Regulation of m-Calpain Activity by α-Synuclein and Its C-terminal Fragment (α-syn61-140)

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The m-calpain activity hydrolyzing a fluorogenic substrate of N-Succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcourmarin (LLVY-AMC) was significantly stimulated by more than two-fold in the presence of 5 μM α-synuclein at 15 ºC. The stimulation was also confirmed with azocasein. The stimulation of the peptide hydrolyzing activity required structural intactness of α-synuclein since the C-terminally or N-terminally modified proteins such as β-synuclein, α-syn1-97, and α-syn61-140 did not increase the proteolytic activity. Instead, however, the N-terminally truncated α-syn61-140 was shown to drastically suppress the calpain activity. Since the N-terminal truncation was known to be the primary cleaving event of calpain-mediated proteolysis of α-synuclein and the α-syn61-140 has been demonstrated to be resistant against the calpain digestion, it has been proposed that the intracellular calpain activity could be regulated in a reciprocal manner by α-synuclein and its proteolyzed C-terminal fragment. Based on the results, a possible physiological function of α-synuclein has been suggested as a calpain regulator which contains both stimulatory and inhibitory activities.

Key Words : α-Synuclein, Calpain, Proteolysis, Calpain regulator, Parkinson's disease

Introduction

α-Synuclein is a pathological protein for Parkinson's disease by participating in the formation of Lewy bodies found in the dopaminergic neurons of substantia nigra pars compacta.[1-3] Physiological function of the protein is undefined although it has been proposed to be involved in synaptic plasticity, neuronal differentiation, regulation in dopaminergic neurotransmission, and cell viability.[4-10] α-Synuclein is a 'natively unfolded' protein which makes the protein versatile for its partner interactions.[11,12] Specific interaction between calmodulin and α-synuclein has been demonstrated in the presence and even absence of calcium.[13] Since calmodulin-binding proteins have been considered to interact with calpain,[14] a possible regulatory function of α-synuclein during calpain-mediated proteolysis has been investigated.

In 1982, DeMartino and Blumenthal reported a heat-stable calpain activator isolated from the cytosolic fraction of bovine brain, which was distinctive from calmodulin.[15] In 1986, Takeyama *et al*.[16] reported a similar calpain activator from the brain microsomal-insoluble fraction, which was also heat-stable with an approximate M_r of 15 KDa. In fact, these characteristics of the activators are the properties also shared by α-synuclein since it is a brain-specific heat-stable protein with M_r of 14 KDa.[17] α-Synuclein tends to interact with membranes through the dramatic structural transition from random structure to amphipathic α-helices.[18] The protein was also demonstrated to experience protein self-oligomerization upon the lipid or fatty acid interaction.[19-22] Therefore, α-synuclein could be found in the particulate fraction from the brain extract as well as in the cytosolic fraction. In addition, the protein also tends to self-interact with each other, which would form possible intracellular inclusion bodies collectable in the particulate fraction.

Calpain is a calcium-activated intracellular cysteine protease, which is a heterodimer with 28 and 80 KDa subunits.[23] The larger subunit contains the active site while the smaller one could play regulatory roles via phospholipid or calmodulin interactions.[24] Calpain has been categorized into two groups - μ- and m-calpain - depending on its calcium requirement for the optimal activity.[25] The protease exerts its activity in association with membranes although most of them are found in a cytosolic fraction.[26,27] Since α-synuclein is prone to associate with membranes, their functional topologies between calpain and α-synuclein have been shared with each other. Since physiological regulatory mechanism for the calpain activity has been one of key questions concerning the intracellular proteolytic system, α-synuclein has been examined as a possible natural regulator of the protease. This investigation could also contribute to elucidation of natural function(s) of the calpain system under physiological condition.

