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Research Report

Role of μ-calpain in proteolytic cleavage of brain L-glutamic acid decarboxylase

Di Sha[a,b,d], Ying Jin[a,b], Heng Wu[a,b], Jianning Wei[a], Chun-Hua Lin[e], Yi-Hsuan Lee[e], Chandana Buddhala[a], Shafi Kuchay[f], Athar H. Chishti[f], Jang-Yen Wu[a,b,c,*]

[a]Department of Biomedical Sciences, Florida Atlantic University, Boca Raton, FL 33431, USA
[b]Center for Complex Systems and Brain Sciences, Florida Atlantic University, Boca Raton, FL 33431, USA
[c]Center of Excellence for Biomedical and Marine Biotechnology, 777 Glades Road, Florida Atlantic University, Boca Raton, FL 33431, USA
[d]Department of Pharmacology, Emory University, School of Medicine, Atlanta, GA 30322, USA
[e]Department of Physiology, Graduate Institute of Medical Sciences, Taipei Medical University, Taipei, Taiwan
[f]Department of Pharmacology, University of Illinois, College of Medicine, Chicago, IL 60607, USA

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ABSTRACT

Glutamic acid decarboxylase (GAD) is the rate-limiting enzyme for γ-aminobutyric acid (GABA) biosynthesis. Previously, we reported the presence of truncated forms of GAD *in vivo* and *in vitro*. In addition, an unidentified endogenous protease responsible for proteolytic cleavage of full-length GAD (fGAD) to its truncated form (tGAD) was also observed. In this communication, we report that μ-calpain is a good candidate for conversion of $fGAD_{67}$ to $tGAD_{67}$. This conclusion is based on the following observations: 1. purified recombinant GAD_{67} is cleaved by μ-calpain at specific sites; 2. in brain synaptosomal preparation, GAD_{67} is cleaved to its truncated form by an endogenous protease which is inhibited by specific calpain inhibitors; 3. in μ-calpain knockout mice, the level of tGAD in the brain is greatly reduced compared with the wild type; 4. when μ-calpain gene is silenced by siRNA, the level of tGAD is also markedly reduced compared to the control group; and 5. μ-calpain is activated by neuronal stimulation and Ca^{2+}-influx. The physiological significance of calpain in regulation of GABA synthesis and GABAergic neurotransmission is also discussed.

In the central nervous system (CNS), γ-aminobutyric acid (GABA) is synthesized by a single enzymatic reaction catalyzed by L-glutamic decarboxylase (EC 4.1.1.15; GAD) (Wu, 1976; Watanabe et al., 2002). Mammalian species express two isoforms of GAD, namely, GAD_{65} and GAD_{67}, referring to GAD with a molecular weight of 65 kDa and 67 kDa, respectively. Previously, we reported the presence of the truncated GAD (tGAD) derived from proteolytic cleavage of the full-length (fGAD) *in vivo* as well as *in vitro* (Wei et al., 2003; Sha et al., 2005). The presence of smaller forms of GAD was also observed

* *Corresponding author*. Florida Atlantic University, Department of Biomedical Science, 777 Glades Road, Boca Raton, FL 33431-0991, USA. Fax: +1 561297 0174.
E-mail address: jwu@fau.edu (J.-Y. Wu).

Abbreviations: GAD, Glutamic acid decarboxylase; GABA, γ-Aminobutyric acid; fGAD, full-length GAD; tGAD, truncated GAD; SELISA, Sandwich Enzyme-Linked ImmunoSorbent-Assay; HRP, horseradish peroxidase; ABTS, 2,2′-azino-bis(3-3 ethylbenziazoline-6-sulfonic acid); PLP, pyridoxal phosphate; AET, 2-aminoethylisothiuronium bromide; GAPDH, hGAD, human GAD; GST, glutathione-S-transferase; PKA, cAMP-dependent protein kinase A; $PKC_ε$, protein kinase $C_ε$; Capn, calpain; CTL, control group; STI, stimulation group; KO, knockout; WT, wild type; anti-*p*S/T, anti-phospho-serine and threonine

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from other laboratories (Legay et al., 1987; Chang and Gottlieb, 1988; Chu and Metzler, 1994; Buss et al., 2001). However, no information was reported regarding the identity of the endogenous proteases responsible for GAD cleavage and their physiological significance. Recently, we found that recombinant human brain GAD_{65} is specifically cleaved at arginine 69 from the N-terminal of $fGAD_{65}$ to produce $tGAD_{65}$, which is ~2–3 fold more active than $fGAD_{65}$ (Wei et al., 2003). In addition, GAD_{67} was found to be cleaved at two specific sites, one at arginine 70 and another at arginine 90, to produce two truncated forms of GAD_{67} (Wei et al., 2003). Based on these observations, it seems that the formation of tGAD is catalyzed by specific proteases instead of a random degradation of GAD. The presence of truncated GAD prompts us to investigate the identity of the protease and to determine the physiological/pathological significance of the cleavage.

