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

Suk Kyung Lee [a,1], Hyunyoung Kim [a,1], You-Hoon Cheong [b], Min-Ju Kim [c], Sangmee Ahn Jo [a,2], Hyung-Seop Youn [d], Sang Ick Park [a,*]

[a] Division of Brain Diseases, Center for Biomedical Science, Korea National Institute of Health, Osong, Chungcheongbuk-do 363-951, South Korea
[b] Department of Bioscience and Biotechnology, Bio/Molecular Informatics Center, Konkuk University, Seoul 143-701, South Korea
[c] Department of Anatomy and Neurobiology, College of Medicine, Hallym University, Chuncheon, Kangwon-do 200-702, South Korea
[d] School of Life Sciences, Gwangju Institute of Science and Technology, Gwangju 500-712, South Korea

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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

* Corresponding author. Fax: +82 43 719 8602.
E-mail address: parksi61@hotmail.com (S.I. Park).
[1] These authors contributed equally to this work.
[2] Present address: Department of Pharmacy, College of Pharmacy, Dankook University, Cheonan-si, Chungcheongnam-do, South Korea.

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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).

Recombinant rhGCPII was mixed with Aβ42 in the presence of various selected candidates, and the mixtures were incubated at 37 °C over 16 h. The cleavage activity of GCPII on Aβ peptides was detected by Western blotting.

3. Results

3.1. 2-PMPA completely blocks NAAG cleavage activity but not Aβ degradation

2-PMPA has been known to act as a specific GCPII inhibitor [11,12]; however, in our previous report, we observed that its inhibitory effect on Aβ degradation was much less than its inhibitory effect on NAAG hydrolysis. Based on the observation, we hypothesized that the Aβ binding site for Aβ degradation could be different from the binding site for NAAG hydrolysis. To test this hypothesis, we first examined the effect of 2-PMPA on Aβ degradation. As expected, 2-PMPA completely inhibited NAAG hydrolysis (Fig. 1A), while the inhibitory effects on Aβ40 and Aβ42 degradation were negligible, regardless of the 2-PMPA dose (Fig. 1B). A similar pattern was also observed in astrocytes overexpressing GCPII (Fig. 1C), although the efficiency of Aβ degradation by GCPII was less than that in the *in vitro* studies. Consistent with these results, our AD mouse model (APP Swedish/PS1ΔE9) showed that the inhibitory effect of 2-PMPA on Aβ degradation was marginal compared to the inhibition of NAAG hydrolysis (Fig. 1D and E).

3.2. The binding cavity of the S1′ pocket on GCPII is too narrow to dock the Aβ peptide

By computational modeling, we found that the S1 pocket is more suitable to accomodate the Aβ peptides because the binding cavity of the S1 pocket has greater space than the S1′ pocket (Fig. 2). Moreover, we observed that some hydrophobic interactions exist between Aβ peptides and amino acids residues within S1 pocket, as well as 3 ionic interactions, which promote binding of Aβ peptides to S1 pocket (Fig. 2B).

