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EXPRESSION OF GLUTAMATE CARBOXYPEPTIDASE II IN HUMAN BRAIN

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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,

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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

Table 2. Specific NAAG-hydrolyzing activity and protein content of individual brain samples

Sample number	CNS compartment	N	GCPII content (ng GCPII/mg total protein)	Activity (pmol/min/mg total protein)	Specific activity (nmol/min/mg)
S2	Frontal cerebral cortex	5	118±50	2.9±1.0	27±11
S4	Temporal gray matter	4	49±30	1.5±0.4	37±13
S6	Temporal white matter	5	282±75	11.8±2.3	43±5
S11	Nucleus caudatus	4	64±29	2.9±0.7	46±7
S24	Spinal cord	5	156±94	6.4±3.1	47±16
S30	Brainstem regions	5	176±61	6.7±1.5	40±8

The sample identification corresponds to the lines in Fig. 3A and B. *N* represents the number of brains from which the samples were taken. Total protein content was determined by Bio-Rad Protein Assay, "GCPII content" represents data from densitometry quantification of the immunoblot intensity of the corresponding sample with ECL detection using a CCD-camera. Pure recombinant GCPII was used as a standard. The activities were measured in duplicates; data represent average values with standard deviation. "Specific activity" represents activity in nanomoles of substrate hydrolyzed per minute divided by GCPII content in mg determined by densitometry as described above.

fuged for 10 min at 16,000×*g* at 4 °C. The supernatants were used for further analysis. Determination of protein concentration in the supernatant was performed by BioRad protein assay (BioRad, Munich, Germany) according to the manufacturer's manual.

Preparation of the monoclonal antibodies GCP-04, GCP-02, and 7E11

Hybridomas secreting monoclonal antibodies GCP-04 and GCP-02 (both IgG1) were prepared by standard methods from mice (F1 hybrids of BALB/c and B10.A strains) immunized with recombinant human glutamate carboxypeptidase II (rhGCPII, extracellular portion, i.e. amino acid residues 44–750), as described previously (Barinka et al., 2002). A hybridoma secreting 7E11 antibody was obtained from the American Tissue Culture Collection. Hybridoma cell supernatants were purified by Protein A (Amersham, Pharmacia Biotech, Uppsala, Sweden) affinity chromatography, and the resulting IgG preparation (stock concentration 1 mg/ml of antibody 7E11 and GCP-02, 7 mg/ml in case of GCP-04) was stored at 5 °C until usage.

Phage display

The epitope mapping of the GCP-04 mAb was carried out using Ph.D.-7 Phage Display Peptide Library Kit (NEB, Beverly, MA, USA) according to the manufacturer's protocol. Polysorp tubes (NUNC, Rochester, NY, USA) were coated with 1 ml of purified mAb GCP-04 (100 μg/ml in Tris-buffered saline (TBS)) overnight at 4 °C. The coating solution was discarded and the tubes were blocked with 1.5 ml of bovine serum albumin (BSA, Fluka Chemie, Buchs, Switzerland) solution (5 mg/ml in 0.1 M $NaHCO_3$ (Lachema, Brno, Czech Republic), 0.02% NaN_3 (Penta, Prague, Czech Republic), pH 8.6) for one hour at 4 °C. The blocking solution was poured off and tubes were washed 6×1 ml with Tris buffered saline containing 0.1% Triton X-100 (TBST). Phages (2×10^{11}) (10 μl of the original library) in 1 ml of TBST were pipetted into the coated tube and rocked gently for 60 min at room temperature. The solution with non-bound phages was discarded and tubes were washed 10×1 ml with TBST. Bound phages were eluted with 1 ml of 200 mM glycine (ICN Biomedicals, Irvine, CA, USA), 1 mg/ml BSA, pH 2.2. The elution proceeded for 10 min at room temperature, and the eluate was then immediately neutralized by addition of 150 μl 1 M Tris–HCl (USB, Cleveland, OH, USA), pH 9.1. The phages were titrated and amplified and the panning procedure was repeated three more times. Following the fourth round of panning, the individual binding clones were amplified and the phage DNA was isolated and sequenced.

Activity assay

One microliter of the supernatant prepared from tissue samples as described above was diluted by reaction buffer (50 mM Tris–HCl, 5 mM NaCl, pH 7.4) with or without the presence of 100 nM inhibitor 2-(phosphonomethyl)pentanedioic acid (2-PMPA) (Slusher et al., 1999) to a final volume of 180 μl and preincubated at 37 °C for 3 min. The reaction was started by addition of 20 μl of radioactive NAAG (1 μM, 1850 Bq/20 μl, tritium label on the glutamate, PerkinElmer, Boston, MA, USA). After 1–7 h (to achieve conversion between 5% and 30%), the reaction was stopped by the addition of 200 μl chilled 200 mM sodium phosphate (pH 7.4, Lachema). Two hundred microliters of the mixture was separated in a glass column on AG 1-X8 Resin (BioRad), the cleaved glutamate was eluted with 2 ml of 1 M formic acid, mixed with scintillation cocktail (Rotiszint ECO Plus, Roth, Karlsruhe, Germany) and measured in a liquid scintillator (LS 6500, Beckman, Fullerton, CA, USA).

A similar reaction setup was used for a direct comparison of pure recombinant GCPII (extracellular part 44–750, Barinka et al., 2002) and GCPIII (extracellular part 36–740, Hlouchova et al., manuscript in preparation) activities. In the assay, 0.37 ng of rhGCPII and 12.5 ng of rhGCPIII (as determined by active site titration) were used in a total reaction volume of 200 μl, so that after a 20 min reaction the substrate conversion was approximately 20% in both cases.

