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EXPRESSION OF GLUTAMATE CARBOXYPEPTIDASE II IN HUMAN BRAIN

P. ŠÁCHA,[a,b] J. ZÁMEČNÍK,[c] C. BAŘINKA,[a] K. HLOUCHOVÁ,[a,b] A. VÍCHA,[d] P. MLČOCHOVÁ,[a,b] I. HILGERT,[e] T. ECKSCHLAGER[d] AND J. KONVALINKA[a,b]*

[a]*Department of Biochemistry, Institute of Organic Chemistry and Biochemistry, Academy of Science of the Czech Republic, Flemingovo n.2, Prague 6, 166 10 Czech Republic*

[b]*Department of Biochemistry, Charles University, Faculty of Natural Science, Hlavova 2030, Prague 2, 128 43 Czech Republic*

[c]*Department of Pathology and Molecular Medicine, Charles University, 2nd Medical Faculty and University Hospital Motol, V Úvalu 84, Prague 5, 150 06, Czech Republic*

[d]*Department of Pediatric Oncology and Hematology, Charles University, 2nd Faculty of Medicine, V Úvalu 84, Prague 5, 150 06, Czech Republic*

[e]*Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Vídeňská 1083, Prague 4, 142 20, Czech Republic*

Abstract—Glutamate carboxypeptidase II (GCPII) is a transmembrane glycoprotein expressed in various tissues. When expressed in the brain it cleaves the neurotransmitter N-acetylaspartylglutamate (NAAG), yielding free glutamate. In jejunum it hydrolyzes folylpoly-gamma-glutamate, thus facilitating folate absorption. The prostate form of GCPII, known as prostate specific membrane antigen (PSMA), is an established cancer marker. The NAAG-hydrolyzing activity of GCPII has been implicated in a number of pathological conditions in which glutamate is neurotoxic (e.g. amyotrophic lateral sclerosis, Huntington's disease, Alzheimer's disease, epilepsy, schizophrenia, and stroke). Inhibition of GCPII was shown to be neuroprotective in tissue culture and in animal models. GCPII is therefore an interesting putative therapeutic target. However, only very limited and controversial data on the expression and localization of GCPII in human brain are available. Therefore, we set out to analyze the activity and expression of GCPII in various compartments of the human brain using a radiolabeled substrate of the enzyme and the novel monoclonal antibody GCP-04, which recognizes an epitope on the extracellular portion of the enzyme and is more sensitive to GCPII than to the homologous GCPIII. We show that this antibody is more sensitive in immunoblots than the widely used antibody 7E11. By Western blot, we show that there are approximately 50–300 ng of GCPII/mg of total protein in human brain, depending on the specific area. Immunohistochemical analysis revealed that astrocytes specifically express GCPII in all parts of the brain. GCPII is enzymatically active and the level of activity follows the expression pattern. Using pure recombinant GCPII and homologous GCPIII, we conclude that GCPII is responsible for the majority of overall NAAG-hydrolyzing activity in the human brain.

Key words: NAALADase, PSMA, metallopeptidase, prostate cancer, immunohistochemistry, epitope mapping.

Glutamate carboxypeptidase II (GCPII, EC 3.4.17.21), also known as prostate specific membrane antigen (PSMA) or folate hydrolase I (FOLH1), is a type II transmembrane glycoprotein, the human form of which has a molecular weight of approximately 100 kDa and consists of 750 amino acids. Glycosylation of the enzyme is critical for its proteolytic activity (Barinka et al., 2002, 2004b). GCPII is a dizinc metallopeptidase. Recently, its crystal structure has been solved by two groups independently (Davis et al., 2005; Mesters et al., 2006).

In the brain, GCPII cleaves N-acetylaspartylglutamate (N-acetyl-L-aspartyl-L-glutamate, NAAG) to N-acetylaspartate and glutamate. NAAG is a highly abundant peptide neurotransmitter and an agonist of metabotropic glutamate receptor 3 (Wroblewska et al., 1997; Neale et al., 2000). The inhibition of the brain form of GCPII has been demonstrated to be neuroprotective in animal models of ischemic brain injury (Slusher et al., 1999; Lu et al., 2000), to attenuate neuropathic pain (Jackson et al., 2001; Yamamoto et al., 2004), and to prolong survival of the experimental animals in the mouse model of amyotrophic lateral sclerosis (Ghadge et al., 2003; for review, see Neale et al., 2005).

In the jejunum, GCPII cleaves pteroylpoly-gamma-glutamate to folate and glutamate, thus enabling the absorption of dietary folates (Halsted et al., 1998). The physiological function of GCPII in prostate is not known. A GCPII variant called PSM′ (N-terminally truncated intracellular form of prostate specific membrane antigen) is transcribed in the prostate. PSM′, which lacks the coding sequence for the intracellular and transmembrane domains due to alternative splicing, is a 693 amino acid protein (Su et al., 1995). The PSMA/PSM′ mRNA ratio is elevated in prostate cancer (Su et al., 1995), and PSMA could serve as a prostate cancer marker (Murphy, 1995). Furthermore, the mRNA in the rat brain is transcribed in six variants of 3900,

*Correspondence to: J. Konvalinka, Department of Biochemistry, Institute of Organic Chemistry and Biochemistry, Academy of Science of the Czech Republic, Flemingovo n.2, Prague 6, 166 10 Czech Republic. Tel: +42–0220183218.
E-mail address: konval@uochb.cas.cz (J. Konvalinka).

