, is overexpressed in prostate cancer and is used as a diagnostic marker of the disease. Inhibition of the form of GCPII expressed in the central nervous system has been shown to protect against ischemic injury in experimental animal models. Human GCPII consists of 750 amino acids, and six individual domains were predicted to constitute the protein structure. Here, we report the analysis of the contribution of these putative domains to the structure/function of recombinant human GCPII. We cloned 13 mutants of human GCPII that are truncated or extended at one or both the N- and C-termini of the GCPII sequence. The clones were used to generate stably transfected *Drosophila* Schneider's cells, and the expression and carboxypeptidase activities of the individual protein products were determined. The extreme C-terminal region of human GCPII was found to be critical for the hydrolytic activity of the enzyme. The deletion of as few as 15 amino acids from the C-terminus was shown to completely abolish the enzymatic activity of GCPII. Furthermore, the GCPII carboxypeptidase activity was abrogated upon removal of more than 60 amino acid residues from the N-terminus of the protein. Overall, these results clearly show that amino acid segments at the N- and C-termini of the ectodomain of GCPII are essential for its carboxypeptidase activity and/or proper folding.

Keywords: NAALADase; PSMA; metallopeptidase; prostate cancer; mutagenesis.

Human glutamate carboxypeptidase II (GCPII; EC 3.4.17.21) is a 750 amino acid type II transmembrane glycoprotein. Its expression is restricted mainly to the nervous system, prostate, small intestine, and kidney [1–3]. The GCPII form expressed in the brain, termed *N*-acetylated-α-linked acidic dipeptidase, plays an important role in neurotransmission, as it cleaves *N*-acetyl-L-aspartyl-L-glutamate (NAAG), the most abundant peptidic transmitter within the human central nervous system [4], and terminates its activity [5]. Inhibition of the brain form of GCPII has been shown to be neuroprotective in animal models of stroke, neuropathic pain, or amyotrophic lateral sclerosis [6–8]. The physiological role of GCPII in the prostate is unknown [9]. Expression of this protein is upregulated in prostate cancer (where it is termed prostate specific membrane antigen, PSMA) and is exploited both as a diagnostic modality of, and a therapeutic target for, carcinomas of prostatic origin [10–12]. The enzyme represents a promising target of therapeutic intervention under various pathological conditions.

GCPII belongs to the M28 peptidase family, which encompasses co-catalytic metallopeptidases requiring two zinc ions for catalytic activity, such as aminopeptidases from *Streptomyces griseus* and *Vibrio proteolyticus* [13]. Additionally, the homology of human GCPII with the transferrin receptor has been reported, with sequence identities of 30.3%, 30.2% and 24.0% for the protease-like, apical, and helical domains of the transferrin receptor, respectively [14]. Rawlings & Barrett made

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Abbreviations: ERAD, endoplasmic reticulum-associated degradation; GCPII, human glutamate carboxypeptidase II; NAAG, *N*-acetyl-L-aspartyl-L-glutamate; rhGCPII, recombinant human glutamate carboxypeptidase II; Z-Leu-Leu-Leucinal (Z-LLnL, MG132), *N*-benzyloxycarbonyl-L-leucinyl-L-leucinyl-L-leucinal; Z-Leu-Leu-Norvalinal (Z-LLnV, MG115), *N*-benzyloxycarbonyl-L-leucinyl-L-leucinyl-L-norvalinal.

Enzyme: human glutamate carboxypeptidase II (EC 3.4.17.21).

(Received 26 March 2004, revised 3 May 2004, accepted 7 May 2004)

predictions about the domain structure and the putative catalytic site of GCPII [16]. Similarly to the transferrin receptor, GCPII probably exists as a homodimer under physiological conditions and the dimerization seems to be essential for its hydrolytic activity [15]. The protein is proposed to consist of six domains: the N-terminal cytoplasmic tail (amino acids 1–18), the helical transmembrane region (amino acids 19–43), and four extracellular domains spanning amino acids 44–150 (domain C), 151–274 (domain D), 275–586 (domain E), and 587–750 (domain F). While the domain spanning amino acids 275–586 is believed to be the catalytic domain, the importance/function of the three remaining extracellular domains is unknown [16]. The putative catalytic domain of GCPII is homologous to aminopeptidases from *S. griseus* and *V. proteolyticus* whose crystal structures have been solved at 1.75 Å and 1.8 Å resolution, respectively [17,18]. By analogy with the *Vibrio* aminopeptidase and the alignment of partial amino acid sequences from human GCPII, human transferrin receptor, yeast aminopeptidase Y, *S. griseus* aminopeptidase, and *Caenorhabditis elegans* mGCP fragment, His377, Asp387, Glu425, Asp453 and His553 were proposed to be the zinc ligands of GCPII [16]. To experimentally verify these amino acid assignments, Speno *et al*. [19] mutated the putative zinc ligands, putative substrate-binding residues and other amino acids situated in the vicinity of these residues. The results confirmed the importance of the amino acid residues, all located at the putative catalytic domain, for the GCPII hydrolytic activity and substrate binding.

Recently, a 3D model of the extracellular region of rat GCPII has been published [20]. In addition to the model of the ligand-free protein, the authors docked several GCPII inhibitors into the GCPII-binding pocket and proposed/analyzed the amino acid residues involved in the ligand–protein interactions. All of the residues identified are situated within the segment spanning Arg212 to Arg538, i.e. the putative catalytic domain (domain E) and the D domain of rat GCPII. The contribution of domains C and F to the GCPII hydrolytic activity/inhibitor binding remains to be established.

The 3D structure of GCPII has not yet been solved and virtually nothing is known about the significance of the individual putative GCPII domains for the carboxypeptidase activity and/or proper folding of the protein. In this work we report cloning and expression of GCPII mutants truncated or extended at both N- and C-termini. We analyzed the expression of individual mutants in *Drosophila* Schneider's S2 cells and their corresponding hydrolytic activities, and identified the minimal catalytically competent fragment. We show that the C-terminal end is necessary for GCPII enzymatic activity and that any polypeptide truncated beyond Lys59 (from the N-terminus) is inactive and probably misfolded.