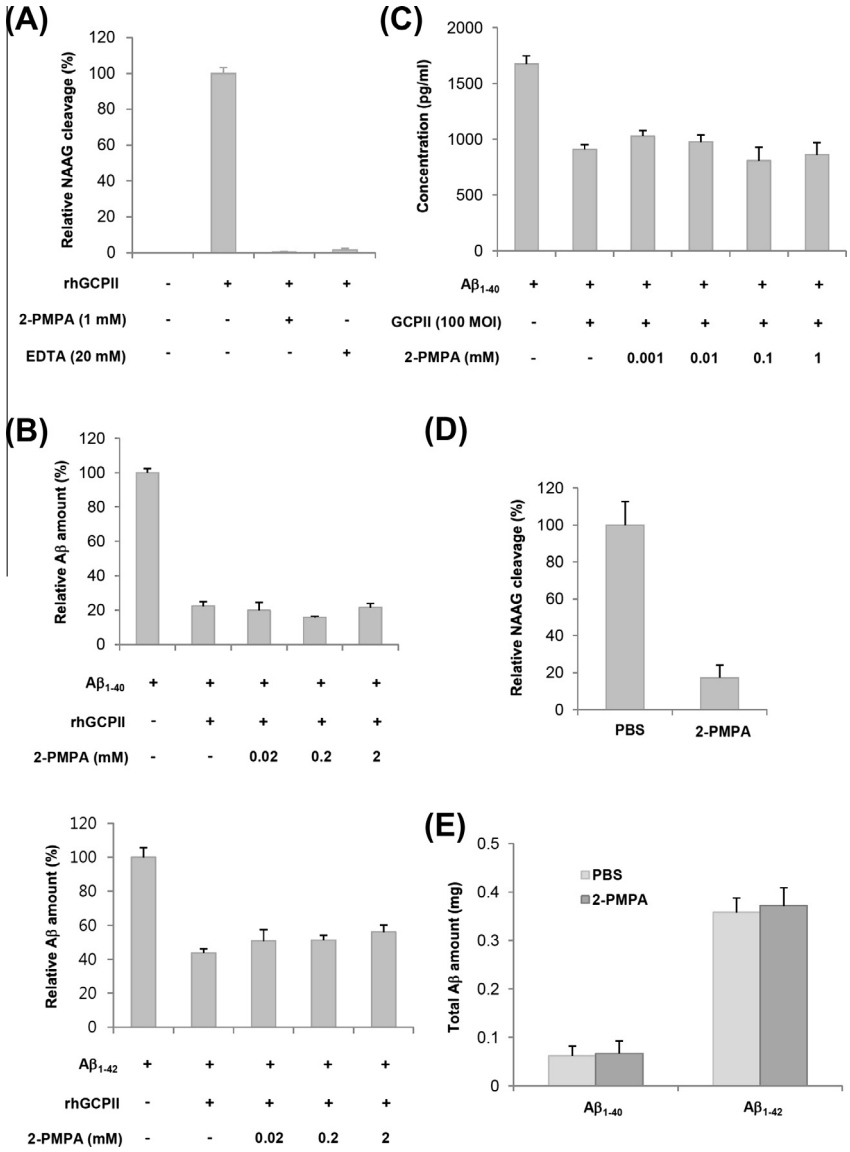

Fig. 1. 2-PMPA does not affect Aβ degradation by GCPII. (A, B) Purified rhGCPII was incubated with Aβ40/Aβ42 or $[H]^3$-labeled NAAG, in the presence or absence of 2-PMPA. Aβ40/Aβ42 and NAAG levels were detected by ELISA and NAAG-cleavage assay, respectively. (C) After GCPII lentiviral infection into rat primary astrocytes, Aβ40 was added to the cell culture dish, along with 2-PMPA at various doses and incubated for indicated time. The remaining Aβ40 concentration in the cell medium was measured by the ELISA assay. (D, E) Membrane fractions of transgenic mouse (APP Swedish/PS1dE9) brain were used for the *in vivo* analysis of NAAG hydrolysis and Aβ degradation by the NAAG and ELISA assays, respectively.