Experimental Section

Preparations of α-synuclein and its related proteins. Recombinant α- and β-synucleins cloned in pRK172 were overexpressed in *E. coli* BL21 (DE3) and purified according to the procedures described previously.[13,17] α-Synuclein was prepared *via* heat-treatment of the cell lysate, DEAE-Sephacel anion-exchange, Sephacryl S-200 gel-permeation, and S-Sepharose cation-exchange chromatography steps. β-Synuclein was obtained *via* the heat-treatment and two

successive anion-exchange chromatography steps using DEAE-Sephacel and Q-Sepharose in 20 mM Tris-Cl, pH 7.5. The C-terminal truncation of α-synuclein to obtain α-syn1-97 was carried out with Endoproteinase Asp-N treatment, and the product was isolated with Q-Sepharose anion-exchange chromatography and C4 reversed-phase HPLC under the experimental conditions described elsewhere.[28] The N-terminally truncated α-syn61-140 was overexpressed as a recombinant glutathione S-transferase fusion protein. Following purification with glutathione-Sepharose chromatography, α-syn61-140 was prepared *via* thrombin digestion of the fusion protein and subsequent Q-Sepharose anion-exchange chromatography according to the previously described procedure.[29] All the proteins were stored in aliquots at −30 ºC following extensive dialyses against 20 mM Mes, pH 6.5.

Calpain activity measurements. Calpain activity was measured with both a fluorogenic peptide of N-Succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (LLVY-AMC) and azocasein as a protein substrate, which were obtained from BIOMOL (Plymouth Meeting, USA) and Sigma (St. Louis, USA), respectively. The peptide cleaving activity of calpain (20 nM) was observed with LLVY-AMC at 150 μM in the presence and absence of various concentrations of α-synuclein at either 15 ºC or 37 ºC in 50 mM Tris-Cl, pH 7.5, containing 1 mM EDTA, 0.1 M KCl, 1 mM DTT, and 20 mM $CaCl_2$. The proteolysis was continuously monitored for more than 15 min with a chemiluminescent spectrometry (Perkin Elmer LS 55) by following the 7-amino-4-methylcoumarin (AMC) generation which emitted fluorescence at 460 nm with an excitation at 380 nm. The LLVY-AMC cleaving activity of m-calpain was also monitored at 25 ºC with various forms of α-synuclein isoforms including β-synuclein, α-syn1-97, and α-syn61-140 at 5 μM in the Tris buffer.

Azocasein cleavage by calpain was performed in the digestion buffer of 50 mM Tris-Cl, pH 7.5, containing 1 mM EDTA, 0.1 M KCl, 1 mM DTT, and 200 mM $CaCl_2$. The protein substrate (0.25%) was incubated with 115 nM m-calpain (Calbiochem, San Diego, USA) for 1 hour at 25 ºC in the presence and absence of 5 μM α-synuclein. The reaction was terminated by addition of a half volume of 20% trichloroacetic acid and left in ice for 15 min. Following centrifugation at 20,000 × g for 10 min at 4 ºC, the supernatant was collected and neutralized with 1 N NaOH at 1 : 1 (v:v) ratio, then the absorbance was measured at 405 nm.

Calpain digestions of α-synuclein and α-syn61-140 were carried out with 0.33 μM m-calpain for the digestion of 6.9 and 60.2 μM synucleins, respectively, at 37 ºC for 30 min in a reaction buffer of 50 mM Tris-Cl, pH 7.5, containing 1 mM EDTA, 0.1 M KCl, 1 mM DTT, and 20 mM $CaCl_2$. The reaction was terminated by mixing with Tricine-SDS-PAGE sample buffer consisted of 450 mM Tris-Cl, pH 8.45, 12% glycerol, 4% SDS, and 0.0025% Coomassie blue G and 0.0025% Phenol red at an 1 : 1 (v:v) ratio. The proteins were analyzed with Novex 10-20% Tricine gel and visualized with Coomassie brilliant blue staining.

Determination of Km and Vmax. Proteolytic cleavages of LLVY-AMC were performed with 20 nM m-calpain in the presence and absence of 5 μM α-synuclein at various concentrations of the peptide substrate at 15 ºC in the digestion buffer of 50 mM Tris-Cl at pH 7.5. Initial velocities of the proteolyses were obtained by monitoring the AMC generation with fluorescence at 460 nm. The reaction rates obtained with the change in the fluorescence intensity within 1 min (FI/min) and the corresponding substrate concentrations were subjected to a double-reciprocal plot from which K_m and V_{max} were obtained from the intercepts of x- and y-axis, respectively.