Materials and methods

Expression plasmids

All of the GCPII variants used in this study are schematically depicted in Fig. 1.

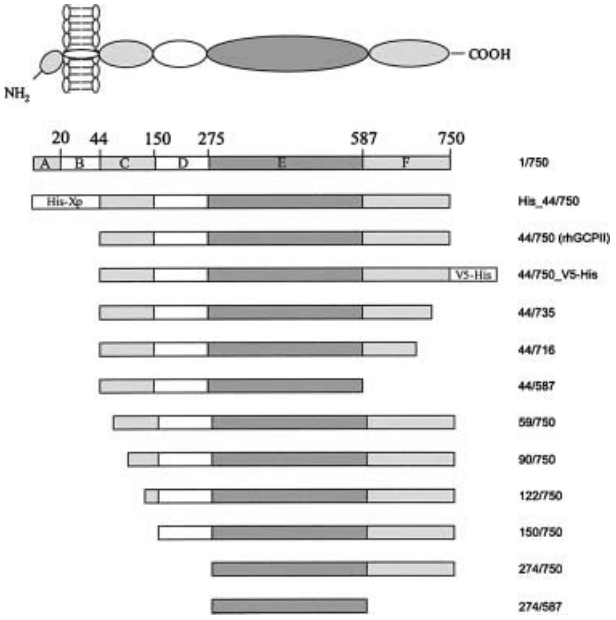

Fig. 1. Schematic diagram of the human glutamate carboxypeptidase II (GCPII) domain structure and GCPII variants used in this study. The figure shows wild-type human GCPII and its truncated or tagged variants. Individual domains are as described previously [16]: A, intracellular segment; B, transmembrane domain; E, putative catalytic domain; polypeptides spanning amino acids 44–150 (domain C), 151–274 (domain D), and 587–750 (domain F) represent domains of unknown function; His, histidine tag; V5, V5 epitope; Xp, Xpress epitope. Numbers before or after a slash correspond to the first or the last amino acid of the truncated variant, respectively, as compared to the full-length wild-type protein.

Truncated constructs. The pMTNAEXST plasmid, described previously [21], was used as the template for generating truncated GCPII constructs. Corresponding primer pairs (20 pmol each), together with 3 U of *Pfu* polymerase (Promega) and 1 ng of the template plasmid, were employed in amplification reactions according to the manufacturer's protocol. The primer sequences, together with thermal cycling parameters, are described in Table 1. Generally, 30 PCR cycles were used for the sequence amplification.

The individual PCR fragments were purified by gel electrophoresis, digested with *Bgl*II/*Xho*I and cloned into pMT/BiP/V5-His A (Invitrogen), in-frame with the BiP leader peptide.

Full-length construct. Sequences of primers and cycling conditions used for generation of the full-length construct (transmembrane, spanning amino acids 1–750) are described in Table 1. The pcDNA3.1/GCPII plasmid [21] was used as a template. The PCR product was digested with *Kpn*I/*Xho*I endonucleases and cloned into a pMT/V5-His A plasmid (Invitrogen).

C-terminally tagged construct. The C-terminally tagged construct was generated similarly to the 44/750 variant. The only exception was usage of the reverse primer (complementary to the C-terminal part of GCPII) that was devoid

Table 1. Primer sequences and thermal cycling parameters.

Variant	Primer pairs (5′→3′)	Cycling conditions		
1–750	AAAGGTACCAAAGATGTGGAATCTCCTTCACG ATTCTCGAGTCATTAGGCTACTTCACTCAAAG	30 s/94 °C;	1 min/57 °C;	5 min/72 °C
44/750	AAACTCGAGAGATCTAAATCCTCCAATGAAGC ATTCTCGAGTCATTAGGCTACTTCACTCAAAG	1 min/94 °C;	1 min/54 °C;	4 min/72 °C
44/735	AAACTCGAGAGATCTAAATCCTCCAATGAAGC ATTCTCGAGTCATTATGCAACATAAATCTGTCTCTT	30 s/94 °C;	1 min/54 °C;	4 min/72 °C
44/716	AAACTCGAGAGATCTAAATCCTCCAATGAAGC AAACTCGAGTTATTATTCAATATCAAACAGAG	30 s/94 °C;	1 min/56 °C;	4 min/72 °C
59/750	AAAAGATCTAAAGCATTTTTGGATGAATTG ATTCTCGAGTCATTAGGCTACTTCACTCAAAG	1 min/94 °C;	1 min/54 °C;	4 min/72 °C
90/750	AAAAGATCTTTTCAGCTTGCAAAGCAA ATTCTCGAGTCATTAGGCTACTTCACTCAAAG	1 min/94 °C;	1 min/57 °C;	4 min/72 °C
122/750	AAAAGATCTAAGACTCATCCCAACTAC ATTCTCGAGTCATTAGGCTACTTCACTCAAAG	1 min/94 °C;	1 min/54 °C;	4 min/72 °C
150/750	AAAAGATCTGGATATGAAAATGTTTCGG ATTCTCGAGTCATTAGGCTACTTCACTCAAAG	30 s/94 °C;	1 min/56 °C;	4 min/72 °C
274/750	ACACTCGAGAGATCTGCAAATGAATATG ATTCTCGAGTCATTAGGCTACTTCACTCAAAG	30 s/94 °C;	1 min/57 °C;	4 min/72 °C
274/587	AAACTCGAGAGATCTAAATCCTCCAATGAAGC CACCTCGAGTTATTATAGCTCAAACACCATCC	30 s/94 °C;	1 min/56 °C;	3 min/72 °C
44/587	AAACTCGAGAGATCTAAATCCTCCAATGAAGC CACCTCGAGTTATTATAGCTCAAACACCATCC	30 s/94 °C;	1 min/56 °C;	3 min/72 °C
44/750_V5-His	AAACTCGAGAGATCTAAATCCTCCAATGAAGC AAACTCGAGGGCTACTTCACTCAAAG	1 min/94 °C;	1 min/57 °C;	4 min/72 °C

of a stop codon, and consequently, the PCR product could be cloned into the pMT/BiP/V5-His A in-frame with the C-terminal V5-His epitope.