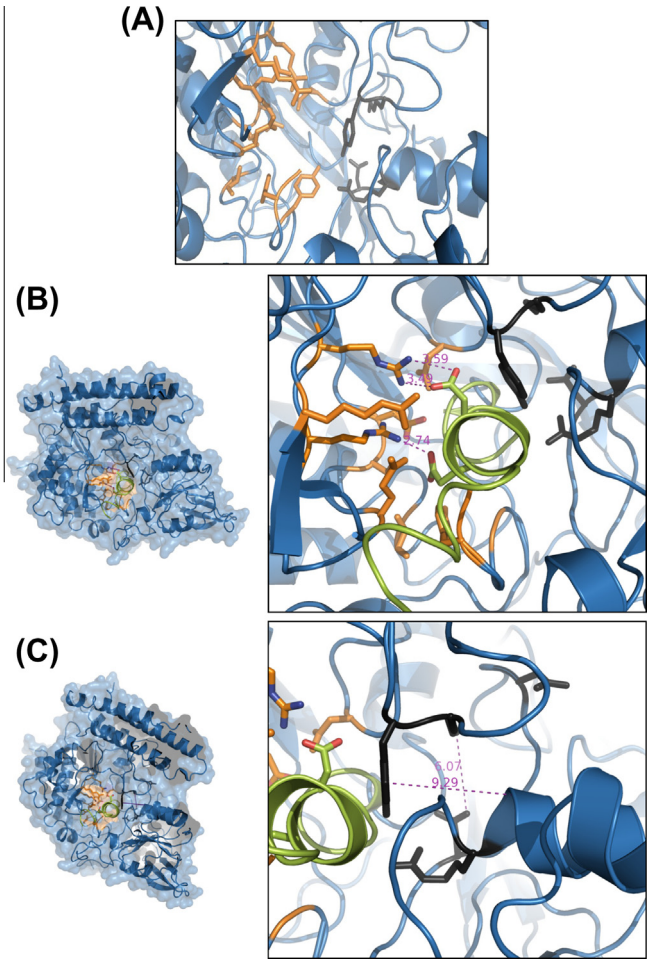

Fig. 2. Computational molecular modeling of GCPII interacting with Aβ peptides. (A) Structure of GCPII active site containing S1 (brown) and S1' pockets (black). (B and C) Possible interaction between GCPII and Aβ peptides (green) within S1 pocket relative to neighboring S1' pocket at two different angles.

3.3. Site-directed mutagenesis of human GCPII affects enzyme activity

To investigate the existence of two distinct binding sites for NAAG hydrolysis and Aβ degradation, we performed site-directed mutagenesis in the S1 and S1′ pockets and examined the effect on GCPII activity. As shown in Table S1, we constructed and generated 2 mutants, R210A and K699S, targeting the functional groups within the S1′ pocket [8], as well as 2 mutants, R536L and G548P, targeting the functional groups within the S1 pocket [13].

Compared to the mutations on S1 pocket, although R536L showed severe effect, the R210A and K699S mutations on S1′ pocket completely abrogated the NAAG hydrolysis activity in PC3 cells (Fig. 3A) [14]. Meanwhile, when we examined the effect of the each mutation on Aβ degradation, K699S mutation exerted similar activity compared to wild type GCPII in the degradation of Aβ40 and Aβ42; however G548P showed significantly impaired activity in the degradation of Aβ (Fig. 3B and C), suggesting that Glysine-548 in the S1 pocket is functionally important for Aβ degradation.

Next, to check whether the binding sites are common to NAAG and Aβ substrates or not, we performed competition assay. The NAAG cleavage activity of GCPII was not changed by the addition of the Ab peptides (Fig. 3D). In the same manner, the Aβ degradation was not affected by addition of the NAAG (Fig. 3E), suggesting that the binding sites for NAAG hydrolysis and Aβ degradation are different from each other.

3.4. Specific inhibitor targeting the S1 pocket of GCPII inhibits Aβ degradation

We used the ChemBridge and ChemDiv compound library databases to develop GCPII S1 pocket-specific chemical inhibitor. We initially selected 396,047 compounds with docking scores above -40 and among those, non-S1 site-selective binders were filtered out through visual scrutiny, which led to 1,974 candidates. Of these candidates, 54 commercially available compounds were chosen, and their inhibitory effects on Aβ degradation were examined by western blotting using 6E10 Aβ antibody (data not shown).