One of the clues to suggest that the endogenous protease responsible for cleavage of GAD is Ca^{2+}-dependent comes from our recent observation that the extent of GAD cleavage is markedly increased when synaptosomes are depolarized in the presence of Ca^{2+}. Hence, we have examined the ability of calpain to cleave GAD. Calpains comprise a family of calcium-dependent, nonlysosomal, neutral, cysteine proteases that are present in most of the mammalian tissues (Goll et al., 2003). The calpain system is composed of three molecules: two Ca^{2+}-dependent proteases, μ-calpain (Calpain 1) and m-calpain (Calpain 2) and a polypeptide, calpastatin, an endogenous specific 5 calpain inhibitor. Both μ- and m-calpain consist of two subunits: an identical 30 kDa regulatory subunit and an 80 kDa catalytic subunit that shares about 60% homology between the two isoforms. The catalytic subunits of μ-calpain and m-calpain come from different but closely related genes (referred to as Capn 1 and Capn 2, respectively). The two forms differ in their responses to calcium *in vitro*. The μ-calpain is fully activated by micromolar concentrations of calcium, while the m-calpain requires a millimolar range of calcium. Calpains play an important role in various cellular processes including remodeling cytoskeletal attachments to the plasma membrane during cell fusion and cell motility, regulating signaling pathways, apoptosis and an involvement in long-term potentiation (Goll et al., 2003). The Capn $1^{-/-}$ mice are viable and fertile, but they have a significant reduction in platelet aggregation and clot retraction (Azam et al., 2001). In contrast, disruption of the catalytic subunit of calpain 2 caused embryonic death at an early stage (Goll et al., 2003). Transgenic mice that lack the regulatory subunit die during embryonic development with defects in vascular development (Arthur et al., 2000).

In this communication, evidence of μ-calpain being the protease responsible for the conversion of $fGAD_{67}$ to $tGAD_{67}$

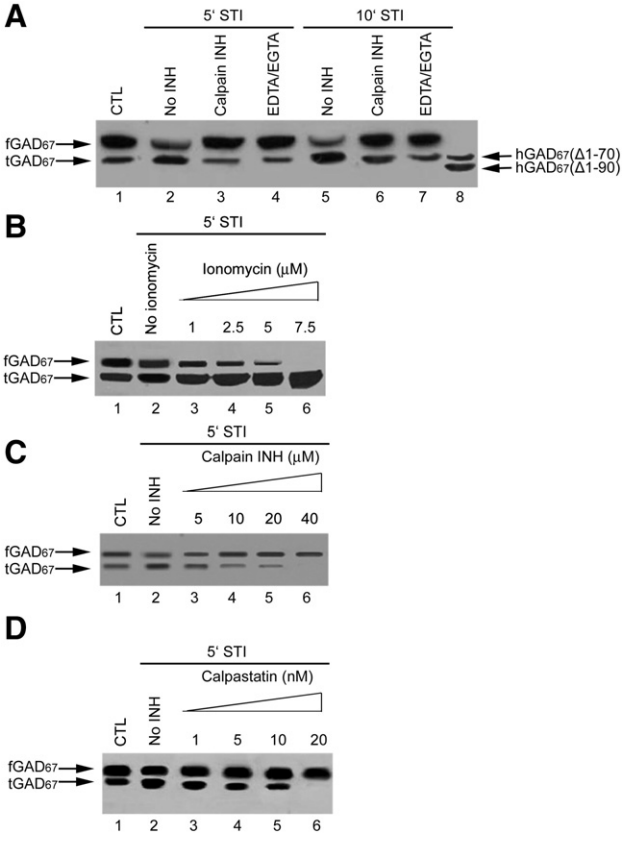

Fig. 1 – Effect of neuronal stimulation on cleavage of GAD_{67} in rat brain synaptosome preparation. Synaptosome was prepared and stimulated as described. A. Effect of EDTA/EGTA and calpain inhibitor I on stimulation-induced cleavage of GAD_{67}. Lane 1: Synaptosomal soluble fraction under the non-stimulation condition; Lane 2: Synaptosomal soluble fraction under the stimulation condition for 5 min; Lane 3: Same as lane 2 except that the sample was incubated with 10 μM of calpain inhibitor I before the stimulation; Lane 4: Same as lane 2 except that the sample was incubated with 2 mM of EDTA/EGTA before the stimulation; Lane 5: Synaptosomal soluble fraction under the stimulation condition for 10 min; Lane 6: Same as lane 2 except that the sample was incubated with 10 μM of calpain inhibitor I before the stimulation; Lane 7: Same as lane 2 except that the sample was incubated with 2 mM of EDTA/EGTA before the stimulation; Lane 8: Mixture of recombinant $hGAD_{67}$ (Δ1–70) and $hGAD_{67}$ (Δ1–90); B. Effect of ionomycin on cleavage of GAD_{67} under stimulation condition. Lane 1: Synaptosomal soluble fraction under the non-stimulation condition; Lane 2: Synaptosomal soluble fraction under the stimulation condition for 5 min; Lanes 3–6: same as Lane 2 except that a different concentration of ionomycin is included during the stimulation reaction; C. Effect of calpain inhibitor I on cleavage of GAD_{67}. Lane 1: Synaptosomal soluble fraction under the non-stimulation condition; Lane 2: Synaptosomal soluble fraction under the stimulation condition for 5 min; Lanes 3–6: same as Lane 2 except that synaptosome is pre-incubated with a different concentration of calpain inhibitor I; D. Effect of calpastatin on cleavage of GAD_{67}. Lane 1: Synaptosomal soluble fraction under the non-stimulation condition; Lane 2: Synaptosomal soluble fraction under the stimulation condition for 5 min; Lanes 3–6: same as Lane 2 except that the synaptosome is pre-incubated with different concentrations of calpastatin. For each panel, upper arrow indicated the position of $fGAD_{67}$ and lower arrow indicated the position of $tGAD_{67}$.