N-terminally tagged construct. The DNA sequence encoding the GCPII variant (amino acids 44–750) in the pMTNAEXST plasmid was excised by digestion with *Bgl*II and *Xho*I restriction enzymes and ligated into *Bam*HI/*Xho*I sites in a pcDNA4/HisA vector (Invitrogen). The resulting plasmid was digested with *Nco*I/*Xho*I endonucleases and the GCPII-coding sequence, N-terminally flanked with His-tag and Xpress epitope, was cloned into the *Nco*I/*Xho*I-digested pMTBiP/V5-His A vector in-frame with the BiP leader peptide. The resulting plasmid was designated pMTHis-NA44/750.

The identities of all sequences were verified by dideoxynucleotide-terminal sequencing using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit v2.0 (Perkin-Elmer) and an ABI Prism 310 Genetic Analyzer (PE Corporation).

Transfection of insect cells and generation of stable cell lines

Schneider's S2 cells (Invitrogen) were maintained in SF900II medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (complete medium; Gibco) at 22–24 °C. Stable cell lines expressing individual mutants were generated by cotransfection with 19 μg of the expression plasmid and 1 μg of a pCoHYGRO selection vector (Invitrogen), using a kit for calcium phosphate-mediated transfection (Invitrogen). Stable transfectants were selected by culture of the cells in complete medium [SF900II + 10% (v/v) fetal bovine serum] supplemented with 400 $\mu g \cdot mL^{-1}$ Hygromycin B (Invitrogen).

Expression of GCPII variants

Stably transfected S2 cells were transferred into six-well plates and grown in serum-free SF900II medium to a density of 8×10^6 cells·mL^{-1}. At this point, protein expression was induced with 0.5 mM $CuSO_4$ (final concentration) (Sigma). Three days later, conditioned media and cells were harvested by centrifugation and stored at −70 °C until further use.

Cell lysates

The cell pellets were resuspended in 50 mM Tris/HCl, pH 7.4, containing 100 mM NaCl and a protease inhibitor cocktail (MiniEDTAfree; Roche), to a concentration of 40×10^6 cells per mL, sonicated three times (20 s each, 10 μm amplitude) on ice (Soniprep 150; Sanyo), and subjected to centrifugation at 15 000 ***g*** for 10 min. The supernatant fraction is referred to as the cell lysate.

Total RNA isolation

Total RNA from stably transfected S2 cells (with protein expression induced by addition of 0.5 mM $CuSO_4$) was isolated using Trizol Reagent (Gibco), according to the manufacturer's protocol, with 5×10^6 cells as the starting material. Isolated total RNA was dissolved in RNAse-free water to a concentration of 1 $\mu g \cdot \mu L^{-1}$.

RT-PCR

To eliminate contaminating chromosomal DNA, 1 μg of total RNA was incubated with DNAse I (1 U; Gibco) for 30 min at room temperature in a total volume of 10 μL. One microlitre of EDTA (25 mM, pH 8.0) was then added and DNAse I inactivated at 65 °C for 10 min. The RNA was further amplified using a pair of sequence-specific primers (forward primer, 5′-ATTCAAGACTCCTTCAA GAGCGTGGCGTGGC-3′; reverse primer, 5′-GCTCA AACACCATCCCTCCTCGAACCTGGG-3′) with cycling conditions comprising 30 min at 55 °C followed by 25 cycles of 30 s at 94 °C, 30 s at 55 °C, and 60 s at 72 °C. The reaction products were analyzed on a 1% (w/v) agarose gel, and a positive signal identified as a 549 bp band.

Proteasome inhibition

Stably transfected S2 cells were cultured in SF900II medium supplemented with 10% (v/v) fetal bovine serum, and protein expression was induced with 0.5 mM $CuSO_4$ at a density of 8×10^6 cells mL^{-1}. Twelve hours postinduction, lactacystine (10 μM final concentration), *N*-benzyloxycarbonyl-L-leucinyl-L-leucinyl-L-norvalinal (*Z*-Leu-Leu-Norvalinal, *Z*-LLnV, MG115; 50 μM final concentration), or *N*-benzyloxycarbonyl-L-leucinyl-L-leucinyl-L-leucinal (*Z*-Leu-Leu-Leucinal, *Z*-LLnL, MG132; 50 μM final concentration) was added to the medium and incubation continued for additional 0, 4, 8 or 12 h. The cells were counted, harvested by centrifugation at 500 ***g*** for 5 min, and frozen at −70 °C until further use.

Antibodies

Hybridomas secreting mAbs (clones GCP-01, GCP-02 and GCP-04, all IgG1) were prepared by standard methods from mice (F1 hybrids of BALB/c and B10.A strains) immunized with recombinant human GCPII (rhGCPII, a major fragment corresponding to the extracellular domain, i.e. amino acid residues 44–750), prepared as described previously [21].

SDS/PAGE and Western blotting

Proteins were resolved by SDS/PAGE [0.1% SDS, 13% polyacrylamide (w/w/v)] and electroblotted onto a nitrocellulose membrane. The membrane was probed with the GCP-02 anti-rhGCPII mouse monoclonal antibody (1 $mg{\cdot}mL^{-1}$) at a 1 : 5000 dilution, followed by incubation with a 1 : 20 000 dilution of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Pierce) for 2 h, then developed using a West Dura™ chemiluminescence substrate (Pierce).