Of the several compounds that specifically inhibited Aβ degradation activity but not NAAG cleavage (data not shown), we finally selected one chemical compound named compound 2 because not only the structure fits well into the S1 pocket (Fig. 4A) but also it showed the strongest inhibition effect (Fig. 4B). As shown in Fig. 4B, compound 2 completely blocked Aβ degradation by GCPII in a dose-dependent manner without any inhibitory effect on NAAG hydrolysis. 2-PMPA did not show any inhibitory effect on Aβ degradation in contrast to its strong inhibitory effect on NAAG hydrolysis (Fig. 4C). By developing the S1 pocket specific-inhibitor in this study, we could demonstrate the existence of the 2 different binding sites and also reveal that the Aβ binding site of GCPII is localized in the S1 pocket.

3.5. The NAAG and Aβ peptides may share common catalytic site of GCPII

To check whether GCPII use same catalytic site in NAAG hydrolysis and Aβ degradation , we substituted Glutamate-424, which is known as catalytic base, to alanine(E/A) or glutamine(E/Q) on GCPII [15–17]. Both E424A and E424Q abolished NAAG hydrolysis activity, but the former's effect was severe than that of latter (Fig. 4D). And also, E424A and E424Q mutants remarkably blocked Aβ degradation activity to a similar extent (Fig. 4E). These results raise the possibility that GCPII could share same catalytic base, Glutamate-424 on GCPII, for NAAG hydrolysis and Aβ degradation, although the binding sites are different from each other. When we roughly compared the catalytic rates of the two reactions, we could almost simultaneously observe the NAAG hydrolysis just after mixing, but it took over 16 h incubation to observe Aβ degradation (Fig. S1).

4. Discussion

Until our recent discovery that GCPII has Aβ degradation activity [2], the main function of GCPII has been known to hydrolyze *N*-acetylaspartylglutamate (NAAG) to *N*-acetylaspartate (NAA) and glutamate. Despite the potential importance of Aβ degradation activity in GCPII in association with Alzheimer's disease, the molecular mechanism by which GCPII degrades Aβ peptides remains unclear. In our previous report [2], we observed that the inhibitory effect of 2-PMPA on Aβ degradation was marginal compared to its complete inhibitory effect on NAAG hydrolysis. This observation raised the possibility of the existence of a separate binding site that is responsible for Aβ degradation, distinct from NAAG hydrolysis, and prompted us to conduct this study.

Knowing that the inhibitor 2-PMPA was initially developed targeting the S1′ pocket, we instead focused on the S1 pocket. Actually. the function of S1 pocket was not well known and thus, it was simply thought as a "fine tuner" for substrate specificity [8]. By developing an S1 pocket-specific inhibitor (compound **2**) in this study, we could easily discriminate two different functions of GCPII and demonstrate that Aβ degradation occurs through binding of Aβ peptides to the S1 pocket. In line with this, functional group mutations located within the S1 pocket significantly abrogated Aβ