Active site titrations

Approximately 1.5 μM GCPII was preincubated with differing concentrations of the tight binding inhibitor 2-(phosphonomethyl)-pentanedioic acid (10–1000 nM) in 20 mM MOPS, 20 mM NaCl, pH 7.4, for 15 min at 37 °C and reacted with 100μM Ac-Asp-Met (Barinka et al., 2002) in the total final volume of 110 μl. The reaction products were derivatized by 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AccQ)-Fluor reagent (Waters) dissolved in acetonitrile and resolved on a HPLC Luna C18(2)-column (250×4.6 mm, 5 μm, Phenomenex). For the active-site titration of GCPIII, approximately 200 nM enzyme was preincubated with differing concentrations of the tight binding inhibitor 2-(phosphonomethyl)-pentanedioic acid (10–3000 nM) in 20 mM MOPS, 20 mM NaCl, pH 7.4, for 15 min at 37 °C, and reacted with 6 μM Ac-Asp-Glu in the total volume of 180 μl. The radioactive assay (see above) was used for product detection. The active-site concentrations of the enzymes were determined from the plots of v_i/v_0 (the ratio of individual reaction rates to rate of uninhibited reaction) against the inhibitor concentration Grafit 5.0 II (Erithacus Software Ltd.), Active Site Titration module.

Western blotting

A defined amount of protein from each sample was resolved on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), electroblotted onto a nitrocellulose membrane, and immunostained with an appropriate mouse mAb (GCP-02, 1 mg/ml, diluted 1:5000, GCP-04, 7 mg/ml, diluted 1:35,000 or 7E11, 1

mg/ml, diluted 1:250), followed by incubation with 1:25,000 horseradish peroxidase-conjugated goat anti-mouse antibody (Pierce, Rockford, IL, USA) for 1 h and developed using West Femto mixture (Pierce). The relative intensities of immunoblot bands were quantified by recording the image with a CCD-camera (LAS-1000, Fujifilm, Stamford, CT, USA) followed by analysis using ImageQuant software (version v2003, Amersham Biosciences, Uppsala, Sweden). Alternatively blots were visualized using X-ray film MEDIX XBU (Foma Bohemia, Hradec Králové, Czech Republic) developed by film developer M35-M X-OMAT Processor (Kodak, Hemel Hempstead, UK) by standard procedures.

Deglycosylation

Twenty micrograms of total protein in a total volume of 20 μl of glycoprotein denaturing buffer (NEB, Boston, MA, USA) was boiled in a water bath for 3 min. Samples were cooled to room temperature. Afterward, 3 μl of 10% NP-40 (NEB), 3 μl G7 Reaction Buffer (NEB), and 0.4 μl peptide N-glycosidase F (500 U/μl, EC 3.5.1.52, NEB) were added and the mixture was incubated overnight at 37 °C.

Morphology and immunohistochemistry

Tissues were fixed in 10% buffered formalin (Lachema) for 24 h and embedded in paraffin. To determine the morphological features of the tissue, routine hematoxylin and eosin staining was carried out. All unstained tissue sections were stored at 4 °C until used to minimize antigen deterioration. Thereafter, 4 μm thick tissue sections aimed for immunohistochemical staining were deparaffinized in xylene and rehydrated through decreasing concentrations of ethanol to water. After blocking of endogenous peroxidase activity, heat-induced epitope retrieval was performed in 10 mM sodium citrate buffer solution (pH 6.0) by warming to 96 °C in a water bath for 40 min. The sections were incubated overnight at 4 °C with anti-GCPII monoclonal mouse antibody GCP-04 (stock concentration 7 mg/ml, diluted 1:1000) or with monoclonal mouse antibody against glial fibrillary acidic protein (GFAP, clone 6F2, DakoCytomation Co., Glostrup, Denmark, diluted 1:1000). The antigen–antibody complexes were visualized by a biotin–streptavidin detection system (ChemMate Detection kit, DakoCytomation Co.) with 3,3′-diaminobenzidine (DAB, Fluka Chemie) as a chromogen. The specificity of staining was confirmed by processing sections cut from the same paraffin block with omission of the primary antibody (data not shown). As a positive control, reactions with the sections of prostate adenocarcinoma were used.

All sections were counterstained with Harris' hematoxylin (Fluka Chemie, Seelze, Germany).

Immunofluorescence

Double immunofluorescence staining was performed using fluorescence-conjugated secondary antibodies. The 5 μm cryosections were incubated for 1 h at room temperature with antibody to GCP-04 (stock concentration 7 mg/ml, diluted 1:1000) and antibody to GFAP (polyclonal, Dako, Denmark, rabbit IgG, diluted 1:1000). Antibodies were diluted by Antibody Diluent (Dako). Thereafter, the slides were washed in TBS for 5 min and subsequently reacted in the dark for 30 min using a mixture of fluorophore-linked secondary bodies (FITC-conjugated anti-mouse IgG, diluted 1:100 and Cy3-conjugated anti-rabbit IgG, diluted 1:100; Jackson ImmunoResearch, Inc., West Grove, PA, USA). The slides were then washed in TBS for 5 min. The nuclei were visualized by staining with DAPI (1 μg/ml) for 10 min and washed in TBS. To reduce the autofluorescence of lipofuscin, the sections were then treated with a solution of Sudan Black B (Sigma) in 70% methanol for 5 min. The slides were mounted using mounting medium Mowiol (Calbiochem, La Jolla, CA, USA) containing n-propylgallate (Sigma-Aldrich, Germany) as an antifading agent. Fluorescence microscopy was then performed sequentially for each field using the Olympus BX51 fluorescent microscope fitted with appropriate filters for FITC, Cy3 and DAPI. The images were captured by Olympus C-5050 in monochrome mode and superimposed using imaging program Analysis D 5.0.

Positive and negative immunohistochemical controls run with each assay; as a positive control, reactions with the sections of prostate adenocarcinoma were used. The specificity of staining was confirmed by processing sections cut from the same block with omission of both primary antibodies. The specificity of the antibodies was further confirmed by preabsorbtion test. Briefly: antibody GCP-04 (stock concentration 7 mg/ml, diluted 1:1000) or antibody to GFAP (polyclonal, Dako, rabbit IgG, diluted 1:1000) was incubated with excess of recombinant protein GCPII (50 μg/ml) for 2 h at 37 °C and centrifuged 13,000×*g*/30 min. Subsequently, the sections were incubated with the supernatant showing no positive staining by GCP-04 antibody.