NAAG-hydrolyzing activities

Radioenzymatic assays using ^{3}H-labelled NAAG (radiolabeled at the terminal glutamate) were performed as described previously [5], with minor modifications. Briefly, 50 mM Tris/HCl, pH 7.4 (at 37 °C), containing 20 mM NaCl and 20 μL of the GCPII sample, were preincubated for 15 min at 37 °C in a final volume of 225 μL. A 25 μL mixture of 950 nM 'cold' NAAG (Sigma) and 50 nM ^{3}H-labelled NAAG (51.9 $Ci{\cdot}mmol^{-1}$; New England Nuclear) was added to each tube and incubation continued for 20 min. The reaction was stopped with 250 μL of ice-cold 200 mM sodium phosphate, pH 7.4, after which the released glutamate was separated from the substrate by ion exchange chromatography and quantified by liquid scintillation.

Determination of kinetic parameters

Michaelis–Menten (saturation) kinetics were measured in a reaction setup similar to that used for the activity measurements (see above) with substrate concentrations ranging from 0.025 to 50 μM NAAG. Initial velocity measurements for each concentration point were carried out in triplicate. Typical turnover of the substrate did not exceed 25%. K_m and k_{cat} values were determined by a nonlinear least-squares fit of the initial velocity vs. substrate concentration using a GRAFIT software package (Erithacus Software Limited).

Large scale expression and purification

The 44/750 variant was expressed in large quantities in spinner flasks and purified by a combination of ion-exchange chromatography, Lentil-Lectin Sepharose chromatography and chromatofocusing, as described previously [21].

Results

Expression and secretion of truncated variants of GCPII

To analyze the contribution of individual domains of human GCPII (as proposed by Rawlings & Barrett [16]) to its carboxypeptidase activity and/or folding, 13 variants encoding the polypeptide chains truncated or extended at one or both N- or C-termini were constructed (Fig. 1) and the resulting plasmids were used for transfection of *Drosophila* Schneider's S2 cells. The expression and carboxypeptidase activities of the individual constructs were analyzed both in cell lysates and conditioned media, and the results are summarized in Fig. 2 and Table 2, respectively.

Of the 13 variants, only 274/587 (the putative catalytic domain) and 274/750 (the polypeptide spanning the putative catalytic domain and the C-terminal-most domain) were not detected in Western blots of the cell lysates, even though the mAb used in the experiment targets an epitope within these sequences (data not shown). The remainder of the constructs were expressed and immunoreactive bands of expected relative molecular weights observed. Analysis of conditioned media revealed that the majority of the constructs detectable in the cell lysates were secreted into the medium. The only exception was the 150/750 variant, which was retained intracellularly. Additionally, and not surprisingly, neither of the variants absent from the cell lysates (274/587 and 274/750) were detected in the conditioned media.

To quantify the amount of the individual GCPII variants, the signal intensities of the blots were recorded with a CCD camera and analyzed using the AIDA image-analyzing software, version 3.28.001 (Raytest Isotopenmessgerate, Straubenhardt, Germany). Subsequently, calculations of the

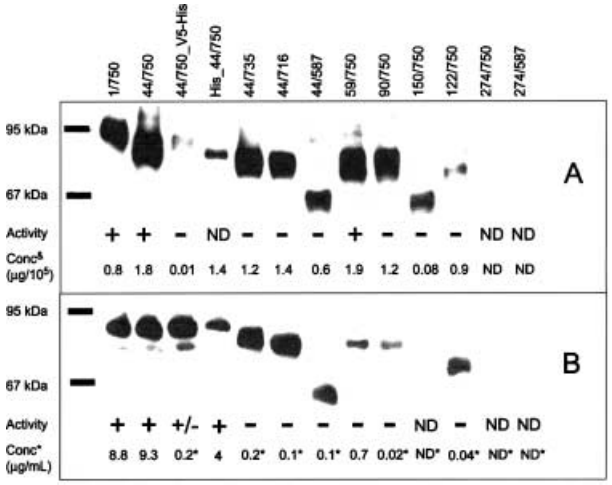

Fig. 2. Western blot analysis of the expression of human glutamate carboxypeptidase II (GCPII) variants in S2 cells. Stably transfected S2 cells were grown in serum-free SF900II medium. Protein expression was induced with 500 μM $CuSO_4$ and conditioned media and cells were harvested 3 days later. Some of the conditioned media, marked with an asterisk (*), were concentrated ×20 using a Microcon ultracentrifugation device (Millipore) prior to Western blot analysis. The proteins were resolved by SDS/PAGE (13% gel), electroblotted onto a nitrocellulose membrane, and immunostained as described in the Materials and methods. Relative band intensities were recorded using a CCD camera, and the concentrations of individual variants was calculated from a calibration curve of known 44/750 concentrations. Carboxypeptidase activities of individual GCPII variants were determined using 100 nM *N*-acetyl-L-aspartyl-L-glutamate (NAAG) as a substrate. (A) Expression of GCPII variants in S2 cells. The cell lysates were mixed with an equal volume of the denaturing SDS buffer and loaded onto a single lane. Activity levels are indicated as follows: (+), measurable NAAG-hydrolyzing activity; (+/–), extremely low activity; (–), no activity; ND, not determined. Conc[&], expression levels of the individual variants in stably transfected induced cells (μg per 10^5 cells). *To visualize and quantify the individual variants in one blot, different numbers of cells were loaded for each mutant. (B) Expression of GCPII variants in conditioned media. Conditioned media were mixed with an equal volume of the denaturing SDS buffer and 10 μL of the mixture was loaded onto a single lane. Activity levels are indicated as follows: (+), measurable NAAG-hydrolyzing activity; (+/–), extremely low activity; (–), no activity; ND, not determined. Conc*, amount of the individual variant in the conditioned medium prior to concentration ($\mu g \cdot mL^{-1}$).

protein quantities from the standard calibration curve of known GCPII (the purified 44/750 variant) concentrations were performed.