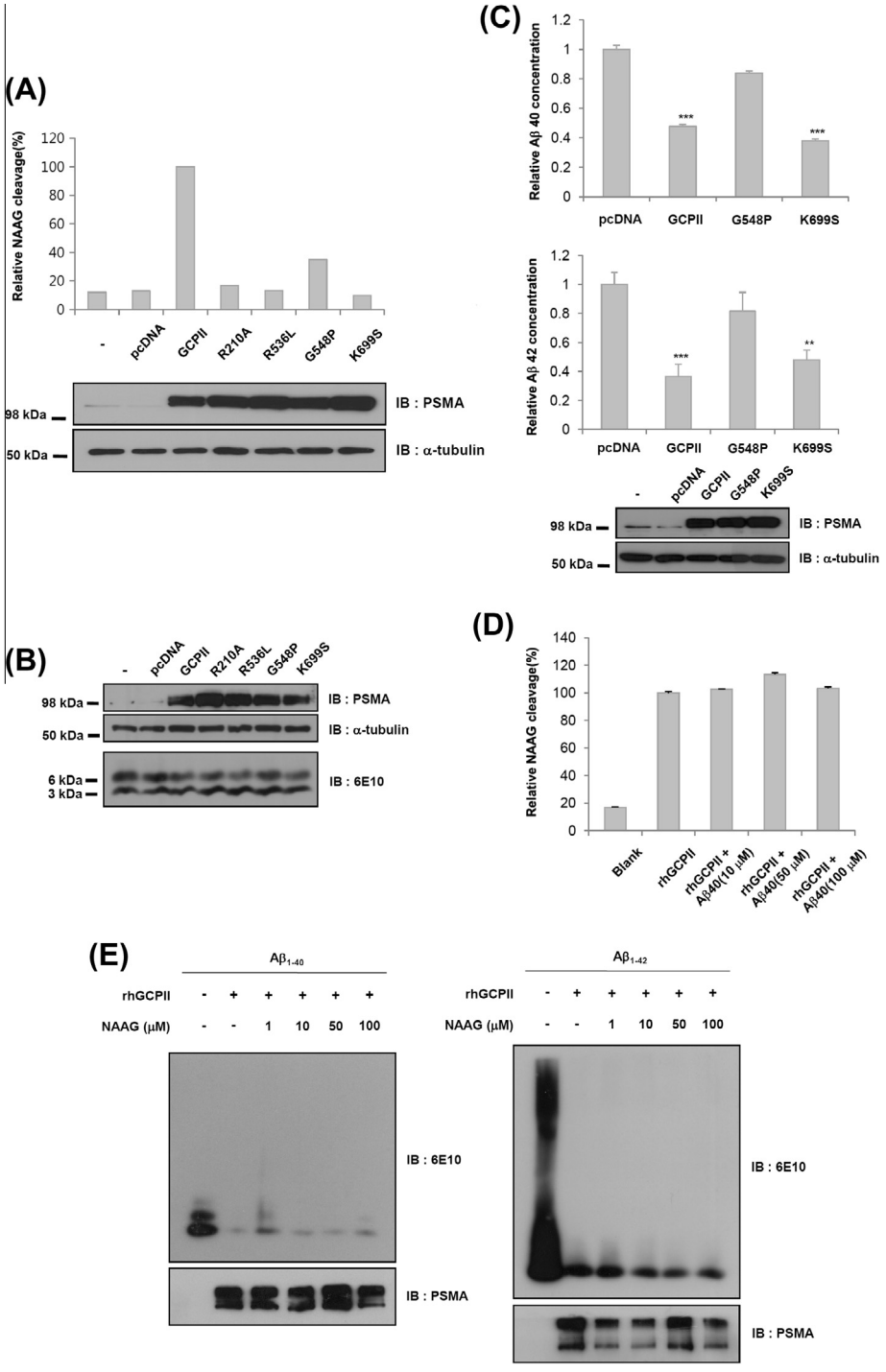

Fig. 3. The mutations effects of S1 and S1′ pocket on NAAG hydrolysis and Aβ degradation. Mutant vectors with amino acid substitution in the S1′ pocket (R210A, K699S) or S1 pocket (R536L, G548P) were transfected into PC3 cells and cultured for 16 h. The cell lysates were incubated with 10 mM $[H]^3$-labeled NAAG for 1 h at 37 °C for the analysis of NAAG hydrolysis (A) or incubated with 2 μM Aβ42 for 24 h at 37 °C for the analysis of Aβ degradation by western blotting (B). α-tubulin was used as an internal loading control. (C) The transfectant PC3 cells were treated with 1 ng of Aβ40 or Aβ42 for 8 h and residual Aβ peptides were detected by ELISA. (D) For competition assay, purified rhGCPII was incubated with 10 mM [H]-labeled NAAG in the presence of gradual increased concentration of Aβ40. (E) Reversely, purified rhGCPII was incubated with 8 uM Aβ40/ Aβ42 in the presence of gradual increased concentration of NAAG. The relative concentrations of Aβ40/42 were determined by statistical analysis of three independent experiments. $^{**}P< 0.01$, $^{***}P< 0.001$ relative to pcDNA vector control. Student's *t*-test (data were represented as mean ± SEM).