RESULTS

Human brain samples for GCPII immunochemical detection and specific activity

In order to analyze the expression and enzymatic activity of GCPII in individual compartments of the human brain, 30 samples from various loci of the brain were obtained from a 48-year-old male. From each brain region, two sets of samples were prepared: formaldehyde-fixed samples for immunohistochemistry and samples frozen on dry ice for subsequent protein extraction and Western blotting (for sample description, see Table 1). In order to make quantitative comparisons between individual brain regions and to assess the variability of GCPII expression among humans, six samples of selected brain regions from an additional four individuals (three males and one female, aged 68–81) were obtained and used for the parallel determination of protein content, GCPII expression, and NAAG-hydrolyzing activity. For the activity determination, samples were homogenized and thoroughly dialyzed against reaction buffer. The supernatants were then used for further analysis of the total protein concentration, for the activity testing, and for Western blot analysis using a specific mAb raised against GCPII (antibody GCP-04; see Tables 1 and 2 and Fig. 3). The specificity of the NAAG hydrolysis was confirmed by the fact that all the NAAG-hydrolyzing activity of the supernatants was completely blocked by 2-PMPA, a selective inhibitor of GCPII (data not shown).

Characterization of GCP-04 antibody

The antibody was obtained by immunization of mice with the extracellular part of GCPII prepared by recombinant expression in insect cells (Barinka et al., 2004a). The epitope mapping of the GCP-04 mAb was performed using a library of phage-displayed random heptapetides (see Experimental Procedures) and revealed that the antibody recognizes the "Φ+−ΨG" linear sequence pattern (where Φ is a hydrophobic amino acid, mainly Trp; + is a positively charged amino acid, Lys or Arg; − is a negatively charged amino acid, Glu or Asp; Ψ is a hydrophobic amino acid, Leu or Phe; G is glycine) that corresponds to amino acids

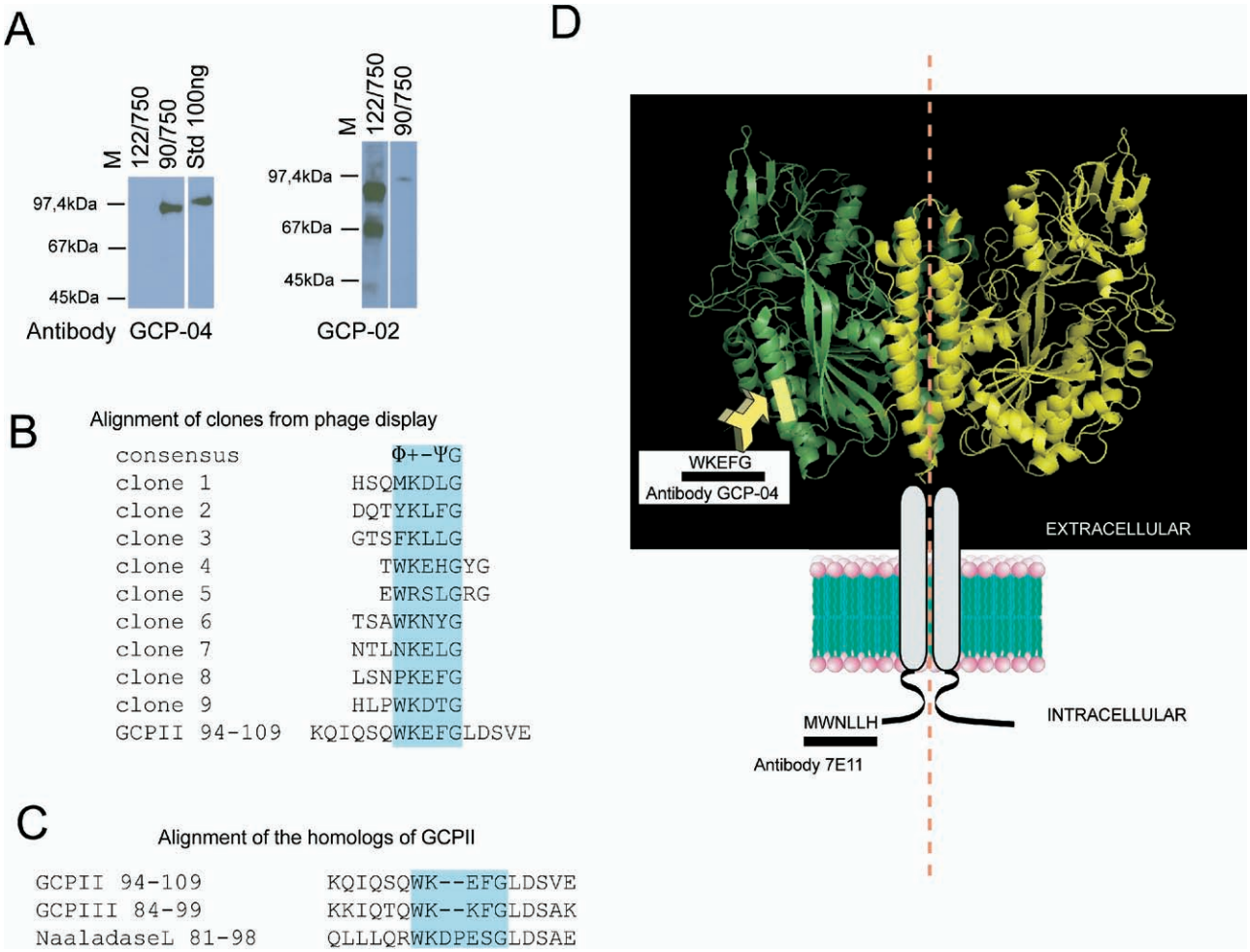

Fig. 1. Epitope mapping of GCP-04 antibody. (A) Western blot analysis comparing immunoreactivity of truncated variants of GCPII and recombinant ectodomain of GCPII (amino acids 44–750, Std), using monoclonal antibodies GCP-04 and GCP-02. Purified recombinant proteins comprised of amino acids 44–750 (Std), 90–750, and 122–750 were separated on SDS PAGE, blotted onto nitrocellulose membrane and visualized using the corresponding mAb. (B) Alignment of sequenced phage clones obtained after four rounds of panning to immobilized GCP-04 (for details see the Experimental Procedures). (C) Alignment of the sequences of known GCPII homologs in the position corresponding to the GCP-04 epitope in GCPII. (D) Graphic representation of the localization of epitopes recognized by monoclonal antibodies 7E11 and GCP-04 in the 3D structure of GCPII (Mesters et al., 2006).