Marked differences in the expression levels of the individual variants were observed in both cell lysates and conditioned media. The highest expression levels in the conditioned media were ≈ 10 $\mu g \cdot mL^{-1}$ for the 44/750 and 1/750 variants. A decrease of more than 80-fold in the secretion of recombinant protein was associated with the deletion of the C-terminal part(s) of the protein, even though the intracellular expression levels remained fairly constant. Likewise, deletions within the N-terminal part of the polypeptide resulted in a noticeable decrease in secretion efficiency, as the amounts of the 59/750, 90/750, and 122/750 variants in the medium were ≈ 14-, 600-, and 250-times lower as compared to the 44/750 variant. Moreover, the 150/750 variant was not secreted at all (Fig. 2).

Table 2. Specific activities of the human glutamate carboxypeptidase II (GCPII) variants and wild-type recombinant human glutamate carboxypeptidase II (rhGCPII). Stably transfected S2 cells were grown in serum-free SF900II medium and protein expression was induced with 500 μM $CuSO_4$. Three days later, the cells and conditioned media were harvested and processed as described in the Materials and methods. Conditioned media were dialyzed and concentrated, if desired. Carboxypeptidase activities of the individual variants were determined using 100 nM *N*-acetyl-L-aspartyl-L-glutamate (NAAG) as a substrate and related to the amounts of the immunoreactive proteins, as determined by Western blot densitometry, using purified rhGCPII as a standard. ND, not detected.

Construct	Cell lysates ($nmol \cdot s^{-1} \cdot mg^{-1}$)	Conditioned medium ($nmol \cdot s^{-1} \cdot mg^{-1}$)
1/750	1.5	5.4
His_44/750	ND	4.1
44/750	6.7	27.7
44/750_V5-His	<0.001	0.002
44/735	<0.001	<0.001
44/716	<0.001	<0.001
44/587	<0.001	<0.001
59/750	0.003	4.0
90/750	<0.001	<0.001
122/750	<0.001	<0.001
150/750	<0.001	ND
274/750	ND	ND
274/587	ND	ND

Analysis of the DNA transcription of mutants 274/587 and 274/750

Regarding the 274/587 and 274/750 variants, no protein products of the expected relative molecular masses were observed in Western blots of either cell lysates or the conditioned media. To analyze whether the cells were really transfected with the plasmids encoding the corresponding GCPII variants and that the DNA was transcribed, we isolated genomic DNA and total RNA from the induced cells and performed PCR or RT-PCR assays, respectively. The experiments using GCPII-specific primers confirmed plasmid integration into the genome of Schneider S2 cells and functional transcription of GCPII-coding sequences (data not shown).

Inhibition of proteasome degradation

As the mRNAs encoding the 274/587 and 274/750 variants, but no corresponding protein products, were detected in the induced, stably transfected S2 cells, we attempted to distinguish between two possible alternatives: either the protein was not translated at all, or it was aberrantly folded and consequently degraded by the endoplasmic reticulum-associated degradation system (ERAD), a ubiquitin-proteasome dependent pathway [22]. To investigate this further, we used three different proteasome inhibitors to block the degradation activity of the cells. The proteasome

was inhibited 12 h postinduction by addition of the commercially available inhibitors lactacystine, *Z*-Leu-Leu-Norvalinal or *Z*-Leu-Leu-Leucinal to the S2 cells stably transfected with 274/587 and 274/750. The presence of recombinant proteins in cell lysates was analyzed at 0, 4, 8 and 12 h following the addition of inhibitors. No immunoreactive bands of expected molecular mass were observed in the cell lysates at any of the time-points (data not shown).

Analysis of carboxypeptidase activities of the individual truncated mutants of GCPII

The carboxypeptidase activities against NAAG, a naturally occurring substrate of GCPII, were analyzed both in conditioned media and the cell lysates. The results are summarized in Fig. 2 and Table 2. Out of the 11 variants with detectable levels of expression, only five GCPII constructs were found to be enzymatically active. These were the 1/750 (the transmembrane full-length protein), the 44/750 (the whole ectodomain of GCPII, rhGCPII), the 59/750 and the His_44/750 variants. An extremely low level of NAAG-hydrolyzing activity, $< 0.01\%$ of the 44/750 variant, was associated with the 44/750_V5-His variant, and no proteolytic activity could be detected with variants N-terminally truncated beyond Lys59 or truncated at the C-terminus. These results clearly show that polypeptide stretches situated both N- and C-terminally of the putative catalytic domain are indispensable for GCPII carboxypeptidase activity.

To further characterize the hydrolytical activities of the GCPII variants, we determined the kinetic parameters (K_m and k_{cat}) of the mutants towards NAAG. The data are summarized in Table 3. The kinetic constants for the 44/750_V5-His protein construct could not be determined owing to a very low specific activity of the truncated enzyme. The Michaelis constants of all the constructs tested were comparable, ranging from 81 nM to 472 nM for the 59/750 and 1/750 variants, respectively. In terms of both k_{cat} and K_m, the full-length 1/750 variant showed values similar to the ectodomain-spanning 44/750 mutant, confirming that the ectodomain is a fully active form of the enzyme. Further truncation at the N-terminus, or addition of the V5-His tag at the C-terminus, significantly compromises the proteolytic activity of the variants, by affecting the turnover number rather than substrate binding (Table 3).

Table 3. Kinetic characterization of the human glutamate carboxypeptidase II (GCPII) variants and wild-type recombinant human glutamate carboxypeptidase II (rhGCPII). The kinetic parameters against *N*-acetyl-L-aspartyl-L-glutamate (NAAG) were determined by saturation kinetics for the mutated variants, and wild-type rhGCPII. k_{cat} values were calculated from known concentrations of the individual proteins, as determined by Western blot densitometry.