Results and Discussion

Stimulation of the m-calpain activity by α-synuclein was examined with a fluorogenic substrate of LLVY-AMC at 150 μM in the 50 mM Tris-Cl buffer at pH 7.5 containing 1 mM EDTA, 20 mM $CaCl_2$, 0.1 M KCl, and 1 mM DTT. The

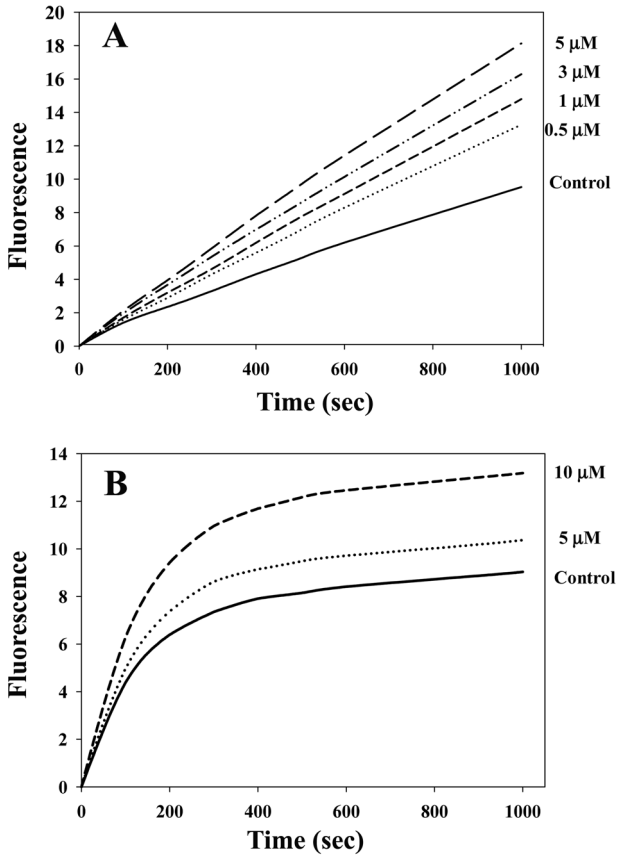

Figure 1. Stimulation of the peptide-hydrolyzing m-calpain activity by α-synuclein. The LLVY-AMC cleaving calpain activity was continuously monitored at either 15 ºC (A) or 37 ºC (B) by measuring the fluorescence of AMC at 460 nm upon excitation at 380 nm in the presence of various concentrations of α-synuclein as indicated on the panels. The fluorogenic substrate (150 μM) was incubated with 20 nM m-calpain in the digestion buffer of 50 mM Tris-Cl, pH 7.5, containing 1 mM EDTA, 0.1 M KCl, 1 mM DTT, and 20 mM $CaCl_2$.

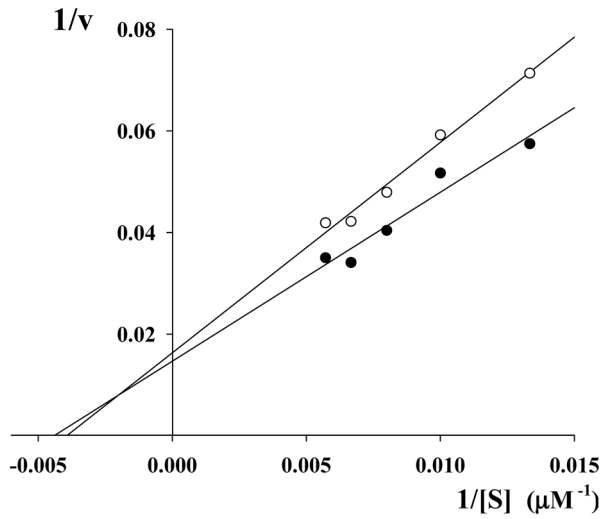

Figure 2. Lineweaver-Burk plots of the LLVY-AMC cleaving m-calpain activity. The peptide hydrolyzing activities were obtained with 20 nM m-calpain at various concentrations of the substrate in the presence (●) and absence (○) of 5 μM α-synuclein at 15 °C in the digestion buffer. The rates were observed with the changes in the fluorescence intensity (FI) due to AMC generation at 460 nm per min. The reaction rates and corresponding substrate concentrations were subjected to the double reciprocal plots.