100–104 of human GCPII (see Fig. 1, panel B). A homologous sequence was identified in human GCPIII, suggesting that GCP-04 also recognizes this homologous protein (Fig. 1, panel C). The epitope location is further corroborated by the fact that in the Western blots, GCP-04 gives a positive signal with mutants truncated up to amino acid 90 of GCPII, but fails to recognize GCPII mutants missing the first 121 amino acids. On the other hand, mAb GCP-02 recognizes all of the truncated variants of GCPII tested and could therefore be used as a control for the expression of both variants (see Fig. 1, panel A).

Interestingly, the GCP-04 interacting amino acid segment is a part of an alpha-helix in the native human GCPII (Mesters et al., 2006, Fig. 1, panel D), and due to this spatial arrangement it is inaccessible to antibody binding. Indeed, the GCP-04 mAb interacts with GCPII only under denaturing conditions. In direct comparison to 7E11, antibody GCP-04 was at least 10 times more sensitive for immunochemical detection of the GCPII antigen. Therefore, SDS PAGE analysis of the brain cell lysates probed with 7E11 yielded only a weak, specific signal (Fig. 2A).

Enzymatic and immunochemical detection of GCPII in the brain

Fig. 3A demonstrates that GCPII expression was observed in all 30 samples from individual brain regions. Panel B of the same figure provides additional immunochemical detection of GCPII in selected brain regions in a total of five human brains: frontal cerebral cortex (lanes S2/1 to S2/4), temporal gray matter (lanes S4/2–S4/4), temporal white matter (lanes S6/1 to S6/4), nucleus caudatus (lanes S11/1, S11/2 and S11/4), spinal cord (lanes S24/1 to S24/4), and the brainstem region (lanes S30/1 to S30/4). In order to provide direct comparison of the GCPII expression in these brain regions in a single brain, samples from

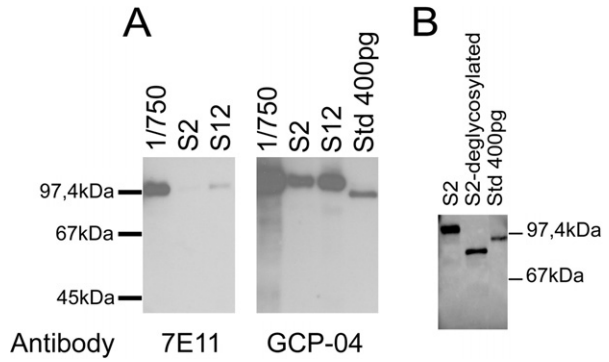

Fig. 2. (A) Comparison of the specificity and selectivity of monoclonal antibodies GCP-04 and 7E11. S2 cells stable transfected by a plasmid encoding the full length (1–750) GCPII (Barinka et al., 2004a) and human brain samples S2 (frontal cerebral cortex) and S12 (globus pallidum; see Table 1) were analyzed on SDS-PAGE, blotted onto nitrocellulose membrane, and visualized either by GCP-04 or 7E11. Std, 400 pg of pure recombinant GCPII (amino acids 44–750) was loaded as a control. (B) Glycosylation of GCPII in human brain. A sample of frontal cerebral cortex was deglycosylated *in vitro* and resolved on 10% SDS PAGE, blotted onto nitrocellulose membrane, and visualized using the mAb GCP-04. Std, 400 pg of pure recombinant GCPII (amino acids 44–750) was loaded as a control. For experimental details see Experimental Procedures.

individual regions of brain five are displayed in the last six lanes (S2/5 to S30/5). The temporal white matter (samples S6 from both panels), the brainstem (S30) and, to a lesser extent, the frontal cerebral cortex, seem to express the highest amounts of GCPII.

For the quantification of the expression level of GCPII in individual samples, we used purified recombinant human GCPII as a standard, loaded six defined concentrations of the protein on each gel, and analyzed the resulting Western blots by densitometry (see the first two lanes in panel A and the first lane in panel B). Using this approach, we were able to identify as little as 40 pg of GCPII in a sample (data not shown). In several samples, small variations in the apparent mobilities of immunoreactive species were observed (e.g. lanes S5 and S6, Fig. 3A). Moreover, additional immunoreactive bands were observed in several samples containing higher amounts of GCPII. While some additional bands could be explained by the cross-reactivity of the mAb with other cellular proteins, we hypothesized that other bands might mirror different glycosylation patterns in individual human brain cell types. Therefore, we subjected the samples to *in vitro* deglycosylation by the peptide N-glycosidase F. The results of the experiment are depicted in Fig. 2B. Upon deglycosylation *in vitro*, the relative mobility of the immunoreactive band from the frontal cerebral cortex sample decreases by approximately 15 kDa. However, the lower-molecular weight components seen in the original samples are still present in the deglycosylated one, with a corresponding shift in the relative mobility. Thus, the heterogeneity observed in the Western blot analysis cannot be accounted for by different glycosylation patterns alone.

In order to compare the specific NAAG-hydrolyzing activities that we attribute to the enzymatic activities of GCPII expressed in selected parts of the brain, the cleared cell lysates were tested for their NAAG-hydrolyzing activity using a radiolabeled substrate. The product of the enzymatic hydrolysis, ^{3}H-labeled free glutamate, was separated and quantified by liquid scintillator. Since the substrate turnover was kept below 30% in all reactions, the product concentrations are directly proportional to the specific enzymatic activities of the individual samples. The results of this analysis are summarized in Table 2. Much higher NAAG-hydrolyzing activities (per mg of total protein)