Construct	k_{cat} (s^{-1})	K_m (nM)	k_{cat}/K_m ($\mu M^{-1}\cdot s^{-1}$)
44/750 (rhGCPII)	5.4 ± 0.3	160 ± 44	33.7 ± 15.4
1/750	8.5 ± 0.4	472 ± 88	18.1 ± 5.1
His_44/750	0.80 ± 0.05	127 ± 47	6.6 ± 4.0
59/750	1.00 ± 0.04	81 ± 11	12.7 ± 2.2

Discussion

Within the last decade, GCPII has been recognized as a promising pharmacological target, and much effort has been invested in developing compounds and strategies targeting or manipulating this enzyme under various pathological conditions. Surprisingly, the 'basic' biochemical characterization of GCPII at the protein level, which might simplify and rationalize the development of modalities useful in clinical practice, is lagging behind the drug discovery activities. Here, we report mapping of the individual predicted domains of human GCPII with regard to their contributions to the GCPII enzymatic activity and folding.

The first critical, important step for analyzing all the GCPII variants used in this study was the development of specific antibodies against human GCPII. As polyclonal rabbit anti-GCPII immunoglobulin cross-reacted slightly with Schneider's autologous S2 cell proteins, and because this cross-reactivity might have interfered with the detection of GCPII variants (especially when the expression level of the variant was very low), several clones of mouse mAbs, specifically recognizing human GCPII, were produced. A polypeptide spanning the putative catalytic domain of human GCPII (amino acids 274–587) expressed in *Escherichia coli* was used to select clones immunoreactive against an epitope within this sequence (data not shown), as all of the variants used in this study comprise the putative catalytic domain.

Carboxypeptidase activities of each of the GCPII constructs that were modified at the C-terminus (either truncated or modified with the V5-His epitope) were either absent or extremely low. An intact C-terminus is therefore indispensable for GCPII enzymatic activity, as the removal of as few as 15 amino acids from the C-terminus completely abolished NAAG-hydrolyzing activity (the 44/736 variant), and the C-terminal extension (addition of the V5-His tag in the case of the 44/750_V5-His variant) reduced the activity by a factor of $> 10^4$. Furthermore, C-terminal modifications also negatively influenced secretion of the truncated variants into the culture medium, suggesting the importance of the C-terminus for the correct folding and procession of GCPII throughout the secretory pathway. These data imply that the putative F domain of GCPII (amino acids 587–750) (Fig. 1), as predicted by Rawlings & Barret [16], might represent an integral part of the GCPII fold, and cannot be deleted without adverse effects on the structure/function of GCPII.

Recently, it has been shown that human GCPII exists in the form of a dimer and that the dimerization is critical for its carboxypeptidase activity [15]. Interestingly, the dimerization of the human transferrin receptor is mediated via contacts of the amino acids forming a helical segment near the C-terminus. As the human transferrin dimerization domain has been reported to be homologous with the C-terminal end of human GCPII [14], it is conceivable that manipulation of the GCPII C-terminus could disrupt the structure of this potential dimerization interface, thus abolishing the enzymatic activity of the protein. Unfortunately, as a result of extremely low yields of the mutants modified at the C-terminus, we were not able to identify an oligomeric status of the variants and confirm these assumptions experimentally.

In contrast to our results, Meighan *et al.* [23] reported expression of the hydrolytically active full-length GCPII flanked with the FLAG-tag at the C-terminus in an HEK293 human embryonic kidney cell line. The authors concluded that this C-terminally modified protein retains hydrolytic activity similar to the wild-type enzyme isolated from LNCaP cells, the cell line naturally expressing GCPII. This discrepancy is difficult to explain. It could be hypothesized that the observed inhibition might be sequence specific, i.e. that either the presence of the 6-His tag compromises carboxypeptidase activity of the 1/750_V5-His construct in an unidentified specific manner or that the inhibition might depend on the length of an epitope attached.

The sequence at the N-terminus of the protein was also shown to be required for the activity and/or secretion of the GCPII carboxypeptidase. As for the N-terminally modified variants, the absence of the intracellular and transmembrane domains does not influence carboxypeptidase activity of GCPII and neither does the attachment of the His-Xpress epitope at the N-terminus of the 44/750 variant. However, the protein was rendered inactive following the deletion of more than 60 amino acids from the N-terminus. Moreover, the amounts of recombinant protein secreted into the media were substantially lower for the variants truncated further at the N-terminus (as compared to the 44/750 variant), and the 150/750 construct was not secreted at all.

The specific activities of the mutants secreted into the medium were generally higher that those retained intracellularly (Table 2). These differences could be attributed to the presence of incorrectly or partially folded protein species in the intracellular fraction, while the extracellular protein consists exclusively of a properly folded enzyme. However, the cause for three orders of magnitude specific activity difference in the case of mutant 59/750 is not clear at present.

The ER is responsible for the quality control of newly synthesized polypeptide chains. Nascent proteins with only a partial fold are cycled via the calnexin-calreticulin-glucosidase I and II system within the ER lumen, providing space and time for the unfolded/partially folded proteins to acquire the correct 3D conformation. The proteins that fail to attain their native conformation are subsequently degraded by the ERAD system [24–26]. As the 150/750 variant was clearly detectable in the cell lysate, but absent from the conditioned medium, it is plausible that the 150/750 variant was not able to fold correctly and consequently was retained in the ER and not allowed to proceed further along the secretory pathway.

Two of the GCPII variants studied, namely the 274/750 and 274/587 constructs, were detected neither in the cell lysates nor in the conditioned media, although the corresponding mRNAs were detected by RT-PCR. Our failure to detect expression of these GCPII variants, even after proteasome inhibition, cannot be explained unequivocally, but may be a result of the fact that mRNAs encoding the respective proteins are not, for an unknown reason, translated in S2 cells. Another possibility could be that the proteasome inhibition was not complete. Similar phenomena were described for the EL4 mouse cell line that was formerly reported to be adapted to conditions of total proteasome inhibition [27]. Additionally, an increase in the proteolytic activity of different cell degradation systems, for example tripeptidyl peptidase II, might compensate for the inhibited proteasome activity [28,29]. Yet another explanation might be that proteasome inhibitors exercise more general effects on the overall metabolism of S2 cells, resulting in an overall decrease of protein synthesis or increase in protein degradation. This interpretation is supported by our control experiment with proteasome inhibition of the S2 cells expressing the 44/587 variant, which lowered, rather than increased, the expression levels of the recombinant protein (data not shown). Similar, negative effects of proteasome inhibitors on recombinant protein expression (reduction of luciferase and beta-galactosidase activity in tissue culture cells treated with proteasome inhibitors) were recently reported by Deroo & Archer [30].