basal proteolytic activity observed in the absence of α-synuclein was proportionally stimulated as the protein level was raised from 0.5 μM to 5 μM at 15 °C (Fig. 1A). In the presence of 5 μM α-synuclein, the activity was enhanced by almost 100%. The dose-dependent stimulation was also obtained at 37 °C although control activity of calpain in the absence of α-synuclein was rapidly deactivated at this temperature, which gave rise to a downward curvature of the fluorescent AMC production as the reaction proceeded (Fig. 1B). This short lifespan of the calpain activity has been recognized, and suggested to be attributable to possible autolysis of the protease.[30] The m-calpain stimulation by α-synuclein was observed throughout various substrate concentrations from 75 to 175 μM (Fig. 2). The double reciprocal plots indicated that α-synuclein has influenced the enzyme by decreasing K_m from 256 to 227 μM with increased V_{max} from 61.3 to 68.5 FI/min. The α-synuclein mediated m-calpain stimulation was observed with a protein substrate of azocasein as well as the peptide substrate of LLVY-AMC. In order to confirm whether the stimulation of the peptide cleaving activity of m-calpain could be applicable to protein substrate, azocasein was employed to examine the stimulatory activity of α-synuclein. It turned out that the azocasein-cleaving calpain activity was augmented by 44% in the presence of 5 μM α-synuclein (Fig. 3).

The stimulation of the LLVY-AMC cleaving activity required structural intactness of α-synuclein since variously modified forms of α-synuclein such as the N-terminally and C-terminally truncated fragments of α-syn61-140 and α-syn1-97, respectively, and β-synuclein with a diversified C-terminal region did not enhance the proteolysis at all. In particular, the deletion and variation in the acidic C-terminus

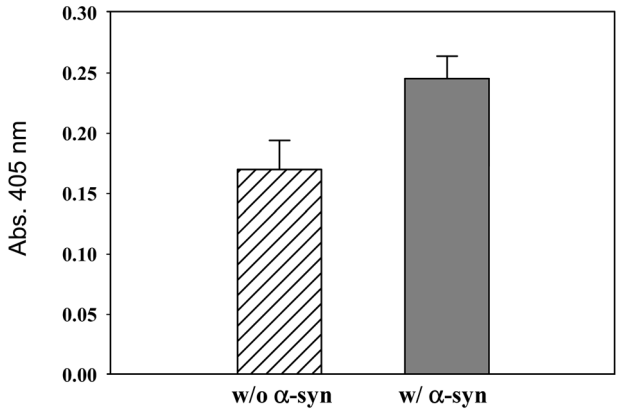

Figure 3. Stimulation of the azocasein-hydrolyzing m-calpain activity by α-synuclein. Azocasein (0.25%) was hydrolyzed with 115 nM m-calpain in the presence (black bar) and absence (oblique bar) of 5 μM α-synuclein for 1 hour at 25 °C in the digestion buffer at pH 7.5. Following TCA precipitation and neutralization with 1 N NaOH, the absorbance of the supernatants were measured at 405 nm.

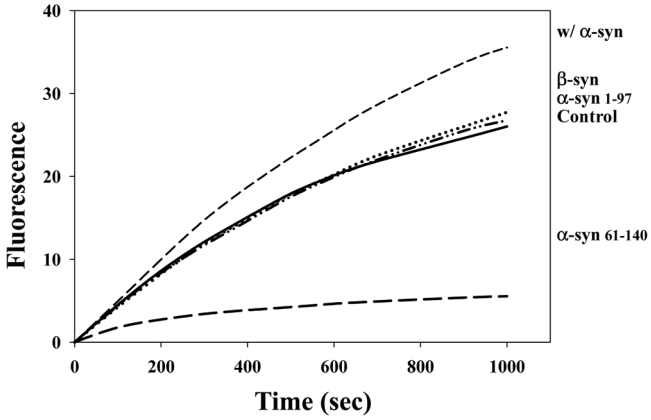

Figure 4. m-Calpain activities in the presence of the modified synucleins. The peptide- hydrolyzing m-calpain activities were monitored with the fluorescence at 460 nm for the reactions of 20 nM m-calpain and 150 mM LLVY-AMC at 25 °C in the presence of 5 mM α-synuclein and its related proteins such as β-synuclein, α-syn1-97, and α-syn61-140 as indicated on the panel.

