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S1 pocket of glutamate carboxypeptidase II: A new binding site for amyloid-β degradation

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ABSTRACT

We recently reported that glutamate carboxypeptidase II (GCPII) has a new physiological function degrading amyloid-**β** (A**β**), distinct from its own hydrolysis activity in N-acetyl-L-aspartyl-L-glutamate (NAAG); however, its underlying mechanism remains undiscovered. Using site-directed mutagenesis and S1 pocket-specific chemical inhibitor (compound 2), which was developed for the present study based on *in sillico* computational modeling, we discovered that the A**β** degradation occurs through S1 pocket but not through S1′ pocket responsible for NAAG hydrolysis. Treatment with compound 2 prevented GCPII from A**β** degradation without any impairment in NAAG hydrolysis. Likewise, 2-PMPA (specific GCPII inhibitor developed targeting S1′ pocket) completely blocked the NAAG hydrolysis without any effect on A**β** degradation. Pre-incubation with NAAG and A**β** did not affect A**β** degradation and NAAG hydrolysis, respectively. These data suggest that GCPII has two distinctive binding sites for two different substrates and that Aβ degradation occurs through binding to S1 pocket of GCPII.

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1. Introduction

Glutamate carboxypeptidase II (GCPII) is a type II membrane integral protein found primarily within the glia of the brain, with an apparent molecular mass of 94–100 kDa. GCPII hydrolyzes extracellular *N*-acetylaspartylglutamate (NAAG) to *N*-acetylaspartate (NAA) and glutamate, playing a role in glutamatergic transmission [1].

GCPII is expressed at different levels and has different functions depending on its location in the body. In the prostate, even if its function is unknown, it is called prostate-specific membrane antigen (PSMA). In the central nervous system, where it metabolizes the brain neurotransmitter NAAG, GCPII is called NAALADase. In the proximal small intestine it is called folate hydrolase (FOLH1) and its role is to remove γ-linked glutamates from poly-γ-glutamated folate [1,3,4]. In the brain, because the GCPII increases cellular glutamate through hydrolysis of neuropeptide NAAG, an event associated with excitotoxicity, the excessive glutamate neurotransmission has been implicated in neuronal injury in many disorders of the central nervous system including dementia [5]. Therefore, a potent GCPII inhibitor, 2-(phosphonomethyl) pentanedioic acid (2-PMPA) has been used as a neuroprotective agent [6]. In addition to NAAG hydrolysis, our recent study revealed a novel function of GCPII in modulating Aβ levels in the brain by degrading several Aβ species, including monomers, oligomers, and fibrils [2]. However, the GCPII inhibitor, 2-PMPA, did not influence the Aβ-degrading action of GCPII, raising the possibility that NAAG and Aβ could have different binding sites on GCPII. Structural analysis of GCPII suggested that the enzyme is composed of two separate binding sites, S1 and S1′ pockets, and only the latter is known to play a role in NAAG hydrolysis, while the former has been thought as a "fine tuner" for substrate specificity [7–9]. The crystal structure of the rhGCPII/glutamate complex showed that α or γ-carboxylate of the S1′-bound glutamate interacts with Arg210 and Lys699 [8]. The mutations of the glutarate-binding residues (R210K and K699S) led to a dramatic increase in the Michaelis–Menten constant value compared to wild-type, ranging from approximately 35-fold for the K699S to almost 700-fold increase for the R210K. However, the mutational analysis of the S1 site (R536L and G548P) showed moderate decrease in the Km values compared to S1′ site mutation [8].

In the current study, by using site-directed mutagenesis and by developing a new chemical inhibitor specifically targeting the S1

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pocket, we found that the Aβ-degradation activity of GCPII occurs through the S1 pocket, distinct from the S1′ pocket, where NAAG binding takes place.

2. Materials and methods

2.1. Cell culture and transfection

PC3 prostate cancer cells were cultured in RPMI Medium 1640 (Gibco) supplemented with 10% Fetal Bovine Serum (FBS; Gibco) and 1% penicillin/streptomycin (Gibco). For primary astrocyte cultures, cells were dissociated from the cortexes of rat brains at 2 d after birth. Astrocytes were maintained in MEM (Sigma, St. Louis, MO, USA) supplemented with 10% FBS, 1% penicillin/streptomycin, and L-glutamine (Gibco). Transient transfections were performed using the Lipofectamine 2000 reagent following the manufacturer's protocol.

2.2. GCPII plasmids and lentivirus production

The hGCPII plasmids and lentiviral hGCPII were prepared as described previously [2]. Briefly, the hGCPII cDNA was amplified from the U87-MG human astrocyte cell line and subcloned into the pcDNA3 vector. Lentiviral hGCPII was prepared by Macrogen Inc (Seoul, Korea).