Unexpectedly, the 1/750 variant of GCPII, i.e. full-length transmembrane protein, was detected in the conditioned medium. This observation contradicts the analysis of conditioned media of LNCaP cells or HEK cells stably transfected with full-length human GCPII, in which 'shedding' of GCPII was not detected (data not shown). We attempted to identify the cleavage site recognized by an unknown 'sheddase' by the N-terminal sequencing, but no sequence was recovered, apparently as a result of the blocking of the N-terminal amino acid. Subsequent Western blot analysis, exploiting the 7E11 mAb recognizing the first six amino acids of the full-length GCPII [31], revealed the absence of the immunoreactive epitope (i.e. the N-terminal end of GCPII) in the species 'shed' into the medium, but not in the species expressed on the cell surface (data not shown). Taken together, S2 cells probably express an unidentified peptidase capable of specific cleavage of the 1/750 variant, releasing soluble protein into the culture medium.

Kinetic parameter comparison of the individual enzymatically active GCPII variants did not reveal any significant differences in either the binding or the turnover of the substrate. The submicromolar values of the Michaelis constants are in good agreement with the data reported previously for both rat and human enzymes [5,32–35].

In conclusion, we analyzed the contribution of the N- and C-terminal regions of GCPII to its enzymatic properties and structure/folding. The results clearly show that the amino acids at the extreme C-terminus of GCPII are crucial for the hydrolytic activity of the enzyme and, furthermore, that no more than 60 amino acids can be deleted from the N-terminus without compromising the carboxypeptidase activity of GCPII. These data thus indicate that current GCPII homology models should be interpreted with some caution, as they might lack elements indispensable for the enzymatic activity of GCPII.

Acknowledgements

The authors wish to thank Jana Starková and Tat'ána Mrázková for excellent technical assistance. This work (performed under the research project Z4055 905) was supported by grant IAA5055108 from the Grant Agency of the Academy of Science of the Czech Republic, grant 301/03/0784 from the Grant Agency of the Czech Republic and by research support from Guilford Pharmaceuticals.