Abbreviations: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; GCPII/III, human glutamate carboxypeptidase II/III; GFAP, glial fibrillary acidic protein; mAb, monoclonal antibody; NAAG, N-acetyl-L-aspartyl-L-glutamate; NAALADase L, N-acetylated-alpha-linked-acidic dipeptidase L; PSMA, prostate specific membrane antigen; PSM′, N-terminally truncated intracellular form of prostate specific membrane antigen; rhGCPII/III, recombinant human glutamate carboxypeptidase II/III; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TBST, Tris buffered saline containing 0.1% Triton X-100; 2-PMPA, 2-(phosphonomethyl)pentanedioic acid.

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3000, 2800, 2100, 750, and 500 nucleotides. However, the function of those variants is not known (Carter et al., 1996).

There are also reports on expression of GCPII in rat kidney studied by immunohistochemistry (Slusher et al., 1992) or by activity determination (Robinson et al., 1987), but the function of the enzyme in that organ remains unknown.

A human homolog labeled GCPIII which shares 81% similarity with GCPII has been described (Pangalos et al., 1999), and its mouse ortholog has been partially characterized (Bzdega et al., 2004). Little is known about its expression level and activity, and no specific antibodies for GCPIII have been described so far. A report on the phenotype of GCPII knock-out mice suggests that GCPIII activity might compensate for the absent GCPII activity (Bacich et al., 2002). Apart from GCPIII, two other variants of GCPII have been described: N-acetylated-alpha-linked-acidic dipeptidase L (NAALADase L) and PSMA-like enzyme (Pangalos et al., 1999; O'Keefe et al., 2004). Neither of these gene products seems to exhibit any proteolytic activity, and their physiological role (if any) remains elusive.

Most of the information about the distribution of GCPII in the brain is derived from studies in rats and mice. Considering the wealth of direct and indirect observation suggesting the important role of GCPII in the pathology of various neurological disorders, little is known about its expression and localization in human brain. In the rat brain, NAAG-hydrolyzing activity was reported (Fuhrman et al., 1994), and GCPII was detected immunochemically (Berger et al., 1999). The reports on the immunochemical detection of GCPII in the human brain are more controversial. In their early analysis of 122 human specimens using the antibody 7E11 Horoszewicz et al. (1987) did not observe any GCPII expression in human samples except for prostate and kidney. Similarly, Lopes et al. (1990) and Silver et al. (1997) could not detect any GCPII expression in human brain by using 7E11 antibody. Chang et al. (1999) could not detect GCPII in human brain by immunohistochemistry, although they employed five different monoclonal antibodies.

On the other hand, Troyer et al. (1995) detected low GCPII expression levels by Western blot analysis of membrane preparations of human cerebral cortex using 7E11 antibody. O'Keefe et al. (2004) reported low mRNA and protein expression in human hippocampus and amygdala using monoclonal antibodies. Furthermore, Berger et al. (1995) described localization of GCPII on neuromuscular junctions in rats using immunohistochemical methods. Another report showed strong cytoplasmic staining in the neurons in the hippocampal region of mouse and human brain (Huang et al., 2004). A low level of mRNA was found in human brain by Israeli et al. (1994), Luthi-Carter et al. (1998b), and Renneberg et al. (1999). However, none of these reports analyzed the expression in various segments of human brain systematically. The expression of GCPII in human brain thus remains controversial and poorly characterized. Therefore, we set out to perform a systematic analysis of GCPII expression, enzymatic activity, and localization in individual segments of human brain using a novel, sensitive monoclonal antibody (mAb).

EXPERIMENTAL PROCEDURES

Tissue samples: dissection and preparation

Samples for the study were obtained from five brains (four males aged 48, 71, 76, and 81 years and one female aged 68 years) during autopsy. The brains were free of metastatic spread of cancer. The autopsies were performed 4–8 h (in one case, 27 h) postmortem. After 2 h at room temperature, the corpses were stored at 5 °C. From one patient (a 48-year-old man who died due to generalized lung cancer and severe bronchopneumonia) samples were taken systematically from different brain compartments as listed in Table 1. Two sets of samples were dissected from each brain location. The first set was fixed in 10% buffered formalin for 24 h at room temperature for the immunohistochemical study. A second set of samples for the quantitative studies was immediately frozen on dry ice and later used for the immunochemical detection by Western blot. In order to analyze the variability in GCPII activity among individuals, additional samples from selected CNS compartments (frontal cerebral cortex, temporal gray matter, temporal white matter, nucleus caudatus, spinal cord and brainstem, see Table 2) were taken from the brains of further four patients and frozen on dry ice. For the activity testing, these frozen samples were thawed, homogenized, and sonicated in the reaction buffer (50 mM Tris–HCl, 5 mM NaCl, pH 7.4) with addition of 1% Triton X-100 and protease inhibitors cocktail (Complete Mini, EDTA-free, Roche, Mannheim, Germany), dialyzed against reaction buffer for 3 days with six exchanges at 4 °C, and centri-

Table 1. Identification of samples taken from various CNS regions

Sample number	CNS compartment
S1	Olfactory bulb
S2	Frontal cerebral cortex
S3	Somatomotoric cerebral cortex
S4	Temporal gray matter
S5	Occipital gray matter
S6	Temporal white matter
S7	Anteroventral thalamic nuclei
S8	Corpus geniculatum lateralis
S9	Ventroposterior thalamic nuclei
S10	Corpus geniculatum mediale
S11	Nucleus caudatus
S12	Globus pallidum
S13	Cerebellum, folia of hemispheres
S14	Nucleus dentatus
S15	Hippocampus
S16	Corpus callosum
S17	Amygdala
S18	Substantia nigra
S19	Pontine nuclei
S20	Cochlear nuclei
S21	Inferior olive
S22	Locus coeruleus
S23	Ventrolateral medulla oblongata
S24	Spinal cord
S25	Supraoptic nucleus
S26	Lateral hypothalamic area
S27	Periventricular nuclei
S28	Nucleus ruber
S29	Superior colliculus
S30	Brainstem regions