of α-synuclein as observed in β-synuclein and α-syn1-97 failed to augment the proteolytic activity, indicating that the C-terminus is critical for the activation. Intriguingly, however, the N-terminally truncated form of α-syn61-140 has drastically suppressed the calpain activity (Fig. 4). This observation clearly indicates that the calpain stimulation observed with α-synuclein requires the intactness of the protein.

More importantly, the suppression of m-calpain activity observed with α-syn61-140 may have physiological or pathological implications since we have found out that the N-terminally truncated fragment was resistant against m-calpain digestion. When the modified protein was incubated with m-calpain, the protein was hardly degraded while the intact α-synuclein experienced a significant digestion by the enzyme (Fig. 5). It was already reported that the major

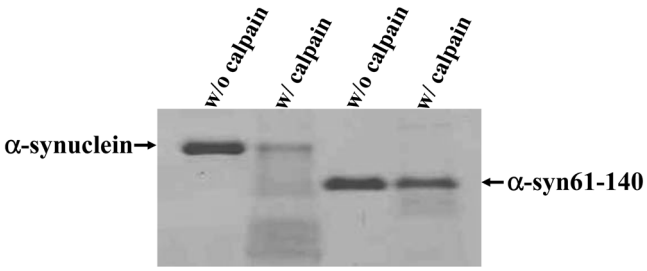

Figure 5. Calpain digestions of α-synuclein and α-syn61-140. α-Synuclein and α-syn61-140 were subjected to m-calpain (0.33 μM) digestion in the digestion buffer at 37 °C for 2 hours. Undigested α-synuclein and α-syn61-140 are shown on lanes 1 and 3, respectively, as indicated by arrows. Digestions of α-synuclein and α-syn61-140 by m-calpain were shown on lanes 2 and 4, respectively.

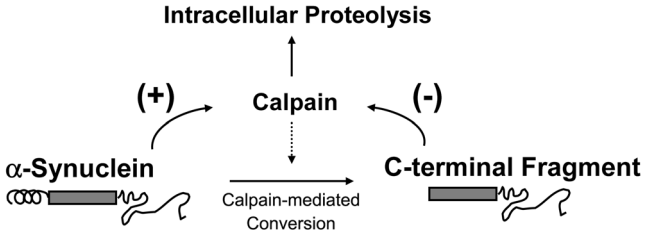

Figure 6. Model of m-calpain regulation by α-synuclein and its C-terminal fragment.

cleavage site of α-synuclein by calpain was located at Glu57-Lys58,[31] which would yield an N-terminally truncated C-terminal fragment including the whole amino acid sequence of α-syn61-140 as one of the major and predominant polypeptides derived from α-synuclein upon the proteolysis. Therefore, it might be suggested that intracellular calpain activity could be regulated by α-synuclein and its proteolyzed fragment(s) in a mutually reciprocal manner.

Taken together, intracellular calpain activity has been proposed to be regulated according to the following model (Fig. 6). The calpain has been stimulated by α-synuclein under a normal situation, which makes the protease actively involved in various proteolytic events within the cell. As the C-terminal fragment has been generated from the stimulatory α-synuclein by the protease, the proteolytic activity could be completely suppressed. When it is needed, however, the enzymatic activity might be regenerated upon a molecular competition between another α-synuclein and the C-terminal fragment bound on the enzyme. Although it is not clear whether α-synuclein is identical to the calpain stimulators previously isolated from the bovine brain by DeMartino and Blumenthal[15] and Takeyama *et al.*,[16] a possible physiological function of α-synuclein has been suggested as a calpain regulator, which might be further implicated in calcium-related intracellular activities in general.

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