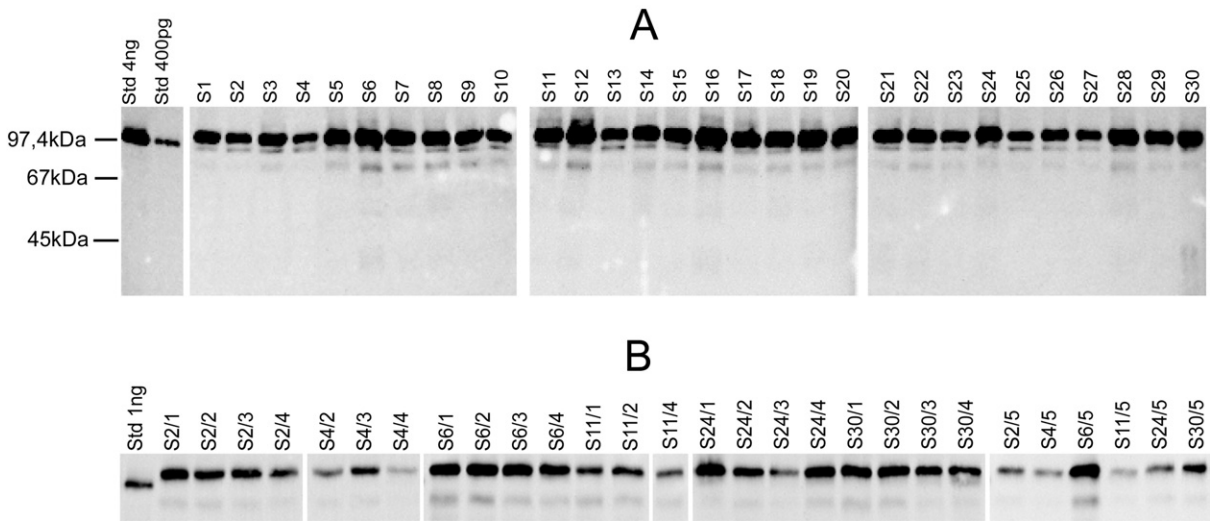

Fig. 3. Western blot analysis of GCPII expression in individual sections of human brain using GCP-04 antibody. (A) Thirty samples from various loci of a single male brain. For sample identification see Table 1. (B) immunodetection of GCPII expression in six brain regions of five human brains: frontal cerebral cortex (lines S2/1 to S2/4), temporal gray matter (lines S4/2–S4/4), temporal white matter (lines S6/1 to S6/4), nucleus caudatus (lines S11/1, S11/2 and S11/4), spinal cord (lines S24/1 to S24/4), and the brainstem region (lines S30/1 to S30/4). For a clear, direct comparison of GCPII expression within a single brain, samples from all six regions of brain five are displayed in the last six lanes (S2/5 to S30/5). For sample identification see Table 1. Std, different amounts of purified recombinant GCPII (amino acids 44–750) for quantitative comparison.

are seen in the astrocyte-rich white matter of the brain. The specific activities (per mg of GCPII) were obtained by quantification of the relative intensities of corresponding immunoblot bands in Fig. 3 photographed by a CCD-camera and analyzed by ImageQuant software. Pure recombinant extracellular GCPII (250 pg to 4 ng) was used as a standard for quantification. The results suggest that the specific activity of GCPII is between 27 and 47 nmol/min/mg GCPII.

GCPII and III: antibody specificity and activity comparison

In order to determine the selectivity of the GCP-04 antibody against individual homologs of the enzyme (namely GCPIII), we probed recombinant GCPII and GCPIII proteins, expressed and purified from insect cells (Barinka et al., 2002; Hlouchova et al., manuscript in preparation), with GCP-04. These results are shown in Fig. 4A. GCP-04, the mAb that we use for histochemical and immunochemical detection throughout this manuscript, is approximately two orders of magnitude less sensitive toward the recombinant human GCPIII than toward recombinant GCPII. The reason for this difference might be a single amino acid substitution in the sequence of GCPIII (Lys92→Glu102; compare Fig. 1C).

In order to quantify the possible contribution of GCPIII activity to the overall NAAG-hydrolyzing activity observed in the assay, we directly compared the NAAG-hydrolyzing activities of pure recombinant GCPII under the assay conditions used for the analysis of the NAAG-hydrolyzing activities of the samples from the human brain. GCPII is approx. 30 times more active than GCPIII under identical conditions (the specific activity of GCPII is 646±123 nmol of hydrolyzed NAAG/min/mg of GCPII while GCPIII converts 19±0.1 nmol of NAAG/min/mg, see Fig. 4B).

Immunohistochemistry of GCPII in human brain

Since only scarce and controversial data have been available on the expression of GCPII in various structures of the human brain and on its subcellular localization, we set out to analyze GCPII expression by immunohistochemistry using the novel mAb described above. Figs. 5 and 6 exemplify the results that we obtained on both male and female brain slices.

GCPII was reported to be expressed predominantly in astrocytes in a rat model (Berger et al., 1999). Consistent with these observations, we noticed intense immunoreactivity of the astrocyte-containing white matter when compared with that of the gray matter. The expression of GCPII was not observed in any other cell type of the CNS. This observation could be best documented on the inferior temporal gyrus (Fig. 5, panel A), medulla oblongata (Fig. 5, panel B) and basal ganglia (Fig. 5, panel C) where the white matter is closely intermixed with gray matter islands. In all three panels, immunopositive astrocytes in the white matter form clearly distinguishable darker areas around or within the surrounding gray matter that seems to express GCPII very slightly or not at all. In the astrocytes, the expression of GCPII was localized to the cytoplasm of the cell bodies near the cellular membrane; pure membranous positivity was observed only rarely. Focal positivity of astrocytic processes was present in the form of small puncta dispersed in the surrounding white matter. Such an observation was made in each sample analyzed by immunohistochemistry (data not shown).