degradation activity without any effect on NAAG hydrolysis. In contrast, functional group mutations within the S1′ pocket abrogated NAAG hydrolysis without any effect on Aβ degradation. Concerning about the effect of R536L mutation, it seems that the hydrogen bond formation between the aspartyl side chain of NAAG and the arginine-536 might disrupt the NAAG hydrolysis function on GCPII [9].

Considering the 3D structure modeling analysis, the S1 pocket has advantage over S1' pocket by providing better environment for the binding of Aβ peptides in the following 2 aspects. First, the S1 pocket has a larger opening than the S1′ pocket to accommodate various size molecules, ranging from small Aβ oligomers to large fibrils (Fig. 2). Second, the S1 pocket allows not only hydrophobic interactions with Aβ peptides but also 3 ionic interactions

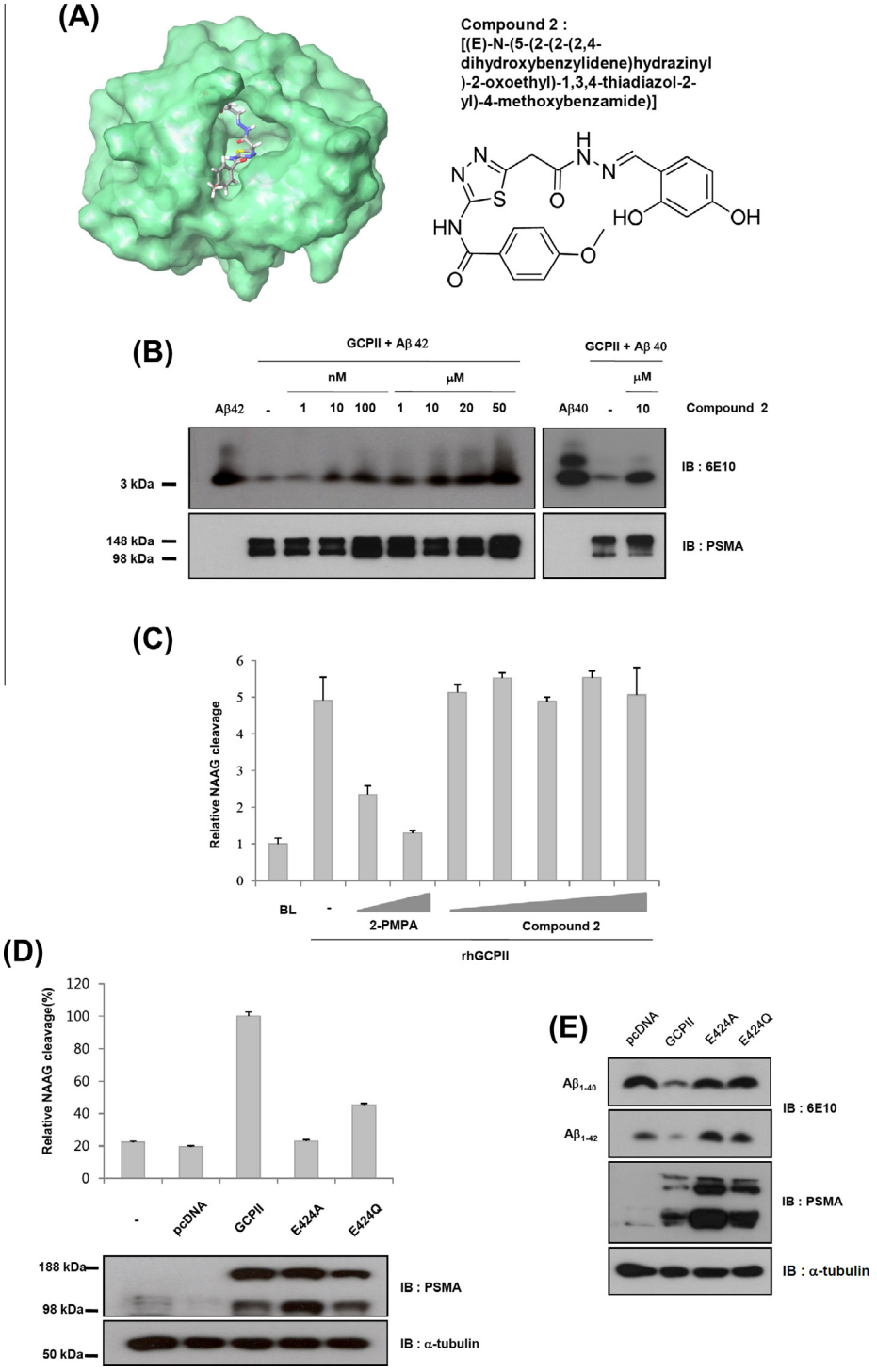

Fig. 4. Compoud 2 specifically inhibits Aβ degradation without any effect on NAAG hydrolysis. (A) Docking mode of compound **2** to S1 pocket of the GCPII and molecular structure of compound **2**. (B)Purified rhGCPII was incubated with Aβ42 in the presence of compound **2** over 16 h at 37 °C and residual Aβ peptides were detected by western blotting. (C)Purified rhGCPII was incubated with 10 mM $[H]^3$-labeled NAAG in the presence of 2-PMPA (50 nM or 1 μM) or compound **2** (10 μM, 20 μM, 50 μM, 100 μM, or 1 mM) and NAAG hydrolysis was analyzed by assay kit. For the mutation effect on NAAG hydrolysis and Aβ degradation, NAAG cleavage assay (D) and western blotting (E) were performed. α-tubulin was used as an internal loading control.

between basic residues (sticks highlighted in pink and blue) within the S1 pocket and an acidic residue (green and pink sticks) within the Aβ peptide (Fig. 2B).