References

1. Chang, S.S., Reuter, V.E., Heston, W.D., Bander, N.H., Grauer, L.S. & 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.
2. Silver, D.A., Pellicer, I., Fair, W.R., Heston, W.D. & Cordon-Cardo, C. (1997) Prostate-specific membrane antigen expression in normal and malignant human tissues. *Clin. Cancer Res.* **3**, 81–85.
3. Troyer, J.K., Beckett, M.L. & Wright, G.L. Jr (1995) Detection and characterization of the prostate-specific membrane antigen (PSMA) in tissue extracts and body fluids. *Int. J. Cancer* **62**, 552–558.
4. Coyle, J.T. (1997) The nagging question of the function of *N*-acetylaspartylglutamate. *Neurobiol. Dis.* **4**, 231–238.
5. Robinson, M.B., Blakely, R.D., Couto, R. & 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.
6. Chen, S.R., Wozniak, K.M., Slusher, B.S. & Pan, H.L. (2002) Effect of 2-(phosphono-methyl)-pentanedioic acid on allodynia and afferent ectopic discharges in a rat model of neuropathic pain. *J. Pharmacol. Exp. Ther.* **300**, 662–667.
7. Ghadge, G.D., Slusher, B.S., Bodner, A., Canto, M.D., Wozniak, K., Thomas, A.G., Rojas, C., Tsukamoto, T., Majer, P., Miller, R.J., Monti, A.L. & Roos, R.P. (2003) Glutamate carboxypeptidase II inhibition protects motor neurons from death in familial amyotrophic lateral sclerosis models. *Proc. Natl Acad. Sci. USA* **100**, 9554–9559.
8. 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. & Jackson, P.F. (1999) Selective inhibition of NAALADase, which converts NAAG to glutamate, reduces ischemic brain injury. *Nat. Med.* **5**, 1396–1402.
9. Bostwick, D.G., Pacelli, A., Blute, M., Roche, P. & Murphy, G.P. (1998) Prostate specific membrane antigen expression in prostatic intraepithelial neoplasia and adenocarcinoma: a study of 184 cases. *Cancer* **82**, 2256–2261.
10. Lamb, H.M. & Faulds, D. (1998) Capromab pendetide. A review of its use as an imaging agent in prostate cancer. *Drugs Aging* **12**, 293–304.
11. Lodge, P.A., Jones, L.A., Bader, R.A., Murphy, G.P. & Salgaller, M.L. (2000) Dendritic cell-based immunotherapy of prostate cancer: immune monitoring of a phase II clinical trial. *Cancer Res.* **60**, 829–833.
12. Mincheff, M., Tchakarov, S., Zoubak, S., Loukinov, D., Botev, C., Altankova, I., Georgiev, G., Petrov, S. & Meryman, H.T. (2000) Naked DNA and adenoviral immunizations for immunotherapy of prostate cancer: a phase I/II clinical trial. *Eur. Urol.* **38**, 208–217.
13. Rawlings, N.D. (1998) Introduction: Metallopeptidases and their clans. In *Handbook of Proteolytic Enzymes* (Barrett, A.J., Rawlings, N.D. & Woessner, J.F., eds), pp. 989–991. Academic Press, New York.
14. Lawrence, C.M., Ray, S., Babyonyshev, M., Galluser, R., Borhani, D.W. & Harrison, S.C. (1999) Crystal structure of the ectodomain of human transferrin receptor. *Science* **286**, 779–782.
15. Schulke, N., Varlamova, O.A., Donovan, G.P., Ma, D.S., Gardner, J.P., Morrissey, D.M., Arrigale, R.R., Zhan, C.C., Chodera, A.J., Surowitz, K.G., Maddon, P.J., Heston, W.D.W. & Olson, W.C. (2003) The homodimer of prostate-specific membrane antigen is a functional target for cancer therapy. *Proc. Natl Acad. Sci. USA* **100**, 12590–12595.
16. Rawlings, N.D. & Barrett, A.J. (1997) Structure of membrane glutamate carboxypeptidase. *Biochim. Biophys. Acta* **1339**, 247–252.
17. Chevrier, B., Schalk, C., Dorchymont, H., Rondeau, J.M., Tarnus, C. & Moras, D. (1994) Crystal structure of *Aeromonas proteolytica* aminopeptidase – a prototypical member of the co-catalytic zinc enzyme family. *Structure* **2**, 283–291.
18. Greenblatt, H.M., Almog, O., Maras, B., SpunginBialik, A., Barra, D., Blumberg, S. & Shoham, G. (1997) *Streptomyces griseus* aminopeptidase: X-ray crystallographic structure at 1.75 angstrom resolution. *J. Mol. Biol.* **265**, 620–636.
19. Speno, H.S., Luthi-Carter, R., Macias, W.L., Valentine, S.L., Joshi, A.R. & Coyle, J.T. (1999) Site-directed mutagenesis of predicted active site residues in glutamate carboxypeptidase II. *Mol. Pharmacol.* **55**, 179–185.
20. Rong, S.B., Zhang, J., Neale, J.H., Wroblewski, J.T., Wang, S. & Kozikowski, A.P. (2002) Molecular modeling of the interactions of glutamate carboxypeptidase II with its potent NAAG-based inhibitors. *J. Med. Chem.* **45**, 4140–4152.
21. Barinka, C., Rinnova, M., Sacha, P., Rojas, C., Majer, P., Slusher, B.S. & Konvalinka, J. (2002) Substrate specificity, inhibition and enzymological analysis of recombinant human glutamate carboxypeptidase II. *J. Neurochem.* **80**, 477–487.
22. Doherty, F.J., Dawson, S. & Mayer, R.J. (2002) The ubiquitin-proteasome pathway of intracellular proteolysis. *Essays Biochem.* **38**, 51–63.
23. Meighan, M.A., Dickerson, M.T., Glinskii, O., Glinsky, V.V., Wright, G.L. & Deutscher, S.L. (2003) Recombinant glutamate carboxypeptidase II (prostate specific membrane antigen-PSMA) – cellular localization and bioactivity analyses. *J. Protein Chem.* **22**, 317–326.
24. Hampton, R.Y. (2002) ER-associated degradation in protein quality control and cellular regulation. *Curr. Opin. Cell Biol.* **14**, 476–482.
25. Jarosch, E., Lenk, U. & Sommer, T. (2003) Endoplasmic reticulum-associated protein degradation. *Int. Rev. Cytol.* **223**, 39–81.
26. Yoshida, Y. (2003) A novel role for N-glycans in the ERAD system. *J. Biochem. (Tokyo)* **134**, 183–190.
27. Princiotta, M.F., Schubert, U., Chen, W.S., Bennink, J.R., Myung, J., Crews, C.M. & Yewdell, J.W. (2001) Cells adapted to the proteasome inhibitor 4-hydroxy5-iodo-3-nitrophenylacetyl-Leu-Leu-leucinal-vinyl sulfone require enzymatically active proteasomes for continued survival. *Proc. Natl Acad. Sci. USA* **98**, 513–518.
28. Geier, E., Pfeifer, G., Wilm, M., Lucchiari-Hartz, M., Baumeister, W., Eichmann, K. & Niedermann, G. (1999) A giant protease with potential to substitute for some functions of the proteasome. *Science* **283**, 978–981.
29. Wang, E.W., Kessler, B.M., Borodovsky, A., Cravatt, B.F., Bogyo, M., Ploegh, H.L. & Glas, R. (2000) Integration of the ubiquitin-proteasome pathway with a cytosolic oligopeptidase activity. *Proc. Natl Acad. Sci. USA* **97**, 9990–9995.
30. Deroo, B.J. & Archer, T.K. (2002) Proteasome inhibitors reduce luciferase and beta-galactosidase activity in tissue culture cells. *J. Biol. Chem.* **277**, 20120–20123.
31. Troyer, J.K., Feng, Q., Beckett, M.L. & Wright, G.L. Jr (1995) Biochemical characterization and mapping of the 7E11-C5.3 epitope of the prostate-specific membrane antigen. *Urol. Oncol.* **1**, 29–37.
32. Luthi-Carter, R., Barczak, A.K., Speno, H. & Coyle, J.T. (1998) Hydrolysis of the neuropeptide N-acetylaspartylglutamate (NAAG) by cloned human glutamate carboxypeptidase II. *Brain Res.* **795**, 341–348.
33. Luthi-Carter, R., Barczak, A.K., Speno, H. & Coyle, J.T. (1998) Molecular characterization of human brain N-acetylated

alpha-linked acidic dipeptidase (NAALADase). *J. Pharmacol. Exp. Ther.* **286**, 1020–1025.

34. Serval, V., Barbeito, L., Pittaluga, A., Cheramy, A., Lavielle, S. & Glowinski, J. (1990) Competitive inhibition of N-acetylated-alpha-linked acidic dipeptidase activity by N-acetyl-L-aspartyl-beta-linked L-glutamate. *J. Neurochem.* **55**, 39–46.
35. Slusher, B.S., Robinson, M.B., Tsai, G., Simmons, M.L., Richards, S.S. & Coyle, J.T. (1990) Rat brain N-acetylated alpha-linked acidic dipeptidase activity. Purification and immunologic characterization. *J. Biol. Chem.* **265**, 21297–21301.