To find out whether all astrocytes express GCPII, we performed immunohistochemical reactions with the anti-GFAP antibody. Although not quantified, the GFAP positive astrocytes clearly outnumbered the GCPII positive

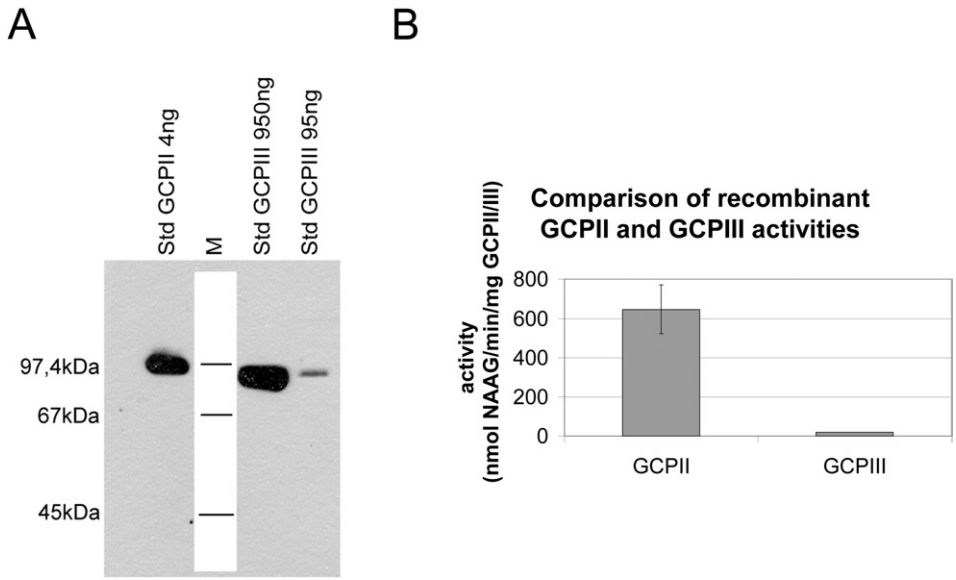

Fig. 4. Comparison of pure recombinant GCPII and GCPIII by Western blot and by activity measurements. (A) Western blot of GCPII and GCPIII: Std GCPII, purified recombinant ectodomain of GCPII (amino acids 44–750), Std GCPIII, different amounts of purified recombinant ectodomain of GCPIII (amino acids 36–740), M, molecular weight marker. Antibody GCP-04 was used for immunodetection. (B) Direct comparison of GCPII and GCPIII NAAG-hydrolyzing activities. For experimental details see Experimental Procedures.

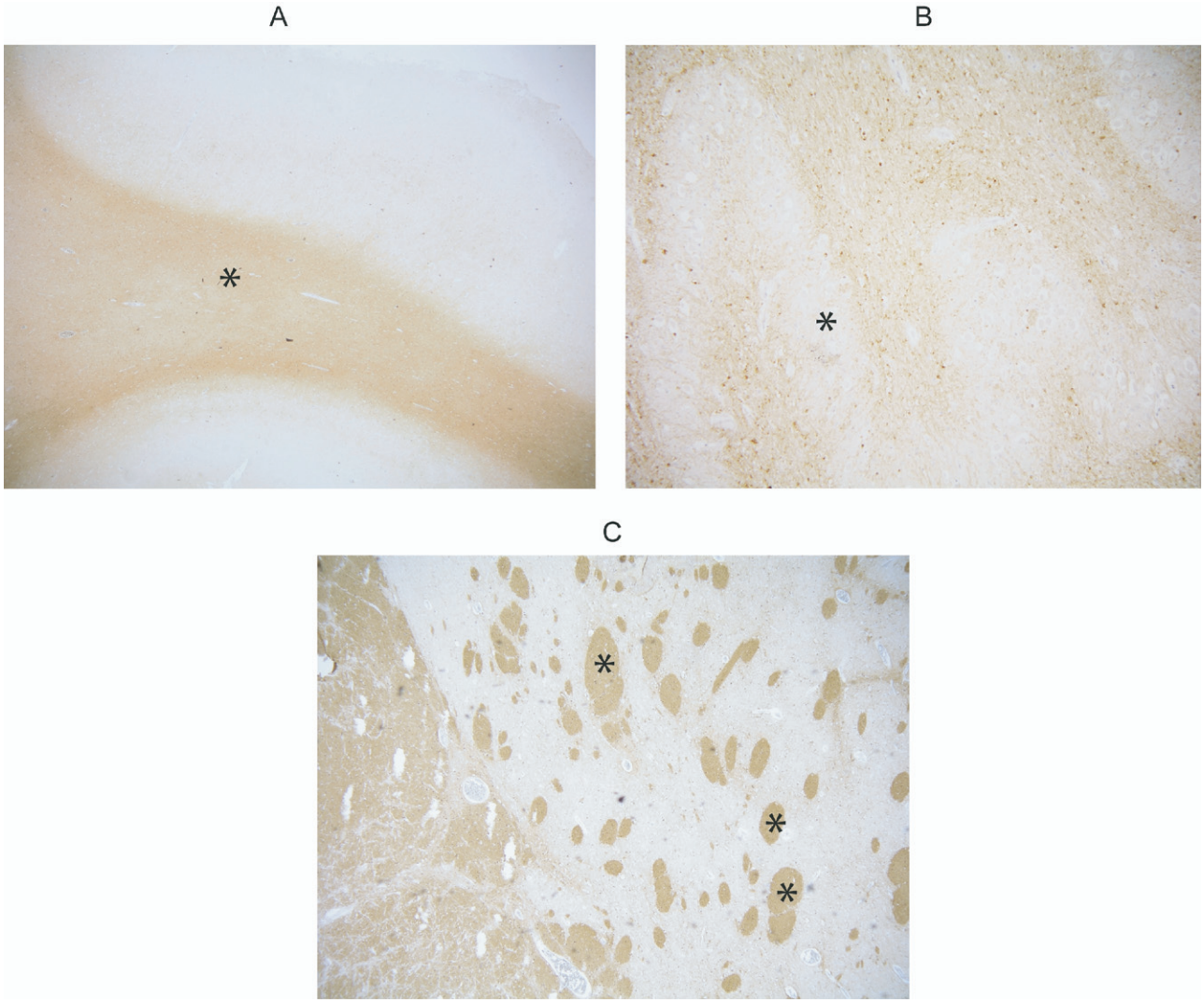

Fig. 5. Localization of GCPII expression in selected regions of the human brain. Immunoperoxidase method, counterstained slightly with hematoxylin. For experimental details see Experimental Procedures. (A) Inferior temporal gyrus. Note the intense immunoreactivity of the white matter (indicated by asterisk *) when compared with that of the cortex. Original magnification 25×. (B) Medulla oblongata. The low reactivity of the inferior olivary nucleus (*), in which GCPII-positive astrocytes are scarce, forms the serpentine pale area in the surrounding white matter. Original magnification 40×. (C) Basal ganglia-the increased reactivity of the white matter bands (*) inside the gray matter of the basal ganglia. Original magnification 40×.

cells in each studied region of the gray matter, indicating that not all astrocytes express GCPII (Fig. 6, panel F). The colocalization of GCPII and GFAP was confirmed by double-labeling immunofluorescence staining using anti-GCPII and anti-GFAP antibodies (Fig. 6, panels C–F). GFAP-positive cells not expressing GCPII are indicated by an asterisk in Fig. 6, panel F.