2.3. Site-directed mutagenesis

The pcDNA-hGCPII plasmid was used as a template, and each mutation was introduced by 2 complementary oligonucleotide primers harboring the desired mutation (Table S1). *Pfu* Ultra High-Fidelity DNA polymerase (Stratagene) was used to extend and incorporate the mutagenic primers, resulting in nicked circular strands. The methylated non-mutated parental DNA template was digested with *Dpn*I (New England Biolabs) for 1 h at 37 °C, and the circular dsDNA was then transformed into DH5α competent cells (RBC Bioscience). The presence of individual mutations was confirmed by sequencing (Cosmo Corporation).

2.4. NAAG cleavage assay

Endogenous GCPII activity was determined by the method described previously [2]. Briefly, the cell lysate or rhGCPII was incubated with 20 μM N-acetyl-L-aspartyl-L [3,4-3H] glutamate ([3H]NAAG; NEN Corporation) in 50 mM HEPES and 150 mM NaCl for 1 h at 37 °C. Following the reaction, the sample mixture was applied to AG 1-X8 anion-exchange resin (Biorad) prepared in 96-well columns (Harvard Apparatus). For elution, the resin bound with the sample mixture was washed with 0.5 mM formate and centrifuged. The eluents were mixed with 1 ml of scintillation solution (Optiphase HiSafe, Wallac), and radioactivity was measured with a scintillation counter (Wallac Inc.).

2.5. Western blotting

Cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris at pH 8.0) with protease inhibitor cocktail (Sigma). The lysates were boiled with β-mercaptoethanol for 10 min and separated by 10% SDS–PAGE. The Aβ peptides were monitered by 4–12% gradient NuPAGE gels (Invitrogen). The proteins were electrotransferred onto polyvinylidenedifluoride (PVDF) membranes (GE Healthcare) and blocked with 5% nonfat dry milk in TBS containing 0.1% Tween 20 (TBS-T) at room temperature (RT) for 1 h. Then, the membrane was incubated with anti-PSMA antibody (Santa Cruz; sc-59674), anti-α-tubulin antibody (Sigma–Aldrich; T6199), or anti-Aβ antibody, 6E10, (Covance; SIG-39300) at 4 °C overnight. After washing 3 times with TBS-T, the blots were incubated with an HRP-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch; 115-035-071) or HRP-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch; 115-035-046) for 2 h at RT. After washing, the proteins were detected using a Pierce ECL Western Blotting Substrate (Thermo Scientific).

2.6. Preparation of Aβ peptides

Synthetic Aβ40 and Aβ42 peptides (Invitrogen) were dissolved in cold hexafluoroisopropanol (HFIP; Sigma) at a concentration of 1 mM. After shaking for 40 min at RT and incubating for 20 min at 4 °C, HFIP solution was removed by centrifugation. The peptide pellets were stored at −70 °C until use. The pellets were dissolved in 5 mM DMSO and distilled water was then added to a final concentration of 100 μM.

2.7. ELISA

Vectors encoding the hGCPII and various mutant hGCPII genes were transfected into PC3 cells in a 12-well plate. After 30 h, the cultured medium was changed by Ab40 or Ab42 peptide-treated medium and the cells were incubated for another 16 h. The medium was collected and assayed for residual Aβ40 or Aβ42 by using ELISA kits (Invitrogen), following the manufacturer's protocol. Cells transfected with the pcDNA3 empty vector were used as a control.

2.8. Transgenic mouse model

Double-transgenic mice (APP Swedish/PS1ΔE9) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained as described previously [2]. Briefly, mouse genotypes were confirmed by PCR with the following primers: mouse prion protein (PrP) and human APP. The primer sequences have been provided in our previous report [2]. All animals were housed according to standard animal care protocols and maintained in a pathogen-free facility at the Korea Centers for Disease Control & Prevention. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Korea Centers for Disease Control & Prevention (Permit Number: KCDC-12-016-2A). To administer GCPII inhibitor into the mouse brain, 2-PMPA (10 mg/kg) dissolved in phosphate-buffered saline (PBS) was intraperitoneally injected into mice twice a week for 1 month. The control mice received an equal volume of PBS. Treatment was started at 8 months of age.

2.9. GCPII/Aβ complex modeling

The binding mode of the Aβ to the S1 or S1′ pocket was investigated through a molecular docking study by using program O (http://xray.bmc.uu.se/alwyn/A-Z_frameset.html) [10] with the solution structure of Aβ (PDB code 1z0q) and ligand-free GCPII (PDB code 2oot). All the molecular graphics were generated using PyMOL (The PyMOL Molecular Graphics System, Version 0.99rc6, Schrödinger, LLC).

2.10. Compound library screening and Aβ cleavage

Three-dimensional (3D) searches were performed with Unity in Sybyl 7.3 (Tripos, Inc.). The ChemBridge and ChemDiv compound library databases, with a total of 396,047 compounds, were used for virtual screening of the GCPII inhibitor. All the compounds in the database were stored as 3D structures converted from their 2D forms by Concord (SYBYL 7.3).

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