Based on the data about catalytic base mutation (E424A) on GCPII, although Aβ peptides and NAAG have separate its own substrate binding sites, they seem to use the same functional amino acid residue for catalysis with substrate-dependent enzyme kinetics. Recently, two groups have reported that GCPII does not process Aβ degradation [18,19], completely contradicting our previous result [2]. The cause of conflicting results could be considered in following several viewpoints. First, the reaction conditions for the of Aβ degradation were different; for example, buffer composition and amount of GCPII enzyme used. Second, GCPII enzyme sources were different. The two groups used same

GCPII, which contains only extracellular part of human GCPII (aa residues 44-750), whereas we used intact whole enzyme, although it was tagged by His for convenient purification through affinity chromatography. Finally, two groups insisted that S1' pocket responsible for NAAG hydrolysis is not enough to accommodate large Aβ peptides, requiring dynamic structural change. With the *in sillico* computational modeling, we developed a new chemical inhibitor (compound 2) targeting the S1 pocket responsible for Aβ degradation, different from 2-PMPA targeting the S1' pocket responsible for NAAG hydrolysis. Using the compound 2, we could definitely discriminate the two different binding sites on GCPII, and also we again strongly assert that GCPII truly has Aβ degradation activity with the help of new finding that Aβ degradation occurs through binding to S1 pocket.

In conclusion, we could substantiate the molecular mechanism of Aβ degradation by demonstrating that S1 pocket serves as binding site of Aβ peptides. We also found that GCPII could be a potential therapeutic strategy for treating neurodegenerative disease through genetic manipulation, such as K669S mutant, which shows high Aβ degradation activity and simultaneously low NAAG hydrolysis activity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.07.059.

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