DISCUSSION

GCPII is a well-established tumor cell marker and a therapeutic target for prostate cancer. It is also a very attractive target for therapeutic intervention in pathological processes in which excessive glutamate is neurotoxic. Therefore, it is surprising that only limited information on the expression of the enzyme in the human CNS is available. Most of the literature reports analyze only the NAAG-hydrolyzing activity in human brain or determine expression of the enzyme detected immunochemically in rodent tissues. Several authors failed to detect the expression of GCPII in the human CNS by immunochemical or immunohistochemical methods using various monoclonal antibodies.

The outcome of the immunochemical protein determination obviously depends on the antibody available. The mAb GCP-04 used throughout this study was prepared using the extracellular part of GCPII, obtained by heterologous expression in a *Drosophila* cell line. Recombinant proteins expressed by insect cells might differ from the authentic, native proteins. Namely, they may show different glycosylation patterns in comparison to their human counterparts. However, analyses of the glycosylation of recombinant GCPII revealed the N-glycosylation sites are

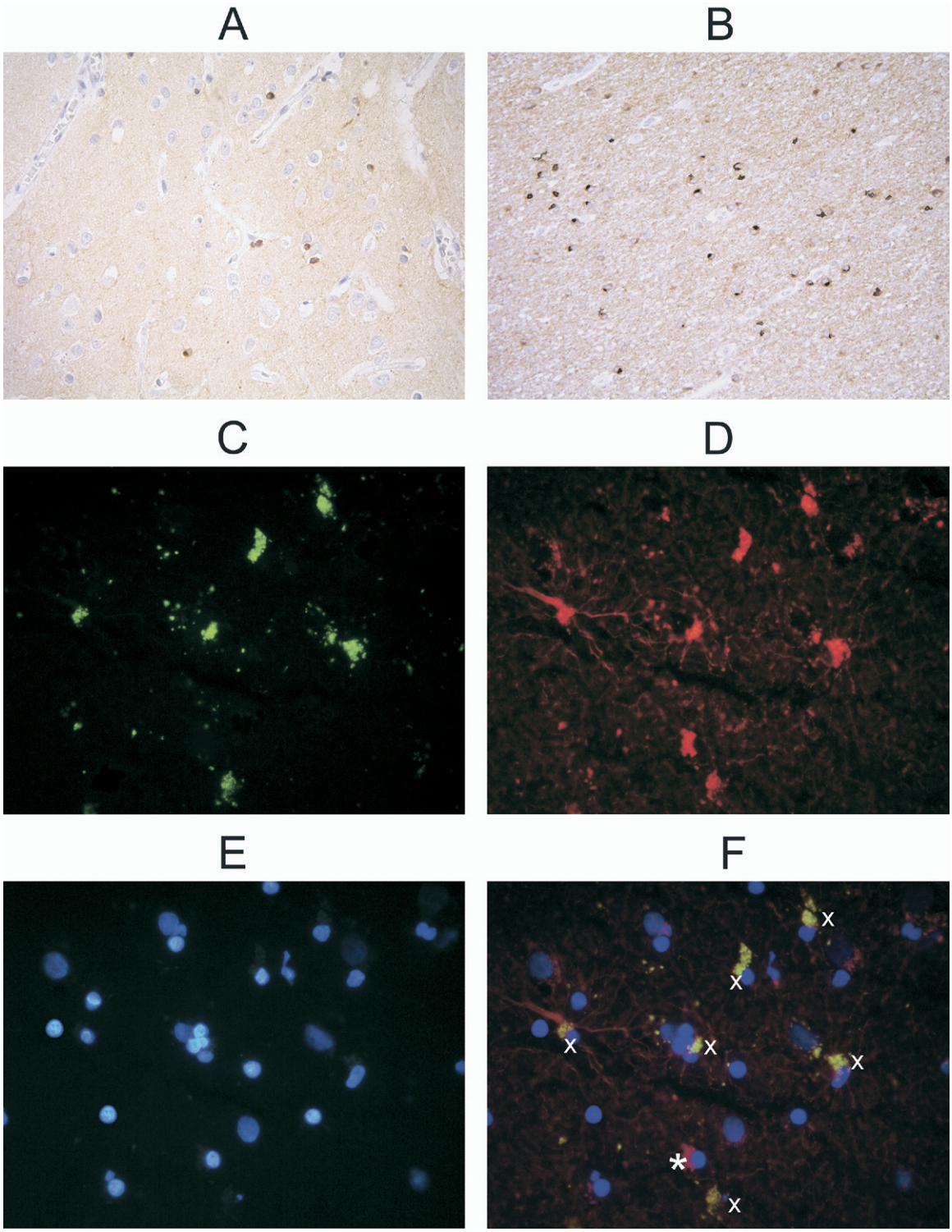

Fig. 6. Localization of GCPII expression in frontal cortex in comparison with GFAP expression. Immunoperoxidase method, counterstained slightly with hematoxylin. Original magnification 400×. (A) Frontal cortex: the immunopositive astrocytes (detected by GCP-04 antibody) are scarce in the gray matter. (B) White matter, stained with GCP-04 antibody. The number of GCPII immunoreactive cells is higher in the white matter than in the cortex (A). (C–F) The double-labeling GCP-04/GFAP immunofluorescence in the human frontal cortex. (C, D) The GCPII (visualized by FITC-labeled GCP-04 antibody, green) is localized to the cytoplasm of a portion of GFAP- positive astrocytes (Cy3, red). (E) The nuclei of the cells are visualized by DAPI staining. (F) The cytoplasmic co-localization of GCPII and GFAP in the superimposed image is indicated by a cross (×); the GFAP positive but GCP-04 negative astrocytes are indicated by asterisks (*). Original magnification 600×.

identical to those of human GCPII and that both proteins show equivalent activity (Ghosh and Heston, 2003; Barinka et al., 2004b).

In an attempt to quantify GCPII expression in individual tissue sections, we performed Western blot analysis using GCP-04. The semi-quantitative analysis of the signal intensities in the blots using a standard calibration curve of known concentrations of rhGCPII yielded concentrations of GCPII in various sections of the CNS ranging from approximately 50–300 ng per mg of total protein. No other quantitative value is available for GCPII expression in human brain; however, this value is slightly lower than the numbers published by Murphy et al. (2000) for GCPII expression in normal human prostate and than values reported by Sokoloff et al. (2000), who reported 519–4254 ng/mg protein in the normal prostate and 1.8–51 ng/mg protein in other tissues.

At least two other homologs of GCPII, GCPIII (formerly referred to as NAALADase II) and NAALADase L (Pangalos et al., 1999), have been described. Since these homologs differ in the N-terminal parts of their amino acid sequence, they lack the epitope recognized by the 7E11 antibody and thus should not cross-react with GCPII in immunochemical determination using this antibody. Epitope mapping using a phage display approach revealed that GCP-04 interacts with the linear epitope comprising amino acids 100–104 of human GCPII. In human NAALADase L, a close homolog of GCPII with 54% sequence similarity, this linear epitope is disrupted by the insertion of two amino acids (see Fig. 1 panel C), and consequently, GCP-04 might not be able to recognize the protein. Another GCPII-like protein described in the literature is PSMA-like protein. Its sequence starts at amino acid 309 (GCPII numbering) (Pangalos et al., 1999; O'Keefe et al., 2004); it thus lacks the epitope recognized by GCP-04. Furthermore, since it also lacks the active-site domain of GCPII, as determined by X-ray crystallography (Mesters et al., 2006), it is highly improbable that it shows any peptidase activity whatsoever.

The closest homolog of GCPII in human is GCPIII. In order to dissect the contributions of GCPII and GCPIII to the overall NAAG-hydrolyzing activity in the human brain, we cloned and expressed human GCPIII in insect cells (Hlouchová et al., manuscript in preparation). As shown in Fig. 4A, antibody GCP-04 is at least 100 times more sensitive to recombinant purified GCPII than to purified GCPIII. A single amino acid substitution in the epitope sequence of GCPIII (Lys92→Glu102) seems to be responsible for a profound decrease in the affinity of GCP-04 mAb toward GCPIII. As the NAAG-hydrolyzing activity is concerned, in the assay conditions used in this paper, GCPII is approx. 30 times more active than GCPIII. A more detailed analysis of the enzymatic activity of GCPIII will be given elsewhere (Hlouchová et al., manuscript in preparation).

It should be noted that these results do not suggest that GCPIII expression and/or activity in human brain is irrelevant. Enzymatic activities of metalloenzymes are highly dependent on the exact assay conditions. Furthermore, no direct comparison has been reported for the antibodies used in previous studies, and it remains questionable as to what extent the GCPII-homologs have interfered with the immunologic detection of GCPII in human tissues in the studies published by other authors previously.

Western blots of brain sections (Fig. 3) show one major and two minor immunoreactive bands. The electrophoretic mobility of the major band corresponds to the expected molecular weight of GCPII. Lower bands are much weaker in intensity and might thus represent a degradation product, cross-reactive human protein, or a truncated GCPII variant (Bzdega et al., 1997). In order to analyze whether or not these two bands represent alternatively glycosylated GCPII species, we deglycosylated the corresponding samples by PNGase F *in vitro* (Fig. 2B). Equivalent heterogeneity of the samples was shown after the deglycosylation, suggesting that the immunoreactive bands do not differ solely in glycosylation state.

The specific expression of GCPII in studied tissues was further confirmed by its enzymatic activity, analyzed as the ability to hydrolyze radiolabeled NAAG. The values summarized in Table 2 range from 1 to 12 pmol/min/mg of total protein. These values are higher than those reported by Tsai et al. (1991) in several sections of CNS (ranging from 0.3 pmol/h/mg in spinal cord to 8.5 pmol/h/mg in the motor cortex). On the other hand, the values reported for the NAAG-hydrolyzing activities in the rat CNS are approximately 10 times higher than the activities reported in this paper (Fuhrman et al., 1994; Blakely et al., 1988; Robinson et al., 1987). These discrepancies could be explained by the fact that these authors analyzed the activity of an isolated membrane fraction of rat CNS (and obtained thus much higher specific activities per milligram of the protein. Furthermore, sample preparation, storage, and assay conditions (especially buffer and salt concentration) strongly influence the activities of GCPII.

It should be noted that the NAAG-hydrolyzing activities of the cleared samples steadily increased with prolonged dialysis in our hands (data not shown). It could be argued that an endogenous inhibitor, slowly dissociating from the enzyme upon dialysis, might be present in the crude sample preparation. The exact method of sample preparation might thus considerably influence the outcome of the analysis and make direct comparison of the enzymatic activities reported by different groups very difficult.

In general, our data support the observations made by Slusher et al. (1992) and Berger et al. (1999) in a rodent model, suggesting that GCPII is mostly expressed in the white matter, specifically in the astrocytes. However, not all of the GFAP-positive astrocytes were immunopositive in our samples, as suggested by Berger et al. (1999) in the rat model. Based on our observations, we can speculate that GCPII is expressed mostly in fibrillary astrocytes (i.e. type II astrocytes), which are known to be present in higher numbers in the white matter when compared with the type I protoplasmic astrocytes of the gray matter. Accordingly, we did not observe a tight link between the expression of GFAP and GCPII, as was proposed by Luthi-Carter et al. (1998a).

CONCLUSION

In conclusion, in this study we report the first systematic analysis of the expression of GCPII in human brain using immunochemical detection. Using the specific mAb GCP-04, we have detected GCPII expression in all parts of the human brain. GCPII seems to be expressed exclusively in astrocytes, especially in those localized in the white matter. The presented immunochemical and histochemical data are generally in agreement with the enzymatic analyses of the corresponding brain samples.

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